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Glycyrrhetinic amides and their cytotoxicity

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

Keywords: Glycyrrhetinic acid; Amides; Cytotoxicity.

1. Introduction

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

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

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

2. Results and Discussion

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

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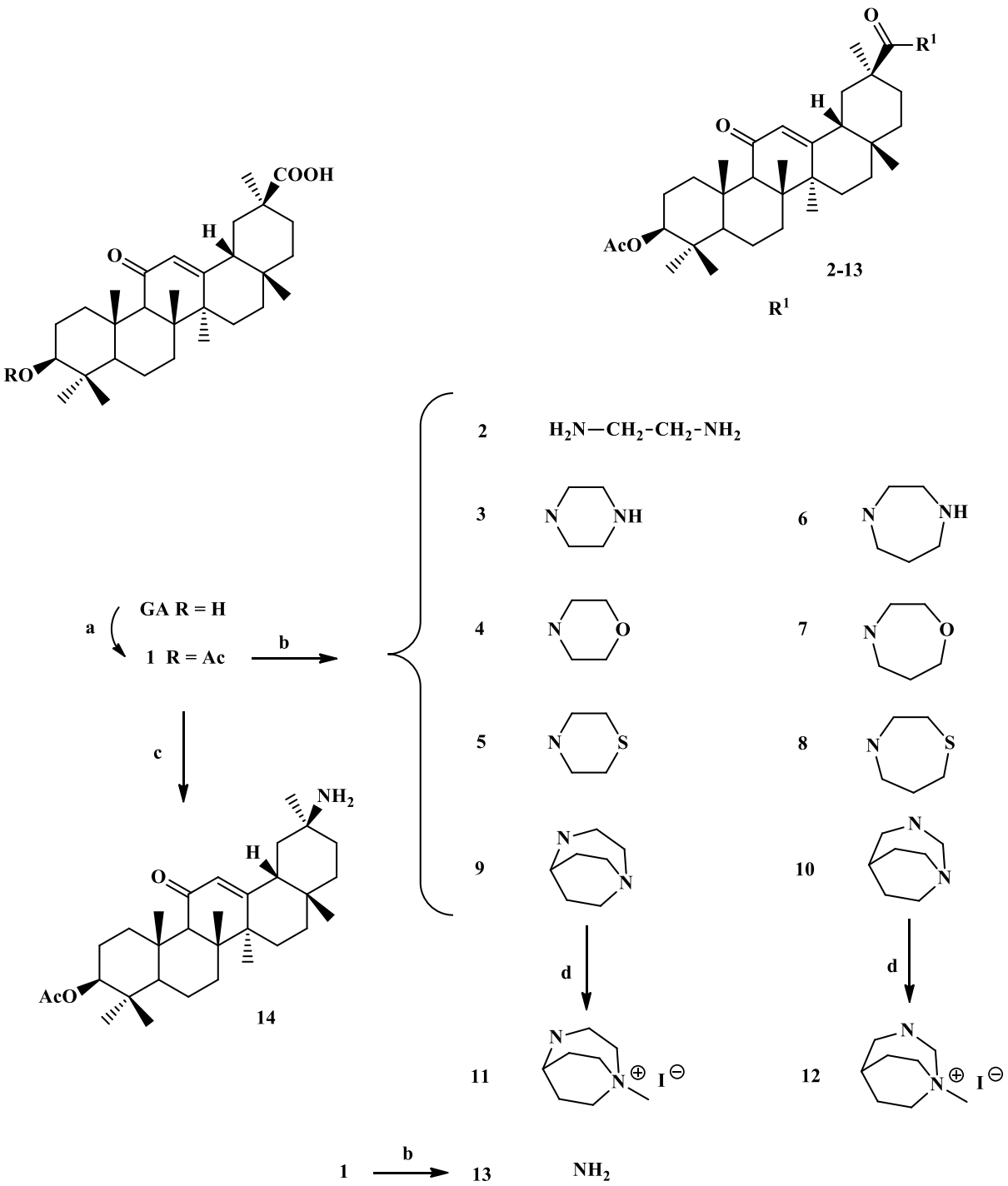

Scheme 1. Reactions and conditions: a) AcCl, NEt_3, DMAP (cat.), DCM, 23°C, 12 h, 91%; b) $(COCl)_2$, DMF (cat.), DCM then: amine, NEt_3, DMAP (cat), DCM, 23°C, 1 d: → **2** (71%), → **3** (64%), → **4** (61%), → **5** (88%), → **6** (67%), → **7** (86%), → **8** (69%),→ **9** (78%), → **10** (99%),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

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

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

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

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

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

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

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

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

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

3. Conclusion

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

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4. Experimental

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

4.1. Cell lines and culture conditions

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

4.2. Cytotoxicity assay (SRB assay)

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

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

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

4.4. Apoptosis test – trypan blue cell counting

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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