SYNTHESE ANTITUMORAKTIVER TRITERPEN-CARBONSÄURE-DERIVATE UND DEREN BIOLOGISCHE EVALUIERUNG

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Vorwort

Diese vorliegende wissenschaftliche Arbeit wurde im Zeitraum von Mai 2021 bis Juli 2023 am Institut für Chemie im Bereich für Bioorganische Chemie der Martin-Luther-Universität Halle-Wittenberg in der Arbeitsgruppe von Prof. Dr. René Csuk angefertigt.

Die Dissertation wurde in kumulativer Form verfasst und die Forschungsergebnisse bereits in internationalen „peer-reviewed“ Fachzeitschriften publiziert.

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Abkürzungsverzeichnis

ATP	Adenosintriphosphat
Bcl-2	B-cell lymphoma 2
DNA	Desoxyribonukleinsäure
HER2	human epidermal growth receptor 2
HPTLC	Hochleistungsdünnschichtchromatographie
IC_{50}	mittlere inhibitorische Konzentration
IR	Infrarot
MCS	maximum common substructure
MS	Massenspektrometrie
NIR	Nah-Infrarot
OA	Oleanolsäure
PI	Propidiumiodid
Rh(B)	Rhodamin (B)
ROS	Reaktive Sauerstoffspezies
SAR	Struktur-Aktivitätsbeziehung
UV	Ultraviolett
VDAC/ANT	Voltage-dependent anion channel/ adenine nucleotide translocase

Einleitung

Die besonders große Notwendigkeit der Entwicklung neuartiger als auch die Weiterentwicklung bestehender Therapieansätze von Krebserkrankungen wird durch aktuelle Statistiken und Prognosen noch einmal hervorgehoben. Allein im Jahr 2020 gab es weltweit circa 20 Millionen neue Fälle, wobei je nach Art des Tumors von einer Verdopplung der Fallzahlen bis 2040 ausgegangen wird. [1-2]

Die vorhandenen Ansätze beschränken sich im Allgemeinen auf operative Methoden, Strahlentherapie, Bio-/Hormontherapie sowie die medikamentöse Behandlung. Aufgrund von Metastasierung oder einer schlechten Zugänglichkeit des Tumorgewebes muss meist oder zumindest zusätzlich auf Letzteres zurückgegriffen werden. Der größte Nachteil dieser Methode liegt jedoch darin, dass dabei nur schlecht zwischen Tumorzellen und gesunden, normalen Zellen unterschieden werden kann, was oft zu erheblichen Nebenwirkungen führt. Um die Selektivität zu erhöhen hat sich der Fokus der zielgerichteten Krebstherapie auf maßgeschneiderte Antikörper sowie sogenannte „small molecules“ gerichtet, welche in der Lage sind tumorspezifische extra- sowie intrazelluläre Ziele anzugreifen. Zu den innovativsten Methoden zählen dabei u.a. auf Mitochondrien gerichtet Wirkstoffe, sogenannte „Mitocane“. Eine weitere Herausforderung besteht in der zielgerichteten und selektiven Anreicherung bzw. Freisetzung des Wirkstoffs in den Tumorzellen. In den letzten Jahren wurde eine Optimierung dessen bspw. durch den Einsatz von Mizellen, Liposomen, Antikörper-/Peptidkonjugaten, speziellen Polymeren oder beladenen Nanopartikeln erreicht. [3-6] Von immer größer werdender Bedeutung für die zielgerichtete Therapie ist zudem die Theranostik, eine therapiebegleitende Diagnostik, die durch exakte Charakterisierung des Krankheitsstadiums sowie Monitoring des erreichten Heilungsfortschritts eine individuelle Abstimmung, also eine personalisierte Medizin, ermöglicht. Dies rückt zudem optische Tags in den Vordergrund, um Bildgebungsverfahren weiter zu verbessern.

Bei all diesen Entwicklungen stehen häufig Naturstoffe als Ausgangsmaterialien oder Vorbilder von davon abgeleiteten Wirkstoffen im Mittelpunkt. Unter allen neuzugelassenen „small molecules“ Antitumorwirkstoffen beträgt dieser Anteil mehr als 50 %. [7] Eine sehr bedeutende Klasse dieser Naturstoffe sind die aus einer verschiedenen Anzahl an Isopren-Einheiten aufgebauten Terpene, welche überwiegend pflanzlicher Herkunft sind. Von besonderem Interesse sind dabei Triterpene bzw. deren abgeleitete Carbonsäuren wie beispielsweise Oleanol-, Ursol-, Maslin-, Corosol- oder Asiasäure.

Zielgerichtete Tumortherapie

Targets

Mithilfe der zielgerichteten Tumortherapie sollen spezifische biochemische Prozesse in der Tumorzelle adressiert werden, um durch deren Hemmung oder auch Förderung das Wachstum bzw. Überleben der Tumorzelle selektiv einzuschränken. Dabei wird zwischen verschiedenen Wirkmechanismen unterschieden.

Kinase Inhibitoren hemmen den Transfer von γ-Phosphatgruppen des ATP und haben so einen entscheidenden Einfluss auf den meist überregulierten Energiehaushalt von Tumorzellen und somit auf das Zellwachstum und deren Proliferation. Daraus ergibt sich eine Möglichkeit den programmierten Zelltod, die Apoptose, zu induzieren. Je nach Substratrest werden die Kinasen nochmals in Tyrosin-Kinasen und Serin/Threonin-Kinasen unterteilt, welche sich ebenfalls in eine Vielzahl von Untergruppen aufteilen. **Inhibitoren des Bcl-2-Regulatorproteins**, welches in Tumorzellen überexprimiert wird und so die Apoptose hemmt, gelten als weiteres Target in der Krebstherapie. Weitere Signalwege/Prozesse, welche von großem Interesse sind, stellen **DNA-Reparaturprozesse** wie bei Topoisomeraseinhibitoren oder **Hormonrezeptoren** wie Estrogenrezeptoren bei einigen Brustkrebsarten dar. Aber auch die **Angiogenese** und die **ROS-Regulierung**, um den erhöhten Nährstoff- und Sauerstoffbedarf von Tumoren einzuschränken, sowie der **Hedgehog-Signalweg**, über den Zellen auf äußere Signale reagieren, sind wichtige Targets. [8-10]

Mitocane

Mitochondrien sind nicht nur verantwortlich für den Energiestoffwechsel der Zelle sondern dienen auch als ein Reservoir für apoptose-regulierende Proteine, was sie vor allem in Anbetracht der Tatsache, dass ihr Metabolismus sowie Membranpotential in malignen Tumorzellen verändert ist, als wichtiges Ziel der Tumortherapie kennzeichnet. Wirkstoffe, die auf diesem Weg Apoptose induzieren, werden Mitocane genannt, welche je nach Wirkmechanismus nochmals in acht Gruppen unterteilt werden. Neben den Hexokinase-Inhibitoren, bereits erwähnten Bcl2-Inhibitoren, Thiol-Redox-Inhibitoren und Wirkstoffen, welche auf VDAC/ANT-Kanäle, den Citrat-Zyklus, die Elektronentransportkette oder die *mt*DNA abzielen, gibt es die lipophilen Kationen mit dem Ziel der inneren Membran der Mitochondrien. [11] Aufgrund eines veränderten Energiebedarfs der Tumorzellen, ist deren Transmembranpotential stark erhöht. [12] Wird von ca. 60 mV ausgegangen, entspricht dies einer 10-fachen Akkumulation lipophiler Kationen. [13]

Bildgebende Verfahren in der Theranostik

Wie bereits zu Beginn erwähnt spielt Theranostik eine große Rolle in der dem Patienten als auch dem Krankheitsverlauf angepassten Behandlung. Wichtig dafür sind bildgebende Verfahren wie das Nahinfrarot (NIR) Fluoreszenz-Imaging, um biologische Prozesse auf molekularer Ebene in lebenden Zellen und Gewebe beobachten zu können. Darunter zählen auch das Tumorwachstum und dessen Reaktion auf eingesetzte Therapien. Durch das Einbringen von NIR-Tags können Verteilung und Effizienz genau untersucht werden und so die Dosis, aber auch die Struktur/Art der Behandlung, schnell auf den Patienten und Krankheitsverlauf angepasst werden. Der Vorteil der NIR-Fluoreszenz liegt in der höheren Eindringtiefe, der verringerten Hintergrundfluoreszenz durch Autofluoreszenz des Gewebes sowie verbessertem Signal-Rausch-Verhältnis als bei der Verwendung von energiereicherer UV-Strahlung. [14] Diese ist in den meisten Fällen auch schädlich für lebende Zellen, was deren Einsatzmöglichkeiten minimiert. Somit werden durch NIR-Fluoreszenz nicht nur genauere, sensitive Untersuchungen auf zellulärer Ebene durch hochaufgelöste Mikroskopie ermöglicht sondern beispielsweise auch die fluoreszenzgeleitete Chirurgie tiefsitzender Tumore und deren endoskopische Untersuchung sowie Lokalisation mittels Fluoreszenztomographie. [14-15] Letztere wird speziell für die in-vivo Diagnostik von Brustkrebs in der Fluoreszenzmammographie verwendet, wodurch auf zusätzliche Strahlenbelastung verzichtet werden kann und die Bildgebung bereits mit Kleingeräten ermöglicht wird. [16] Außerdem erlaubt sie die Verwendung von sogenannten Stratifizierungsmarkern, um schnell zu erkennen, welche Therapien indiziert und wie erfolgsversprechend diese sind. [17]

(Tri)terpene als Grundstruktur

Terpene sind mit über 8000 bzw. 30.000 (Terpenoide) Vertretern eine der größten Klassen von Naturstoffen, welche aus Terpen-Einheiten, die wiederum aus zwei Isopren-Einheiten bestehen, aufgebaut sind oder sich als Terpenoide davon ableiten. [18] Demnach werden sie entsprechend der Anzahl dieser Einheiten als Monoterpene, Sesquiterpene, Diterpene, Triterpene, bis hin zu Polyterpenen klassifiziert. Bereits seit dem Ende des 19. Jahrhunderts wurden eine Vielzahl von Strukturen dieser Gruppe von O. Wallach und L. Ružička genauer charakterisiert und beschrieben, wofür diese jeweils auch mit einem Nobelpreis gewürdigt wurden. [19-20] Seit dem sind vor allem auch die zahlreichen biologischen Eigenschaften zum Gegenstand der Forschung geworden, wobei Triterpene eine wichtige Rolle einnehmen. Zahlreiche Publikationen beschäftigen sich mit ihren anti-inflammatorischen [21-23], antibakteriellen [24-25], antiviralen [26], antiparasitären [27-28], antitumoraktiven [29-30], antidepressiven [31], neuroprotektiven [32-33], kardioprotektiven [34] sowie immunmodulierenden [35-36] Eigenschaften. Sie werden unterteilt in lineare Triterpene, dessen Vertreter Squalen als Grundgerüst der Biosynthese der folgenden gilt, tetracyclische Triterpene, abgeleitet vom Steran-Gerüst wie bspw. Dammarane, Lanostane oder Cycloartane, sowie die pentacyclischen Triterpene, auf denen der Fokus dieser Arbeit liegt.

Pentacyclischen Triterpenen liegen im Wesentlichen zwei Grundgerüste zugrunde, das Hopan-Gerüst sowie das Baccharangerüst, von dem sich wiederrum die Lupane sowie Oleanane und Ursane ableiten. Diese sind zusammen mit ihren typischen Vertretern, der Betulin- und Platansäure sowie Glycyrrhetin-, Oleanol- und Ursolsäure, in **Abbildung 1** dargestellt

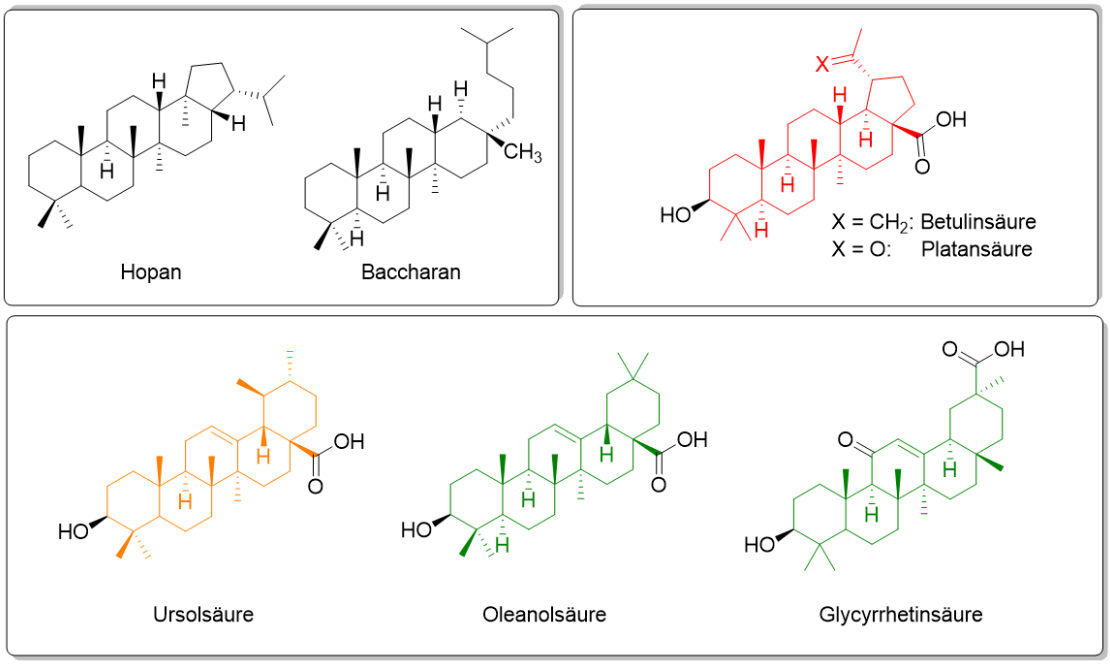


Abbildung 1: *Übersicht über die verschiedenen pentacyclischen Triterpene und ihre Grundstrukturen. (Lupangerüst: rot markiert; Ursangerüst: orange markiert; Oleangerüst: grün markiert).*

Wie anhand der Strukturen bereits zu erkennen ist, bieten die pentacyclischen Triterpencarbonsäuren zahlreiche Modifikationsmöglichkeiten, um ihre cytotoxischen Eigenschaften zu optimieren. Bisherige Untersuchungen konnten zeigen, dass eine Acetylierung der Hydroxygruppe am A-Ring essentiell ist, um antitumoraktive Derivate zu erhalten; das Einfügen einer Acetylgruppe in Nachbarposition scheint zu dem ebenfalls vorteilhaft zu sein. [37] Zudem erwiesen sich Benzylamide wie **EM2** **[38]** (Vgl. **Abbildung 5**), oder Chinolinamide [39-40] als besonders effektiv. Verwendet man für die Bildung eines Amidderivates ein 1,ω-Diamin, erhält man in vielen Fällen nicht nur aktivere Verbindungen sondern bietet zudem die Möglichkeit der Synthese verschiedenster Hybridverbindungen. Während Konjugate mit Safirinium [41] oder BODIPY [42] das Endoplasmatische Retikulum als Wirkungsort haben, zielen jene mit Rhodamin B [43-46], Malachitgrün [47], Phosphoniumsalzen [48-50] sowie F-16 [51-52] auf das Mitochondrium ab. Theoretische Studien konnten für entsprechende Rhodaminderivate pentacyclischer Triterpene dort mittels Molecular Modeling einen Wirkmechanismus an mitochondrialen Enzymen wie NAD(P)H-Chinonreduktasen, Oxidoreduktasen oder Succinatdehydrogenasen postulieren. [53] Änderungen des mitochondrialen Membranpotentials oder ein Anstieg der Konzentration von ROS-Spezies wären ebenfalls denkbar.

Zielstellung

Das Ziel dieser Arbeit war es nun, den Einfluss verschiedenster struktureller Änderungen und deren Einfluss auf die Cytotoxizität zu untersuchen, um eine exaktere SAR beschreiben zu können. Dabei sollten folgende Fragestellungen geklärt werden:

Lässt sich der positive Einfluss des bekannten Diamins Piperazin auf strukturähnliche Verbindungen ausweiten?

Steigert eine Ringerweiterung des cyclischen Amins die Cytotoxizität? Dabei sollte eine geeignete Synthesestrategie für das 1,5-Diazacyclooctan entwickelt werden.

Liefern weitere Linkermoleküle oder Verknüpfungspositionen ähnliche oder sogar bessere Ergebnisse?

Welches Diastereomer der beschriebenen, benachbarten Acetylgruppen des A-Rings ist am geeignetsten und über welche Partialsynthese ist dieses am einfachsten zugänglich?

Welche Abhängigkeit der Antitumoraktivität von der Art des Rhodamins kann beobachtet werden? Dieses sollte zudem anschließend für NIR-Fluoreszenz-Experimente optimiert werden, um neben der klassischen biologischen Evaluierung weitere Untersuchungen zu ermöglichen und auch experimentelle Ansätze für mögliche Wirkmechanismen zu erhalten.

Diskussion und Einordnung der Forschungsergebnisse

Im folgenden Abschnitt dieser Dissertation sollen die wichtigsten Forschungsergebnisse der einzelnen, zugrundeliegenden Publikation zusammenfassend erläutert, diskutiert und in den Gesamtkontext der Entwicklung cytotoxischer Triterpenderivate eigeordnet werden. Genaue Synthesebedingungen, Ausbeuten und spektroskopische Charakterisierungen aller Verbindungen, sowie die Durchführung der biologischen Assays etc. als auch deren gesamte Ergebnisse können den einzelnen Publikationen entnommen werden. Aus Gründen der Übersichtlichkeit wurden relevante Verbindungen abschnittsweise neu nummeriert.

Die Untersuchung verschiedener Glycyrrhetinsäureamide (P1)

Um der Fragestellung nachzugehen, ob verschiedene strukturähnliche Amide von vergleichbarer Cytotoxizität wie das entsprechende Piperazinamid sind, wurden eine Reihe von Amiden der 3-*O*-Acetyl-Glycyrrhetinsäure hergestellt und mittels SRB-Assay untersucht. Diese schien ein geeignetes Ausgangsmaterial zu sein, da im Vergleich zu anderen Triterpencarbonsäure nur wenige Derivate in der Literatur beschrieben sind, sie jedoch zugleich günstig in großen Mengen kommerziell erhältlich ist. [46, 54-56] Sie ist der Hauptbestandteil des Extraktes der Süßholzwurzel. [57-58]

Als Amine wurden verschiedene cyclische Verbindungen mit unterschiedlichen distalen Heteroatomen (N, O, S) und Ringgrößen (6, 7) sowie ein acyclisches Amin und mehrere bicyclische Amine verwendet. Eine Übersicht ist in **Abbildung 2** dargestellt. Die entsprechenden Amide konnten über ein *in-situ* erzeugtes Carbonsäurechlorid mittels Oxalylchlorid in guten Ausbeuten erhalten werden. Zu Vergleichszwecken wurde ebenfalls das freie Amin durch Curtius-Abbau der Carbonsäure hergestellt. Die quartären Ammoniumsalze wurden durch Umsetzen der tertiären Amine mit Methyliodid synthetisiert.

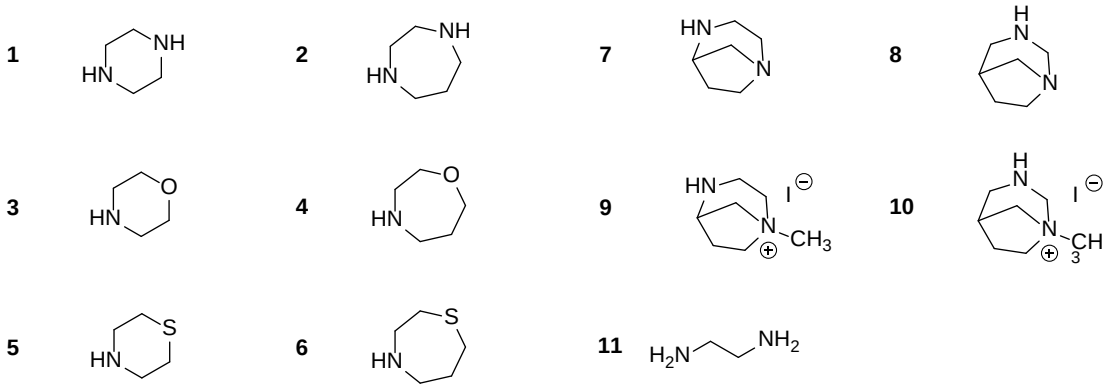


***Abbildung 2**: Übersicht über die zur Synthese verwendeten Amine.*

Für das anschließende SRB Assay wurden verschiedene Tumorzellinien verwendet (A375 – Melanom, HT29 – Kolorektalkarzinom, MCF-7 – Brust-Adenokarzinom, A2780 – Eierstockkarzinom, FaDu – Pharynxkarzinom, sowie vglw. NIH 3T3 – nonmaligne Mausfibroblasten), entsprechende IC_{50}-Werte sind jeweils in Klammern angegeben.

Interessanterweise waren die durch Umsetzung mit Piperazin (4,4 µM – 8,7 µM) und Morpholin (5,11 µM – 18,66 µM) erhaltenen Amide für alle Tumorzellinien aktiv, jedoch auch unselektiv. Ihre ringerweiterten Analoga zeigten, wie das Ausgangsmaterial selbst, jedoch keine Cytotoxizität im Rahmen des Assays (> 30 µM). Auch die vom Thiomorpholin abgeleiteten Amide waren inaktiv. Aus der Gruppe der Diazabicyclo-Derivate zeigte nur das von Amin **8** abgeleitete Amid eine, wenn auch geringe, Aktivität

(19,0 µM – 27,4 µM), jedoch ebenfalls keine Selektivität. Die aktivste Verbindung war das vom Ethylendiamin abgeleitete Derivat (2.0 µM – 5.7 µM).

Zudem konnte mittels Trypan-Blue-Staining gezeigt werden, dass die Cytotoxizität dieses Amids primär auf Apoptose zurückzuführen ist (89,5 % bei 4 µM für A2780 Zellen). Zusammenfassend lässt sich also sagen, dass Diamine für die Synthese cytotoxischer Triterpenderivate zu bevorzugen sind.

Die Untersuchung verschiedener Spacerpositionen und –arten (P2)

Als Ausgangsmaterial für diese Untersuchungen wurde Oleanolsäure gewählt, da sie aufgrund der im Vergleich zur Glycyrrhetinsäure fehlenden Carbonylfunktion am C-Ring eine höhere Vergleichbarkeit mit den weiteren in **Abbildung 1** dargestellten Triterpencarbonsäuren besitzt und bei der geplanten Reaktionsführung keine weiteren Nebenreaktionen eingehen kann. Sie ist Bestandteil vieler Pflanzen wie Oliven [59] oder Rosmarin [60]; aber auch kommerziell in große Mengen erhältlich. Um ein als Mitocan wirkendes Hybridmolekül zu erhalten, wurde das lipophile Kation Rhodamin B (siehe **Abbildung 3**) gewählt, da es ebenfalls kommerziell erhältlich ist und wie eingangs erwähnt, bereits vielfach in Kombination mit pentacyclischen Triterpenen untersucht wurde.

Einerseits sollte der Einfluss der Art der Verknüpfung (Amid bzw. Ester) betrachtet werden, andererseits auch die Auswirkung verschiedener Linker und Positionen ermittelt werden. Als mögliche Positionen kommen die Hydroxygruppe am A-Ring und die Carboxylgruppe an Position 28 in Frage.

Um Letztere näher zu betrachten wurde die Hydroxygruppe als Acetat geschützt und die Carbonsäure über einen Piperazinspacer mit Rhodamin B verknüpft. Eine Übersicht dieses Teils ist **Abbildung 3** zu entnehmen.

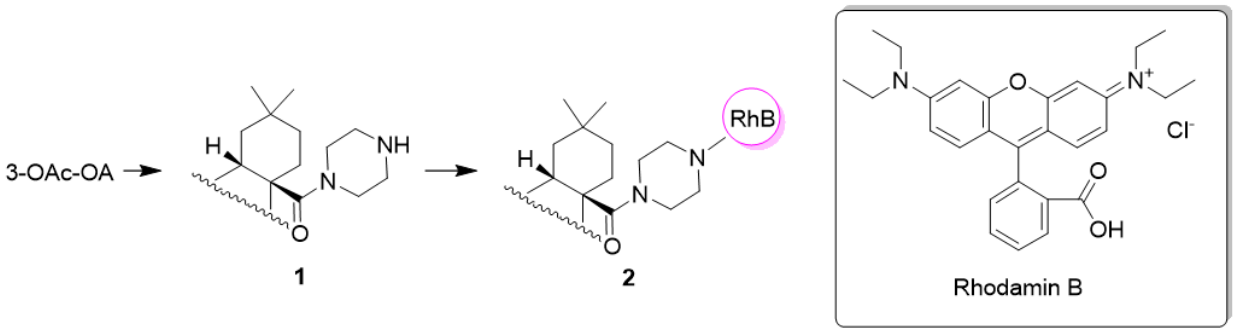


Abbildung 3: *Teil 1 der durchgeführten Modifikationen sowie die Struktur des Rhodamin B*

Um Erstere näher zu betrachten, wurde die Carbonsäure als Methylester geschützt und zuerst Rhodamin B nach einer Aktivierung mittels Oxalylchlorid direkt zum entsprechenden Ester umgesetzt. Um nun einen Linker einzufügen, wurde die Hydroxygruppe mit Bersteinsäureanhydrid verestert, die nun eingeführte Carboxylgruppe wieder als Carbonsäurechlorid aktiviert und mit Piperazin umgesetzt, um so Rhodamin B als Amid zu verknüpfen. Ebenfalls wurde die Hydroxygruppe mittels Jones-Oxidation oxidiert und über eine reduktive Aminierung mittels Ammoniumacetat und Natriumcyanoborhydrid das Amin gebildet. Dabei entstand selektiv das *3*β-Derivat, welches nun direkt mit Rhodamin B zum entsprechenden Amid umgesetzt werden konnte. Eine Übersicht dieses Teils ist **Abbildung 4** zu entnehmen.

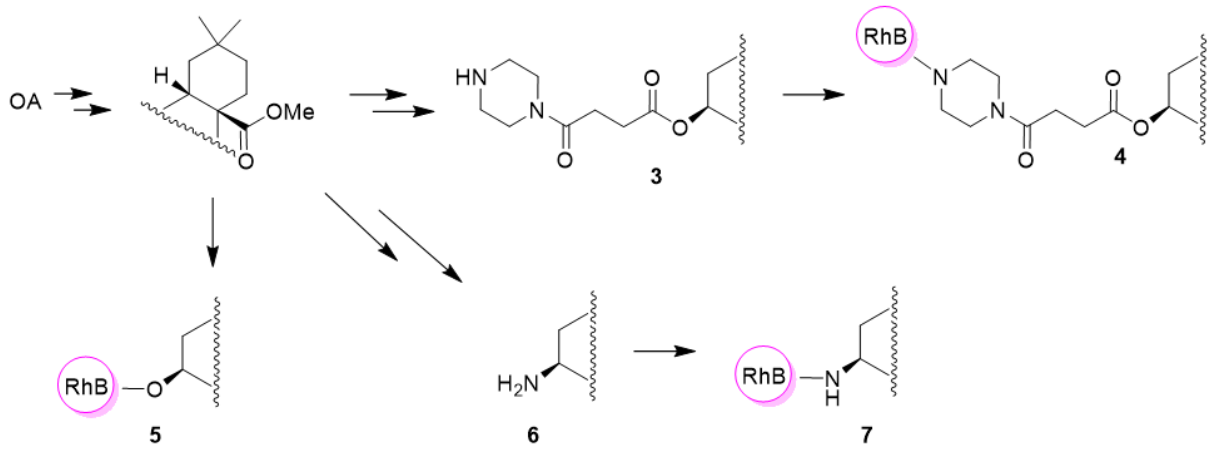


Abbildung 4: *Teil 2 der durchgeführten Modifikationen.*

Anschließend wurden SRB-Assays mit verschiedenen humanen Tumorzelllinien (A375, HT29, MCF-7, A2780) sowie nonmalignen Mausfibroblasten (NIH 3T3) zur Beurteilung der Selektivität durchgeführt.

Ausgehend von einer Aktivität der Ausgangsverbindungen (OA sowie 3-OAc-OA und RhB) von über 30 µM für alle Zelllinien konnte bereits eine deutliche Steigerung für beide freien Piperazinamide **1** und **3** auf unter 2.1 µM bzw. 12.7 µM beobachtet werden. Allerdings fehlt es diesen Verbindungen an Selektivität. Verbindung **6** zeigt eine Aktivität im gleichen Bereich.

Die Überlegenheit der Verknüpfung über Position 28 wird ebenfalls beim Betrachten der Ergebnisse für die jeweiligen Rhodamin-Hybride deutlich. Während sich die IC_{50}-Werte für **4** im Bereich von 0.8 µM bis 1.1 µM befinden, konnten für **2** Ergebnisse unter 0.1 µM erzielt werden. Im Vergleich zu den freien Piperazinamiden konnte jedoch in beiden Fällen eine ca. 10 bis 25-fache Steigerung der Cytotoxizität beobachtet werden. Eine Steigerung der Selektivität blieb jedoch aus.

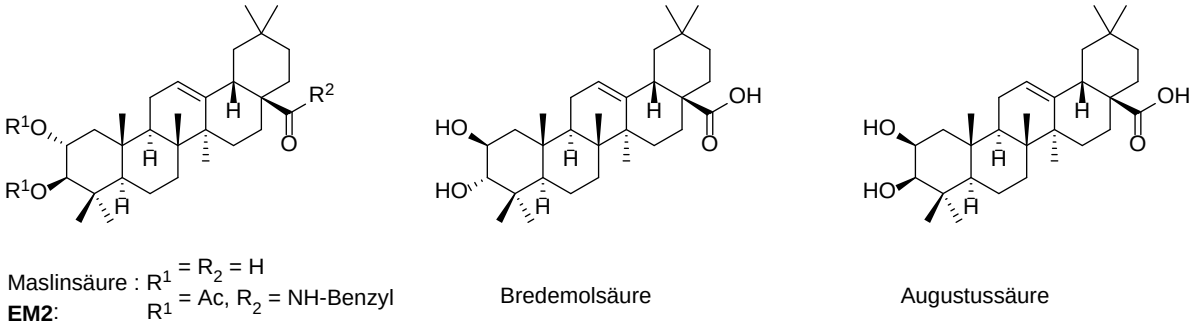
Zwischen den Verbindungen **5** und **7** ist kein signifikanter Unterschied in den IC_{50}-Werten der malignen Zelllinien zu beobachten (1.5 µM – 2.4 µM bzw. 1.1 µM – 2.8 µM), allerdings ist **5** etwas selektiver (IC_{50} [NIH 3T3] = 5.5 µM vs. 2.6 µM).

Dies betont die essentielle Rolle der Verknüpfung des Triterpen-Grundgerüsts mit dem Rhodamin über einen Piperazinspacer an Position 28. Auf diese Weise konnten im nanomolaren Bereich aktive Derivate erhalten werden.

Mittels Annexin-V/PI- Assay, welches mittels Durchflusszytometrie durchgeführt wurde, kann eine Differenzierung von Zellen im nekrotischen, spät apoptotischen, apoptotischen und lebensfähigen Stadium ermöglicht werden. Dadurch lassen sich tiefere Einblicke in den Wirkmechanismus erhalten. Dabei wurde gezeigt, dass nach 48 Stunden, 44,9 % der mit **2** inkubierten A375-Zellen apoptotisch waren, 19,6 % spätapoptotisch und nur 0,9 % nekrotisch.

Untersuchungen zur Stellung der benachbarten Acetylgruppen am A-Ring (P3)

Vergangene Untersuchungen zeigen am Beispiel der Maslinsäure, dass das Einführen einer benachbarten Acetylgruppe an Position 2 im Vergleich zu Oleanolsäure mit einer Steigerung der Antitumoraktivität einhergeht. [38, 61-62] Als mögliche Ursache dafür wird höhere Bioverfügbarkeit vermutet. Deshalb sollte es nun Gegenstand der Forschung sein, den Einfluss der Stellungen dieser beiden benachbarten Acetylgruppen zueinander zu untersuchen. Deshalb wurden analog zu **EM2** verschieden substituierte Benzylamide der Maslin-, Bredemol-, und Augustussäure synthetisiert. Die Konfiguration der Hydroxygruppen dieser ist jeweils *2*α, *3*β; *2*β, *3*α bzw. *2*β, *3*β. (Siehe **Abbildung 5**) Die Partialsynthesen ausgehend von kommerziell erhältlicher Oleanolsäure sind literaturbekannt. [63] Zu den Substituenten des eingesetzten Benzylamins gehören jeweils in *ortho*-, *meta*- und *para*-Position: -Chlor, -Fluor, -Methyl und -Methoxy.



Maslinsäure : R^1 = R_2 = H
EM2: R^1 = Ac, R_2 = NH-Benzyl

Bredemolsäure

Augustussäure

Abbildung 5: *Übersicht der verwendeten Ausgangsverbindungen sowie* ***EM2****.*

Mittels SRB-Assay bereits beschriebener Zelllinien konnte die These bestätigt werden, das Derivate mit zwei Acetylgruppen eine höhere Cytotoxizität aufweisen, als vergleichbare Derivate der Oleanolsäure (IC_{50}-Werte meist < 5 µM). Ein einheitlicher Einfluss der Substituenten am Benzolring konnte nicht festgestellt werden. Es wurde gezeigt, dass Amide der Maslinsäure aktiver sind als solche der Augustussäure und diese wiederrum als solche der Bredemolsäure. Mittels Annexin-V/PI- Assay wurde Apoptose als erwarteter Hauptmechanismus bekräftigt (siehe **Abbildung 6**).

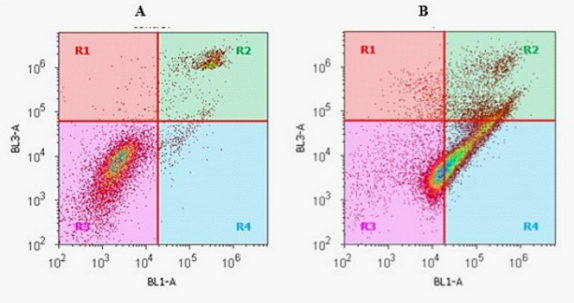


Abbildung 6: *Ergebnisse des durchflusszytometr. Annexin-V/PI- Assays nach 24h mit A375 Zellen; A: Kontrolle; B: Inkubation mit aktivstem Benzylamid; R1: nekrotisch; R2: spät apoptotisch; R3: lebend; R4: apoptotisch*

Untersuchungen zur Corosolsäure (P4)

In diesem Teil der Arbeit sollten die bereits erhaltenen Erkenntnisse auf die Corosolsäure, welche über ein Ursangrundgerüst und wie die Maslinsäure über zwei benachbarte Hydroxygruppe in *2*α, *3*β-Stellung verfügt, übertragen werden. In der Natur kommt sie bspw. in den Blättern der Banaba-Pflanze, auch Königinblume genannt,[64] sowie in Apfelschale[65] vor. Obwohl sie aus dem Banabaextrakt vglw. unkompliziert zu erhalten ist und der Anteil bei einigen im Handel erhältlichen Extrakten darin sogar bis zu 20 % beträgt, ist dieser Weg nur bedingt geeignet, da der Import des Extrakts in großen Mengen durchaus mit Hindernissen verbunden ist und auch die Pflanze selbst im europäischen Raum kaum vorkommt. Hier sind im Handel nur fertige Kapseln mit Banabaextrakt geringerer Corosolsäurekonzentration als Nahrungsergänzungsmittel verfügbar, deren einzelne Öffnung und Extraktion nicht zielführend scheint. Aufgrund dessen sollte die von Wen *et al.* [66] vorgeschlagene Synthese mithilfe der Kenntnisse aus der Herstellung der Maslinsäure optimiert werden. Eine Eliminierung der vorhandenen Hydroxygruppe und anschließende Oxidation der erhaltenen Doppelbindungen mit bspw. $KMnO_4$, OsO_4 oder $RuCl_3/NaIO_4$ erwies sich als ungeeignet, weshalb nach vorheriger Jones-Oxidation eine Hydroxylierung in α-Position mittels *m*CPBA unter sauren Bedingungen durchgeführt wurde. Die erhaltene *2*α-Hydroxy-*3*-Oxoursolsäure konnte mit Natriumborhydrid zur Corosolsäure reduziert werden, wobei nur Spuren der *epi*-Corosolsäure mittels HPTLC-MS detektierbar waren. Eine Zusammenfassung der Synthese ist in **Abbildung 6** zu sehen.

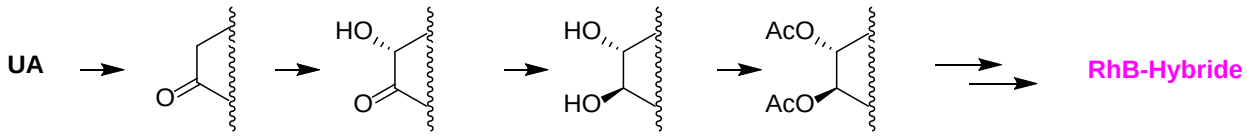
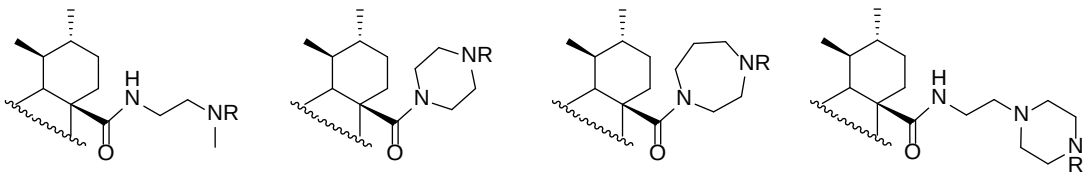


***Abbildung 7**: Überblick über die Synthese der Corosolsäure und weitere Derivatisierungen.*

Für die Synthese der RhB-Hybride wurden vier verschiedene Spacer ausgewählt: Piperazin und das ringvergrößerte Homopiperazin, *1*-(*2*-Aminoethyl)-piperazin als Vergleich für einen zusätzlichen Abstand zum Grundgerüst sowie *N*-Methyl-ethylendiamin. Nicht methyliertes Ethylendiamin ist nicht geeignet, da es aufgrund der freien NH-Gruppe zu einer internen Lactamisierung und somit zum Verlust des essentiellen kationischen Zentrums führt. Die Produkte sind in **Abbildung 8** dargestellt.



R = H	**1**	**2**	**3**	**4**
R = RhB	**5**	**6**	**7**	**8**

***Abbildung 8**: Übersicht über die untersuchten Produkte.*

Anschließend wurde mit den bereits aufgeführten Zelllinien SRB-Assays durchgeführt. Wieder zeigen die gespacerten Carbonsäuren **1** - **4** eine Aktivität im unteren mikromolaren Bereich; Selektivität ist jedoch nicht zu beobachten. Diese konnte neben einer deutlichen Aktivitätssteigerung in den unteren nanomolaren Bereich jedoch für die Rhodamin-Hybride erreicht werden. Am schlechtesten schnitt Verbindung **8** ab (0.05 µM – 0.29 µM) gefolgt von **5** und **6** (0,01 µM – 0.05 µM, Selektivität NIH 3T3/A2780 = 20 bzw. 10) und dem aktivsten Derivat **7** (0,002 µM – 0,008 µM, Selektivität NIH 3T3/A2780 = 60). Somit konnte eine 1400 – fache Aktivitätssteigerung im Vergleich zur acetylierten Corosolsäure und eine 10 – fache Steigerung im Vergleich Piperazin/Homopiperazin erzielt werden.

Es konnte bestätigt werden, dass vicinale Acetylgruppen in *2*α, *3*β-Stellung eine exzellente Ausgangsbedingung für die Synthese cytotoxischer Triterpenderivate darstellen und die dafür geeignetsten Spacer Piperazin und vor allem Homopiperazin sind.

Untersuchungen zu den Grundgerüsten des Triterpens sowie des Rhodamins (P5)

Um einer vollständigen SAR näher zu kommen, galt es nun, den Einfluss verschiedener Triterpen-Grundgerüste sowie des Rhodamins zu beleuchten. Dafür wurden die in **Abbildung 1** bereits dargestellten Carbonsäuren: Betulin- und Platansäure sowie Oleanol- und Ursolsäure gewählt, acetyliert, als Säurechlorid aktiviert und mit Piperazin als auch Homopiperazin umgesetzt. Für die Bildung der Rhodamin-Hybride wurde sich einerseits für das bekannte Rhodamin B, anderseits für das Rhodamin 101 entschieden. Ein Vergleich dieser beiden Strukturen ist **Abbildung 9** zu entnehmen. Man erkennt, dass Rhodamin 101 deutlich polarer (berechn. log $P_{O/W}$ 2.21 vs. 3.96) sowie sterisch anspruchsvoller ist, was es zu einem geeigneten Kandidaten für die geplante Untersuchung machte.

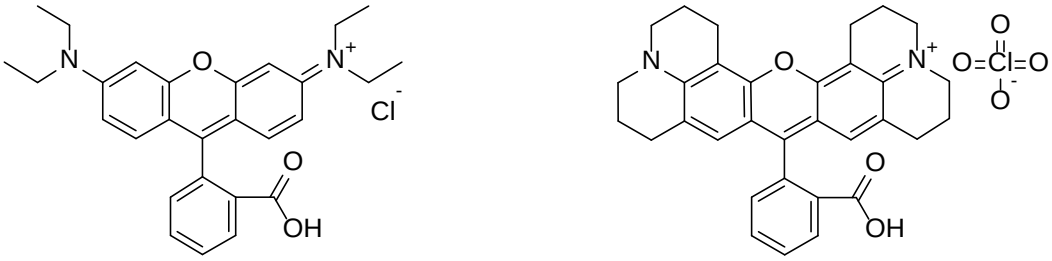


***Abbildung 9**: Vergleich Rhodamin B / Rhodamin 101.*

Vergleicht man nun die Ergebnisse der wieder rum durchgeführten SRB-Assays, lässt sich folgendes feststellen:

Die acetylierten Triterpene weisen bis auf Platansäure (> 30 µM) eine vergleichbar hohe Cytotoxizität bei jedoch fehlender Selektivität auf. (ca. 10-20 µM)

Beim Vergleich der Piperazin- und Homopiperazinamide lässt sich kein Unterschied in der dennoch gesteigerten Aktivität beobachten (ca. 1-3 µM). Lediglich das Homopiperazinderivat der Betulinsäure ist überraschend schlechter (ca. 5-20 µM).

Zwischen den Rhodamin B – Konjugaten lässt sich kaum ein Unterschied zwischen den beiden Spacern feststellen; teilweise sind die Derivate des Homopiperazins inaktiver. Dennoch befinden sich die IC_{50}-Werte aller Derivate im mittleren nanomolaren Bereich. Oleanol- und Ursolsäure sind der Betulin- sowie Platansäure tendenziell zu bevorzugen.

Betrachtet man die Rhodamin 101 – Konjugate, befinden sich die IC_{50}-Werte in der gleichen Größenordnung, die Derivate des Homopiperazins sind in diesem Fall jedoch sichtbar aktiver; Oleanol- und Ursolsäure sind wieder zu bevorzugen. Das aktivste Derivat ist das Homopiperazin-Rhodamin 101 – Konjugat der Ursolsäure mit IC_{50}-Werten bis zu 50 nM (A2780).

Um einen weiteren Einblick in den Wirkmechanismus zu bekommen wurden Färbeversuche mit Acridin Orange, Höchst 33342 sowie der aktivsten Verbindung unternommen. Die mikroskopischen Aufnahmen sind in **Abbildung 10** zu sehen und zeigen die Wirkung als Mitocan.

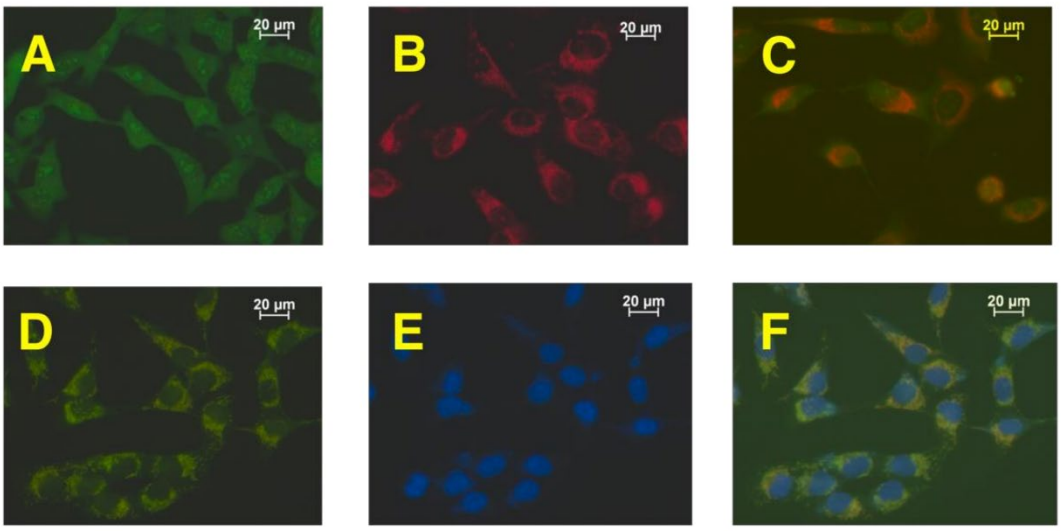


***Abbildung 10**: Färbeversuch der A375-Zellen nach 24 h. A: AO-Färbung, Kontrolle; B: Rh-Hybrid; C: merged A und B; D: AO-Färbung, Kontrolle; E: Höchst 33342-Färbung; F: merged D und E.*

Zusammenfassend lässt sich an dieser Stelle sagen, dass Piperazin und Homopiperazin sehr gut geeignete Spacer darstellen, in vielen Fällen Homopiperazin sogar deutlich besser abschneidet. Die Grundgerüste des Ursan- und Oleantyps sind zu bevorzugen, wobei wenn auch nur leicht, der Ursantyp geeigneter scheint. Für die Wahl des Rhodamins, lässt sich noch keine Empfehlung aussprechen, trotz des vglw. großen strukturellen Unterschiedes im Spektrum der bekannten, einfachen Rhodamine verhalten sich beide sehr ähnlich.

Untersuchungen zum 1,5-Diazacyclooctan-Spacer (P6)

Um an die Erkenntnisse zu den beiden Spacern Piperazin und Homopiperazin anzuknüpfen, war es nun von Interesse die Verwendung des „Bis-Homopiperazins“ zu untersuchen. In der Literatur sind dafür einige Synthesewege wie die Ringöffnung des 1,5-Diaminobicyclo[3.3.0]octans [67], die silica-unterstützte Cyclisierung von 1,3-Diaminopropan bei 350 °C [68] oder dessen Ringschluss mit 1,3-Dibrompropan [69] beschrieben, allerdings verlaufen diese Reaktionen unter drastischen Bedingungen und liefern nur schlechte Ausbeuten vor allem bei höherer Skalierung. Deshalb sollte eine geeignetere Synthesestrategie entwickelt werden.

Da 1,3 bzw. 1,4-Cyclohexandion in großen Mengen kommerziell erhältlich sind, schien es sinnvoll, dieses über das Oxim mittels Beckmann-Umlagerung zum Bislactam umzusetzen und anschließend zu reduzieren. Allerdings gelang die Umlagerung im Gegensatz zu Erfahrungen ausgehend von Cyclohexanon nur in sehr schlechten Ausbeuten.

Als Ausgangsstoffe kommen ebenfalls die kommerziell günstig verfügbaren Verbindungen 1,3-Diaminopropan und Propan-1,3-diol in Frage. Beide konnten erfolgreich tosyliert und anschließend unter basischen Bedingungen (Natriummethanolat) kondensiert werden. Die Abspaltung des Tosylats erfolgte mithilfe von HBr und Thioanisol, und das gewünschte Produkt konnte mit einer Gesamtausbeute von 64 % erhalten werden.

Ebenso lässt sich das Produkt in einer *one-pot*-Reaktion aus 1,3-Dibrompropan mit Hydrazin und Umkristallisation aus Ethanol erhalten. Aufgrund der vielen Nebenreaktionen beträgt die Ausbeute jedoch nur 7,5 %.

Um nun die entsprechenden Rhodamin-Hybride der acetylierten Triterpencarbonsäuren mit dem 1,5-Diazacyclooctan-Spacer zu bilden wurde neben den bekannten Ausgangsmaterialien Ursol-, Oleanol-, Betulin- und Platansäure auch Asiasäure verwendet. Diese leitet sich vom Ursan-Typ ab und besitzt neben den zwei vicinalen Hydroxylgruppen in *2*α, *3*β-Stellung zusätzlich eine Hydroxygruppe an Position 23. (Siehe **Abbildung 11**) Zudem wurde neben Rhodamin B wieder Rhodamin 101 als Vergleich gewählt.

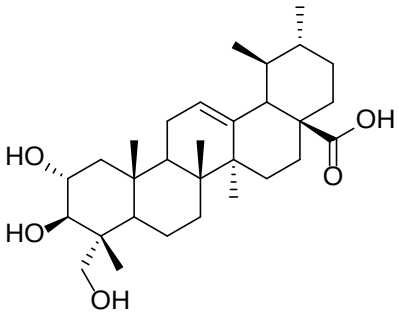


***Abbildung 11**: Struktur der Asiasäure.*

Anschließend wurden mit den Verbindungen SRB-Assays durchgeführt. In diesem Fall wurde allerdings auf andere Ziellinien zurückgegriffen um eine genauere Differenzierung zu ermöglichen. Dabei handelt es sich um vier Brustkrebszellinien: MDA-MB-231, HS578T, MCF-7 und T47D.

Brustkrebs lässt sich in drei molekulare Subtypen einteilen: Der am häufigsten vorkommende luminale A/B-Tumor, welcher immunhistochemisch Hormonrezeptoren aufweist; der HER2-exprimierende Typ, ein häufig sehr aggressiver Tumor; sowie der Basal-Typ, in denen Gene der Zellproliferation vermehrt abgelesen werden. MDA-MB-231 und HS578T sind beides basale, triple-negative Zelllinien, d.h. weder Estrogen- noch Progesteronrezeptoren oder der HER2-Faktor werden gebildet. Aufgrund dessen ist eine zielgerichtete Therapie oft nicht möglich, was mit einer sehr schlechten Prognose für betroffene Patienten einhergeht auch wenn sie sensitiv für Chemotherapie sind. Meist handelt es sich um „high-grade" Tumore, also undifferenziertes bzw. anaplastisches, bösartiges Gewebe. MCF-7 und T47D sind beides luminale A-Typ Zelllinien, welche positiv für Estrogen- und Progesteronrezeptoren sind. Es handelt sich meist um „low-grade" Tumore, das heißt gut differenziertes Gewebe mit hoher Übereinstimmung zum Ursprungsgewebe. Diese sind meist insensitiv für Chemotherapie, reagieren aber gut auf Hormonbehandlung. [70-71]

Vergleicht man die IC_{50}-Werte, so lässt sich feststellen, dass die Derivate der Asiasäure am aktivsten sind, gefolgt von Oleanol- und Ursolsäure. Letzteres ist also in Übereinstimmung mit bisherigen Befunden. Die Ergebnisse der gespacerten Triterpene sind in vergleichbarer Größenordnung wie für die Piperazin- und Homopiperazinanaloga bekannt. Wie erwartet sind für die Rhodaminderivate Aktivitäten im unteren nanomolaren Bereich zu beobachten. Die Rhodamin-Hybride sind generell insensitiv für die HS578 Zelllinie, zeigen höhere Aktivitäten für die beiden luminalen Zelltypen und sind am aktivsten gegenüber der MDA-MB-231 Zelllinie. Die Konjugate des Rhodamins 101 weisen eine tendenziell höhere Cytotoxizität als die des Rhodamins B auf. Das Rhodamin-101-Derivat der Asiasäure konnte sogar erstmals einen IC_{50}-Wert im subnanomolaren Bereich (0,6 nM für MDA-MB-231) liefern.

Der Bis-Homopiperazin-Spacer ist also ein mindestens genauso geeigneter Spacer wie Piperazin und Homopiperazin und kann mit passenden Kopplungspartnern sogar noch bessere Ergebnisse erzielen. Die Analogie: Zwei Acetylgruppen am A-Ring sind besser als nur eine, kann zudem auf die drei Acetylgruppen am Asiasäurederivat ausgeweitet werden.

Die beschriebene aktivste Verbindung sollte nun weiter hinsichtlich der Auswirkung auf Proliferation und Zelltod in der sensitiven MDA-MB-231-Zelllinie und resistenten HS578T-Zelllinie mittels durchflusszytometrischem Annexin-V/Sytox Deep Red Assay untersucht werden. Während geringere Konzentration die Proliferation einschränkten und einen Wachstumsarrest hervorriefen, führten höhere Konzentrationen zur Apoptoseinduktion. Bei den basalen Tumorzellen konnte dieser Wechsel sprunghaft bei 250 nM beobachtet werden, bei der resistenten Linie gab es einen langsamen Abfall der Zellpopulation von 500 nM bis hin zu 2 µM. Dieses unterschiedliche Ansprechverhalten könnte die Selektivität teilweise erklären.

Zudem wurde die subzelluläre Lokalisation mittels mikroskopischer Aufnahmen untersucht. Wie erwartet zeigt die aktivste Verbindung ein identisches Akkumulationsmuster wie der gewählte Farbstoff BioTracker™ 488 Green Mitochondria Dye, was den Wirkmechanismus als Mitocan bestätigt. (siehe **Abbildung 12**)

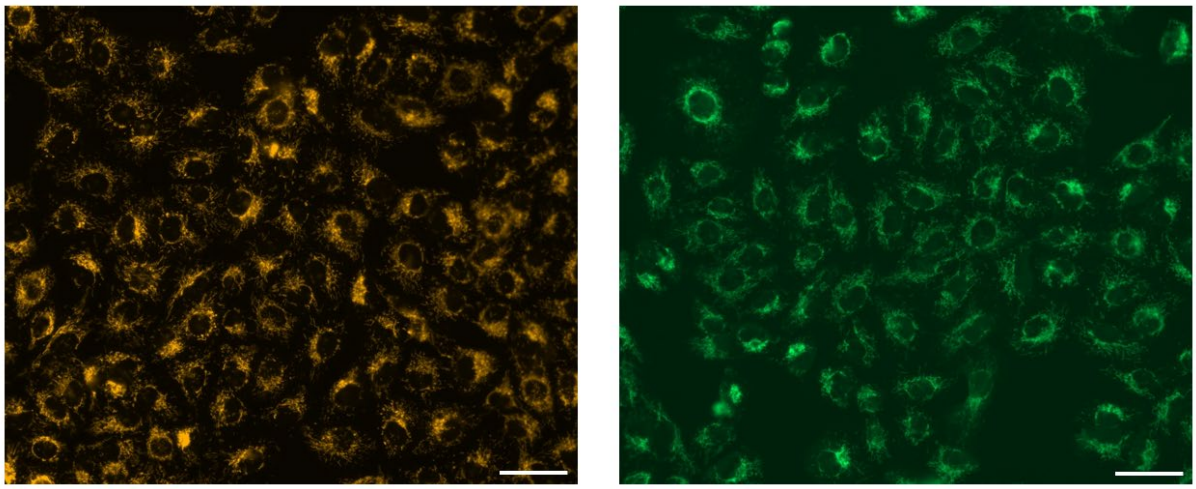


Abbildung 12: *Mikroskopische Aufnahmen; Asiasäure-Rhodamin 101-Hybrid sowie BioTracker™ 488 Green Mitochondria dye, MDA-MC-231 Zellen nach jeweils 6h (100nM) bzw. 30 min (100 nM) beobachtet bei 555 nm/ 592 nm bzw. 475 nm/ 514 nm; scale bar 50 µm.*

Untersuchung weiterer substituierter Rhodamine (P7)

Da die bisherigen Versuche, welche sich lediglich auf Rhodamin B und Rhodamin 101 beschränkten, keine genaue Herleitung einer SAR ermöglichten, sollten nun verschiedene andere Substituenten am Stickstoffatom betrachtet werden, wobei sich für Methyl, *n*-Propyl und *n*-Butyl entschieden wurde.

Diese lassen sich durch die Reaktion des entsprechenden dialkylierten Aminophenols, welches aus der Umsetzung des Alkylhalogenids mit 3-Aminophenol erhalten wird, und Phthalsäureahydrid in der Schmelze in Anwesenheit von kat. Mengen Aluminiumchlorid herstellen. Trotz der relativ drastischen Reaktionsbedingungen lassen sich die Rhodamine nach säulenchromatographischer Aufreinigung mit Ausbeuten von ca. 40% gewinnen. [62] Eine Übersicht der Synthese ist **Abbildung 12** zu entnehmen.

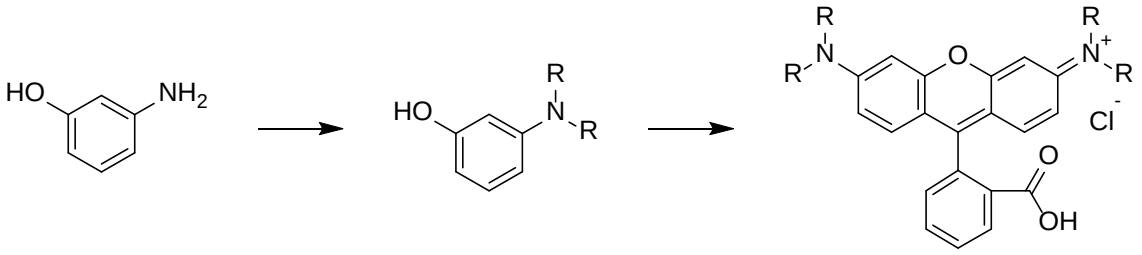


***Abbildung 13**: Übersichtsreaktion zur Synthese der Rhodamine.*

Als exemplarisches Ausgangsmaterial sollte acetylierte Asiasäure dienen, da ihre Derivate in bisherigen SRB-Assays die höchste Aktivität aufwiesen. Die verwendeten Spacer sind Piperazin, Homopiperazin sowie das im letzten Abschnitt beschrieben Bis-Homopiperazin, wodurch auch hier ein weiterer Vergleich ermöglicht wurde.

Anschließend wurden die Rhodamine selbst sowie die Asiasäure-Hybride SRB-Assays mit den klassischen Zelllinien (A375, HT29, MCF7, A2780 sowie NIH 3T3) unterzogen. Während die methyl- und ethylsubstituierten Rhodamine selbst keine Aktivität aufwiesen, erwiesen sich die propyl- und vor allem die butylsubstituierten Varianten im unteren mikromolaren Bereich als cytotoxisch. Rhodamin 101 war dabei vergleichbar mit dem „Propyl-Rhodamin“.

Vergleicht man nun die verschiedenen Spacer der Asiasäure-Rhodamin-Konjugate untereinander, so lässt sich feststellen, dass Homopiperazin dem Piperazin in allen Fällen zu bevorzugen ist, jedoch vergleichbare Werte wie das Bis-Homopiperazin zeigt.

Ein Vergleich der unterschiedlichen Rhodamine lässt keinen einheitlichen Schluss zu. Während in Kombination mit Piperazin das Butyl-Rhodamin am geeignetsten ist, werden mit Homopiperazin und dem klassischen Rhodamin B die besten IC_{50}-Werte erzielt, mit Bis-Homopiperazin jedoch mit dem Propyl-Rhodamin. Mit letzteren wird zudem sogar ein Selektivitätsfaktor von 190 erreicht.

Optimierung der Rhodamin-Konjugate für *in-vivo*-Untersuchungen (P8)

Bisher wurde deutlich gezeigt, welches große Potenzial von Hybridverbindungen aus Rhodaminen als lipophilen Kationen und pentacyclischen Triterpenen, allen voran der Asiasäure, verbunden über einen (Homo)Piperazin, ausgeht. Trotz ihrer Cytotoxizität welche mitunter im subnanomolaren Bereich liegt, sind diese Derivate aufgrund ihrer Absorptions- und Emissionswellenlänge in der UV- sowie sichtbaren Region ungeeignet für *in-vivo*-Untersuchungen. Wie bereits in der Einleitung beschrieben sollten diese dafür in den Nah-Infrarot-Bereich verschoben werden. Vor kurzem wurden sogenannte Changsha-Farbstoffe vorgestellt, welche dem Rhodamin sehr ähnliche Derivate aus Merocyaninen und Benzoesäure sind und über geeignete Fluoreszenzeigenschaften verfügen. [72] Aufgrund der größten Übereinstimmung mit Rhodamin B (MCS = 0.72) war das sogenannte CS-2 für uns von besonderem Interesse. Seine Struktur, so wie der im Folgenden beschriebene Syntheseweg, ist in **Abbildung 13** gezeigt.

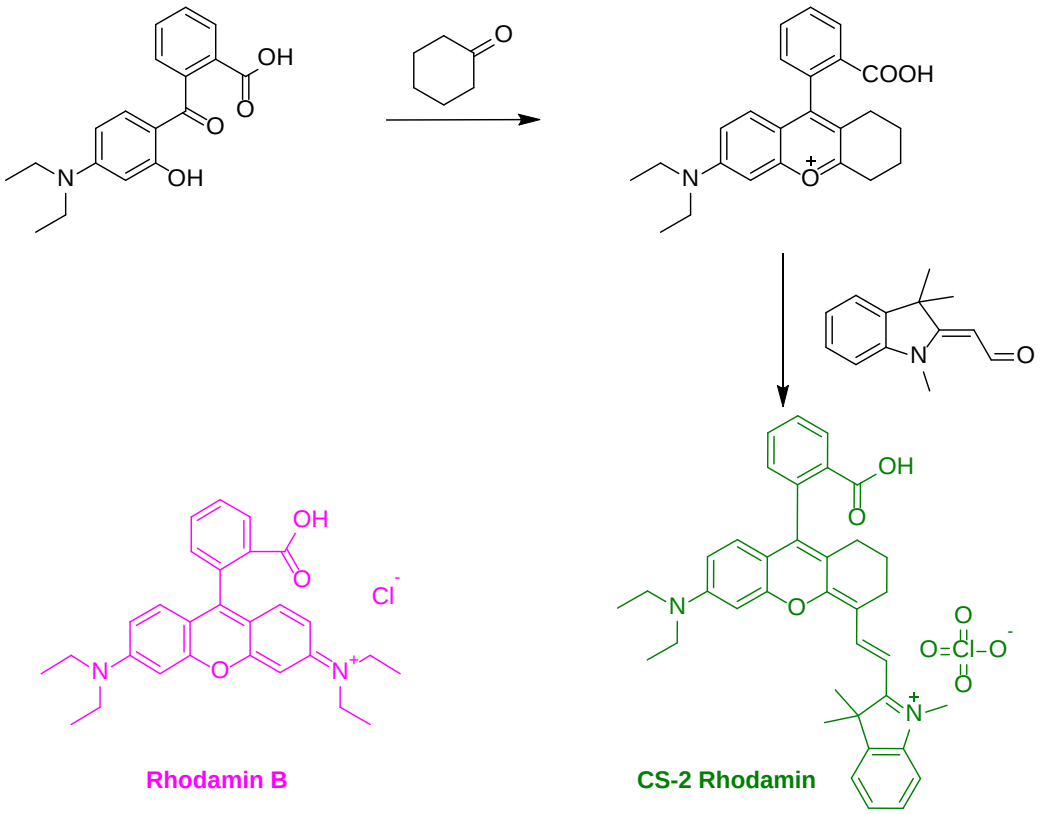


***Abbildung 14**: Syntheseweg für das CS-2-Rhodamin sowie der Vergleich zum Rhodamin B.*

Das gewünschte modifizierte Rhodamin konnte über die Kondensation der kommerziell erhältlichen, substituierten Benzoesäure mit Cyclohexanon und anschließender Umsetzung des Zwischenprodukts mit der entsprechenden Fischer-Base in Acetanhydrid mit einer Gesamtausbeute von ca. 50% erhalten werden.

Anschließend wurden daraus die Hybrid-Verbindungen der acetylierten und gespacerten Triterpene synthetisiert. Dabei fiel die Wahl der Grundgerüste auf Oleanol- sowie Ursolsäure und die von letzterer abgeleitete Reihe Corosol- und Asiasäure. Zudem kamen wieder Piperazin und Homopiperazin zum Einsatz; von Bis-Homopiperazin wurde in diesem Fall abgesehen, da damit bisher keine eindeutige Aktivitätssteigerung zu beobachten war.

Um nun deren Eignung für NIR-Experimente zu bestätigen, wurde eine Reihe von Fluoreszenzspektren aller Verbindungen sowie einiger Rhodamin-B-Analoga aufgenommen. Die Anregungswellenlänge verschob sich von ca. 430 nm zu 580 nm, die Emissionswellenlänge von ca. 590 nm zu 760 nm. Ein Vergleich der normierten Emissions-Spektren für die Asiasäure-Derivate ist in **Abbildung 14** dargestellt.

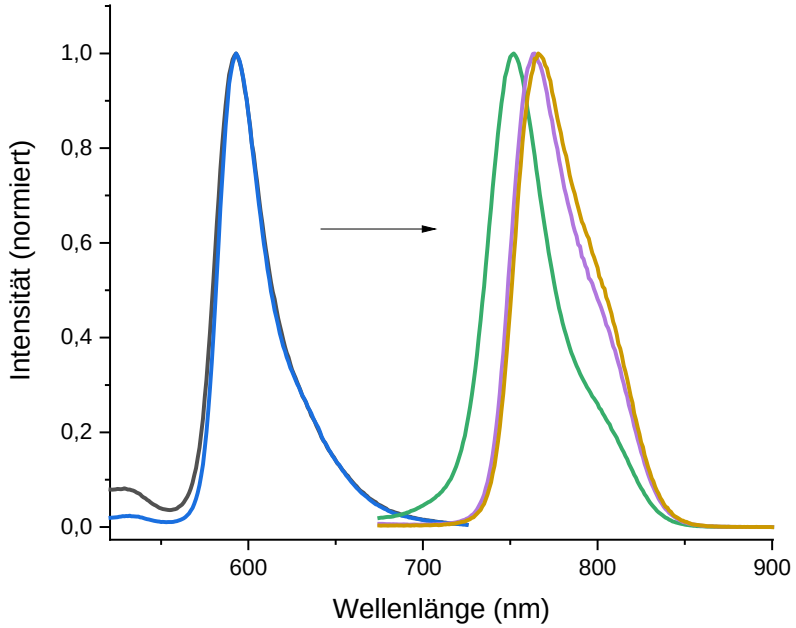


Abbildung 15: *Fluoreszenzemissionsspektren von Rhodamin B (blau) und dem Konjugat mit acet. Asiasäure und Homopiperazin (schwarz) sowie dem CS-2 Rhodamin (orange) und seiner Asiasäure-Konjugate (Piperazin: grün, Homopiperazin: rot).*

Anschließend wurde wieder auf ein SRB-Assay zur Beurteilung der Cytotoxizität zurückgegriffen. Neben klassischen Tumor-Zelllinien wie HT29, MCF-7, A549 (Lungenkarzinom) und A2780 wurden ebenfalls A2780cis-Zellen (eine besonders resistente Form der A2780 Zellen) sowie humane, nonmaligne Fibroblasten (CCD18Co) eingesetzt.

Dabei konnte sehr gut beobachtet werden, wie die Aktivität und Selektivität, welche mit denen bekannter Rhodamin-B-Analoga sehr vergleichbar sind, mit zunehmender Zahl an Acetylgruppen ansteigt. Alle Verbindungen waren am sensitivsten für A2780-Zellen und am resistentesten für MCF7-Zellen bzw. wünschenswerterweise noch stärker resistent gegen die gesunden Fibroblasten. Das aktivste Derivat (Asiasäure + Homopiperazin + CS) lieferte einen IC_{50}-Wert für A2780 von 1,1 nM, für MCF7 von 12.9 nM. Diese Verbindung ist mit einem Selektivitätsfaktor von ca. 60 auch am selektivsten. Die Resistenz der

A2780cis-Zellen konnte mit einer bis zu 5-fachen Effektivität überwunden werden. Mit den in **Abbildung 16** dargestellten mikroskopischen Aufnahmen konnte bestätigt werden, dass sich die CS2-Hybride ebenfalls in den Mitochondrien anreichern und somit trotz ihrer strukturellen Veränderung und verschobenen Fluoreszenzwellenlänge die gleichen biologischen Eigenschaften wie ihre Rhodamin-B-Analoga aufweisen. Somit sind sie für weiterführende Untersuchungen durch ihre verbesserten optischen Eigenschaften besonders geeignet.

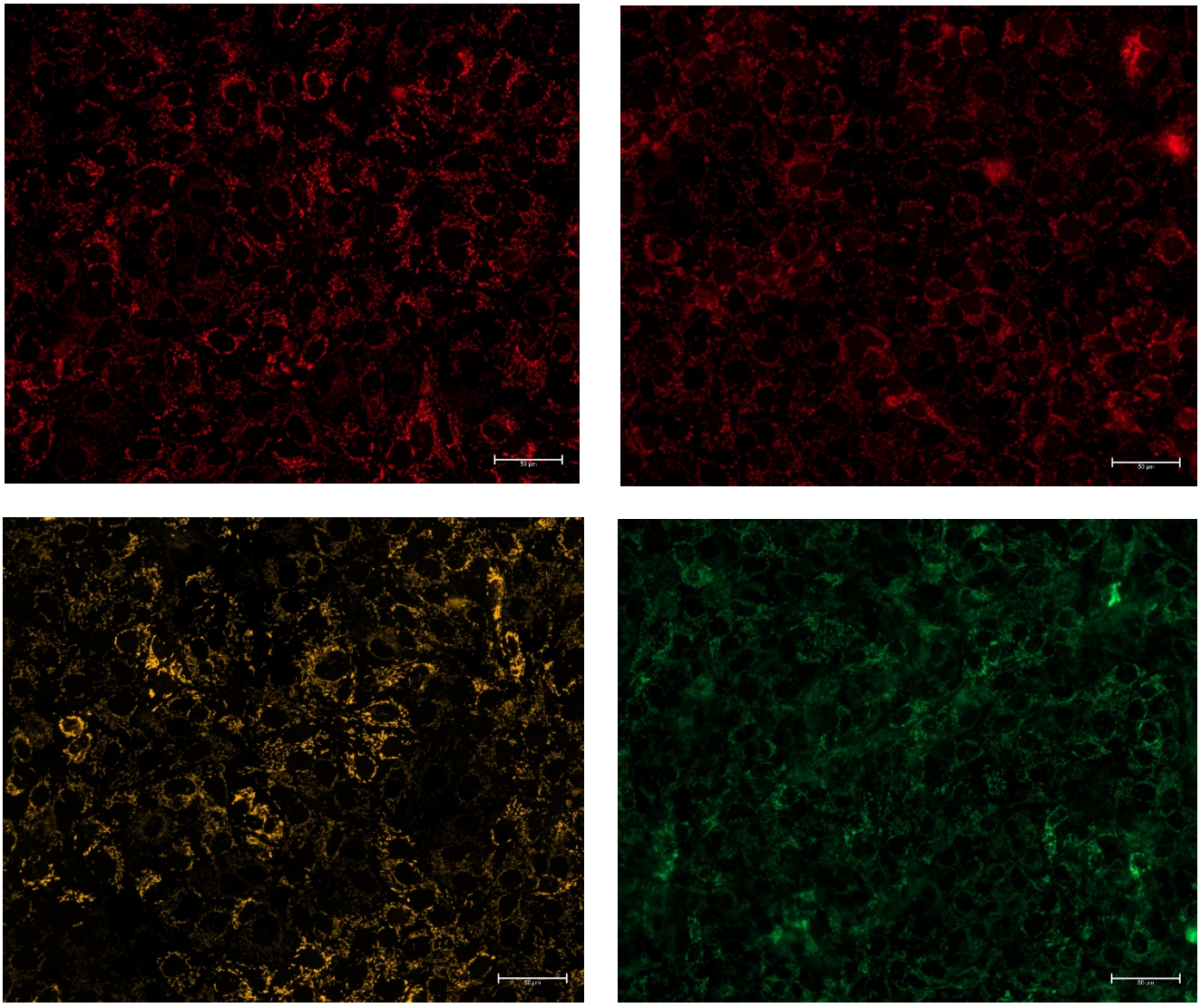


***Abbildung 16**: Mikroskopische Aufnahmen von A549 Zellen mit: Asiasäure-Piperazin/Homopiperazin-CS2-Hybrid oben links bzw. oben rechts, Asiasäure-Homopiperazin-RhB-Hybrid zum Vergleich unten links sowie BioTracker™ 488 Green Mitochondira Dye unten rechts.*

Zusammenfassung und Ausblick

Durch gezielte Modifikationen konnte die Cytotoxizität von Rhodamin-Triterpen-Konjugaten bis hin zu IC_{50}-Werten im subnanomolaren Bereich gesteigert werden und deren Fluoreszenzeigenschaften für die Verwendung von *in-vivo*-Untersuchungen optimiert werden. Dies wurde in zahlreichen biologischen Assays bestätigt als auch näher beleuchtet und lässt nun das Aufstellen einer genauen SAR für die hochaktiven, selektiven sowie resistenzüberwindenen Mitocane zu. Diese wurde in **Abbildung 15** veranschaulicht.

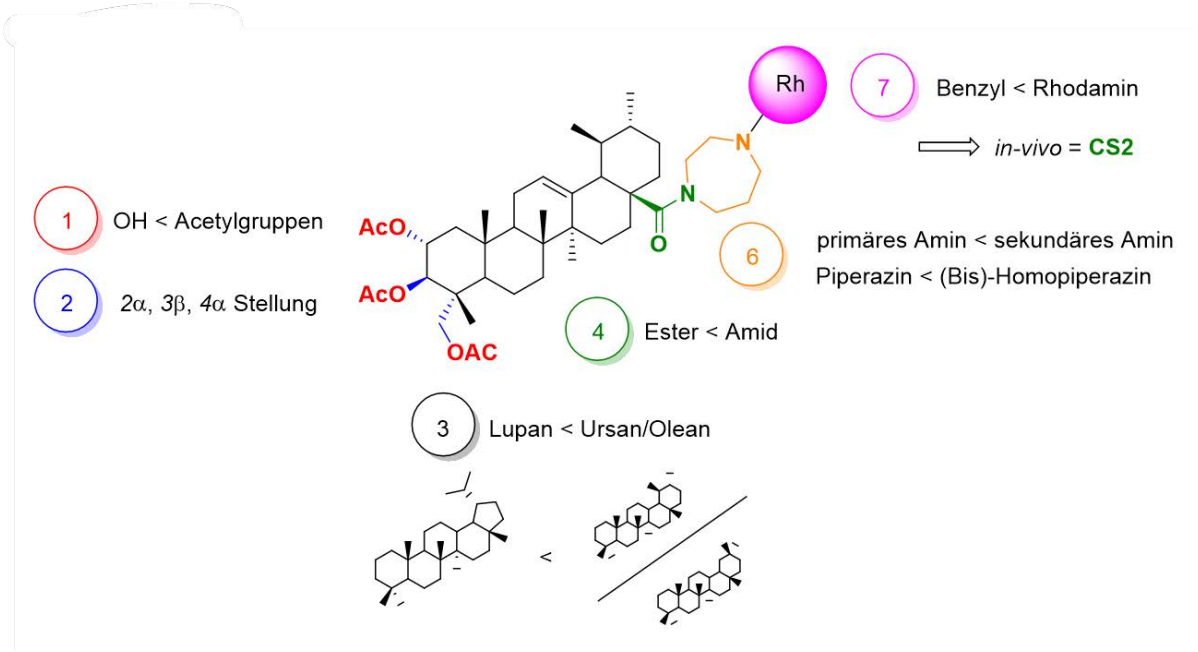


***Abbildung 17**: Zusammenfassung der Struktur-Aktivitäts-Beziehung*

Zukünftig sind detaillierte biologische Experimente bspw. an 3D-Modellen und Tumoren sowie *in-vivo*-Untersuchungen an Mäusen von großem Interesse. Zudem könnten Metabolomics-Studien als auch ein tiefergehendes Molecular Modeling vorgenommen werden, um einen tieferen Einblick auf molekularer Ebene zu erhalten.

Um die Mitocane noch weiter zu optimieren, könnte an ihnen ein weiteres Strukturelement für spezifische Targets, wie beispielsweise überexprimierte Carboanhydrasen in hypoxischen Tumoren, angebracht werden. Des Weiteren könnte sich das Verhalten der mit primären Aminen gespacerten Hybride zu Nutze gemacht werden, um optisch schaltbare Mitocane bspw. für dermale Applikationen zu erhalten. Außerdem sollte an geeigneten Wirkstoff-Carrier-Systemen gearbeitet werden.

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Abbildungsverzeichnis

Anhang

Publikationen

Die dieser Dissertation zugrundeliegenden Publikationen sind nachfolgend aufgelistet:

P1: “Glycyrrhetinic amides and their cytotoxicity”

N. Heise, S. Hoenke, A. Al-Harrasi, H.-P. Deigner, R. Csuk, *Mediterr. J. Chem.* ***2021,*** *11(3), 2*55-263

P2: „Type and position of linkage govern the cytotoxicity of oleanolic acid rhodamine B hybrids”

N. Heise, S. Hoenke, V. Simon, H.-P. Deigner, A. Al-Harrasi, R. Csuk, *Steroids*, **2021,** 172

P3: “Synthesis and cytotoxicity of diastereomeric benzylamides derived from maslinic acid, augustic acid and bredemolic acid”

N. V. Heise, J. Heisig, L. Höhlich, S. Hoenke, R. Csuk, *Results Chem.* ***2023***, 100805

P4: “An improved partial synthesis of corosolic acid and its conversion to highly cytotoxic mitocans”

N. V. Heise, S. Hoenke, I. Serbian, R. Csuk, , Eur. J. Med. Chem. Reports, Volume 6, 2022

P5: “Rhodamine 101 Conjugates of Triterpenoic Amides Are of Comparable Cytotoxicity as Their Rhodamine B Analogs”

N. Heise, D. Major, S. Hoenke, M. Kozubek, I. Serbian, R. Csuk, *Molecules*, **2022**, *27*, 2220

P6: “Mitochondrial targeting 1,5-diazacyclooctane-spacered triterpene rhodamine conjugates exhibit cytotoxicity at sub-nanomolar concentration against breast cancer cells”

N. Heise, S. Becker, T. Müller, A. Güttler, M. Bache, R. Csuk, *Int. J. Mol. Sci.* **2023**, 24(13), 10695

P7: “Developing an amide spacered triterpenoid rhodamine hybrid of nano-molar cytotoxicity combined with excellent tumor cell/non-tumor cell selectivity“

N. Heise, T. Denner, S. Becker, S. Hoenke, R. Csuk, *Heliyon*, **2023**, *Molecules* **2023**, *28*, 6404

P8: “Targeted theranostics: near-infrared triterpenoic acid-rhodamine conjugates as prerequisites for precise cancer diagnosis and therapy”

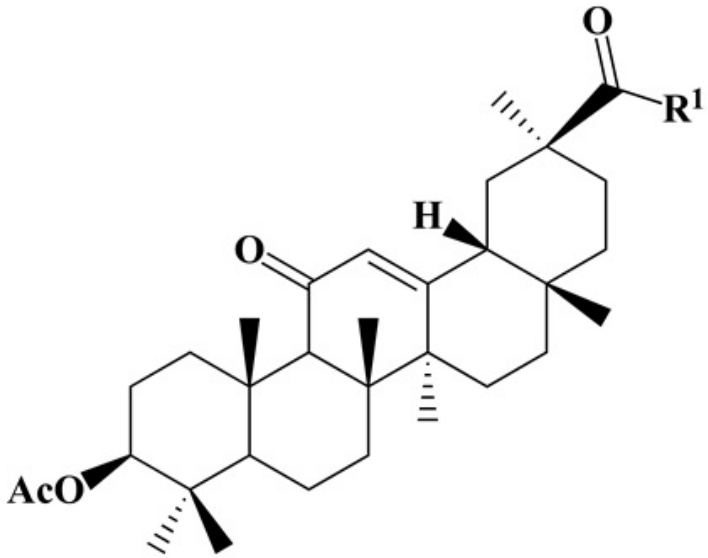
N. Heise, F. Lehmann, R. Csuk, T. Müller, *Eur. J. Med. Chem.* ***2023****, 259, 115663*

Publikation P1

Glycyrrhetinic amides and their cytotoxicity

N. Heise, S. Hoenke, A. Al-Harrasi, H.-P. Deigner, R. Csuk, *Mediterr. J. Chem.* ***2021,*** *11(3),* 255-263

Graphical Abstract



$R^1 = OH$ $EC_{50,\ A2780}$ cells > 30 μM

$R^1 = -NH\text{-}CH_2\text{-}CH_2\text{-}NH_2$, $EC_{50,\ A2780\ cells}$ 2.0 μM

Abstract

3-*O*-Acetyl-glycyrrhetinic amides were prepared, and sulforhodamine B assays investigated their cytotoxicity. Their cytotoxicity strongly depended on the substitution pattern of the respective compounds. Thereby, an ethylenediamine-derived compound **2** performed the best, acting mainly by apoptosis. As far as heterocyclic amides are concerned, ring enlargement and the replacement of the distal nitrogen invariably led to a more or less complete loss of cytotoxic activity. Thus, the presence of a carbonyl function (C-30) seems necessary for providing significant cytotoxicity.

Keywords

Glycyrrhetinic acid, Amides, Cytotoxicity

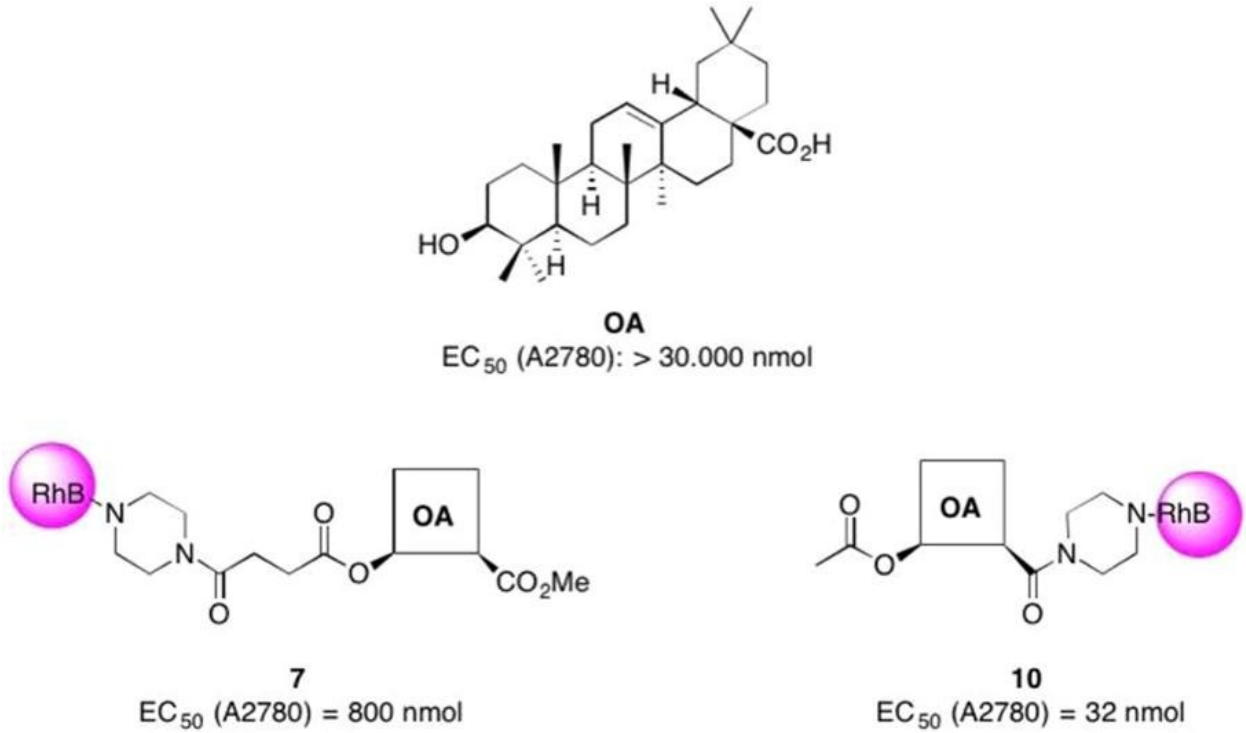
DOI: 10.13171/mjc02110161595Cesuk

Publikation P2

Type and position of linkage govern the cytotoxicity of oleanolic acid rhodamine B hybrids

N. Heise, S. Hoenke, V. Simon, H.-P. Deigner, A. Al-Harrasi, R. Csuk, *Steroids*, **2021,** 172, 108876

Graphical Abstract



Abstract

Oleanolic acid/rhodamine B hybrids exhibit different cytotoxicity depending on the way these two structural elements are linked. While a hybrid holding a piperazinyl spacer at C-28 proved to be cytotoxic in the nano-molar concentration range, hybrids with a direct linkage of the Rho B residue to C-3 of the triterpenoid skeleton are cytotoxic only in the low micro-molar concentration range without any selectivity. This once again underlines the importance of selecting the right spacer and the most appropriate position on the skeleton of the triterpene to achieve the most cytotoxic hybrids possible.

Keywords

Oleanolic acid, Rhodamine B, Hybrids, Cytotoxicity

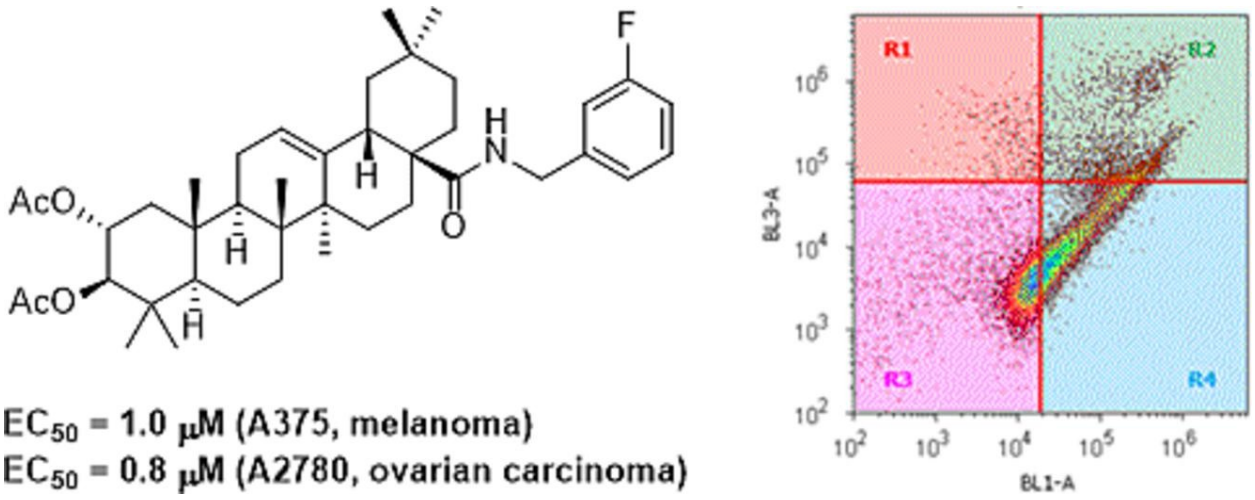
DOI: 10.1016/j.steroids.2021.108876

Publikation P3

Synthesis and cytotoxicity of diastereomeric benzylamides derived from maslinic acid, augustic acid and bredemolic acid

N. V. Heise, J. Heisig, L. Höhlich, S. Hoenke, R. Csuk, *Results Chem.* ***2023***, 5, 100805

Graphical Abstract



Abstract

36 substituted benzylamides were prepared starting from maslinic acid, bredemolic acid, and augustic acid and evaluated for their cytotoxicity in SRB assays employing several human tumor cell lines as well as non-malignant fibroblasts. Thereby, the benzylamides of maslinic acid, however, were found to be more cytotoxic than those obtained from augustic acid or bredemolic acid. The best compound (18, derived from maslinic acid) showed an EC_{50} value of 1.3 µM against A375 melanoma cells. Additional staining experiments revealed that this compound acted rather by apoptosis than by necrosis.

Keywords

Bredemolic acid, Maslinic acid, Augustic acid, Cytotoxicity, Amides

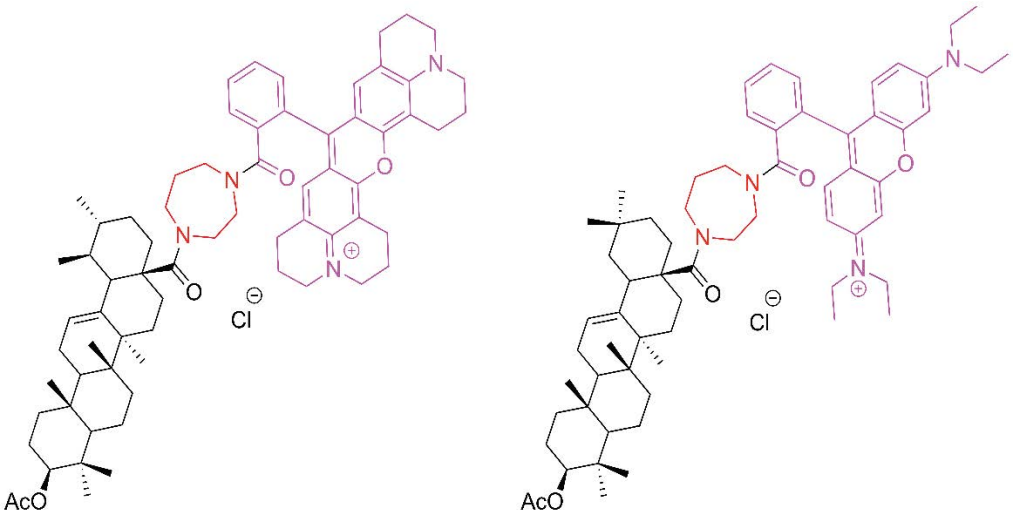
DOI: 10.1016/j.rechem.2023.100805

Publikation P4

Rhodamine 101 Conjugates of Triterpenoic Amides Are of Comparable Cytotoxicity as Their Rhodamine B Analogs

N. Heise, D. Major, S. Hoenke, M. Kozubek, I. Serbian, R. Csuk, *Molecules*, **2022**, *27*, 2220

Graphical Abstract



EC_{50} = 0.05 μM (A2780 ovarian carcinoma)
EC_{50} = 0.06 μM (A375 melanoma)
EC_{50} = 0.08 μM (HT29 colon carcinoma)

EC_{50} = 0.02 μM (A2780 ovarian carcinoma)
EC_{50} = 0.04 μM (A375 melanoma)
EC_{50} = 0.04 μM (HT29 colon carcinoma)

Abstract

Pentacyclic triterpenoic acids (betulinic, oleanolic, ursolic, and platanic acid) were selected and subjected to acetylation followed by the formation of amides derived from either piperazine or homopiperazine. These amides were coupled with either rhodamine B or rhodamine 101. All of these compounds were screened for their cytotoxic activity in SRB assays. As a result, the cytotoxicity of the parent acids was low but increased slightly upon their acetylation while a significant increase in cytotoxicity was observed for piperazinyl and homopiperazinyl amides. A tremendous improvement in cytotoxicity was observed; however, for the rhodamine B and rhodamine 101 conjugates, and compound 27, an ursolic acid derived homopiperazinyl amide holding a rhodamine 101 residue showed an EC_{50} = 0.05 μM for A2780 ovarian cancer cells while being less cytotoxic for non-malignant fibroblasts. To date, the rhodamine 101 derivatives presented here are the first examples of triterpene derivatives holding a rhodamine residue different from rhodamine B.

Keywords

triterpenoic acid; ursolic acid; oleanolic acid; betulinic acid; rhodamine B; rhodamine 101; cytotoxicity

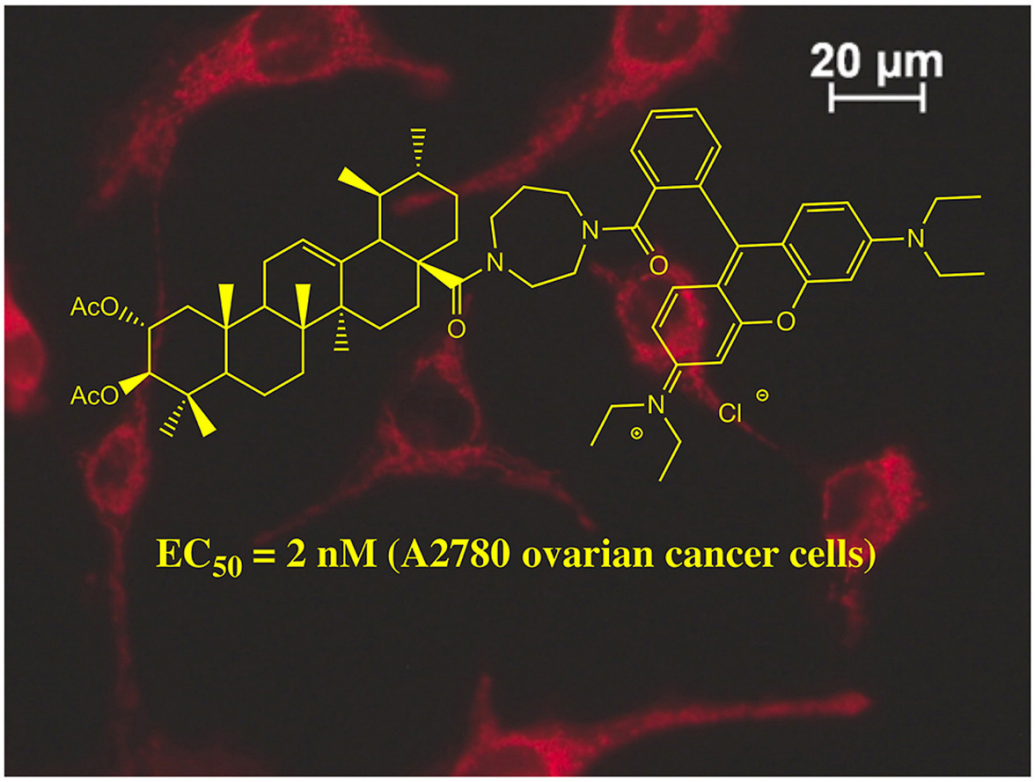
DOI: 10.3390/molecules27072220

Publikation P5

An improved partial synthesis of corosolic acid and its conversion to highly cytotoxic mitocans

N. V. Heise, S. Hoenke, I. Serbian, R. Csuk, *Eur. J. Med. Chem. Reports*, **2022**, 6

Graphical Abstract



Abstract

Ursolic acid was used as a convenient starting material for a three-step partial synthesis of corosolic acid. Corosolic acid was acetylated, and an amide linker was attached followed by a rhodamine B unit at the distal end of the linker. Especially compounds holding a piperazinyl or homopiperazinyl linker held exceptional cytotoxicity for several human tumor cell lines. For example, homopiperazinyl spacered 11 showed EC_{50} = 2 nM for A2780 cells combined with high tumor cell/non-tumor cell selectivity (compound 11: EC_{50} = 0.122 µM for non-malignant NIH 3T3). Thus, compound 11 currently represents one of the most cytotoxic triterpene derivatives holding both superior cytotoxicity combined with high selectivity (SI A2780 vs NIH 3T3 > 60). Staining experiments showed this compound to act as a mitocan. This makes 11 an interesting compound for further studies or as a lead substance.

Keywords

Triterpenes, Corosolic acid, Cytotoxicity, Mitocans

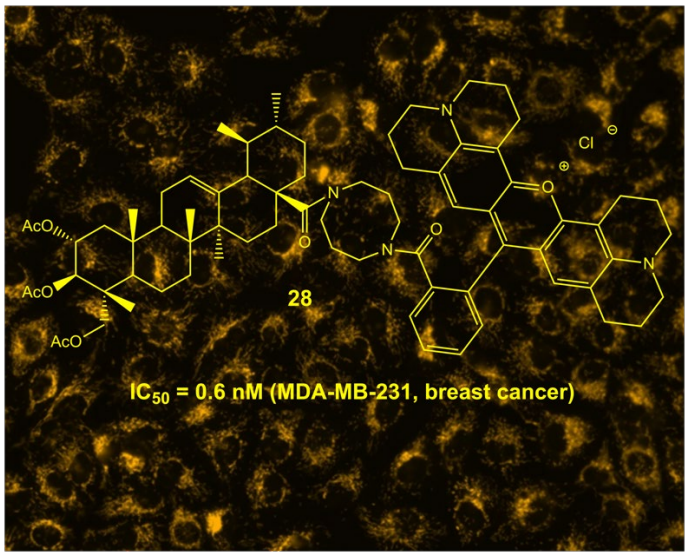
DOI: 10.1016/j.ejmcr.2022.100073

Publikation P6

Mitochondria-targeting 1,5-diazacyclooctane-spacered triterpene rhodamine conjugates exhibit cytotoxicity at sub-nanomolar concentration against breast cancer cells

N. Heise, S. Becker, T. Müller, A. Güttler, M. Bache, R. Csuk, *Int. J. Mol. Sci.* **2023**, 24(13), 10695

Graphical Abstract



Abstract

1,5-Diazacyclooctane was prepared by a simple synthetic sequence and coupled to pentacyclic triterpenoic acids oleanolic acid, ursolic acid, betulinic acid, platanic acid and asiatic acid; these amides were activated with oxalyl chloride and reacted with rhodamine B or rhodamine 101 to yield conjugates. The conjugates were screened in SRB assays with various human breast cancer cell lines (MDA-MB-231, HS578T, MCF-7 andT47D) and found to exert cytotoxic activity even at low concentration. Thereby for an asiatic acid rhodamine 101 conjugate (28) an IC_{50} = 0.60 nM was determined, and to induce apoptosis in MDA-MB-231 and HS578T cells. Extra experiments showed the compound to act as a mitocan and to induce inhibition of proliferation or growth arrest in MDA-MB-231 cells at lower dose followed by an induction of apoptosis at higher doses. Furthermore, differential responses to proliferation inhibition and apoptosis induction may explain differential sensitivity of mammary cell lines to compound 28.

