Bioorganic & Medicinal Chemistry 18 (2010) 2549–2558

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis and biological evaluation of antitumor-active γ-butyrolactone substituted betulin derivatives

René Csuk [a,*], Alexander Barthel [a], Stefan Schwarz [a], Harish Kommera [b], Reinhard Paschke [b]

[a] Bereich Organische Chemie, Martin-Luther-Universität Halle-Wittenberg, Kurt-Mothes-Str. 2, D-06120 Halle (Saale), Germany
[b] BioSolution GmbH, Weinbergweg 22, D-06120 Halle (Saale), Germany

ARTICLE INFO

Article history:
Received 17 September 2009
Revised 13 January 2010
Accepted 21 February 2010
Available online 2 March 2010

Keywords:
Betulin
Betulinic acid
γ-Butyrolactone
Apoptosis
Antitumor activity

ABSTRACT

The plant triterpenes betulin and betulinic acid (BA) are triterpenes featuring interesting pharmacological properties. Starting from substituted betulinic aldehydes, we used them as lead structures for the synthesis of several γ-butyrolactones and butenolides. Their antitumor activity was examined for 15 cancer cell lines using a SRB-assay and their apoptotic action was documented by trypan-blue test and DNA laddering. Several compounds revealed a higher activity than betulinic acid.

1. Introduction

Betulin **I** and betulinic acid **II** (Scheme 1) are naturally occurring lupan-type triterpenes displaying interesting pharmacological properties.[1] They are widespread in the plant kingdom and readily available from different plants, for example from the bark of white birch (*Betula alba*) (Fig. 1).

Initially, for betulinic acid and derivatives an anti-HIV activity in infected lymphocytic cells[2] was reported. However, in contrast to many other compounds, these compounds do not substantially interfere with HIV-1 proteases, reverse transcriptase or integrase. They rather have a unique way of action depending on the position of substituents. For a betulinic acid derivative holding an extra 3′,3′-dimethylsuccinyl-group at the 3-position, a change of the cell maturation was reported (by affecting the CA-SP1 junction in Gag processing); this formed intact virus cells with defective cores.[3,4] However, derivatives showing variations at the carboxylic group, for example RPR103611, were reported[5,6] to inhibit the virus-cell fusion at the interface between gp120 and gp41.

In athymic mice betulin derivatives acted[7] as potent melanoma specific cytotoxic agents leading to a tumor regression up to 80% at a dose of 50 mg/kg; interestingly enough, no toxic side effects were noted even at high concentrations up to 500 mg/kg. It was assumed that cell death is provoked by inducting apoptosis. Thus, betulinic acid is a versatile compound capable of several modes of action. In addition, it decreases the permeability of the mitochondrian transition pore complex,[8] it releases cytochrome c and apoptosis inducing factor (AIF) into the cytosol.[9] Adding permeability stabilizing agents, for example bongkrekic acid, impeded the release of cell proteins and apoptosis. Also, caspases were activated and the generation of the reactive oxygen species was demonstrated.[10] The phosphorylation of the proapoptotic mitogen-activated protein kinases (MAPKs) p38 and SAP/JNK was also observed, therefore suggesting at least a partial contribution of the p38 apoptosis pathway.[11] Betulinic acid is also an inhibitor of topoisomerase I; however, it only prevents the formation of DNA adducts and does not form apoptotic topoisomerase I cleavable complexes.[12] The unique action of betulin and betulinic acid derivatives for potential treatment of HIV infections and their high selectivity for cancer cells at nontoxic doses makes them interesting as targets for the synthesis of derivatives. Here, we present the synthesis of betulin derivatives bearing an extra γ-lactone moiety at C-28. We examined their antitumor potency for 15 different cancer cell lines by an SRB-assay.

2. Chemistry

For the synthesis of compounds holding a γ-lactone at C-28, the corresponding aldehydes are suitable precursors. Thus, the efficient synthesis of substituted betulinic aldehydes (Scheme 2) became a first objective. The betulinic aldehyde **1** was easily accessible by *Swern* oxidation from *O,O′*-bis-(trimethylsilyl)-betulin.[13] This procedure is preferred to procedures using toxic chromium(VI)-

* Corresponding author. Tel.: +49 345 55 25660; fax: +49 345 55 27030.
E-mail address: rene.csuk@chemie.uni-halle.de (R. Csuk).

