






Article

# Small Structural Differences Govern the Carbonic Anhydrase II Inhibition Activity of Cytotoxic Triterpene Acetazolamide Conjugates

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**Abstract:** Acetylated triterpenoids betulin, oleanolic acid, ursolic acid, and glycyrrhetic acid were converted into their succinyl-spacered acetazolamide conjugates. These conjugates were screened for their inhibitory activity onto carbonic anhydrase II and their cytotoxicity employing several human tumor cell lines and non-malignant fibroblasts. As a result, the best inhibitors were derived from betulin and glycyrrhetic acid while those derived from ursolic or oleanolic acid were significantly weaker inhibitors but also of diminished cytotoxicity. A betulin-derived conjugate held a  $K_i = 0.129 \mu\text{M}$  and an  $\text{EC}_{50} = 8.5 \mu\text{M}$  for human A375 melanoma cells.

**Keywords:** triterpenoic acid; carbonic anhydrase II; acetazolamide conjugate; cytotoxicity



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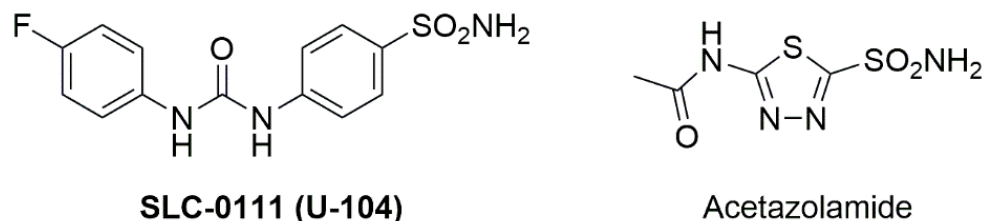
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## 1. Introduction

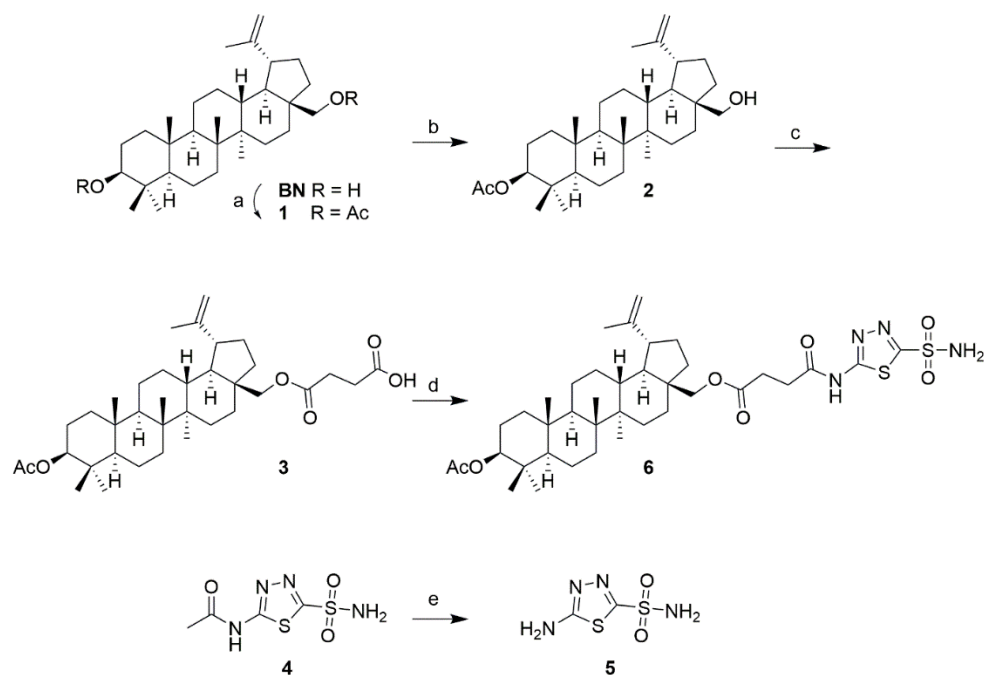
The ubiquitous metalloenzymes carbonic anhydrases (CAs) [1–6] are present in bacteria [7,8] and fungi [9–12], plants and animals. Inhibitors of these enzymes have been clinically exploited for decades, and the discovery of multiple human isoforms [13–18] has led to many new applications and the development of new therapeutic principles, among them antiglaucoma [19–21] and antitumor drugs but also antiepileptic [22–26] and antiobesity drugs [27–29] as well as agents for the management of Alzheimer’s disease [30,31], neuropathic pain, cerebral ischemia, and some forms of arthritis [32–34]. Furthermore, the development of inhibitors for bacterial carbonic anhydrases is thought as a new concept to develop antibacterial drugs [35–42]. In addition, drug conjugates were investigated for their ability to treat a variety of disorders in a multitargeting approach [43–47]. The most investigated compound, however, is SLC-0111 (U-104, Figure 1) [48–53] for the management of advanced, metastatic solid tumors; this compound is now in Phase Ib/II clinical trials [54].



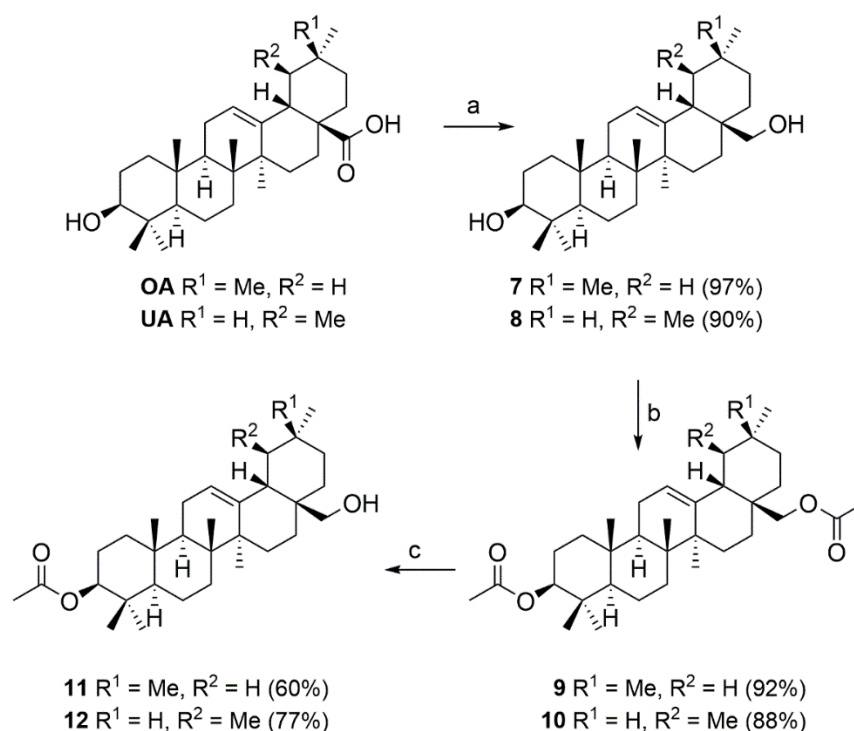
**Figure 1.** Structure of well-established CA inhibitors SLC-0111 and acetazolamide.

For many years, especially CA IX and CA XII were in the focus of scientific interest to combat cancer. Recently, CA II in the endothelium of glial tumors became a potential target for therapy [55–64]. Furthermore, the CA II inhibitor acetazolamide was suggested as a chemosensitizer for treating temozolomide resistant gliomas [65–69]. In addition, CA





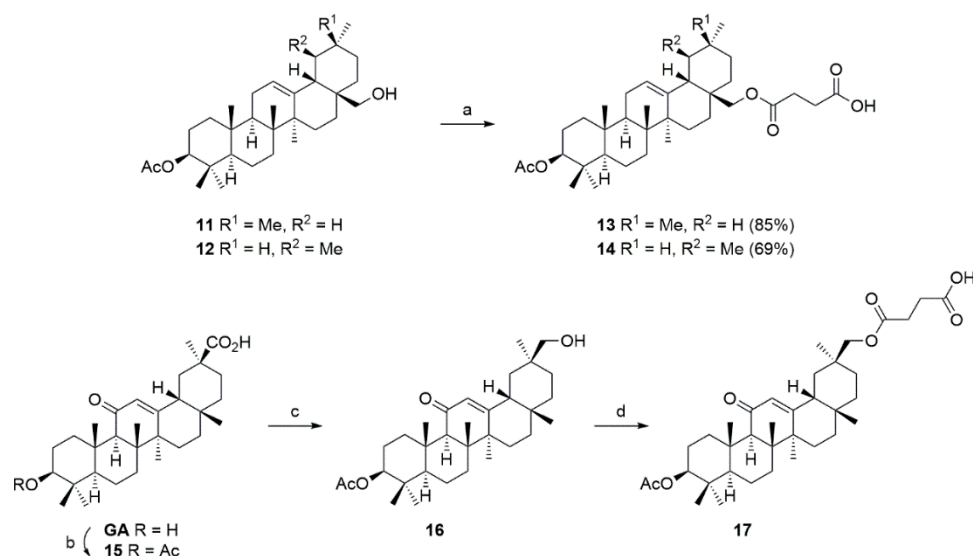
**Scheme 1.** Reactions and conditions: (a)  $\text{Ac}_2\text{O}$ , TEA, DMAP (cat.), DCM, 20 °C, 12 h, 90%; (b)  $\text{CaH}_2$ , MeOH/THF, 20 °C, 12 h, 83%; (c) pyridine, DMAP, succinic anhydride, reflux, 15 h, 71%; (d) THF, 4-methyl-morpholine, ethyl chloroformate, 20 °C, 15 min, then **5**, reflux, 48 h, 88%; (e) conc. HCl, reflux, 3 h, 94%.