Keywords

asiatic acid; breast cancer; mitocans; rhodamine conjugates; triterpenoic acids

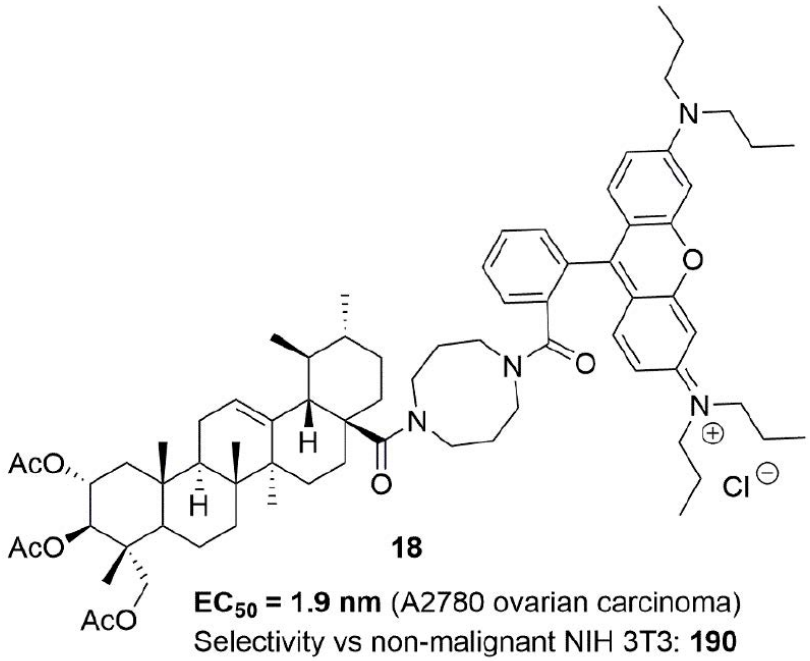
DOI: 10.3390/ijms241310695

Publikation P7

Developing an amide spacered triterpenoid rhodamine hybrid of nano-molar cytotoxicity combined with excellent tumor cell/non-tumor cell selectivity

N. Heise, T. Denner, S. Becker, S. Hoenke, R. Csuk, *Heliyon*, **2023**, *Molecules* **2023**, *28*, 6404

Graphical Abstract



Abstract

Asiatic acid, a pentacyclic triterpene was converted into a series of piperazinyl, homopiperazinyl and 1,5-diazocinyl spacered rhodamine conjugates differing in the type of spacer and the substitution pattern on the rhodamine part of the hybrids. The compounds were tested for cytotoxic activity in SRB assays and compound 12 holding an EC_{50} of 0.8 nM was the most cytotoxic compound of this series, but 18 (holding a ring expanded 1,5-diazocinyl moiety and n-propyl substituents on the rhodamine) was the most selective compound exhibiting a selectivity factor of almost 190 while retaining high cytotoxicity (EC_{50} = 1.9 nM, for A2780 ovarian carcinoma)

Keywords

Asiatic acid, Rhodamine, Cytotoxicity

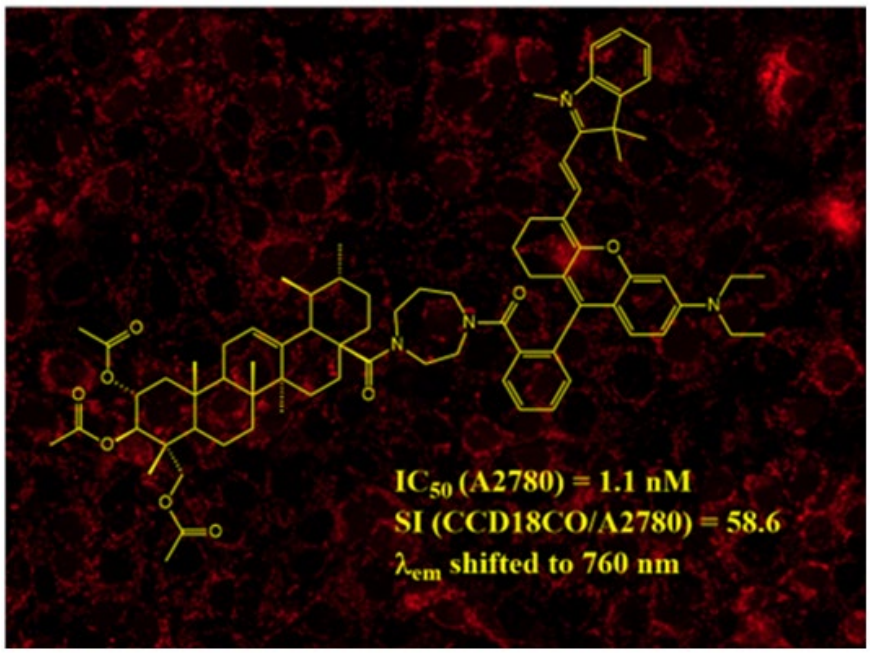
DOI: 10.3390/molecules28176404

Publikation P8

Targeted theranostics: near-infrared triterpenoic acid-rhodamine conjugates as prerequisites for precise cancer diagnosis and therapy

N. Heise, F. Lehmann, R. Csuk, T. Müller, *Eur. J. Med. Chem.* ***2023****, 259, 115663*

Graphical Abstract



Abstract

Pentacyclic triterpenoic acids have shown excellent potential as starting materials for the synthesis of highly cytotoxic agents with significantly reduced toxicity for non-malignant cells. This study focuses on the development of triterpenoic acid-rhodamine conjugates with fluorescence shifted to the near-infrared (NIR) region for theranostic applications in cancer research. Spectral analysis revealed emission wavelengths around λ = 760 nm, enabling stronger signals and deeper tissue penetration. The conjugates were evaluated using SRB assays on tumor cell lines and non-malignant fibroblasts, demonstrating low nanomolar activity and high selectivity, similarly to their known rhodamine B counterparts. Additional staining experiments proved their mode of action as mitocans.

Keywords

asiatic acid; cytotoxicity; rhodamine; NIR-fluorescence; theranostics

DOI: 10.1016/j.ejmech.2023.115663

Erklärung über den Autorenanteil

Publikation 1: Type and position of linkage govern the cytotoxicity of oleanolic acid rhodamine B hybrids

N. Heise, S. Hoenke, V. Simon, H.-P. Deigner, A. Al-Harrasi, R. Csuk

Der Großteil der Synthesen der Verbindungen sowie die Auswertung der spektroskopischen Daten wurde von mir vorgenommen. Die biologische Evaluierung wurde von S. Hoenke durchgeführt. V. Simon stellte einen Teil der Ausgangsmaterialien her. R. Csuk betreute praktische sowie zusammen mit A. Al-Harrasi theoretische Aspekte der Arbeit.

Publikation 2: Glycyrrhetinic amides and their cytotoxicity

Niels Heise, Sophie Hoenke, Ahmed Al-Harrasi, Hans-Peter Deigner, René Csuk

Die Synthese der Verbindungen sowie die Auswertung der spektroskopischen Daten wurde von mir vorgenommen. Die biologische Evaluierung wurde von S. Hoenke durchgeführt. R. Csuk betreute praktische sowiez usammen mit A. Al-Harrasi und H.-P. Deigner theoretische Aspekte der Arbeit.

Publikation 3: Rhodamine 101 Conjugates of Triterpenoic Amides Are of Comparable Cytotoxicity as Their Rhodamine B Analogs

N. Heise, D. Major, S. Hoenke, M. Kozubek, I. Serbian, R. Csuk

Der Großteil der Synthesen der Verbindungen sowie die Auswertung der spektroskopischen Daten wurde von mir vorgenommen. Die biologische Evaluierung wurde von S. Hoenke durchgeführt. D. Major stellte unter der Betreuung von M. Kozubek und I. Serbian einen Teil der Verbindungen her. R. Csuk betreute praktische sowie theoretische Aspekte der Arbeit.

Publikation 4: An improved partial synthesis of corosolic acid and its conversion to highly cytotoxic mitocans

N. V. Heise, S. Hoenke, I. Serbian, R. Csuk

Der Großteil der Synthesen der Verbindungen sowie die Auswertung der spektroskopischen Daten wurde von mir vorgenommen. Die biologische Evaluierung wurde von S. Hoenke durchgeführt. I. Serbian stellte einen Teil der Verbindungen her. R. Csuk betreute praktische sowie theoretische Aspekte der Arbeit.

Publikation 5: Synthesis and cytotoxicity of diastereomeric benzylamides derived from maslinic acid, augustic acid and bredemolic acid

N. V. Heise, J. Heisig, L. Höhlich, S. Hoenke, R. Csuk

Der Großteil der Synthesen der Verbindungen sowie die Auswertung der spektroskopischen Daten wurde von mir vorgenommen. Die biologische Evaluierung wurde von S. Hoenke durchgeführt. J. Heisig sowie L. Höhlich stellten einen Teil der Ausgangsmaterialien her. R. Csuk betreute praktische sowie theoretische Aspekte der Arbeit.

Publikation 6: Mitochondria-targeting 1,5-diazacyclooctane-spacered triterpene rhodamine conjugates exhibit cytotoxicity at sub-nanomolar concentration against breast cancer cells

N. Heise, S. Becker, T. Mueller, M. Bache, R. Csuk, A. Güttler

Der Großteil der Synthesen der Verbindungen sowie die Auswertung der spektroskopischen Daten wurde von mir vorgenommen. Die biologische Evaluierung wurde von T. Müller, A. Güttler sowie M. Bache durchgeführt. S. Becker stellte einen Teil der Ausgangsmaterialien her. R. Csuk betreute praktische sowie theoretische Aspekte der Arbeit.

Publikation 7: Developing an amide spacered triterpenoid rhodamine hybrid of nano-molar cytotoxicity combined with excellent tumor cell/non-tumor cell selectivity

N. Heise, T. Denner, S. Becker, S. Hoenke, R. Csuk

Der Großteil der Synthesen der Verbindungen sowie die Auswertung der spektroskopischen Daten wurde von mir vorgenommen. Die biologische Evaluierung wurde von S. Hoenke durchgeführt. T. Denner und S. Becker stellten einen Teil der Ausgangsmaterialien her. R. Csuk betreute praktische sowie theoretische Aspekte der Arbeit.

Publikation 8: Targeted theranostics: near-infrared triterpenoic acid-rhodamine conjugates as prerequisites for precise cancer diagnosis and therapy

Die Synthesen der Verbindungen sowie die Auswertung der spektroskopischen Daten wurde von mir vorgenommen. Die biologische Evaluierung wurde von T. Müller. F. Lehmann unterstütze bei der Aufnahme der Fluoreszenzspektren. R. Csuk betreute praktische sowie theoretische Aspekte der Arbeit.

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Publikationsliste

1. *"Targeted theranostics: near-infrared triterpenoic acid-rhodamine conjugates as prerequisites for precise cancer diagnosis and therapy"*

   N. Heise, F. Lehmann, R. Csuk, T. Müller, *Eur. J. Med. Chem.* **2023**, in print

2. *"The finally rewarding search for a cytotoxic isosteviol derivative"*

   J. Heisig, **N. V. Heise**, S. Hoenke, D. Ströhl, R. Csk, *Molecules*, **2023**, in print

3. *"Developing an amide spacered triterpenoid rhodamine hybrid of nano-molar cytotoxicity combined with excellent tumor cell/non-tumor cell selectivity"*

   N. Heise, T. Denner, S. Becker, S. Hoenke, R. Csuk, *Heliyon*, **2023**, in print

4. *"Mitochondrial targeting 1,5-diazacyclooctane-spacered triterpene rhodamine conjugates exhibit cytotoxicity at sub-nanomolar concentration against breast cancer cells"*

   N. Heise, S. Becker, T. Müller, A. Güttler, M. Bache, R. Csuk, *Int. J. Mol. Sci*. **2023**, in print

5. *"Acetylcholinesterase inhibitory activity of modified lupane, oleanane, and ursane A-seco-triterpenoids"*

   A. Petrova, O. Kazakova, I. S. Nazarov, R. Csuk, **Niels V. Heise**, *Chem. Biodiversity*, **2023**, 20

6. *"Asiatic acid as a leading structure for derivatives combining sub-nanomolar cytotoxicity, high selectivity, and the ability to overcome drug resistance in human preclinical tumor models"*

   O. Kraft, A.-K. Hartmann, S. Brandt, S. Hoenke, **N. V. Heise**, R. Csuk, T. Mueller, *Eur. J. Med. Chem.* **2023**, 250, 115189

7. *"Synthesis and cytotoxicity of diastereomeric benzylamides derived from maslinic acid, augustic acid and bredemolic acid"*

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8. *"α-Glucosidase and cholinesterase inhibiting potential of a series of semisynthetic nitrogen triterpenic derivative"*

   O. Kazakova, I. Smirnova, H. T. T. Nguyen, **N. V. Heise**, S. Hoenke, I. Serbian, R. Csuk, *Med. Chem. Res-*, **2023**, 32, 485-495

9. *"Small Structural Differences Govern the Carbonic Anhydrase II Inhibition Activity of Cytotoxic Triterpene Acetazolamide Conjugates"*

   T. Denner, **N. Heise**, J. Zacharias, O. Kraft, S. Hoenke, R. Csuk, *Molecules*, **2023**, 28, 1009

10. *"Triterpenoid cholinesterase inhibitors that might improve gait disturbances in Parkinson's disease patients"*

    N. V. Heise, J. Schüler, T. E. Orlamünde, B. Brandes, H.-P. Deigner, A. Al-Harrasi, R. Csuk*, Mediterr. J. Chem. **2022**, 12(2), 188-200*

11. *"New amides derived from sclareolide as anticholinesterase agents"*

    J. G. Silva, T. F. Borgati, S. M.G. Lopes, **N. Heise**, S. Hoenke, R. Csuk, L. C.A. Barbosa, *Bioorg. Chem*. **2023,** 130, 106249

12. *"Synthesis and characterization of steroidal, anellated aminothiophenes by Gewald reaction"*

    O. Kraft, G. C Mittag, S. Hoenke, **N. Heise**, A. Al-Harrasi, R. Csuk, *Mediterr. J. Chem. **2022**, 12(2), 140-148*

13. *"Synthesis and In Silico Docking Study towards M-Pro of Novel Heterocyclic Compounds Derived from Pyrazolopyrimidinone as Putative SARS-CoV-2 Inhibitors"*

    M. Horchani, **N. Heise**, R. Csuk, H. B. Jannet, A. H. Harrath, A. Romdhane, *Molecules,* **2022**, *27*, 5303

14. *"An improved partial synthesis of corosolic acid and its conversion to highly cytotoxic mitocans"*

    N. V. Heise, S. Hoenke, I. Serbian, R. Csuk*, Eur. J. Med. Chem. Reports*, **2022**, 6

15. *"Synthesis and Exploitation of the Biological Profile of Novel Guanidino Xylofuranose Derivatives*"

    A. Fortuna, R. Gonçalves-Pereira, P. J. Costa, R. Jorda, V. Vojáčková, G. Gonzalez, **N. V. Heise**, R. Csuk, M. C. Oliveira, N. M. Xavier, *ChemMedChem,* **2022**, *17*

16. *"Rhodamine 101 Conjugates of Triterpenoic Amides Are of Comparable Cytotoxicity as Their Rhodamine B Analogs"*

    N. Heise, D. Major, S. Hoenke, M. Kozubek, I. Serbian, R. Csuk, *Molecules*, **2022**, *27*, 2220

17. *"Glycyrrhetinic amides and their cytotoxicity"*

    N. Heise, S. Hoenke, A. Al-Harrasi, H.-P. Deigner, R. Csuk, *Mediterr. J. Chem.* **2021,** 11(3), 255-263

18. *"N-methylated diazabicyclo[3.2.2]nonane substituted triterpenoic acids are excellent, hyperbolic and selective inhibitors for butyrylcholinesterase"*

    N. Heise, S. Friedrich, V. Temml, D. Schuster, B. Siewert, R. Csuk, , *Eur. J. Med. Chem.* **2022**, 27

19. *"Stable triterpenoid iminium salts and their activity as inhibitors of butyrylcholinesterase"*

    N. V. Heise, D. Ströhl, T. Schmidt, R. Csuk, *J. Mol. Struct.* **2022**, 1249

20. *"Synthesis and In Silico Docking of New Pyrazolo[4,3-e]pyrido[1,2-a]pyrimidine-based Cytotoxic Agents"*

    M. Horchani, **N. V. Heise**, S. Hoenke, R. Csuk, A. H. Harrath, H. B. Jannet, A. Romdhane, *Int. J. Mol. Sci.* **2021**, *22*, 10258

21. *"Concise Synthesis of Both Enantiomers of Pilocarpine"*

    T. Schmidt, **N. Heise**, K. Merzweiler, H.-P. Deigner, A. Al-Harrasi, R. Csuk, *Molecules*, **2021**, *26*, 3676

22. *„Type and position of linkage govern the cytotoxicity of oleanolic acid rhodamine B hybrids"*

    N. Heise, S. Hoenke, V. Simon, H.-P. Deigner, A. Al-Harrasi, R. Csuk, Steroids, **2021,** 172, 108876

23. *"The Presence of a Cyclohexyldiamine Moiety Confers Cytotoxicity to Pentacyclic Triterpenoids"*

    S. Hoenke, M. A. Christoph, S. Friedrich, **N. V. Heise**, B. Brandes; H.-P. Deigner, A. Al-Harrasi, R. Csuk, *Molecules*, **2021**, *26*, 2102

24. *"A simple but unusual rearrangement of an oleanane to a taraxerane-28,14 β –olide"*

    N. V. Heise, B. Siewert, D. Ströhl, S. Hoenke, O. Kazakova, R. Csuk, *Steroids*, **2021,** *72*

25. *"Betulinic acid derived amides are highly cytotoxic, apoptotic and selective"*

    S. Hoenke, **N. V. Heise**, M. Kahnt, H.-P. Deigner, R. Csuk, , *Eur. J. Med. Chem.* **2020**, *207*

26. *“An unprecedented epimerization and annelation reaction of platanic acid amides”*

N. V. Heise, M. Kahnt, C. Wagner, A. Al-Harrasi, R. Csuk, *J. Mol. Struct.* **2020,** *1220*

Posterbeiträge

Novel dehydroabietyl amine substituted bistetrazoles from Ugi-Azide-4CR act as excellent and selective cholinesterase inhibitors

Niels V. Heise, René Csuk

EFMC-ISMC 2022 – International Symposium on Medicinal Chemistry – Nizza, Frankreich, 04.-08. September 2022

First subnanomolarcytotoxic triterpene-rhodamineconjugates and their modification for NIR-fluorescence experiments

Niels V. Heise, Selina Becker, Thomas Müller, Antje Güttler, Matthias Bache, René Csuk

23rd Tetrahedron Symposium 2023 – Göteborg, Schweden, 27.-30. Juni 2023

Selbständigkeitserklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Arbeit selbstständig und nur unter Verwendung der von mir angegebenen Quellen und Hilfsmittel verfasst habe. Die aus den benutzten Werken, wörtlich oder inhaltlich, entnommenen Stellen wurden als solche kenntlich gemacht. Die Arbeit wurde bisher in gleicher oder ähnlicher Form an keiner anderen Universität oder Hochschule zur Erlangung eines akademischen Grades eingereicht.

Halle (Saale), den 22.06.2023 Niels Valentin Heise

Angehangene Publikationen

Im nachfolgenden sind die Publikationen P1 – P8 angefügt.

P1

Available free online at www.medjchem.com

Mediterranean Journal of Chemistry

Mediterranean Journal of Chemistry **2021**, 11(3), 255-263

Glycyrrhetinic amides and their cytotoxicity

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Abstract: 3-*O*-Acetyl-glycyrrhetinic amides were prepared, and sulforhodamine B assays investigated their cytotoxicity. Their cytotoxicity strongly depended on the substitution pattern of the respective compounds. Thereby, an ethylenediamine-derived compound **2** performed the best, acting mainly by apoptosis. As far as heterocyclic amides are concerned, ring enlargement and the replacement of the distal nitrogen invariably led to a more or less complete loss of cytotoxic activity. Thus, the presence of a carbonyl function (C-30) seems necessary for providing significant cytotoxicity.

Keywords: Glycyrrhetinic acid; Amides; Cytotoxicity.

1. Introduction

Cancer remains one of the leading causes of death; as many cancers are extremely poorly treated, there is still a high demand for cytotoxic compounds. Natural products, particularly the pentacyclic triterpenes, have proven to be valuable starting materials for this purpose. Glycyrrhetinic acid (**GA**, Scheme 1) is a pentacyclic triterpenoid being the main component of the extract of licorice roots. Several interesting biological properties have been attributed to parent **GA** [1-10]. Of particular interest seemed that **GA** is only slightly cytotoxic for different human tumor cell lines due to this acting mainly by apoptosis [11-20]. However, although its cytotoxicity is lower than that of betulinic acid, several derivatives have shown promising and even excellent cytotoxic activity recently [11,13,18,19,21].

While there have been numerous studies on the cytotoxic activity of triterpene carboxylic acids such as oleanolic [22-26], ursolic [27-32], maslinic [33-40], or betulinic acid [41-49], the number of publications on glycyrrhetinic acid derivatives is incomparably smaller. This is all the more surprising as this triterpene carboxylic acid is very readily available even in large quantities from a renewable source and hence an ideal starting material for syntheses.

Amides of triterpene carboxylic acids have been shown in the past to be cytotoxic [11,18,19,21-24,26,33,34,37-39], and of special interest are those holding a heterocyclic ring at the distal amide position. Consequently, we became interested in the synthesis of 3-*O*-acetylated glycyrrhetinic acid amides holding heterocyclic moieties differing in the kind of heteroatoms (N, O, S), ring size (acyclic, 6, 7), and the steric demand of the heterocyclic system.

2. Results and Discussion

Acetylation of **GA** (Scheme 1) gave **1** in 91% [50] whose activation by oxalylchloride in the presence of a catalytic amount of dimethylformamide (DMF) followed by the addition of either ethylenediamine, piperazine, homopiperazine, morpholine, thiomorpholine, homomorpholine, homothiomorpholine, 1,4-diazabicyclo[3.2.2] nonane [24], 1,3-diazabicyclo[3.2.2]nonane [24,51] gave amides **2**–**10**; reaction of **9** and **10** with methyl iodide resulted in the formation of the quaternary ammonium iodides **11** and **12**, respectively. For comparison, primary amide **13** was prepared, and the Curtius degradation [52,53] of **1** gave amine **14**.

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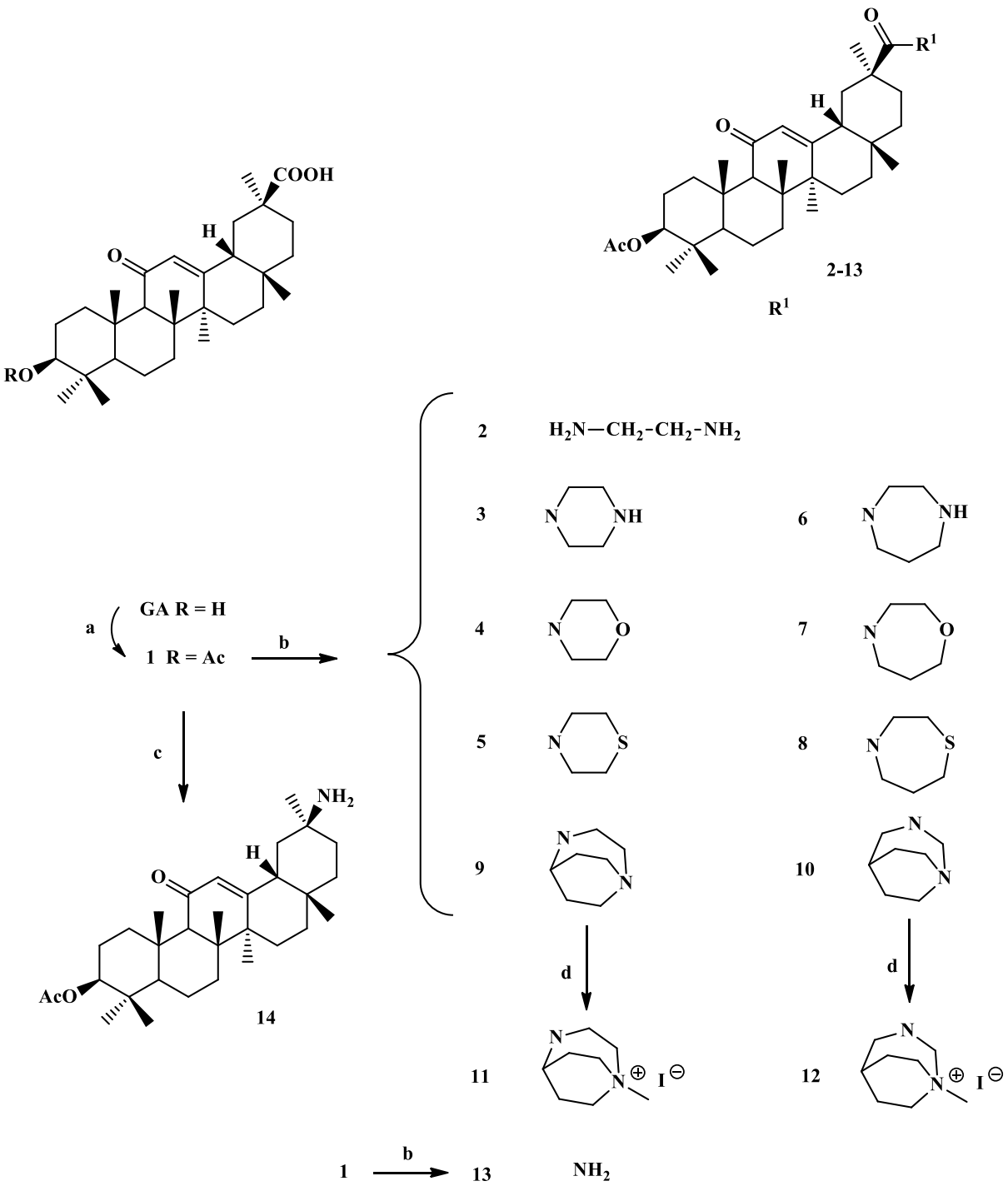

Scheme 1. Reactions and conditions: a) AcCl, NEt_3, DMAP (cat.), DCM, 23°C, 12 h, 91%; b) $(COCl)_2$, DMF (cat.), DCM then: amine, NEt_3, DMAP (cat), DCM, 23°C, 1 d: → **2** (71%), → **3** (64%), → **4** (61%), → **5** (88%), → **6** (67%), → **7** (86%), → **8** (69%),→ **9** (78%), → **10** (99%), → **13** (97%); c) $(COCl)_2$ then NaN_3, AcCN, 23°C, 1 h, then reflux, 12 h, 98%; d) MeI, DCM, 23°C, 1 d, → **10** (50%), → **12** (80%)

To test the cytotoxic activity of the compounds, sulforhodamine B assays were performed employing a selection of different human tumor cell lines [11,22,38,39]. The results of these assays are compiled in Table 1.

Interestingly, compounds piperazine derived **3** [11,54], and morpholine derived compound **4** [18,55-57] are active, while their enlarged ring analogs **6** [55-57] and **7** are not. Also, morpholine-derived **4** was shown to be cytotoxic, while thiomorpholine derived **5** was not active. Diazabiclo-derived compounds **9**–**12** performed poorly in the SRB assays since only **10** held a diminished cytotoxic activity. Amide **13** [52,53,58-60] was not functioning, and amine **14** [61-66] showed EC_{50} values 11.3 and 20.1 µM, respectively.

Table 1. Cytotoxicity of selected compounds [a)].

#	A375	HT29	MCF-7	A2780	FaDu	NIH 3T3
GA	>30	>30	>30	>30	>30	18.7 ± 4.2
1	>30	>30	>30	>30	>30	>30
2	4.1 ± 0.3	4.3 ± 0.4	3.2 ± 0.3	2.0 ± 0.2	5.7 ± 0.6	4.3 ± 0.3
3	5.0 ± 0.3	4.4 ± 0.6	8.4 ± 0.8	8.2 ± 0.5	8.7 ± 0.9	8.7 ± 0.7
4	18.66 ± 1.63	5.11 ± 1.07	10.74 ± 1.00	12.0 ± 0.62	13.4 ± 1.1	12.30 ± 1.02
5	>30	>30	>30	>30	30	>30
6	>30	>30	>30	>30	>30	>30
7	>30	>30	23.4 ± 3.0	22.4 ± 3.9	>30	>30
8	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
9	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
10	21.02 ± 0.4	24.7 ± 1.2	20.3 ± 1.4	19.0 ± 1.1	27.4 ± 2.2	25.5 ± 1.6
11	>30	>30	>30	>30	>30	>30
12	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
13	>30	>30	>30	>30	>30	>30
14	12.4 ± 0.8	17.3 ± 1.0	13.4 ± 0.9	11.3 ± 0.9	19.4 ± 0.9	20.1 ± 0.8
DX	n.d.	0.9±0.2	1.1±0.3	0.02±0.01	n.d.	0.06±0.03

[a] SRB assay EC_{50} values [µM] after 72 h of treatment; averaged from three independent experiments performed each in triplicate; confidence interval CI = 95%. Human cancer cell lines: A375 (melanoma, ATCC CRL_3222), HT29 (colorectal carcinoma, 91072201), MCF-7 (breast adenocarcinoma, CVCL_0031), A2780 (ovarian carcinoma, 93112519), FaDu (pharynx carcinoma, CVCL_1218), NIH 3T3 (non-malignant fibroblasts, ATCC CRL-158); cut-off 30 µM, n.s. not soluble, n.d. not determined. Doxorubicin (**DX**) has been used as a positive standard.

For most active compound **2** [67,68] (EC_{50} 2.0–4.3 µM), several additional assays were performed, e.g., an acridine orange/propidium staining (AO/PI) using A2780 tumor cells. Thereby, a red-colored nucleus indicated necrotic cells while a green fluorescence is indicative for apoptotic cells. Trypan blue staining of the cells followed by automatic cell counting allowed to differentiate between cells with an intact cell membrane and cells without. The results from these assays are compiled in Table 2; parent **GA** and amine **14** were investigated for comparison, too. The compounds show slightly worse cytotoxicity than the positive standard doxorubicin (**DX**). Since no pronounced selectivity was observed, no further experiments with a primary cell line were undertaken.

As a result, parent **GA** and compounds **2** and **14** mainly act by apoptosis after an incubation period of 2 days employing A2780 cells. This parallels previous findings [52] (for **GA** and **14** and A549 cells).

Table 2. Percentage of apoptotic cells (A2780 cells) after 48 h of incubation (at given concentration; 2 x EC_{50}); results from 6-fold determination, trypan blue assay.

	GA	2	15
concentration	60 µM	4 µM	20 µM
% apoptosis	70.1% ± 2.3%	89.5% ± 1.7%	80.4% ± 1.9%

3. Conclusion

The cytotoxicity of 3-*O*-acetyl-glycyrrhetinic amides strongly depends on the substitution pattern of the respective compounds. An ethylenediamine-derived compound **2** performed best, followed by the piperazine derivative **3**. Ring enlargement as well as the replacement of the distal nitrogen led invariably to a more or less complete loss of cytotoxic activity. The presence of a carbonyl function (C-30) seems necessary for providing significant cytotoxicity since amine **14** only held EC_{50} values between 11.3–20.1 µM, respectively. Most active compound **2** ($EC_{50,\ A2780\ cells}$ = 2.0 µM) mainly acted by apoptosis.

Acknowledgments

We like to thank Dr. D. Ströhl, Ms Y. Schiller, and Ms S. Ludwig for the NMR spectra and Ms Th. Schmidt for taking the MS spectra; several MS spectra were recorded by the late Dr. R. Kluge; IR, UV-Vis spectra and optical rotation and microanalyses were measured by Mr M. Schneider. The cell lines were provided by Dr. Th. Müller (Dept. Oncology); some of the biological tests were performed by Dr. L. Fischer. We like to thank Mr S. Friedrich for his help in the lab.

4. Experimental

NMR spectra were recorded using the Varian spectrometers DD2 and VNMRS (400 and 500 MHz, respectively). MS spectra were taken on an Advion expression ^{L} CMS mass spectrometer (positive ion polarity mode, solvent: methanol, solvent flow: 0.2 mL/min, spray voltage: 5.17 kV, source voltage: 77 V, APCI corona discharge: 4.2 µA, capillary temperature: 250°C, capillary voltage: 180 V, sheath gas: N_2). Thin-layer chromatography was performed on pre-coated silica gel plates supplied by Macherey-Nagel. IR spectra were recorded on a Spectrum 1000 FT-IR-spectrometer from Perkin Elmer. The UV/Vis-spectra were recorded on a Lambda 14 spectrometer from Perkin Elmer. The optical rotations were measured either on a JASCO P-2000 or a Perkin-Elmer polarimeter at 20°C. The melting points were determined using the Leica hot stage microscope Galen III and are uncorrected. The solvents were dried according to usual procedures. Glycyrrhetinic acid was bought from "Orgentis Chemicals GmbH" and used as received.

4.1. Cell lines and culture conditions

Following human cancer cell lines A375 (malignant melanoma), HT29 (colon adenocarcinoma), MCF-7 (breast cancer), A2780 (ovarian carcinoma), FaDu (pharynx carcinoma), and non-malignant mouse fibroblasts NIH 3T3 were used. All cell lines were obtained from the Department of Oncology (Martin-Luther-University Halle Wittenberg). Cultures were maintained as monolayers in RPMI 1640 medium with L-glutamine (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich GmbH, Steinheim, Germany) and penicillin/streptomycin (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) at 37°C in a humidified atmosphere with 5% CO_2.

4.2. Cytotoxicity assay (SRB assay)

To evaluate the cytotoxicity of the compounds, the sulforhodamine-B (Kiton-Red S, ABCR GmbH, Karlsruhe, Germany) micro-culture colorimetric assay was used. The assay was carried out as described in the manual of the supplier. The EC_{50} values were averaged from three independent experiments performed in triplicate and calculated from semi-logarithmic dose-response curves applying a non-linear 4P Hills-slope equation.

4.3. Apoptosis test – acridine orange/propidium iodide (AO/PI) test

AO/PI dye and fluorescence microscopy on A2780 cells were performed to test or apoptotic cell death. The assay was carried out as described in the manual of the supplier. In short: Approx. 500000 cells were seeded in cell culture flasks and allowed to grow for 24 hours. After removing the medium, the substance-loaded medium was loaded, and the cells were incubated for 48 hours. The supernatant medium was collected and centrifuged, the pellet was suspended in phosphate-buffered saline (PBS) and centrifuged again. The liquid was removed, the cells re-suspended in PBS, mixed with AO/PI, and investigated using a fluorescence microscope.

4.4. Apoptosis test – trypan blue cell counting

Following the procedure, as described above for the AO/PI test, equal amounts of a trypan blue solution (0.4% in PBS, pH = 7.2) and a suspension of the pellet in PBS were mixed and transferred onto chamber slides (Invitrogen^{TM}), and an automatic cell counter (Invitrogen ^{TM} countess automated cell counter) was used for counting the cells, differing between cells and an intact cell membrane and cells without.

4.5. General procedure for the synthesis of amides 2–10 (GPA)

To a 1 (1 eq.) solution in dry DCM, a drop of dry DMF and oxalyl chloride (4 eq.) were added at 0°C. Stirring at 25°C was continued until the evolution of gases had ceased. The volatiles were removed under reduced pressure. The corresponding amine (3 eq.) was dissolved in dry DCM (20 mL), and a solution of TEA (4.2 eq.), DMAP (cat.) in dry DCM (10 mL), was added. To this mixture, the reaction mixture (dissolved in dry DCM) from above was slowly added at 0°C, and stirring at 23°C was continued for 1 day. Usual aqueous workup followed by liquid column chromatography ($CHCl_3$/MeOH) gave the products **2**–**10**, respectively.

(3β, 20β) 3-Acetyloxy-11-oxoolean-12-en-29-oic acid (1)

Acetylation of **GA** as previously described [50] gave **2** (4.9 g, 91%) as a colorless solid; m.p. 311–313°C (lit.:[50] 310–313°C); $[\alpha]_D^{20}$= +162.7° (*c* 0.85, $CHCl_3$) [lit.:[50] $[\alpha]_D^{20}$= +163.3° (*c* 1.00, $CHCl_3$)];
MS (ESI, MeOH): *m/z* 514 (100%, $[M+H]^+$, 536 (60%, $[M+Na]^+$.

(3β, 20β) 3-Acetyloxy-N-(2-aminoethyl)-11-oxoolean-12-en-29-amide (2)

Following GPA from **1** and ethylenediamine, **2** (398 mg, 71%) [11,67,68] was obtained as a colorless solid; m.p. 114–117°C (lit.:[11] 126°C); $[\alpha]_D^{20}$= +81.2°

(*c* 0.53 MeOH) [lit.:[11] $[\alpha]_D^{20}$= +82° (*c* 0.37, MeOH)]; MS (ESI, MeOH): *m/z* 555 (100%, $[M+Na]^+$.

(3β, 20β) 3-Acetyloxy-30-(1-piperazinyl)-olean-11,29-dione (3)

Following GPA from **2** and piperazine, **3** (364 mg, 64%) was obtained as a colorless solid; m.p. 158–160°C (lit.:[11] 160°C); $[\alpha]_D^{20}$= +123.8° (*c* 0.46 MeOH) [lit.:[11] $[\alpha]_D^{20}$= +120.6° (*c* 0.29, MeOH)]; MS (ESI, MeOH): *m/z* 581 (100%, $[M+H]^+$.

(3β, 20β) 3-Acetyloxy-30-(1-homopiperazinyl)-olean-11,29-dione (4)

Following GPA from **2** and homopiperazine, **4** (318 mg, 61%) was obtained as a colorless solid; m.p. 262–265°C (lit.:[18] 260–264°C); $[\alpha]_D^{20}$= +104.2° (*c* 0.66 $CHCl_3$) [lit.:[18] $[\alpha]_D^{20}$= 109.8° (*c* 0.38, $CHCl_3$)]; MS (ESI, MeOH): *m/z* 596 (100%, $[M+H]^+$.

(3β, 20β) 3-Acetyloxy-30-(morpholinyl)-olean-11,29-dione (5)

Following GPA from **2** (400 mg, 0.8 mmol) and morpholine (0.26 mL, 3.0 mmol), **5** (360 mg, 88%) was obtained as a colorless solid; m.p. 162–165°C; R_F = 0.36 (SiO_2, toluene/EtOAc/heptane/HCOOH, 80:26:10:5); $[\alpha]_D^{20}$= +106.8° (*c* 0.175, $CHCl_3$); UV-Vis ($CHCl_3$): λ_{max} (log ε) = 249.2 nm (4.00);
IR (ATR): ν = 2951*w*, 1729*m*, 1631*m*, 1364*w*, 1244*s*, 1118*m*, 1026*s*, 986*m*, 751*s*, 667*w*, 540*w* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 5.68 (*dd*, *J* = 13.8, 2.7 Hz, 1H, 12-H), 4.51 (*dd*, *J* = 11.6, 4.7 Hz, 1H, 3-H), 3.71 – 3.55 (*m*, 8H, 33-H, 34-H, 35-H, 36-H), 2.79 (*dt*, *J* = 13.5, 3.6 Hz, 1H, 1-H_a), 2.34 (*s*, 1H, 9-H), 2.28 (*dd*, *J* = 13.6, 3.3 Hz, 1H, 18-H), 2.12 – 1.99 (*m*, 2H, 16-H_a, 21-H_a), 2.04 (s, 3H, 32-H), 1.97 (*dt*, *J* = 13.7, 3.5 Hz, 1H, 19-H_a), 1.83 (*td*, *J* = 13.7, 4.6 Hz, 1H, 15-H_a), 1.77 – 1.23 (*m*, 10H, 2-H, 19-H_b, 7-H_a, 6-H_a, 22-H_a, 6-H_b, 7-H_b, 22-H_b, 21-H_b), 1.35 (*s*, 3H, 27-H), 1.21 (*s*, 3H, 29-H), 1.20 – 1.17 (*m*, 1H, 15-H_b), 1.15 (*s*, 3H, 25-H), 1.11 (*s*, 3H, 26-H), 1.10 – 0.96 (*m*, 2H, 1-H_b, 16-H_b), 0.87 (*s*, 6H, 23-H, 24-H), 0.81 (*s*, 3H, 28-H), 0.78 (*d*, *J* = 2.0 Hz, 1H, 5-H) ppm;
^{13}C NMR (101 MHz, $CDCl_3$): δ = 200.1 (C-11), 174.2 (C-30), 171.1 (C-31), 169.6 (C-13), 128.7 (C-12), 80.8 (C-3), 67.1 (C-34, C-35), 61.9 (C-9), 55.2 (C-5), 48.4 (C-18), 46.1 (C-33, C-36), 45.4 (C-8), 44.0 (C-20), 43.8 (C-19), 43.4 (C-14), 39.0 (C-1), 38.2 (C-4), 37.9 (C-22), 37.1 (C-10), 33.5 (C-21), 32.9 (C-7), 31.9 (C-17), 28.6 (C-28), 28.2 (C-23), 27.1 (C-29), 26.9 (C-16), 26.6 (C-15), 23.7 (C-2), 23.2 (C-27), 21.4 (C-32), 18.8 (C-26), 17.5 (C-6), 16.8 (C-24), 16.6 (C-25) ppm;
MS (ESI, MeOH): *m/z* 582 (100%, $[M+H]^+$), 1164 (58%, $[2M+H]^+$), 612 (22%, $[M+MeOH+H]^+$);
analysis calcd for $C_{36}H_{55}NO_5$ (512.35): C 74.32, H 9.53, N 2.41; found: C 74.01, H 7.85, N 2.14.

(3β, 20β) 3-Acetyloxy-30-(thiomorpholinyl)-olean-11,29-dione (6)

Following GPA from **2** (400 mg, 0.8 mmol) and thiomorpholine (0.3 mL, 3.0 mmol), **6** (390 mg, 67%) was obtained as a colorless solid; m.p. 231–233°C; R_F = 0.36 (SiO_2, toluene/EtOAc/heptane/HCOOH, 80:26:10:5); $[\alpha]_D^{20}$= +117.0° (*c* 0.182, $CHCl_3$); UV-Vis ($CHCl_3$): λ_{max} (log ε) = 248.7 nm (4.13);
IR (ATR): ν = 2949*w*, 1728*m*, 1656*m*, 1630*m*, 1364*w*, 1244*s*, 1160*m*, 1026*m*, 986*w*, 958*m*, 751*s*, 667*w* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 5.70 (*s*, 1H, 12-H), 4.51 (*dd*, *J* = 11.7, 4.8 Hz, 1H, 3-H), 3.87 (*ddt*, *J* = 44.1, 13.8, 5.0 Hz, 4H, 33-H, 36-H), 2.79 (*dt*, *J* = 13.6, 3.6 Hz, 1H, 1-H_a), 2.61 (*t*, *J* = 5.1 Hz, 4H, 34-H, 35-H), 2.34 (*s*, 1H, 9-H), 2.30 (*d*, *J* = 3.2 Hz, 1H, 18-H), 2.04 (*s*, 3H, 32-H), 2.11 – 1.93 (*m*, 3H, 16-H_a, 19-H_a, 21-H_a), 1.82 (*td*, *J* = 13.6, 4.5 Hz, 1H, 15-H_a), 1.77 – 1.24 (*m*, 10H, 2-H, 7-H_a, 19-H_b, 6-H_a, 22-H_a, 7-H_b, 6-H_b, 21-H_b), 1.34 (*s*, 3H, 27-H), 1.21 (*s*, 3H, 29-H), 1.18 (*s*, 1H, 16-H_b), 1.15 (*s*, 3H, 25-H), 1.11 (*s*, 3H, 26-H), 1.09 – 0.96 (*m*, 2H, 1-H_b, 15-H_b), 0.87 (*s*, 6H, 23-H, 24-H), 0.80 (*s*, 3H, 28-H), 0.77 (*d*, *J* = 2.0 Hz, 1H, 5-H) ppm;
^{13}C NMR (101 MHz, $CDCl_3$): δ = 200.0 (C-11), 174.1 (C-30), 171.1 (C-31), 169.5 (C-13), 128.7 (C-12), 80.8 (C-3), 61.8 (C-9), 55.2 (C-5), 48.1 (C-18), 48.1 (C-33, C-36), 45.4 (C-8), 44.3 (C-20), 44.2 (C-19), 43.4 (C-14), 39.0 (C-1), 38.2 (C-4), 38.0 (C-22), 37.1 (C-10), 33.2 (C-21), 32.9 (C-7), 31.9 (C-17), 28.6 (C-28), 28.2 (C-23), 27.8 (C-34, C-35), 27.3 (C-29), 26.9 (C-16), 26.5 (C-15), 23.7 (C-2), 23.2 (C-27), 21.4 (C-32), 18.8 (C-26), 17.5 (C-6), 16.8 (C-24), 16.5 (C-25) ppm;
MS (ESI, MeOH): *m/z* 598 (100%, $[M+H]^+$), 1195 (42%, $[2M+H]^+$);
analysis calcd for $C_{36}H_{55}NO_4S$ (597.39): C 72.32, H 9.27, N 2.34; found: C 72.04, H 9.49, N 2.17.

(3β, 20β) 3-Acetyloxy-30-(homomorpholinyl)-olean-11,29-dione (7)

Following GPA from **2** (400 mg, 0.8 mmol) and homomorpholine (220 mg, 1.6 mmol), **7** (400 mg, 86%) was obtained as a colorless solid; m.p. 130–133°C; R_F = 0.32 (SiO_2, toluene/EtOAc/heptane/HCOOH, 80:26:10:5); $[\alpha]_D^{20}$= +111.7° (*c* 0.188, $CHCl_3$); UV-Vis ($CHCl_3$): λ_{max} (log ε) = 249.3 nm (4.02);
IR (ATR): ν = 2947*m*, 1729*m*, 1656*m*, 1619*m*, 1464*w*, 1365*m*, 1244*s*, 1210*w*, 1126*m*, 1074*m*, 1028*m*, 985*m*, 751*m*, 669*w*, 539*w* cm^{-1};
^1H NMR (500 MHz, $CDCl_3$): δ = 5.70 (*s*, 1H, 12-H), 4.49 (*dd*, *J* = 11.8, 4.7 Hz, 1H, 3-H), 3.80 – 3.51 (*m*, 8H, 33-H, 34-H, 36-H, 38-H), 2.77 (*dt*, *J* = 13.6, 3.6 Hz, 1H, 1-H_a), 2.33 (*s*, 1H, 9-H), 2.30 (*d*, *J* = 3.0 Hz, 1H, 18-H), 2.11 – 1.97 (*m*, 3H, 32-H), 2.02 (*s*, 3H, 16-H_a, 19-H_a, 21-H_a), 1.96 – 1.89 (*m*, 2H, 37-H), 1.81 (*td*, *J* = 13.8, 4.8 Hz, 1H, 15-H_a), 1.74 – 1.36 (*m*, 10H, 2-H, 7-H_a, 19-H_b, 6-H_a, 22-H_a, 7-H_b, 6-H_b, 21-H_b, 22-H_b), 1.33 (*s*, 3H, 27-H), 1.21 (*s*, 3H, 29-H_b), 1.19 – 1.15 (*m*, 1H, 16-H_b), 1.13 (*s*, 3H, 25-H), 1.09 (*s*, 3H, 26-H), 1.07 – 0.96 (*m*, 2H, 1-H_b, 15-H_b), 0.85 (*s*, 6H, 23-H, 24-H), 0.79 (*s*, 3H, 28-H), 0.76 (*d*, *J* = 1.8 Hz, 1H, 5-H) ppm;

^{13}C NMR (126 MHz, $CDCl_3$): δ = 199.9 (C-11), 174.7 (C-30), 170.9 (C-31), 169.5 (C-13), 128.5 (C-12), 80.6 (C-3), 70.7 (C-38), 70.4 (C-34), 61.7 (C-9), 55.0 (C-5), 50.7 (C-36), 48.1 (C-18), 46.9 (C-33), 45.2 (C-8), 44.3 (C-20), 44.1 (C-19), 43.3 (C-14), 38.8 (C-1), 38.0 (C-4), 37.9 (C-22), 36.9 (C-10), 33.3 (C-21), 32.8 (C-7), 31.8 (C-17), 30.4 (C-37), 28.5 (C-28), 28.0 (C-23), 27.2 (C-29), 26.8 (C-16), 26.4 (C-15), 23.5 (C-2), 23.0 (C-27), 21.3 (C-32), 18.7 (C-26), 17.4 (C-6), 16.7 (C-24), 16.4 (C-25) ppm;
MS (ESI, MeOH): *m/z* 596 (100%, $[M]^+$);
analysis calcd for $C_{37}H_{57}NO_5$ (595.42): C 74.58, H 9.64, N 2.35; found: C 74.33, H 9.93, N 1.97.

(3β, 20β) 3-Acetyloxy-30-(1,4-thiazepanylamide)-olean-11,29-dione (8)
Following GPA from **2** (400 mg, 0.8 mmol) and homothiomorpholin (240 mg, 1.6 mmol), **8** (330 g, 69%) was obtained as a colorless solid; m.p. 145–148°C; R_F = 0.41 (SiO_2, toluene/EtOAc/heptane/HCOOH, 80:26:10:5); $[\alpha]_D^{20}$= +104.8° (*c* 0.163, $CHCl_3$); UV-Vis ($CHCl_3$): λ_{max} (log ε) = 249.9 nm (3.98);
IR (ATR): ν = 2948*m*, 2873*w*, 1728*m*, 1656*m*, 1619*s*, 1465*w*, 1406*m*, 1365*m*, 1243*s*, 1210*m*, 1161*w*, 1027*m*, 985*m*, 878*w*, 751*s*, 668*w* cm^{-1};
^{1}H NMR (500 MHz, CDCl3): δ = 5.75 (*s*, 1H, 12-H), 4.51 (*dd*, *J* = 11.7, 4.8 Hz, 1H, 3-H), 3.96 – 3.40 (*m*, 4H, 33-H, 36-H), 2.86 – 2.76 (*m*, 3H, 1-H_a, 34-H), 2.75 – 2.63 (*m*, 2H, 38-H), 2.46 – 2.34 (*m*, 2H, 9-H, 18-H), 2.17 – 1.96 (*m*, 3H, 16-H_a, 19-H_a, 21-H_a), 2.04 (*s*, 3H, 32-H), 1.90 – 1.77 (*m*, 1H, 15-H_a), 1.77 – 1.38 (*m*, 12H, 2-H, 7-H_a, 19-H_b, 6-H_a, 22-H_a, 6-H_b, 7-H_b, 37-H, 21-H_b, 22-H_b), 1.35 (*s*, 3H, 27-H), 1.23 (*s*, 3H, 29-H), 1.19 (*m*, 1H, 16-H_b), 1.15 (*s*, 3H, 25-H), 1.11 (*s*, 3H, 26-H), 1.09 – 0.96 (*m*, 2H, 1-H_b, 15-H_b), 0.87 (*s*, 6H, 23-H, 24-H), 0.81 (*s*, 3H, 28-H), 0.78 (*d*, *J* = 2.0 Hz, 1H, 5-H) ppm;
^{13}C NMR (126 MHz, CDCl3): δ = 200.1 (C-11), 175.0 (C-30), 171.1 (C-31), 169.6 (C-13), 128.8 (C-12), 80.8 (C-3), 61.8 (C-9), 55.2 (C-5), 52.0 (C-36), 48.5 (C-33), 48.2 (C-18), 45.4 (C-8), 44.5 (C-20), 44.5 (C-19), 43.5 (C-14), 39.0 (C-1), 38.2 (C-4), 38.2 (C-22), 37.1 (C-10), 33.3 (C-21), 32.9 (C-7), 32.0 (C-17), 28.7 (C-28), 28.2 (C-23), 27.4 (C-29), 27.0 (C-16), 26.6 (C-15), 23.7 (C-2), 23.2 (C-27), 21.4 (C-32), 18.9 (C-26), 17.5 (C-6), 16.8 (C-24), 16.6 (C-25) ppm;
MS (ESI, MeOH): *m/z* 612 (100%, $[M]^+$);
analysis calcd for $C_{37}H_{57}NO_4S$ (611.4): C 72.62, H 9.39, N 2.29; found: C 72.49, H 9.63, N 1.99.

(3β, 20β) 30-(1,4-Diazabicyclo[3.2.2]non-4-yl)-11,30-dioxoolean-12-en-3-yl acetate (9)
Following GPA from **2** (256 mg, 0.51 mmol) and 1,4-diazabicyclo[3.2.2]nonane (250 mg, 1.24 mmol), **9** (244 mg, 78%) was obtained as a colorless solid; m.p. 275–278°C (lit.: 276–279°C); $[\alpha]_D^{20}$= +29.3° (*c* 0.20, $CHCl_3$) [lit.: $[\alpha]_D^{20}$= +28.8° (*c* 0.15, $CHCl_3$)];
MS (ESI, MeOH): *m/z* = 622 (50%, $[M + H]^+$), 654 (95%, $[M + CH_3OH + H]^+$), 1242 (100%, $[2M + H]^+$).

(3β, 20β) 30-(1,3-Diazabicyclo[3.2.2]non-3-yl)-11,30-dioxoolean-12-en-3-yl acetate (10)
Following GPA from **2** (245 mg, 0.48 mmol) and 1,3-diazabicyclo[3.2.2]nonane (250 mg, 1.24 mmol), **10** (276 mg, 99%) was obtained as a colorless solid; m.p. 156–159°C (lit.: 156–160°C); $[\alpha]_D^{20}$= +85.3° (*c* 0.25, $CHCl_3$) [lit.: $[\alpha]_D^{20}$= +84.6° (*c* 0.11, $CHCl_3$)];
MS (ESI, MeOH): *m/z* = 621.3 (100%, $[M + H]^+$), 622.3 (45%; $[M + 2H]^+$); MS (ESI, MeOH): *m/z* = 619 (80%, $[M-H]^-$), 620 2 (35%, $[M]^-$).

3β-Acetyloxy-30-(1-methyl-4-aza-1-azoniabicyclo[3.2.2]non-4-yl)-11,30-dioxoolean-12-ene iodide (11)
This compound was obtained from **9** (168 mg, 0.27 mmol) and MeI (0.25 mL, 1.12 mmol) as an off-white solid (120 mg, 50%); m.p. 201–204°C (lit.: m.p. 205°C (decomp.)); $[\alpha]_D^{20}$= + 55.0° (*c* 0.15, $CHCl_3$) [lit.: $[\alpha]_D^{20}$= +56.5° (*c* 0.10, $CHCl_3$)];
MS (ESI, MeOH): *m/z* = 635 (100%, $[M]^+$) , 636 (40%, $[M + H]^+$).

(3β)Acetyloxy-30-(1-methyl-3-aza-1-azoniabicyclo [3.2.2]non-3-yl)-11,30-dioxoolean-12-ene iodide (12)
This compound was obtained from **10** (175 mg, 0.28 mmol) and MeI (0.25 mL, 1.12 mmol) as an off-white solid (170 mg, 80%); m.p. 262–266°C (lit.: m.p. 261–266°C (decomp.)); $[\alpha]_D^{20}$= +47.0° (c 0.15, $CHCl_3$) [lit.: $[\alpha]_D^{20}$= +48.3° (*c* 0.161, $CHCl_3$)];
MS (ESI, MeOH): *m/z* = 635 (100%, $[M]^+$).

(3β, 20β) 3-Acetyloxy-11-oxoolean-12-en-29-amide (13)
Following GPA and as previously described as an off-white solid (97%); m.p. 309–312°C (lit.:[52] 312–314°C); $[\alpha]_D^{20}$= +121.3° (*c* 0.4, $CHCl_3$) [lit.:[52] $[\alpha]_D^{20}$= +119.05° (*c* 0.41, $CHCl_3$)];
MS (ESI, MeOH): *m/z* 512 (100%, $[M+H]^+$), 534 (50%, $[M+Na]^+$).

(3β, 20β) 20-Amino-3-acetyloxy-30-norolean-12-en-11-one (14)
Obtained as previously [52, 53] described as a colorless solid (98%); m.p. 231–234°C (lit.: [52] 235–237°C); $[\alpha]_D^{20}$= 80.1° (*c* 0.5, $CHCl_3$) [lit.: [52] $[\alpha]_D^{20}$= 80.5° (*c* 0.63, $CHCl_3$)];
MS (ESI, MeOH): *m/z* 484 (100%, $[M+H]^+$).

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Type and position of linkage govern the cytotoxicity of oleanolic acid rhodamine B hybrids

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ABSTRACT

Oleanolic acid/rhodamine B hybrids exhibit different cytotoxicity depending on the way these two structural elements are linked. While a hybrid holding a piperazinyl spacer at C-28 proved to be cytotoxic in the nano-molar concentration range, hybrids with a direct linkage of the Rho B residue to C-3 of the triterpenoid skeleton are cytotoxic only in the low micro-molar concentration range without any selectivity. This once again underlines the importance of selecting the right spacer and the most appropriate position on the skeleton of the triterpene to achieve the most cytotoxic hybrids possible.

1. Introduction

Cancer affects the lives of numerous people every year; this disease ends fatally for many of them. Thus, despite many advances, state-of-the-art therapy and early diagnosis, the number of people suffering from cancer continues to rise. Whereas in 2008 there were around 12.7 million persons affected, by 2020 the figure had already risen to 19.3 million, and a further increase to 28.4 million persons is forecast for 2040. In 2020 alone, 10 million deaths were recorded. In addition to the personal and professional impact on the concerned persons and their families, the costs of treating the disease are also very high. It is estimated that the total cost of cancer worldwide will exceed in 2030 US$ 450 billion [1,2].

Drug targeting by definition refers to the targeted and selective accumulation or release of a drug at one or more desired sites of action. In cancer therapy, this ultimately reflects the ability to distinguish between malignant and normal cells. An insufficient selectivity is the cause of severe side effects. This inevitably leads to a poor compliance of patients because of facing a reduced quality of life due to the drugs. Asa result, an early discontinuation of therapy takes place.

Extending the original concept of drug-targeting directed at different tissues, a new line of research ("third level drug targeting") [3] has emerged in recent years that applies drug-targeting to subcellular entities and compartments ("organelle specific drug targeting") [3–6]. The focus here is on the endoplasmic reticulum, lysosomes and especially mitochondria.

In recent years, derivatives of pentacyclic triterpenoic acids have been identified as promising and, in some cases, highly cytotoxic compounds, starting with studies on the cytotoxic activity of betulinic acid and melanoma [7]. While betulinic acid causes parallel damage in both mitochondrial and lysosomal compartments thereby inducing autophagy [8], we were able to show for triterpene-derived saphirinium derivatives [9] that the endoplasmic reticulum is the target. Rhodamine B derivatives [10–17], as well as several phosphonium salts [18–20] or F-16 conjugates [21] on the other hand, act as mitocans; their targets are the mitochondria [22–26].

Hereby we could show that EC_{50} values in the low micro or even in the nano-molar concentration range for cytotoxicity on human cancer cell lines were observed depending very strongly on the triterpene scaffold, the spacer and the cationic residue. Thus, derivatives of maslinic acid were found to be more active than those derived from betulinic acid [10,14,17]. Rhodamine B derivatives were more cytotoxic than comparable malachite green derivatives [27], and compounds holding a (homo)-piperazinyl spacer [16] were significantly more effective than those with an ethylenediamine spacer [14,28]. The latter derivatives showed EC_{50} values > 30 μM and thus are usually considered non-cytotoxic.

Thus, the question of the spacer and its linkage is of decisive

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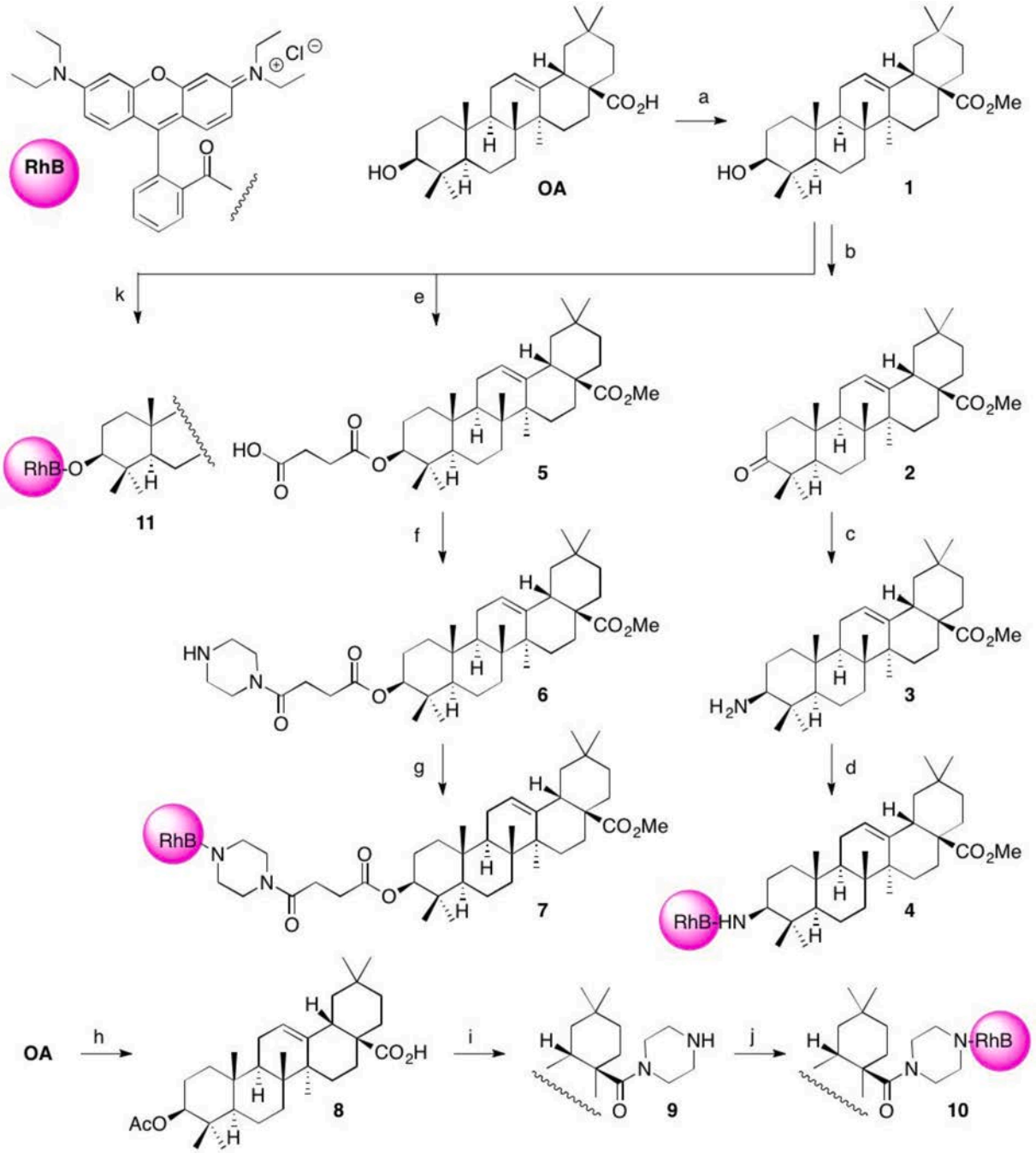
importance. For this comparative study, oleanolic acid (OA) was chosen as the basic structure to access a small library of compounds. OA derivatives had previously been shown to hold good cytotoxicity [29–31]. Furthermore, OA is also readily and commercially available in larger quantities.

2. Results and discussion

Thus, OA was converted into methyl ester 1 (Scheme 1) [32–35], the oxidation of which with freshly prepared Jones reagent afforded ketone 2 in 71% isolated yield [32]. Reductive amination of 2 with ammonium acetate and sodium cyanoborohydride gave the 3β-configured amine 3 [36–40]; the corresponding 3α epimer [36–41] could be determined in trace amounts on TLC and detected by mass spectrometry using HPTLC-ASAP MS but could not be isolated.

Amide 4 was formed in 65% yield from the reaction of rhodamine B (Rho B) in acetonitrile in the presence of EDC and TEA showing an UV absorption λ_{max} = 550 nm being characteristic for the presence of an intact Rho B moiety.

Reaction of 1 with succinic anhydride in the presence of TEA gave chain elongated 5 whose reaction with oxalyl chloride followed by the addition of piperazine furnished 6. The use of a succinyl spacer has been



Scheme 1. Reactions and conditions: a) K_2CO_3, DMF, MeI, 23 °C, 24 h, 77.5%; b) Jones oxidation, 71.3%; c) $(NH_4)_2CO_3$, $NaBH_3CN$, MeOH, 23 °C, 24 h, 65%; d) Rho B, EDC, TEA, 23 °C, 3 d, 65%; e) succinyl anhydride, pyridine, DMAP (cat.), 23 °C, 8 h, 79%; f) $(CO)_2Cl_2$, DMF, then piperazine, DCM, TEA, DMAP, 23 °C, 1d, 94%; g) Rho B, DCM, $(CO)_2Cl_2$, DMF (cat.), 23 °C, 1 h, then 6, TEA, DMAP (cat), 23 °C, 1 d, 75%; h) Ac_2O, DCM, TREA, DMAP (cat), 23 °C, 1 d, 75%; i) $(CO)_2Cl_2$, DMF, then piperazine, DCM, REA, DMAP (cat.), 23 °C, 1 d, 84%; j) Rho B, DCM, $(CO)_2Cl_2$, DMF (cat.), 23 °C, 1 h, the 9, TEA, DMAP (cat.), 23 °C, 1 d, 79%; k) Rho B, DCM, $(CO)_2Cl_2$, DMF (cat.), 23 °C, 1 h, then 1, TEA, DMAP (cat.), 23 °C, 1 d, 41%.

applied very successfully in the past for the synthesis of biologically active triterpene carboxylic acid derivatives. Rho B was activated with oxalyl chloride and allowed to react with 6 to afford 7 as a purple solid showing $\lambda_{max} = 559$ nm.

Acetylation of OA gave well known acetate 3 whose reaction – as described above – yielded piperazinyl amide 9. This compound was coupled with Rho B to afford 10, again as a pink colored solid.

To get an insight onto the influence of an oxygen substituent at position C-3 of the triterpenoid skeleton (as compared to a nitrogen substituent as in 4), compound 1 was coupled with Rho B to yield 11. To evaluate the cytotoxic activity of these compounds, photometric sulforhodamine B assays (SRB) were performed employing several human tumor cell lines as well as non-malignant fibroblasts (NIH 3 T3). The results from these assays are compiled in Table 1.

The results from the SRB assays showed no significant difference between compounds 4 and 11; the cytotoxic effect was independent of whether the RhoB moiety was bound to C-3 via an ester linkage or as an amide. Their EC_{50} values for all tumor cells were low, but these compounds also lacked selectivity. In this series of compounds, 10 performed best. Compounds 7 and 10 showed EC_{50} values in the nano-molar or low micro-molar concentration range. This highlights the importance of the piperazinyl moiety as well as the nature of the attachment of the Rho B residue to the triterpenoid backbone. Compound 10 is thus 25 times more cytotoxic (to A2780 cells) than compound 7 and >1000 times more cytotoxic than parent compound OA.

Molecular modeling calculations, as they have been performed in the past, are of limited value, only. These calculations had shown, using similar compounds as examples, that some mitochondrial enzymes (e.g. NAD(P)H-quinone oxidoreductase or succinate dehydrogenase) could possibly be inhibited; however, the experimental evidence for this is still pending [42]. The cytotoxicity of the compounds could also be caused by changes in the potential of mitochondrial membranes or their ability to increase the concentration of reactive oxygen species. To get a deeper insight, most cytotoxic compound 10 was subjected to flow cytometric measurements (Annexin V/PI assay). Thereby, A375 cells were treated with 2 × EC_{50} concentrations of 10 for 48 h, and the results from these experiments are depicted in Fig. 1.

In Fig. 1, the BL1-A signal corresponds to the FITC signal for annexin V (x-axis); PI was detected at BL3-A (y-axis). As a vonsequence, cells found in R1 areo necrotic cells, those in R2 are late apoptotic, while cells in *R*3 are viable cells, and cells in R4 have died from apoptosis. Thus, from the 48 h incubation of 10, 44.9% of the A375 cells have died by apoptosis and 19.6% by late apoptosis. The number of necrotic cells remained small (0.9%).

Table 1
Cytotoxicity of selected compounds; SRB assay EC_{50} values [μM] after 72 h of treatment; averaged from three independent experiments performed each in triplicate; confidence interval CI = 95%. Human cancer cell lines: A375 (melanoma), HT29 (colorectal carcinoma), MCF-7 (breast adenocarcinoma), A2780 (ovarian carcinoma), NIH 3 T3 (non-malignant fibroblasts); cut-off 30 μM, n.s. not soluble, n.d. not determined. Betulinic acid (BA), oleanolic acid (OA) and doxorubicin (DX) have been used as positive standards.

#	A375	HT29	MCF-7	A2780	NIH 3 T3
OA	>30	>30	>30	>30	>30
Rho B	>30	>30	>30	>30	>30
1	>30	>30	>30	>30	>30
2	3.8 ± 1.0	4.7 ± 0.5	4.5 ± 0.5	3.8 ± 0.2	6.3 ± 0.4
3	2.9 ± 0.3	4.0 ± 0.4	2.9 ± 0.2	2.9 ± 0.5	2.8 ± 0.5
4	1.8 ± 0.1	2.4 ± 0.3	1.5 ± 0.2	1.5 ± 0.1	5.5 ± 0.4
5	n.s.	n.s.	n.s.	n.s.	n.s.
6	9.2 ± 0.6	11.3 ± 0.6	10.7 ± 0.4	12.7 ± 1.3	7.9 ± 0.3
7	1.1 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	1.4 ± 0.1
8	13.0 ± 1.1	20.5 ± 1.7	12.9 ± 1.9	9.4 ± 0.5	17.5 ± 1.5
9	1.9 ± 0.2	1.3 ± 0.1	2.0 ± 0.1	2.1 ± 0.1	2.1 ± 0.1
10	0.06 ± 0.004	0.09 ± 0.01	0.06 ± 0.004	0.032 ± 0.001	0.137 ± 0.006
11	1.6 ± 0.2	2.8 ± 0.4	2.3 ± 0.1	1.1 ± 0.2	2.6 ± 0.2
BA	n.d.	12.7 ± 1.8	18.4 ± 2.0	12.0 ± 1.7	16.1 ± 1.4
DX	n.d.	0.9 ± 0.2	1.1 ± 0.3	0.02 ± 0.01	0.06 ± 0.03

3. Conclusion

In this small study, using OA as the starting material, it was shown that OA Rho B hybrids exhibit different cytotoxicity depending on the way these two structural elements are linked. Hybrids with a direct linkage of the Rho B residue to C–3 of the triterpenoid skeleton (whether as ester or as amide) are cytotoxic in the low micro-molar concentration range but also not selective. In contrast, a hybrid of OA, Rho B and a piperazinyl spacer at C–28 proved to be cytotoxic in the nano-molar concentration range, whereas no increase in cytotoxicity can be observed when binding to C–3 via a succinyl spacer. This once again underlines the importance of selecting the right spacer and the most appropriate position on the skeleton of the triterpene to achieve the most cytotoxic hybrids possible. Compound 10 is significantly more cytotoxic than parent compound OA and acts mainly by apoptosis while the number of necrotic cells remains small.

4. Experimental part

4.1. General

NMR spectra were recorded using the Varian spectrometers DD2 and VNMRS (400 and 500 MHz, respectively, δgiven in ppm, J in Hz; typical experiments: APT ^{13}C, HMBC, HSQC); MS spectra were taken on a Finnigan MAT LCQ 7000 (electrospray, voltage 4.1 kV, sheath gas nitrogen) or an Advion expression^{L} CMS. TLC was performed ion silica gel (Merck 5554, detection with cerium molybdate reagent); melting points are uncorrected (Leica hot stage microscope). IR spectra were recorded on a Perkin Elmer FT-IR spectrometer 1000 or on a Perkin Elmer Spectrum Two (UATR Two unit). The solvents were dried according to usual procedures. Chemicals were obtained from local suppliers; oleanolic acid was bought "Betulinines" (Stříbrná Skalice, Czech Republic) and used as received. SRB assays were performed as previously described [14,34,43].

4.2. Biology

4.2.1. Cell lines and culture conditions

Following human cancer cell lines A375 (malignant melanoma), HT29 (colon adenocarcinoma), MCF-7 (breast cancer), A2780 (ovarian carcinoma), and non-malignant mouse fibroblasts NIH 3 T3 were used. All cell lines were obtained from the Department of Oncology (Martin-Luther-University Halle Wittenberg). Cultures were maintained as monolayers in RPMI 1640 medium with L-glutamine (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich GmbH, Steinheim, Germany) and penicillin/streptomycin (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) at 37 °C in a humidified atmosphere with 5% CO_2.

4.2.2. Cytotoxicity assay (SRB assay)

For the evaluation of the cytotoxicity of the compounds the sulforhodamine-B (Kiton-Red S, ABCR) micro-culture colorimetric assay was used as previously reported. The EC_{50} values were averaged from three independent experiments performed each in triplicate calculated from semi-logarithmic dose–response curves applying a non-linear 4P Hills-slope equation.

4.2.3. Annexin V/PI assay

Approx. 600,000 cells (A375) were seeded in cell culture flasks; they were allowed to grow for 1 day. The medium was removed, and the substance loaded medium was added; incubation lasted for for 48 h. All

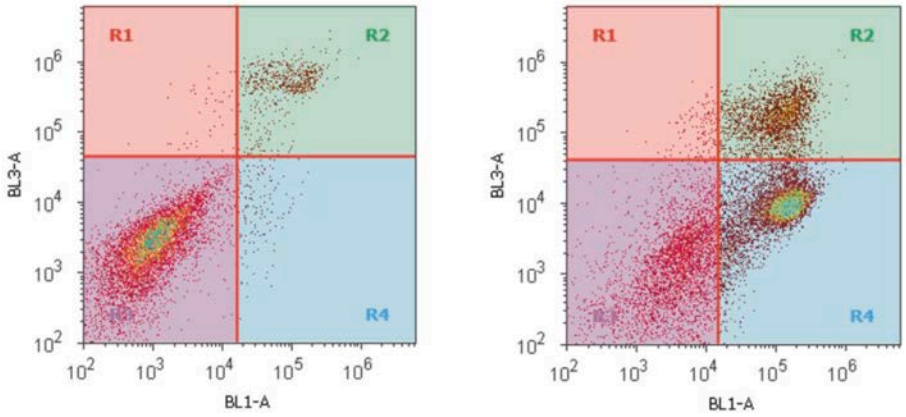


Fig. 1. Annexin V/PI flow cytometry of 10 employing A375 cells (48 h of incubation, 2 × EC_{50} concentration); control experiment (left), incubation with 10 (right).

cells were harvested, centrifuged (1200 rpm, 5 min) and washed twice (PBS (w/w)). Approx. 100,000 cells were washed with annexin V bounding buffer (BD Biosciences®) and treated with a propidium iodide solution (3 μL, 1 mg/mL) and annexin V (5 μL, BD Biosciences®) for 15 min at room temperature in the dark. After adding annexin V bounding buffer (400 μL) the suspension was submitted to a FACS measurement. Calculation was performed as suggested from the supplier (BD Biosciences®).

4.2.4. Synthesess

4.2.4.1. Methyl 3 β -hydroxyolean-12-en-28-oate (1). Oleanolic acid (15.0 g, 32.8 mmol) was dissolved in DMF (200 mL) and potassium carbonate (4.5 g, 32.8 mmol) was added. The reaction mixture was stirred at 23 °C for 30 min. Iodomethane (2.5 mL, 39.7 mmol) was added dropwise, and the mixture was stirred at 23 °C for 24 h. HCl (0.1 m, 65 mL) and water (0.5 L) were added, the precipitate was collected, washed with water (2 × 250 mL) and dried. Recrystallization from ethanol gave 1 as a white solid (11.63 g, 77.5%); m.p. 203 °C (lit.:[44] 200–202 °C); $[\alpha]_D = +66.5°$ (*c* 0.34, $CHCl_3$) [lit.:[44] $[\alpha]_D = 70°$ (*c* 1.0, $CHCl_3$)]; $R_F =$ 0.78 (SiO_2, hexanes/EtOAc, 7:3); UV–Vis ($CHCl_3$): λ_{max} (logε) = 257 nm (3.74) IR (ATR): ν = 3441 s, 2947 m, 1728 m, 1636*w*, 1464*w*, 1386*w*, 1163*w*, 1032*w* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 5.26$ (*m*,1H, 12-H) 3.60 (s, 3H, OMe), 3.19 (dd, 1H, *J* = 11.0, 4.4 Hz, 3-H), 2.84 (dd, 1H, *J* = 13.9, 4.2 Hz, 18-H), 1.95 (ddd, 1H, *J* = 14.5, 14.4, 4.1 Hz, 16-H_a), 1.88–1.82 (*m*, 2H,11-$H_{a,b}$), 1.67 (ddd, 1H, *J* = 13.9, 13.9, 4.4 Hz, 22-H_a), 1.63–1.47 (*m*, 9H, 9-H, 1-H_a, 19-H_a, 6-H_a, 15-H_a, 22-H_b, 16-H_b, 2-H), 1.43–1.22 (*m*, 4H, 21-H_a, 7-H, 6-H_b), 1.19–1.12 (*m*, 2H, 19-H_b, 21-H_b), 1.10 (s, 3H, 27-H), 1.03(dd, 1H, *J* = 14.9, 4.0 Hz, 15-H_b) 0.97–0.92 (*m*, 1H, 1-H_b), 0.96 (s, 3H, 23-H), 0.90 (s, 3H, 30-H), 0.88 (s, 3H, 25-H), 0.87 (s, 3H, 29-H), 0.76 (s, 3H, 24-H), 0.73–0.68 (*m*, 1H, 5-H), 0.70 (s, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): $\delta = 178.2$ (C-28), 143.7 (C-13), 122.3 (C-12), 79.0 (C-3), 55.2 (C-5), 51.5 (OMe), 47.6 (C-9), 46.7 (C-17), 45.8 (C-19), 41.6 (C-14), 41.3 (C-18), 39.2 (C-8), 38.7 (C-4.), 38.4 (C-1), 37.0 (C-10), 33.8 (C-21), 33.1 (C-29), 32.6 (C-7), 32.3 (C-22), 30.6 (C-20), 28.1 (C-23), 27.7 (C-15), 27.1 (C-2), 25.9 (C-27), 23.6 (C-30), 23.4 (C-11), 23.0 (C-16), 18.3 (C-6), 16.8 (C-26), 15.5 (C-24), 15.3 (C-25) ppm; MS (ESI, MeOH): *m/z* 453.1 (68%, $[M + H\text{-}H_2O]^+$), 471.3 (16%, $[M + H]^+$) 493.2 (100%, $[M + H + H_2O]^+$); analysis calcd for $C_{31}H_{50}O_3$ (470.73): C 79.10, H 10.71; found: C 78.85, H 10.97.

4.2.4.2. Methyl 3-oxoolean-12-en-28-oate (2). A solution of 1 (5.61 g, 12.28 mmol) in acetone (300 mL) was heated under reflux for 30 min. After cooling to 0 °C, Jones reagent [prepared from CrO_3 (6.27 g, 62.7 mmol), water (26 mL), and conc. H_2SO_4 (6.23 mL)] was slowly added, and the mixture was stirred for 1 h at 0 °C. Then MeOH (10 mL) was added, and stirring was continued for another 30 min. The solvents were removed under diminished pressure, water was added, and the aqueous phase was extracted with DCM (3 × 25 mL). After evaporation of the DCM, column chromatography (silica gel, hexane/EtOAc, 9:1) furnished 2 as a white solid (5.18 g, 71.3%); m.p. 186 °C (lit.:[45] 184 °C) ; $[\alpha]_D =$ +93.2° (*c* 0.320, $CHCl_3$) [lit.:[46] $[\alpha]_D = 90°$ (*c* 1.2, $CHCl_3$); $R_F = 0.66$ (SiO2, hexanes/EtOAc, 8:2)]; UV–Vis ($CHCl_3$): λ_{max} (log ε) = 310 nm (3.09); IR (ATR): ν = 3432 s, 2941 s, 1726*vs*, 1703 s, 1458 m, 1382*w*, 1364*w*, 1264*w*, 1205 m, 1163 m, 1125*w*, 1040*w*, 1016 m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 5.29$ (*m*, 1H, 12-H), 3.61 (s, 3H, OMe), 2.86 (dd, 1H, *J* = 13.7, 4.3 Hz, 18-H), 2.53 (ddd, 1H, *J* = 16.0, 11.2, 7.3 Hz, 2-H_a), 2.34 (ddd, 1H, *J* = 15.9, 6.7, 3.6 Hz, 2-H_b), 1.99–1.-83 (*m*, 4H, 1-H_a, 16-H_a, 11-H), 1.67 (ddd, 1H, *J* = 14.0, 13.9, 4.6 Hz, 22-H_a), 1.64–1.56 (*m*, 4H, 9-H, 15-H_a, 19-H_a, 16-H_b), 1.53–1.44 (*m*, 4H, 22-H_b, 7-H_a, 6-H), 1.42–1.26 (*m*, 4H, 1-H_b, 21-H_a, 7-H_b, 5-H), 1.20–1.11 (*m*, 2H, 19-H_b, 21-H_b), 1.12 (s, 3H, 27-H), 1.09–1.04 (*m*, 1H, 15-H_b), 1.06 (s, 3H, 23-H), 1.02 (s, 3H, 24-H), 1.02 (s, 3H, 25-H), 0.91 (s, 3H, 30-H), 0.87 (s, 3H, 29-H), 0.76 (s, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, CDCl3): $\delta = 217.6$ (C-3), 178.2 (C-28), 143.8 (C-13), 122.1 (C-12), 55.3 (C-5), 51.5 (OMe), 47.4 (C-9), 46.9 (C-4), 46.7 (C-17), 45.8 (C-19), 41.7 (C-14), 41.4 (C-18), 39.2 (C-8), 39.1 (C-1), 36.7 (C-10), 34.1 (C-2), 33.8 (C-21), 33.1 (C-29), 32.3 (C-7), 32.2 (C-22), 30.7 (C-20), 27.7 (C-15), 26.4 (C-23), 25.8 (C-27), 23.6 (C-30), 23.5 (C-11), 23.0 (C-16), 21.4 (C-24), 19.6 (C-6), 16.7 (C-26), 15.0 (C-25) ppm; MS (ESI, MeOH): *m/z* 469.2 (100%, $[M + H]^+$), 491.0 (32%, $[M + Na]^+$), 408.4 (12%, $[M + Na + H_2O]^+$); analysis calcd for $C_{30}H_{46}O_3$ (454.69): C 79.25, H 10.20; found: C 78.98, H 10.32.

4.2.4.3. Methyl 3 β -aminoolean-12-en-28-oate (3). A suspension of 2 (580 mg, 1.24 mmol)) and ammonium acetate (950 mg, 12.4 mmol) in MeOH (50 mL) stirred at 23 °C for 10 min. A 1 m solution of sodium cyanoborohydride in THF (0.54 mL) was added, and the reaction mixture was stirred at 23 °C for 24 h. Usual aqueous workup and purification by column chromatography (SiO_2, $CHCl_3$/MeOH, 9:1) gave 3 as a white solid (196 mg, 71.4%); m.p. 216–220 °C; $[\alpha]_D = +39.0°$ (*c* 0.134, $CHCl_3$); $R_F = 0.36$ (SiO_2, $CHCl_3$/MeOH, 9:1); IR (ATR): ν = 3413 m, 2946*w*, 1726*w*, 1635 m, 1328 s, 1191*w*, 1040*w*, 824 m cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 5.33–5.23 (*m*, 1H, 12-H), 3.62 (s, 3H, 31-H), 3.20–3.08 (*m*, 1H, 3-H), 2.86 (dd, *J* = 13.7, 4.6 Hz, 1H, 18-H), 2.20–1.17 (*m*, 19H, 16-H, 11-H_a, 7-H, 22-H, 2-H, 11-H_b, 19-H_a, 15-H_a, 6-H, 1-H, 21-H, 9-H), 1.13 (s, 3H, 30-H), 1.09 (s, 3H, 23-H), 1.08–1.03 (*m*, 2H, 15-H_b, 19-H_b), 0.96 (s, 3H, 24-H), 0.93 (s, 6H, 25-H, 26-H), 0.88 (s, 3H, 29-H), 0.81–0.76 (*m*, 1H, 5-H), 0.72 (s, 3H; 27-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$) $\delta = 178.5$ (C-28), 144.1 (C-13), 122.3 (C-12), 58.5 (C-3), 56.3 (C-5), 48.3 (C-9), 46.9 (C-19), 46.7 (C-17), 42.0 (C-14), 41.6 (C-18), 39.5 (C-8), 37.0 (C-4), 35.5 (C-10), 34.1 (C-1), 34.1 (C-21), 33.2 (C-29), 32.6 (C-2), 32.5 (C-7), 32.4 (C-22), 30.9 (C-20), 27.9 (C-15), 27.7 (C-23), 25.9 (C-30), 23.8 (C-26), 23.3 (C-16), 22.9 (C-24), 17.0 (C-27), 15.4 (C-25) ppm; MS (ESI, MeOH): *m/z* 470.1 (100%, $[M + H]^+$); analysis calcd for $C_{30}H_{49}NO_2$ (455.72): C 79.07, H 10.84, N 3.07; found:

C 78.83, H 11.03, N 2.86.

4.2.4.4. 6-(Diethylamino)-N,N-diethyl-9-(2-{[(3 β)-28-methoxy-28-oxoolean-12-en-3-yl]carbamoyl}phenyl)3H-xanthen-3-iminium chloride (4). To a solution of rhodamine B (184.9 mg, 0.386 mmol) in acetonitrile (60 mL), EDC (74.0 mg, 0.386 mmol) and TEA (0.06 mL, 0.386 mmol) were added, and the mixture was stirred at 23 °C for 90 min. Compound 3 (165 mg, 0.351 mmol) was added, and stirring at 23 °C was continued for 3 days. Usual aqueous workup followed by column chromatography (SiO_2, $CHCl_3$/MeOH, 9:1) gave 4 as a purple solid (160 mg, 65%); m.p. 247–250 °C; $R_F = 0.48$ (SiO_2, $CHCl_3$/MeOH, 9:1); UV–Vis ($CHCl_3$): λ_{max} (log ε) = 550 nm (4.97); IR (ATR): ν = 2938*w*, 1645 m, 1587 s, 1409 m, 1332 s, 1178 s, 1130 m, 1073 m, 823 m, 683 m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 7.93$ (d, $J = 7.7$ Hz, 1H, 37-H), 7.67–7.62 (*m*, 1H, 35-H), 7.61–7.51 (*m*, 2H, 38-H, 41-H), 7.47 (d, $J = 9.4$ Hz, 1H, 41′-H), 7.22 (dd, $J = 7.5$, 1.1 Hz, 1H, 36-H), 6.99 (d, $J = 9.4$ Hz, 1H, 42-H), 6.91 (d, $J = 9.4$ Hz, 1H, 42′-H), 6.70–6.65 (*m*, 2H, 44-H, 44′-H), 5.23 (t, $J = 3.7$ Hz, 1H, 12-H), 3.60 (s, 3H, 31-H), 3.64–3.51 (*m*, 8H, 46-H, 46-H'), 3.47–3.37 (*m*, 1H, 18-H), 2.82 (dd, $J = 13.7$, 4.6 Hz, 1H), 1.97–0.93 (*m*, 20H, 16-H, 2-H_a, 22-H_a, 15-H_a, 19-H_a, 22-H_b, 1-H_a, 6-H, 9-H, 7-H_a, 21-H_a, 7-H_b, 21-H_b, 2-H_b, 19-H_b, 15-H_b), 1.35–1.24 (*m*, 12H, 47-H, 47′-H), 1.03 (s, 3H, 30-H), 0.90 (s, 3H, 26-H), 0.86 (d, $J = 1.6$ Hz, 6H, 25-H, 29-H), 0.82 (s, 1H, 1-H_b), 0.81 (s, 3H, 27-H), 0.70 (*m*, 1H, 5-H), 0.67 (s, 3H, 24-H), 0.58 (s, 3H, 23-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 178.4 (C-28), 168.0 (C-32), 160.2 (C-39), 158.0 (C-45′), 157.9 (C-45), 155.7 (C-43′), 155.4 (C-43), 143.8 (C-13), 138.3 (C-34), 133.2 (C-41′), 132.9 (C-41), 130.8 (C-33), 130.5 (C-35), 130.1 (C-38), 129.3 (C-36), 128.6 (C-37), 122.6 (C-12), 114.6 (C-40′), 114.3 (C-42′), 114.2 (C-40), 114.2 (C-42), 96.0 (C-44′), 96.0 (C-44), 57.8 (C-3), 56.3 (C-5), 51.6 (C-31), 47.7 (C-9), 46.9 (C-17), 46.1 (C-46, C-46′), 46.1 (C-19), 41.7 (C-14), 41.5 (C-18), 39.4 (C-1), 39.3 (C-8), 38.3 (C-4), 37.0 (C-10), 34.0 (C-21), 33.2 (C-29), 32.8 (C-7), 32.5 (C-22), 30.8 (C-20), 28.6 (C-23), 27.8 (C-15), 25.9 (C-30), 24.6 (C-2), 23.8 (C-26), 23.5 (C-11), 23.2 (C-16), 18.6 (C-6), 16.9 (C-24), 16.7 (C-27), 15.3 (C-25), 12.8 (C-47, C-47′) ppm; MS (ESI, MeOH): *m/z* 895.2 (100%, $[M + H]^+$); analysis calcd for $C_{59}H_{80}N_3O_4Cl$ (930.74): C 76.14, H 8.66, N 4.52; found: C 75.95, H 8.90, N 4.31.

4.2.4.5. 4-{[(3 β)-28-Methoxy-28-oxoolean-12-en-3-yl]oxy}-4-oxobutanoic acid (5). Compound 1 (2 g, 4.23 mmol) and catalytic amounts of DMAP were added to a solution of succinic anhydride (2.1 g, 21.15 mmol) in dry pyridine (50 mL), and the mixture was stirred under reflux for 8 h. Usual aqueous workup followed by column chromatography (SiO_2, hexanes/EtOAc, 7:3) gave 5 as a white solid (1.9 g, 79%); m.p. 210–212 °C; $[\alpha]_D = +60.8°$ (*c* 0.201, $CHCl_3$); $R_F = 0.29$ (SiO_2, hexanes/EtOAc, 7:3); IR (ATR): ν = 2935 m, 1728 s, 1710 s, 1440*w*, 1381*w*, 1317 m, 1175 s, 1148 m, 1036*w*, 1013*w*, 985 m, 801*w*, 645 m cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 5.27$ (t, $J = 3.7$ Hz, 1H, 12-H), 4.52 (dd, $J = 9.0$, 6.9 Hz, 1H, 3-H), 3.62 (s, 3H, 31-H), 2.86 (dd, $J = 13.9$, 4.5 Hz, 1H, 18-H), 2.73–2.56 (*m*, 4H, 33-H, 34-H), 2.02–1.91 (*m*, 1H, 16-H_a), 1.90–1.83 (*m*, 2H, 11-H), 1.74–1.14 (*m*, 16H, 22-H_a, 16-H_b, 2-H, 19-H_a, 1-H_a, 15-H_a, 9-H, 6-H_a, 7-H, 6-H_b, 21-H_a, 22-H_b, 21-H_b, 19-H_b), 1.12 (s, 3H, 27-H), 1.09–0.99 (*m*, 2H, 15-H_b, 1-H_b), 0.92 (s, 3H, 25-H), 0.92 (s, 3H, 30-H), 0.89 (s, 3H, 29-H), 0.85 (s, 3H, 23-H), 0.85 (s, 3H, 24-H), 0.82–0.76 (*m*, 1H, 5-H), 0.72 (s, 3H, 26-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 178.5 (C-28), 177.8 (C-32), 171.9 (C-35), 144.0 (C-13), 122.4 (C-12), 81.7 (C-3), 55.5 (C-5), 51.7 (C-31), 47.7 (C-9), 46.9 (C-17), 46.0 (C-19), 41.8 (C-14), 41.5 (C-18), 39.4 (C-8), 38.2 (C-1), 37.9 (C-4), 37.1 (C-10), 34.0 (C-21), 33.2 (C-29), 32.8 (C-7), 32.5 (C-22), 30.8 (C-20), 29.5 (C-33), 29.1 (C-34), 28.1 (C-23), 27.8 (C-15), 26.1 (C-27), 23.8 (C-30), 23.6 (C-16), 23.2 (C-2), 18.4 (C-6), 17.0 (C-26), 16.8 (C-24), 15.5 (C-25) ppm; MS (ESI, MeOH): *m/z* 493.1 (38%, $[M + Na]^+$), 1163.3 (100%, $[2\ M + Na]^+$); analysis calcd for $C_{35}H_{54}O_6$ (570.80): C 73.65, H 9.54.

4.2.4.6. Methyl (3 β)-3-{[(4-oxo-4-(piperazin-1-yl)butanoyl]oxy}olean-12-en-28-oate (6). Compound 5 (300 mg, 0.526 mmol) was dissolved in dry DCM (20 mL), and oxalyl chloride (4 eq.) and catalytic quantities of DMF were added. The reaction mixture was stirred at 23 °C for one hour. The volatiles were removed under diminished pressure, the residue was dissolved in dry DCM (15 mL), and piperazine (4 eq.), TEA (1 eq.) and DMAP (cat.) were added. The reaction mixture was stirred at 23 °C for one day. Usual aqueous workup followed column chromatography (SiO_2, hexanes/EtOAc, 7:3) gave 6 as a white solid (295 mg, 94%); m.p. 78–82 °C; $[\alpha]_D = +11.4°$ (*c* 0.163, $CHCl_3$); $R_F = 0.21$ (SiO_2, hexanes/EtOAc, 7:3); IR (ATR): ν = 3442 m, 2946 s, 1725 s, 1628 m, 1328 s, 1173 m, 1036*w*, 822 m cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 5.27$ (t, $J = 3.6$ Hz, 1H, 12-H), 4.50 (dd, $J = 8.8$, 7.1 Hz, 1H, 3-H), 3.68–3.44 (*m*, 8H, 36-H, 37-H, 38-H, 39-H), 3.62 (s, 3H, 31-H), 2.85 (dd, $J = 13.8$, 4.5 Hz, 1H, 18-H), 2.71–2.59 (*m*, 4H, 33-H, 34-H), 2.02–1.91 (*m*, 1H, 16-H_a), 1.91–1.84 (*m*, 2H, 11-H), 1.73–1.14 (*m*, 16H, 22-H_a, 16-H_b, 2-H, 19-H_a, 1-H_a, 15-H_a, 9-H, 6-H_a, 7-H, 6-H_b, 21-H_a, 22-H_b, 21-H_b, 19-H_b), 1.12 (s, 3H, 27-H), 1.08–0.98 (*m*, 2H, 1-H_b, 15-H_b), 0.92 (d, $J = 1.3$ Hz, 6H, 25-H, 30-H), 0.89 (s, 3H, 29-H), 0.86 (s, 3H, 23-H), 0.85 (s, 3H, 24-H), 0.82–0.79 (*m*, 1H, 5-H), 0.72 (s, 3H, 26-H) ppm; ^{13}C NMR (101 MHz, CDCl3): δ = 178.7 (C-28), 173.1 (C-32), 170.6 (C-35), 144.3 (C-13), 122.7 (C-12), 81.7 (C-3), 55.8 (C-5), 52.0 (C-31), 48.0 (C-9) 47.2 (C-17), 46.3 (C-19), 45.5 (C-37, C-38), 42.1 (C-14), 42.1 (C-36, C-39), 41.8 (C-18), 39.8 (C-8), 38.6 (C-1), 38.2 (C-4), 37.4 (C-10), 34.3 (C-21), 33.6 (C-29), 33.1 (C-7), 32.8 (C-22), 31.2 (C-20), 30.0 (C-34), 28.5 (C-23), 28.4 (C-33), 28.2 (C-15), 26.4 (C-27), 24.1 (C-30), 24.0 (C-16), 23.9 (C-11), 23.5 (C-2), 18.7 (C-6), 17.3 (C-26), 17.2 (C-24), 15.8 (C-25) ppm; MS (ESI, MeOH): *m/z* 639.3 (100%, $[M + H]^+$), 1277.2 (12%, $[2\ M + H]^+$), 1299.3 (100%, $[2\ M + Na]^+$); analysis calcd for $C_{39}H_{62}N_2O_5$ (638.92): C 73.31, H 9.78, N 4.38; found: C 73.05, H 9.98, N 4.18.

4.2.4.7. 6-(Diethylamino)-N,N-diethyl-9-(2-{[(3 β)-2-methoxy-28-oxoolean-12-en3-yl]carbamoyl}phenyl)–3H-xanthen-3-iminium chloride (7). Rhodamine B (277 mg, 0.6 mmol) was dissolved in dry DCM (15 mL) and oxalyl chloride (4 eq.) and catalytic quantities of DMF were added. The reaction mixture was stirred at 23 °C for one hour. The volatiles were removed under diminished pressure, the residue was dissolved in dry DCM (15 mL), and compound 6 (200 mg, 0.469 mmol), TEA (1 equiv.) and DMAP (cat.) were added. The reaction mixture was stirred at 23 °C for one day. Usual aqueous workup followed column chromatography (SiO_2, $CHCl_3$/MeOH, 9:1) gave 7 as a purple solid (151 mg, 75.5%); m.p. 165–175 °C; $R_F = 0.44$ (SiO_2, $CHCl_3$/MeOH, 9:1); UV–Vis ($CHCl_3$): λ_{max} (log ε) = 559 nm (4.92); IR (ATR): ν = 3411 m, 2932*w*, 1722*w*, 1633 m, 1588 m, 1411 m, 1334 s, 1245 m, 1179 m, 1074*w*, 1007*w*, 979*w*, 823*w*, 683*w* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.78–6.64 (*m*, 10*H*, 45-H, 43-H, 46-H, 49-H, 49′-H, 44-H, 50-H, 50′-H, 44-H, 44′-H), 5.29–5.22 (*m*, 1H, 12-H), 4.45 (t, $J = 7.9$ Hz, 1H, 3-H), 3.61 (s, 3H, 31-H), 3.76–3.26 (*m*, 16H, 54-H, 54′-H, 36-H, 37-H, 38-H, 39-H), 2.85 (dd, $J = 14.4$, 4.8 Hz, 1H, 18-H), 2.73–2.52 (*m*, 4H, 33-H, 34-H), 2.01–1.90 (*m*, 1H, 16-H_a), 1.86 (dd, $J = 9.5$, 4.0 Hz, 2H, 11-H), 1.74–1.12 (*m*, 16H, 22-H_a, 16-H_b, 2-H, 19-H_a, 1-H_a, 15-H_a, 9-H, 6-H_a, 7-H, 6-H_b, 21-H_a, 22-H_b, 21-H_b, 19-H_b), 1.31 (s, 12H, 55-H, 55′-H), 1.11 (s, 3H, 27-H), 1.08–0.95 (*m*, 2H, 15-H_b, 1-H_b), 0.91 (s, 3H, 30-H), 0.90 (s, 3H, 25-H), 0.89 (s, 3H, 29-H), 0.83 (s, 3H, 23-H), 0.81 (s, 3H, 24-H), 0.78–0.76 (*m*, 1H, 5-H), 0.71 (s, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 178.4 (C-28), 173.1 (C-32), 170.5 (C-35), 168.0 (C-40), 163.1 (C-47), 157.9 (C-53, C-53′), 155.9 (C-51, C-51′), 143.9 (C-13), 137.2 (C-42), 132.6 (C-49, C-49′), 131.0 (C-41), 130.1, 127.8 (C-45), 122.4 (C-12), 115.3 (C-48, C-48′, C-50, C-50′), 96.1 (C-52, C-52′), 81.2 (C-3), 55.5 (C-5), 51.6 (C-31), 47.7 (C-9), 46.9 (C-17), 46.2 (C-54, C-54′), 46.0 (C-19), 45.5 (C-37, C-38), 42.4 (C-36′, C-39), 41.8 (C-14), 41.4 (C-18), 39.4 (C-8), 38.2 (C-1), 37.9 (C-4), 37.0 (C-10), 34.0 (C-21), 33.2 (C-29), 32.7 (C-7), 32.5 (C-22), 32.3 (C-20), 30.8 (C-34), 28.2 (C-23), 28.0 (C-33), 27.8 (C-15), 26.0 (C-27), 23.8 (C-30), 23.6 (C-16), 23.5 (C-11), 23.2 (C-2), 18.3 (C-6), 17.0 (C-26), 16.9 (C-24), 15.5 (C-25), 12.7

(C-55, C-55′) ppm; MS (ESI, MeOH): *m/z* 1064.1 (100%, $[M + H]^+$); analysis calcd for $C_{67}H_{91}N_4O_7Cl$ (1099.92): C 73.16, H 8.34, N 5.09; found: C 72.96, H 8.51, N 4.87.

4.2.4.8. 3 β -Acetyloxy-olean-12-en-28-oic acid (8). This compound was prepared by acetylation of OA as previously reported, and 8 (10 g, 75%) was obtained as a colorless solid; m.p. 265–267 °C (lit.:[47] 264–265 °C); MS (ESI, MeOH): *m/z* 499.2 (13%, $[M + H]^+$), 521.2 (35%, $[M + Na]^+$, 1019.4 $[2 M + Na]^+$).

4.2.4.9. (3 β) 28-Oxo-28-(piperazine-1-yl)-olean-12-en-3-yl acetate (9). As described for the synthesis of 6, from 8 and piperazine, compound 9 (0.42 g, 84%) was obtained as a colorless solid; m.p. 171–175 °C; (lit.: [48,49] m.p. 170–176 °C); MS (ESI, MeOH): *m/z* 567.2 (52%, $[M + H]^+$).