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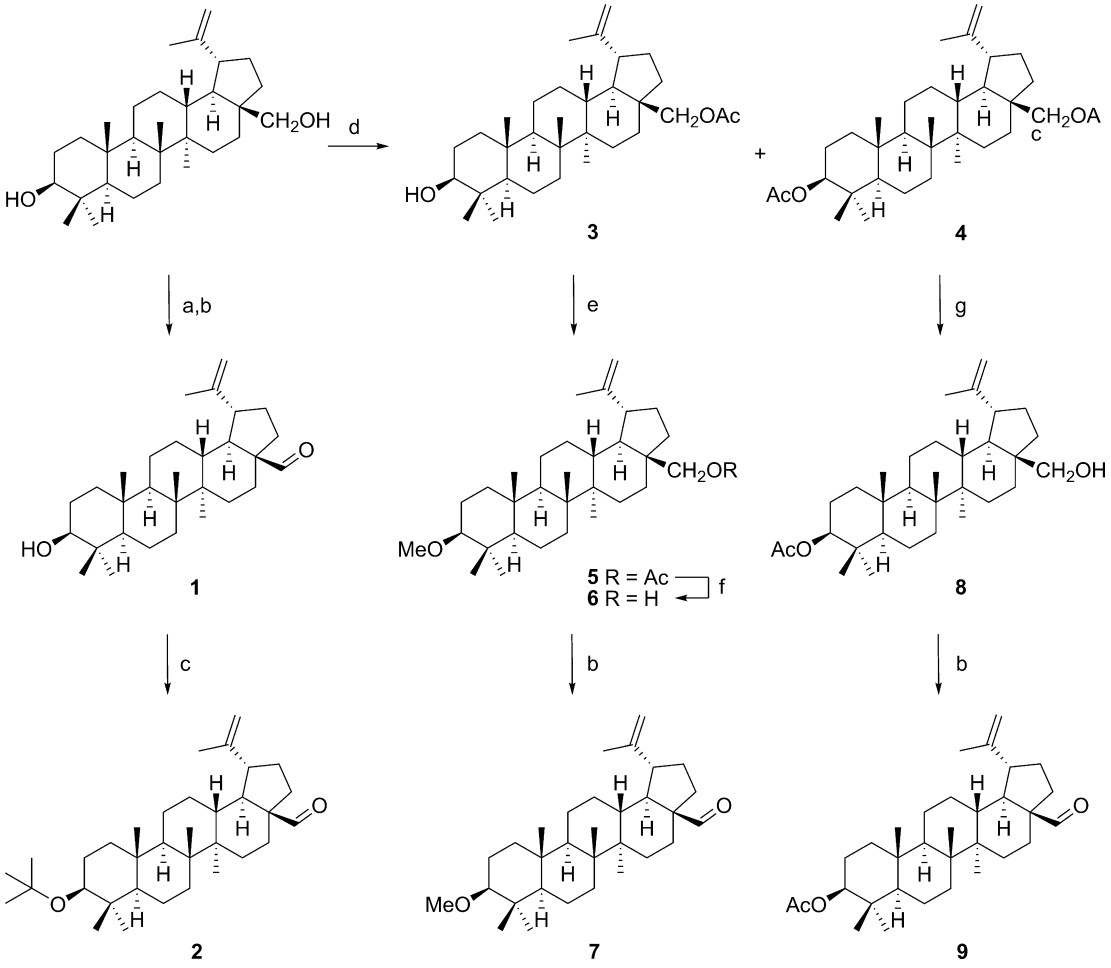
doi:10.1016/j.bmc.2010.02.042



Scheme 1. Synthesis of C3 substituted betulin aldehydes. Reagents and conditions: (a) TMSCl, imidazole, CH_2Cl_2, 0 °C; (b) oxalyl chloride, DMSO, TEA, CH_2Cl_2, −78 °C; (c) Boc_2O, $Mg(ClO_4)_2$, CH_2Cl_2; (d) Ac_2O, TEA, DMAP, CH_2Cl_2, 0 °C; (e) MeI, NaH, DMF; (f) NaOMe, MeOH, THF, reflux; (g) CaH, MeOH, THF.