**Scheme 2.** Reactions and conditions: (a)  $\text{LiAlH}_4$ , THF, reflux, 2 h; (b)  $\text{Ac}_2\text{O}$ , pyridine, 20 °C, 15 h; (c)  $\text{Al}(\text{iPrO})_3$ ,  $^i\text{PrOH}$ , reflux, 4 h.

Compounds **7** and **8** were converted into the corresponding diacetates **9** and **10**, respectively, whose selective de-acetylation gave compounds **11** and **12**. Analogous conditions as described above could now be carried out for the subsequent reactions to yield the target compounds.

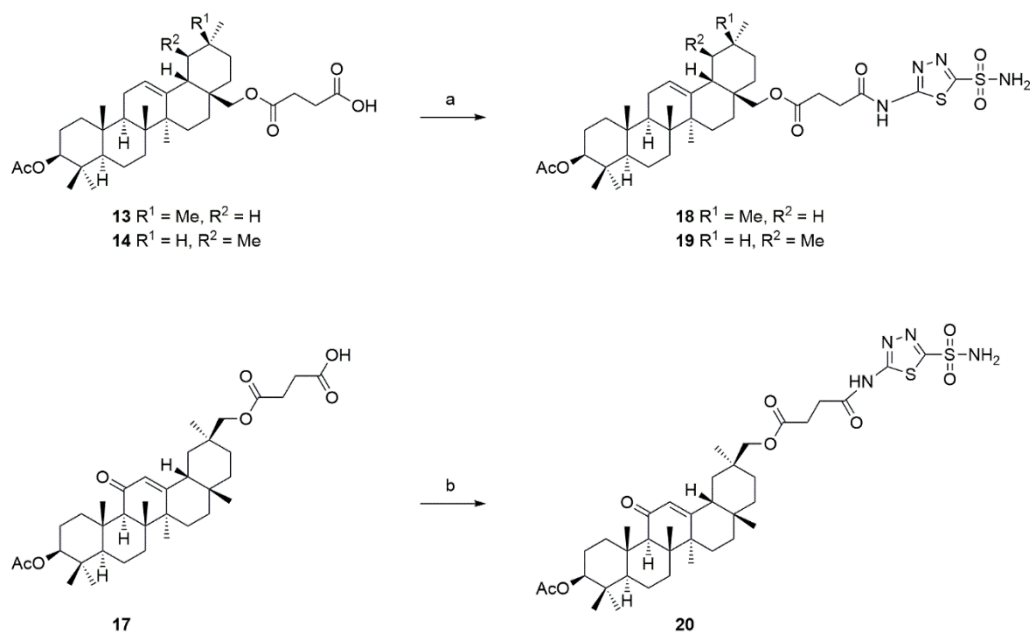
Thus, the mono-acetates **11** and **12** were converted (Scheme 3) to the succinyl derivatives **13** and **14**.



**Scheme 3.** Reactions and conditions: (a) Pyridine, DMAP, succinic anhydride, reflux, 24 h; (b)  $\text{Ac}_2\text{O}$ , pyridine,  $20^\circ\text{C}$ , 15 h; (c) ethyl chloroformate, TEA, THF,  $-12^\circ\text{C}$ , 15 min, then sodium borohydride in water, 15 min; (d) pyridine, DMAP, succinic anhydride, reflux, 24 h.

Since the reduction of **GA** by  $\text{LiAlH}_4$  failed to give good yields, **GA** was first converted into acetate **15**, the reaction of which with ethyl chloroformate/TEA gave an un-isolated mixed anhydride, the reduction of which with  $\text{NaBH}_4$  at room temperature afforded compound **16** in good yields within a few minutes. Its reaction with succinyl anhydride yielded **17**.

The coupling of **13**, **14**, and **17** with **5** (Scheme 4) gave the products **18–20**, respectively.



**Scheme 4.** Reactions and conditions: (a,b) THF, 4-methyl-morpholine, ethyl chloroformate,  $20^\circ\text{C}$ , 15 min, then **5**, reflux, 48 h.

Screening of compounds **6**, **18–20** for their activity was performed with CA II as previously described; the results from the assays are compiled in Table 1. Acetazolamide (**4**) was used as a positive control.

**Table 1.** Inhibition percentage of conjugates (at 10  $\mu$ M concentration) and of standard acetazolamide (**4**).

Compound	Inhibition [%]
<b>4</b>	89.9 $\pm$ 0.6
<b>6</b>	93.0 $\pm$ 0.1
<b>18</b>	49.4 $\pm$ 0.1
<b>19</b>	70.8 $\pm$ 0.2
<b>20</b>	96.8 $\pm$ 0.2

These assays showed glycyrrhetic acid-derived conjugate **20** as the best inhibitor for this enzyme followed by betulin-derived **6**. These compounds were even better inhibitors than gold standard acetazolamide (**4**). Oleanolic and ursolic-derived conjugates showed a diminished ability to inhibit CA II. Parent compounds, i.e., betulin, betulinic acid, ursolic acid, oleanolic acid, and glycyrrhetic acid did not inhibit the enzyme under the conditions of the assay at all. Compounds **2**, **3**, **7–17** showed inhibition rates less than 10%.

For compounds with the highest inhibition percentage, i.e., **6** and **19** and **20**, some extra measurements were performed to determine their respective inhibition constants  $K_i$  values. The results from these experiments are summarized in Table 2; Figure 3 shows the Dixon plot for compound **6**; this compound acts as a competitive inhibitor for the enzyme and holds a rather low  $K_i = 0.129 \mu$ M.

**Table 2.**  $K_i$  values (in  $\mu$ M) for conjugates **6**, **19**, and **20**.

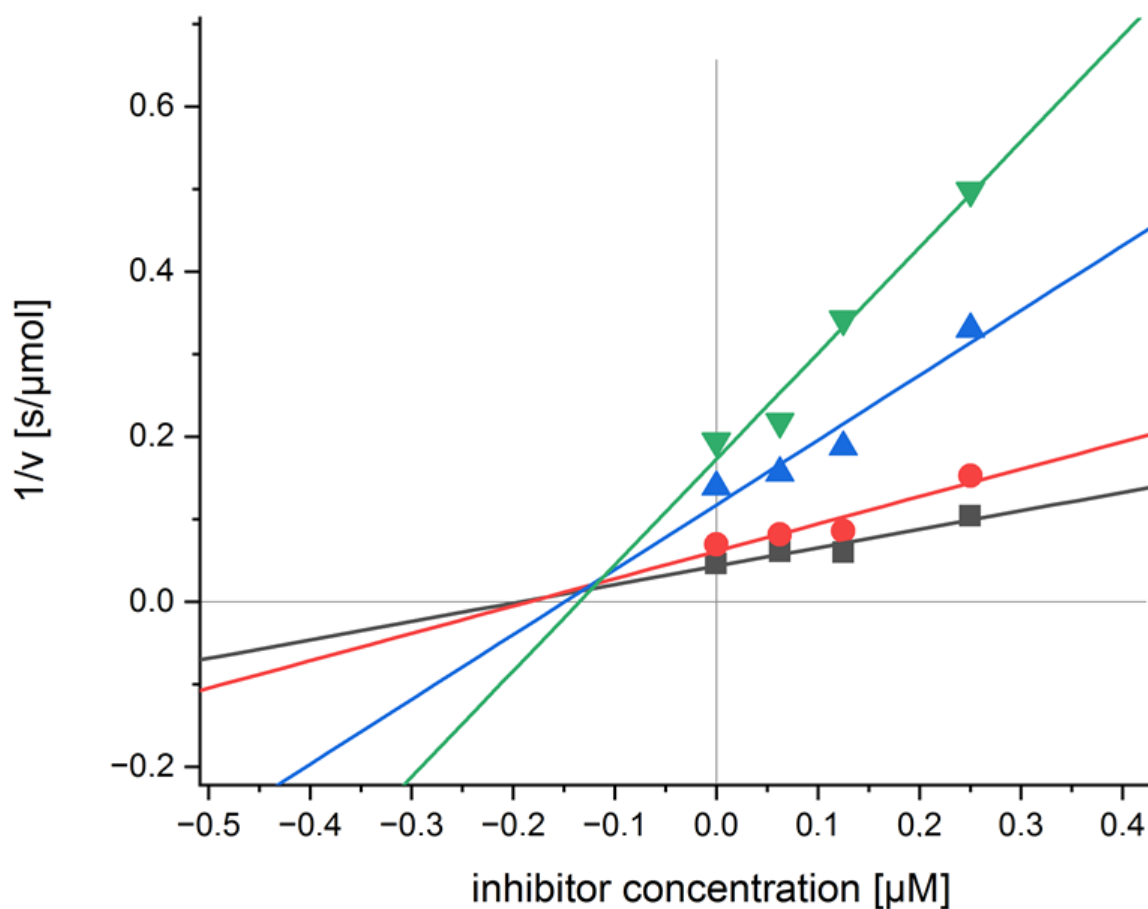
Compound	$K_i$ (in $\mu$ M)
<b>6</b>	0.129 $\pm$ 0.02
<b>19</b>	0.91 $\pm$ 0.17
<b>20</b>	5.22 $\pm$ 0.57

Initial molecular modelling calculations were performed to get some insights in the mode of action of the conjugates. These calculations, however, did not provide any reasonable explanation for the different ability of the conjugates to inhibit the enzyme. While it seems plausible that the acetazolamide moiety interacts with the active site of the enzyme in a manner like parent acetazolamide, it cannot be excluded; however, that the conjugates also act as non-zinc binding inhibitors, thus paralleling previous findings for structurally similar pentacyclic triterpenoid arjunolic acid [79].