4.2.4.10. 9-(2-{4[(3 β)-3-Acetyloxy-28-oxoolean-12-en-28-yl]-piperazine-1-carbonyl}phenyl)-6-(diethylamino)-N,N-diethyl-3H-xanthen-3-iminium chloride (10). As described for the synthesis of 7, from 9 and piperazine, compound 10 (0.67 g, 79%) was obtained as a colorless solid; m.p. 244–247 °C, (lit.:[14] 245–248 °C); MS (ESI, MeOH): *m/z* 991.7 (100%, $[M-Cl]^+$).

4.2.4.11. 6-(Diethylamino)-N,N-diethyl-9-[2-({[(3 β)-28-methoxy-28-oxoolean-12-en-3-yl]oxy}carbonyl)phenyl]–3H-xanthen-3-iminium chloride (11). This compound was prepared as previously described; 11 was obtained as a pink solid (0.68 g, 41%); m.p. 237–240 °C (lit.:[17] 235–240 °C); MS (ESI, MeOH): *m/z* 896.1 (100%, $[M-Cl]^+$).

CRediT authorship contribution statement

Niels Heise: Investigation, Writing - review & editing. **Sophie Hoenke:** Investigation, Writing - review & editing. **Vivienne Simon:** Investigation, Writing - review & editing. **Hans-Peter Deigner:** Conceptualization, Writing - original draft, Writing - review & editing. **Ahmed Al-Harrasi:** Conceptualization, Writing - original draft, Writing - review & editing. **René Csuk:** Conceptualization, Writing - original draft, Writing - review & editing.

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Synthesis and cytotoxicity of diastereomeric benzylamides derived from maslinic acid, augustic acid and bredemolic acid

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ABSTRACT

36 substituted benzylamides were prepared starting from maslinic acid, bredemolic acid, and augustic acid and evaluated for their cytotoxicity in SRB assays employing several human tumor cell lines as well as non-malignant fibroblasts. Thereby, the benzylamides of maslinic acid, however, were found to be more cytotoxic than those obtained from augustic acid or bredemolic acid. The best compound (**18**, derived from maslinic acid) showed an EC_{50} value of 1.3 μM against A375 melanoma cells. Additional staining experiments revealed that this compound acted rather by apoptosis than by necrosis.

1. Introduction

Recently, we observed that the benzylamide of 2,3-di-*O*-acetyl-maslinic acid (**EM2**, Fig. 1) held high cytotoxicity for a panel of different human tumor cell lines combined with a good selectivity for the tumor cells and lower cytotoxicity for non-malignant cell lines. [1,2] Since analogs derived from oleanolic acid or ursolic acid held significant lower cytotoxicity with the tumor cell lines, we deduced that the presence of a second acetoxy group in ring A of pentacyclic triterpene carboxylic acids exerts significant influence on their cytotoxic activity. A possible reason could be a higher bioavailability due to better solubility in physiological solutions. In order to obtain a statement whether this holds true for analogs of **EM2** and whether the higher cytotoxicity holds true for other triterpenoic acids with two vicinal acetoxy groups in ring A but with an altered absolute configuration of these acetoxy groups, we decided to prepare some substituted benzylamides derived from bredemolic acid (**1**) [3–6], maslinic acid (**2**) [7–12] and augustic acid (**3**) [13–15]. Furthermore, the scope of terpenoids has been enlarged recently due to their applications in nanoscience, green chemistry and especially by studies concerning their self-assembly [16].

While numerous studies exist for maslinic acid and its derivatives, dealing with a wide variety of biological properties, only a few studies are known for augustic acid and even less for bredemolic acid. The latter finding might also be since bredemolic acid can be obtained in only low yields from natural sources. However, partial syntheses from oleanolic acids have already been described for **1**–**3**, too. [4] Bredemolic acid and augustic acid differ from maslinic acid by the configuration of the two hydroxy groups in ring A. While maslinic acid is configured (2α, 3 β), bredemolic acid (2β, 3α) configurated and augustic acid holds a (2β, 3β) configuration.

2. Results and discussion

As mentioned above, bredemolic acid (**1**) is accessible by a partial synthesis from oleanolic acid. [4] Its acetylation (Scheme 1) gave the known diacetate **4**. [17] Reaction of **4** with oxalyl chloride followed by addition of substituted benzylamines gave products **5**–**16**. For comparison, analogous reactions were carried out starting from maslinic acid (**2**). This pentacyclic triterpenoic acid was either extracted from pitted olives or obtained by partial synthesis [4] starting from oleanolic acid. Acetylation of **2** gave known [2] diacetate **17** that was converted into amides **18**–**29**, respectively. In similar fashion augustic acid (**3**, also obtained by partial synthesis from oleanolic acid) [4] was acetylated, and di-acetate **30** [18] transformed into amides **31**–**42**.

The cytotoxicity of the compounds was determined in SRB assays; the results of these assays are summarized in Table 1.

Analysis of these data reveals a few structure–activity relationships. The assumption that derivatives with two acetoxy groups on ring A have a significantly higher cytotoxicity than the previously described analogs with an oleanolic acid backbone carrying only one acetoxy group in ring A is confirmed. There is no significant dependence of observed cytotoxicity on the substituent on the benzyl ring. However, it was found that, as a rule, those derivatives derived from maslinic acid are somewhat more cytotoxic than those derived from augustic acid. Bredemolic

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acid-derived derivatives are less cytotoxic. The lowest cytotoxicity was observed for HT29 and MCF-7 tumor cells while the highest cytotoxicity was established for A375 and A2780 cells. All derivatives are significantly more cytotoxic with malignant cell lines than for non-malignant fibroblasts NIH 3T3. This has previously [1,2] been demonstrated also for **EM2**.

Some extra staining experiments employing A375 cells and compound **35** are depicted in Fig. 2 (thereby, cells in R1 are necrotic, in R2 late apoptotic, viable cells are measured in R3 and apoptotic cells are detected in R4). The results indicate that A375 cells die rather by apoptosis than by necrosis.

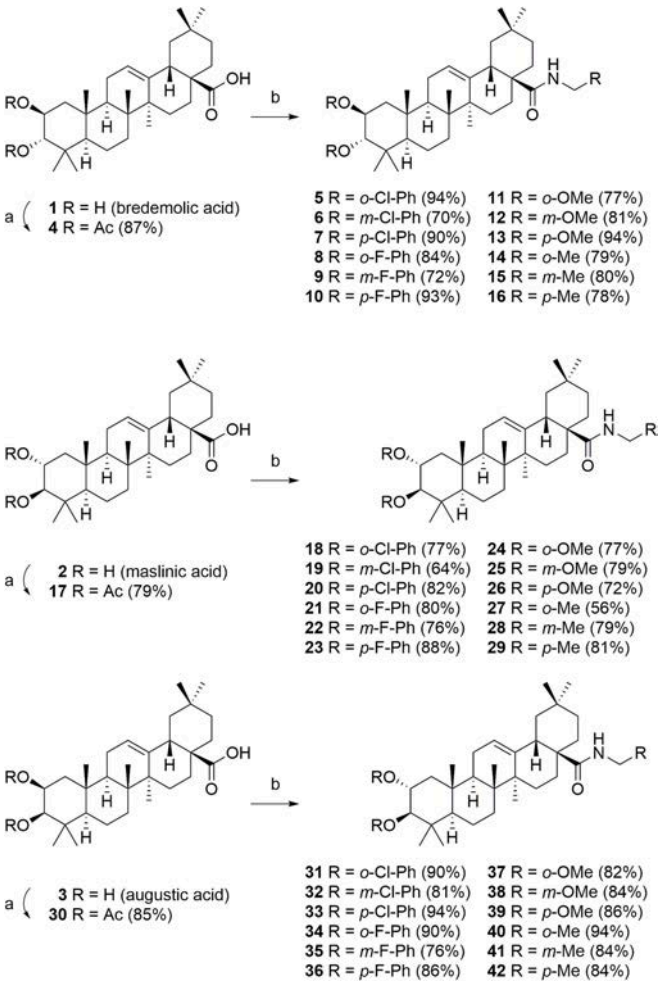
3. Conclusion

Based on our assumption that benzylamides of pentacyclic acetylated triterpenes are expected to exhibit particularly good cytotoxicity toward malignant cancer cell lines [based on a previous observation for a 2,3-di-*O*-acetyl-maslinic acid benzyl amide (**EM2**)], 36 new derivatives were prepared starting from maslinic acid, bredemolic acid, and augustic acid and evaluated for their cytotoxicity in SRB assays employing several human tumor cell lines as well as non-malignant fibroblasts. All compounds were found to be highly cytotoxic to cancer cells, while a significantly lower cytotoxicity was determined for the non-malignant fibroblasts. Benzylamides of maslinic acid, however, were found to be more cytotoxic than those obtained from augustic acid or bredemolic acid. The best compound (**35**) showed an EC_{50} value of 1.3 μM against A375 melanoma cells and 0.8 μM for ovarian carcinoma cells. Additional staining experiments revealed that this compound acted rather by apoptosis than by necrosis.

4. Experimental

4.1. General

NMR spectra were recorded using the Varian spectrometers (Darmstadt, Germany) DD2 and VNMRS (400 and 500 MHz, respectively). MS spectra were taken on a Advion expression^{L} CMS mass spectrometer (Ithaca, NY, USA; positive ion polarity mode, solvent: methanol, solvent flow: 0.2 mL/min, spray voltage: 5.17 kV, source voltage: 77 V, APCI corona discharge: 4.2 μA, capillary temperature: 250 °C, capillary voltage: 180 V, sheath gas: N_2). Thin-layer chromatography was performed on pre-coated silica gel plates supplied by Macherey-Nagel (Düren, Germany). IR spectra were recorded on a Spectrum 1000 FT-IR-spectrometer from Perkin Elmer (Rodgau, Germany). The UV/Vis-spectra were recorded on a Lambda 14 spectrometer from Perkin Elmer (Rodgau, Germany); optical rotations were measured at 20 °C using a JASCO-P2000 instrument (JASCO Germany GmbH, Pfungstadt, Germany). The melting points were determined using the Leica hot stage microscope Galen III (Leica Biosystems, Nussloch, Germany) and are uncorrected. The solvents were dried according to usual procedures. Microanalyses were performed with an Elementar Vario EL (CHNS) instrument (Elementar Analysensysteme GmbH, Elementar-Straβe 1, D-63505, Langenselbold, Germany). As checked by NMR and HPLC, the purity of all products was >95 %.



Scheme 1. Synthesis of amides **5**–**16** from bredemolic acid (**1**) and amides **18**–**29** from maslinic acid (**2**) and bredemolic acid derived benzyl amides 31–42; reactions and conditions: a) Ac_2O, DCM, NEt_3, DMAP (cat.), 21 °C, 12 h; b) $(COCl)_2$, DMF (cat.), then DCM, substituted benzylamine, 21 °C, 12 h, 21 °C.

All dry solvents were distilled over respective drying agents except for DMF which was distilled and stored under argon and molecular sieve. Reactions using air- or moisture-sensitive reagents were carried out under argon atmosphere in dried glassware. Triethylamine was stored over potassium hydroxide. Biological assays were performed as previously reported employing cell lines obtained from the Department of Oncology [Martin-Luther-University Halle Wittenberg; they were bought from ATCC: malignant: A 375, HT29, MCF7, A2780 and HeLa; non-malignant: NIH 3T3]. Oleanolic acid was obtained from Betulinines

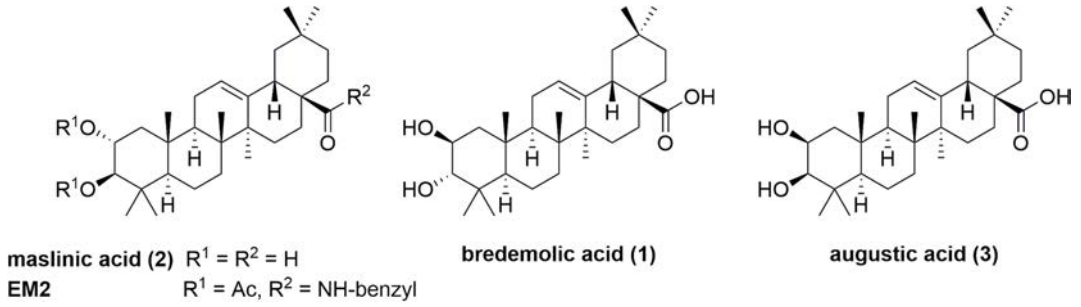


Fig. 1. Structure of bredemolic acid (**1**), maslinic acid (**2**), and augustic acid (**3**) and the benzyl amide of 2,3-di-*O*-acetyl-maslinic acid (**EM2**).

Table 1
Cytotoxicity of benzylamides **5**–**16**, **18**–**29** and **31**–**42** assessed from SRB-assays (EC_{50} values [μM] after 72 h of treatment). Human cancer cell lines: A375 (epithelial melanoma), HT29 (colorectal adenocarcinoma), MCF-7 (breast adenocarcinoma), A2780 (ovarian carcinoma), HeLa cervical carcinoma); non-malignant: NIH 3T3 (fibroblasts); n.d. not determined; n.s. not soluble under the conditions of the assay; positive control: doxorubicin (**DX**).

	A375	HT29	MCF-7	A2780	HeLa	NIH 3T3
5	3.3 ± 0.4	15.8 ± 3.6	> 20	5.4 ± 0.8	11.3 ± 2.7	>30
6	2.9 ± 0.1	10.7 ± 2.8	12.8 ± 2.7	2.9 ± 0.1	8.9 ± 2.7	>30
7	2.9 ± 0.2	11.5 ± 3.8	14.4 ± 3.5	4.2 ± 0.6	6.8 ± 1.8	>30
8	1.8 ± 0.2	6.0 ± 1.8	8.9 ± 1.5	3.2 ± 0.4	4.9 ± 1.0	>30
9	2.4 ± 0.1	10.5 ± 2.2	7.9 ± 1.5	3.2 ± 0.6	7.1 ± 1.1	>30
10	2.3 ± 0.9	6.1 ± 1.8	5.2 ± 2.2	1.6 ± 0.2	6.7 ± 0.9	>30
11	2.6 ± 0.7	7.0 ± 2.4	7.9 ± 2.4	2.5 ± 0.5	7.8 ± 1.0	>30
12	3.0 ± 0.1	8.4 ± 1.6	7.8 ± 1.1	3.1 ± 0.2	6.7 ± 0.8	>30
13	2.7 ± 0.6	10.1 ± 2.3	11.6 ± 1.4	3.3 ± 0.4	6.7 ± 2.0	>30
14	2.1 ± 0.3	4.4 ± 1.2	3.0 ± 0.6	1.7 ± 0.1	4.4 ± 0.5	>30
15	2.3 ± 0.6	6.0 ± 1.8	7.2 ± 1.0	2.7 ± 0.2	5.8 ± 1.1	>30
16	2.6 ± 0.5	7.4 ± 1.9	9.2 ± 1.5	2.4 ± 0.4	7.7 ± 1.9	>30
18	1.8 ± 0.3	6.8 ± 1.1	6.8 ± 0.9	1.9 ± 0.1	4.9 ± 0.5	>30
19	2.3 ± 0.3	13.5 ± 2.9	6.9 ± 1.3	1.6 ± 0.3	6.6 ± 0.4	>30
20	3.3 ± 0.3	>30	>30	2.9 ± 1.5	9.9 ± 0.8	>30
21	2.0 ± 0.1	4.5 ± 0.3	6.5 ± 1.0	2.4 ± 0.2	6.2 ± 0.5	>30
22	1.0 ± 0.1	4.5 ± 0.9	4.0 ± 0.4	0.8 ± 0.1	4.3 ± 0.4	>30
23	1.0 ± 0.1	4.4 ± 0.3	3.8 ± 0.5	0.8 ± 0.1	4.3 ± 0.8	18.3 ± 2.9
24	n.s.	n.s.	n.s.	n.s.	n.s.	>30
25	1.4 ± 0.1	5.1 ± 0.4	4.4 ± 0.7	1.3 ± 0.1	4.7 ± 0.4	>30
26	1.8 ± 0.1	7.2 ± 0.5	5.8 ± 0.8	1.6 ± 0.1	6.1 ± 07	>30
27	n.s.	n.s.	n.s.	n.s.	n.s.	>30
28	1.6 ± 0.2	6.2 ± 0.7	5.2 ± 0.5	1.4 ± 0.1	5.2 ± 0.3	>30
29	1.4 ± 0.2	9.9 ± 2.6	7.3 ± 0.9	1.6 ± 0.4	6.2 ± 0.6	> 30
31	2.5 ± 0.2	6.7 ± 1.1	7.9 ± 1.8	5.2 ± 0.6	7.8 ± 1.4	>30
32	2.2 ± 0.3	6.0 ± 1.9	7.1 ± 2.1	4.1 ± 0.3	13.2 ± 1.6	>30
33	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
34	2.3 ± 0.3	5.9 ± 1.6	6.3 ± 1.7	4.0 ± 0.7	7.4 ± 0.7	>30
35	1.0 ± 0.3	1.9 ± 0.4	2.9 ± 0.9	1.7 ± 0.5	2.9 ± 0.5	>30
36	n.s.	1.7 ± 0.7	3.0 ± 0.9	2.5 ± 0.2	2.8 ± 0.5	>30
37	1.7 ± 0.1	3.3 ± 0.6	4.7 ± 1.3	1.9 ± 0.4	2.9 ± 0.7	>30
38	1.8 ± 0.1	3.8 ± 0.3	3.8 ± 0.8	1.9 ± 0.3	5.8 ± 0.5	>30
39	1.3 ± 0.2	2.5 ± 0.7	4.3 ± 1.5	2.1 ± 0.3	4.1 ± 1.5	>30
40	1.3 ± 0.1	3.2 ± 0.3	3.9 ± 0.7	1.7 ± 0.2	4.8 ± 0.4	>30
41	1.5 ± 0.1	3.2 ± 0.5	4.0 ± 0.8	2.2 ± 0.3	4.8 ± 0.8	>30
42	1.3 ± 0.1	3.3 ± 0.6	4.4 ± 0.5	2.1 ± 0.1	4.4 ± 1.0	>30
DX	n.d.	0.25 ± 0.02	0.1 ± 0.01	0.1 ± 0.01	n.d.	0.01 ± 0.001

(Strbrna Skalice, Czech Republic) and used as received.

For the SRB assay: cells were seeded into 96 well plates on day zero at appropriate cell densities to prevent confluence of the cells during the period of the experiment. After 24 h, the cells were treated with different concentrations (1, 3, 7, 12, 20 and 30 μM), but the final concentration of DMSO/DMF never exceeded 0.5 %, which was non-toxic to the cells. After 72 h treatment, the supernatant media from the 96 well plates were discarded, then the cells were fixed with 10 % trichloroacetic acid and allowed to rest at 4 °C. After 24 h of fixation, the cells were washed in a strip washer and then dyed with SRB solution (200 μL, 10 mM) for 20 min. Then the plates were washed four times with 1 % sacetic acid to remove the excess of the dye and allowed to air-dry overnight. Tris base solution (200 μL, 10 mM) was added to each well. The absorbance was measured with a 96 well plate reader from Tecan Spectra.

4.2. Syntheses

4.2.1. Bredemolic acid (1)

Starting from commercially available oleanolic acid, **1** was prepared as previously reported; m.p. 239–242 °C lit.: [4] 234–237 °C]; $[\alpha]_D$ = +92.8° (*c* 0.145, pyridine) [lit.: [4] + 91.13° (*c* 0,290, pyridine); MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 471.3 (100 %, $[M-H]^-$).

4.2.2. (2β,3α) Diacetyloxy-olean-12-en-28-oic acid (4)

Compound **4** was obtained from **1** following standard procedure for acetylation with acetic anhydride; yield 87 %; m.p. 165–167 °C [lit.: [19] 163–166 °C]; $[\alpha]_D$ = +78.2° (*c* 0.180, $CHCl_3$) [lit.: [19] + 75.98° (*c* 0.50, $CHCl_3$); MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 555.2 (100 %, $[M-H]^-$).

4.2.3. (2β,3α) N-(2-chlorobenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (5)

Compound **5** (115 mg, 94 %) was obtained from **4** (100 mg, 0.18 mmol) according to GPA as a white solid; m.p. 118 °C; R_f = 0.37 (SiO_2, hexanes/ethyl acetate, 8:2); $[\alpha]_D$ = +47.9° (*c* 0.084, MeOH); IR (ATR): ν = 2944w, 1739 s, 1645w, 1515w, 1471w, 1463w, 1366 m, 1241 s, 1227 s, 1027 m, 749w, 606w, 534w cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ = 7.41 – 7.30 (m, 2H, 39-H, 40-H), 7.23 – 7.16 (m, 2H, 37-H, 38-H), 6.44 (t, *J* = 6.0 Hz, 1H, NH), 5.35 (t, *J* = 3.7 Hz, 1H, 12-H), 4.97 (d, *J* = 6.4 Hz, 1H, 3-H), 4.94 – 4.89 (m, 1H, 2-H), 4.55 (dd, *J* = 14.5, 6.0 Hz, 1H, 35-H_a), 4.40 (dd, *J* = 14.5, 5.9 Hz, 1H, 35-H_a), 2.53 (dd, *J* = 12.7, 4.3 Hz, 1H, 18-H), 2.06 (s, 3H, 32-H), 2.02 (s, 3H, 34-H), 1.97 (dt, *J* = 13.7, 3.9 Hz, 1H, 16-H_a), 1.88 – 1.82 (m, 2H, 11-H), 1.80 – 1.73 (m, 1H, 19-H_a), 1.72 – 1.63 (m, 3H, 1-H_a, 16-H_a, 22-H_a), 1.61 – 1.51 (m, 3H, 1-H_a, 9-H, 22-H_a), 1.50 – 1.49 (m, 1H, 15-H_a), 1.48 – 1.40 (m, 2H, 6-H_a, 7-H_a), 1.38 – 1.30 (m, 2H, 6-H_a, 21-H_a), 1.25 – 1.17 (m, 4H, 5-H, 7-H_a, 21-H_a, 19-H_a), 1.16 (s, 3H, 27-H), 1.07 (s, 3H, 25-H), 1.04 – 1.02 (m, 1H, 15-H_a), 1.01 (s, 3H, 24-H), 0.91 – 0.87 (m, 9H, 23-H, 29-H, 20-H), 0.46 (s, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 178.0 (C-28), 170.3 (C-31), 170.1 (C-33), 144.6 (C-13), 136.1 (C-36), 134.1 (C-41), 131.0 (C-40), 129.5 (C-39), 127.3 (C-38), 123.1 (C-12), 76.2 (C-3), 70.7 (C-2), 50.5 (C-5), 48.0 (C-9), 46.8 (C-17), 46.7 (C-19), 42.7 (C-18), 42.4 (C-14), 41.6 (C-35), 41.3 (C-1), 39.5 (C-8), 37.0 (C-4), 36.7 (C-10), 34.3 (C-21), 33.1 (C-30), 32.8 (C-22), 32.2 (C-7), 30.9 (C-20), 27.4 (C-15), 27.0 (C-23), 25.9 (C-27), 23.9 (C-16), 23.7 (C-29), 23.6 (C-11), 22.3 (C-24), 21.5 (C-34), 21.1 (C-32), 18.7 (C-6), 18.2 (C-25), 16.5 (C-26) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 678.5 (100 %, $[M-H]^-$); analysis calcd for $C_{41}H_{58}NO_5Cl$ (680.37): C 72.39, H 8.59, N 2.21; found: C 72.11, H 8.87 N 1.98.

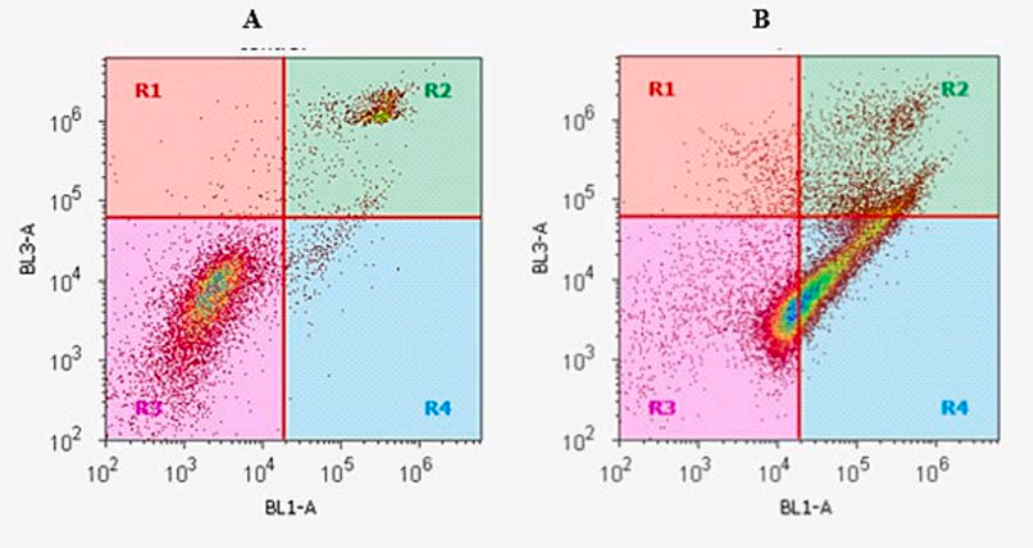


Fig. 2. FITC/Annexin V / Propidium iodide assay employing compound **35** and A375 cells (24 h, 2 × EC_{50} concentration); **A** control; **B** incubation with **35**.

4.2.4. (2β,3α) N-(3-chlorobenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (6)

Compound **6** (85 mg, 70 %) was obtained from **4** (100 mg, 0.18 mmol) according to GPA as a white solid; m.p. 109 °C; $R_f = 0.29$ (SiO_2, hexanes/ethyl acetate, 8:2); $[\alpha]_D = +51.2°$ (*c* 0.054, MeOH); IR (ATR): ν = 2945w, 1740 s, 1643w, 1517w, 1473w, 1433w, 1367 m, 1242 s, 1228 s, 1027 m, 681w, 605w cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.26 – 7.22 (m, 3H, 38-H, 39-H, 41-H), 7.17 – 7.12 (m, 1H, 37-H), 6.18 (t, *J* = 5.6 Hz, 1H, NH), 5.34 (t, *J* = 3.6 Hz, 1H, 12-H), 4.98 (d, *J* = 6.4 Hz, 1H, 3-H), 4.95 – 4.89 (m, 1H, 2-H), 4.54 (dd, *J* = 14.8, 6.1 Hz, 1H, 35-H_a), 4.16 (dd, *J* = 14.9, 5.0 Hz, 1H, 35-H_b), 2.57 – 2.51 (m, 1H, 18-H), 2.06 (s, 3H, 32-H), 2.02 (s, 3H, 34-H), 2.00 – 1.96 (m, 1H, 16-H_a), 1.90 – 1.84 (m, 2H, 11-H), 1.82 – 1.74 (m, 1H, 19-H_a), 1.73 – 1.64 (m, 3H, 1-H_a, 16-H_b, 22-H_a), 1.64 – 1.52 (m, 4H, 1-H_b, 9-H, 15-H_a, 22-H_b), 1.51 – 1.41 (m, 3H, 6-H, 7-H_a), 1.37 (dt, *J* = 13.7, 4.0 Hz, 1H, 21-H_a), 1.31 – 1.19 (m, 4H, 5-H, 7-H_b, 21-H_b, 19-H_b), 1.18 (s, 3H, 27-H), 1.11 (s, 3H, 25-H), 1.09 – 1.03 (m, 1H, 15-H_b), 1.02 (s, 3H, 24-H), 0.91 (s, 9H, 23-H, 29-H, 30-H), 0.66 (s, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 178.3 (C-28), 170.3 (C-31), 170.1 (C-33), 145.0 (C-13), 140.7 (C-36), 134.6 (C-40), 130.1 (C-38), 128.1 (C-41), 127.7 (C-39), 126.1 (C-37), 123.0 (C-12), 76.2 (C-3), 70.6 (C-2), 50.5 (C-5), 48.0 (C-9), 46.8 (C-19), 46.6 (C-17), 43.2 (C-35), 42.6 (C-18), 42.5 (C-14), 41.3 (C-1), 39.7 (C-8), 37.0 (C-4), 36.7 (C-10), 34.3 (C-21), 33.1 (C-30), 32.8 (C-22), 32.1 (C-7), 30.9 (C-20), 27.4 (C-15), 27.0 (C-23), 26.0 (C-27), 24.0 (C-16), 23.7 (C-29), 23.5 (C-11), 22.3 (C-24), 21.4 (C-34), 21.1 (C-32), 18.7 (C-6), 18.2 (C-25), 16.9 (C-26) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 678.2 (100 %, $[M-H]^-$); analysis calcd for $C_{41}H_{58}NO_5Cl$ (680.37): C 72.39, H 8.59, N 2.21; found: C 72.19, H 8.82 N 2.02.

4.2.5. (2β,3α) N-(4-chlorobenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (6)

Compound **7** (110 mg, 90 %) was obtained from **4** (100 mg, 0.18 mmol) according to GPA as a white solid; m.p. 115 °C; $R_f = 0.71$ (SiO_2, hexanes/ethyl acetate, 8:2); $[\alpha]_D = +46.6°$ (*c* 0.037, MeOH); IR (ATR): ν = 2945 m, 1739 s, 1643w, 1517w, 1492w, 1462w, 1367 m, 1242 s, 1227 s, 1091w, 1027 m, 1017 m, 801w, 754w, 605w, 556w, 475w cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.29 (d, *J* = 8.4 Hz, 2H, 38-H, 40-H), 7.18 (d, *J* = 8.4 Hz, 2H, 37-H, 41-H), 6.17 (t, *J* = 5.6 Hz, 1H, NH), 5.32 (t, *J* = 3.6 Hz, 1H, 12-H), 4.98 (d, *J* = 6.4 Hz, 1H, 3-H), 4.95 – 4.86 (m, 1H, 2-H), 4.55 (dd, *J* = 14.8, 6.3 Hz, 1H, 35-H_a), 4.11 (dd, *J* = 14.7, 4.7 Hz, 1H, 35-H_b), 2.53 (dd, *J* = 13.2, 3.8 Hz, 1H, 18-H), 2.06 (s, 3H, 32-H), 2.02 (s, 3H, 34-H), 2.00 – 1.94 (m, 1H, 16-H_a), 1.85 (dd, *J* = 8.8, 3.6 Hz, 2H, 11-H), 1.81 – 1.63 (m, 4H, 1-H_a, 16-H_b, 22-H_a, 19-H_a), 1.63 – 1.53 (m, 4H, 1-H_b, 9-H, 15-H_a, 22-H_b), 1.53 – 1.41 (m, 3H, 6-H, 7-H_a), 1.36 (dt, *J* = 13.5, 4.3 Hz, 1H, 21-H_a), 1.30 – 1.19 (m, 4H, 5-H, 7-H_b, 21-H_b, 19-H_b), 1.18 (s, 3H, 27-H), 1.10 (s, 3H, 25-H), 1.08 – 1.03 (m, 1H, 15-H_b), 1.02 (s, 3H, 24-H), 0.92 – 0.89 (m, 9H, 23-H, 29-H, 30-H), 0.64 (s, 3H, 26-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 178.3 (C-28), 170.3 (C-31), 170.1 (C-33), 145.0 (C-13), 137.2 (C-36), 133.3 (C-39), 129.3 (C-37, 41), 128.9 (C-38, 40), 122.9 (C-12), 76.2 (C-3), 70.6 (C-2), 50.5 (C-5), 48.0 (C-9), 46.8 (C-17), 46.6 (C-19), 43.0 (C-35), 42.5 (C-18), 42.4 (C-14), 41.2 (C-1), 39.6 (C-8), 37.0 (C-4), 36.7 (C-10), 34.3 (C-21), 33.1 (C-30), 32.8 (C-22), 32.1 (C-7), 30.9 (C-20), 27.4 (C-15), 27.0 (C-23), 26.0 (C-27), 24.0 (C-16), 23.7 (C-29), 23.6 (C-11), 22.2 (C-24), 21.4 (C-34), 21.1 (C-32), 18.7 (C-6), 18.2 (C-25), 16.9 (C-26) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 678.5 (100 %, $[M-H]^-$); analysis calcd for $C_{41}H_{58}NO_5Cl$ (680.37): C 72.39, H 8.59, N 2.21; found: C 72.13, H 8.81, N 1.96.

4.2.6. (2β,3α) N-(2-fluorobenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (8)

Compound **8** (150 mg, 84 %) was obtained from **4** (150 mg, 0.27 mmol) according to GPA as a white solid; m.p. 110 °C; $R_f = 0.28$ (SiO_2, hexanes/ethyl acetate, 8:2); $[\alpha]_D = +48.1°$ (*c* 0.066, MeOH); IR (ATR): ν = 2944w, 1739 m, 1652w, 1517w, 1489w, 1457w, 1366 m, 1224 s, 1226 s, 1032 m, 832w, 754 m, 605w cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.31 (dt, *J* = 7.6, 1.8 Hz, 1H, 37-H), 7.25 – 7.21 (m, 1H, 39-H), 7.10 (dd, *J* = 7.5, 1.2 Hz, 1H, 38-H), 7.07 – 7.00 (m, 1H, 40-H), 6.29 (t, *J* = 5.9 Hz, 1H, NH), 5.34 (t, *J* = 3.7 Hz, 1H, 12-H), 4.98 (d, *J* = 6.4 Hz, 1H, 3-H), 4.95 – 4.87 (m, 1H, 2-H), 4.55 (dd, *J* = 14.7, 6.0 Hz, 1H, 35-H_a), 4.33 (dd, *J* = 14.7, 5.2 Hz, 1H, 35-H_b), 2.52 (dd, *J* = 13.1, 4.4 Hz, 1H, 18-H), 2.06 (s, 3H, 32-H), 2.02 (s, 3H, 34-H), 2.00 – 1.93 (m, 1H, 16-H_a), 1.88 – 1.82 (m, 2H, 11-H), 1.80 – 1.62 (m, 4H, 1-H_a, 16-H_b, 19-H_a, 22-H_a), 1.61 – 1.50 (m, 4H, 1-H_b, 9-H, 15-H_a, 22-H_b), 1.50 – 1.40 (m, 3H, 6-H, 7-H_a), 1.35 (dt, *J* = 13.6, 3.8 Hz, 1H, 21-H_a), 1.27 – 1.17 (m, 4H, 5-H, 7-H_b, 19-H_b, 21-H_b), 1.16 (s, 3H, 27-H), 1.07 (s, 3H, 25-H), 1.05 – 1.02 (m, 1H, 15-H_b), 1.01 (s, 3H, 24-H), 0.90 (s, 9H, 23-H, 29-H, 30-H), 0.54 (s, 3H, 26-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 178.1 (C-28), 170.3 (C-31), 170.1 (C-33), 161.44 (d, *J* = 245.8 Hz, C-41), 144.7 (C-13), 130.68 (d, *J* = 4.5 Hz, C-37), 129.31 (d, *J* = 7.7 Hz, C-39), 125.51 (d, *J* = 14.7 Hz, C-36), 124.46 (d, *J* = 3.6 Hz, C-38), 123.0 (C-12), 115.39 (d, *J* = 21.4 Hz, C-40), 76.2 (C-3), 70.7 (C-2), 50.5 (C-5), 48.0 (C-9), 46.7 (C-17), 46.6 (C-19), 44.96 (d, *J* = 49.9 Hz, C-35), 42.6 (C-18), 42.4 (C-14), 41.3 (C-1), 39.6 (C-8), 36.9 (C-4), 36.7 (C-10), 34.3 (C-21), 33.1 (C-30), 32.8 (C-22), 32.1 (C-7), 30.9 (C-20), 27.4 (C-15), 27.0 (C-23), 25.9 (C-27), 23.9 (C-16), 23.7 (C-29), 23.6 (C-11), 22.2 (C-24), 21.4 (C-34), 21.1

(C-32), 18.7 (C-6), 18.2 (C-25), 16.6 (C-26) ppm; ^{19}F NMR (376 MHz, $CDCl_3$): δ = -119.04 ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 662.5 (100 %, $[M-H]^-$); analysis calcd for $C_{41}H_{58}NO_5F$ (663.92): C 74.17, H 8.81, N 2.11; found: C 73.96, H 9.15 N 1.99.

4.2.7. (2β,3α) N-(3-fluorobenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (9)

Compound **9** (129 mg,72 %) was obtained from **4** (150 mg, 0.27 mmol) according to GPA as a white solid; m.p. 113 °C; R_f = 0.32 (SiO_2, hexanes/ethyl acetate, 8:2); $[\alpha]_D$ = +55.1° (*c* 0.054, MeOH); IR (ATR): ν = 2944w, 1739 s, 1643w, 1592w, 1517w, 1450w, 1367 m, 1243 s, 1227 s, 1027 m, 756w, 605w, 521w, 440w cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.30 – 7.27 (m, 1H, H-38), 7.03 (dt, *J* = 7.7, 1.3 Hz, 1H, H-37), 6.98 – 6.93 (m, 2H, H-39, H-41), 6.20 (t, *J* = 5.7 Hz, 1H, NH), 5.34 (t, *J* = 3.6 Hz, 1H, 12-H), 4.98 (d, *J* = 6.4 Hz, 1H, 3-H), 4.94 – 4.87 (m, 1H, 2-H), 4.59 (dd, *J* = 14.9, 6.2 Hz, 1H, 35-H_a), 4.15 (dd, *J* = 14.9, 4.9 Hz, 1H, 35-H_b), 2.55 (dd, *J* = 12.9, 4.5 Hz, 1H, 18-H), 2.06 (s, 3H, 32-H), 2.02 (s, 3H, 34-H), 2.01 – 1.96 (m, 1H, 16-H_a), 1.88 – 1.84 (m, 2H, 11-H), 1.82 – 1.71 (m, 2H, 22-H_a, 19-H_a), 1.72 – 1.65 (m, 2H, 1-H_a, 16-H_b), 1.64 – 1.53 (m, 4H, 1-H_b, 9-H, 15-H_a, 22-H_b), 1.51 – 1.41 (m, 3H, 6-H, 7-H_a), 1.37 (dt, *J* = 13.4, 4.0 Hz, 1H, 21-H_a), 1.30 – 1.20 (m, 4H, 5-H, 7-H_b, 19-H_b, 21-H_b), 1.18 (s, 3H, 27-H), 1.10 (s, 3H, 25-H), 1.09 – 1.04 (m, 1H, 15-H_a), 1.02 (s, 3H, 24-H), 0.91 (m, 9H, 23-H, 29-H, 30-H), 0.67 (s, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 178.3 (C-28), 170.3 (C-31), 170.1 (C-33), 163.13 (d, *J* = 246.6 Hz, C-40), 145.0 (C-13), 141.24 (d, *J* = 7.1 Hz, C-36), 130.30 (d, *J* = 8.2 Hz, C-38), 123.42 (d, *J* = 2.9 Hz, C-37), 122.9 (C-12), 114.77 (d, *J* = 21.9 Hz, C-39), 114.39 (d, *J* = 21.0 Hz, C-41), 76.2 (C-3), 70.6 (C-2), 50.5 (C-5), 48.0 (C-9), 46.8 (C-19), 46.6 (C-17), 43.19 (d, *J* = 1.9 Hz, C-35), 42.6 (C-18), 42.4 (C-14), 41.2 (C-1), 39.6 (C-8), 37.0 (C-4), 36.7 (C-10), 34.3 (C-21), 33.1 (C-30), 32.8 (C-22), 32.1 (C-7), 30.9 (C-20), 27.4 (C-15), 27.0 (C-23), 26.0 (C-27), 24.0 (C-16), 23.7 (C-29), 23.5 (C-11), 22.2 (C-24), 21.4 (C-34), 21.1 (C-32), 18.7 (C-6), 18.2 (C-25), 16.9 (C-26) ppm; ^{19}F NMR (470 MHz, $CDCl_3$): δ = -112.84 ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 662.6 (38 %, $[M-H]^-$); analysis calcd for $C_{41}H_{58}NO_5F$ (663.92): C 74.17, H 8.81, N 2.11; found: C 73.87, H 9.14 N 1.97.

4.2.8. (2β,3α) N-(4-fluorobenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (10)

Compound **10** (167 mg, 93 %) was obtained from **4** (150 mg, 0.27 mmol) according to GPA as a white solid; m.p. 117 °C; R_f = 0.25 (SiO_2, hexanes/ethyl acetate, 8:2); $[\alpha]_D$ = +52.2° (*c* 0.105, MeOH); IR (ATR): ν = 2943w, 1739 m, 1642w, 1509 m, 1462w, 1433w, 1367w, 1223 s, 1156w, 1027 m, 823w, 752w, 605w, 577w, 486w cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.25 – 7.18 (m, 2H, 37-H, 41-H), 7.04 – 6.95 (m, 2H, 38-H, 40-H), 6.16 (t, *J* = 5.6 Hz, 1H, NH), 5.31 (t, *J* = 3.1 Hz, 1H, 12-H), 4.98 (d, *J* = 6.4 Hz, 1H, 3-H), 4.95 – 4.88 (m, 1H, 2-H), 4.54 (dd, *J* = 14.6, 6.2 Hz, 1H, 35-H_a), 4.13 (dd, *J* = 14.6, 4.5 Hz, 1H, 35-H_b), 2.53 (dd, *J* = 13.3, 4.4 Hz, 1H, 18-H), 2.06 (s, 3H, 32-H), 2.02 (s, 3H, 34-H), 2.00 – 1.93 (m, 1H, 16-H_a), 1.85 (dd, *J* = 8.9, 3.5 Hz, 2H, 11-H), 1.80 – 1.63 (m, 5H, 1-H_a, 16-H_b, 22-H, 19-H_a), 1.62 – 1.53 (m, 3H, 1-H_b, 9-H, 15-H_a), 1.52 – 1.41 (m, 3H, 6-H, 7-H_a), 1.36 (dt, *J* = 13.4, 4.2 Hz, 1H, 21-H_a), 1.28 – 1.20 (m, 4H, 5-H, 7-H_b, 21-H_b, 19-H_b), 1.18 (s, 3H, 27-H), 1.10 (s, 3H, 25-H), 1.07 – 1.04 (m, 1H, 15-H_b), 1.02 (s, 3H, 24-H), 0.94 – 0.88 (m, 9H, 23-H, 29-H, 30-H), 0.65 (s, 3H, 26-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 178.2 (C-28), 170.3 (C-31), 170.1 (C-33), 162.27 (d, *J* = 245.9 Hz, C-39), 145.0 (C-13), 134.44 (d, *J* = 3.6 Hz, C-36), 129.65 (d, *J* = 8.3 Hz, C-37, C-41), 122.9 (C-12), 115.62 (d, *J* = 21.7 Hz, C-38, C-40), 76.2 (C-3), 70.6 (C-2), 50.5 (C-5), 48.0 (C-9), 46.8 (C-17), 46.5 (C-19), 43.0 (C-35), 42.5 (C-18), 41.2 (C-1), 39.6 (C-8), 37.0 (C-4), 36.7 (C-10), 34.3 (C-21), 33.1 (C-30), 32.8 (C-22), 32.1 (C-7), 30.9 (C-20), 27.4 (C-15), 27.0 (C-23), 26.0 (C-27), 24.0 (C-16), 23.7 (C-29), 23.6 (C-11), 22.2 (C-24), 21.4 (C-34), 21.1, 18.7 (C-6), 18.2 (C-25), 16.9 (C-26) ppm; ^{19}F NMR (376 MHz, $CDCl_3$): δ = -115.26 ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 662.0 (86 %, $[M-H]^-$); analysis calcd for $C_{41}H_{58}NO_5F$ (663.92): C 74.17, H 8.81, N 2.11; found: C 73.85, H 9.15 N 1.85.

4.2.9. (2β,3α) N-(2-methoxybenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (11)

Compound **11** (233 mg, 77 %) was obtained from **4** (250 mg, 0.45 mmol) according to GPA as a white solid; m.p. 114 °C; R_f = 0.73 (SiO_2, hexanes/ethyl acetate, 5:5); $[\alpha]_D$ = +38.5° (*c* 0.072, MeOH); IR (ATR): ν = 2943w, 1739 m, 1651w, 1514w, 1493w, 1367w, 1240 s, 1227 s, 1027 m, 751 m, 605w, 525w cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.24 – 7.18 (m, 2H, 37-H, 39-H), 7.00 – 6.80 (m, 2H, 28-H, 40-H), 6.47 (t, *J* = 5.6 Hz, 1H, NH), 5.27 (t, *J* = 3.6 Hz, 1H, 12-H), 4.96 (d, *J* = 6.3 Hz, 1H, 3-H), 4.93 – 4.88 (m, 1H, 2-H), 4.45 (dd, *J* = 14.2, 5.7 Hz, 1H, 35-H_a), 4.36 (dd, *J* = 14.2, 5.6 Hz, 1H, 35-H_b), 3.87 (s, 3H, 42-H), 2.49 (dd, *J* = 12.6, 4.3 Hz, 1H, 18-H), 2.05 (s, 3H, 32-H), 2.02 (s, 3H, 34-H), 1.94 (dt, *J* = 14.7, 13.6, 4.8 Hz, 1H, 16-H_a), 1.83 – 1.78 (m, 2H, 11-H), 1.77 – 1.75 (m, 1H, 19-H_a), 1.74 – 1.63 (m, 3H, 1-H_a, 16-H_b, 22-H_a), 1.61 – 1.47 (m, 4H, 1-H_b, 9-H, 15-H_a, 22-H_b), 1.45 – 1.30 (m, 4H, 6-H, 7-H_a, 21-H_a), 1.28 – 1.16 (m, 4H, 5-H, 7-H_b, 19-H_b, 21-H_b), 1.15 (s, 3H, 27-H), 1.07 (m, 1H, 15-H_b), 1.04 (s, 3H, 25-H), 1.00 (s, 3H, 24-H), 0.91 – 0.87 (m, 9H, 23-H, 29-H, 30-H), 0.43 (s, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 177.5 (C-28), 170.3 (C-31), 170.1 (C-33), 157.8 (C-41), 145.0 (C-13), 130.2 (C-39), 128.8 (C-37), 126.6 (C-36), 122.4 (C-12), 120.9 (C-38), 110.2 (C-40), 76.2 (C-3), 70.7 (C-2), 55.4 (C-42), 50.5 (C-5), 48.0 (C-9), 46.6 (C-17), 42.6 (C-18), 42.4 (C-14), 39.6 (C-35), 39.5 (C-8), 36.9 (C-4), 36.7 (C-10), 34.3 (C-21), 33.2 (C-30), 32.8 (C-22), 30.9 (C-20), 25.9 (C-27), 23.7 (C-29), 22.2 (C-24), 21.5 (C-34), 21.1 (C-32), 18.1 (C-25), 16.4 (C-26) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 674.1 (100 %, $[M-H]^-$); analysis calcd for $C_{42}H_{61}NO_6$ (675.95): C 73.63, H 9.10, N 2.07; found: C 73.40, H 9.41, N 1.86.

4.2.10. (2β,3α) N-(3-methoxybenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (12)

Compound **12** (245 mg, 81 %) was obtained from **4** (250 mg, 0.45 mmol) according to GPA as a white solid; m.p. 110 °C; R_f = 0.85 (SiO_2, hexanes/ethyl acetate, 5:5); $[\alpha]_D$ = +49.1° (*c* 0.023, MeOH); IR (ATR): ν = 2942w, 1738 m, 1641w, 1518w, 1489w, 1460w, 1367 m, 1241 s, 1227 s, 1027 m, 756w, 693w, 605w cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.24 (d, *J* = 7.9 Hz, 1H, 38-H), 6.87 – 6.75 (m, 3H, 37-H, 39-H, 41-H), 6.16 (t, *J* = 5.4 Hz, 1H, NH), 5.32 (t, *J* = 3.6 Hz, 1H, 12-H), 4.99 (d, *J* = 6.5 Hz, 1H, 3-H), 4.96 – 4.89 (m, 1H, 2-H), 4.59 (dd, *J* = 14.7, 6.3 Hz, 1H, 35-H_a), 4.12 (dd, *J* = 14.7, 4.5 Hz, 1H, 35-H_b), 3.80 (s, 3H, 42-H), 2.54 (dd, *J* = 13.3, 4.4 Hz, 1H, 18-H), 2.06 (s, 3H, 32-H), 2.02 (s, 3H, 34-H), 2.00 – 1.95 (m, 1H, 16-H_a), 1.89 – 1.84 (m, 2H, 11-H), 1.80 – 1.72 (m, 2H, 19-H_a, 22-H_a), 1.72 – 1.52 (m, 6H, 1-H, 9-H, 15-H_a, 16-H_b, 22-H_b), 1.50 – 1.32 (m, 4H, 6-H, 7-H_a, 21-H_a), 1.30 – 1.20 (m, 4H, 5-H, 7-H_b, 21-H_b, 19-H_b), 1.18 (s, 3H, 27-H), 1.10 (s, 3H, 25-H), 1.08 – 1.04 (m, 1H, 15-H_b), 1.02 (s, 3H, 24-H), 0.93 – 0.88 (m, 9H, 23-H, 29-H, 30-H), 0.69 (s, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 178.1 (C-28), 170.3 (C-31), 170.1 (C-33), 160.0 (C-40), 145.0 (C-13), 140.1 (C-36), 129.9 (C-38), 122.9 (C-12), 120.1 (C-37), 113.5 (C-39), 113.0 (C-41), 76.2 (C-3), 70.7 (C-2), 55.4 (C-42), 50.5 (C-5), 48.0 (C-9), 46.8 (C-19), 46.6 (C-17), 43.7 (C-35), 42.6 (C-18), 42.4 (C-14), 41.3 (C-1), 39.7 (C-8), 37.0 (C-4), 36.7 (C-10), 34.3 (C-21), 33.1 (C-30), 32.8 (C-22), 32.1 (C-7), 30.9 (C-20), 27.4 (C-15), 27.0 (C-23), 26.0 (C-27), 24.0 (C-16), 23.7 (C-29), 23.5 (C-11), 22.3 (C-24), 21.4 (C-34), 21.1 (C-32), 18.8 (C-6), 18.2 (C-25), 16.9 (C-26) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 676.3 (100 %, $[M + H]^+$); analysis calcd for $C_{42}H_{61}NO_6$ (675.95): C 73.63, H 9.10, N 2.07; found: C 73.47, H 9.33, N 1.96.

4.2.11. (2β,3α) N-(4-methoxybenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (13)

Compound **13** (286 mg, 94 %) was obtained from **4** (250 mg, 0.45 mmol) according to GPA as a white solid; m.p. 112 °C; R_f = 0.79 (SiO_2, hexanes/ethyl acetate, 5:5); $[\alpha]_D$ = +44.9° (*c* 0.078, MeOH); IR (ATR): ν = 2943w, 1739 m, 1642w, 1512 m, 1463w, 1367w, 1242 s, 1227 s, 1174w, 1031 m, 821w, 605w, 523w cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ

= 7.17 (d, J = 8.6 Hz, 2H, 37-H, 41-H), 6.86 (d, J = 8.6 Hz, 2H, 38-H, 40-H), 6.10 – 6.08 (m, 1H, NH), 5.29 (t, J = 3.7 Hz, 1H, 12-H), 4.98 (d, J = 6.4 Hz, 1H, 3-H), 4.95 – 4.91 (m, 1H, 2-H), 4.52 (dd, J = 14.5, 6.1 Hz, 1H, 35-H_a), 4.09 (dd, J = 14.4, 4.4 Hz, 1H, 35-H_b), 3.80 (s, 3H, 42-H), 2.58 – 2.44 (m, 1H, 18-H), 2.06 (s, 3H, 32-H), 2.02 (s, 3H, 34-H), 1.99 – 1.94 (m, 1H, 16-H_a), 1.84 (dd, J = 8.9, 3.5 Hz, 2H, 11-H), 1.79 – 1.71 (m, 2H, 19-H_a, 22-H_a), 1.70 – 1.63 (m, 2H, 1-H_a, 16-H_b), 1.63 – 1.52 (m, 4H, 1-H_b, 9-H, 15-H_a, 22-H_b), 1.51 – 1.32 (m, 4H, 6-H, 7-H_a, 21-H_a), 1.30 – 1.20 (m, 3H, 5-H, 7-H_b, 21-H_b), 1.17 (s, 3H, 27-H), 1.15 – 1.12 (m, 1H, 19-H_b), 1.10 (s, 3H, 25-H), 1.08 – 1.03 (m, 1H, 15-H_b), 1.02 (s, 3H, 24-H), 0.93 – 0.87 (m, 9H, 23-H, 29-H, 30-H), 0.68 (s, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 178.0 (C-28), 170.3 (C-31), 170.1 (C-33), 159.1 (C-39), 145.0 (C-13), 130.6 (C-36), 129.3, 129.3 (C-37, 41), 122.8 (C-12), 114.2 (C-38, 40), 76.2 (C-3), 70.7 (C-2), 55.4 (C-42), 50.5 (C-5), 48.0 (C-9), 46.8, 46.5 (C-17), 43.3 (C-35), 42.6 (C-18), 42.4 (C-14), 41.3 (C-1), 39.6 (C-8), 37.0 (C-4), 36.7 (C-10), 34.3 (C-21), 33.1 (C-30), 32.8 (C-22), 32.1 (C-7), 30.9 (C-20), 27.4 (C-15), 27.0 (C-23), 26.0 (C-27), 23.9 (C-16), 23.7 (C-29), 23.5 (C-11), 22.3 (C-24), 21.4 (C-34), 21.1 (C-32), 18.8 (C-6), 18.2 (C-25), 17.0 (C-26) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): m/z = 674.0 (100 %, $[M-H]^-$); analysis calcd for $C_{42}H_{61}NO_6$ (675.95): C 73.63, H 9.10, N 2.07; found: C 73.49, H 9.25, N 1.82.

4.2.12. (2β,3α) N-(2-methylbenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (14)

Compound **14** (235 mg, 79 %) was obtained from **4** (250 mg, 0.45 mmol l) according to GPA as a white solid; m.p. 111 °C; R_f = 0.78 (SiO_2, hexanes/ethyl acetate, 8:2); $[\alpha]_D$ = +46.7° (c 0.101, MeOH); IR (ATR): ν = 2943w, 1739 s, 1644w, 1514w, 1462w, 1466 m, 1242 s, 1226 s, 1027 m, 739 m, 605w cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.23 – 7.11 (m, 4H, 37-H, 38-H, 39-H, 40-H), 6.05 (dd, J = 6.4, 4.2 Hz, 1H, NH), 5.28 (t, J = 3.7 Hz, 1H, 12-H), 4.98 (d, J = 6.4 Hz, 1H, 3-H), 4.95 – 4.91 (m, 1H, 2-H), 4.61 (dd, J = 14.7, 6.4 Hz, 1H, 35-H_a), 4.15 (dd, J = 14.7, 4.1 Hz, 1H, 35-H_b), 2.52 (dd, J = 13.6, 4.7 Hz, 1H, 18-H), 2.31 (s, 3H, 42-H), 2.06 (s, 3H, 32-H), 2.02 (s, 3H, 34-H), 2.00 – 1.95 (m, 1H, 16-H_a), 1.84 – 1.79 (m, 2H, 11-H), 1.78 – 1.75 (m, 1H, 19-H_a), 1.75 – 1.71 (m, 1H, 22-H_a), 1.71 – 1.64 (m, 2H, 1-H_a, 16-H_b), 1.58 (m, 4H, 1-H_b, 9-H, 15-H_a, 22-H_b), 1.47 (dd, J = 13.9, 8.6 Hz, 3H, 6-H, 7-H_a), 1.41 – 1.31 (m, 1H, 21-H_a), 1.30 – 1.19 (m, 3H, 5-H, 7-H_b, 21-H_b), 1.17 (s, 3H, 27-H), 1.15 – 1.12 (m, 1H, 19-H_b), 1.09 (s, 3H, 25-H), 1.07 – 1.03 (m, 1H, 15-H_b), 1.02 (s, 3H, 24-H), 0.92 – 0.87 (m, 9H, 23-H, 29-H, 30-H), 0.67 (s, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 178.0 (C-28), 170.3 (C-31), 170.1 (C-33), 145.1 (C-13), 136.5 (C-36), 136.2 (C-41), 130.6 (C-40), 128.5 (C-37), 127.7 (C-39), 126.3 (C-38), 122.9 (C-12), 76.2 (C-3), 70.7 (C-2), 50.5 (C-5), 48.0 (C-9), 46.8 (C-19), 46.6 (C-17), 42.6 (C-18), 42.4 (C-14), 41.7 (C-35), 41.2 (C-1), 39.6 (C-8), 37.0 (C-4), 36.7 (C-10), 34.3 (C-21), 33.1 (C-30), 32.8 (C-22), 32.1 (C-7), 30.9 (C-20), 27.4 (C-15), 27.0 (C-23), 25.9 (C-27), 24.0 (C-16), 23.7 (C-29), 23.5 (C-11), 22.2 (C-24), 21.4 (C-34), 21.1 (C-32), 19.2 (C-42), 18.7 (C-6), 18.2 (C-25), 16.9 (C-26) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): m/z = 658.7 (100 %, $[M-H]^-$); analysis calcd for $C_{42}H_{61}NO_5$ (659.95): C 76.44, H 9.32, N 2.12; found: C 76.20, H 9.53, N 1.97.

4.2.13. (2β,3α) N-(3-methylbenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (15)

Compound **15** (238 mg, 80 %) was obtained from **4** (250 mg, 0.45 mmol) according to GPA as a white solid; m.p. 127 °C; R_f = 0.76 (SiO_2, hexanes/ethyl acetate, 8:2); $[\alpha]_D$ = +47.9° (c 0.124, MeOH); IR (ATR): ν = 2944w, 1740 m, 1653 m, 1510 m, 1455w, 1367 m, 1241 s, 1227 s, 1032 m, 755 m, 694w, 605w, 535w, 446w cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.23 – 7.19 (m, 1H, 38-H), 7.13 – 7.02 (m, 3H, 27-H, 39-H, 41-H), 6.13 (d, J = 5.4 Hz, 1H, NH), 5.30 (t, J = 3.6 Hz, 1H, 12-H), 4.98 (d, J = 6.5 Hz, 1H, 3-H), 4.95 – 4.91 (m, 1H, 2-H), 4.55 (dd, J = 14.6, 6.1 Hz, 1H, 35-H_a), 4.15 – 4.10 (m, 1H, 35-H_b), 2.53 (dd, J = 13.2, 4.5 Hz, 1H, 18-H), 2.34 (s, 3H, 42-H), 2.06 (s, 3H, 32-H), 2.02 (s, 3H, 34-H), 2.01 – 1.94 (m, 1H, 16-H_a), 1.84 (dd, J = 8.8, 3.6 Hz, 2H, 11-H), 1.78 – 1.75 (m, 1H, 19-H_a), 1.75 – 1.72 (m, 1H, 22-H_a), 1.72 – 1.64 (m, 3H, 1-H_a, 16-H_b, 22-H_b), 1.64 – 1.55 (m, 3H, 1-H_b, 9-H, 15-H_a), 1.52 – 1.33 (m, 4H, 6-H, 7-H_a, 21-H_a), 1.30 – 1.19 (m, 3H, 5-H, 7-H_b, 21-H_b), 1.18 (s, 3H, 27-H), 1.15 – 1.11 (m, 1H, 19-H_b), 1.10 (s, 3H, 25-H), 1.08 – 1.04 (m, 1H, 15-H_b), 1.02 (s, 3H, 24-H), 0.91 – 0.90 (m, 9H, 23-H, 29-H, 30-H), 0.69 (s, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 178.0 (C-28), 170.3 (C-31), 170.1 (C-33), 145.1 (C-13), 138.4 (C-36), 138.4 (C-40), 128.7 (C-38, 41), 128.2 (C-39), 125.0 (C-37), 122.8 (C-12), 76.2 (C-3), 70.6 (C-2), 50.5 (C-5), 48.0 (C-9), 46.8 (C-19), 46.5 (C-17), 43.8 (C-35), 42.6 (C-18), 42.4 (C-14), 41.3 (C-1), 39.6 (C-8), 37.0 (C-4), 36.7 (C-10), 34.3 (C-21), 33.1 (C-30), 32.8 (C-22), 32.1 (C-7), 30.9 (C-20), 27.0 (C-23), 26.0 (C-27), 23.9 (C-16), 23.7 (C-29), 23.5 (C-11), 22.3 (C-24), 21.5 (C-42), 21.4 (C-34), 21.1 (C-32), 18.7 (C-6), 18.2 (C-25), 17.0 (C-26) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): m/z = 658.5 (100 %, $[M-H]^-$); $]^-$); analysis calcd for $C_{42}H_{61}NO_5$ (659.95): C 76.44, H 9.32, N 2.12; found: C 76.17, H 9.55, N 1.83.

4.2.14. (2β,3α) N-(4-methylbenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (16)

Compound **16** (232 mg, 78 %) was obtained from **4** (250 mg, 0.45 mmol) according to GPA as a white solid; m.p. 127 °C; R_f = 0.76 (SiO_2, hexanes/ethyl acetate, 8:2); $[\alpha]_D$ = +49.4° (c 0.166, MeOH); IR (ATR): ν = 2943w, 1740 m, 1651w, 1516w, 1460w, 1432w, 1366 m, 1241 s, 1226 s, 1032 m, 806w, 754w, 605w, 579w, 474w cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.17 – 7.06 (m, 4H, 37-H, 38-H, 40-H, 41-H), 6.12 (t, J = 5.5 Hz, 1H, NH), 5.29 (t, J = 3.6 Hz, 1H, 12-H), 4.98 (d, J = 6.5 Hz, 1H, 3-H), 4.95 – 4.87 (m, 1H, 2-H), 4.56 (dd, J = 14.5, 6.1 Hz, 1H, 35-H_a), 4.10 (dd, J = 14.5, 4.6 Hz, 1H, 35-H_b), 2.56 – 2.48 (m, 1H, 18-H), 2.33 (s, 3H, 42-H), 2.06 (s, 3H, 32-H), 2.02 (s, 3H, 34-H), 1.99 – 1.94 (m, 1H, 16-H_a), 1.84 (dd, J = 8.9, 3.5 Hz, 2H, 11-H), 1.78 – 1.71 (m, 2H, 19-H_a, 22-H_a), 1.71 – 1.64 (m, 2H, 1-H_a, 16-H_b), 1.63 – 1.53 (m, 4H, 1-H_b, 9-H, 15-H_a, 22-H_b), 1.52 – 1.41 (m, 3H, 6-H, 7-H_a), 1.39 – 1.32 (m, 1H, 21-H_a), 1.30 – 1.19 (m, 3H, 5-H, 7-H_b, 21-H_b), 1.17 (s, 3H, 27-H), 1.15 – 1.11 (m, 1H, 19-H_b), 1.09 (s, 3H, 25-H), 1.07 – 1.04 (m, 1H, 15-H_b), 1.02 (s, 3H, 24-H), 0.92 – 0.88 (m, 9H, 23-H, 29-H, 30-H), 0.67 (s, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 178.0 (C-28), 170.3 (C-31), 170.1 (C-33), 145.0 (C-13), 137.2 (C-36), 135.5 (C-39), 129.5 (C-38, 40), 127.9 (C-37, 41), 122.8 (C-12), 76.2 (C-3), 70.7 (C-2), 50.5 (C-5), 48.0 (C-9), 46.8 (C-19), 46.5 (C-17), 43.5 (C-35), 42.6 (C-18), 42.4 (C-14), 41.2 (C-1), 39.6 (C-8), 37.0 (C-4), 36.7 (C-10), 34.3 (C-21), 33.1 (C-30), 32.8 (C-22), 32.1 (C-7), 30.9 (C-20), 27.4 (C-15), 27.0 (C-23), 26.0 (C-27), 23.9 (C-16), 23.7 (C-29), 23.5 (C-11), 22.3 (C-24), 21.4 (C-34), 21.2 (C-42), 21.1 (C-32), 18.7 (C-6), 18.2 (C-25), 16.9 (C-26) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): m/z = 658.3 (100 %, $[M-H]^-$); $]^-$); analysis calcd for $C_{42}H_{61}NO_5$ (659.95): C 76.44, H 9.32, N 2.12; found: C 76.21, H 9.49, N 1.90.

4.2.15. (2α,3β) Dihydroxyolean-12-en-28-acid (maslinic acid) (2) [4373–41–5]

Preserved, pitted green olives (2 kg) were crushed and dried at 110 °C for 24 h. The dried olives (399 g) were extracted with methanol (700 mL) for 4 days. The solid residue was filtered off and extracted two times with methanol (700 mL, each) each for another 4 days. The methanol was evaporated, the residue was washed with hexanes and mixed 1:1 with silica gel and the mixture was grounded. The powder was extracted for 24 h in a Soxhlet extraction with diethyl ether, the extract concentrated and the solid was purified by column chromatography (SiO_2, hexanes/ethyl acetate/chloroform, 6:4:10). The product was recrystallized from ethyl acetate and **2** (1.2 g, 0.3 % on dry weight basis) was obtained as a colorless solid; m.p. 263 – 267 °C, (lit.: [16] 269 – 271 °C); R_f = 0.22 (SiO_2, hexanes/ethyl acetate, 6:4); $[\alpha]_D$ = + 55.0° (c 0.42, $CHCl_3$) (lit: [20] + 60.0° (c 0.1, $CHCl_3$); MS (ESI, MeOH): m/z = 471.5 (43 %, $[M-H]^-$), 517.0 (100 %, $[M + HCO_2]^-$), 943.1 (62 %, $[2M-H]^-$).

4.2.16. (2α,3β) Diacetyloxy-olean-12-en-28-acid (17)

Acetylation of **2** as previously described followed by

chromatography (SiO_2, hexanes/ethyl acetate, 5:1) furnished **15** (2.6 g, 79 %) as a colorless solid; m.p. 179–181 °C (lit.: [2] 170–173 °C); R_f = 0.32 (SiO_2, hexanes/ethyl acetate, 3:1); $[\alpha]_D$ = +28.37° (*c* 0.43, $CHCl_3$) (lit.: [2] + 30°, (*c* 0.83, $CHCl_3$); MS (ESI, MeOH): *m/z* = 557.4 (49 %, $[M + H]^+$), 574.5 (100 %, $[M + NH_4]^+$), 579.5 (51 %, $[M + Na]^+$).

4.2.17. (2α,3β) N-(2-chlorobenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (18)

Following GPA from **17** (0.2 g, 0.36 mmol) followed by chromatography (SiO_2, hexanes/ethyl acetate, 9:1) **18** (187 mg, 77 %) was obtained as a colorless solid; m.p. 129 °C; R_f = 0.44 (SiO_2, hexanes/ethyl acetate, 3:1); $[\alpha]_D$ = -11.3° (*c* 0.174, $CHCl_3$); IR (KBr): ν = 3422vw, 2944 m,: 2864w, 1740 s, 1659 m, 1512 m, 1471 m, 1445 m, 1367 m, 1248vs, 1230vs, 1155w, 1039 s, 990w, 965w, 918w, 824w, 750 s, 667w, 641w, 598w, 527w cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.40 – 7.31 (m, 2H, 38-H + 39-H), 7.23 – 7.17 (m, 2H, 40-H + 41-H), 6.43 (dd, *J* = 5.9, 5.9 Hz, 1H, NH), 5.33 (t, *J* = 3.6 Hz, 1H, 12-H), 5.07 (ddd, *J* = 11.4, 10.2, 4.6 Hz, 1H, 2-H), 4.73 (d, *J* = 10.4 Hz, 1H, 3-H), 4.54 (dd, *J* = 14.5, 5.8 Hz, 1H, 35-H_a), 4.40 (dd, *J* = 14.5, 5.9 Hz, 1H, 35-H_b), 2.57 – 2.49 (m, 1H, 18-H), 2.04 (s, 3H, 34-H_a + 34-H_b + 34-H_c), 2.02 – 1.97 (m, 2H, 1-H_a + 22-H_a), 1.97 (s, 3H, 32-H_a + 32-H_b + 32-H_c), 1.83 (dd, *J* = 8.9, 3.6 Hz, 2H, 11-H_a + 11-H_b), 1.80 – 1.59 (m, 4H, 7-H_a + 7-H_b + 22-H_b + 19-H_a), 1.59 – 1.29 (m, 6H, 6-H_a + 6-H_b + 9-H + 15-H_a + 16-H_a + 21-H_a), 1.28 – 1.13 (m, 3H, 15-H_b + 21-H_b + 19-H_b), 1.11 (s, 3H, 27-H_a + 27-H_b + 27-H_c), 1.07 – 0.99 (m, 2H, 1-H_b + 16-H_b), 0.98 (s, 3H, 26-H_a + 26-H_b + 26-H_c), 0.94 (d, *J* = 2.0 Hz, 1H, 5-H), 0.91 – 0.85 (m, 12H, 23-H_a + 23-H_b + 23-H_c + 24-H_a + 24-H_b + 24-H_c + 29-H_a + 29-H_b + 29-H_c + 30-H_a + 30-H_b + 30-H_c), 0.43 (s, 3H, 25-H_a + 25-H_b + 25-H_c) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 177.9 (C-28), 170.9 (C-33), 170.7 (C-31), 144.6 (C-13), 136.0 (C-36), 134.1 (C-37), 131.0 (C-38), 129.5 (C-39), 129.0 (C-41), 127.3 (C-40), 122.8 (C-12), 80.7 (C-3), 70.1 (C-2), 54.9 (C-5), 47.6 (C-9), 46.7 (C-17), 46.7 (C-19), 44.0 (C-1), 42.6 (C-35), 42.2 (C-14), 41.7, 39.5 (C-4), 39.4 (C-8), 38.2 (C-10), 34.2 (C-21), 33.1 (C-30), 32.8 (C-7), 32.3 (C-15), 30.9 (C-20), 28.5, 27.4 (C-16), 25.8, 23.8 (C-22), 23.7 (C-29), 23.7 (C-11), 21.3 (C-32), 21.0 (C-34), 18.3 (C-6), 17.7, 16.6 (C-25), 16.5 (C-26) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 678.6 (100 %, $([M-H]^-$); analysis calcd for $C_{41}H_{58}NO_5Cl$ (680.37): C 72.38, H 8.59, N 2.21; found: C 72.09, H 8.76, N 2.04.

4.2.18. (2α,3β) N-(3-chlorobenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (19)

Following GPA from **17** (0.2 g, 0.36 mmol) followed by chromatography (SiO_2, hexanes/ethyl acetate, 9:1) **19** (156 mg, 64 %) was obtained as a colorless solid; m.p. 135 °C; R_f = 0.39 (SiO_2, hexanes/ethyl acetate, 9:1); $[\alpha]_D$ = -1.17° (*c* 0.172, $CHCl_3$); IR (KBr): ν = 3422vw, 2944 m, 1739 s, 1654 m, 1647 m, 1598w, 1576w, 1514 m, 1472 m, 1432 m, 1367 m, 1247vs, 1230vs, 1154w, 1097w, 1079w, 1042 s, 1031 s, 989 m, 958w, 918w, 823w, 771 m, 755 m, 703w, 681 m, 597 m cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.25–7.22 (m, 3H, 37-H + 39-H + 40-H), 7.17 – 7.09 (m, 1H, 41-H), 6.17 (dd, *J* = 5.6, 5.6 Hz, 1H, NH), 5.32 (t, *J* = 3.6 Hz, 1H, 12-H), 5.08 (ddd, *J* = 11.5, 10.3, 4.6 Hz, 1H, 2-H), 4.73 (d, *J* = 10.3 Hz, 1H, 3-H), 4.53 (dd, *J* = 14.8, 6.1 Hz, 1H, 35-H_a), 4.21 – 4.06 (m, 1H, 35-H_b), 2.58 – 2.50 (m, 1H, 18-H), 2.05 (s, 3H, 34-H_a + 34-H_b + 34-H_c), 2.04 – 1.98 (m, 2H, 1-H_a + 22-H_a), 1.97 (s, 3H, 32-H_a + 32-H_b + 32-H_c), 1.91 – 1.81 (m, 2H, 11-H_a + 11-H_b), 1.81 – 1.64 (m, 3H, 7-H_a + 19-H_a + 22-H_b), 1.63 – 1.49 (m, 4H, 6-H_a + 7-H_b + 9-H + 16-H_b), 1.48–1.31 (m, 3H, 6-H_b + 15-H_a + 21-H_a), 1.22–1.31 (m, 1H, 15-H_b), 1.22 – 1.17 (m, 2H, 19-H_b + 21-H_b), 1.14 (s, 3H, 27-H_a + 27-H_b + 27-H_c), 1.10 – 1.03 (m, 2H, 1-H_b + 16-H_b), 1.02 (s, 3H, 26-H_a + 26-H_b + 26-H_c), 0.95 (dd, *J* = 11.2, 2.0 Hz, 2H, 5-H), 0.92 – 0.87 (m, 12H, 23-H_a + 23-H_b + 23-H_c + 24-H_a + 24-H_b + 24-H_c + 29-H_a + 29-H_b + 29-H_c + 30-H_a + 30-H_b + 30-H_c), 0.64 (s, 3H, 25-H_a + 25-H_b + 25-H_c) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 178.0 (C-28), 170.8 (C-33), 170.5 (C-31), 144.9 (C-13), 140.5 (C-36), 134.5 (C-38), 129.9 (C-40), 127.9 (C-37), 127.5 (C-39), 125.9 (C-41), 122.5 (C-12), 80.5 (C-3), 69.9 (C-2), 54.8 (C-5), 47.4 (C-9), 46.6 (C-19), 46.4 (C-17), 43.9 (C-1), 43.0 (C-35), 42.3 (C-18), 42.1 (C-14), 39.4 (C-4), 39.3 (C-8), 38.0 (C-10), 34.1 (C-21), 33.0 (C-30), 32.7 (C- 7), 32.2 (C-15), 30.7 (C-20), 28.4 (C-24), 27.2 (C-16), 25.7 (C-27), 23.7 (C-22), 23.6 (C-29), 23.5 (C-11), 21.1 (C-32), 20.9 (C-34), 18.1 (C-6), 17.6 (C-23), 16.9 (C-25), 16.4 (C-26) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 678.6 (100 %, $[M-H]^-$); analysis calcd for $C_{41}H_{58}NO_5Cl$ (680.37): C 72.38, H 8.59, N 2.21; found: C 72.11, H 8.82, N 1.95.

4.2.19. (2α,3β) N-(4-chlorobenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (20)

Following GPA from **17** (0.2 g, 0.36 mmol) followed by chromatography (SiO_2, hexanes/ethyl acetate, 9:1) **20** (201 mg, 82 %) was obtained as a colorless solid; m.p. 130 °C; R_f = 0.38 (SiO_2, hexanes/ethyl acetate, 3:1); $[\alpha]_D$ = -4.77° (*c* 0.186, $CHCl_3$); IR (KBr): ν = 3423vw, 2944 m, 2864w, 1739 s, 1646 m, 1514 m, 1492 m, 1463w, 1432w, 1367 m, 1248vs, 1230vs, 1091 m, 1042 m, 1031 s, 1016 m, 989 m, 958w, 918w, 819w, 800 m, 771 m, 754 m, 654w, 641w, 597w cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.31 – 7.26 (m, 2H, 38-H + 40-H), 7.21 – 7.14 (m, 2H, 37-H + 41-H), 6.16 (dd, *J* = 5.6, 5.6 Hz, 1H, NH), 5.31 (t, *J* = 3.6 Hz, 1H, 12-H), 5.08 (ddd, *J* = 11.5, 10.3, 4.6 Hz, 1H, 2-H), 4.73 (d, J = 10.3, 1H, 3-H), 4.59 – 4.49 (m, 1H, 35-H_a), 4.10 (dd, *J* = 14.8, 4.8 Hz, 1H, 35-H_b), 2.53 (dd, *J* = 13.1, 4.4 Hz, 1H, 18-H), 2.05 (s, 3H, 34-H_a + 34-H_b + 34-H_c), 2.01 (dd, *J* = 5.0, 1.2 Hz, 2H, 1a + 22a), 1.97 (s, 3H, 32-H_a + 32-H_b + 32-H_c), 1.85 (dd, *J* = 8.9, 3.6 Hz, 2H, 11-H_a + 11-H_b), 1.79 – 1.51 (m, 7H, 6-H_a + 7-H_a + 7-H_b + 9-H + 16-H_a + 19-H_a + 22-H_b), 1.51 – 1.30 (m, 3H, 6-H_b + 15-H_a + 21-H_b), 1.30 – 1.15 (m, 3H, 15-H_b + 19-H_b + 21-H_b), 1.14 (s, 3H, 27-H_a + 27-H_b + 27-H_c), 1.10 – 1.03 (m, 2H, 1-H_b + 16-H_b), 1.02 (s, 3H, 26-H_a + 26-H_b + 26-H_c), 0.97 – 0.91 (m, 1H, 5-H), 0.92 – 0.87 (m, 12H, 23-H_a + 23-H_b + 23-H_c + 24-H_a + 24-H_b + 24-H_c + 29-H_a + 29-H_b + 29-H_c + 30-H_a + 30-H_b + 30-H_c), 0.63 (s, 3H, 3H, 25-H_a + 25-H_b + 25-H_c) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 178.2 (C-28), 171.0 (C-33), 170.7 (C-31), 145.1 (C-13), 137.2 (C-36), 133.3 (C-39), 129.3 (C-37, C-41), 128.9 (C-38, C-40), 122.6 (C-12), 80.6 (C-3), 70.1 (C-2), 54.9 (C-5), 47.6 (C-9), 46.7 (C-19), 46.5 (C-17), 44.0 (C-1), 43.0 (C-35), 42.4 (C-18), 42.2 (*c* 14), 39.5 (C-4), 39.5 (C-8), 38.2 (C-10), 34.2 (C-21), 33.1 (C-30), 32.8 (C-7), 32.3 (C-15), 30.9 (C-20), 28.5 (C-24), 27.4 (C-16), 25.8 (C-27), 23.9 (C-22), 23.7 (C-29), 23.7 (C-11), 21.3 (C-32), 21.0 (C-34), 18.3 (C-6), 17.7 (C-23), 17.0 (C-25), 16.5 (C-26) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 678.7 (100 %, $[M-H]^-$); analysis calcd for $C_{41}H_{58}NO_5Cl$ (680.37): C 72.38, H 8.59, N 2.21; found: C 72.13, H 8.73, N 1.98.

4.2.20. (2α,3β) N-(2-fluorobenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (21)

Following GPA from **17** (0.2 g, 0.36 mmol) followed by chromatography (SiO_2, hexanes/ethyl acetate, 9:1) **21** (190 mg, 80 %) was obtained as a colorless solid; m.p. 125 °C; R_f = 0.37 (SiO_2, hexanes/ethyl acetate, 3:1); $[\alpha]_D$ = -5.04° (*c* 0.174, $CHCl_3$); IR (KBr): ν = 3423vw, 2944w, 2865w, 1739 s, 1645 m, 1587w, 1516 m, 1489 m, 1456 m, 1433w, 1367 m, 1301w, 1248 s, 1228vs, 1191 m, 1153w, 1106w, 1041 m, 1032 m, 989w, 959w, 918w, 832w, 806w, 755 s, 641w, 598w, 524w cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.33 – 7.21 (m, 1H, 41-H), 7.13 – 6.97 (m, 3H, 38-H + 39-H + 40-H), 6.28 (dd, *J* = 5.7, 5.7 Hz, 1H, NH), 5.35 – 5.29 (m, 1H, 12-H), 5.07 (dt, *J* = 11.0, 4.6 Hz, 1H, 2-H), 4.73 (d, *J* = 10.3 Hz, 1H, 3-H), 4.53 (dd, *J* = 14.7, 5.8 Hz, 1H, 35-H_a), 4.33 (dd, *J* = 14.7, 5.2 Hz, 1H, 35-H_b), 2.52 (dd, *J* = 13.0, 4.3 Hz, 1H, 18-H), 2.05 (s, 3H, 34-H_a + 34-H_b + 34-H_c), 2.03 – 1.97 (m, 2H, 1-H_a + 22-H_a), 1.97 (s, 3H, 32-H_a + 32-H_b + 32-H_c), 1.83 (dd, *J* = 8.9, 3.6 Hz, 2H, 11-H_a + 11-H_b), 1.80 – 1.65 (m, 3H, 7-H_a + 19-H_a + 22-H_b), 1.65 – 1.45 (m, 4H, 6-H_a + 7-H_b + 15-H_a + 16-H_a), 1.46 – 1.27 (m, 2H, 6-H_b + 21-H_a), 1.27 – 1.13 (m, 3H, 15′-H_b + 19-H_b + 21-H_b), 1.12 (s, 3H, 27-H_a + 27-H_b + 27-H_c), 1.08 – 0.99 (m, 2H, 1-H_b + 16-H_b), 0.98 (s, 3H, 26-H_a + 26-H_b + 26-H_c), 0.93 (dd, *J* = 11.4, 1.9 Hz, 1H, 5-H), 0.91 – 0.87 (m, 12H, 23-H_a + 23-H_b + 23-H_c + 24-H_a + 24-H_b + 24-H_c + 29-H_a + 29-H_b + 29-H_c + 30-H_a + 30-H_b + 30-H_c), 0.51 (s, 3H, 25-H_a + 25-H_b + 25-H_c) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 178.0 (C-28), 170.9 (C-33), 170.7 (C-31),

161.4 (d, J = 245.5 Hz, C-37), 144.8 (C-13), 130.7 (d, J = 4.5 Hz, C-41), 129.3 (d, J = 8.2 Hz, C-39), 125.5 (d, J = 15.1 Hz, C-36), 124.5 (d, J = 3.5 Hz, C-40), 122.7 (C-12), 115.4 (d, J = 21.4 Hz, C-38), 80.7 (C-3), 70.1 (C-2), 54.9 (C-5), 47.6 (C-9), 46.7 (C-17), 46.6 (C-19), 44.0 (C-1), 42.5 (C-18), 42.2 (C-14), 39.5 (C-4), 39.5 (C-8), 38.2 (C-10), 37.7 (d, J = 3.3, C-35), 34.2 (C-21), 33.1 (C-30), 32.7 (C-7), 32.3 (C-15), 30.9 (C-20), 28.5 (C-24), 27.4 (C-16), 25.8 (C-27), 23.8 (C-22), 23.7 (C-29), 23.7, 21.3 (C-32), 21.0 (C-34), 18.3 (C-6), 17.7 (C-23), 16.7 (C-25), 16.5 (C-26) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): m/z = 662.5 (100 %, $[M-H]^-$); analysis calcd for $C_{41}H_{58}NO_5F$ (663.92): C 74.17, H 8.81, N 2.11; found: C 73.81, H 9.03, N 1.92.

4.2.21. (2α,3β) N-(3-fluorobenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (22)

Following GPA from **17** (0.2 g, 0.36 mmol) followed by chromatography (SiO_2, hexanes/ethyl acetate, 9:1) **22** (182 mg, 76 %) was obtained as a colorless solid; m.p. 131 °C; R_f = 0.34 (SiO_2, hexanes/ethyl acetate, 3:1); $[\alpha]_D$ = -0.85° (*c* 0.164, $CHCl_3$); IR (KBr): ν = 3422vw, 2944 m, 1740 s, 1649 m, 1617w, 1592w, 1514 m, 1487 m, 1451 m, 1367 m, 1248vs, 1230vs, 1140w, 1042 s, 1031 s, 990 m, 962w, 918w, 788w, 689w, 598w, 520 m cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.32 – 7.24 (m, 1H, 40-H), 7.02 (d, J = 7.6 Hz, 1H, 41-H), 6.99 – 6.92 (m, 2H, 37-H + 39-H), 6.23 – 6.16 (m, 1H, NH), 5.34 – 5.30 (m, 1H, 12-H), 5.08 (ddd, J = 11.5, 10.2, 4.6 Hz, 1H, 2-H), 4.73 (d, J = 10.3 Hz, 1H, 3-H), 4.58 (dd, J = 14.9, 6.2 Hz, 1H, 35-H_a), 4.14 (dd, J = 14.9, 4.8 Hz, 1H, 35-H_b), 2.55 (dd, J = 13.0, 4.4 Hz, 1H, 18-H), 2.05 (s, 3H, 34-H_a + 34-H_b + 34-H_c), 2.00 (dd, J = 7.2, 4.8 Hz, 2H, 1-H_a + 22-H_a), 1.97 (s, 3H, 32-H_a + 32-H_b + 32-H_c), 1.85 (dd, J = 8.8, 3.6 Hz, 2H, 11-H_a + 11-H_b), 1.81 – 1.63 (m, 4H, 7-H_a + 7-H_b + 19-H_a + 22-H_b), 1.64 – 1.49 (m, 3H, 6-H_a + 9-H + 16-H_a), 1.48 – 1.31 (m, 3H, 6-H_b + 15-H_a + 21-H_a), 1.30 – 1.17 (m, 3H, 15-H_b + 19-H_b + 21-H_b), 1.14 (s, 3H, 27-H_a + 27-H_b + 27-H_c), 1.09 – 1.02 (m, 2H, 1-H_b + 16-H_b), 1.01 (s, 3H, 26-H_a + 26-H_b + 26-H_c), 0.95 (dd, J = 11.4, 2.0 Hz, 1H, 5-H), 0.93 – 0.87 (m, 12H, 23-H_a + 23-H_b + 23-H_c + 24-H_a + 24-H_b + 24-H_c + 29-H_a + 29-H_b + 29-H_c + 30-H_a + 30-H_b + 30-H_c), 0.65 (s, 3H, 25-H_a + 25-H_b + 25-H_c) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 178.2 (C-28), 170.9 (C-33), 170.7 (C-31), 163.1 (d, J = 246.4 Hz, C-38), 145.1 (C-13), 141.2 (d, J = 7.1 Hz, C-36), 130.3 (d, J = 8.3 Hz, C-40), 123.4 (d, J = 2.9 Hz, C-41), 122.7 (C-12), 123.4 (d, J = 2.9 Hz, C-37), 114.5 (d, J = 36.5 Hz, C-39), 80.6 (C-3), 70.1 (C-2), 54.9 (C-5), 47.5 (C-9), 46.7 (C-19), 46.5 (C-17), 44.0 (C-1), 43.2 (d, J = 1.9 Hz, C-35), 42.5 (C-18), 42.2 (C-14), 39.5 (C-4), 39.5 (C-8), 38.2 (C-10), 34.2 (C-21), 33.1 (C-30), 32.8 (C-7), 32.3 (C-15), 30.9 (C-20), 28.5 (C-24), 27.4 (C-16), 25.8 (C-27), 23.9 (C-22), 23.7 (C-29), 23.7 (C-11), 21.3 (C-32), 21.0 (C-34), 18.3 (C-6), 17.7 (C-23), 17.0 (C-25), 16.5 (C-26) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): m/z = 662.5 (100 %, $[M-H]^-$); analysis calcd for $C_{41}H_{58}NO_5F$ (663.92): C 74.17, H 8.81, N 2.11; found: C 73.85, H 9.16, N 1.85.

4.2.22. (2α,3β) N-(4-fluorobenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (23)

Following GPA from **17** (0.2 g, 0.36 mmol) followed by chromatography (SiO_2, hexanes/ethyl acetate, 9:1) **23** (211 mg, 88 %) was obtained as a colorless solid; m.p. 106 °C; R_f = 0.31 (SiO_2, hexanes/ethyl acetate, 3:1); $[\alpha]_D$ = -0.24° (*c* 0.194, $CHCl_3$); IR (KBr): ν = 3419vw, 2944 m, 1739 s, 1645 m, 1604w, 1509 m, 1463 m, 1432w, 1367 m, 1248vs, 1229vs, 1193 m, 1156 m, 1097w, 1042 s, 1031 s, 990 m, 962w, 918w, 851w, 823 m, 751 m, 667w, 640w, 597w, 577 m, 487 m cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.24 – 7.17 (m, 2H, 37-H + 41-H), 7.05 – 6.94 (m, 2H, 38-H + 40-H), 6.15 (dd, J = 5.5, 5.5 Hz, 1H, NH), 5.34 – 5.25 (m, 1H, 12-H), 5.15 – 5.01 (m, 1H, 2-H), 4.73 (d, J = 10.3 Hz, 1H, 3-H), 4.61 – 4.49 (m, 1H, 35-H_a), 4.12 (dd, J = 14.7, 4.5 Hz, 1H, 35-H_b), 2.58 – 2.47 (m, 1H, 18-H), 2.05 (s, 3H, 34-H_a + 34-H_b + 34-H_c), 2.04 – 1.97 (m, 2H, 1-H_a + 22-H_a), 1.97 (s, 3H, 32-H_a + 32-H_b + 32-H_c), 1.84 (dd, J = 8.9, 3.6 Hz, 2H, 11-H_a + 11-H_b), 1.80 – 1.66 (m, 2H, 7-H_a + 19-H_a), 1.66 – 1.48 (m, 5H, 6-H_a + 7-H_b + 9-H + 16-H_a + 22-H_b), 1.48 – 1.30 (m, 3H, 6-H_b + 15-H_a + 21-H_a), 1.30 – 1.15 (m, 3H, 15-H_b + 19-H_b + 21-H_b), 1.13 (s, 3H, 27-H_a + 27-H_b + 27-H_c), 1.10 – 1.03 (m, 2H, 1-H_b + 16-H_b), 1.01 (s, 3H, 26-H_a + 26-H_b + 26-H_c), 0.98 – 0.92 (m, 1H, 5-H), 0.92 – 0.84 (m, 12H, 23-H_a + 23-H_b + 23-H_c + 24-H_a + 24-H_b + 24-H_c + 29-H_a + 29-H_b + 29-H_c + 30-H_a + 30-H_b + 30-H_c), 0.63 (s, 3H, 25-H_a + 25-H_b + 25-H_c) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 178.4 (C-28), 171.3 (C-33), 171.0 (C-31), 162.6 (d, J = 245.6 Hz, C-39), 145.4 (C-13), 134.8 (d, J = 3.2 Hz, C-36), 129.9 (d, J = 8.0 Hz, C-37, C-41), 122.9 (C-12), 115.9 (d, J = 21.4 Hz, C-38, C-40), 81.0 (C-3), 70.4 (C-2), 55.2 (C-5), 47.9 (C-9), 47.1 (C-19), 46.8 (C-17), 44.3 (C-1), 43.3 (C-35), 42.7 (C-18), 42.5 (C-14), 39.8 (C-4), 39.8 (C-8), 38.5 (C-10), 34.6 (C-21), 33.4 (C-30), 33.1 (C-7), 32.6 (C-15), 31.2 (C-20), 28.8 (C-24), 27.7 (C-16), 24.2 (C-22), 24.1 (C-29), 24.0 (C-11), 21.6 (C-32), 21.3 (C-34), 18.6 (C-6), 18.1 (C-23), 17.3 (C-25), 16.9 (C-26) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): m/z = 662.5 (100 %, $[M-H]^-$); analysis calcd for $C_{41}H_{58}NO_5F$ (663.92): C 74.17, H 8.81, N 2.11; found: C 74.92, H 9.03, N 1.92.

4.2.23. (2α,3β) N-(2-methoxybenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (24)

Following GPA from **15** (0.2 g, 0.36 mmol) followed by chromatography (SiO_2, hexanes/ethyl acetate, 9:1) **22** (188 mg, 77 %) was obtained as a colorless solid; m.p. 204 °C; R_f = 0.31 (SiO_2, hexanes/ethyl acetate, 3:1); $[\alpha]_D$ = -18.42° (*c* 0.162, $CHCl_3$); IR (KBr): ν = 3427vw, 2945w, 2916w, 2862w, 1743 s, 1657 m, 1512 m, 1494 m, 1461 m, 1434w, 1369 m, 1250vs, 1224 s, 1119w, 1042 m, 1026 m, 965w, 939w, 919w, 803w, 774 s, 753 m, 639w, 597w, 510 m cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.26 – 7.19 (m, 2H, 39-H, 41-H), 6.92 – 6.81 (m, 2H, 38-H + 40-H), 6.49 (dd, J = 5.7, 5.7 Hz, 1H, NH), 5.25 (t, J = 3.6 Hz, 1H, 12-H), 5.06 (dt, J = 10.9, 4.6 Hz, 1H, 2-H), 4.73 (d, J = 10.3 Hz, 1H, 3-H), 4.49 – 4.32 (m, 2H, 35-H_a + 35-H_b), 3.86 (s, 3H, 42-H_a + 42-H_b + 42-H_c), 2.47 (dd, J = 12.9, 4.3 Hz, 1H, 18-H), 2.04 (s, 3H, 34-H_a + 34-H_b + 34-H_c), 2.02 – 1.98 (m, 1H, 1-H_a), 1.97 (s, 3H, 32-H_a + 32-H_b + 32-H_c), 1.92 (dd, J = 13.7, 3.9 Hz, 1H, 11-H_a), 1.83 – 1.64 (m, 5H, 22-H_a + 22-H_b + 19-H_a + 11-H_b + 7-H_a), 1.62 – 1.44 (m, 4H, 7-H_b + 9-H + 16-H_a + 6-H_a), 1.43–1.24 (m, 3H, 6-H_b + 21-H_a + 15-H_a), 1.23 – 1.13 (m, 3H, 21-H_b + 15-H_b + 19-H_b), 1.10 (s, 3H, 27-H_a + 27-H_b + 27-H_c), 1.06 – 0.97 (m, 2H, 1-H_b, 16-H_b), 0.95 (s, 3H, 26-H_a + 26-H_b + 26-H_c), 0.93 (d, J = 2.1, 1H, 5-H), 0.91–0.85 (m, 12H, 23-H_a + 23-H_b + 23-H_c + 24-H_a + 24-H_b + 24-H_c + 29-H_a + 29-H_b + 29-H_c + 30-H_a + 30-H_b + 30-H_c), 0.39 (s, 3H, 25-H_a + 25-H_b + 25-H_c) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 177.5 (C-28), 170.9 (C-33), 170.7 (C-31), 157.8 (C-37), 144.9 (C-13), 130.2 (C-39), 128.9 (C-41), 126.5 (C-36), 122.2 (C-12), 120.9 (C-40), 110.1 (C-38), 80.6 (C-3), 70.2 (C-2), 55.3 (C-42), 54.9 (C-5), 47.6 (C-9), 46.8 (C-19), 46.6 (C-17), 44.0 (C-1), 42.6 (C-18), 42.1 (C-14), 39.7 (C-35), 39.5 (C-4), 39.4 (C-8), 38.1 (C-10), 34.3 (C-21), 33.1 (C-30), 32.7 (C-7), 32.3 (C-15), 30.8 (C-20), 28.5 (C-24), 27.4 (C-16), 25.8 (C-27), 23.7 (C-29), 23.7 (C-22), 23.7 (C-11), 21.3 (C-32), 21.0 (C-34), 18.3 (C-6), 17.7 (C-23), 16.5 (C-25), 16.5 (C-26) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): m/z = 674.7 (100 %, $[M-H]^-$); analysis calcd for $C_{42}H_{61}NO_6$ (675.95): C 74.63, H 9.10, N 2.07; found: C 74.45, H 9.38, N 1.85.

4.2.24. (2α,3β) N-(3-methoxybenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (25)

Following GPA from **17** (0.2 g, 0.36 mmol) followed by chromatography (SiO_2, hexanes/ethyl acetate, 9:1) **25** (192 mg, 79 %) was obtained as a colorless solid; m.p. 118 °C; R_f = 0.28 (SiO_2, hexanes/ethyl acetate, 3:1); $[\alpha]_D$ = -3.54° (*c* 0.175, $CHCl_3$); IR (KBr): ν = 3422vw, 2943 m, 2865w, 1739 s, 1655 m, 1602w, 1587w, 1514 m, 1490 m, 1464 m, 1434 m, 1367 m, 1248vs, 1230vs, 1191w, 1153 m, 1042 s, 965w, 918w, 872w, 823w, 772w, 735w, 694w, 641w, 597w, 554w, 523w, 464w cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.23 (dd, J = 7.8, 7.8 Hz, 1H, 40-H), 6.85 – 6.76 (m, 3H, 37-H + 39-H + 41-H), 6.15 (dd, J = 6.3, 4.4 Hz, 1H, NH), 5.33 – 5.27 (m, 1H, 12-H), 5.13 – 5.02 (m, 1H, 2-H), 4.73 (d, J = 10.4 Hz, 1H, 3-H), 4.57 (dd, J = 14.7, 6.2 Hz, 1H, 35-H_a), 4.12 (dd, J = 14.7, 4.3 Hz, 1H, 35-H_b), 3.79 (s, 3H, 42-H_a + 42-H_b + 42-H_c), 2.58 – 2.50 (m, 1H, 18-H), 2.05 (s, 3H, 34-H_a + 34-H_b + 34-H_c), 2.02 – 1.98 (m, 2H, 1-H_a + 22-H_a), 1.97 (s, 3H, 32-H_a + 32-H_b + 32-H_c),

1.84 (dd, J = 8.9, 3.6 Hz, 2H, 11-H_a + 11-H_b), 1.81 – 1.64 (m, 3H, 7-H_a + 22-H_b + 19-H_a), 1.64 – 1.48 (m, 4H, 6-H_a + 7-H_b + 9-H + 16-H_a), 1.40 (ddt, J = 28.6, 13.3, 4.1 Hz, 3H, 6-H_b + 15-H_a + 21-H_a), 1.30 – 1.16 (m, 3H, 15-H_b + 21-H_b + 19-H_b), 1.14 (s, 3H, 27-H_a + 27-H_b + 27-H_c), 1.05 (dt, J = 10.2, 4.8 Hz, 2H, 1-H_b + 16-H_b), 1.01 (s, 3H, 26-H_a + 26-H_b + 26-H_c), 0.98 – 0.93 (m, 1H, 5-H), 0.92 – 0.81 (m, 12H, 23-H_a + 23-H_b + 23-H_c + 24-H_a + 24-H_b + 24-H_c + 29-H_a + 29-H_b + 29-H_c + 30-H_a + 30-H_b + 30-H_c), 0.66 (s, 3H, 25-H_a + 25-H_b + 25-H_c) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 177.9 (C-28), 170.8 (C-33), 170.5 (C-31), 159.9 (C-38), 144.9 (C-13), 139.9 (C-36), 129.7 (C-40), 122.5 (C-12), 119.9 (C-41), 113.4 (C-39), 112.8 (C-37), 80.5 (C-3), 70.0 (C-2), 55.2 (C-42), 54.8 (C-5), 47.4 (C-9), 46.6 (C-19), 46.3 (C-17), 43.9 (C-1), 43.5 (C-35), 42.3 (C-18), 42.1 (C-14), 39.4 (C-4), 39.3 (C-8), 38.0 (C-10), 34.1 (C-21), 33.0 (C-30), 32.6 (C-7), 32.2 (C-20), 30.7 (C-15), 28.4 (C-24), 27.3 (C-16), 25.7 (C-27), 23.7 (C-22), 23.6 (C-29), 23.5 (C-11), 21.1 (C-32), 20.9 (C-34), 18.1 (C-6), 17.6 (C-23), 16.9 (C-25), 16.4 (C-26) ppm; MS (ESI, MeOH/$CHCl_3$, (4:1)): m/z = 674.6 (100 %, $[M-H]^-$); analysis calcd for $C_{42}H_{61}NO_6$ (675.95): C 74.63, H 9.10, N 2.07; found: C 74.49, H 9.40, N 1.79.