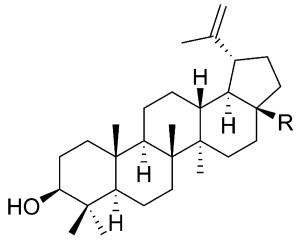


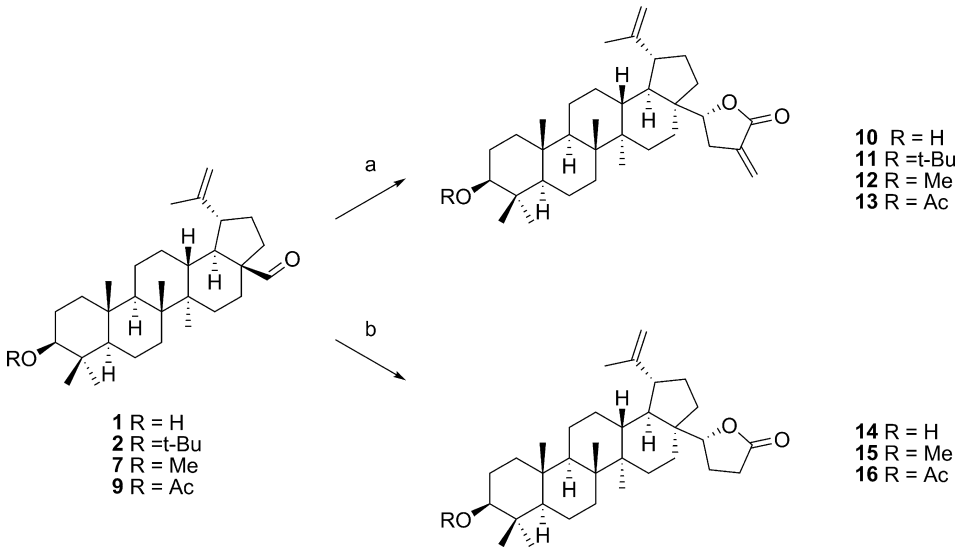
Figure 1. Pharmacological important triterpenes betulin (**I**) and betulinic acid, BA (**II**).

compounds.[14] Aldehydes substituted at C-3 were prepared either after suitable protection or by direct functionalisation of aldehyde **1**. This direct approach was suitable for the synthesis of *tert*-butyl-betulinic aldehyde (**2**) being obtained by the reaction of **1** with di-*tert*-butyldicarbonate in the presence of perchlorate.[15] The synthesis of the 3-*O*-acetyl- and 3-*O*-methyl derivatives was achieved by selective acetylation–deacetylation procedures.[16] Thus, treating betulin with a slightly excess of acetic anhydride gave a mixture of the compounds **3** and **4** in 69% and 25% yield, respectively. The use of an excess of acetic anhydride allowed the exclusive preparation of bisacetylbetulin **4**. The 28-*O*-acetylbetulin **3** afforded on reaction with methyl iodide and sodium hydride in DMF the corresponding 3-*O*-methyl derivative **5**. Deacetylation with sodium methoxide in methanol followed by oxidation yielded the corresponding 3-*O*-methylbetulinic aldehyde **7**.