Previously especially CA IX was extensively studied in the process of tumorigenesis, [15,80] and several derivatives of pentacyclic triterpenoids have been revealed as inhibitors of this isoform, too [81]. The selectivity of the triterpenoid investigated so far toward individual isoforms of CA, however, was not particularly pronounced.

Compounds **6** and **18–20** were screened for their cytotoxic activity in sulforhodamine B assays (SRB), employing several human tumor cell lines. The results from these assays are summarized in Table 3. Expression of CA II and its involvement cancer has previously been established for A375 [82], HT29 [83] as well as for MCF-7 cells [84]. Cell line A2780 and non-malignant fibroblasts (NIH 3T3) were employed for comparison.

As a result, the highest cytotoxicity was established for betulin-derived **6** followed by glycyrrhetic acid-derived **20**. This parallels the finding for the inhibition rates for CA II established for these compounds. A significantly lower cytotoxicity was determined for oleanolic or ursolic acid-derived compounds **18** and **19**, respectively. The malignant/non-malignant cell selectivity, however, was low for all compounds. No cytotoxicity ( $EC_{50} > 30 \mu$ M; cut-off of the assay) was found for parent triterpenic acids.



**Figure 3.** Dixon plot for compound **6** and CA II.

**Table 3.** Cytotoxicity of acetazolamide (**4**) and conjugates **6**, **18–20** assessed from SRB-assays ( $EC_{50}$  values [ $\mu\text{M}$ ] after 72 h of treatment). Human cancer cell lines: A375 (epithelial melanoma), HT29 (colorectal adenocarcinoma), MCF-7 (breast adenocarcinoma), A2780 (ovarian carcinoma); non-malignant: NIH 3T3 (fibroblasts); n.d. not determined; positive control: doxorubicin (**DX**).

	A375	HT29	MCF-7	A2780	NIH 3T3
<b>4</b>	>30	>30	>30	>30	>30
<b>6</b>	$8.5 \pm 0.7$	$10.2 \pm 1.3$	$8.9 \pm 0.7$	$9.3 \pm 1.2$	$9.5 \pm 1.0$
<b>18</b>	$10.1 \pm 0.8$	$14.2 \pm 1.4$	$10.6 \pm 1.4$	$11.8 \pm 1.4$	$14.0 \pm 1.5$
<b>19</b>	$13.7 \pm 1.1$	$15.0 \pm 0.6$	$12.4 \pm 0.8$	$12.5 \pm 1.7$	$14.8 \pm 1.5$
<b>20</b>	$9.2 \pm 0.5$	$13.0 \pm 1.3$	$10.5 \pm 1.2$	$9.8 \pm 0.8$	$11.9 \pm 1.7$
<b>DX</b>	n.d.	$0.25 \pm 0.02$	$0.1 \pm 0.01$	$0.1 \pm 0.01$	$0.01 \pm 0.001$

### 3. Conclusions

Pentacyclic triterpenoids betulin, oleanolic acid, ursolic acid, and glycyrrhetic acid were acetylated at position C-3 and converted into their succinyl-spacered acetazolamide conjugates. Their screening for their inhibitory activity onto carbonic anhydrase II and screening for their cytotoxicity in SRB assays employing several human tumor cell lines and non-malignant fibroblasts showed the conjugates derived from betulin and glycyrrhetic acid to be the best inhibitors while those derived from ursolic or oleanolic acid were significantly weaker inhibitors but also of diminished cytotoxicity. A betulin-derived conjugate held a  $K_i = 0.129 \mu\text{M}$  and an  $EC_{50} = 8.5 \mu\text{M}$  for human A375 melanoma cells.

#### 4. Experimental

NMR spectra were recorded using the Varian spectrometers (Darmstadt, Germany) DD2 and VNMR5 (400 and 500 MHz, respectively). MS spectra were taken on a Advion expressionL CMS mass spectrometer (Ithaca, USA; positive ion polarity mode, solvent: methanol, solvent flow: 0.2 mL/min, spray voltage: 5.17 kV, source voltage: 77 V, APCI corona discharge: 4.2  $\mu$ A, capillary temperature: 250 °C, capillary voltage: 180 V, sheath gas: N<sub>2</sub>). Thin-layer chromatography was performed on pre-coated silica gel plates supplied by Macherey-Nagel (Düren, Germany). IR spectra were recorded on a Spectrum 1000 FT-IR-spectrometer from Perkin Elmer (Rodgau, Germany). The UV/Vis-spectra were recorded on a Lambda 14 spectrometer from Perkin Elmer (Rodgau, Germany); optical rotations were measured using a JASCO-P2000 instrument (JASCO Germany GmbH, Pfungstadt, Germany) The melting points were determined using the Leica hot stage microscope Galen III (Leica Biosystems, Nussloch, Germany) and are uncorrected. The solvents were dried according to usual procedures. Microanalyses were performed with an Elementar Vario EL (CHNS) instrument (Elementar Analysensysteme GmbH, Elementar-Straße 1, D-63505 Langenselbold, Germany). All dry solvents were distilled over respective drying agents except for DMF which was distilled and stored under argon and molecular sieve. Reactions using air- or moisture-sensitive reagents were carried out under argon atmosphere in dried glassware. Triethylamine was stored over potassium hydroxide. Biological assays were performed as previously reported employing cell lines obtained from the Department of Oncology [Martin-Luther-University Halle Wittenberg; they were bought from ATCC: malignant: A 375, HT29, MCF7, and A2780; non-malignant: NIH 3T3]. Oleanolic and ursolic acid were obtained from Betulinines (Strbrna Skalice, Czech Republic) and used as received. Glycyrrhetic acid was bought from Orgentis Chemicals GmbH (Gatersleben).

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

For the CA II assay: Carbonic anhydrase II (bCA II,  $\geq 3000$  W-A units/mg from bovine erythrocytes) as well as 4-nitrophenyl acetate (4-NA) were purchased from Sigma.

A 96-well microplate spectrometer BMG Labtech Spectrostar Omega working in the slow kinetics mode and measuring the absorbance at a distinct wavelength of  $\lambda = 415$  nm with center scanning was used for the enzymatic studies. In short: A mixture of 4-NA solution (125  $\mu$ L, 6 mM in 50 mM Tris-HCl buffer, pH 8), enzyme solution (25  $\mu$ L, 0.3 mg/mL), and compounds solutions (25  $\mu$ L, 3 different concentrations and water as a blank) was incubated at 37 °C for 20 min. The substrate (25  $\mu$ L, [4-NA] = 0.75 mM, 0.50 mM, 0.25 mM, 0.15 mM) was added to start the enzymatic reaction. The absorbance data was recorded under a controlled temperature of 37 °C for 30 min at 1 min intervals at  $\lambda = 415$  nm. The relative inhibition was determined as the quotient of the slopes (compound divided by blank) of the linear ranges.

##### 4.1. Di-O-Acetyl-betulin

Compound 1 was prepared from BN (15.0 g, 34 mmol) by acetylation with acetic anhydride as previously described; re-crystallization from ethanol gave 1 (15.8 g, 90%) as a white solid;  $R_F = 0.73$  (silica gel, hexanes/ethyl acetate, 8:2); m.p.: 221 °C (lit.: [85] 216–218 °C);  $[\alpha]_D = +16.6^\circ$  ( $c = 0.061$ , MeOH), [lit.: [85]  $[\alpha]_D = +19.7^\circ$  (CHCl<sub>3</sub>)]; MS (ESI, MeOH):  $m/z = 467.5$  (100%, [M + H-HOAc]<sup>+</sup>).

#### 4.2. 3-O-Acetyl-betulin

Selective deacetylation of 1 (8.0 g, 15.2 mmol) with cat. amounts of  $\text{CaH}_2$  in MeOH/THF (100 mL, 1:1 *v:v*) for 12 h at 20 °C followed by usual aqueous workup and chromatography (silica gel, hexanes/ethyl acetate, 8:2) gave 2 (6.1 g, 83%) as a colorless solid;  $R_F = 0.40$  (silica gel, hexanes/ethyl acetate, 8:2); m.p.: 256–259 °C (lit.: [85] 258–260 °C);  $[\alpha]_D = +28.8^\circ$  ( $c = 0.039$ ,  $\text{CHCl}_3$ ), [lit.: [85]  $[\alpha]_D = +25.7^\circ$  ( $\text{CHCl}_3$ )]; MS (ESI, MeOH):  $m/z = 992.0$  (100%,  $[\text{2M}+\text{Na}]^+$ ).

