Synthese, Charakterisierung und biologische Evaluierung von

Triterpencarbonsäure- und Naturstoffderivaten

Dissertation

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Inhaltsverzeichnis

Abkürzungsverzeichnis

Abb.	Abbildung
Ac	Acetyl
AT	Österreich
BE	Betulin
Bodipy	Bordifluorid-Dipyrromethen
Bodipy FL	3-Bodipy-Propansäure, (3-{2-[(3,5-Dimethyl-1H-pyrrol-2-yl-κN)methylen]-2H-pyrrol-5-yl-κN}propanoato)(difluor)bor
Bn	Benzyl
BR	Brasilien
Caspase	aus dem Englischen: cysteinyl-aspartate specific protease
CA	Carboanhydrase
Cisplatin	*cis*-Diammindichloroplatin
CT	Chemotherapie
DE	Deutschland
DNA	Desoxyribonukleinsäure
EC_{50}	mittlere effektive Konzentration
Eng.	Englisch
EU	Europäische Union
FACS	Fluorescence Activated Cell Sorting
FDA	Food and Drug Administration
FF	Fluoreszenzfarbstoff
FITC	Fluoresceinisothiocyanat
FR	Frankreich
G0	Ruhephase der Zelle
G1	G1-Phase des Zellzyklus
G2	G2-Phase des Zellzyklus

h	Stunde
HUN	Ungarn
IT	Italien
JP	Japan
Me	Methyl
Mil.	Millionen
miRNA	microRNA
nat.	natürlich
NMR	Kernspinresonanzspektroskopie
NP	Natürliches Produkt
PDT	Photodynamische Therapie
PI	Propidiumiodid
PPARγ	Peroxisom-Proliferator-aktivierter Rezeptor gamma
Rhd B	Rhodamin B
RNA	Ribonukleinsäure
S	Synthese-Phase des Zellzyklus
s.	siehe
SA	Südamerika
SAC	spindle assembly checkpoint
SRB	Sulforhodamin B
USA	Vereinigte Staaten von Amerika

1. Einleitung

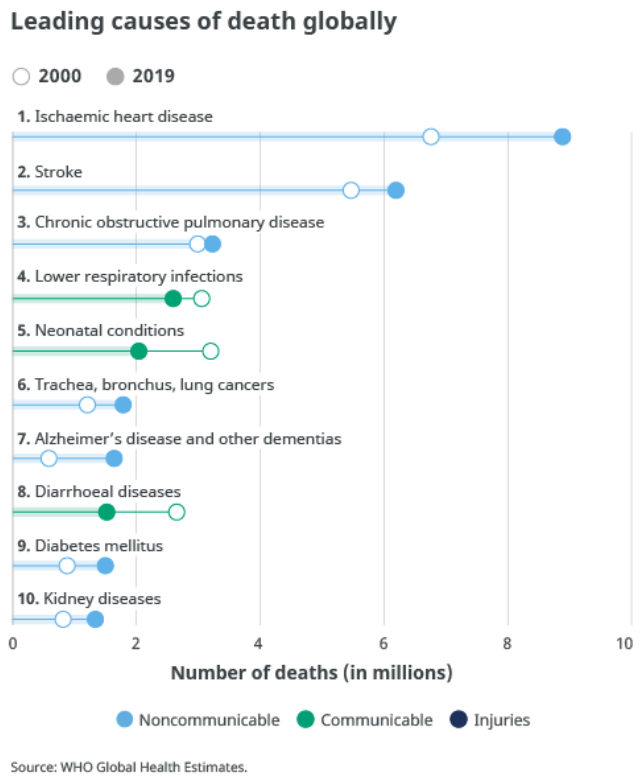


Abb. 1: Die zehn häufigsten Todesursachen weltweit.

Die Naturstoffchemie ist eine nicht erschöpfende Quelle an Stoffen, die für die Medizin genutzt werden kann und somit die allgemeine Lebensqualität aller Menschen verbessert. Viele Therapeutika kommen aus dem marinen Bereich [1] oder stammen von Landtieren [2] und Landpflanzen ab. Naturstoffe bieten eine erstaunliche Vielzahl an Wirkmechanismen und Möglichkeiten diese chemisch zu verändern. Von den ungefähr 250.000 Pflanzenarten sind bisher fast 10% für verschiedene medizinische Anwendungen getestet worden [3]. Die interessanteste Stoffklasse der Naturstoffe sind die sekundären Pflanzenstoffe (eng.: „*Phytochemicals*“), die evolutionär ausgebildet wurden, um Pflanzen vor Pilzbefall, Viren, Bakterien und Fressfeinden zu schützen. Sekundäre Pflanzenstoffe werden vor allem als günstige Möglichkeit der Vorbeugung gegen Krebs [4], Entzündungen [5], Bluthochdruck [6], oxidativen Stress [7], Diabetes [8] und Demenz [9] gesehen. Somit haben diese, durch die Nahrung aufgenommenen Stoffe ein hohes Potential, die nicht übertragbaren Todesursachen weltweit zu verringern (s. Abb. 1) [10]. Viele der Naturstoffe besitzen ein

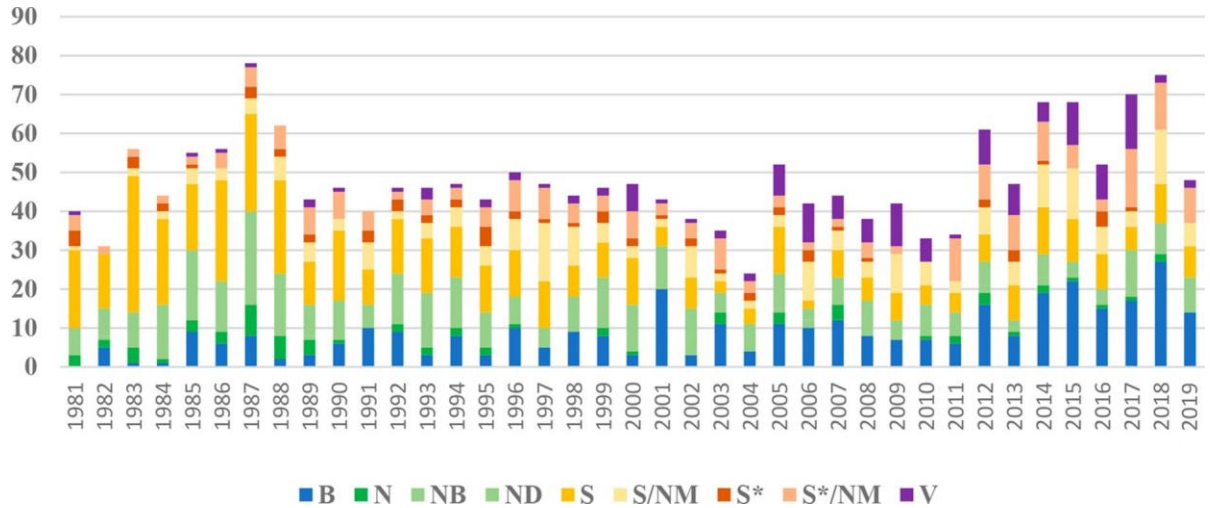


Abb. 2: Zugelassene Medikamente sortiert nach Jahr: B=biologisches Makromolekül, N=unverändertes natürliches Produkt (NP), NB=botanisches Medikament, ND=Derivat eines NPs, S= Totalsynthese Produkt, S= Totalsynthese Produkt mit Pharmakophor eines NPs, S*/NM=synthetische Nachahmung eines NPs, V=Impfstoff.*

hohes Molekulargewicht und widersprechen der „*rule of five*“ von LIPINSKI [11], welche fünf

verschiedene Bedingungen für eine optimale orale Bioverfügbarkeit von Substanzen definiert. Allerdings werden vermehrt Naturstoffe von der FDA zugelassen, die dieser Regel widersprechen [12]. Ein Großteil der neu zugelassen Medikamente basieren auf natürlichen Stoffen oder ähneln diesen stark (s. Abb. 2) [13]. Es wird geschätzt, dass zwischen 1981 und 2019 ungefähr 25% der Neuzulassung für Krebstherapien auf natürliche Produkte zurückzuführen sind [14].

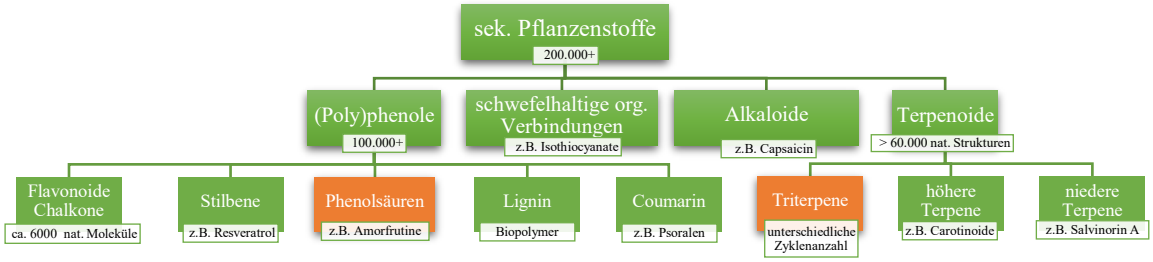


Abb. 3: Klassen von sekundären Pflanzenstoffen. Die in dieser Arbeit behandelten Verbindungklassen sind orange markiert.

Sekundäre Pflanzenstoffe lassen sich in vier Kategorien einteilen: den Alkaloiden, Polyphenolen, Terpenoiden und schwefelorganischen Verbindungen (s. Abb. 3) [15]. Die strukturellen Unterschiede und verschiedenen Wirkmechanismen machen die sekundären Pflanzenstoffe zu einer vielversprechenden Quelle für Ausgangsmaterialien [16] pharmazeutischer Substanzen. In dieser Arbeit wurde der Fokus vornehmlich auf Triterpene und Phenolsäuren gelegt.

1.1 Krebs und die Geschichte der Chemotherapie

Krebs war mit geschätzten 19 Mil. Erkrankungen und 10 Mil. Todesfällen 2020 die führende Todesursache weltweit (s. Abb. 4 und Abb. 5) [17]. Je älter der Mensch wird, desto mehr treten

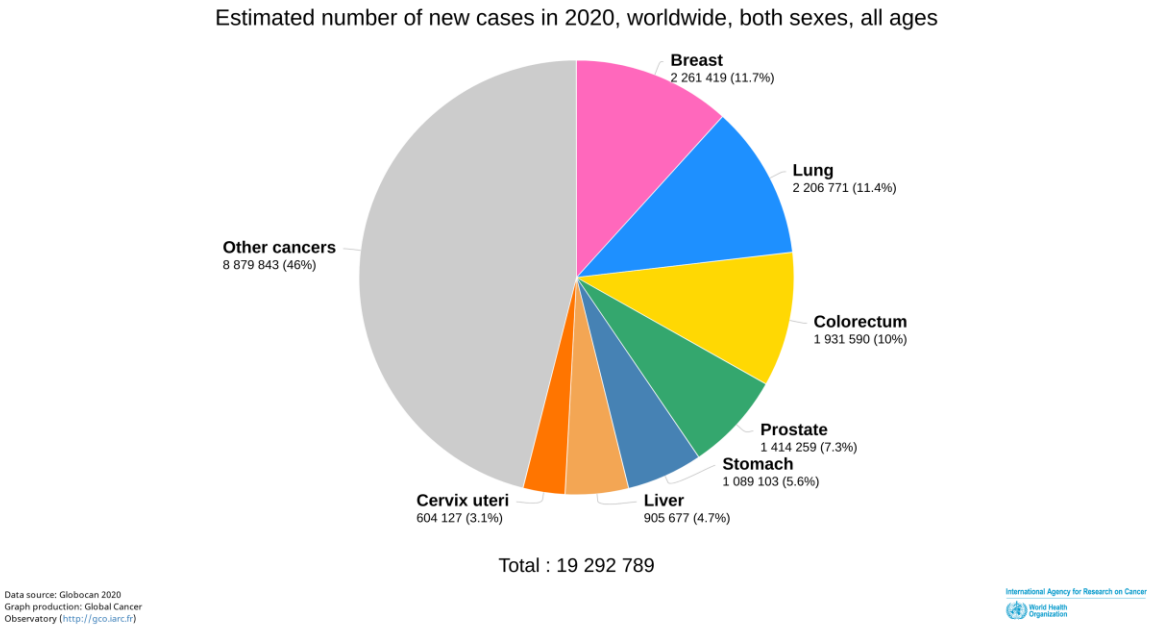


Abb. 4: Kuchen-Diagramm der geschätzten Krebsfälle weltweit im Jahr 2020.

gleichzeitig kleinere Tumore auf, die durch die fortgeschrittene Diagnostik eher entdeckt werden und somit für eine Erhöhung der Krebsfälle sorgen kann, ohne die Schwere der Tumore zu berücksichtigen. Dadurch entsteht eine zusätzliche Belastung für das Gesundheitssystems und höhere finanzielle Aufwände [18]. Eine etablierte und effektive Methode zur Krebsbehandlung ist die Chemotherapie (**CT**), bei der Substanzen oder Substanzgemische über einen gewissen Zeitraum verabreicht werden. Die Suche nach solchen Chemotherapeutika hat sich seit 1940 von einer durch wenig öffentliche Gelder finanzierten Forschung zu einem Multi-Milliarden-Geschäft entwickelt [19]. Das erste Chemotherapeutikum wurde in hohen Dosen verabreicht und bestand aus Senfgas, einem Kampfstoff aus dem ersten Weltkrieg, der die Purin-Base der DNA alkylierte und so den kontrollierten Zelltod einleitete. Diesen natürlichen Prozess nennt man Apoptose. Dieser wird ausgelöst, um die Integrität des Gewebes zu erhalten oder potenziell schädliche Zellen zu eliminieren. Eine Chemotherapie mit Senfgas führt zu starken Nebenwirkungen. Dennoch wurden weitere auf diesem Prinzip basierende DNA-Alkylierungsmittel synthetisiert und schlussendlich herausgefunden, dass eine Dosis-Abhängigkeit besteht und es von Vorteil ist, verschiedene Chemotherapeutika zu verwenden. Einige Meilensteine waren Paclitaxel (Taxol®) und Cis-Platin-Verbindungen. Während der klinischen Entwicklung dieser

Therapeutika entwickelten die Forschenden Methoden, um Nebenwirkungen zu minimieren, dennoch bleiben die Langzeitschäden, wie Leukämie und Organschäden, ein großes Problem der Chemotherapie.

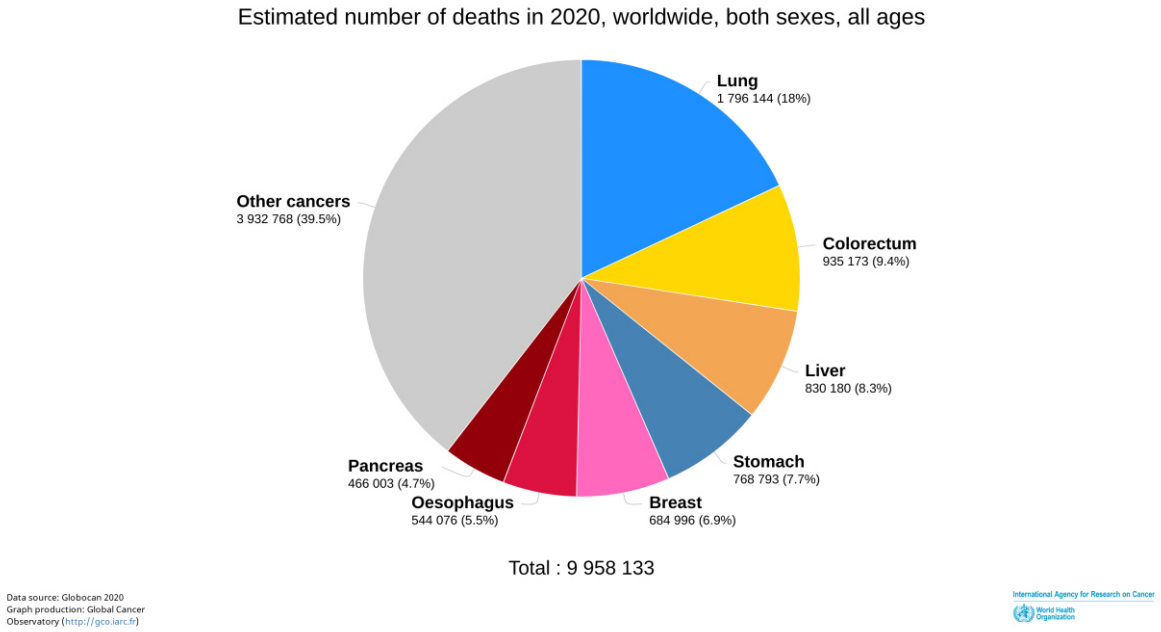


Abb. 5: Tortendiagramm, das die weltweiten Todesfälle durch Krebs im Jahr 2020 nach Krebsarten aufschlüsselt.

Das Ziel der Chemotherapie-Forschung ist die Erhöhung der Überlebensrate und die Verbesserung der Lebensqualität nach der Entdeckung des Krebses. Deswegen müssen neue Chemotherapeutika nicht nur gegenüber gesunden Zellen spezifischer den Tod von Krebszellen induzieren, sondern auch in die Tumorumgebung eingreifen, um die Entstehung von neuen Blutgefäßen (Angiogenese) und eine dauerhafte Entzündungsreaktion des Gewebes zu verhindern. Einige Chemotherapeutika greifen zusätzlich in die Immunantwort ein oder induzieren Differenzierung der Zelle [20]. Eine weitere Möglichkeit ist die generelle Prävention und Inhibierung der Krebszellbildung durch unterstützende Chemotherapeutika. Meistens wird die Chemotherapie eingesetzt, wenn es keine anderen effektiven Behandlungen gibt, oder sie wird unterstützend zu einer Strahlentherapie oder einem chirurgischen Eingriff eingesetzt (neoadjuvant oder adjuvant). In manchen Fällen wird auch eine ortsabhängige Perfusion der Organe durchgeführt [21]. Bei der Behandlung von Krebs wird häufig eine Kombination verschiedener Chemotherapeutika eingesetzt. Eine Übersicht über die damit behandelten Krebsarten ist in Tabelle *1* zu sehen. Hier sind vor allem die schon erwähnten Vorteile der sekundären Pflanzenstoffe hervorzuheben, die viele unterstützende Eigenschaften besitzen. Bei den Chemotherapeutika unterscheidet man zwischen Zytostatika wie: Antimetaboliten, Hormonregulatoren, Alkylierungsmitteln, reversiblen DNA-Bindern, DNA-assoziierten Enzymantagonisten, Zytoskelett-Inhibitoren, Zellsignal-Inhibitoren, Kinaseinhibitoren und Drogen für die photodynamische Therapie (**PDT**) und Radiotherapie [22]. Häufig werden mehrere Präparate mit komplementären pharmakologischen Eigenschaften in der Therapie eingesetzt („*Multi-Drug-Treatment*“).

Tabelle 1: Die häufigsten Anwendungsgebiete der Chemotherapie nach Krebsart aufgeschlüsselt [21].

CT Variante / Krebsart	Hodkin-Lymphom	Non-Hodkin Lymphom	Keimzellkrebs	Chorionkarzinom	Akute Leukämie	Akute lymphatische Leukämie	Burkit-Lymphom	Nephroblastom	Rhabdomyosarkom	Afterkarzinom	Blasenkrebs	Brustkrebs	Speiseröhrenkrebs	Kehlkopfkrebs	Fortgeschrittener Lungenkrebs	Darmkrebs	Magenkrebs	Osteosarkom	anaplastisches Astrozytom
Neoadjuvante										☑	☑	☑	☑	☑	☑	☑	☑	☑	
Adjuvante								☑				☑			☑	☑	☑	☑	☑
Normale	☑	☑	☑	☑	☑	☑	☑	☑	☑										

1.2 Chemotherapie-Strategien und Anwendungen in der Wirkstoffforschung

Für Krebs wurden Kennzeichen (s. Abb. 6) [23] festgelegt, die ihn von gesunden Zellen unterscheiden. Diese Kennzeichen entwickeln sich auf dem Weg von einer normalen Zelle zu einer Tumorzelle und wurden mit der Zeit von sechs (2000) auf acht (2011) und nun auf zehn erhöht. Zusätzlich sind 2022 zwei weitere Aktvierungsmerkmale dazu gekommen: die polymorphen Mikrobiome und die mutationsfreie epigenetische Reprogrammierung. Die Kennzeichen von Krebs bieten Angriffsfläche für unterschiedliche Therapieansätze, während die Aktivierungsmerkmale mit vorbeugenden Praktiken bekämpft werden können.

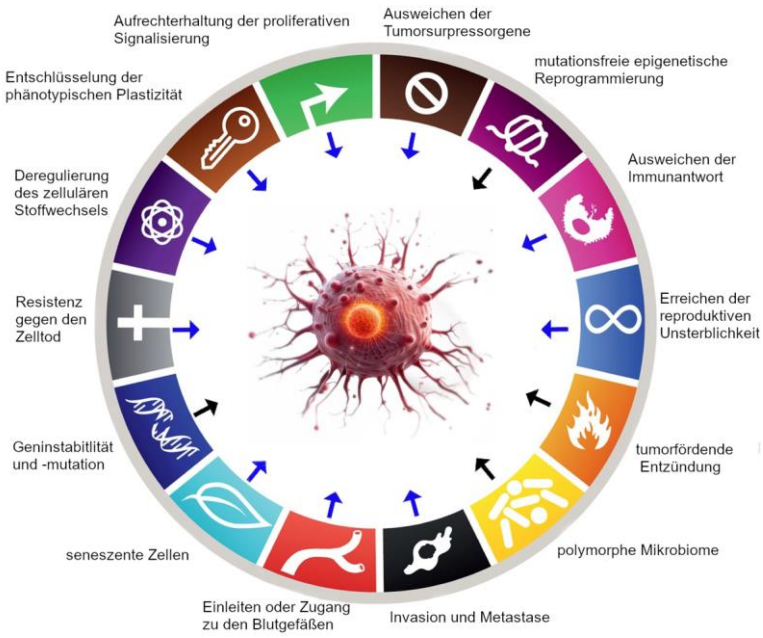


Abb. 6: Kennzeichen von Krebs (blauer Pfeil) und Aktivierungsmerkmale (schwarzer Pfeil). Abbildung wurde aus der Literatur übersetzt und leicht verändert [20].

1.2.1 Der Zellzyklus und Apoptose

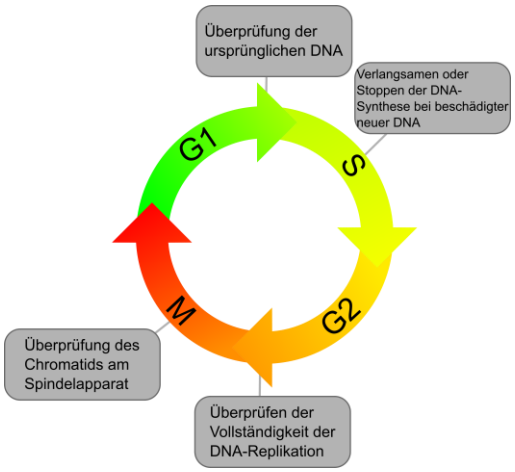


Abb. 7: Schematische Darstellung des Zellzyklus.

Der Zellzyklus ist ein weiterer Punkt, an dem Chemotherapeutika ansetzen können. Dieser Zyklus (s. Abb. 7) besteht aus drei Phasen: Der Interphase (G1+S+G2), der Mitose (M) und der Zytokinese. Entlang des Zellzyklus befinden sich verschiedene Kontrollpunkte. Bei diesen stellt die Zelle sicher, dass die neu gebildete Zelle keine Defekte aufweist. Am G1/S-Kontrollpunkt wird das Genom auf Schäden untersucht und anhand dessen entscheidet sich, ob DNA und Histone in der S-Phase synthetisiert werden. Das Schicksal der Zelle hängt dann von der Zellgröße, Nährstoffverfügbarkeit, DNA-Integrität und Botenstoffen ab. Falls die DNA beschädigt ist, geht die Zelle in die Ruhephase (G0). Nachdem in der S-Phase die DNA synthetisiert wurde, wird am G2/M -Kontrollpunkt überprüft, ob die DNA Schäden aufweist. Danach wird entweder die Mitose oder Apoptose eingeleitet. Bei der Mitose wird am Spindel-Kontrollpunkt (**SAC**) überprüft, ob die Spindel sich korrekt gebildet hat.

Wenn mittels Zytostatika oder Bestrahlung die DNA beschädigt wird, kann sich eine normale Zelle also reparieren, während in beschädigten Tumorzellen das Einleiten der Mitose zur sogenannten mitotischen Krise führen kann. Bei der mitotischen Krise können sich bei der Mitose die Chromosomen nicht trennen und schließlich wird die Apoptose eingeleitet. In Krebszellen sind meistens die Onkogene (wachstumsfördernde Gene) oder Tumorsurpressorgene mutiert und zeitgleich ein oder mehrere Kontrollpunkte abgeschaltet, wobei der wichtigste der G0/G1-Kontrollpunkt ist, was für ein unkontrolliertes Fortschreiten des Zellzyklus sorgt.

Der G2/M-Kontrollpunkt ist ein wichtiger Abschnitt im Zellzyklus, der sicherstellt, dass die Zelle bereit ist, sich zu teilen. An diesem Kontrollpunkt wird überprüft, ob die DNA in der S-Phase vollständig und korrekt kopiert wurde. Wenn die DNA-Fragmente fehlerhaft oder beschädigt sind, können sie zu Chromosomenbrüchen oder Mutationen führen, die das Wachstum von Krebszellen fördern. Wenn beim G2/M-Kontrollpunkt Probleme feststellt werden, bleibt die Zelle im G2-Stadium, bis die Fehler behoben sind. Die Zelle aktiviert Reparaturmechanismen, um sicherzustellen, dass die DNA-Stränge vollständig und genau kopiert werden. Wenn jedoch keine Reparatur möglich ist oder die Fehler zu schwerwiegend sind, sendet die Zelle Signale, um die Apoptose auszulösen. Gelangt die Zelle in die Mitose, bewertet die Zelle beim SAC, ob sich der Spindelapparat richtig gebildet hat und ob alle Chromatiden korrekt an den Spindel-Mikrotubuli befestigt sind.

Zusammengefasst kann man sagen, dass es zur Vermeidung der Übertragung eines veränderten Genoms diese wichtigen drei Kontrollpunkte gibt: den G0/G1-, den G2/M- und den Spindelmontage-Kontrollpunkt (M-Kontrollpunkt). Diese können den Fortschritt des Zellzyklus beeinflussen, indem sie Reparaturmechanismen fördern oder bei irreparablem Schaden den programmierten Zelltod, die Apoptose, auslösen.

Das Einleiten der Apoptose kann mit und ohne Caspasen ausgelöst werden. Die zwei wichtigsten, von Caspase 3 abhängenden Wege sind die extrinsische Aktivierung eines Zelltod-Rezeptors und die Aktivierung über das Mitochondrium (intrinsisch). Das Mitochondrium reagiert auf das Fehlen von Zytokinen, Hormonen und verfügbaren Wachstumsfaktoren und wird durch die folgende Freisetzung von pro-apoptotischen Proteinen vom „Kraftwerk der Zelle zum Arsenal der Zelle“ [24].

Eine wichtige Rolle spielen die Botenstoffe oder Kinasen, die durch ihre unterschiedlichen Signale die unkontrollierten Wachstumswege beschleunigen. Therapeutisch können Medikamente, die intrazellulare Kinasen inhibieren, oder Antikörper, die die Aktivität der Membranrezeptoren stören, benutzt werden [20]. Solche Medikamente werden Signalweginhibitoren genannt und bieten den Vorteil, dass sie weniger Nebenwirkungen haben als konventionelle CT. Allerdings besteht bei ihnen der Nachteil, dass zugleich das Risiko einer Resistenzentwicklung des Krebses auftreten kann.

Eine weitere Klasse der Zytostatika sind die Antimetaboliten, die chemische Analoga der Metaboliten sind und die Biosynthese der Mikrotubuli-Struktur stören [22] oder in die DNA eingebaut werden. Ähnlich wirken Zytostatika, die direkt die DNA angreifen, indem sie diese alkylieren oder vernetzen. Weitere wichtige Klassen an Zytostatika sind Anthracycline [25] und Topoisomeraseinhibitoren [26]. Die Inhibierung von Carboanhydrasen IX/XII (**CA IX/XII**) ist ein neues Ziel für Chemotherapeutika, da sie spezifisch in Tumorzellen überexprimiert sind. Diese Enzyme sorgen für die Regulierung des pH-Werts im extra- und intrazellulären Raum der Tumorzelle [27]. Eine Überexpression sorgt für eine stärkere Ansäuerung und den damit verbundenen Resistenzen gegenüber Medikamenten [28]. Auswirkungen der Inhibierung dieser Enzyme sind sowohl die Unterdrückung des primären Tumorwachstums und der Metastasenbildung als auch die Verringerung der Anzahl der Tumorstammzellen. Dadurch, dass die Isoformen der CA auch in normalen Zellen vertreten sind, ist eine selektive Inhibierung aufgrund von Nebenwirkungen zu bevorzugen. Der Stoff SLC-0111, ein Fluoraromatenharnstoffderivat mit Sulfonaminrest, ist zurzeit der vielversprechendste CA IX-Inhibitor und wurde nach einer Phase I-Studie für eine Phase II-Studie vorgeschlagen [29]. In Verbindung mit anderen Therapien oder Therapeutika zeigen die CA-Inhibitoren einen stark synergetischen Effekt bei der Therapie von hypoxischen Tumoren [30]. Durch das Verwenden von mehreren Zytostatika mit mehreren Wirkmechanismen wird eine Resistenzentwicklung unwahrscheinlicher.

Für die Untersuchung von neuen Wirkstoffen hinsichtlich ihrer Eignung als Zytostatika werden in dieser Arbeit hauptsächlich der SRB-Assay genutzt. Der SRB-Assay SRB-Assay (Sulforhodamin B-Assay) ist ein farbbasierter Zellviabilitätstest, der in der medizinischen Chemie häufig verwendet wird. Er bietet

eine hohe Empfindlichkeit, Reproduzierbarkeit und Robustheit gegenüber verschiedenen Zelllinien und Wirkstoffen. Dieser *in vitro* Assay bietet also eine zuverlässige Möglichkeit, die Wirkungen von Verbindungen auf Zellkulturen zu bewerten und potenzielle Wirkstoffkandidaten zu identifizieren [31]. Die erhaltenen Inhibierungsdaten werden als EC_{50}-Werte, die auch zur Selektivitätsberechnung herangezogen werden, angegeben. Zusätzlich zu dieser Untersuchung wird auch die Inhibierung der CA gemessen, Zellzyklusanalysen mittels FACS durchgeführt und der Annexin V-FITC/PI-Assay verwendet, um weitere Informationen über die Wirkmechanismen zu erhalten.

1.3 Triterpene als Wirkstoffe

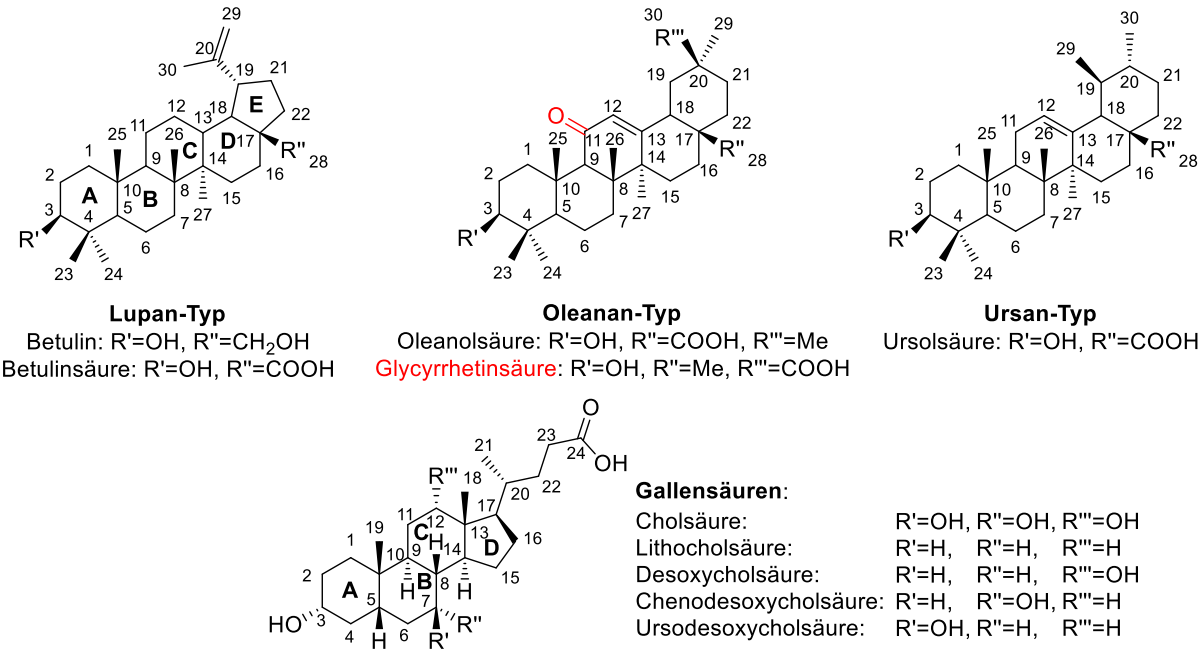


Abb. 8: Struktur der penta- und tetrazyklischen Grundgerüste mit Nummerierung. Die Doppelbindungen gehören nicht zum Grundgerüst, aber zur jeweiligen Verbindung. Rot gekennzeichnet ist die Keto-Gruppe der Glycyrrhetinsäure.

Als eine besonders interessante Gruppe der sekundären Pflanzenstoffe haben sich pentazyklische und tetrazyklische Triterpene herausgestellt. Diese bestehen aus 6 Isopreneinheiten und entstehen durch die Zyklisierung des all-*trans* Squalens oder Squalenoxids mit eventueller Oxidation des Zyklisierungsproduktes [32]. Einige für diese Arbeit wichtigen Vertreter sind in Abb. 8 zu sehen.

Betulin (**BE**) zeigt eine hemmende Wirkung gegen viele Krebszelllinien und leitet die Apoptose durch verschiedene Wirkmechanismen ein [33]. Da Betulin in der äußeren Rinde der gemeinen Birke (*Betula alba*) mit einem Gehalt von 20-45% (je nach Spezies), enthalten ist, lässt sich Betulin einfach in hoher Reinheit gewinnen [34] und durch Oxidation an der Position 28 zur Betulinsäure (**BA**) umsetzen. Betulinsäure und einige ihrer Derivate zeigen zusätzlich eine Anti-Malaria- und Anti-HIV-Aktivität. Oleanolsäure (**OA**) ist in China als Medikament zugelassen (s. Tabelle *2*) [35]. Ursolsäure (**UA**) besitzt eine geringere Toxizität als Oleanolsäure und ähnliche Aktivitäten, jedoch ist die Wasserlöslichkeit niedriger. Gallensäuren sind amphiphile, tetrazyklische Triterpene, die einen wichtigen Bestandteil des menschlichen Metabolismus ausmachen und durch ihre physikochemischen Eigenschaften als Trägersystem für Medikamente oder als Medikament-Drogen-Konjugat eingesetzt werden können [36]. Eine der größten Hürden für den Einsatz natürlicher Produkte in Therapien ist die effektive Gewinnung dieser. Zukunftsfähige krebstherapeutische Medikamente müssen auch hinsichtlich der Regulierung des Lipidmetabolismus, des Knochenmetabolismus, der Glucosehomöostase, der Entzündung, der Autoimmunität, des Tumorwachstums, des oxidativen Stresses und der Neurogenese untersucht werden.

Zudem müssen die Zielproteine der Substanzen identifiziert werden und ADMET-Parameter (Absorption/Resorption, Distribution, Metabolismus, Exkretion/Ausscheidung, Toxizität) bestimmt werden. Einige Triterpene sind in klinischen Studien getestet worden oder werden noch getestet [37]. Eine Übersicht der verschiedenen biologischen Aktivitäten der ausgewählten Triterpene ist in Tabelle 3 zu sehen.

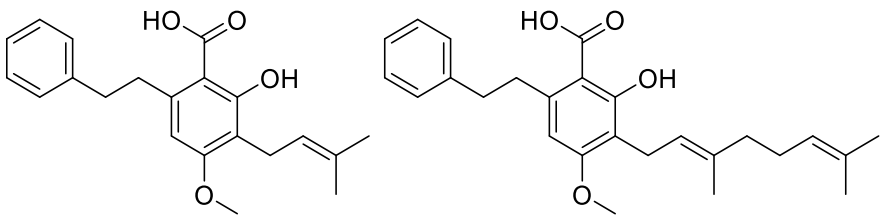
Tabelle 2: Registrierte und auf Triterpenen basierende Medikamente (ausgenommenen Anabolika- und Vitamin D-Analoga) [35,38,21].

Substanz	Anwendung	Region oder Zulassung
Ursodesoxycholsäure	Gallensteine [39], Gallengrieß [39], Cholangitis [40]	Welt
Obeticholsäure	Cholangitis [41]	FDA/EU
Chenodesoxycholsäure	Gallensteine, Cerebrotendinöse Xanthomatose [42]	FDA/EU
Tauroursodesoxycholsäure	Gallensteine [43]	Italien/Türkei/China
Fusidinsäure	Antibiotikum [44]	Welt
Drospirenon	Geburtenkontrolle [45]	Welt
Rocuronium	Muskelrelaxans [46]	Welt
Vecuronium	Muskelrelaxans [47]	Welt
Pancuronium	Muskelrelaxans [48]	Welt
Pipecuronium	Muskelrelaxans [49]	Europa/China
(Acetyl)-Digitoxin	Herzversagen und –krankheiten [50]	DE/USA/AT/HUN/(Fr)
Digoxin	Herzversagen und –krankheiten [51]	Welt
Deslanoside	Herzversagen und –krankheiten [52]	USA/IT/BR/JP
Oleanolsäure	Leberkrankheiten	China
Asiaticosid	Wundheilung	China, Europa
Glycyrrhizinat	Leberkrankheiten	China, Japan
Isoglycyrrhizinat	Leberkrankheiten	China
Aescin	Hydrocephalus	China, Deutschland
Abirateronacetat	Prostatakrebs	FDA/EU/UK/Argentinien
Fulvestrant	Brustkrebs	FDA/EU/SA/Indien
Megestrol acetat	Brustkrebs/Endometriumkarzinom/Gewichtsverlust	FDA/EU

Tabelle 3: Grundgerüste und deren derzeitiger Forschungstands hinsichtlich ihrer Wirkungen.

Triterpen	Nachgewiesene hemmende und positive Wirkung							
	Krebs	AD	Entz.	OP	Bakterien/ Mikroben	HIV	Malaria	Diabetes
BE	☑[33,53]	☑[54]	☑[53,55]	☑[56]	☑[53]	☒[53,57,58]	☒[53]	☑[59,58,53]
BA	☑[60,55]	☑[61]	☑[53,55]	☑[62]	☑[60]	☑[60,55]	☑[60]	☑[63]
OA	☑[35]	☑[35]	☑[35,64]	☑[65,35]	☑[66,67]	☑[68]	☑[69]	☑[35]
GA	☑[70]	☑[71]	☑[72]	☑[73]	☑[72]	☒[74]	☑[75]	☑[76]
UA	☑[77]	☑[78]	☑[77]	☑[79,77]	☑[67,77]	☑[77]	☑[77]	☑[77]
Gallensäuren	☑[80,81]	☑[82]	☑[82]	☒[83]	☑[80]	☒[84]	☑[85]	☑[86]

1.4 Amorfrutine als Wirkstoffe



Amorfrutin A Amorfrutin B

Abb. 9: Chemische Struktur von Amorfrutin A und B.

Amorfrutine gehören zu der Klasse der Polyphenole und stellen eine faszinierende Gruppe von Wirkstoffen dar, die in der Natur vorkommen und in der Naturheilkunde verwendet werden [87]. Besonders hervorzuheben ist Amorfrutin B (s. Abb. 9), das in Mausmodellen eine bedeutende Schutzwirkung auf Gehirnneuronen vor Hypoxie/Ischämie zeigt [88]. Diese Eigenschaft wird durch die Hemmung von Apoptose und Autophagie mittels Genmethylierung und miRNA-regulierter Steuerung erreicht. Die Ergebnisse der Untersuchung unterstützen die potenzielle Anwendung von Amorfrutin B als Anti-Schlaganfall-Therapeutika. Neben diesen Effekten haben Amorfrutin A und B *in vitro* antimikrobielle Aktivität gegen *Staphylococcus aureus* und *Mycobacterium smegmatis* demonstriert [89]. Zudem zeigten Amorfrutine in Tierexperimenten eine sehr gute Verträglichkeit und waren nicht leberschädlich, was ihre Sicherheit als potenzielle Therapeutika betont [90]. Die Wirkung auf die Modulation von PPARγ-Genexpressionsnetzwerken in Adipozyten (Zellen des Fettgewebes) ist eine mögliche Erklärung für die Insulinresistenz, die sich in Mausmodellen für Typ-2-Diabetes gezeigt hat [87]. Die Einstufung als mögliches Anti-Diabetika fügt sich in das breite Spektrum an Anwendungen ein, die diese Polyphenole bieten. Trotz dieser vielversprechenden Eigenschaften ist die Verfügbarkeit von Amorfrutinen aus der Natur limitiert.

Zusammenfassend repräsentieren Amorfrutine, speziell Amorfrutin A und B, einen vielversprechenden Bereich der pharmazeutischen Forschung. Die wissenschaftlichen Voruntersuchungen unterstreichen die vielseitigen Anwendungsmöglichkeiten der Amorfrutine in der pharmazeutischen Chemie und bieten daher eine Basis, um neuartige Wirkstoffe zu synthetisieren. Von der Schutzwirkung von Neuronen bis hin zur antimikrobiellen Wirkung und der günstigen Verträglichkeit in Tierversuchen bieten diese Polyphenole ein breites Potenzial für therapeutische Anwendungen. Ihre Erforschung wird wahrscheinlich zu weiteren Entdeckungen und möglichen neuen Therapieansätzen führen.

1.5 Fluoreszenzmarkierungen mittels Bodipy und Rhodamin

Seit der Herstellung von Fluorescein im Jahr 1871 hat sich die Verwendung von Fluoreszenzfarbstoffen (**FF**) in viele Anwendungsbereiche ausgebreitet. Die Hauptanwendungsgebiete der organischen Vertreter sind hierbei die Fluoreszenzspektroskopie und Biochemie bzw. Medizin. Fluoreszenzfarbstoffe lassen sich nach ihren Emissionsbereichen in drei Kategorien einteilen: Sichtbares Licht (λ_{Em} < 700 nm), naher Infrarot Bereich-I (NIR-I, 700 nm < λ_{Em} < 1000 nm) und naher Infrarot Bereich-II (λ_{Em} >1000 nm) [91]. Im sichtbaren Licht sind am meisten Fluoresceinisothiocyanate, Cyanine, Rhodamine, BODIPYs, Cumarine, Chinoline vertreten. Diese zeichnen sich gegenüber anderen Fluoreszenzfarbstoff-Klassen vor allem durch eine höhere Wasserlöslichkeit aus. Im NIR-I sind Fluoreszenzfarbstoffe wie NIR-760, IRDye800CW, Indocyaningrün, Methylenblau und AZA-Bodipys vertreten. Im Gegensatz zu den vorhergenannten Fluoreszenzfarbstoffen bieten diese besonderen Vorteile, wie ein besseres Signal-Rausch-Verhältnis und eine tiefere Eindringtiefe in Gewebe (~ 1,2 cm). NIR-II Fluoreszenzfarbstoffe sind in diesem Bereich meist besser, haben aber eine schlechtere Wasserlöslichkeit und sind in der medizinischen Chemie dadurch seltener vertreten. Typische Anwendungen in der medizinischen Chemie beinhalten: Proteinspezifische Fluoreszenzproben, Färben von Organellen, Diagnostik, Verfolgen der Verteilung und Pharmakokinetik von Medikamenten, photodynamische/photothermale Therapien und FF-Prodrug-Konjugate [92].

Rhodamine und Bodipys besitzen eine hohe Photostabilität und Quantenausbeute [93]. Rhodamin-123 wurde klinisch zur Diagnostik von Prostata-Krebs untersucht und sammelte sich bei verträglichen Dosen im Tumorgewebe an [94]. Rhodamine besitzen eine positive Ladung und werden meist in den Mitochondrien, welche ein exzellentes Ziel für Chemotherapeutika bieten, angereichert und zeichnen sich dementsprechend in Verbindung mit photodynamischer Therapie oder als Konjugate als sehr gute Zytostatika aus [95]. Rhodamin B kann auch als Photosensibilisator benutzt werden. Ein Photosensibilisator ist ein Molekül, das Energie in Form von Lichtquanten aufnimmt und wieder abgibt. Wenn man ein anderes Molekül über eine Amidbindung mit Rhodamine B verknüpft, kann man die Lactambildung unter unterschiedlichen pH-Werten ausnutzen, um die Zytotoxizität zu steuern (s. Abb. 10) [96]. Im basischen Milieu besitzt das Molekül ein komplett anderes Absorptionsspektrum als im sauren Milieu, welches häufig in Tumorumgebungen vorherrscht.

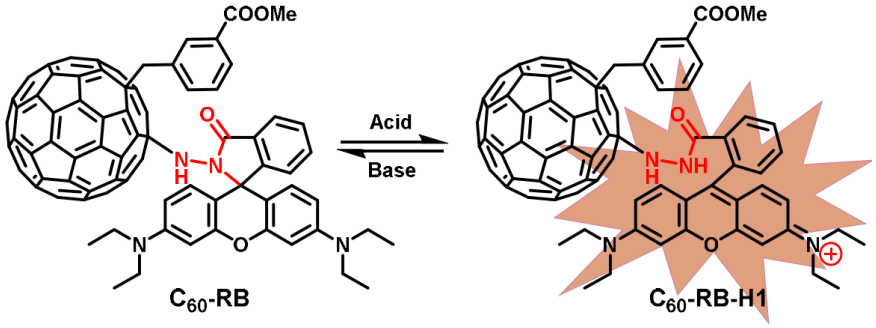


Abb. 10: Beispiel eines pH-abhängigen Photosensibilisators auf Rhodamine-Basis [90].Rot markiert sind links die Lactam-Gruppe und rechts die Amid-Gruppe.

Bodipys sind dagegen meist ungeladen, besitzen selbst in Wasser Quantenausbeuten von fast 100% und lassen sich nicht durch Polarität oder den pH-Wert beeinflussen. Durch verschiedene Substitutionsmuster lassen sich die photoelektrischen Eigenschaften einstellen, sodass ein weiter Absorptions- und Emissionsbereich abgedeckt werden kann. Substituiert man den verbrückenden Kohlenstoff an der *meso*-Position mit einem Stickstoffatom, so erhält man aza-Bodipys, die im Vergleich zu normalen Bodipys in ihren spektralen Eigenschaften rotverschoben sind [97]. Zudem können Bodipys sich selbst über supramolekulare Wechselwirkungen zu Nanopartikeln zusammen oder in Lipide einbauen, was zu einer höheren Wasserlöslichkeit und verbesserten Anreicherung in Tumoren führen kann [98]. Außerdem wird die Anwendung von aza-Bodipys in photodynamischen und photothermalen Therapien diskutiert [99].

2. Zielstellung

Durch die vielseitigen Wirkmechanismen von sekundären Pflanzenstoffen, insbesondere der Triterpene, eröffnen sich für die medizinische Anwendung viele Möglichkeiten. Ziel dieser Arbeit ist vor allem das Herstellen und Untersuchen neuer Verbindungen hinsichtlich ihrer Zytotoxizität, Inhibierung bestimmter Enzyme und Wirkmechanismen. Es werden verschiedene Thesen, aufgeteilt in die verschiedenen Veröffentlichungen, aufgestellt und überprüft. Hierbei muss auch auf die Machbarkeit der Derivatisierung und Verfügbarkeit der sekundären Pflanzenstoffe geachtet werden. Neben der machbaren Synthese ausgehend von verfügbaren Edukten, sollen auch Fluoreszenzfarbstoffe eingesetzt werden, um, mittels Fluoreszenzmikroskopie, Wirkmechanismen entschlüsseln zu können und bzw. die Zytotoxizität zu steigern. Außerdem werden verschiedene Derivatisierungsstrategien auf ihre Sinnhaftigkeit und die Anwendbarkeit auf andere sekundäre Pflanzenstoffe untersucht.

3. Einordnung der Forschungsergebnisse

3.1 Synthese und biologische Evaluierung von Triterpen-Bodipy Konjugaten [100]

In vorherigen Arbeiten zeigte eine Derivatisierung von pentazyklischen Triterpencarbonsäuren mit Rhd B vielversprechende Zytotoxizitäten, allerdings ohne nennenswerte Selektivitäten [101]. Die nanomolaren Zytotoxizitätswerte wurden auf die positive Ladung des Rhd B und den Transport des Konjugats in das Mitochondrium erklärt. Für die Synthese wurden Piperazin-Amidspacer an das Triterpengrundgerüst angebaut und festgestellt, dass diese schon stark zytotoxisch gegenüber allen Zelllinien sind. Es stellte sich also die Frage, wie sich andere FF in Konjugation mit pentazyklischen Triterpenen in ihrer Zytotoxizität und Metabolismus verhalten. Aufgrund der vielen Vorteile von Bodipys wurde Bodipy FL ausgewählt und pentazyklische Triterpen-Bodipy FL-Konjugate mit Piperazin- und Ethylendiamin-

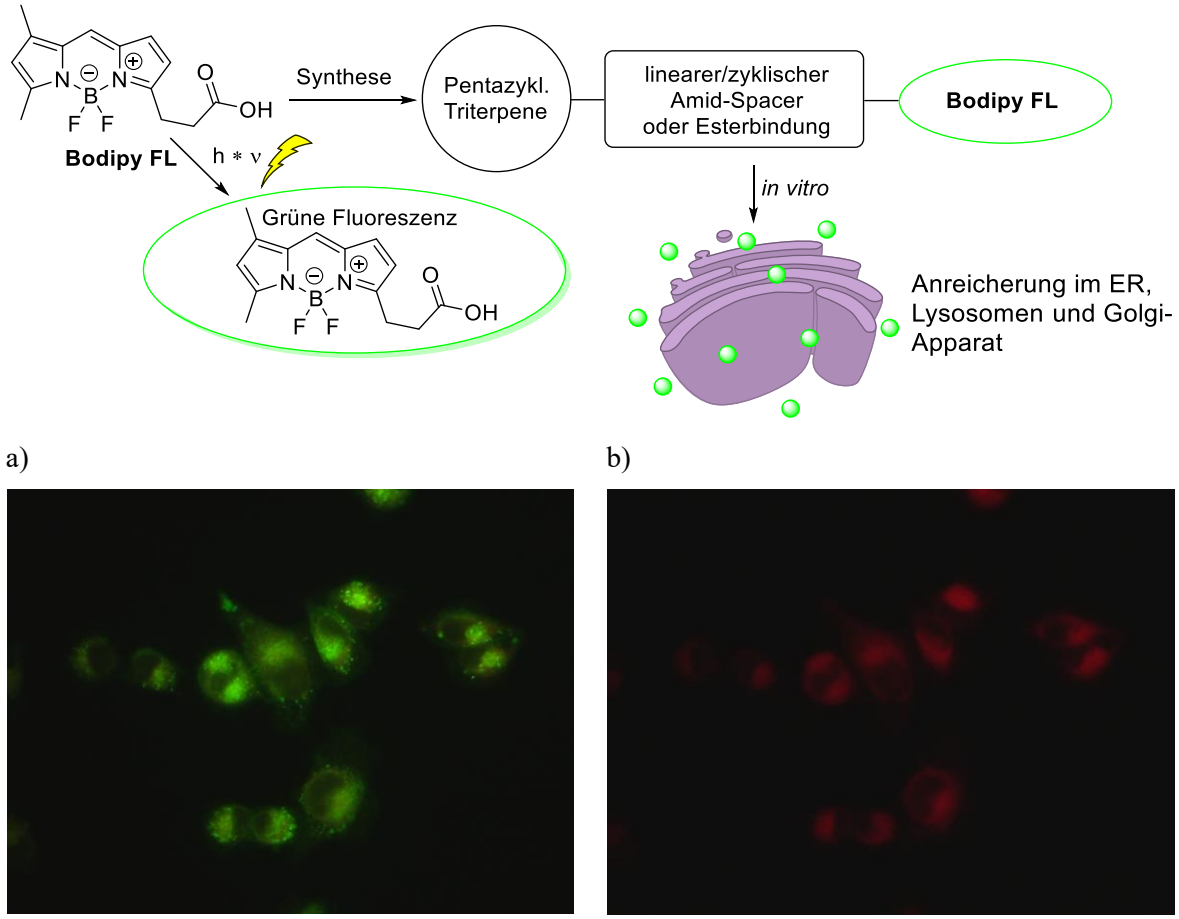


Abb. 11: Schematische Darstellung der synthetisierten Strukturen und Fluoreszenzmikroskopie-Aufnahmen zur Bestätigung der Anreicherung im ER: a) maligne Zellen mit Triterpen-Spacer-FF-Konjugat Fluoreszenz, b) gleiche maligne Zellen mit ER-Tracker Fluoreszenz.

Amid-Spacern synthetisiert und auf ihre biologischen Aktivitäten untersucht (s. Abb. 11). Dabei konnten wir feststellen, dass die Auswahl des Amid-Spacers einen starken Unterschied in den Zytotoxizitäten verursachte, der sich gegensätzlich zu den Rhd B-Konjugaten verhielt. Bei den Bodipy-Ethylendiamin-Spacer-Konjugaten waren nicht nur die Aktivitäten, sondern auch Selektivitäten höher als bei den

Piperazin-Spacer-Konjugaten. Dafür besitzen manche der Rhd B-Piperazin-Spacer-Konjugate eine deutlich höhere Zytotoxizität und bessere Löslichkeit. Untersuchungen mittels eines Fluoreszenzsmikroskops zeigten, dass sich die Bodipy-Spacer-Triterpen-Konjugate im Endoplasmatischen Retikulum ansammeln (s. Abb. 11). Nach der erfolgreichen Synthese und biologischen Evaluierung der Bodipy-Konjugate stellte sich die Frage, ob die hohe Zytotoxizität und Selektivität dieser Art der FF-Konjugate auf dem Prinzip beruhen, dass sie aufgrund ihrer Konjugation mit einem FF an andere Wirkorte transportiert und metabolisiert werden. Dafür wurde ein asymmetrischer aza-Bodipy-FF geplant und synthetisiert, der durch zwei weitere Phenylringe eine höhere Konjugation und somit Rotverschiebung besitzt. Außerdem wurde eine Hydroxygruppe eingebaut, um die Löslichkeit zu erhöhen. Als Amid-Spacer wurde Piperazin ausgewählt und direkt in einem frühen Schritt in die Synthese des FF eingebaut. Erstaunlicherweise zeigten die Zytotoxizitätstests, dass die hergestellten Konjugate im getesteten Konzentrationsbereich nicht zytotoxisch sind [102]. Es zeigte sich zudem, dass nur die zwei ersten Synthesestufenprodukte und das FF-Piperazin-Amid zytotoxisch sind. Nach diesen Ergebnissen wurde postuliert, dass der NIR-Farbstoff eventuelle Vorteile in der PDT bringen und unter Bestrahlung zytotoxisch gegenüber Krebszellen sein könnte. Durch *in vitro* Versuche konnte gezeigt werden, dass die Bestrahlung mit rotem Licht die Zytotoxizität stark anstiegen ließ. Eine weitere Beobachtung war, dass, wie bei den Triterpenen, die Zytotoxizität des FFs durch die Amidierung mit Piperazin stieg und somit der Trend bestätigt werden konnte. Zusammengefasst konnten neuartige zyklische Triterpen-Bodipy-Konjugate synthetisiert und deren biologische Aktivität, hinsichtlich Zellaufnahme und Wirkmechanismus, bei Tests mit malignen Zelllinien untersucht werden.

3.2 Synthese von Triterpenpiperazinylamiden mit unterschiedlichen Polaritäten [103]

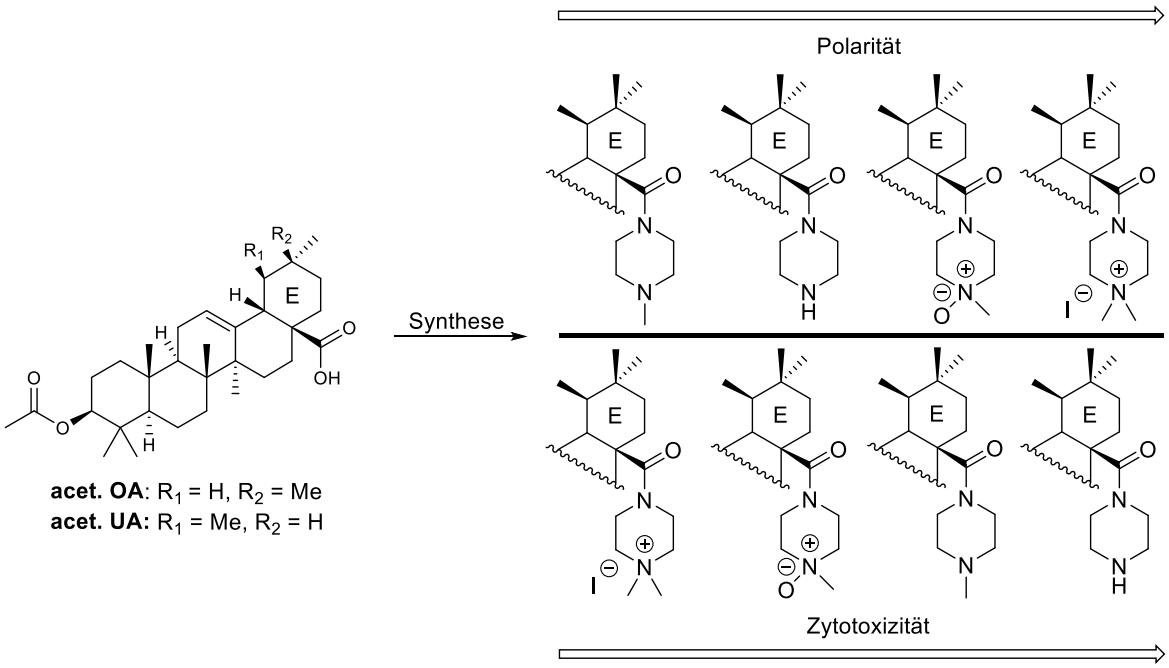


Abb. 12: Synthetisierte Verbindungen zur Untersuchung der Abhängigkeit der Zytotoxizität von der Polarität.

Aus den vorangegangenen Erkenntnissen wurde die Hypothese aufgestellt, dass eine positive Ladung oder ein Piperazinylamid in Verbindung mit einem lipophilen Triterpen vorteilhaft für die Zytotoxizität dieser Verbindungen ist. Die Veränderung der elektronischen Struktur durch die chemische Modifikation der Aminofunktion des Piperazinylrests an einem Triterpen-Grundgerüst kann dazu genutzt werden diese These zu untersuchen. Daraufhin wurden *N*- und *N*,*N*-dimethylierte Piperazinylamide, sowie die *N*-Methyl-*N*-oxide von UA und OA synthetisiert und mit dem SRB-Essay untersucht (s. Abb. 12). Es stellte sich heraus, dass die sekundären Amine des Piperazinylamids den anderen Derivaten in ihrer Zytotoxizität überlegen waren und eine Veränderung an der sekundären Aminofunktion, der These widersprechend, keine Vorteile bot. Die Polarität des Amidrests und die Zytotoxizität verhielten sich in diesem Fall gegensätzlich. Die Hypothese, dass die erhöhte Polarität von lipophilen Kationen immer zu einer erhöhten Zytotoxizität führt, konnte in dieser Arbeit widerlegt werden.

3.3 Synthese von Gallensäure-Rhd B-Konjugaten („Mitocans“) [104]

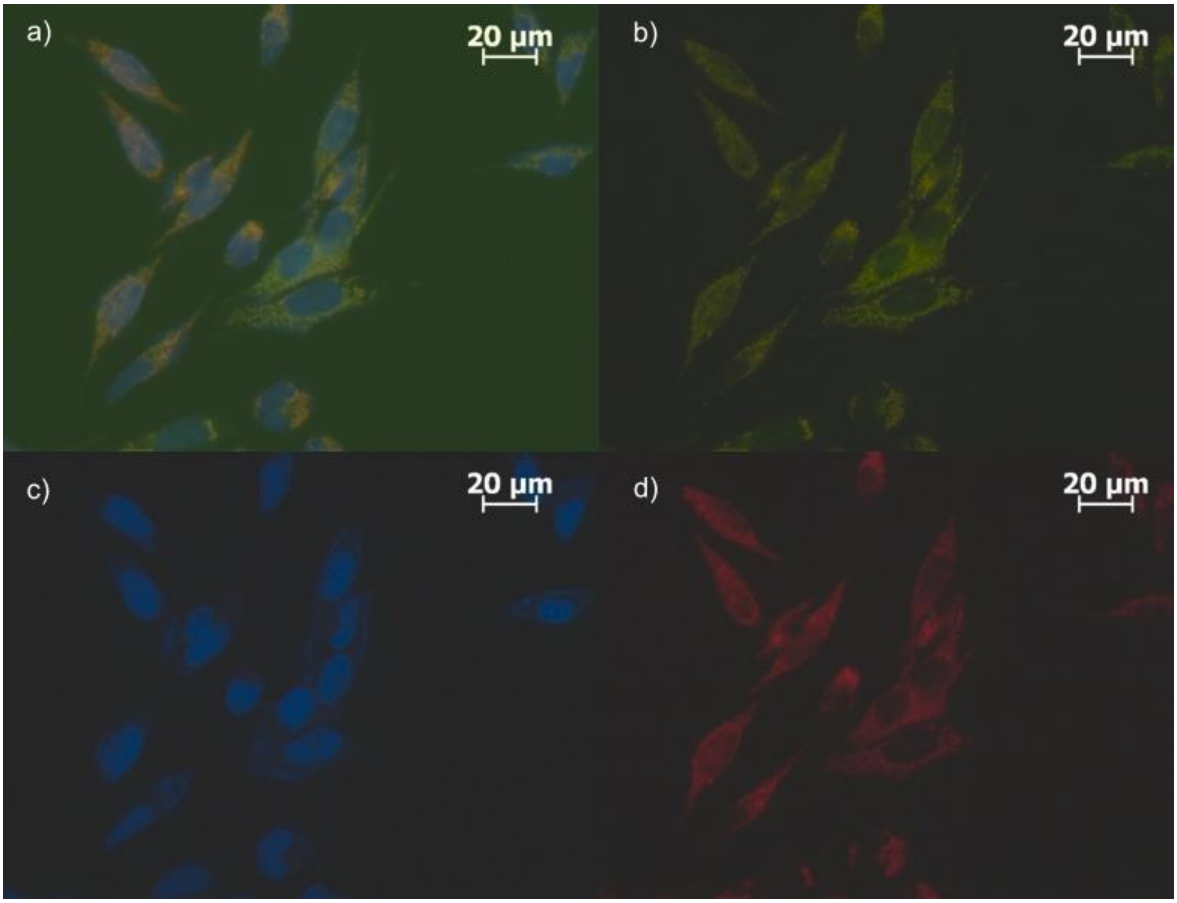


Abb. 13: Fluoreszenzbilder von A2780-Tumorzellen, die mit einem Gallensäure-Rhd B-Konjugat, Rhodamin 123 bzw. Hoechst 33,342 behandelt wurden: a) zusammengeführte Bilder, b) Rhodamin 123, c) Hoechst 33,342, d) Gallensäure-Rhd B-Konjugat.

Eine noch nicht so stark untersuchte Gruppe von Triterpenen und deren mögliche Anwendungen für die Krebs- und Alzheimerforschung sind die Gallensäuren: Litho-, Desoxy-, Chenodesoxy -, und Ursodesoxycholsäure. Einige von ihnen leiten die Apoptose ein, während Usodesoxycholsäure diese unterdrückt [105]. Manche Gallensäuren besitzen zudem tumorunterdrückende Eigenschaften, dies macht eine Erforschung von Derivaten für Krebstherapien besonders interessant [106]. Gallensäuren sind eine amphiphile Substanzklasse und besitzen somit ein interessantes Lösungsverhalten. Nach den Ergebnissen der vorherigen Arbeiten stellte sich die Frage, ob auch ein Gallensäure-Rhd B-Konjugat eine vorteilhafte Zytotoxizität zeigen würde. Gallensäuren sind in den benötigten Mengen und zu einem guten Preis verfügbar und in dieser Arbeit in wenigen Schritten mit einem Piperazin-Amid-Spacer mit Rhd B derivatisiert worden. Im ersten Schritt wurden die Hydroxylgruppen der Gallensäuren acetyliert und anschließend mit Piperazin amidiert. Diese tetrazyklischen Triterpenpiperazinylamide zeigten sehr ähnliche Zytotoxizitäten wie die vorher synthetisierten pentazyklischen Triterpenpiperazinylamide. Auf ähnliche Weise verhielt es sich auch mit den im nächsten Schritt synthetisierten Rhd B-Konjugaten, die stark zytotoxisch waren. Diese Ergebnisse stützen die These, dass die Synthese von lipophilen Rhd B-

Triterpen-Konjugate eine Strategie zur Synthese von zytotoxischen Verbindungen ist. Zusammengefasst waren die Konjugate zytostatisch, was auf einem Zellstillstand in der G1-Phase und einer Anreicherung in den Mitochondrien der Tumorzellen zurückzuführen war (s. Abb. 13).

3.4 Apoptose-auslösende aromatische *N*-Heterozyklische Triterpenamide [107]

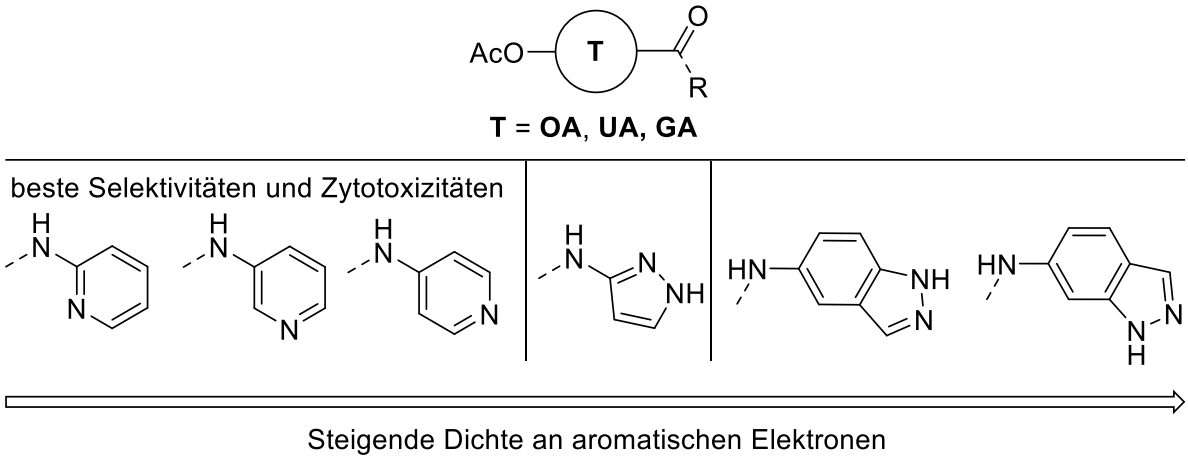


Abb. 14: Überblick über die N-heterozyklischen Triterpenamide.