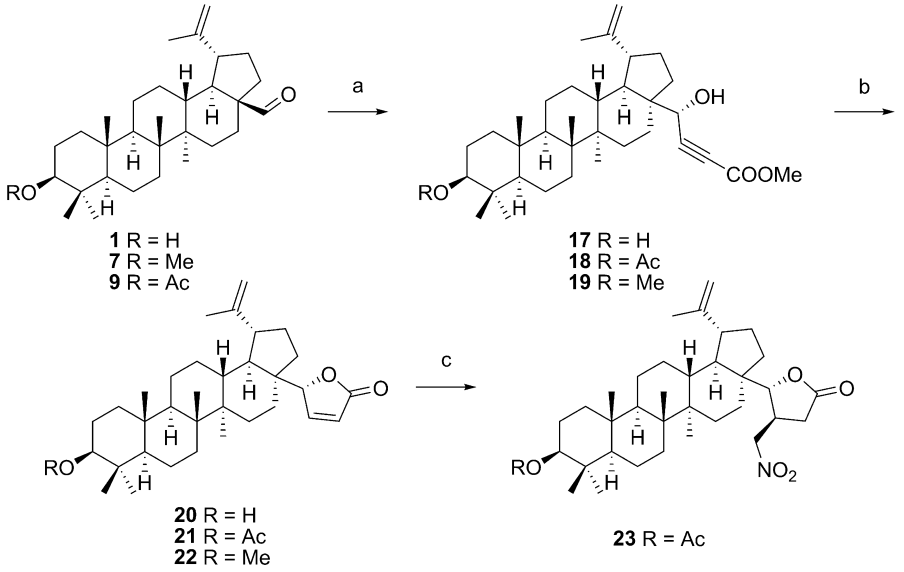
Treating the 3,28-*O*,*O*′-bis-acetylbetulin **4** with catalytic amounts of calcium hydride in a MeOH–THF mixture enabled the selective deprotection of the primary hydroxy group thus yielding 3-*O*-acetylbetulin **8**. Subsequent *Swern* oxidation gave the 3-*O*-acetylbetulinic aldehyde **9**.

The aldehydes were transformed into various saturated and unsaturated γ-lactones (Scheme 3). We focused on the synthesis of α-methylene-γ-lactones. These were obtained in good yields from Dreiding–Schmidt reactions using methyl 2-(bromomethyl)-acrylate and zinc.[17] Although we expected the formation of two diastereoisomers, the (28*R*)-diastereomer was exclusively formed during the reaction. The γ-butyrolactones **14–16** were obtained by a SmI_2 induced reductive coupling using methyl acrylate.[18]

Again, the (28*R*)-isomer was exclusively obtained and a small amount of the corresponding betulin. From 2-substituted acrylates, mixtures of two isomeric compounds were obtained, whereas 3-substituted acrylates gave no reaction at all. For the synthesis of the butenolides **20–22**, a two-step-procedure was applied (Scheme 3). Therefore, the aldehydes were allowed to react with methyl propiolate followed by hydrogenation in the presence of *Lindlar* catalyst.[19] Michael addition of nitromethane with the butenolide **21** proceeded stereoselectively yielding the *trans*-isomer **23**[20] only. Thus, butenolides are suitable starting materials for further derivatization; this is currently under investigation in our laboratory.



Scheme 2. Synthesis of the γ-butyrolactones. Reagents and conditions: (a) methyl 2-(bromomethyl)acrylate, zinc, THF, ultrasound; (b) (1) samarium, 1,2-diiodoethane, THF, ultra sonic, (2) methyl acrylate, ^{t}BuOH, THF, 0 °C.



Scheme 3. Synthesis of betulin derivatives carrying a butenolide. Reagents and conditions: (a) LDA, methyl propiolate, THF, −78 °C; (b) H_2, Lindlar catalyst, quinoline, 1 atm; (c) DBU, nitromethane.

3. Results and discussion

The compounds **10–23** were assayed for their cytotoxic activity against 15 human cancer cell lines using a colorimetric[21] SRB-assay; a summary of the IC_{50}-values is shown in Table 1. The activity of the compounds depends on the modification at C-28 but also from substituents at the C-3. The α-methylene-γ-butyrolactone having an OH-group at C-3 shows an average IC_{50}-value of 10.3 μM. Its matching 3-*O*-methyl derivative **12**, however, shows a slightly decreased IC_{50}-value of 9.1 μM whereas the presence of a 3-*O*-*tert*-butyl group (as in **11**) results in a dramatically loss of activity. This finding is in perfect agreement with results from Salvador and co-workers showing that there is a size limitation at position C-3.[22] The low IC_{50} values for the α-methylene-γ-butyrolactone **13** and for the γ-butyrolactone **16** reveal the valuable effect of a 3-*O*-acetyl moiety. This result is consistent with the reported data[23] for the antitumor activity of acetyl derivatives of betulin. Interestingly, the butenolides **20–22** showed—compared with their precursors **17**–**19**—a decreased activity. Our most active compound in this study was the propiolate **18** having an acetyl-group at C-3 with an average IC_{50}-value of 4.0 μM (Table 2).