#### 4.3. 4-[(3 $\beta$ )-3-(Acetyloxy)lup-20(29)-en-28-yl]oxy-4-oxobutanoic Acid

To a solution of 2 (4.0 g, 8.2 mmol) in dry pyridine (50 mL), DMAP (cat.) and succinic anhydride (1.64 g, 16.4 mmol) were added. The reaction mixture was stirred under reflux for 15 h. Usual aqueous work up and chromatography (silica gel, hexanes/ethyl acetate, 7:3) gave 3 [86–91] (3.4 g, 71%) as a white solid; m.p. 189–191 °C (lit.: [86–88] 190–191 °C);  $[\alpha]_D = +12.1^\circ$  ( $c = 0.198$ , MeOH);  $R_F = 0.15$  (silica gel, hexanes/ethyl acetate, 8:2); IR (ATR):  $\nu = 2943m, 2871w, 1732s, 1713s, 1455w, 1361w, 1366m, 1244s, 1159m, 1027w, 979m, 883w, 754m \text{ cm}^{-1}$ ;  $^1\text{H NMR}$  (500 MHz,  $\text{CDCl}_3$ ):  $\delta = 4.68$  (s, 1H, 29- $\text{H}_a$ ), 4.58 (s, 1H, 29- $\text{H}_b$ ), 4.46 (dd,  $J = 10.6, 5.6$  Hz, 1H, 3- $\text{H}_a$ ), 4.30 (d,  $J = 11.0$  Hz, 1H, 28- $\text{H}_a$ ), 3.88 (d,  $J = 11.1$  Hz, 1H, 28- $\text{H}_b$ ), 2.75–2.54 (m, 4H, 34-H, 35-H), 2.42 (td,  $J = 11.0, 5.8$  Hz, 1H, 19-H), 2.03 (s, 3H, 32-H), 2.01–1.88 (m, 1H, 21- $\text{H}_a$ ), 1.85–1.79 (m, 1H, 16- $\text{H}_a$ ), 1.75 (dd,  $J = 12.5, 7.9$  Hz, 1H, 22- $\text{H}_a$ ), 1.68 (s, 3H, 30-H), 1.72–1.54 (m, 7H, 1- $\text{H}_a$ , 13-H, 15- $\text{H}_a$ , 12- $\text{H}_a$ , 2-H, 9-H), 1.50 (s, 1H, 6- $\text{H}_a$ ), 1.44–1.35 (m, 5H, 6- $\text{H}_b$ , 11- $\text{H}_a$ , 21- $\text{H}_b$ , 7-H), 1.34–1.14 (m, 3H, 16- $\text{H}_b$ , 18-H, 11- $\text{H}_b$ ), 1.02 (s, 3H, 23-H), 1.12–0.90 (m, 4H, 22- $\text{H}_b$ , 12- $\text{H}_b$ , 15- $\text{H}_b$ , 1- $\text{H}_b$ ), 0.96 (s, 3H, 27-H), 0.84 (s, 3H, 24-H), 0.84 (s, 3H, 26-H), 0.83 (s, 3H, 25-H), 0.78 (m, 1H, 5-H). ppm;  $^{13}\text{C NMR}$  (126 MHz,  $\text{CDCl}_3$ ):  $\delta = 177.8$  (C-36), 172.6 (C-33), 171.3 (C-31), 150.2 (C-20), 110.0 (C-29), 81.1 (C-3), 63.3 (C-28), 55.5 (C-5), 50.4 (C-18), 48.9 (C-9), 47.9 (C-19), 46.6 (C-17), 42.8 (C-14), 41.0 (C-8), 38.5 (C-1), 37.9 (C-4), 37.7 (C-13), 37.2 (C-10), 34.2 (C-22), 29.9 (C-16), 29.1 (C-35), 28.1 (C-24), 27.2 (C-15), 25.3 (C-12), 23.8 (C-2), 21.4 (C-32), 20.9 (C-11), 19.3 (C-30), 18.3 (C-6), 16.6 (C-25), 16.2 (C-23), 14.9 (C-27) ppm; MS (ESI, MeOH):  $m/z = 583.6$  (100%,  $[\text{M}-\text{H}]^-$ ); analysis calcd for  $\text{C}_{36}\text{H}_{56}\text{O}_6$  (584.83): C 73.93, H 9.65; found: C 73.67, H 9.88.

#### 4.4. 5-Amino-1,3,4-thiadiazole-2-sulfonamide

A solution of acetazolamide (4, 9.0 g, 40.7 mmol) in conc. HCl (60 mL) was heated under reflux for 3 h. After neutralization with NaOH, saturation with NaCl and extraction with THF ( $4 \times 100$  mL) followed by removal of the organic solvent, 5 (6.9 g, 94 %) was obtained as a white solid; m.p. 195 °C decomp. (lit.: [92] 215.5–216);  $R_F = 0.3$  (silica gel,  $\text{CHCl}_3/\text{MeOH}$ , 9:1); UV-Vis (MeOH):  $\lambda_{\text{max}}$  ( $\log \epsilon$ ) = 278 nm (3.80) IR (ATR):  $\nu = 3427w, 3321m, 3173w, 2870w, 2636w, 1601s, 1496s, 1448m, 1338s, 1172m, 1139m, 1098w, 1058w, 941m, 647s, 581s, 484w \text{ cm}^{-1}$ ;  $^1\text{H NMR}$  (500 MHz,  $\text{DMSO}-d_6$ ):  $\delta = 8.04$  (s, 2H,  $\text{NH}_2$ ), 7.84 (s, 2H,  $\text{NH}_2$ ) ppm;  $^{13}\text{C NMR}$  (126 MHz,  $\text{DMSO}-d_6$ ):  $\delta = 171.7, 157.9$ . ppm; MS (ESI, MeOH):  $m/z = 179.0$  (100%,  $[\text{M}-\text{H}]^-$ ).

#### 4.5. (3 $\beta$ )-3-(Acetyloxy)lup-20(29)-en-28-yl 4-[[5-(aminosulfonyl)-1,3,4-thiadiazol-2-yl]amino]-4-oxobutanoate

Compound 3 (500 mg, 0.85 mmol) was dissolved in dry THF (50 mL), 4-methylmorpholine (172 mg, 1.7 mmol) and ethyl chloroformate (185 mg, 1.7 mmol.) were added. The reaction mixture was stirred at 20 °C for 15 min. Compound 5 (184 mg, 1.02 mmol) was added, and the mixture was heated under reflux for another 48 h. The solvent was removed, the residue dissolved in  $\text{CHCl}_3$ , washed with 2 M NaOH, water and brine and dried ( $\text{MgSO}_4$ ). Chromatography (silica gel,  $\text{CHCl}_3/\text{MeOH}$ , 9:1) gave 4 (560 mg, 88%) as a white solid; m.p. 161–164 °C;  $R_F = 0.55$  (silica gel, hexanes/ethyl acetate, 7:3); UV-Vis ( $\text{CHCl}_3$ ):  $\lambda_{\text{max}}$  ( $\log \epsilon$ ) = 264 nm (3.92) IR (ATR):  $\nu = 2944m, 1733m, 1701m, 1531w, 1371m, 1245s, 1173s, 1018w, 979m, 882w, 609m, 504w \text{ cm}^{-1}$ ;  $^1\text{H NMR}$  (500 MHz,  $\text{DMSO}-d_6$ ):  $\delta = 8.30$  (s, 2H,  $\text{NH}_2$ ), 4.69 (s, 1H, 29- $\text{H}_a$ ), 4.55 (s, 1H, 29- $\text{H}_b$ ), 4.36 (dd,  $J = 11.4, 4.7$  Hz, 1H, 3-H), 4.23 (d,  $J = 10.9$  Hz, 1H, 28- $\text{H}_a$ ), 3.78 (d,  $J = 11.1$  Hz, 1H, 28- $\text{H}_b$ ), 2.87–2.78 (m, 2H, 35-H), 2.76–2.65 (m, 2H, 34-H),



2.43 (s, 1H, 19-H), 1.99 (s, 3H, 32-H), 1.85 (m, 1H, 21-H<sub>a</sub>), 1.63 (s, 3H, 30-H), 1.76–1.43 (m, 9H, 16-H<sub>a</sub>, 22-H<sub>a</sub>, 12-H<sub>a</sub>, 13-H, 1-H<sub>a</sub>, 9-H, 15-H<sub>a</sub>, 2-H), 1.42–1.12 (m, 9H, 6-H, 11-H<sub>a</sub>, 21-H<sub>b</sub>, 7-H, 18-H, 16-H<sub>b</sub>, 11-H<sub>b</sub>), 0.95 (s, 3H, 23-H), 0.93 (s, 3H, 27-H), 1.09–0.73 (m, 5H, 22-H<sub>b</sub>, 12-H<sub>b</sub>, 1H<sub>b</sub>, 15-H<sub>b</sub>, 5-H), 0.79 (s, 9H, 24-H, 25-H, 26-H) ppm; <sup>13</sup>C NMR (126 MHz, DMSO-*d*<sub>6</sub>): δ = 171.9 (C-31), 171.3 (C-33), 170.1 (C-36), 164.3 (C-38), 161.0 (C-37), 149.8 (C-20), 110.0 (C-29), 79.9 (C-3), 61.9 (C-28), 54.6 (C-5), 49.5 (C-18), 48.1 (C-9), 47.0 (C-19), 46.0 (C-17), 42.2 (C-14), 40.4 (C-8), 37.4 (C-4), 37.0 (C-13), 36.6 (10), 34.2, 34.0 (22), 33.5 (7), 30.9 (34), 30.0 (35), 29.1 (16), 28.9, 28.5 (21), 27.6 (24), 26.6 (C-15), (C-12), 23.4 (C-2), 21.0 (C-32), 20.3 (C-11), 18.7 (C-30), 17.7 (C-6), 16.4 (C-25), 15.8 (C-26), 15.5 (C-23), 14.5 (C-27) ppm; MS (ESI, MeOH): *m/z* = 745.7 (100%, [M-H]<sup>−</sup>); analysis calcd for C<sub>38</sub>H<sub>58</sub>N<sub>4</sub>O<sub>7</sub>S<sub>2</sub> (747.03): C 61.10, H 7.83, N 7.50; found: C 60.85, H 8.03, N 7.33.