4.2.25. (2α,3β) N-(4-methoxybenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (26)

Following GPA from **15** (0.2 g, 0.36 mmol) followed by chromatography (SiO_2, hexanes/ethyl acetate, 9:1) **24** (176 mg, 72 %) was obtained as a colorless solid; m.p. 130 °C; R_f = 0.25 (SiO_2, hexanes/ethyl acetate, 3:1); $[\alpha]_D$ = -6.39° (*c* 0.178, $CHCl_3$); IR (KBr): ν = 3419vw, 2943w, 1740 s, 1654w, 1613w, 1512 m, 1463w, 1433w, 1367 m, 1301w, 1246vs, 1231vs, 1175 m, 1109w, 1032 s, 965w, 918w, 822w, 773w, 640w, 596w, 582w, 523w cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.19 – 7.13 (m, 2H, 37-H + 41-H), 6.88 – 6.82 (m, 2H, 38-H + 40-H), 6.11 – 6.05 (m, 1H, NH), 5.30 – 5.25 (m, 1H, 12-H), 5.08 (ddd, J = 11.5, 10.3, 4.6 Hz, 1H, 2-H), 4.73 (d, J = 10.2 Hz, 1H, 3-H), 4.51 (dd, J = 14.4, 6.1 Hz, 1H, 35-H_a), 4.09 (dd, J = 14.5, 4.3 Hz, 1H, 35-H_b), 3.80 (s, 3H, 42-H_a + 42-H_b + 42-H_c), 2.51 (dd, J = 13.2, 4.4 Hz, 1H, 18-H), 2.05 (s, 3H, 34-H_a + 34-H_b + 34-H_c), 2.04 – 1.98 (m, 2H, 1-H_a + 22-H_b), 1.97 (s, 3H, 32-H_a + 32-H_b + 32-H_c), 1.83 (dd, J = 8.8, 3.7 Hz, 2H, 11-H_a + 11Hb), 1.79 – 1.62 (m, 3H, 7-H_a + 19-H_a + 22-H_b), 1.62 – 1.45 (m, 5H, 6-H_a + 7-H_b + 9-H + 16-H_a + 15-H_a), 1.45 – 1.24 (m, 3H, 6-H_b + 21-H_b + 15-H_b), 1.24 – 1.15 (m, 2H, 21-H_b + 19-H_b), 1.13 (s, 3H, 27-H_a + 27-H_b + 27-H_c), 1.09 – 1.03 (m, 2H, 1-H_b + 16-H_b), 1.01 (s, 3H, 26-H_a + 26-H_b + 26-H_c), 1.00 – 0.92 (m, 1H, 5-H), 0.92 – 0.86 (m, 12H, 23-H_a + 23-H_b + 23-H_c + 24-H_a + 24-H_b + 24-H_c + 29-H_a + 29-H_b + 29-H_c + 30-H_a + 30-H_b + 30-H_c), 0.66 (s, 3H, 25-H_a + 25-H_b + 25-H_c) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 178.0 (C-28), 170.9 (C-33), 170.7 (C-31), 159.1 (C-39), 145.1 (C-13), 130.5 (C-36), 129.3 (C-37, C-41), 122.5 (C-12), 114.2 (C-38, C-40), 80.7 (C-3), 70.1 (C-2), 55.4 (C-42), 54.9 (C-5), 47.6 (C-9), 46.7 (C-19), 46.4 (C-17), 44.0 (C-1), 43.3 (C-35), 42.5 (C-18), 42.2 (C-14), 39.5 (C-4), 39.5 (C-8), 38.2 (C-10), 34.3 (C-21), 33.1 (C-30), 32.7 (C-7), 32.3 (C-15), 30.9 (C-20), 28.5 (C-24), 27.4 (C-16), 25.8 (C-27), 23.8 (C-22), 23.8 (C-29), 23.7 (C-11), 21.3 (C-32), 21.0 (C-34), 18.3 (C-6), 17.7 (C-23), 17.1 (C-25), 16.5 (C-26) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): m/z = 674.6 (100 %, $[M-H]^-$); analysis calcd for $C_{42}H_{61}NO_6$ (675.95): C 74.63, H 9.10, N 2.07; found: C 74.45, H 9.36, N 1.75.

4.2.26. (2α,3β) N-(2-methylbenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (27)

Following GPA from **17** (0.2 g, 0.36 mmol) followed by chromatography (SiO_2, hexanes/ethyl acetate, 9:1) **27** (132 mg, 56 %) was obtained as a colorless solid; m.p. 261 °C (decomp.); R_f = 040 (SiO_2, hexanes/ethyl acetate, 3:1); $[\alpha]_D$ = -8.99° (*c* 0.184, $CHCl_3$); IR (KBr): ν = 3429w, 2971w, 2945 m, 2916w, 2870w, 1744 s, 1656 s, 1504 m, 1464w, 1433w, 1369 m, 1252Vs, 1223 s, 1190w, 1043 m, 1031 m, 821w, 803w, 771 s, 639w, 599w, 515w, 493w, 452w cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.24 – 7.10 (m, 4H, 38-H + 39-H + 40-H + 41-H), 6.04 (dd, J = 5.2, 5.2 Hz, 1H, NH), 5.27 (t, J = 3.6 Hz, 1H, 12-H), 5.12 – 5.02 (m, 1H, 2-H), 4.73 (d, J = 10.3 Hz, 1H, 3-H), 4.60 (dd, J = 14.7, 6.3 Hz, 1H, 35-H_a), 4.15 (dd, J = 14.6, 4.0 Hz, 1H, 35-H_b), 2.52 (dd, J = 12.9, 4.4 Hz, 1H, 18-H), 2.31 (s, 3H, 42-H_a + 42-H_b + 42-H_c), 2.05 (s, 3H, 34-H_a + 34-H_b + 34-H_c), 2.03 – 1.97 (m, 2H, 1-H_a + 22-H_a), 1.97 (s, 3H, 32-H_a + 32-H_b + 32-H_c), 1.86 – 1.78 (m, 2H, 11-H_a + 11-H_b), 1.78 – 1.63 (m, 3H, 7-H_a + 19-H_a + 22-H_a), 1.63 – 1.51 (m, 4H, 6-H_a + 7-H_b + 9-H + 16-H_a), 1.49 – 1.31 (m, 3H, 6-H_b + 15-H_a + 21-H_b), 1.30 – 1.15 (m, 3H, 15-H_b + 19-H_b + 21-H_b), 1.13 (s, 3H, 27-H_a + 27-H_b + 27-H_c), 1.04 (dd, J = 13.2, 4.1 Hz, 2H, 1-H_b + 16-H_b), 1.00 (s, 3H, 26-H_a + 26-H_b + 26-H_c), 0.97 – 0.91 (m, 1H, 5-H), 0.92 – 0.87 (m, 12H, 23-H_a + 23-H_b + 23-H_c + 24-H_a + 24-H_b + 24-H_c + 29-H_a + 29-H_b + 29-H_c + 30-H_a + 30-H_b + 30-H_c), 0.64 (s, 3H, 25-H_a + 25-H_b + 25-H_c) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 177.9 (C-28), 170.9 (C-33), 170.7 (C-31), 145.1 (C-13), 136.5 (C-36), 136.2 (C-37), 130.6 (C-38), 128.5 (C-41), 127.7 (C-39), 126.3 (C-40), 122.6 (C-12), 80.6 (C-3), 70.1 (C-2), 54.9 (C-5), 46.7 (C-19), 46.6 (C-17), 44.0 (C-1), 42.5 (C-18), 42.2 (C-14), 41.7 (C-35), 39.5 (C-4), 39.5 (C-8), 38.2 (C-10), 34.3 (C-21), 33.1 (C-30), 32.7 (C-7), 32.3 (C-15), 30.9 (C-20), 28.5 (C-24), 27.4 (C-16), 25.8 (C-27), 23.9 (C-22), 23.7 (C-29), 23.6 (C-11), 21.3 (C-32), 21.0 (C-34), 19.2 (C-42), 18.3 (C-6), 17.7 (C-23), 17.0 (C-25), 16.5 (C-26) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): m/z = 658.5 100 %, ($[M-H]^-$); analysis calcd for $C_{42}H_{61}NO_5$ (659.45): C 76.44, H 9.32, N 2.12; found: C 76.19, H 9.62, N 1.96.

4.2.27. (2α,3β) N-(3-methylbenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (28)

Following GPA from **17** (0.2 g, 0.36 mmol) followed by chromatography (SiO_2, hexanes/ethyl acetate, 9:1) **28** (187 mg, 79 %) was obtained as a colorless solid; m.p. 121 °C; R_f = 0.36 (SiO_2, hexanes/ethyl acetate, 3;1); $[\alpha]_D$ = -2.85° (*c* 0.164, $CHCl_3$); IR (KBr): ν = 3425vw, 2944 m, 2864w, 1740 s, 1645 m, 1610w, 1514 m, 1462 m, 1433w, 1367 m, 1247vs, 1229vs, 1192w, 1154w, 1096w, 1042 s, 1031 s, 989 m, 965w, 918w, 822w, 773w, 757w, 699w, 640w, 597w cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.25 – 7.18 (m, 1H, 40-H), 7.11 – 7.01 (m, 3H, 37-H + 39-H + 41-H), 6.12 (dd, J = 5.3, 5.3 Hz, 1H, NH), 5.28 (t, J = 3.6 Hz, 1H, 12-H), 5.08 (dt, J = 11.0, 4.6 Hz, 1H, 2-H), 4.74 (d, J = 10.3 Hz, 1H, 3-H), 4.59 – 4.49 (m, 1H, 35-H_a), 4.13 (dd, J = 14.6, 4.3 Hz, 1H, 35-H_b), 2.53 (dd, J = 13.2, 4.4 Hz, 1H, 18-H), 2.34 (s, 3H, 42-H_a + 42-H_b + 42-H_c), 2.05 (s, 3H, 34-H_a + 34-H_b + 34-H_c), 2.03 – 1.98 (m, 2H, 1-H_a + 22-H_a), 1.97 (s, 3H, 32-H_a + 32-H_b + 32-H_c), 1.83 (dd, J = 8.9, 3.6 Hz, 2H, 11-H_a + 11-H_b), 1.80 – 1.68 (m, 2H, 7-H_a + 19-H_a), 1.69 – 1.51 (m, 5H, 6-H_a + 7-H_b + 9-H + 16-H_a + 22-H_b), 1.51 – 1.31 (m, 3H, 6-H_b + 15-H_a + 21-H_b), 1.31 – 1.16 (m, 3H, 15-H_b + 19 + Hb + 21-H_b), 1.14 (s, 3H, 27-H_a + 27-H_b + 27-H_c), 1.11 – 1.03 (m, 2H, 1-H_b + 16-H_b), 1.01 (s, 3H, 26-H_a + 26-H_b + 26-H_c), 0.98 – 0.91 (m, 1H, 5-H), 0.93 – 0.87 (m, 12H, 23-H_a + 23-H_b + 23-H_c + 24-H_a + 24-H_b + 24-H_c + 29-H_a + 29-H_b + 29-H_c + 30-H_a + 30-H_b + 30-H_c), 0.67 (s, 3H, 25-H_a + 25-H_b + 25-H_c) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 178.0 (C-28), 170.9 (C-33), 170.7 (C-31), 145.1 (C-13), 138.4 (C-36), 138.3 (C-38), 128.7 (C-37, C-40), 128.3 (C-39), 125.0 (C-41), 122.6 (C-12), 80.6 (C-3), 70.1 (C-2), 54.9 (C-5), 47.6 (C-9), 46.7 (C-19), 46.5 (C-17), 44.0 (C-1), 43.8 (C-35), 42.5 (C-18), 42.2 (C-14), 39.5 (C-4), 39.5 (C-8), 38.2 (C-10), 34.3 (C-21), 33.1 (C-30), 32.8 (C-7), 32.3 (C-15), 30.9 (C-20), 28.5 (C-24), 27.4 (C-16), 25.8 (C-27), 23.8 (C-22), 23.7 (C-29), 23.6 (C-11), 21.5 (C-42), 21.3 (C-32), 21.0 (C-42), 18.3 (C-6), 17.7 (C-23), 17.1 (C-25), 16.5 (C-26) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): m/z = 658.5 (100 %, $[M-H]^-$), analysis calcd for $C_{42}H_{61}NO_5$ (659.45): C 76.44, H 9.32, N 2.12; found: C 76.21, H 9.65, N 2.00.

4.2.28. (2α,3β) N-(4-methylbenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (29)

Following GPA from **17** (0.2 g, 0.36 mmol) followed by chromatography (SiO_2, hexanes/ethyl acetate, 9:1) **29** (191 mg, 81 %) was obtained as a colorless solid; m.p. 136 °C; R_f = 0.34 (SiO_2, hexanes/ethyl acetate, 3:1); $[\alpha]_D$ = -7.56° (*c* 0.173, $CHCl_3$); IR (KBr): ν = 3427vw, 2944 m, 2864w, 1740 s, 1647 m, 1515 m, 1462w, 1432w, 1367 m,

1248vs, 1230vs, 1185w, 1154w, 1042 m, 1031 m, 959w, 918w, 807w, 755w, 640w, 598w, 578w, 475 m cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.18 – 7.07 (m, 4H, 37-H + 38-H + 40-H + 41-H), 6.16 – 6.06 (dd, J = 5.3, 5.3 Hz, 1H, NH), 5.32 – 5.25 (m, 1H, 12-H), 5.12 – 5.02 (m, 1H, 2-H), 4.74 (d, J = 10.3 Hz, 1H, 3-H), 4.55 (dd, J = 14.5, 6.1 Hz, 1H, 35-H_a), 4.11 (dd, J = 14.6, 4.3 Hz, 1H, 35-H_b), 2.56 – 2.47 (m, 1H, 18-H), 2.33 (s, 3H, 42-H_a + 42-H_b + 42-H_c), 2.05 (s, 3H, 34-H_a + 34-H_b + 34-H_c), 2.03 – 1.97 (m, 2H, 1-H_a + 22-H_a), 1.97 (s, 3H, 32-H_a + 32-H_b + 32-H_c), 1.82 (dd, J = 8.9, 3.6 Hz, 2H, 11-H_a + 11-H_b), 1.79 – 1.68 (m, 3H, 7-H_a + 19-H_a + 22-H_b), 1.68 – 1.49 (m, 4H, 6-H_b + 7-H_b + 9-H + 16-H_b), 1.49 – 1.30 (m, 3H, 6-H_b + 15-H_a + 21-H_a), 1.31 – 1.15 (m, 3H, 15-H_b + 19-H_b + 21-H_b), 1.13 (s, 3H, 27-H_a + 27-H_b + 27-H_c), 1.09 – 1.02 (m, 2H, 1-H_b + 16-H_b), 1.01 (s, 3H, 26-H_a + 26-H_b + 26-H_c), 0.99 – 0.91 (m, 1H, 5-H), 0.92 – 0.86 (m, 12H, 23-H_a + 23-H_b + 23-H_c + 24-H_a + 24-H_b + 24-H_c + 29-H_a + 29-H_b + 29-H_c + 30-H_a + 30-H_b + 30-H_c), 0.65 (s, 3H, 25-H_a + 25-H_b + 25-H_c) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 178.0 (C-28), 170.9 (C-33), 170.7 (C-31), 145.1 (C-13), 137.2 (C-36), 135.4 (C-39), 129.5 (C-38, 40), 127.9 (C-37, C-41), 122.6 (C-12), 80.6 (C-3), 70.1 (C-2), 54.9 (C-5), 47.6 (C-9), 46.7 (C-19), 46.4 (C-17), 44.0 (C-1), 43.5 (C-35), 42.5 (C-18), 42.2 (C-14), 39.5 (C-4), 39.5 (C-8), 38.2 (C-10), 34.2 (C-21), 33.1 (C-30), 32.7 (C-7), 32.3 (C-15), 30.9 (C-20), 28.5 (C-24), 27.4 (C-16), 25.8 (C-27), 23.8 (C-22), 23.8 (C-29), 23.6 (C11), 21.3 (C-42), 21.2 (C-32), 21.0 (C-34), 18.3 (C-6), 17.7 (C-23), 17.0 (C-25), 16.5 (C-26) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): m/z = 658.5 (100 %, $[M-H]^-$); analysis calcd for $C_{42}H_{61}NO_5$ (659.45): C 76.44, H 9.32, N 2.12; found: C 76.21, H 9.51, N 1.86.

4.2.29. Augustic acid 26707-60-8 (3)

Augustic acid was prepared as previously reported; m.p. 310–314 °C (lit.: [4] 40 308–310 °C); R_f = 0.49 (SiO_2, hexanes/ethyl acetate, 1:1); $[\alpha]_D$ = +88.05° (c 0.31, THF) (lit.: [4] 43 + 93.5° (c 0.17, pyridine); MS (ESI, MeOH): m/z = 471.3 (100 %, $[M-H]^-$), 943.3 (30 %, $[2\ M-H]^-$).

4.2.30. (2β,3β) Diacetyloxy-olean-12-en-28-acid (30)

Acetylation as previously reported provided **30** (5.1 g, 55 %) as a colorless solid; m.p. 322 °C (decomp.); R_f = 0.24 (SiO_2, hexanes/ethyl acetate, 8:2); $[\alpha]_D$ = +83.81° (c 0.32 $CHCl_3$); MS (ESI, MeOH): m/z = 557.2 (13 %, $[M + H]^+$), 574.3 (100 %, $([M + NH_4]^+$).

4.2.31. (2β,3β) N-(2-chlorobenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (31)

Following GPA from **30** (180 mg, 0.32 mmol) followed by chromatography (SiO_2, hexanes/ethyl acetate, 9:1) **31** (196 mg, 90 %) was obtained as a colorless solid; m.p. 101–104 °C; R_f = 0.47 (SiO_2, hexanes/ethyl acetate, 3:1); $[\alpha]_D$ = +28.99° (c 0.15, $CHCl_3$); IR (KBr): ν = 3359vw, 2928 m, 2869w, 1742 s, 1647 m, 1515 m, 1469 m, 1444 m, 1363 m, 1247vs, 1232vs, 1192 m, 1158w, 1054 m, 1030 s, 1009 m, 991 m, 945 m, 822w, 748 m, 605 m cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.43 – 7.30 (m, 2H, 38-H + 39-H), 7.24 – 7.17 (m, 2H, 40-H + 41-H), 6.45 (dd, J = 6.0, 6.0 Hz, 1H, NH), 5.34 (t, J = 3.6 Hz, 1H, 12-H), 5.30 (dd, J = 3.7, 3.7 Hz, 1H, 2-H), 4.60 (d, J = 3.9 Hz, 1H, 3-H), 4.55 (dd, J = 14.6, 5.9 Hz, 1H, 35-H_a), 4.40 (dd, J = 14.6, 5.8 Hz, 1H, 35-H_b), 2.53 (dd, J = 13.1, 4.3 Hz, 1H, 18-H), 2.05 (s, 3H, 34-H_a + 34-H_b + 34-H_c), 2.02 (s, 3H, 32-H_a + 32-H_b + 32-H_c), 1.99 – 1.90 (m, 2H, 1-H_a + 22-H_a), 1.90 – 1.82 (m, 2H, 11-H_a + 11-H_b), 1.81 – 1.63 (m, 3H, 7-H_a + 19-H_a + 22-H_b), 1.62 – 1.45 (m, 4H, 6-H_a + 7-H_b + 9-H + 16-H_a), 1.45 – 1.36 (m, 3H, 6-H_b + 15-H_a + 21-H_a), 1.36 – 1.14 (m, 4H, 1-H_b + 15-H_b + 19-H_b + 21-H_b), 1.12 (s, 6H, 23-H_a + 23-H_b + 23-H_c + 27-H_a + 27-H_b + 27-H_c), 1.04 (s, 3H, 25-H_a + 25-H_b + 25-H_c), 1.02 – 0.92 (m, 2H, 5-H + 16-H_b), 0.90 (s, 6H, 29-H_a + 29-H_b + 29-H_c + 30-H_a + 30-H_b + 30-H_c), 0.88 (s, 3H, 24-H_a + 24-H_b + 24-H_c), 0.48 (s, 3H, 26-H_a + 26-H_b + 26-H_c) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 177.9 (C-28), 170.7 (C-33), 170.2 (C-31), 144.6 (C-13), 135.9 (C-36), 133.9 (C-37), 130.9 (C-38), 129.3 (C-39), 128.8 (C-41), 127.1 (C-40), 122.8 (C-12), 77.9 (C-3), 69.6 (C-2), 55.1 (C-5), 48.0 (C-9), 46.6 (C-17), 46.5 (C-19), 42.4 (C-18), 42.1 (C-14), 41.9 (C-1), 41.5 (C-35), 39.4 (C-8), 37.3 (C-4), 36.6 (C-10), 34.1 (C-21), 33.0 (C-30), 32.7 (C-7), 32.3 (C-15), 30.7 (C-20), 29.0 (C-24), 27.2 (C-16), 25.7 (C-27), 23.6 (C-22), 23.6 (C- 29), 23.5 (C-11), 21.3 (C-34), 20.8 (C-32), 17.9 (C-6), 17.6 (C-25), 16.6 (C-26), 15.9 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): m/z = 678.5 (100 %, $[M-H]^-$); analysis calcd for $C_{41}H_{58}NO_5Cl$ (680.37): C 72.38, H 8.59, N 2.21; found: C 72.07, H 8.83, N 1.92.

4.2.32. (2β,3β) N-(3-chlorobenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (32)

Following GPA from **30** (180 mg, 0.32 mmol) followed by chromatography (SiO_2, hexanes/ethyl acetate, 9:1) **35** (178 mg, 81 %) was obtained as a colorless solid; m.p. 118.8 °C; R_f = 0.41 (SiO_2, hexanes/ethyl acetate, 3:1); $[\alpha]_D$ = +34.98° (c 0.158, $CHCl_3$); IR (KBr): ν = 3382vw, 2945 m, 2867w, 1741 s, 1647 m, 1599w, 1574w, 1517 m, 1472 m, 1432 m, 1397w, 1364 m, 1248vs, 1232vs, 1196 m, 1161w, 1078w, 1056 m, 1030 m, 991 m, 945w, 822w, 787w, 755 m, 703w, 681 m, 625w, 605 m cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.26–7.21 (m, 3H, 37-H + 39-H + 40-H), 7.17 – 7.10 (m, 1H, 41-H), 6.19 (dd, J = 5.5, 5.5 Hz, 1H, NH), 5.36 – 5.27 (m, 2H, 2-H, 12-H), 4.61 (d, J = 3.8 Hz, 1H, 3-H), 4.58 – 4.48 (m, 1H, 35-H_a), 4.17 (dd, J = 14.9, 4.8 Hz, 1H, 35-H_b), 2.54 (dd, J = 13.1, 4.3 Hz, 1H, 18-H), 2.04 (s, 3H, 34-H_a + 34-H_b + 34-H_c), 2.02 (s, 3H, 32-H_a + 32-H_b + 32-H_c), 1.97 (ddd, J = 14.8, 8.9, 3.4 Hz, 2H, 1-H_a + 22-H_a), 1.91 – 1.83 (m, 2H, 11-H_a + 11-H_b), 1.80 – 1.63 (m, 3H, 7-H_a + 19-H_a + 22-H_b), 1.63 – 1.44 (m, 6H, 6-H_a + 6-H_b + 7-H_b + 9-H + 15-H_a + 16-H_a), 1.41 – 1.25 (m, 3H, 1-H_b + 15-H_b + 21-H_a), 1.25–1.15 (m, 2H, 19-H_b + 21-H_b), 1.15 (s, 3H, 23-H_a + 23-H_b + 23-H_c), 1.14 (s, 3H, 27-H_a + 27-H_b + 27-H_c), 1.05 (s, 3H, 25-H_a + 25-H_b + 25-H_c), 1.01 (q, J = 5.3, 4.5 Hz, 1H, 16-H_b), 0.99 – 0.92 (m, 1H, 5-H), 0.91 (s, 6H, 29-H_a + 29-H_b + 29-H_c + 30-H_a + 30-H_b + 30-H_c), 0.90 (s, 3H, 24-H_a + 24-H_b + 24-H_c), 0.67 (s, 3H, 26-H_a + 26-H_b + 26-H_c) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 178.3 (C-28), 170.9 (C-33), 170.4 (C-31), 145.1 (C-13), 140.6 (C-36), 134.6 (C-38), 130.1 (C-40), 128.1 (C-37), 127.6 (C-39), 126.1 (C-41), 122.9 (C-12), 78.0 (C-3), 69.7 (C-2), 55.3 (C-5), 48.1 (C-9), 46.7 (C-17), 46.6 (C-19), 43.2 (C-35), 42.5 (C-18), 42.4 (C-14), 42.0 (C-1), 39.6 (C-8), 37.5 (C-4), 36.8 (C-10), 34.2 (C-21), 33.1 (C-30), 32.8, 32.5 (C-15), 30.9 (C-20), 29.2 (C-24), 27.3 (C-16), 25.9 (C-27), 23.9 (C-22), 23.7 (C-29), 23.6 (C-11), 21.4 (C-34), 21.0 (C-32), 18.1 (C-6), 17.8 (C-25), 17.2 (C-26), 16.0 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): m/z = 678.6 (100 %, $[M-H]^-$); analysis calcd for $C_{41}H_{58}NO_5Cl$ (680.37): C 72.38, H 8.59, N 2.21; found: C 72.11, H 8.86, N 2.00.

4.2.33. (2β,3β) N-(4-chlorobenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (33)

Following GPA from **30** (180 mg, 0.32 mmol) followed by chromatography (SiO_2, hexanes/ethyl acetate, 9:1) **33** (206 mg, 94 %) was obtained as a colorless solid; m.p. 128.2 °C; R_f = 0.40 (SiO_2, hexanes/ethyl acetate, 3:1); $[\alpha]_D$ = +34.95° (c 0.157, $CHCl_3$); IR (KBr): ν = 3408vw, 2945 m, 1742 s, 1645 m, 1514 m, 1492 m, 1463 m, 1432w, 1364 m, 1247vs, 1232vs, 1192 m, 1161w, 1091 m, 1056 m, 1030 s, 1015 m, 991 m, 945w, 820 m, 800w, 606w cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.32 – 7.26 (m, 2H, 38-H + 40-H), 7.22 – 7.15 (m, 2H, 37-H + 41-H), 6.17 (dd, J = 5.5, 5.5 Hz, 1H, NH), 5.35 – 5.27 (m, 2H, 2-H + 12-H), 4.61 (d, J = 3.9 Hz, 1H, 3-H), 4.55 (dd, J = 14.8, 6.2 Hz, 1H, 35-H_a), 4.12 (dd, J = 14.8, 4.7 Hz, 1H, 35-H_b), 2.57 – 2.49 (m, 1H, 18-H), 2.05 (s, 3H, 34-H_a + 34-H_b + 34-H_c), 2.03 (s, 3H, 32-H_a + 32-H_b + 32-H_c), 1.97 (ddd, J = 14.9, 8.2, 3.5 Hz, 2H, 1-H_a + 22-H_a), 1.90 – 1.82 (m, 2H, 11-H_a + 11-H_b), 1.78 – 1.62 (m, 3H, 7-H_a + 19-H_a + 22-H_b), 1.62 – 1.43 (m, 5H, 6-H_a + 6-H_b + 7-H_b + 9-H + 15-H_a + 16-H_a), 1.39 – 1.15 (m, 5H, 1-H_b + 15-H_b + 19-H_b + 21-H_a + 21-H_b), 1.15 (s, 3H, 23-H_a + 23-H_b + 23-H_c), 1.14 (s, 3H, 27-H_a + 27-H_b + 27-H_c), 1.05 (s, 3H, 25-H_a + 25-H_b + 25-H_c), 1.03 – 0.99 (m, 1H, 16-H_b), 0.98 – 0.93 (m, 1H, 5-H), 0.91 (s, 3H, 30-H_a + 30-H_b + 30-H_c), 0.90 (s, 6H, 24-H_a + 24-H_b + 24-H_c + 29-H_a + 29-H_b + 29-H_c), 0.66 (s, 3H, 26-H_a + 26-H_b + 26-H_c) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 178.3 (C-28), 170.9 (C-33), 170.4 (C-31), 145.1 (C-13), 137.2 (C-36), 133.3 (C-39), 129.3 (C-37, C-41), 128.9

(C-38, C-40), 122.8 (C-12), 78.0 (C-3), 69.7 (C-2), 55.3 (C-5), 48.1 (C-9), 46.7 (C-17), 46.5 (C-19), 43.0 (C-35), 42.5 (C-18), 42.3 (C-14), 42.0 (C-1), 39.6 (C-8), 37.5 (C-4), 36.8 (C-10), 34.2 (C-21), 33.1 (C-30), 32.8 (C-7), 32.4 (C-15), 30.9 (C-20), 29.2 (C-24), 27.3 (C-16), 25.9 (C-27), 23.9 (C-22), 23.7 (C-29), 23.6 (C-11), 21.4 (C-34), 21.0 (C-32), 18.1 (C-6), 17.8 (C-25), 17.2 (C-26), 16.1 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): m/z = 678.7 (100 %, $[M-H]^-$); analysis calcd for $C_{41}H_{58}NO_5Cl$ (680.37): C 72.38, H 8.59, N 2.21; found: C 72.17, H 8.77, N 1.93.

4.2.34. (2β,3β) N-(2-fluorobenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (34)

Following GPA from **30** (180 mg, 0.32 mmol) followed by chromatography (SiO_2, hexanes/ethyl acetate, 9:1) **34** (192 mg, 90 %) was obtained as a colorless solid; m.p. 106.6 °C; R_f = 0.44 (SiO_2, hexanes/ethyl acetate, 3:1); $[\alpha]_D$ = +37.02° (*c* 0.174, $CHCl_3$); IR (KBr): ν = 3418vw, 2945 m, 2864w, 1742 s, 1658 m, 1587w, 1514 m, 1489 m, 1457 m, 1433w, 1364 m, 1247vs, 1229vs, 1191 m, 1161w, 1107w, 1056 m, 1030 m, 1011 m, 990 m, 945w, 822w, 754 s, 685w, 667w, 606 m, 510w cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.34 – 7.28 (m, 1H, 41-H), 7.28 – 7.22 (m, 1H, 39-H), 7.09 (ddd, *J* = 7.5, 7.5, 1.2 Hz, 1H, 40-H), 7.06 – 7.00 (m, 1H, 38-H), 6.29 (dd, *J* = 5.8, 5.8 Hz, 1H, NH), 5.33 (t, *J* = 3.6 Hz, 1H, 12-H), 5.32 – 5.29 (m, 1H, 2-H), 4.60 (d, *J* = 3.9 Hz, 1H, 3-H), 4.55 (dd, *J* = 14.7, 5.9 Hz, 1H, 35-H_a), 4.33 (dd, *J* = 14.7, 5.3 Hz, 1H, 35-H_b), 2.52 (dd, *J* = 13.0, 4.0 Hz, 1H, 18-H), 2.04 (s, 3H, 34-H_a + 34-H_b + 34-H_c), 2.02 (s, 3H, 32-H_a + 32-H_b + 32-H_c), 1.96 (dt, *J* = 14.9, 3.6 Hz, 2H, 1-H_a + 22-H_a), 1.88 – 1.82 (m, 2H, 11-H_a + 11-H_b), 1.78 – 1.62 (m, 3H, 7-H_a + 19-H_a + 22-H_b), 1.62 – 1.41 (m, 6H, 6-H_a + 6-H_b + 7-H_b + 9-H + 15-H_a + 16-H_a), 1.40 – 1.14 (m, 5H, 1-H_b + 15-H_b + 19-H_b + 21-H_a + 21-H_b), 1.13 (s, 3H, 27-H_a + 27-H_b + 27-H_c), 1.12 (s, 3H, 23-H_a + 23-H_b + 23-H_c), 1.04 (s, 3H, 25-H_a + 25-H_b + 25-H_c), 1.03 – 0.97 (m, 1H, 16-H_b), 0.97 – 0.91 (m, 1H, 5-H), 0.90 (s, 3H, 30-H_a + 30-H_b + 30-H_c), 0.89 (s, 3H, 29-H_a + 29-H_b + 29-H_c), 0.89 (s, 3H, 24-H_a + 24-H_b + 24-H_c), 0.56 (s, 3H, 26-H_a + 26-H_b + 26-H_c) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 178.1 (C-28), 170.8 (C-33), 170.4 (C-31), 161.4 (d, *J* = 245.6 Hz, C-37), 144.8 (C-13), 130.7 (d, *J* = 4.4 Hz, C-41), 129.3 (d, J = 8.2 HZ, C-39), 125.5 (d, *J* = 15.0 Hz, C-36), 124.5 (d, *J* = 3.5 Hz, C-40), 122.9 (C-12), 115.4 (d, *J* = 21.4 Hz, C-38), 78.0 (C-3), 69.7 (C-2), 55.3 (C-5), 48.1 (C-9), 46.6 (C-19), 42.5 (C-18), 42.3 (C-17), 42.0 (C-1), 39.5 (C-14), 37.9 (C-35), 37.8 (C-8), 37.4 (C-4), 36.8 (C-10), 34.2 (C-21), 33.1 (C-30), 32.7 (C-7), 32.5 (C-15), 30.8 (C-20), 29.2 (C-24), 27.3 (C-16), 25.9 (C-27), 23.8 (C-22), 23.7 (C-29), 23.7 (C-11), 21.4 (C-34), 21.0 (C-32), 18.1 (C-6), 17.8 (C-25), 16.8 (C-26), 16.0 (C-23) ppm; MS (ESI): m/z = 662.6 (100 %, $[M-H]^-$); analysis calcd for $C_{41}H_{58}NO_5F$ (663.92): C 74.17, H 8.81, N 2.11; found: C 73.75, H 9.03, N 1.95.

4.2.35. (2β,3β) N-(3-fluorobenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (35)

Following GPA from **30** (180 mg, 0.32 mmol) followed by chromatography (SiO_2, hexanes/ethyl acetate, 9:1) **35** (164 mg, 76 %) was obtained as a colorless solid; m.p. 120–122 °C; R_f = 0.40 (SiO_2, hexanes/ethyl acetate, 3:1); $[\alpha]_D$ = +38.62° (*c* 0.172, $CHCl_3$); IR (KBr): ν = 3401vw, 2945 m, 2866w, 1742 s, 1646 m, 1616w, 1592w, 1513 m, 1487w, 1450 m, 1364 m, 1248vs, 1232vs, 1191 m, 1159w, 1139w, 1056 m, 1030 m, 1009 m, 991 m, 944 m, 915w, 887w, 822w, 772 m, 741w, 684 m, 605w, 578w, 520w, 494w, 439w cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.33 – 7.24 (m, 1H, 40-H), 7.03 (d, *J* = 7.8 Hz, 1H, 41-H), 6.99 – 6.92 (m, 2H, 37-H + 39-H), 6.24 – 6.17 (m, 1H, NH), 5.35 – 5.28 (m, 2H, 2-H + 12-H), 4.62 – 4.60 (m, 1H, 3-H), 4.60 – 4.54 (m, 1H, 35-H_a), 4.20 – 4.10 (m, 1H, 35-H_b), 2.55 (dd, *J* = 13.1, 4.4 Hz, 1H, 18-H), 2.04 (s, 3H, 34-H_a + 34-H_b + 34-H_c), 2.02 (s, 3H, 32-H_a + 32-H_b + 32-H_c), 2.01 – 1.91 (m, 2H, 1-H_a + 22-H_a), 1.90 – 1.82 (m, 2H, 11-H_a + 11-H_b), 1.81 – 1.64 (m, 3H, 7-H_a + 19-H_a + 22-H_b), 1.64 – 1.44 (m, 6H, 6-H_a + 6-H_b + 7-H_b + 9-H + 15-H_a + 16-H_a), 1.44 – 1.33 (m, 1H, 21-H_a), 1.33 – 1.18 (m, 4H, 1-H_b + 15-H_b + 19-H_b + 21-H_b), 1.15 (s, 6H, 23-H_a + 23-H_b + 23-H_c + 27-H_a + 27-H_b + 27-H_c), 1.05 (s, 3H, 25-H_a + 25-H_b + 25-H_c), 1.03 – 0.93 (m, 2H, 5-H + 16-H_b), 0.91 (s, 6H, 29-H_a + 29-H_b + 29-H_c + 30-H_a + 30-H_b + 30-H_c), 0.90 (s, 3H, 24-H_a + 24-H_b + 24-H_c), 0.68 (s, 3H, 26-H_a + 26-H_b + 26-H_c) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 178.6 (C-28), 171.2 (C-33), 170.7 (C-31), 163.4 (d, *J* = 246.4 Hz, C-38), 145.4 (C-13), 141.5 (d, *J* = 7.0 Hz, C-36), 130.6 (d, *J* = 8.2 Hz, C-40), 123.73 (d, *J* = 2.8 Hz, C-41), 123.1 (C-12), 115.0 (d, *J* = 40.9 Hz, C-37), 114.8 (d, *J* = 40.3 Hz, C-39), 78.3 (C-3), 70.0 (C-2), 55.6 (C-5), 48.4 (C-9), 47.0 (C-19), 46.9 (C-17), 43.5 (d, *J* = 1.9 Hz, C-35), 42.8 (C-18), 42.7 (C-14), 42.3 (C-1), 39.9 (C-8), 37.8 (C-4), 37.1 (C-10), 34.5 (C-21), 33.4 (C-30), 33.1 (C-7), 32.8 (C-15), 31.2 (C-20), 29.5 (C-24), 27.6 (C-16), 26.2 (C-27), 24.2 (C-22), 24.1 (C-29), 23.9, 21.7 (C-34), 21.3 (C-32), 18.4 (C-6), 18.1 (C-25), 17.5 (C-26), 16.4 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): m/z = 662. (100 %, $[M-H]^-$); analysis calcd for $C_{41}H_{58}NO_5F$ (663.92): C 74.17, H 8.81, N 2.11; found: C 73.84, H 9.13, N 1.86.

4.2.36. (2β,3β) N-(4-fluorobenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (36)

Following GPA from **30** (180 mg, 0.32 mmol) followed by chromatography (SiO_2, hexanes/ethyl acetate, 9:1) **39** (186 mg, 86 %) was obtained as a colorless solid; m.p. 123–126 °C; R_f = 0.38 (SiO_2, hexanes/ethyl acetate, 3:1); $[\alpha]_D$ = +40.72° (*c* 0.153, $CHCl_3$); IR (KBr): ν = 3411vw, 2945 m, 1742 s, 1645 m, 1605w, 1509 s, 1463w, 1433w, 1364 m, 1247vs, 1231vs, 1193 m, 1156 m, 1096w, 1056 m, 1030 m, 1015 m, 991 m, 946w, 822 m, 686w, 667w, 605w, 577 m, 487 m, 429w cm^{-1};^1H NMR (400 MHz, $CDCl_3$): δ = 7.25 – 7.19 (m, 2H, 37-H + 41-H), 7.03 – 6.97 (m, 2H, 38-H + 40-H), 6.16 (dd, *J* = 5.4, 5.4 Hz, 1H, NH), 5.33 – 5.27 (m, 2H, 2-H + 12-H), 4.60 (d, *J* = 3.9 Hz, 1H, 3-H), 4.53 (dd, *J* = 14.6, 6.1 Hz, 1H, 35-H_a), 4.17 – 4.08 (m, 1H, 35-H_b), 2.56 – 2.49 (m, 1H, 18-H), 2.04 (s, 3H, 34-H_a + 34-H_b + 34-H_c), 2.02 (s, 3H, 32-H_a + 32-H_b + 32-H_c), 2.00 – 1.92 (m, 2H, 1-H_a + 22-H_a), 1.87 – 1.82 (m, 2H, 11-H_a + 11-H_b), 1.80 – 1.62 (m, 3H, 7-H_a + 19-H_a + 22-H_b), 1.62 – 1.43 (m, 6H, 6-H_a + 6-H_b + 7-H_b + 9-H + 15-H_a + 16-H_a), 1.37 – 1.17 (m, 5H, 1-H_a + 15-H_b + 19-H_b + 21-H_a + 21-H_b), 1.14 (s, 3H, 27-H_a + 27-H_b + 27-H_c), 1.14 (s, 3H, 23-H_a + 23-H_b + 23-H_c), 1.05 (s, 3H, 25-H_a + 25-H_b + 25-H_c), 1.03 – 0.93 (m, 2H, 5-H + 16-H_b), 0.90 (s, 3H, 30-H_a + 30-H_b + 30-H_c), 0.89 (s, 6H, 24-H_a + 24-H_b + 24-H_c + 29-H_a + 29-H_b + 29-H_c), 0.66 (s, 3H, 26-H_a + 26-H_b + 26-H_c) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 178.0 (C-28), 170.7 (C-33), 170.2 (C-31), 162.1 (d, *J* = 245.6 Hz, C-39), 145.0 (C-13), 134.3 (d, *J* = 3.0 Hz, C-36), 129.5 (d, *J* = 8.1 Hz, C-37, C-41), 122.6 (C-12), 115.4 (d, *J* = 21.5 Hz, C-38, C-40), 77.9 (C-3), 69.5 (C-2), 55.1 (C-5), 47.9 (C-9), 46.5 (C-19), 46.3 (C-17), 42.9 (C-35), 42.3 (C-18), 42.2 (C-14), 41.9 (C-1), 39.5 (C-8), 37.3 (C-4), 36.6 (C-10), 34.1 (C-21), 32.9 (C-30), 32.6 (C-7), 32.3 (C-15), 30.7 (C-20), 29.0 (C-24), 27.2 (C-16), 25.7 (C-27), 23.8 (C-22), 23.6 (C-29), 23.5 (C-11), 21.3, 20.8 (C-32), 17.9 (C-6), 17.6 (C-25), 17.0 (C-26), 15.9 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): m/z = 662.7 (100 %, $[M-H]^-$); analysis calcd for $C_{41}H_{58}NO_5F$ (663.92): C 74.17, H 8.81, N 2.11; found: C 73.73, H 9.05, N 1.84.

4.2.37. (2β,3β) N-(2-methoxybenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (37)

Following GPA from **30** (180 mg, 0.32 mmol) followed by chromatography (SiO_2, hexanes/ethyl acetate, 9:1) **37** (180 mg, 82 %) was obtained as a colorless solid; m.p. 122–126 °C; R_f = 0.35 (SiO_2, hexanes/ethyl acetate, 3:1); $[\alpha]_D$ = +27.41° (*c* 0.162, $CHCl_3$); IR (KBr): ν = 3430vw, 2944 m, 2866w, 1741 s, 1654 m, 1604w, 1493 m, 1463 m, 1437w, 1364 m, 1242vs, 1193 m, 1161w, 1120w, 1054 m, 1029 s, 990w, 945w, 822w, 751 s, 685w, 666w, 606w, 574w, 513w cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.25 – 7.19 (m, 2H, 39-H + 41-H), 6.92 – 6.82 (m, 2H, 38-H + 40-H), 6.49 (dd, *J* = 5.7, 5.7 Hz, 1H, NH), 5.29 (dd, *J* = 3.8, 3.6 Hz, 1H, 2-H), 5.27 (t, *J* = 3.6 Hz, 1H, 12-H), 4.59 (d, *J* = 3.9 Hz, 1H, 3-H), 4.46 (dd, *J* = 14.2, 5.5 Hz, 1H, 35-H_a), 4.36 (dd, *J* = 14.2, 5.6 Hz, 1H, 35-H_b), 3.87 (s, 3H, 42-H_a + 42-H_b + 42-H_c), 2.48 (dd, *J* = 13.3, 4.0 Hz, 1H, 18-H), 2.05 (s, 3H, 34-H_a + 34-H_b + 34-H_c), 2.02 (s, 3H, 32-H_a + 32-H_b + 32-H_c), 2.00 – 1.87 (m, 2H, 1-H_a + 22-H_a), 1.85 – 1.78 (m, 2H, 11-H_a + 11-H_b), 1.77 – 1.63 (m, 3H, 7-H_a + 19-H_a + 22-

H_b), 1.62 – 1.45 (m, 4H, 6-H_a + 7-H_b + 9-H + 16-H_a), 1.44 – 1.36 (m, 3H, 6-H_b + 15-H_a + 21-H_a), 1.37 – 1.24 (m, 1H, 1-H_b), 1.24 – 1.13 (m, 3H, 15-H_b + 19-H_b + 21-H_b), 1.11 (s, 3H, 27-H_a + 27-H_b + 27-H_c), 1.09 (s, 3H, 23-H_a + 23-H_b + 23-H_c), 1.04 (s, 3H, 25-H_a + 25-H_b + 25-H_c), 0.97 – 0.91 (m, 2H, 5-H + 16-H_b), 0.89 (s, 6H, 29-H_a + 29-H_b + 29-H_c + 30-H_a + 30-H_b + 30-H_c), 0.88 (s, 3H, 24-H_a + 24-H_b + 24-H_c), 0.45 (s, 3H, 26-H_a + 26-H_b + 26-H_c) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 177.6 (C-28), 170.8 (C-33), 170.4 (C-31), 157.8 (C-37), 145.0 (C-13), 130.2 (C-39), 128.8 (C-41), 126.5 (C-36), 122.4 (C-12), 120.9 (C-40), 110.1 (C-38), 78.0 (C-3), 69.8 (C-2), 55.4 (C-5), 55.3 (C-42), 48.1 (C-9), 46.7 (C-19), 46.6 (C-17), 42.5 (C-18), 42.3 (C-14), 42.0 (C-1), 39.7 (C-35), 39.5 (C-8), 37.4 (C-4), 36.7 (C-10), 34.3 (C-21), 33.1 (C-30), 32.7 (C-7), 32.5 (C-15), 30.8 (C-20), 27.3 (C-16), 25.9 (C-27), 23.7 (C-29), 23.7 (C- 22), 23.7 (C-11), 21.4 (C-34), 21.0 (C-32), 18.0 (C-6), 17.8 (C-25), 16.6 (C-26), 16.0 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 674.7 (100 %, $[M-H]^-$); analysis calcd for $C_{42}H_{61}NO_6$ (675.95): C 74.63, H 9.10, N 2.07; found: C 74.50, H 8.88, N 1.83.

4.2.38. (2β,3β) N-(3-methoxybenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (38)

Following GPA from **30** (180 mg, 0.32 mmol) followed by chromatography (SiO_2, hexanes/ethyl acetate, 9:1) **38** (182 mg, 84 %) was obtained as a colorless solid; m.p. 113.7 °C; R_f = 0.33 (SiO_2, hexanes/ethyl acetate, 3:1); $[\alpha]_D$ = +35.40° (*c* 0.132, $CHCl_3$); IR (KBr): ν = 3406vw, 2945 m, 1741 s, 1649 m, 1602w, 1587w, 1514 m, 1489 m, 1464 m, 1455 m, 1434 m, 1365 m, 1248vs, 1232vs, 1192 m, 1154 m, 1031 s, 991 m, 945 m, 913w, 873w, 822w, 771 m, 757 m, 691 m, 667w, 605 m cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.26 – 7.21 (m, 1H, 40-H), 6.87 – 6.76 (m, 3H, 37-H + 39-H + 41-H), 6.16 (dd, *J* = 6.7, 5.91 Hz, 1H, NH), 5.34 – 5.28 (m, 2H, 2-H + 12-H), 4.61 (d, *J* = 4.0 Hz, 1H, 3-H), 4.58 (d, *J* = 6.3 Hz, 1H, 35-H_a), 4.15 – 4.08 (m, 1H, 35-H_b), 3.79 (s, 3H, 42-H_a + 42-H_b + 42-H_c), 2.54 (dd, *J* = 12.9, 4.5 Hz, 1H, 18-H), 2.06 – 2.03 (m, 3H, 34-H_a + 34-H_b + 34-H_c), 2.02 (s, 3H, 32-H_a + 32-H_b + 32-H_c), 2.00 – 1.93 (m, 2H, 1-H_a + 22-H_a), 1.90 – 1.80 (m, 2H, 11-H_b + 11-H_b), 1.79 – 1.68 (m, 3H, 7-H_a + 19-H_a + 22-H_b), 1.68 – 1.42 (m, 6H, 6-H_a + 6-H_b + 7-H_b + 9-H + 15-H_a + 16-H_a), 1.42 – 1.25 (m, 3H, 1-H_b + 15-H_b + 21-H_a), 1.25 – 1.15 (m, 2H, 19-H_b + 21-H_b), 1.14 (s, 6H, 23-H_a + 23-H_b + 23-H_c + 27-H_a + 27-H_b + 27-H_c), 1.05 (s, 3H, 25-H_a + 25-H_b + 25-H_c), 1.04 – 0.93 (m, 2H, 5-H + 16-H_b), 0.91 (s, 3H, 30-H_a + 30-H_b + 30-H_c), 0.90 (s, 6H, 24-H_a + 24-H_b + 24-H_c + 29-H_a + 29-H_b + 29-H_c), 0.71 (s, 3H, 26-H_a + 26-H_b + 26-H_c) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 177.9 (C-28), 170.7 (C-33), 170.2 (C-31), 159.9 (C-38), 145.0 (C-13), 140.0 (C-36), 129.7 (C-40), 122.6 (C-12), 119.9 (C-41), 113.2 (C-39), 112.9 (C-37), 77.9 (C-3), 69.5 (C-2), 55.2 (C-42), 55.2, 48.0 (C-9), 46.5 (C-19), 46.4 (C-17), 43.5 (C-35), 42.3 (C-18), 42.2 (C-14), 39.5 (C-8), 37.3 (C-4), 36.6 (C-10), 34.1 (C-21), 33.0 (C-30), 32.6 (C-7), 32.3 (C-15), 30.7 (C-20), 29.0 (C-24), 27.2 (C-16), 25.8 (C-27), 23.8 (C-22), 23.6 (C-29), 23.5 (C-11), 21.3 (C-34), 20.8 (C-32), 17.9 (C-6), 17.6 (C-25), 17.0 (C-26), 15.9 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 674.6 (100 %, $[M-H]^-$); analysis calcd for $C_{42}H_{61}NO_6$ (675.95): C 74.63, H 9.10, N 2.07; found: C 74.41, H 9.37, N 1.86.

4.2.39. (2β,3β) N-(4-methoxybenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (39)

Following GPA from **30** (180 mg, 0.32 mmol) followed by chromatography (SiO_2, hexanes/ethyl acetate, 9:1) **39** (210 mg, 96 %) was obtained as a colorless solid; m.p. 118 °C; R_f = 0.29 (SiO_2, hexanes/ethyl acetate, 3:1); $[\alpha]_D$ = +32.02° (*c* 0.169, $CHCl_3$); IR (KBr): ν = 3419vw, 2944w, 2882w, 1742 s, 1649w, 1613w, 1512 m, 1463 m, 1433w, 1364 m, 1301w, 1246vs, 1192 m, 1175 m, 1111w, 1055 m, 1031 s, 990w, 946w, 821 m, 753w, 686w, 667w, 604w, 582w, 524w cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.19 – 7.13 (m, 2H, 37-H + 41-H), 6.90 – 6.81 (m, 2H, 38-H + 40-H), 6.13 – 6.05 (m, 1H, NH), 5.33 – 5.25 (m, 2H, 2-H + 12-H), 4.61 (d, *J* = 3.9 Hz, 1H, 3- H), 4.55 – 4.48 (m, 1H, 35-H_a), 4.09 (dd, *J* = 14.4, 4.2 Hz, 1H, 35-H_b), 3.80 (s, 3H, 42-H_a + 42-H_b + 42-H_c), 2.56 – 2.48 (m, 1H, 18-H), 2.04 (s, 3H, 34-H_a + 34-H_b + 34-H_c), 2.03 (s, 3H, 32-H_a + 32-H_b + 32-H_c), 1.96 (ddd, *J* = 14.9, 7.9, 3.5 Hz, 2H, 1-H_a + 22-H_a), 1.88 – 1.81 (m, 2H, 11-H_a + 11-H_b), 1.79 – 1.63 (m, 3H, 7-H_a + 19-H_a + 22-H_b), 1.62 – 1.42 (m, 6H, 6-H_a + 6-H_b + 7-H_b + 9-H + 15-H_a + 16-H_a), 1.41 – 1.25 (m, 3H, 1-H_b + 15-H_b + 21-H_a), 1.25 – 1.16 (m, 2H, 19-H_b + 21-H_b), 1.15 (s, 3H, 23-H_a + 23-H_b + 23-H_c), 1.14 (s, 3H, 27-H_a + 27-H_b + 27-H_c), 1.05 (s, 3H, 25-H_a + 25-H_b + 25-H_c), 1.03 – 0.93 (m, 2H, 5-H + 16-H_b), 0.92 – 0.89 (m, 6H, 24-H_a + 24-H_b + 24-H_c + 30-H_a + 30-H_b + 30-H_c), 0.89 (s, 3H, 29-H_a + 29-H_b + 29-H_c), 0.71 (s, 3H, 26-H_a + 26-H_b + 26-H_c) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 178.3 (28), 171.1 (C-33), 170.6 (C-31), 159.3 (C-39), 145.4 (C-13), 130.9 (C-36), 129.5 (C-37, C-41), 123.0 (C-12), 114.4 (C-38, C-40), 78.3 (C-3), 70.0 (C-2), 55.7 (C-5), 55.5 (C-42), 48.4 (C-9), 46.9 (C-17), 46.7 (C-19), 43.5 (C-35), 42.7 (C-18), 42.6 (C-14), 42.3 (C-1), 39.9 (C-8), 37.7 (C-4), 37.0 (C-10), 34.5 (C-21), 33.4 (C-30), 33.0 (C-7), 32.7 (C-15), 31.1 (C-20), 29.5 (C-24), 27.6 (C-16), 26.2 (C-27), 24.1 (C-22), 24.0 (C-29), 23.9 (C-11), 21.7 (C-34), 21.3 (C-32), 18.3 (C-6), 18.0 (C-25), 17.5 (C-26), 16.3 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 674.5 (100 %, $[M-H]^-$); analysis calcd for $C_{42}H_{61}NO_6$ (675.95): C 74.63, H 9.10, N 2.07; found: C 74.41, H 9.37, N 1.85.

4.2.40. (2β,3β) N-(2-methylbenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (40)

Following GPA from **30** (180 mg, 0.32 mmol) followed by chromatography (SiO_2, hexanes/ethyl acetate, 9:1) **40** (200 mg, 94 %) was obtained as a colorless solid; m.p. 114–117 °C; R_f = 0.42 (SiO_2, hexanes/ethyl acetate, 3:1); $[\alpha]_D$ = +34.59° (*c* 0.159, $CHCl_3$); IR (KBr): ν = 3410vw, 2945 m, 2866w, 1742 s, 1658 m, 1513 m, 1462 m, 1433w, 1364 m, 1247vs, 1231vs, 1191 m, 1158w, 1055 m, 1029 m, 1012 m, 990 m, 945w, 911w, 822w, 739 m, 685w, 667w, 605w, 514w cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.23 – 7.12 (m, 4H, 38-H + 39-H + 40-H + 41-H), 6.06 (dd, *J* = 6.4, 4.2 Hz, 1H, NH), 5.33 – 5.24 (m, 2H, 2-H + 12-H), 4.67 – 4.55 (m, 2H, 3-H + 35-H_a), 4.15 (dd, *J* = 14.7, 4.1 Hz, 1H, 35-H_b), 2.57 – 2.48 (m, 1H, 18-H), 2.31 (s, 3H, 42-H_a + 42-H_b + 42-H_c), 2.04 (s, 3H, 34-H_a + 34-H_b + 34-H_c), 2.02 (s, 3H, 32-H_a + 32-H_b + 32-H_c), 2.01 – 1.83 (m, 2H, 1-H_a + 22-H_a), 1.82 (dd, *J* = 8.8, 3.6 Hz, 2H, 11-H_a + 11-H_b), 1.80 – 1.68 (m, 3H, 7-H_a + 19-H_a + 22-H_b), 1.68 – 1.56 (m, 3H, 6-H_a + 7-H_b + 16-H_a), 1.56 – 1.44 (m, 3H, 6-H_b + 9-H + 15-H_a), 1.43 – 1.17 (m, 5H, 1-H_b + 15-H_b + 19-H_b + 21-H_a + 21-H_b), 1.14 (s, 6H, 23-H_a + 23-H_b + 23-H_c + 27-H_a + 27-H_b + 27-H_c), 1.05 (s, 3H, 25-H_a + 25-H_b + 25-H_c), 1.03 – 0.93 (m, 2H, 5-H + 16-H_b), 0.90 (s, 3H, 30-H_a + 30-H_b + 30-H_c), 0.90 (s, 6H, 24-H_a + 24-H_b + 24-H_c + 29-H_a + 29-H_b + 29-H_c), 0.68 (s, 3H, 26-H_a + 26-H_b + 26-H_c) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 177.9 (C-28), 170.7 (C-33), 170.2 (C-31), 145.0 (C-13), 136.4 (C-36), 136.0 (C-37), 130.5 (C-38), 128.3 (C-41), 127.6 (C-39), 126.2 (C-40), 122.7 (C-12), 69.5 (C-2), 55.1 (C-5), 47.9 (C-9), 46.5 (C-19), 46.4 (C-17), 42.4 (C-18), 42.2 (C-14), 41.9 (C-1), 41.6 (C-35), 39.4 (C-8), 37.3 (C-4), 36.6 (C-10), 34.1 (C-21), 32.9 (C-30), 32.6 (C-7), 32.3 (C-15), 30.7 (C-20), 29.0 (C-24), 27.2 (C-16), 25.7 (C-27), 23.8 (C-22), 23.6 (C-29), 23.5 (C-11), 21.3 (C-34), 20.8 (C-32), 19.1 (C-42), 17.9 (C-6), 17.6 (C-25), 17.0 (C-26), 15.9 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 658.6 (100 %, $[M-H]^-$); analysis calcd for $C_{42}H_{61}NO_5$ (659.45): C 76.44, H 9.32, N 2.12; found: C 76.19, H 9.64, N 1.92.

4.2.41. (2β,3β) N-(3-methylbenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (41)

Following GPA from **30** (180 mg, 0.32 mmol) followed by chromatography (SiO_2, hexanes/ethyl acetate, 9:1) **41** (178 mg, 84 %) was obtained as a colorless solid; m.p. 116–119 °C; R_f = 0.40 (SiO_2, hexanes/ethyl acetate, 3:1); $[\alpha]_D$ = +40.98° (*c* 0.166, $CHCl_3$); IR (KBr): ν = 3405vw, 2943 m, 2877w, 1742 s, 1646 m, 1514 m, 1462 m, 1433 m, 1364 m, 1246vs, 1231vs, 1192 m, 1158 m, 1056 m, 1030 s, 1010 m, 991 m, 973 m, 945 m, 912w, 822w, 772 m, 737w, 691 m, 667w, 625w, 604 m, 578w cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.22 (dd, *J* = 7.4, 7.4 Hz, 1H, 40-H), 7.12 – 7.01 (m, 3H, 37-H + 39-H + 41-H), 6.18 – 6.10 (m, 1H, NH), 5.33 – 5.27 (m, 2H, 2-H + 12-H), 4.61 (d, *J* = 3.9 Hz, 1H, 3-H), 4.59 – 4.50 (m, 1H, 35-H_a), 4.13 (dd, *J* = 14.6, 4.3 Hz, 1H, 35-H_b), 2.58 – 2.49

(m, 1H, 18-H), 2.34 (s, 3H, 42-H_a + 42-H_b + 42-H_c), 2.05 (s, 3H, 34-H_a + 34-H_b + 34-H_c), 2.02 (s, 3H, 32-H_a + 32-H_b + 32-H_c), 2.00–1.91 (m, 2H, 1-H_a + 22-H_a), 1.89 – 1.81 (m, 2H, 11-H_a + 11-H_b), 1.81 – 1.56 (m, 6H, 6-H_a + 7-H_a + 7-H_b + 16-H_a + 19-H_a + 22-H_b), 1.56 – 1.43 (m, 3H, 6-H_b + 9-H + 15-H_a), 1.43 – 1.16 (m, 5H, 1-H_b + 15-H_b + 19-H_b + 21-H_b + 21-H_b), 1.14 (s, 6H, 23-H_a + 23-H_b + 23-H_c + 27-H_a + 27-H_b + 27-H_c), 1.05 (s, 3H, 25-H_a + 25-H_b + 25-H_c), 1.03 – 0.94 (m, 2H, 5-H + 16-H_b), 0.90 (s, 3H, 30-H_a + 30-H_b + 30-H_c), 0.90 (s, 6H, 24-H_a + 24-H_b + 24-H_c + 29-H_a + 29-H_b + 29-H_c), 0.71 (s, 3H, 26-H_a + 26-H_b + 26-H_c) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 178.1 (C-28), 170.8 (C-33), 170.4 (C-31), 145.1 (C-13), 138.4 (C-36), 138.3 (C-38), 128.7 (C-37, C-40), 128.2 (C-39), 124.9 (C-41), 122.7 (C-12), 78.0 (C-3), 77.5, 77.2, 76.8, 69.7 (C-2), 55.3 (C-5), 48.1 (C-9), 46.7 (C-19), 46.5 (C-17), 43.8 (C-35), 42.5 (C-18), 42.3 (C-14), 42.0 (C-1), 39.6 (C-8), 37.5 (C-4), 36.8 (C-10), 34.3 (C-21), 33.1 (C-30), 32.8 (C-7), 32.5 (C-15), 30.9 (C-20), 29.2 (C-24), 27.3 (C-16), 25.9 (C-27), 23.9 (C-22), 23.7 (C-29), 23.6 (C-11), 21.5 (C-34), 21.4 (C-42), 21.0 (C-32), 18.1 (C-6), 17.8 (C-25), 17.2, 16.0 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): m/z = 658.8 (100 %, $[M-H]^-$); analysis calcd for $C_{42}H_{61}NO_5$ (659.45): C 76.44, H 9.32, N 2.12; found: C 76.22, H 9.54, N 1.87.

4.2.42. (2β,3β) N-(4-methylbenzyl) 2,3-bis(acetyloxy)-olean-12-en-28-amide (42)

Following GPA from **30** (180 mg, 0.32 mmol) followed by chromatography (SiO_2, hexanes/ethyl acetate, 9:1) **42** (185 mg, 84 %) was obtained as a colorless solid; m.p. 124.6 °C; R_f = 0.40 (SiO_2, hexanes/ethyl acetate, 3:1); $[\alpha]_D$ = +35.56° (*c* 0.181, $CHCl_3$); IR (KBr): ν = 3409vw, 2945 m, 2877w, 1743 s, 1645 m, 1515 m, 1462 m, 1432w, 1364 m, 1247vs, 1232vs, 1192 m, 1159w, 1056 m, 1030 m, 991 m, 972w, 945w, 912w, 807 m, 754 m, 686w, 666w, 603w, 579w, 475 m cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.13 (s, 4H, 37-H + 38-H + 40-H + 41-H), 6.12 (dd, *J* = 6.3, 4.3 Hz, 1H, NH), 5.33 – 5.27 (m, 2H, 2-H + 12-H), 4.61 (d, *J* = 3.9 Hz, 1H, 3-H), 4.57 (dd, *J* = 14.6, 6.2 Hz, 1H, 35-H_a), 4.10 (dd, *J* = 14.6, 4.3 Hz, 1H, 35-H_b), 2.56 – 2.49 (m, 1H, 18-H), 2.34 (s, 3H, 42-H_a + 42-H_b + 42-H_c), 2.05 (s, 3H, 34-H_a + 34-H_b + 34-H_c), 2.03 (s, 3H, 32-H_a + 32-H_b + 32-H_c), 2.00–1.93 (m, 2H, 1-H_a + 22-H_a), 1.88 – 1.82 (m, 2H, 11-H_a + 11-H_b), 1.80 – 1.67 (m, 3H, 7-H_a + 19-H_a + 22-H_b), 1.66 – 1.56 (m, 3H, 6-H_a + 7-H_b + 16-H_a), 1.56 – 1.42 (m, 3H, 6-H_b + 9-H + 15-H_a), 1.42 – 1.24 (m, 3H, 1-H_b + 15-H_b + 21-H_a), 1.24 – 1.16 (m, 2H, 19-H_b + 21-H_b), 1.15 (s, 3H, 23-H_a + 23-H_b + 23-H_c), 1.14 (s, 3H, 27-H_a + 27-H_b + 27-H_c), 1.05 (s, 3H, 25-H_a + 25-H_b + 25-H_c), 1.04 – 0.91 (m, 2H, 5-H + 16-H_b), 0.90 (s, 6H, 24-H_a + 24-H_b + 24-H_c + 30-H_a + 30-H_b + 30-H_c), 0.89 (s, 3H, 29-H_a + 29-H_b + 29-H_c), 0.70 (s, 3H, 26-H_a + 26-H_b + 26-H_c) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 178.3 (C-28), 171.1 (C-33), 170.6 (C-31), 145.4 (C-13), 137.4 (C-36), 135.7 (C-39), 129.7 (C-38, C-40), 128.1 (C-37, C-41), 123.0 (C-12), 78.3 (C-3), 70.0 (C-2), 55.5 (C-5), 48.4 (C-9), 46.9 (C-19), 46.7 (C-17), 43.8 (C-35), 42.7 (C-18), 42.6 (C-14), 42.3 (C-1), 39.9 (C-8), 37.7 (C-4), 37.0 (C-10), 34.5 (C-21), 33.4 (C-30), 33.0 (C-7), 32.7 (C-15), 31.1 (C-20), 29.5 (C-24), 27.6 (C-16), 26.2 (C-27), 24.1 (C-22), 24.0 (C-29), 23.9 (C-11), 21.7 (C-34), 21.5 (C-42), 21.2 (C-32), 18.3, 18.0 (C-25), 17.4 (C-26), 16.3 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): m/z = 658.6 (100 %, $[M-H]^-$); analysis calcd for $C_{42}H_{61}NO_5$ (659.45): C 76.44, H 9.32, N 2.12; found: C 76.25, H 9.56, N 1.89.

CRediT authorship contribution statement

Niels V. Heise: Investigation. **Julia Heisig:** Investigation. **Linda Höhlich:** Investigation. **Sophie Hoenke:** Investigation. **René Csuk:** Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rechem.2023.100805.

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An improved partial synthesis of corosolic acid and its conversion to highly cytotoxic mitocans

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ABSTRACT

Ursolic acid was used as a convenient starting material for a three-step partial synthesis of corosolic acid. Corosolic acid was acetylated, and an amide linker was attached followed by a rhodamine B unit at the distal end of the linker. Especially compounds holding a piperazinyl or homopiperazinyl linker held exceptional cytotoxicity for several human tumor cell lines. For example, homopiperazinyl spacered **11** showed EC_{50} = 2 nM for A2780 cells combined with high tumor cell/non-tumor cell selectivity (compound **11**: EC_{50} = 0.122 μM for non-malignant NIH 3T3). Thus, compound **11** currently represents one of the most cytotoxic triterpene derivatives holding both superior cytotoxicity combined with high selectivity ($SI_{A2780\ vs\ NIH\ 3T3} > 60$). Staining experiments showed this compound to act as a mitocan. This makes **11** an interesting compound for further studies or as a lead substance.

1. Introduction

The significance of triterpenes for the development of cytotoxic compounds is undisputed and has become increasingly important in recent years [1–14]. This is, on the one hand, due to the fact that the number of people suffering from cancer [15] has increased significantly and, on the other hand, some triterpene-derived compounds have emerged as hopeful candidates for further development.

In the course of our own investigations, we have been able to access numerous compounds that exhibited good cytotoxicity in a variety of human tumor cell lines. In order to obtain compounds with enhanced cytotoxicity, hydroxyl groups present in ring A must be appropriately protected. Acetates and amino(oxo)acetates [16] were particularly suitable for this purpose, whereas chloroacetyl groups at these positions increased the cytotoxicity but at the same time drastically reduced the tumor cell/non-tumor cell selectivity of the compounds [17–19]. Furthermore, the carboxyl group on ring E must be retained [20–28]. Particularly advantageous seems its conversion into an amide [29], especially as a phenyl, benzyl or (iso)-quinolinyl amide [30–36]. Two compounds in particular should be mentioned here, first of all **EM2**, a diacetylated benzylamide of maslinic acid [31], and a iso-quinolinylamide **IQAA** [36] derived from augustic acid (Fig. 1).

We observed that the presence of at least two acetyl groups in ring A led to compounds of higher cytotoxicity [37]; at the same time, the introduction of a (homo)-piperazinyl residue [38–40] (as an amide) gave access to a further increase in cytotoxicity. If, in addition, a cationic lipophilic group was introduced at the distal end of this moiety, compounds with cytotoxicity even in the nano-molar range were obtained.

On the one hand, compounds holding a rhodamine B or a rhodamine 101 residue proved to be particularly advantageous [6,38], whereas malachite green-substituted compounds [41] were significantly less active, and triterpenes provided with a safirinium ring could enter the cell and reached the endoplasmic reticulum of cancer cells but not their mitochondria [42]; these compounds were probably weaker in their cytotoxic activity for this reason. Since a (2α, 3β) configuration in ring A seemed to be particularly advantageous (as shown in examples of maslinic acid as well as in the comparison between euscaphic and tormentillic acid) [6], it was obvious to also investigate to what extent oleanolic acid-derived compounds (such as maslinic acid derivatives) differ from ursolic acid-derived compounds when the (2α, 3β) configuration of the acetyl groups in ring remained the same. Corosolic acid [43–49] (**CA**, Scheme 1) was selected as an ideal comparative molecule meeting all these criteria. Maslinic acid and corosolic acid share a calculated maximum common structural size (MCS Tanimoto index of 0.94); these two pentacyclic triterpene carboxylic acids differ only in the arrangement of the two methyl groups in ring E.

Corosolic acid is a pentacyclic triterpene with an ursane backbone, and was first isolated from rose-bay willow (Chamaenerion

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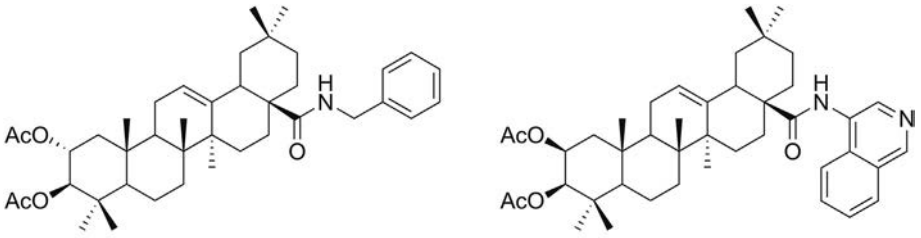


EM2
EC_{50} = 0.5 μM (A2780)
EC_{50} = 33.8 μM (NIH 3T3)

IQAA
EC_{50} = 1.2 μM (A2780)
EC_{50} = > 60 μM (NIH 3T3)

Fig. 1. Structure of cytotoxic maslinic acid derived **EM2** and augustic acid derived **IQAA** and their EC_{50} values for A2780 ovarian cancer cells and non-malignant fibroblasts NIH 3T3.

angustifolium) in 1965 by A.T. Glen et al. [50] Corosolic acid occurs in the plant Eriobotrya japonica (loquat) [51–54] and Lagerstroemia espiosa (banaba) [55–57] but also in apple peels [58–63] and can be obtained from it by extraction, although yields are usually low, also due to a complicated purification process. Alternatively, a partial synthesis starting from ursolic acid was proposed by Wen et al. several years ago [64,65]. Following our own partial syntheses of triterpenes with oleanane backbone (maslinic, augustic, bredemolic and *epi*-maslinic acid) [66,67] we modified these known synthesis strategies to make **CA** accessible in larger amounts. Further conversions involve the formation of amides and their respective linkage with a rhodamine B residue. The cytotoxicity of these compounds should be demonstrated in sulforhodamine B (SRB) assays.

2. Results and discussion

2.1. Chemistry

The introduction of vicinal hydroxyl groups can be accomplished in a variety of ways; the often-used sequence of elimination of an already present hydroxyl group followed by epoxidation of the corresponding alkene and subsequent ring opening seemed unattractive to us for reasons of atom economy as defined by B.M. Trost. This sequence leads to *trans*-oriented hydroxyl groups while *cis*-oriented 1,2-diols can be obtained – *inter alia* – from the reaction of the alkene with reagents such as $KMnO_4$, OsO_4, $RuCl_3/NaIO_4$. To provide a short synthesis (that could even be scaled-up) we therefore decided to consider a hydroxylation reaction in the α-position to a keto group as an alternative. Various methods for this hydroxylation reactions have already been described in the literature, including many Davies and Rubottom type oxidations but also Oxone has been used [68–70]. Previously, we [67] have used some of these strategies to access *inter alia* maslinic acid and augustic acid, respectively.

Thus, in a first step, commercially available ursolic acid (**UA**) was to be oxidized at the C-3 position, and a Jones Oxidation of ursolic acid (**UA**, Scheme 1) gave well known ursonic acid (**1**) in 97% isolated yield. Reaction of **1** with *m*CPBA in a 3:1 mixture of MeOH/DCM in the presence of sulfuric acid provided 78% of the corresponding 2α-hydroxy-3-oxoursolic acid (**2**). This method has been patented [71–75] and also been used in Wen's approach of 2007 [64,65]; usually from the reaction of ketones with *m*CPBA [76] Baeyer-Villiger reactions can be expected to occur leading to a lactone [77,78]. Investigation of the crude reaction mixture by ESI-MS, however, showed the presence very small amounts of a product with M = 470 (corresponding to $C_{30}H_{46}O_4$, probably the lactone from the Baeyer-Villiger reaction; not isolated). Reduction of **2** with sodium borohydride in a THF/MeOH (3:1) mixture gave **CA** (**3**). Under these conditions, reduction occurs rather selectively to form **CA** holding a (2α, 3β) configuration; traces of an epimeric (2α, 3α) configurated triterpene (i.e. 3-*epi*-corosolic acid) could be detected by HPTLC/ESI-MS but this compound was not isolated. Acetylation of **CA** with acetic anhydride in pyridine in the presence of cat. amounts of DMAP afforded diacetate **4**.

2.2. Biology

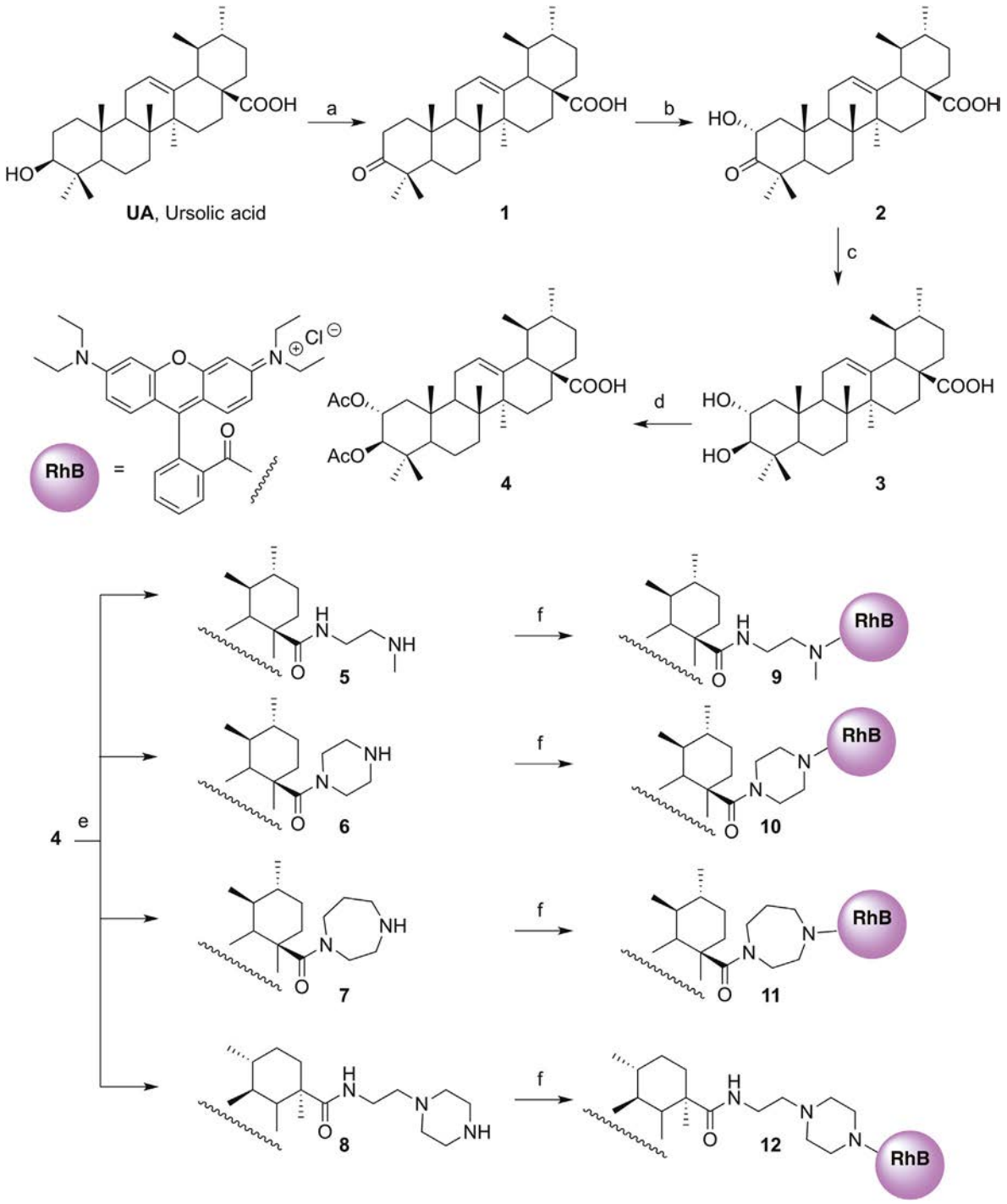
Except for a 2,3-bis(acetyloxy)urs-12-en-28-amide no amides have been reported so far from corosolic acid. Thus, for the synthesis of the amides, **4** was allowed to react with oxalyl chloride followed by the addition of the corresponding amine (*N*-methyl-ethylenediamine, piperazine, homopiperazine, 1-(2-aminoethyl)-piperazine) to afford amides **5**–**8**, respectively. Their reaction with activated rhodamine B (from the reaction of rhodamine B with oxalyl chloride) afforded rhodamine conjugates **9**–**12**.

The rhodamine conjugates are characterized by their deep violet color. This indicates the presence of an intact cationic rhodamine B moiety. A lipophilic cationic residue at this position is a prerequisite for obtaining compounds of high cytotoxicity probably due to interactions with the mitochondrial membrane(s).

To assess the cytotoxicity of the compounds, sulforhodamine B assays (SRB) were performed employing several human tumor cell lines (A375, HT29, MCF-7, A2780, HeLa) as well as non-malignant fibroblasts (NIH 3T3). The results from these assays are compiled in Table 1.

As a result of these assays: while **4** is only slightly cytotoxic and also relatively unselective with respect to the cytotoxicity for the cancer cell lines and non-malignant fibroblasts, an approximately 5-fold higher cytotoxicity was found for the piperazinylamide **6** and homopiperazinylamide **7**. However, selectivity is again not pronounced: SI $EC_{50,\ NIH\ 3T3}/EC_{50,\ A2780}$ = 0.9 for A2780 cells, for MCF7 SI = 1.2 and for A375 cells SI = 1.4. A dramatic improvement in cytotoxicity but also selectivity occurred when moving to the rhodamine B conjugates. For the human tumor cell line A2780 an approximately 140-fold increase in cytotoxicity for **10** as compared to **6** was determined. Finally, an approximately 1400-fold increase in cytotoxicity was shown for the homopiperazinyl conjugate **11**. A similar trend is also seen for the other tumor cell lines. Furthermore, there is a clear increase in selectivity in the transition from piperazinyl to homopiperazinyl spacered compounds. While the cell selectivity (A2780 vs NIH 3T3) for the piperazinyl-spaced compound **10** is approximately 10, this value increases six-fold to 61 for the homopiperazinyl-spacered conjugate **11**. This compound is ca 9400 times more cytotoxic than acetylated corosolic acid **4**. The comparison between compounds **6** and **8** but also between **10** and **12** shows that an additional spacer (as present in **8** and **10**) significantly reduces cytotoxicity. The presence of an additional methyl group in **9** avoids an internal lactam formation (as already known from the corresponding ethylenediamine-spaced compounds). Thus, the cationic character of the rhodamine B residue is preserved and good cytotoxicity is thus achieved albeit not as high as in the piperazinyl or homopiperazinyl spacered compounds. This emphasizes the importance of a very careful selection of the spacer to achieve optimal cytotoxicity and selectivity.

We have previously shown that triterpene-rhodamine B conjugates act as mitocans [6]. To demonstrate this also for **11** – the most cytotoxic



Scheme 1. Synthesis of corosolic acid (**CA**, **3**) and **CA**-derived mitocans **9**–**12**: a) CrO_3, H_2SO_4, acetone, 0 °C, 1h, 97%; b) *m*CPBA, H_2SO_4, MeOH, DCM, 0 °C, 5 h, 78%; c) $NaBH_4$, THF, MeOH, 30 min, 21 °C, 81%; d) Ac_2O, py, DMAP (cat.), 10 h, 21 °C, 72%; e) $(COCl)_2$, DCM, DMF (cat.), 0 °C → 21 °C, then amine (5 equiv.), 0 °C → 21 °C, 2 h: → **5** (from *N*-methyl-ethylenediamine, 73%), → **6** (from piperazine, 86%), → **7** (from homopiperazine, 76%), → **8** (from 1-(2-aminoethyl)-piperazine, 65%); f) Rho B, $(COCl)_2$, DMF (cat.), THF, then **5**–**8**, DCM, 0 °C → 21 °C, 2 h: → **9** (from *N*-methyl-ethylenediamine, 86%), → **10** (from piperazine, 92%), → **11** (from homopiperazine,77%), → **12** (from 1-(2-aminoethyl)-piperazine, 62%).

compound of this study – A375 cells were stained with acridine orange (AO), rhodamine 123 as well as Hoechst 33,342. Thereby, Hoechst 33, 342 binds selectively to DNA while rhodamine 123 is usually applied for dying mitochondria. The results of these staining experiments prove that **11** also acts as a mitocan since this compound is localized within the cells exactly in the same area as rhodamine 123 while a location in the nucleus can be ruled out from staining experiments using Hoechst 33,342.

A summary of our previous results and the results from this investigation of hitherto known structural prerequisites to obtain pentacyclic triterpenoids of high cytotoxicity and good tumor/non-tumor cell selectivity is depicted in Fig. 2. Thus, ring A should hold vicinal hydroxyl groups preferentially – but protected as acetates to provide better bioavailability. It seems that a (2α, 3β) configuration adds to cytotoxicity and selectivity. A carboxyl group attached to ring E is necessary for high cytotoxicity as well as an amide linker preferentially derived from a cyclic secondary diamine with homopiperazine being

Table 1
Cytotoxicity of compounds **4**–**12** (EC_{50}-values in μM from SRB-assays) after 72 h of treatment; the values are averaged from three independent experiments performed each in triplicate, confidence interval CI = 95%; mean ± standard mean error); n.d. not determined; n.s. not soluble; doxorubicin (**DX**) was used as a positive control. Cell lines: malignant: A375 (melanoma), HT29 (colon adenocarcinoma), MCF7 (breast adenocarcinoma), A2780 (ovarian carcinoma), HeLa (cervical cancer); non-malignant: NIH 3T3 (fibroblasts).

Compound	A375	HT29	MCF7	A2780	HeLa	NIH 3T3
4	25.6 ± 1.4	27.0 ± 0.8	14.3 ± 2.2	18.7 ± 1.4	23.5 ± 1.1	17.8 ± 1.2
6	2.0 ± 0.1	2.3 ± 0.1	2.3 ± 0.2	2.8 ± 0.4	2.7 ± 0.2	2.7 ± 0.2
5	3.7 ± 0.3	2.9 ± 0.1	3.2 ± 0.2	3.6 ± 0.5	4.3 ± 0.6	4.8 ± 0.3
7	2.4 ± 0.3	1.5 ± 0.2	2.0 ± 0.2	2.6 ± 0.3	2.9 ± 0.4	1.6 ± 0.2
8	4.7 ± 0.4	3.0 ± 0.2	3.9 ± 0.3	4.7 ± 0.8	5.9 ± 0.4	4.1 ± 0.4
10	0.05 ± 0.01	0.05 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.13 ± 0.02	0.20 ± 0.01
9	0.04 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.01 ± 0.01	0.22 ± 0.03	0.23 ± 0.04
11	0.005 ± 0.001	0.008 ± 0.003	0.012 ± 0.004	0.002 ± 0.001	0.053 ± 0.02	0.122 ± 0.005
12	0.12 ± 0.01	0.12 ± 0.02	0.10 ± 0.01	0.05 ± 0.01	0.29 ± 0.04	0.36 ± 0.04
DX	n.d.	0.9 ± 0.2	1.1 ± 0.3	0.02 ± 0.01	n.d.	1.7 ± 0.3

slightly better than piperazine. The presence of a rhodamine unit at the distal nitrogen of the spacer is mandatory; previous studies showed rhodamines (B or 101) being superior to other lipophilic cationic moieties at this position.

From Fig. 3 it can be seen that **11** has comparable cell selectivity to **EM2**, but this compound is several orders of magnitude more cytotoxic.

3. Conclusion

Corosolic acid can be obtained in a partial synthesis from ursolic acid in high yields. Acetylation followed by attaching an amide linker (piperazine or homopiperazine) and a rhodamine B unit led to a series of cytotoxic conjugates holding exceptional high cytotoxicity for a variety of different human tumor cell lines; for example, **11** showed $EC_{50} = 2$ nM for A2780 cells) combined with high tumor cell/non-tumor cell selectivity (compound **11**: $EC_{50} = 0.122$ μM for non-malignant fibroblasts NIH 3T3). Thus, compound **11** currently represents one of the most cytotoxic triterpene derivatives holding both superior cytotoxicity combined with high selectivity index (for example, $SI_{A2780\ vs\ NIH\ 3T3} > 60$). This makes **11** an interesting compound for further studies or as a lead substance.

4. Experimental part

4.1. General

General information about equipment is provided in the supplementary materials file as well as a description of the biological assays. The strong coloration of the solutions of the rhodamine B conjugates prevents the measurement of the corresponding $[\alpha]_D$ values. Ursolic acid was bought from "Betulinines" (Stříbrná Skalice, Czech Republic) and used as received.

4.2. Syntheses

4.2.1. General procedure for the synthesis of amides 5–8 (GPA)

To an ice-cold solution of **4** (5.0 g, 8.98 mmol) in dry DCM (200 mL) oxalyl chloride (2.3 mL, 27 mmol) as well as dry DMF (0.2 mL) were slowly added. After completion of the addition, the mixture was allowed to warm to 21 °C, and stirring was continued until the evolution of gases has ceased. The volatiles were removed under reduced pressure, the residue re-dissolved in dry DCM (200 mL), the solvent evaporated again (until all of the excess of oxalyl chloride has been removed). To an ice-cold solution, the corresponding amine (5 eq.) in dry DCM (100 mL), a solution of the acyl chloride (1.0 g, 1.73 mmol) in dry DCM (100 mL) was slowly added, and stirring at 21 °C was continued for 2h. Usual aqueous work-up followed by column chromatography (silica gel, $CHCl_3$/MeOH, 9:1) furnished the amides **5**–**8** each as a colorless solid.

4.2.2. General procedure for the synthesis of the rhodamine conjugates 9–12 (GPB)

To a solution of the amide **5**–**8** (500 mg) in dry DCM (100 mL) rhodamine B chloride (from rhodamine B and oxalyl chloride as described in GPA, 1.2 eq.) was added at 0 °C, and stirring at 21 °C was continued for another 4h. The solvents were removed under reduced pressure, and the residue was subjected to column chromatography (silica gel, $CHCl_3$/MeOH 9:1) to yield conjugates **9**–**12**.

4.2.3. 3-Oxo-urs-12-en-28-oic acid (1, ursonic acid))

To an ice-cold solution of **UA** (20.0 g, 43.8 mmol) in acetone (1500 mL), Jones reagent [freshly prepared from CrO_3 (5.0 g, 48.8 mmol) and sulfuric acid (98%, 5.0 mL, distilled water (20 mL)] was slowly added, and stirring at 0 °C was continued for another hour. The reaction was quenched by the careful addition of MeOH (8.0 mL), and stirred for another 30 min. The solvent was removed under diminished pressure, the residue dissolved in diethyl ether (1000 mL), and the organic phase was washed with water (4 x 300 mL), brine (2 x 150 mL) and dried ($MgSO_4$). Evaporation of the solvent furnished **1** (19.32 g, 97%) as a colorless, amorphous solid being pure enough for the next step. An analytical

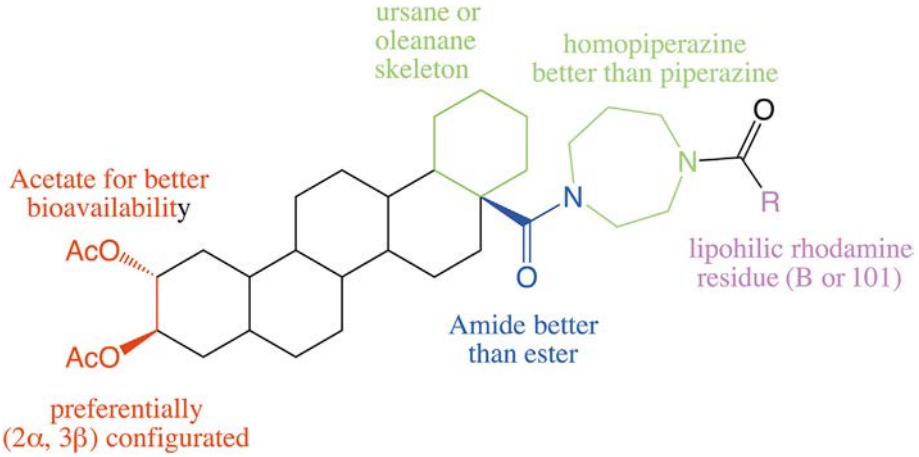


Fig. 2. Hitherto known prerequisites to obtain pentacyclic triterpenes of high cytotoxicity and significant tumor cell/non-tumor cell selectivity.

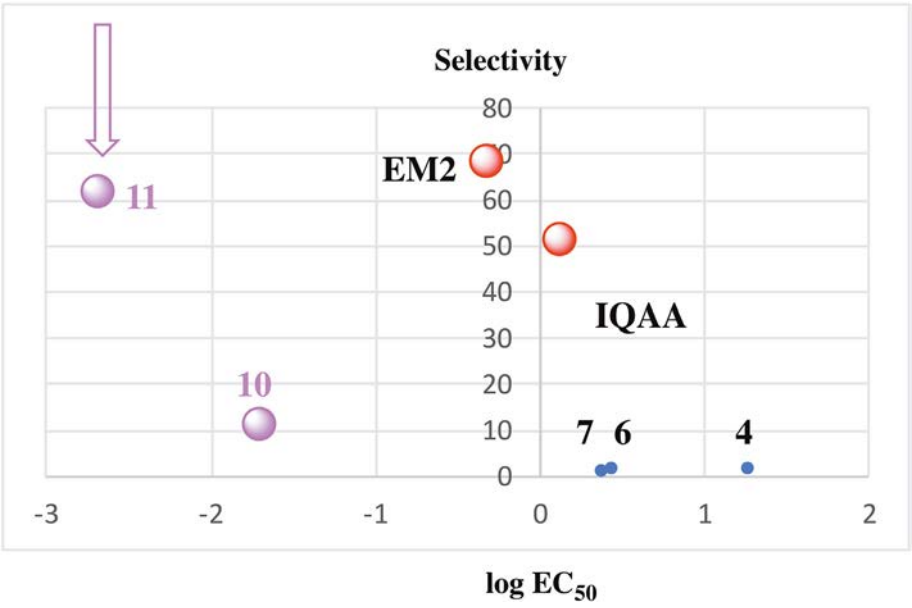


Fig. 3. Cytotoxicity (as log EC_{50}) and selectivity (malignant A2780 cells *vs* non-malignant NIH 3T3 fibroblasts) for selected compounds; this highlights the outstanding performance of **11**.

sample was obtained by column chromatography (silica gel, *n*-hexane/ethyl acetate, 3:2); m.p. 267–270 °C (lit.: [79]: 271–275 °C; $[\alpha]_D$ = +81.4° (*c* 0.5, $CHCl_3$), [lit.: [80]:$[\alpha]_D$ = +83° (*c* 0.1, $CHCl_3$)]; MS (ESI, MeOH): *m/z* = 453.5 ($[M - H]^-$, 100%).

4.2.4. 2α-Hydroxy-3-oxo-urs-12-en-28-oic acid (2)

To an ice-cold solution of **1** (18.0 g, 39.6 mmol) in MeOH/DCM (800 mL, 3:1), sulfuric acid (98%, 1.0 mL) was carefully added followed by the slow addition of *m*CPBA (70%, 12.66 g, 51.4 mmol). The mixture was stirred at 0 °C for 5h (TLC indicated completion of the reaction), diluted with DCM (500 mL), and washed with an aqueous solution of sodium hydrogencarbonate/sodium thiosulfate (1:1, 400 mL), water (2 x 300 mL) and brine (2 x 200 mL) and dried (MgSO4). The volatiles were removed under diminished pressure, and the residue was subjected to column chromatography (silica gel, *n*-hexane/ethyl acetate, 4:1) to yield **2** (14.6 g, 78%) as an amorphous solid; $[\alpha]_D$ = +41.5° (*c* 0.50, $CHCl_3$), [lit.: [81]:$[\alpha]_D$ = +42.2° (*c* 0.48, $CHCl_3$)]; MS (ESI, MeOH): *m/z* = 469.5 ($[M - H]^-$, 100%).

4.2.5. (2α, 3β) 2,3-Dihydroxy-urs-12-en-28-oic acid (corosolic acid, CA, 3)

To an ice-cold solution of **2** (7.1 g, 15.08 mmol) in THF/MeOH (3:1, 400 mL), sodium borohydride (1.5 g, 39.6 mmol) was added in several small portions. The mixture was stirred for 30 min. Usual aqueous work-up followed by column chromatography (silica gel, *n*-hexane/ethyl acetate 7:1 → 4:1) gave **3** (5.77 g, 81%) as a colorless solid; m.p. 246–248 °C (lit.: [65]: 253–255 °C); $[\alpha]_D$ = +48.5° (*c* 1.00, $CHCl_3$) [lit.: [82]: $[\alpha]_D$ = +49.9° (*c* 0.13, $CHCl_3$)]; IR (ATR): ν = 3363 m, 2924 s, 2869 m, 1687 s, 1589 m, 1457 m, 1389 m, 1377 m, 1347 m, 1308 m, 1277 m, 1231 m, 1186 m, 1147 m, 1107 m, 1087 m, 1049 s, 1032 m 993 m, 961 m, 943 m, 662 m, 586 m, 571 m cm^{-1}; ^1H NMR (500 MHz, pyridine-d_5): δ = 5.42 (dd, *J* = 3.5 Hz, 1H, 12-H), 4.05 (ddd, *J* = 11.3, 9.3, 4.4 Hz, 1H, 2-H), 3.35 (d, *J* = 9.4 Hz, 1H, 3-H), 2.63–2.54 (m, 1H, 18-H), 2.34–2.23 (m, 1H, 15-Ha), 2.23–2.16 (m, 1H, 1-Ha), 2.13–2.04 (m, 1H, 16-Ha), 2.04–1.78 (m, 5H, 11-Ha + 11-Hb + 16-Hb + 22-Ha + 22-Hb), 1.74–1.68 (m, 1H, 9-H), 1.61–1.50 (m, 2H, 7-Ha + 6-Ha), 1.49–1.30 (m, 5H, 21-Ha + 21-Hb + 7-Hb + 6-Hb + 19-H), 1.26–1.15 (m, 2H, 1-Hb + 15-Hb), 1.23 (s, 3H, 23-H), 1.18 (s, 3H, 27-H), 1.04 (s, 3H, 24-H), 1.01 (s, 3H, 26-H), 0.99–0.96 (m, 2H, 5-H + 20-H), 0.95 (s, 3H, 30-H), 0.93 (s, 3H, 25-H), 0.92 (d, *J* = 6.4 Hz, 3H, 29-H) ppm; ^{13}C NMR (126 MHz, pyridine-d_5): δ = 179.6 (C-28), 139.0 (C-13), 125.3 (C-12), 83.6 (C-3), 68.3 (C-2), 55.7 (C-5), 53.3 (C-18), 47.8 (C-9), 47.8 (C-17), 47.7 (C-1), 42.3 (C-14), 39.8 (C-8), 39.6 (C-4), 39.2 (C-19), 39.2 (C-20), 38.2 (C-10), 37.2 (C-22), 33.3 (C-7), 30.8 (C-21), 29.1 (C-23), 28.4 (C-15), 24.7 (C-16), 23.7 (C-27), 23.5 (C-11), 21.2 (C-30), 18.6 (C-6), 17.5 (C-24), 17.3 (C-26), 17.2 (C-29), 16.7 (C-25) ppm; MS (ESI, MeOH): *m/z* = 471.1 ($[M - H]^-$, 100%); analysis calcd for $C_{30}H_{48}O_4$ (472.35): C 76.23, H 10.24; found: C 76.01 H 10.43.

4.2.6. 2α,3β-Bis(acetyloxy)urs-12-en-28-oic acid (4)

Acetylation of **3** (7.0 g, 14.8 mmol) in dry pyridine (100 mL) with acetic anhydride (20 mL) in the presence of DMAP (cat.) for 10h followed by usual aqueous work-up and column chromatography (silica gel, *n*-hexane/ethyl acetate, 4:1) gave **4** (5.9 g, 72%) as a colorless solid; m.p. 237–241 °C (lit.: [83]: 242–244 °C); $[\alpha]_D$ = +24.85° (*c* 1.51, $CHCl_3$) [lit.: [83]: $[\alpha]_D$ = +20.7° (*c* 1.0, $CHCl_3$)]; IR (ATR): ν = 2926 m, 2871 w, 1741 s, 1695 m, 1455 w, 1368 m, 1247 vs, 1230 vs, 1153 w, 1106 w, 1031 s, 961 m, 755 m cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 5.22 (dd, *J* = 3.6 Hz, 1H, H-12), 5.10 (td, *J* = 11.3, 4.6 Hz, 1H, H-2), 4.75 (d, *J* = 10.3 Hz, 1H, H-3), 2.22–2.15 (m, 1H, H-18), 2.05 (s, 2H, H-1a), 2.08–1.80 (m, 3H, H-16a + H-16b + H-15a), 2.04 (s, 3H, H-32), 1.97 (s, 3H, H-34), 1.76–1.61 (m, 4H, H-22a + H-22b + H-11a + H-11b), 1.61–1.44 (m, 4H, H-9 + H-6a + H-21a + H-7a), 1.40–1.25 (m, 4H, H-6b + H-7b + H-21b + H-19), 1.16–1.07 (m, 2H, H-1b + H-15b), 1.06 (s, 3H, H-25), 1.06 (s, 3H, H-27), 1.05–0.95 (m, 2H, H-20 + H-5), 0.95 (s, 3H, H-30), 0.89 (s, 3H, H-23), 0.89 (s, 3H, H-24), 0.84 (d, *J* = 6.2 Hz, 3H, H-29), 0.75 (s, 3H, H-26) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 183.8 (C-28), 170.8 (C-31), 170.6 (C-33), 138.0 (C-13), 125.4 (C-12), 80.6 (C-3), 70.1 (C-2), 54.9 (C-5), 52.4 (C-18), 47.9 (C-9), 47.4 (C-17), 44.1 (C-1), 42.0 (C-14), 39.5 (C-8), 39.3 (C-4), 39.0 (C-19), 38.8 (C-20), 38.1 (C-10), 36.7 (C-22), 32.7 (C-7), 30.6 (C-21), 28.4 (C-23), 27.9 (C-15), 24.0 (C-16), 23.5 (C-27), 23.3 (C-11), 21.1 (C-30), 21.1 (C-34), 20.9 (C-32), 18.1 (C-6), 17.6 (C-24), 17.1 (C-26), 16.9 (C-29), 16.5 (C-25) ppm; MS (ESI, MeOH): *m/z* = 555.7 ($[M - H]^-$, 100%); %); analysis calcd for $C_{34}H_{52}O_4$ (556.77): C 73.34, H 9.41; found: C 73.12 H 9.57.