Based on the promising cytotoxicity of the compounds, we selected compounds **10** and **21** to be examined whether the cell death was mediated by apoptosis. Therefore, the floating cells (obtained after treatment with IC_{90}-concentrations for 24 h) were analyzed by a trypan-blue exclusion test and DNA gel electrophoresis[24,25] (Figure 2). In this test, apoptotic cells are characterized by an intact cell membrane and therefore they exclude the blue dye. The programed cell death is characterized by fragmentation of DNA into smaller parts of 180 bp; these can be observed as DNA laddering using gel electrophoresis.

4. Conclusion

We synthesized a series of novel betulin derived γ-butyrolactones and butenolides. Their antitumor activity was examined for 15 cancer cell lines using an SRB-assay and their apoptotic action

Table 1
Cytotoxicity (IC_{50} values, μM) in a panel of various cancer cell lines: the values for melanoma (518A2), zervic cancer (A431), head and neck tumor (A253, FADU), lung carcinoma (A549), ovarian cancer (A2780), colon cancer (DLD-1, HCT-8, HCT-116, HT-29, SW-480), anaplastic thyroid cancer (8505C, SW-1736), mamma carcinoma (MCF-7) and Liposarcoma were obtained by an SRB-assay after 96 h of treatment and are the average from at least two independent experiments

Cell line	BA	10	11	12	13	14	15	16
518A2	11.9	11.3	26.6	12.6	8.1	21.8	NA	8.2
A431	15.4	9.6	29.5	8.1	6.2	22.2	NA	4.6
A253	11.1	11.0	NA	9.0	5.7	20.7	18.7	3.9
FADU	10.4	12.2	NA	13.4	8.5	25.5	22.6	9.0
A549	14.9	11.9	NA	8.6	4.1	25.0	18.9	4.7
A2780	11.0	10.4	17.4	8.6	3.6	20.2	24.6	4.9
DLD-1	17.5	11.7	14.4	8.8	9.5	26.8	NA	10.4
HCT-8	17.8	9.0	NA	9.3	7.1	23.4	26.4	4.6
HCT-116	13.3	8.3	NA	7.0	6.3	17.8	18.8	7.9
HT-29	16.1	8.8	NA	9.8	5.3	15.2	NA	10.6
SW480	6.4	9.7	NA	7.0	5.6	22.4	21.3	5.1
8505C	6.7	11.6	27.5	6.4	3.7	22.4	19.5	2.6
SW1736	11.6	7.9	19.0	10.5	5.8	24.8	22.7	6.0
MCF-7	14.9	9.4	NA	10.6	7.5	20.1	NA	5.7
Lipo	9.7	12.1	NA	7.4	5.7	24.5	NA	8.6

Variation was ±10%; NA: no activity <30 μM.

Table 2
Cytotoxicity (IC_{50} values, μM) in a panel of various cancer cell lines: cf. comments to Table 1

Cell line	17	18	19	20	21	22	23
518A2	6.0	5.9	7.5	6.9	3.6	18.4	22.4
A431	4.0	3.4	5.0	6.1	5.3	10.9	11.2
A253	5.3	2.7	4.8	5.9	3.9	11.1	4.7
FADU	6.2	5.0	7.2	6.4	8.5	NA	6.2
A549	5.9	3.1	4.4	6.3	4.2	12.7	22.1
A2780	6.9	3.0	4.3	7.1	3.7	16.9	15.3
DLD-1	6.0	3.6	5.7	8.4	6.1	17.6	NA
HCT-8	2.6	6.0	8.2	7.7	4.0	15.1	11.3
HCT-116	4.8	3.5	3.5	3.5	3.5	3.5	10.1
HT-29	4.9	5.0	4.7	5.9	12.7	16.7	17.4
SW480	5.9	3.6	5.5	6.1	5.3	13.8	9.8
8505C	5.9	3.6	3.7	7.1	5.7	18.0	28.7
SW1736	5.2	2.3	5.0	7.7	3.4	15.9	7.0
MCF-7	5.8	6.0	7.3	6.4	4.0	NA	25.8
Lipo	5.9	4.2	5.5	6.9	6.4	NA	15.3