#### 4.6. (3β) Olean-12-en-3,28-diol (Erythrodiol)

To a solution of OA (5.0 g, 10.7 mmol, 1.00 eq.) in dry THF (150 mL), LiAlH<sub>4</sub> (2.0 g, 53.6 mmol, 5.00 eq.) was slowly added. Stirring under reflux was continued for another 2 h. After cooling to 20 °C, the reaction was quenched (slow addition of 20 mL MeOH), and aq. HCl (6 M, 50 mL) was added. The reaction mixture was extracted with ethyl acetate (3 × 75 mL); the combined organic phases were washed with aq. NaOH (1 M, 2 × 50 mL), brine (50 mL) and dried (MgSO<sub>4</sub>). The solvent was removed under reduced pressure, and the residue was subjected to chromatography (silica gel, chloroform/hexanes/ethyl acetate, 10:8:2) to yield 7 (4.61 g, 97%) as a colorless solid; m.p. 218–219 °C (lit.: [93] 217–219 °C); R<sub>F</sub> = 0.17 (silica gel, chloroform/hexanes/ethyl acetate, 10:8:2); [α]<sub>D</sub> = +72.1° (*c* = 0.113, MeOH) (lit.: [94] [α]<sub>D</sub> = +75.0° (*c* = 0.325, CHCl<sub>3</sub>)).

#### 4.7. (3β) Urs-12-ene-3,28-diol (Uvaol, 8)

Following the procedure given for the synthesis of 7, from UA (5.00 g, 10.7 mmol), dry THF (150 mL) and LiAlH<sub>4</sub> (2.0 g, 53.6 mmol) followed by chromatography (silica gel, (chloroform/hexanes/ethyl acetate, 10:8:2) 8 (4.36 g, 90%) was obtained as a colorless solid; m.p. 227–229 °C (lit.: [93] 225–227 °C); R<sub>F</sub> = 0.18 (silica gel, chloroform/hexanes/ethyl acetate, 10:8:2); [α]<sub>D</sub> = +60.5° (*c* = 0.109, MeOH); (lit.: [95] [α]<sub>D</sub> = +62.6° (*c* = 0.62, CHCl<sub>3</sub>)).

#### 4.8. (3β)-Olean-12-ene-3,28-diyl Diacetate

Acetylation of 7 (4.00 g, 9.04 mmol) in dry pyridine (16 mL) with acetic anhydride (2.6 mL, 27.1 mmol) for 15 h at 20 °C followed by usual aqueous work up and chromatography (silica gel, (hexanes/ethyl acetate, 9:1) gave 9 (4.37 g, 92%) as a colorless solid; m.p. 184–186 °C (lit.: [96] 184–186 °C); R<sub>F</sub> = 0.43 (silica gel, hexanes/ethyl acetate, 9:1); [α]<sub>D</sub> = +62.2° (*c* = 0.124, CHCl<sub>3</sub>); (lit.: [97] [α]<sub>D</sub> = +56.0° (*c* = 1.0, CHCl<sub>3</sub>)).

#### 4.9. (3β)-Urs-12-ene-3,28-diyl Diacetate

Acetylation of 8 (3.48 g, 7.86 mmol) as described above for the synthesis of 9 gave 10 (3.66 g, 88%) as a colorless solid; m.p. 151–153 °C (lit.: [98] 150–151 °C); R<sub>F</sub> = 0.41 (silica gel, hexanes/ethyl acetate, 9:1); [α]<sub>D</sub> = +51.4° (*c* = 0.115, CHCl<sub>3</sub>).

#### 4.10. (3β)-28-Hydroxyolean-12-en-3-yl Acetate

A solution of 9 (3.1 g, 5.89 mmol) and aluminum isopropoxide (12.3 g, 58.8 mmol) in isopropanol (150 mL) was heated under reflux for 4 h. Usual aq. work-up followed by chromatography (silica gel, hexanes/ethyl acetate, 8:2) gave 11 (1.71 g, 60%) as a colorless solid; m.p. 234–236 °C (lit.: [99] 233–234 °C); R<sub>F</sub> = 0.55 (silica gel, hexanes/ethyl acetate, 8:2); [α]<sub>D</sub> = +67.4° (*c* = 0.122, CHCl<sub>3</sub>) (lit.: [100] [α]<sub>D</sub> = +71° (*c* = 0.70, CHCl<sub>3</sub>)).

#### 4.11. (3β)-28-Hydroxyolean-12-en-3-yl Acetate

As described above for the synthesis of 11, from 10 (2.7 g, 5.13 mmol) and aluminum propoxide (10.7 g, 51.3 mmol) in isopropanol (150 mL) followed by chromatography (silica gel, hexanes/ethyl acetate, 8:2) 12 (1.92 g, 77%) was obtained as a colorless solid;

m.p. 265–267 °C (lit.: [101] 258–261 °C);  $R_F = 0.51$  (silica gel, hexanes/ethyl acetate, 8:2);  $[\alpha]_D = +63.1^\circ$  ( $c = 0.139$ ,  $\text{CHCl}_3$ ) (lit.: [102]  $[\alpha]_D = +70.5^\circ$  ( $c = 0.145$ ,  $\text{CHCl}_3$ )).

#### 4.12. 4-[(3 $\beta$ )-3-(Acetyloxy)-olean-12-en-28-yl]oxy-4-oxobutanoic Acid

To a solution of 11 (0.45 g, 0.928 mmol) in dry pyridine (15 mL) succinic anhydride (0.188 g, 1.86 mmol) and cat. DMAP were added, and the mixture was stirred for 1 day under reflux. Usual aq. work-up followed by chromatography (silica gel, hexanes/ethyl acetate (1% HCOOH), 8:2  $\rightarrow$  7:3) gave 13 (0.460 g, 85%) as a colorless solid; m.p. 124–126 °C;  $R_F = 0.47$  (silica gel, hexanes/ethyl acetate, 7:3);  $[\alpha]_D = +49.6^\circ$  ( $c = 0.126$ ,  $\text{CHCl}_3$ ); IR (ATR):  $\nu = 2946m, 2864w, 1734s, 1712s, 1463w, 1432w, 1387m, 1364m, 1244s, 1160s, 1095w, 1027m, 1004m, 986m, 967m \text{ cm}^{-1}$ ;  $^1\text{H NMR}$  (500 MHz,  $\text{CDCl}_3$ ):  $\delta = 8.01$  (brs, 1H, COO-H), 5.19 (t,  $J = 3.6$  Hz, 1H, 12-H), 4.55–4.44 (m, 1H, 3-H), 4.07 (d,  $J = 11.0$  Hz, 1H, 28-H<sub>a</sub>), 3.72 (d,  $J = 11.0$  Hz, 1H, 28-H<sub>b</sub>), 2.71–2.59 (m, 4H, 34-H + 35-H), 2.04 (s, 3H, 32-H), 2.01–1.99 (m, 1H, 18-H), 1.97–1.78 (m, 3H, 16-H<sub>a</sub> + 11-H), 1.77–1.46 (m, 9H, 19-H<sub>a</sub> + 15-H<sub>a</sub> + 2-H + 1-H<sub>a</sub> + 9-H + 6-H<sub>a</sub> + 7-H<sub>a</sub> + 22-H<sub>a</sub>), 1.45–1.21 (m, 4H, 6-H<sub>b</sub> + 22-H<sub>b</sub> + 7-H<sub>b</sub> + 21-H<sub>a</sub>), 1.19–1.10 (m, 5H, 16-H<sub>b</sub> + 21-H<sub>b</sub> + 27-H), 1.12–0.95 (m, 3H, 19-H<sub>b</sub> + 1-H<sub>b</sub> + 15-H<sub>b</sub>), 0.94 (s, 3H, 25-H), 0.93 (s, 3H, 26-H), 0.88 (s, 3H, 30-H), 0.86 (s, 6H, 29-H + 24-H), 0.85 (s, 3H, 23-H), 0.84–0.80 (m, 1H, 5-H) ppm;  $^{13}\text{C NMR}$  (126 MHz,  $\text{CDCl}_3$ ):  $\delta = 177.9$  (C-36), 172.2 (C-33), 171.3 (C-31), 143.7 (C-13), 123.0 (C-12), 81.1 (C-3), 71.3 (C-28), 55.4 (C-5), 47.6 (C-9), 46.3 (C-19), 42.7 (C-18), 41.8 (C-14), 39.9 (C-8), 38.4 (C-1), 37.8 (C-4), 36.9 (C-10), 36.0 (C-17), 34.1 (C-21), 33.3 (C-30), 32.6 (C-7), 31.6 (C-22), 31.0 (C-20), 29.1 (C-34), 29.1 (C-35), 28.2 (C-24), 26.1 (C-27), 25.7 (C-15), 23.7 (C-29), 23.7 (C-11), 23.7 (C-2), 22.3 (C-16), 21.4 (C-32), 18.4 (C-6), 16.8 (C-26), 16.8 (C-23), 15.7 (C-25) ppm; MS (ESI, MeOH/ $\text{CHCl}_3$ , 4:1):  $m/z = 583.9$  (100%,  $[\text{M}-\text{H}]^-$ ); analysis calcd for  $\text{C}_{36}\text{H}_{56}\text{O}_6$  (584.84): C 73.93, H 9.65; found: C 73.71, H 9.86.