4.2.7. (2α, 3β) 2,3-Bis(acetyloxy)-N-[2-(methylamino)ethyl]-olean-12-en-28-amide (5)

Following GPA, **5** (73%) was obtained as a colorless solid; m.p. 143.2–146.2 °C; $[\alpha]_D$ = +8.9° (*c* 0.06, $CHCl_3$); IR (ATR): ν = 2926 m, 2869 w, 1740 s, 1639 w, 1520 w, 1453 m, 1390 w, 1368 m, 1248 vs,

1230 vs, 1189 w, 1031 s, 752 m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.10–6.98 (m, 1H, amide-H), 5.39–5.33 (m, 1H, H-12), 5.09 (td, J = 10.9, 4.6 Hz, 1H, H-2), 4.75 (d, J = 10.3 Hz, 1H, H-3), 3.71–3.60 (m, 1H, H-35a), 3.49–3.38 (m, 1H, H-35b), 3.09–3.03 (m, 1H, H-36), 2.66 (s, 3H, H-37), 2.17 (d, J = 10.2 Hz, 1H, H-18), 2.05 (s, 3H, H-32), 2.07–1.85 (m, 3H, H-1a + H-16 a + H-16b), 1.97 (s, 3H, H-34), 1.83–1.23 (m, 10H, H-22a + H-11a + H-11b + H-6a + H-22b + H-7a + H-7b + H-21 a + H-21b + H-19), 1.08 (s, 3H, H-25), 1.05 (s, 3H, H-27), 1.23–0.94 (m, 5H, H-15 a + H-15b + H-1b + H-5 + H-20), 0.94 (s, 3H, H-30), 0.90 (s, 3H, H-24), 0.90 (s, 3H, H-23), 0.86 (d, J = 6.6 Hz, 3H, H-29), 0.75 (s, 3H, H-26) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 179.7 (C-28), 170.8 (C-31), 170.5 (C-33), 139.1 (C-13), 125.3 (C-12), 80.5 (C-3), 70.0 (C-2), 54.8 (C-5), 52.7 (C-18), 50.0 (C-36), 47.8 (C-9), 47.4 (C-17), 44.1 (C-1), 42.3 (C-14), 39.6 (C-8), 39.6 (C-19), 39.3 (C-4), 38.7 (C-20), 38.0 (C-10), 37.3 (C-22), 36.8 (C-35), 33.5 (C-37), 32.6 (C-7), 30.8 (C-21), 28.4 (C-23), 27.8 (C-15), 24.4 (C-11), 23.4 (C-27), 23.4 (C-16), 21.2 (C-30), 21.1 (C-34), 20.9 (C-32), 18.1 (C-6), 17.7 (C-24), 17.1 (C-29), 17.0 (C-26), 16.6 (C-25) ppm; MS (ESI, MeOH): m/z = 613.1 ([M+H]$^+$, 100%); %); analysis calcd for $C_{37}H_{60}N_2O_5$ (612.90): C 72.51, H 9.87, N 4.57; found: C 72.30, H 10.03, N 4.29.

4.2.8. (2α, 3β) 2,3-Bis(acetyloxy)-28-(1-piperazinyl)-urs-12-en-28-one (6)

Following GPA, **6** (86%) was obtained as a colorless solid; m.p. 189–111 °C (lit.: [84]: m.p. 191–192 °C); $[\alpha]_D$ = +10.26° (*c* 0.06, $CHCl_3$); IR (ATR): ν = 2924 w, 2870 w, 1740 s, 1628 m, 1454 w, 1395 m, 1367 m, 1249 vs, 1229 vs, 1184 w, 1155 w, 1104 w, 1036 m, 1009 m, 752 m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.26–5.17 (m, 1H, H-12), 5.15–5.05 (m, 1H, H-2), 4.74 (dd, J = 10.3, 1.2 Hz, 1H, H-3), 4.00–3.84 (m, 2H, H-35), 3.70–3.54 (m, 2H, H-35′), 3.18–3.07 (m, 2H, H-36), 2.54–2.35 (m, 2H, H-36′), 2.21–2.13 (m,1H, H-18), 2.05 (s, 3H, H-32), 2.09–1.89 (m, 3H, H-1a + H-16 a + H-16b), 1.97 (s, 3H, H-34), 1.85–1.21 (m, 10H, H-22a + H-11a + H-11b + H-6a + H-22b + H-7a + H-7b + H-21 a + H-21b + H-19), 1.07 (s, 3H, H-25), 1.06 (s, 3H, H-27), 1.21–0.95 (m, 5H, H-15 a + H-15b + H-1b + H-5 + H-20), 0.95 (s, 3H, H-30), 0.90 (s, 3H, H-24), 0.90 (s, 3H, H-23), 0.86 (d, J = 6.4 Hz, 3H, H-29), 0.75 (s, 3H, H-26) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 175.6 (C-28), 170.8 (C-31), 170.5 (C-33), 141.6 (C-13), 124.8 (C-12), 80.7 (C-3), 70.0 (C-2), 54.9 (C-5), 51.5 (C-18), 47.6 (C-9), 47.5 (C-17), 44.1 (C-1), 43.6 (C-35 + C-35′), 42.6 (C-36 + C-36’), 42.2 (C-14), 39.4 (C-8), 39.3 (C-19), 38.7 (C-4), 38.4 (C-20), 38.1 (C-10), 34.3 (C-22), 32.8 (C-7), 30.3 (C-21), 28.5 (C-23), 28.1 (C-15), 23.4 (C-27), 23.3 (C-16), 21.2 (C-30), 21.1 (C-34), 20.9 (C-32), 18.2 (C-6), 17.7 (C-24), 17.4 (C-29), 17.3 (C-26), 16.5 (C-25) ppm; MS (ESI, MeOH): m/z = 625.0 ([M+H]$^+$, 100%); analysis calcd for $C_{38}H_{60}N_2O_5$ (624.91): C 73.04, H 9.68, N 4.48; found: C 72.88, H 9.88, N 4.24.

4.2.9. (2α, 3β) 2,3-Bis(acetyloxy)-28-(1-piperazinyl)-urs-12-en-28-one (7)

Following GPA, **7** (76%) was obtained as a colorless solid; m.p. 164.8–167.4 °C; $[\alpha]_D$ = +9.02° (*c* 1.57, $CHCl_3$); IR (ATR): ν = 2925 m, 2869 w, 1739 s, 1620 m, 1455 w, 1394 m, 1368 m, 1285 w, 1248 vs, 1230 vs, 1183 w, 1174 w, 1154 w, 1030 m, 751 m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.30–5.17 (m, 1H, H-12), 5.15–5.05 (m, 1H, H-3), 4.74 (d, J = 10.3 Hz, 1H, H-2), 3.99–3.49 (m, 4H, H-35 + H-35‘), 3.29–3.11 (m, 3H, H-36), 2.49–2.39 (m, 2H, H-36’), 2.26–2.11 (m, 1H, H-18), 2.05 (s, 3H, H-32), 2.08–1.88 (m, 3H, H-1a + H-16 a + H-16b), 1.97 (s, 3H, H-34), 1.84–1.22 (m, 10H, H-22a + H-11a + H-11b + H-6a + H-22b + H-7a + H-7b + H-21 a + H-21b + H-19), 1.07 (s, 3H, H-25), 1.05 (s, 3H, H-27), 1.20–0.96 (m, 5H, H-15 a + H-15b + H-1b + H-5 + H-20), 0.95 (s, 3H, H-30), 0.90 (s, 3H, H-24), 0.89 (s, 3H, H-23), 0.86 (d, J = 6.5 Hz, 3H, H-29), 0.72 (s, 3H. H-26) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 176.5 (C-28), 170.8 (C-31), 170.5 (C-33), 138.9 (C-13), 124.9 (C-12), 80.7 (C-3), 70.0 (C-2), 54.9 (C-5), 49.0 (C-35 + C-35′), 47.5 (C-17) 47.4 (C37 + C37′), 44.1 (C-1), 39.4 (C-8), 39.3 (C-19), 39.3 (C-4), 38.6 (C-20), 38.1 (C-10), 34.3 (C-22), 32.7 (C-7), 30.4 (C-21), 28.4 (C-23), 27.7 (C-15), 26.4 (C-36), 23.4 (C-16), 23.3 (C-11), 21.6 (C-27), 21.2 (C-30), 21.1 (C-34), 20.9 (C-32), 18.2 (C-6), 17.7 (C-24), 17.4 (C-29), 17.3 (C-26), 16.5 (C-25) ppm; MS (ESI, MeOH): m/z = 639.1 ([M+H]$^+$, 100%); analysis calcd for $C_{39}H_{62}N_2O_5$ (638.93): C 73.31, H 9.78, N 4.38; found: C 72.97, H 9.90, N 4.13.

4.2.10. (2α, 3β) 2,3-Bis(acetyloxy)-N-[2-(1-piperazinyl)ethyl]-urs-12-en-28-amide (8)

Following GPA, **8** (65%) was obtained as a colorless solid; m.p. 150.6–154.6 °C (lit.: [84]: 154–155 °C); $[\alpha]_D$ = +8.0° (*c* 0.06, $CHCl_3$); IR (ATR): ν = 2936 m, 1739 s, 1645 m, 1512 w, 1505 w, 1455 m, 1391 w, 1368 m, 1304 w, 1248 vs, 1230 vs, 1188 w, 1151 w, 1141 w, 1087 w, 1031 s, 751 m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.27–5.23 (m, 1H, H-12), 5.10 (m, 1H, H-2), 4.75 (d, J = 10.4, 1.7 Hz, 1H, H-3), 3.76–3.60 (m, 1H, H-35a), 3.45–3.31 (m, 1H, H-35b), 3.25–3.18 (m, 2H, H-36), 2.84–2.72 (m, 4H, H-38 + H-38′), 2.58–2.45 (m, 4H, H-37 + H-37′), 2.22–2.15 (m, 1H, H-18), 2.05 (s, 3H, H-32), 2.08–1.91 (m, 3H, H-1a + H-16 a + H-16b), 1.97 (s, 3H, H-34), 1.88–1.23 (m, 10H, H-22a + H-11a + H-11b + H-6a + H-22b + H-7a + H-7b + H-21 a + H-21b + H-19), 1.23–0.92 (m, 5H, H-15 a + H-15b + H-1b + H-5 + H-20), 1.09 (s, 3H, H-25), 1.05 (s, 3H, H-27), 0.96 (s, 3H, H-30), 0.91 (s, 3H, H-24), 0.90 (s, 3H, H-23), 0.87 (d, J = 7.0 Hz, 3H, H-29), 0.76 (s, 3H, H-26) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 177.8 (C-28), 170.8 (C-31), 170.5 (C-33), 140.0 (C-13), 124.8 (C-12), 80.5 (C-3), 69.9 (C-2), 56.6 (C-37 + C-37′), 54.8 (C-5), 52.4 (C-18), 49.4 (C-38 + C-38’), 47.8 (C-17), 47.4 (C-9), 44.1 (C-1), 43.6 (C-36), 42.5 (C-14), 39.7 (C-8), 39.6 (C-19), 39.3 (C-4), 39.1 (C-20), 38.0 (C-10), 37.3 (C-22), 35.8 (C35), 32.5 (C-7), 30.9 (C-21), 28.4 (C-23), 27.8 (C-15), 24.8 (C-11), 23.5 (C-27), 23.2 (C-16), 21.2 (C-30), 21.1 (C-34), 20.9 (C-32), 18.1 (C-6), 17.7 (C-24), 17.2 (C-29), 17.0 (C-26), 16.6 (C-25) ppm; MS (ESI, MeOH): m/z = 668.1 ([M+H]$^+$, 100%); analysis calcd for $C_{40}H_{65}N_3O_5$ (667.98): C 71.92, H 9.81, N 6.29; found: C 71.77, H 10.02, N 6.13.

4.2.11. 9-[2-[[4-(2α,3β)-2,3-bis(acetyloxy)-28-oxours-12-en-28-yl]-N-[2-(methylamino)ethyl] carbonyl]phenyl]-3,6-bis(diethylamino)-xanthylium chloride (9)

Following GPB, **9** (86%) was obtained as a violet solid; m.p. 227–231 °C; IR (ATR): ν = 2972 w, 2925 w, 1738 m, 1644 m, 1632 m, 1587 vs, 1528 m, 1508 m, 1481 m, 1466 m, 1433 m, 1412s, 1394 m, 1380 m, 1367 m, 1336 s, 1273 m, 1246 s, 1198 m, 1179 vs, 1159 m, 1132 m, 1093 w, 1073 m, 1037 m, 1010 m, 746 s, 683 m, 662 m cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.68–7.55 (m, 2H, H-42 + H-41), 7.36–7.29 (m, 2H, H-40 + H-43), 7.25–7.15 (m, 2H, H-48 + H-48‘), 7.00–6.90 (m, 2H, H-47 + H-47‘), 6.80–6.74 (m, 2H, H-50 + H-50‘), 6.69–6.65 (m, 1H, amide-H), 5.37–5.32 (m, 1H, H-12), 5.10–4.99 (m, 1H, H-3), 4.75–4.69 (m, 1H, H-2), 3.76–3.47 (m, 8H, H-52 + H-52’ + H-52’’ + H-52’’’), 3.46–3.30 (m, 2H, H-36), 3.28–3.15 (m, 2H, H-35), 2.99 (s, 2H, H-37), 2.36 (d, J = 10.9 Hz, 1H, H-18), 2.11–1.91 (m, 3H, H-1a + H-16 a + H-16b), 2.03 (s, 3H, H-32), 1.95 (s, 3H, H-34), 1.91–1.35 (m, 11H, H-22a + H-11a + H-11b + H-9 + H-6a + H-22b + H-7a + H-7b + H-21 a + H-21b + H-19), 1.31 (q, J = 6.9 Hz, 12H, H-53 + H-53’ + H-53’’ + H-53’’’), 1.27–0.79 (m, 5H, H-15 a + H-15b + H-1b + H-5 + H-20), 1.03 (s, 3H, H-25), 1.02 (s, 3H, H-27), 0.90 (s, 3H, H-30), 0.87 (s, 3H, H-24), 0.87 (s, 3H, H-23), 0.78 (d, J = 6.6 Hz, 3H, H-29), 0.67 (s, 3H, H-26) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 178.7 (C-28), 170.8 (C-31), 170.5 (C-33), 168.7 (C-38), 157.7 (C-51 + C-51′), 155.7 (C-45), 155.6 (C-49 + C-49′), 138.9 (C-13), 135.9 (C-44), 132.1 (C-48 + C-48′), 130.3 (C-39), 130.1 (C-43),130.0 (C-41), 129.7 (C-42), 127.9 (C-40), 125.1 (C-12), 114.8 (C-47), 114.2 (C-47′), 113.8 (C-45), 113.7 (C-45′), 96.4 (C-50), 95.9 (C-50′), 80.5 (C-3), 70.0 (C-2), 54.8 (C-18), 54.8 (C-5), 47.5 (C-9), 47.4 (C-17), 47.0 (C-35 + C-35′), 46.1 (C-52 + C-52′), 44.1 (C-1), 42.2 (C-36 + C-36′), 42.1 (C-14), 39.6 (C-8), 39.4 (C-19), 39.3 (C-4), 38.8 (C-37), 38.4 (C-20), 38.1 (C-10), 34.5 (C-22), 32.9 (C-7), 30.8 (C-21), 28.4 (C-23), 27.9 (C-15), 23.3 (C-27), 23.3 (C-16), 21.3 (C-30), 21.1 (C-34), 20.9 (C-32), 18.1 (C-6), 17.6 (C-24), 17.1 (C-29), 16.9 (C-26), 16.5 (C-25), 12.6 (C-53 + C-53′) ppm; MS (ESI, MeOH): m/z = 1037.2 ([M − Cl]$^+$, 100%); analysis calcd for $C_{65}H_{89}N_4O_7Cl$ (1073.90): C 72.70, H 8.35, N 5.22; found: C 72.47, H 8.51, N 4.97.

4.2.12. 9-[2-[[4-(2α,3β)-2,3-bis(acetyloxy)-28-oxours-12-en-28-yl]-1-piperazinyl]carbonyl]phenyl]-3,6-bis(diethylamino)-xanthylium chloride (10)

Following GPB, **10** (92%) was obtained as a violet solid; m.p. 241–248 °C; IR (ATR): ν = 2972 w, 2924 w, 2870 w, 1738 m, 1632 m, 1587 vs, 1529 w, 1507 w, 1482 m, 1466 m, 1431 m, 1412 s, 1394 m, 1380 m, 1336 s, 1272 m, 1245 s, 1197 m, 1179 vs, 1159 m, 1133 m, 1094 w, 1073 m, 1039 m, 1033 m, 1005 m, 921 m, 745 s, 683 m, 663 m cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.67–7.60 (m, 2H, H-41 + H-40), 7.53–7.47 (m, 1H, H-39), 7.32–7.27 (m, 1H, H-42), 7.25–7.18 (m, 2H, H-47+H-47′), 7.00–6.95 (m, 1H, H-46), 6.95–6.89 (m, 1H, H-46‘), 6.76–6.72 (m, 2H, H-49 + H-49’), 5.10 (dd, J = 3.6, 3.6 Hz, 1H, H-12), 5.03 (td, J = 10.9, 4.7 Hz, 1H, H-2), 4.68 (d, J = 10.3 Hz, 1H, H-3), 3.65–3.54 (m, 8H, H-51 + H-51‘ + H-51‘’+ H-51‘’‘), 3.47–2.99 (m, 8H, H-35 + H-35‘ + H-36 + H-36‘), 2.34–2.23 (m, 1H, H-18), 1.99 (s, 3H, H-32), 2.13–1.75 (m, 3H, H-1a + H-16 a + H-16b), 1.91 (s, 3H, H-34), 1.76–1.56 (m, 2H, H-22a + H-9), 1.54–1.21 (m, 7H, H-6a + H-22b + H-7a + H-7b + H-21 a + H-21b + H-19), 1.27 (t, J = 7.0 Hz, 12H, H-52 + H-52’ + H-52’’ + H-52′′′), 0.99 (s, 3H, H-25), 0.97 (s, 3H, H-27), 1.07–0.76 (m, 5H, H-15 a + H-15b + H-1b + H-5 + H-20), 0.87 (s, 3H, H-30), 0.84 (s, 3H, H-24), 0.83 (s, 3H, H-23), 0.78 (d, J = 6.2 Hz, 3H, H-29), 0.61 (s, 3H, H-26) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 175.8 (C-28), 170.7 (C-31), 170.4 (C-33), 167.6 (C-37), 157.7 (C-50 + C-50‘), 155.7 (C-44), 155.6 (C-48), 155.6 (C-48‘), 138.5 (C-13), 135.0 (C-43), 132.1 (C-47 + C-47‘), 130.6 (C-38), 130.3 (C-42), 130.2 (C-40), 130.2 (C-41), 127.6 (C-39), 124.7 (C-12), 114.4 (C-46), 114.2 (C-46‘), 113.7 (C-45), 113.7 (C-45‘), 96.3 (C-49), 96.3 (C-49‘), 80.5 (C-3), 70.0 (C-2), 54.9 (C-18), 54.8 (C-5), 47.4 (C-9), 47.3 (C-17), 47.2 (C-36 + C-36‘), 46.1 (C-51 + C-51‘), 44.0 (C-1), 42.1 (C-35 + C-35‘), 42.1 (C-14), 39.4 (C-8), 39.3 (C-19), 39.3 (C-4), 38.6 (C-20), 38.0 (C-10), 34.2 (C-22), 32.6 (C-7), 30.3 (C-21), 28.4 (C-23), 28.0 (C-15), 23.6 (C-27), 23.3 (C-16), 21.1 (C-30), 21.1 (C-34), 20.8 (C-32), 18.1 (C-6), 17.6 (C-24), 17.3 (C-29), 16.8 (C-26), 16.5 (C-25), 12.6 (C-52 + C-52‘ + C-52’’ + C-52′′′) ppm; MS (ESI, MeOH): m/z = 1049.2 ([M − Cl]$^+$, 100%); analysis calcd for $C_{66}H_{89}N_4O_7Cl$ (1085.91): C 73.00, H 8.26, N 5.15; found: C 72.76, H 8.56, N 4.96.

4.2.13. 9-[2-[[4-(2α,3β)-2,3-bis(acetyloxy)-28-oxours-12-en-28-yl]-1-homopiperazinyl]carbonyl] phenyl]-3,6-bis(diethylamino)-xanthylium chloride (11)

Following GPB, **11** (77%) was obtained as a violet solid; m.p. 249–253 °C; IR (ATR): ν = 2972 w, 2925 w, 2871 w, 1737 m, 1645 w, 1626 m, 1586 vs, 1529 w, 1508 w, 1482 m, 1467 m, 1431 m, 1412 s, 1394 m, 1379 m, 1367 m, 1335 s, 1273 m, 1245 s, 1197 m, 1179 vs, 1159 m, 1132 m, 1073 m, 1038 m, 1009 m, 921 m, 743 s, 683 m cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.67–7.60 (m, 2H, H-42 + H-41), 7.57–7.47 (m, 1H, H-40), 7.32–7.26 (m, 1H, H-43), 7.23–7.16 (m, 2H, H-48 + H-48′), 7.16–7.04 (m, 1H, H-49), 6.99–6.92 (m, 1H, H-49′), 6.82–6.71 (m, 1H, H-47), 6.71–6.67 (m, 1H, H-47′), 6.67–6.62 (m, 2H, H-50 + H-50), 5.10–5.01 (m, 1H, H-12), 4.97 (td, J = 11.0, 4.6 Hz, 1H, H-2), 4.61 (d, J = 10.3 Hz, 1H, H-3), 3.96–3.74 (m, 4H, H-37 + H-37′), 3.64–3.42 (m, 8H, H-52 + H-52’ + H-52’’ + H-52′′′), 3.20–2.86 (m, 4H, H-35 + H-35′), 2.37–2.24 (m, 1H, H-18), 1.92 (s, 3H, H-32), 1.98–1.73 (m, 3H, H1a + H16a + H16b), 1.84 (s, 3H, H-34), 1.72–1.25 (m, 11H, H-22a + H-11a + H-11b + H-9 + H-6a + H-22b + H-7a + H-7b + H-21a + H-21b + H-19), 1.21 (t, J = 6.8 Hz, 12H, H-53 + H-53’ + H-53’’ + H-53′′′), 1.18–0.86 (m, 5H, H-15a + H-15b + H-1b + H-5 + H-20), 0.92 (s, 3H, H-27), 0.92 (s, 3H, H-25), 0.81 (s, 3H, H-30), 0.77 (s, 3H, H-23), 0.77 (s, 3H, H-24), 0.74 (s, 3H, H-29), 0.59 (s, 3H, H-26) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 176.2 (C-28), 170.7 (C-31), 170.4 (C-33), 167.9 (C-38), 157.6 (C-51 + C-51′), 155.6 (C-45), 155.6 (C-49), 155.5 (C-49′), 138.6 (C-13), 135.7 (C-44), 132.4 (C-48 + C-48′), 130.2 (C-39), 130.1 (C-43), 129.9 (C-41), 129.6 (C-42), 126.7 (C-40), 124.5 (C-12), 114.5 (C-47), 114.4 (C-47′), 113.8 (C-46), 113.7 (C-46′), 96.2 (C-50), 96.1 (C-50′), 80.5 (C-3), 70.0 (C-2), 54.9 (C-18), 54.8 (C-5), 47.4 (C-9), 46.1 (C-37 + C-37′), 46.0 (C-17), 44.00 (C-1), 42.2 (C-35 + C-35′), 42.1 (C-14), 39.2 (C-8), 39.2 (C-19), 39.0 (C-4), 38.6 (C-20), 38.3 (C-10), 34.6 (C-22), 32.7 (C-7), 30.4 (C-21), 30.3 (C-36), 28.3 (C-23), 27.8 (C-15), 23.4 (C-27), 23.2 (C-16), 21.2 (C-30), 21.0 (C-34), 20.8 (C-32), 18.0 (C-6), 17.6 (C-24), 17.3 (C-29), 16.8 (C-26), 16.4 (C-25), 12.60 (C-53 + C-53’) ppm; MS (ESI, MeOH): m/z = 1063.2 ([M − Cl]$^+$, 100%); analysis calcd for $C_{67}H_{91}N_4O_7Cl$ (1099.94): C 73.16, H 8.34, N 5.09; found: C 72.96, H 8.47, N 4.84.

4.2.14. 9-[2-[[4-(2α,3β)-2,3-bis(acetyloxy)-28-oxours-12-en-28-yl]-N[2-(1-piperaziny)ethyl] carbonyl]phenyl]-3,6-bis(diethylamino)-xanthylium chloride (12)

Following GPB, **12** (62%) was obtained as a violet solid; m.p.: 206–211 °C; IR (ATR): ν = 2972 w, 2926 w, 1738 m, 1634 m, 1586 vs, 1528 m, 1508 m, 1482 m, 1466 m, 1431 m, 1412s, 1394 m, 1380 m, 1367 m, 1336 s, 1273 m, 1245 s, 1197 m, 1179 vs, 1159 m, 1132 s, 1093 w, 1073 m, 1029 m, 1011 m, 921 m, 745 s cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.72–7.58 (m, 3H, H-43 + H-42 + H-41), 7.32–7.27 (m, 1H, H-44), 7.23–7.16 (m, 2H, H-51 + H-51‘), 7.03–6.95 (m, 2H, H-50 + H-50‘), 6.76–6.69 (m, 2H, H-51 + H-51‘), 5.30–5.22 (m, 1H, H-12), 5.06 (ddd, J = 11.2, 4.6 Hz, 1H, H-2), 4.71 (d, J = 10.3 Hz, 1H, H-3), 3.71–3.50 (m, 8H, H-53 + H-53‘ + H-53‘’+ H-53‘’‘), 3.47–3.36 (m, 2H, H-36), 3.26–3.13 (m, 2H, H-35), 2.20–2.14 (m, 1H, H-18), 2.01 (d, J = 3.2 Hz, 3H, H-32), 2.10–1.80 (m, 3H, H-1a + H-16a + H-16b), 1.93 (d, J = 6.4 Hz, 3H, H-34), 1.78–1.34 (m, 18H, H-11a + H-22a + H-11b + H-6a + H-22b + H-7a + H-7b + H-21 a + H-21b + H-19, H-37 + H-37’ + H-38 + H-38′), 1.30 (t, J = 7.1 Hz, 12H, H-54 + H-54‘ + H-54‘’+ H-54‘’‘), 1.26–0.91 (m, 5H, H-15a + H-15b + H-1b + H-5 + H-20), 1.02 (s, 3H, H-25), 0.99 (s, 3H, H-27), 0.89 (s, 3H, H-30), 0.86 (s, 3H, H-24), 0.86 (s, 3H, H-23), 0.81 (d, J = 6.4 Hz, 3H, H-29), 0.69 (s, 3H, H-26) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 178.0 (C-28), 170.7 (C-31), 170.4 (C-33), 167.2 (C-39), 157.7 (C-52 + C-52‘), 156.1 (C-46), 155.7 (C-50), 155.6 (C-50‘), 139.4 (C-13), 135.1 (C45), 132.1 (C-49 + C-49‘), 130.8 (C-40), 130.2 (C-44), 130.1 (C-42), 129.9 (C-43), 128.0 (C-41), 124.8 (C-12), 114.3 (C-48 + C-48’), 113.8 (C-47), 113.8 (C-47‘), 96.2 (C-51 + C-51‘), 80.6 (C-3), 70.0 (C-2), 56.7 (C-18), 54.8 (C-5), 47.5 (C-9), 47.4 (C-17), 46.2 (C-53 + C-53‘), 44.1 (C-1), 42.3 (C-35), 42.3 (C-14), 39.6 (C-8), 39.6 (C-19), 39.3 (C-4), 38.7 (C-20), 38.0 (C-10), 37.2 (C-38 + C-38‘), 32.6 (C-7), 30.9 (C-21), 28.4 (C-23), 27.7 (C-15), 24.4 (C-27), 23.4 (C-37 + C-37‘), 23.3 (C-16), 21.2 (C-30), 21.1 (C-34), 20.9 (C-32), 18.1 (C-6), 17.6 (C-24), 17.1 (C-29), 17.0 (C-26), 16.5 (C-25), 12.7 (C-54 + C-54‘ + C-54’’ + C-54′′′) ppm; MS (ESI, MeOH): m/z = 1092.3 ([M − Cl]$^+$, 100%); analysis calcd for $C_{68}H_{94}N_5O_7Cl$ (1128.98): C 72.34, H 8.39, N 6.20; found: C 72.05, H 8.57, N 5.98.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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Rhodamine 101 Conjugates of Triterpenoic Amides Are of Comparable Cytotoxicity as Their Rhodamine B Analogs

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Abstract: Pentacyclic triterpenoic acids (betulinic, oleanolic, ursolic, and platanic acid) were selected and subjected to acetylation followed by the formation of amides derived from either piperazine or homopiperazine. These amides were coupled with either rhodamine B or rhodamine 101. All of these compounds were screened for their cytotoxic activity in SRB assays. As a result, the cytotoxicity of the parent acids was low but increased slightly upon their acetylation while a significant increase in cytotoxicity was observed for piperazinyl and homopiperazinyl amides. A tremendous improvement in cytotoxicity was observed; however, for the rhodamine B and rhodamine 101 conjugates, and compound **27**, an ursolic acid derived homopiperazinyl amide holding a rhodamine 101 residue showed an EC_{50} = 0.05 μM for A2780 ovarian cancer cells while being less cytotoxic for non-malignant fibroblasts. To date, the rhodamine 101 derivatives presented here are the first examples of triterpene derivatives holding a rhodamine residue different from rhodamine B.

Keywords: triterpenoic acid; ursolic acid; oleanolic acid; betulinic acid; rhodamine B; rhodamine 101; cytotoxicity

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1. Introduction

Despite significant progress, cancer therapy still falls short of the expectations placed in it many years ago [1,2]. The prognosis for a complete cure is very good for some types of cancer, but still poor for many others, especially when regular screening is taken into account. The high cost of therapy [3–5] is often offset especially for cancers that are difficult to treat by only a slight increase in life expectancy and, at the same time, a significantly reduced quality of life. Thus, the survival rate [6] for testicular cancer is approximately 98% while for pancreatic cancer it is about 1%. The main reason for the reduced quality of life and, thus, a reduced compliance by the affected patients is more or less often insufficient selectivity of the chemotherapeutic agents used. As a consequence, there has been no lack of attempts to improve the efficacy but also to reduce the side effects caused by antitumor drugs (such as weight loss, hair loss, etc.). Furthermore, many different strategies have been tested for a successful drug targeting of tumors—whereby the real problem is usually not the solid primary tumor but the metastases that have already formed and spread throughout the body. These attempts [7] included the use of micelles [8], antibodies [9], liposomes [10], polymers but also of drug-loaded nanoparticles [7].

Although first described several years ago, so-called mitocans (i.e., mitochondria targeting anticancer drugs) [11–16] are currently experiencing a scientific renaissance. Mitocans, which specifically induce a programmed cell death in tumor cells, can be considered as one of the most innovative therapeutic approaches of drug targeting of the "next generation". In the past, we could already show with some examples that compounds derived from pentacyclic triterpenes (such as ursolic, oleanolic, betulinic, platanic, glycyrrhetinic, β-boswellic, tormentic, euspaphic, or asiatic acid) exhibit high cytotoxicity, and can act as

mitocans [17–22]. These mitocans might cause either membrane permeabilization but also the opening of the mitochondrial permeability pore [16,17]. However, the deactivation of mitochondrial enzymes cannot completely be ruled out [17].

This cytotoxicity (but also to some extent their pronounced tumor/non-tumor cell selectivity) seems to depend on many parameters. On the one hand, this concerns a dependence on the type of terpene (Figure 1) used: compounds derived from dehydroabietylamine [23] were—by and large—less cytotoxic than those with a pentacyclic triterpenoid backbone [17]. Amides at position C-28 were mostly more active than the analogous esters [22], whereas a direct attachment of a rhodamine B moiety to the triterpenoid backbone resulted in compounds of significantly lowered selectivity [17]. Therefore, the use of a suitable spacer is of crucial importance. Furthermore, triterpene/rhodamine B hybrids holding an ethylenediamine spacer [20] were significantly less active than those with a piperazine spacer; in some cases, the use of a homopiperazine spacer [17] proved successful. However, the presence of a distal cationic center alone is not sufficient for achieving good cytotoxic activity [17,24–26]. Only special delocalized lipophilic cations are useful for a successful mitochondria-targeted chemotherapy. Thereby, quaternary ammonium salts [24] but also malachite green-derived compounds [27] proved to be significantly less cytotoxic than their rhodamine B analogs [17]. Furthermore, the presence of a rhodamine residue is of crucial importance, which is why we decided to extend our studies to rhodamine B and other rhodamines, and to investigate especially the synthesis and cytotoxic activity of (homo)-piperazinyl-spaced triterpenes holding a rhodamine 101 residue in more detail, and to compare their cytotoxic activity with those carrying a rhodamine B unit.

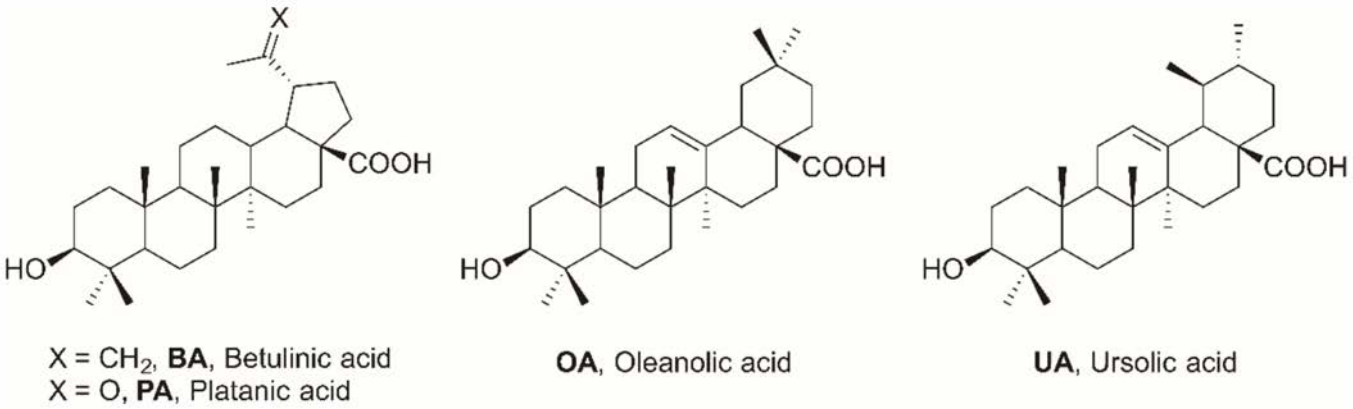
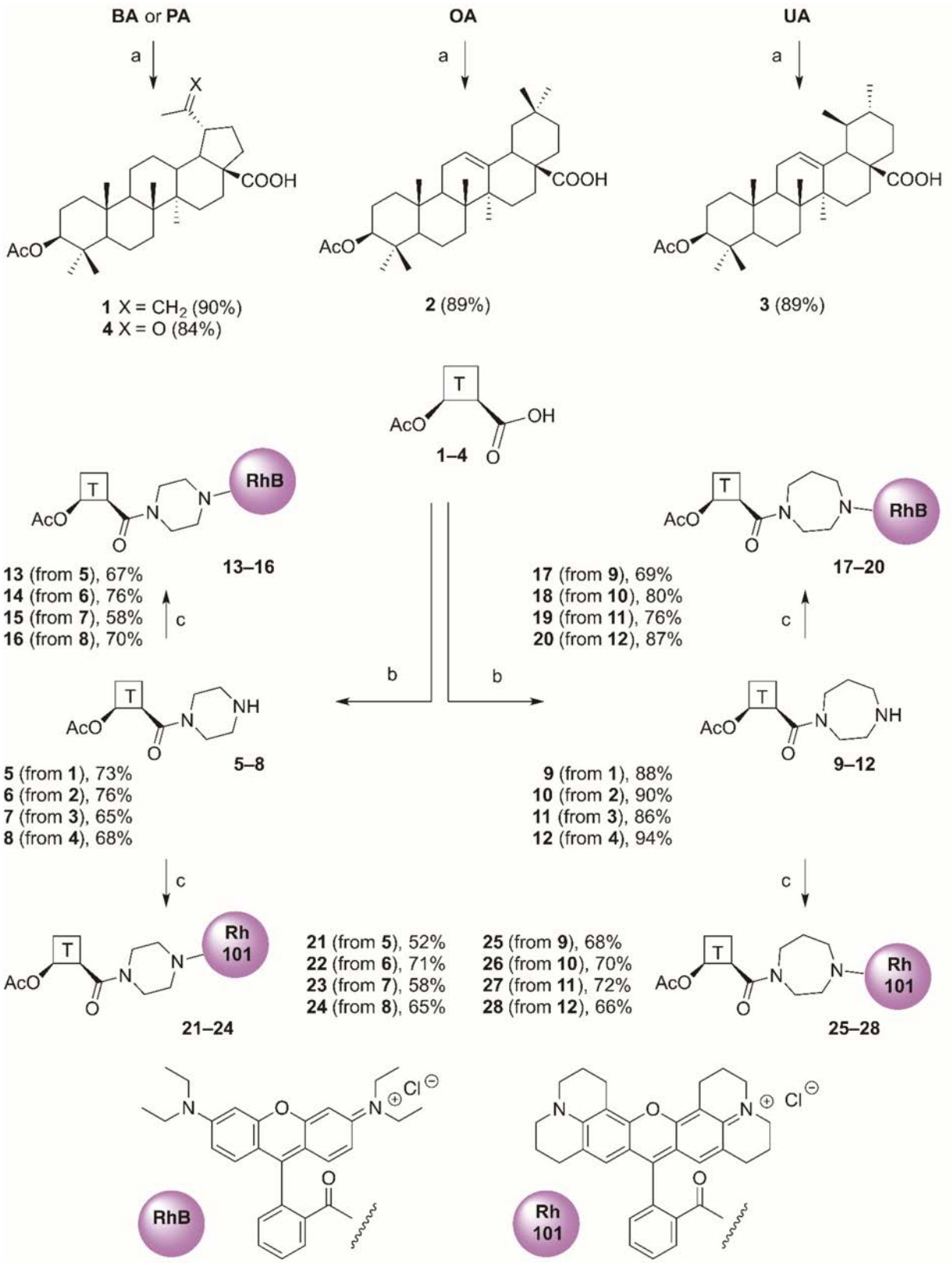


Figure 1. Structure of betulinic acid (**BA**), platanic acid (**PA**), oleanolic acid (**OA**), and **UA** (ursolic acid).

2. Results

Acetylation (Scheme 1) of betulinic (**BA**, Figure 1), oleanolic (**OA**), ursolic (**UA**), and platanic (**PA**) acid gave well known acetates **1**–**4**; their carboxyl group was activated with oxalyl chloride followed by the addition of either piperazine or homopiperazine to furnish amides **5**–**8** and **9**–**12**, respectively. Activation of rhodamine B or rhodamine 101 with oxalyl chloride and reaction with amides **5**–**12** furnished piperazine/rhodamine B derived conjugates **13**–**16** and **17**–**20** as well as rhodamine 101 derived hybrids **21**–**24** and **25**–**28**, respectively. All of these conjugates were violet in color, hence, indicating the presence of an intact cationic rhodamine moiety. This is regarded as a prerequisite for obtaining high cytotoxicity due to interaction with the mitochondrial membrane(s).

The cytotoxicity of the compounds was determined in sulforhodamine B (SRB) assays employing several human tumor cell lines (A375, HT29, MCF-7, A2780, FaDu) as well as two non-malignant cell lines (NIH 3T3, HEK293). The results from these assays are summarized in Tables 1–4.



Scheme 1. Reactions and conditions: (**a**) Ac_2O, NEt_3, DMAP (cat.), DCM, 20 °C, 1 d; (**b**) $(COCl)_2$, DMF (cat.), DCM, then DCM (homo)piperazine, 20 °C, 1 h; (**c**) rhodamine B or rhodamine 101, $(COCl)_2$, NEt_3, DMAP (cat.), DCM, 20 °C, 1 d; T—triterpenoic acid; OA—oleanolic acid; UA—ursolic acid; BA—betulinic acid; PA—platanic acid; Rh B—rhodamine B; Rh 101—rhodamine 101.

Table 1. Cytotoxicity of parent compounds **BA**, **OA**, **UA**, and **PA** as well as of their corresponding acetates **1**–**4** (EC_{50}-values in μM from SRB-assays) after 72 h of treatment; the values are averaged from three independent experiments performed each in triplicate, confidence interval CI = 95%; mean ± standard mean error); n.d. not determined; doxorubicin (**DX**) was used as a positive control. Cell lines: malignant: A375 (melanoma), HT29 (colon adenocarcinoma), MCF-7 (breast adenocarcinoma), A2780 (ovarian carcinoma), FaDu (hypopharyngeal carcinoma); non-malignant: NIH 3T3 (fibroblasts).

Compound	A375	HT29	MCF-7	A2780	FaDu	NIH 3T3
BA	17.7 ± 0.4	14.4 ± 2.3	10.2 ± 1.2	8.8 ± 0.9	13.7 ± 0.9	16.1 ± 1.4
OA	>30	>30	>30	>30	>30	>30
UA	15.4 ± 1.0	10.6 ± 0.7	12.7 ± 0.1	11.7 ± 0.6	18.2 ± 1.7	13.1 ± 1.1
PA	>30	>30	>30	>30	>30	>30
1	19.2 ± 1.7	21.3 ± 2.0	11.0 ± 0.5	18.3 ± 0.5	7.2 ± 1.2	>30
2	13.0 ± 1.1	20.5 ± 1.7	12.9 ± 1.9	9.4 ± 0.5	11.8 ± 0.9	17.5 ± 1.5
3	11.4 ± 1.4	17.3 ± 1.5	12.1 ± 1.2	8.3 ± 0.9	10.7 ± 0.8	16.4 ± 1.7
4	>30	>30	>30	>30	>30	>30
DX	n.d.	0.9 ± 0.2	1.1 ± 0.3	0.02 ± 0.01	1.7 ± 0.3	0.06 ± 0.03

Table 2. Cytotoxicity of 3-*O*-acetylated triterpenoic amides **5**–**8** (piperazinyl amimides) and **9**–**12** (homopiperazinyl amides) (EC_{50}-values in μM from SRB-assays) after 72 h of treatment; the values are averaged from three independent experiments performed each in triplicate, confidence interval CI = 95%; mean ± standard mean error); n.d. not determined; n.s. not soluble; doxorubicin (**DX**) was used as a positive control. Cell lines: malignant: A375 (melanoma), HT29 (colon adenocarcinoma), MCF-7 (breast adenocarcinoma), A2780 (ovarian carcinoma); non-malignant: NIH 3T3 (fibroblasts).

Compound	A375	HT29	MCF-7	A2780	NIH 3T3
5	1.5 ± 0.3	1.0 ± 0.1	1.4 ± 0.1	1.9 ± 0.1	0.9 ± 0.1
6	1.4 ± 0.2	1.3 ± 0.1	1.7 ± 0.2	1.7 ± 0.1	1.7 ± 0.1
7	2.0 ± 0.4	1.9 ± 0.3	1.7 ± 0.2	2.1 ± 0.1	2.1 ± 0.1
8	1.9 ± 0.4	3.9 ± 0.2	2.7 ± 0.3	2.6 ± 0.4	1.3 ± 0.1
9	18.7 ± 1.6	5.1 ± 1.1	10.7 ± 1.0	12.0 ± 0.6	18.7 ± 1.6
10	1.9 ± 0.9	1.9 ± 0.1	1.6 ± 0.1	2.2 ± 0.1	1.8 ± 0.2
11	3.2 ± 0.2	2.0 ± 0.1	2.4 ± 0.1	2.9 ± 0.24	0.9 ± 0.1
12	0.9 ± 0.1	2.3 ± 0.2	1.8 ± 0.2	1.6 ± 0.1	0.6 ± 0.1
DX	n.d.	0.9 ± 0.2	1.1 ± 0.3	0.02 ± 0.01	1.7 ± 0.3

Table 3. Cytotoxicity of 3-*O*-acetylated triterpenoic amides holding a distal rhodamine B unit **13**–**20** (EC_{50}-values in μM from SRB-assays) after 72 h of treatment; the values are averaged from three independent experiments performed each in triplicate, confidence interval CI = 95%; mean ± standard mean error); n.d. not determined; n.s. not soluble; doxorubicin (**DX**) was used as a positive control. Cell lines: malignant: A375 (melanoma), HT29 (colon adenocarcinoma), MCF-7 (breast adenocarcinoma), A2780 (ovarian carcinoma); non-malignant: NIH 3T3 (fibroblasts).

Compound	A375	HT29	MCF-7	A2780	NIH 3T3
13	0.09 ± 0.01	0.151 ± 0.022	0.081 ± 0.005	0.050 ± 0.004	0.208 ± 0.034
14	0.09 ± 0.01	0.091 ± 0.010	0.062 ± 0.004	0.032 ± 0.001	0.137 ± 0.006
15	0.04 ± 0.02	0.083 ± 0.007	0.075 ± 0.005	0.038 ± 0.002	0.137 ± 0.009
16	0.08 ± 0.03	0.099 ± 0.016	0.070 ± 0.002	0.036 ± 0.001	0.171 ± 0.006
17	0.76 ± 0.09	0.28 ± 0.01	0.22 ± 0.02	0.22 ± 0.01	0.33 ± 0.07
18	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.004	0.02 ± 0.003	0.14 ± 0.02
19	0.51 ± 0.05	0.50 ± 0.07	0.39 ± 0.04	0.45 ± 0.03	0.40 ± 0.03
20	0.24 ± 0.017	0.30 ± 0.027	0.15 ± 0.050	0.12 ± 0.018	0.34 ± 0.056
DX	n.d.	0.9 ± 0.2	1.1 ± 0.3	0.02 ± 0.01	1.7 ± 0.3

Table 4. Cytotoxicity of 3-*O*-acetylated triterpenoic amides holding a distal rhodamine 101 unit **13**–**20** (EC_{50}-values in μM from SRB-assays) after 72 h of treatment; the values are averaged from three independent experiments performed each in triplicate, confidence interval CI = 95%; mean ± standard mean error); n.d. not determined; n.s. not soluble; doxorubicin (**DX**) was used as a positive control. Cell lines: malignant: A375 (melanoma), HT29 (colon adenocarcinoma), MCF-7 (breast adenocarcinoma), A2780 (ovarian carcinoma), HeLa (cervical cancer); non-malignant: NIH 3T3 (fibroblasts), HEK293 (human embryonic kidney).

Compound	A375	HT29	MCF7	A2780	HeLa	NIH 3T3	HEK293
21	0.14 ± 0.02	0.36 ± 0.06	0.21 ± 0.03	0.09 ± 0.01	0.12 ± 0.03	0.56 ± 0.07	0.05 ± 0.05
22	0.15 ± 0.01	0.25 ± 0.03	0.23 ± 0.02	0.11 ± 0.01	0.20 ± 0.05	0.36 ± 0.05	0.07 ± 0.02
23	0.16 ± 0.02	0.21 ± 0.07	0.16 ± 0.03	0.08 ± 0.02	0.12 ± 0.02	0.37 ± 0.05	0.04 ± 0.01
24	0.25 ± 0.04	0.26 ± 0.04	0.17 ± 0.02	0.17 ± 0.03	0.21 ± 0.02	0.26 ± 0.04	0.10 ± 0.04
25	0.17 ± 0.05	0.43 ± 0.08	0.22 ± 0.04	0.19 ± 0.04	0.27 ± 0.14	0.56 ± 0.07	0.12 ± 0.04
26	0.07 ± 0.01	0.13 ± 0.03	0.12 ± 0.03	0.07 ± 0.03	0.10 ± 0.05	0.26 ± 0.05	0.05 ± 0.01
27	0.06 ± 0.04	0.08 ± 0.02	0.08 ± 0.03	0.05 ± 0.02	0.09 ± 0.06	0.24 ± 0.03	0.04 ± 0.01
28	0.20 ± 0.03	0.35 ± 0.05	0.20 ± 0.05	0.17 ± 0.04	0.31 ± 0.15	0.39 ± 0.05	0.12 ± 0.03
DX	n.d.	0.9 ± 0.2	1.1 ± 0.3	0.02 ± 0.01	n.d.	1.7 ± 0.3	n.d.

Table 1 shows the results from the SRB assays for the parent compounds and their acetates. Except for **BA** and **UA**, all other triterpenoids held EC_{50} values > 30 μM (cut-off of the assay) for the cancer cell lines but also for the non-malignant fibroblasts NIH 3T3. Acetates **1**–**4** showed slightly improved cytotoxicity (except **PA** derived **4**); by-and-large, EC_{50} values between 7.2 μM (**1** for FaDu cells) and 21.3 (**1** for A375 cells) were observed. Highest cytotoxicity was found for **1** and FaDu cells, for **2** with respect to A2780 and for **3** also with A2780 cells, respectively. Interestingly, **PA** derived acetate **4** was not cytotoxic at all within the limits of the assay.

Significant improvement was observed for the piperazinyl amides (Table 2), and EC_{50} values between 1.00 (**5** for HT29) and 3.86 (for **PA** derived **8** and HT29 cells) were determined. Except for the latter, all EC_{50} values were smaller than 3 μM. While **5** was cytotoxic with an EC_{50} = 1.5 μM for A375 cells, its homopiperazinyl analog **9** was significantly less active (EC_{50} = 18.7 μM). However, by-and-large, the cytotoxicity of the homopiperazinyl derivatives **9**–**12** was of the same order as that of the piperazinyl analogs **5**–**8**.

A dramatic improvement of cytotoxicity, however, was observed for the piperazinyl and homopiperazinyl spacered triterpenoic acid–rhodamine B conjugates **13**–**20** (Table 3).

Thereby, all of the compounds showed high cytotoxicity for all human tumor cell lines; EC_{50} values ranged from EC_{50} = 0.02 μM (compound **18** and A2780 cells) to EC_{50} = 0.76 μM (compound **17** and A375 cells). Thus, the former compound was as cytotoxic as standard doxorubicin.

Previously, we have shown the high cytotoxicity of several rhodamine B conjugates. Hence, it became of interest to investigate whether this high cytotoxicity is limited to rhodamine B conjugates or can also be found in conjugates holding a rhodamine 101 scaffold. As a result (Table 4), conjugates holding either a piperazinyl or homopiperazinyl spacer were only slightly less cytotoxic than those holding a rhodamine B moiety.

Interestingly enough, in this series of compounds, **UA** derived **27** (carrying a homopiperazine spacer and a rhodamine 101 residue) held the highest cytotoxicity, and the EC_{50} values for this compound were as low as EC_{50} = 0.05 μM (A2780 cells). Cytotoxicity for non-malignant fibroblasts NIH 3T3 were approximately five times lower for both rhodamine scaffolds. Extra staining experiments of A375 cells (acridine orange (AO), Hoechst 33342, rhodamine 123 (Figure 2)) showed **26** to act as a mitocan.

A summary of the hitherto known structural prerequisites to obtain pentacyclic triterpenoids of high cytotoxicity is depicted in Figure 3.

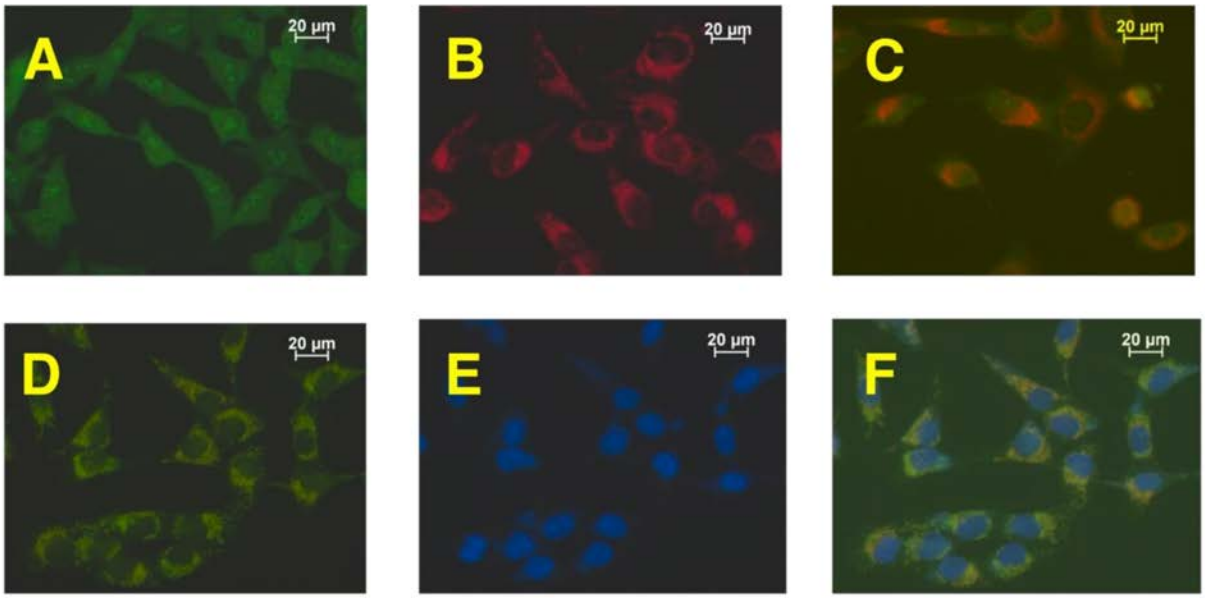


Figure 2. Staining experiments: A375 cells, 24 h; (**A**) control (AO); (**B**) in the presence of **26**; (**C**) merged (AO, **26**); (**D**) control (AO); (**E**) Hoechst 33,342 staining; (**F**) merged (Hoechst 33,342).

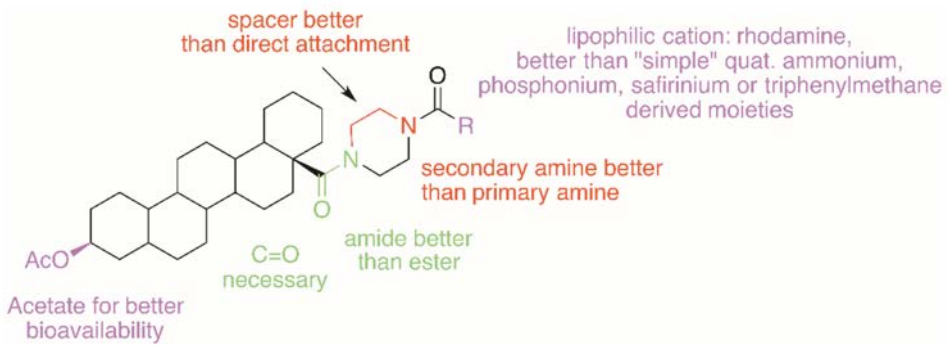


Figure 3. Hitherto known SAR parameters for obtaining pentacyclic triterpenoids of high cytotoxicity.

3. Conclusions

Four representative pentacyclic triterpenoic acids (**BA**, **OA**, **UA,** and **PA**) were selected for a systematic evaluation of cytotoxic derivatives. As a result—and as exemplified for A2780 cancer cells—the cytotoxicity of the parent acids is low but increased slightly upon their acetylation. A significant increase in cytotoxicity was observed when acetates **1–4** were transformed into their piperazinyl amides **5**–**8**. For the latter, compounds EC_{50} values between EC_{50} = 2.6 to 1.7 µM have been determined. The same trend was observed for the homopiperazinyl derivatives **9**–**12**. Interestingly, betulinic acid derived **9** (EC_{50} = 12.0 µM) was significantly less cytotoxic than its piperazinyl derivative **5** (EC_{50} = 1.9 µM). A tremendous improvement in cytotoxicity was observed, however, for the rhodamine conjugates, and EC_{50} values between EC_{50} = 0.05–0.032 µM were observed for the piperazinyl rhodamine B conjugates. The corresponding piperazinyl rhodamine 101 conjugates were of comparable bioactivity (EC_{50} = 0.09–0.17 µM). A similar trend was observed for the homopiperazinyl rhodamine conjugates, and EC_{50} = 0.02–0.45 µM (for rhodamine B derived **17**–**20**) and EC_{50} = 0.05–0.19 µM (for rhodamine 101 derived **25**–**28**) were determined. Thus, it can be concluded that an optimal combination of pentacyclic triterpene, a suitable spacer and a lipophilic cationic residue must be found to achieve good cytotoxic activity. It was shown that both piperazinyl and homopiperazinyl spacers are equally suitable to serve as anchors for the binding of either rhodamine B or rhodamine

101. Furthermore, the conjugates derived from either rhodamine B or rhodamine 101 are of comparable cytotoxicity.

4. Experimental

NMR spectra were recorded using the Varian spectrometers (Darmstadt, Germany) DD2 and VNMRS (400 and 500 MHz, respectively). MS spectra were taken on a Advion expression^{L} CMS mass spectrometer (Ithaca, NY, USA); positive ion polarity mode, solvent: methanol, solvent flow: 0.2 mL/min, spray voltage: 5.17 kV, source voltage: 77 V, APCI corona discharge: 4.2 µA, capillary temperature: 250 °C, capillary voltage: 180 V, sheath gas: N_2). Thin-layer chromatography was performed on pre-coated silica gel plates supplied by Macherey-Nagel (Düren, Germany). IR spectra were recorded on a Spectrum 1000 FT-IR-spectrometer from Perkin Elmer (Rodgau, Germany). The UV/Vis-spectra were recorded on a Lambda 14 spectrometer from Perkin Elmer (Rodgau, Germany); optical rotations were measured at 20 °C using a JASCO-P2000 instrument (JASCO Germany GmbH, Pfungstadt, Germany) The melting points were determined using the Leica hot stage microscope Galen III (Leica Biosystems, Nussloch, Germany) and are uncorrected. The solvents were dried according to usual procedures. Microanalyses were performed with an Elementar Vario EL (CHNS) instrument (Elementar Analysensysteme GmbH, Elementar-Straße 1, D-63505, Langenselbold, Germany).

4.1. General Procedure for the Synthesis of Acetates **1**–**4** *(GPA)*

To a solution of the triterpenoic acid (**OA**, **UA**, **BA**, **PA**, 1 equiv.) in dry DCM, acetic anhydride (3 equiv.), triethylamine (3 equiv.), and DMAP (cat.) were added, and the mixture was stirred at 20 °C for 1 day. Usual aqueous work-up followed by re-crystallization from ethanol furnished products **1**–**4**.

4.2. 3β-Acetyloxy-lup-20(29)-en-28-oic Acid (**1**)

Following GPA, compound **1** (4.90 g, 90%) was obtained from **BA** as a colorless solid; R_f = 0.59 (n-hexane/ethyl acetate, 4:1); m.p. 277–279 °C (lit.: [28] 277–278 °C); $[\alpha]_D$ = +20.7° (*c* 0.42, $CHCl_3$) [(lit.: [29] $[\alpha]_D$ = +20.7° (*c* 0.42, $CHCl_3$)]; MS (ESI, MeOH): *m*/*z* 487.1 (32%, $[M - H]^-$, 995.0 (100%, $[2M - H]^-$, 1018.6 (29% $[2M - 2H + Na]^-$).

4.3. 3β-Acetyloxy-olean-12-en-oic Acid (**2**)

Following GPA, compound **2** (2.45 g, 89%) was obtained from **OA** as a colorless solid; R_f = 0.72 (toluene/ethyl acetate/heptane/formic acid, 80:26:10:5); m.p. 286–289 °C (lit.: [30] 264–265 °C); $[\alpha]_D$ = +69.4° (*c* 0.30, $CHCl_3$) [(lit.: [31] $[\alpha]_D$ = +74.0° (*c* 1.0, $CHCl_3$)]; MS (ESI, MeOH): *m*/*z* 499.4 ([100%, $M + H]^+$), 516.3 (36%, $[M + NH_4]^+$, 521.5 [35%, $[M + Na]^+$.

4.4. 3β-Acetyloxy-urs-12-en-oic Acid (**3**)

Following GPA, compound **3** (2.41 g, 89%) was obtained from **UA** as a colorless solid; R_f = 0.57 (n-hexane/ethyl acetate, 3:1); m. p. 281–283 °C (lit.: [32] 280 °C); $[\alpha]_D$ = +66.5° (*c* 0.42, $CHCl_3$) [lit.: [33] $[\alpha]_D$ = +63.5° (*c* 0.5, $CHCl_3$)]; MS (ESI, MeOH): *m*/*z* 499.3 (17%, $[M + H]^+$), 521.2 (31%, $[M + H]^+$), 1019.4 (100%, $[2M + Na]^+$).

4.5. 3β-Acetyloxy-20-oxo-30-norlupan-28-oic Acid (**4**)

Following GPA, compound **4** (6.9 g, 84%) was obtained from **PA** as a colorless solid; R_f = 0.50 (toluene/ethyl acetate/heptane/formic acid, 80:26:10.5); m.p. 265–268 °C (lit.: [34] 252–255 °C); $[\alpha]_D$ = −9.4° (*c* 0.32, $CHCl_3$) [(lit.: [34] $[\alpha]_D$ = −9.5° (*c* 0.5, $CHCl_3$)]; MS (ESI, MeOH): *m*/*z* 999.5 (100%, $[2M - H]^-$).

4.6. General Procedure for the Synthesis of Acetylated (homo)piperazinyl Amides **5**–**12** *(GPB)*

To a solution of the acetylated triterpenoic acid **1**–**4** (1 equiv.) in dry DCM, DMF (cat.) and oxalyl chloride (4 equiv.) were added followed by the addition of (homo)piperazine

(4 equiv.). After stirring for 1h at 20 °C followed by usual aqueous work-up and column chromatography, products **5**–**12** were obtained.

4.7. 3β-Acetyloxy-28-(1-piperazinyl)-lup-20(29)en-28-one (**5**)

Following GPB from **1** (2.5 g, 5 mmol) and piperazine (1.6 g, 20.0 mmol), compound **5** (2.07 g, 73%) was obtained as a colorless solid; R_f = 0.40 (SiO_2, $CHCl_3$/MeOH, 9:1); m.p. = 166–173 °C (lit.: [35] 162–167 °C); $[\alpha]_D = -1.4°$ (*c* 0.21, MeOH), [lit.: [35] $[\alpha]_D = -1.8°$ (*c* 0.32, MeOH); MS (ESI, MeOH): *m*/*z* (%) = 567.3 ([100%, M + H]$^+$).

4.8. 3β-Acetyloxy-28-(1-piperazinyl)-olean-12-en-28-one (**6**)

Following GPB from **2** (2.5 g, 5.0 mmol) and piperazine (1.6 g, 29 mmol), **6** (2.16 g, 76%) was obtained as a colorless solid; R_f = 0.40 (SiO_2, $CHCl_3$/MeOH, 9:1); m.p. = 172–175 °C (lit.: [35] 173–175 °C); $[\alpha]_D = +23.4°$ (*c* 0.18, $CHCl_3$), [lit.: [35] $[\alpha]_D = +26.6°$ (*c* 0.35, MeOH); MS (ESI, MeOH): *m*/*z* (%) = 567.4 (100%, [M + H]$^+$).

4.9. 3β-Acetyloxy-28-(1-piperazinyl)-urs-12-en-28-one (**7**)

Following GPB from **3** (2.5 g, 5.0 mmol) and piperazine (1.6 g, 20.0 mmol), **7** (1.84 g, 65%) was obtained as a colorless solid; R_f = 0.40 (SiO_2, $CHCl_3$/MeOH, 9:1); m.p. = 187–190 °C (lit.: [35] 187–188 °C); $[\alpha]_D = +25.1°$ (*c* 0.24, MeOH), [lit.: [35] $[\alpha]_D = +24.5°$ (*c* 0.29, MeOH); MS (ESI, MeOH): *m*/*z* (%) = 567.4 (100%, [M + H]$^+$).

4.10. 3β-Acetyloxy-28-(1-piperazinyl)-30-norlupane-20,28-dione (**8**)

Following GPB from **4** (2.5 g, 5.0 mmol) and piperazine (1.6 g, 20.0 mmol), **17** (1.93 g, 68%) was obtained as a colorless solid; R_f = 0.40 (SiO_2, $CHCl_3$/MeOH, 9:1); m.p. = 127–130 °C (lit.: [20] 115–125 °C); $[\alpha]_D = -20.3°$ (*c* 0.13, $CHCl_3$); MS (ESI, MeOH): *m*/*z* (%) = 569.3 (100%, [M + H]$^+$).

4.11. 3β-Acetyloxy-28-(1-homopiperazinyl)-lup-20(29)en-28-one (**9**)

Following GPB from **1** (1.0 g, 2.02 mmol) and homopiperazine (9.8 g, 8.0 mmol), **9** (1.02 g, 88%) was obtained as a colorless solid; R_f = 0.4 (SiO_2, $CHCl_3$/MeOH, 9:1); m.p. 190–193°C (lit.: [21] 196–199 °C); $[\alpha]_D = +107.6°$ (*c* 0.20, $CHCl_3$), [lit.: [21] $[\alpha]_D = +109.8°$ (*c* 0.38, $CHCl_3$); MS (ESI, MeOH): *m*/*z* = 581.4 (100%, [M + H]$^+$).

4.12. 3β-Acetyloxy-28-(1-homopiperazinyl)-olean-12-en-28-one (**10**)

Following GPB from **2** (1.0 g, 2.02 mmol) and homopiperazine (0.8 g, 8.0 mmol), **10** (1.4 g, 90%) was obtained as a colorless solid; R_f = 0.4 (SiO_2, $CHCl_3$/MeOH, 9:1); m.p. 182–185 °C (lit.: [21] 187–190 °C); $[\alpha]_D = 12.4°$ (*c* 0.14, $CHCl_3$), [lit.: [21] $[\alpha]_D = +9.9°$ (*c* 0.35, $CHCl_3$); MS (ESI, MeOH): *m*/*z* = 581.4 (100%, [M + H]$^+$).

4.13. 3β-Acetyloxy28-(1-homopierazinyl)-urs-12-en-28-one (**11**)

Following GPB from **3** (1.0 g, 2.02 mmol) and homopiperazine (0.8 g, 8.0 mmol), **11** (1.1 g, 86%) was obtained as a colorless solid; R_f = 0.4 (SiO_2, $CHCl_3$/MeOH, 9:1); m.p. 171–175 °C (lit.: [21] 178–180 °C); $[\alpha]_D = +27.0°$ (*c* 0.21, $CHCl_3$), [lit.: [21] $[\alpha]_D = +29.7°$ (*c* 0.34, $CHCl_3$); MS (ESI, MeOH): *m*/*z* = 581.4 (100%, [M + H]$^+$, 100%).

4.14. 3β-Acetyloxy-28-(1-homopiperazinyl)-30-norlupane-20,28-dione (**12**)

Following GPB from **4** (1.0 g, 2.02 mmol) and homopiperazine (0.8 g, 8.0 mmol), **12** (1.1 g, 94%) was obtained as a colorless solid; R_f = 0.4 (SiO_2, $CHCl_3$/MeOH, 9:1); m.p. 162–165 °C; $[\alpha]_D = -29.2°$ (*c* 0.16, $CHCl_3$); IR (ATR): ν = 2941m, 2866w, 1732m, 1708m, 1622m, 1464m, 1453m, 1408m, 1367m, 1244vs, 1191m, 1149m, 1133m, 1109w, 1026m, 979m, 947w, 934w, 900w, 750s, 665m, 610w, 557m, 506m, 455w, 410w cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.44 (dd, *J* = 10.5, 5.5 Hz, 1H, 3-H), 3.89–3.60 (m, 4H, 33-H), 3.40–3.09 (m, 5H, 19-H, 33-H), 2.62 (td, *J* = 12.1, 4.1 Hz, 1H, 13-H), 2.25 (s, 2H, 35-H), 2.17 (s, 3H, 29-H), 2.14–2.06 (m, 2H, 16-H_a + 18-H), 2.02 (s, 3H, 32-H), 1.97–1.84 (m, 2H, 21-H_a + 22-H_a),

1.68–1.14 (m, 15H + 1-H_a + 2-H + 6-H_a + 6-H_b + 7-H + 9-H + 11-H_a + 11-H_b + 15-H + 16-H_b + 21-H_b + 22-H_b), 1.06–0.98 (m, 2H, 12-H), 0.96 (s, 4H, 1-H_b + 27-H), 0.89 (s, 3H, 26-H), 0.82 (s, 3H, 24-H), 0.82 (s, 3H, 25-H), 0.81 (s, 3H, 23-H), 0.79–0.74 (m, 1H, 5-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 213.0 (C-20), 175.0 (C-28), 171.2 (C-31), 81.0 (C-3), 55.4 (C-5), 54.9 (C-17), 52.6 (C-18), 50.6 (C-9), 49.9 (C-19), 46.6 (C-33 + C-34), 41.8 (C-14), 40.6 (C-8), 38.3 (C-1), 37.8 (C-4), 37.1 (C-10), 36.0 (C-13), 35.7 (C-22), 34.1 (C-7), 31.7 (C-16), 30.3 (C-29), 29.9 (C-15), 28.6 (C-21), 27.9 (C-24), 27.3 (C-12), 25.7 (C-35), 23.6 (C-2), 21.3 (C-32), 21.1 (C-11), 18.1 (C-6), 16.4 (C-23), 16.2 (C-25), 15.9 (C-26), 14.7 (C-27) ppm; MS (ESI, MeOH): m/z = 583.3 (100%, $[M + H]^+$; analysis calcd for $C_{36}H_{58}N_2O_4$ (582.87): C 74.18, H 10.03, N 4.81; found: C 73.95, H 10.34, N 4.63.

4.15. General Procedure for the Synthesis of the Rhodamine Conjugates **13**–**28** *(GPC)*

To a solution of the respective rhodamine (1.15 eq.) in dry DCM at 0 °C, DMF (cat.) and oxalyl chloride (4 eq.) were added (vide supra). This acid chloride was slowly added to the solution of the respective triterpene (1 eq. in DCM) in the presence of triethylamine (1 eq.). After stirring for 1 day at 20 °C, aqueous work-up was carried out as usual and the residue was purified by column chromatography.

4.16. 9-[2-[[4-(3β-Acetyloxy-28-oxo-lup-20(29)en-28-yl)-1-piperazinyl]carbonyl]phenyl]-3,6-bis(diethylamino)-xanthylium chloride (**13**)

Following GPC from **5** (180 mg, 0.32 mmol) and rhodamine B, **13** (220 mg, 67%) was obtained as a pink solid; R_f = 0.39 (SiO_2, $CHCl_3$/MeOH, 9:1); m.p. 247–252 °C (lit.: [20] m.p. 246–250 °C); MS (ESI, MeOH): m/z = 991.6 (100%, $[M - Cl]^+$).

4.17. 9-[2-[[4-(3β-Acetyloxy-28-oxo-olean-12-en-28-yl)-1-piperazinyl]carbonyl]phenyl]-3,6-bis(diethylamino)-xanthylium chloride (**14**)

Following GPC from **6** (180 mg, 0.32 mmol) and rhodamine B, **14** (250 mg, 76%) was obtained as a pink solid; R_f = 0.40 (SiO_2, $CHCl_3$/MeOH, 9:1); m.p. 246–252 °C (lit.: [20] 245–248 °C); MS (ESI, MeOH): m/z = 991.9 (100%, $[M - Cl]^+$).

4.18. 9-[2-[[4-(3β-Acetyloxy-28-oxo-ursan-12-en-28-yl)-1-piperazinyl]carbonyl]phenyl]-3,6-bis(diethylamino)-xanthylium chloride (**15**)

Following GPC from **7** (180 mg, 0.32 mmol) and rhodamine B, **15** (190 mg, 58%) was obtained as a pink solid; R_f = 0.40 (SiO_2, $CHCl_3$/MeOH, 9:1); m.p. 245–251 °C (lit.: [20] 243–245 °C); MS (ESI, MeOH): m/z = 991.7 (100%, $[M - Cl]^+$).

4.19. 9-[2-[[4-(3β-Acetyloxy-20,28-dioxo-30-norlupan-28-yl)-1-piperazinyl]carbonyl]phenyl]-3,6-bis(diethylamino)-xanthylium chloride (**16**)

Following GPC from **8** (180 mg, 0.32 mmol) and rhodamine B, **16** (230 mg, 70%) was obtained as a pink solid; R_f = 0.37 (SiO_2, $CHCl_3$/MeOH, 9:1); m.p. 247–254 °C (lit.: [20] 235–243 °C); MS (ESI, MeOH): m/z = 993.7 (100%, $[M - Cl]^+$).

4.20. 9-[2-[[4-(3β-Acetyloxy-28-oxo-30-norlupan-28-yl)-1-homopiperazinyl]carbonyl]phenyl]-3,6-bis(diethylamino)-xanthylium chloride (**17**)

Following GPC from **9** (260 mg, 0.32 mmol) and rhodamine B, **17** (229 mg, 69%) was obtained as a pink solid; R_f = 0.50 (SiO_2, MeCN/CH_2Cl_2/H_2O, 10:1:1); m.p. 261–266 °C (lit.: [21] 256–260 °C); MS (ESI, MeOH): m/z = 1005.7 (100%, $[M - Cl]^+$).

4.21. 9-[2-[[4-(3β-Acetyloxy-28-oxo-olean-12-en-28-yl]-1-homopiperazinyl]carbonyl]phenyl]-3,6-bis(diethylamino)-xanthylium chloride (**18**)

Following GPC from **10** (270 mg, 0.32 mmol) and rhodamine B, **18** (267 mg, 80%) was obtained as a pink solid; R_f = 0.52 (SiO_2, MeCN/CH_2Cl_2/H_2O, 10:1:1); m.p. 235–241 °C (lit.: [21] 238–245 °C); MS (ESI, MeOH): *m*/*z* = 1005.8 (100%, $[M - Cl]^+$).

4.22. 9-[2-[[4-(3β-Acetyloxy-28-oxo-urs-12-en-28-yl]-1-homopiperazinyl]carbonyl]phenyl]-3,6-bis(diethylamino)-xanthylium chloride (**19**)

Following GPC from **11** (270 mg, 0.32 mmol) and rhodamine B, **19** (253 mg, 76%) was obtained as a pink solid; R_f = 0.51 (SiO_2, MeCN/CH_2Cl_2/H_2O, 10:1:1); m.p. 238–246 °C (lit.: [21] 238–245 °C); MS (ESI, MeOH): *m*/*z* = 1005.7 (100%, $[M - Cl]^+$).

4.23. 9-[2-[[4-(3β-Acetyloxy-20,28-dioxo-30-norlupan-28-yl)-1-homopiperazinyl]carbonyl]phenyl]-3,6-bis(diethylamino)-xanthylium chloride (**20**)

Following GPC from **10** (125 mg, 0.21 mmol) and rhodamine B, **18** (190 mg, 87%); was obtained as a pink solid; m.p. 248–250 °C (decomp.); R_f = 0.30 (SiO_2, $CHCl_3$/MeOH, 9:1); UV-Vis ($CHCl_3$): λ_{max} (log ε) = 562 nm (5.05); IR (ATR): ν = 2937w, 1730w, 1585s, 1466m, 1411m, 1334s, 1273m, 1244m, 1178s, 1131m, 1072m, 977w, 920w, 822w, 746m, 683m cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.66–7.57 (*m*, 2H, 42-H, 43-H), 7.42–7.36 (*m*, 1H, 40-H), 7.31–7.27 (*m*, 1H, 41-H), 7.25–7.15 (*m*, 2H, 50-H), 6.99–6.83 (*m*, 2H, 49-H), 6.79–6.68 (*m*, 2H, 47-H), 4.40 (*dd*, *J* = 10.4, 5.6 Hz, 1H, 3-H), 3.85–3.31 (*m*, 16H, 32-H, 33-H, 34-H, 36-H, 51-H), 3.27–3.12 (*m*, 1H, 13-H), 2.72–2.57 (*m*, 1H, 19-H), 2.11 (*s*, 3H, 29-H), 2.18–2.06 (*m*, 1H, 16-H_a), 1.98 (*s*, 3H, 31-H), 1.96 (*s*, 1H, 18-H), 1.95–0.97 (*m*, 21H, 22-H_a, 21-H_a, 35-H, 1-H_a, 2-H, 16-H_b, 21-H_b, 6-H, 22-H_b, 11-H_a, 7-H, 11-H_b, 9-H, 15-H, 12-H), 1.32–1.26 (*m*, 12H, 52-H), 0.92 (*s*, 3H, 27-H), 0.89 (*s*, 3H, 24-H), 0.94–0.86 (*m*, 1H, 1-H_b), 0.79 (*s*, 9H, 23-H, 25-H, 26-H), 0.73 (*s*, 1H, 5-H_b) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 212.8 (C-20), 174.7 (C-28), 170.7 (30 C-), 168.3 (C-37), 157.5 (C-48), 157.5 (C-44), 155.4 C- (46), 136.5 (C-38) 135.9 (C-39), 132.1 (C-50), 130.1 (C-41), 129.6 (C-42), 129.4 (C-43), 126.3 (C-40), 113.9 (C-49), 113.4 (C-45), 96.2 (C-47), 80.7 (C-3), 55.3 (C-5), 54.7 (C-17), 52.7 (C-18), 50.4 (C-9), 50.0 (C-19), 46.0 (C-51), 41.6 (C-14), 40.4 (C-8), 38.2 (C-1), 37.6 (C-4), 36.9 (C-10), 35.8 (C-13), 35.3 (C-22), 34.1 (C-7), 31.7 (C-35), 31.2 (C-16), 30.0 (C-29), 29.7 (C-15), 28.7 (C-21), 27.7 (C-23), 27.2 (C-12), 23.5 (C-2), 21.1 (C-31), 21.0 (C-11), 18.0 (C-6), 16.3 (C-25), 16.1 (C-26), 16.0 (C-24), 14.4 (C-27), 12.5 (C-52). ppm; MS (ESI, MeOH): *m*/*z* 1007 (100%, $[M]^+$); analysis calculated for $C_{64}H_{85}ClN_4O_6$ (1041.84): C 73.78, H 8.22, N 5.38; found: C 73.55, H 8.41, N 5.19.

4.24. 3β-Acetyloxy-28-[4-[3-(2,3,6,7,12,13,16,17-octahydro-1H,5H,11H,15H-pyrido[3,2,1-ij]pyrido[1″,2″,3″:1′,8′]quinolino[6′,5′:5,6]pyrano[2,3-f]quinolin-4-ium-9-yl)benzoyl]piperazine-1-yl]-28-oxo-lup-20(29)-en chloride (**21**)

Following GPC from **5** (0.25 g, 0.44 mmol) and rhodamine 101 (0.25 g, 0.51 mmol), **21** (0.24 g, 52%) was obtained as a pink colored solid; R_f = 0.4 (SiO_2, $CHCl_3$/MeOH, 9:1); m.p. >300 °C; IR (ATR): ν = 3400w, 2939w, 2862w, 1728w, 1630m, 1595m, 1542w, 1493m, 1434m, 1360m, 1294vs, 1267s, 1248s, 1195s, 1182s, 1099s, 1035m, 1003m, 978m, 896w, 827w, 772m, 747m, 637m, 560m, 506m, 421s cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.67 (dd, *J* = 5.7, 3.3 Hz, 2H, 37-H, 39-H), 7.53–7.48 (m, 1H, 38-H), 7.31 (dd, *J* = 5.7, 3.2 Hz, 1H, 40-H), 6.68 (d, *J* = 6.0 Hz, 2H, 48-H), 4.68 (s, 1H, 29-H_a), 4.56 (s, 1H, 29-H_b), 4.44 (dd, *J* = 10.2, 5.9 Hz, 1H, 3-H), 3.60–3.48 (m, 4H, 49-H), 3.48–3.40 (m, 4H, 52-H), 3.40–3.31 (m, 8H, 33-H + 34-H), 3.04–2.97 (m, 4H, 54-H), 2.91 (dt, *J* = 11.9, 6.1 Hz, 1H, 19-H), 2.81–2.74 (m, 1H, 13-H), 2.74–2.60 (m, 4H, 51-H), 2.14–2.04 (m, 4H, 53-H), 2.02 (s, 3H, 32-H), 1.98–1.91 (m, 4H, 50-H), 1.77 (q, *J* = 33.5, 10.4 Hz, 4H, 12-H + 16-H_a + 22-H_a), 1.65 (s, 3H, 30-H), 1.63–1.44 (m, 8H, 1-H + 2-H + 6-H_a + 11-H + 16-H_b + 18-H), 1.44–1.08 (m, 10H, 6-H_b + 7-H + 9-H + 15-H + 21-H + 22-H_b), 0.92 (s, 3H, 27-H), 0.88 (s, 3H, 26-H), 0.82 (s, 6H, 23-H + 24-H), 0.81 (s, 3H, 25-H), 0.77 (s, 1H, 5-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 174.1 (C-28), 171.0 (C-31), 167.9 (C-35), 162.7 (C-20), 152.0 (C-43), 151.2 (C-44), 150.9 (C-46), 134.8 (C-41), 131.8 (C-36),

130.7 (C-40), 130.2 (C-39), 129.7 (C-37), 127.5 (C-38), 126.5 (C-48), 123.6 (C-45), 123.6 (C-45), 113.2 (C-42), 109.4 (C-29), 105.4 (C-47), 80.9 (C-3), 55.5 (C-5), 54.6 (C-17), 52.5 (C-18), 51.0 (C-49), 50.7 (C-9), 50.5 (C-52,), 45.7 (C-19), 41.9 (C-14), 40.6 (C-8), 38.4 (C-1), 37.8 (C-4), 37.1 (C-10), 36.9 (C-13), 35.8 (C-22), 34.3 (C-7), 32.5 (C-16), 31.2 (C-21), 29.8 (C-15), 27.9 (C-24), 27.7 (C-51), 25.5 (C-12), 23.7 (C-2), 21.3 (C-32), 20.6 (C-11, C-50), 19.9 (C-54), 19.6 (C-53), 19.5 (C-30), 18.1 (C-6), 16.5 (C-23), 16.2 (C-25), 16.1 (C-26), 14.6 (C-27) ppm; MS (ESI, MeOH): m/z = 1039.3 (100%, $[M - Cl]^+$); analysis calculated for $C_{68}H_{87}N_4O_5Cl$ (1075.92): C 75.91, H 8.15, N 5.21; found: C 75.77, H 8.36, N 5.05.

4.25. 3β-Acetyloxy-28-[4-[3-(2,3,6,7,12,13,16,17-octahydro-1H,5H,11H,15H-pyrido[3,2,1-ij]pyrido[1″,2″,3″:1′,8′]quinolino[6′,5′:5,6]pyrano[2,3-f]quinolin-4-ium-9-yl)benzoyl]piperazine-1-yl]-28-oxoolean-12-en chloride (**22**)

Following GPC from **6** (0.25 g, 0.44 mmol) and rhodamine 101 (0.25 g, 0.51 mmol), **22** (0.33 g, 71%) was obtained as a pink solid; R_f = 0.4 (SiO_2, $CHCl_3$/MeOH, 9:1); m.p. >300 °C; IR (ATR): ν = 3398w, 2941w, 2859w, 1728w, 1631m, 1594s, 1543w, 1494s, 1458m, 1435m, 1361m, 1295Vs, 1267s, 1248s, 1196s, 1182s, 1100s, 1077m, 1035m, 1002m, 897w, 862w, 773w, 733m, 640m, 560m, 498m, 421s cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ = 7.70–7.66 (m, 2H, 37-H, 39-H), 7.53–7.49 (m, 1H, 38-H), 7.34–7.30 (m, 1H, 40-H), 6.67 (d, *J* = 7.5 Hz, 2H, 48-H), 5.23 (t, *J* = 3.5 Hz, 1H, 12-H), 4.48 (dd, *J* = 9.9, 6.0 Hz, 1H, 3-H), 3.63–3.51 (m, 4H, 49-H), 3.52–3.40 (m, 4H, 52-H), 3.39–3.29 (m, 8H, 33-H + 34-H), 3.09–3 (m, 4H, 54-H), 3.00–2.96 (m, 1H, 18-H), 2.76–2.62 (m, 4H, 51-H), 2.15–2.07 (m, 5H, 16-H_a + 53-H), 2.04 (s, 3H, 32-H), 2.01–1.91 (m, 4H, 50-H), 1.89–1.81 (m, 1H, 11-H), 1.71–1.48 (m, 10H, 1-H_a + 2-H + 6-H_a + 7-H + 9-H + 15-H_a + 16-H_b + 19-H_a), 1.48–1.13 (m, 7H, 6-H_b + 19-H_b + 21-H + 22-H_a + 22-H_b), 1.11 (s, 3H, 27-H), 1.08–0.94 (m, 2H, 1-H_b + 15-H_b), 0.91 (s, 3H, 25-H), 0.89 (s, 3H, 29-H), 0.88 (s, 3H, 30-H), 0.86 (s, 3H, 24-H), 0.85 (s, 3H, 23-H), 0.82 (s, 1H, 5-H), 0.66 (s, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 176.4 (C-28), 171.0 (C-31), 167.8 (C-35), 152.8 (C-43), 152.0 (C-44), 152.0 (C-44), 151.3 (C-46), 151.2 (C-46), 144.4 (C-13), 134.8 (C-41), 131.8 (C-36), 130.8 (C-40), 130.2 (C-39), 129.7 (C-37), 127.4 (C-36), 126.5 (C-48), 126.5 (C-48), 123.6 (C-45), 123.5 (C-45), 121.7 (C-12), 113.3 (C-42), 105.5 (C-47), 105.5 (C-47), 80.8 (C-3), 55.3 (C-5), 51.0 (C-49, 49), 50.6 (C-52), 47.6 (C-9, 33, 34), 47.5 (C-17), 46.2 (C-19), 43.5 (C-18), 41.8 (C-14), 39.1 (C-8), 38.0 (C-1), 37.7 (C-4), 37.0 (C-10), 33.9 (C-21), 32.9 (C-30), 32.8 (C-22), 30.3 (C-20), 30.0 (C-7), 28.0 (C-24), 27.8 (C-15), 27.7 (C-51), 25.9 (C-27), 24.0 (C-29), 23.5 (C-2), 23.3 (C-11), 22.5 (C-16), 21.3 (C-32), 20.6 (C-50), 19.9 (C-54), 19.7 (C-53), 18.2 (C-6), 16.9 (C-26), 16.7 (C-23), 15.4 (C-25) ppm; MS (ESI, MeOH): m/z = 1039.3 (100%, $[M - Cl]^+$); analysis calculated for $C_{68}H_{87}N_4O_5Cl$ (1075.64): C 75.91, H 8.15, N 5.21; found: C 75.72, H 8.29, N 5.01.

4.26. 3β-Acetyloxy-28-[4-[3-(2,3,6,7,12,13,16,17-octahydro-1H,5H,11H,15H-pyrido[3,2,1-ij] pyrido[1″,2″,3″:1′,8′]quinolino[6′,5′:5,6]pyrano[2,3-f]quinolin-4-ium-9-yl)benzoyl]piperazine-1-yl]-28-oxo-urs-12-en chloride (**23**)

Following GPC from **7** (0.25 g, 0.44 mmol) and rhodamine 101 (0.25 g, 0.51 mmol), **23** (0.27 g, 58%) was obtained as a pink solid; R_f = 0.4 (SiO_2, $CHCl_3$/MeOH, 9:1); m.p. >300 °C; IR (ATR): ν = 3392w, 2926w, 2867w, 1728w, 1626m, 1594s, 1542w, 1493s, 1457m, 1435m, 1361m, 1294vs, 1266s, 1246s, 1196s, 1181s, 1099s, 1035m, 1004m, 897w, 863w, 773m, 732m, 653m, 561m, 420s cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.71–7.65 (m, 2H, 37-H, 39-H), 7.54–7.48 (m, 1H, 38-H), 7.34–7.30 (m, 1H, 40-H), 6.67 (d, *J* = 4.7 Hz, 2H, 48-H), 5.17 (s, 1H, 12-H), 4.48 (dd, *J* = 10.4, 5.5 Hz, 1H, 3-H), 3.56 (dt, *J* = 17.2, 6.1 Hz, 4H, 49-H), 3.51–3.41 (m, 4H, 52-H), 3.33 (s, 8H, 33-H + 34-H), 3.02 (q, *J* = 6.0 Hz, 4H, 54-H), 2.68 (ddt, *J* = 22.7, 15.6, 7.3 Hz, 4H, 51-H), 2.39–2.32 (m, 1H, 18-H), 2.15–2.06 (m, 4H, 53-H), 2.04 (s, 3H, 32-H), 1.99–1.94 (m, 4H, 50-H), 1.91–1.87 (m, 1H + 11-H_a), 1.79–1.57 (m, 7H + 1-H_a + 2-H + 11-H_b + 16-H + 22-H_a), 1.56–1.40 (m, 5H, 6-H_a + 7-H_a + 9-H + 21-H_a + 22-H_b), 1.40–1.20 (m, 4H, 6-H_b + 7-H_b + 19-H + 21-H_b), 1.05 (s, 6H, 1-H_b + 15-H + 27-H), 0.99–0.95 (m, 1H, 20-H), 0.93 (s, 3H, 30-H), 0.91 (s, 3H, 25-H), 0.86 (s, 3H, 24-H), 0.86 (s, 3H, 26-H), 0.84 (s, 3H, 29-H), 0.81–0.80 (m, 1H, 5-H), 0.67 (s, 3H, 23-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 174.8

(C-28), 171.0 (C-31), 167.8 (C-35), 152.8 (C-46), 152.0 (C-43), 151.3 (C-44), 151.2 (C-44), 134.8 (C-41), 130.8 (C-40), 130.2 (C-37), 129.8 (C-39), 127.5 (C-38), 126.5 (C-48), 125.2 (C-12), 123.6, 123.5 (C-45), 113.2 (C-42), 105.5 (C-47), 105.5 (C-47), 80.8 (C-3), 55.3 (C-5, 18), 51.0 (C-49), 50.6 (C-52), 48.6 (C-17), 47.5 (C-9, C-33, C-34), 42.1 (C-14), 39.8 (C-19), 39.4 (C-8), 38.7 (C-20), 34.4 (C-22), 32.9 (C-7), 30.4 (C-21), 28.1 (C-15), 28.1 (C-24), 27.7 (C-51), 27.7 (C-51), 23.5 (C-2, 16, 27), 23.3 (C-11), 21.3 (C-30), 21.2 (C-32), 20.6 (C-50), 19.9 (C-54), 19.7 (C-53), 18.2 (C-6), 17.4 (C-29), 16.9 (C-26), 16.7 (C-23), 15.5 (C-25) ppm; MS (ESI, MeOH): m/z = 1039.4 ($[M - Cl]^+$, 100%); analysis calculated for $C_{68}H_{87}N_4O_5Cl$ (1075.64): C 75.91, H 8.15, N 5.21; found: C 75.64, H 8.29, N 4.96.

4.27. 3β-Acetyloxy-28-[4-[3-(2,3,6,7,12,13,16,17-octahydro-1H,5H,11H,15H-pyrido[3,2,1-ij]pyrido[1″,2″,3″:1′,8′]quinolino[6′,5′:5,6]pyrano[2,3-f]quinolin-4-ium-9-yl)benzoyl]piperazine-1-yl]-20,28-dioxo-30-norlupan-12-en chloride (**24**)

Following GPC from **8** (0.25 g, 0.44 mmol) and rhodamine 101 (0.25 g, 0.51 mmol), **24** (0.30 g, 65%) was obtained as a pink solid; R_f = 0.4 (SiO_2, $CHCl_3$/MeOH, 9:1); m.p. >300 °C; IR (ATR): ν = 3396w, 2940w, 2863w, 1727w, 1627m, 1594s, 1543w, 1493s, 1459m, 1446m, 1439m, 1419m, 1361m, 1295Vs, 1267s, 1195s, 1182s, 1099s, 1035m, 1004m, 979m, 897w, 863w, 773m, 732m, 561m, 505m, 420s cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.70–7.67 (m, 2H, 37-H, 39-H), 7.53–7.49 (m, 1H, 38-H), 7.33–7.30 (m, 1H, 40-H), 6.66 (d, *J* = 6.8 Hz, 2H, 48-H), 4.44 (dd, *J* = 10.9, 5.2 Hz, 1H, 3-H), 3.62–3.51 (m, 4H, 49-H), 3.51–3.43 (m, 4H, 52-H), 3.43–3.29 (m, 8H, 33-H + 34-H), 3.15 (td, *J* = 11.1, 3.1 Hz, 1H, 19-H), 3.06–2.98 (m, 4H, 54-H), 2.78–2.63 (m, 4H, 51-H), 2.58 (td, *J* = 12.1, 3.8 Hz, 1H, 13-H), 2.14 (s, 3H, 29-H), 2.13–2.08 (m, 4H, 53-H), 2.08–2.04 (m, 1H, 18-H), 2.02 (s, 3H, 32-H), 1.97 (q, *J* = 6.9 Hz, 5H, 16-H_a + 50-H), 1.88–1.80 (m, 2H, 21-H_a + 22-H_a), 1.67–1.54 (m, 4H, 1-H_a + 2-H + 16-H_b), 1.54–0.98 (m, 14H, 6-H_a + 6-H_b + 7-H + 9-H + 11-H_a + 11-H_b + 12-H + 15-H + 21-H_b + 22-H_b), 0.95 (s, 4H, 1-H_b + 27-H), 0.87 (s, 3H, 26-H), 0.82 (s, 3H, 24-H), 0.82 (s, 3H, 25-H), 0.81 (s, 3H, 23-H), 0.79–0.75 (m, 1H, 5-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) δ = 212.6 (C-20), 173.9 (C-28), 170.9 (C-31), 167.8 (C-35), 152.8 (C-43), 152.0 (C-44), 152.0 (C-44), 151.3 (C-46), 151.2 (C-46), 134.7 (C-41), 131.9 (C-36), 130.8 (C-40), 130.3 (C-39), 129.7 (C-37), 127.4 (C-38), 126.5 (C-48), 123.6 (C-45), 123.5 (C-45), 113.2 (C-42), 105.5 (C-47), 55.4 (C-5), 54.5 (C-17), 52.5 (C-18), 51.0 (C-49), 51.0 (C-49), 50.6 (C-52), 50.6 (C-52), 50.5 (C-9), 50.0 (C-19), 41.7 (C-14), 40.5 (C-8), 38.3 (C-1), 37.8 (C-4), 37.1 (C-10), 35.9 (C-13), 35.6 (C-22), 34.2 (C-7), 32.0 (C-16), 30.2 (C-29), 29.8 (C-15), 28.7 (C-21), 27.9 (C-24), 27.7 (C-51), 27.4 (C-12), 23.6 (C-2), 21.3 (C-32), 21.1 (C-11), 20.6 (C-50), 19.9 (C-54), 19.7 (C-53), 18.1 (C-6), 16.5 (C-23), 16.2 (C-25), 16.0 (C-26), 14.6 (C-27) ppm; MS (ESI, MeOH): m/z = 1041.3 ($[M - Cl]^+$, 100%); analysis calculated for $C_{67}H_{85}N_4O_6Cl$ (1077.89): C 74.66, H 7.95, N 5.20; found: C 74.50, H 8.14, N 5.03.

4.28. 3β-Acetyloxy-28-[4-[3-(2,3,6,7,12,13,16,17-octahydro-1H,5H,11H,15H-pyrido[3,2,1-ij]pyrido[1″,2″,3″:1′,8′]quinolino[6′,5′:5,6]pyrano[2,3-f]quinolin-4-ium-9-yl)benzoyl]homopiperazine-1-yl]-28-oxo-lup-20(29)-en chloride (**25**)

Following GPC from **9** (0.35 g, 0.60 mmol) and rhodamine 101 (0.25 g, 0.51 mmol), **25** (0.37 g, 68%) was obtained as a pink solid; R_f = 0.4 (SiO_2, $CHCl_3$/MeOH, 9:1); m.p. >300 °C; IR (ATR): ν = 2940w, 2865w, 1730w, 1624m, 1595s, 1542w, 1493s, 1459m, 1376m, 1361m, 1294vs, 1268s, 1247m, 1196s, 1180s, 1098s, 1035m, 1018m, 978m, 895w, 772w, 747m, 622m, 421s cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.63–7.51 (m, 2H, 37-H, 39-H), 7.41 (t, *J* = 9.2 Hz, 1H, 38-H), 7.21 (d, *J* = 7.4 Hz, 1H, 40-H), 6.68 (dd, *J* = 26.0, 20.3 Hz, 2H, 48-H), 4.70 (d, *J* = 18.2 Hz, 1H, 29-H_a), 4.55 (d, *J* = 17.7 Hz, 1H, 29-H_b), 4.45–4.38 (m, 1H, 3-H), 3.85–3.62 (m, 4H, 33-H), 3.55–3.45 (m, 8H, 49-H + 52-H), 3.44–3.24 (m, 4H, 34-H), 3.08–2.91 (m, 5H, 19-H + 54-H), 2.89–2.80 (m, 1H, 13-H), 2.78–2.59 (m, 4H), 2.07 (s, 7H, 16-H_a + 53-H + 55-H), 1.99 (s, 3H, 32-H), 1.93 (s, 4H, 50-H), 1.83 (d, *J* = 4.6 Hz, 2H, 21-H_a + 22-H_a), 1.66 (s, 2H, 12-H), 1.62 (s, 3H, 30-H), 1.55 (dd, *J* = 22.6, 11.1 Hz, 3H, 1-H + 2-H), 1.50–1.40 (m, 3H, 6-H_a + 16-H_b + 18-H), 1.40–1.30 (m, 5H, 7-H + 11-H_a + 21-H_b + 22-H_b), 1.30–1.04 (m, 5H, 6-H_b + 9-H + 11-H_b + 15-H_a + 15-H_b), 0.93–0.89 (m, 5H, 1-H + 12-H + 27-H), 0.88 (s, 3H, 26-H),

0.82–0.78 (m, 9H, 23-H + 24-H + 25-H), 0.77–0.72 (m, 1H, 5-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 175.5 (C-28), 170.9 (C-31), 168.9 (C-35), 152.8, 152.0 (C-43), 151.9 (C-43), 151.3 (C-44), 151.3 (C-44), 151.2 (C-20), 151.1 (C-46), 136.0 (C-41), 131.1 (C-36), 130.5 (C-40), 129.8 (C-39), 129.5 (C-37), 127.6 (C-38), 126.6 (C-48), 126.4 (C-48), 123.4 (C-45), 123.4 (C-45), 112.9 (C-42), 109.4 (C-29), 105.4 (C-47), 105.3 (C-47), 80.9 (C-3), 55.5 (C-5), 54.8 (C-17), 52.7 (C-18), 51.0 (C-49), 50.5 (C-52), 46.1 (C-33 + 34), 45.9 (C-19), 41.9 (C-14), 40.7 (C-8), 38.4 (C-1), 37.8 (C-4), 37.1 (C-10), 36.8 (C-13), 36.0 (C-22), 34.3 (C-7), 32.3 (C-16), 31.5 (C-21), 29.9 (C-15), 28.9 (C-55), 27.9 (C-24), 27.5 (C-51), 25.5 (C-12), 23.6 (C-2), 21.3 (C-32), 21.1 (C-11), 20.6 (C-50), 19.9 (C-54), 19.7 (C-53), 19.5 (C-30), 18.2 (C-6), 16.4 (C-23), 16.2 (C-25), 16.1 (C-26), 14.6 (C-27) ppm; MS (ESI, MeOH): *m*/*z* = 1052.9 ([M − Cl]$^+$, 100%); analysis calculated for $C_{69}H_{89}N_4O_5Cl$ (1089.94): C 76.04, H 8.23, N 5.14; found: C 75.76, H 8.31, N 5.02.