was documented by trypan-blue test and DNA laddering. These studies reveal the favorable effect of a 3-*O*-acetyl moiety. Our most active compound was a 3-O-acetylated propiolate **18** showing an average IC_{50}-value of 4.0 μM. Several of these novel compounds revealed a higher activity than betulinic acid.

5. Experimental

5.1. Chemistry

Melting points are uncorrected (*Leica* hot stage microscope), optical rotations were obtained using a Perkin–Elmer 341 polarimeter (1 cm micro cell, 20 °C), NMR spectra were recorded using the Varian spectrometers Gemini 200, Gemini 2000 or Unity 500 (δ given in ppm, J in hertz, internal Me_4Si), IR spectra (film or KBr pellet) on a Perkin–Elmer FT-IR spectrometer Spectrum 1000, MS spectra were taken on a Intectra GmbH AMD 402 (electron impact, 70 eV) or on a Finnigan MAT TSQ 7000 (electrospray, voltage 4.5 kV, sheath gas nitrogen) instrument; for elemental analysis a Foss-Heraeus Vario EL instrument was used; TLC was performed on silica gel (Merck 5554, detection by UV absorption or by treatment with a solution of 10% sulfuric acid, ammonium molybdate and cerium(IV) sulfate) followed by gentle heating. The solvents were dried according to usual procedures.

5.1.1. General procedure for the *Swern* oxidation (GP1)

To a solution of oxalyl chloride (6.0 g, 47.1 mmol) in dry dichloromethane (100 ml) at −78 °C a solution of dry DMSO (7.8 ml) in dry (100 ml) was slowly added and stirring at −78 °C was continued for another 30 min. At this temperature a solution of the corresponding betulinol (25.0 g, 42.6 mmol) in dry dichloromethane (50 ml) was slowly added and stirring at this temperature was continued for another 2 h. Then, dry TEA (14 ml) was added and the reaction mixture was allowed to warm to room temperature. Diluted aq HCl (10%, 100 ml) was added under vigorous stirring, the phases were separated, the organic layer was washed with aq Na_2CO_3 (2 × 50 ml), water (2 × 50 ml), and brine (2 × 50 ml), the solvents were removed, and the residue was subjected to chromatography (silica gel, hexane–ethyl acetate 9:1) to afford the corresponding betulinic aldehyde.

5.1.2. General procedure for the synthesis of α-methylene-γ-butyrolactones (GP2)

A suspension of zinc (0.54 g, 8.20 mmol) in THF was activated[26] with a few crystals iodine under sonification. After the consumption of the iodine, the corresponding aldehyde (1.35 mmol), methyl 2-bromomethylacrylate (0.54 g, 3.00 mmol) and hydroquinone (10 mg) were added. The suspension was sonificated until TLC revealed the absence of starting material. The precipitate was filtered off, washed with THF and the solution was concentrated in vacuo. Purification was achieved by column chromatography (silica gel, hexane–ethyl acetate, 8:2).