#### 4.13. 4-[(3 $\beta$ )-3-(Acetyloxy)urs-12-en-28-yl]oxy-4-oxobutanoic Acid

Following the procedure given for 11, from 12 (1.35 g, 2.79 mmol), 14 (1.12 g, 69%) was obtained as a colorless solid; m.p. 112–114 °C;  $R_F = 0.50$  (silica gel, hexanes/ethyl acetate, 7:3);  $[\alpha]_D = +42.7^\circ$  ( $c = 0.131$ ,  $\text{CHCl}_3$ ); IR (ATR):  $\nu = 2948m, 2925m, 1734s, 1712s, 1456m, 1432w, 1388m, 1370m, 1269m, 1244s, 1158s, 1095w, 1025m, 1006m, 985m, 967m \text{ cm}^{-1}$ ;  $^1\text{H NMR}$  (500 MHz,  $\text{CDCl}_3$ ):  $\delta = 8.01$  (brs, 1H, COO-H), 5.13 (dd,  $J = 3.7$  Hz, 1H, 12-H), 4.53–4.44 (m, 1H, 3-H), 4.10 (d,  $J = 11.0$  Hz, 1H, 28-H<sub>a</sub>), 3.64 (d,  $J = 11.0$  Hz, 1H, 28-H<sub>b</sub>), 2.70–2.60 (m, 4H, 34-H + 35-H), 2.04 (s, 3H, 32-H), 1.98–1.88 (m, 2H, 16-H<sub>a</sub> + 11-H), 1.76–1.68 (m, 1H, 15-H<sub>a</sub>), 1.67–1.59 (m, 2H, 1-H<sub>a</sub> + 2-H), 1.59–1.48 (m, 4H, 22-H<sub>a</sub> + 7-H<sub>a</sub> + 9-H + 6-H<sub>a</sub>), 1.47–1.28 (m, 6H, 21-H<sub>a</sub> + 18-H + 6-H<sub>b</sub> + 19-H + 7-H<sub>b</sub> + 22-H<sub>b</sub>), 1.27–1.13 (m, 2H, 21-H<sub>b</sub> + 16-H<sub>b</sub>), 1.08 (s, 3H, 27-H), 1.08–1.06 (m, 1H, 1-H<sub>b</sub>), 0.99–0.96 (m, 1H, 15-H<sub>b</sub>), 0.97 (s, 3H, 26-H), 0.96 (s, 3H, 25-H), 0.93 (d,  $J = 5.8$  Hz, 3H, 29-H), 0.91–0.88 (m, 1H, 20-H), 0.86 (s, 3H, 24-H), 0.86 (s, 3H, 23-H), 0.85–0.83 (m, 1H, 5-H), 0.80 (d,  $J = 5.3$  Hz, 3H, 30-H) ppm;  $^{13}\text{C NMR}$  (126 MHz,  $\text{CDCl}_3$ ):  $\delta = 178.0$  (C-36), 172.2 (C-33), 171.3 (C-31), 138.3 (C-13), 125.7 (C-12), 81.1 (C-3), 71.8 (C-28), 55.4 (C-5), 54.4 (C-18), 47.7 (C-9), 42.1 (C-14), 40.1 (C-8), 39.5 (C-20), 39.3 (C-19), 38.6 (C-1), 37.8 (C-17), 37.1 (C-4), 36.9 (C-10), 35.8 (C-22), 32.8 (C-7), 30.6 (C-21), 29.2 (C-34), 29.2 (C-35), 28.2 (C-24), 26.1 (C-15), 23.7 (C-2), 23.5 (C-11), 23.5 (C-16), 23.5 (C-27), 21.4 (C-32), 21.4 (C-29), 18.3 (C-6), 17.4 (C-30), 16.9 (C-26), 16.8 (C-23), 15.9 (C-25) ppm; MS (ESI, MeOH/ $\text{CHCl}_3$ , 4:1):  $m/z = 607.9$  (100%,  $[\text{M} + \text{Na}]^+$ ); analysis calcd for  $\text{C}_{36}\text{H}_{56}\text{O}_6$  (584.84): C 73.93, H 9.65; found: C 73.68, H 9.91.

#### 4.14. (3 $\beta$ , 20 $\beta$ )-3-Acetyloxy-11-oxoolean-12-en-29-oic Acid

Acetylation of GA (2.50 g, 5.31 mmol) as described above followed by chromatography (silica gel, hexanes/ethyl acetate, 8:2) gave 15 (2.15 g, 79%) as a colorless solid; m.p. 305–307 °C (lit.: [96] 316–318 °C);  $R_F = 0.41$  (silica gel, hexanes/ethyl acetate, 9:1);  $[\alpha]_D = +163.3^\circ$  ( $c = 0.142$ ,  $\text{CHCl}_3$ ); (lit.: [103]  $[\alpha]_D = +165.1^\circ$  ( $c = 0.7$ ,  $\text{CHCl}_3$ )).

#### 4.15. (3 $\beta$ , 20 $\beta$ ) 3-Acetyloxy-29-hydroxyolean-12-en-11-one

To a solution of 15 (1.3 g, 2.76 mmol) and triethylamine (1.1 mL, 7.60 mmol) in dry THF (15 mL), at  $-12\text{ }^{\circ}\text{C}$ , ethyl chloroformate (1.1 mL, 11.1 mmol) was added, and the mixture was stirred for 15 min. The precipitate was filtered off, and the filtrate was slowly added to a freshly prepared solution of sodium borohydride (0.522 g, 13.8 mol) in water (2.5 mL). Stirring at room temperature was continued for another 15 min followed by usual aq. work-up and chromatography (silica gel,  $\text{CHCl}_3/\text{Et}_2\text{O}/\text{hexanes}/\text{HCOOH}$ , 25:25:43:7) to yield 16 (1.09 g, 82%) as a colorless solid; m.p.  $264\text{--}266\text{ }^{\circ}\text{C}$ ;  $R_F = 0.46$  (silica gel,  $\text{CHCl}_3/\text{Et}_2\text{O}/\text{hexanes}/\text{HCOOH}$ , 25:25:43:7);  $[\alpha]_D = +91.6^{\circ}$  ( $c = 0.129$ ,  $\text{CHCl}_3$ ); UV-Vis ( $\text{CHCl}_3$ ):  $\lambda_{\text{max}}$  ( $\log \epsilon$ ) = 249 nm (4.07); IR (ATR):  $\nu = 3569w, 3550w, 2925m, 2862w, 1725s, 1695m, 1651s, 1626m, 1465w, 1455m, 1386m, 1366m, 1325w, 1279w, 1246s, 1209m, 1173s, 1143m, 1095w, 1048m, 1025s, 1001m, 985m\text{ cm}^{-1}$ ;  $^1\text{H NMR}$  (500 MHz,  $\text{CDCl}_3$ ):  $\delta = 5.58$  (s, 1H, 12-H), 4.50 (dd,  $J = 11.8, 4.7$  Hz, 1H, 3-H), 4.13 (d,  $J = 11.0$  Hz, 1H, 30-H<sub>a</sub>), 4.03 (d,  $J = 11.0$  Hz, 1H, 30-H<sub>b</sub> (30)), 2.78 (dt,  $J = 13.6, 3.6$  Hz, 1H, 1-H<sub>a</sub>), 2.35 (s, 1H, 9-H), 2.13–2.06 (m, 2H, 18-H + 16-H<sub>a</sub>), 2.04 (s, 3H, 32-H), 1.86–1.76 (m, 1H, 15-H<sub>a</sub>), 1.74–1.53 (m, 7H, 2-H<sub>a</sub> + 22-H<sub>a</sub> + 7-H<sub>a</sub> + 19-H<sub>a</sub> + 2-H<sub>b</sub> + 21-H<sub>a</sub> + 6-H<sub>a</sub>) 1.50–1.36 (m, 3H, 6-H<sub>b</sub> + 7-H<sub>b</sub> + 22-H<sub>b</sub> + 19-H<sub>b</sub>), 1.36 (s, 3H, 27-H), 1.21–1.16 (m, 1H, 15-H<sub>b</sub>), 1.15 (s, 3H, 25-H), 1.12 (s, 3H, 26-H), 1.07–0.98 (m, 2H, 1-H<sub>b</sub> + 16-H<sub>b</sub>), 0.95 (s, 3H, 29-H), 0.87 (s, 6H, 28-H + 23-H), 0.86 (s, 3H, 24-H), 0.81–0.77 (m, 1H, 5-H), 0.80 (d,  $J = 5.7$  Hz, 3H, 30-H) ppm;  $^{13}\text{C NMR}$  (126 MHz,  $\text{CDCl}_3$ ):  $\delta = 200.4$  (C-11), 171.4 (C-31), 169.8 (C-13), 128.5 (C-12), 80.9 (C-3), 67.0 (C-30), 61.8 (C-9), 55.2 (C-5), 47.0 (C-18), 45.6 (C-8), 43.5 (C-14), 40.2 (C-19), 38.9 (C-1), 38.2 (C-4), 37.1 (C-10), 36.0 (C-22), 34.3 (C-20), 32.8 (C-7), 32.4 (C-17), 30.2 (C-21), 28.6 (C-28), 28.2 (C-24), 27.9 (C-29), 26.7 (C-16), 26.5 (C-15), 23.7 (C-2), 23.5 (C-27), 21.4 (C-32), 18.8 (C-26), 17.5 (C-6), 16.8 (C-23), 16.5 (C-25) ppm; MS (ESI,  $\text{MeOH}/\text{CHCl}_3$ , 4:1):  $m/z = 497.9$  (100%,  $[\text{M}-\text{H}]^-$ ); analysis calcd for  $\text{C}_{32}\text{H}_{50}\text{O}_4$  (498.74): C 77.06, H 10.10; found: C 76.81, H 10.35.