Nach der Piperazinylamid-Studie kam die Frage auf, ob eine andere elektronische Verteilung (Polarität) des Amidrestes bessere zytotoxische Ergebnisse zeigt. Aromatische *N*-Heterozyklen besitzen je nach Struktur eine andere Verteilung der Elektrondichte und können entweder als elektronenreich oder -arm beschrieben werden. Bei den in dieser Arbeit untersuchten Triterpencarbonsäuren handelt es sich um Glycyrrhetin-, Ursol- und Oleanolsäure. Als elektronenarme *N*-Heterozyklenamine wurden die Strukturisomere des Pyridins ausgewählt, während als elektronenreiche Amine Pyrazol und zwei Strukturisomere des Indazols ausgewählt wurden. Mehrere dieser Gerüstmoleküle zeigten attraktive chemotherapeutische Aktivitäten und sind Bestandteil zugelassener Chemotherapeutika wie Crizotinib, Bosutinib, Sorafenib, Pazopanib, Regorafenib und Linifanib [108]. Die Derivate der Glycyrrhetinsäure zeigten im Vergleich eine geringere zytotoxische Wirkung, während die Ursol- und Oleanolsäure hohe Zytotoxizitäten aufwiesen. Die besten Selektivitäten und EC_{50}-Werte zeigten die Ursolsäurederivate vor allem gegenüber der malignen Zelllinie A375 (Melanom des Epithels). Je nach Amidrest (s. Abb. 14) zeigte sich ein Einfluss auf die Selektivität und Zytotoxizität, so zeigten die Derivate mit der 2-Aminopyridn-Einheit die besten Selektivitäten und Zytotoxizität gegenüber malignen Zelllinien. Analysen zum Wirkmechanismus der synthetisierten Wirkstoffe zeigten eine stark apoptotische Wirkung dieser. So konnten wir auch durch Fluoreszenzmikroskopie zeigen, dass die Zellen durch Apoptose sterben, was eine attraktive Eigenschaft für Cytostatika ist (s. Abb. 15).

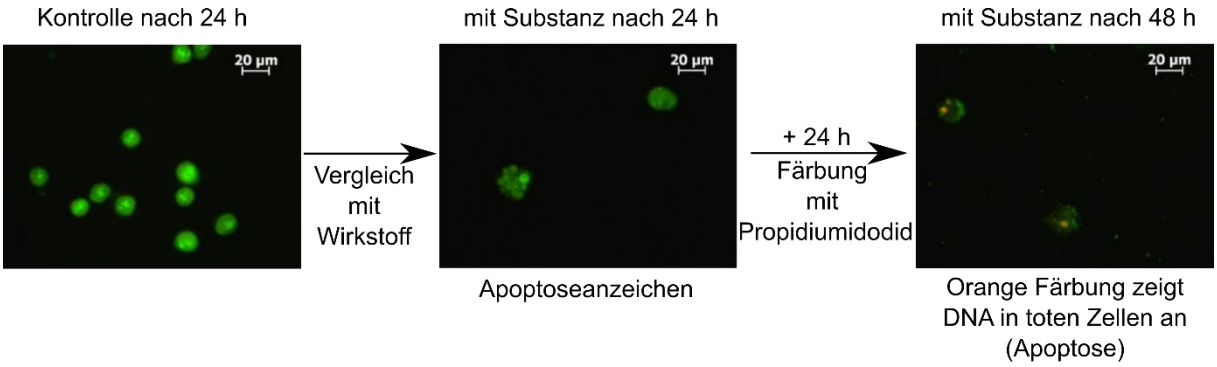


Abb. 15: Fluoreszenzmikroskopie-Untersuchung des Zelltodmechanismus des 4-Aminopyridinylamids der Oleanolsäure. Ein Ausbleiben der Orangefärbung in den kleinen, abgetrennten grüngefärbten Zellresten nach 48 h spricht für einen kontrollierten Abbau der DNA.

3.5 „Missing Links“ – Selektive und kostengünstige Amid- und Harnstoffderivate von Triterpenen als mögliche Cytostatika [109]

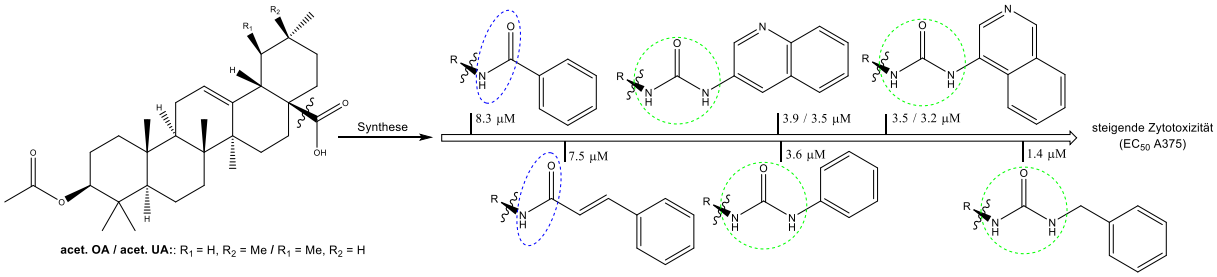


Abb. 16: Überblick über die Struktur-Wirkungs-Beziehung der synthetisierten Amid- (blau) und Harnstoffderivate (grün).

Basierend auf vorrangegangenen Arbeiten, die eine Struktur-Wirkungs-Beziehung zwischen aminischen Amiden und deren Positivierung untersuchten, und einer anderen Arbeit, die Harnstoffderivate der Oleanol-, Ursol- und Maslinsäure untersuchte [110], wurde die Synthese für die in diesen Untersuchungen fehlenden Strukturen geplant. Diese vorrausgegangen Untersuchungen zeigten erhebliche Unterschiede in der Zytotoxizität und Selektivität je nach aromatischem Rest und der Verbindungsklasse.

In dieser Arbeit konnten wir zeigen, dass die Harnstoffderivate den Verbindungen mit Amidbindung in ihrer Zytotoxizität und Selektivität überlegen waren (s. Abb. 15). Das Benzyl-Harnstoff-Derivat der Ursolsäure zeigte die höchste Zytotoxizität mit einer EC_{50} von 1.4 µM und die beste Selektivität gegenüber der nicht malignen Zelllinie NIH 3T3 mit einem Selektivitätsfaktor von 45. Ein Test der **CA II** Inhibierung zeigte, dass, außer dem Benzylamids der acetylierten Oleanolsäure mit einer Inhibierung von 79.8%, keine der synthetisierten Verbindungen eine Inhibierung des Enzyms verursachte. Die sehr guten Ausbeuten und unkomplizierte Synthese machen das Benzylharnstoff-Derivat der Ursolsäure zu einem attraktiven Kandidaten für weitere Tests. Die Ergebnisse zeigten auch, dass die Ursolsäurederivate zytotoxischer als die Oleanolsäurederivate sind.

3.6 Synthese von Amorfrutin A und B und Evaluierung der Zytotoxizität [111]

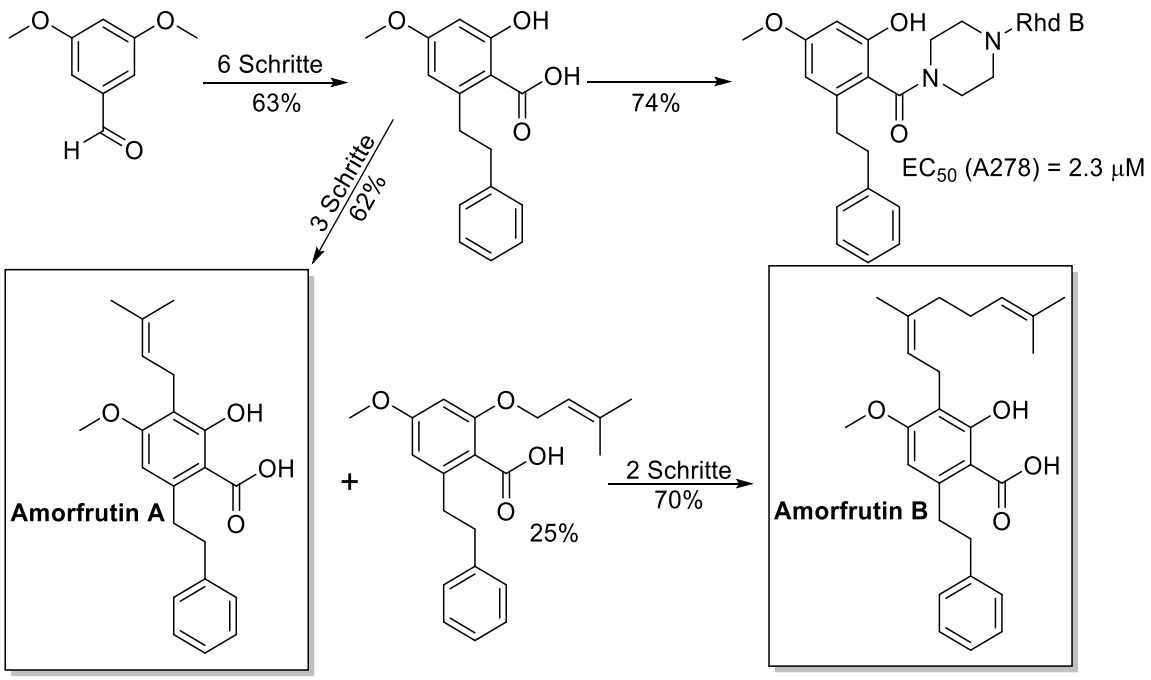


Abb. 17: Hervorzuhebende Synthesen der wichtigsten Substanzen.

Amorfrutine lassen sich schlecht aus natürlichen Quellen gewinnen und sind von hohem wissenschaftlichem Interesse für die Diabetes-Forschung [112]. In dieser Arbeit zeigen wir eine neue effiziente Syntheseroute für die Amorfrutine A und B, als auch die Synthese von zytotoxischen Verbindungen mit dem Amorfrutin Grundkörper, unter denen die Rhodamin B-piperazinyl-Verbindung die höchste Zytotoxizität zeigte (s. Abb. 17). Die Vorteile dieser Syntheseroute für die Amorfrutine sind die einfach zugänglichen Edukte und die Verwendung von Nebenprodukten zur Synthese des wertvollen Amorfrutins B. Die stark zytotoxische Rhodamin B Verbindung, die aus dieser Arbeit entstanden ist, wies allerdings keine Selektivitäten auf und bietet somit keinen Mehrwert gegenüber den anderen, in dieser Dissertation vorgestellten, Substanzen. Jedoch konnte auch in dieser Arbeit das Theorem, dass Naturstoff-Piperazin-Rhodamin B-Konjugate zytotoxisch sind, bestätigt werden.

4. Zusammenfassung der Dissertation

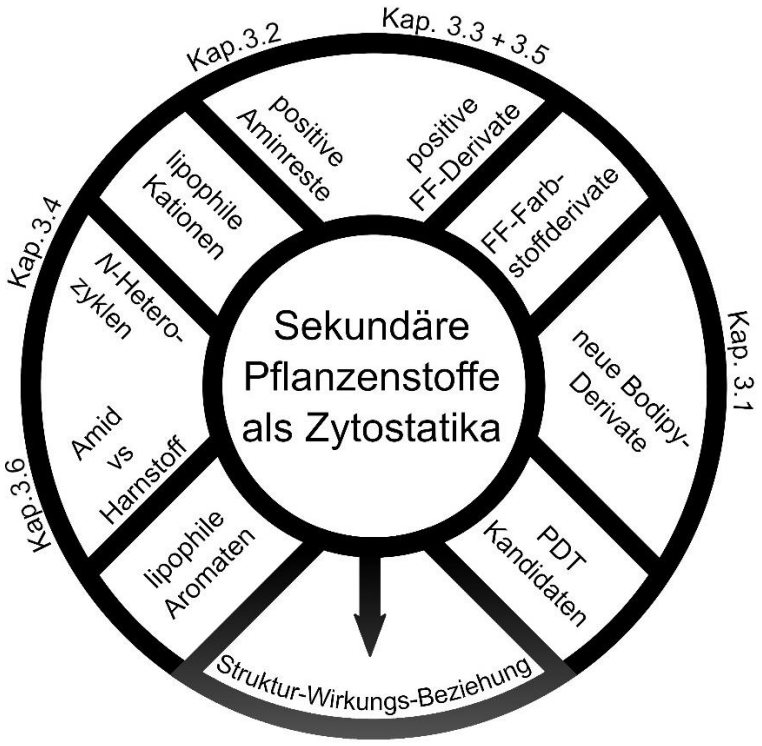


Abb. 18: Zusammenfassung und Einordnung der Veröffentlichungen aus dieser Arbeit.

Um das volle Potential von Triterpenen hinsichtlich der Eignung als Zytostatika zu untersuchen, wurden mehrere Ansätze zur Derivatisierung durchgeführt und analysiert. Es wurden viele unterschiedliche Derivate der am besten zugänglichen Triterpene, als auch neue Fluoreszenzfarbstoffe und die Amorfrutine A und B synthetisiert und analysiert. Hierfür wurden sowohl der SRB- und Annexin V-FITC/PI-Assay, als auch Zellzyklus- und Fluoreszenzmikroskopie-Untersuchungen durchgeführt, um die Mechanismen der zytotoxischen Wirkung einzuschätzen.

Die wichtigsten Erkenntnisse aus diesen Untersuchungen sind in Veröffentlichungen zusammengefasst und konnten im wissenschaftlichen Kontext eingeordnet werden (s. Abb. 18). Die Derivatisierung von Triterpenen mit Fluoreszenzfarbstoff-, Amin- und *N*-Heterozyklen-Resten zeigte eine interessante Struktur-Wirkungs-Beziehung innerhalb der einzelnen Studien, als auch im Vergleich zu anderen Studien. Hervorzuheben ist, dass die Derivatisierungsstrategien immer zu besser zytotoxischen Verbindungen führten, und alle untersuchten Verbindungen vermehrt den kontrollierten Zelltod (Apoptose) und kaum Nekrose einleiteten. Die interessantesten Verbindungen dieser Arbeit zeigten auch hohe Selektivitäten gegenüber nicht malignen Zelllinien, während andere enormes Potenzial für die PDT zeigen. Außerdem wurde ein Beitrag zur Vereinfachung der Synthese von Amorfrutin A und B geleistet und neue Strategien zur Erlangung von zytotoxischen Derivaten von Triterpenen auf die Amorfrutin-Grundstruktur angewendet. Zusammengefasst konnte ein Beitrag im Bereich der Naturstoffchemie und medizinischen Chemie geleistet werden.

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6. Abbildungsverzeichnis

7. Tabellenverzeichnis

8. Anhang

Die vollständigen Publikationen werden der Arbeit hinter der Selbständigkeitserklärung für die Prüfung der Arbeit angefügt. Die danach veröffentlichte Version wird nur die Publikationen enthalten, die öffentlich zugänglich sind. Folgend sind die Abstracts der für diese Arbeit relevanten Publikationen aufgelistet.

8.1 "Design, synthesis and cytotoxicity of BODIPY FL labelled triterpenoids"

Abstract:

Several triterpenoid acids (betulinic, oleanolic, ursolic, glycyrrhetinic) and triterpene betulin were used as starting material to synthesize BODIPY FL adducts, and these compounds were screened for their cytotoxic activity employing several human tumor cell lines. The cytotoxicity of the compounds strongly depended on the chosen spacer between the triterpenoid core and the BODIPY FL unit. Thus, 3-*O*-acetyl-betulinic acid derived BODIPY FL conjugate holding an ethylendiamine spacer was cytotoxic for human breast adenocarcinoma cells MCF7 but not cytotoxic for all other cell lines.

Keywords: BODIPY FL; Betulinic acid; Cytotoxicity; Glycyrrhetinic acid; Oleanolic acid; Triterpenoids; Ursolic acid.

DOI: https://doi.org/10.1016/j.ejmech.2019.111858

8.2 "The presence of a cationic center is not alone decisive for the cytotoxicity of triterpene carboxylic acid amides"

Abstract:

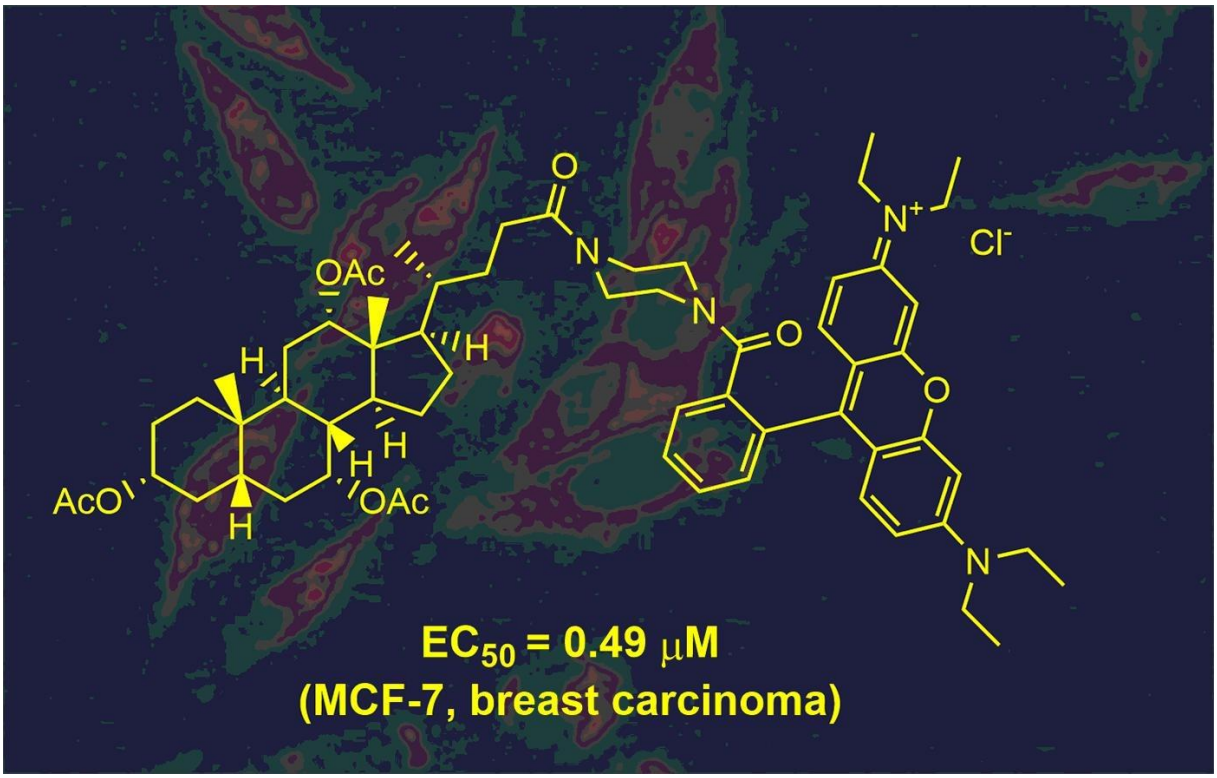
3-O-Acetyl-ursolic acid (2) and 3-O-acetyl oleanolic acid (8) were converted into piperazinylamides holding a distal NH, NMe or a NMe_2 group. These compounds as well as the corresponding N-methyl-N-oxides were accessed. Their cytotoxicity was assessed in SRB assays employing a panel of human tumor cell lines and non-malignant fibroblasts (NIH 3T3). As a result, compounds holding a quaternary distal N-substituent were less cytotoxic that those holding a NH-moiety. Hence, the presence of a distal cationic center seems not to be a sufficient criterion for obtaining triterpenoids of high cytotoxicity and selectivity.

Keywords: Amides; Cytotoxicity; N-oxide; Oleanolic acid; Ursolic acid.

DOI: https://doi.org/10.1016/j.steroids.2020.108713

8.3 "Converting bile acids into mitocans"

Graphical abstract:



Abstract:

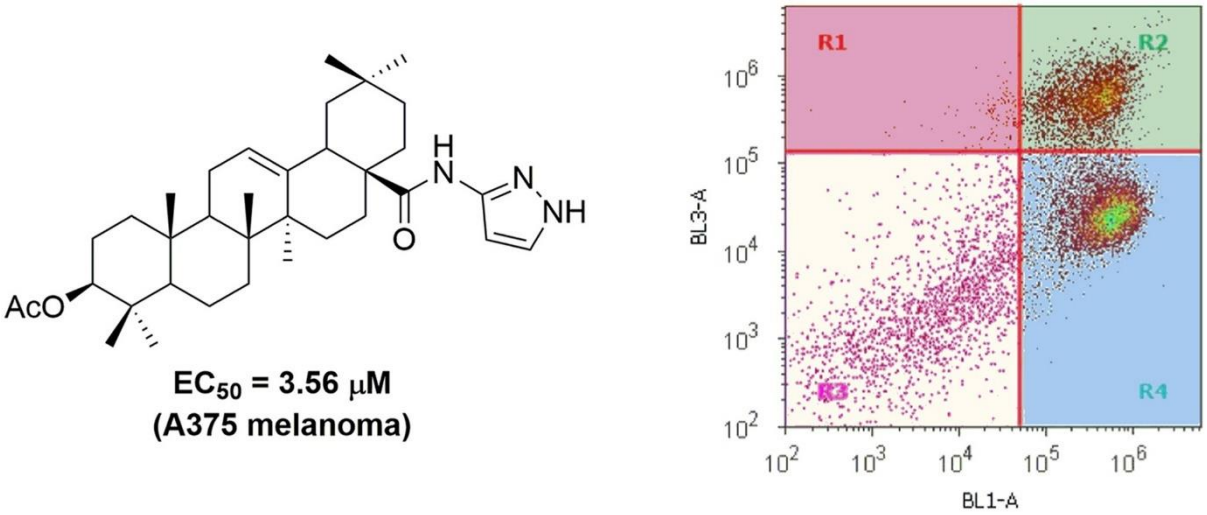
Cholic acid (1, CD), deoxycholic (3, DCA), chenodeoxycholic acid (5, CDCA), ursodeoxycholic acid (7, UDCA), and lithocholic acid (9, LCA) were acetylated and converted into their piperazinyl spacered rhodamine B conjugates 16–20. While the parent bile acids showed almost no cytotoxic effects for several human tumor cell lines, the piperazinyl amides were cytostatic but an even superior effect was observed for the rhodamine B conjugates. Extra staining experiments showed these compounds as mitocans; they led to a cell arrest in the G1 phase.

Keywords: Bile acids; Rhodamine B conjugates; Mitocans

DOI: https://doi.org/10.1016/j.steroids.2022.109148

8.4 "Synthesis and cytotoxicity of apoptosis-inducing *N*-heterocyclic triterpene amides"

Graphical abstract:



Abstract:

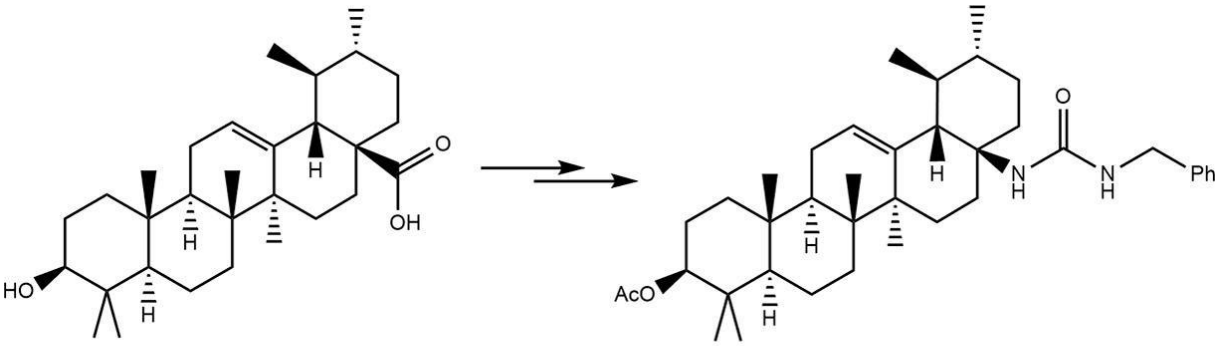
The modification of lipophilic triterpenes to enhance their cytotoxicity, is a viable strategy for finding new anti-cancer agents. Herein we report the synthesis, analysis of 18 pentacyclic triterpenoic acid *N*-heterocyclic amides and their cytotoxicity, tumor cell/non-tumor cell selectivity, as well as their putative mode of action. EC_{50} values were measured by SRB-assays, and found to be as low as 3.13 μM, with a selectivity as high as S = 5.05. Moreover, supportive assays were performed to further analyze their cytotoxicity; these experiments showed the compounds to act mainly by apoptosis.

Keywords: Triterpenoic acids; Cytotoxicity; Amides

DOI: https://doi.org/10.1016/j.ejmcr.2022.100085

8.5 "Selective and low-cost triterpene urea and amide derivatives of high cytotoxicity and selectivity"

Graphical abstract:



Ursolic acid

EC_{50} = 1.4 µM (A375, melanoma)
EC_{50} = 70 µM (NIH 3T3, fibroblasts)
Selectivity = ~ 50 fold more cytotoxic for malignant melanoma cells

Abstract:

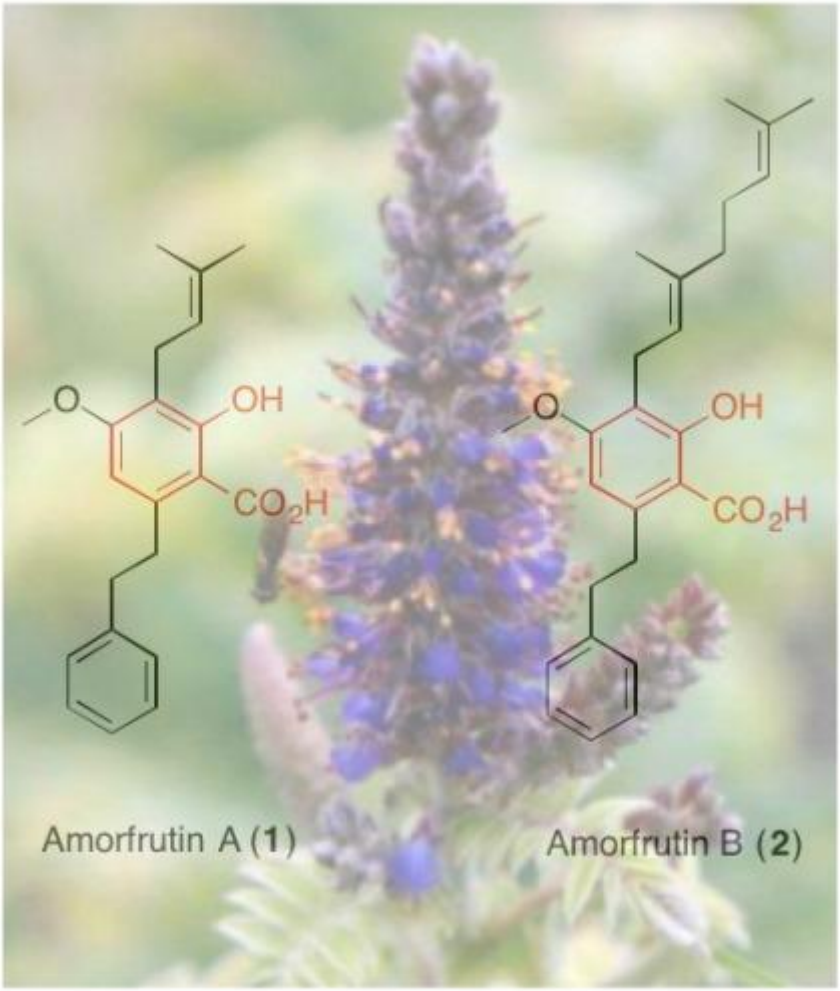
Phytochemicals play a vital role in drug discovery, especially for the development of anti-cancer drugs. Thereby, convenient syntheses, high cytotoxicity but also good tumor cell/non-tumor cell selectivity, are called for. An interesting group of phytochemicals is represented by pentacyclic triterpenoic acids and derivatives thereof. Herein we report the synthesis of some ursolic and oleanolic acid derived amides and urea derivatives and the results from sulforhodamine (SRB) assays to assess their cytotoxic activity for several human tumor cell lines. As a result, an ursolic acid derived benzyl urea **16** showed a rather low EC_{50} = 1.4 µM for A375 melanoma cell while being not cytotoxic (EC_{50} = 70 µM) for non-malignant fibroblasts (NIH 3T3).

Keywords: Triterpenoic Acids; Cytotoxicity; Urea

DOI: https://doi.org/10.1016/j.rechem.2022.100610

8.6 "A unified strategy for the synthesis of amorfrutins A and B and evaluation of their cytotoxicity"

Graphical abstract:



Abstract:

3,5-Dimethoxy-benzaldehyde was used as a starting material to synthesize a central intermediate, 2-hydroxy-4-methoxy-6-phenethylbenzoic acid that was converted very quickly and with good yields into amorfrutins A and B. Furthermore, this compound was also used as a starting material to synthesize a piperazinyl-rhodamine B conjugate. The latter compound showed good cytotoxicity (EC_{50} = 2.3–5.1 mM) and promising selective cytotoxicity (S = 2.1–4.6) for human tumor cell lines as compared to non-malignant fibroblasts (NIH 3T3).

DOI: http://dx.doi.org/10.13171/mjc10902011171546rc

9. Lebenslauf

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10. Publikationsliste

Non-cytotoxic aza-BODIPY triterpene conjugates to target the endoplasmic reticulum
S. Hoenke, **B. Brandes**, R. Csuk, *Eur. J. Med. Chem. Rep.* **2023**, *7*, 100099.

Converting bile acids into mitocans
B. Brandes, S. Hoenke, C. Schultz, H.-P. Deigner, R. Csuk, *Steroids* **2023**, *189*, 109148.

Triterpenoid cholinesterase inhibitors that might improve gait disturbances in Parkinson's disease patients
N. V. Heise, J.-A. Schüler, T. E. Orlamünde, **B. Brandes**, H.-P. Deigner, A. Al-Harrasi, R. Csuk, *Mediterr. J. Chem.* **2022**.

Synthesis and cytotoxicity of apoptosis-inducing *N*-heterocyclic triterpene amides
B. Brandes, S. Hoenke, N. Starke, I. Serbian, H.-P. Deigner, Ah. Al-Harrasi, R. Csuk, *Eur. J. Med. Chem. Rep.* **2022**, *6*, 100085.

Selective and low-cost triterpene urea and amide derivatives of high cytotoxicity and selectivity
B. Brandes, T. E. Orlamünde, S. Hoenke, T. C. Denner, A. Al-Harrasi, R. Csuk, *Results Chem.* **2022**, *4*, 100610.

Design, synthesis, and characterization of hole transport materials for perovskite solar cells
B. Brandes, B. Weber, R. Csuk, *Mediterr. J. Chem.* **2022**.

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The Presence of a Cyclohexyldiamine Moiety Confers Cytotoxicity to Pentacyclic Triterpenoids
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A unified strategy for the synthesis of amorfrutins A and B and evaluation of their cytotoxicity
B. Brandes, S. Hoenke, M. Türk, B. Weber, H.-P. Deigner, A. Al-Harrasi, R. Csuk, *Mediterr. J. Chem.,* **2020**, *10*, 858.

The presence of a cationic center is not alone decisive for the cytotoxicity of triterpene carboxylic acid amides
B. Brandes, L. Koch, S. Hoenke, H.-P. Deigner, R. Csuk, *Steroids* **2020**, *163*, 108713.

A hitherto unreported impurity in Terazosin – elucidation of the structure, synthesis and cytotoxicity
B. Brandes, S. Hoenke, H.-P. Deigner, R. Csuk, *Mediterr. J. Chem.,* **2020**, *10*.

Design, synthesis and cytotoxicity of BODIPY FL labelled triterpenoids
B. Brandes, S. Hoenke, L. Fischer, R. Csuk, *Eur. J. Med. Chem.* **2020**, *185*, 111858.

An efficient and robust synthesis of amorfrutin A
B. Weber, **B. Brandes**, D. Powroznik, R. Kluge, R. Csuk, *Tetrahedron Lett.* **2019**, *60*, 1379.

11. Erklärung des Autorenanteils

Publikation Kap 3.1: “Design, synthesis and cytotoxicity of BODIPY FL labelled triterpenoids”

B. Brandes, S. Hoenke, L. Fischer, R. Csuk, *Eur. J. Med. Chem.* 2020, *185*, 111858.

Synthese und Charakterisierung: **B. Brandes**

Biologische Assays und Mikroskopie: S. Hoenke und L. Fischer

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Planung und Bereitstellung von Ressourcen: R. Csuk

Publikation Kap 3.2: “The presence of a cationic center is not alone decisive for the cytotoxicity of triterpene carboxylic acid amides”

B. Brandes, L. Koch, S. Hoenke, H.-P. Deigner, R. Csuk, *Steroids* **2020**, *163*, 108713.

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Biologische Assays: S. Hoenke

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Planung und Bereitstellung von Ressourcen: R. Csuk

Publikation Kap 3.3: “Converting bile acids into mitocans”

B. Brandes, S. Hoenke, C. Schultz, H.-P. Deigner, R. Csuk, *Steroids* **2023**, *189*, 109148.

Synthese und Charakterisierung: **B. Brandes** und L. Koch

Biologische Assays und Mikroskopie: S. Hoenke

Verfassen und Überprüfung der Veröffentlichung: **B. Brandes**, S. Hoenke, H.-P. Deigner und R. Csuk

Planung und Bereitstellung von Ressourcen: R. Csuk

Publikation Kap 3.4: “Synthesis and cytotoxicity of apoptosis-inducing N-heterocyclic triterpene amides”

B. Brandes, S. Hoenke, N. Starke, I. Serbian, H.-P. Deigner, A. Al-Harrasi, R. Csuk, *Eur. J. Med. Chem. Rep.* **2022**, *6*, 100085.

Synthese und Charakterisierung: **B. Brandes** und N. Starke

Biologische Assays und Mikroskopie: S. Hoenke

Verfassen und Überprüfung der Veröffentlichung: **B. Brandes**, S. Hoenke, H.-P. Deigner, A. Al-Harrasi und R. Csuk

Planung und Bereitstellung von Ressourcen: I. Serbian und R. Csuk

Publikation Kap 3.5: “Selective and low-cost triterpene urea and amide derivatives of high cytotoxicity and selectivity”

B. Brandes, T. E. Orlamünde, S. Hoenke, T. C. Denner, A. Al-Harrasi, R. Csuk, *Results Chem.* **2022**, *4*, 100610.

Synthese und Charakterisierung: **B. Brandes** und T. E. Orlamünde

Biologische Assays und Mikroskopie: S. Hoenke und T. C. Denner

Verfassen und Überprüfung der Veröffentlichung: **B. Brandes**, A. Al-Harrasi und R. Csuk

Planung und Bereitstellung von Ressourcen: R. Csuk

Publikation Kap 3.6: “A unified strategy for the synthesis of amorfrutins A and B and evaluation of their cytotoxicity”

B. Brandes, S. Hoenke, M. Türk, B. Weber, H.-P. Deigner, A. Al-Harrasi, R. Csuk, *Mediterr. J. Chem.* **2020**, *10*, 858.

Synthese und Charakterisierung: **B. Brandes**, M. Türk und B. Weber.

Biologische Assays und Mikroskopie: S. Hoenke.

Verfassen und Überprüfung der Veröffentlichung: **B. Brandes**, H.-P. Deigner, A. Al-Harrasi und R. Csuk

Planung und Bereitstellung von Ressourcen: R. Csuk

12. Selbstständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und nur unter Zuhilfenahme der angegebenen Quellen und Hilfsmittel verfasst habe. Die aus den angegebenen Werken, wörtlich oder inhaltlich, entnommenen Stellen sind kenntlich gemacht. Die Arbeit wurde keiner anderen Prüfungsbehörde vorgelegt und auch nicht veröffentlicht.

Halle (Saale), 24.08.2023

Benjamin Brandes

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Research paper

Design, synthesis and cytotoxicity of BODIPY FL labelled triterpenoids

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ABSTRACT

Several triterpenoid acids (betulinic, oleanolic, ursolic, glycyrrhetinic) and triterpene betulin were used as starting material to synthesize BODIPY FL adducts, and these compounds were screened for their cytotoxic activity employing several human tumor cell lines. The cytotoxicity of the compounds strongly depended on the chosen spacer between the triterpenoid core and the BODIPY FL unit. Thus, 3-*O*-acetyl-betulinic acid derived BODIPY FL conjugate holding an ethylendiamine spacer was cytotoxic for human breast adenocarcinoma cells MCF7 but not cytotoxic for all other cell lines.

1. Introduction

Since their first description [1] at the end of the 80's of the last century, more than 11.000 publications or patents for the synthesis and application of BODIPYs (boron dipyrromethene complexes) have appeared [2–5]. The range of applications covers their use as molecular probes [6–9] through their application in solar cells [10], as diagnostics [11–15], as photosensitizers [16–20], as cytotoxic agents [21–32] and for the visualization of small molecules in cells [33–37]. The latter is of great importance in particular, as it is now possible to investigate the cellular uptake and distribution of a molecule in the cell or even in individual compartments of a cell using high-resolution fluorescence microscopy. The advantages of BODIPYs are their high photo-stability, their low polarity and charge neutrality as well as the high quantum yield of fluorescence. An almost uncountable number of molecules have been labelled with BODIPYs, but it was not until 2018 that a first paper was published dealing with the fluorescence labelling of triterpenes by BODIPYs, and this work was also limited to the labelling of betulinic acid derivatives [38]. Thereby, these derivatives were accessed by solid phase synthesis. This allowed the rapid synthesis of the derivatives, however, the amount of a single compound to be obtained was extremely small.

To investigate possible uptake, transport and metabolisation processes of triterpenes in malignant as well as non-malignant cells, it is of great importance that the triterpene-BODIPY adducts can be synthesized in good yields. In this paper, we describe the synthesis of triterpene carboxylic acid (Fig. 1) derived BODIPY derivatives from betulinic acid (**BA**), oleanolic acid (**OA**), ursolic acid (**UA**), glycyrrhetinic acid (**GA**) and betulin (**BN**). BODIPY-FL propanoic acid (**7**) was selected as a model compound to prove the concept.

Most compounds in medical chemistry fall within the 500 Da rule, one of Lipinksi's rule of five. All triterpene-BODIPY adducts planned and synthesized in this study, however, have molar masses above 750 Da since a more or less flexible diamide spacer was inserted between the BODIPY residue and the respective triterpene. This strategy might allow to draw some conclusions about recognition of the compounds and perhaps also influence the ability of cells to incorporate them.

2. Results and discussion

2.1. Chemistry

Since BODIPY-FL (**7**) is commercially available at an extremely high price, and although several syntheses have previously been described [38,39], we became interested in developing a robust

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0223-5234/

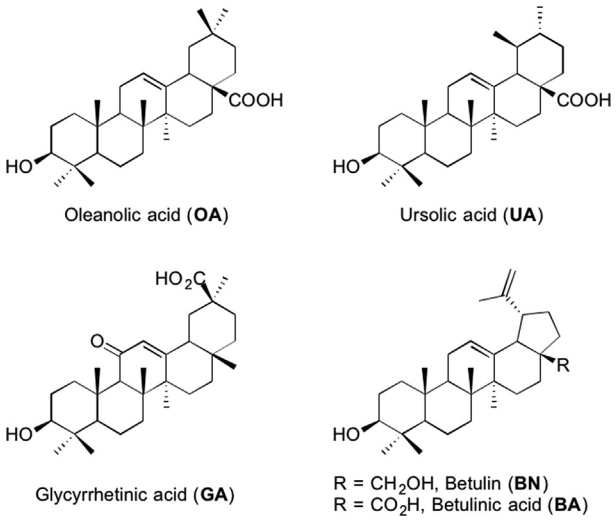


Fig. 1. Structure of parent triterpenecarboxylic acids: oleanolic, ursolic, glycyrrhetinic and betulinic acid as well of betulin.

synthesis that should allow to get hold of larger amounts of this valuable substance. Starting from commercially 1*H*-pyrrole-2-carbaldehyde (**1**, Scheme 1) its olefination reaction with benzyl(-dimethoxyphosphoryl)acetate (**2**)/NaH in dry THF gave an 86% yield of acrylate **3** whose hydrogenation yielded the propanoate **4**. From the reaction of **4** with 3,5-dimethyl-1*H*-pyrrole-2-carbaldehyde (**5**)/phosphoryl chloride and BF_3 diethyl etherate in the presence of diisopropylamine, complex **6** was obtained as a reddish solid. De-protection by hydrogenation finally led to BODIPY-FL (**7**) in 95% isolated yield (see Scheme 2).

Acetylation of parent betulinic acid (**BA**), oleanolic acid (**OA**), ursolic acid (**UA**) and glycyrrhetinic acid (**GA**) gave acetates **8**–**11**, respectively. Their reaction with oxalyl chloride and piperazine yielded amides **12**–**15**, and from the reaction of **8**–**11** with oxalyl chloride and ethylene diamine amides **16**–**19** were obtained. Amides **12**–**19** served as starting material for the synthesis of the BODIPYs adducts. Thus, their reaction with oxalyl chloride in the presence of a catal. amount of DMF followed by the addition of *in situ* prepared chloride of **7** (from **7** and oxalyl chloride) gave triterpene-BODIPY adducts **20**–**27**, respectively.

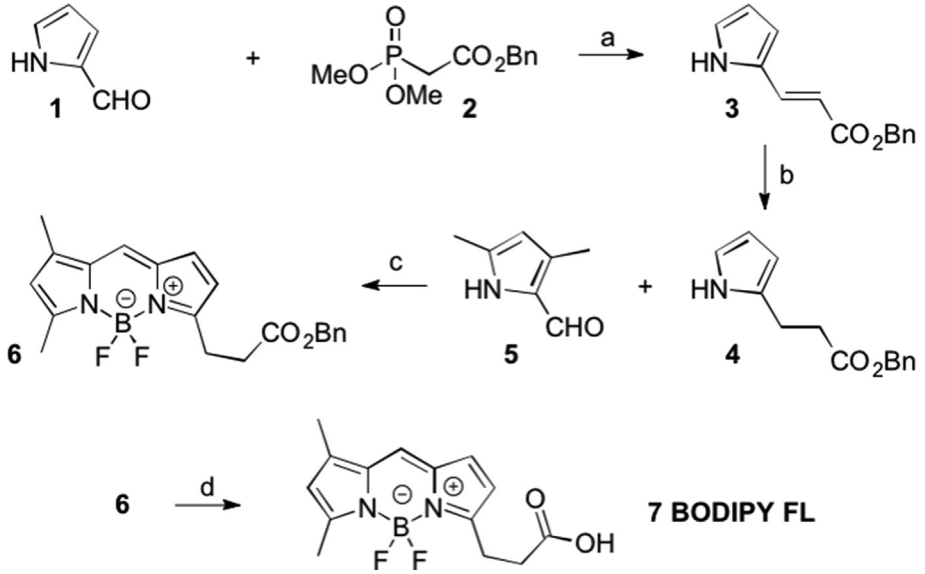
Similar to the synthesis of the amides **20**–**27**, the synthesis of betulin (**BN**) derived esters (Scheme 3) was performed. Thus, **BN** was converted into its 3-*O*-acetyl **28** and 28-*O*-acetyl derivative **29**. Their esterification with **7** yielded adducts **30** and **31**, respectively.

The triterpene-BODIPY adducts **20**–**27** are characterized in their ^{19}F NMR spectra by a signal between $\delta = 143.6$–144.8 ppm, and in the ^{13}C NMR spectra the amide carbonyl carbon was detected between $\delta = 170.7$ and 172.9 ppm, while for the esters **30** and **31** the carbonyl carbon was found at $\delta = 172.3$ and 173.0 ppm, respectively. Triterpene/BODIPY amidoethyl and piperazinyl adducts showed different splitting patterns in their ^{19}F NMR spectra. While triterpene/BODIPY piperazinyl amides gave similar splitting patterns as compared to BODIPY FL, amidoethyl compounds gave more complex spectra due to their different tautomeric properties (see supplementary materials file).

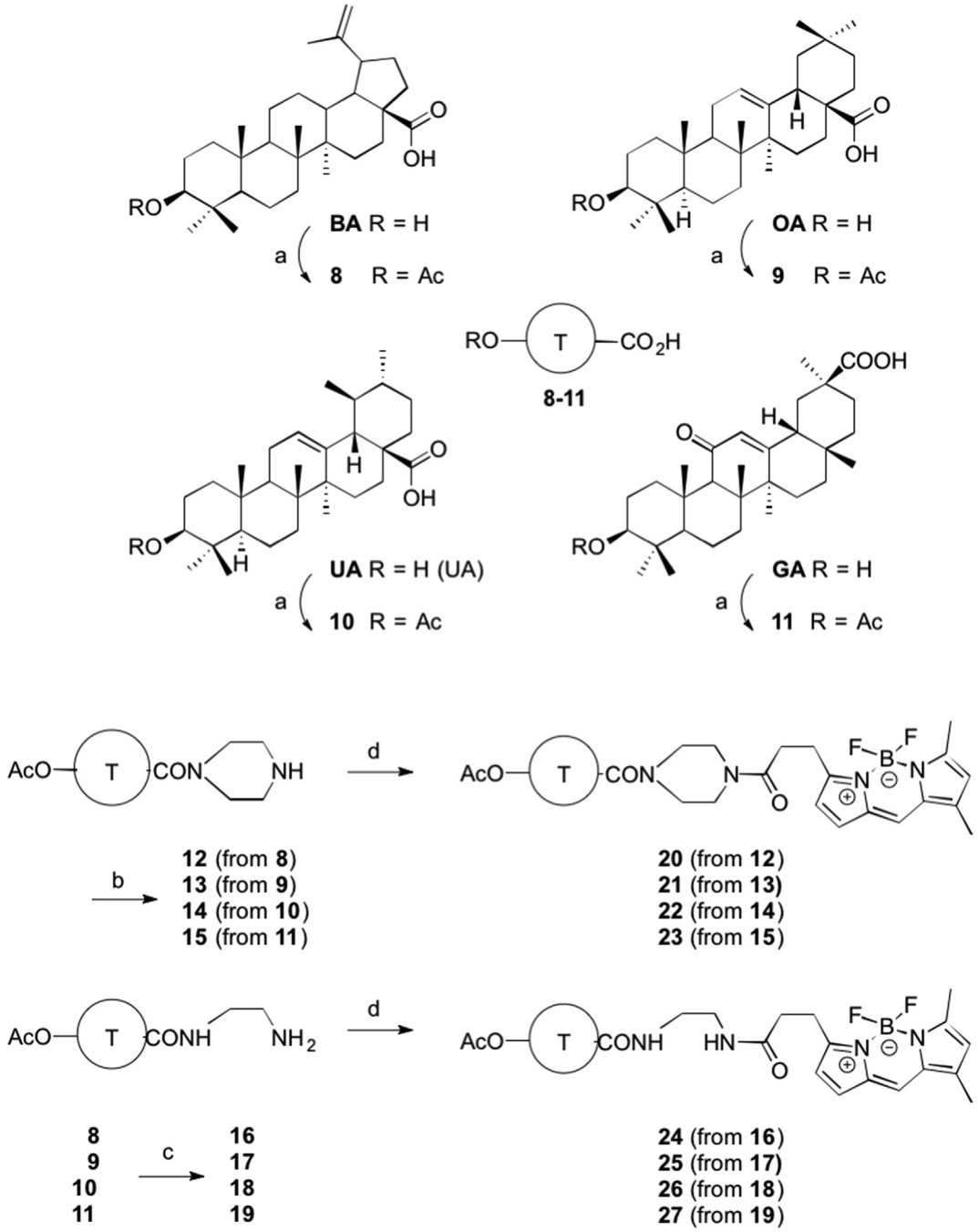
2.2. Biological evaluation

Compounds **8**–**31** and parent triterpenoids (**BA**, **OA**, **UA**, **GA**, **BN**) were screened for their cytotoxic activity. The results of the sulforhodamine B assays (SRB) are summarized in Table 1.

BODIPY FL (**7**) was not cytotoxic for all human tumor cell lines as well as for non-malignant mouse fibroblasts (NIH 3T3). While the parent triterpenoids showed only weak (**BA**, **UA**) or no cytotoxicity (**OA**, **GA**, **BN**), strong cytotoxicity was observed for all 3-*O*-acetylated piperazinyl derivdes amides **12**–**15** (1.0–4.4 μM for HT-29 human colorectal adenocarcinoma), and a similar high cytotoxicity was found for the 3-*O*-acetylated ethylenediamine derived amides **16**–**19**. Interestingly, piperazine amide derived BODIPY adducts **20**–**23** were not cytotoxic at all for HT-29 cells within the limits of the assay (30 μM cut-off), while their ethylenediamine spacered analogs **24**–**27** were also not cytotoxic to HT-29 cells but cytotoxic to MCF7 human breast adenocarcinoma cells. More importantly, betulinic acid derived compound **24** and glycyrrhetinic acid derived **27** showed no cytotoxic effect to non-malignant fibroblasts. The large difference in cytotoxicity depending on the chosen spacer proves once more the importance and



Scheme 1. Synthesis of BODIPY-FL (**7**): a) NaH, THF, 0 °C → 25 °C, 90 min, 86%; b) H_2 (1 at), Pd/C (10%), Ph_2S (0.5 mol%), $PhCF_3$, 25 °C, 4 h, 87%; c) $POCl_3$, $BF_3 \cdot Et_2O$, $PhCF_3$, DIPA, 25 °C, 12 h, 75%; d) H_2 (1 at), Pd/C (10%), MeOH, 25 °C, 2 h, 95%.

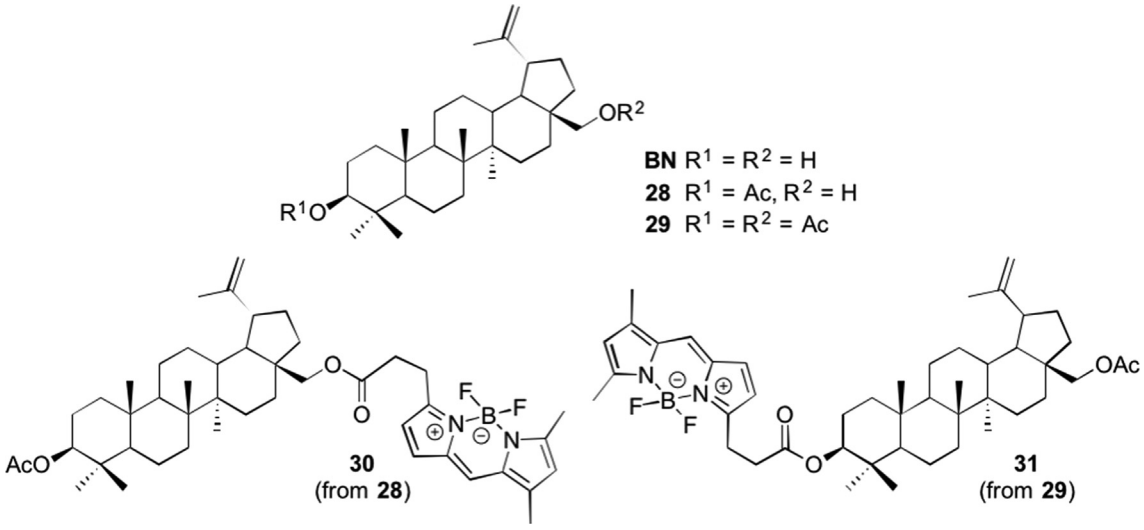


Scheme 2. Synthesis of compounds **8**–**27**: a) $(Ac)_2O$, cat. DMAP, pyridine, 25 °C, 12 h b) $(COCl)_2$, cat. DMF, 0 °C → 25 °C, 90 min, → piperazine, DCM, −21 °C, 30 min → 25 °C, 12 h c) $(COCl)_2$, cat. DMF, 0 °C → 25 °C, 90 min, → ethylenediamine, DCM, −21 °C, 30 min → 25 °C, 12 h d) BODIPY FL, $(COCl)_2$, cat. DMF, 0 °C → 25 °C, 90 min, → respective amine, DCM, −21 °C, 30 min → 25 °C, 12 h.

significance of a suitable spacer between the triterpene and the additionally introduced group. Triterpenes carrying a BODIPY moiety showed good cytotoxicity and partly high selectivity with ethylenediamine as a spacer, but the opposite is true for previously investigated derivatives holding a rhodamine B residue [40–44]. Thereby, only piperazinyl or homopiperazinyl-spacered derivatives were active, whereas ethylenediamine spacered products showed no cytotoxicity at all. Our findings are in excellent agreement with an earlier report by Krajcovicova et al. [38] showing also that length of the linker affects the activity of conjugates. In addition, it has been established [45] that the cytotoxicity of triterpenoid derived aminocarboxamides strongly depends on their aminoalkyl substituents.

3. Conclusion

Several triterpenoid acids (betulinic, oleanolic, ursolic, glycyrrhetinic) and triterpene betulin were used as starting material to synthesize BODIPY FL adducts, and these compounds were screened for their cytotoxic activity employing several human tumor cell lines. As a prerequisite, a modified synthesis for BODIPY FL was developed thus allowing a facile up-scaling and avoiding some



Scheme 3. Synthesis of betulin derived compounds **28**–**31**: d) BODIPY FL, $(COCl)_2$, cat. DMF, 0 °C → 25 °C, 90 min, → respective alcohol, DCM, −21 °C, 30 min → 25 °C, 12 h.

Table 1
Cytotoxicity of compounds **7**, **12**–**31** and their parent compounds (**BA**, **OA**, **UA**, **GA** and **BN**); EC_{50} values from SRB assays after 96 h of treatment in μM; the values are averaged from three independent experiments each performed in triplicate; confidence interval CI = 95%; n.d. not determined.

Compound	A375	HT-29	MCF7	A2780	FaDu	NIH 3T3
BA	n.d.	14.4 ± 2.3	10.2 ± 1.2	8.8 ± 0.9	n.d.	16.1 ± 1.4
OA	n.d.	>30	>30	>30	>30	>30
UA	n.d.	10.6 ± 0.7	12.7 ± 0.1	11.7 ± 0.6	n.d.	13.1 ± 1.1
GA	n.d.	>30	>30	>30	>30	18.7 ± 4.2
BN	>30	>30	>30	>30	>30	>30
7	>30	>30	>30	>30	>30	>30
12	n.d.	1.0 ± 0.1	1.4 ± 0.1	1.9 ± 0.1	1.3 ± 0.2	0.9 ± 0.1
13	n.d.	1.3 ± 0.1	1.7 ± 0.2	1.7 ± 0.1	1.6 ± 0.3	1.7 ± 0.1
14	n.d.	1.9 ± 0.1	2.0 ± 0.1	2.1 ± 0.1	1.9 ± 0.2	2.1 ± 0.1
15	n.d.	4.4 ± 0.6	8.4 ± 0.8	8.2 ± 0.5	8.7 ± 0.9	8.7 ± 0.7
16	n.d.	1.0 ± 0.3	1.3 ± 0.1	1.4 ± 0.2	1.2 ± 0.1	1.4 ± 0.1
17	n.d.	2.0 ± 0.2	1.7 ± 0.2	3.1 ± 0.1	4.3 ± 0.4	2.1 ± 0.1
18	n.d.	1.8 ± 0.1	2.0 ± 0.1	2.3 ± 0.1	3.7 ± 0.4	2.6 ± 0.3
19	n.d.	4.3 ± 0.4	3.2 ± 0.3	2.0 ± 0.2	5.7 ± 0.6	4.3 ± 0.3
20	>30	>30	>30	>30	>30	>30
21	29.8 ± 2.0	>30	>30	>30	>30	>30
22	21.6 ± 0.9	>30	24.4 ± 2.8	>30	>30	>30
23	8.3 ± 1.0	>30	7.9 ± 1.2	7.8 ± 1.5	8.6 ± 2.1	9.1 ± 2.3
24	>30	>30	11.3 ± 1.4	>30	>30	>30
25	4.3 ± 0.4	>30	2.3 ± 0.3	10.3 ± 1.3	7.0 ± 0.5	3.4 ± 0.6
26	4.0 ± 0.4	>30	2.2 ± 0.2	8.7 ± 1.6	5.7 ± 1.3	3.0 ± 0.5
27	23.9 ± 3.5	26.5 ± 2.9	7.2 ± 1.4	18.4 ± 5.2	>30	>30
28	>30	>30	20.2 ± 2.0	28.5 ± 1.9	>30	>30
29	>30	>30	>30	>30	>30	>30
30	>30	>30	28.9 ± 2.3	>30	>30	>30
31	n.d.	3.1 ± 0.8	4.3 ± 0.7	2.7 ± 0.2	4.7 ± 0.2	9.2 ± 0.9

disadvantages of previous syntheses. The cytotoxicity of the triterpene BODIPY conjugates strongly depended on the chosen spacer between the triterpenoid core and the BODIPY FL unit. These conjugates showed good cytotoxicity and partly high selectivity with ethylenediamine as a spacer; activity was diminished for piperazinyl analogs. Eventually, a 3-*O*-acetyl-betulinic acid derived BODIPY FL conjugate **24** holding an ethylendiamine spacer was cytotoxic for human breast adenocarcinoma cells MCF7 but not cytotoxic for all other cell lines.

4. Experimental part

4.1. General

Betulinic acid, ursolic acid, oleanolic acid were obtained from Betulinines (Stribrna Skalice, Czech Republic) and glycyrrhetinic acid was bought from Orgentis GmbH (Neugatersleben, Germany) and used as received. Equipment and lab equipment was used as previously described. Details can be found in the supplementary materials file.

4.2. Syntheses

4.2.1. General procedure (GP) for the synthesis of BODIPY amides or esters

To an ice-cold solution of BODIPY FL (**7**, 1 equiv.) in dry DCM (50 mL/mmol) under argon atmosphere, dry DMF (2 drops) and oxalyl chloride (1.5 eq) were slowly added. Stirring at room temperature was continued until the evolution of gases has ceased. The solvents were removed under reduced pressure, the residue

dissolved in dry THF (2 x 250 mL/mmol) and removed under reduced pressure (the residue consisted of **7** as carboxyl chloride). To a solution of the corresponding triterpene (**22**–**29**, 1.1 equiv.) in dry DCM (40 mL/mmol) at −21 °C under argon, dry triethylamine (1.1 equiv.) and a solution of the BODIPY FL acid chloride (vide supra) in dry DCM (25 mL/mmol) was slowly added within 30 min (for the synthesis of esters, catal. amounts of DMAP were added prior to the addition of the carboxylic acid chloride). The reaction mixture was allowed to warm to room temperature and stirred for 2 days. For work-up, the solvents were removed under reduced pressure, and the residue was subjected to column chromatography (silica gel, *n*-hexane/ethyl acetate gradients) to obtain the final products.

4.2.2. Benzyl(E)-3-(1H-pyrrol-2-yl)acrylate (3)

To an ice-cold solution of benzyl(dimethoxyphosphoryl)acetate (**2**, 10.0 g, 38.7 mmol) in dry THF (80 mL), NaH (1.86 g, 46.5 mmol, as 60% dispersion in paraffin oil) was slowly added [39]. After stirring for additional 5 min at room temperature, at 0 °C a solution of 1*H*-pyrrole-2-carbaldehyde (**1**, 1.70 g, 42.6 mmol) in dry THF (12 mL) was added, and stirring at room temperature was continued for another 90 min. For work-up, an aqueous solution of citric acid (50 mL, 5%) and water (100 mL) were added at 0 °C. The mixture was extracted with ethyl acetate (3 x 100 mL), the combined organic phases were washed with water (100 mL) and brine (50 mL). The organic layer was dried ($MgSO_4$), and the solvent was removed under reduced pressure. The residue was subjected to column chromatography (silica gel, *n*-hexane/ethyl acetate, 9:1) and **3** (7.6 g, 86%) was obtained as a white solid; m.p. 25–30 °C; $R_F = 0.25$ (*n*-hexane/ethyl acetate, 9:1); IR (KBr): $\tilde{\nu}$ = 1097*m*, 1123*s*, 2261*vs*, 1231*s*, 1254*m*, 1264*m*, 1296*m*, 1306*m*, 1327*m*, 1355*m*, 1371*m*, 1407*m*, 1444*m*, 1496*w*, 1546*w*, 1621*vs*, 1646*s*, 1686*m*, 2831*vw*, 2871*vw*, 2954*w*, 2987*vw*, 3033*w*, 3112*w*, 3330 m cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 11.49 (s, 1H, N*H*), 7.47 (d, J = 15.8 Hz, 1H, 6-H), 7.38–7.27 (m, 5H, Ph), 7.04–6.98 (m, 1H, 2-H), 6.60–6.54 (m, 1H, 3-H), 6.23 (d, J = 15.9 Hz, 1H, 7-H), 6.17–6.11 (m, 1H, 4-H), 5.15 (s, 2H, 9-H_2) ppm; ^{13}C NMR (101 MHz, DMSO-d_6): δ = 167.2 (C-8), 137.1 (C-10), 135.6 (C-6), 128.9 (C-12+C-12'), 128.5 (C-13), 128.5 (C-5), 128.4 (C-11, C-11'), 124.1 (C-2), 115.5 (C-3), 110.6 (C-4), 110.2 (C-7), 65.6 (C-9) ppm; MS (ESI, MeOH): m/z = 228.1 (89%, $[M+H]^+$), 250.1 (100%, $[M+Na]^+$); analysis calcd for $C_{14}H_{13}NO_2$ (227.26): C 73.99, H 5.77, N 6.16; found: C 73.72, H 5.96, N 5.98.

4.2.3. Benzyl-3-(1H-pyrrol-2-yl)propanoate (4)

Compound **3** (5.8 g, 25.5 mmol) was hydrogenated (1 at) with Pd/C (580 mg, 10%) in MeOH (50 mL) containing Ph_2S (21 µl, 150 µmol, 0.5 mol%) for 4 h. For work-up, the catalyst was filtered off (Celite), and the solvent was removed under reduced pressure. The residue was subjected to column chromatography (silica gel, *n*-hexane/ethyl acetate, 95:5) to yield **4** (5.1 g, 87%) as a colorless oil [39]; $R_F = 0.30$ (*n*-hexane/ethyl acetate, 9:1); IR (KBr): $\tilde{\nu}$ = 1030*m*, 1067*m*, 1082*w*, 1100*m*, 1118*m*, 1161*s*, 1214*m*, 1260*m*, 1311*m*, 1329*w*, 1365*w*, 1387*m*, 1417*m*, 1447*m*, 1456*w*, 1473*w*, 1496*w*, 1577*w*, 1682*vw*, 1721*s*, 2957*w*, 3036*vw*, 3385 m cm^{-1}; UV/vis (MeOH): λ_{max} (log ε) = 250 (3.47), 271 (2.99), 363 (2.95) nm; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.51 (s, 1H, N*H*), 7.60–7.02 (m, Ph), 6.63–6.49 (*m*, 1H, 2-H), 5.88–5.84 (m, 1H, 3-H), 5.75–5.69 (m, 1H, 4-H), 5.08 (s, 2H, 9-H_2), 2.85–2.75 (m, 2H, 6-H_2), 2.68–2.59 (m, 2H, 7-H_2) ppm; ^{13}C NMR (101 MHz, DMSO-d_6): δ = 172.6 (C-8), 136.7 (C-10), 130.4 (C-5), 128.8 (C12 + C12'), 128.4 (C-13), 128.3 (C11 + C11'), 116.7 (C-2), 107.6 (C-3), 104.9 (C-4), 65.8 (C-9), 34.3 (C-7), 23.1 (C-6) ppm; MS (ESI, MeOH): m/z = 230.1 (100%, $[M+H]^+$), 252.1 (83%, $[M+Na]^+$); analysis calcd for $C_{14}H_{15}NO_2$ (229.27): C 73.34, H 6.59, N 6.11; found: C 73.11, H 6.71, N 6.02.

4.2.4. (Benzyl 3-{2-[(3,5-dimethyl-1H-pyrrol-2-yl-κN) methylidene]-2H-pyrrol-5-yl- κN}propanoatato)borondifluoride (6)

To a mixture of **4** (3.685 g, 16.1 mmol) and 3,5-dimethyl-1*H*-pyrrole-2-carbaldehyde (**5**, 2.07 g, 16.8 mmol) in $PhCF_3$ (100 mL) at 0 °C, phosphoryl chloride (1.51 mL, 16.3 mmol) was slowly added. After an additional stirring at 25 °C, at 0 °C $BF_3 \cdot Et_2O$ (8.03 mL, 63 mmol) and DIPA (11.23 mL, 66 mmol) were slowly added, and stirring at room temperature was continued overnight. For work-up, at 0 °C, an aq. solution of $NaHCO_3$ (30 mL, 5%) and ethyl acetate (100 mL) were added. The filtrate was evaporated, and the residue was subjected to column chromatography (silica gel, ethyl acetate/*n*-hexane, 19:1) to yield **6** (4.6 g, 75%) [39] as a reddish solid; m.p. 118–121 °C (decomp.); $R_F = 0.23$ (*n*-hexane/ethyl acetate, 9:1); IR (KBr): $\tilde{\nu}$ = 1024*s*, 1050*vs*, 1083*s*, 1134*vs*, 1165*s*, 1196*m*, 1248*s*, 1316*w*, 1359*m*, 1389*m*, 1423*m*, 1460*m*, 1489*m*, 1530*m*, 1603*vs*, 1730*s*, 2894*vw*, 2926*vw*, 2953*vw*, 3036*vw*, 3062*vw*, 3106*vw* cm^{-1}; UV/vis (MeOH): λ_{max} (log ε) = 213 (3.31), 223 (3.46), 241 (3.62), 396 (3.61), 556 (4.48) nm; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.68 (s, 1H, 5-H), 7.38–7.27 (m, 5H, Ph), 7.05 (d, J = 4.0 Hz, 1H, 7-H), 6.34 (d, J = 4.0 Hz, 1H, 8-H), 6.30 (s, 1H, 2-H), 5.11 (s, 2H, 15-H_2), 3.12 (t, J = 7.7 Hz, 2H, 12-H_2), 2.78 (dd, J = 8.6, 6.7 Hz, 2H, 13-H_2), 2.45 (s, 3H, 10-H_3), 2.25 (s, 3H, 11-H_3) ppm; ^{13}C NMR (101 MHz, DMSO-d_6): δ = 172.2 (C-14), 160.2 (C-1), 156.7 (C-9), 145.0 (C-4), 136.6 (C-16), 135.1 (C-3), 133.4 (C-6), 129.2 (C-7), 128.9 (C-19), 128.5 (C-18+C-18'), 128.4 (C-17+C-17'), 126.0 (C-5), 120.9 (C-2), 117.0 (C-8), 66.1 (C-15), 32.7 (C-13), 23.9 (C-12), 15.0 (C-10), 11.5 (C-11) ppm; ^{19}F NMR (376 MHz, DMSO-d_6): δ = −143.3 (q, J = 34.4, 33.9, 33.1 Hz, B–F_2) ppm; MS (ESI, MeOH): m/z = 363.1 (57%, $[M-F]^+$), 405.2 (100%, $[M+Na]^+$); analysis calcd for $C_{21}H_{21}BF_2N_2O_2$ (382.21): C 69.99, H 5.54, N 7.33; found: C 69.67, H 5.78, N 7.12.