4.29. 3β-Acetyloxy-28-[4-[3-(2,3,6,7,12,13,16,17-octahydro-1H,5H,11H,15H-pyrido[3,2,1-ij] pyrido[1′′,2′′,3′′:1′,8′]quinolino[6′,5′:5,6]pyrano[2,3-f]quinolin-4-ium-9-yl)benzoyl]homopiperazine-1-yl]-28-oxo-olean-12-en chloride (**26**)

Following GPC from **10** (0.25 g, 0.60 mmol) and rhodamine 101 (0.25 g, 0.51 mmol), **26** (0.38 g, 70%) was obtained as a pink solid; R_f = 0.4 (SiO_2, $CHCl_3$/MeOH, 9:1); m.p. > 300 °C; IR (ATR): ν = 3369vw, 2942w, 2863w, 1729w, 1623m, 1595s, 1543w, 1493s, 1459m, 1361m, 1294vs, 1268s, 1246s, 1195s, 1180s, 1150m, 1098s, 1035m, 985m, 896w, 862w, 771m, 746m, 622m, 575w, 560w, 498m, 421s cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.63–7.58 (m, 2H, 37-H + 39-H), 7.55–7.50 (m, 1H, 38-H), 7.23–7.17 (m, 1H, 40-H), 6.72–6.60 (m, 2H, 48-H), 5.25–5.21 (m, 1H, 12-H), 4.45 (t, *J* = 7.8 Hz, 1H, 3-H), 3.95–3.60 (m, 4H, 33-H + 34-H), 3.58–3.43 (m, 8H, 49-H + 52-H), 3.41–3.11 (m, 4H, 34-H), 3.06 (s, 1H, 18-H), 3.03–2.95 (m, 4H, 54-H), 2.74–2.61 (m, 4H, 51-H), 2.14–2.02 (m, 7H, 16-H_a + 53-H + 55-H), 2.00 (s, 3H, 32-H), 1.94 (dq, *J* = 11.1, 5.3 Hz, 4H, 50-H), 1.88–1.79 (m, 2H + 11-H), 1.71–1.41 (m, 10H, 1-H_a + 2-H + 6-H_a + 7-H + 9-H + 15-H_a + 16-H_b + 19-H_a), 1.41–1.26 (m, 3H, 6-H_b, 21 + 22-H_a), 1.26–1.12 (m, 3H, 19-H_b + 22-H_b), 1.09 (s, 3H, 27-H), 1.06–0.96 (m, 2H, 1-H_b + 15-H_b), 0.94 (s, 3H, 29-H), 0.88 (d, *J* = 2.0 Hz, 6H, 25-H + 30-H), 0.82 (s, 3H, 24-H), 0.81 (s, 3H, 23-H), 0.79–0.76 (m, 1H, 5-H), 0.66 (s, 3H, 26-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 176.1 (C-28), 171.0 (C-31), 169.0 (C-35), 152.8 (C-43), 152.0 (C-44), 151.9 (C-44), 151.3 (C-46), 151.2 (C-46), 144.8 (C-13), 136.0 (C-41), 130.4 (C-40), 129.9 (C-39), 129.5 (C-37), 127.5 (C-36), 126.5 (C-48), 126.2 (C-48), 123.6 (C-45), 123.5 (C-45), 121.4 (C-12), 113.3 (C-42), 105.5 (C-47), 105.4 (C-47), 80.9 (C-3), 55.3, 51.0 (C-49), 50.5 (C-52), 47.7 (C-17), 47.6 (C-9), 47.6 (C-33 + 34), 46.6 (C-19), 43.5 (C-18), 42.0 (C-14), 39.0 (C-8), 38.0 (C-1), 37.7 (C-4), 37.0 (C-10), 34.0 (C-21), 32.9 (C-30), 32.7 (C-22), 30.5 (C-7), 30.3 (C-20, C-55), 28.0 (C-24), 27.8 (C-15), 27.6 (C-51), 25.9 (C-27), 24.2 (C-29), 23.5 (C-2), 23.3 (C-11), 22.5 (C-16), 21.3 (C-32), 20.6 (C-50), 19.9 (C-54), 19.7 (C-53), 19.6 (C-53), 18.2 (C-6), 17.0 (C-26), 16.6 (C-23), 15.4 (C-25) ppm; MS (ESI, MeOH): *m*/*z* = 1053.0 (100%, [M − Cl]$^+$); analysis calculated for $C_{69}H_{89}N_4O_5Cl$ (1089.94): C 76.04, H 8.23, N 5.14; found: C 75.81, H 8.40, N 4.86.

4.30. 3β-Acetyloxy-28-[4-[3-(2,3,6,7,12,13,16,17-octahydro-1H,5H,11H,15H-pyrido[3,2,1-ij]pyrido[1′′,2′′,3′′:1′,8′]quinolino[6′,5′:5,6]pyrano[2,3-f]quinolin-4-ium-9-yl)benzoyl]homopiperazine-1-yl]-28-oxo-urs-12-en chloride (**27**)

Following GPC from **11** (0.35 g, 0.60 mmol) and rhodamine 101 (0.25 g, 0.51 mmol), **27** (0.39 g, 72%) was obtained as a pink solid; R_f = 0.4 (SiO_2, $CHCl_3$/MeOH, 9:1); m.p. >300 °C; IR (ATR): ν = 2934w, 2866w, 1730w, 1622w, 1594w, 1543w, 1493w, 1459w, 1435w, 1376w, 1361w, 1293w, 1268w, 1246w, 1195w, 1180w, 1143w, 1098w, 1035w, 985w, 966w, 941w, 896w, 862w, 772w, 744w, 715w, 661w, 622w, 574w, 560w, 496w cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.59 (dd, *J* = 5.7, 3.3 Hz, 2H, 37-H + 39-H), 7.43–7.34 (m, 1H, 38-H), 7.22 (dd, *J* = 5.7, 3.3 Hz, 1H, 40-H), 6.61 (d, *J* = 24.5 Hz, 2H, 48-H), 5.18–5.11 (m, 1H-H), 4.44 (dd, *J* = 11.0, 5.2 Hz, 1H, 3-H), 4.08–3.66 (m, 4H, 34-H), 3.59–3.41 (m, 8H, 49-H + 52-H), 3.38–3.07 (m, 4H, 33-H), 3.02–2.93 (m, 4H, 54-H), 2.67 (h, *J* = 9.5, 8.8 Hz, 4H, 51-H), 2.45–2.34 (m, 1H, 18-H), 2.11–2.03 (m, 4H,55-H + 53-H), 2.00 (s, 3H, 32-H), 1.96–1.81 (m, 6H, 11-H + 50-H),

1.79–1.52 (m, 7H, 1-H_a + 2-H + 16-H + 22-H), 1.44 (dt, *J* = 14.3, 7.8 Hz, 4H, 6-H_a + 7-H_a + 9-H + 21-H_a), 1.37–1.11 (m, 4H, 6-H_b + 7-H_b + 19-H + 21-H_b), 1.01 (d, *J* = 11.5 Hz, 6H, 1-H_b + 15-H + 27-H), 0.92 (s, 1H, 20-H), 0.88 (s, 6H, 25-H + 30-H), 0.81 (s, 6H, 24-H + 29-H), 0.80 (s, 3H, 23-H), 0.75 (s, 1H, 5-H), 0.66 (s, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 176.4 (C-28), 170.9 (C-31), 168.1 (C-35), 152.9 (C-46), 151.9 (C-43), 151.3 (C-44), 138.7 (C-13), 135.7 (C-41), 131.4 (C-36), 130.4 (C-40), 129.8, 129.6 (C-37), 129.5 (C-39), 127.5 (C-38), 126.8 (C-48), 125.0 (C-12), 123.7 (C-45), 113.3 (C-42), 105.3 (C-47), 105.1 (C-47), 80.9 (C-3), 55.3 (C-5, 18), 51.0 (C-49), 50.5 (C-52), 48.8 (C-17), 47.5 (C-9, 33, 34), 42.8 (C-14), 39.3 (C-8, 19), 38.7 (C-20), 38.2 (C-1), 37.6 (C-4), 36.9 (C-10), 34.4 (C-22), 32.9 (C-7), 30.4 (C-21), 30.3 (C-55), 28.0 (C-15, 24), 27.6 (C-51), 27.5 (C-51), 23.5 (C-2, 16, 27), 23.2 (C-11), 21.3 (C-30), 21.2 (C-32), 20.6 (C-50), 20.6 (C-50), 19.9 (C-54), 19.7 (C-53), 19.6 (C-53), 18.1, 17.4 (C-29), 17.0 (C-26), 16.7 (C-23), 15.5 (C-25) ppm; MS (ESI, MeOH): *m*/*z* = 1053.1 (100%, $[M - Cl]^+$); analysis calculated for $C_{69}H_{89}N_4O_5Cl$ (1089.94): C 76.04, H 8.23, N 5.14; found: C 75.76, H 8.51, N 4.97.

4.31. 3β-Acetyloxy-28-[4-[3-(2,3,6,7,12,13,16,17-octahydro-1H,5H,11H,15H-pyrido[3,2,1-ij]pyrido[1′′,2′′,3′′:1′,8′]quinolino[6′,5′:5,6]pyrano [2,3-f]quinolin-4-ium-9 -yl)benzoyl]homopiperazine-1-yl]-20,28-dioxo-30-norlupan-12-en chloride (**28**)

Following GPC from **12** (0.35 g, 0.60 mmol) and rhodamine 101 (0.25 g, 0.51 mmol), **28** (0.35 g, 66%) was obtained as a pink solid; R_f = 0.4 (SiO_2, $CHCl_3$/MeOH, 9:1); m.p. > 300 °C; IR (ATR): ν = 3383vw, 2940w, 2863w, 1731w, 1623m, 1594s, 1542w, 1493s, 1459m, 1436m, 1376m, 1361m, 1294vs, 1268s, 1247m, 1195s, 1180s, 1143m, 1099s, 1075m, 1035m, 1018m, 978w, 897w, 862w, 746m, 623w, 561m, 506m, 421s cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.63–7.57 (m, 2H, 37-H + 39-H), 7.41 (d, *J* = 23.5 Hz, 1H, 38-H), 7.29–7.21 (m, 1H, 40-H), 6.67 (dd, *J* = 27.4, 18.9 Hz, 2H, 48-H), 4.42 (dt, *J* = 10.6, 5.4 Hz, 1H, 3-H), 3.93–3.58 (m, 4H, 33-H), 3.58–3.40 (m, 8H, 49-H, 52-H), 3.40–3.08 (m, 5H, 19-H, 34-H), 3.06–2.91 (m, 4H, 54-H), 2.70 (d, *J* = 27.3 Hz, 5H, 13-H + 51-H), 2.12 (s, 3H, 29-H), 2.10–2.02 (m, 7H, 16-H_a + 53-H + 55-H), 2.00 (s, 4H, 18-H + 32-H), 1.98–1.88 (m, 5H, 22-H_a + 50-H), 1.87–1.80 (m, 1H, 21-H_a), 1.66–1.52 (m, 4H, 1-H + 16-H_b), 1.45 (q, *J* = 16.9, 12.1 Hz, 3H, 6-H_a + 21-H_b + 22-H_b), 1.39–1.30 (m, 5H, 6-H_b + 7-H + 11-H_a + 12-H_a), 1.29–1.19 (m, 4H, 9-H + 11-H_b + 15-H), 0.95–0.93 (m, 2H, 1-H + 12-H_b), 0.91 (s, 6H, 26-H + 27-H), 0.83–0.78 (m, 9H, 23-H + 24-H + 25-H), 0.76–0.72 (m, 1H, 5-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 213.0 (C-20), 174.5 (C-28), 170.9 (C-31), 168.9 (C-35), 152.6 (C-43), 151.9 (C-44), 151.3 (C-46), 136.1 (C-41), 131.1 (C-36), 130.5 (C-40), 129.8 (C-39), 129.5 (C-37), 127.5 (C-38), 126.7 (C-48), 126.2 (C-48), 123.7 (C-45), 123.5 (C-45), 112.9 (C-42), 105.3 (C-47), 80.9 (C-3), 55.5 (C-5), 54.9 (C-17), 52.8 (C-18), 51.0 (C-49), 50.6 (C-9), 50.5 (C-52), 50.2 (C-19), 48.0 (C-33 + 34), 41.8 (C-14), 40.6 (C-8), 38.4 (C-1), 37.8 (C-4), 37.1 (C-10), 36.0 (C-13), 35.6 (C-22), 34.2 (C-7), 31.9 (C-16), 30.2 (C-29), 29.9 (C-15), 29.6 (C-55), 28.9 (C-21), 27.9 (C-24), 27.6 (C-51), 27.6 (C-51), 27.3 (C-12), 23.6 (C-2), 21.3 (C-32), 21.2 (C-11), 20.6 (C-50), 19.9 (C-54), 19.7 (C-53), 19.6 (C-53), 18.2 (C-6), 16.5 (C-23), 16.2 (C-25), 16.1 (C-26), 14.7 (C-27) ppm; MS (ESI, MeOH): *m*/*z* = 1055.0 (100%, $[M - Cl]^+$); analysis calcd for $C_{68}H_{87}N_4O_6Cl$ (1091.92): C 74.80, H 8.03, N 5.13; found: C 74.61, H 8.27, N 4.95.

4.32. Cytotoxicity Assay (SRB)

The cell lines were obtained from Department of Oncology (Martin-Luther-University Halle Wittenberg; they were bought from ATCC; A 375 (CRL-1619), HT29 (HTB-38), MCF7 (HTM-22), A2780 (HTP-77), FaDu (HTP-43), NIH 3T3 (CRL-1658), HEK-293 (CRL-1573)). Cultures were maintained as monolayers in RPMI 1640 medium with L-glutamine (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) supplemented with 10% heat inactivated fetal bovine serum (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and penicillin/streptomycin (1%, Capricorn Scientific GmbH, Ebsdorfergrund, Germany) at 37 °C in a humidified atmosphere with 5% CO_2. The cytotoxicity of the compounds was evaluated using the sulforhodamine-B (Kiton-Red S, ABCR) micro culture colorimetric assay using confluent cells in 96-well plates with the seeding of the cells on day 0 applying appropriate cell densities to prevent confluence of the cells during the period of the experiment. On day 1, the cells were treated with six different concentrations (1, 3, 7, 12, 20, and 30 μM);

thereby, the final concentration of DMSO was always <0.5%, generally regarded as non-toxic to the cells. On day 4, the supernatant medium was discarded; the cells were fixed with 10% trichloroacetic acid. After another day at 4 °C, the cells were washed in a strip washer and dyed with the SRB solution (100 µL, 0.4% in 1% acetic acid) for about 20 min to be followed by washing of the plates (four times, 1% acetic acid) and air-drying overnight. Furthermore, tris base solution (200 µL, 10 mM) was added to each well and absorbance was measured at λ = 570 nm employing a reader (96 wells, Tecan Spectra, Crailsheim, Germany). The EC_{50} values were averaged from three independent experiments performed each in triplicate calculated from semi logarithmic dose response curves applying a non-linear four-parameter Hills-slope equation (GraphPad Prism5; variables top and bottom were set to 100 and 0, respectively).

4.33. Acridine Orange (AO) Staining

On the first day, A375 cells were counted and seeded 1×10^5 in a Petri dish (diameter 4 cm) with coverslips (22 mm × 22 mm) in 2 mL medium. After 24 h, the medium was removed, and treatment was performed with 2 mL of new medium (control) and 2 mL each of 2 times the EC_{50} concentration of compounds **27**. After 24 h, the medium was removed from the samples, the coverslip was rinsed with 1 mL of PBS (with Ca^{2+} and Mg^{2+}), placed on a slide containing 20 µL of AO solution (2.5 µg/mL in PBS), and measured directly on the fluorescence microscope.

4.34. Hoechst 33,3342 and Rhodamine 123 Staining

On day 1, A375 cells were counted and seeded 1×10^5 in a Petri dish (diameter 4 cm) with coverslips (22 mm × 22 mm) in 2 mL medium. After 24 h, the medium was removed, and treatment was performed with 1 mL of new medium containing 1 µL of compound **27** (0.08 mM solution). After another 24 h, additional treatment was performed with 1 µL rhodamine (1 mg/mL in EtOH) and 2 µL Hoechst 33342 (100 µg/mL in DMSO) for (at least) 30 min. The medium was then removed, rinsed once with PBS (with Ca^{2+} and Mg^{2+}), placed on a slide containing 20 µL PBS (with Ca^{2+} and Mg^{2+}), and measured directly on the fluorescence microscope.

Author Contributions: Conceptualization, R.C.; validation, R.C., N.V.H. and M.K.; investigation, N.V.H., S.H. and M.K.; writing—original draft preparation, R.C.; writing—review and editing, N.V.H., D.M., S.H., M.K., I.S. and R.C. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the compounds are available from the authors.

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Mitochondria-Targeting 1,5-Diazacyclooctane-Spacered Triterpene Rhodamine Conjugates Exhibit Cytotoxicity at Sub-Nanomolar Concentration against Breast Cancer Cells

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Abstract: 1,5-Diazacyclooctane was prepared by a simple synthetic sequence and coupled to pentacyclic triterpenoic acids oleanolic acid, ursolic acid, betulinic acid, platanic acid and asiatic acid; these amides were activated with oxalyl chloride and reacted with rhodamine B or rhodamine 101 to yield conjugates. The conjugates were screened in SRB assays with various human breast cancer cell lines (MDA-MB-231, HS578T, MCF-7 andT47D) and found to exert cytotoxic activity even at low concentration. Thereby for an asiatic acid rhodamine 101 conjugate (28) an IC_{50} = 0.60 nM was determined, and found to induce apoptosis in MDA-MB-231 and HS578T cells. Extra experiments showed the compound to act as a mitocan and to induce inhibition of proliferation or growth arrest in MDA-MB-231 cells at lower doses followed by an induction of apoptosis at higher doses. Furthermore, differential responses to proliferation inhibition and apoptosis induction may explain differential sensitivity of mammary cell lines to compound 28.

Keywords: asiatic acid; breast cancer; mitocans; rhodamine conjugates; triterpenoic acids

1. Introduction

Breast cancer is the most common type of tumor disease and despite recent advances in cancer therapy it remains the leading cause of tumor-related death in women [1–8]. While traditional treatments like surgery, chemotherapy, radiation and hormone therapy are effective [9], they often cause severe side effects and may not be suitable for all patients. Therefore, there is a need to develop new and effective treatment options. One highly promising approach is the use of natural product derived compounds as anticancer agents, especially pentacyclic triterpenoids have emerged as a class of phytochemicals with potential anticancer activity. Several studies have demonstrated their ability to cause apoptosis, reduce clonogenic survival, migration, and enhance radiosensitivity of human breast cancer cells [10–13]. These effects have been attributed to their ability to modulate various signaling pathways involved in cancer progression.

Pentacyclic triterpenoic acids linked with lipophilic cations like rhodamines [13–27] are known to act as mitocans even at low nanomolar concentrations by inhibiting their synthesis of ATP [21]. In this context, the mitochondrial targeting function of rhodamine seems particularly worth mentioning [28–30]. Thereby, the use of an amine spacer is crucial for enhancing their cytotoxicity, whereby secondary amines are favored over primary

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amines to prevent lactamization and maintain their cationic structures. Furthermore, incorporating a homopiperazinyl spacer leads to more cytotoxic compounds than those analogues with a piperazinyl spacer. Therefore, we have been interested in the use of a 1,5-diazacyclooctane spacer and its influence on the cytotoxicity of different pentacyclic triterpenoic acid conjugates of rhodamine B and rhodamine 101.

2. Results

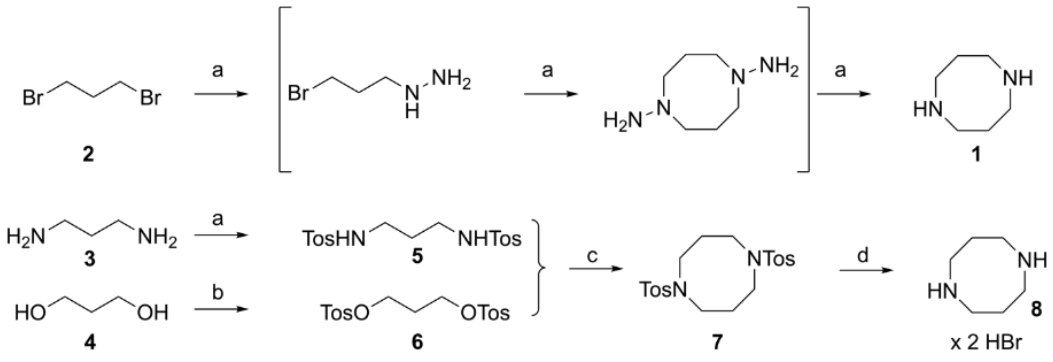
Since the first preparation of octahydro-1,5-diazocine (**1**, 1,5-diazacyclooctane, a "bis-homo-piperazine", Scheme 1) in 1939 by W. L. C. Veer [31] several routes have been suggested to this compound, among them the ring cleavage reaction of 1,5-diaminobicyclo[3.3.0]octane, the condensation of propane-1,3-diamine with 1,3-dibromopropane, and the silica supported intramolecular cyclization of propane-1,3-diamine at 350 °C [32–43].

As an alternative, one could also imagine the reduction of the bis-lactam 1,5-diazocane-2,6-dione; the latter compound is accessible either via Staudinger ring closure reactions, Beckmann and Schmidt rearrangements, however, usually under very drastic conditions (e.g., fuming sulfuric acid) [44–48]. All these routes are not very suitable, since their mostly drastic conditions make the preparation of larger amounts on a laboratory scale quite difficult.

Special attention therefore deserves the only recently proposed [49] route starting from propane-1,3-diamine and propane-1,3-diol, two starting materials that are available in larger quantities and commercially cheap. In this process, both starting materials are first tosylated and then condensed by a double nucleophilic substitution. An alternative is the reaction of 1,3-dibromopropane (**2**) with hydrazine. This route would have the advantage of yielding the desired product in a one-pot procedure. However, it very quickly became apparent that many by-products were formed in this reaction, so that the maximum yield of pure **1** was 7.5% only. Working with larger quantities of hydrazine poses an additional risk.

However, the published synthesis using propane-1,3-diamine (**3**) and propane-1,3-diol (**4**) could not be reproduced in terms of the yields obtained either, so we decided to optimize this synthetic route on our own.

Thus, propane-1,3-diamine (**3**) was tosylated (Scheme 1) to yield **5** in 83% yield, while the tosylation of propane-1,3-diol (3) gave 87% of the di-tosylate **6**. These two compounds were condensed in the presence of sodium methoxide (which proved to result in higher yields than using sodium ethoxide) to afford 84% of **7**. De-tosylation was performed with hydrobromic acid in the presence of thioanisole and the desired octahydro-1,5-diazocine was obtained as di-hydrobromide (**8**) in 92% isolated yield.



Scheme 1. Synthesis of octahydro-1,5-diazocine (**1**) and its dihydrobromide (**8**): Reactions and conditions: a) NH_2-NH_2, EtOH, reflux, 4 h; then HBr, benzaldehyde, 7.5%; b) TosCl, no solvent, 80 °C, 30 min, 83%; c) TosCl, pyridine, 0 °C, 30 min, 87%; d) NaOMe, MeOH, DMF, 80 °C, 12 h, 84%; e) HBr (33% glacial AcOH), 80 °C, 3 h, 92%.

The starting materials for the preparation of the spacered rhodamine conjugates were the triterpene carboxylic acids oleanolic acid (OA, Figure 1), ursolic acid (UA), and the lupanes betulinic acid (BA) and platanic acid (PA); in previous works, asiatic acid (AA) had been shown to be particularly suitable with respect to cytotoxic activity and was therefore included in this study as a model featuring a tri-hydroxylated triterpene carboxylic acid [21].

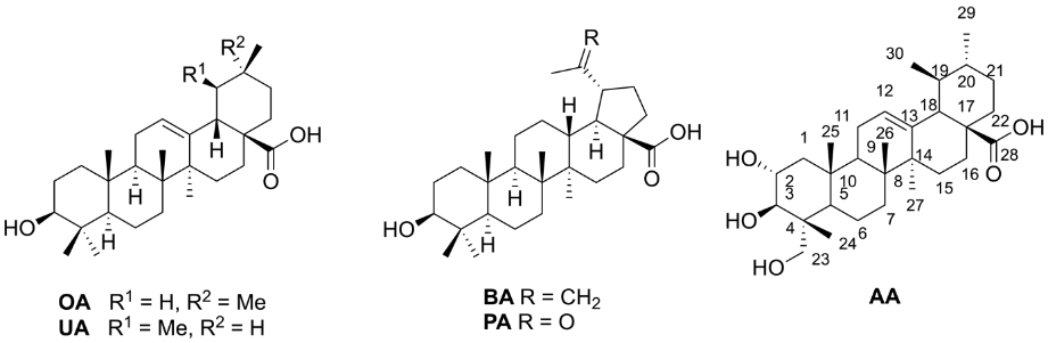
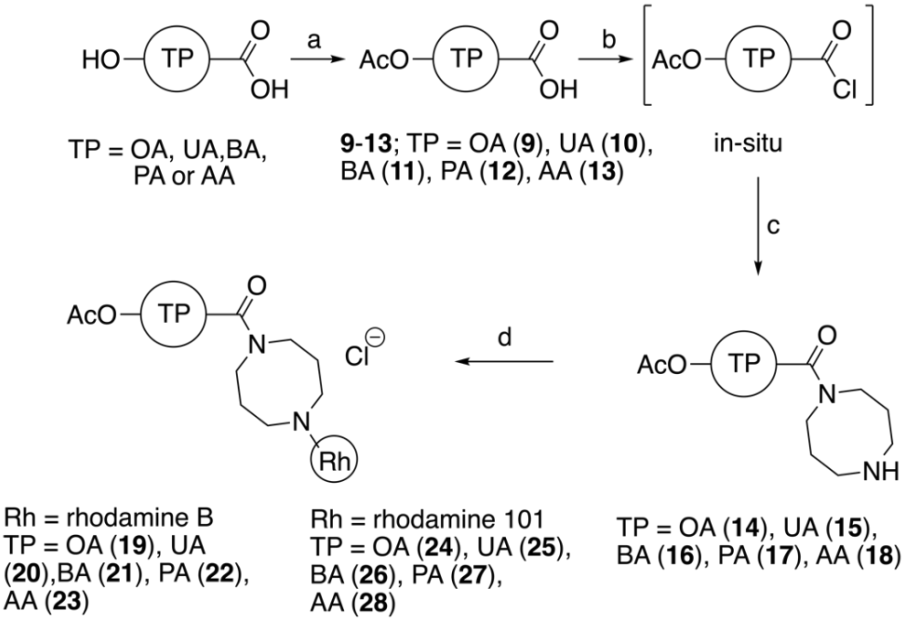
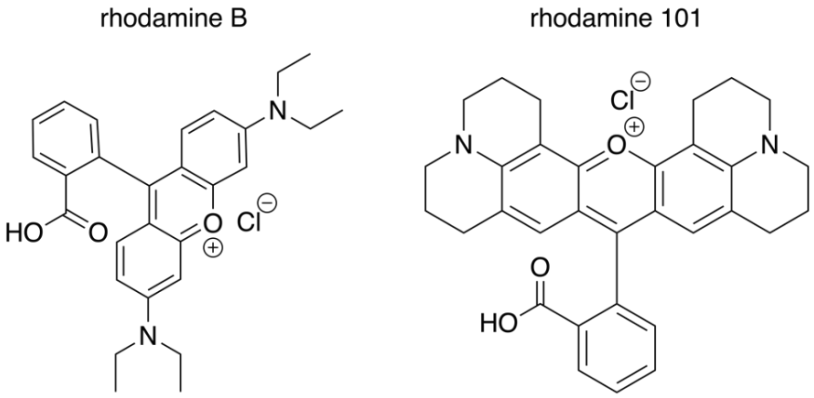


Figure 1. Structure of triterpenoic acid oleanolic acid (OA), ursolic acid (UA), betulinic acid (BA), platanic acid (PA) and asiatic acid (AA); for the latter a numbering scheme is depicted.

The triterpenoic acids were acetylated to yield the acetates 9–13 (Scheme 2). Rhodamine B and rhodamine 101 were chosen as representative examples of rhodamines. The former compound has been shown in previous studies to be an essential component of mitocan-acting triterpene carboxylic acid amide conjugates, the latter differs from the former in having a somewhat higher lipophilicity (consensus log $P_{o/w}$ 2.21 and 3.96, respectively; from www.swiss.adme.ch), which we consider advantageous for possible interactions with biological membranes. Thus, reaction of acetates 9–13 with oxalyl chloride followed by the addition of 8 furnished amides 14–18. Rhodamine B and rhodamine 101 were transformed with oxalyl chloride *in situ* into their corresponding acid chlorides that were reacted with amides 14–18 to yield rhodamine B derived conjugates 19–23 and rhodamine 101 derived hybrids 24–28.

Compounds 14–28 were screened in sulforhodamine B assays employing breast cancer cell lines MDA-MB-231, HS578T, MCF-7 and T47D (Table 1). Breast cancer could be distinguished in different molecular subtypes: luminal-like (luminal A or B), HER2-enriched and basal-like, which differ in biology, treatment response, patients' survival, and clinical outcome. These subtypes are also found in cell lines and our investigated breast cancer cell lines have been characterized before. Breast cancer cell lines MDA-MB-231 and HS578T are basal and so-called triple negative, which means neither estrogen receptor (ER) and progesterone receptor (PR) nor human epidermal growth factor receptor 2 (HER2) are expressed. Basal breast cancers are mostly high-grade tumors and no therapeutic targeted therapy can be applied, thus resulting in poor prognosis for patients although they are relatively sensitive for chemotherapy. MCF-7 and T47D breast cancer cells are luminal A and positive for ER and PR. Breast cancers with this type are often low-grade tumors, which are characterized by chemotherapy resistance, but hold good response to hormone therapy resulting in better clinical outcome compared to basal breast cancers.





Scheme 2. Synthesis of the rhodamine B and rhodamine 101 conjugates; reactions and conditions: a) Ac_2O, DCM, NEt_3, DMAP (cat.), 21 °C, 24h; b) $(COCl)_2$, DCM, DMF (cat.), in-situ; c) DCM, **8**, NEt_3, DMAP (cat.), 20 °C, 1h; d) $(COCl)_2$, DCM, DMF (cat.), then rhodamine B or rhodamine 101, 20 °C, 1h.

As a result, amides of triterpenoic acids 14–18 (Table 1) show cytotoxicity at low micromolar range for all investigated breast cancer cell lines. IC_{50} values about 0.5–50 μM were determined. As expected, conjugation of rhodamine B (compounds 19-23) or rhodamine 101 (compounds 24–28) led to increased cytotoxicity (in nanomolar range) in all breast cancer cell lines (Table 2). In investigated breast cancer cell lines IC_{50} values of all homopiperazinyl-spacered rhodamine B derivatives are in a range between 25 nM and 350 nM, and for rhodamine 101 conjugates in a range between 0.6 nM and 1.3 μM. An asiatic acid derivatized rhodamine 101 amide (compound 28) is the most cytotoxic conjugate in all screened breast cancer cells. The IC_{50} values are a in a low nanomolar range (0.6–126 nM). Comparing breast cancer cell lines, HS578T cell line is the most resistant cell line for rhodamine B or rhodamine 101 conjugates (IC_{50} between 215 nM and 356 nM and between 126 nM and 1.3 μM). Our previous work showed that compounds of this class are also highly able to discriminate between malignant and non-malignant cells [13, 23] and affect the mitochondrial ATP synthesis [23]. Future studies will also investigate whether changes in the expression of programmed death ligand-1 (PD-L1) can be observed [50].

In addition to studying the cytotoxicity of 28 in above mentioned cell lines, we investigated its ability to overcome resistance. While the IC_{50} of 28 in A2780 cells was 0.72 nM,

the resistant A2780cis cells exhibited an IC_{50} of 1.82 nM. Although complete resistance reversal was not achieved, the results highlight the promising potential to partially overcome resistance. We also assessed its selectivity by comparing the cytotoxicity in non-malignant fibroblasts CCD18Co. The IC_{50} value of 28 in CCD18Co cells was 503.2 nM, which was approximately 800-fold higher than the IC_{50} value observed in the MDA-MB-231 cells.

The most cytotoxic compound 28 was used for further investigations of proliferation and cell death in sensitive MDA-MB-231 and resistant HS578T breast cancer cells. In MDA-MB-231 cells compound 28 caused a strong inhibition of proliferation (under 20% compared to control cells) after treatment with at least 250 nM (Figure 2). However, in HS578T cells treatment with 250 nM of compound 28 resulted in a less decrease of proliferation about 50%, but with 500 nM compound 28 cell number was reduced up to 20% compared to control cells (Figure 2).

Table 1. Cytotoxicity of compounds 14–28 determined by SRB assay in four different breast cancer cell lines (MDA-MB-231, HS578T, MCF-7 and T47D). IC_{50} values were calculated after 96 h treatment. The data represent values of at least three independent experiments, which were done each in triplicate.

Compound	MDA-MB-231	HS578T	MCF-7	T47D
14 (μM)	2.88 ± 0.11	3.39 ± 0.92	3.03 ± 0.22	3.86 ± 0.93
15 (μM)	38.91 ± 14.08	15.18 ± 7.18	26.09 ± 10.76	49.67 ± 13.92
16 (μM)	3.36 ± 0.22	4.14 ± 0.13	3.59 ± 0.21	4.39 ± 0.88
17 (μM)	2.58 ± 0.37	2.77 ± 0.41	2.82 ± 0.57	3.78 ± 0.74
18 (μM)	0.46 ± 0.21	2.80 ± 0.16	1.53 ± 0.23	1.97 ± 0.29
19 (nM)	35.87 ± 19.42	280.06 ± 31.25	147.26 ± 68.02	190.96 ± 113.70
20 (nM)	71.76 ± 46.35	215.54 ± 96.53	155.25 ± 64.67	269.61 ± 76.07
21 (nM)	126.46 ± 40.55	351.94 ± 127.31	221.96 ± 90.61	261.83 ± 49.91
22 (nM)	134.05 ± 76.38	356.46 ± 92.90	120.63 ± 43.11	187.07 ± 60.55
23 (nM)	55.99 ± 19.44	275.88 ± 64.62	25.97 ± 21.28	32.71 ± 24.35
24 (nM)	1140.71 ± 255.22	1341.56 ± 74.91	1189.47 ± 325.25	1316.63 ± 713.38
25 (nM)	69.68 ± 8.43	341.79 ± 36.15	138.65 ± 111.56	232.17 ± 65.43
26 (nM)	135.93 ± 71.83	538.92 ± 27.80	239.90 ± 3.63	251.17 ± 56.18
27 (nM)	62.91 ± 22.03	440.34 ± 206.56	103.85 ± 19.75	129.25 ± 38.29
28 (nM)	0.60 ± 0.11	125.79 ± 7.61	3.96 ± 1.95	8.18 ± 6.51

Cell death analyses were done by use of FITC annexin V-Sytox Deep Red staining in MDA-MB-231 (IC_{50} = 0.6 nM) and HS578T (IC_{50} = 126 nM) breast cancer cell lines to discriminate apoptotic and necrotic cells. An example for the evaluation of cell death via annexin V-Sytox Deep Red staining in sensitive breast cancer cell line MDA-MB-231 and resistant breast cancer cell line HS578T is shown in Figure 3 A. Cells stained negative for both annexin V and Sytox Deep Red were viable (Q3). Early apoptotic cells stained positive for annexin V but negative for Sytox Deep Red (Q4), whereas late apoptotic or dead cells stained positive for both annexin V and Sytox Deep Red (Q2). Necrotic cells are indicated as negative for annexin V but positive for Sytox Deep Red (Q1).

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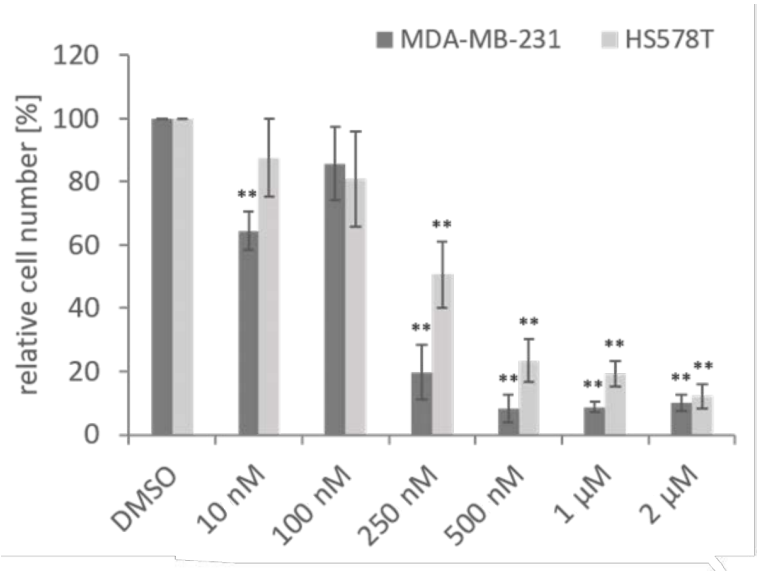

Figure 2. Relative cell number of MDA-MB-231 and HS578T breast cancer cells. Cells were seeded in 6 well plates and treated with different concentrations of compound 28. After 72 h the number of viable cells was counted. Data represent mean values (± SD) of at least three independent experiments. All data were referred to DMSO treated cells (= 100%). Significant p values are highlighted with asterisks (** $p \leq 0.01$). 169 170 171 172 173

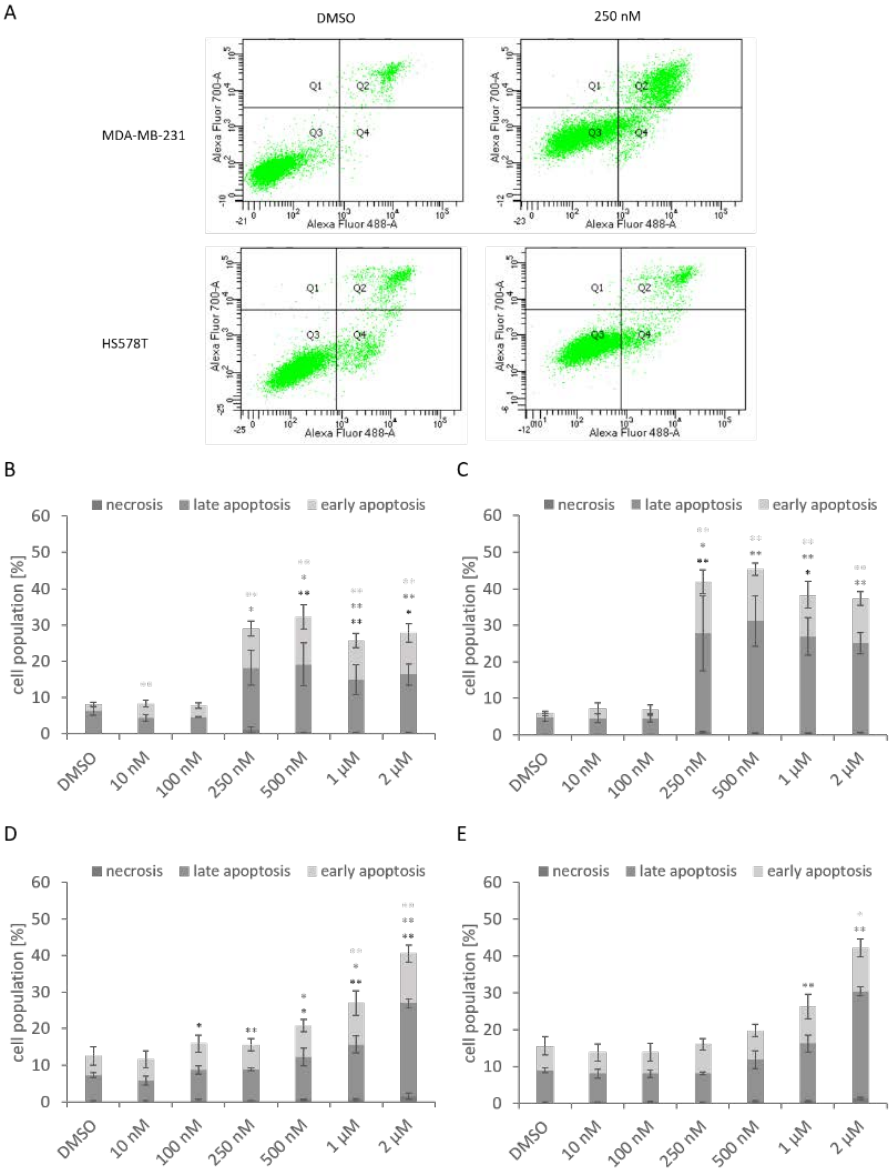

Figure 3. FITC Annexin V (Alexa 488)-Sytox Deep Red (Alexa 700) staining of MDA-MB-231 and HS578T cells. (A) Dot Plots of MDA-MB-231 and HS578T cell line after treatment with 250 nM compound 28 (B–E). Quantitative analysis of cell death of MDA-MB-231 (B + C) and HS578T cells (D + E) after treatment with different concentrations of compound 28 for 48 h (B + D) and 72 h (C + E). Data represent mean values (± SD) of at least three independent experiments. Significant p values are highlighted with asterisks (* $p \leq 0.05$; ** $p \leq 0.01$).

Analysis of subcellular localization of compound 28 (Figure 4, left) compared to the mitochondrial targeting compound BioTracker™ 488 Green Mitochondria Dye (Figure 4, right) in MDA-MB-231 cells shows identical pattern of accumulation indicating the mitochondrial targeting of 28. Using a quantitative analysis of the respective integrated fluorescence intensity, an mitochondrial uptake of about 56% could be determined.

In summary, determination of proliferation and cell death indicates, that compound 28 induces inhibition of proliferation or growth arrest at lower dose and with increasing dose treatment with compound 28 causes an induction of apoptosis. Furthermore, differential responses to proliferation inhibition and apoptosis induction may explain differential sensitivity of mammary cell lines to compound 28.

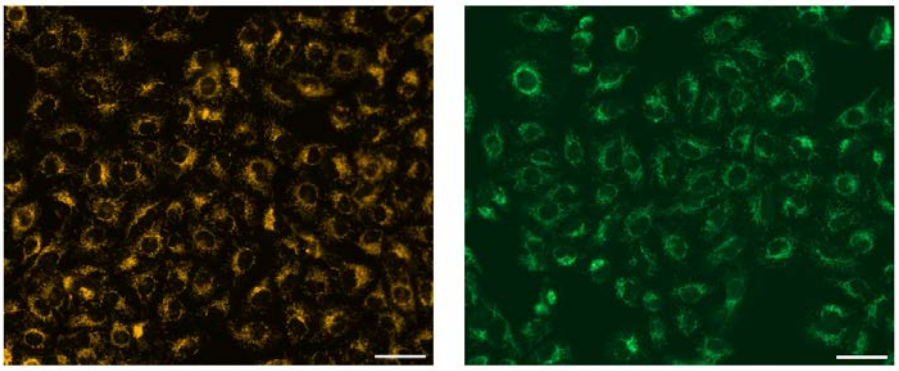

Figure 4. Analysis of subcellular localization of compound 28 was performed in MDA-MB-231 cells using BioTracker™ 488 Green Mitochondria Dye; Cells treated with 100 nM 28 for 6h or 100 nM BioTracker488 for 30min, observed: BioTracker (475 nm/514 nm), AS101 (555 nm/592 nm). Scale bar: 50 μm.

3. Disussion

1,5-Diazacyclooctane was synthesized through a straightforward synthetic pathway and subsequently linked with pentacyclic triterpenoic acids, namely oleanolic acid, ursolic acid, betulinic acid, platanic acid, and asiatic acid. These resulting amides were activated with oxalyl chloride and reacted with either rhodamine B or rhodamine 101 to form conjugates. These conjugates were then subjected to screening using SRB assays on various breast cancer cell lines, namely MDA-MB-231, HS578T, MCF-7, and T47D. The findings revealed that the conjugates exhibited cytotoxic activity even at low concentrations. Notably, the asiatic acid rhodamine 101 conjugate 28 displayed an IC_{50} = 0.60 nM and demonstrated the ability to induce apoptosis in MDA-MB-231 and HS578T cells. Further investigations demonstrated that the compound acted as a mitocan, resulting in inhibition of proliferation or growth arrest in MDA-MB-231 cells at lower doses, followed by the induction of apoptosis at higher doses. Moreover, the differential responses observed in terms of proliferation inhibition and apoptosis induction could potentially explain the varying sensitivity of mammary cell lines to compound 28.

4. Materials and Methods

4.1. General

NMR spectra were recorded using the Varian spectrometers (Darmstadt, Germany) DD2 and VNMRS (400 and 500 MHz, respectively). MS spectra were taken on a Advion expression^{L} CMS mass spectrometer (Ithaca, NY, USA; positive ion polarity mode, solvent: methanol, solvent flow: 0.2 mL/min, spray voltage: 5.17 kV, source voltage: 77 V, APCI corona discharge: 4.2 μA, capillary temperature: 250 °C, capillary voltage: 180 V, sheath gas: N_2). Thin-layer chromatography was performed on pre-coated silica gel plates supplied by Macherey-Nagel (Düren, Germany). IR spectra were recorded on a Spectrum 1000 FT-IR-spectrometer from Perkin Elmer (Rodgau, Germany). The UV/Vis-spectra were recorded on a Lambda 14 spectrometer from Perkin Elmer (Rodgau, Germany); optical rotations were measured at 20 °C using a JASCO-P2000 instrument (JASCO Germany GmbH, Pfungstadt, Germany). The melting points were determined using the Leica hot stage microscope Galen III (Leica Biosystems, Nussloch, Germany) and are uncorrected. The solvents were dried according to usual procedures. Microanalyses were performed with an Elementar Vario EL (CHNS) instrument (Elementar Analysensysteme GmbH, Elementar-Straße 1, D-63505, Langenselbold, Germany).

All dry solvents were distilled over respective drying agents except for DMF which was distilled and stored under argon and molecular sieve. Reactions using air- or mois-

ture-sensitive reagents were carried out under argon atmosphere in dried glassware. Triethylamine was stored over potassium hydroxide. Biological assays were performed as previously reported. The parent triterpenoic acids were obtained from local vendors.

4.2. General procedure for acetylation (GP 1)

To a solution of the parent triterpenoic acid (1 equiv.) in dry DCM, acetic anhydride (3 equiv.), dry triethylamine (3 equiv.) and DMAP (catal. amounts) were added, and the mixture was stirred at 20 °C for one day. Usual aqueous work-up followed by re-crystallization from ethanol furnished the corresponding acetates 9–13. Their respective m.p., $[\alpha]_D^{20}$ values, ^{1}H and ^{13}C NMR spectra as well as ESI MS data correspond to literature values.

4.3. General procedure for the synthesis of amides 14–18 (GP 2)

To a solution of acetates 9–13 (1 equiv.) in dry DCM (100 mL), oxalyl chloride (5 equiv.) and DMF (2 drops) were added, and the mixture was stirred at 20 °C for 2 hours. The volatiles were removed under diminished pressure, and the residue was dissolved in dry DCM (100 mL). This solution was slowly added to a solution of the corresponding amine (3 equiv) in dry acetonitrile (100 mL) in the presence of DMAP (catal. amounts). The mixture was stirred at 20 °C for 1 day, the volatiles were removed under diminished pressure, and the residue was subjected to column chromatography (silica gel) to afford products 14–18.

4.4. General procedure for the synthesis of the rhodamine conjugates 19–28 (GP 3)

To a solution of the rhodamine (rhodamine B or rhodamine 101, 1 equiv.) in dry DCM (100 mL) oxalyl chloride (7 equiv.) and dry DMF (2 drops) were added, and the mixture was stirred at 20 °C for 1 hour. The volatiles were removed under diminished pressure, and the residue was dissolved in dry DCM (100 mL). A solution of the corresponding amine (1 equiv.) in dry DCM (100 mL) was added, followed by the addition of catal. amounts of triethylamine and DMAP. The mixture was stirred at 20 °C for 1 hour (TLC showed completion of the reaction), the solvents were removed *in vacuo*, and the residue was subjected to column chromatography (silica gel, $CHCl_3$/MeOH) to afford products 19–28.

4.5. N,N′-Ditosyl-1,3-propanediamine (5)

Tosyl chloride (40.0 g, 210 mmol) was molten in a beaker at 80 °C, and 1,3-propanediamine (3, 8.9 mL, 106 mmol) was added dropwise; to complete the reaction, the mixture was stirred for an additional 30 min at 80 °C. After cooling to 20 °C, aq. HCl (2 M) was added, and the precipitate was washed with water followed by a re-crystallization from ethanol to furnish 5 (33.7 g, 83%) as a colorless solid; m.p. 138 °C (lit: [49] 137–140 °C); R_f = 0.75 (silica gel, hexanes/ethyl acetate, 4:6); UV-Vis ($CHCl_3$): λ_{max} (log ε) = 228 nm (4.16); IR (ATR): ν = 3271*w*, 1595*w*, 1431*w*, 1305*s*, 1214*w*, 1154*s*, 1088*m*, 1024*w*, 980*m*, 858*m*, 815*s*, 698*s*, 550*s*, 568*s*, 489*m* cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.73 (*m*, 4H, 4-H, 8-H), 7.32–7.25 (*m*, 4H, 5-H, 7-H), 3.02 (*t*, *J* = 5.8 Hz, 4H, 2-H), 2.42 (*s*, 6H, 9-H), 1.67 (*p*, *J* = 6.2 Hz, 2H, 1-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 143.6 (C-6), 136.8 (C-3), 129.8 (C-5, C-7), 127.0 (C-4, C-8), 39.8 (C-2), 29.9 (C-1), 21.5 (C-9) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m*/*z* = 405.0 (100%, $[M+Na]^+$).

4.6. 1,3-Propanediol ditosylate (6)

A mixture of 1,3-propanediol (4,16.0 g, 210 mmol) and tosyl chloride (88.0 g, 461 mmol) in dry pyridine (70 mL) was stirred at 0 °C for 1 h. The product was precipitated by adding aq. HCl (2 M), filtered off and dried. Compound 6 (69.9 g, 87%) was obtained as a colorless solid; m.p. 92 °C (lit.: [51] 92–93 °C); R_f = 0.49 (hexanes/ethyl acetate, 6:4); UV-Vis ($CHCl_3$): λ_{max} (log ε) = 225 nm (4.11); IR (ATR): ν = 2978*w*, 1599*m*, 1496*w*, 1470*w*,

1421*w*, 1352*s*, 1293*m*, 1254*w*, 1190*m*, 1172*s*, 1095*m*, 1029*m*, 1021*m*, 941*s*, 892*w*, 852*s*, 810*s*, 739*s*, 660*s*, 580*s*, 568*s*, 549*s*, 488*m* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.75–7.23 (*m*, 8H, 4-H, 5-H, 7-H, 8-H), 4.06 (*t*, *J* = 6.0 Hz, 4H, 2-H), 2.46 (s, 6H, 9-H), 1.99 (*p*, *J* = 6.0 Hz, 2H, 1-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 145.1 (C-6), 132.6 (C-3), 130.0 (C-5, C-7), 127.9 (C-4, C-8), 65.9 (C-2), 28.7 (C-1), 21.6 (C-9) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m*/*z* = 407.3 (100%, $[M+Na]^+$).

4.7. 1,5-Bis (p-toluenesulfonyl)-1,5-diazacyclooctane (7)

To a solution of sodium methanolate (8.0 g, 148 mmol) in dry MeOH (100 ml) 5 (5.0 g, 13 mmol) was added, and the mixture was heated under reflux for 4 hours. The solvent was removed, the residue was dissolved in dry DMF (100 mL) and 6 (5.0 g, 13 mmol) was added. The mixture was stirred at 80 °C for 12 hours. The product was precipitated by adding aq. HCl (2 M), filtered off and 7 (4.7 g, 84%) was obtained as a colorless solid; m.p. 214–216 °C (lit. [33]: 214–215 °C); R_f = 0.33 (hexane/ethyl acetate, 7:3); UV-Vis ($CHCl_3$): λ_{max} (log ε) = 232 nm (4.32); IR (ATR): ν = 2953*w*, 1597*w*, 1456*m*, 1378*m*, 1321*s*, 1182*m*, 1150*s*, 1088*s*, 1017*m*, 1059*s*, 987*s*, 927*m*, 837*m*, 812*s*, 723*s*, 644*s*, 627*m*, 543*s*, 487*m*, 462*m*, 408*m* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.68 (*d*, *J* = 8.3 Hz, 4H, 5-H, 9-H), 7.33–7.30 (*m*, 4H, 6-H, 8-H), 3.31–3.24 (*m*, 8H, 1-H, 3-H), 2.43 (*s*, 6H, 10-H), 2.04 (*p*, *J* = 5.9 Hz, 4H, 2-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 143.4 (C-7), 135.6 (C-4), 129.8 (C-6, C-8), 127.1 (C-5, C-9), 47.0 (C-1, C-3), 30.2 (C-2), 21.5 (C-10) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m*/*z* = 445.2 (100%, $[M+Na]^+$).

4.8. 1,5-Diazacyclooctane dihydrobromide (8)

4.8.1. Procedure A

A solution of 7 (2.5 g, 6 mmol) and thioanisole (2.4 mL, 18 mmol) in HBr (33% in glacial acetic acid, 150 mL) was stirred at 80 °C for 3 hours. The volatiles were removed under diminished pressure, DCM (30 mL) was added, the solution was washed with water (3 x 100 mL), followed by decolorization (activated charcoal). The solution was filtered, the solvent removed, and 8 (1.5 g, 5.5 mmol, 92%) was obtained as a colorless solid.

4.8.2. Procedure B

A solution of hydrazine (75 mL, 1.5 mol) in EtOH (200 mL) was heated under reflux and 1,3-dibromopropane (75 mL, 0.75 mol) was added slowly within 4 hours. Stirring was continued for another hour, the solids were filtered off, washed with ethanol (3 x 50 mL), and discarded. The pH of the filtrate [combined with the EtOH washings and additional water (150 mL)] was adjusted to pH =3 by adding aqu. HBr (48% in water). Benzaldehyde (60 mL, 0.6 mol) was added, and the precipitate formed upon addition was filtered off, washed with water (3 x 50 mL), and discarded. The combined filtrates were extracted with ether (1000 mL), and the aq. layer was concentrated under diminished pressure resulting in the formation of a red solid. Ethanol (250 mL) was added, and shaking of this suspension was continued for another 5 min. The yellowish solid was filtered off, washed with ethanol (250 mL) and ether (5 x 100 mL), and 8 (15.6 g, 7.5%) was obtained as a colorless solid; m.p. = 220–225 °C (lit.: [51, 52] >250 °C); R_f = 0.8 ($CHCl_3$:MeOH, 95:5); IR (ATR): ν = 2971*s*, 2728*s*, 2418*m*, 1577*s*, 1461*s*, 1331*m*, 1095*s*, 1027*m*, 890*m*, 696*m*, 547*m*, 491*m*, cm^{-1}; ^1H NMR (400 MHz, D_2O): δ = 3.36–3.31 (*m*, 8H, 1-H, 3-H, 4-H, 6-H), 2.22–2.16 (*m*, 4H, 2-H, 5-H) ppm; ^{13}C NMR (101 MHz, D_2O): δ = 43.8 (C-1, C-3, C-4, C-6), 20.8 (C-2, C-5) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m*/*z* = 115.0 (100%, $[M+H\text{-}2\ HBr]^+$).

4.9. (3ß)28-(1,5-Diazocan-1-yl)-28-oxoolean-12-en-3-yl acetate (14)

Following GP 2 from 3-*O*-acetyl-oleanolic acid (9, 500 mg, 1.0 mmol), followed by chromatography (silica gel, $CHCl_3$/MeOH (2% → 10%) compound 14 (425 mg, 71%) was obtained as a colorless solid; m.p. = 207–210 °C (decomp.); R_f = 0.52 ($CHCl_3$/MeOH, 95 :5); $[\alpha]_D^{20}$ = + 3.8° (*c* 0.088, $CHCl_3$); IR (ATR): ν = 2954*m*, 1732*s*, 1626*m*, 1464*m*, 1368*s*, 1245*s*, 1026*s*,

750*s*, 662*w* cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.25 (*m*, 1H, 12-H), 4.50 (*m*, 1H, 3-H), 3.67–3.11 (*m*, 8H, 33-H, 35-H, 36-H, 38-H), 3.03 (*d*, *J* = 13.8 Hz 1H, 18-H), 2.16–2.12 (m, 1H, 16-H), 2.04 (*s*, 3H, 32-H), 1.87–1.17 (*m*, 23H, 11-H, 34-H, 37-H, 19-H_a, 2-H, 1-H_a, 9-H, 6-H_a, 15-H, 7-H, 21-H, 6 H_b, 22-H, 19-H_b), 1.13 (*s*, 3H, 27-H), 1.01–0.98 (m, 1H, 1-H_b), 0.97-0.93 (*s*, 3H, 25-H), 0.92 (*s*, 3H, 30-H), 0.89 (*s*, 3H, 29-H), 0.85 (*s*, 3H, 23-H), 0.84 (*s*, 3H, 24-H), 0.82-0.81 (*m*, 1H, 5-H), 0.72 (*s*, 3H, 26-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 177.0 (C-28), 171.2 (C-31), 144.7 (C-13), 121.7 (C-12), 81.1 (C-3), 55.5 (C-5), 47.9 (C-33, C-35, C-36, C-38), 47.8 (C-9),47.4 (C-17), 46.6 (C-19), 43.9 (C-18), 42.6 (C-14), 39.2 (C-8), 38.2 (C-1), 37.8 (C-4), 37.1 (C-10), 34.2 (C-21), 33.1 (C-7), 33.0 (C-29), 30.5 (C-34, C-37), 30.4 (C-20),29.8 (C-22), 28.2 (C-23), 27.6 (C-15), 26.0 (C-27), 24.1 (C-30), 23.7 (C-2), 23.5 (C-11), 22.8 (C-16), 21.4 (C-32), 18.3 (C-6), 17.3 (C-26), 16.8 (C-24), 15.6 (C-25) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m*/*z* = 596.3 (100%, $[M+H]^+$); analysis calcd for $C_{38}H_{62}N_2O_3$ (594.93): C 76.72, H 10.50, N 4.71; found: C 76.47, H 10.74, N 4.50.

4.10. (3ß) 28-(1,5-Diazocan-1-yl)-28-oxours-12-en-3-yl acetate (15)

Following GP 2 from 3-*O*-acetyl-ursolic acid (10, 500 mg, 1.0 mmol), followed by chromatography (silica gel, $CHCl_3$/MeOH (2% → 10%) compound 15 (413 mg, 69%) was obtained as an off-white solid; m.p. = 232–235 °C (decomp.); R_f = 0.37 ($CHCl_3$/MeOH, 95:5); $[\alpha]_D^{20}$ = + 0.45° (*c* 0.088, $CHCl_3$); IR (ATR): ν = 2942*m*, 1731*m*, 1627*m*, 1456*m*, 1370*s*, 1245*s*, 1026*s*, 750*s*, 662*m* cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.18–5.13 (*m*, 1H, 12-H), 4.46–4.39 (*m*, 1H, 3-H), 3.74–3.01 (*m*, 8H, 33-H, 35-H, 36-H, 38-H), 2.39 (*d*, *J* = 11.3 Hz, 1H, 18-H), 1.99 (*s*, 3H, 32-H), 1.88–1.82 (*m*, 2H, 11-H), 1.74–1.67 (*m*, 1H, 20-H), 1.73–1.05 (*m*, 23H, 2-H, 6-H, 15-H, 16-H, 21-H, 7-H, 9-H, 22-H, 1H_a, 19-H, 34-H, 37-H), 1.02 (*s*, 3H, 27-H), 0.99–0.95 (m, 1H, 1H_b), 0.92 (*s*, 3H, 23-H), 0.88 (*s*, 3H, 30-H), 0.86 (*s*, 3H, 25-H), 0.81 (*s*, 3H, 29-H), 0.80 (*s*, 3H, 24-H), 0.77-0.74 (*m*, 1H, 5-H), 0.72 (*s*, 3H, 26-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 174.9 (C-28), 171.0 (C-31), 125.3 (C-12), 80.9 (C-3), 55.2 (C-5), 55.0 (C-18), 48.7 (C-33, C-35, C-36, C-38), 48. 6 (C-17), 47.7 (C-9), 43.4 (C- 8), 43.5 (C-14), 39.4 (C-19), 38.7 (C-20), 38.6 (C-1), 37.6 (C-4), 37.0 (C-10), 33.9 (C-22), 32.9 (C-7), 30.6 (C-21), 28.1 (C-23), 27.3 (C-34, C-37), 27.0 (C-15), 26.4 (C-16), 23,4 (C-27), 23.5 (C-2), 23.3 (C-11), 21.2 (C-32), 21.0 (C-30), 18.3 (C-6), 16.7 (C-29), 16.39 (C-26), 15.62 (C-24), 15.40 (C-25) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m*/*z* = 596.2 (100%, $[M+H]^+$); analysis calcd for $C_{38}H_{62}N_2O_3$ (594.93): C 76.72, H 10.50, N 4.71; found: C 76.58, H 10.76, N 4.49.

4.11. (3ß) 28-(1,5-Diazocan-1-yl)-28-oxolup-20(29)-en-3-yl acetate (16)

Following GP 2 from 3-*O*-acetyl-betulinic acid (11, 500 mg, 1.0 mmol), followed by chromatography (silica gel, ethyl acetate/MeOH (10% → 50%) compound 16 (430 mg, 72%) was obtained as a colorless solid; m.p. 223-234 °C (decomp.); R_f = 0.43 ($CHCl_3$/MeOH, 9:1); $[\alpha]_D^{20}$ = - 8.0° (*c* 0.064, $CHCl_3$); IR (ATR): ν = 3408*w*, 2942*m*, 1731*m*, 1632*s*, 1455*m*, 1373*s*, 1246*s*, 1195*m*, 1026*m*, 979*m*, 882*m*, 730*s* cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.68 (*m*, 1H, 29-H_a), 4.56–4.53 (*m*, 1H, 29-H_b), 4.42 (*dd*, J = 10.1, 6.2 Hz, 1H, 3-H), 4.02–3.19 (*m*, 8H, 33-H, 35-H, 36-H, 38-H), 2.85 (*m*, 2H, 13-H, 19-H), 2.13–2.08 (*m*, 1H, 16-H_a), 2.00 (*s*, 3H, 32-H), 1.96–1.93 (*m*, 1H, 22-H_a), 1.78–1.74 (*m*, 1H, 21-H_a1.65–1.63 (*m*, 5H, 1-H_a, 12-H_a, 30-H), 1.60–1.05 (*m*, 2-H, 16-H_b, 18-H, 6-H_a, 7-H, 21-H, 11-H, 22-H_b,34-H, 37-H, 9-H, 15-H), 0.96–0.94 (*m*, 1H, 1-H_b), 0.92 (*s*, 3H, 27-H), 0.90–0.89 (*m*, 1H, 12-H_b), 0.87 (*s*, 3H, 25-H), 0.80 (*s*, 3H,m 24-H), 0.79 (*s*, 3H, 23-H), 0.76–0.74 (*m*, 1H, 5-H), 0.72 (*s*, 3H, 26-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 175.4 (C-28), 171.0 (C-31), 150.8 (C-20), 109.4 (C-29), 80.9 (C-3), 55.5 (C-5), 55.2 (C-33, C-35, C-36, C-38), 55.0 (C-17), 52.9 (C-18), 50.7 (C-9), 45.6 (C-19), 42.0 (C-14), 40.7 (C-8), 38.8 (C-4), 38.4 (C-1), 37.8 (C-10), 37.1 (C-7), 36.9 (C-13), 36.1 (C-22), 34.3 (C-34, C-37), 32.3 (C-16), 31.4 (C-21), 30.1 (C-15), 25.5 (C-23), 23.7 (C-12), 23.7 (C-2), 21.3 (C-32), 21.1 (C-11), 19.7 (C-30), 18.1 (C-6), 16.4 (C-24), 16.2 (C-25), 16.0 (C-26), 14.6 (C-27) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m*/*z* = 596.1 (100%, $[M+H]^+$); analysis calcd for $C_{38}H_{62}N_2O_3$ (594.93): C 76.72, H 10.50, N 4.71; found: C 76.46, H 10.77, N 4.53.

4.12. (3ß)28-(1,5-Diazocan-1-yl)-30-nor-20,28-dioxolup-3-yl-acetate (17)

Following GP 2 from 3-*O*-acetyl-platanic acid (12, 500 mg, 1.0 mmol), followed by chromatography (silica gel, ethyl acetate/MeOH (10% → 50%) compound 8 (425 mg, 70%) was obtained as a colorless solid; m.p. = 210-214 °C (decomp.); R_f = 0.44 ($CHCl_3$/MeOH, 9:1); $[\alpha]_D^{20}$ = -26.6° (*c* 0.028, $CHCl_3$); IR (ATR): ν = 3396*w*, 2942*m*, 2866*m*, 1731*m*, 1626*s*, 1466*m*, 1411*m*, 1369*m*, 1197*s*, 1245*m*, 1120*m*, 1025*m*, 978*m* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 4.39 (*dd*, *J* = 10.6, 5.5 Hz, 1H, 3-H), 3.78–3.06 (*m*, 9H, 19-H, 32-H, 34-H, 35-H, 37-H), 2.66–2.56 (*m*, 1H, 13-H), 2.10 (*s*, 3H, 29-H), 2.08–1.98 (*m*, 2H, 16-H_a, 18-H), 1.97 (*s*, 3H, 31-H), 1.94–1.90 (*m*, 1H, 22-H_a), 1.82–1.76 (*m*, 1H, 21-H_a), 1.70–1.05 (*m*, 19H, 1-H_a, 16-H_b, 2-H, 22-H_b, 21-H_b, 6-H_a, 11-H_a, 7-H, 6H_b, 9-H, 15-H, 11-H_b, 33-H, 36-H), 0.98–0.95 (*m*, 2H, 12-H), 0.92 (*s*, 3H, 27-H), 0.91–0.85 (*m*, 1H, 1-H_b), 0.83 (*s*, 3H, 24-H), 0.79–0.77 (*m*, 6H, 23-H, 25-H), 0.76 (*s*, 3H, 26-H), 0.72–0.71 (*m*, 1H, 5-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 212.5 (C-20), 175.3 (C-28), 170.9 (C-30), 80.8 (C-3), 55.4 (C-5), 55.1 (C-32, C-34, C-35, C-37), 52.7 (C-18), 50.6 (C-9), 49.9 (C-19), 46.1 (C-17), 41.8 (C-8), 40.6 (C-14), 38.3 (C-1), 37.7 (C-4), 37.1 (C-10), 35.9 (C-13), 35.8 (C-22), 34.1 (C-7), 31.8 (C-16), 30.3 (C-29), 30.0 (C-15), 28.8 (C-21), 27.9 (C-23), 27.3 (C-12), 23.6 (C-2), 21.3 (C-31), 21.1 (C-11), 18.1 (C-6), 16.4 (C-26), 16.2 (C-25), 15.9 (C-24), 14.6 (C-27) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m*/*z* = 597.3 (100%, $[M+H^+]$); analysis calcd for $C_{37}H_{60}N_2O_4$ (596.90): C 74.45, H 10.13, N 4.69; found: C 74.21, H 10.32, N 4.43.

4.13. (2α,3ß,4α)28-(1,5-Diazocan-1-yl)-28-oxours-12-ene-2,3,23-triyl triacetate (18)

Following GP 2 from 2,3,24-tri-*O*-acetyl-asiatic acid (13, 400 mg, 0.8 mmol), followed by chromatography [silica gel, $CHCl_3$/MeOH (2% → 50%)] compound 18 (425 mg, 74%) was obtained as colorless solid; m.p. = 187–190 °C (decomp.); R_f = 0.38 ($CHCl_3$/MeOH, 9:1); $[\alpha]_D^{20}$ = -30.2° (*c* 0.015, $CHCl_3$); IR (ATR): ν = 2925*w*, 1741*s*, 1623*w*, 1368*m*, 1231*s*, 1042*m*, 748*w* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 5.22–5.17 (*m*, 1H, 12-H), 5.14–5.08 (*m*, 1H, 2-H), 5.04–5.01 (*m*, 1H, 3-H), 3.80 (*m*, 1H, 23-H_a), 3.51 (*m*, 1H, 23-H_b), 3.32–2.67 (*m*, 8H, 37-H, 39-H, 40-H, 42-H), 2.40–2.34 (*m*, 1H, 18-H), 2.03 (*s*, 3H, 36-H), 2.02–2.00 (*m*, 1H, 1-H_a), 1.97 (*s*, 3H, 34-H), 1.92 (*s*, 3H, 32-H), 1.88–1.69 (*m*, 5H, 11-H, 16-H 22-H_a), 1.60–1.45 (*m*, 4H, 22-H_b, 9-H, 21-H_a, 7-H_a), 1.53–1.45 (*m*, 2H, 16-H_a, 16-H_b), 1.33–1.14 (*m*, 10H, 19-H, 5-H, 21-H_b, 7-H_b, 15-H, 38-H, 41-H), 1.11–1.09 (*m*, 1H, 1-H_b), 1.05 (*s*, 3H, 27-H), 1.02 (s, 3H, 25-H), 0.99-0.96 (*m*, 1H, 20-H), 0.91 (*s*, 3H, 30-H), 0.84 (*s*, 3H, 24-H), 0.82 (*s*, 3H, 29-H), 0.70 (*s*, 3H, 26-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 176.8 (C-28), 170.8 (C-35), 170.3 (C-33), 170.3 (C-31), 138.7 (C-13), 124.9 (C-12), 74.8 (C-3), 69.8 (C-2), 65.2 (C-23), 55.6 (C-18), 47.6 (C-9), 47.5 (C-5), 46.1 (C-37, C-39, C-40, C-42), 43.7 (C-1), 42.3 (C-14), 41.9 (C-4), 39.5 (C-8), 39.3 (C-19), 38.5 (C-20), 37.8 (C-10), 34.8 (C-22), 34.7 (C-7), 31.9 (C-21), 29.6 (C-15), 23.3 (C-11), 23.2 (C-16), 22.6 (C-38, C-41), 21.2 (C-30), 21.0 (C-36), 20.8 (C-32), 20.7 (C-34), 17.8 (C-6), 17.4 (C-27), 17.3 (C-29), 17.1 (C-25), 13.9 (C-26), 8.7 (C-24) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m*/*z* = 711.8 (100%, $[M+H^+]$); analysis calcd for $C_{42}H_{66}N_2O_7$ (711.00): C 70.95, H 9.36, N 3.94; found: C 70.69, H 9.51, N 3.75.

4.14. (3ß)-3-Acetyloxy-28-(5-{2-[3,6-bis(diethylamino)xanthen-10-ium-9-yl]benzoyl}-1,5-diazocan-1-yl)-28-oxoolean-12-ene chloride (19)

Following GP 3 from 14 (150 mg, 0.14 mmol) and rhodamine B (100 mg, 0.2 mmol) followed by chromatography (silica gel, ethyl acetate/MeOH, 10% → 40%) 19 (100 mg, 72%) was obtained as a pink solid; m.p. = 211–216 °C; R_f = 0.44 ($CHCl_3$/MeOH, 9:1); UV-Vis ($CHCl_3$): λ_{max} (log ε) = 562 nm (4.53); IR (ATR): ν = 2926*m*, 2605*w*, 2498*w*, 1729*w*, 1587*s*, 1466*s*, 1412*s*, 1336, 1245*s*, 1180*s*, 1132*m*, 1073*s*, 1009*m*, 921*w*, 748*m*, 683*m* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.67–7.57 (*m*, 2H, 43-H, 44-H), 7.53–7.47 (*m*, 1H, 42-H), 7.34–7.26 (*m*, 3H, 45-H, 48-H), 7.14–6.65 (*m*, 4H, 49-H, 51-H), 5.25–5.15 (*m*, 1H, 12-H), 4.51–4.40 (*m*, 1H, 3-H), 3.78–3.20 (*m*, 16H, 33-H, 35-H, 36-H, 38-H, 53-H), 3.05–2.95 (*m*, 1H, 18-H), 2.07–2.02 (*m*, 1H, 16-H_a), 2.01–1.99 (*m*, 3H, 32-H), 1.89–1.81 (*m*, 2H, 11-H), 1.67–1.37 (*m*, 14H, 19-H_a, 1-H_a, 2-H, 9-H, 6-H_a, 15-H_a, 22-H_a, 21-H_a, 6-H_b, 34-H, 37-H), 1.30–1.24 (*m*, 12H, 54-H), 1.23–1.13 (*m*, 6H, 16-H_b, 7-H 22-H_b, 21-Hb, 19-H_b,), 1.08 (*s*, 3H, 27-H), 0.99 (*m*, 2H, 1-H_b, 15-H_b),

0.87 (*s*, 3H, 25-H), 0.86 (*s*, 3H, 29-H), 0.84 (*s*, 3H, 30-H), 0.82 (*s*, 3H, 23-H), 0.80 (*s*, 3H, 24-H), 0.79–0.76 (*m*, 1H, 5-H), 0.68 (*s*, 3H, 26-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 170.9 (C-28, C-31), 168.7 (C-39), 157.7 (C-52), 155.8 (C-46), 155.7 (C-50), 145.4 (C-13), 136.6 (C-41), 132.5 (C-49), 130.4 (C-40), 130.1 (C-42), 130.0 (C-44), 129.4 (C-43), 127.7 (C-45), 121.2 (C-129, 113.9 (C-47), 96.1 (C-48, C-51), 80.9 (C-3), 55.3 (C-5), 48.4 (C-17), 47.6 (C-9), 46.6 (C-19), 46.2 (C-53), 46.1 (C-33, C-35, C-36, C-38), 44.7 (C-18), 42.0 (C-14), 39.1 (C-8), 38.0 (C-1), 37.6 (C-4), 37.0 (C-10), 34.1 (C-21), 32.9 (C-30), 32.8 (C-22), 30.3 (C-20), 29.6 (C-7), 28.0 (C-15), 28.0 (C-23), 25.8 (C-27), 24.0 (C-29), 23.5 (C-2), 23.3 (C-11), 22.6 (C-16), 21.3 (C-32), 18.2 (C-6), 17.2 (C-26), 16.6 (C-24), 15.4 (C-25), 12.7 (C-54) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m*/*z* = 1021.4 (98%, $[M\text{-}Cl]^+$); analysis calcd for $C_{66}H_{91}N_4O_5Cl$ (1055.93): C 75.07, H 8.69, N 5.31; found: C 74.87, H 8.82, N 5.08.

4.15. (3ß)-3-Acetyloxy-28-(5-{2-[3,6-bis(diethylamino)xanthen-10-ium-9-yl]benzoyl}-1,5-diazocan-1-yl)-28-oxours-12-ene chloride (20)

Following GP 3 from 15 (150 mg, 0.14 mmol) and rhodamine B (100 mg, 0.2 mmol) followed by chromatography (silica gel, ethyl acetate/MeOH, 10% → 40%) 20 (94 mg, 63%) was obtained as a pink solid; m.p. = 194–197 °C (decomp.); R_f = 0.41 ($CHCl_3$/MeOH, 9:1); UV-Vis ($CHCl_3$): λ_{max} (log ε) = 560 nm (5.54); IR (ATR): ν = 2932*w*, 1726*w*, 1586*s*, 1465*m*, 1411*s*, 1335*s*, 1272*m*, 1245*s*, 1179*s*, 1132*m*, 1073*m*, 1009*m*, 921*m*, 823*w*, 746*m*, 683*m*, 663*m*, 498*m* cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.68–7.56 (*m*, 2H, 43-H, 44-H), 7.53–7.49 (*m*, 1H, 42-H), 7.32–7.25 (*m*, 3H, 45-H, 48-H), 7.18–6.54 (*m*, 4H, 49-H, 51-H), 5.24–5.11 (*m*, 1H, 12-H), 4.50–4.39 (*m*, 1H, 3-H), 4.12–2.76 (*m*, 16H, 33-H, 35-H, 36-H, 38-H,53-H), 2.43–2.33 (*m*, 1H, 18-H), 2.09–2.07 (*m*, 1H, 16-H_a), 2.00 (*s*, 3H, 32-H), 1.91–1.84 (*m*, 2H, 11-H), 1.77–1.36 (*m*, 14H, 1-H_a, 2-H, 21-H_a, 6-H_a, 9-H, , 22-H_a, 19-H, 6-H_b, 16-H_b, 34-H, 37-H), 1.29 (*t*, J = 7.1 Hz, 12H, 54-H), 1.23 (*m*, 6H, 7-H, 15-H, 21-H_b, 22-H_b), 1.03 (s, 4H, 1-H_b, 27-H), 0.96–0.93 (*m*, 1H, 20-H), 0.90 (*s*, 3H, 29-H), 0.88 (*s*, 3H, 25-H), 0.83 (*s*, 3H, 30-H), 0.82 (*s*, 3H, 23-H), 0.81 (*s*, 3H, 24-H), 0.77–0.75 (*m*, 1H, 5-H), 0.69 (*s*, 3H, 26-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 170.9 (C-28), 157.7 (C-31), 157.7 (C-39), 155.8 (C-50), 155.7 (C-46) 155.6 (C-52), 136.6 (C-40), 132.9 (C-48), 130.1 (C-44), 130.0 (C-42), 129.4 (C-43), 127.0 (C-45), 125.0 (C-12), 113.9 (C-47), 96.2 (C-49, C-51), 80.9 (C-3), 55.3 (C-18), 55.3 (C-5), 49.4 (C-17), 47.5 (C-9), 46.2 (C-33, C-35, C-36, C-38), 46.1 (C-53), 42.4 (C-14), 39.6 (C-19), 39.3 (C-8), 38.6 (C-20), 38.2 (C-1), 37.6 (C-4), 36.9 (C-10), 32.7 (C-22), 31.9 (C-7), 30.5 (C-21), 29.6 (C-15), 29.3 (C-16), 28.0 (C-23), 23.2 (C-2), 23.1 (C-11), 23.0 (C-27), 18.1 (C-6), 17.4 (C-30), 17.2 (C-26), 16.7 (C-24), 15.5 (C-25), 12.7 (C-54) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m*/*z* = 1020.4 (100%, $[M\text{-}Cl]^+$); analysis calcd for $C_{66}H_{91}N_4O_5Cl$ (1055.93): C 75.07, H 8.69, N 5.31; found: C 74.83, H 8.91, N 5.03.

4.16. (3ß)-3-Acetyloxy-28-(5-{2-[3,6-bis(diethylamino)xanthen-10-ium-9-yl]benzoyl}-1,5-diazocan-1-yl)-28-oxolup-20(29)-ene chloride (21)

Following GP 3 from 16 (300 mg, 0.5 mmol) and rhodamine B (200 mg, 0.4 mmol) followed by chromatography (silica gel, $CHCl_3$/MeOH, 9:1) 21 (3536 mg, 69%) was obtained as a pink solid; m.p. = 212–218 °C; R_f = 0.49 ($CHCl_3$/MeOH, 9:1); UV-Vis ($CHCl_3$): λ_{max} (log ε) = 562 nm (4.43); IR (ATR): ν = 2936*w*, 1730*w*, 1587*s*, 1465*m*, 1411*s*, 1335*s*, 1244*s*, 1179*s*, 1132*m*, 1073*m*, 978*w*, 921*w*, 684*m* cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.52–7.47 (*m*, 2H, 43-H, 44-H), 7.43–7.37 (*m*, 1H, 42-H), 7.22–6.99 (*m*, 3H, 45-H, 48-H), 6.97–6.71 (*m*, 2H, 49-H), 6.64–6.57 (*m*, 2H, 51-H), 4.58–4.50 (*m*, 1H, 29-H_a), 4.42–4.37 (*m*, 1H, 29-H_b), 4.30 (*m*, 1H, 3-H), 3.73–2.93 (*m*, 16H, 33-H, 35-H, 36-H, 38-H, 53-H), 2.83–2.68 (*m*, 2H, 19-H, 13-H), 2.00–1.94 (*m*, 1H, 16-H_a), 1.87 (*s*, 3H, 32-H), 1.83–1.54 (*m*, 4H, 22-H_a, 15-H, 21-H_a), 1.53–1.51 (*m*, 2H, 12-H_a, 1-H_a), 1.50 (*s*, 3H, 30-H), 1.47–1.42 (*m*, 2H, 2-H), 1.41–1.36 (*m*, 1H, 18-H), 1.35–1.29 (*m*, 2H, 16-H_a, 6-H_a), 1.28–1.12 (*m*, 21H, 11-H_a, 6-H_b, 7-H, 22-H_b, 54-H, 34-H, 37-H), 1.11–1.09 (*m*, 2H, 11-H_b, 9-H, 21-H_b), 0.83–0.74 (*m*, 8H, 1-H_b, 12-H_b, 23-H, 27-H), 0.69–0.65 (*m*, 9H, 26-H, 25-H, 24-H), 0.63–0.59 (*m*, 1H, 5-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 170.8 (C-28), 168.5 (C-39), 167.6 (C-31), 157.6 (C-50), 155.6 (C-46), 155.5 (C-52), 151.2 (C-

20), 136.4 (C-40), 132.3 (C-41), 130.8 (C-48), 130.0 (C-43), 129.4 (C-42), 128.6 (C-45), 127.0 (C-44), 114.6 (C-49), 113.6 (C-47), 108.9 (C-29), 96.0 (C-51), 80.8 (C-3), 55.2 (C-5), 55.1 (C-38, C-36, C-35, C-33), 53.0 (C-18), 50.6 (C-9), 46.1 (C-53), 45.8 (C-19), 41.9 (C-17), 40.6 (C-8), 40.6 (C-14), 38.3 (C-1), 37.7 (C-10), 37.0 (C-4), 36.8 (C-13), 36.0 (C-22), 34.2 (C-7), 32.0 (C-16), 31.3 (C-21), 30.2 (C-34, C-37), 29.8 (C-15), 27.8 (C-24), 25.5 (C-12), 25.4 (C-37, C-34), 23.6 (C-2), 21.2 (C-32), 21.0 (C-11), 19.6 (C-30), 18.1 (C-6), 16.1 (C-25), 15.9 (C-26), 14.6 (C-23), 14.5 (C-27), 12.6 (C-54) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m*/*z* = 1020.5 (100%, $[M\text{-}Cl]^+$); analysis calcd for $C_{66}H_{91}N_4O_5Cl$ (1055.93): C 75.07, H 8.69, N 5.31; found: C 74.86, H 8.90, N 5.09.

4.17. (3ß)-3-Acetyloxy-28-(5-{2-[3,6-bis(diethylamino)xanthen-10-ium-9-yl]benzoyl}-1,5-diazocan-1-yl)-28-oxolup-20-oxo chloride (22)

Following GP 3 from 17 (300 mg, 0.50 mmol) and rhodamine B (300 mg, 0.6 mmol) followed by chromatography (silica gel, ethyl acetate/MeOH, 9:1) 22 (350 mg, 69%) was obtained as a pink solid; m.p. = 198–201 °C; R_f = 0.51 ($CHCl_3$/MeOH, 9:1); UV-Vis ($CHCl_3$): λ_{max} (log ε) = 558 nm (4.73); IR (ATR): *ν* = 2934*w*, 1721*m*, 1585*s*, 1410*m*, 1466*s*, 1334*s*, 1272*s*, 1245*s*, 1131*s*, 1072*s*, 1009*s*, 977*m*, 921*m*, 823*m*, 755*m*, 682*s* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.67–7.57 (*m*, 2H, 42-H, 43-H), 7.53–7.47 (*m*, 1H, 41-H), 7.35–7.27 (*m*, 3H, 44-H, 47-H), 7.11–6.64 (*m*, 4H, 48-H, 50-H), 4.46–4.36 (*m*, 1H, 3-H), 3.88–3.20 (*m*, 16H, 32-H, 34-H, 35-H, 37-H, 52-H), 3.16–3.05 (*m*, 1H, 18-H), 2.76–2.51 (*m*, 1H, 13-H), 2.11–2.04 (*m*, 4H, 16-H_a, 29-H), 1.98 (*s*, 4H, 19-H, 31-H), 1.78 (*s*, 2H, 21-H_a, 22-H_a), 1.62–1.48 (*m*, 4H, 1-H_a, 2-H, 16-H_a), 1.42 (*m*, 7H, 6-H_a, 22-H_b, 21-H_b, 11-H, 7-H_a, 6-H_b), 1.28 (*t*, *J* = 6.8 Hz, 14H, 7-H_b, 9-H, 53-H), 1.23–1.04 (*m*, 6H, 33-H, 36-H, 15-H), 0.94 (*s*, 2H, 12-H), 0.91 (*s*, 4H, 1-H_b, 24-H), 0.82 (*s*, 3H, 27-H), 0.78 (*m*, 10H, 23-H, 25-H, 26-H, 5-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 212.8 (C-20), 170.9 (C-28, C-30, C- 38), 157.7 (C-49), 155.7 (C-45), 155.6 (C-51), 136.6 (C-39), 136.5 (C-40), 132.4 (C-47), 130.1 (C-42), 129.7 (C-43), 129.4 (C-44), 127.1 (C-41), 114.5 (C-48), 113.7 (C-46), 96.2 (C-50), 80.8 (C-3), 55.4 (C-5), 55.3 (C-32, C-34, C-35, C-37), 53.0 (C-19), 50.6 (C-9), 50.3 (C-18), 49.5 (C-17), 46.2 (C-52), 41.9 (C-14), 40.6 (C-8), 38.3 (C-1), 37.7 (C-4), 37.1 (C-10), 35.8 (C-13), 35.6 (C-22), 34.2 (C-7), 31.6 (C-16), 30.1 (C-29), 29.9 (C-15), 28.8 (C-21), 27.9 (C-23), 27.4 (C-12), 23.6 (C-2), 22.6 (C-33, C-36), 21.1 (C-11), 18.1 (C-6), 16.4 (C-25), 16.2 (C-26), 14.7 (C-24), 14.0 (C-27), 12.7 (C-53) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m*/*z* = 1022.4 (100%, $[M\text{-}Cl]^+$); analysis calcd for $C_{65}H_{89}N_4O_6Cl$ (1057.90): C 73.80, H 8.48, N 5.30; found: C 73.55, H 8.67, N 5.07.

4.18. (2α,3ß,4α)2,3,23-Tris (acetyloxy)-28-(5-{2-[3,6-bis(diethylamino)xanthen-10-ium-9-yl]benzoyl}-1,5-diazocan-1-yl)-28-oxours-12-en chloride (23)

Following GP 3 from 18 (300 mg, 0.4 mmol) and rhodamine B (250 mg, 0.5 mmol) followed by chromatography (silica gel, ethyl acetate/MeOH, 9:1) 23 (184 mg, 60%) was obtained as a pink solid; m.p. = 225 °C; R_f = 0.44 ($CHCl_3$/MeOH, 9:1); UV-Vis ($CHCl_3$): λ_{max} (log ε) = 562 nm (4.50); IR (ATR): *ν* = 2927*w*, 1793*m*, 1587*s*, 1467*m*, 1411*m*, 1336*s*, 1244*s*, 1179*s*, 1042*m*, 921*w*, 684*m*, 436*w* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.66–7.57 (*m*, 2H, 47-H, 48-H), 7.52–7.47 (*m*, 1H, 46-H), 7.27-7.20 (m, 49-H, 52-H), 7.17–6.63 (*m*, 4H, 53-H, 55-H), 5.20–5.00 (*m*, 3H, 12-H, 2-H, 3-H), 3.82–3.76 (*m*, 1H, 23-H_a), 3.73–2.93 (*m*, 17H, 37-H, 39-H, 40-H, 42-H, 57-H, 23-H_b), 2.44–2.33 (*m*, 1H, 18-H), 2.04 (*s*, 3H, 34-H), 2.02–1.99 (*m*, 1H, 1-H_a), 1.97 (*s*, 3H, 36-H), 1.93 (*s*, 3H, 32-H), 1.90–1.32 (*m*, 15H, 11-H, 9-H, 15-H, 16-Ha, 21-H_a, 22-H_a, 20-H, 38-H, 41-H, 6-H), 1.28 (*t*, *J* = 7.1 Hz, 13H, 5-H, 58-H), 1.25–1.10 (*m*, 5H, 7-H, 16-H_b, 21-H_b, 22-H_b), 1.09–1.07 (*m*, 1H, 1-H_b), 1.04 (*s*, 3H, 30-H), 1.01 (*s*, 3H, 27-H), 0.97–0.92 (*m*, 1H, 19-H), 0.88 (*s*, 3H, 29-H), 0.84 (*s*, 3H, 25-H), 0.81 (*s*, 3H, 26-H), 0.69 (*s*, 3H, 24-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 176.2 (C-43), 170.8 (C-35), 170.4 (C-31, C-33), 170.3 (C-28), 157.7 (C-54), 155.7 (C-56), 155.6 (C-50), 138.6 (C-13), 136.6 (C-45), 132.3 (C-52), 130.1 (C-47), 130.0 (C-49), 129.3 (C-48), 127.2 (C-46), 125.9 (C-12), 113.9 (C-51), 96.2 (C-53, C-55), 74.8 (C-3), 69.9 (C-2), 65.3 (C-23), 55.5 (C-18), 53.4 (C-37, C-39, C-40, C-42), 47.6 (C-5), 47.5 (C-9), 46.2 (C-57), 46.1 (C-17), 43.7 (C-1), 42.5 (C-4), 41.9 (C-14), 38.9 (C-8), 38.7 (C-20), 38.6 (C-19), 37.8 (C-10), 32.6 (C-22), 30.5 (C-21), 29.6 (C-7), 28.4 (C-15), 23.4 (C-27), 23.3

(C-11), 21.2 (C-29), 21.0 (C-32), 20.8 (C-36), 20.7 (C-34), 17.8 (C-6), 17.4 (C-26), 17.2 (C-30), 17.0 (C-24), 13.9 (C-25), 12.6 (C-58) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m*/*z* = 1036.5 (100%, $[M\text{-}Cl]^+$); analysis calcd for $C_{70}H_{95}N_4O_9Cl$ (1172.00): C 71.74, H 8.17, N 4.78; found: C 71.49, H 8.35, N 4.47.

4.19. 3β-Acetyloxy-28-[4-[3-(2,3,6,7,12,13,16,17-octahydro-1H,5H,11H,15H-pyrido [3,2,1-ij]pyrido[1′′,2′′,3′′:1′,8′]quinolino[6′,5′:5,6]pyrano[2,3-f]quinolin-4-ium-9-yl)benzoyl]1,5-diazocan-1-yl]-28-oxo-olean-12-en chloride (24)

Following GP 3 from 14 (100 mg, 0.14 mmol) and rhodamine 101 (200 mg, 0.4 mmol) followed by chromatography (silica gel, ethyl acetate/MeOH, 10% → 50%) 24 (114 mg, 75%) was obtained as a pink solid; m.p. = 205–210 °C; R_f = 0.41 ($CHCl_3$/MeOH, 9:1); UV-Vis ($CHCl_3$): λ_{max} (log ε) = 580 nm (4.23); IR (ATR): *ν* = 2942*w*, 1727*m*, 1595*s*, 1493*m*, 1459*m*, 1362*m*, 1295*s*, 1196*s*, 1035*m*, 746*m*, 420*m* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.81–7.61 (*m*, 2H, 45-H), 7.54–7.43 (*m*, 1H, 42-H), 7.29 (*s*, 1H, 44-H), 6.86–6.47 (*m*, 2H, 48-H), 5.27–5.21 (*m*, 1H, 12-H), 4.51–4.44 (*m*, 1H, 3-H), 3.79–3.15 (*m*, 16H, 33-H, 35-H, 36-H, 38-H, 52-H, 57-H), 3.09–2.90 (*m*, 5H, 18-H, 55-H,), 2.76–2.47 (*m*, 4H, 50-H), 2.15–2.06 (*m*, 4H, 56-H), 2.03 (*s*, 3H, 32-H), 1.97 (*s*, 5H, 16-H_a, 51-H), 1.85 (*s*, 2H, 11-H), 1.70–1.15 (*m*, 20H, 19-H_a, 21-H, 2-H, 1-H_a, 9-H, 6-H_a, 7-H_a, 6-H_b, 22-H_a, 7-H_b, 15-H, 22-H_b, 19-H_b, 34-H, 37-H), 1.12 (*s*, 3H, 30-H), 1.07–0.97 (*m*, 2H, 1-H_b, 16-H_b), 0.91 (*s*, 3H, 25-H), 0.90 (*s*, 3H, 27-H), 0.88 (*s*, 3H, 29-H), 0.85 (*s*, 3H, 23-H), 0.83 (*s*, 3H, 24-H), 0.81–0.79 (*m*, 1H, 5-H), 0.72 (*s*, 3H, 26-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 171.0 (C-28, C-31, C-39), 164.1 (C-53), 152.0 (C-46), 139.9 (C-58), 134.6 (C-41), 131.0 (C-40), 130.5 (C-44), 129.4 (C-45, C-42), 126.9 (C-43), 125.9 (C-48), 123.5 (C-47), 121.4 (C-12), 113.0 (C-49), 105.3 (C-54), 81.0 (C-3), 55.4 (C-5), 51.1 (C-33, C-35, C-36, C-38), 50.5 (C-52, C-57), 48.2 (C-17), 47.6 (C-9), 46.6 (C-19), 43.7 (C-18), 43.3 (C-14), 39.1 (C-8), 38.1 (C-1), 37.7 (C-4), 37.0 (C-10), 33.9 (C-22), 33.0 (C-29), 32.9 (C-7), 30.5 (C-20), 30.3 (C-21), 29.7 (C-15), 28.0 (C-23), 27.8 (C-16), 27.6 (C-50), 25.8 (C-30), 24.1 (C-27), 23.5 (C-2), 23.4 (C-11), 21.3 (C-32), 20.6 (C-51), 19.9 (C-55), 19.7 (C-56), 18.2 (C-6), 17.2 (C-26), 16.7 (C-24), 15.4 (C-25) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m*/*z* = 1068.6 (100%, $[M\text{-}Cl]^+$); analysis calcd for $C_{70}H_{91}N_4O_5Cl$ (1103.97): C 76.16, H 8.31, N 5.08; found: C 75.81, H 8.52, N 4.89.

4.20. 3β-Acetyloxy-28-[4-[3-(2,3,6,7,12,13,16,17-octahydro-1H,5H,11H,15H-pyrido[3,2,1-ij]pyrido[1′′,2′′,3′′:1′,8′]quinolino[6′,5′:5,6]pyrano[2,3-f]quinolin-4-ium-9-yl)benzoyl]1,5-diazocan-1-yl]-28-oxo-urs-12-en chloride (25)

Following GP 3 from 15 (150 mg, 0.2 mmol) and rhodamine 101 (150 mg, 0.3 mmol) followed by chromatography (silica gel, ethyl acetate/MeOH, 10% → 50%) 25 (94 mg, 62%) was obtained as a pink solid; m.p. = 199–202 °C; R_f = 0.43 ($CHCl_3$:Methanol, 9:1); UV-Vis ($CHCl_3$): λ_{max} (log ε) = 571 nm (3.94); IR (ATR): *ν* = 3388*w*, 2925*m*, 1728*m*, 1597*s*, 1495*m*, 1459*m*, 1362*s*, 1297*s*, 1246*s*, 1195*s*, 1100*s*, 1024*s*, 421*s* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 8.36–8.06 (*m*, 1H, 43-H), 7.75–7.63 (*m*, 2H, 42-H, 45-H), 7.24–7.11 (*m*, 1H, 44-H), 6.81–6.50 (*m*, 2H, 48-H), 5.26–5.16 (*m*, 1H, 12-H), 4.49–4.43 (*m*, 1H, 3-H), 3.71–3.21 (*m*, 16H, 33-H, 35-H, 36-H, 38-H, 52-H, 57-H), 3.17–2.90 (*m*, 4H, 55-H), 2.81–2.57 (*m*, 4H, 50-H), 2.23–2.06 (*m*, 4H, 56-H), 2.01 (*s*, 3H, 32-H), 1.98–1.84 (*m*, 6H, 51-H, 11-H_a, 16-H_a), 1.66–1.19 (*m*, 22H, 1-H_a, 11-H_b, 21-H_a, 6-H_a, 22-H_a, 19-H, 6-H_b, 21-H_b, 22-H_b, 2-H, 15-H, 7-H, 16-H_b, 18-H, 34-H, 37-H), 1.13–1.10 (*m*, 3H, 29-H), 1.05 (*s*, 3H, 27-H), 1.04–0.99 (*m*, 2H, 1-H_b, 20-H), 0.92 (*s*, 3H, 24-H), 0.85 (*s*, 3H, 25-H), 0.83 (*s*, 3H, 23-H), 0.82 (*s*, 3H, 30-H), 0.79–0.77 (*m*, 1H, 5-H), 0.72 (*s*, 3H, 26-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 171.0 (C-28), 169.3 (C-31, C-39), 151.2 (C-53), 150.9 (C-46), 135.2 (C-58), 132.3 (C-42), 131.3 (C-43), 130.2 (C-45), 129.1 (C-44), 127.1 (C-48), 125.1 (C-12), 112.6 (C-49), 111.6 (C-47), 105.6 (C-54), 80.9 (C-3), 55.3 (C-5), 47.7 (C-33, C-35, C-36, C-38), 47.5 (C-18), 47.5 (C-9), 45.3 (C-17), 43.3 (C-52, C-57), 41.7 (C-14), 39.6 (C-8), 39.5 (C-19), 38.6 (C-20), 38.2 (C-1), 37.7 (C-4), 36.9 (C-10), 33.1 (C-22), 31.9 (C-7), 30.5 (C-21), 29.7 (C-15), 28.0 (C-16), 27.8 (C-23), 27.5 (C-50), 25.0 (C-34, C-37), 23.5 (C-11), 23.4 (C-27), 23.3 (C-51), 22.6 (C-2), 21.3 (C-32), 19.9 (C-55), 19.7 (C-56), 18.7 (C-29), 18.1 (C-6), 17.3 (C-30), 16.7 (C-26), 15.5 (C-24), 14.1 (C-25) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m*/*z* =

1068.4 (100%, $[M\text{-}Cl]^+$); analysis calcd for $C_{70}H_{91}N_4O_5Cl$ (1103.97): C 76.16, H 8.31, N 5.08; found: C 75.87, H 8.59, N 4.83.

4.21. 3β-Acetyloxy-28-[4-[3-(2,3,6,7,12,13,16,17-octahydro-1H,5H,11H,15H-pyrido[3,2,1-ij] pyrido[1″,2″,3″:1′,8′]quinolino[6′,5′:5,6]pyrano[2,3-f]quinolin-4-ium-9-yl)benzoyl] 1,5-diazocan-1-yl]-28-oxo-lup-20(29)-en chloride (26)

Following GP 3 from 16 (200 mg, 0.14 mmol) and rhodamine 101 (200 mg, 0.4 mmol) followed by chromatography (silica gel, $CHCl_3$/MeOH, 9:1) 26 (103 mg, 68%) was obtained as a pink solid; m.p. = 203–206 °C; R_f = 0.44 ($CHCl_3$/MeOH, 9:1); UV-Vis ($CHCl_3$): λ_{max} (log ε) = 578 nm (4.33); IR (ATR): ν = 2931*w*, 1721*w*, 1595*s*, 1493*s*, 1361*m*, 1294*s*, 1246*s*, 1180*s*, 1035*s*, 746*m*, 622*m*, 421*s* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.68–7.53 (*m*, 2H, 43-H, 45-H), 7.52–7.45 (*m*, 1H, 42-H), 7.31–7.26 (*m*, 1H, 44-H), 6.76–6.59 (*m*, 2H, 48-H), 4.70–4.62 (*m*, 1H, 29-H_a), 4.54–4.49 (*m*, 1H, 29-H_b), 4.44–4.38 (*m*, 1H, 3-H), 3.81–3.02 (*m*, 16H, 33-H, 35-H, 36-H, 38-H, 52-H, 57-H), 3.00–2.89 (*m*, 4H, 55-H), 2.88–2.72 (*m*, 2H, 13-H, 18-H), 2.71–2.51 (*m*, 4H, 50-H), 2.16–2.01 (*m*, 5H, 16-H_a, 56-H), 1.99 (*s*, 3H, 32-H), 1.96–1.87 (*m*, 5H, 21-H_a, 51-H), 1.85–1.74 (*m*, 2H, 15-H_a, 22-H_a), 1.70–1.66 (*m*, 1H, 12-H_a), 1.62 (*s*, 4H, 1-H_a, 30-H), 1.60–1.53 (*m*, 2H, 2-H), 1.50–1.47 (*m*, 1H, 9-H), 1.47–1.40 (*m*, 2H, 6-H_a, 16-H_b), 1.36–1.06 (*m*, 13H, 11-H_a, 21-H_b, , 6-H_b, 7-H, 15-H_b, 19-H, 22-H_b, 11-H_b, 34-H, 37-H), 0.94 (*s*, 2H, 1-H_b, 12-H_b), 0.90 (*s*, 3H, 24-H), 0.86 (*s*, 3H, 25-H), 0.81 (*s*, 3H, 27-H), 0.79 (*s*, 3H, 23-H), 0.73 (*s*, 3H, 26-H), 0.70–0.59 (*m*, 1H, 5-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 170.9 (C-28), 168.8 (C-31, C-39), 151.9 (C-53), 151.3 (C-46), 151.2 (C-20), 138.4 (C-40, C-41), 136.5 (C-58), 130.4 (C-44), 129.4 (C-45, C-42), 127.1 (C-48), 127.0 (C-43) 123.5 (C-47), 113.1 (C-49), 109.1 (C-29), 105.3 (C-54), 81.0 (C-3), 55.5 (C-5), 53.1 (C-9), 52.9, 50.9 (C-33, C-35, C-36, C-38), 50.7 (C-19), 50.5 (C-52, C-57), 49.4 (C-17), 45.9 (C-13), 42.0 (C-14), 40.7 (C-14), 40.6 (C-8), 38.4 (C-1), 37.8 (C-4), 37.1 (C-10), 36.9 (C-18), 36.1 (C-21), 34.3 (C-7), 32.1 (C-16), 31.4 (C-22), 29.9 (C-15), 27.9 (C-23), 27.5 (C-50), 25.5 (C-12), 23.7 (C-2), 22.6 (C-34, C-37), 21.3 (C-32), 21.1 (C-11), 20.6 (C-51), 19.8 (C-55), 19.6 (C-56), 18.7 (C-30), 18.2 (C-6), 16.5 (C-25), 16.4 (C-26), 14.7 (C-24), 14.6 (C-27) ppm; MS (ESI, MeOH/CHCl3, 4:1): *m*/*z* = 1067.2 (100%, $[M\text{-}Cl]^+$); analysis calcd for $C_{70}H_{91}N_4O_5Cl$ (1103.97): C 76.16, H 8.31, N 5.08; found: C 75.98, H 8.52, N 4.83.