#### 4.16. 4-[(3 $\beta$ , 20 $\beta$ )3-(Acetyloxy)-11-oxoolean-12-en-30-yl]oxy]-4-oxobutanoic Acid

Following the procedure described above, from 16 (0.270 g, 0.541 mmol) 17 (0.315 g, 97%) was obtained as a colorless solid; m.p.  $109\text{--}111\text{ }^{\circ}\text{C}$ ;  $R_F = 0.44$  (silica gel, hexanes/ethyl acetate, 1:1);  $[\alpha]_D = +110.5^{\circ}$  ( $c = 0.118$ ,  $\text{CHCl}_3$ ); UV-Vis ( $\text{CHCl}_3$ ):  $\lambda_{\text{max}}$  ( $\log \epsilon$ ) = 254 nm (4.05); IR (ATR):  $\nu = 2949m, 2928m, 2871w, 1730s, 1657m, 1465w, 1456w, 1388m, 1365m, 1322w, 1244s, 1209m, 1158m, 1091w, 1049w, 1028m, 1001m, 986\text{ m cm}^{-1}$ ;  $^1\text{H NMR}$  (400 MHz,  $\text{CDCl}_3$ ):  $\delta = 8.01$  (brs, 1H, COO-H), 5.64 (s, 1H, 12-H), 4.70 (d,  $J = 10.9$  Hz, 1H, 30-H<sub>a</sub>), 4.51 (dd,  $J = 11.5, 4.9$  Hz, 1H, 3-H), 3.46 (d,  $J = 10.9$  Hz, 1H, 30-H<sub>b</sub>), 2.85 (dt,  $J = 13.6, 3.5$  Hz, 1H, 1-H<sub>a</sub>), 2.79–2.71 (m, 2H, 34-H), 2.58–2.49 (m, 2H, 35-H), 2.37 (s, 1H, 9-H), 2.36–2.25 (m, 1H, 18-H), 2.12–2.06 (m, 1H, 16-H<sub>a</sub>), 2.04 (s, 3H, 32-H), 1.87–1.75 (m, 1H, 15-H<sub>a</sub>), 1.74–1.36 (m, 9H, 2-H + 7-H<sub>a</sub> + 6-H<sub>a</sub> + 19-H<sub>a</sub> + 6-H<sub>b</sub> + 22-H<sub>a</sub> + 7-H<sub>b</sub> + 21-H<sub>a</sub>), 1.35 (s, 3H, 27-H), 1.33–1.14 (m, 4H, 21-H<sub>b</sub> + 22-H<sub>b</sub> + 19-H<sub>b</sub> + 16-H<sub>b</sub>), 1.12 (s, 6H, 25-H + 26-H), 1.10–0.98 (m, 2H, 1-H<sub>b</sub> + 15-H<sub>b</sub>), 0.95 (s, 3H, 29-H), 0.87 (s, 9H, 28-H + 23-H + 24-H), 0.83–0.77 (m, 1H, 5-H) ppm;  $^{13}\text{C NMR}$  (101 MHz,  $\text{CDCl}_3$ ):  $\delta = 202.2$  (C-11), 174.8 (C-36), 172.9 (C-33), 171.8 (C-13), 171.1 (C-31), 128.1 (C-12), 80.7 (C-3), 67.7 (C-30), 61.9 (C-9), 55.2 (C-5), 46.7 (C-18), 45.5 (C-8), 43.4 (C-14), 39.2 (C-19), 39.0 (C-1), 38.3 (C-4), 37.4 (C-10), 36.1 (C-22), 34.9 (C-20), 32.7 (C-7), 32.3 (C-17), 31.3 (C-21), 29.5 (C-35), 28.9 (C-34), 28.9 (C-28), 28.2 (C-24), 28.2 (C-29), 26.6 (C-16), 26.6 (C-15), 23.7 (C-2), 23.3 (C-27), 21.4 (C-32), 19.0 (C-26), 17.5 (C-6), 16.8 (C-23), 16.7 (C-25) ppm; MS (ESI,  $\text{MeOH}/\text{CHCl}_3$ , 4:1)  $m/z = 600.0$  (96%,  $[\text{M} + \text{H}]^+$ ); analysis calcd for  $\text{C}_{36}\text{H}_{54}\text{O}_7$  (598.82): C 72.21, H 9.09; found: C 71.97, H 9.32.

#### 4.17. (3 $\beta$ ) 3-(Acetyloxy)olean-12-en-28-yl-4-[[5-(aminosulfonyl)-1,3,4-thiadiazol-2-yl]amino]-4-oxobutanoate

To a solution of 13 (0.250 g, 0.427 mmol) in dry THF (20 mL) at  $-15\text{ }^{\circ}\text{C}$ , 4-methylmorpholine (0.1 mL, 0.641 mmol) and ethyl chloroformate (0.05 mL, 0.513 mmol) were added, and the mixture was stirred for 10 min at this temperature. Then 5-amino-1,3,4-thiadiazole-2-sulfonamide 5 (0.092 g, 0.513 mmol) was added, and the mixture was heated under reflux for 4 h. The solvents were removed under diminished pressure, and the residue was

subjected to chromatography (silica gel, (CHCl<sub>3</sub>/MeOH, 95:5) to yield 18 (0.166 g, 52%) as a colorless solid; m.p. 198–200 °C; R<sub>F</sub> = 0.36 (silica gel, CHCl<sub>3</sub>/MeOH, 9:1); [α]<sub>D</sub> = +19.2° (c = 0.122, CHCl<sub>3</sub>); UV-Vis (CHCl<sub>3</sub>): λ<sub>max</sub> (log ε) = 262 nm (4.45); IR (ATR): ν = 3332w, 3234w, 2947m, 2864m, 1734m, 1704s, 1545m, 1536m, 1463w, 1432w, 1422w, 1371s, 1329m, 1245s, 1216m, 1173s, 1092m, 1027m, 1004m, 985m, 967m, 755m, 651m, 637m, 606 s, 504m cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>): δ = 13.07 (*brs*, 1H, CON-H), 8.30 (d, *J* = 6.2 Hz, 2H, SN-H), 5.15 (t, *J* = 3.7 Hz, 1H, 12-H), 4.38 (dd, *J* = 11.7, 4.5 Hz, 1H, 3-H), 3.96 (d, *J* = 10.9 Hz, 1H, 28-H<sub>a</sub>), 3.60 (d, *J* = 10.9 Hz, 1H, 28-H<sub>b</sub>), 2.89–2.76 (m, 2H, 34-H), 2.72–2.65 (m, 2H, 35-H), 2.02–1.97 (m, 1H, 18-H), 1.99 (s, 3H, 32-H), 1.86 (*td*, *J* = 13.9, 4.6 Hz, 1H, 16-H<sub>a</sub>), 1.82–1.75 (m, 2H, 11-H), 1.70 (dd, *J* = 13.4, 13.4 Hz, 1H, 19-H<sub>a</sub>), 1.62–1.13 (m, 11H, 15-H<sub>a</sub> + 2-H + 1-H<sub>a</sub> + 9-H + 6-H<sub>a</sub> + 21-H<sub>a</sub> + 22-H<sub>a</sub>, 6-H<sub>b</sub> + 22-H<sub>b</sub> + 7-H<sub>b</sub>), 1.11 (s, 3H, 27-H), 1.09–0.93 (m, 4H, 16-H<sub>b</sub> + 7-H<sub>b</sub> + 19-H<sub>b</sub> + 1-H<sub>b</sub>), 0.88 (s, 3H, 25-H), 0.85 (d, 3H, 30-H), 0.84 (d, 3H, 29-H), 0.83 (s, 3H, 24-H), 0.83–0.80 (m, 7H, 26-H + 23-H + 5-H); ppm; <sup>13</sup>C NMR (126 MHz, DMSO-*d*<sub>6</sub>): δ = 171.6 (C-33), 171.2 (C-36), 170.1 (C-31), 164.3 (C-38), 161.0 (C-37), 143.4 (C-13), 122.2 (C-12), 79.9 (C-3), 70.0 (C-28), 54.5 (C-5), 46.7 (C-9), 45.7 (C-19), 41.8 (C-18), 41.1 (C-14), 39.2 (C-8), 37.7 (C-1), 37.3 (C-4), 36.3 (C-10), 35.4 (C-17), 33.4 (C-7), 32.9 (C-30), 31.8 (C-21), 31.0 (C-22), 30.5 (C-20), 30.0 (C-34), 28.4 (C-35), 27.7 (C-24), 25.7 (C-27), 25.1 (C-15), 23.4 (C-29), 23.2 (C-2), 23.0 (C-11), 21.5 (C-16), 20.9 (C-32), 17.7 (C-6), 16.6 (C-26), 16.2 (C-23), 15.2 (C-25) ppm; MS (ESI, MeOH/CHCl<sub>3</sub>, 4:1): *m/z* = 770.1 (100%, [M + Na]<sup>+</sup>); analysis calcd for C<sub>38</sub>H<sub>58</sub>S<sub>2</sub>N<sub>4</sub> (747.02): C 61.10, H 7.83, N 7.50; found: C 60.81, H 8.03, N 7.39.