4.2.5. Boron, difluoro[methyl 5-methyl-2-[(5-methyl-2H-pyrrol-2-ylidene)methyl]-1H-pyrrole-3-acetato-N^1,N^2], BODIPY FL (7)

Hydrogenation (1 at) of **6** (1.0 g, 2.6 mmol) with Pd/C (200 mg, 10%) in MeOH (50 mL) for 2 h, followed by work-up as described above and column chromatography (silica gel, $CHCl_3$/MeOH, 19:1) gave **7** (0.73 g, 95%) as a red solid; m.p. 110–113 °C (decomp.); $R_F = 0.37$ ($CHCl_3$/MeOH 19:1); IR (KBr): $\tilde{\nu}$ = 1075*s*, 1105*m*, 1133*vs*, 1166*s*, 1195*m*, 1216*m*, 1244*s*, 1295*w*, 1317*w*, 1363*m*, 1382*w*, 1424*m*, 1437*m*, 1488*m*, 1528*m*, 1540*m*, 1595*vs*, 1701*s*, 2565*vw*, 2634*w*, 2715*w*, 2925*w*, 3107*vw* cm^{-1}; UV/vis (MeOH): λ_{max} (log ε) = 394 (3.48), 556 (4.35) nm; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 12.26 (br, 1H, COO*H*), 7.68 (s, 1H, 5-H), 7.07 (d, J = 4.0 Hz, 1H, 7-H), 6.36 (d, J = 4.0 Hz, 1H, 8-H), 6.29 (s, 1H, 2-H), 3.06 (t, J = 7.8 Hz, 2H, 12-H_2), 2.65–2.60 (m, 2H, 13-H_2), 2.45 (s, 3H, 11-H_3), 2.24 (s, 3H, 10-H_3) ppm; ^{13}C NMR (126 MHz, DMSO-d_6): δ = 173.9 (C-14), 159.9 (C-1), 157.4 (C-9), 144.8 (C-4), 135.0 (C-3), 133.5 (C-6), 129.3 (C-7), 125.9 (C-5), 120.8 (C-2), 117.0 (C-8), 32.8 (C-13), 24.0 (C-12), 15.0 (C10), 11.4 (C-11) ppm; ^{19}F NMR (470 MHz, DMSO-d_6): δ = −143.4 (*q*, J = 33.7, 32.6 Hz, B–F_2) ppm; MS (ESI, MeOH): m/z = 273.9 (30%, $[M-F]^+$), 314.9 (100%, $[M+Na]^+$); analysis calcd for $C_{14}H_{15}BF_2N_2O_2$ (292.09): C 57.57, H 5.18, N 9.59; found: C 57.30, H 5.37, N 9.41.

4.2.6. 3-O-acetyl-betulinic acid (8)

This compound was prepared from betulinic acid as previously reported; m.p. 281–283 °C (decomp.) (lit.: 280–285 °C) [44]; $R_F = 0.71$ (toluene/ethyl acetate/heptane/HCOOH, 80:26:10:5); $[\alpha]_D$ = +25.3° (*c* 0.4, $CHCl_3$) [lit.: $[\alpha]_D$ = 26.4° (*c* 0.54, $CHCl_3$) [46].

4.2.7. 3-O-acetyl-oleanolic acid (9)

This compound was prepared from oleanolic acid as previously reported; m.p. 269–272 °C (decomp.) (lit.: 266–269 °C) [44]; $R_F = 0.60$ (toluene/ethyl acetate/heptane/HCOOH, 80:26:10:5); $[\alpha]_D$ = 74.4° (*c* 0.9, $CHCl_3$) [lit.: $[\alpha]_D$ = 74.0° (*c* 1.0, $CHCl_3$)] [47].

4.2.8. 3-O-acetyl-ursolic acid (10)

This compound was prepared from ursolic acid as previously reported; m.p. 281–283 °C (decomp.) (lit.: 280–285 °C) [44]; $R_F = 0.70$ (toluene/ethyl acetate/heptane/HCOOH, 80:26:10:5); $[\alpha]_D = 74.1°$ (*c* 0.4, $CHCl_3$) [lit.: $[\alpha]_D = 72.3°$ (*c* 0.5, $CHCl_3$)] [48].

4.2.9. 3-O-acetyl-glycyrrhetinic acid (11)

This compound was prepared from glycyrrhetinic acid as previously reported; m.p. 317–319 °C (decomp.) (lit.: 316–318 °C [44]); $R_F = 0.51$ (*n*-hexane/ethyl acetate, 7:3; $[\alpha]_D = 165.1°$ (*c* 0.7, $CHCl_3$) [lit.: 163.3° (c 1, $CHCl_3$)] [49].

4.2.10. (3β) 3-O-acetyl-betulinic acid piperazinyl amide (12)

This compound was prepared from **8** as previously reported; m.p. 162–167 °C (decomp.) (lit.: 177–181 °C [41]; $R_F = 0.4$ ($CHCl_3$/MeOH 9:1); $[\alpha]_D = -1.8°$ (*c* 0.32, MeOH) [41].

4.2.11. (3β) 3-O-acetyl-oleanolic acid-piperazinyl amide (13)

This compound was prepared from **9** as previously reported; m.p. 173–175 °C (decomp.) (lit.: 170–176 °C [41]; $R_F = 0.47$ ($CHCl_3$/MeOH 9:1); $[\alpha]_D = = 26.6°$ (*c* 0.35, MeOH); MS (ESI, MeOH): *m/z* = 567.3 (100%, $[M+H]^+$).

4.2.12. (3β) 3-O-acetyl-ursolic acid-piperazinyl amide (14)

This compound was prepared from **10** as previously reported; m.p. 187–188 °C (decomp.) (lit.: 158–161 °C [41]); $R_F = 0.19$ ($CHCl_3$/MeOH 19:1); $[\alpha]_D = 24.5°$ (*c* 0.29, MeOH), 33.9° (*c* 0.33, CH_2Cl_2).

4.2.13. (3β) 3-O-acetyl-glycyrrhetinic acid-piperazinyl amide (15)

This compound was prepared from **11** as previously reported; m.p. 147 °C (decomp.) (lit.: 160 °C (decomp.) [41]; $R_F = 0.09$ ($CHCl_3$/MeOH 19:1); $[\alpha]_D = 120.6°$ (*c* 0.31, MeOH), 114.0° (*c* 0.335, CH_2Cl_2).

4.2.14. (3β) 3-O-acetyl-betulinic acid 2-aminoethylamide (16)

Following the procedure reported for the synthesis of **12**, from **8** (541 mg, 1 mmol) and ethylene diamine (280 mg, 3 mmol) **16** (446 mg, 83%) was obtained as a colorless solid; m.p. 142 °C (decomp.); $R_F = 0.16$ ($CHCl_3$/MeOH, 9:1); $[\alpha]_D = -7.6°$ (*c* 0.31, MeOH); IR (ATR) $\tilde{\nu}$ = 544*w*, 744*vw*, 821*w*, 883*w*, 979*w*, 1031*w*, 1086*vw*, 1169*w*, 1196*w*, 1247*m*, 1330*vs*, 1423*s*, 1516*w*, 1634*m*, 1733*m*, 2871*w*, 2944*m*, 3418*w* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 7.25–7.18$ (t, $J = 4.4$ Hz, 1H, CON*H*), 4.71 (d, $J = 2.1$ Hz, 1H, 29-H_a), 4.58 (s, 1H, 29-H_b), 4.46 (dd, $J = 10.2$, 6.0 Hz, 1H, 3-H), 3.73–3.56 (m, 1H, 33-H_a), 3.53–3.39 (m, 1H, 33-H_b), 3.10 (t, $J = 5.3$ Hz, 2H, 34-H_2), 3.06–3.01 (m, 1H, 19-H), 2.47–2.36 (m, 1H, 13-H), 2.16 (m, 1H, 16-H_a), 2.03 (s, 3H, 32-H_3), 1.86 (d, $J = 7.0$ Hz, 2H, 21-H_a+22-H_a), 1.67 (s, 3H, 30-H_3), 1.65–1.30 (m, 14H, 1-H_a+2-H_2+6-H_2+7-H_2+11-H_a+12-H_a+15-H_a+16-H_b+18-H+21-H_b+22-H_b), 1.29–0.96 (m, 5H, 1-H_b+9-H+11-H_b+12-H_b+15-H_b), 0.94 (s, 3H, 27-H_3), 0.90 (s, 3H, 26-H_3), 0.83 (s, 6H, 23-H_3+25-H_3), 0.82 (s, 3H, 24-H_3), 0.80–0.74 (m, 1H, 5-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 178.2 (C-28), 171.0 (C-31), 150.6 (C-20), 109.6 (C-29), 80.9 (C-3), 55.8 (C-17), 55.4 (C-5), 50.5 (C-9), 50.2 (C-18), 46.7 (C-19), 42.4 (C-14), 40.7 (C-8), 40.6 (C-33), 38.4 (C-4), 38.2 (C-34), 37.8 (C-10), 37.7 (C-13), 37.6 (C-13), 37.1 (C-22), 34.3 (C-7), 30.9 (C-21), 29.4 (C-15), 27.9 (C-24), 25.5 (C-12), 23.7 (C-2), 21.3 (C-32), 21.0 (C-11), 19.4 (C-30), 18.2 (C-6), 16.5 (C-23), 16.2 (C-25), 16.1 (C-26), 14.6 (C-27) ppm; MS (ESI, MeOH): *m/z* = 541.3 (100%, $[M+Na]^+$), 1081.3 (6%, $[2M + H]^+$); analysis calcd for $C_{34}H_{56}N_2O_3$ (540.82): C 75.51, H 10.44, N 5.18; found: C 75.29, H 10.71, N 5.00.

4.2.15. (3β) 3-O-acetyl-oleanolic acid 2-aminoethylamide (17)

This compound was prepared from **9** as previously reported; m.p. 190.5 °C (decomp.) (lit.: 198.7–201.3 °C [41]; $R_F = 0.12$ ($CHCl_3$/MeOH, 9:1); $[\alpha]_D = 54.3°$ (*c* 0.315, MeOH); MS (ESI, MeOH): *m/z* = 541.3 (100%, $[M+H]^+$); IR (ATR: $\tilde{\nu}$ = 558*w*, 611*w*, 653*w*, 668*w*, 701*vw*, 733*m*, 818*vw*, 827*w*, 900*w*, 923*w*, 950*w*, 970*w*, 987*m*, 1008*m*, 1028*m*, 1097*w*, 1148*w*, 1188*v*, 1214*w*, 1246*vs*, 1364*m*, 1370*m*, 1389*w*, 1434*w*, 1464*m*, 1525*m*, 1634*m*, 1732*s*, 2876*w*, 2940*m*, 3366*vw* cm^{-1}; ^1H NMR (500 MHz, CD_3OD) $\delta = 7.32$ (t, $J = 5.3$ Hz, 1H, CON*H*), 5.37 (t, $J = 3.7$ Hz, 1H, 12-H), 4.45 (dd, $J = 11.3$, 4.9 Hz, 1H, 3-H), 3.42–3.26 (m, 3H, 33-H_a+34-H_2), 3.20–3.12 (m, 1H, 33-H_b), 2.79–2.72 (m, 1H, 18-H_a), 2.11–2.00 (m, 1H, 16-H_a), 2.02 (s, 3H, 32-H_3), 2.00–1.84 (m, 2H, 11-H_2), 1.78 (t, $J = 13.5$ Hz, 1H, 19-H_a), 1.72–1.49 (m, 10H, 1-H_a+2-H_2+6-H_a+7-H_a+9-H+15-H_a+16-H_a+22-H_2), 1.48–1.35 (m, 2H, 6-H_b+21-H_a), 1.34–1.25 (m, 1H, 7-H_b), 1.24–1.11 (m, 2H, 19-H_b+21-H_b), 1.18 (s, 3H, 27-H_3), 1.11–1.01 (m, 2H, 1-H_b+15-H_b), 0.98 (s, 3H, 25-H_3), 0.94 (s, 3H, 29-H_3), 0.91 (s, 3H, 30-H_3), 0.88 (s, 3H, 23-H_3), 0.87 (s, 3H, 24-H_3), 0.86–0.84 (m, 1H, 5-H), 0.78 (s, 3H, 26-H_3) ppm; ^{13}C NMR (126 MHz, CD_3OD) δ = 180.9 (C-28), 172.9 (C-31), 145.2 (C-13), 124.1 (C-12), 82.5 (C-3), 56.7 (C-5), 48.9 (C-9), 47.7 (C-17), 47.6 (C-19), 42.9 (C-18), 42.5 (C-8), 40.8 (C-33), 40.7 (C-14), 39.3 (C-1), 38.7 (C-4), 38.6 (C-34), 38.1 (C-10), 35.1 (C-21), 34.3 (C-22), 33.7 (C-7), 33.5 (C-30), 31.6 (C-20), 28.6 (C-15), 28.5 (C-24), 26.5 (C-27), 24.5 (C-11), 24.5 (C-2), 24.1 (C-16), 24.0 (C-29), 21.1 (C-32), 19.3 (C-6), 17.8 (C-26), 17.1 (C-23), 15.9 (C-25) ppm; $]^+$); analysis calcd for $C_{34}H_{56}N_2O_3$ (540.82): C 75.51, H 10.44, N 5.18; found: C 75.29, H 10.73, N 4.98.

4.2.16. (3β) 3-O-acetyl-ursolic acid 2-aminoethylamide (18)

Following the procedure reported for the synthesis of **16**, from **10** (499 mg, 1 mmol) and ethylene diamine (280 mg, 3 mmol) **18** (530 mg, 98%) [50–52] was obtained as colorless solid; m.p. 198 °C (decomp.); $R_F = 0.16$ ($CHCl_3$/MeOH 9:1); $[\alpha]_D = 36.0°$ (*c* 0.35, MeOH), 29.6° (*c* 0.375, CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 1531*m*, 1627*m*, 1713*w*, 1733*m*, 2927*m*, 3394*w* cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta = \delta$ 7.55 (t, $J = 5.9$ Hz, 1H, CON*H*), 5.34 (t, $J = 3.6$ Hz, 1H, 12-H), 4.56 (br, 2H, N*H*$_2$), 4.46 (dd, $J = 11.1$, 5.2 Hz, 1H, 3-H), 3.46 (dt, $J = 14.1$, 6.4 Hz, 1H, 33-H_a), 3.34–3.24 (m, 1H, 33-H_b), 2.99 (t, $J = 6.4$ Hz, 2H, 34-H_2), 2.17–2.04 (m, 2H, 16-H_a+18-H), 2.02 (s, 3H, 32-H_3), 2.00–1.92 (m, 2H, 11-H_2), 1.83–1.27 (m, 15H, 1-H_a+2-H_2+6-H_2+7-H_2+9-H+15-H_a+16-H_b+19-H+21-H_2+22-H_2), 1.14 (s, 3H, 27-H_3), 1.13–1.01 (m, 2H, 1-H_b+15-H_b), 0.99 (s, 3H, 25-H_3), 0.97 (s, 3H, 30-H_3), 1.01–0.95 (m, 1H, 20-H), 0.98–0.83 (m, 10H, 5-H+23-H_3+24-H_3+29-H_3), 0.80 (s, 3H, 26-H_3) ppm; ^{13}C NMR (101 MHz, CD_3OD): δ = 180.4 (C-28), 171.5 (C-31), 138.4 (C-13), 125.8 (C-12), 81.0 (C-3), 55.2 (C-5), 52.6 (C-18), 47.6 (C-17), 47.4 (C-9), 41.8 (C-14), 39.5 (C-8), 39.4 (C-19), 39.3 (C-34), 38.8 (C-20), 38.0 (C-1), 37.3 (C-4), 37.3 (C-22), 37.2 (C-33), 36.6 (C-10), 32.5 (C-7), 30.4 (C-21), 27.5 (C-15), 27.2 (C-24), 23.8 (C-16), 23.1 (C-2), 22.9 (C-11), 22.7 (C-27), 20.1 (C-30), 19.7 (C-32), 17.9 (C-6), 16.4 (C-26), 16.3 (C-29), 15.8 (C-23), 14.6 (C-25) ppm; MS (ESI, MeOH): *m/z* = 541.4 (100%, $[M+Na]^+$), 1103.5 (3%, $[2M + Na]^+$); analysis calcd for $C_{34}H_{56}N_2O_3$ (540.82): C 75.51, H 10.44, N 5.18; found: C 75.36, H 10.69, N 5.03.

4.2.17. (3β) 3-O-acetyl-glycyrrhetinic acid 2-aminoethylamide (19)

Following the procedure given for the synthesis of **17**, from **11** (499 mg, 1 mmol) and ethylene diamine (280 mg, 3 mmol) **19** (528 mg, 95%) [49] was obtained as a colorless solid; m.p. 116 °C; $R_F = 0.16$ ($CHCl_3$/MeOH, 9:1); $[\alpha]_D = 82.0°$ (*c* 0.37, MeOH); IR (ATR): $\tilde{\nu}$ = 1531*m*, 1627*m*, 1713*w*, 1733*m*, 2927*m*, 3394*w* cm^{-1}; UV/vis (MeOH): λ_{max} (log ε) = 261 (3.76) nm; ^1H NMR (400 MHz, CD_3OD): $\delta = 7.96$ (t, $J = 5.7$, 5.3 Hz, 1H, CON*H*), 5.62 (s, 1H, 12-H), 4.58 (s, 2H, N*H*$_2$), 4.48 (dd, $J = 11.8$, 4.6 Hz, 1H, 3-H), 3.54–3.38 (m, 2H, 34-H_2), 3.08–3.00 (m, 2H, 33-H_2), 2.75 (dt, $J = 13.5$, 3.6 Hz, 1H, 1-H_a), 2.49 (s, 1H, 9-H), 2.23–2.10 (m, 2H, 16-H_a+18-H), 2.03 (s, 3H, 32-H_3), 1.98–1.82 (m, 3H, 7-H_a+15-H_a+21-H_a), 1.82–1.73 (m, 2H, 2-H_a+19-H_a), 1.76–1.70 (m, 1H, 21-H_b), 1.73–1.55 (m, 2H, 2-H_b+6-H_a), 1.59–1.43 (m, 3H, 6-H_b+7-H_b+19-H_b), 1.43 (s, 3H, 27-H_3), 1.42–1.30 (m, 2H, 22-H_2), 1.34–1.22 (m, 1H, 15-H_b), 1.16 (s, 3H, 25-H_3), 1.14 (s,

6H, 26-H_3+28-H_3), 1.12−0.98 (m, 2H, 1-H_b+16-H_b), 0.90 (s, 3H, 23-H_3), 0.89 (s, 3H, 29-H_3), 0.92−0.79 (m, 1H, 5-H), 0.82 (s, 3H, 24-H_3) ppm; ^{13}C NMR (101 MHz, CD_3OD): $\delta = 201.1$ (C-11), 179.0 (C-30), 171.5 (C-31), 171.4 (C-13), 127.6 (C-12), 80.8 (C-3), 61.6 (C-9), 54.7 (C-5), 48.3 (C-18), 45.3 (C-8), 43.4 (C-20), 43.3 (C-14), 41.1 (C-21), 39.4 (C-33), 38.3 (C-1), 38.3 (C-4) 37.3 (C-22), 37.2 (C-34), 36.8 (C-10), 32.3 (C-19), 31.5 (C-17), 30.5 (C-7), 28.0 (C-28), 27.7 (C-24), 27.1 (C-29), 26.1 (C-15), 26.0 (C-16), 23.1 (C-2), 22.3 (C-27), 19.7 (C-32), 17.9 (C-26), 17.0 (C-6), 15.7 (C-25), 15.5 (C-23) ppm; MS (ESI, MeOH): m/z = 555.4 (100%, $[M+Na]^+$), 1109.5 (3%, $[2M + H]^+$); analysis calcd for $C_{34}H_{54}N_2O_4$ (554.80): C 73.61, H 9.81, N 5.05; found: C 73.40, H 10.02, N 4.88.

4.2.18. N-(3β-Acetyloxy-lup-20(29)en-28-oyl)piperazinyl 3-{2-[(3,5-dimethyl-1H-pyrrol-2-yl-κN)methylidene]-2H-pyrrol-5-yl-κN}propionamide)boron difluoride (20)

Reaction of **7** (60 mg, 0.21 mmol) with **12** (131 mg, 0.23 mmol) according to the GP gave **20** (90 mg, 51%) as a red amorphous solid; $R_F = 0.30$ (*n*-hexane/ethyl acetate, 2:1); IR (ATR): $\tilde{\nu} = 1187s$, 1222*m*, 1244*s*, 1315*w*, 1372*m*, 1407*m*, 1437*m*, 1486*w*, 1528*w*, 1603*vs*, 1636*m*, 1731*m*, 2867*w*, 2941*w* cm^{-1}; UV/vis (MeOH): λ_{max} ($\log \varepsilon$) = 395 (3.97), 557 (4.85) nm; ^{1}H NMR (500 MHz, $CDCl_3$) $\delta = 7.08$ (s, 1H, 42-H), 6.87 (d, $J = 4.0$ Hz, 1H, 40-H), 6.30 (d, $J = 4.0$ Hz, 1H, 39-H), 6.12 (s, 1H, 45-H), 4.72 (d, $J = 2.4$ Hz, 1H, 29-H_a), 4.59−4.57 (m, 1H, 29-H_b), 4.50−4.43 (m, 1H, 3-H), 3.49 (m, 8H, 33-H_2+33'-H_2+34-H_2+34'-H_2), 3.28 (t, $J = 7.6$ Hz, 2H, 36-H_2), 2.96 (td, $J = 11.0$, 3.6 Hz, 1H, 19-H), 2.87−2.81 (m, 1H, 13-H), 2.79 (dd, $J = 8.7$, 6.8 Hz, 2H, 36-H_2), 2.56 (s, 3H, 47-H_3), 2.25 (s, 3H, 48-H_3), 2.09−2.05 (m, 1H, 16-H_a), 2.03 (s, 3H, 32-H_3), 1.95−1.87 (m, 1H, 22-H_a), 1.87−1.78 (m, 1H, 21-H_a), 1.68 (s, 3H, 30-H_3), 1.74−1.19 (m, 15H, 1-H_a+2-H_2+6-H_2+7-H_2+9-H+11-H_2+12-H_a+15-H_a+16-H_b+18-H+22-H_b), 1.18−1.12 (m, 1H), 0.95 (s, 3H, 27-H_3), 1.01−0.86 (m, 2H, 1-H_b+12-H_b), 0.92 (s, 3H, 26-H_3), 0.85−0.82 (m, 9H, 23-H_3+24-H_3+25-H_3), 0.80−0.76 (m, 1H, 5-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) δ = 174.0 (C-28), 171.2 (C-31), 171.0 (C-35), 160.5 (C-46), 157.7 (C-38), 151.3 (C-20), 144.1 (C-41), 135.3 (C-44), 133.5 (C-41), 128.2 (C-40), 123.9 (C-42), 120.6 (C-45), 117.9 (C-39), 109.5 (C-29), 81.1 (C-3), 55.7 (C-5), 54.8 (C-17), 52.8 (C-18), 50.9 (C-9), 45.8 (C-19), 42.1 (C-14), 41.9 (C-33+C-33'+C-34+C-34'), 40.9 (C-8), 38.6 (C-1), 38.0 (C-4), 37.3 (C-10), 37.1 (C-13), 36.1 (C-22), 34.5 (C-7), 33.2 (C-36), 32.7 (C-16), 31.5 (C-21), 30.0 (C-15), 28.1 (C-24), 25.8 (C-12), 25.0 (C-37), 23.9 (C-2), 21.5 (C-32), 21.3 (C-11), 19.8 (C-30), 18.4 (C-6), 16.6 (C-23), 16.4 (C-25), 16.3 (C-26), 15.1 (C-47), 14.8 (C-27), 11.5 (C-48) ppm; ^{19}F NMR (470 MHz, $CDCl_3$) $\delta = -144.6$ (q, $J = 34.3$, 34.0, 32.7 Hz, B−F_2) ppm; MS (ESI, MeOH): m/z = 821.7 (92%, $[M - F]^+$), 841.1 (100%, $[M+H]^+$) 863.5 (100%, $[M+Na]^+$); analysis calcd for $C_{50}H_{71}BF_2N_4O_4$ (840.93): C 71.41, H 8.51, N 6.66; found: C 71.20, H 8.74, N 6.37.

4.2.19. N-(3β-Acetyloxy-olean-12-en-28-oyl)piperazinyl 3-{2-[(3,5-dimethyl-1H-pyrrol-2-yl-κN)methylidene]-2H-pyrrol-5-yl-κN}propionamide)boron difluoride (21)

Reaction of **7** (60 mg, 0.21 mmol) and **13** (131 mg, 0.23 mmol) according to the GP gave **21** (93 mg, 48%) as a red amorphous solid; $R_F = 0.31$ (*n*-hexane/ethyl acetate, 2:1); IR (ATR): $\tilde{\nu} = 1172s$,1244*s*, 1293*w*, 1315*w*, 1364*m*, 1370*m*, 1406*m*, 1435*w*, 1455*m*, 1486*m*, 1529*w*, 1602*vs*, 1731*m*, 2861*w*, 2944*w* cm^{-1}; UV/vis (MeOH): λ_{max} ($\log \varepsilon$) = 400 (3.64), 559 (4.43) nm; ^{1}H NMR (500 MHz, $CDCl_3$) $\delta = 7.07$ (s, 1H, 42-H), 6.87 (d, $J = 4.0$ Hz, 1H, 40-H), 6.30 (d, $J = 4.0$ Hz, 1H, 39-H), 6.12 (s, 1H, 45-H), 5.25 (t, $J = 3.7$ Hz, 1H, 12-H), 4.52−4.45 (m, 1H, 3-H), 3.70−3.32 (m, 8H, 33-H_2+33'-H_2+34-H_2+34'-H_2), 3.28 (t, $J = 7.6$ Hz, 2H, 37-H_2), 3.06 (d, $J = 13.4$ Hz, 1H, 18-H), 2.82−2.75 (m, 2H, 36-H_2), 2.55 (s, 3H, 47-H_3), 2.25 (s, 3H, 48-H_3), 2.17−2.07 (m, 1H, 16-H_a), 2.04 (s, 3H, 32-H_3), 1.95−1.80 (m, 2H, 11-H_2), 1.74−1.53 (m, 8H, 19-H_a+1-H_a+2-H_2+7-H_2+15-H_a+16-H_b), 1.55−1.49 (m, 2H, 6-H_a+9-H), 1.49−1.29 (m, 3H, 6-H_b+21-H_a+22-H_a), 1.29−1.22 (m, 1H, 22-H_b), 1.21−1.14 (m, 2H, 19-H_b+21-H_b), 1.13 (s, 3H, 27-H_3), 1.09−0.99 (m, 2H, 1-H_b+15-H_b), 0.92 (s, 6H, 25-H_3+29-H_3), 0.89 (s, 3H, 30-H_3), 0.86 (s, 3H, 23-H_3), 0.85 (s, 3H, 24-H_3), 0.84−0.80 (m, 1H, H-5), 0.71 (s, 3H, 26-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) δ = 175.3 (C-28), 171.0 (C-31), 170.8 (C-35), 160.3 (C-46), 157.5 (C-38), 144.5 (C-13), 143.9 (C-43), 135.1 (C-44), 133.3 (C-41), 128.0 (C-40), 123.7 (C-42), 121.6 (C-12), 120.5 (C-45), 117.8 (C-39), 80.9 (C-3), 55.4 (C-5), 47.7 (C-9), 47.5 (C-17), 46.3 (C-19), 45.5 (C-34+C-34'), 41.8 (C-14), 41.7 (C-33+C-33'), 39.1 (C-8), 38.1 (C-1), 37.7 (C-4), 37.0 (C-10), 33.9 (C-21), 33.0 (C-30), 33.0 (C-36), 32.8 (C-22), 30.4 (C-20), 30.0 (C-7), 28.0 (C-24), 27.9 (C-15), 25.9 (C-27), 24.8 (C-37), 24.0 (C-29), 23.5 (C-2), 23.4 (C-11), 22.8 (C-16), 21.3 (C-32), 18.2 (C-6), 16.9 (C-26), 16.7 (C-23), 15.4 (C-25), 14.9 (C-47), 11.3 (C-48) ppm; ^{19}F NMR (470 MHz, $CDCl_3$) $\delta = -144.4$ to -144.8 (m, B−F_2) ppm; MS (ESI, MeOH): m/z = 821.7 (13%, $[M - F]^+$), 841.5 (100%, $[M+H]^+$) 863.5 (53%, $[M+Na]^+$); analysis calcd for $C_{50}H_{71}BF_2N_4O_4$ (840.93): C 71.41, H 8.51, N 6.66; found: C 71.33, H 9.02, N 6.51.

4.2.20. N-(3β-Acetyloxy-urs-12-en-28-oyl)piperazinyl 3-{2-[(3,5-dimethyl-1H-pyrrol-2-yl-κN)methylidene]-2H-pyrrol-5-yl- κN} propionamide)boron difluoride (22)

Reaction of **7** (60 mg, 0.21 mmol) and **14** (142 mg, 0.23 mmol) according to the GP gave **22** (120 mg, 68%) as a red amorphous solid; $R_F = 0.30$ (*n*-hexane/ethyl acetate, 2:1); IR (ATR): $\tilde{\nu} = 1372m$, 1407*m*, 1437*m*, 1486*m*, 1528*w*, 1602*vs*, 1635*m*, 1732*m*, 2866*w*, 2961*w* cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) $\delta = 7.07$ (s, 1H, 42-H), 6.87 (d, $J = 4.0$ Hz, 1H, 40-H), 6.30 (d, $J = 4.0$ Hz, 1H, 39-H), 6.12 (s, 1H, 45-H), 5.21 (s, 1H, 12-H), 4.49 (dd, $J = 9.6$, 6.4 Hz, 1H, 3-H), 3.61−3.57 (m, 4H, 33-H_2+33'-H_2), 3.53−3.37 (m, 4H, 34-H_2+34'-H_2), 3.28 (t, $J = 7.6$ Hz, 2H, 37-H_2), 2.78 (t, $J = 7.6$ Hz, 2H, 36-H_2), 2.56 (s, 3H, 47-H_3), 2.48−2.36 (m, 1H, 18-H), 2.25 (s, 3H, 48-H_3), 2.21−2.09 (m, 1H, 16-H_a), 2.04 (s, 3H, 32-H_3), 1.95−1.42 (m, 14H, 1-H_a+2-H_2+6-H_a+7-H_2+9-H+11-H_2+15-H_a+16-H_b+21-H_a+22-H_2), 1.41−1.32 (m, 2H, 6-H_b+19-H), 1.32−1.28 (m, 1H, 21-H_b), 1.07 (s, 3H, 27-H_3), 1.13−0.97 (m, 3H, 1-H_b+15-H_b+20-H), 0.95 (s, 3H, 30-H_3), 0.93 (s, 3H, 25-H_3), 0.89−0.86 (m, 6H, 24-H_3+29-H_3), 0.85 (s, 3H, 23-H_3), 0.85−0.80 (m, 1H, 5-H), 0.72 (s, 3H, 26-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) δ = 171.1 (C-28), 171.0 (C-31), 170.8 (C-35), 160.4 (C-46), 157.6 (C-38), 143.9 (C-43), 138.5 (C-13), 135.1 (C-44), 133.3 (C-41), 128.1 (C-40), 125.2 (C-12), 123.7 (C-42), 120.5 (C-45), 117.8 (C-39), 80.9 (C-3), 55.3 (C-5), 50.3 (C-18), 48.7 (C-17), 47.5 (C-9), 45.4 (C-34+C-34'), 42.6 (C-14), 41.6 (C-33+C-33'), 39.5 (C-19), 39.4 (C-8), 38.7 (C-20), 38.2 (C-1), 37.7 (C-4), 36.9 (C-10), 34.3 (C-22), 33.0 (C-7+C-36), 30.5 (C-21), 28.2 (C-15), 28.1 (C-24), 24.8 (C-37) 23.8 (C-27), 23.5 (C-16), 23.3 (C-2+C-11), 21.2 (C-30), 21.0 (C-32), 18.2 (C-6), 17.4 (C-29), 16.9 (C-26), 16.7 (C-23), 15.5 (C-25), 14.9 (C-47), 11.3 (C−C-48); ^{19}F NMR (470 MHz, $CDCl_3$) $\delta = -144.4$ to -144.8 (m, B−F_2) ppm; MS (ESI, MeOH): m/z = 821.7 (14%, $[M - F]^+$), 841.4 (100%, $[M+H]^+$), 861.0 (21%, $[2M + Ca]^{2+}$), 863.5 (44%, $[M+Na]^+$); analysis calcd for $C_{50}H_{71}BF_2N_4O_4$ (840.93): C 71.41, H 8.51, N 6.66; found: C 71.29, H 8.72, N 6.41.

4.2.21. N-(3β-Acetyloxy-11-oxo-olean-12-en-28-oyl)piperazinyl 3-{2-[(3,5-dimethyl-1H-pyrrol-2-yl-κN)methylidene]-2H-pyrrol-5-yl-κN}propionamide)boron difluoride (23)

Reaction of **7** (60 mg, 0.21 mmol) and **15** (134 mg, 0.23 mmol) according to the GP gave **33** (117 mg, 65%) as a red amorphous solid; $R_F = 0.33$ (*n*-hexane/ethyl acetate, 2:1); [lit.: $[\alpha]_D = 18.8°$ (*c* 0.31, $CHCl_3$); IR (ATR): $\tilde{\nu} = 1438m$, 1486*w*, 1529*w*, 1606*vs*, 1635*m*, 1728*w*, 2872*w*, 2950*w* cm^{-1}; UV/vis ($CHCl_3$): λ_{max} ($\log \varepsilon$) = 396 (3.55), 559 (4.33) nm; ^{1}H NMR (500 MHz, $CDCl_3$) $\delta = 7.09$ (s, 1H, 42-H), 6.90 (d, $J = 4.0$ Hz, 1H, 40-H), 6.30 (d, $J = 4.0$ Hz, 1H, 39-H), 6.12 (s, 1H, 45-H), 5.67 (s, 1H, 12-H), 4.52 (dd, $J = 11.8$, 4.7 Hz, 1H, 3-H), 3.67−3.39 (m, 8H, 33-H_2+33'-H_2+34-H_2+34'-H_2), 3.28 (t, $J = 7.5$ Hz, 2H, 37-H_2),

2.83−2.76 (m, 3H, 1-H_a+36-H_2), 2.56 (s, 3H, 47-H_3), 2.35 (s, 1H, 9-H), 2.30 (d, J = 12.8 Hz, 1H, 18-H), 2.25 (s, 3H, 48-H_3), 2.04 (s, 3H, 32-H), 2.10−2.00 (m, 2H, 16-H_a+19-H_a), 2.00−1.93 (m, 1H, 21-H_a), 1.83 (td, J = 13.7, 4.7 Hz, 1H, 15-H_a), 1.74−1.67 (m, 1H, 2-H_a), 1.69−1.55 (m, 4H, 2-H_b+6-H_a+7-H_a+21-H_b), 1.50−1.37 (m, 5H, 6-H_b+7-H_b+19-H_b+22-H_2), 1.35 (s, 3H, 27-H_3), 1.20 (s, 3H, 29-H_3), 1.23−1.18 (m, 1H, 15-H_b), 1.16 (s, 3H, 25-H_3), 1.11 (s, 3H, 26-H_3), 1.09−0.98 (m, 2H, 1-H_b+16-H_b), 0.88 (s, 6H, 23-H_3+24-H_3), 0.80 (s, 3H, 28-H_3), 0.80−0.77 (m, 1H, H-5) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) δ = 199.8 (C-11), 174.2 (C-30), 171.0 (C-31), 170.7 (C-35), 169.3 (C-13), 160.3 (C-46), 157.4 (C-38), 143.9 (C-43), 135.1 (C-44), 133.4 (C-41), 128.6 (C-12), 128.1 (C-40), 123.8 (C-42), 120.4 (C-45), 117.8 (C-39), 80.6 (C-3), 61.7 (C-9), 55.0 (C-5), 48.0 (C-18), 45.6 (C-33+C-33‘), 45.3 (C-8), 43.9 (C-21+C-20), 43.3 (C-14), 41.7 (C-34+C-34‘), 38.8 (C-1), 38.0 (C-4), 37.7 (C-22), 37.0 (C-10), 33.0 (C-36), 32.9 (C-19), 32.8 (C-7), 31.8 (C-17), 28.4 (C-28), 28.0 (C-24), 27.0 (C-29), 26.7 (C-16), 26.4 (C-15), 24.8 (C-37), 23.6 (C-2), 23.1 (C-27), 21.3 (C-32), 18.7 (C-26), 17.4 (C-6), 16.7 (C-23), 16.4 (C-25), 14.9 (C-47), 11.3 (C-48) ppm; ^{19}F NMR (470 MHz, $CDCl_3$) δ = −144.4 to −144.8 (m, B−F_2) ppm; MS (ESI, MeOH): m/z = 835.7 (38%, $[M-F]^+$), 855.2 (32%, $[M+H]^+$), 875 (14%, $[2M+Ca]^{2+}$), 877.6 (100%, $[M+Na]^+$); analysis calcd for $C_{50}H_{69}BF_2N_4O_5$ (854.91): C 70.24, H 8.14, N 6.55; found: C 70.03, H 8.37, N 6.39.

4.2.22. N-(3β-Acetyloxy-lup-20(29)en-28-oyl)acetamidoethyl 3-{2-[(3,5-dimethyl-1H-pyrrol-2-yl-κN)methylidene]-2H-pyrrol-5-yl-κN}propionamide)boron difluoride (24)

Reaction of **7** (60 mg, 0.21 mmol) with **16** (124 mg, 0.23 mmol) according to the GP gave **24** (106 mg, 62%) as a red amorphous solid; R_F = 0.08 (*n*-hexane/ethyl acetate, 2:1); IR (ATR): $\tilde{\nu}$ = 1133*vs*, 1174*m*, 1193*m*, 1247*s*, 1317*m*, 1372*m*, 1393*w*, 1440*m*, 1445*m*, 1464*m*, 1487*m*, 1528*m*, 1557*w*, 1574*w*, *1604vs*, 1639*m*, 1729*w*, 2870*w*, 2943 m cm^{-1}; UV/vis (CH_2Cl_2): λ_{max} (log ε) = 398 (3.68), 559 (4.49) nm; ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.08 (s, 1H, 42-H), 6.87 (d, J = 4.0 Hz, 1H, 40-H), 6.32 (t, J = 4.8 Hz, 1H, 35-N*H*), 6.27 (d, J = 4.0 Hz, 1H, 39-H), 6.24 (t, J = 5.8 Hz, 1H, 28-N*H*), 6.12 (s, 1H, 45-H), 4.71 (d, J = 2.4 Hz, 1H, 29-H_a), 4.59−4.55 (m, 1H, 29-H_b), 4.45 (dd, J = 10.7, 5.6 Hz, 1H, 3-H), 3.36−3.21 (m, 6H, 33-H_2+34-H_2+37-H_2), 3.08 (td, J = 11.0, 4.0 Hz, 1H, 19-H), 2.65 (t, J = 7.5 Hz, 2H, 36-H_2), 2.56 (s, 3H, 47-H_3), 2.42 (ddd, J = 12.8, 11.3, 3.6 Hz, 1H, 13-H), 2.25 (s, 3H, 48-H_3), 2.03 (s, 3H, 32-H_3), 1.95−1.82 (m, 2H, 16-H_a, 21-H_a), 1.71−1.66 (m, 1H, 22-H_a), 1.66 (s, 3H, 30-H_3), 1.64−1.18 (m, 16H, 1-H_a+2-H_2+6-H_2+7-H_2+9-H+11-H_2+12-H_a+15-H_a+16-H_b+18-H+21-H_b+22-H_b), 1.14−1.05 (m, 1H, 15-H_b), 1.04−0.82 (m, 2H, 1-H_b+12-H_b), 0.92 (s, 3H, 27-H_3), 0.89 (s, 3H, 26-H_3), 0.82 (s, 3H, 23-H_3), 0.81−0.79 (m, 6H, 24-H_3+25-H_3), 0.77−0.73 (m, 1H, 5-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) δ = 177.2 (C-28), 172.9 (C-35), 171.0 (C-31), 160.6 (C-46), 156.9 (C-38), 150.9 (C-20), 144.1 (C-43), 135.2 (C-44), 133.3 (C-41), 128.1 (C-40), 123.8 (C-41), 120.6 (C-40), 117.3 (C-39), 109.3 (C-29), 81.0 (C-3), 55.6 (C-17), 55.5 (C-5), 50.5 (C-9), 50.1 (C-18), 46.7 (C-19), 42.4 (C-14), 40.7 (C-8), 40.2 (C-33), 40.0 (C-34), 38.4 (C-1), 38.3 (C-22), 37.8 (C-4), 37.6 (C-13), 37.1 (C-10), 35.7 (C-36), 34.3 (C-7), 33.5 (C-16), 30.9 (C-21), 29.4 (C-15), 27.9 (C-23), 25.6 (C-12), 24.8 (C-37), 23.7 (C-2), 21.3 (C-32), 20.9 (C-11), 19.4 (C-30), 18.1 (C-6), 16.4 (C-24), 16.1 (C-25), 16.1 (C-26), 14.9 (C-47), 14.6 (C-27), 11.3 (C-48) ppm; ^{19}F NMR (470 MHz, $CDCl_3$) δ = −144.0 to −144.8 (*m*, B−F_2) ppm; MS (ESI, MeOH): m/z = 795.6 (52%, $[M-F]^+$), 815.2 (87%, $[M+H]^+$) 837.5 (100%, $[M+Na]^+$); analysis calcd for $C_{48}H_{69}BF_2N_4O_4$ (814.89): C 70.74, H 8.53, N 6.88; found: C 70.51, H 8.76, N 6.62.

4.2.23. N-(3β-Acetyloxy-olean-12-en-28-oyl)acetamidoethyl 3-{2-[(3,5-dimethyl-1H-pyrrol-2-yl-κN)methylidene]-2H-pyrrol-5-yl-κN}propionamide)boron difluoride (25)

A solution containing **7** (60 mg, 0.21 mmol), DCC (48 mg, 0.23 mmol) and NHS (27 mg, 0.23 mmol) in dry DMF (5 mL) was stirred at room temperature overnight. The filtered solution was slowly added to a solution of **17** (124 mg, 0.23 mmol) in dry DMF (5 mL), and stirring at room temperature was continued for another two days. Usual work-up followed by chromatography (silica gel, ethyl acetate, *n*-hexane; gradient) gave **25** (112 mg, 64%) as an amorphous red solid; R_F = 0.09 (*n*-hexane/ethyl acetate, 2:1); IR (ATR): $\tilde{\nu}$ = 1086*s*, 1133*s*, 1174*s*, 1192*m*, 1211*m*, 1245*s*, 1311*w*, 1347*w*, 1365*m*, 1370*m*, 1388*w*, 1436*m*, 1448*m*, 1463*m*, *1488m*, 1528*m*, 1575*m*, 1604*vs*, 1627*m*, 1732*m*, 2852*w*, 2928*m*, 3325*w* cm^{-1}; UV/vis (CH_2Cl_2): λ_{max} (log ε) = 559 (4.3) nm; ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.07 (s, 1H, 42-H), 6.87 (d, J = 4.0 Hz, 1H, 40-H), 6.42−6.34 (m, 1H, 28-N*H*), 6.36−6.31 (m, 1H, 35-N*H*), 6.27 (d, J = 4.0 Hz, 1H, 39-H), 6.11 (s, 1H, 45-H), 5.38 (t, J = 3.6 Hz, 1H, 12-H), 4.48 (dd, J = 9.5, 6.4 Hz, 1H, 3-H), 3.52−3.10 (m, 4H, 33-H_2+34-H_2), 3.27 (t, J = 7.8, 7.2 Hz, 2H, 37-H_2), 2.62 (t, J = 7.6 Hz, 2H, 36-H_2), 2.55 (s, 3H, 47-H_3), 2.58−2.53 (m, 1H, 18-H), 2.25 (s, 3H, 48-H_3), 2.04 (s, 3H, 32-H_3), 1.98−1.86 (m, 3H, 11-H_a+16-H_a+22-H_a), 1.77−1.65 (m, 2H, 2-H_a+19-H_a), 1.65−1.55 (m, 6H, 1-H_a+7-H_2+9-H+11-H_b+16-H_b), 1.55−1.39 (m, 2H, 6-H_a+15-H_a), 1.42−1.33 (m, 2H, 2-H_b+6-H_b), 1.35−1.23 (m, 2H, 21-H_2), 1.13 (s, 3H, 27-H_3), 1.20−0.99 (m, 4H, 1-H_b+15-H_b+19-H_b+22-H_b), 0.91 (s, 3H, 25-H_3), 0.90 (s, 3H, 29-H_3), 0.89 (s, 3H, 30-H_3), 0.85 (s, 3H, 24-H_3), 0.84 (s, 3H, 23-H_3), 0.83−0.79 (m, 1H, 5-H), 0.73 (s, 3H, 26-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) δ = 179.3 (C-28), 172.6 (C-31), 171.0 (C-35), 160.3 (C-46), 157.2 (C-38), 144.4 (C-13), 143.9 (C-43), 135.1 (C-44), 133.3 (C-41), 128.1 (C-40), 123.8 (C-42), 122.8 (C-12), 120.4 (C-45), 117.2 (C-39), 80.9 (C-3), 55.2 (C-5), 47.5 (C-9), 46.6 (C-19), 46.3 (C-17), 41.9 (C-14), 41.8 (C-18), 40.0 (C-33), 39.9 (C-34), 39.3 (C-8), 38.1 (C-1), 37.7 (C-4), 36.9 (C-10), 35.8 (C-36), 34.1 (C-21), 33.9 (C-22), 33.0 (C-30), 32.7 (C-7), 30.7 (C-20), 28.0 (C-24), 27.3 (C-15), 25.7 (C-27), 24.9 (C-2), 24.8 (C-37), 23.6 (C-11), 23.5 (C-29), 23.5 (C-16), 21.3 (C-32), 18.2 (C-6), 16.8 (C-26), 16.6 (C-23), 15.4 (C-25), 14.9 (C-47), 11.3 (C-48) ppm; ^{19}F NMR (470 MHz, $CDCl_3$) δ = −144.4 to −144.8 (m, B−F_2) ppm; MS (ESI, MeOH): m/z = 815.3 (100%, $[M+H]^+$) 837.6 (80%, $[M+Na]^+$); analysis calcd for $C_{48}H_{69}BF_2N_4O_4$ (814.89): C 70.74, H 8.53, N 6.88; found: C 70.49, H 8.77, N 6.65.

4.2.24. N-(3β-Acetyloxy-urs-12-en-28-oyl)acetamidoethyl 3-{2-[(3,5-dimethyl-1H-pyrrol-2-yl-κN)methylidene]-2H-pyrrol-5-yl-κN}propionamide)boron difluoride (26)

Reaction of **7** (60 mg, 0.21 mmol) and **18** (124 mg, 0.23 mmol) according to the GP gave **26** (80 mg, 47%) as an amorphous red solid; R_F = 0.19 (*n*-hexane/ethyl acetate, 1:1); [lit.: $[\alpha]_D$ = 15.4° (*c* 0.305, CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 1603*vs*, 1645*m*, 1731*w*, 2872*w*, 2926*w* cm^{-1}; UV/vis (CH_2Cl_2): λ_{max} (log ε) = 559 (4.76) nm; ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.07 (s, 1H, 42-H), 6.87 (d, J = 4.0 Hz, 1H, 40-H), 6.35−6.29 (m, 2H, 28-N*H*+36-N*H*), 6.27 (d, J = 4.0 Hz, 1H, 39-H), 6.11 (s, 1H, 45-H), 5.31 (t, J = 3.6 Hz, 1H, 12-H), 4.52−4.45 (m, 1H, 3-H), 3.26 (t, J = 7.4 Hz, 2H, 37-H_2), 3.42−3.08 (m, 4H, 33-H_2+34-H_2), 2.62 (t, J = 7.6 Hz, 2H, 36-H_2), 2.55 (s, 3H, 47-H_3), 2.25 (s, 3H, 48-H_3), 2.04 (s, 3H, 32-H_3), 2.02−1.86 (m, 4H, 11-H_2+16-H_a+18-H), 1.82−1.74 (m, 1H, 22-H_a), 1.70−1.61 (m, 5H, 1-H_a+2-H_2+15-H_a+16-H_b), 1.57−1.47 (m, 1H, 9-H), 1.50−1.44 (m, 2H, 7-H_a+21-H_a), 1.46−1.40 (m, 1H, 6-H_b), 1.40−1.35 (m, 2H, 19-H+22-H_b), 1.38−1.22 (m, 3H, 6-H_b+7-H_b+21-H_b), 1.07 (s, 3H, 27-H_3), 1.09−0.99 (m, 2H, 1-H_b+15-H_b), 0.95−0.91 (m, 7H, 20-H+25-H_3+30-H_3), 0.87−0.85 (m, 6H, 24-H_3+29-H_3), 0.84 (s, 3H, 23-H_3), 0.83−0.78 (m, 1H, 5-H), 0.74 (s, 3H, 26-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) δ = 179.2 (C-28), 172.5 (C-35), 171.0 (C-21), 160.3 (C-46), 157.3 (C-38), 143.9 (C-43), 139.3 (C-13), 135.1 (C-44), 133.3 (C-41), 128.1 (C-40), 125.7 (C-12), 123.7 ((C-42), 120.4 (C-45), 117.2 (C-39), 80.9 (C-3), 55.2 (C-5), 53.4 (C-18), 47.7 (C-17), 47.4 (C-9), 42.3 (C-14), 40.0 (C-33), 39.9 (C-34), 39.7 (C-19), 39.5 (C-8), 39.9 (C-20), 38.3 (C-1), 37.7 (C-4), 37.3 (C-22), 36.8 (C-10), 35.8 (C-36), 32.6

(C-7), 30.9 (C-21), 28.0 (C-24), 27.8 (C-15), 24.8 (C-16), 24.7 (C-37), 23.5 (C-2), 23.3 (C-11), 23.3 (C-27), 21.3 (C-32), 21.2 (C-30), 18.1 (C-6), 17.2 (C-29), 16.7 (C-26), 16.7 (C-23), 15.5 (C-25), 14.9 (C-47), 11.3 (C-48) ppm; ^{19}F NMR (470 MHz, $CDCl_3$) $\delta = -144.4$ to -144.8 (m, B–F_2) ppm; MS (ESI, MeOH): $m/z = 795.3$ (16%, $[M - F]^+$), 815.3 (56%, $[M+H]^+$), 837.6 (100%, $[M+Na]^+$); analysis calcd for $C_{48}H_{69}BF_2N_4O_4$ (814.89): C 70.74, H 8.53, N 6.88; found: C 70.50, H 8.79, N 6.67.

4.2.25. N-(3β-Acetyloxy-11-oxo-olean-12-en-28-oyl) acetamidoethyl 3-{2-[(3,5-dimethyl-1H-pyrrol-2-yl-κN) methylidene]-2H-pyrrol-5-yl- κN}propionamide)boron difluoride (27)

Reaction of **7** (60 mg, 0.21 mmol) and **19** (127 mg, 0.23 mmol) according to the GP gave **27** (105 mg, 60%) as an amorphous red solid; $R_F = 0.27$ (ethyl acetate); IR (ATR): $\tilde{\nu} = 1323m$, 1365*m*, 1388*w*, 1441*m*, 1486*w*, 1530*m*, 1605*s*, 1646*m*, 1725*w*, 2854*w*, 2925 m cm^{-1}; UV/vis (CH_2Cl_2): λ_{max} (log ε) = 559 (4.15) nm; ^{1}H NMR (500 MHz, $CDCl_3$) $\delta = 7.08$ (s, 1H, 42-H), 6.88–6.83 (m, 1H, 40-H), 6.59–6.44 (m, 2H, 28-N*H*+36-N*H*), 6.31–6.24 (m, 1H, 39-H), 6.10 (s, 1H, 45-H), 5.72 (s, 1H, 12-H), 4.50 (dd, $J = 11.8$, 4.6 Hz, 1H, 3-H), 3.41–3.16 (m, 6H, 33-H_2+34-H_2+37-H_2), 2.82–2.71 (m, 1H, 1-H_a), 2.70–2.63 (m, 2H; 36-H_2), 2.54 (s, 3H, 47-H_3), 2.40–2.30 (m, 2H, 18-H+19-H_a), 2.24 (s, 3H, 48-H_3), 2.04 (d, $J = 1.1$ Hz, 3H, 32-H_3), 2.04–1.73 (m, 3H, 15-H_a+16-H_a+21-H_a), 1.74–1.48 (m, 6H, 2-H_2+6-H_a+7-H_a+21-H_b), 1.38–1.15 (m, 8H, 1-H_b+6-H_b+7-H_b+15-H_b+16-H_b+22-H_2), 1.25 (s, 3H, 27-H_3), 1.14 (s, 3H, 25-H_3), 1.13–1.07 (m, 6H, 26-H_3+28-H_3), 0.88–0.84 (m, 6H, 23-H_3+24-H_3), 0.83–0.75 (m, 1H, 5-H), 0.78 (s, 3H, 29-H_3) ppm; ^{19}F NMR (470 MHz, $CDCl_3$) $\delta = -143.6$ to -144.8 (m, B–F_2) ppm; MS (ESI, MeOH): $m/z = 809.6$ (76%, $[M - F]^+$), 849.1 (70%, $[2M + Ca]^{2+}$), 851.3 (79%, $[M+Na]^+$), 1263.1 (100%, $[3M + Ca]^{2+}$), 1677.4 (47%, $[4M + Ca]^{2+}$); analysis calcd for $C_{48}H_{67}BF_2N_4O_5$ (828.88): C 69.55, H 8.14, N 6.76; found: C 69.41, H 8.29, N 6.51.

4.2.26. 3-O-acetyl-betulin (28)

This compound was prepared from **BN** as previously reported; m.p. 263–265 °C (lit.: 260–263 °C [53]; $R_F = 0.37$ (DCM); $[\alpha]_D = 24.8°$ (*c* 1.0, $CHCl_3$) [lit.: $[\alpha]_D = 25.7°$ (*c* 0.92, $CHCl_3$)] [54].

4.2.27. 28-O-acetyl-betulin (29)

This compound was prepared from **BN** as previously reported; m.p. 206–209 °C (lit.: 209–212 °C) [55]; $R_F = 0.22$ (*n*-hexane/ethyl acetate, 9:1); $[\alpha]_D = 8.9°$ (*c* 1.2, $CHCl_3$) [lit.: $[\alpha]_D = 8.5°$ (*c* 1.58, $CHCl_3$)] [54].

4.2.28. O-(3β-Acetyloxy-lup-20(29)en-28-oyl)3-{2-[(3,5-dimethyl-1H-pyrrol-2-yl-κN)methylidene]-2H-pyrrol-5-yl- κN}propylester) boron difluoride (30)

Reaction of **7** (46 mg, 0.15 mmol) and **28** (70 mg, 0.16 mmol) according to the GP gave **30** (80 mg, 70%) as an amorphous red solid; $R_F = 0.27$ (*n*-hexane/ethyl acetate, 9:1); IR (ATR): $\tilde{\nu} = 1367m$, 1391*w*, 1449*m*, 1488*m*, 1530*w*, 1605*vs*, 1728*m*, 2872*w*, 2944 m cm^{-1}; UV/vis (CH_2Cl_2): λ_{max} (log ε) = 559 (4.29) nm; ^{1}H NMR (500 MHz, $CDCl_3$) $\delta = 7.06$ (s, 1H, 40-H), 6.86 (d, $J = 4.0$ Hz, 1H, 38-H), 6.26 (d, $J = 4.0$ Hz, 1H, 37-H), 6.10 (s, 1H, 43-H), 4.67 (d, $J = 2.3$ Hz, 1H, 29-H_a), 4.57 (t, $J = 1.9$ Hz, 1H, 29-H_b), 4.46 (dd, $J = 10.4$, 5.8 Hz, 1H, 3-H), 4.26 (dd, $J = 11.1$, 1.9 Hz, 1H, 28-H_a), 3.86 (d, $J = 11.0$ Hz, 1H, 28-H_b), 3.36–3.24 (m, 2H, 35-H_2), 2.77 (t, $J = 7.5$ Hz, 2H, 34-H_2), 2.55 (s, 3H, 45-H_3), 2.46–2.38 (m, 1H, 19-H), 2.24 (s, 3H, 46-H_3), 2.03 (s, 3H, 32-H_3), 1.99–1.89 (m, 1H, 21-H_a), 1.78 (ddd, $J = 13.3$, 4.4, 2.4 Hz, 1H, 16-H_a), 1.67 (s, 3H, 30-H_3), 1.76–1.52 (m, 8H, 1-H_a+2-H_2+12-H_a+13-H+15-H_a+18-H+22-H_a), 1.53–1.47 (m, 1H, 6-H_a), 1.46–1.32 (m, 5H, 6-H_b+7-H_2+11-H_a+21-H_a), 1.32–1.14 (m, 2H, 9-H+11-H_b), 1.10–1.03 (m, 3H, 12-H_b+15-H_b+22-H_b), 1.01 (s, 3H, 25-H_3), 0.96 (s, 3H, 27-H_3), 0.98–0.93 (m, 1H, 1-H_b), 0.90–0.81 (m, 9H, 23-H_3+24-H_3+25-H_3), 0.81–0.73 (m, 1H, 5-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) $\delta = 173.0$ (C-33), 171.0 (C-31), 160.4 (C-44), 157.3 (C-36), 143.7 (C-20), 135.2 (C-41), 133.3 (C-42), 128.0 (C-39), 123.8 (C-38), 120.4 (C-40), 116.6 (C-37), 109.7 (C-29), 80.9 (C-29), 62.9 (C-28), 55.4 (C-5), 50.3 (C-9), 48.7 (C-18), 47.7 (C-19), 46.4 (C-17), 42.7 (C-14), 40.9 (C-8), 38.4 (C-1), 37.8 (C-4), 37.6 (C-13), 37.1 (C-10), 34.5 (C-22), 34.1 (C-7), 33.5 (C-34), 29.7 (C-16), 29.6 (C-21), 27.9 (C-23), 27.0 (C-15), 25.2 (C-12), 24.0 (C-35), 23.7 (C-2), 21.3 (C-32), 20.8 (C-11), 19.1 (C-30), 18.2 (C-6), 16.5 (C-24), 16.1 (C-25), 16.0 (C-26), 14.9 (C-45), 14.7 (C-27), 11.3 (C-46) ppm; ^{19}F NMR (470 MHz, $CDCl_3$) $\delta = -145.19$ to -145.51 (m, B–F_2) ppm; MS (ESI, MeOH): $m/z = 739.3$ (38%, $[M - F]^+$), 776.1 (70%, $[M + NH_4]^+$), 781.4 (100%, $[M+Na]^+$); analysis calcd for $C_{46}H_{65}BF_2N_2O_4$ (758.83): C 72.81, H 8.63, N 3.69; found: C 72.55, H 8.74, N 3.41.

4.2.29. O-(28β-Acetyloxy-lup-20(29)en-3-oyl) 3-{2-[(3,5-dimethyl-1H-pyrrol-2-yl-κN)methylidene]-2H-pyrrol-5-yl- κN} propylester)boron difluoride (31)

Reaction of **7** (60 mg, 0.21 mmol) and **29** (112 g, 0.23 mmol) according to the GP gave **31** (109 mg, 68%) as an amorphous red solid; $R_F = 0.22$ (*n*-hexane/ethyl acetate, 9:1); [lit.: $[\alpha]_D = 11.0°$ (*c* 0.25, $CHCl_3$); IR (ATR): $\tilde{\nu} = 1364m$, 1374*m*, 1388*m*, 1455*m*, 1485*m*, 1530*w*, 1606*s*, 1737*s*, 2870*m*, 2941 m cm^{-1}; UV/vis ($CHCl_3$): λ_{max} (log ε) = 559 (4.00) nm; ^{1}H NMR (500 MHz, $CDCl_3$) $\delta = 7.07$ (s, 1H, 40-H), 6.87 (d, $J = 4.0$ Hz, 1H, 38-H), 6.27 (d, $J = 4.0$ Hz, 1H, 37-H), 6.10 (s, 1H, 43-H), 4.69 (d, $J = 2.3$ Hz, 1H, 29-H_a), 4.61–4.56 (m, 1H, 29-H_b), 4.49 (dd, $J = 11.0$, 5.0 Hz, 1H, 3-H), 4.24 (dd, $J = 11.1$, 2.0 Hz, 1H, 28-H_a), 3.85 (dt, $J = 11.2$, 1.9 Hz, 1H, 28-H_b), 3.29 (t, $J = 7.5$ Hz, 2H, 35-H_2), 2.75 (t, $J = 7.5$ Hz, 2H, 34-H_2), 2.56 (s, 3H, 45-H_3), 2.44 (td, $J = 11.1$, 5.8 Hz, 1H, 19-H), 2.24 (s, 3H, 46-H_3), 2.06 (s, 3H, 32-H_3), 2.01–1.89 (m, 1H, 21-H_a), 1.89–1.80 (m, 1H, 16-H_a), 1.80–1.71 (m, 1H, 22-H_a), 1.68 (s, 3H, 30-H_3), 1.73–1.54 (m, 7H, 1-H_a+2-H_2+12-H_a+13-H+15-H_a+18-H), 1.55–1.47 (m, 1H, 6-H_a), 1.44–1.34 (m, 5H, 6-H_b+7-H_2+11-H_a+21-H_b), 1.33–1.20 (m, 3H, 9-H+11-H_b+16-H_b), 1.03 (s, 3H, 25-H_3), 1.14–0.92 (m, 3H, 12-H_b+15-H_b+22-H_b), 0.97 (s, 3H, 27-H_3), 0.89–0.83 (m, 1H, 1-H_b), 0.82 (s, 3H, 23-H_3), 0.82 (s, 3H, 26-H_3), 0.81–0.74 (m, 1H, 5-H), 0.76 (s, 3H, 24-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) $\delta = 172.3$ (C-33), 171.6 (C-31), 160.2 (C-44), 157.5 (C-36), 150.1 (C-20), 143.6 (C-41),135.3 (C-42), 133.3 (C-39), 128.0 (C-38), 123.7 (C-40), 120.3 (C-43), 116.6 (C-37), 109.8 (C-29), 81.2 (C-3), 62.8 (C-28), 55.4 (C-5), 50.3 (C-9), 48.8 (C-18), 47.7 (C-19), 46.3 (C-17), 42.7 (C-14), 40.9 (C-10), 38.4 (C-1), 37.8 (C-4), 37.6 (C-13), 37.0 (C-8), 34.7 (C-22), 34.1 (C-7), 33.6 (C-34), 29.8 (C-16), 29.6 (C-21), 27.9 (C-23), 27.4 (C-2), 27.1 (C-15), 25.2 (C-12), 24.0 (C-35), 21.0 (C-32), 20.8 (C-11), 19.1 (C-30), 18.1 (C-6), 16.0 (C-26+C-25), 15.3 (C-24), 14.9 (C-45), 14.7 (C-27), 11.26 (C-46) ppm; ^{19}F NMR (470 MHz, $CDCl_3$) $\delta = -145.4$ to -145.8 (m, B–F_2) ppm; MS (ESI, MeOH): $m/z = 739.2$ (37%, $[M - F]^+$), 776.1 (26%, $[M + NH_4]^+$), 781.5 (100%, $[M+Na]^+$); analysis calcd for $C_{46}H_{65}BF_2N_2O_4$ (758.83): C 72.81, H 8.63, N 3.69; found: C 72.69, H 8.82, N 3.44.

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.ejmech.2019.111858.

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The presence of a cationic center is not alone decisive for the cytotoxicity of triterpene carboxylic acid amides

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ABSTRACT

3-*O*-Acetyl-ursolic acid (**2**) and 3-*O*-acetyl oleanolic acid (**8**) were converted into piperazinylamides holding a distal NH, NMe or a NMe_2 group. These compounds as well as the corresponding *N*-methyl-*N*-oxides were accessed. Their cytotoxicity was assessed in SRB assays employing a panel of human tumor cell lines and non-malignant fibroblasts (NIH 3T3). As a result, compounds holding a quaternary distal *N*-substituent were less cytotoxic that those holding a NH-moiety. Hence, the presence of a distal cationic center seems not to be a sufficient criterion for obtaining triterpenoids of high cytotoxicity and selectivity.

1. Introduction

Attempts to successfully treat cancer are more necessary than ever, as 9.6 million people worldwide died in 2018, and globally about 1 in 6 deaths is due to cancer [1]. Many of the novel therapeutics are derived from natural products, such as best-sellers vincristine [2–4], paclitaxel [5–7], etoposide [8,9], irinotecan [10–12], docetaxel [13–16] and topotecan [17–20]. In recent years, terpenes, especially triterpene carboxylic acids [21–40], have again become the focus of scientific interest. In particular, esters and amides of betulinic acid, oleanolic acid, ursolic acid and maslinic acid showed sufficiently high cytotoxicity towards a large number of human tumor cell lines [24,25,27,28,30,41–48]. The attachment of a cationic residue to the triterpenoid skeleton proved to be particularly advantageous, since this improved the notoriously poor solubility of triterpene carboxylic acids and – as a consequence – also the bioavailability. Furthermore, particularly in the case of derivatives derived from rhodamine B an increased inward transport into mitochondria was observed thus triggering apoptosis [47–49]. A slightly increased cytotoxicity has also been reported for triterpenoids holding an extra ammonium [50,51] or phosphonium group [52–54]. In extension to these findings we became interested in the synthesis and biological evaluation of ursolic and oleanolic acid derived amides holding a distal secondary, tertiary or quaternary amino group.

2. Results and discussion

2.1. Chemistry

Acetylation (Scheme 1) of ursolic acid (**1**) gave 3-*O*-acetyl-ursolic acid (**2**) [55]. Reaction of **2** with oxalyl chloride in DCM in the presence of catal. amounts DMF followed by a condensation reaction with piperazine gave amide (**3**).[56–58] Under similar conditions from *N*-methyl-piperazine compound **4** [59] was obtained in almost quantitative yield. Reaction of **3** with an excess of methyl iodide for 20 h gave 82% of **5**, while the oxidation of **4** with hydrogen peroxide for two days yielded 96% of *N*-oxide **6**. While a few ammonium salts of triterpenoids have been investigated for their cytotoxic potential, the number of complex molecules holding a piperazinyl derived *N*-oxide remained small over the years. Thus, a *N*-oxide derived from the aminosteroid RM-133 was shown of negligible low cytotoxicity against HL-60, PANC-1 and OVCAR-3 cancer cells [60].

In a similar way as described above, acetylation of oleanolic acid (**7**) gave 3-*O*-acetyl-oleanolic acid (**8**) [61]. Activation with oxalyl chloride followed by its reaction with piperazine or *N*-methyl-piperazine gave amides **9** [55] and **10** [62], respectively. Quaternisation of **9** gave ammonium salt **11** while from the hydrogen peroxide oxidation of **10***N*-oxide **12** was obtained.

3. Biology

The compounds were subjected to sulforhodamine B (SRB) assays to assess their cytotoxicity employing several human tumor cell lines, i.e.