4.22. 3β-Acetyloxy-28-[4-[3-(2,3,6,7,12,13,16,17-octahydro-1H,5H,11H,15H-pyrido[3,2,1-ij] pyrido[1″,2″,3″:1′,8′]quinolino[6′,5′:5,6]pyrano[2,3-f]quinolin-4-ium-9-yl)benzoyl] 1,5-diazocan-1-yl]-30-nor-20,28-dioxo-lup-20(29)-en chloride (27)

Following GP 3 from 17 (200 mg, 0.3 mmol) and rhodamine 101 (100 mg, 0.2 mmol) followed by chromatography (silica gel, $CHCl_3$/MeOH, 9:1) 27 (132 mg, 60%) was obtained as a pink solid; m.p. = 208–210 °C; R_f = 0.49 ($CHCl_3$:MeOH, 9:1); UV-Vis ($CHCl_3$): λ_{max} (log ε) = 577 nm (4.66); IR (ATR): ν = 3350*w*, 1596*s*, 11495*s*, 1298*s*, 1197*s*, 1138*s* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.63–7.52 (*m*, 2H, 41-H, 44-H), 7.50–7.43 (*m*, 1H, 42-H), 7.24–7.20 (*m*, 1H, 43-H), 6.72–6.59 (*m*, 2H, 47-H), 4.42–4.33 (*m*, 1H, 3-H), 3.86–3.01 (*m*, 17H, 18-H, 32-H, 34-H, 35-H, 37H, 49-H, 51-H), 2.97–2.85 (*m*, 4H, 54-H), 2.76–2.54 (*m*, 4H, 49-H), 2.52–2.43 (*m*, 1H, 13-H), 2.18–2.06 (*m*, 4H, 21-H_b, 29-H), 2.07–1.99 (*m*, 4H, 55-H), 1.96 (*s*, 4H, 19-H, 31-H), 1.94–1.64 (*m*, 7H, , 50-H, 22-H_a, 16-H_a, 15-H_a), 1.60–1.14 (*m*, 18H, 1-H_a, 2-H, , 21-H_b, 22-H_b, 6-Ha, 16-H_b, 11-H_a, 7-H, 6-H_b, 11-H_b, 15-H_b), 1.08–0.96 (*m*, 3H, 1-H_b, 12-H), 0.90 (*s*, 3H, 24-H), 0.85 (*s*, 3H, 25-H), 0.80 (*s*, 3H, 27-H), 0.76 (*s*, 3H, 23-H), 0.71 (s, 3H, 26-H), 0.69–0.65 (m, 1H, 5-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 213.2 (C-20), 170.9 (C-28, C-30, C-38), 151.9 (C-52), 151.2 (C-45), 136.6 (C-57), 136.3 (C-39, C-40), 130.4 (C-43), 129.4 (C-41, C-44), 126.8 (C-42), 126.7 (C-47), 123.5 (C-46), 113.0 (C-48), 105.2 (C-53), 80.8 (C-3), 55.4 (C-5), 53.0 (C-19), 50.9 (C-32, C-34, C-35, C-37), 50.6 (C-9), 50.4 (C-51, C-56), 50.3 (C-18), 49.8 (C-17), 44.1 (C-13), 41.8 (C-14), 40.6 (C-8), 38.3 (C-1), 37.7 (C-10), 37.1 (C-4), 35.8 (C-22), 34.2 (C-7), 31.8 (C-21), 30.1 (C-29), 29.9 (C-15), 28.8 (C-16), 27.9 (C-23), 27.5 (C-49), 27.3 (C-12), 23.6 (C-2), 22.6 (C-33, C-36), 21.2 (C-31), 21.1 (C-11), 20.6 (C-50), 19.9 (C-54), 19.6 (C-55), 18.1 (C-6), 16.4 (C-26), 15.9 (C-25), 14.7 (C-24), 14.0 (C-27) ppm; MS (ESI, MeOH/$CHCl_3$,

4:1): *m*/*z* = 1070 (100%, $[M\text{-}Cl]^+$); analysis calcd for $C_{69}H_{89}N_4O_6Cl$ (1105.94): C 74.94, H 8.11, N 5.07; found: C 74.73, H 8.35, N 4.81.

4.23. (2α,3ß,4α)2,3,23-Tris(acetoxy)-28-[4-[3-(2,3,6,7,12,13,16,17-octahydro-1H,5H,11H,15H-pyrido[3,2,1-ij]pyrido[1'',2'',3'':1',8']quinolino[6',5':5,6]pyrano[2,3-f]quinolin-4-ium-9-yl)benzoyl]1,5-diazocan-1-yl]-28-oxo-olean-12-en chloride (28)

Following GP 3 from 18 (200 mg, 0.3 mmol) and rhodamine 101 (200 mg, 0.4 mmol) followed by chromatography (silica gel, $CHCl_3$/MeOH, 9:1) 28 (232 mg, 64%) was obtained as a pink solid; m.p. = 193–196 °C; R_f = 0.45 ($CHCl_3$:MeOH, 9:1); UV-Vis ($CHCl_3$): λ_{max} (log ε) = 578 nm (4.50); IR (ATR): ν = 2924*w*, 1739*w*, 1594*s*, 1493*s*, 1459*m*, 1361*m*, 1293*s*, 1195*s*, 1180*s*, 1090*s*, 1035*s*, 729*m*, 622*m*, 421*s* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ =7.67–7.58 (*m*, 2H, 47-H, 49-H), 7.52–7.48 (*m*, 1H, 46-H), 7.29–7.27 (*m*, 1H, 48-H), 6.77–6.65 (*m*, 2H, 52-H), 5.17–5.03 (*m*, 3H, 12-H, 2-H, 3-H), 3.83–3.79 (*m*, 1H, 23-H_a), 3.60–3.16 (*m*, 17H, 23-H_b, 37-H, 39-H, 40-H, 42-H, 56-H, 61-H), 3.00–2.94 (*m*, 4H, 59-H), 2.77–2.63 (*m*, 4H, 54-H), 2.46–2.36 (*m*, 1H, 18-H), 2.07 (*s*, 4H, 60-H), 2.06 (*s*, 3H, 36-H), 2.04–2.01 (*m*, 1H, 1-H_a), 1.99 (*s*, 3H, 34-H), 1.95 (*s*, 7H, 32-H, 55-H), 1.91–1.86 (*m*, 2H, 11-H), 1.60–1.57 (*m*, 1H, 9-H), 1.46–1.42 (*m*, 2H, 21-H_a, 22-H_b), 1.35–1.30 (*m*, 4H, 6-H, 19-H, 5-H), 1.26–1.22 (*m*, 12H, 16-H_a, 38-H, 41-H, , 22-H_b, 7-H, 15-H, 21-H_b, 16-H_b), 1.11–1.09 (*m*, 1H, 1-H_b), 1.04 (*s*, 3H, 24-H), 0.98–0.94 (*m*, 1H, 20-H), 0.91 (*s*, 3H, 29-H), 0.85 (*s*, 3H, 25-H), 0.83 (*s*, 3H, 27-H), 0.82 (*s*, 3H, 30-H), 0.72 (*s*, 3H, 26-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 176.3 (C-28), 170.8 (C-35, C-43), 170.4 (C-33), 170.3 (C-31), 152.0 (C-57), 151.3 (C-50), 139.1 (C-62), 136.6 (C-44), 130.3 (C-45), 129.6 (C-48), 129.2 (C-46) 129.1 (C-49), 127.0 (C-47, C-52), 124.5 (C-12), 123.4 (C-51), 113.0 (C-53), 105.2 (C-58), 74.9 (C-3), 69.9 (C-2), 65.3 (C-23), 55.6 (C-18), 51.0 (C-34, C-37, C-40, C-42), 50.5 (C-56, C-61), 47.7 (C-5), 47.5 (C-9), 46.2 (C-17), 43.7 (C-1), 41.9 (C-4, C-14), 39.5 (C-19), 38.6 (C-20), 37.8 (C-10), 32.6 (C-22), 31.9 (C-7), 30.6 (C-21), 29.7 (C-15), 29.6 (C-16), 27.6 (C-54), 23.3 (C-11), 22.6 (C-38, C-41), 22.6 (C-27), 21.2 (C-29), 21.0 (C-32), 20.8 (C-36), 20.7 (C-34), 20.6 (C-55), 19.9 (C-59), 19.7 (C-60), 17.9 (C-6), 17.4 (C-30), 17.1 (C-24), 17.0 (C-26), 14.1 (C-25) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m*/*z* = 1084.3 (100%, $[M\text{-}Cl]^+$); analysis calcd for $C_{74}H_{95}N_4O_9Cl$ (1220.04): C 72.85, H 7.85, N 4.59; found: C 72.63, H 8.01, N 4.39.

4.24. Cell culture

Breast cancer cell lines were obtained from Department of Radiobiology (MLU Halle-Wittenberg) and previously described. MDA-MB-231, HS578T and MCF-7 and T47D were cultured as monolayer in RPMI (Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (Capricorn Scientific, Ebsdorfergrund, Germany), 2% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and 1% sodium pyruvate (Gibco, Thermo Fisher Scientific) at 37 °C and 5% CO_2. All cell lines were regularly tested for mycoplasma contamination.

4.25. SRB assay

Breast cancer cells were seeded in 96 well plates with different cell numbers depending on cell line in triplicate and after 24 h treated with different concentrations of compound 14–28. Treatment ended after 96 h when cells were fixed with 10% trichloroacetic acid (Carl Roth GmbH, Karlsruhe, Germany) for 1h at 4 °C. Afterwards cells were washed with ice water four times and stained with 4.4% SRB solution (Sigma–Aldrich) for 10 min at room temperature. After washing cells with 1% acetic acid (Carl Roth GmbH), cells were air-dried overnight and then dissolved with 300 µl 20 mM Tris base solution (Sigma-Aldrich). Excitation was measured at 540 nm with Spark plate reader (Tecan Treading AG, Männedorf, Switzerland) and IC_{50} values were calculated by dose response curve fitting using Origin 2019 (OriginLab Corp., Northampton, MA, USA).

4.26. Cell death

For determination of apoptotic and necrotic cell death after treatment with compound 28 Annexin V-Sytox Deep Red staining was performed. Therefore MDA-MB-231 and HS578T cell were seeded in 6-well plates. After 24 h cells were treated with different concentrations of compound 28 (10 nM, 100 nM, 250 nM, 500 nM, 1 μM and 2 μM) for 24 h, 48 h and 72 h at 37 °C and 5% CO_2. For analysis of cell death detached cells were collected in tubes and living cells were detached by accutase (Biowest, Nuaillé, France) and collected in the same tube. After several washing steps cells were resuspended in 1x annexin V binding puffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$) and stained with 5 μl Annexin V-FITC (BioLegend, San Diego, CA, USA) and 1 μl 100 μM Sytox Deep Red Nucleic Acid Stain (Invitrogen, Thermo Fisher Scientific) for 15 min. Afterwards 400 μl 1x annexin V binding puffer were added to each tube. Gating was realized by use of unstained, single annexin V-FITC or single Sytox Deep Red Nucleic Acid-stained cells, respectively. For quantification of necrotic and apoptotic cells 10,000 cells were analyzed by LSRFortessa™ flow cytometer (BD Biosciences, Heidelberg, Germany).

4.27. Proliferation

MDA-MB-231 and HS578T cells were seeded in 6-well plates and treated with different concentrations (10 nM, 100 nM, 250 nM, 500 nM, 1 μM and 2 μM) of compound 28 after 24 h. Cell number of dead and viable cells was measured by use of CASY cell counter (OMNI Life Science, Bremen, Germany) after 72 h.

4.28. Staining

Analysis of subcellular localization of compound AS101 was performed in MDA-MB-231 cells using the mitochondrial targeting compound BioTracker™ 488 Green Mitochondria Dye (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) for comparison. Cells were seeded in a μ-Plate 96 Well Black plate (ibiTreat: #1.5 polymer coverslip bottom, ibidi GmbH, Gräfelfing, Germany) at cell density of 50.000 per well. After 24h cells were treated with 100nM AS101 for 6h or 100nM BioTracker488 for 30min, followed by rinsing and supplementation with RPMI 1640 w/o Phenol-red (Pan-Biotech GmbH, Aidenbach, Germany). Live cell imaging was performed on an Axio Observer 7 (Carl Zeiss Microscopy Deutschland GmbH, Oberkochen, Germany) using the settings for Ex/Em as followed: BioTracker (475nm/514nm), AS101 (555nm/592). Scale bar: 50μm.

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Developing an amide spacered triterpenoid rhodamine hybrid of nano-molar cytotoxicity combined with excellent tumor cell/non-tumor cell selectivity

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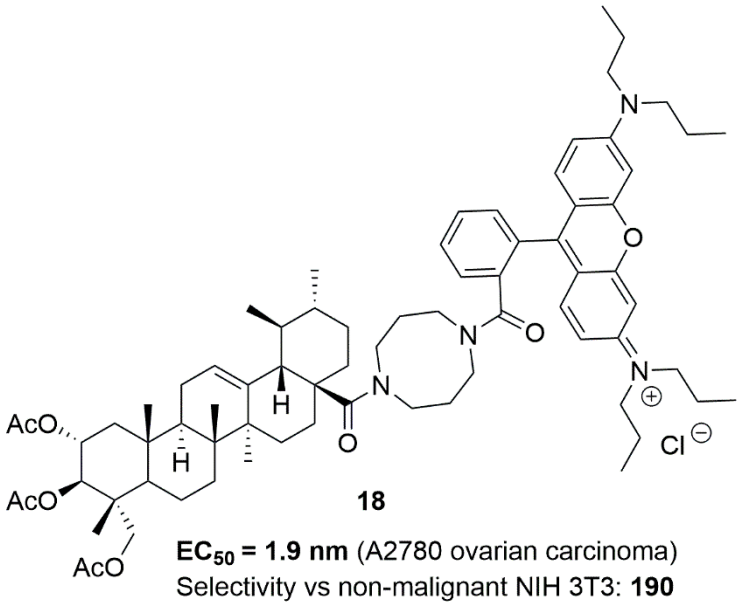
Graphical abstract



Keywords: Asiatic acid; rhodamine; cytotoxicity

Abstract: Asiatic acid, a pentacyclic triterpene was converted into a series of piperazinyl, homopiperazinyl and 1,5-diazocinyl spacered rhodamine conjugates differing in the type of spacer and the substitution pattern on the rhodamine part of the hybrids. The compounds were tested for cytotoxic activity in SRB assays and compound **12** holding an EC_{50} of 0.8 nM was the most cytotoxic compound of this series, but **18** (holding a ring expanded 1,5-diazocinyl moiety and *n*-propyl substituents on the rhodamine) was the most selective compound exhibiting a selectivity factor of almost 190 while retaining high cytotoxicity (EC_{50} = 1.9 nM, for A2780 ovarian carcinoma)

Introduction

Previous studies revealed acetylated conjugates of triterpene carboxylic acids with secondary cyclic amines and rhodamine B or rhodamine 101 in the low nano-molar concentration range to be cytotoxic to several different human cancer cell lines.[1-12] Additional studies showed that these compounds act as mitocans.[13] Furthermore, it could be determined that conjugates of triterpenes holding only one acetyl group in ring A (e. g., derived from oleanolic acid, ursolic acid, glycyrrhetinic acid, etc.) are strongly cytotoxic, but are surpassed in efficacy by those compounds bearing 2 or 3 acetyl groups, such as in maslinic acid,[14-22] madecassic acid,[23] tormentic acid,[24, 25] euscaphic acid [3] and corosolic acid,[26] but also conjugates of asiatic acid.[4, 27] Most recently, an asiatic acid rhodamine hybrid was established as an excellent cytotoxic agent holding cytotoxic activity in a sub-nanomolar concentration.[27] Furthermore, it was established that this compound inhibited the mitochondrial synthesis of ATP, and to be cytotoxic even to several multi-resistant human cancer cell lines. In addition, a first trend was established showing those amide spacered triterpenoid-rhodamine hybrids holding two acetyl groups in ring A to be more cytotoxic than those with only one acetyl groups, and compounds with a 2α, 3β configuration of these acetyl groups were shown to be superior to those compounds holding acetyl groups at positions C-2 and C-3 in a different configuration.[3] A clear indication of whether rhodamine B conjugates are superior to those with a rhodamine 101 moiety has not yet been obtained.[2] Only recently it was discovered for piperazinyl spacered rhodamine conjugates that the substitution pattern on the rhodamine skeleton exerts some effects onto the cytotoxicity of the compounds as well.[5] However, a systematic study further investigating the dependence of the observed cytotoxicity on the type of cyclic spacer [29] in combination with spacers of different ring sizes is also missing. It was only shown that the conjugates must contain lipophilic cations.[30-37] While lipophilic cations derived from structurally simple ammonium salts showed only moderate cytotoxicity [38, 39] diminished cytotoxicity was also established for BODIPY conjugates [33, 40, 41] as hybrids holding a malachite green [42] scaffold. Conjugates holding an ethylenediamine spacer and a rhodamine B moiety were also not active.[10] The latter compounds do not form cationic structures under physiologic conditions but rather exist as neutral compounds.[43] However, it does not seem to have any influence on cytotoxicity whether the triterpene had an ursane or oleanane backbone, as a comparison of analogous compounds from maslinic acid and corosolic acid showed.[3]

Results and discussion

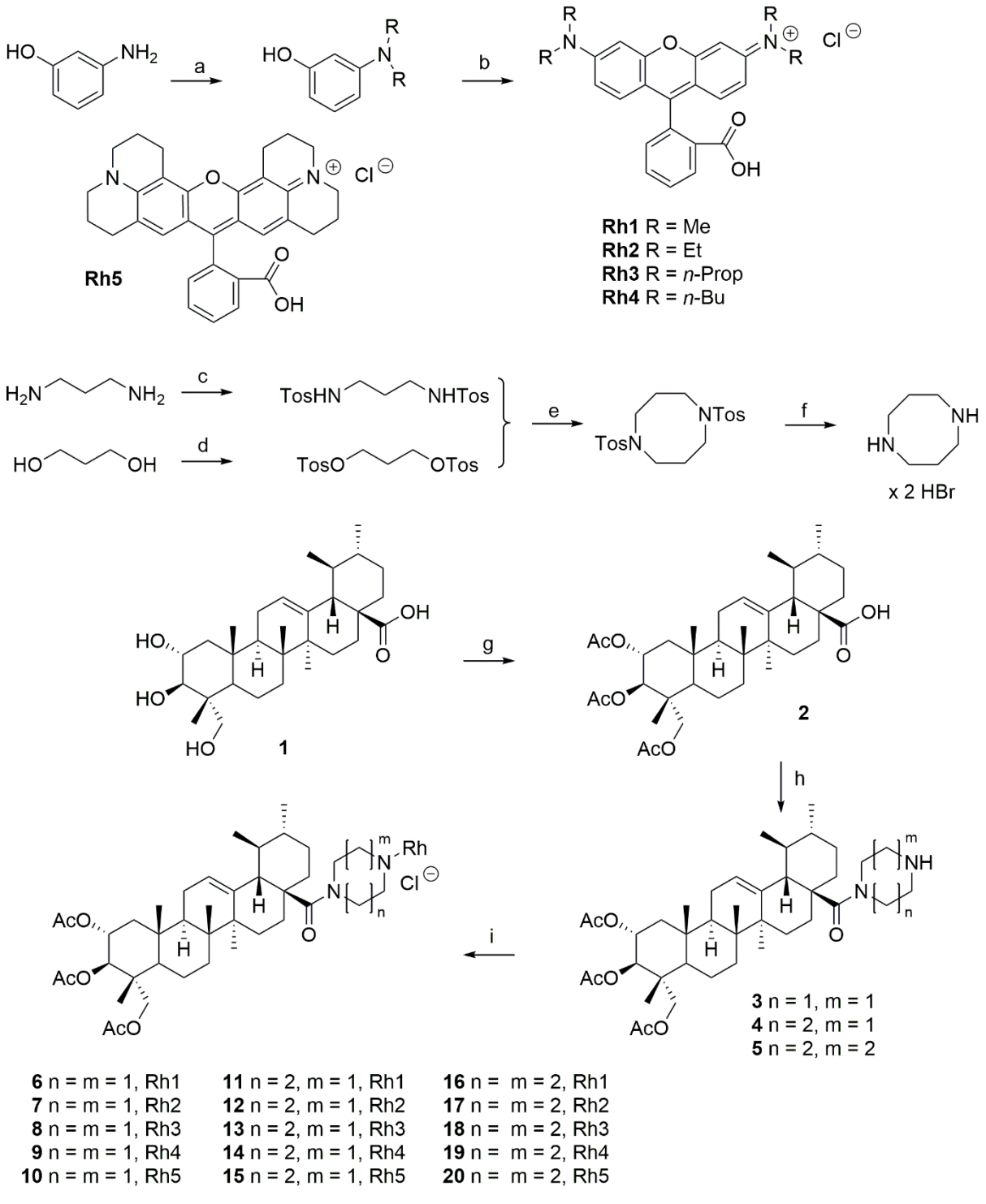
From these premises, it was concluded that asiatic acid (**1**, Scheme 1) should be an ideal starting material for systematic studies. Asiatic acid holds 3 hydroxyl groups at positions C-2, C-3, and C-23; it is commercially available at reasonable prices. In addition, the two hydroxyl groups in ring A are configured 2α,3β.

Since the amide-bound spacer must be a secondary amine group, piperazine, homopiperazine (perhydro-1,4-diazepane), and the ring-extended analog octahydro-1,5-diazocine (a "bis-homopiperazine") were selected. While the first two amines are commercially available, the latter is not commercially available but can be synthesized by a number of different routes reported in the literature.[44-53] Many different routes were tried, but in the end only one proved to be truly feasible.[51] Tosylation of 1,3-propane-diamine (Scheme 1) and 1,3-propanediol with *p*-toluenesulfonyl chloride gave the corresponding di-tosylates. Their reaction with sodium methoxide in refluxing methanol afforded 1,5-bis(*p*-toluenesulfonyl)-1,5-diazacyclooctane in 84% isolated yield. Its treatment with HBr (33% in glacial acetic acid) in the presence of an excess of thioanisole [54] afforded octahydro-1,5-diazocine as dihydromide in 92% isolated yield. This meant that also the third spacer to be used in this study was now readily available and could be prepared in larger quantities.

A further problem arose in the selection of the differently substituted rhodamines. While rhodamine B and rhodamine 101 are commercially available, this is not the case for analogs differing in the substituents at the two nitrogen substituents of the rhodamine. The syntheses of the alkyl substituted rhodamines were performed as previously reported.[4, 5, 27] In short, the reaction of 3-aminophenol with an excess of the corresponding alkyl halide and potassium carbonate in DMF at 100 °C for 3–8 h gave 3-(dialkylamino)-phenols in 50–70% yield. Their reaction with phthalic anhydride in the presence of aluminum chloride (catalytic amounts) at 200 °C followed by chromatography (silica gel, $CHCl_3$/MeOH mixtures) yielded the rhodamines Rh1-Rh4 each as a violet solid.

Thus, all the building blocks were now available. Asiatic acid (**1**) was acetylated (Scheme 1) as described earlier, und tri-*O*-acetyl-asiatic acid (**2**) was obtained in 97% yield.[4, 27] Activation of **2** with oxalyl chloride furnished an intermediary acid chloride that was allowed to react with piperazine, homopiperazine and its ring-expanded homolog, octahydro-1,5-diazocine to yield amides **3**–**5**. Reaction with the rhodamines Rh1–Rh5 (previously activated with oxalyl chloride)

gave the piperazine-derived conjugates **6**–**10**, the homopiperazine derivatives **11**–**15**, and the ring expanded compounds **16**–**20**. All the conjugates exhibited their typical purple color, clearly demonstrating that they are present in a cationic form.[10]



Scheme 1. Synthesis of starting material (rhodamines Rh1–Rh4; structure of Rh5) and compounds **2**–**20**; reactions and conditions: a) DMF, K_2CO_3, DMF; for R = Me (from MeI, 52%), for R = Et (from EtBr, 67%), for R = *n*-Prop (from *n*-Prop-Br, 61%), for R = *n*-Bu (from *n*-Bu-Br, 68%), 3–8 h, 21 °C; b) phthalic anhydride, $AlCl_3$ (cat.), 5–60 min, 200 °C: for R =

Me 35%, R = Et 45%, R = *n*-Prop 42%, R = *n*-Bu 47%; c) TosCl, H_2N-$(CH_2)_3$-NH_2, no solvent, 80 °C, 30min, 83%; d) TosCl, HO-$(CH_2)_3$-OH, pyridine, 0 °C, 30 min, 87%; e) NaOMe, MeOH, DMF, 80 °C, 12 h, 84%; f) HBr (33% in glacial acetic acid), 80 °C, 3 h, 92%; g) Ac_2O, DCM, NEt_3, DMAP (cat.), 21 °C, 24 h, 97%; h) DCM, $(COCl)_2$, DMF (cat), NEt_3; then piperazine (→ **3**, 86%), homopiperazine (→ **4**, 79%) or 1,5-diazocinyl dihydrobromide (→ **5**, 68%); i) Rh1–Rh5, $(COCl)_2$, NEt_3, DCM, DMF (cat.), 21 °C, 3 h, then 3–5, NEt_3, DMAP (cat.), 1h: **6** (59%), **7** (80%), **8** (57%), **9** (49%), **10** (55%), **11** (49%), **12** (57%), **13** (64%), **14** (58%), **15** (63%), **16** (55%), **17** (60%), **18** (68%), **19** (70%), **20** (64%).

To assess the cytotoxicity of the compounds sulforhodamine (SRB) assays were performed. First, the cytotoxicity of the different rhodamines was determined; the results from these assays are compiled in Table 1.

Table 1. Cytotoxicity of rhodamines Rh1–Rh5 (in μM) from SRB assays after 72 h of treatment: averaged from three independent experiments each in triplicate; confidence interval CI = 95%. Human tumor cell lines: A375 (melanoma), HT29 (colorectal carcinoma), MCF7 (breast adenocarcinoma), HeLa (cervical adenocarcinoma), A2780 (ovarian carcinoma), NIH 3T3 (nob malignant fibroblasts, murine); cut-off of the assay 30 μM; n.d. not determined; doxorubicin (**DX**) has been used as a positive standard.

	A375	HT29	MCF7	A2780	HeLa	NIH 3T3
Rh1	>30	>30	>30	>30	>30	>30
Rh2	>30	>30	>30	>30	>30	>30
Rh3	8.2 ± 0.2	9.3 ± 0.4	6.4 ± 0.4	6.7 ± 0.7	8.0 ± 0.7	12.0 ± 0.7
Rh4	4.3 ± 0.1	4.9 ± 0.3	3.08 ± 0.06	3.4 ± 0.1	4.3 ± 0.3	4.3 ± 0.1
Rh5	11.2 ±1.8	18.5 ±1.8	8.2 ± 0.8	8.0 ±1.5	11.8 ± 1.1	11.9 ±1.3
DX	n.d.	0.9 ± 0.2	1.1 ± 0.3	0.02 ± 0.01	n.d.	11.9 ±1.3

Thereby methyl- and ethyl (= rhodamine B) substituted rhodamines Rh1 and Rh2 proved to be not cytotoxic within the limits of the assay (EC_{50} > 30 μM) for all human tumor cell lines but also for the non-malignant fibroblasts (NIH 3T3). Increased cytotoxicity, however, was observed for the rhodamines carrying propyl or butyl moieties. Thereby the butyl substituted rhodamine Rh4 was higher cytotoxic than the propyl substituted analog Rh3. Rh5 (= rhodamine 101) was about as cytotoxic as the propyl substituted rhodamine Rh3.

The cytotoxic activity of the conjugates **6**–**20** was also investigated in SRB assays employing the human tumor cell lines A375, HT29, MCF7, A2780, HeLa and, for comparison, non-malignant fibroblasts NIH 3T3. The results from these assays are summarized in Table 2.

Table 2. Cytotoxicity of conjugates **6**–**20** (in μM) from SRB assays after 72 h of treatment: averaged from three independent experiments each in triplicate; confidence interval CI = 95%. Human tumor cell lines: A375 (melanoma), HT29 (colorectal carcinoma), MCF7 (breast adenocarcinoma), HeLa (cervical adenocarcinoma), A2780 (ovarian carcinoma), NIH 3T3 (nob malignant fibroblasts, murine); cut-off of the assay 30 μM; n.d. not determined; doxorubicin (**DX**) has been used as a positive standard.

	A375	HT29	MCF7	A2780	HeLa	NIH 3T3
6	0.15 ± 0.008	0.23 ± 0.08	0.15 ± 0.03	0.043 ± 0.008	0.70 ± 0.2	0.55 ± 0.06
7	0.027 ± 0.009	0.026 ± 0.009	0.034 ± 0.009	0.0056 ± 0.0006	0.20 ± 0.1	0.33 ± 0.08
8	0.032 ± 0.003	0.031 ± 0.007	0.034 ± 0.005	0.0055 ± 0.0005	0.16 ± 0.05	0.44 ± 0.09
9	0.016 ± 0.003	0.026 ± 0.008	0.05 ± 0.01	0.0049 ± 0.0007	0.21 ± 0.05	0.40 ± 0.1
10	0.045 ± 0.004	0.05 ± 0.02	0.05 ± 0.01	0.009 ± 0.002	0.22 ± 0.05	0.25 ± 0.05
11	0.039 ± 0.003	0.02 ± 0.01	0.028 ± 0.005	0.0032 ± 0.0004	0.40 ± 0.1	0.30 ± 0.1
12	0.0028 ± 00005	0.0055 ± 0.0023	0.0071 ± 0.0021	0.0008 o± 0.0001	0.0177 ± 0.0049	0.0650 ± 0.0262
13	0.0039 ± 0.0006	0.006 ± 0.001	0.010 ± 0.001	0.0011 ± 0.0001	0.041 ± 0.009	0.10 ± 0.05
14	0.014 ± 0.001	0.025 ± 0.005	0.049 ± 0.006	0.0045 ± 0.0004	0.15 ± 0.05	0.20 ± 0.1
15	0.008 ± 0.001	0.010 ± 0.006	0.019 ± 0.006	0.0031 ± 0.0005	0.08 ± 0.03	0.14 ± 0.05
16	0.07 ± 0.01	0.05 ± 0.02	0.05 ± 0.01	0.006 ± 0.001	0.50 ± 0.2	0.40 ± 0.09
17	0.03 ± 0.01	0.03 ± 0.01	0.025 ± 0.02	0.007 ± 0.002	0.06 ± 0.01	0.45 ± 0.02
18	0.0074 ±0.0009	0.012 ±0.004	0.017 ±0.002	0.0019 ± 0.0002	0.07 ± 0.02	0.36 ± 0.05
19	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.005	0.006 ± 0.002	0.17 ± 0.08	0.24 ± 0.08
20	0.02 ± 0.01	0.01 ± 0.005	0.0039 ± 0.002	0.004 ± 0.001	0.11 ± 0.06	0.32 ± 0.02
DX	n.d.	0.9 ± 0.2	1.1 ± 0.3	0.02 ± 0.01	n.d.	11.9 ±1.3

As a result, for the piperazinyl spacered compounds **6**–**10** the cell line A2780 (ovarian carcinoma) proved to be the most sensitive, and the lowest EC_{50} values were determined ranging between 0.043 μM to 0.0049 μM. As compared to non-malignant NIH 3T3 fibroblasts a selectivity factor of 82 was determined showing this compound to be more cytotoxic to the cancer cells than to the non-malignant cells. In this series of compounds, also the butyl substituted rhodamine conjugated asiatic acid hybrid **9** proved best for A375 (melanoma) and HT29 (colon adenocarcinoma) cells.

Interestingly, for the homopiperazinyl conjugates **11**–**15** those compounds performed best holding a rhodamine B (Rh2) unit, and for A2780 cells a low EC_{50} value of 0.8 nm was determined. The selectivity factor (compared to NIH 3T3) was about 81. Also, A375, HT29,

MCF7 (breast adenocarcinoma) and HeLa (cervix carcinoma) cells responded well to this compound, and low EC_{50} values between 0.0028 and 0.0177 μM were observed.

Increasing the ring size of the spacer using the 1,5-diazocinyl spacer changed the dependance of cytotoxicity with respect to the residue attached to the rhodamine core inasmuch as no longer the rhodamine B derivatives performed best but the *N*, *N*-dipropyl substituted compound **18**; thereby, for A2780 cells an EC_{50} = 1.9 nM was determined, and an excellent selectivity factor of almost 190 was thereby observed.

Rhodamine 101 (Rh5) conjugates behaved - more or less - like the alkyl substituted rhodamine conjugates, and the lowest EC_{50} values were again measured for A2780 cells, and thereby the lowest EC_{50} value was 0.004 μM (selectivity factor = 80).
To sum up, all human tumor cell lines were shown to be very sensitive for the spacered asiatic acid rhodamine hybrids, and a significant influence of the substitution pattern of the rhodamine onto the cytotoxic effect was observed. A similar behavior was established for the influence of the amide spacer connecting the triterpenoid skeleton and the rhodamine part. Hereby, for example, for A2780 cells and propyl substituents the EC_{50} decreased for the piperazinyl spacered **8** (5.5 nM) to EC_{50} = 1.1 nM (for **13**) but increased again slightly for **18** (EC_{50} = 1.9 nM). Although there is no generalization possible to answer the question which spacer/rhodamine combination performed best with the respect to all cell lines, it seems that homopiperazinyl and 1,5-diazocinyl derivatives are superior to those carrying a “simple” piperazinyl moiety. Investigations employing 3D spheroid models as well as multi-resistant tumor cell lines (such as MDA-MB 231) will be carried out in due course to investigate the potential of these conjugates in more detail.

Conclusion

A series of piperazinyl, homopiperazinyl and 1,5-diazocinyl spacered rhodamine conjugates was prepared from Asiatic acid, a naturally occurring pentacyclic triterpene. The hybrids were tested for cytotoxic activity in SRB assays with respect to a different ring size of the spacer and to a different substitution pattern of the rhodamine scaffold. Thereby compound **12** holding an EC_{50} of 0.8 nM was the most cytotoxic compound of this series but **18** with EC_{50} = 1.9 nM was the most selective compound exhibiting a selectivity factor of almost 190.

Experimental

TLC was carried out on silica gel (Macherey-Nagel, detection under UV light and with cerium molybdate reagent). The used solvents were dried according to usual procedures. Melting points are uncorrected (Leica hot stage microscope, or BÜCHI melting point M-565). IR spectra were recorded on a Perkin Elmer FT-IR spectrometer Spectrum 1000 or on a Perkin-Elmer Spectrum Two (UATR Two Unit). NMR spectra were recorded using the Agilent spectrometers DD2 500 MHz and VNMRS 400 MHz (δ given in ppm, J in Hz; typical experiments: APT, H-H-COSY, HMBC, HSQC, NOESY), MS spectra were taken on an Advion Expression CMS instrument, and elemental analyses were conducted on a Foss-Heraeus Vario EL (CHNS) unit. Rh5 (rhodamine 101) as well as asiatic acid (**1**) were obtained from local vendors and used as received.

Synthesis of substituted rhodamines Rh1–Rh4

The synthesis was performed as previously published.[5]

Synthesis of 1,5-diazocinyl dihydrobromide.

This compound was prepared as described in the literature.[51]

General procedure for the synthesis of triterpenoic amides 3–5 (GP A)

To a solution of **1** (1 eq.) in dry DCM (10 mL), oxalyl chloride (4 eq.), DMF (0.24 eq.) and NEt_3 (0.24 eq.) were added. After stirring for 2h at room temperature, the solvent was removed under reduced pressure, re-evaporated with DCM (3 x 10 mL), and the residue was dissolved in dry DCM (10 mL). A solution of the piperazine, homopiperazine or 1,5-diazocinyl dihydromide (3 eq.) in dry DCM, NEt_3 (1 eq.; 4 eq. when using 1,5-diazocinyl dihydromide) and DMAP (cat.) was added, and stirring at room temperature was continued until completion of the reaction (as indicated by TLC). The solvent was removed under reduced pressure, and the crude product was subjected to column chromatography to yield compounds **3–5**.

General procedure for synthesis of rhodamine B conjugates 6–20 (GP B)

A solution of the corresponding rhodamine Rh1–Rh5 (1.5 eq.), oxalyl chloride (6 eq.), DMF (0.2 eq.) and NEt_3 (0.2 eq.) in dry DCM (30 mL) was stirred at ambient temperature for 3h. The solvent was removed under reduced pressure, re-evaporated with dry DCM (3 x 30 mL), and the residue was dissolved in dry DCM (30 mL). To this solution compounds **3–5** (1 eq.), NEt_3 (1.5 eq.) and DMAP (cat.) were added. After stirring for 1h, the solvent was removed under

reduced pressure, and the resulting solid was subjected to column chromatography to yield compounds **6–20** each as a purple solid.

Tri-*O*-acetyl-asiatic acid [(2α, 3β, 4α) 2,3,23-Tris(acetyloxy)-urs-12-en-28-oic acid] (2)

Acetylation of asiatic acid (**1**, 11.2 g, 21.8 mmol) in dry DCM (250 mL) with Ac_2O (20.0 mL, 210 mmol) in the presence of NEt_3 (26 mL, 180 mmol) and catal. DMAP for 24 h followed by usual aq. workup and chromatography (SiO_2, hexanes/ethyl acetate, 7:3) gave **2** (97%) as a colorless solid; m.p. 160-162 °C (lit.: [4, 27]160-163 °C);); $[\alpha]_D^{20}$ = +35.8° (*c* 0.25, $CHCl_3$) (lit.:[4, 27] $[\alpha]_D^{20}$ = +34.71° (*c* 0.35, $CHCl_3$); R_F = 0.29 (hexanes/ethyl acetate, 7:3); MS (ESI, MeOH): *m/z* = 615.2 (15% $[M+H]^+$, 637.3 (100%, $[M+Na]^+$).

(2α, 3β, 4α) 28-Oxo-28-piperazin-1-yl-urs-12-ene-2,3,23-triyl triacetate (3)

Following GP A, from **2** (200 mg, 0.32 mmol) and piperazine (85 mg, 0.97 mmol) **3** (190 mg, 86%) was obtained as a colorless solid; m.p. 158–160 °C (lit.: [4]157–160 °C); $[\alpha]_D^{20}$ = +17.20° (c 0.31, $CHCl_3$), lit.:); $[\alpha]_D^{20}$ = +17.48° (*c* 0.27, $CHCl_3$); R_F = 0.33 (SiO_2, $CHCl_3$/MeOH, 9:1); MS (ESI, MeOH): *m/z* = 683.4 (100%, $[M+H]^+$).

(2α, 3β, 4α) 28-(1,4-Diazepan-1-yl)-28-oxo-urs-12-ene-2,3,23-triyl triacetate (4)

Following GP A, from **2** (500 mg, 0.81 mmol) and homopiperazine (210 mg, 2.43 mmol) **4** (445 mg, 79%) was obtained as a colorless solid; m.p. 185–187 °C (lit.:[4] 185.3–186.3 °C);); $[\alpha]_D^{20}$ = = +14.5° (*c* 0.21, $CHCl_3$), lit.:); $[\alpha]_D^{20}$ = +14.38° (*c* 0.145, $CHCl_3$); R_F = 0.40 (SiO_2, $CHCl_3$/MeOH, 9:1); MS (ESI, MeOH): *m/z* = 697.4 (100%, $[M+H]^+$).

(2α, 3β, 4α) 28-(1,5-Diazocin-1-yl)-28-oxo-urs-12-ene-2,3,23-triyl triacetate (5)

Following GP A from **2** (400 mg, 0.80 mmol) and 1,5-diazocinyl dihydromide (660 mg, 2.4 mmol) **5** (390 mg, 68%) was obtained as a colorless solid; m.p. = 188–191 °C (decomp.); R_F = 0.38 ($CHCl_3$/MeOH, 9:1); $[\alpha]_D^{20}$ = -30.2° (*c* 0.015, $CHCl_3$); IR (ATR): *ν* = 2925*w*, 1741*s*, 1623*w*, 1368*m*, 1231*s*, 1042*m*, 748*w* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 5.22 – 5.17 (*m*, 1H, 12-H), 5.14 – 5.08 (*m*, 1H, 2-H), 5.04 – 5.01 (*m*, 1H, 3-H), 3.80 (*m*, 1H, 23-H_a), 3.51 (*m*, 1H, 23-H_b), 3.32 – 2.67 (*m*, 8H, 37-H, 39-H, 40-H, 42-H), 2.40 – 2.34 (*m*, 1H, 18-H), 2.03 (*s*, 3H, 36-H), 2.02 – 2.00 (*m*, 1H, 1-H_a), 1.97 (*s*, 3H, 34-H), 1.92 (*s*, 3H, 32-H), 1.88 – 1.69 (*m*, 5H, 11-H, 16-H 22-H_a), 1.60 – 1.45 (*m*, 4H, 22-H_b, 9-H, 21-H_a, 7-H_a), 1.53 – 1.45 (*m*, 2H, 16-H_a, 16-H_b), 1.33 – 1.14 (*m*, 10H, 19-H, 5-H, 21-H_b, 7-H_b, 15-H, 38-H, 41-H), 1.11 – 1.09 (*m*, 1H, 1-H_b), 1.05 (*s*, 3H, 27-H), 1.02 (s, 3H, 25-H), 0.99-0.96 (*m*, 1H, 20-H), 0.91 (*s*, 3H, 30-H),

0.84 (*s*, 3H, 24-H), 0.82 (*s*, 3H, 29-H), 0.70 (*s*, 3H, 26-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 176.8 (C-28), 170.8 (C-35), 170.3 (C-33), 170.3 (C-31), 138.7 (C-13), 124.9 (C-12), 74.8 (C-3), 69.8 (C-2), 65.2 (C-23), 55.6 (C-18), 47.6 (C-9), 47.5 (C-5), 46.1 (C-37, C-39, C-40, C-42), 43.7 (C-1), 42.3 (C-14), 41.9 (C-4), 39.5 (C-8), 39.3 (C-19), 38.5 (C-20), 37.8 (C-10), 34.8 (C-22), 34.7 (C-7), 31.9 (C-21), 29.6 (C-15), 23.3 (C-11), 23.2 (C-16), 22.6 (C-38, C-41), 21.2 (C-30), 21.0 (C-36), 20.8 (C-32), 20.7 (C-34), 17.8 (C-6), 17.4 (C-27), 17.3 (C-29), 17.1 (C-25), 13.9 (C-26), 8.7 (C-24) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m*/*z* = 711.2 (100%, [M+H$^+$); analysis calcd for $C_{42}H_{66}N_2O_7$ (711.00): C 70.95, H 9.36, N 3.94; found: C 70.71, H 9.63, N 3.75.

(2α,3β,4α)-9-[2-{[4-(2,3,23-Tris(acetyloxy)-urs-12-en-28-oyl)-piperazinyl]carbonyl}phenyl]-3,6-bis(dimethylamino)-xanthylium chloride (6)

Following GB B from **3** (150 mg, 0.22 mmol) followed by chromatography (SiO_2, $CHCl_3$/MeOH, 9:1) **6** (140 mg, 59%) was obtained as a violet solid; m.p. = 248–250°C; R_f = 0.62 (SiO_2, $CHCl_3$/MeOH, 8:2); UV-Vis (MeOH): λ_{max} (log ε) = 552 nm (4.71); IR (ATR): ν = 2923*m*, 2869*w*, 1737*m*, 1630*m*, 1592*vs*, 1534*w*, 1493*m*, 1457*w*, 1406*m*, 1365*m*, 1340*s*, 1231*s*, 1184*vs*, 1124*m*, 1042*m*, 1004*m*, 926*m*, 823*m*, 699*m*, 596*w*, 580*w*, 517*w* cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.71–7.60 (*m*, 2H, 44-H, 46-H), 7.55–7.48 (*m*, 1H, 47-H), 7.39–7.29 (*m*, 1H, 45-H), 7.28–7.22 (*m*, 2H, 51-H), 7.07–6.93 (*m*, 2H, 50-H), 6.86–6.77 (*m*, 2H, 53-H), 5.19–5.08 (*m*, 2H, 12-H, 2-H), 5.04 (*d*, *J* = 10.3 Hz, 1H, 3-H), 3.83 (*d*, *J* = 11.6 Hz, 1H, 24-H$_a$), 3.54 (*d*, *J* = 11.9 Hz, 1H, 24-H$_b$), 3.43–3.19 (*m*, 8H, 37-H, 38-H, 39-H, 40-H), 3.32 (*s*, 12H, 55-H, 56-H), 2.41–2.22 (*m*, 1H, 18-H), 2.05 (*s*, 3H, 32-H), 2.04–2.00 (*m*, 1H, 1-H$_a$), 1.99 (*s*, 3H, 34-H), 1.94 (*s*, 3H, 36-H), 1.93–1.85 (*m*, 3H, 11-H, 16-H$_a$), 1.71–1.63 (*m*, 2H, 16-H$_b$, 22-H$_a$), 1.60–1.54 (*m*, 1H, 9-H), 1.53–1.19 (*m*, 9H, 22-H$_b$, 21-H$_a$, 7-H$_a$, 6-H, 19-H, 5-H, 21-H$_b$, 7-H$_b$), 1.10 (*d*, *J* = 12.2 Hz, 1H, 1-H$_b$), 1.04 (*s*, 4H, 25-H, 15-H$_a$), 1.02 (*s*, 4H, 27-H, 15-H$_b$), 0.89 (*s*, 4H, 20-H, 30-H), 0.85 (*s*, 3H, 23-H), 0.82–0.79 (*m*, 3H, 29-H), 0.63 (*s*, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 175.9 (C-28), 170.9 (C-35), 170.5 (C-31), 170.4 (C-33), 167.7 (C-41), 157.6 (C-54), 156.4 (C-48, C-52), 144.1 (C-13), 135.2 (C-43), 131.8 (C-51), 130.5 (C-42), 130.4 (C-46), 130.4 (C-44), 130.2 (C-45), 127.8 (C-47), 124.7 (C-12), 114.5 (C-50), 114.0 (C-49), 96.9 (C-53), 74.9 (C-3), 70.0 (C-2), 65.3 (C-24), 55.1 (C-18), 48.7 (C-17), 47.7 (C-9), 47.6 (C-5), 43.8 (C-1), 42.2 (C-37, C-38, C-39, C-40), 42.2 (C-14), 42.0 (C-4), 41.2 (C-55, C-56), 39.5 (C-19), 38.7 (C-20), 37.9 (C-8, C-10), 34.2 (C-22), 32.4 (C-7), 30.4 (C-21), 28.1 (C-15), 23.4 (C-11, C-16), 23.4 (C-27), 21.2 (C-36), 21.1 (C-30), 21.0 (C-34), 20.8 (C-32), 17.9 (C-6), 17.4 (C-29), 17.1 (C-25), 16.9 (C-26), 14.0 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$): *m*/z = 1052 (100%, [M-

Cl]$^+$); analysis calcd for $C_{64}H_{834}N_4O_9Cl$ (1087.84): C 70.66, H 7.69, N 5.15; found: C 70.39, H 7.91, N 4.96.

(2α,3β,4α)-9-[2-{[4-(2,3,23-Tris(acetyloxy)-urs-12-en-28-oyl)-piperazinyl]carbonyl}phenyl]-3,6-bis(diethylamino)-xanthylium chloride) (7)

Following GP B, from **3** (70 mg, 0.10 mmol) and rhodamine B (72 mg, 0.15 mmol) **7** (93 mg, 80%) was obtained as a purple solid; m.p. 241–243 °C (lit.: 244–246 °C); R_f = 0.28 (SiO_2, $CHCl_3$/MeOH, 9:1); MS (ESI, MeOH): *m/z* = 1107.7 (100%, [M-Cl]$^+$).

(2α,3β,4α)-9-[2-{[4-(2,3,23-Tris(acetyloxy)-urs-12-en-28-oyl)-piperazinyl]carbonyl}phenyl]-3,6-bis(dipropylamino)-xanthylium chloride (8)

Following GP B, from **3** (150 mg, 0.22 mmol) followed by chromatography (SiO_2, $CHCl_3$/MeOH 9:1) gave **8** (150 mg, 57%) as a violet solid; m.p. 226–228 °C; R_f = 0.55 (SiO_2, $CHCl_3$/MeOH 8:2); UV-Vis (MeOH): λ_{max} (log ε) = 563 nm (4.72); IR (ATR): ν = 2926*m*, 2872*w*, 1739*m*, 1633*m*, 1589*vs*, 1545*w*, 1456*m*, 1410*m*, 1366*m*, 1336*s*, 1301*w*, 1230*vs*, 1178*s*, 1132*m*, 1100*m*, 1041*m*, 1004*m*, 962*w*, 940*w*, 918*m*, 825*w*, 758*w*, 597*w*, 576*w*, 506*w* cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.71–7.62 (*m*, 2H, 45-H, 46-H), 7.56–7.48 (*m*, 1H, 47-H), 7.37–7.32 (*m*, 1H, 44-H), 7.31–7.22 (*m*, 2H, 51-H), 7.01–6.94 (*m*, 2H, 50-H), 6.76–6.69 (*m*, 2H, 53-H), 5.19–5.09 (*m*, 2H, 12-H, 2-H), 5.06 (*d*, *J* = 10.3 Hz, 1H, 3-H), 3.83 (*d*, *J* = 11.8 Hz, 1H, 24-H_a), 3.56 (*d*, *J* = 11.9 Hz, 1H, 24-H_b), 3.49 (*t*, *J* = 7.9, 7.2 Hz, 8H, 55-H, 56-H), 3.46–3.24 (*m*, 8H, 37-H, 38-H, 39-H, 40-H), 2.39–2.30 (*m*, 1H, 18-H), 2.07 (s, 3H, 32-H), 2.05–2.01 (*m*, 1H, 1-H_a), 2.00 (*s*, 3H, 34-H), 1.96 (*s*, 3H, 36-H), 1.94–1.87 (*m*, 3H, 11-H, 16-H_a), 1.76–1.66 (*m*, 10H, 57-H, 59-H, 16-H_b, 22-H_a), 1.62–1.56 (*m*, 2H, 9-H, 22-H_b), 1.50–1.17 (*m*, 8H, 21-H_a, 7-H_a, 6-H, 19-H, 5-H, 21-H_b, 7-H_b), 1.12–1.08 (*m*, 2H, 1-H_b, 15-H_a), 1.05 (*s*, 3H, 25-H), 1.03 (*s*, 4H, 27-H, 15-H_b), 1.00 (*t*, *J* = 7.4 Hz, 12H, 58-H), 0.92–0.90 (*m*, 4H, 20-H, 30-H), 0.86 (*s*, 3H, 23-H), 0.84–0.81 (*m*, 3H, 29-H), 0.67 (*s*, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 176.1 (C-28), 171.0 (C-35), 170.6 (C-31), 170.4 (C-33), 167.9 (C-41), 157.8 (C-54), 156.3 (C-48), 156.2 (C-52), 143.2 (C-13), 135.1 (C-43), 132.3 (C-51), 130.8 (C-42), 130.5 (C-44), 130.4 (C-45), 130.4 (C-46), 127.8 (C-47), 124.7 (C-12), 114.6 (C-50), 114.0 (C-49), 96.6 (C-53), 74.9 (C-3), 70.0 (C-2), 65.4 (C-24), 54.7 (C-18), 53.9 (C-55, C-56), 49.0 (C-17), 47.7 (C-9), 47.6 (C-5), 47.4 (C-37, C-38, C-39, C-40), 43.8 (C-1), 42.2 (C-14), 42.0 (C-4), 39.5, 39.3 (C-19), 38.7 (C-20), 37.9 (C-8, C-10), 34.3 (C-22), 32.6 (C-7), 30.5 (C-21), 28.1 (C-15), 23.4 (C-11, C-16), 23.4 (C-27), 21.3 (C-36), 21.2 (C-30), 21.0 (C-34), 20.9 (C-32), 20.7 (C-57, C-59), 18.0 (C-6), 17.4 (C-29), 17.1 (C-25), 17.0 (C-26), 14.0 (C-23), 11.4 (C-58, C-60) ppm; MS (ESI,

MeOH): *m*/z = 1164.5 (100%, $[M\text{-}Cl]^+$); analysis calcd for $C_{72}H_{99}N_4O_9Cl$ (1200.03): C 72.06, H 8.32, N 4.67; found: C 71.85, H 8.53, N 4.40.

(2α,3β,4α)-9-[2-{[4-(2,3,23-Tris(acetyloxy)-urs-12-en-28-oyl)-piperazinyl]carbonyl}phenyl]-3,6-bis(dibutylamino)-xanthylium chloride (9)

Following GP B, from **3** (200 mg, 0.29 mmol) followed by chromatography (SiO_2, $CHCl_3$/MeOH, 9:1) **9** (180 mg, 0.14 mmol, 49%) was obtained as a violet solid; m.p. 222–226 °C; R_f = 0.44 (SiO_2, $CHCl_3$/MeOH, 8:2); UV-Vis (MeOH): λ_{max} (log ε) = 567 nm (4.91); IR (ATR): ν = 2929*m*, 2870*w*, 1740*m*, 1633*w*, 1587*vs*, 1528*w*, 1506*w*, 1462*m*, 1429*w*, 1411*s*, 1394*m*, 1366*m*, 1340*s*, 1290*m*, 1219*vs*, 1188*w*, 1176*s*, 1133*m*, 1109*m*, 1042*m*, 1004*m*, 962*w*, 922*m*, 823*w*, 755*w*, 732*w*, 705*w*, 664*w*, 641*w*, 597*w*, 509*w* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.69–7.64 (*m*, 2H, 45-H, 46-H), 7.55–7.50 (*m*, 1H, 47-H), 7.35–7.30 (*m*, 1H, 44-H), 7.28–7.21 (*m*, 2H, 51-H), 7.07–6.91 (*m*, 2H, 50-H), 6.70 (*s*, 2H, 53-H), 5.15 (*s*, 1H, 12-H), 5.13–5.09 (*m*, 1H, 2-H), 5.04 (*d*, *J* = 10.3 Hz, 1H, 3-H), 3.81 (*d*, *J* = 11.7 Hz, 1H, 24-H_a), 3.60–3.55 (*m*, 1H, 24-H_b), 3.54–3.48 (*m*, 8H, 55-H, 59-H), 3.39–3.24 (*m*, 8H, 37-H, 38-H, 39-H, 40-H), 2.37–2.29 (*m*, 1H, 18-H), 2.05 (*s*, 3H, 32-H), 2.04–2.01 (*m*, 1H, 1-H_a), 1.99 (*s*, 3H, 34-H), 1.94 (*s*, 3H, 36-H), 1.92–1.86 (*m*, 4H, 11-H, 16-H), 1.75–1.70 (*m*, 1H, 22-H_a), 1.69–1.62 (*m*, 8H, 56-H, 60-H), 1.60–1.55 (*m*, 1H, 9-H), 1.50–1.19 (*m*, 17H, 22-H_b, 21-H_a, 57-H, 61-H, 7-H_a, 6-H, 19-H, 5-H, 21-H_b, 7-H_b), 1.10 (*d*, *J* = 11.9 Hz, 1H, 1-H_b), 1.04 (*s*, 3H, 25-H), 1.02 (*s*, 5H, 27-H, 15-H), 0.96 (*t*, *J* = 6.8 Hz, 12H, 58-H, 62-H), 0.93–0.92 (*m*, 1H, 20-H), 0.90 (*s*, 3H, 30-H), 0.85 (*s*, 3H, 23-H), 0.81 (*d*, *J* = 6.3 Hz, 3H, 29-H), 0.66 (*s*, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 175.9 (C-28), 170.9 (C-35), 170.5 (C-31), 170.4 (C-33), 167.8 (C-41), 157.7 (C-54), 156.1 (C-48), 156.0 (C-52), 143.9 (C-13), 135.0 (C-43), 132.3 (C-51), 130.7 (C-42), 130.5 (C-44), 130.4 (C-46), 130.4 (C-45), 127.8 (C-47), 124.7 (C-12), 114.5 (C-50), 113.9 (C-49), 96.5 (C-53), 74.9 (C-3), 70.0 (C-2), 65.3 (C-24), 55.2 (C-18), 52.0 (C-55, C-59), 47.7 (C-9), 47.6 (C-5), 47.4 (C-37, C-38), 43.8 (C-1), 42.3 (C-39, C-40), 42.2 (C-14), 42.0 (C-4, C-17), 39.5 (C-19), 38.7 (C-20), 37.9 (C-8, C-10), 34.2 (C-22), 32.6 (C-7), 30.5 (C-21), 29.6 (C-56, C-60), 28.1 (C-15), 23.4 (C-11, C-16), 23.4 (C-27), 21.2 (C-30), 21.1 (C-36), 20.9 (C-34), 20.8 (C-32), 20.3 (C-57, C-61), 18.0 (C-6), 17.4 (C-29), 17.1 (C-25), 16.9 (C-26), 14.0 (C-23), 13.9 (C-58, C-62) ppm; MS (ESI, MeOH): *m*/z = 1219.4 (100%, $[M\text{-}Cl]^+$); analysis calcd for $C_{76}H_{107}N_4O_9Cl$ (1256.16): C 72.67, H 8.59, N 4.46; found: C 72.49, H 8.75, N 4.20.

(2α,3β,4α)-2,3,23-Triacetoxy-28-[3-(2,3,6,7,12,13,16,17-octahydro-1H,5H,11H,15H-pyrido[3.2.1-ij]pyrido[1",2",3":1',8']quinolino[6',5':5,6]pyrano[2,3-f]quinoline-4-ium-9-yl)benzoyl]-piperazin-1-yl]-28-oxo-olean-12-ene chloride (10)

Following GP B from **3** (150 mg, 0.22 mmol) followed by chromatography (SiO_2, $CHCl_3$/MeOH, 9:1) **10** (144 mg, 55%) was obtained as a violet solid; m.p. 244–247 °C; R_f = 0.38 (SiO_2, $CHCl_3$/MeOH, 8:2); UV-Vis (MeOH): λ_{max} (log ε) = 581 nm (4.69); IR (ATR): ν = 2924*m*, 2867*w*, 1739*s*, 1630*m*, 1595*s*, 1546*w*, 1493*m*, 1458*w*, 1446*w*, 1364*s*, 1296*s*, 1233*vs*, 1196*s*, 1182*s*, 1097*s*, 1035*s*, 1004*m*, 962*w*, 773*w*, 735*w*, 641*w*, 622*w*, 598*w*, 422*w* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.70–7.62 (*m*, 2H, 45-H, 46-H), 7.55–7.46 (*m*, 1H, 47-H), 7.30–7.27 (*m*, 1H, 44-H), 6.72–6.62 (*m*, 2H, 50-H), 5.19–5.15 (*m*, 1H, 12-H), 5.12 (*td*, *J* = 11.1, 4.5 Hz, 1H, 2-H), 5.05 (*d*, *J* = 10.3 Hz, 1H, 3-H), 3.83 (*d*, *J* = 11.7 Hz, 1H, 24-H_a), 3.69–3.21 (*m*, 17H, 24-H_b, 57-H, 58-H, 37-H, 38-H, 39-H, 40-H), 3.08–2.92 (*m*, 4H, 55-H), 2.78–2.60 (*m*, 4H, 60-H), 2.45–2.25 (*m*, 1H, 18-H), 2.14–2.08 (*m*, 4H, 56-H), 2.06 (*s*, 3H, 32-H), 2.04–2.01 (*m*, 1H, 1-H_a), 1.99 (*s*, 3H, 34-H), 1.95 (*s*, 3H, 36-H), 1.93–1.86 (*m*, 7H, 59-H, 11-H, 16-H_a), 1.74–1.64 (*m*, 2H, 22-H_a, 16-H_b), 1.62–1.50 (*m*, 2H, 9-H, 22-H_b), 1.46–1.14 (*m*, 8H, 21-H_a, 7-H_a, 6-H, 19-H, 5-H, 21-H_b, 7-H_b), 1.14–1.07 (*m*, 2H, 1-H_b, 15-H_a), 1.06 (*s*, 3H, 25-H), 1.03 (*s*, 4H, 27-H, 15-H_b), 0.96–0.92 (*m*, 1H, 20-H), 0.92–0.89 (*m*, 3H, 30-H), 0.86 (*s*, 3H, 29-H), 0.84–0.80 (*m*, 3H, 23-H), 0.67 (*s*, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 175.9 (C-28), 170.9 (C-35), 170.5 (C-31), 170.4 (C-33), 168.0 (C-41), 153.1 (C-54,C- 62), 152.1 (C-48), 151.3 (C-52, C-72), 143.9 (C-13), 134.9 (C-43), 131.8 (C-42), 130.8 (C-44), 130.3 (C-46), 129.9 (C-45), 127.6 (C-47), 126.7 (C-50, C-70), 124.8 (C-12), 123.7 (C-51, C-71), 113.3 (C-49, C-61), 105.5 (C-53, C-73), 74.9 (C-3), 70.0 (C-2), 65.3 (C-24), 55.2 (C-18), 51.1 (C-58, C-83), 50.6 (C-57, C-85), 49.0 (C-17), 47.7 (C-9), 47.6 (C-5), 43.8 (C-1), 42.2 (C-14), 42.2 (C-37, C-38, C-39, C-40), 42.0 (C-4), 39.5 (C-19), 38.7 (C-20), 37.9 (C-8, C-10), 34.3 (C-22), 32.6 (C-7), 30.5 (C-21), 28.1 (C-15), 27.7 (C-60, C-81), 23.5 (C-27), 23.4 (C-11, C-16), 21.3 (C-30), 21.1 (C-36), 21.0 (C-34), 20.8 (C-32), 20.7 (C-59, C-82), 20.0 (C-55, C-87), 19.8 (C-56, C-86), 18.0 (C-6), 17.4 (C-29), 17.1 (C-25), 16.9 (C-26), 14.0 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$): *m*/z = 1156.3 (100%, $[M\text{-}Cl]^+$); analysis calcd for $C_{68}H_{78}N_4O_5Cl$ (1040.47): C 78.50, H 8.43, N 5.36; found: C 78.34, H 8.59, N 5.24.

(2α,3β,4α)-9-[2-{[4-(2α,3β,23-tris(acetyloxy)-urs-12-en-28-oyl)-homopiperazinyl]carbonyl}phenyl]-3,6-bis(dimethylamino)-xanthylium chloride (11)

Following GP B from **4** (200 mg, 0.29 mmol) followed by chromatography (SiO_2, $CHCl_3$/MeOH, 9:1) **11** (156 mg, 49%) was obtained as a violet solid; m.p. 258–260 °C R_f =

0.38 (SiO_2, $CHCl_3$/MeOH, 8:2); UV-Vis (MeOH): λ_{max} (log ε) = 553 nm (4.70); IR (ATR): ν = 2925*m*, 2870*w*, 1738*m*, 1592*vs*, 1534*w*, 1493*m*, 1406*m*, 1365*m*, 1341*s*, 1304*w*, 1231*s*, 1185*s*, 1135*m*, 1042*m*, 1032*m*, 963*w*, 926*m*, 823*w*, 700*m*, 602*w*, 580*w*, 516*w* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.69–7.57 (*m*, 2H, 45-H, 47-H), 7.47–7.38 (*m*, 1H, 48-H), 7.34–7.28 (*m*, 1H, 46-H), 7.26–7.11 (*m*, 2H, 52-H), 7.02–6.72 (*m*, 4H, 54-H, 51-H), 5.23–5.09 (*m*, 2H, 12-H, 2-H), 5.05 (*d*, *J* = 10.3 Hz, 1H, 3-H), 3.82 (*d*, *J* = 11.7 Hz, 1H, 24-H_a), 3.55 (*d*, *J* = 11.9 Hz, 1H, 24-H_b), 3.51–2.79 (*m*, 22H, 56-H, 57-H, 37-H, 38-H, 39-H, 40-H, 41-H), 2.49–2.32 (*m*, 1H, 18-H), 2.05 (*s*, 3H, 32-H), 2.04–2.01 (*m*, 1H, 1-H_a), 1.99 (*s*, 3H, 34-H), 1.95 (*s*, 3H, 36-H), 1.94–1.82 (*m*, 3H, 11-H, 16-H_a), 1.82–1.62 (*m*, 2H, 16-H_a, 22-H_a), 1.63–1.54 (*m*, 1H, 9-H), 1.50–1.17 (*m*, 9H, 22-H_b, 21-H_a, 7-H_a, 6-H, 19-H, 5-H, 21-H_b, 7-H_b), 1.11 (*d*, *J* = 12.0 Hz, 1H, 1-H_b), 1.05 (*s*, 3H, 25-H), 1.02 (*s*, 4H, 27-H, 15-H_a), 1.00–0.96 (*m*, 2H, 20-H, 15-H_a), 0.95–0.88 (*m*, 3H, 30-H), 0.85 (*s*, 6H, 23-H, 29-H), 0.69 (*s*, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 173.3 (C-28), 171.0 (C-35), 170.6 (C-31), 170.5 (C-33), 168.1 (C-42), 157.7 (C-55), 157.6 (C-49), 157.5 (C-53), 144.9 (C-13), 135.0 (C-44), 132.0 (C-52), 130.5 (C-43), 130.3 (C-47), 130.2 (C-45), 129.7 (C-46), 126.9 (C-48), 124.8 (C-12), 114.1 (C-51), 113.9 (C-50), 96.8 (C-54), 75.0 (C-3), 70.0 (C-2), 65.4 (C-24), 55.4 (C-18), 49.0 (C-17), 47.8 (C-9), 47.7 (C-5), 43.8 (C-1), 42.4 (C-14), 42.0 (C-4), 41.4 (C-37, C-38, C-39, C-40, C-41), 41.2 (C-56, C-57), 39.4 (C-19), 38.8 (C-20), 37.9 (C-8, C-10), 34.0 (C-22), 32.4 (C-7), 30.6 (C-21), 27.9 (C-15), 23.4 (C-27), 23.4 (C-11, C-16), 21.3 (C-30), 21.2 (C-36), 21.0 (C-34), 20.9 (C-32), 18.0 (C-6), 17.5 (C-29), 17.1 (C-25), 17.0 (C-26), 14.0 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$): *m*/z = 1065.5 (100%, $[M\text{-}Cl]^+$); analysis calcd for $C_{65}H_{85}N_4O_9Cl$ (1101.86): C 70.85, H 7.78, N 5.08; found: C 70.59, H 7.93, N 4.88.

(2α,3β,4α)-9-[2-{[4-(2α,3β,23-tris(acetyloxy)-urs-12-en-28-oyl)-homopiperazinyl]carbonyl}phenyl]-3,6-bis(diethylamino)-xanthylium chloride (12)

Following GP B from **4** (180 mg, 0.26 mmol) followed by chromatography (SiO_2, $CHCl_3$/MeOH, 9:1) **12** (224 mg, 64%) was obtained as a violet solid; m.p. 253–257 °C (lit.: 254–258 °C; R_f = 0.30 (SiO_2, $CHCl_3$/MeOH, 8:1); MS (ESI, MeOH/$CHCl_3$): *m/z* = 1121.4 (100%, $[M\text{-}Cl]^+$).

(2α,3β,4α)-9-[2-{[4-(2α,3β,23-tris(acetyloxy)-urs-12-en-28-oyl)-homopiperazinyl]carbonyl}phenyl]-3,6-bis(dipropylamino)-xanthylium chloride (13)

Following GP B from **4** (200 mg, 0.29 mmol) followed by chromatography (SiO_2, $CHCl_3$/MeOH, 9:1) **13** (224 mg, 64%) was obtained as a violet solid; m.p. 224–225 °C; R_f =

0.33 (SiO_2, $CHCl_3$/MeOH, 8:2); UV-Vis (MeOH): λ_{max} (log ε) = 566 nm (4.91); IR (ATR): ν = 2928*m*, 2873*w*, 1739*m*, 1627*m*, 1587*vs*, 1528*w*, 1507*w*, 1470*m*, 1412*s*, 1365*m*, 1337*s*, 1301*m*, 1230*vs*, 1177*s*, 1133*m*, 1100*m*, 1041*m*, 1032*m*, 997*w*, 963*w*, 939*w*, 919*w*, 824*w*, 780*w*, 757*w*, 597*w*, 575*w*, 507*w* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.64–7.57 (*m*, 2H, 46-H, 47-H), 7.46–7.37 (*m*, 1H, 48-H), 7.30–7.15 (*m*, 3H, 45-H, 52-H), 6.78–6.67 (*m*, 2H, 54-H), 5.20–5.15 (*m*, 1H, 2-H), 5.12 (*td*, *J* = 11.0, 4.7 Hz, 2H, 12-H), 5.04 (*d*, *J* = 10.3 Hz, 1H, 3-H), 4.16–3.86 (*m*, 4H, 37-H, 38-H), 3.80 (*d*, *J* = 11.7 Hz, 1H, 24-H_a), 3.54 (*d*, *J* = 11.8 Hz, 1H, 24-H_b), 3.53–3.38 (*m*, 8H, 56-H, 59-H), 3.34–2.97 (*m*, 8H, 39-H, 40-H), 2.49–2.35 (*m*, 1H, 18-H), 2.05 (*s*, 3H, 32-H), 2.03–2.00 (*m*, 1H, 1-H_a), 1.98 (*s*, 3H, 34-H), 1.94 (*s*, 3H, 36-H), 1.93–1.83 (*m*, 3H, 11-H, 16-H_a), 1.79–1.65 (*m*, 10H, 57-H, 60-H, 16-H_b, 22-H_a), 1.62–1.53 (*m*, 1H, 22-H_b, 9-H), 1.42–1.18 (*m*, 10H, 21-H_a, 7-H_a, 6-H, 19-H, 5-H, 21-H_b, 7-H_b, 41-H), 1.10–1.07 (*m*, 1H, 1-H_b), 1.04 (s, 3H, 25-H), 1.01 (*s*, 4H, 27-H, 15-H_a), 1.00–0.96 (*m*, 13H, 58-H, 61-H, 20-H), 0.93–0.88 (*m*, 4H, 30-H, 15-H_b), 0.85 (*s*, 6H, 23-H, 29-H), 0.68 (*s*, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 176.6 (C-28), 170.9 (C-35), 170.5 (C-33), 170.4 (C-31), 168.1 (C-42), 157.8 (C-55), 156.3 (C-49), 156.2 (C-53), 142.6 (C-13), 136.0 (C-44), 132.6 (C-52), 130.7 (C-43), 130.2 (C-47), 130.2 (C-45), 129.7 (C-46), 126.9 (C-48), 124.6 (C-12), 114.9 (C-51), 113.8 (C-50), 96.6 (C-54), 77.4, 77.2, 76.9, 75.0 (C-3), 70.0 (C-2), 65.4 (C-24), 55.8 (C-18), 53.9 (C-56, C-59), 53.8 (C-37, C-38, C-39, C-40, C-41), 48.9 (C-17), 47.8 (C-9), 47.7 (C-5), 43.8 (C-1), 42.4 (C-14), 42.0 (C-4), 39.4 (C-19), 38.8 (C-20), 37.9 (C-8, C-10), 34.0 (C-22), 32.7 (C-7), 30.5 (C-21), 28.0 (C-15), 23.4 (C-11, C-16), 23.4 (C-27), 21.3 (C-30), 21.1 (C-36), 21.0 (C-34), 20.8 (C-32), 20.8 (C-57, C-60), 18.0 (C-6), 17.5 (C-29), 17.1 (C-25), 17.0 (C-26), 14.0 (C-23), 11.4 (C-58, C-61) ppm; MS (ESI, MeOH/$CHCl_3$): *m*/z = 1178.4 (100%, $[M\text{-}Cl]^+$); analysis calcd for $C_{73}H_{101}N_4O_9Cl$ (1214.08): C 72.22, H 8.39, N 4.61; found: C 71.97, H 8.48, N 4.39.

(2α,3β,4α)-9-[2-{[4-(2α,3β,23-tris(acetyloxy)-urs-12-en-28-oyl)-homopiperazinyl]carbonyl}phenyl]-3,6-bis(dibutylamino)-xanthylium chloride (14)

Following GP B, from **4** (284 mg, 0.4 mmol) followed by chromatography (SiO_2, $CHCl_3$/MeOH, 9:1) **14** (2198 mg, 58%) was obtained as a violet solid; m.p. 210–213 °C; R_f = 0.36 (SiO_2, $CHCl_3$/MeOH, 8:2); UV-Vis (MeOH): λ_{max} (log ε) = 567 nm (4.94); IR (ATR): ν = 2927*m*, 2870*w*, 1741*m*, 1626*w*, 1588*vs*, 1528*w*, 1507*w*, 1462*m*, 1412*s*, 1394*w*, 1366*m*, 1339*s*, 1291*m*, 1220*s*, 1187*s*, 1177*s*, 1133*m*, 1109*m*, 1043*m*, 1033*m*, 963*w*, 921*m*, 823*w*, 756*w*, 704*w*, 597*w* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.68–7.54 (*m*, 2H, 46-H, 47-H), 7.46–7.36 (*m*, 1H, 48-H), 7.29–7.25 (*m*, 2H, 45-H), 7.25–7.14 (*m*, 2H, 52-H), 7.09–6.98 (*m*, 2H, 51-H), 6.79–6.64 (*m*, 2H, 54-H), 5.18–5.15 (*m*, 1H, 12-H), 5.12 (*td*, *J* = 11.0, 4.7 Hz, 1H, 2-H), 5.03 (*d*, *J* =

10.3 Hz, 1H, 3-H), 4.26–2.89 (*m*, 20H, 24-H, 37-H, 38-H, 39-H, 40-H, 41-H, 56-H, 60-H), 2.49–2.33 (*m*, 1H, 18-H), 2.04 (*s*, 3H, 32-H), 2.03–2.00 (*m*, 1H, 1-H_a), 1.98 (*s*, 3H, 34-H), 1.94 (*s*, 3H, 36-H), 1.92–1.82 (*m*, 4H, 11-H, 16-H), 1.81–1.72 (*m*, 2H, 22-H), 1.71–1.61 (*m*, 8H, 57-H, 61-H), 1.61–1.54 (*m*, 1H, 9-H), 1.48–1.18 (*m*, 16H, 58-H, 62-H, 21-H_a, 7-H_a, 6-H, 19-H, 5-H, 21-H_b, 7-H_b), 1.12–1.07 (*m*, 1H, 1-H_b), 1.04 (*m*, 5H, 25-H, 15-H), 1.01 (*s*, 3H, 27-H), 0.96 (*t*, *J* = 7.2 Hz, 13H, 59-H, 63-H, 20-H), 0.93–0.88 (*m*, 3H, 30-H), 0.84 (*s*, 6H, 23-H, 29-H), 0.68 (*s*, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 176.4 (C-28), 170.9 (C-35), 170.5 (C-31) 170.4 (C-33), 168.0 (C-42), 157.8 (C-55), 156.1 (C-49), 156.0 (C-53), 143.9 (C-13), 136.0 (C-44), 132.6 (C-52), 130.2 (C-43), 130.2 (C-45), 129.8 (C-47), 129.7 (C-46), 126.9 (C-48), 124.6 (C-12), 114.7 (C-51), 113.8 (C-50), 96.4 (C-54), 75.0 (C-3), 70.0 (C-2), 65.4 (C-24), 55.5 (C-18), 52.1 (C-37, C-38, C-39, C-40, C-41), 52.0 (C-56, C-60), 49.0 (C-17), 47.8 (C-9), 47.7 (C-5), 43.8 (C-1), 42.4 (C-14), 42.0 (C-4), 39.4 (C-19), 38.8 (C-20), 37.9 (C-8, C-10), 34.0 (C-22), 32.6 (C-7), 30.5 (C-21), 29.6 (C-57, C-62), 27.6 (C-15), 23.4 (C-11, C-16), 23.4 (C-27), 21.3 (C-30), 21.1 (C-36), 20.9 (C-34), 20.8 (C-32), 20.3 (C-58, C-63), 17.9 (C-6), 17.5 (C-29), 17.1 (C-25), 17.0 (C-26), 14.0 (C-23), 13.9 (C-59, C-64) ppm; MS (ESI, MeOH/$CHCl_3$): *m*/z = 1234.3 (100%, $[M\text{-}Cl]^+$); analysis calcd for $C_{77}H_{109}N_4O_9Cl$ (1270.19): C 72.81, H 8.65, N 4.41; found: C 72.61, H 8.86, N 4.21.

(2α,3β,4α)-2,3,23-Triacetoxy-28-[3-(2,3,6,7,12,13,16,17-octahydro-1H,5H,11H,15H-pyrido[3.2.1-ij]pyrido[1",2",3":1',8']quinolino[6',5':5,6]pyrano[2,3-f]quinoline-4-ium-9-yl)benzoyl]-1,4-diazepan-1-yl]-28-oxo-olean-12-ene chloride (15)

Following GP B, from **4** (115 mg, 0.17 mmol) followed by chromatography (SiO_2, $CHCl_3$/MeOH, 9:1) **15** (104 mg, 0.1 mmol, 63%) was obtained as a violet solid; m.p. 287–289 °C; R_f = 0.19 (SiO_2, $CHCl_3$/MeOH, 9:1); UV-Vis (MeOH): λ_{max} (log ε) = 582 nm (4.82); IR (ATR): ν = 2924*m*, 2866*w*, 1738*s*, 1621*m*, 1595*vs*, 1545*w*, 1493*m*, 1458*w*, 1445*w*, 1435*w*, 1363*s*, 1294*vs*, 1232*s*, 1179*vs*, 1100*s*, 1034*s*, 962*w*, 743*m*, 640*w*, 623*w*, 598*w*, 420*m* cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.72–7.52 (*m*, 2H, 46-H, 47-H), 7.47–7.34 (*m*, 1H, 48-H), 7.25–7.20 (*m*, 1H, 45-H), 6.79–6.55 (*m*, 2H, 51-H), 5.22–5.17 (*m*, 1H, 12-H), 5.14 (*td*, *J* = 11.1, 4.5 Hz, 1H, 2-H), 5.05 (*d*, *J* = 10.3 Hz, 1H, 3-H), 4.50–3.89 (*m*, 4H, 37-H, 38-H), 3.82 (*d*, *J* = 11.6 Hz, 1H, 24-H_a), 3.69–3.15 (*m*, 13H, 24-H_b, 39-H, 40-H, 58-H, 59-H), 3.05–2.95 (*m*, 4H, 56-H), 2.80–2.63 (*m*, 4H, 61-H), 2.49–2.39 (*m*, 1H, 18-H), 2.15–2.08 (*m*, 4H, 57-H), 2.06 (*s*, 3H, 32-H), 2.04–2.02 (*m*, 1H, 1-H_a), 2.00 (*s*, 3H, 34-H), 1.96 (*s*, 3H, 36-H), 1.94–1.84 (*m*, 5H, 60-H, 11-H_a), 1.85–1.64 (*m*, 4H, 11-H_b, 16-H, 22-H_a), 1.63–1.56 (*m*, 2H, 9-H, 22-H_b), 1.51–1.14 (*m*, 10H, 21-H_a, 7-H_a, 6-H, 19-H, 5-H, 21-H_b, 7-H_b, 41-H), 1.14–1.08 (*m*, 1H, 1-H_b), 1.06 (*s*, 4H,

25-H, 15-H_a), 1.02 (*s*, 4H, 27-H, 15-H_b), 0.97–0.92 (*m*, 1H, 20-H), 0.92–0.88 (*m*, 3H, 30-H), 0.86 (*s*, 6H, 23-H, 29-H), 0.70 (*s*, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 175.5 (C-28), 170.8 (C-35), 170.4 (C-31), 170.3 (C-33), 168.0 (C-42), 152.9 (C-55), 151.9 (C-49), 151.3 (C-53), 143.8 (C-13), 135.0 (C-44), 131.8 (C-43), 130.4 (C-45), 129.6 (C-46), 129.5 (C-47), 126.7 (C-48), 126.6 (C-51), 124.7 (C-12), 123.7 (C-52), 113.4 (C-50), 105.1 (C-54), 74.9 (C-3), 69.9 (C-2), 65.3 (C-24), 55.6 (C-18), 51.0 (C-37, C-38, C-39, C-40), 51.0 (C-59), 50.6 (C-58), 48.9 (C-17), 47.7 (C-9), 47.6 (C-5), 43.7 (C-1), 42.0 (C-4), 41.9 (C-14), 39.4 (C-19), 38.7 (C-20), 37.8 (C-8, C-10), 34.3 (C-22), 32.5 (C-7), 30.5 (C-21), 29.7 (C-41), 27.6 (C-15), 27.6 (C-61), 23.4 (C-27), 23.3 (C-11, C-16), 21.2 (C-30), 21.0 (C-36), 20.9 (C-34), 20.7 (C-32), 20.6 (C-60), 19.9 (C-56), 19.7 (C-57), 17.8 (C-6), 17.4 (C-29), 17.3 (C-25), 17.0 (C-26), 13.9 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$): *m*/z = 1169.7 (100%, $[M\text{-}Cl]^+$); analysis calcd for $C_{69}H_{89}N_4O_5Cl$ (1054.49): C 78.59, H 8.51, N 5.31; found: C 78.22, H 8.79, N 5.06.

(2α,3β,4α)-9-[2-[{4-(2α,3β,23-Tris(acetyloxy)-urs-12-en-28-oyl)-1,5-diazocan-1-yl]carbonyl}phenyl]-3,6-bis(dimethylamino)-xanthylium chloride (16)

Following GP B from **5** (670 mg, 0.94 mmol) followed by chromatography (SiO_2, $CHCl_3$/MeOH, 9:1) **16** (523 mg, 55%) was obtained as a violet solid; m.p. 224–226 °C; .R_f = 0.50 (SiO_2, $CHCl_3$/MeOH, 8:2); UV-Vis (MeOH): λ_{max} (log ε) = 556 nm (4.72); IR (ATR): ν = 2922*s*, 2852*m*, 1741*m*, 1624*w*, 1593*vs*, 1534*w*, 1508*w*, 1493*m*, 1437*w*, 1407*m*, 1365*m*, 1343*s*, 1285*w*, 1231*s*, 1185*vs*, 1135*m*, 1085*w*, 1043*m*, 1033*m*, 925*m*, 820*m*, 757*w*, 699*m*, 518*w*, 492*w* cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.70–7.58 (*m*, 2H, 46-H, 48-H), 7.57–7.46 (*m*, 1H, 49-H), 7.37–7.27 (*m*, 3H, 47-H, 53-H), 7.06–6.87 (*m*, 2H, 52-H), 6.84–6.65 (*m*, 2H, 55-H), 5.23–5.17 (*m*, 1H, 12-H), 5.14 (*td*, *J* = 11.2, 5.3 Hz, 1H, 2-H), 5.06 (*d*, *J* = 10.3 Hz, 1H, 3-H), 3.82 (*d*, *J* = 11.8 Hz, 1H, 24-H_a), 3.56 (*d*, *J* = 11.9 Hz, 1H, 24-H_b), 3.52–2.83 (*m*, 8H, 37-H, 38-H, 41-H, 42-H), 3.40–3.29 (*m*, 12H, 57-H, 58-H), 2.48–2.38 (*m*, 1H, 18-H), 2.07 (*s*, 3H, 32-H), 2.05–2.02 (*m*, 1H, 1-H_a), 2.00 (*s*, 3H, 34-H), 1.96 (*s*, 3H, 36-H), 1.94–1.73 (*m*, 4H, 11-H, 16-H), 1.63–1.55 (*m*, 1H, 9-H), 1.54–1.14 (*m*, 15H, 21-H_a, 7-H_a, 22-H_a, 6-H, 39-H, 40-H, 19-H, 5-H, 22-H_b, 21-H_b, 7-H_b, 15-H_a), 1.10 (*s*, 2H, 1-H_b, 15-H_b), 1.08–1.02 (*m*, 6H, 25-H, 27-H), 1.01–0.95 (*m*, 1H, 20-H), 0.93–0.89 (*m*, 3H, 30-H), 0.86 (*s*, 3H, 23-H), 0.85–0.82 (*m*, 3H, 29-H), 0.72 (*s*, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 175.8 (C-28), 171.0 (C-35), 170.6 (C-31), 170.5 (C-33), 167.9 (C-43), 157.7 (C-56), 157.5 (C-54), 156.4 (C-50), 145.0 (C-13), 136.8 (C-45), 131.2 (C-53), 130.6 (C-44), 130.4 (C-46, C-48), 130.1 (C-47), 127.9 (C-49), 124.6 (C-12), 114.4 (C-52), 114.1 (C-51), 96.9 (C-55), 77.4, 77.2, 76.9, 76.9, 75.0 (C-3), 70.1 (C-2), 65.4 (C-24), 55.6 (C-18), 48.0 (C-17), 47.8 (C-9), 47.7 (C-5), 43.9 (C-1), 42.7 (C-37, C-

38, C-41, C-42), 42.2 (C-14), 42.0 (C-4), 41.4 (C-57, C-58), 39.4 (C-19), 38.8 (C-20), 38.0 (C-8, C-10), 34.1 (C-22), 32.0 (C-7), 29.8 (C-21), 27.4 (C-15), 25.0, 23.4 (C-11, C-16), 23.4 (C-27), 22.8 (C-39, C-40), 21.2 (C-36), 21.0 (C-34), 20.9 (C-32), 18.0 (C-6), 17.5 (C-29), 17.2 (C-25), 17.0 (C-26), 14.0 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$): *m*/z = 1079.5 (100%, $[M\text{-}Cl]^+$); analysis calcd for $C_{66}H_{87}N_4O_9Cl$ (1115.89): C 71.04, H 7.86, N 5.02; found: C 70.86, H 8.03, N 4.77.

(2α,3β,4α)-9-[2-[{4-(2α,3β,23-Tris(acetyloxy)-urs-12-en-28-oyl)-1,5-diazocan-1-yl]carbonyl}phenyl]-3,6-bis(diethylamino)-xanthylium chloride (17)

Following GP B from **5** (300 mg, 0.4 mmol) followed by chromatography (SiO_2, $CHCl_3$/MeOH, 9:1) **17** (188 mg, 60%) was obtained as a purple solid; m.p. 223–226 °C; R_f = 0.44 (SiO_2, $CHCl_3$/MeOH, 9:1); UV-Vis (MeOH): λ_{max} (log ε) = 562 nm (4.5); IR (ATR): ν = 2927*w*, 1793*m*, 1587*s*, 1467*m*, 1411*m*, 1336*s*, 1244*s*, 1179*s*, 1042*m*, 921*w*, 684*m*, 436*w* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.66–7.57 (*m*, 2H, 47-H, 48-H), 7.52–7.47 (*m*, 1H, 46-H), 7.27-7.20 (*m*, 49 H, 52-H), 7.17–6.63 (*m*, 4H, 53-H, 55-H), 5.20–5.00 (*m*, 3H, 12-H, 2-H, 3-H), 3.82–3.76 (*m*, 1H, 24-H_a), 3.73–2.93 (*m*, 17H, 37-H, 39-H, 40-H, 42-H, 57-H, 24-H_b), 2.44–2.33 (*m*, 1H, 18-H), 2.04 (*s*, 3H, 34-H), 2.02–1.99 (*m*, 1H, 1-H_a), 1.97 (*s*, 3H, 36-H), 1.93 (*s*, 3H, 32-H), 1.90–1.32 (*m*, 15H, 11-H, 9-H, 15-H, 16-H_a, 21-H_a, 22-H_a, 19-H, 38-H, 41-H, 6-H), 1.28 (*t*, *J* = 7.1 Hz, 13H, 5-H, 58-H), 1.25–1.10 (*m*, 5H, 7-H, 16-H_b, 21-H_b, 22-H_b), 1.09–1.07 (*m*, 1H, 1-H_b), 1.04 (*s*, 3H, 30-H), 1.01 (*s*, 3H, 27-H), 0.97–0.92 (*m*, 1H, 20-H), 0.88 (*s*, 3H, 29-H), 0.84 (*s*, 3H, 25-H), 0.81 (*s*, 3H, 26-H), 0.69 (*s*, 3H, 23-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 176.2 (C-43), 170.8 (C-35), 170.4 (C-31, C-33), 170.3 (C-28), 157.7 (C-54), 155.7 (C-56), 155.6 (C-50), 138.6 (C-13), 136.6 (C-45), 132.3 (C-52), 130.1 (C-47), 130.0 (C-49), 129.3 (C-48), 127.2 (C-46), 125.9 (C-12), 113.9 (C-51), 96.2 (C-53, C-55), 74.8 (C-3), 69.9 (C-2), 65.3 (C-24), 55.5 (C-18), 53.4 (C-37, C-39, C-40, C-42), 47.6 (C-5), 47.5 (C-9), 46.2 (C-57), 46.1 (C-17), 43.7 (C-1), 42.5 (C-4), 41.9 (C-14), 38.9 (C-8), 38.7 (C-20), 38.6 (C-19), 37.8 (C-10), 32.6 (C-22), 30.5 (C-21), 29.6 (C-7), 28.4 (C-15), 23.4 (C-27), 23.3 (C-11), 21.2 (C-29), 21.0 (C-32), 20.8 (C-36), 20.7 (C-34), 17.8 (C-6), 17.4 (C-26), 17.2 (C-30), 17.0 (C-23), 13.9 (C-25), 12.6 (C-58) ppm; MS (ESI, MeOH/$CHCl_3$): *m*/z = 1036.3 (100%, $[M\text{-}Cl]^+$); analysis calcd for $C_{70}H_{95}N_4O_9Cl$ (1172.00): C 71.74, H 8.17, N 4.78; found: C 71.48, H 8.22, N 5.37.

(2α,3β,4α)-9-[2-[{4-(2α,3β,23-Tris(acetyloxy)-urs-12-en-28-oyl)-1,5-diazocan-1-yl]carbonyl}phenyl]-3,6-bis(dipropylamino)-xanthylium chloride (18)

Following GP B from **5** (500 mg, 0.7 mmol) followed by chromatography (SiO_2, $CHCl_3$/MeOH, 9:1) **18** (612 mg, 68%) was obtained as a violet solid; m.p. 207–209 °C; R_f = 0.53 (SiO_2, $CHCl_3$/MeOH, 8:2); UV-Vis (MeOH): λ_{max} (log ε) = 564 nm (4.47); IR (ATR): ν = 2927*m*, 2873*w*, 1740*s*, 1696*w*, 1589*s*, 1528*w*, 1504*w*, 1457*m*, 1432*w*, 1412*m*, 1367*m*, 1338*m*, 1301*w*, 1230*vs*, 1194*w*, 1178*m*, 1133*m*, 1101*w*, 1042*m*, 1032*m*, 964*w*, 939*w*, 918*w*, 825*w*, 641*w*, 598*w*, 508*w* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.70–7.57 (*m*, 2H, 47-H, 48-H), 7.57–7.47 (*m*, 1H, 49-H), 7.39–7.27 (*m*, 3H, 46-H, 53-H), 7.15–6.88 (*m*, 2H, 52-H), 6.79–6.62 (*m*, 2H, 55-H), 5.27–5.18 (*m*, 1H, 12-H), 5.14 (*td*, *J* = 11.2, 5.5 Hz, 1H, 2-H), 5.06 (*d*, *J* = 10.3 Hz, 1H, 3-H), 3.82 (*d*, *J* = 11.6 Hz, 1H, 24-H_a), 3.76–2.76 (*m*, 17H, 24-H_b, 57-H, 37-H, 38-H, 41-H, 42-H), 2.48–2.31 (*m*, 1H, 18-H), 2.07 (s, 3H, 32-H), 2.05–2.02 (*m*, 1H, 1-H_a), 2.00 (*s*, 3H, 34-H), 1.96 (*s*, 3H, 36-H), 1.95–1.84 (*m*, 4H, 11-H, 16-H), 1.81–1.65 (*m*, 8H, 58-H), 1.65–1.56 (*m*, 1H, 9-H), 1.56–1.22 (*m*, 14H, 22-H, 21-H_a, 7-H_a, 39-H, 40-H, 6-H, 19-H, 5-H, 21-H_b, 7-H_b), 1.15–1.10 (*m*, 1H, 1-H_b), 1.10–1.06 (*m*, 4H, 25-H, 15-H_a), 1.04 (*s*, 4H, 27-H, 15-H_b), 1.01 (*t*, *J* = 7.0 Hz, 12H, 59-H), 0.97–0.94 (*m*, 1H, 20-H), 0.92 (*s*, 3H, 30-H), 0.88–0.85 (*m*, 3H, 23-H), 0.85 (*s*, 3H, 29-H), 0.77–0.69 (*m*, 3H, 26-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 176.1 (C-28), 171.0 (C-35), 170.6 (C-33), 170.5 (C-31), 168.2 (C-43), 157.8 (C-56), 156.3 (C-50), 156.2 (C-54), 144.0 (C-13), 136.1 (C-45), 132.4 (C-53), 130.7 (C-44), 130.3 (C-48), 130.0 (C-46), 129.5 (C-47), 127.3 (C-49), 124.7 (C-12), 115.0 (C-52), 114.0 (C-51), 96.4 (C-55), 75.0 (C-3), 70.1 (C-2), 65.4 (C-24), 55.2 (C-18), 53.9 (C-37, C-38, C-41, C-42), 53.9 (C-57#, C-57), 49.2 (C-17), 47.8 (C-5), 47.7 (C-9), 43.9 (C-1), 42.0 (C-14), 42.0 (C-4), 39.5 (C-19), 39.5 (C-8), 38.8 (C-20), 38.0 (C-10), 34.0 (C-22), 32.7 (C-7), 30.7 (C-21), 29.8 (C-39, C-40), 28.3 (C-15), 23.5 (C-16), 23.5 (C-27), 23.4 (C-11), 21.4 (C-30), 21.2 (C-32), 21.0 (C-34), 20.9 (C-36), 20.9 (C-58#, C-58), 18.0 (C-6), 17.5 (C-29), 17.4 (C-25), 17.2 (C-26), 14.0 (C-23), 11.5 (C-59#, 59) ppm; MS (ESI, MeOH/$CHCl_3$): *m*/z = 1191.3 (100%, $[M\text{-}Cl]^+$); analysis calcd for $C_{74}H_{103}N_4O_9Cl$ (1228.11): C 72.37, H 8.45, N 4.56; found: C 72.11, H 8.64, N 4.40.

(2α,3β,4α)-9-[2-[{4-(2α,3β,23-Tris(acetyloxy)-urs-12-en-28-oyl)-1,5-diazocan-1-yl]carbonyl}phenyl]-3,6-bis(dibutylamino)-xanthylium chloride (19)

Following GP B from **5** (400 mg, 0.56 mmol) followed by chromatography (SiO_2, $CHCl_3$/MeOH, 9:1) **19** (520 mg, 70%) was obtained as a violet solid;.m.p. 215–218 °C; R_f = 0.43 (SiO_2, $CHCl_3$/MeOH, 8:2); UV-Vis (MeOH): λ_{max} (log ε) = 566 nm (4.79); IR (ATR): ν = 2930*m*, 2871*w*, 1741*m*, 1633*w*, 1589*vs*, 1528*w*, 1461*m*, 1412*s*, 1394*w*, 1367*m*, 1338*s*, 1292*m*, 1221*vs*, 1178*s*, 1133*m*, 1109*m*, 1043*m*, 964*w*, 921*m*, 825*w*, 757*w*, 597*w* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.68–7.54 (*m*, 2H, 47-H, 48-H), 7.52–7.46 (*m*, 1H, 49-H), 7.31–7.18 (*m*, 3H,

53-H, 46-H), 7.04–6.76 (*m*, 2H, 52-H), 6.74–6.57 (*m*, 2H, 55-H), 5.19–5.14 (*m*, 1H, 12-H), 5.11 (*td*, *J* = 10.9, 4.9 Hz, 1H, 2-H), 5.03 (*d*, *J* = 10.3 Hz, 1H, 3-H), 3.79 (*d*, *J* = 11.7 Hz, 1H, 24-H_a), 3.67–2.76 (*m*, 17H, 24-H_b, 57-H, 37-H, 38-H, 41-H, 42-H), 2.41–2.33 (*m*, 1H, 18-H), 2.04 (*s*, 3H, 32-H), 2.02–1.99 (*m*, 1H, 1-H_a), 1.97 (*s*, 3H, 34-H), 1.93 (*s*, 3H, 36-H), 1.91–1.80 (*m*, 3H, 11-H, 16-H_a), 1.79–1.71 (*m*, 1H, 16-H_b), 1.69–1.59 (*m*, 8H, 58-H), 1.59–1.54 (*m*, 1H, 9-H), 1.50–1.16 (*m*, 19H, 59-H, 22-H, 7-H_a, 21-H_a, 6-H, 19-H, 39-H, 40-H, 5-H, 7-H_b, 21-H_b, 15-H_a), 1.09–1.05 (*m*, 2H, 1-H_b, 15-H_b), 1.03 (*s*, 3H, 25-H), 1.01 (*s*, 3H, 27-H), 0.95 (*t*, J = 6.1 Hz, 13H, 60-H, 20-H), 0.90–0.86 (*m*, 3H, 30-H), 0.83 (*s*, 3H, 23-H), 0.82–0.78 (*m*, 3H, 29-H), 0.69 (*s*, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 176.1 (C-28), 170.9 (C-35), 170.5 (C-31), 170.4 (C-33), 168.0 (C-43), 157.7 (C-56), 156.1 (C-50), 156.1 (C-54), 144.1 (C-13), 136.6 (C-45), 132.2 (C-53), 130.2 (C-48), 130.1 (C-46), 129.4 (C-44), 129.3 (C-47), 127.4 (C-49), 124.5 (C-12), 113.8 (C-52), 113.5 (C-51), 96.3 (C-55), 74.9 (C-3), 70.0 (C-2), 65.3 (C-24), 55.4 (C-18), 52.0 (C-57, C-57), 52.0 (C-37, C-38, C-41, C-42), 49.0 (C-17), 47.7 (C-9), 47.6 (C-5), 43.8 (C-1), 42.1 (C-14), 41.9 (C-4), 39.8 (C-39, C-40), 39.4 (C-19), 38.7 (C-20), 37.9 (C-10), 37.9 (C-8), 34.3 (C-22), 32.6 (C-7), 30.6 (C-21), 29.7 (C-58#, C-58), 28.1 (C-15), 23.4 (C-11, C-16), 23.3 (C-27), 21.3 (C-30), 21.1 (C-36), 20.9 (C-34), 20.8 (C-32), 20.2 (C-59#, C-59), 17.9 (C-6), 17.4 (C-29), 17.1 (C-25), 17.0 (C-26), 14.0 (C-23), 13.9 (C-60, 61) ppm; MS (ESI, MeOH/$CHCl_3$): *m*/z = 1247.3 (100%, $[M\text{-}Cl]^+$); analysis calcd for $C_{78}H_{111}N_4O_9Cl$ (1284.22): C 72.95, H 8.71, N 4.36; found: C 72.70, H 8.96, N 4.17.