4.18. (3β)-3-(Acetyloxy)urs-12-en-28-yl-4-[[5-(aminosulfonyl)-1,3,4-thiadiazol-2-yl]amino] 4-oxobutanoate

Following the procedure given above for the synthesis of 18, from 14 (0.250 g, 0.427 mmol) 21 (0.217 g, 68%) was obtained as a colorless solid; m.p. 190–192 °C; R<sub>F</sub> = 0.33 (silica gel, CHCl<sub>3</sub>/MeOH, 9:1); [α]<sub>D</sub> = +27.4° (c = 0.129, CHCl<sub>3</sub>); UV-Vis (CHCl<sub>3</sub>): λ<sub>max</sub> (log ε) = 263 nm (4.98); IR (ATR): ν = 3244w, 2948m, 2925m, 2871m, 1733m, 1705s, 1531m, 1457w, 1431w; 1414w, 1367s, 1245s, 1174s, 1094m, 1027m, 1006m, 985m, 967m, 655m, 603s, 508m cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>): δ = 13.07 (*brs*, 1H, CON-H), 8.30 (s, *J* = 2H, SN-H), 5.10 (t, *J* = 3.2 Hz, 1H, H-12), 4.38 (dd, *J* = 11.5, 4.7 Hz, 1H, H-3), 3.94 (d, *J* = 10.8 Hz, 1H, 28-H<sub>a</sub>), 3.54 (d, *J* = 10.9 Hz, 1H, 28-H<sub>b</sub>), 2.84–2.78 (m, 2H, 34-H), 2.71–2.65 (m, 2H, 35-H), 1.99 (s, 3H, 32-H), 1.92–1.80 (m, 3H, 16-H<sub>a</sub> + 11-H), 1.65–1.53 (m, 3H, 15-H<sub>a</sub> + 2-H<sub>a</sub> + 1-H<sub>a</sub>), 1.53–1.42 (m, 5H, 2-H<sub>a</sub> + 9-H + 7-H<sub>a</sub> + 6-H<sub>a</sub> + 22-H<sub>a</sub>), 1.40–1.06 (m, 7H, 18-H + 6-H<sub>b</sub> + 21-H<sub>a</sub> + 22-H<sub>b</sub> + 7-H<sub>b</sub> + 21-H<sub>b</sub> + 16-H<sub>b</sub>), 1.04 (s, 3H, 27-H), 1.01–0.96 (m, 1H, 1-H<sub>b</sub>), 0.90 (s, 3H, 23-H), 0.87 (d, *J* = 6.7, 3H, 29-H), 0.86 (s, 3H, 26-H), 0.85–0.83 (m, 2H, 15-H<sub>b</sub> + 1-H<sub>b</sub>), 0.83 (s, 6H, 24-H + 25-H), 0.83–0.80 (m, 1H, 20-H), 0.87 (d, *J* = 5.3 Hz, 3H, 30-H) ppm; <sup>13</sup>C NMR (126 MHz, DMSO-*d*<sub>6</sub>): δ = 171.4 (C-33), 171.2 (C-36), 170.1 (C-31), 164.3 (C-38), 161.0 (C-37), 138.0 (C-13), 124.8 (C-12), 79.9 (C-3), 70.4 (C-28), 54.5 (C-5), 53.4 (C-18), 46.8 (C-9), 41.4 (C-14), 39.4 (C-8), 38.7 (C-20), 38.5 (C-19), 37.9 (C-1), 37.3 (C-4), 36.4 (C-17), 36.3 (C-10), 35.1 (C-2), 32.1 (C-7), 30.0 (C-34), 29.9 (C-21), 28.4 (C-35), 27.7 (C-24), 25.5 (C-15), 23.3 (C-2), 23.0 (C-27), 22.9 (C-11), 22.8 (C-16), 21.0 (C-29), 20.9 (C-32), 17.1 (C-6), 17.1 (C-30), 16.6 (C-23), 16.3 (C-26), 15.2 (C-25) ppm; MS (ESI, MeOH/CHCl<sub>3</sub>, 4:1): *m/z* = 770.1 (100%, [M + Na]<sup>+</sup>); analysis calcd for C<sub>38</sub>H<sub>58</sub>S<sub>2</sub>N<sub>4</sub>O<sub>7</sub> (747.02): C 61.10, H 7.83, N 7.50; found: C 60.89, H 8.07, N 7.36.

4.19. (3β, 20β) 3-(Acetyloxy)-11-oxoolean-12-en-30-yl-4-[[5-(aminosulfonyl)-1,3,4-thiadiazol-2-yl]amino]-4-oxobutanoate

Following the procedure given above for the synthesis of 18, from 17 (0.250 g, 0.417 mmol) 20 (0.215 g, 68%) was obtained as a colorless solid; m.p. 179–181 °C; R<sub>F</sub> = 0.41 (silica gel, CHCl<sub>3</sub>/MeOH, 9:1); [α]<sub>D</sub> = +83.5° (c = 0.114, CHCl<sub>3</sub>); UV-Vis (CHCl<sub>3</sub>): λ<sub>max</sub> (log ε) = 256 nm (4.25); IR (ATR): ν = 3252w, 2950m, 2872w, 1727m, 1709m, 1643m, 1528m, 1466w, 1455w, 1365s, 1323m, 1247s, 1215m, 1173s, 1088w, 1049w, 1028m, 1001m, 985m, 754s, 667m, 654m, 604s, 509m cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>): δ = 13.08 (*brs*, 1H, CON-H), 8.30 (s, *J* = 2H, SN-H), 5.48 (s, 1H, 12-H), 4.42 (dd, *J* = 11.8, 4.5 Hz, 1H, 3-H), 4.05 (d, *J* = 11.0 Hz, 1H, 30-H<sub>a</sub>), 3.92 (d, *J* = 11.0 Hz, 1H, 30-H<sub>b</sub>), 2.82 (dd, *J* = 7.5, 5.5 Hz 2H, 35-H),

2.71 (dd,  $J = 7.6, 5.5$  Hz, 2H, 34-H), 2.61 (dt,  $J = 13.4, 3.6$  Hz, 1H, 1-H<sub>a</sub>), 2.38 (s, 1H, 9-H), 2.16–2.04 (m, 2H, 18-H + 16-H<sub>a</sub>), 2.00 (s, 3H, 32-H), 1.79–1.35 (m, 7H, 16-H<sub>b</sub> + 7-H<sub>a</sub> + 19-H<sub>a</sub> + 2-H<sub>a</sub> + 6-H<sub>a</sub> + 2-H<sub>b</sub> + 6-H<sub>b</sub>). 1.34 (s, 3H, 27-H), 1.34–1.31 (m, 3H, 7-H<sub>b</sub> + 21-H<sub>b</sub> + 22-H<sub>b</sub>), 1.24–1.06 (m, 4H, 19-H<sub>b</sub> + 22-H<sub>b</sub> + 15-H<sub>b</sub> + 1-H<sub>b</sub>), 1.06 (s, 1H, 25-H) 1.04 (s, 1H, 26-H), 0.86–0.98 (m, 2H, 15-H<sub>b</sub> + 5-H), 0.85 (s, 3H, 29-H), 0.82 (s, 6H, 23-H + 24-H), 0.80 (s, 3H, 28-H) ppm; <sup>13</sup>C NMR (126 MHz, DMSO-*d*<sub>6</sub>):  $\delta = 198.9$  (C-11), 171.8 (C-36), 171.1 (C-33), 170.1 (C-31), 170.0 (C-13), 127.4 (C-12), 79.7 (C-3), 67.0 (C-30), 60.8 (C-9), 53.7 (C-5), 46.0 (C-18), 44.9 (C-8), 43.0 (C-14), 39.7 (C-19), 37.8 (C-1), 37.5 (C-4), 36.5 (C-10), 35.4 (C-22), 34.0 (C-20), 31.9 (C-7), 31.8 (C-17), 29.9 (C-35), 29.3 (C-21), 28.3 (C-34), 28.1 (C-28), 27.7 (C-24), 27.2 (C-29), 25.9 (C-16), 25.9 (C-15), 23.2 (C-2), 23.1 (C-27), 20.9 (C-32), 18.3 (C-26), 16.9 (C-6), 16.6 (C-23), 16.1 (C-25) ppm; MS (ESI, MeOH/CHCl<sub>3</sub>, 4:1):  $m/z = 784.0$  (100%, [M + Na]<sup>+</sup>); analysis calcd for C<sub>38</sub>H<sub>56</sub>S<sub>2</sub>N<sub>4</sub>O<sub>8</sub> (761.00): C 59.97, H 7.42, N 7.36; found: C 59.71, H 7.71, N 7.09.

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

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