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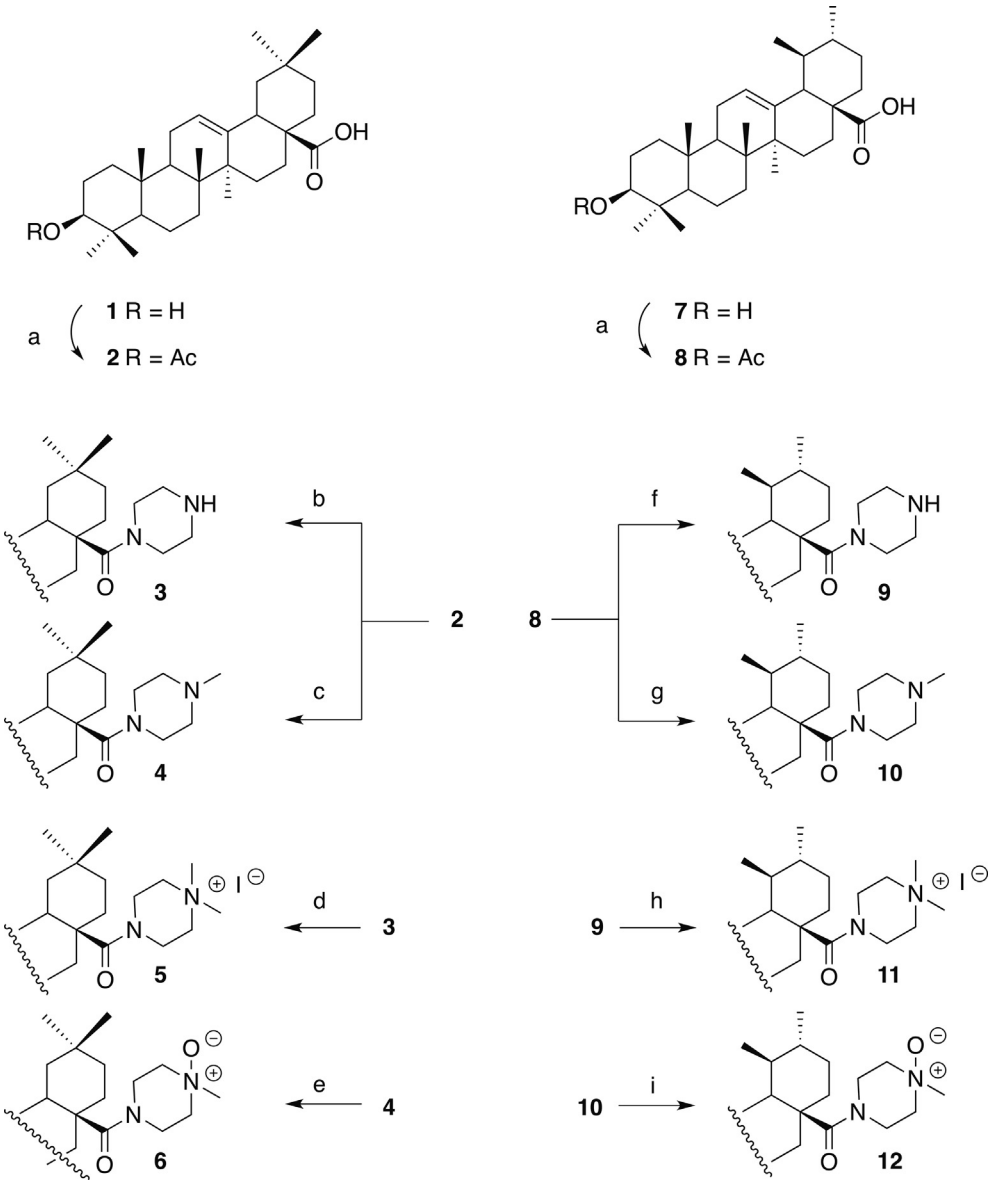

Scheme 1. Reactions and conditions: a) Ac_2O, pyridine, 25 °C, 1 d, 77% (of **2**), 82% (of **8**); b) $(COCl)_2$, catal. DMF, DCM, 0 °C → r.t., 2 h; 2) piperazine, NEt_3, DCM, 17 h, r.t., 84%; c) $(COCl)_2$, catal. DMF, DCM, 0 °C → r.t., 2 h; 2) *N*-methylpiperazine, NEt_3, DCM, 17 h, r.t., 99%; d) K_2CO_3, MeI, ACN, 20 h, r.t. 82%; e) H_2O_2, MeOH, 50 °C, 2 d, 96%; f) $(COCl)_2$, catal. DMF, DCM, 0 °C → r.t., 2 h; 2) piperazine, NEt_3, DCM, 17 h, r.t., 80%; c) $(COCl)_2$, catal. DMF, DCM, 0 °C → r.t., 2 h; 2) *N*-methylpiperazine, NEt_3, DCM, 17 h, r.t., 86%; d) K_2CO_3, MeI, ACN, 20 h, r.t. 53%; e) H_2O_2, MeOH, 50 °C, 2 d, 99%.

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%; cut-off 30 μM; n.d. not detected/not determined, n.s. not soluble under the conditions of the assay. Doxorubicin (**DX**) and betulinic acid (**BA**) were used as a positive standard.

	A375	HT29	MCF-7	A2780	FaDu	NIH 3T3
1	12.3 ± 0.9	10.6 ± 0.7	12.7 ± 0.1	11.7 ± 0.6	14.7 ± 1.1	13.1 ± 1.1
3	1.8 ± 0.1	2.4 ± 0.1	1.9 ± 0.2	2.3 ± 0.2	2.2 ± 0.2	2.7 ± 0.2
4	4.1 ± 0.2	7.3 ± 0.2	5.5 ± 0.4	5.14 ± 0.7	5.3 ± 0.2	11.1 ± 0.2
5	> 30	> 30	> 30	> 30	> 30	> 30
6	12.9 ± 1.3	14.6 ± 1.8	8.8 ± 0.7	11.1 ± 1.6	11.2 ± 0.7	15.3 ± 1.0
7	> 30	> 30	> 30	> 30	> 30	> 30
9	1.8 ± 0.7	1.3 ± 0.2	1.7 ± 0.2	1.7 ± 0.1	1.6 ± 0.3	1.7 ± 0.1
10	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
11	> 30	> 30	> 30	> 30	> 30	> 30
12	11.8 ± 1.4	12.0 ± 2.0	9.6 ± 0.8	11.2 ± 1.0	11.1 ± 1.0	18.2 ± 2.1
BA	14.4 ± 1.3	18.4 ± 2.0	12.0 ± 1.7	12.7 ± 1.8	13.8 ± 2.1	16.1 ± 1.4
DX	1.0 ± 0.8	0.9 ± 0.2	1.1 ± 0.3	0.01 ± 0.01	0.8 ± 0.2	0.01 ± 0.001

A375 (melanoma), HT29 (colorectal carcinoma), MCF-7 (breast adenocarcinoma), A2780 (ovarian carcinoma) and FaDu (hypopharyngeal carcinoma) as well as non-malignant fibroblasts (NIH 3T3). The results from these assays (EC_{50} values after an incubation time of 72 h) are compiled in Table 1.

As a result, cytotoxicity decreases for compounds holding a quaternary nitrogen substituent (EC_{50} > 30 μM). Slightly better results were obtained for the *N*-oxides **6** and **12**. Furthermore, the results from these assays show the substitution pattern of the distal piperazinyl nitrogen of significant influence onto the cytotoxicity of the compounds. Thus, derivatives holding a remote NH moiety were more cytotoxic than their *N*-methylated analogs. While the exact mechanism still remains unclear it appears that the presence of a lipophilic cation in the conjugates is not a sufficient for achieving good cytotoxicity. The acetylated piperazinylamides **3** and **9** showed cytotoxic effects comparable to those of doxorubicin but with a slightly worse selectivity. However, they represent a promising starting point for the further development of cytostatics derived from triterpene carboxylic acids.

4. Conclusion

Ursolic acid (**1**) and oleanolic acid (**7**) were converted into their respective 3-*O*-acetates. The latter compounds were transformed into piperazinylamides. These compounds as well as their *N*- and *N*,*N*-dimethylated analogs and *N*-methyl-*N*-oxides were accessed in good yields and subjected to SRB assays to assess their cytotoxicity employing a panel of human tumor cell lines and non-malignant fibroblasts (NIH 3T3). As a result, and contrary to some expectations, compounds holding a quaternary distal *N*-substituent were less cytotoxic that those holding a NH-moiety. Hence, the presence of a distal cationic center seems not to be a sufficient criterion for assessing triterpenoids of high cytotoxicity. Furthermore the selectivity was diminished for these compounds; the best result was obtained for compound, a *N*-methyl-piperazinyl derivative of ursolic acid holding a selectivity factor of 2.7 for A375 melanoma cells/non-malignant fibroblasts NIH 3T3.

5. Experimental

NMR spectra were recorded using the Varian spectrometers Gemini 2000 or Unity 500 (δ given in ppm, *J* in Hz; typical experiments: H−H−COSY, HMBC, HSQC, NOESY), MS spectra were taken on a Finnigan MAT LCQ (electrospray, voltage 4.1 kV, sheath gas nitrogen) instrument. The optical rotations were measured on a Jasco P-200 polarimeter at 20 °C; TLC was performed on silica gel (Merck 5554, detection with cerium molybdate reagent); melting points are uncorrected (*Büchi* Melting Point M-565), and elemental analyses were performed on a Foss-Heraeus Vario EL (C-HNS) unit. IR spectra were recorded on a Perkin Elmer FT-IR spectrometer Spectrum Two; for UV/Vis a Perkin Elmer Lambda 750S instrument was used. The solvents were dried according to usual procedures. The purity of the compounds was determined by HPLC and found to be > 96%. Ursolic acid (**1**) and oleanolic acid (**7**) and were obtained from Betulinines (Stříbrná Skalice, Czech Republic) in bulk quantities and used as received.

6. 3β-Acetyloxy-urs-12-en-28-oic acid (2)

This compound was obtained from the acetylation of ursolic acid (**1**, 10.01 g, 17.6 mmol) with acetic anhydride in dry pyridine followed by recrystallization from MeOH; **2** (8.43 g, 77%) was obtained as a colorless solid. An analytical sample showed m.p. 279–281 °C (lit.: [63] 279–280 °C).

7. 28-Oxo-28-(1-piperazinyl)urs-12-en-3 β-yl acetate (3)

To a solution of **2** (0.5 g, 1.0 mmol) in dry DCM (50 mL) at 0 °C oxalyl chloride (0.385 mL, 2.0 mmol) and a catal. amount of DMF was added. The mixture was stirred for 2 h at room temperature. The volatiles were removed under diminished pressure, dry THF (25 mL) was added, and evaporated. The residue was dissolved in dry DCM (15 mL) and added within 30 min to a solution of dry piperazine (330 mg, 3.8 mmol), triethylamine (142 mL, 1.9 mmol) in dry DCM (30 mL) at −15 °C. After stirring at room temperature for 17 h followed by usual aqueous workup and chromatography (silica gel, $CHCl_3$/MeOH, gradient 0% MeOH → 5% MeOH), **3** (418 mg, 84%) was obtained as a colorless solid; m.p. 162 °C (decomp.) (lit.: [64] 158–161 °C); $[\alpha]_D$ = +38.2° (*c* 0.191, $CHCl_3$); R_F = 0.6 ($CHCl_3$/MeOH, 9:1); IR (KBr): ν = 698 m, 751 m, 979 m, 1027 m, 1080w, 1109w, 1139w, 1162w, 1193 m, 1244 s, 1367 m, 1454 m, 1518 m, 1646 m, 1709 m, 1732 m, 2868w, 2943 m cm^{-1}; UV/Vis ($CHCl_3$): λ_{max} (log ε) = 226 (3.66) nm; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.23–5.18 (m, 1H, 12-H), 4.52–4.45 (m, 1H, 3-H), 3.79–3.75 (m, 4H, 33-H_2 + 33′-H_2), 2.95–2.89 (m, 4H, 34-H_2 + 34′-H_2), 2.41 (d, *J* = 10.9, 1H, 18-H_1), 2.26–2.09 (m, 1H, 16-H_a), 2.04 (s, 3H, 32-H_3), 1.95–1.86 (m, 3H, 11-H_2 + 16-H_b), 1.78–1.65 (m, 2H, 22-H_2), 1.66–1.56 (m, 3H, 1-H_a + 2-H_2), 1.56–1.20 (m, 8H, 6-H_2 + 7-H_2 + 9-H + 19-H + 21-H_2), 1.12–0.98 (m, 5H, 1-H_2 + 15-H_b + 27-H_3), 0.97–0.91 (m, 7H, 20-H + 25-H_3 + 30-H_3), 0.90–0.78 (m, 11H, 5-H + 15-H_b + 23-H_3 + 24-H_3 + 29-H_3), 0.73 (s, 3H, 26-H_3) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 175.6 (C-28), 171.2 (C-31), b138.7 (C-13), 125.4 (C-12), 81.1 (C-3), 55.5 (C-5), 55.0 (C-18), 48.7 (C-17), 47.7 (C-9), 45.5 (C-33 + C-33′ + C-34 + C-34′), 42.3 (C-14), 39.6 (C-8), 39.6 (C-19), 38.9 (C-20), 38.4 (C-1), 37.8 (C-4), 37.1 (C-10), 34.5 (C-22), 33.1 (C-7), 30.6 (C-21), 28.2 (C-15), 28.2 (C-23), 23.7 (C-2), 23.7 (C-27), 23.5 (C-11), 21.5 (C-32), 21.5 (C-16) 21.4 (C-30), 18.3 (C-6), 17.6 (C-29), 17.3 (C-26), 16.9 (C-24), 15.6 (C-25) ppm; MS (ESI, MeOH): *m/z* = 567.5 ($[M+H]^+$); analysis calcd for $C_{36}H_{58}N_2O_3$ (566.87): C 76.28, H 10.31, N 4.94; found: C 75.97, H 10.51, N 4.76.

8. 28-(4-Methyl-piperazin-1-yl)-28-oxours-12-en-3 β-yl acetate (4)

Following the procedure given for the synthesis of **3**, from **2** (1.0 g, 2.0 mmol) and *N*-methylpiperazine (450 mL, 5.0 mmol), **4** (1.1 g, 99%) was obtained as a colorless solid; m.p. 139–141 °C (decomp.); $[\alpha]_D$ = 34.6° (*c* 0.16, $CHCl_3$); R_F = 0.59 ($CHCl_3$/MeOH, 9:1); IR (KBr): ν = 698 m, 751 m, 979 m, 1027 m, 1080w, 1109w, 1139w, 1162w, 1193 m 1244 s, 1367 m, 1454 m, 1518 m, 1646 m, 1709 m, 1732 m, 2868w, 2943 m cm^{-1}; UV/Vis ($CHCl_3$): λ_{max} (log ε) = 226 (3.60) nm; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.23–5.18 (m, 1H, 12-H), 4.52–4.45 (m, 1H, 3-H), 3.85–3.65 (m, 4H, 33-H_2 + 33′-H_2), 2.57 (s, 4H, 33-H_2 + 33′-H_2), 2.44–2.39 (m, 4H, 18-H + 35-H_3), 2.20–2.07 (m, 1H, 16-H_a), 2.05–2.02 (s, 3H, 32-H_3), 1.94–1.88 (m, 3H, 11-H_2 + 16-H_b), 1.75–1.68 (m, 1H, 22-H_a), 1.68–1.57 (m, 5H, 1-H_2 + 2H_2 + 22-H_b), 1.55–1.42 (m, 4H, 6-H_a + 7-H_a + 9-H + 21-H_a), 1.42–1.33 (m, 2H, 6-H_b + 19-H), 1.33–1.25 (m, 2H, 7-H_b + 21-H_b), 1.06 (s, 3H, 27-H_3), 1.09–1.03 (m, 1H, 15-H_b), 1.02–0.96 (m, 2H, 15-H_b + 20-H), 0.96–0.92 (m, 6H, 25-H_3 + 30-H_3), 0.90–0.79 (m, 9H, 23-H_3 + 24-H_3 + 29-H_3), 0.84–0.79 (m, 1H, 5-H) 0.73 (s, 3H, 26-H_3) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 175.6 (C-28), 171.2 (C-31), 138.6 (C-13), 125.4 (C-12), 81.1 (C-3), 55.5 (C-5), 54.7 (C-18 + C-34 + C-34′), 48.7 (C-17), 47.7 (C-9), 45.3 (C-35), 44.5 (C-33 + C-33′), 42.3 (C-14), 39.6 (C-19), 39.6 (C-8), 38.9 (C-20), 38.4 (C-1), 37.8 (C-4), 37.1 (C-10), 34.5 (C-22), 33.1 (C-7), 30.6 (C-21), 28.3 (C-15), 28.2 (C-23), 23.8 (C-27), 23.7 (C-2), 23.4 (C-11), 23.3 (C-32), 21.4 (C-16), 21.4 (C-30), 18.3 (C-6), 17.6 (C-29), 17.0 (C-26), 16.9 (C-24), 15.6 (C-25) ppm; MS (ESI, MeOH): *m/z* = 580.3 ($[M+H]^+$); analysis calcd for $C_{37}H_{60}N_2O_3$ (580.90): C 76.50, H 10.41, N 4.82; found: C 76.39, H 10.63, N 4.61.

9. 3 β-Acetyloxy-28-(4,4-dimethylpiperazin-4-ium-1-yl)-28-oxours-12-ene iodide (5)

To a solution of **3** (130 mg, 0.23 mmol) in dry ACN (5 mL) at 25 °C

finely ground K_2CO_3 (63 mg, 0.46 mmol), methyl iodide (260 μL, 0.8 mmol) were added, and the mixture was stirred at room temperature for 20 h. The volatiles were removed under diminished pressure, and the residue was subjected to chromatography (silica gel, $CHCl_3$/MeOH, gradient 0% MeOH → 5% MeOH) to yield **5** (136 mg, 82%) as a colorless solid; m.p. 303 °C (decomp.); $[\alpha]_D$ = +17.6° (*c* 0.29, $CHCl_3$); R_F = 0.37 ($CHCl_3$/MeOH, 9:1); IR (KBr): ν = 961 m, 1014 m, 1025 s, 1090w, 1106w, 1147 m, 1153w, 1206 m, 1260 s, 1301w, 1362 s, 1387 m, 1466 m, 1639 s, 1691w, 1733 s, 2910 m, 2941 m, 2953 m, 2966 m cm^{-1}; UV/Vis ($CHCl_3$): λ_{max} (log ε) = 229 (3.99) nm; ^{1}H NMR (500 MHz, CD_3OD): ^{1}H NMR (500 MHz, CD_3OD) δ 5.27 (t, *J* = 3.9 Hz, 1H, 12-H), 4.51 (dd, *J* = 11.4, 5.0 Hz, 1H, 3-H), 4.11–4.08 (m, 2H, 33-H_2), 4.02–3.99 (m, 2H, 33′-H_2), 3.50–3.44 (m, 4H, 34-H_2 + 34′-H_2), 3.32–3.28 (m, 6H, 35-H_3 + 36-H_3), 2.43 (d, *J* = 11.1 Hz, 1H, 18-H), 2.35–2.26 (m, 1H, 16-H_a), 2.05 (s, 3H, 32-H_3), 2.02–1.96 (m, 2H, 11-H_2), 1.96–1.83 (m, 4H, 1-H_2 + 16-H_b + 21-H_a), 1.78–1.55 (m, 8H, 2-H_2 + 6-H_a + 7-H_2 + 9-H + 20-H + 21-H_b), 1.55–1.33 (m, 4H, 6-H_b + 19-H + 22-H_2), 1.14 (s, 3H, 27-H_3), 1.23–1.07 (m, 2H, 15-H_2), 1.0–0.97 (m, 6H. 25-H_3 + 30-H_3), 0.98–0.95 (m, 3H, 29-H_3), 0.90–0.87 (m, 6H, 23-H_3 + 24-H_3), 0.87–0.85 (m, 1H, 5-H), 0.84–0.80 (m, 3H, 26-H_3) ppm; ^{13}C NMR (125 MHz, CD_3OD): δ = 182.5 (C-28), 171.5 (C-31), 125.4 (C-13), 125.3 (C-12), 81.1 (C-3), 61.1 (C-34 + C-34′), 55.3 (C-5), 55.0 (C-18), 50.7 (C-35 + C-36), 48.7 (C-17), 47.4 (C-9), 41.9 (C-14), 39.4 (C-8), 39.2 (C-33 + C-33′), 39.2 (C-19), 38.0 (C-20), 37.3 (C-4), 36.7 (C-10), 33.9 (C-1), 32.6 (C-22), 30.1 (C-7), 28.4 (C-21), 28.0 (C-15), 27.2 (C-23), 23.2 (C-16), 23.0 (C-2), 22.9 (C-11), 22.8 (C-27), 20.0 (C-30), 19.7 (C-32), 17.9 (C-6), 16.5 (C-29), 16.0 (C-26), 15.8 (C-24), 25.3 (C-25) ppm; MS (ESI, MeOH): *m/z* = 595.5 ($[M]^+$); analysis calcd for $C_{38}H_{63}N_2O_3I$ (722.84): C 63.14, H 8.79, N 3.88; found: C 62.84, H 8.99, N 3.59.

10. 28-(4-Methyl-4-oxido-piperazin-1-yl)-28-oxours-12-en-3 β-yl acetate (6)

To a solution of **4** (500 mg, 086 mmol) in MeOH (15 mL) at room temperature an aqueous solution of H_2O_2 (35%, 370 μL, 4.3 mmol) was added. The mixture was stirred at 50 °C for 24 h, another H_2O_2 (35%, 370 μL, 4.3 mmol) was added, and stirring was continued for another 24 h. The volatiles were removed under reduced pressure and the residue was subjected to chromatography (silica gel, $CHCl_3$/MeOH, gradient 0% MeOH → 30% MeOH) to yield **6** (492 mg, 96%) as a colorless solid; m.p. 203 °C (decomp.); $[\alpha]_D$ = +29.9° (*c* 0.29, $CHCl_3$); R_F = 0.25 ($CHCl_3$/MeOH, 9:1); IR (KBr): ν = 984 m, 1005w, 1026 m, 1105w, 1132w, 1207w, 1245 s, 1370 m, 1391 m, 1453 m, 1630 m, 1731 m, 2872 m, 2926 m, 2945 m cm^{-1}; UV/Vis ($CHCl_3$): λ_{max} (log ε) = 227 (3.36) nm; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.21–5.16 (m, 1H, 12-H), 4.50–4.43 (m, 1H, 3-H), 4.35–4.28 (m, 1H, 34-H_a), 4.27–4.20 (m, 1H, 35-H_a), 3.88–3.64 (m, 2H, 34-H_b + 34-H_b), 3.62–3.35 (m, 2H, 33-H_a + 36-H_a), 3.34 (s, 3H, 37-H_3), 3.26–3.08 (m, 2H, 33-H_b + 36-H_b), 2.39–2.34 (m, 1H, 18-H), 2.21–2.11 (m, 1H, 16-H_a), 2.02 (s, 3H, 32-H_3), 1.92–1.86 (m, 2H, 11-H_2), 1.76–1.66 (m, 3H, 15-H_a + 22-H_2), 1.65–1.54 (m, 4H, 2-H_2 + 20-H + 21-H_a), 1.54–1.41 (m, 6H, 1-H_a + 6-H_a + 9-H + 16-H_b + 19-H + 21-H_b), 1.41–1.23 (m, 3H, 7-H_2 + 6-H_b), 1.12–1.07 (m, 1H, 1-H_b), 1.05 (s, 3H, 27-H_3), 1.03–0.95 (m, 1H, 15-H_b), 1.0–0.89 (m, 6H, 30-H_3 + 25-H_3), 0.88–0.77 (m, 9H, 23-H_3 + 24-H_3 + 29-H_3), 0.82–0.78 (m, 1H, 5-H_b), 0.69 (s, 3H, 26-H_3) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 175.6 (C-28), 170.9 (C-31), 138.2 (C-13), 125.2 (C-12), 80.8 (C-3), 65.6 (C-36 + C-33), 59.8 (C-37), 55.2 (C-5), 54.9 (C-18), 48.5 (C-17), 47.4 (C-9), 42.0 (C-14), 40.2 (C-35 + C-34), 39.3 (C-19), 39.3 (C-8), 38.6 (C-20), 38.1 (C-1), 37.5 (C-4), 36.7 (C-10), 34.4 (C-22), 32.7 (C-7), 30.2 (C-21), 28.0 (C-15), 27.9 (C-23), 25.2 (C-27), 23.4 (C-16), 23.2 (C-11), 21.1 (C-32), 21.0 (C-30), 18.3 (C-2), 18.3 (C-6), 17.2 (C-29), 16.7 (C-26), 16.6 (C-24), 15.3 (C-25) ppm; MS (ESI, MeOH): *m/z* = 597.4 ($[M+H]^+$), 1194.6 ($[2M+H]^+$); analysis calcd for $C_{37}H_{60}N_2O_4$ (596.90): C 74.45, H 10.13, N 4.69; found: C 74.32, H 10.36, N 4.44.

11. 3 β-Acetyloxy-olean-12-en-28-oic acid (8)

This product (**8**, 9.0 g, 82%) was obtained from the acetylation of oleanolic acid (**7**, 10.01 g, 17.6 mmol) with acetic anhydride in dry pyridine followed by re-crystallization from MeOH as a colorless solid; an analytical sample showed m.p. 258–260 °C, lit.: [65] 264–265 °C.

12. 28-Oxo-28-(1-piperazinyl)-olean-12-en-3 β-yl acetate (9)

Following the synthesis of **3**, from **8** (500 mg, 1.0 mmol), **9** (400 mg, 80%) was obtained as a colorless solid; m.p. 171 °C (decomp.), lit. 170–176 [64].

13. 28-(4-Methyl-piperazin-1-yl)-28-oxoolean-12-en-3 β -yl acetate 10)

Following the synthesis of **4**, from **8** (470 mg, 0.94 mmol) and 4-methylpiperazine (220 μL, 2.4 mmol) followed by chromatography (silica gel, $CHCl_3$/MeOH, gradient 0% MeOH → 5% MeOH), **10** (472 mg, 86%) was obtained as a colorless solid; m.p. 216–218 °C (decomp.) (lit.: [62] 218–220 °C); $[\alpha]_D$ = +44.0° (*c* 0.305, $CHCl_3$); R_F = 0.56 ($CHCl_3$/MeOH, 9:1); IR (KBr): ν = 1004 s, 1023 m, 1076w, 1143 m, 1167w, 1205 m, 1244 s, 1286w, 1370 m, 1392 m, 1398w, 1411 m, 1457 m, 1616 s, 1734 s, 2746w, 2791w, 2848w, 2941 m cm^{-1}; UV/Vis ($CHCl_3$): λ_{max} (log ε) = 227 (3.65) nm; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.39–5.13 (m, 1H, 12-H), 4.65–4.32 (m, 1H, 3-H), 3.75–3.70 (m, 4H, 33-H_2 + 33′-H_2), 3.23–2.98 (m, 1H, 18-H), 2.66–2.26 (m, 7H, 34-H_2 + 34′-H_2 + 35-H_3), 2.20–2.00 (m, 5H, 2-H_a + 16-H_a + 32-H_3), 1.99–1.78 (m, 2H, 11-H_2), 1.76–1.48 (m, 8H, 1-H_a + 2-H_b + 6-H_a, 9-H, 16-H_b, 19-H_a, 22-H_2), 1.50–1.22 (m, 4H, 6-H_b + 7-H_2 + 21-H_a), 1.22–1.12 (m, 5H, 19-H_b + 21-H_b + 27-H_3), 1.11–0.97 (m, 3H, 1-H_b, 15-H_2), 0.95–0.80 (m, 16H, 5-H + 23-H_3 + 24-H_3, 25-H_3, 29-H_3, 30-H_3), 0.74–0.70 (m, 3H, 26-H_3) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 175.0 (C-28), 171.0 (C-31), 144.7 (C-13), 121.5 (C-12), 80.9 (C-3), 55.4 (C-5), 55.0 (C-34 + C-34′), 47.7 (C-9), 47.5 (C-17), 46.4 (C-19), 45.8 (C-35), 44.9 (C-33 + C-33′), 43.6 (C-18), 41.8 (C-14), 39.1 (C-8), 38.1 (C-1), 37.7 (C-4), 37.0 (C-10), 34.0 (C-21), 33.0 (C-29), 32.8 (C-7), 30.4 (C-20), 30.1 (C-22), 28.0 (C-23), 27.9 (C-15), 25.9 (C-27), 24.1 (C-30), 23.5 (C-11), 23.4 (C-16), 22.8 (C-2), 21.3 (C-32), 18.2 (C-6), 16.9 (C-26), 16.7 (C-24), 15.4 (C-25) ppm; MS (ESI, MeOH): *m/z* = 581.5 ($[M+H]^+$), 1161.6 ($[2M+H]^+$), 1183.8 ($[2M+Na]^+$); analysis calcd for $C_{37}H_{60}N_2O_3$ (580.90): C 76.50, H 10.41, N 4.82; found: C 76.32, H 10.60, N 4.67.

14. 3 β-Acetyloxy-28-(4,4-dimethylpiperazin-4-ium-1-yl)-28-oxoolean-12-ene iodide (11)

Following the procedure given for the synthesis of **5**, from **9** (500 mg, 0.88 mmol), **11** (337 mg, 53%) was obtained as a colorless solid; m.p. 241 °C (decomp.); $[\alpha]_D$ = +17.82° (*c* 0.19, $CHCl_3$); R_F = 0.4 ($CHCl_3$/MeOH, 9:1); IR (KBr): ν = 950 m, 960 m, 984 m, 1003 m, 1025 m, 1085w, 1102w, 1152 m, 1165w, 1206 m, 1257 s, 1302w 1364 m, 1388 m, 1468 m, 1637 s, 1731 s, 2871w, 2941 m cm^{-1}; UV/Vis ($CHCl_3$): λ_{max} (log ε) = 229 (4.01) nm; ^{1}H NMR (500 MHz, CD_3OD): δ = 5.29–5.24 (m, 1H, 12-H), 4.53–4.46 (m, 1H, 3-H), 4.22–4.11 (m, 2H, 33-H_2), 4.07–3.92 (m, 2H, 36-H_2), 3.55–3.45 (m, 4H, 34-H_2 + 35-H_2), 3.31 (s, 6H, 37-H_3 + 38-H_3), 3.11–3.04 (m, 1H, 18-H), 2.32–2.22 (m, 1H, 16-H_a), 2.06 (s, 3H, 32-H_3), 1.99–1.92 (m, 2H, 11-H_2), 1.88–1.44 (m, 13H, 1-H_a + 2-H_2 + 6-H_2 + 7-H_2 + 9-H + 15-H_a + 16-H_a + 19-H_a + 21-H_a + 22-H_a), 1.42–1.33 (m, 1H, 22-H_b), 1.31–1.26 (m, 1H, 21-H_b), 1.25–1.19 (m, 5H, 15-H_b + 19-H_b + 27-H_3), 1.15–1.06 (m, 1H, 1-H_b), 1.02 (s, 3H, 25-H_3), 0.99 (s, 3H, 29-H_3), 0.97 (s, 3H, 30-H_3), 0.95–0.89 (m, 7H, 5-H + 29-H_3 + 30-H_3), 0.80 (s, 3H, 26-H_3) ppm; ^{13}C NMR (125 MHz, CD_3OD): δ = 177.7 (C-28), 172.9 (C-31), 145.7 (C-13), 123.1 (C-12), 82.5 (C-3), 62.6 (C-34), 61.5 (C-35), 56.8 (C-5), 52.1 (C-37 + C-38), 47.7 (C-9), 48.0 (C-17), 47.5 (C-19),

45.0 (C-18), 43.1 (C-14), 40.5 (C-36 + C-33), 40.5 (C-8), 39.3 (C-1), 38.7 (C-4), 38.2 (C-10), 35.0 (C-21), 33.8 (C-22), 33.4 (C-30), 31.2 (C-20), 30.8 (C-7), 29.1 (C-15), 28.6 (C-24), 26.4 (C-27), 24.5 (C-2), 24.5 (C-11), 24.3 (C-29), 23.3 (C-16), 21.1 (C-32), 19.3 (C-6), 17.5 (C-26), 17.1 (C-23), 16.0 (C-25) ppm; MS (ESI, MeOH): m/z = 595.5 ($[M]^+$); analysis calcd for $C_{38}H_{63}N_2O_3I$ (722.84): C 63.14, H 8.79, N 3.88; found: C 62.96, H 8.95, N 3.62.

15. 28-(4-Methyl-4-oxido-piperazin-1-yl)-28-oxoolean-12-en-3 β -yl acetate (12)

Following the procedure given for the synthesis of **6**, from **10** (473 mg, 0.82 mmol), **12** (480 mg, 99%) was obtained as a colorless solid; m.p. 191 °C (decomp.); $[\alpha]_D$ = +28.9° (c 0.25, $CHCl_3$); R_F = 0.3 ($CHCl_3$/MeOH, 9:1); IR (KBr): ν = 973 m, 1007 m, 1027 m, 1136w, 1193w, 1200 m, 1245 s, 1365 m, 1391 m, 1447 m, 1633 m, 1731 m, 2876w, 2944 m cm^{-1}; UV/Vis ($CHCl_3$): λ_{max} (log ε) = 226 (334) nm; ^{1}H NMR (500 MHz, $CDCl_3$): = 5.29–5.22 (m, 1H, 12-H), 4.52–4.43 (m, 1H, 3-H), 4.43–4.35 (m, 1H, 33-H_a) 4.30–4.22 (m, 1H, 36-H_a), 3.99–3.88 (m, 1H, 36-H_b), 3.80–3.67 (m, 1H, 33-H_b), 3.43–3.29 (m, 5H, 37-H_3 + 34-H_a + 35-H_a), 3.28–3.09 (m, 2H, 34-H_b + 35-H_b), 3.09–3.00 (m, 1H, 18-H), 2.21–2.08 (m, 1H, 16-H_a), 2.05–2.02 (m, 3H, 32-H_3), 1.95–1.78 (m, 2H, 11-H_2), 1.78–1.49 (m, 10H, 1-H_a + 2-H_2 + 6-H_a + 7-H_2 + 9-H + 15-H_a + 16-H_b + 19-H_a), 1.49–1.30 (m, 2H, 6-H_b + 22-H_a), 1.30–1.16 (m, 4H, 19-H_b + 21-H_2 + 22-H_b), 1.12 (s, 3H, 27-H_3), 1.09–0.95 (m, 2H, 1-H_b + 15-H_b), 0.94–0.91 (m, 6H, 25-H_3 + 30-H_3), 0.90 (s, 3H, 29-H_3), 0.85 (s, 3H, 23-H_3), 0.84 (s, 3H, 24-H_3), 0.83–0.79 (m, 1H, 5-H), 0.69 (s, 3H, 26-H_3) ppm; ^{13}C NMR (125 MHz, CDCl3): δ = 175.4 (C-28), 171.0 (C-31), 144.3 (C-13), 121.8 (C-12), 80.9 (C-3), 66.0 (C-35), 65.8 (C-34), 60.3 (C-37), 55.4 (C-5), 47.6 (C-9), 47.6 (C-17), 46.2 (C-19), 43.5 (C-18), 41.8 (C-14), 40.5 (C-33), 39.5 (C-36), 39.1 (C-8), 38.1 (C-1), 37.7 (C-4), 37.0 (C-10), 33.9 (C-21), 33.0 (C-29), 32.7 (C-22), 30.4 (C-20), 30.2 (C-7), 28.0 (C-23), 27.9 (C-15), 25.9 (C-27), 24.0 (C-30), 23.5 (C-2), 23.4 (C-11), 22.8 (C-16), 21.3 (C-32), 18.2 (C-6), 16.9 (C-26), 16.6 (C-24), 15.4 (C-25) ppm; MS (ESI, MeOH): m/z = 597.5 ($[M+H]^+$); analysis calcd for $C_{37}H_{60}N_2O_4$ (596.90): C 74.45, H 10.13, N 4.69; found: C 74.27, H 10.31, N 4.47.

CRediT authorship contribution statement

Benjamin Brandes: Investigation, Writing - review & editing. **Lukas Koch:** Investigation. **Sophie Hoenke:** Investigation. **Hans-Peter Deigner:** Conceptualization, Writing - review & editing. **René Csuk:** Conceptualization, Writing - original draft, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.steroids.2020.108713.

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Converting bile acids into mitocans

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ABSTRACT

Cholic acid (**1**, CD), deoxycholic (**3**, DCA), chenodeoxycholic acid (**5**, CDCA), ursodeoxycholic acid (**7**, UDCA), and lithocholic acid (**9**, LCA) were acetylated and converted into their piperazinyl spacered rhodamine B conjugates **16**–**20**. While the parent bile acids showed almost no cytotoxic effects for several human tumor cell lines, the piperazinyl amides were cytostatic but an even superior effect was observed for the rhodamine B conjugates. Extra staining experiments showed these compounds as mitocans; they led to a cell arrest in the G1 phase.

1. Introduction

Recently rhodamine B conjugates of substituted, spacered of pentacyclic triterpenes attracted increased scientific interest, since some of them held most promising cytotoxic properties [1–8]. Cancer is the leading death cause with million deaths worldwide [9], resulting in both an additional burden on health institutions especially in poorer counties but also a social burden for individuals and their family. Since lipophilic derived cations are accumulated in the mitochondria, they have been used for the synthesis of various drug conjugates [4,6,10–15]. Hereby, we report the synthesis of novel tetracyclic triterpene, bile acid derived rhodamine B conjugates and their biological evaluation, thereby employing derivatives of bile acids litho-, deoxy-, chenodeoxy-, and ursodeoxycholic acid (Fig. 1), respectively.

Bile acids are natural detergents. They facilitate the absorption of fat in the intestine but they are also essential in the maintenance of the intestine epithelium homeostasis. Depending on type and concentration they show a dual behavior both as pro-survival or pro-death molecules [16–18]. Recently, cytotoxic activity [19] was established for bile acid-paclitaxel hybrids [20], a camptothecin conjugate [21] as well as for a dihydroartemisinine [22] analog. Furthermore, some bile carboxylamides have been shown to exert pro-apoptotic effects in human colon adenocarcinoma cells DLD-1, HCT-116 and HT29. Pro-apoptotic activity has also been observed on multiple myeloma as well as on glioblastoma multiforme [23]. We have previously demonstrated the cytotoxic activity of triterpenoid-rhodamine B conjugates [4]. It was therefore reasonable to extend our investigations to bile acids, especially since these compounds show a priori a better solubility in biological systems than triterpenoids by comparison due to their amphiphilic nature. In addition, bile acids have emerged as important starting materials for a variety of different bioactive conjugates [24,25].

2. Results and discussion

2.1. Chemistry

Cholic acid (**1**, CA), deoxycholic (**3**, DCA), chenodeoxycholic acid (**5**, CDCA), ursodeoxycholic acid (**7**, UDCA), and lithocholic acid (**9**, LCA) were bought from commercial vendors and acetylated to their respective acetates **2**, **4**, **6**, **8**, and **10** with acceptable yields (Scheme 1). Subsequently, these acetylated bile acids (**ABA**) were allowed to react with oxalyl chloride in the presence of catalytic amounts of DMF followed by adding piperazine to yield piperazinyl amides **11**–**15** (55–66 %, Scheme 2).

Finally, the desired rhodamine B conjugates were easily synthesized employing the well-established EDC·HCl / HOBt method to afford the conjugates in **16**–**20** in 47 %–66 % isolated yields, respectively.

2.2. Biology

The cytotoxicity of the synthesized compounds was evaluated using photometric SRB assays employing several human tumor cell lines (cut-off at 30 μM; Table 1), and the results of which are summarized in Table 1. While the parent bile acids **2**, **4**, **6** and **8** as well as rhodamine B (**Rhd B**) showed no activity towards the cell lines used in the SRB-assay, their acetates held increased biological activity towards the cell lines A375, MCF7, and A2780. Compound **10** proved to be insoluble under the conditions of the assay. At first glance, this seems surprising since

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compounds **2**, **4**, **6** and **8** were readily soluble. However, the poor solubility of **10** also follows the solubility behavior reported for the unsubstituted **GA**s. Here, too, LCA is the most poorly soluble with 0.38 mg/L while CA, for example, has a solubility of 175 mg/L in water. [26] For the piperazinyl amides **12** and **13**, however, even lower EC_{50} values were observed. The observation that piperazinyl amides hold lower EC_{50} values than their corresponding carboxylic acids parallels earlier results having been observed also in the field of terpenoid, in particular of triterpenoid carboxylic acids [1,2,27]. Finally, for the rhodamine B conjugates **16**–**20** and the malignant cell lines very low EC_{50}-values in the range of 0.2–1.2 μM were measured. These conjugates, however, showed only low selectivity for the non-malignant cell line NIH 3 T3 with **16** being the best overall compound holding a tumor cell/non-tumor cell selectivity ranging from S = 1.33 – 3.27. The best selectivity for this series of compounds was measured for the breast adenocarcinoma cell line MCF-7; this again, parallels previous finding for similar steroid conjugates [28].

To further investigate the mode of action of the piperazinyl amides **12** and **13**, as well as of the rhodamine B conjugate **16**, annexin-V-FITC/PI staining assays were used to assess the triggered mode of cell death onto the tumor cell line A2780 at double the EC_{50} concentrations (Fig. 2). Thereby, the samples (sixfold sample repetitions) were incubated for 24 h and 48 h with and without added compounds. Two technical repetitions in reference to the control group were used for calculation. Interestingly, compound **16** showed no significant difference from the control group (Table 2). In contrast to this compound, compounds **12** and **13** showed a significantly lower number of necrotic and vital cells and, in addition, they also held significantly more apoptotic cells at 24 h incubation. These trends continued after 48 h of incubation with compound **12** being the best with an average of 34 % more apoptotic and −26 % vital cells in comparison to the control cell line.

Furthermore, cell cycles were analyzed by FACS employing the ovarian cancer cell line A2780 applying an incubation time of 24 h and 48 h, respectively. Analysis of the results from these experiments showed compounds **12** and **16** to lock treated cells in the G1-phase and a decreased number of cells was found in the S-phase at 24 h incubation (Fig. 3; Fig. 4).

This parallels most recent findings for **12**; R. Yang et al. have previously shown that this compound arrests HepG2 hepatoma cells in G0/G1 and induces apoptosis by the PI3K/AKT/mTor pathway [29]. Compound **12** also induced nearly 39 % of apoptosis as compared to the control cell line (Fig. 3). After an incubation time of 48 h, cells treated with **12** showed 51.38 % apoptotic cells, while the number of cells in the G1 phase had decreased (Fig. 4). The increased cytostatic effect of compound **16** seems to be the result of fewer cells being able to enter the S and G2/M phase.

To assess whether **16** acts as a mitocan (an acrynom describing compounds exerting their anti-cancer activity via their molecular targets within mitochondria), some extra staining experiments were performed, the results of which are depicted in Fig. 5. The dye rhodamine 123 is known to stain mitochondria specifically; this dye emits green light after excitation; compound **16** emits red light.

Consequently, a merged image of the two excitations would cause an observed orange color, and gives evidence whether **16** is accumulated in the mitochondria of A2780 cells (Fig. 5). A microscopic investigation indeed showed a good match of the rhodamine 123 dye with **16**, and an orange color was observed. Moreover, staining with Hoechst 33,342, a blue-emitting nucleus targeting dye, showed **16** not to enter the nucleus of the cancer cells.

3. Conclusion

Several bile acids, i.e. cholic acid (**1**), deoxycholic (**3**), chenodeoxycholic acid (**5**), ursodeoxycholic acid (**7**), and lithocholic acid (**9**) were converted into their acetylated piperazinyl amides. The latter were coupled with rhodamine B to yield conjugates. These conjugates were cytostatic for a panel of human tumor cell lines; they led to a cell arrest in G1, and are accumulated in the mitochondria of the tumor cells. The conjugates do not enter the nucleus. Albeit their cytostatic effect is lower than that of pentacyclic triterpenoid analogs, they represent interesting starting materials for the development of analogs of even higher cytotoxicity and improved tumor cell/non-malignant cell selectivity while still retaining their good solubility properties.

4. Experimental part

4.1. Methods and equipment

Cholic (**1**), deoxycholic (**2**), chenodeoxycholic (**3**), ursodeoxycholic (**4**), and lithocholic acid (**5**) were obtained from Carl Roth and abcr GmbH and were used as received. Equipment and lab equipment was used as previously described. Details can be found in the Supplementary materials file. For the Annexin V-FITC/PI assay as well as for the cell cycle analysis the Attune® Cytometric Software (1.2.5) and MSExcel were used for calculations.

4.2. General procedure (GP1) for the acetylation of bile acids

The bile acid (**1**–**5**, 1 eq) and cat. amounts of DMAP were dissolved in a minimal amount of dry pyridine (20 mL), and acetic anhydride (3–7 eq, depending on the number of hydroxyl groups) was added. After stirring at 25 °C for 24 h, the solution was diluted with DCM (100 mL), washed with HCl (0.1 M, 50 mL), and water (2 × 100 mL). The organic phase was dried ($MgSO_4$), the solvent evaporated under reduced pressure, and the residue was purified by column chromatography (SiO_2, *n*-hexane/ethyl acetate).

4.3. General procedure (GP2) for the synthesis of acetylated piperazinyl amides

The acetylated bile acid (**2**, **4**, **6**, **8**, **10**, 1 eq) was dissolved in a minimal amount of dry DCM (10 mL) under argon and cooled to 0 °C. Cat. amount of dry DMF and oxalyl chloride (4 eq) were added, and the

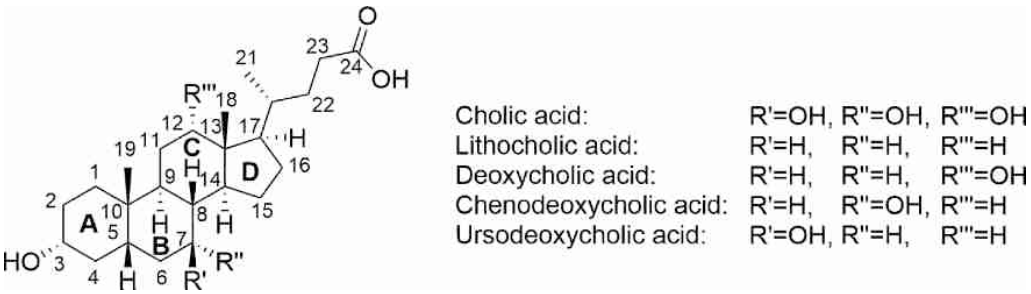


Fig. 1. Structure and numbering scheme of bile acids.

reaction was allowed to warm to 25 °C. After stirring for an additional 2 h, the volatiles were removed under reduced pressure. To a solution containing piperazine (3.8 eq) and triethylamine (1.1 eq) in a minimal amount of dry DCM at –21 °C under argon was added dropwise the acid chloride, dissolved in dry DCM (20 mL). The reaction mixture was stirred at 25 °C for 24 h, quenched with water (100 mL), extracted with DCM (3 × 100 mL), the combined organic phases were dried ($MgSO_4$), and the solvent was evaporated under reduced pressure to obtain a solid which was purified by column chromatography (SiO_2, $CHCl_3$/MeOH, 9:1).

4.4. General procedure (GP3) for the amidation with rhodamine B

Rhodamine B (1 eq), 1-hydroxybenztriazole hydrate (1.1 eq), and *N*-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (1.1 eq) were dissolved in minimal amounts of DMF under argon. After stirring for 24 h, compound **11**–**15**, (1 eq) dissolved in a minimal amount of DMF, was added. Stirring was continued for 24 h, and for work-up aqueous HCl (1 M) and $CHCl_3$ were added; evaporation of the organic layer under reduced pressure afforded the crude product, which was purified by column chromatography (SiO_2, $CHCl_3$/MeOH, 9:1).

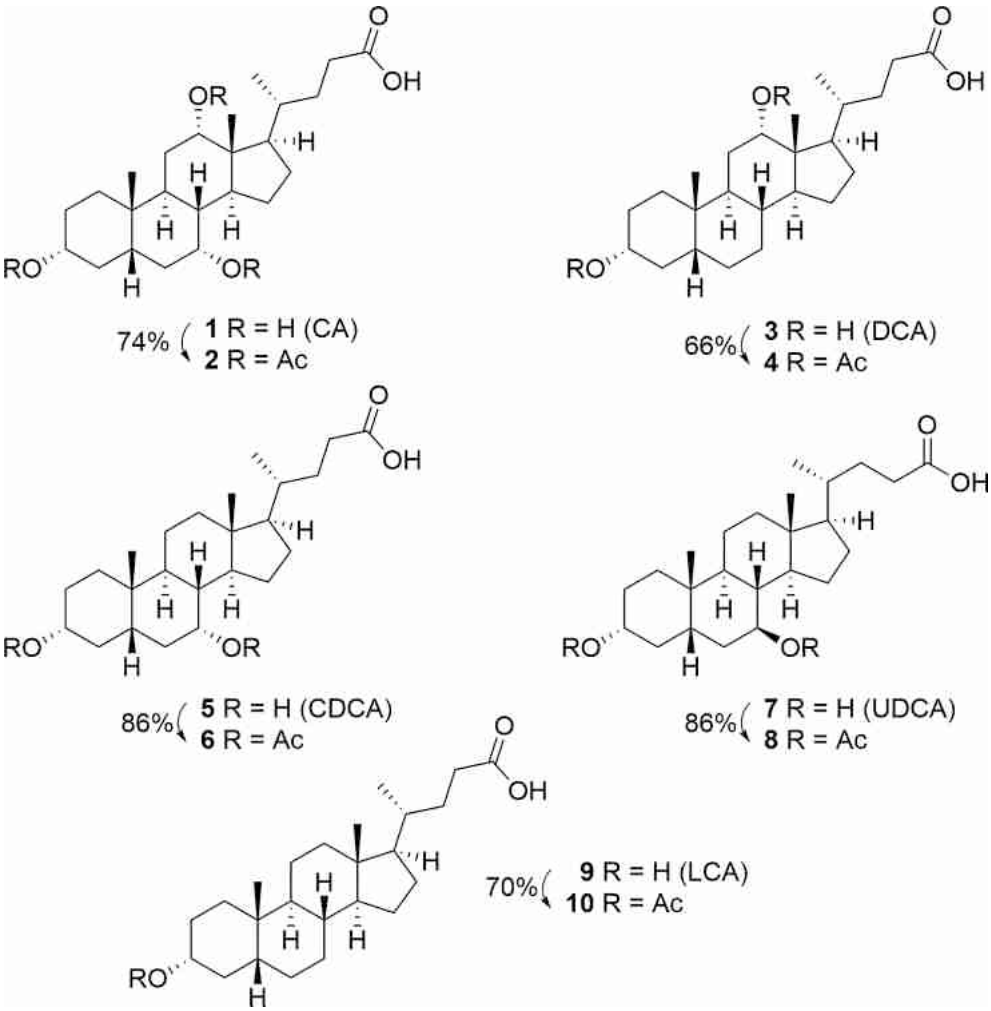
4.5. Syntheses

4.5.1. 3α,7α,12α-Tris(acetyloxy)-5β-cholic acid (2)

Following GP1, from cholic acid (**1**, 3.0 g, 7.3 mmol) **2** (2.2 g, 74 %) was obtained as a white solid; m.p. 92 °C (lit.: [30] 69–70 °C); R_F = 0.3 (*n*-hexane/ethyl acetate, 2:3); $[\alpha]_D$ = +69.6° (*c* 0.249, $CHCl_3$) [lit.: [31] $[\alpha]_D$ = +22.9° (*c* 0.34, $CHCl_3$)]; IR (ATR): $\tilde{\nu}$ = 498*vw*, 584*vw*, 608*w*, 660*vw*, 638*vw*, 722*vw*, 800*w*, 890*w*, 938*w*, 965*w*, 1023 *m*, 1062*w*, 1128*vw*, 1152*w*, 1233 *s*, 1365 *m*, 1377 *m*, 1439*w*, 1731 *s*, 2872*w*, 2941 *m* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.08 (t, *J* = 2.9 Hz, 1H, H-12), 4.90 (q, *J* = 3.2 Hz, 1H, 7-H), 4.57 (tt, *J* = 11.4, 4.3 Hz, 1H, 3-H), 2.31 (dddd, *J* = 70.3, 15.8, 9.6, 5.8 Hz, 2H, 23-H_2), 2.13 (s, 3H, 28-H_3), 2.08 (s, 3H, 30-H_3), 2.04 (s, 3H, 26-H_3), 2.04 – 1.75 (m, 7H, 1-H_a + 4-H_a + 6-H_a + 9-H + 14-H + 16-H_a + 22-H_a), 1.75 – 1.49 (m, 7H, 1-H_b + 2-H_a + 6-H_b + 8-H + 11-H_2 + 17-H), 1.49 – 0.96 (m, 8H, 2-H_b + 4-H_b + 5-H + 15-H_2 + 16-H_b + 20-H + 22-H_b), 0.91 (s, 3H, 19-H_3), 0.82 (d, *J* = 6.5 Hz, 3H, 21-H_3), 0.72 (s, 3H, 18-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 179.5 (C-24), 170.7 (C-25), 170.7 (C-27), 170.6 (C-29), 75.5 (C-12), 74.3 (C-3), 70.9 (C-7), 47.5 (C-17), 45.2 (C-13), 43.6 (C-14), 41.1 (C-5), 37.9 (C-8), 34.9 (C-1), 34.8 (C-4), 34.7 (C-20), 34.5 (C-10), 34.5 (C-6), 31.4 (C-23), 30.9 (C-22), 30.7 (C-22), 29.0 (C-9), 27.3 (C-16), 27.0 (C-2), 25.7 (C-11), 23.0 (C-15), 22.7 (C-19), 21.7 (C-30), 21.6 (C-28), 21.6 (C-26), 17.6 (C-21), 12.4 (C-18) ppm; MS (ESI, MeOH): *m/z* = 552.13 (20 %, $[M + NH_4]^+$), 557.27 (100 %, $[M + Na]^+$), 573.27 (7 %, $[M + K]^+$).

4.5.2. 3α,12α-Bis(acetyloxy)-5β-cholan-24-oic acid (4)

Following GP1 from deoxycholic acid (**3**, 3.0 g, 7.6 mmol) **4** (1.96 g, 66 %) was obtained as a white solid; m.p. 89 °C (lit.: [30] 92–93 °C); R_F = 0.47 (toluene/ethyl acetate/heptane/HCOOH, 80:26:10:5); $[\alpha]_D$ = +90.6° (*c* 0.263, $CHCl_3$) [lit.: [32] $[\alpha]_D$ = +80.5° (*c* 1.00, $CHCl_3$)]; IR (ATR): $\tilde{\nu}$ = 579*w*, 617*w*, 665*w*, 681*w*, 754*w*, 800*w*, 851*w*, 888*w*, 915*w*,



Scheme 1. Synthesis of bile acid derived acetates 2, 4, 6, 8 and 10; conditions: Ac_2O, cat. DMAP, pyridine, 25 °C, 1 day.

954*w*, 971 *m*, 1025 *m*, 1070*w*, 1091*w*, 1160 *m*, 1193 *m*, 1240 *s*, 1363 *m*, 1378 *m*, 1449*w*, 1467*w*, 1707 *m*, 1733 *s*, 2868*w*, 2941 *m* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 5.08$ (s, 1H, 12-H), 4.70 (dt, $J = 11.3$, 6.6 Hz, 1H, 3-H), 2.49 – 2.18 (m, 2H, 23-H_2), 2.10 (s, 3H, 28-H_3), 2.03 (s, 3H, 26-H_3), 1.96 – 1.75 (m, 4H, 4-H_a + 6-H_a + 16-H_a + 22-H_a), 1.75 – 1.51 (m, 9H, 1-H_a + 2-H_a + 4-H_b + 11-H_2 + 14-H + 15-H_a + 17-H + 20-H), 1.50 – 0.94 (m, 10*H*, 1-H_b + 2-H_b + 6-H_b + 7-H_2 + 8-H + 9-H + 15-H_b + 16-H_b + 22-H_b), 0.90 (s, 3H, 19-H_3), 0.81 (d, $J = 6.4$ Hz, 3H, 21-H_3), 0.72 (s, 3H, 18-H_3) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 179.6$ (C-24), 170.8 (C-25), 170.7 (C-27), 76.1 (C-12), 74.4 (C-3), 49.6 (C-14), 47.7 (C-17), 45.2 (C-13), 42.0 (C-5), 35.8 (C-9), 34.9 (C-1), 34.8 (C-8), 34.6 (C-20), 34.2 (C-10), 32.4 (C-4), 31.0 (C-23), 30.8 (C-22), 27.5 (C-16), 27.0 (C-6), 26.8 (C-2), 26.0 (C-7), 25.8 (C-11), 23.6 (C-15), 23.2 (C-19), 21.6 (C-26), 21.5 (C-28), 17.7 (C-21), 12.6 (C-18) ppm; MS (ESI, MeOH): m/z = 357.20 (18 %, $[M + H–2HOAc]^+$), 494.20 (38 %, $[M + NH_4]^+$), 499.27 (100 %, $[M + Na]^+$), 915.53 (23 %, $[2M\text{-}HOAc + Na]^+$).

4.5.3. 3α,7α-Bis(acetyloxy)-5β-cholan-24-oic acid (6)

Following GP1 from chenodeoxycholic acid (**5**, 3.0 g, 7.6 mmol) **6** (2.6 g, 86 %) was obtained as a white solid; m.p. 100 °C (lit.: [33] 99 °C); $R_F = 0.28$ (*n*-hexane/ethyl acetate, 2:1); $[\alpha]_D = +15.6°$ (*c* 0.309, $CHCl_3$) [lit.: [34] $[\alpha]_D = +13.2°$ (*c* 1.16, $CHCl_3$)]; IR (ATR): $\tilde{\nu} =$ 450*w*, 609*w*, 888*vw*, 938*w*, 967*w*, 1023 *m*, 1067*w*, 1140*w*, 1233 *s*, 1245 *s*, 1363 *m*, 1376 *m*, 1439*w*, 1732 *s*, 2870*w*, 2938 *m* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 4.97 – 4.78$ (m, 1H, 7-H), 4.59 (dt, $J = 11.4$, 6.9 Hz, 1H, 3-H), 2.33 (dddd, $J = 54.0$, 15.8, 9.8, 5.8 Hz, 2H, 23-H_2), 2.05 (s, 3H, 26-H_3), 2.04 – 2.01 (m, 4H, 28-H_3 + 6-H_a), 2.01 – 1.65 (m, 6H, 2-H_a + 4-H_a + 8-H + 12-H_a + 16-H_a + 22-H_a), 1.65 – 1.40 (m, 5H, 2-H_b + 5-H + 9-H + 11-H_a + 20-H), 1.40 – 0.98 (m, 8H, 11-H_b + 12-H_b + 14-H + 15-H_2 + 16-H_b + 17-H + 22-H_b), 0.94 (s, 3H, 19-H_3), 0.93 (s, 3H, 21-H_3), 0.65 (s, 3H, 18-H_3) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 179.6$ (C-24), 170.8 (C-25), 170.6 (C-27), 74.3 (C-3), 71.4 (C-7), 55.9 (C-14), 50.6 (C-17), 42.9 (C-13), 41.1 (C-5), 39.7 (C-12), 38.1 (C-9), 35.4 (C-20), 35.1 (C-1), 34.9 (C-6), 34.8 (C-10), 34.2 (C-8), 31.5 (C-4), 31.0 (C-23), 30.9 (C-22), 28.2 (C-16), 26.9 (C-2), 23.7 (C-15), 22.8 (C-19), 21.7 (C-26), 21.6 (C-28), 20.8 (C-11), 18.5 (C-21), 11.9 (C-18) ppm; MS (ESI, MeOH): m/z = 552.13 (20 %, $[M + NH_4]^+$), 557.27 (100 %, $[M + Na]^+$), 573.27 (7 %, $[M + K]^+$).

4.5.4. 3α,7β-Bis(acetyloxy)-5β-cholan-24-oic acid (8)

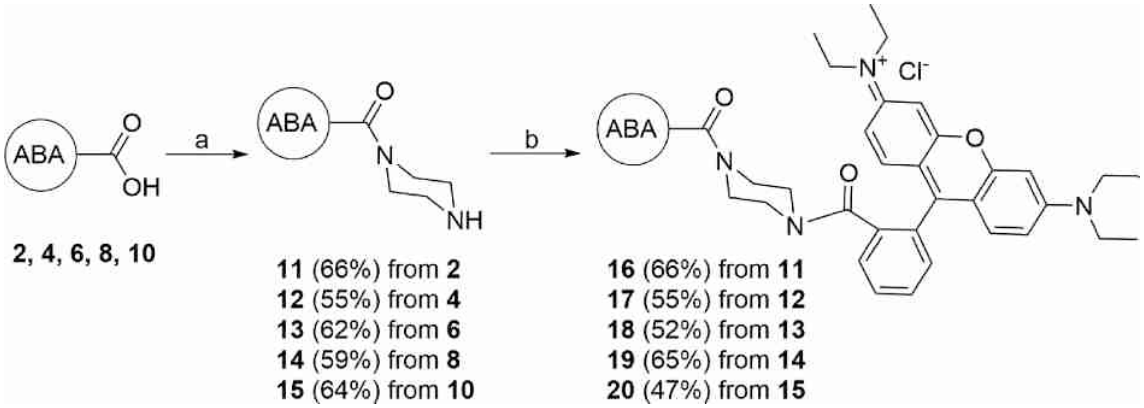
Following GP1 from ursodeoxycholic acid (**7**, 3.0 g, 7.6 mmol) **8** (2.4 g, 86 %) was obtained as a white solid; m.p. 110 °C (lit.: [35] 98–102 °C); $R_F = 0.31$ (hexane/ethyl acetate, 2:1); $[\alpha]_D = +53.8°$ (*c* 0.231, $CHCl_3$); IR (ATR): $\tilde{\nu} =$ 2946 *m*, 2872*w*, 1732 *s*, 1707 *m*, 1451*w*, 1381*w*, 1364 *m*, 1236*vs*, 1167*w*, 1123*w*, 1094*w*, 1075*w*, 1043 *m*, 1021 *m*, 987*w*, 977*w*, 956*w*, 932*w*, 925*w*, 908*w*, 892*w*, 864*w*, 853*w*, 800*vw*, 774*w*, 693*vw*, 666*vw*, 620*w*, 608*w*, 593*vw*, 568*vw*, 544*vw*, 530*vw*, 515*vw*, 504*vw*, 471*w*, 453*vw*, 416*vw* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 4.76$ (td, $J = 11.1$, 5.3 Hz, 1H, 7-H), 4.66 (tt, $J = 10.8$, 5.1 Hz, 1H, 3-H), 2.38 (ddd, $J = 15.5$, 10.0, 5.2 Hz, 1H, 23-H_a), 2.25 (ddd, $J = 15.9$, 9.5, 6.7 Hz, 1H, 23-H_b), 2.01 (s, 3H, 26-H_3), 2.01 – 1.98 (m, 1H,12-H_a), 1.97 (s, 3H, 28-H_3), 1.84 – 1.66 (m, 8H, 1-H_2 + 2-H_a + 4-H_a + 6-H_a + 8-H + 16-H_a + 22-H_a), 1.66 – 1.59 (m, 1H, 6-H_b), 1.58 – 1.49 (m, 2H, 5-H + 9-H), 1.48 – 1.12 (m, 10*H*, 2-H_b + 11-H_2 + 12-H_2 + 14-H + 15-H_2 + 16-H_b + 22-H_b), 1.10 – 0.99 (m, 2H, 4-H_b + 17-H), 0.96 (s, 3H, 19-H_3), 0.92 (d, $J = 6.5$ Hz, 3H, 21-H_3), 0.67 (s, 3H, 18-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): $\delta = 179.7$ (C-24), 170.6 (C-27), 170.6 (C-25), 73.6 (C-7), 73.6 (C-3), 55.2 (C-14), 55.0 (C-17), 43.6 (C-13), 42.0 (C-5), 40.0 (C-8), 39.9 (C-12), 39.4 (C-9), 35.2 (C-20), 34.5 (C-4), 34.0 (C-10), 32.9 (C-1 + C6), 30.9 (C-23), 30.7 (C-22), 28.4 (C-16), 26.4 (C-2), 25.6 (C-15), 23.2 (C-19), 21.8 (C-28), 21.4 (C-26), 21.2 (C-11), 18.3 (C-21), 12.1 (C-18) ppm; MS (ESI, $MeOH/CHCl_3$, 4:1, positive): $m/z = 499.1$ (100 %, $[M + Na]^+$); MS (ESI, $MeOH/CHCl_3$, 4:1): $m/z = 475.2$ (80 %, $[M–H]^-$), 951.3 (100 %, $[2\ M–H]^-$).

4.5.5. 3α-Acetyloxy-5β-cholan-24-oic acid (10)

Following GP1 from lithocholic acid (**9**, 3.0 g, 8.0 mmol) **10** (2.1 g, 70 %) was obtained as a white solid; m.p. 172 °C (lit.: [36] 170–171 °C); $R_F = 0.32$ (*n*-hexane/ethyl acetate, 2:1); $[\alpha]_D = +44.0°$ (*c* 0.310, $CHCl_3$) [lit.: [37] $[\alpha]_D = +41.6°$ (*c* 0.01, $CHCl_3$)]; IR (ATR): $\tilde{\nu} =$ 419*vw*, 449*vw*, 483*vw*, 616 *m*, 661*vw*, 887*vw*, 906*vw*, 931*vw*, 949*vw*, 981*vw*, 1026 *m*, 1065*vw*, 1096*vw*, 1164*w*, 1240 *s*, 1362 *m*, 1379 *m*, 1448 *m*, 1706 *s*, 1735 *s*, 2866 *m*, 2931 *m* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 4.71$ (td, $J =$ 11.3, 5.6 Hz, 1H, 3-H), 2.32 (dddd, $J = 56.0$, 15.8, 9.9, 5.8 Hz, 2H, 23-H_2), 2.03 (s, 3H, 26-H_3), 2.00 – 1.73 (m, 6H, 1-H_a + 4-H_a + 6-H_a + 12-H_a + 16-H_a + 22-H_a), 1.73 – 1.49 (m, 3H, 2-H_a + 4-H_b + 15-H_a), 1.48 – 0.96 (m, 17H, 1-H_b + 2-H_b + 5-H + 6-H_b + 7-H_2 + 8-H + 9-H + 11-H_2 + 12-H_b + 14-H + 15-H_b + 16-H_b + 17-H + 20-H + 22-H_b), 0.93 (d, $J =$ 2.0 Hz, 3H, 19-H_3), 0.91 (s, 3H, 21-H_3), 0.65 (s, 3H, 18-H_3) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 180.0$ (C-24), 170.9 (C-25), 74.6 (C-3), 56.7 (C-14), 56.2 (C-17), 42.9 (C-13), 42.1 (C-5), 40.6 (C-9), 40.3 (C-20), 36.0 (C-12), 35.5 (C-8), 35.2 (C-1), 34.7 (C-10), 32.4 (C-4), 31.1 (C-23), 30.9 (C-22), 28.3 (C-16), 27.2 (C-6), 26.8 (C-2), 26.5 (C-7), 24.3 (C-15), 23.5 (C-19), 21.6 (C-26), 21.0 (C-11), 18.4 (C-21), 12.2 (C-18) ppm; MS (ESI, MeOH): $m/z = 359.20$ (18 %, $[M\text{-}HOAc + H]^+$), 436.20 (17 %, $[M + NH_4]^+$), 441.27 (100 %, $[M + Na]^+$), 799.53 (32 %, $[2\ M\text{-}HOAc + Na]^+$), 859.33 (15 %, $[2\ M + Na]^+$), 875.40 (40 %, $[2\ M + Na]^+$).