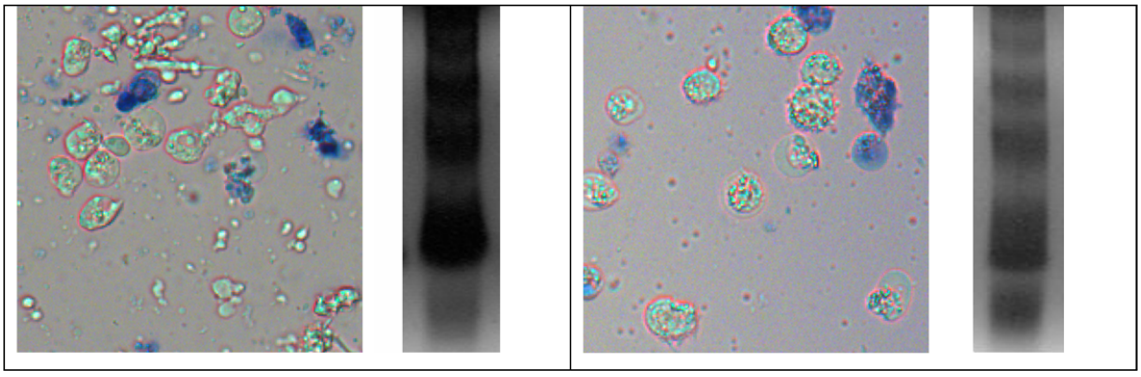


Figure 2. Trypan-blue exclusion test and DNA laddering of compound **10** for adenocarcinoma cell line HCT-8 (left) and compound **21** for ovarian cancer cell line A2780 (right) after treatment with IC_{90}-concentrations for 24 h.

5.1.3. General procedure for the synthesis of γ-butyrolactones (GP3)

A mixture of samarium (0.6 g, 4.0 mmol) and 1,2-diiodoethane (1.1 g, 4.0 mmol) in dry THF (20 ml) was sonificated under argon until a homogeneous deep blue solution was formed. After cooling to 0 °C, a solution of the corresponding aldehyde (1.36 mmol), methyl acrylate (0.12 g, 1.36 mmol) and *t*-BuOH (0.10 g, 1.36 mmol) in dry THF (5 ml) was added. The mixture was stirred over night and then treated with brine (50 ml). The phases were separated and the aq layer was extracted with ethyl acetate (2 × 50 ml). The combined extracts were concentrated in vacuo and the residue was subjected to column chromatography (silica gel, hexane–ethyl acetate, 8:2).

5.1.4. General procedure for the synthesis of 3-substituted methyl propiolates (GP4)

To a solution of DIA (416 mg, 4.12 mmol) in dry THF (20 ml) *n*-BuLi (2.6 ml, 1.6 M in hexane) was added at -20 °C. After 15 min, the solution was cooled to −78 °C and a solution of methyl propiolate (0.34 g, 4.12 mmol) in dry THF (2 ml) was added dropwise. Stirring at this temperature was continued for additional 30 min; then a solution of the corresponding aldehyde (1.03 mmol) was added. After 1 h at −78 °C, the reaction was quenched by the addition of brine (100 ml). The phases were separated and the aq layer was extracted with ethyl acetate (2 × 50 ml). The combined extracts were dried over Na_2SO_4 and evaporated to dryness. The residue was subjected to column chromatography (silica gel, hexane–ethyl acetate, 8:2).

5.1.5. General procedure for the synthesis of butenolides (GP5)

Hydrogen was bubbled for 30 min through a mixture containing the corresponding methyl propiolates (0.35 mmol), Pd/$BaSO_4$ (20 mg) and quinoline (200 mg) in ethyl acetate (10 ml). After completion of the reaction (as observed by TLC), the reaction was filtered over Celite and the solvent removed in vacuo. The residue was subjected to column chromatography (silica gel, hexane–ethyl acetate, 8:2).

5.1.6. 3-*O*-*tert*-Butylbetulinic aldehyde (2)