(2α,3β,4α)-2,3,23-Triacetoxy-28-[3-(2,3,6,7,12,13,16,17-octahydro-1H,5H,11H,15H-pyrido[3.2.1-ij]pyrido[1”,2”,3”:1’,8’]quinolino[6’,5’:5,6]pyrano[2,3-f]quinoline-4-ium-9-yl)benzoyl]-1,5-diazocan-1-yl]-28-oxo-olean-12-ene chloride (20)

Following GP B from **5** (200 mg, 0.3 mmol) followed by chromatography (SiO_2, $CHCl_3$/MeOH, 9:1) **20** (232 mg, 64%)was obtained as a violet solid; m.p. 193–196 °C; R_f = 0.45 (SiO_2, $CHCl_3$/MeOH 9:1); UV-Vis (MeOH): λ_{max} (log ε) = 578 nm (4.5); IR (ATR): ν = 2924*w*, 1739*w*, 1594*s*, 1493*s*, 1459*m*, 1361*m*, 1293*s*, 1195*s*, 1180*s*, 1090*s*, 1035*s*, 729*m*, 622*m*, 421*s* cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.67–7.58 (*m*, 2H, 47-H, 49-H), 7.52–7.48 (*m*, 1H, 46-H), 7.29–7.27 (*m*, 1H, 48-H), 6.77–6.65 (*m*, 2H, 52-H), 5.17–5.03 (*m*, 3H, 12-H, 2-H, 3-H), 3.83–3.79 (*m*, 1H, 24-H_a), 3.60–3.16 (*m*, 17H, 24-H_b, 37-H, 39-H, 40-H, 42-H, 56-H, 61-H), 3.00–2.94 (*m*, 4H, 59-H), 2.77–2.63 (*m*, 4H, 54-H), 2.46–2.36 (*m*, 1H, 18-H), 2.07 (*s*, 4H, 60-H), 2.06 (*s*, 3H, 36-H), 2.04–2.01 (*m*, 1H, 1-H_a), 1.99 (*s*, 3H, 34-H), 1.95 (*s*, 7H, 32-H, 55-H), 1.91–1.86 (*m*, 2H, 11-H), 1.60–1.57 (*m*, 1H, 9-H), 1.46–1.42 (*m*, 2H, 21-H_a, 22-H_b), 1.35–1.30 (*m*, 4H, 6-H, 19-H, 5-H), 1.26–1.22 (*m*, 12H, 16-H_a, 38-H, 41-H, 22-H_b, 7-H, 15-H, 21-H_b, 16-H_b), 1.11–

1.09 (*m*, 1H, 1-H_b), 1.04 *(s*, 3H, 23-H), 0.98–0.94 (*m*, 1H, 20-H), 0.91 (*s*, 3H, 29-H), 0.85 (*s*, 3H, 25-H), 0.83 (*s*, 3H, 27-H), 0.82 (*s*, 3H, 30-H), 0.72 (*s*, 3H, 26-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 176.3 (C-28), 170.8 (C-35, C-43), 170.4 (C-33), 170.3 (C-31), 152.0 (C-57), 151.3 (C-50), 139.1 (C-62), 136.6 (C-44), 130.3 (C-45), 129.6 (C-48), 129.2 (C-46) 129.1 (C-49), 127.0 (C-47, C-52), 124.5 (C-12), 123.4 (C-51), 113.0 (C-53), 105.2 (C-58), 74.9 (C-3), 69.9 (C-2), 65.3 (C-24), 55.6 (C-18), 51.0 (C-34, C-37, C-40, C-42), 50.5 (C-56, C-61), 47.7 (C-5), 47.5 (C-9), 46.2 (C-17), 43.7 (C-1), 41.9 (C-4, C-14), 39.5 (C-19), 38.6 (C-20), 37.8 (C-10), 32.6 (C-22), 31.9 (C-7), 30.6 (C-21), 29.7 (C-15), 29.6 (C-16), 27.6 (C-54), 23.3 (C-11), 22.6 (C-38, C-41), 22.6 (C-27), 21.2 (C-29), 21.0 (C-32), 20.8 (C-36), 20.7 (C-34), 20.6 (C-55), 19.9 (C-59), 19.7 (C-60), 17.9 (C-6), 17.4 (C-30), 17.1 (C-24), 17.0 (C-26), 14.1 (C-25) ppm; MS (ESI, MeOH/$CHCl_3$): *m*/z = 1084.6 (100%, $[M\text{-}Cl]^+$); analysis calcd for $C_{70}H_{91}N_4O_5Cl$ (1068.52): C 78.69, H 8.58, N 5.24; found: C 78.32, H 8.86, N 5.09.

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P8

Targeted theranostics: near-infrared triterpenoic acid-rhodamine conjugates as prerequisites for precise cancer diagnosis and therapy

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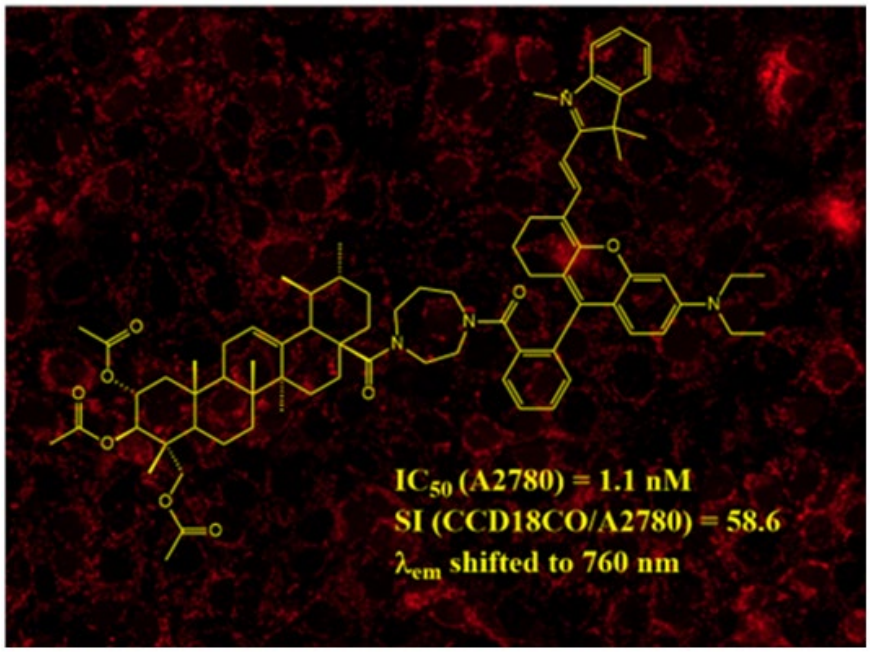
Keywords: asiatic acid; cytotoxicity; rhodamine; NIR-fluorescence; theranostics

Graphical abstract:



Abstract

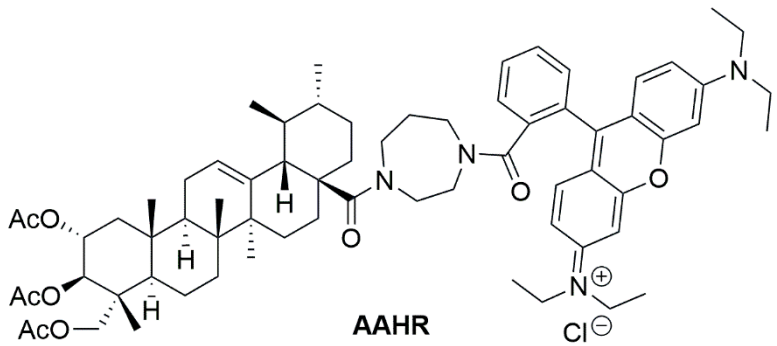
Pentacyclic triterpenoic acids have shown excellent potential as starting materials for the synthesis of highly cytotoxic agents with significantly reduced toxicity for non-malignant cells. This study focuses on the development of triterpenoic acid-rhodamine conjugates with fluorescence shifted to the near-infrared (NIR) region for theranostic applications in cancer research. Spectral analysis revealed emission wavelengths around λ = 760 nm, enabling

stronger signals and deeper tissue penetration. The conjugates were evaluated using SRB assays on tumor cell lines and non-malignant fibroblasts, demonstrating low nanomolar activity and high selectivity, similarly to their known rhodamine B counterparts. Additional staining experiments proved their mode of action as mitocans.

1. Introduction

Cancer is a major public health concern, and despite significant advances in cancer research and treatment, cancer remains a leading cause of death. [1] Diagnosis and treatment, or the course of treatment and the decision to continue or stop treatment, are usually carried out using different methods and at different times. In recent years, theranostics has emerged as a promising approach for the simultaneous identification and treatment of cancer. [2, 3] This has several advantages: First, theranostics allows targeted therapies directly to cancer cells while sparing healthy tissue. Thereby the risk of side effects associated with traditional treatments is avoided or minimized. Second, theranostics allows to create personalized treatment plans for individual patients, hence improving the overall efficacy of the treatments, and finally, theranostics enables to monitor the effectiveness of treatment in more-or-less real-time; this fact ensures most effective treatments while again minimizing the risk of side effects.

Pentacyclic triterpenoic acids have been shown to be excellent starting materials for the synthesis of highly cytotoxic agents while being significantly less cytotoxic for non-malignant tissue. Thereby conjugates consisting of a di- or tri-acetylated triterpene, a suitable amide spacer at the distal position of the triterpene and a rhodamine proved to be especially promising molecules. [4-11] For example, an asiatic acid derived conjugate (AAHR, Fig. 1) held cytotoxic activity in sub-nanomolar concentration, and was also able to be effective even in multiple resistant cell lines, was active in 3D spheroids and acted as a mitocan by shutting down mitochondrial ATP production. [12]



AAHR

Fig. 1. Structure of mitocanic homopiperacinyl spacered asiatic acid rhodamine B conjugate **AAHR** of superior cytotoxic activity

Despite their superior cytotoxicity, these compounds, however, proved unsuitable for *in vivo* studies because their absorption and emission wavelengths were limited to the ultraviolet and visible regions. In recent years, near-infrared (NIR) fluorescence imaging has emerged as a powerful tool for *in vivo* imaging of biological processes and molecular events in living cells and tissues, including tumor growth and response to therapy. [13-16] By incorporating fluorescent tags, biodistribution and efficacy can be monitored *in vivo* in real time, allowing rapid optimization of their structure and dosing regimen. They offer deeper tissue penetration, reduced background fluorescence and improved signal-to-noise ratio. However, the development of efficient NIR fluorescent probes remains challenging due to the lack of suitable fluorophores and design strategies. [17, 18] Hereby, we report the development of a new class of triterpenoic acid-rhodamine conjugates with fluorescence shifted to the NIR region. This has been achieved by redesigning the fluorophore so that it retains its photophysical properties but emits in the NIR when excited by higher wavelength light sources but it also keeps its cytotoxic properties. Fluorescence wavelengths are highly dependent on the substituents attached to the amino group of the rhodamine. [19] Quite recently, a new type of NIR functional dyes has been introduced, i.e., the Changsha (CS) NIR dyes. These dyes are hybrids of a merocyanine and a benzoic acid derivative. [20] Due to its high structural similarity to rhodamine B (calculated maximum common substructure MCS = 0.72), we decided that the dye CS-2 (Fig. 2) should be suited to label triterpenoic acids thus allowing us to investigate whether these hybrids would be able to act as cytotoxic agents (preferentially as a mitocan) and to work as a probe for malignant cells.

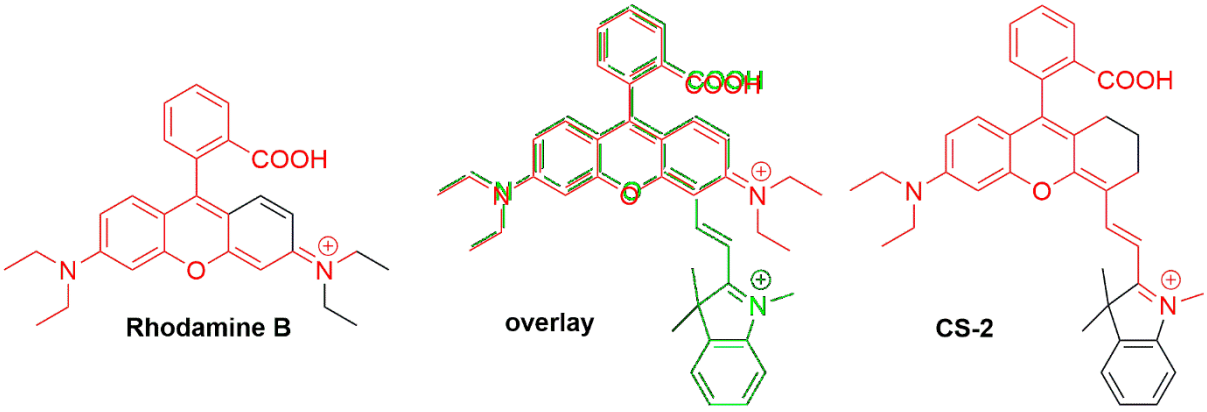


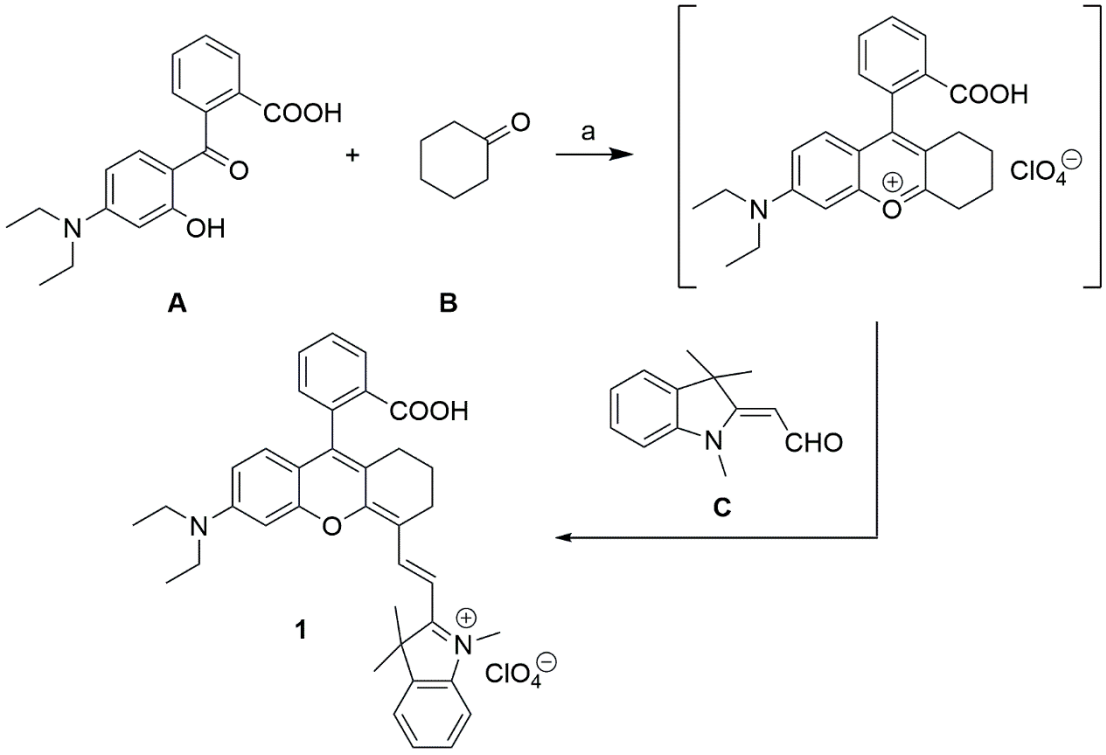
Fig. 2. Comparison of rhodamine B and CS-2 (**1**).

Changsha-type dyes have previously been used for biological imaging of HClO in living mice [21], to measure lysosomal pH inside endothelial and breast cancer cells [22], to image glutathione in HepG2 cells [23-25], and as a chemo-dosimeter for Mg^{2+}, Al^{3+} and Cu^{2+} ions but also for some markers of hepatotoxicity [26-28]. Recently a glycyrrhetinic acid decorated

conjugate was shown to be taken up in lysosomes of liver cancer cell lines HepG2 and Huh7 thereby allowing imaging of hepatocellular carcinoma. [29]

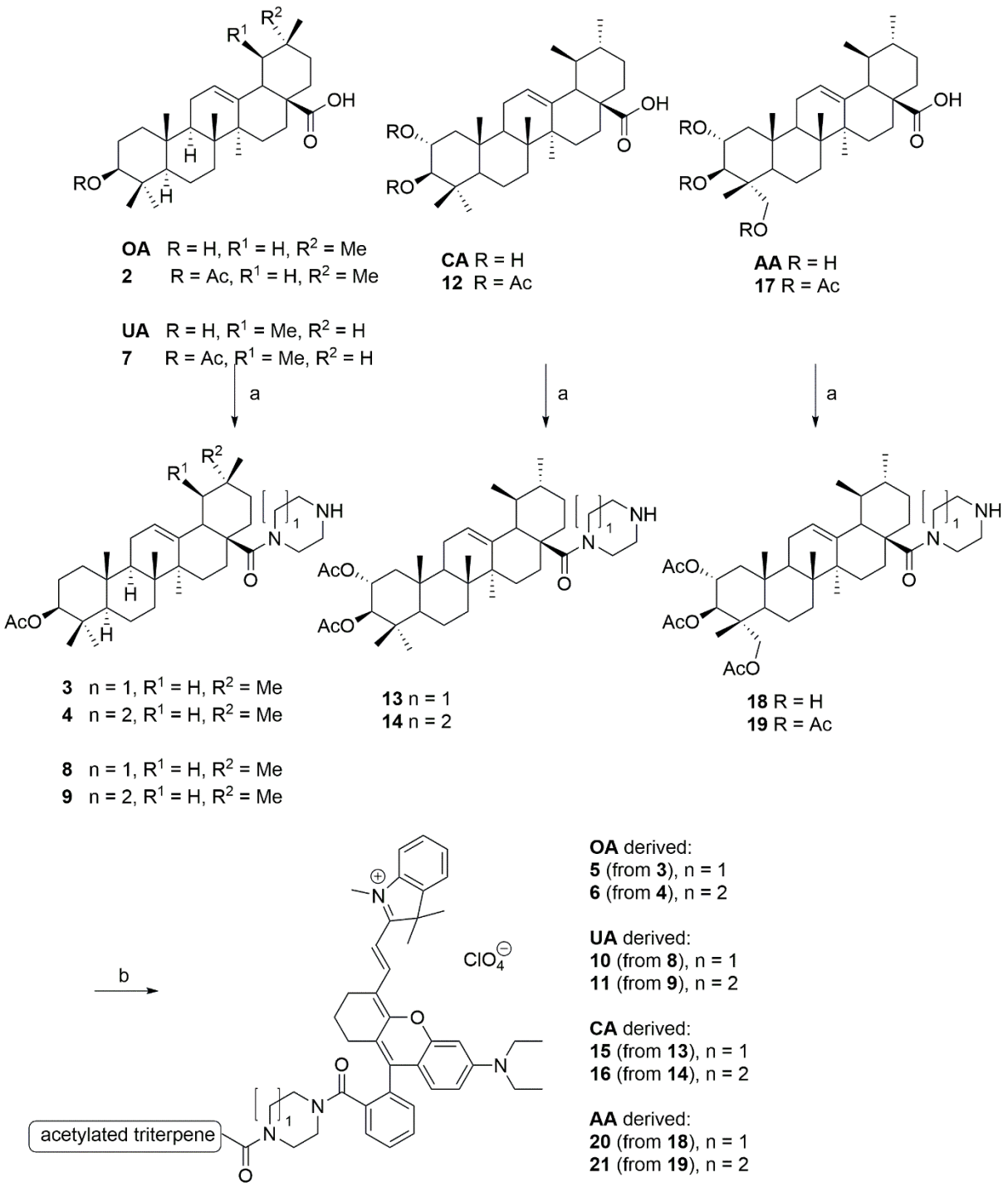
2. Results and discussion

The synthesis of CS-2 (**1)** was accomplished according to literature. [20] Thus, condensation of a commercially available substituted benzoic acid (**A**, Scheme 1) with cyclohexanone (**B**) under acidic conditions followed by reaction with the corresponding Fisher base **C** in acetic anhydride afforded the substituted CS-2 rhodamine **1** as a dark green solid in 50% overall yield.



Scheme 1. Synthesis of the Changsha dye CS-2 **1**. Reactions and conditions: a) conc. H_2SO_4, 90 °C, 90 min; then $HClO_4$; b) Ac_2O, 50 °C; over-all yield: 50%.

Acetylation of triterpenoic acids, oleanolic acid (**OA**, Scheme 2), ursolic acid (**UA**), corosolic acid (**CA**) and asiatic acid (**AA**) gave the known acetates **2**, **7**, **12**, and **17**, respectively. Their carboxyl group was activated with oxalyl chloride followed by the addition of either piperazine or homopiperazine to afford the corresponding amides **3**, **4**, **8**, **9**, **13**, **14**, **18** and **19**. Activation of the CS-2 rhodamine **1** with oxalyl chloride and its reaction with the amine-spaced triterpenoic acids yielded the desired conjugates **5**, **6**, **10**, **11**, **15**, **16**, **20** and **21**, respectively.



Scheme 2. Synthesis of the (homo)-piperazinyl spacered triterpenoic acid CS-2 hybrids; reactions and conditions: a) $(COCl)_2$, DMF (cat.), DCM, 1 h; b) DCM, NEt_3, 1 d, 23 °C

To demonstrate their suitability for NIR experiments, fluorescence spectra were recorded and compared with those of conjugates derived from rhodamine B. Thereby, the samples were irradiated at a fixed excitation wavelength of λ = 580 nm or λ = 430 nm to obtain the emission spectra. The wavelength of maximum fluorescence intensity was used as the fixed emission wavelength to obtain the excitation spectrum of each compound (Fig. 3).

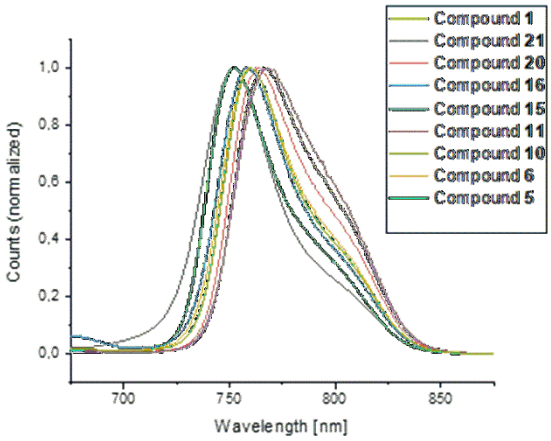


Fig. 3. Emission spectra of parent dye CS-2 (**1**) and spacered triterpenoic acid CS-2 hybrids **5**, **6**, **10**, **11**, **15**, **16**, **20**, and **21** (in MeOH solution; excitation wavelengths λ = 500/430 nm).

The maximum excitation wavelength of the rhodamine B conjugates (Fig. 4) has been shifted from long-wave UV in CS-2 conjugates to λ = 565 nm; this wavelength should result in deeper tissue penetration without photo-damaging the biological probes. This is particularly important for applications such as fluorescence-guided surgery and imaging of deep-seated tumor cells. In addition, biological probes excited by higher wavelength light sources often exhibit less background fluorescence, resulting in an improved signal-to-noise ratio and better image quality. These observations are based on the fact that many biological samples such as tissues and cells, naturally fluoresce at lower wavelengths, which can interfere with the detection of fluorescent probes excited at UV wavelengths.

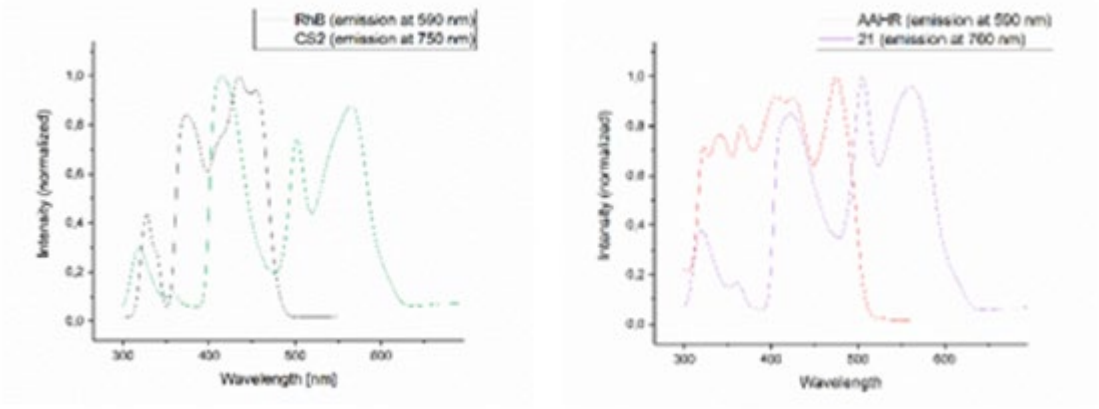


Fig. 4. Comparison maximum excitation wavelengths (vs normalized intensity) of rhodamine B and CS-2 (**1**, left) and a rhodamine B hybrid (**AAHR**) and the corresponding CS-2 conjugate **21** (right).

The wavelength of the emitted light has been shifted from around λ = 590 nm in rhodamine B conjugates to λ = 760 nm for the CS-2 conjugates. (Fig. 5)

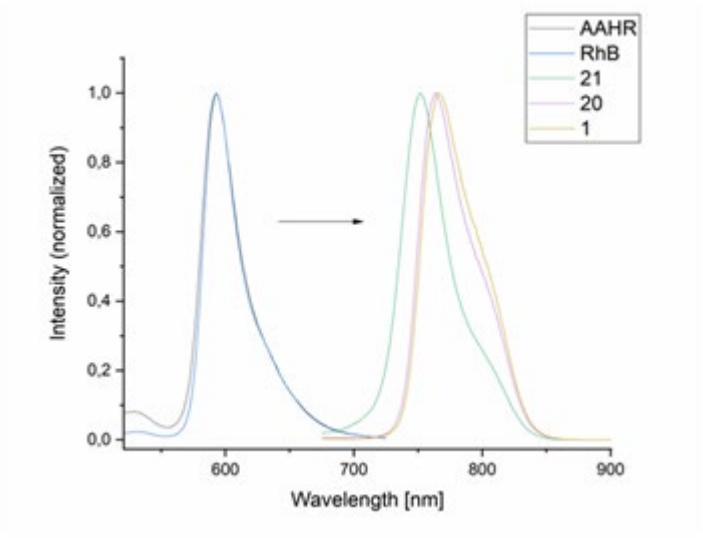


Fig. 5. Comparison emitted light in rhodamine B and CS-2 hybrids

As a result, the latter conjugates should be able to generate stronger signals from deeper tissue depths, making them extremely valuable for detecting and monitoring biological processes deep inside living organisms using highly sensitive imaging systems such as high-resolution microscopes and endoscopic imaging devices.

The CS-2 conjugates **5**, **6**, **10**, **11**, **15**, **16**, **20**, **21** were analyzed using SRB cytotoxicity assays, thereby using a selection of human tumor cell lines representing different solid tumor entities and non-malignant human fibroblasts (CCD18Co) to assess both their anti-proliferative/cytotoxic activity and their tumor cell/non-tumor cell selectivity. In addition, our panel included the cell line pair A2780/ A2780cis, a well-known model of acquired drug resistance to conventional drugs such as cisplatin and doxorubicin. The results from these assays are summarized in Table 1 and in Figures 6 and 7.

As a result, a compound and cell line dependent cytotoxic activity in the nanomolar range could be established. In general, conjugates from asiatic acid were more cytotoxic than their analogues derived from corosolic, oleanolic and ursolic acid, thereby resulting in single to three-digit nanomolar IC_{50} values. In each case, the homopiperazinyl linker led to an increased cytotoxic activity independently of the type of triterpene carboxylic acid. Furthermore, the A2780 cell line was the most sensitive model compared to the least sensitive model MCF7, with up to 12-fold differences in their IC_{50} values. The most active compounds (**16**, **20**, **21**) were also the most selective, as assessed by two different selectivity indices based on the most and least sensitive cell lines. The relative resistance of A2780Cis compared to A2780 was

reproduced in additional assays using doxorubicin, resulting in an approximately 10-fold difference in their IC_{50} values. The compounds were able to reduce this resistance by up to 5-fold. The analysis of subcellular localization for the CS2-hybrides proved the expected mitochondrial targeting. Thereby compounds **16**, **20** and **21** mostly resemble the accumulation pattern of BioTracker 488 and well known **AAHR**, whereas those derivatives holding less acetoxy groups also seem to accumulate partially in the nucleus.

Table 1. SRB cytotoxicity assay: IC_{50} values [nM] after 72 h of treatment; averaged from three to four independent experiments. Human cancer cell lines: A2780 (ovarian carcinoma), A2780Cis (resistant derivative of A2780), A549 (lung carcinoma), HT29 (colorectal carcinoma), MCF7 (breast carcinoma), CCD18Co (non-malignant human fibroblasts). Doxorubicin (**Dox**) has been used as a positive standard. Resistance index (RI): IC_{50} ratio of A2780Cis / A2780, Selectivity index 1 (SI 1): IC_{50} ratio of CCD18Co / A2780, Selectivity index 2 (SI 2): IC_{50} ratio of CCD18Co / MCF7.

#	A2780	A2780CIS	A549	HT29	MCF7	CCD18CO	RI	SI 1	SI 2
5	102.2 ± (21.2)	203.2 ± (43.5)	151.4 ± (12.2)	268.1 ± (47.8)	530.4 ± (82.0)	1229.0 ± (668.0)	2.0	12.0	2.3
6	32.8 ± (14.4)	116.6 ± (55.2)	58.5 ± (20.8)	158.2 (24.8)	336.9 ± (64.6)	250.6 ± (135.4)	3.6	7.6	0.7
10	123.0 ± (19.3)	223.3 ± (33.7)	210.1 ± (29.3)	376.8 ± (59.0)	495.8 ± (69.7)	606.7 ± (253.9)	1.8	4.9	1.2
11	73.2 ± (3.3)	166.8 ± (35.9)	86.2 ± (18.0)	233.1 ± (13.2)	418.0 ± (49.5)	256.0 ± (138.5)	2.3	3.5	0.6
15	33.2 ± (7.1)	75.4 ± (34.2)	87.5 ± (19.5)	127.7 ± (16.0)	147.4 ± (42.0)	441.6 ± (252.0)	2.3	13.3	3.0
16	7.7 ± (2.3)	25.2 (11.4)	34.8 ± (6.5)	49.3 ± (18.7)	77.6 ± (3.6)	375.3 ± (274.8)	3.3	49.0	4.8
20	3.5 ± (0.0)	6.7 ± (0.0)	17.0 ± (8.5)	11.3 ± (5.6)	19.9 ± (5.1)	118.8 ± (46.3)	1.9	34.0	6.0
21	1.1 ± (0.3)	3.1 ± (0.5)	5.6 ± (0.6)	5.2 ± (1.9)	12.9 ± (1.5)	64.1 ± (22.1)	2.9	58.6	5.0
1	1514.0 ± (0.0)	1140.0 ± (0.0)	1578.0 ± (0.0)	1838.0 ± (0.0)	1273.0 ± (0.0)	6505.0 ± (0.0)	0.8	4.3	5.1
DOX	8.5 ± (2.4)	80.4 ± (24.6)	21.5 ± (4.1)	107.8 ± (33.0)	34.2 ± (1.8)	757.1 ± (166.7)	9.4	88.8	22.2

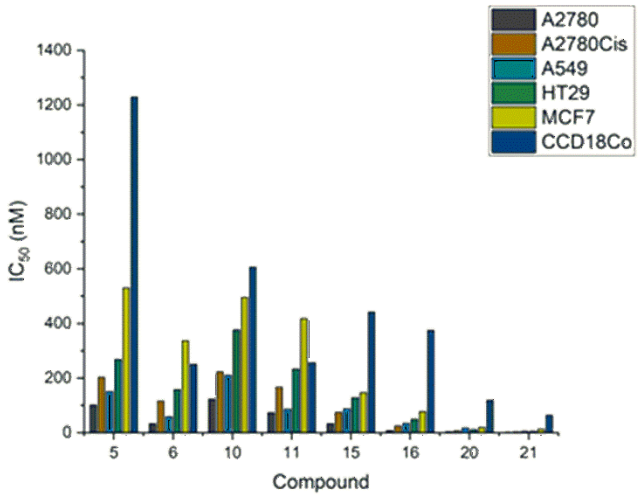


Fig. 6. Cytotoxicity (IC_{50} values, nM) of CS-2 triterpene conjugates for different cancer cell lines (A2780, A2780cis, A549, HT29, MCF7) as well as non-malignant human fibroblasts CCD18Co.

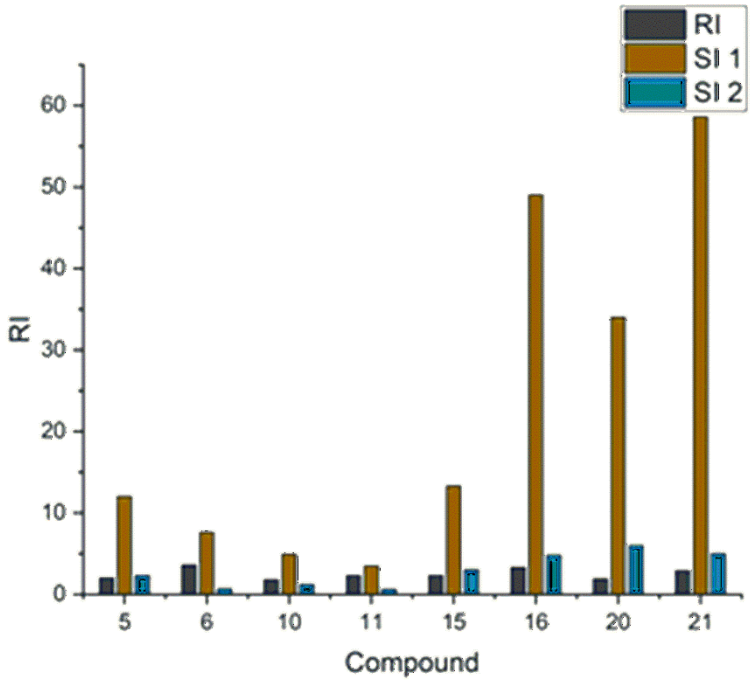


Fig. 7. Comparison of resistance/selectivity index values for the CS-2 hybrids; selectivity index 1 (SI 1): IC_{50} ratio of CCD18Co / A2780, selectivity index 2 (SI 2): IC_{50} ratio of CCD18Co / MCF7.

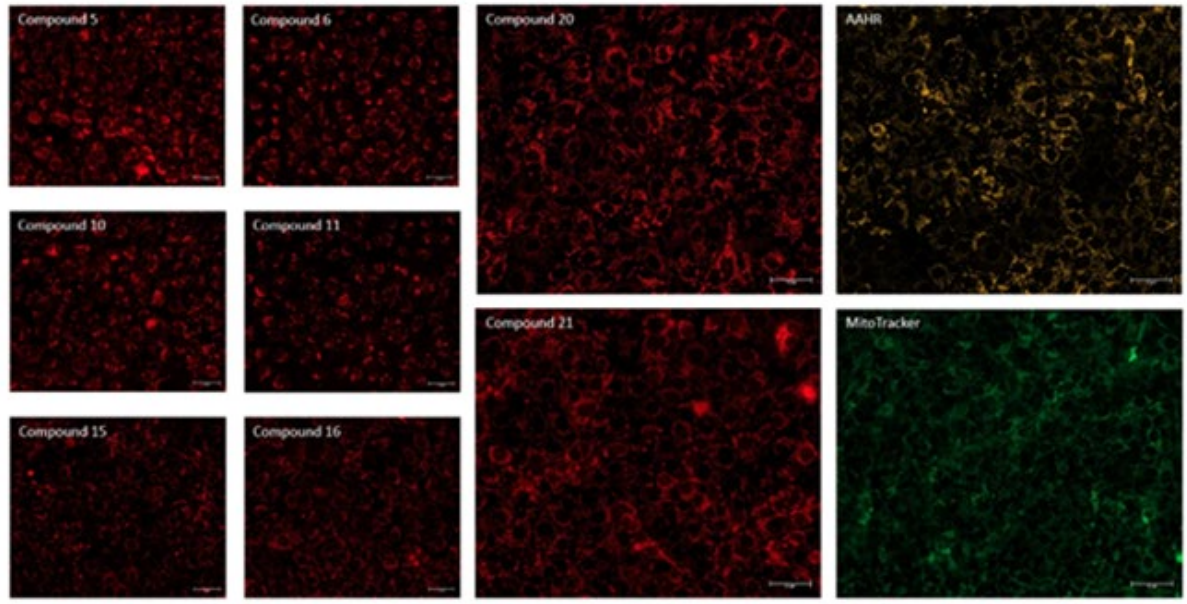


Fig. 8. Analysis of subcellular localization of CS2-compounds compared to the mitochondrial targeting compound BioTracker™ 488 Green Mitochondria Dye (Mito, lower right) and **AAHR** (lower, left) in A549 cells showing different pattern of accumulation among CS2-compounds, with **16**, **20** and **21** most resembling those of BioTracker and AAHR, indicating mitochondrial targeting. Scale bar: 50µm.

3. Conclusion

Our studies confirm the initial hypothesis that not only the triterpene backbone and the type of amide, but also the number of acetoxy groups and the linkage position of the rhodamine residue significantly influence the cytotoxic activity and tumor cell selectivity. Furthermore, the best performing compound **21** showed very similar characteristics in terms of cytotoxicity and selectivity as well as subcellular accumulation pattern compared to the chemical/structural congener containing the rhodamine B residue (**AAHR**) reported in our previous study. Thus, the specific modification of the rhodamine to obtain near-infrared spectral properties does not affect the efficacy of the compounds and proves these compounds as useful probes for further biological evaluation.

4. Experimental part

4.1. General

Fluorescence spectra were recorded using an Edinburgh Instruments FS5 spectrofluorometer. The measured methanolic dye solutions were placed in a 10 mm quartz cuvette with a filling volume of 500 µL. The samples were irradiated with a 150 W CW ozone-free xenon lamp at room temperature. The emitted photons were counted in 1 nm steps for 0.5 seconds. To record

the spectra of CS-2 rhodamine solutions, the width of the excitation and emission light paths was set to 3 mm and 2.5 mm respectively. The samples were irradiated at a fixed excitation wavelength of λ = 580 nm to obtain the emission spectra. The wavelength of maximum fluorescence intensity was used as the fixed emission wavelength to obtain the excitation spectrum of each individual CS-2 rhodamine dye. To obtain the spectra of the modified rhodamine B solutions, the width of the excitation and emission light paths was reduced to 1 mm due to the very high intensity of the fluorescent light. The samples were irradiated at a fixed excitation wavelength of λ = 430 nm to obtain the emission spectra. The wavelength of maximum fluorescence intensity was used as the fixed emission wavelength to obtain the excitation spectrum of each individual rhodamine B dye.

TLC was carried out on silica gel (Macherey-Nagel, detection under UV light and with cerium molybdate reagent). The used solvents were dried according to usual procedures. Melting points are uncorrected (Leica hot stage microscope, or BÜCHI melting point M-565). IR spectra were recorded on a Perkin Elmer FT-IR spectrometer Spectrum 1000 or on a Perkin-Elmer Spectrum Two (UATR Two Unit). NMR spectra were recorded using the Agilent spectrometers DD2 500 MHz and VNMRS 400 MHz (δ given in ppm, J in Hz; typical experiments: APT, H-H-COSY, HMBC, HSQC, NOESY), MS spectra were taken on an Advion Expression CMS instrument, and elemental analyses were conducted on a Foss-Heraeus Vario EL (CHNS) unit. Rhodamine B as well as the triterpenoic acids were obtained from local vendors and used as received. The SRB assays were performed as previously reported.

4.2. Cell culture

The human cancer cell lines A2780 (ECACC #93112519), A2780Cis (ECACC # 93112517), A549 (ATCC - CCL-185), HT29 (ATCC - HTB-38), MCF7 (ATCC - HTB-22) were cultivated in RPMI1640 medium, non-malignant human fibroblasts CCD18Co (ATCC - CRL-1459) were grown in MEME (both from Sigma-Aldrich, St. Louis, MO, USA). Both media were supplemented with 10% fetal bovine serum (Biowest, Nuaillé, France) and 1% penicillin-streptomycin (Sigma-Aldrich).

4.3. SRB assay

Cytotoxic activities of compounds were analyzed using the SRB cytotoxicity assay. Cells were seeded in 96-well plates and after 24h were treated with serial dilutions of compounds for 72 h. All subsequent steps were performed according to the previously described SRB assay protocol.

[30] Dose-response curves and calculation of IC50 values including standard deviations were carried out using GraphPad Prism8.

4.4. Staining

Analysis of subcellular localization of compounds was performed in A549 cells using the mitochondrial targeting compound BioTracker™ 488 Green Mitochondria Dye (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and the established compound AAHR for comparison. Cells were seeded in a µ-Plate 96 Well Black plate (ibiTreat: #1.5 polymer coverslip bottom, ibidi GmbH, Gräfelfing, Germany) at cell density of 30.000 per well. After 24h cells were treated with 20nM of compounds for 24h or 100nM BioTracker488 for 30min, followed by rinsing and supplementation with RPMI 1640 w/o Phenol-red (Pan-Biotech GmbH, Aidenbach, Germany). Live cell imaging was performed on an Axio Observer 7 (Carl Zeiss Microscopy Deutschland GmbH, Oberkochen, Germany) using the settings for Ex/Em as followed: BioTracker (475nm/514nm), AAHR (555nm/592nm), CS2-compounds (735nm/785nm). Scale bar: 50µm.

*4.5. General procedure for the synthesis of acetates **2, 7, 12 and 17** (GPA)*

To a solution of the triterpenoic acid (**OA**, **UA**, **CA**, **AA**) in DCM, acetic anhydride (3 equiv.), triethylamine (3 equiv.), and DMAP (cat.) were added, and the mixture was stirred at room temperature for 24 hours. Usual aqueous work-up followed by re-crystallization from ethanol gave products **2, 7, 12 and 17**.

4.6. General procedure for the synthesis of acetylated (homo)piperazinyl amides (GPB)

To a solution of the acetylated triterpenoic acid **2,7, 12 and 17** (1 equiv.) in dry DCM, DMF (cat.) and oxalyl chloride (4 equiv.) were added, and the reaction mixture was allowed to stir until the evolution of volatiles had ceased (about 1 h). The solvent was removed under reduced pressure, and the residue was re-dissolved in dry THF, and the solvent was removed again under reduced pressure again. The triterpenoic acyl chloride was dissolved in dry DCM and added dropwise to a solution of the amine (4 equiv.) in dry DCM. After stirring for 1h at room temperature, followed by usual aqueous work-up and column chromatography, products **3**/**4, 8**/**9, 13**/**14** and **18**/**19** were obtained.

4.7. General procedure for the synthesis of the rhodamine conjugates (GPC)

The acyl chloride was prepared as described (GPB) above starting from **1**. This acid chloride was slowly added to the solution of the respective triterpenoic amide (1.25 eq. in DCM) in the presence of triethylamine (2 eq.). After stirring for 1 day at room temperature, the crude product was purified by column chromatography to yield **5**/**6**, **10**/**11**, **15**/**16** and **20**/**21**, respectively.

4.8. Syntheses

*4.8.1. 2-[(1E)-2-[9-(2-carboxyphenyl)-6-(diethylamino)-2,3-dihydro-1H-xanthen-4-yl]ethenyl]-1,3,3-trimethyl-3H-indolium perchlorate (**1**) [20]*

A solution of cyclohexanone (6.6 mL, 64.0 mmol) in concentrated sulphuric acid (80 mL) was cooled to 0 °C, 2-(4-(diethylamino)-2-hydroxybenzoyl)-benzoic acid (10.0 g, 32.0 mmol) was added, and the reaction mixture was heated at 90 °C for 90 min. After cooling to room temperature, the reaction mixture was poured into ice cold water, perchloric acid (70%, 10 mL) was added, and the precipitate was filtered off. It was redissolved in acetic anhydride (250 mL). (*Z*)-2-(1,3,3-trimethylindolin-2-ylidene)-acetaldehyde (6.4 g, 32 mmol) was added, and the mixture was stirred at 50 °C for 30 min. After quenching with water (100 mL), the solvents were removed under reduced pressure; purification by column chromatography ($CHCl_3$:MeOH, 95:5) gave **1** (10.5 g, 50 %) as a dark green solid; m.p. >300 °C (decomp.); R_F = 0.11 (SiO_2, $CHCl_3$/MeOH, 95:5); UV-Vis (MeOH): λ_{max} (log ε) = 293 nm (4.75), 387 nm (4.78), 474 nm (4.45), 654 nm (4.75), 706 nm (5.18); IR (ATR): ν = 2931*w*, 1721*w*, 1624*w*, 1575*w*, 1549*w*, 1528*w*, 1438*s*, 1399*w*, 1365*m*, 1309*m*, 1268*m*, 1248*s*, 1223*m*, 1167*m*, 1142*m*, 1099*s*, 1062*s*, 1040*s*, 1016*s*, 994*m*, 924*m*, 859*w*, 814*m*, 796*m*, 748*m*, 710*w*, 675*w*, 621*m* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 8.56 (*d*, *J* = 14.1 Hz, 1H, 25-H), 8.22 (*d*, *J* = 7.0 Hz, 1H, 3-H), 7.71 (*dd*, *J* = 7.5-H, 1.2 Hz, 1H, 5-H), 7.58 (*dd*, *J* = 7.8 Hz, 1.2 Hz, 1H, 4-H), 7.41 (*dd*, *J* = 7.4 Hz, 0.6 Hz, 1H, 30-H), 7.36 (*td*, *J* = 7.8 Hz, 1.1 Hz, 1H, 31-H), 7.22 (*d*, *J* = 7.4 Hz, 1H, 32-H), 7.15 (*s*, 1H, 13-H), 7.13 (*s*, 1H, 6-H), 6.67 (*d*, *J* = 9.1 Hz, 1H, 33-H), 6.60 (*d*, *J* = 2.2 Hz, 1H, 10-H), 6.57 (*d*, *J* = 3.1 Hz, 1H, 11-H), 6.05 (*d*, *J* = 14.1 Hz, 1H, 26-H), 3.65 (*s*, 3H, 37-H), 3.50 (*q*, *J* = 7.1 Hz, 4H, 21-H, 22-H), 2.64 (*t*, *J* = 5.8 Hz, 2H, 19-H), 2.30 – 2.22 (*m*, 2H, 17-H), 1.78 (*s*, 3H, 36-H), 1.77 (*s*, 3H, 35-H), 1.84 – 1.70 (*m*, 2H, 18-H), 1.25 (*t*, *J* = 7.1 Hz, 6H, 23-H, 24-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 173.3 (C-27), 168.5 (C-1), 163.4 (C-14), 156.0 (C-8), 152.3 (C-15), 152.1 (C-12), 143.0 (C-34), 142.1 (C-25), 140.7 (C-29), 136.3 (C-7), 133.5 (C-5), 132.0 (C-3), 129.5 (C-6), 129.5 (C-4), 129.1 (C-2), 128.8 (C-31), 128.3 (C-30), 125.2 (C-32), 122.3 (C-33), 121.2 (C-20), 116.0 (C-16), 113.6 (C-9), 112.4 (C-10), 110.6 (C-13), 99.5 (C-26), 96.0, 49.3 (C-28), 45.3 (C-21, 22), 31.7 (C-37), 28.6 (C-35, C-36), 26.7 (C-17), 24.3 (C-19), 20.6 (C-

18), 12.6 (C-23, C-24) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 559.2 (100%, $[M]^+$); anal. Calcd for $C_{37}H_{39}N_2O_7Cl$ (659.18), C 67.42, H 5.96, N 4.25; found: C 67.23, H 6.13, N 4.01.

4.8.2. 3β-Acetyloxy-olean-12-en-oic acid (**2**)

Following GPA, compound **2** (1.24 g, 92%) was obtained from **OA** as a colorless solid; R_f = 0.70 (toluene/ethyl acetate/heptane/formic acid, 80:26:10:5); m.p. 285–287 °C (lit.: 286–289 °C [6]); $[\alpha]_D^{20}$ = +66.1° (*c* 0.20, $CHCl_3$) [(lit.: $[\alpha]_D^{20}$ = +69.4° (*c* 0.30, $CHCl_3$) [6]]; MS (ESI, MeOH): *m*/*z* (%) = 499.2 ([100%, M + H]$^+$).

4.8.3. 3β-Acetyloxy-28-(1-piperazinyl)-olean-12-en-28-one (**3**)

Following GPB from **2** (4.0 g, 8.0 mmol) and piperazine (2.8 g, 32.0 mmol), **3** (3.95 g, 87%) was obtained as a colorless solid; R_f = 0.12 (SiO_2, $CHCl_3$/MeOH, 95:5); m.p. = 173–175 °C (lit.: 172–175 °C [6]); $[\alpha]_D^{20}$ = +28.0° (*c* 0.26, $CHCl_3$), [lit.: $[\alpha]_D^{20}$ = +23.4° (*c* 0.18, $CHCl_3$) [6]]; MS (ESI, MeOH): *m*/*z* (%) = 567.3 (100%, [M + H]$^+$).

4.8.4. 3β-Acetyloxy-28-(1-homopiperazinyl)-olean-12-en-28-one (**4**)

Following GPB from **2** (4.0 g, 8.0 mmol) and homopiperazine (3.2 g, 32.0 mmol), **4** (4.2 g, 91%) was obtained as a colorless solid; R_f = 0.12 (SiO_2, $CHCl_3$/MeOH, 95:5); m.p. 183–185 °C (lit.: 182–185 °C [6]); $[\alpha]_D^{20}$ = 12.0° (*c* 0.14, $CHCl_3$), [lit.: $[\alpha]_D^{20}$ = +12.4° (*c* 0.14, $CHCl_3$) [6]]; MS (ESI, MeOH): *m*/*z* (%) = 581.3 (100%, [M + H]$^+$).

*4.8.5. (E)-2-(2-[6-(diethylamino)-9-[2-[4-[3β-Acetyloxy-28-oxo-olean-12-en-28-ylpiperazine-1-carbonyl]phenyl]-2,3-dihydro-1H-xanthen-4-yl]vinyl)-1,3,3-trimethyl-3H-indol-1-ium perchlorate (**5**)*

Following GPC from **3** (283 mg, 0.5 mmol) and **1** (250 mg, 0,38 mmol), **5** (280 mg, 61%) was obtained as a green solid; m.p. >300 °C (decomp.); R_F = 0.10 (SiO_2, $CHCl_3$/MeOH, 95:5); UV-Vis (MeOH): λ_{max} (log ε) = 296 nm (4.41), 385 nm (4.44), 477 nm (4.23), 659 nm (4.71), 715 nm (5.08); IR (ATR): ν = 2928*w*, 1728*w*, 1624*m*, 1578*w*, 1551*w*, 1440*m*, 1400*w*, 1365*m*, 1309*w*, 1268*w*, 1247*s*, 1226*m*, 1167*m*, 1143*m*, 1096*s*, 1064*s*, 1043*m*, 1000*m*, 925*m*, 860*w*, 811*m*, 747*m*, 671*w*, 622*m* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 8.58 (*d*, *J* = 13.7 Hz, 1H, 57-H), 7.62 – 7.54 (*m*, 2H, 39-H, 40-H), 7.46 – 7.36 (*m*, 3H, 41-H, 42-H, 61-H), 7.28 – 7.17 (*m*, 3H, 52-H, 62-H, 64-H), 6.81 (*dd*, *J* = 18.3 Hz, 9.1 Hz, 1H, 63-H), 6.76 – 6.65 (*m*, 1H, 55-H), 6.55 (*dd*, *J* = 9.4 Hz, 2.0 Hz, 1H, 54-H), 6.07 (*t*, *J* = 13.1 Hz, 1H, 58-H), 5.22 (*s*, 1H, 12-H), 4.49 – 4.45 (*m*, 1H, 3-H), 3.71 (*d*, *J* = 7.0 Hz, 3H, 67-H), 3.54 – 3.47 (*m*, 4H, 70-H, 72-H), 3.46

– 3.05 (*m*, 8H, 33-H, 34-H, 35-H, 36-H), 3.00 (*d*, *J* = 15.3 Hz, 1H, 18-H), 2.71 – 2.61 (*m*, 2H, 46-H), 2.57 – 2.46 (*m*, 1H, 16-H_b), 2.40 – 2.28 (*m*, 1H, 16-H_a), 2.14 – 2.05 (*m*, 1H, 2-H_a), 2.03 (*s*, 3H, 32-H), 1.93 – 1.81 (*m*, 4H, 11-H, 47-H), 1.78 (*s*, 3H, 69-H), 1.78 (*s*, 3H, 68-H), 1.75 – 1.30 (*m*, 13H, 1-H_a, 2-H_b, 6-H, 7-H_a, 9-H, 15-H_a, 19-H_a, 21-H, 22-H_a, 48-H), 1.28 – 1.22 (*m*, 6H, 71-H, 73-H), 1.10 (*s*, 3H, 27-H), 1.19 – 0.98 (*m*, 5H, 1-H_b, 7-H_b, 15-H_b, 19-H_b, 22-H_b), 0.90 – 0.89 (*m*, 3H, 25-H), 0.87 (*s*, 3H, 30-H), 0.86 (*s*, 3H, 29-H), 0.85 (*s*, 3H, 23-H), 0.83 (*s*, 3H, 24-H), 0.83 – 0.78 (*m*, 1H, 5-H), 0.67 (*s*, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 175.8 (C-28), 174.1 (C-59), 171.1 (C-31), 168.2 (C-37), 163.0 (C-51), 155.8 (C-44), 152.1 (C-53), 152.1 (C-50), 144.8 (C-13), 142.9 (C-60), 142.7 (C-57), 140.8 (C-65), 134.5 (C-43), 133.3 (C-38), 130.3 (C-42), 129.7 (C-41), 129.6 (C-39), 129.3 (C-40), 129.0 (C-63), 127.6 (C-64), 125.6 (C-62), 122.7 (C-45), 122.4 (C-61), 121.6 (C-12), 116.0 (C-49), 113.5 (C-56), 112.1 (C-55), 110.9 (C-52), 99.9 (C-58), 96.0 (C-54), 81.0 (C-3), 55.4 (C-5), 49.5 (C-17), 47.8 (C-9), 47.6 (C-66), 47.1 (C-33, 36), 46.8 (C-34, 35), 46.4 (C-19), 45.3 (C-70, 72), 43.7 (C-18), 42.0 (C-14), 39.2 (C-8), 38.2 (C-1), 37.8 (C-10), 37.1 (C-4), 34.0, 33.1 (C-29), 32.9 (C-7), 31.9 (C-67), 30.4 (C-20), 30.1 (C-48), 29.9 (C-21), 28.5 (C-23), 28.2 (C-68, C-69), 27.9 (C-15), 27.2 (C-16), 26.0 (C-27), 24.4 (C-46), 24.1 (C-30), 23.6 (C-11), 23.5 (C-2), 21.4 (C-32), 20.7 (C-47), 18.3 (C-6), 17.0 (C-24), 16.8 (C-26), 15.5 (C-25), 12.6 (C-71, C-73) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* (%) = 1108.2 (100%, $[M]^+$); anal. calcd for $C_{73}H_{95}N_4O_9Cl$ (1208.03), C 72.58, H 7.93, N 4.64; found: C 72.39, H 8.07, N 4.42.

*4.8.6. (E)-2-(2-[6-(diethylamino)-9-[2-[4-[3β-Acetyloxy-28-oxo-olean-12-en-28-ylhomopiperazine-1-carbonyl]phenyl]-2,3-dihydro-1H-xanthen-4-yl]vinyl)-1,3,3-trimethyl-3H-indol-1-ium perchlorate (**6**)*

Following GPC from **4** (290 mg, 0.5 mmol) and **1** (250 mg, 0,38 mmol), **6** (225 mg, 49%) was obtained as a green solid; m.p. >300 °C (decomp.); R_F = 0.10 (SiO_2, $CHCl_3$/MeOH, 95:5); UV-Vis (MeOH): λ_{max} (log ε) = 300 nm (4.09), 391 nm (4.23), 478 nm (3.79), 659 nm (4.47), 718 nm (4.83); IR (ATR): ν = 2939*w*, 1729*w*, 1624*m*, 1577*w*, 1551*w*, 1441*m*, 1401*w*, 1365*m*, 1309*w*, 1268*w*, 1247*s*, 1227*m*, 1247*s*, 1227*m*, 1168*m*, 1143*m*, 1104*s*, 1065*s*, 1043*m*, 925*m*, 860*w*, 811*m*, 748*m*, 670*w*, 622*m* cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.57 (*d*, *J* = 13.9 Hz, 1H, 58-H), 7.59 – 7.47 (*m*, 2H, 40-H, 41-H), 7.48 – 7.34 (*m*, 3H, 42-H, 43-H, 63-H), 7.25 – 7.15 (*m*, 3H, 53-H, 62-H, 65-H), 6.87 – 6.77 (*m*, 1H, 64-H), 6.70 – 6.48 (*m*, 2H, 55-H, 56-H), 6.07 (*dd*, *J* = 39.5 Hz, 13.7 Hz, 1H, 59-H), 5.33 – 5.17 (*m*, 1H, 12-H), 4.47 (*t*, *J* = 7.2 Hz, 1H, 3-H), 3.74 – 3.68 (*m*, 3H, 68-H), 3.58 – 3.43 (*m*, 4H, 71-H, 72-H), 3.88 – 3.03 (*m*, 8H, 33-H, 34-H, 35-H, 37-H), 2.64 (*s*, 2H, 47-H), 2.32 (*s*, 3H, 16-H, 18-H), 2.11 (*s*, 1H, 2-H_b), 2.03 (*s*,

3H, 32-H), 1.97 – 1.81 (*m*, 5H, 11-H, 15-H_b, 49-H), 1.78 (*s*, 3H, 70-H), 1.77 (*s*, 3H, 69-H), 1.70 – 1.32 (*m*, 14H, 1-H_b, 2-H_a, 6-H, 7-H_b, 9-H, 19-H_b, 21-H, 22-H_b, 36-H, 48-H), 1.26 (*q*, *J* = 6.8 Hz, 6H, 73-H, 74-H), 1.10 (*s*, 3H, 27-H), 1.21 – 0.99 (*m*, 5H, 1-H_a, 7-H_a, 15-H_a, 19-H_a, 22-H_a), 0.91 (*s*, 3H, 30-H), 0.90 (*s*, 3H, 25-H), 0.86 (*s*, 3H, 29-H), 0.84 (*s*, 3H, 23-H), 0.83 (*s*, 3H, 24-H), 0.82 – 0.79 (*m*, 1H, 5-H), 0.70 (*s*, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 176.4 (C-28), 174.5 (C-60), 171.1 (C-31), 169.1 (C-38), 163.2 (C-52), 155.9 (C-45), 152.2 (C-51), 152.1 (C-54), 144.9 (C-13), 143.0, 142.9 (C-58, 61), 140.8 (C-66), 135.5 (C-44), 132.6 (C-39), 129.8 (C-43), 129.6 (C-42), 129.1 (C-41), 129.0 (C-40), 128.9 (C-64), 126.8 (C-65), 125.6 (C-63), 122.8 (C-46), 122.3 (C-62), 121.5 (C-12), 116.1 (C-50), 113.6 (C-57), 111.8 (C-56), 110.9 (C-53), 99.9 (C-59), 95.8 (C-55), 81.1 (C-3), 55.5 (C-5), 49.5 (C-17), 47.9 (C-67), 47.8 (C-9), 46.8 (C-37), 46.7 (C-33), 46.7, 46.6 (C-19), 46.5 (C-35), 46.5 (C-34), 45.3 (C-71, C-72), 45.2, 43.7 (C-18), 42.1 (C-14), 39.2 (C-8), 38.2 (C-1), 37.8 (C-10), 37.1 (C-4), 34.2 (C-22), 33.2 (C-29), 32.9 (C-7), 32.0 (C-68), 30.5 (C-20), 29.8 (C-36), 29.8 (C-48), 29.2 (C-21), 28.5 (C-23), 28.1 (C-69, 70), 28.0 (C-15), 27.1 (C-16), 25.9 (C-27), 24.4 (C-47), 24.2 (C-30), 23.6 (C-2), 23.5 (C-11), 21.4 (C-32), 20.6 (C-49), 18.3 (C-6), 17.1 (C-24), 16.8 (C-26), 15.5 (C-25), 12.6 (C-73, 74) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* (%) = 1122.1 (100%, $[M]^+$); anal. calcd for $C_{74}H_{97}N_4O_9Cl$ (1222.06), C 72.73, H 8.00, N 4.58; found: C 72.50, H 8.29, N 4.19.

4.8.7. 3β-Acetyloxy-urs-12-en-oic acid (**7**)

Following GPA, compound **7** (1.62 g, 94%) was obtained from **UA** as a colorless solid; R_f = 0.68 (*n*-hexane/ethyl acetate, 7:3); m.p. 281–283 °C (lit.: 281–283 °C [6]); $[\alpha]_D$ = +64.2° (*c* 0.15, $CHCl_3$) [lit.: $[\alpha]_D^{20}$ = +66.5° (*c* 0.42, $CHCl_3$) [6]]; MS (ESI, MeOH): *m*/*z* (%) = 499.3 (100%, $[M + H]^+$).

4.8.8. 3β-Acetyloxy-28-(1-piperazinyl)-urs-12-en-28-one (**8**)

Following GPB from **7** (2.5 g, 5.0 mmol) and piperazine (1.6 g, 20.0 mmol), **7** (2.44 g, 86%) was obtained as a colorless solid; R_f = 0.11 (SiO_2, $CHCl_3$/MeOH, 95:5); m.p. = 188–190 °C (lit.: 187–190 °C [6]); [$[\alpha]_D^{20}$ = +27.2° (*c* 0.10, MeOH), [lit.: $[\alpha]_D^{20}$ = +25.1° (*c* 0.24, MeOH) [6]]; MS (ESI, MeOH): *m*/*z* (%) = 567.4 (100%, $[M + H]^+$).

4.8.9. 3β-Acetyloxy28-(1-homopierazinyl)-urs-12-en-28-one (**9**)

Following GPB from **7** (1.0 g, 2.0 mmol) and homopiperazine (1.6 g, 16.0 mmol), **9** (2.0 g, 86%) was obtained as a colorless solid; R_f = 0.12 (SiO_2, $CHCl_3$/MeOH, 95:5); m.p. 172–175

°C (lit.: 171–175 °C [6]); $[\alpha]_D^{20}$ = +31.1° (*c* 0.15, $CHCl_3$), [lit.: $[\alpha]_D^{20}$ = +27.0° (*c* 0.21, $CHCl_3$) [6]]; MS (ESI, MeOH): *m/z* (%) = 581.4 (100%, $[M + H]^+$).

*4.8.10. (E)-2-(2-[6-(diethylamino)-9-[2-[4-[3β-Acetyloxy-28-oxo-ursan-12-en-28-ylpiperazine-1-carbonyl]phenyl]-2,3-dihydro-1H-xanthen-4-yl]vinyl)-1,3,3-trimethyl-3H-indol-1-ium perchlorate (**10**)*

Following GPC from **8** (283 mg, 0.5 mmol) and **1** (250 mg, 0.38 mmol), **10** (240 mg, 52%) was obtained as a green solid; m.p. >300 °C (decomp.); R_F = 0.10 (SiO_2, $CHCl_3$/MeOH, 95:5); UV-Vis (MeOH): λ_{max} (log ε) = 298 nm (4.38), 390 nm (4.42), 477 nm (4.08), 659 nm (4.68), 717 nm (5.04); IR (ATR): ν = 2925*w*, 1729*w*, 1624*m*, 1577*w*, 1551*w*, 1440*m*, 1399*w*, 1365*m*, 1309*w*, 1268*w*, 1247*s*, 1227*m*, 1167*m*, 1143*m*, 1095*s*, 1065*s*, 1043*m*, 1004*m*, 925*m*, 860*w*, 811*m*, 748*m*, 672*w*, 622*m* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 8.57 (*d*, *J* = 14.1 Hz, 1H, 57-H), 7.63 – 7.53 (*m*, 2H, 39-H, 40-H), 7.47 – 7.34 (*m*, 3H, 41-H, 42-H, 61-H), 7.25 – 7.16 (*m*, 3H, 52-H, 62-H, 64-H), 6.81 (*t*, *J* = 9.1 Hz, 1H, 63-H), 6.75 – 6.64 (*m*, 1H, 55-H), 6.54 (*s*, 1H, 54-H), 6.07 (*dd*, *J* = 14.0 Hz, 8.1 Hz, 1H), 5.24 – 5.11 (*m*, 1H, 12-H), 4.53 – 4.42 (*m*, 1H, 3-H), 3.70 (*d*, *J* = 3.9 Hz, 3H, 67-H), 3.50 (*q*, *J* = 7.0 Hz, 4H, 70-H, 72-H), 3.64 – 3.20 (*m*, 8H, 33-H, 34-H, 35-H, 36-H), 2.68 – 2.62 (*m*, 2H, 46-H), 2.58 – 2.46 (*m*, 1H, 16-H_b), 2.39 – 2.25 (*m*, 3H, 16-H_a, 48-H), 2.03 (*s*, 3H, 32-H), 1.78 (*s*, 6H, 68-H, 69-H), 1.94 – 1.66 (*m*, 5H, 11-H, 22-H_b, 47-H), 1.64 – 1.28 (*m*, 14H, 1-H_a, 2-H, 6-H, 7-H, 9-H, 15-H_a, 18-H, 19-H, 21-H, 22-H_a), 1.25 (*t*, *J* = 7.1 Hz, 6H, 71-H, 73-H), 1.04 (*s*, 3H, 27-H), 0.92 – 0.90 (*m*, 6H, 25-H, 29-H), 0.88 (*s*, 4H, 1-H_b, 5-H, 15-H_b, 20-H), 0.86 – 0.83 (*m*, 6H, 24-H, 30-H), 0.83 (*s*, 3H, 23-H), 0.67 (*s*, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 176.0 (C-28), 174.1 (C-59), 171.1 (C-31), 168.2 (C-37), 163.0 (C-51), 155.8 (C-44), 152.1 (C-53), 152.0 (C-50), 142.9 (C-60), 142.8 (C-57), 140.8 (C-13), 140.8 (C-65), 134.5 (C-43), 133.2 (C-38), 130.3 (C-42), 129.7 (C-41), 129.2 (C-39), 129.2 (C-40), 128.9 (C-63), 127.6 (C-64), 125.6 (C-62), 125.2 (C-12), 122.7 (C-45), 122.3 (C-61), 116.0 (C-49), 113.3 (C-56), 112.1 (C-55), 110.9 (C-52), 100.1 (C-58), 96.1 (C-54), 81.0 (C-3), 77.4, 77.2, 76.9, 55.4 (C-5), 49.5 (C-17), 48.7 (C-33, 36), 47.7 (C-18), 47.6 (C-9), 47.4 (C-34, 35), 46.5 (C-66), 45.3 (C-70, 72), 42.0 (C-14), 39.4 (C-19), 38.8 (C-20), 38.3 (C-1), 37.8 (C-8), 37.1 (C-4), 37.0 (C-10), 34.4 (C-22), 32.9 (C-7), 31.9 (C-67), 30.5 (C-21), 28.5 (C-68, C-69), 28.2 (C-15), 28.2 (C-23), 27.9 (C-48), 27.1 (C-16), 24.4 (C-46), 24.1 (C-27), 23.6 (C-11), 23.4 (C-2), 21.4 (C-32), 21.3 (C-29), 20.6 (C-47), 18.3 (C-6), 17.5 (C-30), 17.0 (C-26), 16.8 (C-24), 15.6 (C-25), 12.6 (C-71), 12.6 (C-73) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* (%) = 1108.2 (100%, $[M]^+$); anal. calcd for $C_{73}H_{95}N_4O_9Cl$ (1208.03), C 72.58, H 7.93, N 4.64; found: C 72.39, H 8.12, N 4.40.

*4.8.11. (E)-2-(2-[6-(diethylamino)-9-[2-[4-[3β-Acetyloxy-28-oxo-ursan-12-en-28-ylhomopiperazine-1-carbonyl]phenyl]-2,3-dihydro-1H-xanthen-4-yl]vinyl)-1,3,3-trimethyl-3H-indol-1-ium perchlorate (**11**)*

Following GPC from **9** (290 mg, 0.5 mmol) and **1** (250 mg, 0.38 mmol), **11** (225 mg, 49%) was obtained as a green solid; m.p. >300 °C (decomp.); R_F = 0.10 (SiO_2, $CHCl_3$/MeOH, 95:5); UV-Vis (MeOH): λ_{max} (log ε) = 295 nm (4.27), 390 nm (4.29), 477 nm (397), 659 nm (4.63), 718 nm (5.01); IR (ATR): ν = 2925*w*, 1729*w*, 1624*m*, 1577*w*, 1551*w*, 1441*m*, 1400*w*, 1365*m*, 1309*w*, 1268*w*, 1247*s*, 1227*m*, 1167*m*, 1143*m*, 1097*s*, 1064*s*, 1043*m*, 1018*m*, 925*m*, 860*w*, 811*m*, 748*m*, 670*w*, 622*m* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 8.57 (*d*, *J* = 13.4 Hz, 1H, 58-H), 7.58 – 7.48 (*m*, 2H, 40-H, 41-H), 7.46 – 7.32 (*m*, 3H, 42-H, 43-H, 62-H), 7.21 (*dd*, *J* = 28.5-H, 7.5 Hz, 3H, 53-H, 63-H, 65-H), 6.85 – 6.77 (*m*, 1H, 64-H), 6.70 – 6.46 (*m*, 2H, 55-H, 56-H), 6.14 – 5.99 (*m*, 1H, 59-H), 5.26 – 5.15 (*m*, 1H, 12-H), 4.50 – 4.44 (*m*, 1H, 3-H), 4.21 – 2.94 (*m*, 8H, 33-H, 34-H, 35-H, 37-H), 3.70 (*d*, *J* = 6.3 Hz, 3H, 68-H), 3.56 – 3.44 (*m*, 4H, 71-H, 73-H), 2.64 (*s*, 2H, 47-H), 2.42 (*s*, 1H, 16-H_b), 2.31 (*s*, 3H, 16-H_a, 49-H), 2.02 (*s*, 3H, 32-H), 2.15 – 1.69 (*m*, 5H, 11-H, 15-H_b, 48-H), 1.80 – 1.74 (*m*, 8H, 69-H, 70-H), 1.67 – 1.30 (*m*, 14H, 1-H_b, 2-H, 6-H, 7-H, 9-H, 18-H, 19-H, 21-H, 22-H), 1.26 (*t*, *J* = 6.5 Hz, 6H, 72-H, 74-H), 1.14 – 0.89 (*m*, 4H, 1-H_a, 5-H, 15-H_a, 20-H), 1.03 (*s*, 3H, 27-H), 0.93 – 0.88 (*m*, 9H, 23-H, 25-H, 29-H), 0.86 – 0.81 (*m*, 6H, 24-H, 30-H), 0.71 (*s*, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 176.6 (C-28), 174.1 (C-60), 171.1 (C-31), 168.6 (C-38), 163.1 (C-52), 155.7 (C-45), 152.2 (C-54), 152.1 (C-51), 143.0 (C-61), 142.9 (C-58), 140.8 (C-66), 140.7 (C-13), 135.5 (C-44), 132.7 (C-39), 129.9 (C-43), 129.8 (C-42), 129.2 (C-40), 129.1 (C-41), 128.9 (C-64), 127.0 (C-65), 125.6 (C-63), 125.1 (C-12), 122.9 (C-46), 122.4 (C-62), 116.1 (C-50), 113.3 (C-57), 111.9 (C-56), 110.9 (C-53), 99.7 (C-59), 96.1 (C-55), 81.0 (C-3), 55.4 (C-5), 49.4 (C-17), 49.0 (C-33), 49.0 (C-37), 47.9 (C-67), 47.8 (C-18), 47.7 (C-9), 46.6 (C-35), 46.5 (C-34), 45.3 (C-71, C-73), 42.2 (C-14), 39.8 (C-19), 38.8 (C-20), 38.3 (C-1), 37.8 (C-8), 37.0 (C-4, 10), 33.6 (C-22), 32.9 (C-7), 31.9 (C-68), 30.6 (C-21), 29.8 (C-36), 28.5 (C-69), 28.5 (C-70), 28.2 (C-15), 28.2 (C-23), 27.1 (C-16), 27.1 (C-49), 24.3 (C-47), 24.2 (C-27), 23.6 (C-2), 23.4 (C-11), 21.4 (C-29, 32), 20.6 (C-48), 18.3 (C-6), 17.6 (C-30), 17.1 (C-26), 16.8 (C-24), 15.6 (C-25), 12.6 (C-72, C-24) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* (%) = 1122.4 (100%, $[M]^+$); anal. calcd for $C_{74}H_{97}N_4O_9Cl$ (1222.06), C 72.73, H 8.00, N 4.58; found: C 72.47, H 8.24, N 4.38.

*4.8.12. 2α,3β-Bis(acetyloxy)urs-12-en-28-oic acid (**12**)*

To a solution of banaba leaf extract powder (10.0 g, 20 % corosolic acid, KAN Phytochemicals Pvt. Ltd) in pyridine (80 mL), acetic anhydride (20 mL) was added, and the reaction mixture was stirred at room temperature for 12-24 hours (TLC). The solution was precipitated in aq. HCl (2 M). The solid was subjected to column chromatography (hexanes: ethyl acetate 15 % → 40 %), and compound **12** (1.95 g) was obtained as an off-white solid; R_f = 0.59 (*n*-hexane/ethyl acetate, 6:4); m. p. 235–238 °C (lit.: 237-241 ° C [5]); $[\alpha]_D^{20}$ = +20.5° (*c* 0.25, $CHCl_3$) [lit.: $[\alpha]_D^{20}$ = +24.8° (*c* 0.15, $CHCl_3$) [5]]; MS (ESI, MeOH): *m*/*z* (%) = 625.5 (100%, $[M + H]^+$).

*4.8.13. (2α, 3β) 2,3-Bis(acetyloxy)-28-(1-piperazinyl)-urs-12-en-28-one (**13**)*

Following GPB from **12** (2.2 g, 4.0 mmol) and piperazine (1.36 g, 16.0 mmol), **13** (2.2 g, 88%) was obtained as a colorless solid; R_f = 0.14 (SiO_2, $CHCl_3$/MeOH, 95:5); m.p. 190–192 °C (lit.: 189–191 °C [5]); $[\alpha]_D^{20}$ = +8.3° (*c* 0.25, $CHCl_3$), [lit.: $[\alpha]_D^{20}$ = +10.3° (*c* 0.06, $CHCl_3$) [5, 6]]; MS (ESI, MeOH): *m*/*z* (%) = 639.4 (100%, $[M + H]^+$).

*4.8.14. (2α, 3β) 2,3-Bis(acetyloxy)-28-(1-homopiperazinyl)-urs-12-en-28-one (**14**)*

Following GPB from **12** (2.2 g, 4.0 mmol) and homopiperazine (1.6 g, 16.0 mmol), **14** (1.0 g, 78%) was obtained as a colorless solid; R_f = 0.13 (SiO_2, $CHCl_3$/MeOH, 95:5); m.p. 164–166 °C (lit.: 164–167 °C [5]); $[\alpha]_D^{20}$ = +12.7° (*c* 0.20, $CHCl_3$), [lit.: $[\alpha]_D^{20}$ = +9.0° (*c* 0.15, $CHCl_3$) [5]; MS (ESI, MeOH): *m*/*z* (%) = 639.2 (100%, $[M + H]^+$).

*4.8.15. (E)-2-(2-[6-(diethylamino)-9-[2-[4-[(2α,3β)-bis(acetyloxy)-28-oxo-ursan-12-en-28-ylpiperazine-1-carbonyl]phenyl]-2,3-dihydro-1H-xanthen-4-yl]vinyl)-1,3,3-trimethyl-3H-indol-1-ium perchlorate (**15**)*

Following GPC from **13** (312 mg, 0.5 mmol) and **1** (80 mg, 0.13 mmol), **15** (100 mg, 62%) was obtained as a green solid; m.p. >300 °C (decomp.); R_F = 0.10 (SiO_2, $CHCl_3$/MeOH, 95:5); UV-Vis (MeOH): λ_{max} (log ε) = 298 nm (4.10), 390 nm (4.14), 476 nm (3.80), 659 nm (4.40), 716 nm (4.76); IR (ATR): ν = 2927*w*, 1738*m*, 1625*m*, 1578*w*, 1552*w*, 1442*m*, 1397*w*, 1366*m*, 1310*w*, 1268*w*, 1248*s*, 1228*m*, 1168*m*, 1144*m*, 1108*s*, 1067*s*, 1042*m*, 1019*m*, 927*m*, 860*w*, 811*m*, 749*m*, 672*w*, 622*m* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 8.57 (*d*, *J* = 14.1 Hz, 1H, 59-H), 7.63 – 7.51 (*m*, 2H, 41-H, 44-H), 7.48 – 7.34 (*m*, 3H, 63-H, 65-H, 66-H), 7.25 – 7.15 (*m*, 3H, 42-H, 43-H, 64-H), 6.81 (*s*, 1H, 54-H), 6.68 (*s*, 1H, 57-H), 6.53 (*s*, 1H, 56-H), 6.09 – 6.03 (*m*, 1H, 60-H), 5.19 (*t*, *J* = 3.5 Hz, 1H, 12-H), 5.12 – 5.02 (*m*, 1H, 2-H), 4.72 (*d*, *J* = 10.3 Hz,

1H, 3-H), 3.69 (*d*, *J* = 5.7 Hz, 3H, 69-H), 3.64 – 3.54 (*m*, 4H, 35-H, 38-H), 3.50 (*q*, *J* = 7.0 Hz, 4H, 72-H, 74-H), 2.88 – 2.75 (*m*, 4H, 36-H, 37-H), 2.70 – 2.60 (*m*, 2H, 48-H), 2.45 – 2.25 (*m*, 5H, 16-H, 18-H, 50-H), 2.19 – 2.07 (*m*, 1H, 1-H_a), 2.03 (*s*, 3H, 34-H), 1.95 (*s*, 3H, 32-H), 2.01 – 1.80 (*m*, 4H, 11-H, 49-H), 1.77 (*s*, 6H, 70-H, 71-H), 1.72 (*s*, 3H, 15-H_b, 22-H), 1.60 – 1.27 (*m*, 8H, 6-H, 7-H, 9-H, 19-H, 21-H), 1.24 (*t*, *J* = 7.0 Hz, 6H, 73-H, 75-H), 1.12 – 0.97 (*m*, 4H, 1-H_b, 5-H, 15-H_a, 20-H), 1.04 (*s*, 3H, 25-H), 0.91 (*d*, *J* = 3.8 Hz, 3H, 29-H), 0.88 (*s*, 3H, 24-H), 0.88 – 0.87 (*m*, 6H, 23-H, 27-H), 0.84 (*d*, *J* = 6.4 Hz, 3H, 30-H), 0.74 (*s*, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 176.0 (C-28), 175.3 (C-61), 170.9 (C-31), 170.6 (C-33), 168.1 (C-39), 163.0 (C-53), 155.8 (C-46), 152.1 (C-52), 152.0 (C-55), 142.9 (C-62), 142.7 (C-59), 140.8 (C-13), 140.8 (C-67), 134.4 (C-45), 133.2 (C-40), 130.3 (C-44), 129.7 (C-43), 129.2 (C-41), 129.2 (C-42), 128.9 (C-65), 127.7 (C-66), 125.6 (C-64), 124.8 (C-12), 122.7 (C-47), 122.3 (C-63), 116.0 (C-51), 113.4 (C-58), 112.1 (C-57), 110.9 (C-54), 100.1 (C-60), 96.0 (C-56), 80.8 (C-3), 70.2 (C-2), 55.0 (C-5), 55.0 (C-18), 49.5 (C-17), 47.7 (C-9), 47.5 (C-35), 46.5 (C-38), 46.2 (C-36), 46.1 (C-68), 45.3 (C-72, C-24), 45.1 (C-37), 44.2 (C-1), 42.3 (C-14), 39.6 (C-19), 39.4 (C-8), 38.8 (C-20), 38.2 (C-4), 38.2 (C-10), 34.3 (C-22), 32.9 (C-7), 32.0 (C-69), 30.6 (C-21), 28.5 (C-23), 28.5 (C-70), 28.5 (C-71), 28.3 (C-15), 28.0 (C-50), 27.1 (C-16), 24.4 (C-48), 23.5 (C-11), 21.3 (C-32), 21.3 (C-27), 21.2 (C-34), 21.0 (C-29), 20.6 (C-49), 18.3 (C-6), 17.8 (C-24), 17.5 (C-30), 16.9 (C-26), 16.6 (C-25), 12.6 (C-73), 12.6 (C-75) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* (%) = 1166.5 (100%, $[M]^+$); anal. calcd for $C_{75}H_{97}N_4O_{11}Cl$ (1266.07), C 71.15, H 7.72, N 4.43; found: C 70.92, H 7.93, N 4.18.

*4.8.16. (E)-2-(2-[6-(diethylamino)-9-[2-[4-[(2α,3β)-bis(acetyloxy)-28-oxo-ursan-12-en-28-ylhomopiperazine-1-carbonyl]phenyl]-2,3-dihydro-1H-xanthen-4-yl]vinyl)-1,3,3-trimethyl-3H-indol-1-ium perchlorate (**16**)*

Following GPC from **14** (319 mg, 0.5 mmol) and **1** (250 mg, 0,38 mmol), **16** (235 mg, 48%) was obtained as a green solid; m.p. >300 °C (decomp.); R_F = 0.10 (SiO_2, $CHCl_3$/MeOH, 95:5); UV-Vis (MeOH): λ_{max} (log ε) = 296 nm (4.36), 390 nm (4.41), 480 nm (4.11), 659 nm (4.58), 718 nm (5.01); IR (ATR): ν = 2928*w*, 1738*w*, 1624*m*, 1577*w*, 1552*w*, 1441*m*, 1399*w*, 1366*m*, 1309*w*, 1268*w*, 1247*s*, 1226*m*, 1167*m*, 1143*m*, 1099*s*, 1065*s*, 1042*m*, 1018*m*, 925*m*, 860*w*, 811*m*, 747*m*, 669*w*, 622*m* cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.56 (*d*, *J* = 13.0 Hz, 1H, 60-H), 7.58 – 7.48 (*m*, 2H, 42-H, 45-H), 7.45 – 7.31 (*m*, 3H, 43-H, 64-H, 67-H), 7.25 – 7.21 (*m*, 1H, 65-H), 7.20 – 7.15 (*m*, 2H, 44-H, 55-H), 6.85 – 6.75 (*m*, 1H, 66-H), 6.70 – 6.44 (*m*, 2H, 57-H, 58-H), 6.16 – 5.97 (*m*, 1H, 61-H), 5.23 – 5.15 (*m*, 1H, 12-H), 5.07 (*td*, *J* = 10.9Hz, 4.6 Hz, 1H, 2-H), 4.72 (*d*, *J* = 10.3 Hz, 1H, 3-H), 3.75 – 3.65 (*m*, 3H, 70-H), 3.51 (m, 4H, 73-H,

74-H), 4.32 – 2.82 (*m*, 8H, 35-H, 36-H, 37-H, 39-H), 2.77 – 2.53 (*m*, 3H, 16-H_b, 49-H), 2.53 – 2.20 (*m*, 4H, 16-H_a, 18-H, 51-H), 2.03 (*s*, 3H, 34-H), 1.95 (*s*, 3H, 32-H), 2.19 – 1.69 (*m*, 8H, 1-H_b, 11-H, 15-H_b, 38-H, 50-H), 1.80 – 1.74 (*m*, 6H, 71-H, 72-H), 1.68 – 1.15 (*m*, 10H, 6-H, 7-H, 9-H, 19-H, 21-H, 22-H), 1.29 – 1.22 (*m*, 6H, 75-H, 76-H), 1.12 – 0.81 (*m*, 4H, 1-H_a, 5-H, 15-H_a, 20-H), 1.08 – 1.03 (*m*, 3H, 30-H), 1.02 (*s*, 3H, 25-H), 0.96 – 0.91 (*m*, 3H, 29-H), 0.88 – 0.86 (*m*, 9H, 23-H, 24-H, 27-H), 0.71 (*s*, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 176.6 (C-28), 174.1 (C-62), 170.9 (C-31), 170.6 (C-33), 168.5 (C-40), 163.1 (C-54), 155.8 (C-47), 152.2 (C-53), 152.1 (C-56), 142.9 (C-63), 142.9 (C-60), 140.8 (C-68), 140.7 (C-13), 135.4 (C-46), 132.7 (C-41), 129.9 (C-45), 129.8 (C-44), 129.1 (C-42), 129.0 (C-43), 128.9 (C-66), 126.9 (C-67), 125.5 (C-65), 124.7 (C-12), 124.0 (C-48), 122.4 (C-64), 116.1 (C-52), 113.4 (C-59), 112.0 (C-58), 110.9 (C-55), 100.7 (C-61), 95.7 (C-57), 80.7 (C-3), 70.2 (C-2), 55.0 (C-5, 18), 49.5 (C-39), 49.4 (C-17), 48.9 (C-37), 47.8 (C-35), 47.6 (C-9), 46.7 (C-36), 45.3 (C-73, 74), 45.2 (C-69), 44.2 (C-1), 42.7 (C-14), 39.4 (C-8), 39.3 (C-19), 38.7 (C-20), 38.2 (C-4, 10), 33.7 (C-22), 32.9 (C-7), 31.9 (C-70), 30.6 (C-21), 29.8 (C-38), 28.5 (C-71, C-72), 28.5 (C-23), 28.0 (C-15), 27.9 (C-51), 27.1 (C-16), 24.3 (C-49), 23.4 (C-11), 21.4 (C-32), 21.3 (C-34), 21.2 (C-27), 21.0 (C-29), 20.6 (C-50), 18.2 (C-6), 17.7 (C-24), 17.5 (C-26), 16.9 (C-30), 16.6 (C-25), 12.6 (C-75), 12.6 (C-76) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* (%) = 1180.3 (100%, $[M]^+$); anal. calcd for $C_{76}H_{99}N_4O_{11}Cl$ (1280.10), C 71.31, H 7.80, N 4.38; found: C 71.04, H 7.99, N 4.17.

*4.8.17. 2α,3β,23-Tris(acetyloxy)-urs-12-en-28-oic acid (**17**)*

Following GPA, compound **7** (1.85 g, 89%) was obtained from **AA** (1.65 g, 3.5 mmol) as a colorless solid; R_f = 0.33 (*n*-hexane/ethyl acetate, 7:3); m.p. 161–162 °C (lit.: 159–161 °C [10]); $[\alpha]_D^{20}$ = +33.8° (*c* 0.20, $CHCl_3$) [lit.: $[\alpha]_D^{20}$ = +35.9° (*c* 0.34, $CHCl_3$) [10]]; MS (ESI, MeOH): *m/z* (%) = 615.3 (100%, $[M + H]^+$).

4.8.18. *2α,3β,23-Tris(acetyloxy)-28-(1-piperazinyl)-urs-12-en-28-one* (**18**)

Following GPB from **17** (2.4 g, 4.0 mmol) and piperazine (1.36 g, 16.0 mmol), **19** (2.4 g, 89%) was obtained as a colorless solid; R_f = 0.12 (SiO_2, $CHCl_3$/MeOH, 95:5); m.p. 156–159 °C (lit.: 157–160 °C [10]); $[\alpha]_D^{20}$ = +20.1° (*c* 0.25, $CHCl_3$), [lit.: $[\alpha]_D^{20}$ = +17.7° (*c* 0.27, $CHCl_3$) [10]]; MS (ESI, MeOH): *m/z* (%) = 683.2 (100%, $[M + H]^+$).

*4.8.19. 2α,3β,23-Tris(acetyloxy)-28-(1-homopiperazinyl)-urs-12-en-28-one (**19**)*

Following GPB from **17** (2.2 g, 4.0 mmol) and homopiperazine (1.6 g, 16.0 mmol), **19** (2.51 g, 91%) was obtained as a colorless solid; R_f = 0.15 (SiO_2, $CHCl_3$/MeOH, 95:5); m.p. 185-186 °C (lit.: 185-186 °C [12]); $[\alpha]_D^{20}$ = +15.9° (*c* 0.15, $CHCl_3$), [lit.: $[\alpha]_D^{20}$ = +14.4° (*c* 0.14, $CHCl_3$) [12]]; MS (ESI, MeOH): *m*/*z* (%) = 683.0 (100%, $[M + H]^+$).

*4.8.20. (E)-2-(2-[6-(diethylamino)-9-[2-[4-[(2α,3β, 23)-tris(acetyloxy)-28-oxo-ursan-12-en-28-ylpiperazine-1-carbonyl]phenyl]-2,3-dihydro-1H-xanthen-4-yl]vinyl)-1,3,3-trimethyl-3H-indol-1-ium perchlorate (**20**)*

Following GPC from **18** (334 mg, 0.5 mmol) and **1** (125 mg, 0.19 mmol), **20** (125 mg, 50%) was obtained as a green solid; m.p. >300 °C (decomp.); R_F = 0.10 (SiO_2, $CHCl_3$/MeOH, 95:5); UV-Vis (MeOH): λ_{max} (log ε) = 295 nm (4.22), 390 nm (4.34), 475 nm (4.04), 658 nm (4.52), 716 nm (4.89); IR (ATR): ν = 2926*w*, 1739*w*, 1624*m*, 1577*w*, 1551*w*, 1440*m*, 1399*w*, 1365*m*, 1309*w*, 1268*w*, 1247*s*, 1226*m*, 1154*m*, 1143*m*, 1095*s*, 1065*s*, 1042*s*, 1004*m*, 925*m*, 860*w*, 811*m*, 749*m*, 672*w*, 622*m* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 8.57 (*d*, *J* = 14.1 Hz, 1H, 59-H), 7.62 – 7.52 (*m*, 2H, 41-H, 44-H), 7.41 (*dt*, *J* = 17.8 Hz, 8.7 Hz, 3H, 43-H, 63-H, 66-H), 7.27 – 7.17 (*m*, 3H, 42-H, 54-H, 64-H), 6.82 (*t*, *J* = 11.6 Hz, 1H, 65-H), 6.77 – 6.67 (*m*, 1H, 57-H), 6.53 (*s*, 1H, 56-H), 6.06 (*d*, *J* = 13.9 Hz, 1H, 60-H), 5.17 (*s*, 1H, 12-H), 5.13 (*td*, *J* = 11.0-H, 4.5 Hz, 1H, 2-H), 5.06 (*dd*, *J* = 10.3 Hz, 1.8 Hz, 1H, 3-H), 3.88 – 3.78 (*m*, 1H, 23-H_b), 3.70 (*d*, *J* = 5.1 Hz, 3H, 69-H), 3.56 (*dd*, *J* = 11.8 Hz, 7.9 Hz, 1H, 23-H_a), 3.50 (*q*, *J* = 6.9 Hz, 4H, 72-H, 74-H), 3.32 (*s*, 8H, 37-H, 38-H), 2.65 (*s*, 2H, 48-H), 2.52 (*s*, 1H, 16-H_b), 2.33 (*s*, 2H, 16-H_a, 18-H), 2.07 (*s*, 3H, 36-H), 2.00 (*s*, 3H, 34-H), 1.96 (*s*, 3H, 32-H), 1.90 (*s*, 4H, 1-H_a, 11-H, 15-H_b), 1.82 (*s*, 3H, 22-H_a, 49-H), 1.77 (*s*, 6H, 70-H, 71-H), 1.59 (*d*, *J* = 9.2 Hz, 1H, 9-H), 1.51 – 1.28 (*m*, 11H, 5-H, 6-H, 7-H, 19-H, 21-H, 22-H_b, 50-H), 1.25 (*t*, *J* = 7.0 Hz, 6H, 73-H, 75-H), 1.16 – 0.92 (*m*, 3H, 1-H_b, 15-H_a, 20-H), 1.06 (*s*, 3H, 25-H), 1.04 (*s*, 3H, 27-H), 0.91 (*d*, *J* = 5.9 Hz, 3H, 29-H), 0.86 (*s*, 3H, 24-H), 0.83 (*d*, *J* = 6.0 Hz, 3H, 30-H), 0.68 (*s*, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 176.0 (C-28), 174.0 (C-61), 171.0 (C-35), 170.5 (C-33), 170.4 (C-31), 168.2 (C-39), 163.0 (C-53), 155.8 (C-46), 152.1 (C-52), 152.1 (C-55), 142.9 (C-62), 142.7 (C-59), 140.8 (C-67), 140.8 (C-13), 134.4 (C-45), 133.2 (C-40), 130.3 (C-44), 130.3 (C-43), 129.7 (C-41), 129.3 (C-42), 128.9 (C-65), 127.6 (C-66), 125.6 (C-64), 124.8 (C-12), 122.6 (C-47), 122.3 (C-63), 116.0 (C-51), 113.5 (C-58), 112.2 (C-57), 111.0 (C-54), 100.0 (C-60), 96.1 (C-56), 75.0 (C-3), 70.0 (C-2), 55.8 (C-18), 49.5 (C-17), 48.7 (C-37), 47.7 (C-5), 47.6 (C-9), 47.5 (C-38), 46.3 (C-68), 45.3 (C-72, C-24), 43.8 (C-1), 42.0 (C-14), 39.5 (C-8), 39.4 (C-19), 38.8 (C-20), 37.9 (C-4, 10), 34.3 (C-22), 32.6 (C-7), 32.0 (C-69), 30.5 (C-21), 28.5 (C-70),

28.5 (C-71), 28.1 (C-15), 28.1 (C-50), 27.1 (C-16), 24.4 (C-48), 23.4 (C-11), 21.3 (C-27, 29), 21.2 (C-36), 21.0 (C-32), 20.9 (C-34), 20.6 (C-49), 18.0 (C-6), 17.4 (C-25), 17.1 (C-26), 17.1 (C-30), 14.0 (C-24), 12.6 (C-73, 75) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* (%) = 1224.0 (100%, $[M]^+$); anal. calcd for $C_{77}H_{99}N_4O_{13}Cl$ (1324.10), C 69.85, H 7.54, N 4.23; found: C 69.67, H 7.81, N 4.02.

*4.8.21. (E)-2-(2-[6-(diethylamino)-9-[2-[4-[(2α,3β,23)-tris(acetyloxy)-28-oxo-ursan-12-en-28-ylhomopiperazine-1-carbonyl]phenyl]-2,3-dihydro-1H-xanthen-4-yl]vinyl)-1,3,3-trimethyl-3H-indol-1-ium perchlorate (**21**)*

Following GPC from **19** (341 mg, 0.5 mmol) and **1** (250 mg, 0,38 mmol), **21** (335 mg, 66%) was obtained as a green solid; m.p. >300 °C (decomp.); R_F = 0.10 (SiO_2, $CHCl_3$/MeOH, 95:5); UV-Vis (MeOH): λ_{max} (log ε) = 297 nm (4.28), 390 nm (4.32), 477 nm (3.98), 660 nm (4.68), 719 nm (5.06); IR (ATR): ν = 2926*w*, 1739*w*, 1624*m*, 1577*w*, 1551*w*, 1441*m*, 1366*w*, 1309*w*, 1268*w*, 1248*s*, 1225*m*, 1167*m*, 1143*m*, 1103*s*, 1064*s*, 1042*m*, 1018*m*, 925*m*, 860*w*, 811*m*, 747*m*, 668*w*, 622*m* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 8.60 – 8.52 (*m*, 1H, 62-H), 7.59 – 7.48 (*m*, 2H, 46-H, 47-H), 7.44 – 7.30 (*m*, 4H, 44-H, 45-H, 66-H, 70-H), 7.25 – 7.22 (*m*, 1H, 67-H), 7.21 – 7.16 (*m*, 1H, 57-H), 6.89 – 6.75 (*m*, 1H, 68-H), 6.69 – 6.46 (*m*, 2H, 59-H, 60-H), 6.16 – 5.97 (*m*, 1H, 63-H), 5.20 – 5.18 (*m*, 1H, 12-H), 5.14 (*td*, *J* = 11.0 Hz, 4.6 Hz, 1H, 2-H), 5.05 (*d*, *J* = 10.3 Hz, 1H, 3-H), 3.82 (*d*, *J* = 11.6 Hz, 1H, 23-H_b), 3.69 (*d*, *J* = 6.0 Hz, 3H, 69-H), 3.56 (*d*, *J* = 12.0 Hz, 1H, 23-H_a), 3.54 – 3.43 (*m*, 4H, 75-H, 76-H), 4.21 – 2.98 (*m*, 8H, 37-H, 38-H, 39-H, 41-H), 2.75 – 2.53 (*m*, 2H, 51-H), 2.51 – 2.38 (*m*, 1H, 16-H_b), 2.36 – 2.20 (*m*, 2H, 16-H_a, 18-H), 2.06 (*s*, 3H, 36-H), 2.00 (*s*, 3H, 34-H), 1.95 (*s*, 3H, 32-H), 2.15 – 1.69 (*m*, 6H, 1-H_a, 11-H, 15-H_a, 52-H), 1.77 (*d*, *J* = 4.8 Hz, 6H, 73-H, 74-H), 1.67 – 1.53 (*m*, 5H, 9-H, 40-H, 53-H), 1.53 – 1.18 (*m*, 10H, 5-H, 6-H, 7-H, 19-H, 21-H, 22-H), 1.26 (*t*, *J* = 6.6 Hz, 6H, 77-H, 78-H), 1.06 (*s*, 3H, 27-H), 1.16 – 0.88 (*m*, 3H, 1-H_b, 15-H_b, 20-H), 1.04 (*d*, *J* = 8.8 Hz, 3H, 30-H), 0.90 (*d*, *J* = 6.0 Hz, 3H, 29-H), 0.86 (*s*, 6H, 24-H, 25-H), 0.72 (*s*, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 173.8 (C-28), 170.9 (C-35), 170.5 (C-31), 170.4 (C-33), 169.1 (C-64), 168.6 (C-42), 163.1 (C-56), 155.7 (C-49), 152.2 (C-58), 152.1 (C-55), 142.9 (C-65), 142.6 (C-62), 140.8 (C-71), 140.8 (C-13), 135.4 (C-48), 132.7 (C-43), 129.9 (C-47), 129.8 (C-46), 129.2 (C-44), 129.1 (C-45), 128.9 (C-68), 126.9 (C-70), 125.5, 124.6 (C-12, 67), 123.3 (C-50), 122.3 (C-66), 116.1 (C-54), 113.4 (C-61), 112.0 (C-60), 110.9 (C-57), 99.6 (C-63), 95.8 (C-59), 75.0 (C-3), 70.0 (C-2), 65.4 (C-23), 55.2 (C-18), 49.4 (C-17), 49.0 (C-41), 48.2 (C-37), 48.1 (C-39), 47.8 (C-38), 47.8 (C-9), 47.7 (C-5), 46.4 (C-72), 45.3 (C-75, 76), 43.8 (C-1), 42.0 (C-14), 39.4 (C-8), 38.9 (C-19), 38.7 (C-20), 37.9 (C-4, 10), 33.8 (C-22), 32.7 (C-7), 31.9 (C-69), 30.6 (C-

21), 29.7 (C-40), 28.5 (C-73), 28.5 (C-74), 28.0 (C-53), 28.0 (C-15), 27.1 (C-16), 24.3 (C-51), 23.4 (C-11), 21.4 (C-27), 21.3 (C-29), 21.2 (C-36), 21.0 (C-34), 20.9 (C-32), 20.6 (C-52), 18.0 (C-6), 17.5 (C-26), 17.5 (C-25), 17.1 (C-30), 14.0 (C-24), 12.6 (C-78), 12.6 (C-77) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* (%) = 1238.2 (100%, $[M]^+$); anal. calcd for $C_{78}H_{101}N_4O_{13}Cl$ (1338.13), C 70.01, H 7.61, N 4.19; found: C 69.82, H 7.80, N 3.97.

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