4.5.6. 3α,7α,12α-Tris(acetyloxy)-24-(1-piperazinyl)-5β-cholan-24-one (11)

Following GP2 from **2** (500 mg, 1.0 mmol) **11** (330 mg, 66 %) was obtained as a white solid; m.p. 136 °C; $R_F = 0.10$ ($CHCl_3$/MeOH, 95:5); $[\alpha]_D = +21.1°$ (*c* 0.131, $CHCl_3$); IR (ATR): $\tilde{\nu} =$ 450*w*, 608*w*, 751*w*, 965*w*, 1023 *m*, 1151*vw*, 1233 *s*, 1365 *m*, 1376 *m*, 1435 *m*, 1645 *m*, 1730 *m*,



Scheme 2. Synthesis of the acetylated bile acid (ABA) conjugates 16–20: a) cat. DMF, $(COCl)_2$, DCM, 0 °C → 25 °C, 2 h → piperazine, TEA, DCM, −21 °C → 25 °C, 24 h; b) rhodamine B, HOBt, EDC·HCl, DMF, 25 °C, 24 h.

Table 1
Cytotoxicity of synthesized compounds and rhodamine B (Rhd B) 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), FaDu (hypopharyngeal carcinoma); non-malignant: NIH 3 T3 (fibroblasts); n.s. not soluble; n.d. not determined; S (selectivity) calculated $S = EC_{50\ of\ NIH\ 3T3} / EC_{50\ of\ tumor\ cell\ line}$. Positive control: doxorubicin (DX).

	A375	HT29	MCF-7	A2780	FaDu	NIH 3T3
Rhd B	>30	>30	>30	>30	>30	>30
2	23.7 ± 3.3	>30	28 ± 4	23.8 ± 2.1	>30	>30
4	>30	>30	>30	24.8 ± 3.1	>30	>30
6	>30	>30	>30	>30	>30	>30
8	>30	>30	>30	>30	>30	>30
10	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
11	23.7 ± 2.6 (S = 0.94)	14.2 ± 1.5 (S = 1.57)	21.3 ± 2.5 (S = 1.04)	17.9 ± 2.2 (S = 1.25)	23.3 ± 3.6 (S = 0.96)	22.3 ± 2.7
12	4.1 ± 0.3 (S = 0.85)	3.2 ± 0.3 (S = 1.09)	4.1 ± 0.2 (S = 0.85)	3.4 ± 0.2 (S = 1.03)	3.0 ± 0.4 (S = 1.17)	3.5 ± 0.3
13	4.5 ± 0.4 (S = 0.87)	4.8 ± 0.4 (S = 0.81)	4.6 ± 0.3 (S = 0.85)	4.2 ± 0.5 (S = 0.93)	3.8 ± 0.6 (S = 1.03)	3.9 ± 0.4
14	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
15	6.61 ± 0.3 (S = 0.55)	3.59 ± 0.1 (S = 1.01)	4.12 ± 0.3 (S = 0.88)	5.50 ± 0.4 (S = 0.66)	6.67 ± 0.2 (S = 0.54)	3.62 ± 0.4
16	1.0 ± 0.1 (S = 1.6)	1.0 ± 0.1 (S = 1.6)	0.49 ± 0.05 (S = 3.27)	0.6 ± 0.1 (S = 2.67)	1.2 ± 0.3 (S = 1.33)	1.6 ± 0.1
17	0.66 ± 0.05 (S = 1.11)	0.68 ± 0.04 (S = 1.07)	0.32 ± 0.03 (S = 2.28)	0.36 ± 0.04 (S = 2.03)	0.4 ± 0.2 (S = 1.33)	0.73 ± 0.02
18	0.7 ± 0.1 (S = 0.93)	0.6 ± 0.1 (S = 1.08)	0.207 ± 0.004 (S = 3.14)	0.24 ± 0.03 (S = 2.71)	0.5 ± 0.1 (S = 1.3)	0.65 ± 0.09
19	0.42 ± 0.04 (S = 1.05)	0.39 ± 0.07 (S = 1.13)	0.20 ± 0.01 (S = 2.2)	0.214 ± 0.002 (S = 2.06)	0.5 ± 0.2 (S = 0.88)	0.44 ± 0.08
20	0.73 ± 0.05 (S = 1.18)	0.71 ± 0.03 (S = 1.21)	0.32 ± 0.03 (S = 2.69)	0.41 ± 0.06 (S = 2.1)	0.8 ± 0.1 (S = 1.08)	0.86 ± 0.03
DX	n.d.	0.91 ± 0.01 (S = 0.45)	1.10 ± 0.3 (S = 0.37)	0.01 ± 0.01 (S = 41.0)	n.d.	0.41 ± 0.07

2872*w*, 2939 *m* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 5.09$ (s, 1H, 12-H), 4.90 (d, $J = 2.7$ Hz, 1H, 7-H), 4.61 – 4.51 (m, 1H, 3-H), 3.64 – 3.36 (m, 4H, 32-H_2 + 32'–H_2), 2.87 – 2.78 (m, 4H, 31-H_2 + 31'–H_2), 2.40 – 2.15 (m, 2H, 23-H_2), 2.12 (s, 3H, 26-H_3), 2.07 (s, 3H, 30-H_3), 2.03 (s, 4H, 28-H_3 + 9-H), 2.01 – 1.81 (m, 4H, 1-H_a + 6-H_a + 14-H + 16-H_a), 1.81 – 1.55 (m, 8H, 1-H_a + 2-H_a + 4-H_a + 6-H_b + 8-H + 11-H_a + 17-H + 22-H_a), 1.54 – 0.96 (m, 8H, 2-H_b + 4-H_b + 5-H + 11-H_b + 15-H_2 + 16-H_b + 20-H + 22-H_b), 0.90 (s, 3H, 19-H_3), 0.82 (d, $J = 6.6$ Hz, 3H, 21-H_3), 0.72 (s, 3H, 18-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): $\delta = 172.0$ (C-24), 170.7 (C-25), 170.7 (C-27), 170.5 (C-29), 75.6 (C-12), 74.2 (C-3), 70.8 (C-7), 47.8 (C-17), 47.0 (C-31), 46.5 (C-31'), 46.0 (C-32), 45.3 (C-13), 43.5 (C-14), 42.7 (C-32'), 41.1 (C-5), 37.9 (C-8), 35.2 (C-20), 34.8 (C-1), 34.8 (C-4), 34.5 (C-10), 31.4 (C-6), 31.4 (C-22), 30.5 (C-23), 29.0 (C-9), 27.4 (C-16), 27.0 (C-2), 25.7 (C-11), 23.0 (C-15), 22.7 (C-19), 21.7 (C-30), 21.6 (C-28), 21.6 (C-26), 17.9 (C-21), 12.4 (C-18) ppm; MS (ESI, MeOH): *m/z* = 423.40 (8 %, $[M\text{-}3HOAc + H]^+$), 483.33 (12 %, $[M\text{-}2HOAc + H]^+$), 543.27 (12 %, $[M\text{-}HOAc + H]^+$), 603.27 (100 %, $[M + H]^+$), 615.40 (32 %, $[M + C + H]^+$), 633.20 (35 %, $[M + HCHO + H]^+$); analysis calcd for $C_{34}H_{54}N_2O_7$ (602.81): C 67.74, H 9.03, N 4.64; found: C 67.51, H 9.30, N 4.42.

4.5.7. 3α,12α-Bis(acetyloxy)-24-(1-piperazinyl)-5β-cholan24-one (12)

Following GP2 from **4** (500 mg, 1.0 mmol) **12** (273 mg, 55 %) was obtained as a white solid [29]; m.p. 87 °C; $R_F = 0.38$ ($CHCl_3$/MeOH, 9:1); $[\alpha]_D = +91.1°$ (*c* 0.297, $CHCl_3$); IR (ATR): $\tilde{\nu} = 497vw$, 608*w*, 617*w*, 661*vw*, 755*vw*, 887*w*, 971*w*, 1025 *s*, 1090*w*, 1161*w*, 1195*w*, 1241*vs*, 1319*w*, 1363 *m*, 1377 *m*, 1434 *m*, 1446 *m*, 1644 *m*, 1732 *m*, 2867*w*, 2938*w* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 5.09$ (t, $J = 2.9$ Hz, 1H, 12-H), 4.70 (ddt, $J = 16.0$, 10.8, 4.6 Hz, 1H, 3-H), 3.80 – 3.57 (m, 4H, 29-H_2 + 29'–H_2), 3.13 – 3.01 (m, 4H, 30-H_2 + 30'–H_2), 2.28 (dddd, $J = 64.6$, 15.4, 10.3, 5.3 Hz, 2H, 23-H_2), 2.10 (s, 3H, 28-H_3), 2.03 (s, 3H, 26-H_3), 1.94 – 1.74 (m, 4H, 6-H_a + 16-H_a + 22-H_2), 1.74 – 1.51 (m, 8H, 1-H_a + 2-H_a + 4-H_2 + 8-H + 14-H + 15-H_a + 17-H), 1.50 – 0.94 (m, 12H, 1-H_b + 2-H_b + 5-H + 6-H_b + 7-H_2 + 9-H + 11-H_2 + 15-H_b + 16-H_b + 20-H), 0.90 (s, 3H, 19-H_3), 0.82 (d, $J = 6.4$ Hz, 3H, 21-H_3), 0.72 (s, 3H, 18-H_3) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 172.3$ (C-24), 170.7 (C-25), 170.6 (C-27), 76.0 (C-12), 74.3 (C-3), 49.6 (C-14), 48.0 (C-17), 45.3 (C-29), 45.2 (C-13), 45.0 (C-29'), 44.7 (C-30), 42.0 (C-5), 40.7 (C-30'), 35.8 (C-9), 35.2 (C-20), 34.9 (C-1), 34.6 (C-8), 34.2 (C-10), 32.4 (C-4), 31.3 (C-22), 30.5 (C-23), 27.6 (C-16), 27.0 (C-6), 26.8 (C-2), 26.0 (C-7), 25.8 (C-11), 23.6 (C-15), 23.2 (C-19), 21.6 (C-26), 21.6 (C-28), 17.9 (C-21), 12.6 (C-18) ppm; MS (ESI, MeOH): *m/z* = 425.33 (15 %, $[M\text{-}2HOAc + H]^+$), 485.27 (38 %, $[M\text{-}HOAc + H]^+$), 545.27 (100 %, $[M + H]^+$); analysis calcd for $C_{32}H_{52}N_2O_5$ (544.78): C 70.55, H 9.62, N 5.14; found: C 70.39, H 9.95, N 4.97.

4.5.8. 3α,7α-Bis(acetyloxy) 24-(1-piperazinyl)-5β-cholan-24-one (13)

Following GP2 from **6** (500 mg, 1.1 mmol) **13** (310 mg, 62 %) was obtained as a white solid [29]; m.p. 85 °C; $R_F = 0.33$ ($CHCl_3$/MeOH, 9:1); $[\alpha]_D = +16.2°$ (*c* 0.324, $CHCl_3$); IR (ATR): $\tilde{\nu} = 419vw$, 449*w*, 553*vw*, 609*w*, 794*vw*, 888*w*, 938*w*, 968*w*, 1022 *m*, 1067*w*, 1140*w*, 1233 *s*, 1245 *s*, 1319 *m*, 1363 *m*, 1375 *m*, 1435 *m*, 1636 *m*, 1729 *s*, 2867*w*, 2938 *m* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 4.86$ (s, 1H, 7-H), 4.58 (dt, $J = 11.3$, 6.8 Hz, 1H, 3-H), 3.56 (d, $J = 60.6$ Hz, 4H, 29-H_2 + 29'–H_2), 2.93 (d, $J = 14.6$ Hz, 4H, 30-H_2 + 30'–H_2), 2.43 – 2.12 (m, 2H, 23-H_2), 2.04 (s, 3H, 28-H_3), 2.02 (s, 4H, 26-H_3 + 6-H_a), 2.01 – 1.90 (m, 2H, 12-H_a + 4-H_a), 1.90 – 1.66 (m, 5H, 1-H_a + 2-H_a + 16-H_a + 20-H + 22-H_a), 1.65 – 0.99 (m, 16H, 1-H_b + 2-H_b + 4-H_b + 5-H + 6-H_b + 8-H + 9-H + 11-H_2 + 12-H_b + 14-H + 15-H_2 + 16-H_b + 17-H + 22-H_b), 0.94 (s, 3H, 19-H_3), 0.92 (s, 3H, 21-H_3), 0.64 (s, 3H, 18-H_3) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 172.2$ (C-24), 170.8 (C-25), 170.6 (C-27), 74.3 (C-3), 71.4 (C-7), 56.0 (C-17), 50.6 (C-14), 46.0 (C-30), 45.8 (C-30'), 45.4 (C-29), 42.9 (C-13), 41.8 (C-29'), 41.1 (C-5), 39.7 (C-12), 38.1 (C-8), 35.8 (C-9), 35.0 (C-1), 34.9 (C-6), 34.8 (C-10), 34.2 (C-20), 31.5 (C-22), 31.5 (C-4), 30.4 (C-23), 28.3 (C-16), 26.9 (C-2), 23.7 (C-15), 22.8 (C-19), 21.7 (C-28), 21.6 (C-26), 20.8 (C-11), 18.6 (C-21), 11.9 (C-18) ppm; MS (ESI, MeOH): *m/z* = 425.33 (17 %, $[M\text{-}2HOAc + H]^+$), 485.27 (27 %, $[M\text{-}HOAc + H]^+$), 545.27 (100 %, $[M + H]^+$); analysis calcd for $C_{32}H_{52}N_2O_5$ (544.78): C 70.55, H 9.62, N 5.14; found: C 70.19, H 9.98, N 4.94.

4.5.9. 3α-Acetyloxy-24-(1-piperazinyl)-5β-cholan-24-one (14)

Following GP2 from **8** (500 mg, 1.2 mmol) **14** (330 mg, 59 %) was obtained as a white solid; m.p. 79 °C; $R_F = 0.10$ ($CHCl_3$/MeOH, 95:5); $[\alpha]_D = +39.5°$ (*c* 0.324, $CHCl_3$) [lit.: [38] $[\alpha]_D = +20.5°$ (*c* 0.98, $CHCl_3$)]; IR (ATR): $\tilde{\nu} = 483vw$, 557*w*, 614*w*, 792*w*, 887*w*, 1026 *m*, 1240 *s*, 1362 *m*, 1379 *m*, 1446 *m*, 1640 *m*, 1733 *m*, 2864 *m*, 2929 *m* cm^{-1}; ^1H

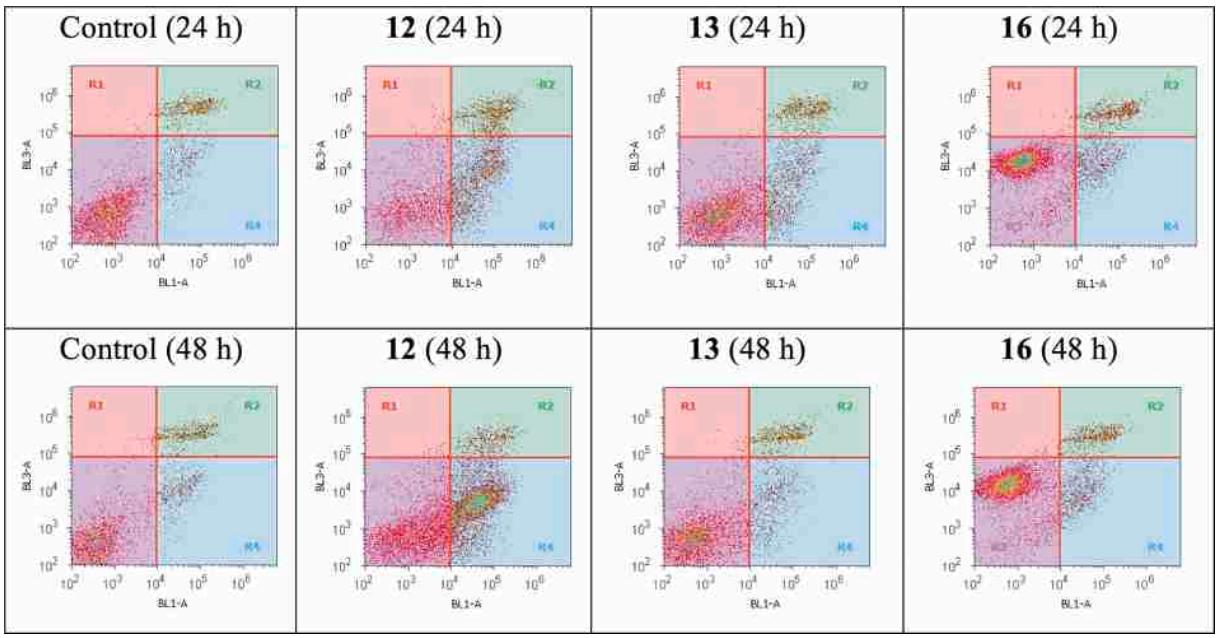


Fig. 2. Annexin V-FITC/PI assay plots. Representative examples of density plots determined by flow cytometry after 24 h and 48 h, respectively; R1: necrotic, R2: secondary necrotic/late-stage apoptotic, *R*3: vital, R4: apoptotic.

Table 2
Annexin V-FITC/PI assay average difference to control results tested for significance with T-test (red = $P > 0.05$; orange = $P < 0.05$; green = $P < 0.01$).

Incubation time	Necrotic			Secondary necrotic Late-stage apoptotic			vital			apoptotic		
	12	**13**	**16**	**12**	**13**	**16**	**12**	**13**	**16**	**12**	**13**	**16**
24 h	−3%	−3%	−1%	1 %	1 %	1 %	−24 %	−11 %	0 %	27 %	13 %	0 %
48 h	−3%	−2%	−1%	−5%	−3%	1 %	−26 %	−4%	−4%	34 %	9 %	4 %

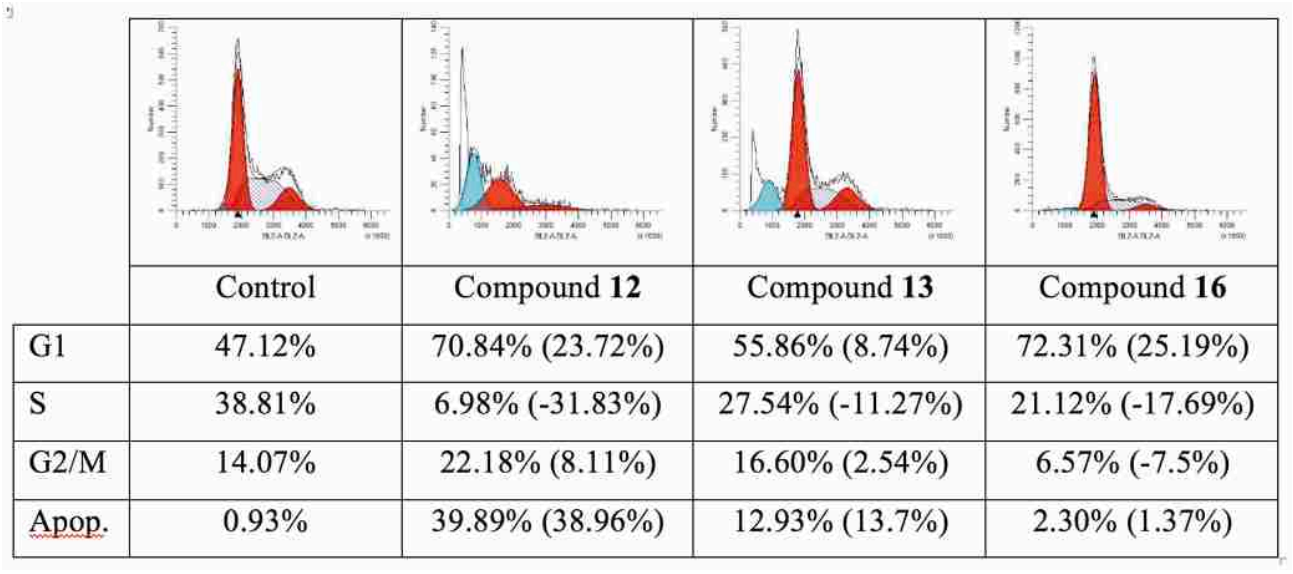
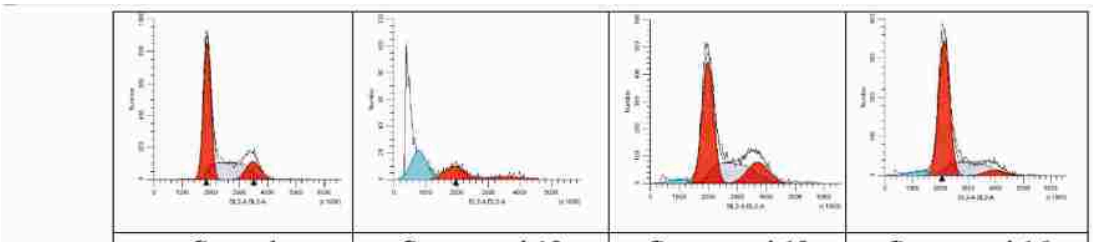


Fig. 3. Cell cycle analysis of cell line A2780 with compounds 12, 13, and 16 in comparison with a control group after an incubation of 24 h. Left red = G1; right red = G2/M; blue striped = S phase; light blue subG1/apoptosis. In brackets the difference to control is shown.

NMR (400 MHz, $CDCl_3$): $\delta = 4.71$ (d, $J = 4.9$ Hz, 1H, 3-H), 3.50 (d, $J = 60.9$ Hz, 4H, 27-H_2 + 27'–H_2), 2.93 – 2.76 (m, 4H, 28-H_2 + 28'–H_2), 2.44 – 2.11 (m, 2H, 23-H_2), 2.02 (s, 3H, 26-H_3), 2.00 – 1.48 (m, 9H, 1-H_a + 2-H_a + 4-H_2 + 6-H_a + 12-H_a + 15-H_a + 16-H_a + 22-H_a), 1.48 – 0.97 (m, 17H, 1-H_b + 2-H_b + 5-H + 6-H_b + 7-H_2 + 8-H + 9-H + 11-H_2 + 12-H_b + 14-H + 15-H_b + 16-H_b + 17-H + 20-H + 22-H_b), 0.94 (s, 3H, 19-H_3), 0.92 (s, 3H, 21-H_3), 0.64 (s, 3H, 18-H_3) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 172.3$ (C-24), 170.8 (C-25), 74.5 (C-3), 56.7 (C-14), 56.3 (C-17), 47.1 (C-27), 46.6 (C-27'), 46.1 (C-28), 42.9 (C-28'), 42.8 (C-13), 42.1 (C-5), 40.6 (C-9), 40.3 (C-12), 36.0 (C-8), 35.9 (C-20), 35.2 (C-1),



	Control	Compound **12**	Compound **13**	Compound **16**
G1	54.81%	64.26% (9.45%)	52.73% (-2.08%)	69.01% (14.2%)
S	31.59%	9.02% (-22.21%)	29.89% (-1.7%)	25.33% (-6.26%)
G2/M	13.60%	26.72% (13.12%)	17.38% (3.78%)	5.66% (-7.94%)
Apop.	0.30%	51.38% (51.08%)	3.00% (2.7%)	5.63% (5.33%)

Fig. 4. Cell cycle analysis of cell line A2780 with compounds 12, 13, and 16 compared to a control group after an incubation of 48 h. Left red = G1; right red = G2/M; blue striped = S phase; light blue subG1/apoptosis. In brackets the difference to control is shown.

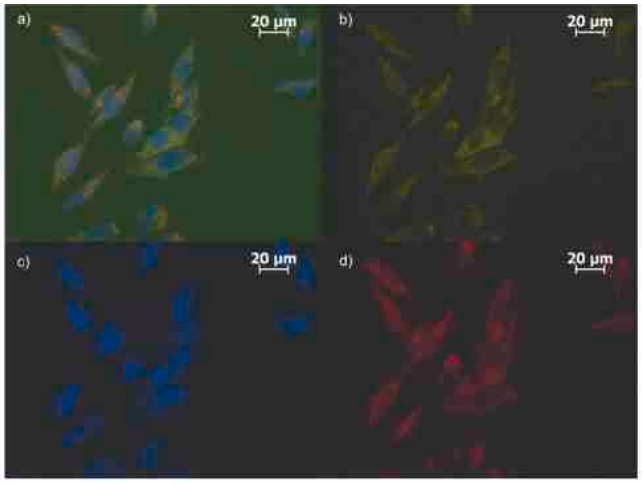


Fig. 5. Fluorescence images of A2780 tumor cells treated with 16, rhodamine 123 and Hoechst 33,342, respectively: a) merged imaged, b) rhodamine 123, c) Hoechst 33,342, d) compound 16.

34.7 (C-10), 32.4 (C-4), 31.6 (C-22), 30.5 (C-23), 28.4 (C-6), 27.2 (C-16), 26.8 (C-2), 26.5 (C-7), 24.4 (C-15), 23.5 (C-19), 21.6 (C-26), 21.0 (C-11), 18.7 (C-21), 12.2 (C-18) ppm; MS (ESI, MeOH): m/z = 427.37 (3 %, [M-HOAc + H]$^+$), 487.33 (100 %, [M + H]$^+$), 509.27 (2 %, [M + Na]$^+$), 973.33 (2 %, [2 M + H]$^+$); analysis calcd for $C_{30}H_{50}N_2O_3$ (486.73): C 74.03, H 10.35, N 5.76; found: C 73.76, H 10.59, N 5.47.

4.5.10. 3α,7β-Bis(acetyloxy)-24-(1-piperazinyl)- 5β-cholan-24-one (15)

Following GP2 from **10** (500 mg, 1.0 mmol) **15** (365 mg, 64 %) was obtained as a white solid; m.p. 94.3 °C; R_F = 0.20 ($CHCl_3$/MeOH/NH_4OH, 98:1.8:0.2); $[\alpha]_D$ = +48.2° (*c* 0.188, $CHCl_3$); IR (ATR): $\tilde{\nu}$ = 468*w*, 477*w*, 559*w*, 608 *m*, 693*w*, 799*w*, 891*w*, 956*w*, 1021 *s*, 1042 *m*, 1122 *w*, 1237*vs*, 1319*w*, 1364 *m*, 1380*w*, 1433 *m*, 1636 *m*, 1729 *s*, 2871*w*, 2944*w* cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): 4.75 (tt, J = 9.0, 4.1 Hz, 1H, 7-H), 3.71 – 3.45 (m, 5H, 3-H, 29-H_2 + 29′-H_2), 3.17 – 2.84 (m, 4H, 30-H_2 + 30′-H_2), 2.35 (ddt, J = 15.5, 10.6, 5.4 Hz, 1H, 23-H_a), 2.19 (ddt, J = 15.3, 10.5, 6.2 Hz, 1H, 23-H_b), 2.03 – 1.96 (m, 4H, 12-H_a + 26-H_3), 1.96 (s, 3H, 28-H_3), 1.86 – 1.57 (m, 9H, 1-H_a + 2-H_a + 4-H_2 + 6-H_2 + 8-H + 16-H_a + 22-H_a), 1.57 – 1.36 (m, 4H, 5-H + 9-H + 11-H_a + 20-H), 1.37 – 1.09 (m, 8H, 2-H_b + 11-H_b + 15-H_2 + 16-H_b + 17-H + 22-H_b), 1.09 – 0.99 (m, 2H, 1-H_b + 14-H), 0.99 – 0.89 (m, 6H, 19-H_3 + 21-H_3), 0.66 (s, 3H, 18-H_3) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 172.2 (C-24), 170.7 (C-27), 170.6 (C-25), 73.8 (C-7), 71.2 (C-3), 55.2 (C-17), 54.9 (C-14), 45.5 (C-30), 45.1 (C-30′), 43.6 (C-13), 42.2 (C-5), 41.4 (C-29 + C-29′), 40.0 (C-8), 39.9 (C-12), 39.4 (C-9), 37.1 (C-4), 35.4 (C-20), 34.8 (C-1), 33.9 (C-10), 33.0 (C-6), 31.3 (C-22), 30.1 (C-23), 28.5 (C-16), 26.4 (C-2), 25.6 (C-15), 23.2 (C-19), 21.8 (C-28), 21.4 (C-26), 21.2 (C-11), 18.6 (C-21), 12.1 (C-18) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): m/z = 545.1 (74 %, [M + H]$^+$), 567.1 (100 %, [M + Na]$^+$); analysis calcd for $C_{32}H_{52}N_2O_5$ (544.78): C 70.65, H 9.62, N 5.14; found: C 70.39, H 9.95, N 4.97.

4.5.11. 9-[2-[[4-(3α,7α,12α-Bis(acetyloxy)-5β-cholan-24-oyl)-1-piperazinyl]carbonyl] phenyl]-3,6-bis(diethylamino]-xanthylium chloride (16)

Following GP3, from **11** (100 mg, 166 µmol), rhodamine B (88 mg, 182 µmol), 1-hydroxybenztriazole hydrate (30.5 mg, 199 µmol) and *N*-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (39 mg, 199 µmol) **16** (112 mg, 66 %) was obtained as a purple solid; m.p. 118 °C; R_F = 0.55 ($CHCl_3$/MeOH, 9:1); IR (ATR): $\tilde{\nu}$ = 448*w*, 498*w*, 583*w*, 607*w*, 666*w*, 683 *m*, 772 *m*, 798 *m*, 922*w*, 938*w*, 966*w*, 1022 *s*, 1073 *s*, 1094 *m*, 1132 *m*, 1160 *m*, 1180 *s*, 1197 *m*, 1245*vs*, 1338 *s*, 1363 *m*, 1378 *m*, 1394 *m*, 1414 *m*,1466 *m*, 1529*w*, 1588 *s*, 1634 *m*, 1644 *m*, 1728 *s*, 2869*w*, 2925 *m*, 2959 *m* cm^{-1}; UV/vis (MeOH): λ^{max} (log ε) = 562 (4.79), 355 (3.61), 309 (3.93), 282 (4.00), 262 (4.29) nm; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.77 – 7.64 (m, 3H, 35-H + 37-H + 38-H), 7.58 – 7.46 (m, 1H, 36-H), 7.18 – 7.03 (m, 4H, 42-H + 42′-H), 7.03 – 6.89 (m, 2H, 45-H + 45′+H), 4.95 (d, J = 4.3, 1H, 12-H), 4.77 (s, 1H, 3-H), 4.44 (tt, J = 11.1, 4.2, 1H, 7-H), 3.70 – 3.57 (m, 8H, 47-H_2 + 47′-H_2 + 47″–H_2 + 47‴–H_2), 3.45 – 3.16 (m, 8H, 31-H_2 + 31′-H_2 + 32-H_2 + 32′-H_2), 2.33 – 2.08 (m, 2H, 23-H_2), 2.07 – 2.02 (m, 3H, 26-H_3), 1.98 (s, 3H, 30-H_3), 1.97 – 1.95 (m, 4H, 6-H_a + 28-H_3), 2.01 – 1.86 (m, 2H, 1-H_a + 9-H), 1.85 – 1.71 (m, 2H, 14-H + 16-H_a), 1.72 – 1.53 (m, 6H, 2-H_a + 4-H_a + 8-H + 11-H_a + 17-H + 22-H_a), 1.53 – 1.39 (m, 4H, 1-H_b + 5-H + 6-H_b + 11-H_b), 1.38 – 1.24 (m, 3H, 2-H_b + 15-H_a + 20-H), 1.24 – 1.14 (m, 14H, 16-H_b + 22-H_b + 48-H_3 + 48′-H_3 + 48″–H_3 + 48‴–H_3), 1.13 – 0.93 (m, 2H, 4-H_b + 15-H_b), 0.93 – 0.82 (m, 3H, 19-H_3), 0.80 – 0.69 (m, 3H, 21-H_3), 0.70 – 0.64 (m, 3H, 18-H_3) ppm; ^{13}C NMR (101 MHz, DMSO-d_6): δ = 175.3 (C-24), 170.3 (C-27), 170.2 (C-25), 170.1 (C-29), 166.8 (C-33), 157.5 (C-46 + C-46′), 156.1 (C-40), 155.6 (C-44 + C-44′), 135.7 (C-39), 132.2 (C-42 + C-42′), 131.2 (C-34), 130.9 (C-36), 130.5 (C-35 + C-38), 128.0 (C-37), 114.7 (C-43 + C-32′), 113.5 (C-41 + C-41′), 96.4 (C-45 + C-45′), 75.0 (C-12), 73.8 (C-7), 70.6 (C-3), 47.6 (C-17), 45.8 (C-47 + C-47′+C-47″+C-47‴), 45.1 (C-13 + C-31 + C-31′), 43.5 (C-14), 41.3 (C-32 + C-32′), 40.6 (C-5), 37.3 (C-8), 34.8 (C-1), 34.7 (C-20), 34.4 (C-10), 34.4 (C-4), 31.2 (C-6), 31.1 (C-22), 29.9 (C-23), 28.8 (C-9), 27.1 (C-

16), 26.9 (C-2), 25.5 (C-11), 22.7 (C-15), 22.6 (C-19), 21.7 (C-30), 21.6 (C-28), 21.5 (C-26), 17.9 (C-21), 12.9 (C-48 + C-48′+C-48″+C-48‴), 12.4 (C-18) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 1027.9 (50 %, [M-Cl]$^+$), 1028.9 (40 %, [M-Cl + H]$^+$); analysis calcd for $C_{62}H_{83}N_4O_9Cl$ (1063.82): C 70.00, H 7.86, N 5.27; found: C 69.73, H 8.01, N 4.98.

4.5.12. 9-[2-[[4-(3α,12α-Bis(acetyloxy)-5β-cholan-24-oyl)-1-piperazinyl]carbonyl] phenyl]-3,6-bis(diethylamino]-xanthylium chloride (17)

Following GP3, from **12** (100 mg, 184 μmol), rhodamine B (97 mg, 202 μmol), 1-hydroxybenztriazole hydrate (34 mg, 220 μmol) and *N*-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (43 mg, 220 μmol) **17** (102 mg, 55 %) was obtained as a purple solid; m.p. 156 °C; R_F = 0.56 ($CHCl_3$/MeOH, 9:1); IR (ATR): $\tilde{\nu}$ = 498*w*, 543*w*, 578*w*, 619*w*, 666*w*, 683 *m*, 747*w*, 784*w*, 823*w*, 922*w*, 972*w*, 1007 *m*, 1026 *m*, 1074 *m*, 1132 *m*, 1160 *m*, 1179*vs*, 1195 *m*, 1244*vs*, 1273 *m*, 1336 *s*, 1380 *m*, 1394 *m*, 1412 *s*, 1448 *m*, 1466 *m*, 1504 *m*, 1507 *m*, 1528*w*, 1556*w*, 1587*vs*, 1632 *m*, 1730 *m*, 2868*w*, 2934*w* cm^{-1}; UV/vis (MeOH): λ^{max} (log ε) = 562 (4.91), 357 (3.76), 308 (4.10), 281 (4.14), 261 (4.44) nm; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.78 – 7.65 (m, 3H, 33-H + 35-H + 36-H), 7.51 (ddd, *J* = 9.3, 5.6, 1.8, 1H, 34-H), 7.19 – 7.04 (m, 4H, 40-H + 40′-H + 41-H + 41′-H), 6.93 (d, *J* = 2.3, 2H, 43-H + 43′-H), 4.94 (d, *J* = 4.1, 1H, 12-H), 4.56 (td, *J* = 11.1, 5.7, 1H, 3-H), 3.73 – 3.59 (m, 8H, 45-H_2 + 45′-H_2 + 45″–H_2 + 45‴–H_2), 3.49 – 3.18 (m, 8H, 29-H_2 + 29′-H_2 + 30-H_2 + 30′-H_2), 2.32 – 2.08 (m, 2H, 23-H_2), 2.01 (s, 3H, 28-H_3), 1.95 (s, 3H, 26-H_3), 1.84 – 1.64 (m, 3H, 4-H_a + 6-H_a + 16-H_a), 1.65 – 1.49 (m, 8H, 1-H_a + 2-H_a + 8-H + 11-H_a + 14-H + 15-H_a + 17-H + 22-H_a), 1.49 – 1.32 (m, 5H, 4-H_b + 5-H + 7-H_a + 9-H + 11-H_b), 1.31 – 1.23 (m, 1H, 20-H), 1.24 – 1.15 (m, 15H, 2-H_b + 6-H_b-16-H_b + 46-H_3 + 46′-H_3 + 46″–H_3 + 46‴–H_3), 1.15 – 0.93 (m, 4H, 1-H_b + 7-H_b + 15-H_b + 22-H_b), 0.86 (s, 3H, 19-H_3), 0.76 – 0.63 (m, 3H, 21-H_3), 0.65 (s, 3H, 18-H_3) ppm; ^{13}C NMR (101 MHz, DMSO-d_6): δ = 171.5 (C-24), 170.2 (C-25), 170.1 (C-27), 167.0 (C-31), 157.5 (C-44 + C-44′), 156.1 (C-38), 155.6 (C-42 + C-42′), 135.7 (C-37), 132.2 (C-40 + C40′), 131.2 (C-32), 130.9 (C-34), 130.3 (C-33), 130.2 (C-36), 128.0 (C-35), 114.7 (C-41 + C-41′), 113.5 (C-39 + C-39′), 96.4 (C-43 + C-43′), 75.4 (C-12), 73.8 (C-3), 49.5 (C-14), 47.7 (C-17), 47.3 (C-29 + C29′), 45.8 (C-45 + C-45′+C-45″+C-45‴), 45.0 (C-13), 41.8 (C-30 + C-30′), 41.5 (C-5), 35.5 (C-9), 34.8 (C-20), 34.7 (C-1), 34.3 (C-8), 34.0 (C-10), 32.3 (C-4), 31.1 (C-22), 29.8 (C-23), 27.2 (C-6), 26.9 (C-16), 26.6 (C-2), 26.0 (C-7), 25.6 (C-11), 23.5 (C-15), 23.2 (C-19), 21.6 (C-26), 21.4 (C-28), 17.9 (C-21), 12.9 (C-46 + C-46′+C-46″+C-46‴), 12.6 (C-18) ppm; MS (ESI, propan-2-ol): *m/z* = 970.2 (100 %, [M-Cl]$^+$), 971.3 (70 %, [M-Cl + H]$^+$); analysis calcd for $C_{60}H_{81}N_4O_7Cl$ (1005.78): C 71.65, H 8.12, N 5.57; found: C 71.43, H 8.39, N 5.26.

4.5.13. 9-[2-[[4-(3α,7α-Bis(acetyloxy)-5β-cholan-24-oyl)-1-piperazinyl] carbonyl]phenyl]-3,6-bis(diethylamino]-xanthylium chloride (18)

Following GP3, from **13** (100 mg, 184 μmol), rhodamine B (97 mg, 202 μmol), 1-hydroxybenztriazole hydrate (34 mg, 220 μmol) and *N*-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (43 mg, 220 μmol) **18** (92 mg, 52 %) was obtained as a purple solid; m.p. 144 °C; R_F = 0.53 ($CHCl_3$/MeOH, 9:1); IR (ATR): $\tilde{\nu}$ = 484*w*. 490w, 498*w*, 546*w*, 580*w*, 611*w*, 666*w*, 683 *m*, 760*w*, 822*w*, 922*w*, 970 *m*, 979 *m*, 1006 *s*, 1024 *s*, 1049 *m*, 1072 *m*, 1133 *m*, 1160 *m*, 1179*vs*, 1245 *s*, 1273 *s*, 1337 *s*, 1378 *m*, 1394 *m*, 1413 *s*, 1448 *m*, 1467 *m*, 1481 *m*, 1529 *m*, 1558*w*, 1587*vs*, 1630 *m*, 1726 *m*, 2870*w*, 2936*w*, 3402*w* cm^{-1}; UV/vis (MeOH): λ^{max} (log ε) = 560 (4.87), 355 (3.71), 309 (4.04), 280 (4.06), 261 (4.39) nm; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 7.77 – 7.64 (m, 3H, 33-H + 35-H + 36-H), 7.54 – 7.46 (m, 1H, 34-H), 7.16 – 7.05 (m, 4H, 40-H + 40′-H + 41-H + 41′-H), 6.92 (d, J = 2.3, 2H, 43-H + 43′-H), 4.77 – 4.72 (m, 1H, 7-H), 4.50 – 4.40 (m, 1H, 3-H), 3.68 – 3.58 (m, 8H, 45-H_2 + 45′-H_2 + 45″–H_2 + 45‴–H_2), 3.44 – 3.17 (m, 8H, 29-H_2 + 29′-H_2 + 30-H_2 + 30′-H_2), 2.26 (ddd, J = 15.2, 10.0, 5.3, 1H, 23-H_a), 2.20 – 2.10 (m, 1H, 23-H_b), 1.96 (s, 3H, 28-H_3), 1.95 (s, 3H, 26-H_3), 2.00 – 1.86 (m, 3H, 4-H_a + 6-H_a + 12-H_a), 1.82 – 1.65 (m, 3H, 1-H_a + 16-H_a + 20-H), 1.64 – 1.53 (m, 3H, 2-H_a + 8-H + 22-H_a), 1.49 (d, J = 12.2, 1H, 6-H), 1.45 – 1.38 (m, 3H, 4-H_b + 5-H + 11-H_a), 1.38 – 1.21 (m, 4H, 2-H_b + 9-H + 14-H + 15-H_a), 1.18 (t, J = 7.1, 12H, 46-H_3 + 46′-H_3 + 46″–H_3 + 46‴–H_3), 1.21 – 1.14 (m, 2H, 11-H_b + 16-H_b), 1.15 – 1.06 (m, 3H, 12-H_b + 17-H + 22-H_b), 1.06 – 0.97 (m, 2H, 1-H_b + 15-H_b), 0.88 (s, 3H, 19-H_3), 0.87 – 0.83 (m, 3H, 21-H_3), 0.58 (s, 3H, 18-H_3) ppm; ^{13}C NMR (126 MHz, DMSO-d_6): δ = 171.6 (C-24), 170.3 (C-25), 170.1 (C-27), 167.0 (C-31), 157.5 (C-44 + C44′), 156.1 (C-38), 155.6 (C-42 + C42′), 135.7 (C-37), 132.2 (C-40 + C-40′), 131.1 (C-32), 130.8 (C-34), 130.3 (C-33), 130.2 (C-36), 128.0 (C-35), 114.7 (C-41 + C-41′), 113.5 (C-35 + C-35′), 96.4 (C-43 + C-43′), 73.8 (C-3), 71.0 (C-7), 55.8 (C-17), 50.5 (C-14), 47.3 (C-29 + C-29′), 45.9 (C-45 + C-45′+C45″+C-45‴), 42.7 (C-13), 41.5 (C-30 + C-30′), 40.7 (C-5), 40.5 (C-10), 39.5 (C-12), 37.5 (C-8), 35.4 (C-9), 34.8 (C-1), 34.7 (C-6), 34.1 (C-20), 31.3 (C-4), 31.2 (C-22), 29.8 (C-23), 28.0 (C-16), 26.9 (C-2), 23.5 (C-15), 22.8 (C-19), 21.7 (C-28), 21.6 (C-26), 20.7 (C-11), 18.8 (C-21), 12.9 (C-46 + C-46′+C-46″+C-46‴), 12.0 (C-18) ppm; MS (ESI, propan-2-ol): *m/z* = 969.3 (100 %, [M-Cl]$^+$), 970.3 (80 %, [M-Cl + H]$^+$); analysis calcd for $C_{60}H_{81}N_4O_7Cl$ (1005.78): C 71.65, H 8.12, N 5.57; found: C 71.42, H 8.40, N 5.39.

4.5.14. 9-[2-[[4-(3α-Acetyloxy-5β-cholan-24-oyl)-1-piperazinyl] carbonyl]phenyl]-3,6-bis(diethylamino]-xanthylium chloride (19)

Following GP3, from **14** (100 mg, 205 μmol), rhodamine B (108 mg, 202 μmol), 1-hydroxybenztriazole hydrate (38 mg, 247 μmol) and *N*-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (48 mg, 247 μmol) **19** (121 mg, 65 %) was obtained as a purple solid; m.p. 147 °C; R_F = 0.58 ($CHCl_3$/MeOH, 9:1); IR (ATR): $\tilde{\nu}$ = 498*w*, 666*w*, 683 *m*, 822*w*, 921*w*, 977*w*, 1007 *m*, 1026 *m*, 1073 *m*, 1095*w*, 1132 *m*, 1160 *m*, 1179*vs*, 1196 *m*, 1243 *s*, 1272 *m*, 1336 *s*, 1381 *m*, 1394 *m*, 1412 *s*, 1449 *m*, 1466 *m*, 1480 *m*, 1508*w*, 1528*w*, 1587*vs*, 1632 *m*, 1731*w*, 2864*w*, 2928*w* cm^{-1}; UV/vis (MeOH): λ^{max} (log ε) = 562 (4.90), 356 (3.71), 309 (4.03), 282 (4.06), 262 (4.37) nm; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 7.77 – 7.71 (m, 2H, 31-H + 34-H), 7.72 – 7.66 (m, 1H, 33-H), 7.54 – 7.47 (m, 1H, 32-H), 7.17 – 7.05 (m, 4H, 38-H + 38′-H + 39-H + 39′-H), 6.93 (d, *J* = 2.3, 2H, 41-H + 41′-H), 4.58 (tt, *J* = 11.2, 4.7, 1H, 3-H), 3.70 – 3.59 (m, 8H, 43-H_2 + 43′-H_2 + 43″–H_2 + 43‴–H_2), 3.47 – 3.17 (m, 9H, 12-H_a + 27-H_2 + 27′-H_2 + 28-H_2 + 28′-H_2), 3.11 – 2.95 (m, 1H, 12-H_b), 2.39 – 2.08 (m, 2H, 23-H_2), 1.95 (s, 3H, 26-H_3), 1.93 – 1.87 (m, 1H, 15-H_a), 1.84 – 1.68 (m, 4H, 1-H_a + 4-H_a + 11-H_a + 16-H_a), 1.66 – 1.55 (m, 1H, 22-H_a), 1.55 – 1.39 (m, 3H, 4-H_b + 5-H + 6-H_a), 1.38 – 1.29 (m, 5H, 2-H_a + 7-H_a + 8-H + 9-H + 20-H), 1.27 – 1.11 (m, 17H, 7-H_b + 11-H_b + 15-H_b + 16-H_b + 22-H_b + 44-H_3 + 44′-H_3 + 44″–H_3 + 44‴–H_3), 1.13 – 0.93 (m, 5H, 1-H_b + 2-H_b + 6-H_b + 14-H + 17-H), 0.92 – 0.82 (m, 6H, 19-H_3 + 21-H_3), 0.61 – 0.57 (m, 3H, 18-H_3) ppm; ^{13}C NMR (126 MHz, DMSO-d_6): δ = 171.7 (C-24), 171.6 (C-29), 170.2 (C-25), 157.5 (C-42 + C-42′), 156.1 (C-36), 155.6 (C-40 + C-40′), 135.7 (C-35), 132.2 (C-38 + C-38′), 131.2 (C-30), 130.9 (C-32), 130.3 (C-31), 130.2 (C-34), 128.0 (C-33), 114.7 (C-39 + C-39′), 113.5 (C-37 + C-37′), 96.4 (C-41 + C-41′), 73.9 (C-3), 56.3 (C-14), 56.0 (C-17), 45.9 (C-43 + C-43′+C-43″+C-43‴), 45.1 (C-27 + C-27′), 42.7 (C-12), 41.6 (C-5), 40.6 (C-28 + C-28′), 40.3 (C-9), 40.0 (C-13 + C-15), 35.8 (C-8), 35.4 (C-20), 35.0 (C-1), 34.6 (C-10), 32.3 (C-4), 31.3 (C-22), 29.9 (C-23), 28.2 (C-16), 27.0 (C-11), 26.4 (C-2), 24.3 (C-6), 23.5 (C-19), 21.5 (C-26), 20.9 (C-7), 18.8 (C-21), 12.9 (C-44 + C-44′+C-44″+C-44‴), 12.3 (C-18) ppm; MS (ESI, propan-2-ol): *m/z* = 911.7 (100 %, [M-Cl]$^+$), 912.7 (65 %, [M-Cl + H]$^+$); analysis calcd for $C_{57}H_{91}N_4O_5Cl$ (946.67): C 72.25, H 9.69, N 5.92; found: C 71.96, H 9.95, N 5.66.

4.5.15. 9-[2-[[4-(3α,7β-Bis(acetyloxy)-5β-cholan-24-oyl)-1-piperazinyl] carbonyl]phenyl]-3,6-bis(diethylamino]-xanthylium chloride (20)

Following GP3, from **15** (100 mg, 188 μmol), rhodamine B (99 mg, 207 μmol), 1-hydroxybenztriazole hydrate (35 mg, 226 μmol) and *N*-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (44 mg, 226 μmol) **20** (88 mg, 47 %) was obtained as a purple solid; m.p. 169 °C; R_F = 0.59 ($CHCl_3$/MeOH, 9:1); IR (ATR): $\tilde{\nu}$ = 683 *m*, 822*w*, 922*w*, 957*w*, 977 *m*, 1007 *s*, 1023 *s*, 1043 *m*, 1074 *m*, 1095*w*, 1132 *m*, 1160 *m*, 1179*vs*,

1197 *m*, 1241 *s*, 1273 *s*, 1336 *s,* 1383 *m,* 1395 *m,* 1412 *s,* 1466 *m*, 1481 *m*, 1508 *m*, 1529 *m*, 1587*vs*, 1633 *m*, 1727 *m*, 2871*w*, 2938*w* cm^{-1}; UV/vis (MeOH): λ^{max} (log ε) = 558 (4.94), 355 (3.85), 306 (4.14), 257 (4.48), nm; ^1H NMR (500 MHz, DMSO-d_6): $\delta = 7.76 - 7.71$ (m, 2H, 33-H + 36-H), 7.70 – 7.66 (m, 1H, 35-H), 7.55 – 7.47 (m, 1H, 34-H), 7.16 – 7.06 (m, 4H, 40-H + 40′-H + 41-H + 41′-H), 6.93 (d, $J = 2.3$, 2H, 43-H + 43′-H), 4.69 – 4.59 (m, 1H, 7-H), 4.53 (ddd, $J = 15.6$, 10.3, 4.8, 1H, 3-H), 3.68 – 3.60 (m, 8H, 45-H_2 + 45′-H_2 + 45″–H_2 + 45‴–H_2), 3.46 – 3.13 (m, 8H, 29-H_2 + 29′-H_2 + 30-H_2 + 30′-H_2), 2.30 – 2.09 (m, 2H, 23-H_2), 1.95 (s, 3H, 26-H_3), 1.91 (s, 3H, 28-H_3), 1.93 – 1.87 (m, 1H, 12-H_a), 1.79 – 1.45 (m, 11*H*, 1-H_a + 2-H_a + 4-H_a + 5-H + 6-H_2 + 8-H + 9-H + 16-H_a + 22-H_2), 1.45 – 1.25 (m, 4H, 2-H_b + 11-H_a + 15-H_a + 20-H), 1.26 – 1.12 (m, 5H, 11-H_b + 12-H_b + 14-H + 16-H_b + 15-H_b), 1.18 (t, $J = 7.1$, 12H, 46-H_3 + 46′-H_3 + 46″–H_3 + 46‴–H_3), 1.16 – 0.96 (m, 3H, 1-H_b + 4-H_b + 17-H), 0.90 (s, 3H, 19-H_3), 0.90 – 0.81 (m, 3H, 21-H_3), 0.60 (s, 3H, 18-H_3) ppm; ^{13}C NMR (126 MHz, DMSO-d_6): $\delta = 171.1$ (C-24), 169.8 (C-27), 169.8 (C-25), 166.5 (C-31), 157.0 (C-44 + C-44′), 155.6 (C-38), 155.1 (C-42 + C-42′), 135.3 (C-37), 131.8 (C-40 + C-40′), 130.7 (C-32), 130.4 (C-34), 129.8 (C-36), 129.7 (C-33), 127.5 (C-35), 114.3 (C-41 + C-41′), 113.0 (C-39 + C-39′), 95.9 (C-43 + C-43′), 72.9 (C-3), 72.8 (C-7), 54.4 (C-17), 54.4 (C-14), 46.8 (C-29 + C-29′), 45.4 (C-45 + C-45′+C-45″+C-45‴), 43.1 (C-13), 41.2 (C-5 + C-30 + C-30′), 39.7 (C-8), 39.5 (C-10), 39.2 (C-12), 38.6 (C-9), 34.8 (C-20), 33.9 (C-1), 32.6 (C-6), 32.5 (C-22), 30.8 (C-4), 29.3 (C-23), 28.0 (C-16), 26.0 (C-2), 25.3 (C-15), 22.8 (C-19), 21.5 (C-28), 21.0 (C-26), 20.8 (C-11), 18.4 (C-21), 12.4 (C-46 + C-46′+C-46″+C-46‴), 11.8 (C-18) ppm; MS (ESI, MeOH): $m/z = 969.5$ (100 %, $[M\text{-}Cl]^+$), 970.5 (78 %, $[M\text{-}Cl + H]^+$); analysis calcd for $C_{60}H_{81}N_4O_7Cl$ (1005.78): C 71.65, H 8.12, N 5.57; found: C 71.43, H 8.39, N 5.37.

CRediT authorship contribution statement

Benjamin Brandes: Investigation, Writing – review & editing. **Sophie Hoenke:** Investigation, Writing – original draft. **Christian Schultz:** Investigation, Writing – original draft. **Hans-Peter Deigner:** Conceptualization, Writing – original draft, Writing – review & editing. **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

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Synthesis and cytotoxicity of apoptosis-inducing *N*-heterocyclic triterpene amides

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ABSTRACT

The modification of lipophilic triterpenes to enhance their cytotoxicity, is a viable strategy for finding new anti-cancer agents. Herein we report the synthesis, analysis of 18 pentacyclic triterpenoic acid *N*-heterocyclic amides and their cytotoxicity, tumor cell/non-tumor cell selectivity, as well as their putative mode of action. EC_{50} values were measured by SRB-assays, and found to be as low as 3.13 μM, with a selectivity as high as S = 5.05. Moreover, supportive assays were performed to further analyze their cytotoxicity; these experiments showed the compounds to act mainly by apoptosis.

1. Introduction

Phytochemicals, such as pentacyclic triterpenoic acids, are a cheap and diverse source of bioactive compounds; their cytotoxicity and anti-cancer activity have been determined [1,2]. Furthermore, for several of them chemo-preventive effects have been reported, too [3]. For some triterpenoic acid derived amides potent cytotoxicity for several human tumor cell lines have been reported; they induced apoptosis rather than necrosis in the tumor cells [4–6]. Recently, a platanic acid-derived homopiperazinyl amide [7] showed an impressive EC_{50}-value of 0.8–1.1 μM for epithelial melanoma cells, and an augustic acid-derived 4-isoquinolinyl amide showed cytotoxicity (EC_{50} = 1.2–2.6 μM) for several tumor cell lines with excellent selectivity against non-malignant murine fibroblasts NIH 3T3 (EC_{50} > 50 μM) [6]. As a consequence, the synthesis of acetylated triterpenoic acid amides seems to be a viable strategy to access new cytotoxic agents. To proof this concept, glycyrrhetinic (**1**, **GA**, Scheme 1) ursolic (**2**, **UA**), and oleanolic acid (**3**, **OA**) were chosen as starting materials, and *N*-heterocyclic amines **7**–**12** (Fig. 1) were selected as representative molecules for coupling; all of these are commercially available. The amines represent pyridine and indazole structural isomers as well as pyrazole amines; several of these scaffolds showed attractive chemotherapeutic activities and are part of approved chemotherapeutics such as Crizotinib, Bosutinib, Sorafenib, Pazopanib, Regorafenib, and Linifanib [8–10]. Herein, we report the design, synthesis, analysis, and biological evaluation of novel *N*-heterocyclic triterpenoic amides.

2. Results and discussion

2.1. Chemistry

First, the hydroxyl group of glycyrrhetinic (**1**), ursolic (**2**), and oleanic (**3**) at position C-3 was acetylated (Scheme 1) in excellent yields (86–90%) to access their respective acetates **4**–**6**. For the subsequent synthesis of the amides, acetates **4**–**6** were transformed with oxalyl chloride in the presence of catalytic amounts of DMF *in situ* into their respective acid chlorides followed by adding the amines **7**–**12** (Scheme 2) to afford amides **13**–**30**. These amides were then subjected to sulforhodamine B (SRB) assays to assess their cytotoxic activity.

2.2. Biology

SRB assays were used to evaluate the cytotoxicity of the compounds employing several human tumor cell lines (cut-off concentration 30 μM); the results of these SRB assays are summarized in Table 1. **UA** and **OA** derived acetates **5** and **6** were of minor cytotoxicity for the cell lines A375, MCF7, HT29, and A2780. A correlation between cytotoxicity (measured) and the respective octanol-water partition coefficient

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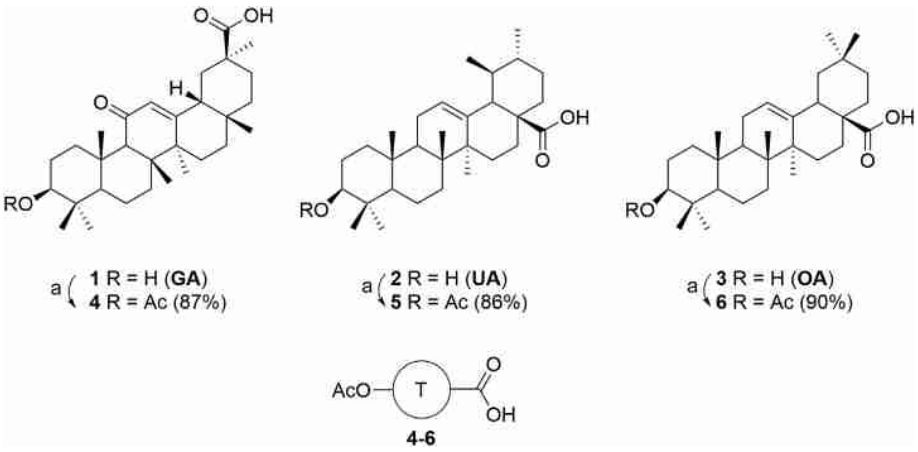


Scheme 1. Structure and synthesis of acetylated triterpenoic acids (**T**): a) cat. DMAP, $(Ac)_2O$, pyridine, 21 °C, 3 h.

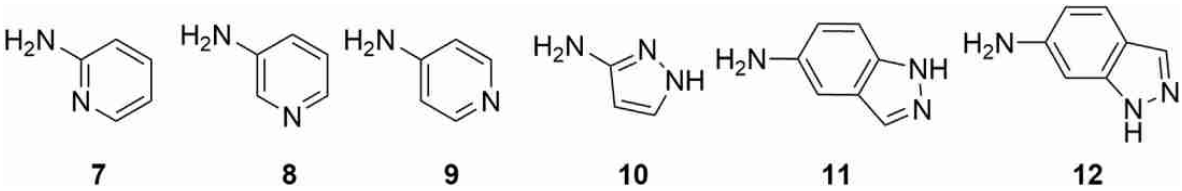
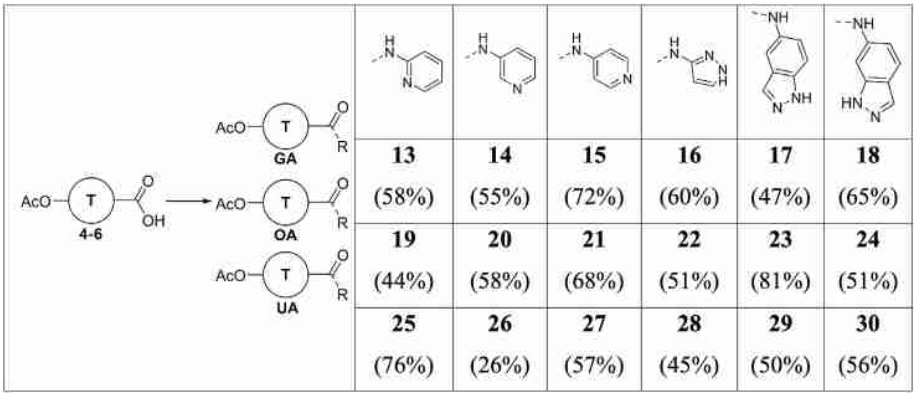


Fig. 1. *N*-heterocyclic amines selected for the synthesis of triterpenoid amides.



Scheme 2. Synthesis of N-heterocyclic triterpenoic amides: cat. DMF, $(COCl)_2$, 0 °C → 21 °C, 2 h → corresponding amine (**7**-**12**), THF/DCM, 21 °C, 24 h.

(calculated, SwissADME) could not be established neither for the starting materials, their acetates, nor for the amides **13**–**30**. The cytotoxicity of the amides seems to be primarily determined by their structure. As a result, almost all the amides **13**–**30** showed better EC_{50} values than their acetylated precursors, thus proving the viability of the concept that amides are of superior cytotoxicity as compared to the corresponding carboxylic acids. This parallels previous findings for amides of the lupane series [11]. Interestingly, apart from two amides (**17** and **18**), the amides were more cytotoxic for the malignant A375 cells. Moreover, the position of the nitrogen in the pyridine subunit seems also to affect both cytotoxicity and selectivity. Again, this is incomplete agreement with previous results obtained for quinolinyl or isoquinolinyl amides [7,11]. Hereby, the **UA** derived pyridinyl amide **26** held the highest cytotoxicity with an EC_{50} value of 3.13 μM for A375 epithelial melanoma cells. For the structural isomer **25** the best selectivity of all tested compounds (S = 5.05) was determined for this cell line. Interestingly, most of the amides showed diminished selectivity but **GA** derived amide **13** also held enhanced selectivity for all malignant cell lines as compared to non-malignant fibroblasts.

Further investigations of compounds **21** and **22** using Annexin V-FITC/PI assays showed these compounds to have – by and large – a smaller number of vital cells (−15/-17%) after 24 h which is probably due to cells being in an apoptotic rather than in a necrotic state (Fig. 2). The number of apoptotic ells increased drastically after 48 h of treatment showing an average of −65% (**21**) and −60% (**22**) less vital cells than counted in the untreated control group.

Moreover, investigation of the cell cycle of A2780 cells treated with **21** and **22** was performed by FACS-analysis applying incubation times of 24 h and 48 h, respectively. Notably, the average cell count difference between the control group and those cell populations having been treated with **21** or **22** in the G1-phase is 24–25% higher than for the control group after 24 h (Fig. 3). In reverse, this means that fewer cells are passing the checkpoint into the S-phase and counts in the S- and G2/M-phase decrease. There is no remarkable difference in the apoptosis between all analyzed cell groups, but this changes significantly to an increase of 52/53% after 48 h of incubation (Fig. 4). The comparison of these results (Figs. 5 and 6) shows that the treated cells stop cell division and are arrested in the G1-or G0-phase with an increasing effect at 48 h

Table 1
Cytotoxicity (from SRB assays, EC_{50} values [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) of starting materials **GA** (**1**), **UA** (**2**), **OA** (**3**) [12], their acetates (**4**–**6**) [13–15] and amides **13**–**30**. Human cancer cell lines: A375 (epithelial melanoma), HT29 (colorectal adenocarcinoma), MCF-7 (breast adenocarcinoma), A2780 (ovarian carcinoma), FaDu (hypopharyngeal carcinoma), HeLa (cervical carcinoma); non-malignant: NIH 3T3 (mouse fibroblasts); n.s. not soluble; n.d. not determined; S selectivity against NIH 3T3; doxorubicin (**DX**) and staurosporine (**ST**) were used as positive controls.

Compound	A375	HT29	MCF-7	A2780	HeLa	NIH 3T3
1	n.d.	>30	>30	>30	n.d.	18.7 ± 4.2
2	n.d.	10.6 ± 0.7	12.7 ± 0.1	11.7 ± 0.6	n.d.	13.1 ± 1.1
3	n.d.	>30	>30	>30	n.d.	>30
4	>30	>30	>30	>30	n.d.	>30
5	11.4 ± 1.4	17.3 ± 1.5	12.1 ± 1.2	8.3 ± 0.9	n.d.	16.4 ± 1.7
6	13.1 ± 1.1	20.5 ± 1.7	12.9 ± 1.9	9.4 ± 0.5	n.d.	17.5 ± 1.5
13	11.96 ± 0.9 (S = 2.51)	21.0 ± 2.3 (S = 1.43)	19.3 ± 1.9 (S = 1.55)	16.0 ± 1.3 (S = 1.88)	19.0 ± 2.9 (S = 1.58)	>30
14	>30	>30	>30	>30	>30	>30
15	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
16	>20	>30	>30	>30	>30	>30
17	14.03 ± 0.6 (S = 0.97)	17.53 ± 0.4 (S = 0.78)	12.82 ± 0.7 (S = 1.06)	15.01 ± 0.7 (S = 0.91)	16.20 ± 0.9 (S = 0.84)	13.61 ± 0.5
18	22.1 ± 1.4 (S = 1.22)	23.9 ± 2.0 (S = 1.13)	19.4 ± 2.1 (S = 1.39)	17.4 ± 1.7 (S = 1.55)	23.6 ± 1.0 (S = 1.14)	27.0 ± 1.3
19	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
20	8.50 ± 0.8 (S = 1.04)	25.1 ± 3.2 (S = 0.35)	10.52 ± 1.2 (S = 0.84)	11.6 ± 1.2 (S = 0.76)	15.52 ± 0.9 (S = 0.57)	8.83 ± 0.5
21	4.3 ± 1.3 (S = 1.07)	22.8 ± 3.3 (S = 0.20)	7.57 ± 0.5 (S = 0.61)	8.32 ± 0.4 (S = 0.55)	11.43 ± 0.9 (S = 0.40)	4.61 ± 0.7
22	3.56 ± 0.1 (S = 1.59)	12.79 ± 0.8 (S = 0.44)	10.61 ± 0.8 (S = 0.53)	7.51 ± 0.4 (S = 0.75)	8.04 ± 0.5 (S = 0.71)	5.67 ± 0.9
23	8.77 ± 0.4 (S = 1.23)	>30	9.99 ± 0.6 (S = 1.08)	10.75 ± 0.4 (S = 1.00)	13.8 ± 1.0 (S = 0.78)	10.75 ± 0.4
24	>20	>30	>30	>30	>20	>30
25	4.52 ± 0.4 (S = 5.05)	17.02 ± 1.1 (S = 1.34)	15.70 ± 1.3 (S = 1.45)	8.18 ± 0.7 (S = 2.79)	15.10 ± 2.0 (S = 1.51)	22.83 ± 4.5
26	3.13 ± 0.3 (S = 2.20)	7.3 ± 1.1 (S = 0.94)	6.26 ± 0.7 (S = 1.10)	5.56 ± 0.6 (S = 1.24)	10.64 ± 0.4 (S = 0.65)	6.88 ± 0.9
27	4.07 ± 0.2 (S = 1.34)	9.4 ± 1.0 (S = 0.58)	7.83 ± 0.7 (S = 0.70)	6.90 ± 0.6 (S = 0.79)	7.44 ± 0.4 (S = 0.73)	5.45 ± 0.8
28	6.66 ± 0.1 (S = 1.53)	16.56 ± 0.5 (S = 0.62)	11.61 ± 0.8 (S = 0.88)	11.01 ± 0.8 (S = 0.93)	12.08 ± 0.8 (S = 0.85)	10.22 ± 0.8
29	4.44 ± 0.1 (S = 0.95)	11.06 ± 0.6 (S = 0.38)	8.38 ± 0.9 (S = 0.50)	7.91 ± 0.5 (S = 0.53)	7.38 ± 0.6 (S = 0.57)	4.22 ± 0.7
30	6.1 ± 1.7 (S = 1.93)	11.1 ± 1.2 (S = 1.06)	8.06 ± 0.3 (S = 1.46)	7.88 ± 0.9 (S = 1.49)	15.75 ± 1.5 (S = 0.75)	11.76 ± 1.0
ST	n.d.	0.9 ± 0.01	1.1 ± 0.3.	0.01 ± 0.01	n.d.	0.45 ± 0.04
DX	n.d.	0.2 ± 0.02	0.1 ± 0.01	0.1 ± 0.01	n.d.	0.01 ± 0.001

and thus leading to fewer cells in the S- and G2/M-phase and higher amounts of apoptotic cells. This is in accordance with the results obtained from the Annexin V-FITC/PI assay (Fig. 2); for the latter, the average apoptosis difference after 24 h was 46% for **21** and 37% for **22**, respectively.

As a further proof for inducing apoptosis some extra microscopic experiments were performed. Thereby, samples treated with **21** or **22** showed a significantly lower number of cells than the respective control groups (Fig. 7, a/d). For example, after treatment of the cells with compound **21** for 24 h, apoptotic cells were visible as indicated by blebbing and the occurrence of cell fragments (Fig. 7, b). Treatment of the cells for 48 h led to late apoptosis; propidium iodide (PI) inclusions in the shrunken parts of the nucleus of the cells was established which gave them an orange or red color (Fig. 7, e).

Treatment with compound **22** for 24 h led to cell shrinkage and blebbing, thus resulting in single cell fragments (Fig. 7, c). These still had an intact membrane, so that PI could not enter the cells. These observations strongly suggest an early stage of apoptosis. After 48 h, the presence of PI in some cells indicate late apoptosis (Fig. 7, f). The red fluorescence was visible in small spots signifying the nucleus fragmentation. Details can be found in the experimental part.

3. Conclusion

We successfully synthesized 18 *N*-heterocyclic triterpenoic acid amides and assayed them for their cytotoxicity and selectivity for several malignant human cell lines. Our findings highly suggest the viability of modifying lipophilic core structures with *N*-heterocyclic residues to further enhance the cytotoxicity and selectivity of these hybrids. While these compounds overall proved to be cytotoxic and selective especially for A375 epithelial melanoma cells, the selectivity for the HT29 and HeLa cell lines were diminished. However, these compounds induce favorable apoptotic cell death, with little to no necrosis, and EC_{50} as low as 3.13 μM for the ursolic acid derived amide **26** was determined. In addition, the highest selectivity of S = 5.05 could be obtained for the structural analogue **25** thereby holding an EC_{50} value of 4.52 μM for the same cell line. In conclusion, when aiming for selectivity and high cytotoxicity, 2-aminopyridine (**7**) seems to be a promising candidate for further lead optimization of cytotoxic drugs.

4. Experimental part

4.1. General

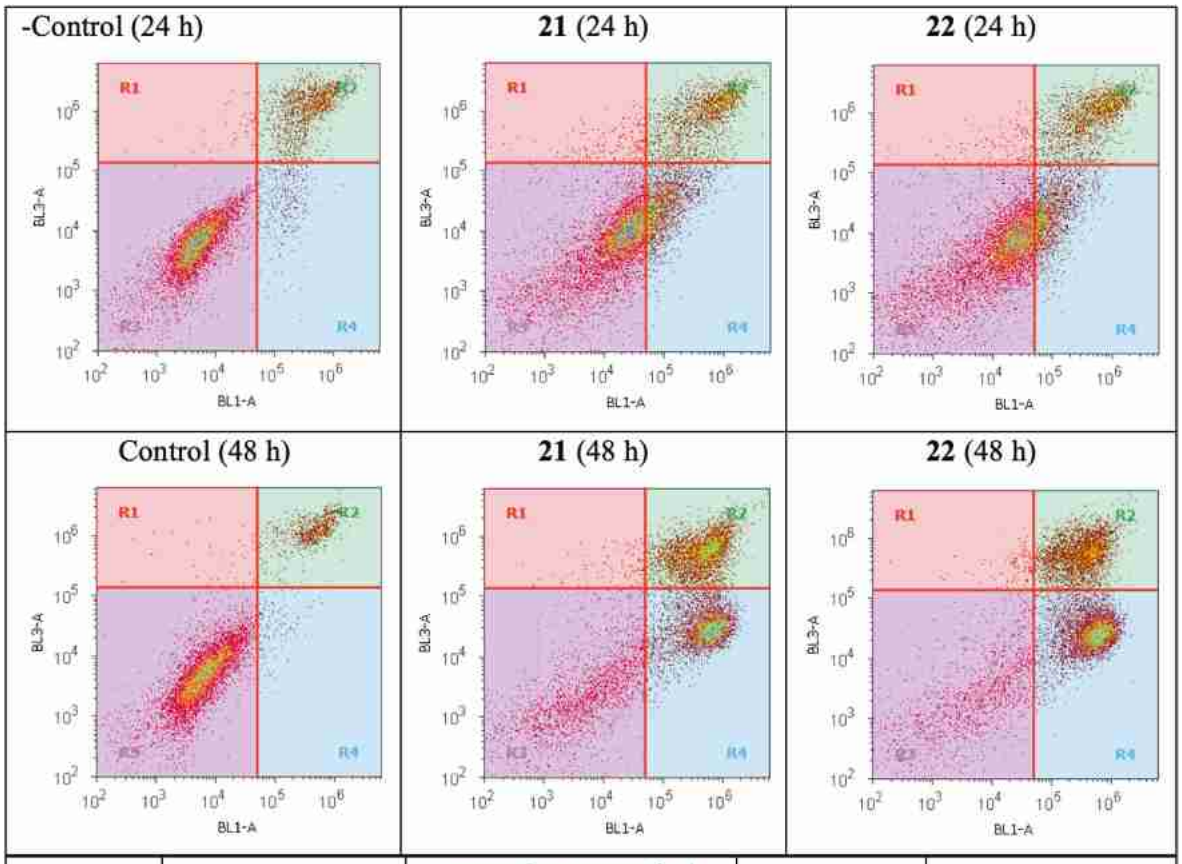
Ursolic and oleanolic acid were obtained from Betulinines (Stribrna Skalice, Czech Republic) and glycyrrhetinic acid was bought from Orgentis GmbH (Neugatersleben, Germany) and used as received. Amines were purchased from TCI, abcr, and Sigma Aldrich. Equipment and lab equipment was used as previously described.

4.2. Flow cytometry

For flow cytometry investigation, 4×10^5 A375 cells were seeded in a flask and allowed to grow for 24 h. The medium was removed, and the cells were treated with 10 mL fresh medium as control and medium mixed with compounds **21** and **22** (double EC_{50} concentration), respectively. After 24 h and 48 h, the cells were harvested by trypsinization, and all solutions were collected in a tube. Each sample was centrifuged (1500 rpm, 5 min, 4 °C), the supernatant was discarded, the pellet was suspended in 1 mL PBS (with Ca^{2+} and Mg^{2+}); this was repeated twice. Thereafter the cells were counted using the Attune® FACS machine (Life technologies™, Darmstadt, Germany).

4.3. Cell cycle

For Cell Cycle investigation [16] 1×10^6 cells were collected by centrifugation and fixed with ethanol (70%, 4 °C, 24 h). The samples were centrifuged (4500 rpm, 5 min, 4 °C), washed with PBS once and centrifuged again. Staining Solution (10 μL PI (1 mg/mL) in 1 mL PBS) with RNAse (100 μL, 100 μg/mL) was added. After incubation for at least



Incubation time in h	necrotic (R1)		secondary necrotic / late-stage apoptotic (R2)		vital (R3)		apoptotic (R4)	
	21	**22**	**21**	**22**	**21**	**22**	**21**	**22**
24	1%	1%	1%	1%	-15%	-17%	13%	15%
48	0%	0%	19%	19%	-65%	-60%	46%	37%
	average difference to control							

Fig. 2. Representative Annexin V-FITC/PI assay density plots determined by flow cytometry after 24 h and 48 h. R1: necrotic (red), R2: secondary necrotic/late-stage apoptotic (green), R3: vital (pink), R4: apoptotic (blue). The difference to the control group was calculated from the arithmetic means of each three biological and two technical replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

30 min at room temperature, the cells were analyzed. The data was collected from the BL-2A and BL-2H channel, plotting A against H for exclusion of doublet cells. For each cell cycle distribution 20,000 events were collected in technical triplicates, each sample was measured in duplicates/triplicates, depending on the sample volume. Cell cycle distribution was calculated using ModFit LT (Verity Software House, Topsham, US).

4.4. Annexin V/PI assay

For this assay [16] 1×10^5 cells were collected by centrifugation and re-suspended in Annexin V binding buffer (100 µL, BioLegend ®, San Diego, US). After treatment with propidium iodide solution (3 µL, 1 mg/mL) and Annexin V-FITC (5 µL, BioLegend ®, San Diego, US) for 15 min in the dark at room temperature, Annexin V binding buffer (400 µL) was added, und the samples were analyzed by Attune ® FACS machine. After gating for living cells, the data from detectors BL-1A and BL-3A were collected (10,000 events) in technical triplicates. The assay was performed in duplicates; cell distribution was calculated using Attune ® Software.

4.5. Microscopy

On the first day, 4×10^5 A375 cells were seeded in a flask. After 24 h, the medium was removed, and the cells were treated with 10 mL fresh medium as control and medium mixed with compounds **21** and **22** (double EC_{50} concentration), respectively. After 24 h and 48 h, the cells were harvested by trypsinization, and all solutions were collected in a tube. Each sample was centrifuged (1500 rpm, 5 min, 4 °C), the supernatant was discarded, the pellet was suspended in 1 mL PBS (with Ca^{2+} and Mg^{2+}), and centrifuged again. The cells were now taken up in 150 µL PBS. For the microscopic investigation, 10 µL cell suspension was mixed with 10 µL AO/PI solution (5 µg/mL each in PBS), placed on a slide, and measured directly with the microscope.

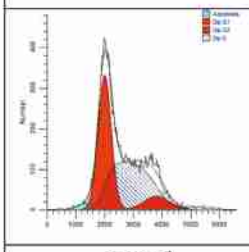
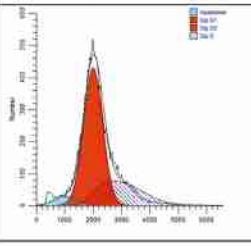
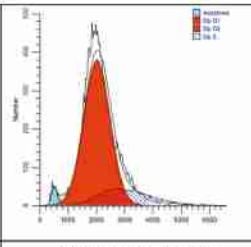
	Cell cycle analysis of A2780 after 24 h		
			
	control	difference to **21**	difference to **22**
G1	44%	24%	25%
S	38%	-13%	-13%
G2/M	14%	-12%	-12%
Apop.	1%	1%	1%

Fig. 3. FACS cell cycle analysis after 24 h of incubation of A2780 cell line highlighting the differences between the control group and the tested compounds (**21** and **22**). Average values are displayed and were taken from at least two biological and two technical replicates. Representative graphs were chosen from one biological replicate group.

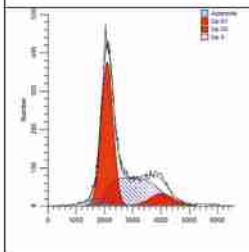
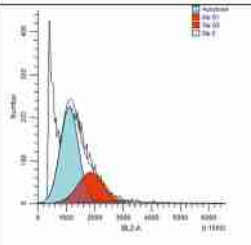
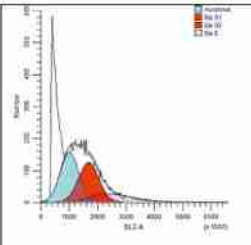
	Cell cycle analysis of A2780 after 48 h		
			
	average control	difference to **21**	difference to **22**
G1	61%	30%	25%
S	28%	-22%	-7%
G2/M	12%	-8%	-8%
Apop.	1%	53%	52%

Fig. 4. FACS cell cycle analysis after 48 h of incubation of A2780 cell line highlighting the differences between the control group and the tested compounds (**21** and **22**). Average values are displayed and were taken from at least two biological and technical replicates. Representative graphs were chosen from one biological replicate group.

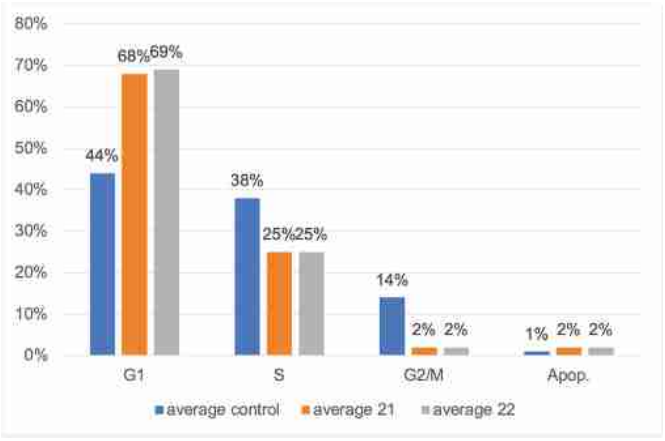


Fig. 5. Cell cycle analysis of A2780 cells after incubation with **21** or **22** for after 24 h.

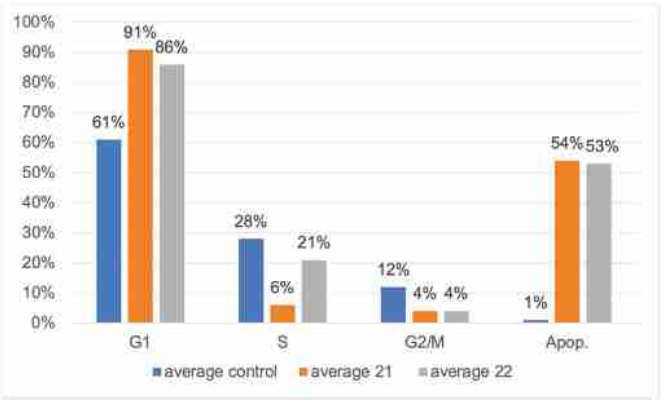


Fig. 6. Cell cycle analysis of A2780 cells after incubation with **21** or **22** for 48 h.

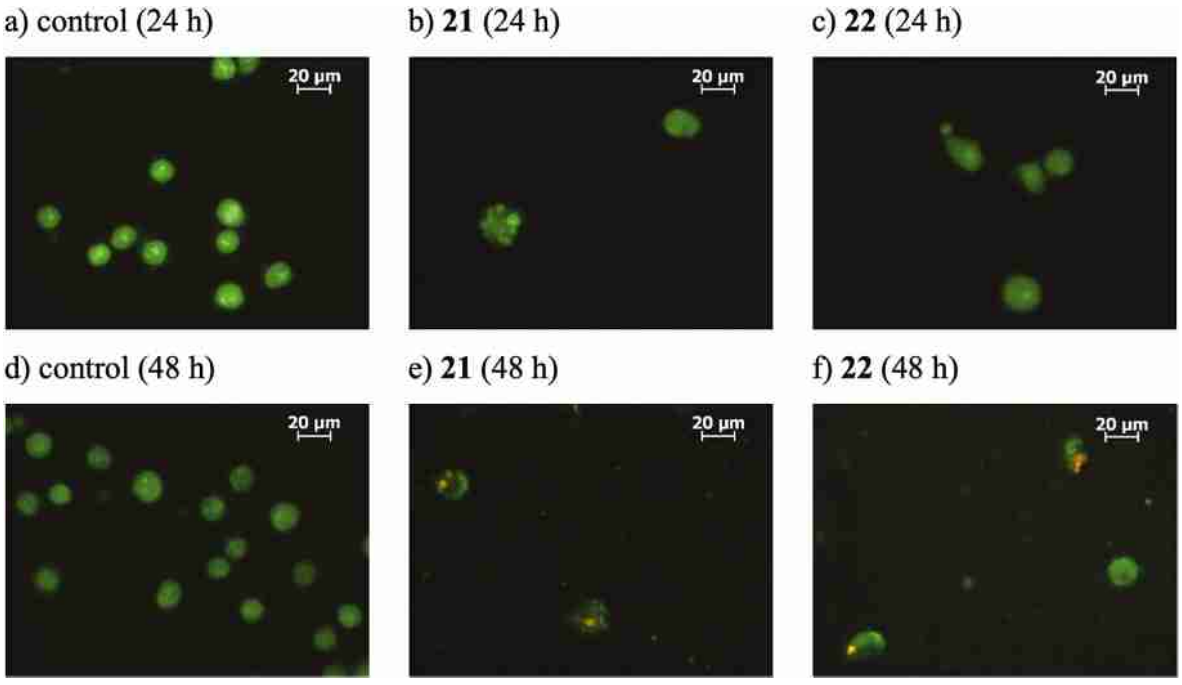


Fig. 7. Microscopic investigations of cells stained with AO/PI. The cells were treated with and without compounds **21** and **22**.

4.6. Syntheses

4.6.1. General procedure (GP1) for the acetylation of triterpene carboxylic acids

The triterpene carboxylic acids (**1**–**3**, 5.0 g) were dissolved in dry pyridine (20 mL). Under stirring, acetic anhydride (2.0 mL, 18.1 mmol) and cat. amounts of DMAP were added. After stirring at 21 °C for 3 h, the reaction solution was checked with TLC and poured into HCl (0.1 M, 50 mL). Then the product was filtered off and dried under reduced pressure. Analytical samples were obtained by re-crystallization or column chromatography.

4.6.2. General procedure (GP2) for the synthesis of the amides

The acetylated triterpene carboxylic acid (**4**–**6**, 5.0 g) was dissolved in dry DCM (20 mL), and the mixture was cooled to 0 °C. Under stirring, oxalyl chloride (20 eq, 1.62 mL, 19 mmol) and cat. amounts of dry DMF were added. The reaction was allowed to warm to 21 °C, and stirring was continued until the gas evolution had ceased (2 h). Excessive oxalyl chloride and solvent were removed under reduced pressure while re-dissolving the obtained solid twice in dry THF. Part of the obtained acid chloride (0.5 g) was dissolved in dry DCM (20 mL) and triethylamine (0.12 mL 0.94 mmol) was added. Then the corresponding amine (**7**–**12**, 1 eq) dissolved in dry THF/DCM was added to the acid chloride solution under stirring. The reaction mixture was stirred at 21 °C for 24 h, quenched with HCl (1 M, 30 mL), extracted with DCM (3 × 30 mL), the combined organic phases were dried ($MgSO_4$), and the solvent was evaporated under reduced pressure to obtain a solid which was purified by column chromatography (SiO_2, hexanes/ethyl acetate, 11:9).

4.6.3. 3-O-Acetyl-glycyrrhetinic acid (4)

Following GP1, **4** (4.77 g; 87%) was obtained as a white solid; m.p. 308–310 °C (lit.: [17]: 310–313 °C); $R_F = 0.56$ (hexanes/ethyl acetate, 11:9); $[\alpha]_D = +165.1°$ (*c* 1.0, $CHCl_3$) [lit.: [18]: $[\alpha]_D = +163.3°$ (*c* 1.0, $CHCl_3$)]; IR (ATR): $\tilde{\nu} = 2952br$, 1731*s*, 1654*s*, 1293 *m*, 1227*s*, 1075*s*, 753 s cm^{-1}; MS (ESI, MeOH): $m/z = 511.1$ ($[M - H]^-$, 100%), 1023.9 ($[2M - H]^-$, 64%).

4.6.4. 3-O-Acetyl-oleanolic acid (5)

Following GP1, **5** (4.7 g, 86%) was obtained as a white solid; m.p. 261 °C (lit.: [19]: 260–261 °C); $R_F = 0.6$ (hexanes/ethyl acetate, 6:4); $[\alpha]_D = +72.5°$ (*c* 1.0, $CHCl_3$) [lit.: [20]: $[\alpha]_D = +74°$ (*c* 1.0, $CHCl_3$)]; IR (ATR): $\tilde{\nu} = 2946br$, 1679*s*, 1455*s*, 1380 *m*, 1034*s*, 748 m cm^{-1}; MS (ESI, MeOH): $m/z = 497.0$ ($[M - H]^-$, 100%).

4.6.5. 3-O-Acetyl-ursolic acid (6)

Following GP1, **6** (4.95 g; 90%) was obtained as a white solid; m.p. 264–266 °C (lit.: [21]: 242.7–244.1 °C); $[\alpha]_D = +72.5°$ (*c* 1.0, $CHCl_3$) [lit.: [21]: $[\alpha]_D = +71.2°$ (*c* 1.0, $CHCl_3$)]; $R_F = 0.65$ (hexanes/ethyl acetate, 6:4); IR (ATR): $\tilde{\nu} = 2948br$, 1683*s*, 1467 *m*, 1371 *m*, 1034*s*, 938 *m*, 748 m cm^{-1}; MS (ESI, MeOH): $m/z = 497.1$ ($[M - H]^-$, 100%).

4.6.6. N-2-pyridinyl-3β-acetoxy-11-oxoolean-12-en-30-ic acid amide (13)

Following GP2, **13** (326 mg, 58%) [22–24] was obtained as a white solid; m.p. 265–268 °C (lit.: [22]: 266–267 °C); $R_F = 0.12$ (hexanes/ethyl acetate, 11:9); $[\alpha]_D = +168.0°$ (*c* 0.124, $CHCl_3$) [lit.: [22]: $[\alpha]_D = +178°$ (*c* 0.12, $CHCl_3$)]; IR (ATR): $\tilde{\nu} = 2950br$, 1727 *m*, 1656*s*, 1511 *m*, 1428*s*, 1293 *m*, 1244*s*, 753 s cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 8.40$ (dt, $J =$ 8.5, 1.0 Hz, 1H, 37-H), 8.24 (ddd, $J = 5.2, 1.9, 0.9$ Hz, 1H, 34-H), 7.84 (ddd, $J = 8.8, 7.4, 1.9$ Hz, 1H, 35-H), 7.13 (ddd, $J = 7.4, 5.2, 1.0$ Hz, 1H, 36-H) 5.75 (s, 1H, 12-H), 4.52 (dd, $J = 11.7, 4.7$ Hz, 1H, 3-H), 2.81 (m, 1H, 1-H_a), 2.37 (s, 1H, 9-H), 2.28 (m, 1H, 18-H), 2.14 (m, 1H, 16-H_a), 2.12–1.98 (m, 2H, 15-H_a+19-H_a), 2.06 (s, 3H, 32-H_3) 1.90–1.79 (m, 1H, 15-H_b), 1.78–1.54 (m, 5H, 19-H_b, 2-H_a+2-H_b+6-H_a+7-H_a), 1.52–1.36 (m, 5H, 16-H_b+7-H_b+21-H_a+12-H_b+ 22-H_a), 1.39 (s, 3H, 27-H_3), 1.31 (s, 3H, 32-H_3), 1.28–1.18 (m, 1H, 6-H_b), 1.17 (s, 3H, 25-H_3), 1.13 (s, 3H, 26-H_3), 1.07 (m, 2H, 1-H_b+22-H_b), 0.89 (s, 3H, 23-H_3), 0.88 (s, 3H, 24-H_3), 0.82 (s, 3H, 28-H_3), 0.81 (s, 1H, 5-H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 199.9 (C-11), 174.7 (C-30), 170.9 (C-31), 168.8 (C-13), 140.8 (C-33), 138.4 (C-37), 136.8 (C-35). 130.5 (C-36) 128.5 (C-21), 124.7 (C-34) 80.5 (C-3), 61.7 (C-9), 54.9 (C-5), 47.8 (C-18), 45.4 (C-8), 45.0 (C-17), 43.2 (C-14), 41.3 (C-19), 38.8 (C-1), 38.0 (C-4), 37.5 (C-21), 36.9 (C-10), 32.7 (C-7), 31.9 (C-22), 31.4 (C-16), 28.9 (C-29), 28.3 (C-28), 28.0 (C-24), 26.4 (C-15), 23.6 (C-2), 23.3 (C-27), 21.3 (C-32), 18.7 (C-26), 17.3 (C-6), 16.7 (C-23), 16.3 (C25) ppm; MS (ESI, MeOH): $m/z = 589.0$ ($[M - H]^+$, 57%), 1199.2 ($[2\,M + Na^+]^+$, 100%); analysis

calcd for $C_{37}H_{52}N_2O_4$ (588.83): C 75.47, H 8.90, N 4.76; found: C 75.19, H 9.03, N 4.57.

4.6.7. N-3-pyridinyl-3β-acetoxy-11-oxoolean-12-en-30-ic acid amide (14)

Following GP2, **14** (306 mg, 55%) was obtained as a white solid; m.p. 190–192 °C; $R_F = 0.11$ (hexanes/ethyl acetate, 11:9); $[\alpha]_D = +155.0°$ (*c* 0.136, $CHCl_3$); IR (ATR): $\tilde{\nu} = 2950br$, 1727 *m*, 1655 *m*, 1479 *m*, 1244*s*, 1027 *m*, 751*s*, 706 m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 9.03$ (d, $J =$ 2.8 Hz, 1H, 37-H), 8.70–8.52 (m, 1H, 36-H), 8.32 (dd, $J = 5.0$, 1.4 Hz, 1H, 34-H), 7.46 (dd, $J = 8.5$, 5.0 Hz, 1H, 35-H), 5.73 (s, 1H, 12-H), 4.53 (dd, $J = 11.7$, 4.7 Hz, 1H, 3-H), 2.80 (m, 1H, 1-H_a), 2.38 (s, 1H, 9-H), 2.27 (m, 2H, 18-H), 2.21 (m, 1H, 19-H_a), 2.12–2.04 (m, 2H, H-15-H_a+19-H_a), 2.06 (s, 3H, 32-H_3) 1.90–1.80 (m, 1H, 15-H_b), 1.78–1.56 (m, 5H, 19-H_b, 2-H_a+2-H_b+6-H_a+7-H_a), 1.56–1.38 (m, 5H, 16-H_b+7-H_b+21-H_a+21-H_b+22-H_a), 1.40 (s, 3H, 27-H_3), 1.32 (s, 3H, 29-H_3), 1.28–1.18 (m, 1H, 6-H_b), 1.16 (s, 3H, 25-H_3), 1.13 (s, 3H, 26-H_3), 1.10–1.02 (m, 2H, 1-H_b+22-H_b), 0.89 (s, 3H, 23-H_3), 0.88 (s, 3H, 24-H_3), 0.82 (s, 3H, 28-H_3), 0.81 (s, 1H, 5-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 199.9$ (C-11), 175.5 (C-30), 170.9 (C-31), 168.6 (C-13), 150.9 (C-36), 140.4 (C-37), 128.7 (C-12), 119.5 (C-34), 114.8 (C-35) 80.6 (C-3), 61.7 (C-9), 55.1 (C-5), 47.9 (C-18), 45.3 (C-8), 44.9 (C-17), 43.2 (C-14), 41.4 (C-19), 38.8 (C-1), 38.0 (C-4), 37.5 (C-21), 36.9 (C-10), 32.7 (C-7), 31.9 (C-22), 31.5 (C-16), 28.9 (C-29), 28.5 (C-28), 28.1 (C-24), 26.4 (C-15), 23.5 (C-2), 23.4 (C-27), 21.3 (C-32), 18.7 (C-26), 17.3 (C-6), 16.7 (C-23), 16.4 (C25) ppm; MS (ESI, MeOH): m/z = 587.2 ([M − H]$^-$, 100%), 623.2 ([M + Cl^-]$^-$, 34%)%); analysis calcd for $C_{37}H_{52}N_2O_4$ (588.83): C 75.47, H 8.90, N 4.76; found: C 75.26, H 8.15, N 4.63.

4.6.8. N-4-pyridinyl-3β-acetoxy-11-oxoolean-12-en-30-ic acid amide (15)

Following GP2, **15** (403 mg, 72%) was obtained as a white solid; m.p. 296–297 °C; $R_F = 0.14$ (hexanes/ethyl acetate, 11:9); $[\alpha]_D = +157.2°$ (*c* 0.117, $CHCl_3$); IR (ATR): $\tilde{\nu} = 2949br$, 1727 *m*, 1655 *m*, 1588*s*, 1505 *m*, 1245*s*, 751 s cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 8.50–8.43 (m, 2H, 36-H+35-H), 7.96 (d, $J = 6.3$ Hz, 2H, 34-H+37-H), 5.71 (s, 1H, H-12), 4.52 (dd, $J = 11.7$, 4.7 Hz, 1H, 3-H), 2.79 (m, 1H, 1-H_a), 2.38 (s, 1H, 9-H), 2.24 (m, 2H, 18-H+16-H_a), 2.12–2.04 (m, 2H, 15-H_a+19-H_a), 2.05 (s, 3H, 32-H_3) 1.90–1.80 (m, 1H, 15-H_b), 1.78–1.56 (m, 3H, 19-H_b, 6-H_a+7-H_a), 1.56–1.38 (m, 7H, 16-H_b+7-H_b+21-H_a+21-H_b+22-H_a+2-H_a+2-H_b), 1.40 (s, 3H, 27-H_3), 1.32 (s, 3H, 29-H_3), 1.28–1.18 (m, 1H, 6-H_b), 1.16 (s, 3H, 25-H_3), 1.13 (s, 3H, 26-H_3), 1.10–1.0 (m, 2H, 1-H_b+22-H_b), 0.89 (s, 3H, 23-H_3), 0.88 (s, 3H, 26-H_3), 0.81 (s, 3H, 28-H_3), 0.8 (s, 1H, 5-H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 199.9 (C-11), 175.8 (C-30), 170.8 (C-31), 146.6 (C-35+C-36), 128.6 (C-12), 114.5 (C-34+C-37) 80.3 (C-3),61.8 (C-9), 54.9 (C-5), 48.0 (C-18), 45.5 (C-8), 45.3 (C-17), 43.2 (C-14), 41.3 (C-19), 38.9 (C-1), 37.9 (C-4), 37.5 (C-21), 36.9 (C-10), 32.6 (C-7), 31.9 (C-22), 31.4 (C-16), 28.6 (C-29), 28.4 (C-28), 28.0 (C-24), 26.3 (C-15), 23.5 (C-2), 23.4 (C-27), 21.3 (C-32), 18.7 (C-26), 17.3 (C-6), 16.7 (C-23), 16.4 (C-25) ppm; MS (ESI, MeOH): m/z = 587.0 ([M − H]$^-$, 100%), 623.1 ([M + Cl^-]$^-$, 16%); analysis calcd for $C_{37}H_{52}N_2O_4$ (588.83): C 75.47, H 8.90, N 4.76; found: C 75.29, H 9.13, N 4.58.

4.6.9. N-3-pyrazolyl-3β-acetoxy-11-oxoolean-12-en-30-ic acid amide (16)

Following GP2, **16** (348 mg, 60%) was obtained as a white solid; m.p. 257–259 °C; $R_F = 0.15$ (hexanes/ethyl acetate, 11:9); $[\alpha]_D = +136.0°$ (*c* 0.115, $CHCl_3$); IR (ATR): $\tilde{\nu} = 2950br$, 1729 *m*, 1652 *m*, 1465 *m*, 1364 *m*, 1243*s*, 985 *m*, 755 s cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 7.67$ (d, $J =$ 2.6 Hz, 1H, 35-H), 6.73 (d, $J = 2.6$ Hz, 1H, 34-H), 5.8 (s, 1H, 12-H), 4.52 (dd, $J = 11.7$, 4.7 Hz, 1H, 3-H), 2.80 (m, 1H, 1-H_a), 2.38 (s, 1H, 9-H), 2.28–2.19 (m, H, 18-H), 2.16–1.95 (m, 3H, 16-H_a+15-H_a+19-H_a), 2.06 (s, 3H, 32-H_3) 1.90–1.80 (m, 1H, 15-H_b), 1.79–1.58 (m, 5H, 19-H_b, 2-H_a+2-H_b+6-H_a+7-H_a), 1.54–1.34 (m, 4H, 16-H_b+21-H_a+21-H_b+22-H_a), 1.40 (s, 3H, 27-H_3), 1.27 (s, 3H, 29-H_3), 1.28–1.18 (m, 2H, 6-H_b+7-H_b), 1.16 (s, 3H, 25-H_3), 1.12 (s, 3H, 26-H_3), 1.10–1.00 (m, 2H, 1-H_b+15-H_b), 0.89 (s, 3H, 23-H_3), 0.88 (s, 3H, 24-H_3), 0.83 (s, 1H, 5-H), 0.81 (s, 3H, 28-H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 200.3$ (C-11), 174.0 (C-30), 171.1 (C-31), 168.6 (C-13), 145.1 (C-33), 131.2 (C-35), 128.8 (C-12), 96.7 (C-34), 80.6 (C-3), 61.8 (C-9), 54.9 (C-5), 47.8 (C-18), 45.3 (C-8), 44.5 (C-17), 43.2 (C-14), 41.2 (C-19), 38.9 (C-1), 38.0 (C-4), 37.5 (C-21), 36.9 (C-10), 32.7 (C-7), 31.9 (C-22), 31.4 (C-16), 29.0 (C-29), 28.4 (C-28), 28.0 (C-24), 26.4 (C-15), 23.6 (C-2), 23.4 (C-27), 21.3 (C-32), 18.7 (C-26), 17.4 (C-6), 16.7 (C-23), 16.4 (C-25) ppm; MS (ESI, MeOH): m/z = 576.1 ([M − H]$^-$, 100%), 639.1 ([M+2-propanol + H]$^-$, 17%); analysis calcd for $C_{35}H_{51}N_3O_4$ (577.81): C 72.75, H 8.90, N 7.27; found: C 72.55, H 9.01, N 7.06.

4.6.10. N-5-indazolyl-3β-acetoxy-11-oxoolean-12-en-30-ic acid amide (17)

Following GP2, **17** (278 mg, 47%) was obtained as a yellow solid; m.p. 226–228 °C; $R_F = 0.18$ (hexanes/ethyl acetate, 11:9); $[\alpha]_D = +184.5°$ (*c* 0.131, $CHCl_3$); IR (ATR): $\tilde{\nu} = 2950br$, 1715 *m*, 1651*s*, 1465 *m*, 1245*s*, 942 *m*, 753 s cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 8.28–8.24 (m, 1H, 37-H), 8.16 (s, 1H, 39-H), 7.50 (dd, $J = 9.0$, 1.9 Hz, 1H, 34-H), 7.44 (d, $J = 9.0$ Hz, 1H, 35-H), 5.75 (s, 1H, 12-H), 4.5 (dd, $J = 11.7$, 4.7 Hz, 1H, 3-H), 2.81 (m, 1H, 1-H_a), 2.38 (s, 1H, 9-H), 2.23 (m, 2H, 18-H+16-H_a), 2.16–2.01 (m, 2H, 15-H_a+19-H_a), 2.06 (s, 3H, 32-H_3) 1.90–1.80 (m, 1H, 15-H_b), 1.78–1.56 (m, 5H, 19-H_b+6-H_a+7-H_a+2-H_a+2-H_b), 1.56–1.38 (m, 5H, 16-H_b+7-H_b+21-H_a+21-H_b+22-H_a), 1.42 (s, 3H, 27-H_3), 1.32 (s, 3H, 29-H_3), 1.28–1.18 (m, 1H, 6-H_b), 1.16 (s, 3H, 25-H_3), 1.14 (s, 3H, 26-H_3), 1.10–0.92 (m, 2H, 1-H_b+22-H_b), 0.89 (s, 3H, 23-H_3), 0.88 (s, 3H, 24-H_3), 0.84 (s, 3H, 28-H_3), 0.81 (s, 1H, 5-H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 200.3$ (C-11), 174.7 (C-30), 171.1 (C-31), 170.4 (C-33), 133.4 (C-37), 128.3 (C-12), 125.6 (C-34), 112.3 (C-39), 111.3 (C-35), 80.7 (C-3),61.8 (C-9), 54.9 (C-5), 48.5 (C-18), 45.5 (C-8), 44.5 (C-17), 43.3 (C-14), 41.5 (C-19), 38.9 (C-1), 38.1 (C-4), 37.5 (C-21), 36.9 (C-10), 32.7 (C-7), 31.9 (C-22), 31.6 (C-16), 29.2 (C-29), 28.6 (C-28), 27.9 (C-24), 26.5 (C-15), 23.5 (C-2), 23.3 (C-27), 21.3 (C-32), 18.7 (C-26), 17.3 (C-6), 16.6 (C-23), 16.4 (C-25) ppm; MS (ESI, MeOH): m/z = 626.1 ([M − H]$^-$, 100%), 662.0 ([M + Cl^-]$^-$, 15%); analysis calcd for $C_{39}H_{53}N_3O_4$ (627.87): C 74.61, H 8.51, N 6.69; found: C 74.48, H 8.78, N 6.51.

4.6.11. N-6-indazolyl-3β-acetoxy-11-oxoolean-12-en-30-ic acid amide (18)

Following GP2, **18** (386 mg, 65%) was obtained as a yellow; m.p. 217–219 °C; $R_F = 0.29$ (hexanes/ethyl acetate, 11:9); $[\alpha]_D = 166.6°$ (*c* 0.139, $CHCl_3$); IR (ATR): $\tilde{\nu} = 2952br$, 1712 *m*, 1650*s*, 1503 *m*, 1364w, 1249s, 985*w*, 944*w*, 752 s cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 8.58–8.49 (m, 1H, 36-H), 7.48 (d, $J = 8.8$ Hz, 1H, 34-H), 6.81 (s, 1H, 38-H), 6.67 (dd, $J = 8.7$, 1.9 Hz, 1H, 39-H), 5.33 (s, 1H, 12-H), 4.5 (dd, $J =$ 11.7, 4.7 Hz, 1H, 3-H), 2.81 (m, 1H, 1-H_a), 2.38 (s, 1H, 9-H), 2.29 (m, 2H, 18-H+16-H_a), 2.17–2.01 (m, 2H, 15-H_a+19-H_a), 2.07 (s, 3H, 32-H_3) 1.90–1.80 (m, 2H, 15-H_b+ 19-H_b), 1.78–1.56 (m, 4H, 6-H_a+7-H_a+2-H_a+2-H_b), 1.56–1.38 (m, 5H, 16-H_b+7-H_b+21-H_a+21-H_b+22-H_a), 1.41 (s, 3H, 27-H_3), 1.32 (s, 3H, 29-H_3), 1.25–1.18 (m, 1H, 6-H_b), 1.16 (s, 3H, 25-H_3), 1.13 (s, 3H, 26-H_3), 1.10–0.99 (m, 2H, 1-H_b+22-H_b), 0.89 (s, 3H, 23-H_3), 0.88 (s, 3H, 24-H_3), 0.82 (s, 3H, 28-H_3), 0.75 (s, 1H, 5-H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 200.1$ (C-11), 174.7 (C-30), 171.1 (C-31), 169.5 (C-33), 121.3 (C-38), 128.5 (C-12), 80.6 (C-3),61.7 (C-9), 54.9 (C-5), 48.4 (C-18), 45.3 (C-8), 44.76 (C-17), 43.3 (C-14), 41.6 (C-19), 38.7 (C-1), 38.0 (C-2), 37.3 (C-21), 36.9 (C-10), 32.6 (C-7), 31.9 (C-22), 31.5 (C-16), 29.1 (C-29), 28.4 (C-28), 28.0 (C-24), 26.5 (C-15), 23.5 (C-2), 23.4 (C-27), 21.3 (C-32), 18.7 (C-26), 17.4 (C-6), 16.7 (C-23), 16.4 (C-25) ppm; MS (ESI, MeOH): $m/z = 626.2$ ([M − H]$^-$, 100%), 662.0 ([M + Cl^-]$^-$, 10%); analysis calcd for $C_{39}H_{53}N_3O_4$ (627.87): C 74.61, H 8.51, N 6.69; found: C 74.40, H 8.77, N 6.41.

4.6.12. N-2-pyridinyl-3β-acetoxyolean-12-en-28-ic acid amide (19)

Following GP2, **19** (253 mg, 44%) [25] was obtained as a white solid; m.p. 218.9 °C; $R_F = 24$ (hexanes/ethyl acetate, 9:1); $[\alpha]_D = +42.7°$ (*c* 0.19, $CHCl_3$); IR (ATR): $\tilde{\nu} = 3360$ *w*, 2955 *m*, 2937 *m*, 2930 *m*, 2860 *w*, 1729 *s*, 1684 *s*, 1595 *w*, 1575 *m*, 1515 *s*, 1459 *m*, 1430 *s*, 1367 *m*, 1302 *m*, 1293 *m*, 1244 *vs*, 1172 *m*, 1148 *m*, 1097 *w*, 1024 *m*, 1010 *m*, 991 *m*, 968

m, 898 *w*, 780 *s*, 743 *w*, 654 *s*, 594 *m*, 516 *m*, 468 *w*, 410 m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 9.46 (s, 1H, N*H*), 8.45 (d, $J = 8.6$ Hz, 1H, 37-H), 8.21 (ddd, $J = 5.4$, 1.9, 0.9 Hz, 1H, 34-H), 7.84 (ddd, $J = 8.9$, 7.3, 1.9 Hz, 1H, 35-H), 7.11 (ddd, $J = 7.4$, 5.4, 1.1 Hz, 1H, 36-H), 5.52 (t, $J = 3.7$ Hz, 1H, 12-H), 4.51–4.41 (m, 1H, 3-H), 2.92–2.79 (m, 1H, 18-H_a), 2.15 (td, $J = 14.1$, 4.0 Hz, 1H, 16-H_a), 2.03 (s, 3H, 32-H_3), 2.01–1.82 (m, 3H, 2-H_a+11-H_a+16-H_b), 1.82–1.67 (m, 3H, 7-H_2), 1.66–1.53 (m, 5H, 1-H_a+2-H_b+9-H+11-H_b+15-H_a), 1.52–1.35 (m, 3H, 6-H_a+21-H_a+22-H_a), 1.34–1.18 (m, 4H, 6-H_b+19-H_b+21-H_b+22-H_b), 1.17 (s, 3H, 27-H_3), 1.12 (dt, $J = 14.0$, 3.5 Hz, 1H, 15-H_b), 1.09–0.98 (m, 1H, 1-H_b), 0.94 (s, 3H, 29-H_3), 0.92 (s, 3H, 30-H_3), 0.88 (s, 3H, 25-H_3), 0.84 (s, 3H, 24-H_3), 0.81 (s, 3H, 23-H_3), 0.85–0.78 (m, 1H, 5-H), 0.64 (s, 3H, 26-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): 177.4 (C-28), 170.9 (C-31), 150.9 (C-33), 143.6 (C-13), 143.6 (C-34), 141.1 (C-35), 123.3 (C-12), 119.2 (C-36), 115.1 (C-37), 80.8 (C-3), 55.2 (C-5), 47.9 (C-17), 47.5 (C-9), 46.3 (C-19), 41.9 (C-14), 41.5 (C-18), 39.4 (C-8), 38.1 (C-1), 37.7 (C-4), 36.9 (C-10), 34.1 (C-21), 33.0 (C-30), 32.4 (C-7+C-22), 30.6 (C-20), 28.0 (C-24), 27.5 (C-15), 25.8 (C-27), 23.6 (C-16), 23.6 (C-29), 23.5 (C-11), 23.5 (C-2), 21.3 (C-32), 18.1 (C-6), 16.6 (C-26), 16.6 (C-23), 15.4 (C-25) ppm; MS (ESI, MeOH): $m/z = 575.1$ ($[M+H]^+$, 100%), 1171.0 ($[2\,M + Na^+]^+$, 12%); analysis calcd for $C_{37}H_{54}N_2O_3$ (574.85): C 77.31, H 9.47, N 4.87; found: C 77.05, H 9.61, N 4.57.

4.6.13. N-3-pyridinyl-3β-acetoxyolean-12-en-28-ic acid amide (20)

Following GP2, **20** (325 mg, 58%) was obtained as a white solid; m.p. 193–194 °C; $R_F = 0.15$ (hexanes/ethyl acetate, 3:2); $[\alpha]_D = 24.3°$ (*c* 0.109, $CHCl_3$); IR (ATR): $\tilde{\nu} = 2924br$, 1732 *m*, 1586*s*, 1505*s*, 1369 *m*, 1325 *m*, 1244*s*, 1026 *m*, 826 *m*, 581*w*, 536 m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 8.59$ (d, $J = 3.0$ Hz, 1H, 37-H), 7.72 (d, $J = 2.8$ Hz, 3H, 36-H), 7.14 (d, $J = 3.1$ Hz, 1H, 34-H), 6.93 (d, $J = 2.8$ Hz, 3H, 35-H), 5.58 (s, 1H, 12-H), 4.49 (dd, $J = 11.7$, 4.7 Hz, 1H, 3-H), 2.89 (m, 1H, 18-H), 2.21–2.1 (m, 1H, 16-H_a), 2.05 (s, 3H, 32-H_3), 1.98 (m, 1H, 16-H_b), 1.88 (m, 2H, 11-H_a+11-H_b), 1.84–1.69 (s, 2H, 15-H_a, 22-H_a) 1.68–1.55 (m, 6H, 2-H_a+2-H_b+6-H_a+1-H_a+19-H_a+9-H), 1.50–1.32 (m, 4H, 7-H_a+7-H_b+6-H_b+21-H_a), 1.32–1.15 (m, 4H, 21-H_b+19-H_b+1-H_b+15-H_b), 1.17 (s, 3H, 27-H_3), 0.98 (s, 3H, 25-H_3), 0.95 (s, 3H, 30-H_3), 0.90 (s, 3H, 29-H_3), 0.86 (s, 3H, 24-H_3), 0.83 (s, 3H, 23-H_3), 0.68–0.63 (m, 1H, 5-H), 0.66 (s, 3H, 26-H_3) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 176.5$ (C-36), 174.7 (C-37), 170.8 (C-31), 165.4 (C-33), 142.7 (C-13), 123.9 (C-12), 80.8 (C-3), 55.1 (C-5), 47.6 (C-9), 47.5 (C-19), 47.5 (C-17), 41.7 (C-14), 41.3 (C-18), 39.4 (C-8), 39.3 (C-1), 37.7 (C-4), 36.9 (C-10), 36.8 (C-21), 36.8 (C-29), 32.9 (C-7), 32.3 (C-22), 30.7 (C-20), 30.7 (C-24), 27.9 (C-15), 23.7 (C-27), 23.6 (C-30), 21.3 (C-32), 18.2 (C-6), 16.7 (C-26), 16.7 (C-23), 15.4 (C-25) ppm; MS (ESI, MeOH): $m/z = 598.1$ ($[M + Na–2H]^-$, 100%); analysis calcd for $C_{37}H_{54}N_2O_3$ (574.85): C 77.31, H 9.47, N 4.87; found: C 77.17, H 9.63, N 4.62.

4.6.14. N-4-pyridinyl-3β-acetoxyolean-12-en-28-ic acid amide (21)

Following GP2, **21** (376 mg, 68%) was obtained as a white solid; m.p. 155–158 °C; $R_F = 0.25$ (hexanes/ethyl acetate, 3:2); $[\alpha]_D = +24.5°$ (*c* 0.132, $CHCl_3$); IR (ATR): $\tilde{\nu} = 2924br$, 1733 *m*, 1680*w*, 1532 *m*, 1479 *m*, 1369 *m*, 1245*s*, 1026 *m*, 706 m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 8.26 (dd, $J = 5.2$, 1.3 Hz, 2H, 36-H+35-H), 7.53 (dd, $J = 8.6$, 5.2 Hz, 2H, 34-H +37-H), 5.5 (s, 1H, 12-H), 4.51 (dd, $J = 11.7$, 4.7 Hz, 1H, 3-H), 2.34 (m, 1H, 18-H), 2.19–2.11 (m, 1H, 16-H_a), 2.05 (s, 3H, 32-H_3), 2.07–1.84 (m, 3H, 11-H_a+16-H_b+11-H_b), 1.85–1.73 (s, 1H, 15-H_a) 1.68–1.56 (m, 7H, 2-H_a+2-H_b+6-H_a+1-H_a+19-H_a+9-H+22-H_a), 1.44–1.21 (m, 4H, 7-H_a+7-H_b+6-H_b+21-H_a), 1.19–1.05 (m, 2H, 21-H_b+19-H_b), 1.14 (s, 3H, 27-H_3), 1.02–0.92 (m, 2H, 1-H_b+15-H_b) 0.99 (s, 3H, 25-H_3), 0.95 (s, 3H, 30-H_3), 0.90 (s, 3H, 29-H_3), 0.86 (s, 3H, 24-H_3), 0.83 (s, 3H, 23-H_3), 0.88–0.80 (m, 1H, 5-H), 0.68 (s, 3H, 26-H_3) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 177.6$ (C-36+C-35), 170.8 (C-31), 139.4 (C-34+C-37), 126.0 (C-12), 80.8 (C-3), 55.2 (C-5), 49.1 (C-19), 47.5 (C-9), 42.4 (C-17), 39.7 (C-18), 39.6 (C-8), 38.3 (C-1), 37.7 (C-4), 36.8 (C-10), 36.7 (C-21), 32.6 (C-29), 30.8 (C-20), 28.0 (C-24), 27.9 (C-15), 24.5 (C-27), 23.6 (C-30), 23.5 (C-11), 21.3 (C-32), 18.2 (C-6), 17.1 (C-26), 16.7 (C-23), 15.5 (C-25) ppm; MS (ESI, MeOH): $m/z = 673.1$ ($[M - H]^-$, 100%), 609.1 ($[M + Cl^-]^-$, 89%); analysis calcd for $C_{37}H_{54}N_2O_3$ (574.85): C 77.31, H 9.47, N 4.87; found: C 77.19, H 9.54, N 4.30.

4.6.15. N-3-pyrazolyl-3β-acetoxyolean-12-en-28-ic acid amide (22)

Following GP2, **22** (282 mg, 51%) was obtained as a white solid; m.p. 138–141 °C; $R_F = 0.19$ (hexanes/ethyl acetate, 6:4); $[\alpha]_D = 36.3°$ (*c* 0.128, $CHCl_3$); IR (ATR): $\tilde{\nu} = 2944br$, 1731 *m*, 1524 *m*, 1479 *m*, 1367 *m*, 1246*s*, 1026 *m*, 730*s*, 706 m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 8.28$ (d, $J = 5.1$ Hz, 1H, 35-H), 7.51–7.45 (m, 1H, 34-H), 5.54 (s, 1H, 12-H), 4.49 (dd, $J = 11.7$, 4.7 Hz, 1H, 3-H), 2.85 (m, 1H, 18-H), 2.19–2.1 (m, 1H, 16-H_a), 2.05 (s, 3H, 32-H_3), 2.01–1.94 (m, 2H, 11-H_a + H-16$_b$), 1.92–1.85 (m, 1H, 11-H_b), 1.85–1.69 (s, 2H, 15-H_a+22-H_a) 1.68–1.56 (m, 6H, 2-H_a+2-H_b+6-H_a+1-H_a+19-H_a+9-H), 1.54–1.38 (m, 4H, 7-H_a+7-H_b+6-H_b+21-H_a), 1.32–1.15 (m, 2H, 21-H_b+19-H_b), 1.20 (s, 3H, 27-H_3), 1.18–1.01 (m, 2H, 1-H_b+15-H_b) 0.97 (s, 3H, 25-H_3), 0.95 (s, 3H, 30-H_3), 0.91 (s, 3H, 29-H_3), 0.86 (s, 3H, 24-H_3), 0.84 (s, 3H, 23-H_3), 0.88–0.81 (m, 1H, 5-H), 0.70 (s, 3H, 26-H_3) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 177.6 (C-35), 171.1 (C-31), 144,1 (C-13), 124.9 (C-34), 122.8 (C-12), 80.3 (C-3), 55.1 (C-5), 47.7 (C-19), 47.4 (C-9), 46.4 (C-17), 42.0 (C-14), 41.9 (C-18), 39.4 (C-8), 38.1 (C-1), 37.7 (C-4), 36.9 (C-10), 34.2 (C-21), 32.9 (C-29), 32.3 (C-7), 32.2 (C-22), 30.7 (C-20), 27.9 (C-24), 27.4 (C-15), 25.7 (C-27), 23.7 (C-30), 23.6 (C-2), 23.5 (C-11), 21.3 (C-32), 18.1 (C-6), 16.9 (C-26), 16.6 (C-23), 15.4 (C-25) ppm; MS (ESI, MeOH): $m/z = 609.2$ ($[M + FA-H]^-$, 100%); analysis calcd for $C_{35}H_{53}N_3O_3$ (563.83): C 74.56, H 9.48, N 7.45; found: C 74.33, H 9.67, N 7.46.

4.6.16. N-5-indazolyl-3β-acetoxyolean-12-en-28-ic acid amide (23)

Following GP2, **23** (480 mg, 81%) was obtained as a yellow solid; m.p. 206–208 °C; $R_F = 0.20$ (hexanes/ethyl acetate, 2:1); $[\alpha]_D = 39.5°$ (*c* 0.131, $CHCl_3$); IR (ATR): $\tilde{\nu} = 2945br$, 1717 *m*, 1656 *m*, 1502*s*, 1463 *m*, 1366 *m*, 1245*s*, 1027 *m*, 753 s cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 8.33$ (dd, $J = 2.0$, 0.8 Hz, 1H, 37-H), 8.17 (d, $J = 1.0$ Hz, 1H, 39-H), 7.62 (dt, $J = 9.0$, 0.9 Hz, 1H, 34-H), 7.33 (ddd, $J = 9.0$, 4.6, 2.0 Hz, 1H, 35-H), 5.6 (s, 1H, 12-H), 4.5 (dd, $J = 11.7$, 4.7 Hz, 1H, 3-H), 2.72 (m, 1H, 18-H), 2.17–2.07 (m, 1H, 16-H_a), 2.05 (s, 3H, 32-H_3), 2.07–1.90 (m, 2H, 11-H_a+16-H_b), 1.89–1.70 (m, 4H, 15-H_a+11-H_b+2-H_a+2-H_b) 1.68–1.56 (m, 5H, 6-H_a+1-H_a+19-H_a+9-H+22-H_a), 1.55–1.39 (m, 3H, 7-H_a+7-H_b+6-H_b), 1.38–1.19 (m, 3H, 21-H_b+19-H_b+21-H_a), 1.2 (s, 3H, 27-H_3), 1.17–1.03 (m, 2H, 1-H_b+15-H_b) 0.98 (s, 3H, 25-H_3), 0.96 (s, 3H, 30-H_3), 0.91 (s, 3H, 29-H_3), 0.86 (s, 3H, 24-H_3), 0.82 (s, 3H, 23-H_3), 0.88–0.75 (m, 1H, 5-H), 0.71 (s, 3H, 26-H_3) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 176.7 (C-35), 171.2 (C-31), 145.3 (C-13), 136.9 (C-33), 133.3 (C-37), 124,1 (C-38), 123.4 (C-34), 121.8 (C-12), 111.5 (C-39), 110.9 (C-35), 80.8 (C-3), 55.2 (C-5), 47.5 (C-9), 47.2 (C-19), 46.7 (C-17), 42.6 (C-18), 42.2 (C-14), 39.5 (C-8), 38.2 (C-1), 37.7 (C-4), 36.8 (C-10), 34.2 (C-21), 32.9 (C-29), 32.4 (C-7), 32.2 (C-22), 30.8 (C-20), 28.0 (C-24), 27.4 (C-15), 25.7 (C-27), 24.2 (C-30),23.7 (C-2), 23.6 (C-11), 23.5 (C-16), 21.3 (C-32), 18.1 (C-6), 16.9 (C-26), 16.6 (C-23), 15.5 (C-25) ppm; MS (ESI, MeOH): $m/z = 612.2$ ($[M - H]^-$, 100%), 648.2 ($[M + Cl^-]^-$, 10%); analysis calcd for $C_{39}H_{55}N_3O_3$ (613.89): C 76.31, H 9.03, N 6.85; found: C 76.05, H 9.27, N 6.66.

4.6.17. N-6-indazolyl-3β-acetoxyolean-12-en-28-ic acid amide (24)

Following GP2, **24** (306 mg, 51%) was obtained as a yellow solid; m.p. 194–197 °C; $R_F = 0.6$ (hexanes/ethyl acetate, 3:2); $[\alpha]_D = +27.3°$ (*c* 0.107, $CHCl_3$); IR (ATR): $\tilde{\nu} = 2944br$, 1732 *m*, 1573 *m*, 1464 *m*, 1364 *m*, 1244*s*, 1026 *m*, 754 s cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 8.57$–8.51 (m, 1H, 36-H), 7.48 (d, $J = 8.8$ Hz, 1H, 34-H), 6.81 (s, 1H, 38-H), 6.67 (dd, $J = 8.7$, 1.9 Hz, 1H, 39-H), 5.33 (s, 1H, 12-H), 4.49 (dd, $J = 11.7$, 4.7 Hz, 1H, 3-H), 2.84 (m, 1H, 18-H), 2.22–2.13 (m, 1H, 16-H_a), 2.05 (s, 3H, 32-H_3), 2.10–1.98 (m, 2H, 11-H_a), 1.85–1.77 (m, 6H, 15-H_a+11-H_b+2-H_a+2-H_b+16-H_b) 1.77–1.51 (m, 5H, 6-H_a+1-H_a+19-H_a+9-H+22-H_a), 1.51–1.24 (m, 3H, 7-H_a+7-H_b+6-H_b), 1.23–1.12 (m, 2H, 19-H_b+21-H_a), 1.2 (s, 3H, 27-H_3), 1.11–0.90 (m, 3H, 21-H_b+1-H_b+15-H_b) 1.01 (s, 3H,

25-H_3), 0.95 (s, 3H, 30-H_3), 0.92 (s, 3H, 29-H_3), 0.87 (s, 3H, 24-H_3), 0.84 (s, 3H, 23-H_3), 0.89–0.78 (m, 1H, 5-H), 0.68 (s, 3H, 26-H_3) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 175.1 (C-33), 170.8 (C-31), 143.8 (C-13), 140.2 (C-36), 132.2 (C-38), 123.7 (C-39), 122.2 (C-12), 119.7 (C-34), 80.9 (C-3), 55.3 (C-5), 47.6 (C-9), 47.5 (C-19), 42.3 (C-18), 41.9 (C-14), 39.2 (C-8), 38.1 (C-1), 37.7 (C-4), 36.9 (C-10), 34,1 (C-21), 33.1 (C-29), 32.6 (C-7), 32.5 (C-22), 30.6 (C-20), 27.9 (C-24), 27.4 (C-15), 25.8 (C-27), 24.0 (C-2), 23.5 (C-11), 23.4 (C-16), 21.3 (C-32), 18.1 (C-6), 16.7 (C-26), 16.6 (C-23), 15.4 (C-25) ppm; MS (ESI, MeOH): *m/z* = 612.4 ([M − H]$^-$, 74%); analysis calcd for $C_{39}H_{55}N_3O3$ (613.89): C 76.31, H 9.03, N 6.85; found: C 76.13, H 9.24, N 6.63.

4.6.18. N-2-pyridinyl-3β-acetoxyolean-12-en-28-ic acid amide (25)

Following GP2, **25** (440 mg, 76%) [25] was obtained as a white solid; m.p. 221 °C; R_F = 0.16 (hexanes/ethyl acetate, 9:1); $[\alpha]_D$ = +27.1° (*c* 16.3, $CHCl_3$); IR (ATR): 3424 *vw*, 2975 *w*, 2967 *w*, 2937 *w*, 2927 *w*, 2846 *w*, 1729 *s*, 1679 *m*, 1592 *w*,1576 *m*,1514 *s*, 1455 *m*, 1430 *s*, 1393 *w*, 1368 *m*, 1302 *m*, 1296 *m*, 1280 *w*, 1249 *vs*, 1167 *w*, 1146 *w*, 1096 *w*, 1049 *w*, 1025 *m*, 1004 *w*, 991 *m*, 971 *w*, 900 *w*, 803 *w*, 784 *s*, 667 *w*, 654 *w*, 609 *w*, 602 *w*, 582 *m*, 525 *w*, 516 *m*, 410 *w* cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.28 (s, 1H, NH), 8.56 (dd, *J* = 8.8, 5.5 Hz, 1H, 37-H), 8.17 (ddd, *J* = 5.6, 1.9, 0.8 Hz, 1H, 34-H), 7.97 (ddd, *J* = 9.0, 7.3, 1.8 Hz, 1H, 35-H-H), 7.20 (ddd, *J* = 7.2, 4.5, 1.1 Hz, 1H, 36-H), 5.49 (t, *J* = 3.7 Hz, 1H, 12-H), 4.48 (dd, *J* = 11.0, 5.0 Hz, 1H, 3-H), 2.36 (d, *J* = 10.7 Hz, 1H, 18-H), 2.18 (td, *J* = 14.1, 4.5 Hz, 1H, 16-H_a), 2.03 (s, 3H, 32-H_3), 2.01–1.95 (m, 2H, 2-H_a+16-H_b), 1.93 (dq, *J* = 8.0, 3.3, 2.8 Hz, 1H, 11-H_a), 1.90–1.87 (m, 1H, 22-H_a), 1.78 (td, *J* = 14.0, 4.7 Hz, 1H, 15-H_a), 1.72–1.39 (m, 9H, 1-H_a+2-H_b+6-H_a+7-H_a+9-H+11-H_b+19-H+21-H_a+22-H_b), 1.39–1.20 (m, 3H, 6-H_b+7-H_b+21-H_b), 1.15 (ddd, *J* = 13.0, 5.2, 2.6 Hz, 1H, 15-H_b), 1.10 (s, 3H, 27-H_3), 1.12–0.98 (m, 2H, 1-H_b+20-H), 0.96 (d, *J* = 6.4 Hz, 3H, 30-H_3), 0.92 (d, *J* = 6.4 Hz, 3H, 29-H_3), 0.88 (s, 3H, 25-H_3), 0.84 (s, 3H, 24-H_3), 0.87–0.73 (m, 1H, 5-H), 0.81 (s, 3H, 23-H_3), 0.64 (s, 3H, 26-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 177.6 (C-28), 170.9 (C-31), 150.5 (C-33), 143.3 (C-35), 140.5 (C-34), 138.0 (C-13), 126.3 (C-12), 118.9 (C-36), 116.1 (C-37), 80.8 (C-3), 55.2 (C-5), 52.7 (C-18), 49.6 (C-17), 47.4 (C-9), 42.1 (C-14), 39.6 (C-19), 39.5 (C-8), 38.6 (C-20), 38.2 (C-1), 37.6 (C-4), 36.8 (C-10), 36.6 (C-22), 32.7 (C-7), 30.7 (C-21), 28.0 (C-24), 27.8 (C-15), 24.3 (C-16), 23.5 (C-27), 23.5 (C-11), 23.3 (C-2), 21.3 (C-32), 21.1 (C-30), 18.1 (C-6), 17.0 (C-29), 16.7 (C-26), 16.7 (C-23), 15.4 (C-25) ppm; MS (ESI, MeOH): *m/z* = 575.0 ([M+H]$^+$, 100%), 1171.1 ([2 M + Na^+]$^+$, 30%)%); analysis calcd for $C_{37}H_{54}N_2O_3$ (574.85): C 77.31, H 9.47, N 4.87; found: C 77.03, H 9.65, N 4.65.

4.6.19. N-3-pyridinyl-3β-acetoxyurs-12-en-28-ic acid amide (26)

Following GP2, **26** (509 mg, 89%) was obtained as a white solid; m.p. 176–179 °C; R_F = 0.5 (hexanes/ethyl acetate, 4:3); $[\alpha]_D$ = +28.1° (*c* 0.185, $CHCl_3$); IR (ATR): $\tilde{\nu}$ = 2925 *m*, 1732 *m*, 1681 *m*, 1586*w*, 1524 *m*, 1480 *m*, 1455 *m*, 1416 *m*, 1390 *m*, 1370 *m*, 1326*w*, 1245*vs*, 1194 *m*, 1145*w*, 1026 *m*, 1006 *m*, 985 *m*, 967 *m*, 902*w*, 796 *m*, 752*s*, 707*s*, 665 m cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.99 (s, 1H, 37-H), 8.79 (s, 1H, 33-NH), 8.68 (d, *J* = 8.6 Hz, 1H, 36-H), 8.26 (dd, *J* = 5.1, 1.4 Hz, 1H, 34-H), 7.44 (dd, *J* = 8.5, 5.1 Hz, 1H, 35-H), 5.49 (t, *J* = 3.6 Hz, 1H, 12-H), 4.48 (dd, *J* = 10.9, 5.3 Hz, 1H, 3-H), 2.28–2.22 (m, 1H, 18-H), 2.17–2.08 (m, 2H, 2-H_a+16-H_a), 2.03 (s, 3H, 32-H_3), 2.02–1.89 (m, 3H, 11-H_a+16-H_b+22-H_a), 1.80–1.70 (m, 2H, 2-H_b+15-H_a), 1.69–1.59 (m, 3H, 1-H_a+11-H_b+22-H_b), 1.59–1.43 (m, 5H, 6-H_a+7-H_a+9-H+19-H+21-H_a), 1.43–1.30 (m, 1H, 21-H_b), 1.30–1.22 (m, 2H, 6-H_b+7-H_b), 1.12 (s, 3H, 27-H_3), 1.16–1.01 (m, 3H, 1-H_b+15-H_b+20-H), 0.97 (d, *J* = 6.3 Hz, 3H, 30-H_3), 0.93 (d, *J* = 6.4 Hz, 3H, 29-H_3), 0.89 (s, 3H, 25-H_3), 0.84 (s, 3H, 24-H_3), 0.83 (dd, *J* = 29.0, 2.4 Hz, 1H, 5-H), 0.82 (s, 3H, 32-H_3), 0.66 (s, 3H, 26-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 177.7 (C-28), 171.0 (C-31), 139.7 (C-34), 139.4 (C-13), 137.5 (C-37), 130.4 (C-36), 129.0 (C-33), 126.1 (C-12), 124.9 (C-35), 80.8 (C-3), 55.2 (C-5), 53.4 (C-18), 49.0 (C-17), 47.4 (C-9), 42.4 (C-14), 39.7 (C-19), 39.6 (C-8), 38.8 (C-20), 38.3 (C-1), 37.6 (C-4), 36.8 (C-10), 36.7 (C-22), 32.6 (C-7), 30.8 (C-21), 28.0 (C-24), 27.9 (C-15), 24.6 (C-16), 23.5 (C-2), 23.5 (C-11), 23.4 (C-27), 21.3 (C-32), 21.2 (C-30), 18.1 (C-6), 17.2 (C-29), 16.9 (C-26), 16.7 (C-23) 15.5 (C-25) ppm; MS (ESI, MeOH/$CHCl_3$): *m/z* = 574.1 ([M − H]$^-$, 100%); analysis calcd for $C_{37}H_{54}N_2O_3$ (574.85): C 77.31, H 9.47, N 4.87; found: C 77.17, H 9.61, N 4.59.

4.6.20. N-4-pyridinyl-3β-acetoxyursol-12-en-28-ic acid amide (27)

Following GP2, **27** (108 mg, 19%) was obtained as a white solid; m.p. 168–171 °C; R_F = 0.09 (hexanes/ethyl acetate, 3:2); $[\alpha]_D$ = +34.4° (*c* 0.138, $CHCl_3$); IR (ATR): $\tilde{\nu}$ = 2944*br*, 1731 *m*, 1586*s*, 1504*s*, 1366 *m*, 1326 *m*, 1245*s*, 1026 *m*, 824 *m*, 751*s*, 579 *m*, 537 m cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.42 (d, *J* = 6.0 Hz, 2H, 35-H+36-H), 7.80 (d, *J* = 6.0 Hz, 2H, 34-H+37-H), 5.50 (t, *J* = 3.6 Hz, 1H, 12-H), 4.47 (dd, *J* = 11.7, 4.7 Hz, 1H, 3-H), 2.16 (s, 3H, 32-H_3), 2.19–2.08 (m, 1H, 16-H_a), 2.08–1.84 (m, 5H, 11-H_a+11-H_b+15-H_a+22-H_a+22-H_b), 1.76–1.43 (m, 6H, 2-H_a+2-H_b+1-H_a+6-H_a+21-H_a+7-H_a), 1.42–1.21 (m, 2H, 16-H_b+8-H_b) 1.12 (s, 3H, 23-H_3), 1.15–1.01 (m, 1H, 22-H_b), 0.97 (s, 3H, 31-H_3), 0.92 (s, 3H, 30-H_3), 0.87 (s, 3H, 23-H_3), 0.86 (s, 3H, 24-H_3), 0.86 (s, 3H, 29-H_3), 0.88–0.79 (m, 1H, 5-H), 0.62 (s, 3H, 26-H_3) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.1 (C-28), 170.8 (C-31), 148.1 (C-1), 146.9 (C-33), 139.7 (C-35+C-36), 126.9 (C-12), 114,1 (C-34+C-37), 80.7 (C-3), 55.2 (C-5), 53.7 (C-18), 49.3 (C-17), 47.4 (C-9), 42.4 (C-14), 39.7 (C-8), 39.5 (C-19), 38.8 (C-1), 38.3 (C-20), 37.6 (C-4), 36.8 (C-10), 36.5 (C-22), 32.6 (C-7), 30.7 (C-21), 28.0 (C-24), 27.9 (C-15), 24.8 (C-16), 23.5 (C-2), 21.3 (C-32), 21.1 (C-30), 18.0 (C-6), 17.2 (C-26), 16.7 (C-23), 15.5 (C-25) ppm; MS (ESI, MeOH): *m/z* = 573.2 ([M − H]$^-$, 100%), 609.2 ([M + Cl^-]$^-$, 21%); analysis calcd for $C_{37}H_{54}N_2O_3$ (574.85): C 77.31, H 9.47, N 4.87; found: C 77.01, H 9.67, N 4.68.

4.6.21. N-3-pyrazolyl-3β-acetoxyursol-12-en-28-ic acid amide (28)

Following GP2, **28** (245 mg, 45%) was obtained as a white solid using GP2 and **10**; m.p. 188–191 °C; R_F = 0.1 (hexanes/ethyl acetate, 3:2); $[\alpha]_D$ = +27.5° (*c* 0.115, $CHCl_3$); IR (ATR): $\tilde{\nu}$ = 2925*br*, 1732 *m*, 1565 *m*, 1455 *m*, 1369 *m*, 1244*s*, 1026*s*, 754 s cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.51 (d, *J* = 2.4 Hz, 1H, 35-H), 6.50 (d, *J* = 2.4 Hz, 1H, 34-H), 5.48 (t, *J* = 3.6 Hz, 1H, 12-H), 4.47 (dd, *J* = 11.7, 4.7 Hz, 1H, 3-H), 2.17–2.05 (m, 2H, 16-H_a+16-H_b), 2.03 (s, 3H, 32-H_3), 2.01–1.87 (m, 3H, 11-H_a+11-H_b+15-H_a), 1.87–1.77 (m, 1H, 22-H_a), 1.77–1.65 (m, 1H, 22-H_b), 1.65–1.41 (m, 6H, 2-H_a+2-H_b+1-H_a+6-H_a+21-H_a+7-H_a), 1.41–1.21 (m, 3H, 9-H+6-H_b+ 21-H_b) 1.11 (s, 3H, 27-H_3), 1.15–1.01 (m, 2H, 15-H_b+1-H_b), 0.97 (s, 3H, 25-H_3), 0.91 (s, 3H, 30-H_3), 0.88 (s, 3H, 23-H_3), 0.85 (s, 3H, 24-H_3), 0.82 (s, 3H, 29-H_3), 0.86–0.76 (m, 1H, 5-H), 0.65 (s, 3H, 26-H_3) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 176.2 (C-28), 170.8 (C-31), 151.9 (C-33), 145.2 (C-13), 139.0 (C-35), 126.4 (C-12), 96.1 (C-34), 80.7 (C-3), 55.1 (C-5), 53.6 (C-18), 48.4 (C-17), 47.4 (C-9), 42.3 (C-14), 39.7 (C-19), 39.5 (C-1), 38.9 (C-20), 37.6 (C-4), 36.9 (C-10), 36.7 (C-22), 32.6 (C-7), 30.8 (C-21), 27.9 (C-24), 27.8 (C-15), 25.0 (C-16), 23.5 (C-16), 21.3 (C-32), 21.1 (C-30), 18.1 (C-6), 17.2 (C-7), 16.7 (C-23), 15.5 (C-25) ppm; MS (ESI, MeOH): *m/z* = 562.0 ([M − H]$^-$, 100%), 598.1 ([M + Cl^-]$^-$, 96%); analysis calcd for $C_{35}H_{53}N_3O_3$ (563.83): C 74.56, H 9.48, N 7.45; found: C 74.41, H 9.65, N 7.35.

4.6.22. N-6-indazolyl-3β-acetoxyurs-12-en-28-ic acid amide (29)

Following GP2, **29** (292 mg, 50%) was obtained as an off-white solid; m.p. 189–191 °C; R_F = 0.48 (hexanes/ethyl acetate, 3:2); $[\alpha]_D$ = +41.6° (*c* 0.115, $CHCl_3$); IR (ATR): $\tilde{\nu}$ = 2925*br*, 1718 *m*, 1653 *m*, 1509 *m*, 1465 *m*, 1359 *m*, 1244*s*, 1026 *m*, 942 *m*, 841*w*, 753 s cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.18 (d, *J* = 1.0 Hz, 1H, 36-H), 8.03 (s, 1H, 34-H), 7.71 (dd, *J* = 8.7, 4.9 Hz, 1H, 38-H), 7.09 (dd, *J* = 8.8, 1.7 Hz, 1H, 39-H), 5.55 (t, *J* = 3.6 Hz, 1H, 12-H), 4.48 (dd, *J* = 11.7, 4.7 Hz, 1H, 3-H), 2.21–1.95 (m, 3H, 16-H_a+16-H_b+22-H_a), 2.03 (s, 3H, 32-H_3), 1.95–1.83 (m, 2H, 11-H_a+11-H_b), 1.83–1.68 (m, 1H, 22-H_b), 1.68–1.41 (m, 6H, 2-H_a+2-H_b+1-H_a+6-H_a+21-H_a+7-H_a), 1.41–1.21 (m, 2H, 9-H+6-H_b) 1.14 (s, 3H, 27-H_3), 1.19–1.02 (m, 3H, 15-H_b+1-H_b+21-H_b), 0.99 (s, 3H, 25-H_3), 0.95 (s, 3H, 30-H_3), 0.88 (s, 3H, 23-H_3), 0.83 (s, 3H, 24-H_3), 0.81 (s, 3H, 29-H_3), 0.91–0.78 (m, 1H, 5-H), 0.66 (s, 3H, 26-H_3) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 176.9 (C-28), 171.1 (C-31), 140.2 (C-13), 139.0 (C-33),

126.4 (C-12), 121.8 (C-36), 118.8 (C-38), 116.9 (C-39), 100.1 (C-34), 80.8 (C-3), 55.1 (C-5), 54.3 (C-18), 48.9 (C-17), 47.4 (C-9), 42.6 (C-14), 39.9 (C-19), 39.6 (C-1), 39.1 (C-20), 38.3 (C-4), 37.7 (C-10), 36.7 (C-22), 32.6 (C-7), 30.8 (C-21), 28.0 (C-24), 27.9 (C-15), 25.2 (C-16), 23.6 (C-2), 21.3 (C-32), 21.2 (C-30), 18.0 (C-6), 17.3 (C-26), 16.6 (C-23), 15.5 (C-25) ppm; MS (ESI, MeOH): $m/z = 612.0$ ([M − H]$^-$, 100%), 648.1 ([M + Cl$^-$]$^-$, 18%); analysis calcd for $C_{39}H_{55}N_3O_3$ (613.89): C 76.31, H 9.03, N 6.85; found: C 76.19, H 9.24, N 6.61.

4.6.23. N-5-indazolyl-3β-acetoxyurs-12-en-28-ic acid amide (30)

Following GP2, **30** (333 mg, 56%) was obtained as a white solid; m.p. 238–241 °C; R_F = 0.35 ($CHCl_3$/MeOH/NH_4OH, 98:1.8:0.2); $[\alpha]_D$ = +30.1° (*c* 0.170, $CHCl_3$); IR (ATR): $\tilde{\nu} = 2946$ *m,* 2925 *m,* 2872*w,* 1731 *m,* 1717 *m,* 1650 *m,* 1593*w,* 1535 *m,* 1502*s,* 1453 *m,* 1390 *m,* 1369 *m,* 1311*w,* 1245*vs,* 1146*w,* 1104*w,* 1078*w,* 1027 *m,* 1006 *m,* 985 *m,* 967 *m,* 944 *m,* 901*w,* 875*w,* 831*w,* 806 *m,* 753*vs,* 665 *m,* 608 *m,* 557*w,* 536*w,* 426 m cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 8.50$ (d, $J = 1.9$ Hz, 1H, 39-H), 8.30 (s, 1H, 37-H), 7.92 (s, 1H, NH), 7.80 (d, $J = 9.1$ Hz, 1H, 34-H), 7.34 (dd, $J = 9.2$, 1.9 Hz, 1H, 35-H), 5.57–5.53 (m, 1H, 12-H), 4.48 (dt, $J = 11.6$, 3.6 Hz, 1H, 3-H), 2.16–1.94 (m, 5H, 2-H_a+11-H_a+16-H_a+18-H), 2.03 (s, 3H, 32-H_3), 1.86 (d, $J = 13.3$ Hz, 1H, 16-H_b), 1.78–1.67 (m, 2H, 2-H_b+15-H_a), 1.68–1.54 (m, 5H, 1-H_a+9-H+11-H_b+21-H_a+22-H_b), 1.54–1.45 (m, 3H, 6-H_a+7-H_a+19-H), 1.43–1.21 (m, 3H, 6-H_b+7-H_b+21-H_b), 1.14 (s, 3H, 27-H_3), 1.21–1.03 (m, 2H, 1-H_b+15-H_b), 1.01–0.98 (m, 4H, 20-H+30-H_3), 0.95 (t, J = 5.8 Hz, 3H, 29-H_3), 0.90–0.87 (m, 3H, 25-H_3), 0.84 (s, 3H, 24-H_3), 0.84–0.79 (m, 4H, 5-H+23-H_3), 0.66–0.62 (m, 3H, 26-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): $\delta = 176.9$ (C-28), 171.0 (C-31), 140.3 (C-13), 136.4 (C-38), 134.5 (C-33), 128.0 (C-37), 126.4 (C-35), 126.2 (C-12), 120.8 (C-36), 112.8 (C-34), 110.3 (C-39), 80.8 (C-3), 55.2 (C-5), 54.3 (C-18), 48.8 (C-17), 47.4 (C-9), 42.7 (C-14), 39.9 (C-19), 39.6 (C-8), 39.1 (C-20), 38.3 (C-1), 37.6 (C-4), 37.0 (C-22), 36.8 (C-10), 32.6 (C-7), 30.8 (C-21), 28.0 (C-15), 27.9 (C-24), 25.2 (C-16), 23.6 (C-11), 23.5 (C-2), 23.3 (C-27), 21.3 (C-32), 21.1 (C-30), 18.0 (C-6), 17.3 (C-29), 16.9 (C-26), 16.7 (C-23), 15.6 (C-25) ppm; MS (ESI, MeOH/$CHCl_3$): $m/z = 613.1$ ([M − H]$^-$, 100%); analysis calcd for $C_{39}H_{55}N_3O_3$ (613.89): C 76.31, H 9.03, N 6.85; found: C 76.18, H 9.31, N 6.58.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmcr.2022.100085.

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Selective and low-cost triterpene urea and amide derivatives of high cytotoxicity and selectivity

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ABSTRACT

Phytochemicals play a vital role in drug discovery, especially for the development of anti-cancer drugs. Thereby, convenient syntheses, high cytotoxicity but also good tumor cell/non-tumor cell selectivity, are called for. An interesting group of phytochemicals is represented by pentacyclic triterpenoic acids and derivatives thereof. Herein we report the synthesis of some ursolic and oleanolic acid derived amides and urea derivatives and the results from sulforhodamine (SRB) assays to assess their cytotoxic activity for several human tumor cell lines. As a result, an ursolic acid derived benzyl urea **16** showed a rather low $EC_{50} = 1.4$ μM for A375 melanoma cell while being not cytotoxic ($EC_{50} = 70$ μM) for non-malignant fibroblasts (NIH 3T3).

Introduction

According to the WHO, cancer is still among the most common causes of death worldwide. There were an estimated 18.1 million - cancer cases around the world in 2020 and the number of persons affected by this disease is estimated to be still rising within the next years. One of the best-established methods – besides surgery – for its therapy remains chemotherapy, involving treating the patient with a cytotoxic agent aiming to kill – more or less selective – the tumor cells while not affecting the non-malignant tissues. However, chemotherapy is very often far away from selectivity; this might result in serious side effects that can be mitigated at least in part by using combinations of different chemotherapeutics or to rely on agents, such as some phytochemicals, that are assumed to have some extra beneficial effects [1]. As a consequence, today about 48 % of all anti-cancer therapies involve the use of phytochemicals [2]. Thereby, pentacyclic triterpenoic acids which are known for their anti-cancer activity and anti-inflammatory properties seem most promising. The cytotoxic effects of these triterpenes can be enhanced by suitable modification. In this work oleanolic (**1**, Fig. 1) and ursolic acid (**2**) were converted into amides and urea derivatives, and analyzed by SRB assays to assess their cytotoxicity [3–5].

Most recently, inhibitors of the enzyme carbonic anhydrase II (CA2) [6–8] have come into the focus for the treatment of cancer [9–12]. Inhibitors of this enzyme [13–15] had appeared in the past mainly for the treatment of ocular diseases, e.g., glaucoma [16–24]. Based on findings that CA2 is amplified in recurrent glioblastoma and temozolomide-resistant glioblastoma stem-like cells, it was reported [25] that the CA2 inhibitor brinzolamide could be used in combination therapies to break the chemo-resistance of glioblastoma. This seems to be of particular interest because glioblastoma is the most common brain tumor; despite aggressive therapy, a median survival of only about 15 months is usually achieved [26–32].

Results and discussion

Chemistry

In previous works [3,33], acetylation proved to be an efficient way to increase cytotoxicity, and parent materials **1** and **2** were converted into their respective acetates **3** and **4** (Scheme 1). Their treatment with diphenylphosphoryl azide (DPPA) [34,35] furnished isocyanates **5** and **6** with excellent yields of 96 % each. Decarboxylative hydrolysis of the isocyanates with diluted hydrochloric acid at 50 °C for one day gave the corresponding amines **7** and **8**.

Isocyanates **5** and **6** were reacted with the aniline, benzylamine, 3-amino-quinoline and 4-amino-isoquinoline to afford urea **13**–**20** while from the reaction of **7** and **8**, amides **9**–**12** were obtained. It should be

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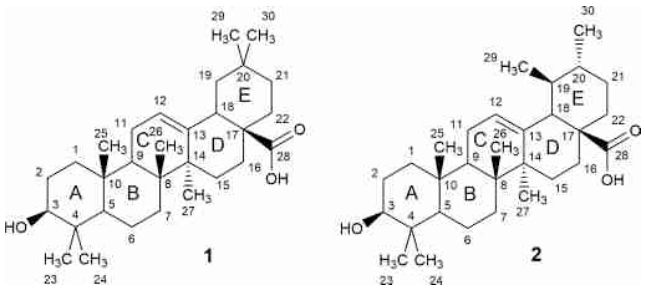


Fig. 1. Structure and numbering scheme of oleanolic (**1**) and ursolic acid (**2**).

noted that isocyanates **5** and **6** are stable under dry conditions and at 5 °C; when dissolved in DCM, however, they are slowly converted to amines **7** and **8** upon exposition to air humidity.

Biology

For the evaluation of their cytotoxicity, SRB assays were carried out employing five human tumor cell lines and non-malignant fibroblasts (NIH 3T3). The results from these assays are compiled in Table 1. (Table 1). As a result, all the compounds (except **11**) were cytotoxic in malignant cell lines A375 (melanoma) and A2780 (ovarian carcinoma). Compared to amides **9**, **11**, and **12**, for urea derivatives **13**–**20** superior EC_{50} values and selectivity were observed with benzyl urea **15** and **16** being the most promising compounds. For them the lowest EC_{50} value of 1.4 μM was determined for A375 cells. Furthermore, for **16** a superior tumor cell/non-tumor cell selectivity of ~ 50 was found. In addition, these compounds also showed promising cytotoxicity in A2780 ovarian carcinoma cells. The most noticeable difference to similar compounds of previous works is the better availability of the starting materials and the simplicity of synthesis [3–5]. Moreover, these results indicate that ursolic acid derived compounds hold overall lower EC_{50} values than their oleanolic acid derived analogs.

As stated above, inhibitory activity for CA2 in addition to the cytotoxic activity might be beneficial in the treatment of glioblastoma. Therefore, the inhibitory activity of the compounds for CA2 was also investigated. These investigations, however, showed only the oleanolic acid derived phenyl amide **9** to act as a strong inhibitor of CA2 with 79.8 ± 9 % inhibition of the enzyme (at a concentration of 8.0 mM). This however doesn't seem to affect the cytotoxicity of this compound as measured in the SRB assays but might have an influence when tested *in vivo* especially under hypoxic conditions and employing glioblastoma cells.

Conclusions

Twelve new triterpenoic urea and amide derivates holding good cytotoxicity in several human tumor cell lines have been synthesized and screened in SRB assays. Of special interest are compounds **15** and **16** – two benzyl urea derivatives derived from ursolic and oleanolic acid. Their EC_{50} values for epithelial melanoma (A375) and ovarian carcinoma (A2780) were as low as 1.4 μM and 2.6 μM, respectively. Thereby, especially **16** was selective cytotoxic for the melanoma cells while being approximately 50 times less cytotoxic for the non-malignant fibroblasts. Hence, we regard this compound as an interesting candidate for further extensive testing. Furthermore, ursolic acid derived compounds seem to be more cytotoxic than their oleanolic acid derived analogs. Interestingly enough, benzamide **9** proved also to be a good inhibitor of the enzyme carbonic anhydrase II – this makes this compound maybe a lead compound for the development of compounds targeting glioblastoma.

Experimental part

General

The melting points were measured with a Büchi Melting Point M-565 apparatus. For MS spectra, an Advion Expression^{L} CMS instrument was used. UV–vis-spectra were obtained and determined by transmission in quartz cuvettes using an Agilent Cary 60 spectroscope. Specific optical rotations were measured using a Perkin-Elmer polarimeter 341, and IR measurements were performed on a Bruker Tensor 27 in ATR mode. For NMR spectroscopy, Agilent spectrometers DD2 500 MHz and VNMRS 400 MHz were used. Solvents were dried according to usual procedures, chemicals obtained from local suppliers. For carbonic anhydrase II inhibition measurements, to a Tris-HCl buffer solution (125 μL, 50 mM, pH = 8), containing NaCl (5.84 mg/mL), enzyme solution (bovine CA2, 0.3 mg/mL, 25 μL), and the test substance solution (25 μL) were added. After incubation of the mixture at 37 °C for 20 min, 4-nitrophenyl acetate (25 μL) was added. The absorbance was then measured at 37 °C and λ = 415 nm. SRB assays were performed as previously described [33].

Syntheses

Oleanoic acid (1) and ursolic acid (2)

These compounds were obtained from Betulinines (Strbrna Skalice, Czech Republic) and used as received.

3-O-Acetyl-oleanolic acid (3) and 3-O-acetyl-ursolic acid (4)

The compounds were prepared as previously described [36].

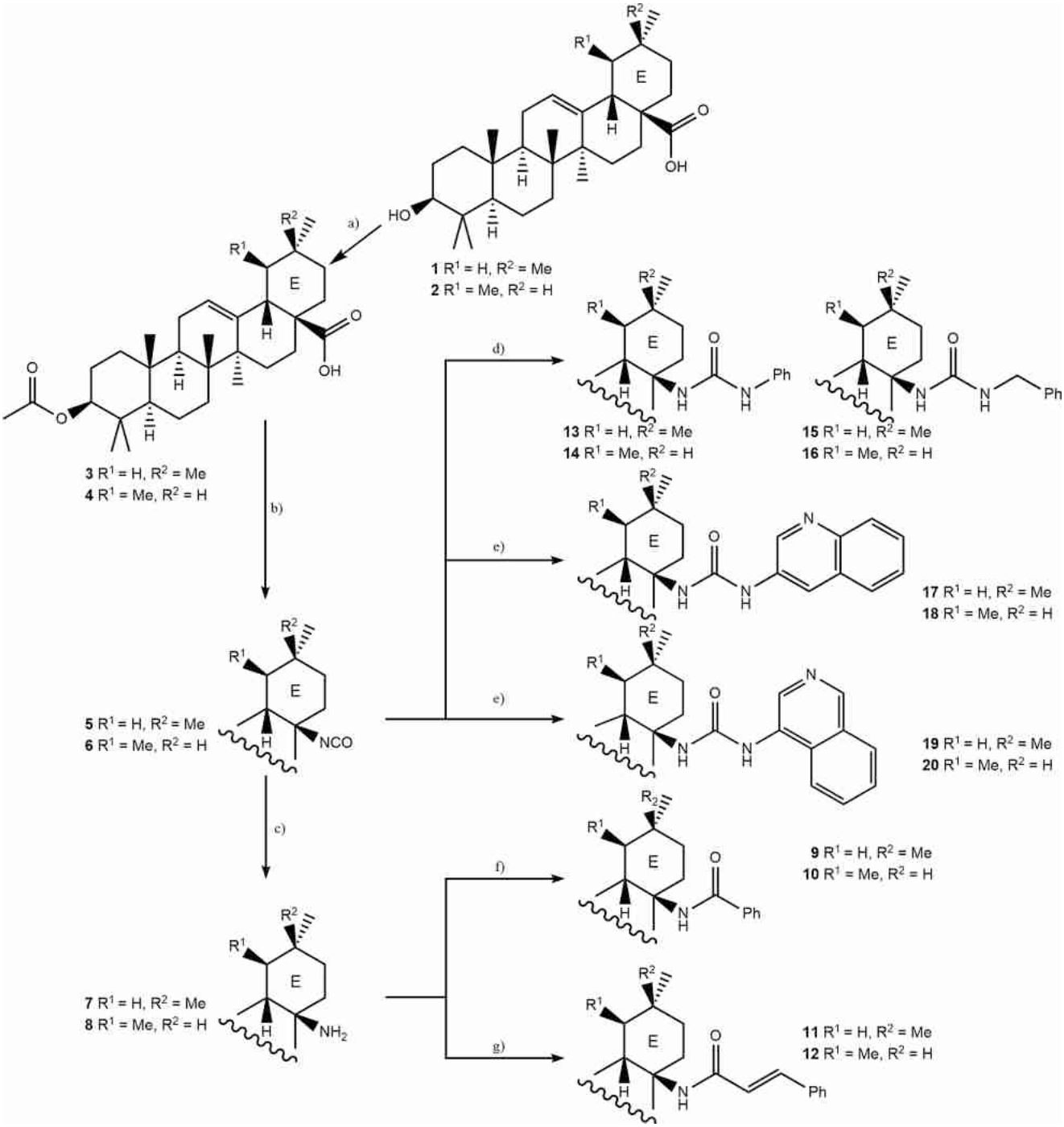
3 β-Acetyloxy-17 β-isocyanato-28-norolean-12-ene (5)

After stirring a solution of **3** (330 mg, 0.66 mmol) in toluene (10 mL) and Et_3N (0.14 mL, 100 mg, 1.0 mmol) for 15 min, diphenyl phosphoryl azide (0.17 mL, 218 mg, 0.8 mmol) was added, and the reaction mixture was stirred for 12 h at 21 °C. The solution was diluted with Et_2O (100 mL) and washed with HCl (0.1 M, 100 mL), H_2O (2 × 100 mL), and brine (50 mL). The organic phase was dried ($MgSO_4$) and evaporated under reduced pressure. The residue was purified by column chromatography (SiO_2, hexanes/ethyl acetate, 97:3) and **5** (310 mg, 96 %) was obtained as a white solid; m.p. 199.5 °C (decomp.); R_F = 0.66 (hexanes/ethyl acetate, 9:1); $[\alpha]_D = 71.9°$ (*c* 0.186, $CHCl_3$); IR (ATR): $\tilde{\nu}$ = 575*w*, 608*w*, 652*w*, 658*w*, 868*w*, 900*w*, 950*w*, 959*w*, 971*w*, 986 *m*, 1010 *m*, 1027 *m*, 1096*w*, 1185*w*, 1212*w*, 1246*vs*, 1363 *m*, 1371 *m*, 1378 *m*, 1387*w*, 1441*w*, 1464*w*, 1730 *m*, 2250 *s*, 2932 *m*, 2943 *m*, 2970*w* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.33–5.28 (m, 1H, 12-H), 4.53–4.45 (m, 1H, 3-H), 2.36 (dd, *J* = 13.7, 4.5 Hz, 1H, 18-H), 2.04 (d, *J* = 2.4 Hz, 3H, 32-H_3), 2.03–1.92 (m, 2H, 16-H_a + 16-H_b), 1.94–1.85 (m, 1H, 11-H_a), 1.69 (m, 2H, 2-H_a + 15-H_a), 1.67–1.54 (m, 6H, 1-H_a + 9-H + 11-H_b + 19-H_a + 22-H_a + 22-H_b), 1.56–1.49 (m, 2H, 1-H_b + 6-H_b), 1.50–1.35 (m, 3H, 2-H_b + 6-H_a + 7-H_a), 1.37–1.15 (m, 3H, 7-H_b + 19-H_b + 21-H_a), 1.12 (s, 3H, 27-H_3), 1.06 (m, 1H, 15-H_b), 0.94 (s, 3H, 25-H_3), 0.91 (s, 3H, 30-H_3), 0.90 (s, 3H, 29-H_3), 0.86 (m, 6H, 23-H_3 + 24-H_3), 0.82 (s, 1H, 5-H), 0.76 (s, 3H, 26-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 171.0 (C-31), 143.0 (C-13), 124.0 (C-28), 123.0 (C-12), 80.9 (C-3), 62.1 (C-17), 55.3 (C-5), 48.8 (C-18), 47.2 (C-1), 45.8 (C-9), 41.6 (C-19), 39.3 (C-8), 38.1 (C-22), 37.7 (C-4), 36.9 (C-10), 35.5 (C-21), 32.9 (C-30), 32.6 (C-7), 32.2 (C-2), 30.6 (C-20), 28.0 (C-24), 27.4 (C-15), 25.8 (C-27), 23.5 (C-29), 23.4 (C-11), 23.0 (C-16), 21.3 (C-32), 18.2 (C-6), 16.9 (C-26), 16.7 (C-23), 15.3 (C-25) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 1013.7 (22 %, $[2\ M + Na]^+$); analysis calcd for $C_{32}H_{49}NO_3$ (495.75): C 77.53, H 9.96, N 2.83; found: C 77.39, H 10.13, N 2.77.

3 β-Acetyloxy-17 β-isocyanato-28-norurs-12-ene (6)

Following the same procedure as for the synthesis of **5**, **6** was synthesized from **4** (330 mg, 0.67 mmol), and **6** (310 mg, 96 %) was obtained as a white solid; m.p. 181.6 °C (decomp.); R_F = 0.66 (hexanes/

ethyl acetate, 9:1); $[\alpha]_D = 63.0°$ (*c* 0.177, $CHCl_3$); IR (ATR): $\tilde{\nu} = 575w$, 662*w*, 864*w*, 901*w*, 977 *m*, 985 *m*, 1005 *m*, 1026 *m*, 1245*vs*, 1370 *m*, 1388*w*, 1456*w*, 1729 *s*, 2238 *s*, 2254 *s*, 2929 *m* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 5.27$ (m, $J = 3.9$ Hz, 1H, 12-H), 4.53–4.46 (m, 1H, 3-H), 2.14 (dd, $J = 11.3$, 1.9 Hz, 1H, 9-H), 2.04 (s, 3H, 32-H_3), 1.96–1.90 (m, 3H, 11-H_a + 16-H_a + 22-H_a), 1.78–1.73 (m, 2H, 18-H + 22-H_b), 1.69–1.58 (m, 5H, 1-H_a + 2-H_a + 2-H_b + 11-H_b + 21-H_a), 1.58–1.48 (m, 1H, 6-H_a), 1.53 (s, 1H, 7-H_a), 1.44–1.36 (m, 2H, 6-H_b + 7-H_b), 1.34–1.25 (m, 2H, 16-H_b + 19-H), 1.22–1.17 (m, 1H, 16-H_b), 1.11–1.05 (m, 3H, 1-H_b + 15-H_a + 15-H_b), 1.06 (s, 3H, 27-H_3), 1.03 (s, 3H, 23-H_3), 0.98 (s, 1H, 20-H), 0.95 (s, 3H, 29-H_3), 0.93 (s, 3H, 30-H_3), 0.88 (s, 3H, 24-H_3), 0.86 (s, 3H, 25-H_3), 0.83 (s, 1H, 5-H), 0.78 (s, 3H, 26-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): $\delta = 170.9$ (C-31), 137.6 (C-13), 126.8 (C-12), 126.2 (C-28), 80.9 (C-3), 60.6 (C-18), 55.3 (C-5), 52.7 (C-9), 41.9 (C-14), 41.7 (C-22), 40.9 (C-19), 39.8 (C-10), 39.5 (C-18), 38.9 (C-20), 38.3 (C-1), 37.7 (C-4), 36.4 (C-2), 33.1 (C-7), 31.7 (C-21), 28.2 (C-16), 28.1 (C-24), 27.3 (C-15), 24.2 (C-17), 23.3 (C-11), 23.1 (C-27), 21.3 (C-32), 20.7 (C-30), 18.2 (C-6), 17.4 (C-23), 16.9 (C-26), 16.7 (C-25), 15.6 (C-29) ppm; MS (ESI, $MeOH/CHCl_3$, 4:1): $m/z = 1013.5$ (28 %, $[2\ M + Na]^+$); analysis calcd for $C_{32}H_{49}NO_3$ (495.75): C 77.53, H 9.96, N 2.83; found: C 77.32, H



Scheme 1. A) cat. dmap, ac_2O, pyridine, 21 °C, 3 h → DCM, 0.1 M HCl, 21 °C, 30 min, 89 % (**3**) / 90 % (**4**); b) NEt_3, toluene, DPPA, 12 h, 21 °C, 96 % (**5**, **6**); c) THF, 2 M HCl, 50 °C, 24 h, 47 % (**7**) / 90 % (**8**); d) toluene, NEt_3, aniline (**13**, **14**) / benzylamine (**15**, **16**), 21 °C, 12 h, 46 % (**13**), 80 % (**14**), 100 % (**15**/**16**); e) toluene, NEt_3, 3-aminoquinoline (**17**, **18**), 4-aminoisoquinoline (**19**, **20**), 21 °C, 12 h, 50 % (**17**), 30 % (**18**), 50 % (**19**), 30 % (**20**); f) DCM, benzoyl chloride, NEt_3, DMAP, 21 °C, 2 h, 86 % (**9**), 100 % (**10**); g) DCM, cinnamic acid chloride, NEt_3, DMAP, 0 °C → 21 °C, 2 h, 89 % (**11**), 54 % (**12**).

Table 1
Cytotoxicity compounds **9**, **11**, **12**, **14**–**20** against various cell lines. These were assessed by SRB-assay (EC_{50} values [μM] after 72 h of treatment; measurements were performed in triplicate with three technical replicates each). Human cancer cell lines: A375 (epithelial melanoma), HT29 (colorectal adenocarcinoma), MCF-7 (breast adenocarcinoma), A2780 (ovarian carcinoma), HeLa (cervical carcinoma); non-malignant: NIH 3T3 (murine fibroblasts); S: Selectivity compared to NIH 3T3 (minimum selectivity was calculated using the cut-off concentration except for **16**). Doxorubicin (**DX**) and staurosporine (**ST**) were used as positive controls. Additionally, **CA2** inhibition is shown at 8.0 mmol concentration of the compound. Compounds **10** and **13** were not soluble under the conditions of the assays.

	A375	HT29	MCF-7	A2780	HeLa	NIH 3T3	CA2 [%]
9	8.3 ± 0.9 (S > 2.4)	>30	>30	11.1 ± 2.1 (S > 1.8)	>30	>20	79.8 ± 9
11	>30	>30	>30	>30	>30	>30	<5
12	7.5 ± 1.1 (S > 4)	>30	>30	15.6 ± 3.4 (S > 1.9)	>30	>20	<5
14	3.6 ± 0.6 (S > 8.3)	>30	>30	7.1 ± 1.8 (S > 4.2)	>30	>30	<5
15	1.4 ± 0.3 (S > 14.3)	>30	6.9 ± 1.4 (S > 2.9)	2.6 ± 0.6 (S > 7.7)	6.2 ± 1.8 (S > 3.2)	>20	<5
16	1.4 ± 0.1 (S ~ 50)	9.8 ± 2.5 (S ~ 6.4)	5.9 ± 1.0 (S ~ 10.7)	2.6 ± 0.2 (S ~ 24.2)	3.9 ± 0.8 (S ~ 16.2)	70 ± 0.1	<5
17	3.9 ± 0.4 (S = 2.2)	>30	14.9 ± 2.6 (S = 0.6)	7.2 ± 1.1 (S = 1.2)	10.0 ± 1.7 (S = 0.8)	8.4 ± 1.5	<5
19	3.5 ± 0.4 (S = 1.0)	10.5 ± 1.2 (S = 0.3)	6.8 ± 0.5 (S = 0.5)	5.5 ± 0.6 (S = 0.6)	8.1 ± 0.8 (S = 0.4)	3.5 ± 0.7	<5
20	3.2 ± 0.3 (S > 9.4)	9.42 ± 1.4 (S > 3.2)	7.3 ± 1.3 (S > 4.1)	4.1 ± 0.6 (S > 7.3)	6.6 ± 1.1 (S > 4.5)	>30	<5
ST	n.d.	0.9 ± 0.01 (S = 0.5)	1.1 ± 0.3 (S = 0.4)	0.01 ± 0.01 (S = 45)	n.d.	0.45 ± 0.04	n.d.
DX	n.d.	0.2 ± 0.02 (S = 0.05)	0.1 ± 0.01 (S = 0.1)	0.1 ± 0.01 (S = 0.1)	n.d.	0.01 ± 0.001	n.d.

10.21, N 2.73.

*3 β-Acetyloxy-17 β-amino-28-norolean-12-ene (**7**)*

Compound **5** (250 mg, 0.5 mmol) was dissolved in THF (15 mL) and aqu. HCl (2 M,1.2 mL, 2.5 mmol) was added. The mixture was stirred for 24 h at 50 °C. For work-up, Et_2O (100 mL) and aqu. NaOH (2 M, 1.5 mL) were added. The organic phase was washed with H_2O (2 × 100 mL) and brine (50 mL) and dried ($MgSO_4$). Column chromatography (SiO_2, $CHCl_3$/MeOH, 95:5) gave **7** (110 mg, 47 %) as a white solid; m.p. 216.1 °C (decomp.); R_F = 0.51 ($CHCl_3$/MeOH, 9:1); $[\alpha]_D$ = 84.4° (*c* 0.025, $CHCl_3$); IR (ATR): $\tilde{\nu}$ = 660*w*, 816 *m*, 968*w*, 987 *m*, 1004 *m*, 1026 *m*, 1244*vs*, 1364 *m*, 1388*w*, 1463*w*, 1733 *s*, 2856*w*, 2946 *m* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 5.40 (t, *J* = 3.6 Hz, 1H, 12-H), 4.55–4.43 (m, 1H, 3-H), 4.15–4.06 (m, 2H, 28-NH_2), 2.32 (m, 1H, 9-H), 2.12 (m, 1H, 15-H_a), 2.04 (s, 3H, 32-H_3), 1.88 (d, *J* = 3.6 Hz, 1H, 11-H_a), 1.79 (m, 1H, 2-H_a + 16-H_a), 1.74–1.59 (m, 5H, 1-H_a + 2-H_b + 19-H_a + 19-H_b + 21-H_a), 1.64–1.34 (m, 8H, 1-H_b + 6-H_a + 6-H_b + 11-H_b + 18-H + 21-H_b + 22-H_a + 22-H_b), 1.37–1.28 (m, 3H, 7-H_a + 7-H_b + 15-H_b), 1.20–1.14 (m, 1H, 16-H_b), 1.12 (s, 3H, 27-H_3), 0.98 (s, 3H, 29-H_3), 0.97 (s, 3H, 26-H_3), 0.95 (s, 3H, 25-H_3) 0.90 (s, 3H, 30-H_3), 0.87 (s, 3H, 24-H_3), 0.86 (s, 3H, 23-H_3), 0.85–0.80 (m, 1H, 5-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 171.0 (C-31), 141.4 (C-13), 125.9 (C-12), 80.8 (C-3), 55.2 (C-5), 54.4 (C-17), 47.5 (C-9), 47.3 (C-18), 47.1 (C-19), 41.4 (C-14), 39.8 (C-8), 38.2 (C-1), 37.7 (C-4), 36.8 (C-10), 35.2 (C-7), 34.6 (C-21), 32.8 (C-30), 32.4 (C-22), 31.0 (C-20), 28.0 (C-24), 25.9 (C-16), 25.7 (C-27), 25.1 (C-15), 23.9 (C-29), 23.7 (C-2), 23.5 (C-11), 21.3 (C-32), 18.2 (C-6), 17.1 (C-26), 16.7 (C-23), 15.5 (C-5) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 470.5 (60 %, $[M + H]^+$); analysis calcd for $C_{31}H_{51}NO_2$ (469.75): C 79.26, H 10.94, N 2.98; found: C 78.98, H 11.15, N 2.78.

*3 β-Acetyloxy-17 β-amino-28-norurs-12-ene (**8**)*

The synthesis of **8** (843 mg, 90 %) [37] was performed as described for **7** using **6** (1.0 g, 2.0 mmol) as a starting material; colorless solid; m.p. 220.0 °C (decomp.); R_F = 0.48 ($CHCl_3$/MeOH, 9:1); $[\alpha]_D$ = 65.8° (*c* 0.031, $CHCl_3$); IR (ATR): $\tilde{\nu}$ = 781*w*, 830*w*, 841*w*, 970 *m*, 985 *m*, 1004 *m*, 1023 *m*, 1244*vs*, 1368 *m*, 1387*w*, 1456*w*, 1733 *s*, 2855*w*, 2911 *m*, 2924 *m*, 2957*w*, 2978*w* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.32 (t, *J* = 3.7 Hz, 1H, 12-H), 4.52–4.47 (m, 1H, 3-H), 3.10 (s, 2H, 28-NH_2), 2.09–2.07 (m, 1H, 16-H_a), 2.03 (s, 3H, 32-H_3), 1.96–1.91 (m, 1H, 11-H_a), 1.91–1.82 (m, 2H, 2-H_a + 15-H_a), 1.77–1.72 (m, 2H, 22-H_a + 2-H_b), 1.67–1.61 (m, 3H, 1-H_a + 1-H_b + 11-H_b), 1.58–1.50 (m, 6H, 6-H_a + 7-H_a + 9-H + 18-H + 21-H_a + 22-H_b), 1.42–1.35 (m, 2H, 6-H_b + 7-H_b), 1.33–1.27 (m, 1H, 19-H), 1.27–1.20 (m, 2H, 16-H_b + 21-H_b), 1.13–1.06 (m, 1H, 15-H_b), 1.08 (s, 3H, 27-H_3), 1.00 (s, 3H, 23-H_3), 1.02–0.95 (m, 1H, 20-H), 0.97 (s, 3H, 29-H_3), 0.93–0.92 (m, 3H, 30-H_3), 0.87 (s, 3H, 24-H_3), 0.86 (s, 3H, 25-H_3), 0.83–0.78 (m, 1H, 5-H), 0.80 (s, 3H, 26-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = δ 170.9 (C-31), 137.4 (C-13), 128.0 (C-12), 80.8 (C-3), 60.4 (C-18), 55.2 (C-5), 53.2 (C-17), 47.4 (C-9), 41.8 (C-14), 40.7 (C-19), 39.9 (C-18), 39.8 (C-22), 39.1 (C-20), 38.4 (C-1), 37.7 (C-4), 36.8 (C-10), 32.6 (C-7), 31.4 (C-21), 28.0 (C-24), 27.9 (C-16), 25.8 (C-15), 23.7 (C-2), 23.5 (C-11), 23.2 (C-27), 21.3 (C-32), 20.9 (C-30), 18.2 (C-6), 17.3 (C-26), 17.0 (C-23), 16.7 (C-25), 15.6 (C-29) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 470.4 (42 %, $[M + H]^+$); analysis calcd for $C_{31}H_{51}NO_2$ (469.75): C 79.26, H 10.94, N 2.98; found: C 79.03, H 11.16, N 2.81.

*N-[3β-Acetyloxy-17β-amino-28-norolean-12-en-17-yl]-benzamide (**9**)*

Compound **7** (110 mg, 0.23 mmol) was dissolved in dry DCM (4 mL), benzoyl chloride (0.1 mL, 0.86 mmol), NEt_3 (1.0 mL) and DMAP (cat. amounts) were added. The solution was stirred for 2 h at 21 °C. For work-up, Et_2O (50 mL) was added, and the mixture was washed with aqueous HCl (0.1 M, 50 mL), H_2O (2 × 50 mL), and brine (25 mL). After drying ($MgSO_4$), **9** (109 mg, 86 %) was obtained after column chromatography (SiO_2, hexanes/ethyl acetate, 9:1) as a colorless solid; m.p. 260.4 °C (decomp.); R_F = 0.25 (hexanes/ethyl acetate, 9:1); $[\alpha]_D$ = 46.3° (*c* 0.194, $CHCl_3$); IR (ATR): $\tilde{\nu}$ = 478*w*, 505*w*, 676*w*, 691*w*, 709 *m*, 970*w*, 986 *m*, 1004 *m*, 1014 *m*, 1027 *m*, 1217*w*, 1244*vs*, 1318*w*, 1366 *m*, 1387*w*, 1434*w*, 1446*w*, 1463 *m*, 1482 *m*, 1512 *m*, 1666 *m*, 1733 *m*, 2872*w*, 2946 *m* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.74–7.68 (m, 2H, 35-H + 39-H), 7.49–7.41 (m, 1H, 37-H), 7.42–7.36 (m, 2H, 36-H + 38-H), 5.87 (s, 1H, 28-H), 5.40 (t, *J* = 3.6 Hz, 1H, 12-H), 4.52–4.45 (m, 1H, 3-H), 2.60–2.52 (m, 1H, 22-H_a), 2.37–2.28 (m, 2H, 9-H + 16-H_a), 2.04 (s, 3H, 32-H_3), 2.07–1.95 (m, 1H, 16-H_b), 1.95–1.87 (m, 1H, 11-H_a), 1.87–1.78 (m, 2H, 2-H_a + 19-H_a), 1.78–1.69 (m, 3H, 2-H_b + 15-H_a + 21-H_a), 1.65–1.54 (m, 3H, 1-H_a + 11-H_b + 18-H), 1.54–1.42 (m, 2H, 6-H_a + 22-H_b), 1.42–1.24 (m, 4H, 6-H_b + 7-H_a + 7-H_b + 21-H_b), 1.26–1.18 (m, 1H, 19-H_b), 1.17 (s, 3H, 27-H_3), 1.09–1.00 (m, 2H, 1-H_b + 15-H_b), 0.98 (s, 3H, 29-H_3), 0.93 (s, 3H, 30-H_3), 0.91 (s, 3H, 23-H_3), 0.85 (s, 3H, 24-H_3), 0.86–0.81 (m, 1H, 5-H), 0.83 (s, 3H, 25-H_3), 0.77 (s, 3H, 26-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 171.0 (C-31), 166.1 (C-33), 143.1 (C-13), 135.4 (C-34), 131.0 (C-37), 128.4 (C-36 + C-38), 126.6 (C-35 + C-39), 124.6 (C-12), 80.8 (C-3), 56.9 (C-17), 55.2 (C-5), 47.4 (C-9), 47.3 (C-18), 46.6 (C-19), 41.7 (C-14), 39.5 (C-8), 38.1 (C-1), 37.7 (C-4), 36.8

(C-20), 35.1 (C-7), 32.9 (C-30), 32.3 (C-21 + C-22), 30.8 (C-10), 28.0 (C-24), 26.2 (C-15), 25.8 (C-27), 23.9 (C-29), 23.6 (C-11), 23.5 (C-2), 21.9 (C-16), 21.3 (C-32), 18.1 (C-6), 16.9 (C-26), 16.6 (C-25), 15.4 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 574.4 (78 %, $[M + H]^+$), 1169.9 (100 %, $[2\,M + Na]^+$); analysis calcd for $C_{38}H_{55}NO_3$ (573.86): C 79.53, H 9.66, N 2.44; found: C 79.38, H 9.84, N 2.19.

*N-[3β-Acetyloxy-17β-amino-28-norurs-12-en-17-yl]-benzamide **(10)***

The synthesis of **10** (160 mg, 100 %) was accomplished from **8** (122 mg, 0.26 mmol) following the procedure given for the synthesis of **9**; m.p. 248.3 °C (decomp.); R_F = 0.24 (hexanes/ethyl acetate, 9:1); $[\alpha]_D$ = 25.4° (*c* 0.188, $CHCl_3$); IR (ATR): $\tilde{\nu}$ = 522 *m,* 529 *m,* 539 *m,* 609*w,* 667*w,* 693 *m,* 715 *s,* 801*w,* 985 *m,* 991*w,* 1005*w,* 1025 *m,* 1246*vs,* 1291 *m,* 1320 *m,* 1368 *m,* 1452 *m,* 1483 *m,* 1510 *m,* 1579*w,* 1601*w,* 1666 *s,* 1730 *s,* 2852*w,* 2927*w,* 3412*w* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.72–7.65 (m, 2H, 35-H + 39-H), 7.50–7.42 (m, 2H, 36-H + 38-H), 7.39 (dd, *J* = 8.2, 6.7 Hz, 1H, 37-H), 5.91 (s, 1H, 28-H), 5.38 (t, *J* = 3.6 Hz, 1H, 12-H), 4.48 (dd, *J* = 10.8, 4.9 Hz, 1H, 3-H), 2.87–2.79 (m, 1H, 22-H_a), 2.46–2.39 (m, 1H, 16-H_a), 2.04 (s, 3H, 32-H_3), 2.02–1.99 (m, 1H, 16-H_b), 1.99–1.96 (m, 1H, 11-H_a), 1.80–1.72 (m, 1H, 15-H_a), 1.67–1.61 (m, 3H, 1-H_a + 11-H_b + 18-H), 1.57–1.54 (m, 2H, 9-H + 21-H_a), 1.54–1.51 (m, 1H, 22-H_b), 1.51–1.48 (m, 2H, 6-H_a + 19-H), 1.48–1.46 (m, 1H, 7-H_a), 1.35–1.22 (m, 3H, 6-H_b + 7-H_b + 21-H_b), 1.11 (s, 3H, 27-H_3), 1.09–1.06 (m, 1H, 15-H_b), 1.05 (d, *J* = 2.3 Hz, 1H, 1-H_b), 1.13–0.98 (m, 1H, 20-H), 0.96 (s, 3H, 29-H_3), 0.91 (s, 3H, 23-H_3), 0.87 (s, 3H, 30-H_3), 0.85 (s, 3H, 24-H_3), 0.82 (s, 3H, 25-H_3), 0.81–0.80 (m, 1H, 5-H), 0.72 (s, 3H, 26-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 171.0 (C-31), 165.9 (C-33), 138.5 (C-13), 135.4 (C-34), 131.0 (C-37), 128.4 (C-36), 128.4 (C-38), 127.3 (C-12), 126.6 (C-35 + C-39), 80.8 (C-3), 59.1 (C-18), 57.5 (C-17), 55.2 (C-5), 47.5 (C-9), 42.1 (C-14), 39.9 (C-19), 39.7 (C-8), 39.3 (C-20), 38.3 (C-1), 37.6 (C-4), 36.8 (C-10), 36.5 (C-22), 32.4 (C-7), 31.3 (C-21), 28.0 (C-24), 26.7 (C-15), 23.5 (C-2), 23.5 (C-11), 23.2 (C-16), 23.2 (C-27), 21.3 (C-32), 20.8 (C-29), 18.1 (C-6), 17.5 (C-30), 16.8 (C-26), 16.7 (C-25), 15.5 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 574.5 (100 %, $[M + H]^+$), 1170.0 (28 %, $[2\,M + Na]^+$); analysis calcd for $C_{38}H_{55}NO_3$ (573.86): C 79.53, H 9.66, N 2.44; found: C 79.40, H 9.86, N 2.27.

*N-[3β-Acetyloxy-17β-amino-28-norolean-12-en-17-yl]-cinnamic acid amide **(11)***

Cinnamic acid chloride (58.5 mg, 0.35 mmol, freshly prepared from cinnamic acid and oxalyl chloride), NEt_3 (0.06 mL), and cat. amounts of DMAP were added to an ice-cold solution of **7** (150 mg, 0.32 mmol) in dry DCM (10 mL). The reaction mixture was allowed to warm to 21 °C and stirred for an additional 2 h. Usual aq. work-up followed by column chromatography (SiO_2, hexanes/ethyl acetate, 9:1) gave **11** (0.283 mmol, 89 %) as a white solid; m.p. 143.8 °C; R_F = 0.21 (hexanes/ethyl acetate, 9:1); $[\alpha]_D$ = 51.4° (*c* 0.195, $CHCl_3$); IR (ATR): $\tilde{\nu}$ = 472 *m,* 491 *m,* 568 *m,* 686 *m,* 710 *m,* 763 *m,* 972 *m,* 985 *m,* 1004 *m,* 1026 *m,* 1217 *m,* 1244*vs,* 1336 *m,* 1365 *m,* 1387*w,* 1449 *m,* 1463 *m,* 1504 *m,* 1537 *m,* 1625 *m,* 1662 *m,* 1673 *m,* 1733 *m,* 2872*w,* 2946 *m* cm^{-1}; UV/vis ($CHCl_3$): λ_{max} (log ε) = 275 (4.36) nm; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.65–7.63 (m, 1H, 39-H), 7.62–7.59 (m, 1H, 35-H), 7.51–7.43 (m, 2H, 38-H + 40-H), 7.36–7.31 (m, 2H, 37-H + 41-H), 6.34 (d, *J* = 15.5 Hz, 1H, 34-H), 5.52 (s, 1H, 28-H), 5.41–5.35 (m, 1H, 12-H), 4.53–4.44 (m, 1H, 3-H), 2.48 (d, *J* = 13.8 Hz, 1H, 22-H_a), 2.28–2.24 (m, 2H, 9-H + 16-H_a), 2.04 (s, 3H, 32-H_3), 2.02–1.96 (m, 1H, 16-H_b), 1.96–1.83 (m, 2H, 2-H_a + 11-H_a), 1.86–1.66 (m, 4H, 2-H_b + 15-H_a + 19-H_a + 22-H_b), 1.70–1.55 (m, 2H, 1-H_a + 11-H_b), 1.62–1.38 (m, 3H, 6-H_a + 18-H + 21-H_a), 1.40–1.17 (m, 5H, 6-H_b + 7-H_a + 7-H_b + 19-H_b + 21-H_b), 1.16 (s, 3H, 27-H_3), 1.09–1.00 (m, 2H, 1-H_b + 15-H_b), 0.97 (s, 3H, 30-H_3), 0.94 (s, 3H, 23-H_3), 0.92 (s, 3H, 29-H_3), 0.87 (s, 3H, 25-H_3), 0.86 (s, 3H, 24-H_3), 0.89–0.81 (m, 1H, 5-H), 0.84 (s, 3H, 26-H_3) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 171.0 (C-31), 165.4 (C-33), 142.9 (C-13), 141.2 (C-35 + C-39), 134.8 (C-36), 129.7 (C-37), 128.8 (C-41), 127.9 (C-38 + C-40), 124.9 (C-12), 120.9 (C-34), 80.8 (C-3), 57.4 (C-17), 55.2 (C-5), 47.4 (C-18), 47.1 (C-9), 46.3 (C-19), 41.5 (C-14), 39.6 (C-8), 38.1 (C-1), 37.7 (C-4), 36.8 (C-20), 35.0 (C-7), 32.8 (C-29), 32.2 (C-22), 32.2 (C-21), 30.7 (C-10), 28.0 (C-24), 26.2 (C-15), 25.8 (C-27), 24.0 (C-30), 23.6 (C-2), 23.5 (C-11), 21.3 (C-16), 21.3 (C-32), 18.1 (C-6), 16.8 (C-26), 16.6 (C-25), 15.4 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 599.2 (97 %, $[M-H]^-$); analysis calcd for $C_{40}H_{57}NO_3$ (599.90): C 80.09, H 9.58, N 2.33; found: C 79.82, H 9.78, N 2.11.

*N-[3β-Acetyloxy-17β-amino-28-norurs-12-en-17-yl]-cinnamic acid amide **(12)***

Following the procedure given for **11**, from **8** (150 mg, 0.32 mmol) **12** (104 mg, 54 %) was obtained as a white solid; m.p. 155.7 °C; R_F = 0.25 (hexanes/ethyl acetate, 9:1); $[\alpha]_D$ = 44.7° (*c* 0.116, $CHCl_3$); IR (ATR): $\tilde{\nu}$ = 480*w,* 496*w,* 565 *m,* 687*w,* 713 *m,* 763 *m,* 972 *m,* 985 *m,* 1005 *m,* 1026 *m,* 1220 *m,* 1244*vs,* 1341 *m,* 1369 *m,* 1450 *m,* 1504 *m,* 1537 *m,* 1622 *m,* 1659 *m,* 1733 *m,* 2870*w,* 2924 *m,* 2947 *m* cm^{-1}; UV/vis ($CHCl_3$): λ_{max} (log ε) = 274 (4.33) nm; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.61–7.58 (m, 1H, 39-H), 7.57–7.55 (m, 1H, 35-H), 7.49–7.43 (m, 2H, 38-H + 40-H), 7.36–7.30 (m, 2H, 37-H + 41-H), 6.30 (s, 1H, 28-H), 6.26 (s, 1H, 34-H), 5.37–5.32 (m, 1H, 12-H), 4.53–4.45 (m, 1H, 3-H), 2.81–2.72 (m, 1H, 22-H_a), 2.39–2.31 (m, 1H, 16-H_a), 2.03 (s, 3H, 32-H_3), 2.01–1.90 (m, 3H, 2-H_a + 11-H_a + 16-H_b), 1.78–1.70 (m, 2H, 2-H_b + 15-H_a), 1.67–1.58 (m, 3H, 1-H_a + 1-H_b + 11-H_b), 1.60–1.45 (m, 7H, 6-H_a + 7-H_a + 9-H + 18-H + 19-H + 21-H_a + 22-H_b), 1.41–1.27 (m, 2H, 6-H_b + 7-H_b), 1.29–1.21 (m, 1H, 21-H_b), 1.10 (s, 3H, 27-H_3), 1.10–1.04 (m, 1H, 15-H_b), 0.95 (s, 6H, 23-H_3 + 30-H_3), 0.94 (s, 1H, 20-H), 0.90 (s, 3H, 25-H_3), 0.86 (s, 3H, 29-H_3), 0.86 (s, 3H, 24-H_3), 0.84 (s, 3H, 26-H_3), 0.82–0.81 (m, 1H, 5-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 171.0 (C-31), 164.8 (C-33), 140.7 (C-35 + C-39), 138.3 (C-13), 135.0 (C-36), 129.5 (C-37), 128.7 (C-41), 127.8 (C-38), 127.5 (C-12 + C-40), 121.5 (C-34), 80.8 (C-3), 58.9 (C-18), 57.5 (C-17), 55.2 (C-5), 47.4 (C-9), 41.9 (C-14), 39.8 (C-19), 39.8 (C-8), 39.2 (C-20), 38.3 (C-1), 37.7 (C-4), 36.8 (C-10), 36.6 (C-22), 32.4 (C-7), 31.3 (C-21), 28.0 (C-24), 26.7 (C-15), 23.5 (C-11), 23.3 (C-27), 23.3 (C-2), 23.0 (C-16), 21.3 (C-32), 20.8 (C-30), 18.1 (C-6), 17.5 (C-29), 16.7 (C-26), 16.7 (C-25), 15.5 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 601.2 (100 %, $[M + H]^+$); analysis calcd for $C_{40}H_{57}NO_3$ (599.90): C 80.09, H 9.58, N 2.33; found: C 79.87, H 9.70, N 2.11.

*N-[3β-Acetyloxy-17β-amino-28-norolean-12-en-17-yl]-phenyl urea **(13)***

To a solution of **5** (124 mg, 0.25 mmol) in dry toluene (10 mL), aniline (0.37 mmol, 34 μL), and NEt_3 (1.0 mL), were added. The reaction mixture was stirred at 21 °C for 12 h, and diluted with Et_2O (100 mL). The organic phase was washed with H_2O (2 × 100 mL) and brine (50 mL) and dried ($MgSO_4$). After flash chromatography (SiO_2, $CHCl_3$), **13** (68 mg, 46 %) was obtained as a colorless solid; m.p. 221.6 °C (decomp.); R_F = 0.19 ($CHCl_3$); $[\alpha]_D$ = 53.7° (*c* 0.211, $CHCl_3$); IR (ATR): $\tilde{\nu}$ = 478 *m,* 505 *m,* 609 *m,* 652 *m,* 665 *m,* 692 *s,* 748 *s,* 897*w,* 968 *m,* 986 *m,* 1009 *m,* 1026 *s,* 1096*w,* 1215 *s,* 1244*vs,* 1310 *m,* 1365 *m,* 1440 *m,* 1464 *m,* 1498 *s,* 1543 *s,* 1599 *m,* 1659 *m,* 1693 *m,* 1698 *m,* 1732 *m,* 2872*w,* 2946 *m,* 3375*w* cm^{-1}; UV/vis ($CHCl_3$): λ_{max} (log ε) = 276 (3.10) nm; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.30–7.21 (m, 1H, 38-H), 7.29–7.23 (m, 2H, 36-H + 40-H), 7.03 (m, 2H, 37-H + 39-H), 6.10 (s, 1H, 28-H), 5.38–5.23 (m, 1H, 12-H), 4.52–4.48 (m, 1H, 34-H), 4.52–4.46 (m, 1H, 3-H), 2.47–2.39 (m, 1H, 22-H_a), 2.19–2.09 (m, 2H, 9-H + 16-H_a), 2.04 (s, 3H, 32-H_3), 1.97–1.86 (m, 2H, 2-H_a + 16-H_b), 1.87–1.79 (m, 1H, 11-H_a), 1.77 (s, 1H, 19-H_a), 1.77–1.67 (m, 1H, 15-H_a), 1.65–1.58 (m, 3H, 1-H_a + 2-H_b + 11-H_b), 1.61–1.49 (m, 2H, 6-H_a + 18-H), 1.49–1.45 (m, 1H, 21-H_a), 1.41–1.35 (m, 1H, 6-H_b), 1.36–1.27 (m, 3H, 7-H_a + 21-H_b + 22-H_b), 1.29–1.23 (m, 1H, 7-H_b), 1.20–1.15 (m, 1H, 19-H_b), 1.13 (s, 3H, 27-H_3), 1.09–0.98 (m, 2H, 1-H_b + 15-H_b), 0.96 (s, 3H, 29-H_3), 0.91 (s, 3H, 23-H_3), 0.91 (s, 3H, 30-H_3), 0.86 (s, 3H, 24-H_3), 0.85 (s, 3H, 25-H_3), 0.86–0.82 (m, 1H, 5-H), 0.75 (s, 3H, 26-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 171.0 (C-31), 154.2 (C-33), 143.1 (C-13), 138.8 (C-35), 129.1 (C-38), 124.5 (C-12), 123.4 (C-37 + C-39), 120.8 (C-36 + C-40), 80.8 (C-3), 56.0 (C-17), 55.2 (C-5), 47.6 (C-9), 47.4 (C-18), 46.3 (C-19),

41.5 (C-14), 39.6 (C-8), 38.1 (C-1), 37.7 (C-4), 36.8 (C-20), 35.2 (C-7), 33.1 (C-22), 32.9 (C-30), 32.3 (C-21), 30.7 (C-10), 28.0 (C-24), 26.2 (C-15), 25.7 (C-27), 24.0 (C-29), 23.6 (C-2), 23.5 (C-11), 22.3 (C-16), 21.3 (C-32), 18.1 (C-6), 16.7 (C-26), 16.6 (C-25), 15.3 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 587.5 (100 %, $[M-H]^-$), 623.5 (86 %, $[M + Cl]^-$); analysis calcd for $C_{38}H_{56}N_2O_3$ (588.88): C 77.51, H 9.59, N 4.76; found: C 77.35, H 9.73, N 4.56.

*N-[3β-Acetyloxy-17β-amino-28-norurs-12-en-17-yl]-phenyl urea **(14)***

Following the procedure given for the synthesis of **13**, **14** (237 mg, 81 %) was prepared from **6** (248 mg, 0.5 mmol) and obtained as a white solid; m.p. 149.5 °C (decomp.); R_F = 0.23 ($CHCl_3$); $[\alpha]_D$ = 49.9° (*c* 0.180, $CHCl_3$); IR (ATR): $\tilde{\nu}$ = 504 *m,* 667 *m,* 692 *s,* 748 *s,* 968 *m,* 985 *m,* 1005 *m,* 1026 *m,* 1244*vs,* 1314 *m,* 1370 *m,* 1440 *m,* 1454 *m,* 1467 *m,* 1498*vs,* 1542 *s,* 1600 *m,* 1659 *m,* 1731 *m,* 2870*w,* 2924 *m,* 2947 *m,* 3374*w* cm^{-1}; UV/vis ($CHCl_3$): λ_{max} (log ε) = 278 (3.17) nm; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.30–7.28 (m, 2H, 37-H + 39-H), 7.28–7.27 (m, 1H, 38-H), 7.23–7.22 (m, 2H, 36-H + 40-H), 5.22–5.15 (m, 1H, 12-H), 4.81 (s, 1H, 28-H), 4.52–4.46 (m, 1H, 3-H), 4.27 (s, 1H, 34-H), 2.61–2.52 (m, 1H, 22-H_a), 2.18–2.10 (m, 1H, 16-H_a), 2.03 (s, 3H, 32-H_3), 1.96–1.92 (m, 1H, 16-H_b), 1.93–1.88 (m, 1H, 11-H_a), 1.83 (d, *J* = 3.0 Hz, 1H, 2-H_a), 1.80–1.73 (m, 1H, 15-H_a), 1.61 (m, 2H, 1-H_a + 11-H_b), 1.52 (s, 1H, 6-H_a), 1.57–1.45 (m, 4H, 2-H_b + 7-H_a + 9-H + 21-H_a), 1.43–1.31 (m, 2H, 19-H + 22-H_b), 1.39–1.37 (m, 2H, 6-H_b + 18-H), 1.36–1.34 (m, 1H, 21-H_b), 1.23–1.14 (m, 1H, 7-H_b), 1.11–1.04 (m, 1H, 1-H_b), 1.06 (s, 3H, 27-H_3), 1.03–0.97 (m, 1H, 15-H_b), 0.96–0.95 (m, 1H, 20-H_b), 0.93 (s, 3H, 23-H_3), 0.92 (s, 6H, 29-H_3 + 30-H_3), 0.87 (s, 3H, 24-H_3), 0.86 (s, 3H, 25-H_3), 0.84–0.83 (m, 1H, 5-H), 0.80 (s, 3H, 26-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 171.0 (C-31), 157.1 (C-33), 139.3 (C-35), 138.5 (C-13), 128.6 (C-39), 128.6 (C-37), 127.4 (C-38), 127.3 (C-36), 127.2 (C-40), 127.0 (C-12), 80.8 (C-3), 59.1 (C-18), 56.4 (C-17), 55.2 (C-5), 47.5 (C-9), 42.0 (C-14), 39.8 (C-19), 39.8 (C-8), 39.2 (C-20), 38.3 (C-1), 37.7 (C-4), 37.6 (C-22), 36.8 (C-10), 32.6 (C-21), 31.4 (C-7), 28.1 (C-24), 26.7 (C-15), 23.8 (C-16), 23.5 (C-2), 23.5 (C-11), 23.2 (C-27), 21.3 (C-32), 20.8 (C-29), 18.1 (C-6), 17.5 (C-26), 16.9 (C-25), 16.7 (C-30), 15.5 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 589.5 (100 %, $[M + H]^+$); analysis calcd for $C_{38}H_{56}N_2O_3$ (588.88): C 77.51, H 9.59, N 4.76; found: C 77.36, H 9.70, N 4.54.

*N-[3 β-Acetyloxy-17 β-amino-28-norolean-12-en-17-yl]-benzyl urea **(15)***

To a solution of **5** (248 mg, 0.5 mmol) in dry toluene (10 mL), benzylamine (81 μL, 79.3 mg, 0.74 mmol) and NEt_3 (1.2 mL) were added. The reaction mixture was stirred at 21 °C for 12 h. Usual aqu. work-up followed by flash chromatography (SiO_2, $CHCl_3$) gave **15** (316 mg, 100 %) as a colorless solid; m.p. 159.0 °C; R_F = 0.1 ($CHCl_3$); $[\alpha]_D$ = 50.3° (*c* 0.177, $CHCl_3$); IR (ATR): $\tilde{\nu}$ = 594*w,* 609*w,* 652*w,* 664*w,* 698 *m,* 743 *m,* 968*w,* 986 *m,* 1013 *m,* 1026 *m,* 1243*vs,* 1302*w,* 1365 *m,* 1454 *m,* 1463 *m,* 1497 *m,* 1547 *m,* 1550 *m,* 1638 *m,* 1735 *m,* 2946 *m,* 3361*w* cm^{-1}; UV/vis ($CHCl_3$): λ_{max} (log ε) = 284 (1.89) nm; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.34–7.29 (m, 2H, 38-H + 40-H), 7.30 (s, 1H, 39-H), 7.29–7.21 (m, 2H, 37-H + 41-H), 5.26–5.21 (m, 1H, 12-H), 5.17 (s, 1H, 28-H), 4.52–4.45 (m, 1H, 3-H), 4.38–4.21 (m, 3H, 34-H + 35-H_a + 35-H_b), 2.34–2.26 (m, 1H, 22-H_a), 2.09–2.00 (m, 2H, 9-H + 16-H_a), 2.04 (s, 3H, 32-H_3), 1.94–1.87 (m, 1H, 16-H_b), 1.89–1.83 (m, 2H, 2-H_a + 11-H_a), 1.78–1.67 (m, 3H, 2-H_b + 15-H_a + 19-H_a), 1.64–1.59 (m, 1H, 11-H_b), 1.61–1.57 (m, 3H, 1-H_a + 18-H + 22-H_b), 1.64–1.52 (m, 1H, 6-H_a), 1.51–1.46 (m, 1H, 21-H_a), 1.44–1.35 (m, 1H, 6-H_b), 1.34–1.28 (m, 1H, 21-H_b), 1.30–1.25 (m, 1H, 7-H_a), 1.24–1.19 (m, 1H, 7-H_b), 1.16–1.10 (m, 1H, 19-H_b), 1.12 (s, 3H, 27-H_3), 1.09–0.96 (m, 2H, 1-H_b + 15-H_b), 0.93 (s, 3H, 23-H_3), 0.89 (s, 3H, 29-H_3), 0.88 (s, 3H, 30-H_3), 0.87 (s, 3H, 24-H_3), 0.86 (s, 3H, 25-H_3), 0.92–0.81 (m, 1H, 5-H), 0.84 (s, 3H, 26-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 171.0 (C-31), 157.3 (C-33), 143.1 (C-13), 138.7 (C-36), 128.7 (C-38 + C-40), 127.5 (C-39), 126.9 (C-37 + C-41), 124.4 (C-12), 80.8 (C-3), 56.2 (C-17), 55.2 (C-5), 47.6 (C-9), 47.4 (C-18), 46.3 (C-19), 44.6 (C-35), 41.6 (C-14), 39.6 (C-8), 38.1 (C-1), 37.7 (C-4), 36.8 (C-20), 35.2 (C-7), 33.3 (C-22), 32.8 (C-30), 32.3 (C-21), 30.7 (C-10), 28.0 (C-24), 26.2 (C-15), 25.7 (C-27), 23.9 (C-29), 23.6 (C-2), 23.5 (C-11), 22.5 (C-16), 21.3 (C-32), 18.2 (C-6), 16.9 (C-26), 16.7 (C-25), 15.4 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 603.1 (100 %, $[M + H]^+$); analysis calcd for $C_{39}H_{58}N_2O_3$ (602.90): C 77.70, H 9.70, N 4.65; found: C 77.51, H 9.83, N 4.57.

*N-[3 β-Acetyloxy-17 β-amino-28-norurs-12-en-17-yl]-benzyl urea **(16)***

Following the procedure given for **15**, **16** (394 mg, 100 %) was synthesized from **6** (248 mg, 0.5 mmol) and obtained as a white solid; m. p. 157.4 °C; R_F = 0.15 ($CHCl_3$); $[\alpha]_D$ = 43.2° (*c* 0.203, $CHCl_3$); IR (ATR): $\tilde{\nu}$ = 594 *m,* 609 *m,* 654*w,* 697 *m,* 744*w,* 968 *m,* 985 *m,* 1005 *m,* 1026 *m,* 1243*vs,* 1312*w,* 1369 *m,* 1453 *m,* 1498 *m,* 1548 *m,* 1552 *m,* 1637 *m,* 1734 *m,* 2870*w,* 2924 *m,* 2946 *m,* 3339*w* cm^{-1}; UV/vis ($CHCl_3$): λ_{max} (log ε) = 278 (1.79) nm; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.26–7.19 (m, 2H, 37-H + 41-H), 7.19–7.12 (m, 2H, 38-H + 40-H), 7.02–6.95 (m, 1H, 39-H), 6.60 (s, 1H, 28-H), 5.20–5.15 (m, 1H, 12-H), 4.75 (s, 1H, 34-H), 4.51–4.44 (m, 1H, 3-H), 3.41–3.22 (m, 1H, 35-H_a), 3.16–3.02 (m, 1H, 35-H_b), 2.69–2.61 (m, 1H, 22-H_a), 2.24–2.17 (m, 1H, 16-H_a), 2.04 (s, 3H, 32-H_3), 1.96–1.84 (m, 3H, 2-H_a + 11-H_a + 16-H_b), 1.85–1.75 (m, 1H, 15-H_a), 1.66–1.56 (m, 2H, 1-H_a + 11-H_b), 1.55–1.47 (m, 6H, 2-H_b + 6-H_a + 7-H_a + 9-H + 21-H_a + 22-H_b), 1.44–1.41 (m, 2H, 18-H + 19-H), 1.37–1.28 (m, 2H, 6-H_b + 21-H_b), 1.27–1.13 (m, 1H, 7-H_b), 1.12–1.04 (m, 1H, 1-H_b), 1.05 (s, 3H, 27-H_3), 1.04–0.98 (m, 1H, 15-H_b), 0.93 (s, 3H, 29-H_3), 0.90 (s, 3H, 23-H_3), 0.86 (s, 3H, 24-H_3), 0.85 (s, 3H, 30-H_3), 0.81 (s, 3H, 25-H_3), 0.80 (s, 1H, 5-H), 0.79 (s, 3H, 26-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 171.1 (C-31), 154.4 (C-33), 139.2 (C-36), 138.4 (C-13), 129.0 (C-40), 128.9 (C-41), 127.0 (C-12), 123.0 (C-39), 120.5 (C-37 + C-38), 80.9 (C-3), 59.1 (C-18), 56.3 (C-17), 55.2 (C-5), 47.5 (C-9), 41.9 (C-14), 40.9 (C-35), 39.8 (C-19), 39.7 (C-8), 39.2 (C-20), 38.3 (C-1), 37.7 (C-4), 37.4 (C-22), 36.8 (C-10), 32.6 (C-21), 31.4 (C-7), 28.0 (C-24), 26.7 (C-15), 23.7 (C-16), 23.5 (C-2), 23.5 (C-11), 23.2 (C-27), 21.3 (C-32), 20.8 (C-29), 18.1 (C-6), 17.5 (C-25), 16.7 (C-26), 16.7 (C-30), 15.5 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$ 4:1): *m/z* = 603.5 (100 %, $[M + H]^+$); analysis calcd for $C_{39}H_{58}N_2O_3$ (602.90): C 77.70, H 9.70, N 4.65; found: C 77.47, H 9.83, N 4.46.

*N-[3 β-Acetyloxy-17 β-amino-28-norolean-12-en-17-yl]-3-quinolyl urea **(17)***

To a solution of **5** (248 mg, 0.5 mmol) in dry toluene (10 mL), 3-aminoquinoline (107 mg, 0.74 mmol) and NEt_3 (1.2 mL) were added. The reaction mixture was stirred at 90 °C in a microwave reactor for 5 h. The reaction mixture was diluted with Et_2O (100 mL), washed with aqueous HCl (0.1 M, 100 mL), H_2O (2 × 100 mL), and brine (50 mL), and dried ($MgSO_4$). Flash chromatography (SiO_2, hexanes/ethyl acetate, 3:1) provided **17** (160 mg, 50 %) as a colorless solid; m.p. 178.3 °C; R_F = 0.16 (hexanes/ethyl acetate, 3:1); $[\alpha]_D$ = 59.8° (*c* 0.187, $CHCl_3$); IR (ATR): $\tilde{\nu}$ = 475 *m,* 610 *m,* 662 *m,* 749 *m,* 781 *m,* 897 *m,* 968 *m,* 986 *m,* 1009 *m,* 1026 *m,* 1183 *m,* 1211 *s,* 1243*vs,* 1302 *m,* 1364 *m,* 1464 *m,* 1489 *m,* 1523 *m,* 1547 *m,* 1609*w,* 1702 *m,* 1734 *m,* 2872*w,* 2945 *m,* 3391*vw* cm^{-1}; UV/vis ($CHCl_3$): λ_{max} (log ε) = 251 (4.49), 328 (3.45), 340 (3.41), 379 (2.94) nm; ^1H NMR (500 MHz, $CDCl_3$): δ = 10.61 (s, 1H, 34-H), 9.66 (d, *J* = 2.3 Hz, 1H, 40-H), 9.16 (s, 1H, 36-H), 8.36 (d, *J* = 8.5 Hz, 1H, 44-H), 7.96 (d, *J* = 8.3 Hz, 1H, 41-H), 7.78 (t, *J* = 7.7 Hz, 1H, 43-H), 7.73 (t, *J* = 7.6 Hz, 1H, 42-H), 5.78 (s, 1H, 28-H), 5.46–5.41 (m, 1H, 12-H), 4.52–4.45 (m, 1H, 3-H), 2.44–2.31 (m, 1H, 22-H_a), 2.14–2.09 (m, 1H, 16-H_a), 2.11–2.07 (m, 1H, 9-H), 2.04 (s, 3H, 32-H_3), 2.06–2.02 (m, 1H, 22-H_b), 2.00–1.92 (m, 1H, 15-H_a), 1.94–1.83 (m, 1H, 16-H_b), 1.85–1.81 (m, 1H, 11-H_a), 1.82–1.79 (m, 1H, 2-H_a), 1.80–1.76 (m, 1H, 19-H_a), 1.64–1.56 (m, 3H, 1-H_a + 11-H_b + 18-H), 1.66–1.54 (m, 1H, 2-H_b), 1.52–1.46 (m, 2H, 6-H_a + 21-H_a), 1.41–1.32 (m, 3H, 6-H_b + 7-H_a + 21-H_b), 1.28–1.25 (m, 1H, 7-H_b), 1.26–1.22 (m, 1H, 19-H_b), 1.18 (s, 3H, 27-H_3), 1.12–1.05 (m, 2H, 1-H_b + 15-H_b), 1.05 (s, 3H, 29-H_3), 0.94 (s, 3H, 23-H_3), 0.93 (s, 3H, 30-H_3), 0.89 (s, 3H, 25-H_3), 0.86 (s, 3H, 24-H_3), 0.85–0.82 (m, 1H, 5-H), 0.80 (s, 3H, 26-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = (126 MHz, $CDCl_3$): δ = 171.0 (C-31), 154.1 (C-33), 142.8 (C-13), 137.0 (C-38), 135.0 (C-36), 132.9 (C-35), 131.1 (C-43), 130.0 (C-

39), 129.9 (C-42), 129.3 (C-40), 128.1 (C-41), 124.2 (C-12), 120.7 (C-44), 80.9 (C-3), 56.3 (C-17), 55.2 (C-5), 47.7 (C-9), 47.4 (C-18), 46.7 (C-19), 41.5 (C-14), 39.8 (C-8), 38.2 (C-1), 37.7 (C-4), 36.8 (C-20), 35.5 (C-7), 33.1 (C-22), 32.9 (C-30), 32.7 (C-21), 30.9 (C-10), 28.0 (C-24), 26.2 (C-15), 25.9 (C-27), 24.2 (C-29), 23.8 (C-2), 23.5 (C-11), 22.3 (C-16), 21.3 (C-32), 18.3 (C-6), 16.7 (C-25), 16.6 (C-26), 15.4 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$ 4:1): *m/z* = 640.7 (100 %, $[M + H]^+$); analysis calcd for $C_{41}H_{57}N_3O_3$ (639.93): C 76.95, H 8.98, N 6.57; found: C 76.74, H 9.11, N 6.42.

*N-[3 β-Acetyloxy-17 β-amino-28-norurs-12-en-17-yl]-3-quinolyl urea **(18)***

As described for **17**, **18** (100 mg, 30 %) was obtained from **6** (248 mg, 0.5 mmol) as a white solid; m.p. 148.6 °C (decomp.) R_F = 0.31 (hexanes/ethyl acetate, 2:1); $[\alpha]_D = 33.7°$ (*c* 0.041, $CHCl_3$); IR (ATR): $\tilde{\nu}$ = 471 *m,* 607 *m,* 653 *m,* 664 *m,* 724 *m,* 752 *m,* 768 *m,* 803 *m,* 900 *m,* 968 *m,* 985 *m,* 1007 *m,* 1025 *s,* 1094 *m,* 1188 *m,* 1244*vs,* 1365 *m,* 1455 *m,* 1519 *m,* 1546 *m,* 1704 *m,* 1733 *m,* 2854 *m,* 2923 *s,* 3318*w* cm^{-1}; UV/vis ($CHCl_3$): λ_{max} (log ε) = 250 (4.41), 321 (3.27), 344 (3.25), 378 (3.54) nm; ^1H NMR (500 MHz, $CDCl_3$): δ = 9.96 (s, 1H, 34-H), 9.44 (s, 1H, 40-H), 9.07 (s, 1H, 36-H), 8.29 (d, *J* = 8.4 Hz, 1H, 44-H), 7.89 (d, *J* = 8.2 Hz, 1H, 41-H), 7.73 (t, *J* = 8.6, 6.9, 1.4 Hz, 1H, 43-H), 7.67 (t, *J* = 7.6 Hz, 1H, 42-H), 5.54 (s, 1H, 28-H), 5.40 (t, *J* = 3.6 Hz, 1H, 12-H), 4.52–4.44 (m, 1H, 3-H), 2.47–2.41 (m, 1H, 22-H), 2.18–2.11 (m, 1H, 16-H_a), 2.11–2.00 (m, 1H, 16-H_b), 2.04 (s, 3H, 32-H_3), 2.01–1.89 (m, 2H, 2-H_a + 11-H_a), 1.86–1.80 (m, 1H, 18-H), 1.80–1.69 (m, 1H, 22-H_b), 1.65–1.57 (m, 2H, 1-H_a + 11-H_b), 1.59–1.51 (m, 3H, 2-H_b + 7-H_a + 9-H), 1.53–1.44 (m, 3H, 6-H_a + 19-H + 21-H_a), 1.46–1.38 (m, 1H, 15-H_a), 1.40–1.33 (m, 1H, 21-H_b), 1.34–1.19 (m, 2H, 6-H_b + 7-H_b), 1.11 (s, 3H, 27-H_3), 1.09–1.06 (m, 2H, 1-H_b + 15-H_b), 1.02 (s, 3H, 30-H_3), 0.96 (s, 1H, 20-H), 0.95 (s, 3H, 29-H_3), 0.87 (s, 3H, 25-H_3), 0.85 (s, 3H, 23-H_3), 0.84 (s, 3H, 24-H_3), 0.83–0.81 (m, 1H, 5-H), 0.80 (s, 3H, 26-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 171.0 (C-31), 153.9 (C-33), 138.7 (C-13), 137.5 (C-36), 135.7 (C-38), 134.8 (C-35), 130.3 (C-43), 129.7 (C-39), 129.4 (C-42), 127.9 (C-40 + C-41), 127.5 (C-12), 122.1 (C-44), 80.9 (C-3), 58.1 (C-18), 56.9 (C-17), 55.2 (C-5), 47.6 (C-9), 45.2 (C-14), 41.9 (C-8), 39.9 (C-19), 39.1 (C-20), 38.4 (C-1), 37.6 (C-4), 37.3 (C-22), 32.9 (C-21), 31.5 (C-7), 29.5 (C-10), 28.0 (C-24), 26.8 (C-15), 24.4 (C-16), 23.7 (C-2), 23.5 (C-11), 23.4 (C-27), 21.3 (C-32), 20.8 (C-29), 18.2 (C-6), 17.5 (C-25), 17.1 (C-30), 16.7 (C-26), 15.6 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 640.7 (100 %, $[M + H]^+$); analysis calcd for $C_{41}H_{57}N_3O_3$ (639.93): C 76.95, H 8.98, N 6.57; found: C 76.75, H 9.14, N 6.38.

*N-[3 β-Acetyloxy-17 β-amino-28-norolean-12-en-17-yl]-4-isoquinolyl urea **(19)***

As described above, from **5** (248 mg, 0.5 mmol) and 4-aminoisoquinoline (107 mg, 0.74 mmol), **19** (160 mg, 50 %) was obtained as a colorless solid; m.p. 179.0 °C (decomp.); R_F = 0.06 (hexanes/ethyl acetate, 3:1); $[\alpha]_D = 45.5°$ (*c* 0.028, $CHCl_3$); IR (ATR): $\tilde{\nu}$ = 476 *m,* 500 *m,* 524 *m,* 661 *m,* 752 *m,* 779 *m,* 847 *m,* 901*w,* 970 *m,* 986 *m,* 1006 *m,* 1026 *s,* 1096 *m,* 1243*vs,* 1365 *m,* 1430 *m,* 1463 *m,* 1534 *s,* 1612*w,* 1707 *m,* 1731 *m,* 2874*w,* 2945 *m,* 3277*w* cm^{-1}; UV/vis ($CHCl_3$): λ_{max} (log ε) = 307 (3.45), 357 (3.70) nm; ^1H NMR (500 MHz, $CDCl_3$): δ = 9.68 (s, 1H, 34-H), 9.17 (s, 1H, 38-H), 8.85–8.79 (m, 2H, 36-H + 44-H), 8.01 (d, *J* = 8.3 Hz, 1H, 41-H), 7.90 (t, *J* = 7.9 Hz, 1H, 43-H), 7.69 (t, *J* = 7.6 Hz, 1H, 42-H), 7.02 (s, 1H, 28-H), 5.32 (t, *J* = 3.7 Hz, 1H, 12-H), 4.51–4.44 (m, 1H, 3-H), 2.81 (s, 1H, 22-H_a), 2.07–1.99 (m, 1H, 16-H_a), 2.06–2.02 (m, 1H, 9-H), 2.05–2.02 (m, 1H, 22-H_b), 2.03 (s, 3H, 32-H_3), 1.95–1.85 (m, 2H, 15-H_a + 16-H_b), 1.85–1.80 (m, 1H, 19-H_a), 1.80–1.71 (m, 2H, 2-H_a + 11-H_a), 1.66–1.52 (m, 4H, 1-H_a + 2-H_b + 11-H_b + 18-H), 1.50–1.44 (m, 2H, 6-H_a + 21-H_a), 1.38–1.34 (m, 3H, 6-H_b + 7-H_a + 21-H_b), 1.28–1.22 (m, 2H, 7-H_b + 19-H_b), 1.16–1.12 (s, 3H, 27-H_3), 1.08–1.03 (m, 2H, 1-H_b + 15-H_b), 1.01 (s, 3H, 30-H_3), 0.98 (s, 3H, 29-H_3), 0.89 (s, 3H, 25-H_3), 0.87 (s, 3H, 23-H_3), 0.84 (s, 3H, 24-H_3), 0.85–0.82 (m, 1H, 5-H), 0.80 (s, 3H, 26-H_3) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 640.8 (100 %, $[M + H]^+$); analysis calcd for $C_{41}H_{57}N_3O_3$ (639.93): C 76.95, H 8.98, N 6.57; found: C 76.80, H 9.16, N 6.52.

*N-[3 β-Acetyloxy-17 β -amino-28-norurs-12-en-17-yl]-4-isoquinolyl urea **(20)***

As described above for **19**, **20** (110 mg, 30 %) was obtained from **6** (248 mg, 0.5 mmol) as a white solid; m.p. 176.9 °C (decomp.) R_F = 0.05 (hexanes/ethyl acetate, 3:1); $[\alpha]_D = 21.5°$ (*c* 0.033, $CHCl_3$); IR (ATR): $\tilde{\nu}$ = 416 *m,* 459 *s,* 479 *s,* 522 *s,* 577 *s,* 660 *s,* 750 *s,* 772 *s,* 830 *s,* 1026 *s,* 1094 *m,* 1244*vs,* 1292 *m,* 1370 *m,* 1402 *m,* 1432 *m,* 1455 *m,* 1538 *s,* 1657 *m,* 1729 *m,* 2871 *m,* 2925 *m,* 3174*w,* 3314*w* cm^{-1}; UV/vis ($CHCl_3$): λ_{max} (log ε) = 250 (4.41), 321 (3.27), 344 (3.25), 378 (3.54) nm; ^1H NMR (500 MHz, $CDCl_3$): δ = 9.00 (s, 1H, 34-H), 8.71 (s, 1H, 36-H), 8.61 (s, 1H, 38-H), 8.09–7.99 (m, 1H, 44-H), 7.94 (t, *J* = 8.2 Hz, 1H, 41-H), 7.77–7.65 (m, 1H, 43-H), 7.65–7.55 (m, 1H, 42-H), 6.94 (s, 1H, 28-H), 5.00 (s, 1H, 12-H), 4.51–4.38 (m, 1H, 3-H), 2.67–2.61 (m, 1H, 22-H_a), 2.20–2.10 (m, 1H, 16-H_a), 2.04 (s, 3H, 32-H_3), 1.95–1.85 (m, 1H, 16-H_b), 1.84–1.73 (m, 3H, 2-H_a + 11-H_a + 15-H_a), 1.60–1.57 (m, 2H, 2-H_b + 11-H_b), 1.56–1.52 (m, 2H, 1-H_a + 22-H_b), 1.53–1.46 (m, 1H, 7-H_a), 1.45 (s, 1H, 6-H_a), 1.47–1.38 (m, 3H, 9-H + 18-H + 21-H_a), 1.40–1.33 (m, 1H, 19-H), 1.31–1.27 (m, 1H, 21-H_b), 1.29–1.25 (m, 1H, 6-H_b), 1.18–1.14 (m, 1H, 7-H_b), 1.01 (s, 3H, 27-H_3), 0.99 (s, 3H, 30-H_3), 0.98–0.97 (m, 2H, 1-H_b + 15-H_b), 0.92–0.90 (m, 4H, 20-H + 29-H_3), 0.87 (s, 3H, 23-H_3), 0.84 (s, 3H, 24-H_3), 0.82 (s, 3H, 25-H_3), 0.78–0.77 (m, 1H, 5-H), 0.74 (s, 3H, 26-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 171.0 (C-31), 154.4 (C-33), 137.9 (C-13), 137.4 (C-38), 131.4 (C-35), 130.9 (C-43), 129.9 (C-40), 128.7 (C-39), 127.9 (C-41), 127.8 (C-42), 127.1 (C-12), 122.4 (C-36), 121.7 (C-44), 80.8 (C-3), 58.9 (C-18), 56.6 (C-17), 55.2 (C-5), 47.4 (C-9), 46.3 (C-14), 41.9 (C-8), 39.8 (C-19), 39.1 (C-20), 38.3 (C-1), 37.6 (C-4), 37.2 (C-22), 32.6 (C-21), 31.4 (C-7), 29.7 (C-10), 28.0 (C-24), 26.7 (C-15), 23.9 (C-16), 23.5 (C-2), 23.3 (C-11), 23.1 (C-27), 21.3 (C-32), 20.7 (C-29), 18.0 (C-6), 17.3 (C-26), 16.8 (C-30), 16.7 (C-25), 15.4 (C-23) ppm; MS (ESI, MeOH/$CHCl_3$ 4:1): *m/z* = 640.6 (100 %, $[M + H]^+$); analysis calcd for $C_{41}H_{57}N_3O_3$ (639.93): C 76.95, H 8.98, N 6.57; found: C 76.75, H 9.06, N 6.42.

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.

Data availability

Data will be made available on request.

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A unified strategy for the synthesis of amorfrutins A and B and evaluation of their cytotoxicity

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Abstract: 3,5-Dimethoxy-benzaldehyde was used as a starting material to synthesize a central intermediate, 2-hydroxy-4-methoxy-6-phenethylbenzoic acid that was converted very quickly and with good yields into amorfrutins A and B. Furthermore, this compound was also used as a starting material to synthesize a piperazinyl-rhodamine B conjugate. The latter compound showed good cytotoxicity (EC_{50} = 2.3–5.1 μM) and promising selective cytotoxicity (S = 2.1–4.6) for human tumor cell lines as compared to non-malignant fibroblasts (NIH 3T3).

Keywords: Amorfrutin A; Amorfrutin B; synthesis; cytotoxicity.

1. Introduction

The vital role of secondary natural products for the development of new drugs is undisputed [1-3]. For thousands of years, people have been using the almost inexhaustible reservoir of plant ingredients [4] of the so-called "God's pharmacy" [5]. For example, in infectious diseases and cancer 75 and 60% of new drugs originate from natural sources [1-3]. The global market for pharmaceuticals is about 1.1 trillion US$; thereby, 35% of the medicines have developed from natural products [1-3]. Cancer, infectious diseases, and complex non-communicable diseases are still the most frequent causes of death worldwide [6]. Of particular interest are phenolic compounds; they are widely dispersed throughout the plant kingdom, and more than 10.000 different phenolic structures have been isolated so far. For many of them, cytotoxic or anticancer activity has been reported. But also for cardiovascular diseases, which are often also associated with type II diabetes mellitus and obesity, there is an unsustainable burden on society [7]. A new strategy for early invention and prevention consists of the timely application of antidiabetic and lipid-lowering compounds such as the 2-hydroxybenzoic acid-derived amorfrutins [8-10].

Amorfrutins A (**1**) and B (**2**) (Fig. 1) were initially isolated from parts of the bastard indigo-bush *Amorpha fruticosa* [11,12]. Still, these and other amorfrutins have also been found in other plants, such as the licorice species *Glycyrrhiza foetida* [13-16]. *A. fruticosa* is an indigenous American shrub, while *G. foetida* is a photoautotrophic plant in the family of Fabaceae. The physiological effects of amorfrutins can be attributed, in part, to selective activation of the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) [14,17-21]. PPARγ regulates genes of glucose and fatty acid metabolism. However, the complex also appears to be important in treating cancer [12,16,22], inflammations [23,24,] and for impending the age-related decline of metabolism [13,15,25-27].

Several syntheses have been published to access amorfrutins A and B whereby the former has been the focus of scientific interest. In contrast, the number of syntheses for the latter has remained small [28-34]. However, of particular interest are synthetic strategies that allow in principle to synthesize as many as possible of the previously known amorfrutins and, if necessary, analogs in a unified manner [31,33].

A most recently published synthesis of amorfrutin B from amorfrutin A methyl ester seems particularly worth mentioning since it holds a critical step in a Johnson-Claisen rearrangement reaction [33,35]. However, this method's elegance is diminished by the available length of the synthesis and the sometimes only moderate yields.

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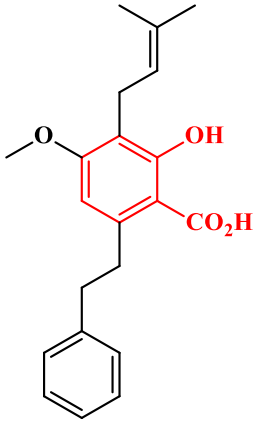
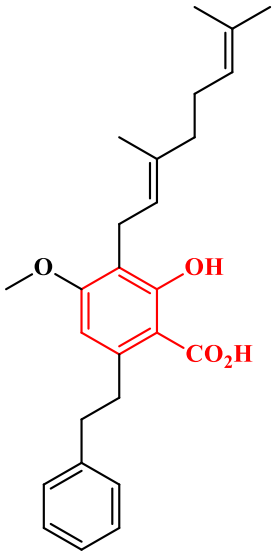


Amorfrutin A (1)



Amorfrutin B (2)

Figure 1. Structure of amorfrutins A (**1**) and B (**2**) highlighting the 2-hydroxybenzoic acid core structure

2. Results and discussion

In the course of our syntheses, we could show that compound **3** (Scheme 1) is easily accessible in large quantities from commercial 3,5-dimethoxy-benzaldehyde in only 6 steps in a total yield of 63% [34]. Thus, **3** seems to be an ideal starting material for synthesizing the two amorfrutins A and B.

Regarding the synthesis of amorfrutins, **3** was converted into methyl ester **4** by reaction with MeI in the presence of Cs_2CO_3 in 98% yield. The reaction of **4** [34,36] with K.H. and prenyl chloride gave a mixture of **5** (as a product of C-alkylation) [34,37], and **6** (as an etherification product) [13]. Whereas, from the reaction with geranyl chloride, a mixture of **7** (from a C-alkylation) [17] and **8** (C- and O-alkylation) was obtained. Both mixes were easily separated by column chromatography. Hydrolysis of **5** furnished amorfrutin A (**1**) while from the hydrolysis of **7**, amorfrutin B (**2**) was obtained in 84% isolated yield. The side products of the former reactions, **6** and **8** were transformed by their reaction with $CeCl_3$ in acetonitrile in the presence of NaI very easily into starting material **4** and amorfrutin B **2**, respectively.

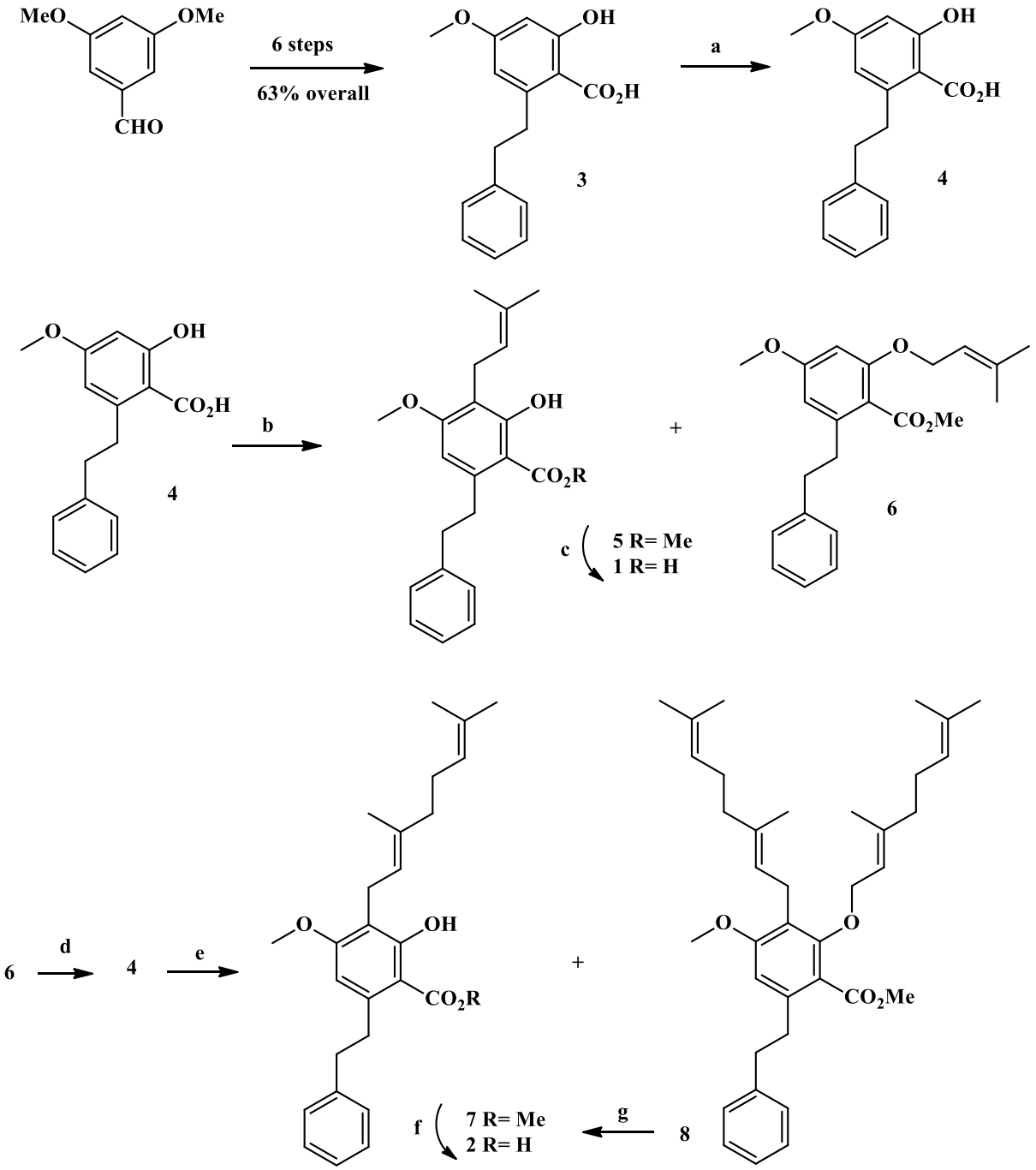
Table 1. Cytotoxicity of compounds **1-10** and rhodamine B (**Rho**) [a].

Compound	A375	HT29	MCF-7	A2780	FaDu	NIH 3T3
Rho	> 30	> 30	> 30	> 30	> 30	> 30
1	> 30	> 30	> 30	> 30	> 30	> 30
2	23.8 ± 2.0	> 30	26.2 ± 1.4	25.0 ± 2.0	27.1 ± 0.9	> 30
3-5	> 30	> 30	> 30	> 30	> 30	> 30
6	> 30	> 30	> 30	22.9 ± 1.8	> 30	> 30
7	> 30	> 30	28.9 ± 3.8	20.5 ± 1.9	> 30	> 30
8	19.6 ± 2.3	> 30	12.0 ± 1.2	19.8 ± 2.1	22.0 ± 2.3	> 30
9	> 30	> 30	17.8 ± 3.9	26.4 ± 2.1	> 30	> 30
10	4.7 ± 0.3	5.1 ± 0.2	3.4 ± 0.5	2.3 ± 0.3	3.7 ± 0.2	1.1 ± 0.1
STA	0.2 ± 0.02	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.05	0.008 ± 0.001	0.2 ± 0.02

[a] (EC_{50} values in µM from SRB assays after 72h of treatment, the values are averaged from three independent experiments performed each in triplicate, confidence interval CI = 95%; mean ± standard mean error, cut-off 30 µM). Human cancer cell lines: A375 (epithelial melanoma), HT29 (colorectal adenocarcinoma), MCF-7 (breast adenocarcinoma), A2780 (ovarian carcinoma), FaDu (squamous cell carcinoma); non-malignant: NIH 3T3 (mouse fibroblasts). Staurosporine (**STA**) was used as a positive standard;

Selectivity S = $EC_{50\ \text{tumor cell line}}$ / $EC_{50\ \text{NIH 3T3}}$.

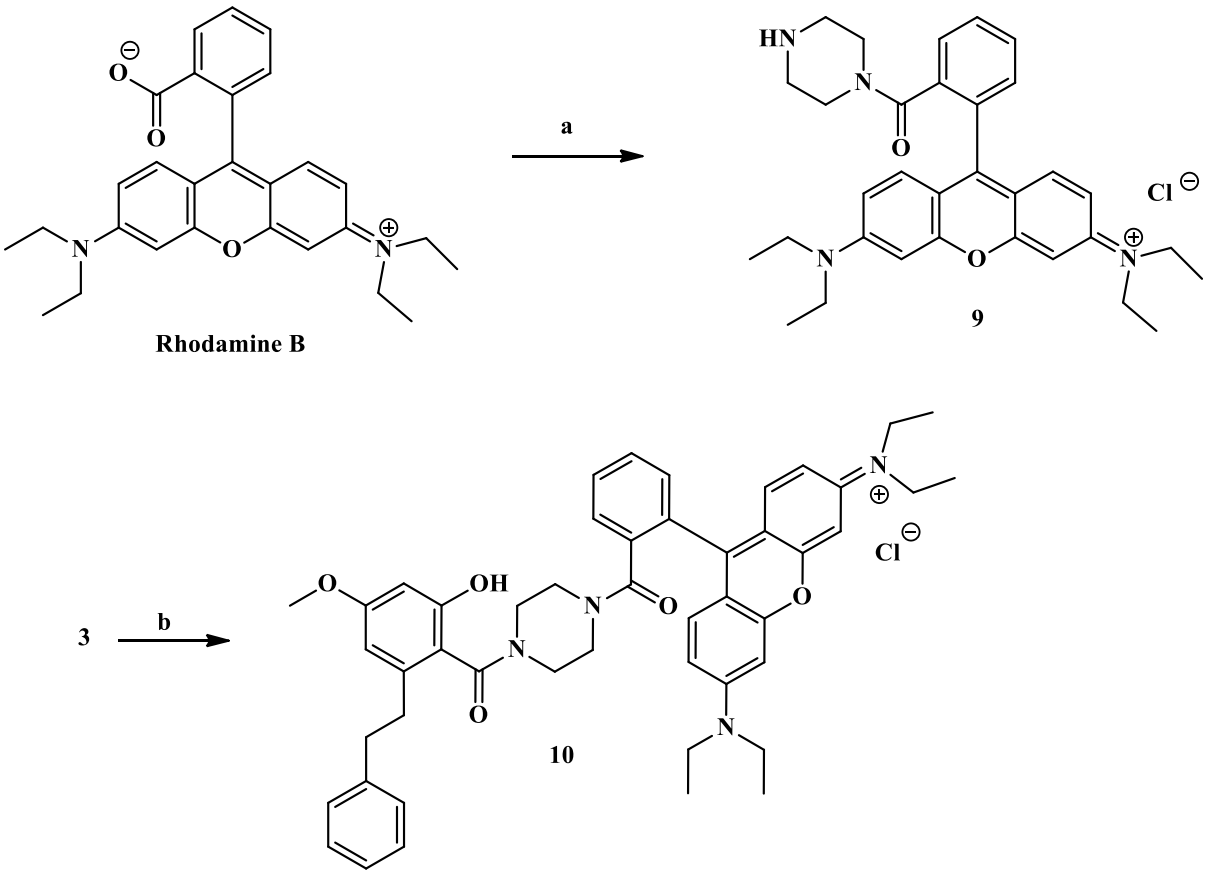
Relatively little is known about the possible cytotoxicity of amorfrutins A and B. Thus, these two compounds were investigated in an SRB assay. This showed amorfrutin A (**1**, Table 1) not to be cytotoxic for several human tumor cell lines as well as for non-malignant mouse fibroblasts (NIH 3T3). EC_{50} values between 23.8 µM (for A375 cells) and 27.1 µM (for FaDu cells), however, were observed for amorfrutin B (**2**).



Scheme 1. Synthesis of amorfrutin A (**1**) and amorfrutin B (**2**) from central intermediate **4**. Reactions and conditions: a) Cs_2CO_3, DMF, MeI, 25°C, 12 h, 96%; b) KH, toluene, prenyl chloride, 75°C, 2 h, 72% (of **5**) and 25% (of **6**); c) KOH, MeOH, H_2O, reflux, 8 h, 90%; d) $CeCl_3$ x 7 H_2O, ACN, NaI, 25°C, 6 h, 95%; e) KH, toluene, geranyl chloride, 70°C, 12 h, 74% (of **7**) and 11% (of **8**); f) KOH, MeOH, H_2O, reflux, 10 h, 84%; g) $CeCl_3$ x 7 H_2O, ACN, NaI, 25°C, 6 h, 84%

We were recently able to show several not cytotoxic (EC50 > 30 µM) di- and triterpene derived carboxylic acids and hydroxycinnamic acid derivatives. Their transformation into a piperazinyl amide and the latter's reaction with rhodamine B led to analogs of significantly increased cytotoxicity [38-46]. Hence, the reaction of **3** with the piperazinyl-rhodamine B conjugate **9** [38,47-49]gave **10**.

Hybrid compound **10** held good cytotoxicity (Table 1) in low micro-molar concentration for all human tumor cell lines, combined with promising selectivity.



Scheme 2. Synthesis of compounds **9** and **10**: reactions and conditions: a) DCM, $(COCl)_2$, DMF, piperazine, 24 h, 25°C, 67%; b) EDC x HCl, HOBt, DCM, **9**, 12 h, 25°C, 74%

3. Conclusion

From 3,5-dimethoxy-benzaldehyde, a central intermediate, 2-hydroxy-4-methoxy-6-phenethyl-benzoic acid is accessible in only 6 steps, which can be converted very easily and with good yields into the amorfrutins A and B. This intermediate, however, can also serve as a starting material for the synthesis of amides. In this case, piperazinyl-rhodamine B conjugate showed good cytotoxicity for several human tumor cell lines (A375, HT29, MCF-7, A2780, FaDu) as well as promising tumor/non-tumor cell selectivity.

Acknowledgments

The authors are grateful to Dr. D. Ströhl, Y. Schiller, and S. Ludwig for multiple NMR spectra and to the late Dr. R. Kluge for M.S. measurements. Th. Schmidt recorded additional M.S. spectra. M. Schneider and S. Ludwig performed I.R. and U.V./Vis spectra as well as elemental analyses. The cell lines were provided by Dr. T. Müller (Dept. of Oncology).

4. Experimental

Instrumentation was previously described [38–46]. Starting materials were obtained from local suppliers in bulk, and the solvents (technical grade) were re-distilled and dried according to usual procedures. All reactions were performed under argon using oven-dried glassware. The routine aqueous workup included the dilution of the reaction mixture with the solvent (used for the reaction), aqueous extraction, re-extraction of the aqueous phase (twice), drying of the combined organic phases ($MgSO_4$), and evaporation of the organic phase under reduced pressure.

4.1. Cytotoxic evaluation

The 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 Chemie GmbH, Steinheim, Germany) and penicillin/streptomycin (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) at 37°C in a humidified atmosphere with 5% CO_2. The compounds' cytotoxicity was evaluated using the sulforhodamine-B (Kiton-Red S, ABCR) micro-

culture colorimetric assay, as previously reported [38–46]. In short, the cells were seeded into 96 well plates on day zero at appropriate cell densities to prevent the confluence of the cells during the experiment. After 24 hours, 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 hours of fixation, the cells were washed in a strip washer and then dyed with SRB solution (200 µL, 10 mM) for 20 minutes. The plates were then washed four times with 1 % acetic acid to remove the dye's excess 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.

Methyl 2-hydroxy-4-methoxy-6-phenethylbenzoate (4)

From **3**: A suspension of **3** (2.00 g, 7.34 mmol) and Cs_2CO_3 (4.8 g, 7.8 mmol) in dry DMF (40 mL) was stirred for 10 min; iodomethane (0.72 mL, 10.8 mmol) was added, and the stirring was continued for 12 h. Usual workup gave **4** (2.02 g, 96%) as a colorless oil pure enough for the transformations to follow.

From **6**: To a solution of **6** (150 mg, 0.42 mmol) in acetonitrile (5 mL) $CeCl_3 \times 7\ H_2O$ (190 mg, 0.51 mmol) and NaI (80 mg, 0.53 mmol) were added. The mixture was stirred at 25°C for 12 h. Usual aqueous work-up followed by chromatography (silica gel, *n*-hexane/ethyl acetate, 6:1) gave **4** (115 mg, 95%); m.p. 49-51°C (lit.: [34] 50-52°C); R_F = 0.45 (*n*-hexane/ethyl acetate, 12:1);
IR (ATR): $\tilde{\nu}$ = 2952w, 1650m, 1614m, 1575m, 1496w, 1434m, 1380w, 1325s, 1203m, 1253s, 1203s, 1156s, 1110m, 1047m, 956m, 802m, 748m, 698s, 638w, 599w cm^{-1};
UV/Vis ($CHCl_3$): λ_{max} (log ε) = 230 (4.30), 265 (4.19), 304 (3.82) nm;
^{1}H NMR (500 MHz, $CDCl_3$) δ = 11.75 (s, 1H, OH), 7.34 – 7.16 (m, 5H, 10-H, 11-H, 12-H), 6.38 (d, J = 2.6 Hz, 1H, 5-H), 6.29 (d, J = 2.6 Hz, 1H, 3-H), 3.96 (s, 3H, COOMe), 3.79 (s, 3H, OMe), 3.21 – 3.14 (m, 2H, 7-H_2), 2.88 – 2.82 (m, 2H, 8-H_2) ppm;
^{13}C NMR (126 MHz, $CDCl_3$): δ = 171.9 (COOH), 165.8 (C-4), 164.2 (C-2), 146.8 (C-6), 142.1 (C-9), 128.6 (C-10), 128.5 (C-11), 126.1 (C-12), 111.0 (C-5), 104.8 (C-1), 99.4 (C-3), 55.4 (OMe), 52.2 (COOMe), 39.0 (C-7), 38.4 (C-8) ppm;
MS (ESI, MeOH): m/z = 287.1 (100, $[M+H]^+$);
analysis calcd for $C_{17}H_{18}O_4$ (286.32): C 71.31, H 6.34; found: C 71.11, H 6.60.

Methyl 2-hydroxy-4-methoxy-3-(3-methyl-2-buten-1-yl)-6-phenylethylbenzoate (5) and methyl 4-methoxy-2-[(3-methylbut-2-en-1-yl)oxy]-6-phenethylbenzoate (6)

A solution of **4** (1.0 g, 3.49 mmol) in dry toluene (30 mL) and K.H. (154 mg, 3.84 mmol; K.H. was obtained as a suspension in mineral oil. Before the reaction, the suspension was washed in a Schlenk-frit with dry *n*-hexane to remove the oil. The pure K.H. was dried in a stream of dry argon) was stirred at 25°C for 20 min, followed by 20 min at 70°C. At 25°C, prenyl chloride (440 mg, 4.21 mmol) was added, and the mixture was stirred at 75°C for 2 hours. Usual workup followed by chromatography (silica gel, *n*-hexane/ethyl acetate, 12:1) gave **5** (890 mg, 72%) and **6** (301 mg, 24%).

Data for **5**: colorless solid; m.p. 67-69°C (lit.: [34] m.p. 67.9 C); R_F = 0.58 (*n*-hexane/ethyl acetate, 12:1);
IR (ATR): $\tilde{\nu}$ = 2924m, 1655m, 1603m, 1573m, 1494w, 1435m, 1405m, 1288s, 1224s, 1154s, 1112s, 1004m, 962w, 804s, 773m, 737s, 700s, 656m, 616w, 558w, 522m cm^{-1};
UV/Vis (MeOH): λ_{max} (log ε) = 226 (4.37), 270 (3.99), 308 (3.55) nm;
^{1}H NMR (500 MHz, $CDCl_3$) δ = 11.72 (s, 1H, OH), 7.33 – 7.17 (m, 5H, 10-H, 11-H, 12-H), 6.21 (s, 1H, 5-H), 5.23 – 5.17 (m, 1H, 14-H), 3.96 (s, 3H, COOMe), 3.80 (s, 3H, OMe), 3.34 (d, J = 7.0 Hz, 2H, 13-H_2), 3.21 – 3.14 (m, 2H, 7-H_2), 2.88 – 2.82 (m, 2H, 8-H_2), 1.78 (s, 3H, CH_3), 1.68 (s, 3H, CH_3) ppm;
^{13}C NMR (126 MHz, $CDCl_3$): δ = 172.3 (COOH), 162.0 (C-4), 161.4 (C-2), 144.2 (C-6), 142.2 (C-9), 131.8 (C-15), 128.5 (C-10, C-11), 126.1 (C-12), 122.5 (C-14), 115.4 (C-3), 106.1 (C-5), 105.3 (C-1), 55.6 (OMe), 52.1 (COOMe), 39.4 (C-7), 38.6 (C-8), 25.9 (CH_3), 22.1 (C-13)), 17.9 (CH_3) ppm;
MS (ESI, MeOH): m/z = 355.1 (15, $[M+H]^+$), 377.2 (100, $[M+Na]^+$);
analysis calcd for $C_{22}H_{26}O_4$ (354.5): C 74.55, H 7.39; found: C 74.32, H 7.54.

Data for **6**: pale yellowish oil; R_F = 0.45 (*n*-hexane/ethyl acetate, 12:1);
IR (ATR): $\tilde{\nu}$ = 2963w, 1602m, 1495w, 1435m, 1364m, 1259s, 1218m, 1204m, 1159s, 1103s, 1032m, 955w, 883w, 850w, 797s, 748m, 725w, 697s, 604m cm^{-1};
UV/Vis ($CHCl_3$): λ_{max} (log ε) = 229 (4.25) nm;
^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.33 – 7.15 (m, 5H, 10-H, 11-H, 12-H), 6.34 (d, J = 2.2 Hz, 1H, 5-H), 6.23 (d, J = 2.2 Hz, 1H, 3-H), 5.43 (t, *J* = 6.3 Hz, 1H, 14-H), 4.53 (d, J = 6.5 Hz, 2H, 13-H_2), 3.89 (s, 3H, COOMe), 3.75 (s, 3H, OMe), 2.94 – 2.80 (m, 4H, 7-H_2, 8-H_2), 1.77 (s, 3H, CH_3), 1.72 (s, 3H, CH_3) ppm;
^{13}C NMR (101 MHz, $CDCl_3$): δ = 168.9 (COOH), 161.4 (C-4), 157.6 (C-2), 141.9 (C-6), 141.8 (C-9), 137.5 (C-15), 128.6 (C-10), 128.5 (C-11), 126.1 (C-12), 119.9 (C-14), 117.0 (C-1), 106.2 (C-5), 98.1 (C-3), 66.1 (C-13)), 55.5 (OMe), 52.2 (COOMe), 37.8 (C-7), 36.4 (C-8), 25.7 (CH_3), 18.4 (CH_3) ppm; MS (ESI, MeOH): m/z = 355.0 (20, $[M+H]^+$), 377.1 (100, $[M+Na]^+$); analysis calcd for $C_{22}H_{26}O_4$ (354.5): C 74.55, H 7.39; found: C 74.31, H 7.58.

Amorfrutin A, 2-hydroxy-4-methoxy-3-(3-methylbut-2-en-1-yl)-6-phenethylbenzoic acid (1)
To a solution of KOH (760 mg, 13.56 mmol) in MeOH/H_2O (7:1, 14 mL) a solution of **5** (800 mg, 2.26 mmol) in MeOH (20 mL) was added. After heating under reflux for 8 h followed by usual aqueous work-up and chromatography (silica gel, n-hexane/ethyl acetate, 4:1) amorfrutin A (**1**, 696 mg, 91%) was obtained as an off-white solid; m.p. 111-113°C (lit.: [34] m.p. 113.7°C); R_F = 0.33 (*n*-hexane/ethyl acetate, 4:1);
IR (ATR): $\tilde{\nu}$ = 2925w, 1610s, 1495w, 1453m, 1435w, 1267s, 1228s, 1175s, 1115s, 1040m cm^{-1};
UV/Vis (MeOH): λ_{max} (log ε) = 224 (4.35), 265 (3.83), 305 (3.42) nm;
^{1}H NMR (500 MHz, $CDCl_3$): δ = 11.90 (s, 1H, OH), 7.35 –7.15 (m, 5H, 10-H, 11-H, 12-H), 6.25 (s, 1H, 5-H), 5.26 – 5.15 (m, 1H, 14-H), 3.82 (s, 3H, OMe), 3.38 (d, J = 7.0 Hz, 2H, 13-H_2), 3.30 – 3.23 (m, 2H, 7-H_2), 2.98 – 2.90 (m, 2H, 8-H_2), 1.81 (s, 3H, CH_3), 1.71 (s, 3H, CH_3) ppm;
^{13}C NMR (126 MHz, $CDCl_3$): δ = 177.1 (COOH), 163.4 (C-4), 162.0 (C-2), 145.7 (C-6), 142.3 (C-9), 131.9 (C-15), 128.5 (C-10), 128.4 (C-11), 126.0 (C-12), 122.6 (C-14), 115.7 (C-3), 106.7 (C-5), 103.7 (C-1), 55.6 (OMe), 39.6 (C-7), 38.5 (C-8), 25.7 (CH_3), 22.0 (C-13), 17.9 (CH_3) ppm;
ESI-MS (MeOH): m/z = 341.0 (50, $[M+H]^+$), 363.1 (100, $[M+Na]^+$);
analysis calcd for $C_{21}H_{24}O_4$ (340.41): C 74.09; H 7.11; found: C 73.91; H 7.30.

Methyl (*E*)-3-(3,7-dimethylocta-2,6-dien-1-yl)-2-hydroxy-4-methoxy-6-phenethylbenzoate (7) and methyl 3-((*E*)-3,7-dimethylocta-2,6-dien-1-yl)-2-(((*E*)-3,7-dimethylocta-2,6-dien-1-yl)oxy)-4-methoxy-6 phenethylbenzoate (8)
To a suspension of K.H. (110 mg, 2.74 mmol) in dry toluene (100 mL), a solution of **4** (714 mg, 2.49 mmol) in dry toluene (50 mL) was slowly added, and the mixture was stirred at 23°C for 15 min followed by the addition of geranyl chloride (560 µL, 3.04 mmol). The stirring at 70°C was continued for 12 h. Usual aqueous workup followed by chromatography (silica gel, *n*-hexane/ethyl acetate, 12:1) gave **7** (778 mg, 74%) and **8** (114 mg, 8%) each as a colorless oil.

Data for **7**: R_F = 0.67 (*n*-hexane/ethyl acetate, 9:1);
IR (ATR): ν = 3085*vw*, 3061*vw*, 3027*w*, 2952*w*, 2916*w*, 2854*w*, 1725*w*, 1651*s*, 1609*m*, 1573*m*, 1496*w*, 1452*m*, 1436*m*, 1404*m*, 1376*m*, 1280*vs*, 1226*s*, 1194*m*, 1155*s*, 1112*s*, 1077*w*, 1043*w*, 1030*w*, 1007*m*, 962*w*, 912*w*, 882*w*, 836*w*, 807*m*, 770*m*, 748*m*, 699*s*, 659*w*, 608*m*, 557*w*, 488*w*, 470*w* cm^{-1};
^{1}H NMR (400 MHz, $CDCl_3$): δ = 11.73 (*s*, 1H, OH), 7.34 – 7.26 (*m*, 2H, 11-H), 7.25 – 7.17 (*m*, 3H, 10-H, 12-H), 6.22 (*s*, 1H, 5-H), 5.22 (*tq*, *J* = 7.0, 1.3 Hz, 1H, 17-H), 5.08 (*tq*, *J* = 7.0, 1.4 Hz, 1H, 21-H), 3.96 (*s*, 3H, CO_2Me), 3.80 (*s*, 3H, OMe), 3.40 –3.33 (*d*, *J* = 7.0 Hz, 2H, 16-H_2), 3.22 – 3.16 (*m*, 2H, 7-H_2), 2.90 – 2.83 (*m*, 2H, 8-H_2), 2.10 – 2.02 (*m*, 2H, 20-H_2), 2.00 – 1.94 (*m*, 2H, 19-H_2), 1.79 (*d*, *J* = 1.3 Hz, 3H, Me), 1.65 (*d*, *J* = 1.4 Hz, 3H, Me), 1.58 (*d*, *J* = 1.3 Hz, 3H, Me) ppm;
^{13}C NMR (101 MHz, $CDCl_3$): δ = 172.1 (CO_2Me), 161.9 (C-4), 161.3 (C-2), 144.0 (C-6), 142.0 (C-9), 135.0 (C-18), 131.1 (C-22), 128.4 (C-10), 125.9 (C-11), 124.5 (C-12), 122.9 (C-21), 122.2 (C-17), 115.3 (C-3), 105.9 (C-1), 105.2 (-C5), 55.4 (OMe), 52 (CO_2Me), 39.8 (C-19), 39.3 (C-8), 38.4 (C-7), 26.7 (C-20), 25.7 (Me), 22.0 (C-16), 17.6 (Me), 16.1 (Me) ppm;
MS (ESI, MeOH): m/z = 391.2 (92%, $[M+H-MeOH]^+$), 423 (40%, $[M+H]^+$), 445.2 (80%, $[M+Na]^+$), 461.2 (40%, $[M+K]^+$), 477.1 (22%, $[M+Na+MeOH]^+$);
analysis calcd for $C_{22}H_{34}O_4$ (422.57): C 76.74, H 8.11; found: C 76.50, H 8.32.

Data for **8**: R_F = 0.46 (*n*-hexane/ethyl acetate);
IR (ATR): ν = 3086*vw*, 3062*vw*, 3026*vw*, 2931*w*, 2859*w*, 1725*s*, 1602*s*, 1584*m*, 1496*w*, 1453*m*, 1432*m*, 1382*w*, 1346*w*, 1317*m*, 1282*m*, 1260*s*, 1225*m*, 1195*s*, 1157*vs*, 1099*s*, 1081*m*, 1049*s*, 1030*m*, 993*w*, 949*w*, 923*w*, 881*w*, 827*m*, 817*m*, 788*w*, 748*m*, 700*s*, 639*w*, 609*w*, 574*w*, 561*w*, 490*w*, 461*w* cm^{-1};
UV/Vis ($CHCl_3$): λ_{max} (log ε) = 228 (4.52), 283 (3.71) nm;
^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.32 – 7.24 (*m*, 2H, 11-H), 7.24 – 7.15 (*m*, 3H, 10-H, 12-H), 6.34 (*d*, *J* = 2.3 Hz, 1H, 5-H), 6.25 (*d*, *J* = 2.2 Hz, 1H, 3-H), 5.43 (*t*, *J* = 6.4 Hz, 1H, 17-H), 5.09 (*t*, *J* = 6.8 Hz, 1H, 21-H), 4.55 (*d*, *J* = 6.3 Hz, 2H, 16-H_2), 3.88 (*s*, 3H, CO_2Me), 3.74 (*s*, 3H, OMe), 2.93 – 2.78 (*m*, 4H, 8-H_2, 7-H_2), 2.14 – 2.01 (*m*, 4H, 20-H_2), 19-H_2), 1.71 (*s*, 3H, Me), 1.68 (*s*, 3H, Me), 1.60 (*s*, 4H, Me) ppm;
^{13}C NMR (101 MHz, $CDCl_3$): δ = 168.8 (CO_2Me), 161.2 (C-4), 157.5 (C-2), 141.8 (C-6), 141.6 (C-18), 140.6 (C-9), 131.8 (C-22), 128.4 (C-10), 128.3 (C-11), 126 (C-12), 123.8 (C-21), 119.6 (C-17), 116.8 (C-1), 106.1 (C-5), 98 (C-3), 66 (C-16), 55.3 (OMe), 52.0 (CO_2Me), 39.5 (C-19), 39.3 (C-8), 38.4 (C-7), 26.3 (C-20), 25.6 (Me), 17.7 (Me), 16.7 (Me) ppm; MS (ESI, MeOH): m/z 581.4 (70%, $[M+Na]^+$), 559.2 (100%, $[M+H]^+$);
analysis calcd for $C_{37}H_{50}O_4$ (558.80): C 79.53, H 9.02; found: C 79.37, H 9.25.

Amorfrutin B, (*E*)-3-(3,7-dimethylocta-2,6-dien-1-yl)-2-hydroxy-4-methoxy-6-phenethyl benzoic acid (2)
From **7**: A solution of **7** (100 mg, 0.24 mmol) in MeOH (5 mL) was added to a 40°C solution of KOH (84 mg, 1.5 mmol) in MeOH/H_2O (7:1, 40 mL), and the mixture was heated under reflux for 10 h. Usual workup followed by chromatography (silica gel, *n*-hexane/ethyl acetate, 3:1) gave **2** (81 mg, 84%) as a colorless solid.

From **8**: Following the procedure given for the synthesis of **4** (from **6**) from **8** compound **2** (72%) was obtained. An analytical sample showed: m.p. 74-76°C

(lit: [50] 80.2-83.1°C); R_F = 0.23 (*n*-hexane/ethyl acetate, 3:1);
IR (ATR): ν = 3064*w,* 3028*w,* 2961*m,* 2925*b,* 2855*w,* 2671*w,* 2592*w,* 2537*w,* 1633*s,* 1607*s,* 1571*m,* 1496*m,* 1453*m,* 1430*m,* 1401*m,* 1380*m,* 1348*w,* 1266*vs,* 1221*s,* 1188*m,* 1170*m,* 1149*m,* 1113*s,* 1077*m,* 1046*w,* 1030*w,* 1003*w,* 983*w,* 923*w,* 902*m,* 858*w,* 837*m,* 819*m,* 804*m,* 773*m,* 749*s,* 734*m,*696*s,* 679*m,* 663*w,* 608*m,* 562*w,* 533*w,* 494*m* cm^{-1}; UV/Vis ($CHCl_3$): λ_{max} (log ε) = 229 (4.07), 277 (3.76), 313 (3.26) nm;
^{1}H NMR (400 MHz, $CDCl_3$): δ = 11.56 (*s*, 2H, COOH + OH), 7.33 – 7.27 (*m*, 2H, 11-H), 7.23 – 7.18 (*m*, 3H, 10-H, 12-H), 6.22 (*s*, 1H, 5-H), 5.21 (*t*, *J* = 5.9 Hz, 1H, 16-H), 5.08 (*t*, *J* = 6.9 Hz, 1H, 20-H), 3.79 (*s*, 3H, OMe), 3.36 (*d*, *J* = 7.0 Hz, 2H, 15-H_2), 3.32 – 3.23 (*m*, 2H, 7-H_2), 2.98 – 2.89 (*m*, 2H, 8-H_2), 2.11 – 1.93 (*m*, 4H, 19-H_2, 18-H_2), 1.81 – 1.76 (*m*, 3H, Me), 1.68 – 1.62 (*m*, 3H, Me), 1.60 – 1.56 (*m*, 3H, Me) ppm;
^{13}C NMR (101 MHz, $CDCl_3$): δ = 175.6 (COOH), 162.9 (C-4), 162.2 (C-2), 145.8 (C-6), 141.9 (C-9), 135.2 (C-17), 131.1 (C-21), 128.5 (C-10), 128.4 (C-11), 125.9 (C-12), 124.5 (C-20), 122.1 (C-16), 115.5 (C-3), 106.5 (C-5), 103.7 (C-1), 55.5 (OMe), 39.8 (C-18), 39.2 (C-8), 38.1 (C-7), 26.8 (C-19), 25.7 (Me), 21.9 (C-15), 17.7 (Me), 16.1 (Me) ppm;
MS (ESI, MeOH): m/z = 391.2 (100%, $[M+H-H_2O]^+$), 409 (46%, $[M+H]^+$), 431.2 (98%, $[M+Na]^+$) and m/z = 363.2 (22%, $[M-H-CO_2]^-$), 407.2 (100%, $[M-H]^-$);
analysis calcd for $C_{26}H_{32}O_4$ (408.54): C 76.44, H 7.90; found: C 76.20, H 8.03.

3,6-Bis(diethylamino)-9-[2-(1-piperazinylcarbonyl)phenyl]-xanthylium chloride (9)
This compound was prepared as previously reported from rhodamine B, oxalyl chloride and piperazine [38] in 67% yield as a dark purple solid; m.p. > 350°C; R_F = 0.15 (chloroform/methanol, 8:2); λ_{max} (log ε) = 260 (0.23), 354 (0.06), 561 (0.82) nm;
IR (ATR) *v* = 3401*br*, 1589*m*, 1529*w*, 1411*s*, 1328*s*, 1275*s*, 1246*m*, 1180*s*, 1132*m*, 1074*m*, 1011*w*, 977*m*, 922*m*, 820*m*, 683*m*;
^{1}H NMR (500 MHz, CD_3OD): δ = 7.79 - 7.74 (*m*, 3H, 3-H + 4-H + 5-H), 7.52 (*m*, 1H, 6-H), 7.28 - 7.25 (*d*, 1H, 10-H), 7.10 - 7.09 (*m*, 1H, 11-H), 6.98 – 6.97 (*d*, 1H, 13-H), 3.72 – 3.59 (*m*, 6H, 15-H_a + 15-H_b + 17-H_a + 17-H_b + 20-H_a + 20-H_b), 3.08 – 3.05 (*t*, 4H, 18-H_a + 18-H_b + 19-H_a + 19-Hb), 1.33 – 1.30 (*t*, 3H, 16-H_a + 16-H_b + 16-H_c) ppm;
^{13}C NMR (126 MHz, CD_3OD): δ = 169.53 (C-1), 159.2 (C-8), 157.3 (C-12), 156.7 (C-14), 135.7 (C-7), 133.0 (C-10), 132.3 (C-2), 131.8 (C-6), 131.5 (C-5), 131.4 (C-4), 128.9 (C-3), 115.4 (C-11), 114.8 (C-9), 97.4 (C-13), 46.9 (C-15), 46.8 (C-17 + C-20), 44.5 (C-18 + C-19), 12.8 (C16) ppm;
MS (ESI, MeOH): *m/z* = 256.4 (24%, $[M+H]^{2+}$), 511.3 (100%, $[M]^+$);
analysis calcd for $C_{32}H_{39}ClN_4O_2$ (547.14): C 70.25, H 7.18, N 10.24; found: C 70.07, H 7.30, N 10.01.

***N*-(6-(Diethylamino)-9-(2-(4-(2-hydroxy-4-methoxy-6-phenethylbenzoyl)piperazine-1-carbonyl)phenyl)-3*H*-xanthen-3-ylidene)-*N*-ethylethanaminium chloride (10)**
To a solution of **3** (181 mg, 0.66 mmol) in dry DCM (30 mL), EDC HCl (153 mg, 0.79 mmol), HOBt (120 mg, 0.79 mmol) and **9** (361 mg, 0.66 mg) were added; the mixture was stirred for 12h. Usual work-up followed by chromatography (silica gel, $CHCl_3$/MeOH, 1% → 15%) gave **10** (392 mg, 74%) as a pink amorphous solid; R_F = 0.2 ($CHCl_3$/MeOH, 9:1);
IR (ATR): ν = 3061*w*, 2974*w*, 2932*w*, 2869*w*, 1629*m*, 1584*vs*, 1558*m*, 1528*m*, 1506*m*, 1481*m*, 1464*m*, 1456*m*, 1410*s*, 1393*s*, 1333*vs*, 1271*s*, 1245*s*, 1196*m*, 1178*vs*, 1158*s*, 1130*s*, 1072*s*, 1048*m*, 1000*s*, 977*m*, 921*m*, 868*m*, 821*s*, 787*m*, 754*m*, 702*m*, 682*s*, 664*m*, 642*m*, 620*m*, 581*m*, 546*m*, 523*m*, 497*m* cm^{-1};
UV/Vis ($CHCl_3$): λ_{max} (log ε) = 229 (4.57), 261 (4.48), 357 (3.85), 563 (5.01) nm;
^{1}H NMR (400 MHz, $CDCl_3$): δ = 10.08 (s, 1H, OH), 7.66 (s, 1H, 23-H), 7.60 (dd, *J* = 5.9, 2.9 Hz, 2H, 21-H, 24-H), 7.43 (s, 1H, 22-H), 7.33 – 7.26 (m, 2H, 11-H), 7.25 –7.20 (m, 2H, 28-H, 37-H), 7.20 – 7.12 (m, 3H, 10-H, 12-H), 6.78 (dd, *J* = 9.4, 2.4 Hz, 2H, 29-H, 36-H), 6.70 (d, *J* = 2.4 Hz, 2H, 31-H, 34-H), 6.66 (s, 1H, 3-H), 6.27 (d, *J* = 2.3 Hz, 1H, 5-H), 4.37 (s, 2H, 15a-H_2, 17a-H_2), 3.94 (s, 2H, 16a-H_2, 18a-H_2), 3.73 (s, 3H, OMe), 3.67 – 3.50 (m, 8H, 39-H_2, 41-H_2, 43-H_2, 45-H_2), 3.06 – 2.96 (m, 2H, 7-H_2), 2.89 (s, 4H, 15b-H_2, 16b-H_2, 17b-H_2, 18b-H_2), 1.32 (dt, *J* = 17.3, 7.0 Hz, 12H, 4 x Me) ppm;
^{13}C NMR (101 MHz, $CDCl_3$): δ = 173.2 (CO), 168.3 (CO), 167.4 (C-4), 161 (C-2), 157.8 (C-27, C-38), 157.5 (C-25), 155.3 (C-30, C-35), 142 (C-6), 140.8 (C-9), 135.4 (C-20), 132.3 (C-28, C-37), 131.4 (C-21), 131.2 (C-26), 130.3 (C-10), 129.8 (C-22), 128.6 (C-23), 128.3 (C-11), 127.4 (C-24), 125.8 (C-12), 115.2 (C-1), 113.9 (C-32), 113.5(C-33), 113.3 (C-29, C-36), 107.6 (C-5), 99.1 (C-3), 96.1 (C-31, C-34), 55.3 (OMe), 45.9 (C-39, C-41, C-43, C-45), 41.8 (C-7), 37.5 (C-8), 35.4 (C-15, C-16, C-17, C-18), 12.6 (4 x Me) ppm;
MS (ESI, MeOH): m/z = 765.3 (100%, $[M-Cl]^+$;
analysis calcd for $C_{48}H_{53}N_4O_5Cl$ (801.43): C 71.94, H 6.67, N 6.99; found: C 71.77, H 6.81, N 6.67.

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