Prior to use $MgClO_4$, was dried in vacuo at 130 °C (oil bath temperature) for 2 h. To a solution of betulin aldehyde (1.0 g, 2.27 mmol) and $MgClO_4$ (1.1 g, 5.0 mmol), di-*tert*-butyldicarbonate (5.0 g, 23 mmol) was added and the mixture was stirred for 1 h at room temperature. When TLC revealed the absence of starting material, the solution was washed with diluted HCl otherwise another portion of di-*tert*-butyldicarbonate (5.0 g, 23 mmol) was added. The organic layer was dried over Na_2SO_4 and concentrated in vacuo. After purification by column chromatography (silica gel, hexane–ethyl acetate, 95:5) **2** (1.0 g, 90%) was obtained as a colourless solid; mp 184 °C; $[\alpha]_D$ +24.1 (*c* 4.4, $CHCl_3$); R_f 0.87 (silica gel, hexane–ethyl acetate, 8:2); IR (KBr): ν = 2948s, 2875 m, 1727 m, 1642w, 1453w, 1364w, 1252w, 1191w, 1056w, 1021w cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 9.66 (d, 1H, *J* = 1.5 Hz, C*H*O), 4.74 (d, 1H, *J* = 2.0 Hz, CH_a (30)), 4.61 (dd, 1H, *J* = 2.0, 1.5 Hz, CH_b (30)), 2.96 (dd, 1H, *J* = 7.5, 5.9 Hz, C*H*O^{t}Bu (3)), 2.84 (ddd, 1H, *J* = 11.0, 11.1, 5.8 Hz, C*H* (19)), 2.08–1.85 (m, 3H, C*H* (13)+CH_a (21)+CH_a (16)), 1.78–1.52 (m, 7H, CH_a (22)+CH_a (12)+C*H* (18)+CH_a (1)+CH_2 (2)+CH_a (6)), 1.68 (s, 3H, CH_3 (29)), 1.52–1.12 (m, 11H, CH_b (21)+CH_a (15)+CH_2 (11)+CH_b (6)+CH_2 (7)+CH_b (22)+CH_b (12)+CH_b (16)+C*H* (9)), 1.16 (s, 9H, *t*-Bu), 1.08–0.98 (m, 1H, CH_b (15)), 0.96 (s, 3H, CH_3 (27)), 0.90 (s, 3H, CH_3 (25)), 0.87 (*s*, 3H, CH_3 (23)), 0.89–0.82 (m, 1H, CH_b (1)), 0.81 (s, 3H, CH_3 (26)), 0.72 (s, 3H, CH_3 (24)), 0.68 (d, 1H, *J* = 9.5 Hz, C*H* (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 206.7 (*C*=O), 149.7 (C20, *C*=CH_2), 110.1 (C30, CH_2=C), 78.5 (C3, *C*HO^{t}Bu), 72.8 (*t*-Bu, *C*), 59.3 (C17, $C_{quart.}$), 56.1 (C5, *C*H), 50.6 (C9, *C*H), 48.1 (C18, *C*H), 47.5 (C19, *C*H), 42.5 (C14, $C_{quart.}$), 40.8 (C8, $C_{quart.}$), 39.1 (C1, CH_2), 39.0 (C4, $C_{quart.}$), 38.7 (C13, *C*H), 37.1 (C10, $C_{quart.}$), 34.5 (C7, CH_2), 33.2 (C22, CH_2), 29.9 (C16, CH_2), 29.3 (C21, CH_2), 29.2 (*t*-Bu, CH_3), 28.8 (C15, CH_2), 28.7 (C23, CH_3), 27.2 (C2, CH_2), 25.6 (C12, CH_2), 20.8 (C11, CH_2), 19.0 (C29, CH_3), 18.5 (C6, CH_2), 16.4 (C26, CH_3), 16.3 (C25, CH_3), 15.9 (C24, CH_3), 14.2 (C27, CH_3) ppm. MS (i.e., 70 eV): *m/z* (%) = 496 (6), 383 (100), 355 (31), 245 (50), 189 (47), 165 (71). Anal. Calcd for $C_{34}H_{56}O_2$ (496.81): C, 82.20; H, 11.36. Found: C, 81.96; H, 11.49.

5.1.7. 28-*O*-Acetylbetulin (3) and *O,O′*-diacetylbetulin (4)

To a suspension of betulin (15 g, 34 mmol), DMAP (0.1 g) in dry CH_2Cl_2 (150 ml) and TEA (4.2 g, 40.8 mmol) was added dropwise a solution of Ac_2O (4.2 g, 40.8 mmol) in dry CH_2Cl_2 (30 ml) at 0 °C. Stirring was continued for 1 h at room temperature. The precipitate was filtered off and washed with CH_2Cl_2 (2 × 50 ml). The filtrate was washed with saturated Na_2CO_3 solution (100 ml) and brine (100 ml), dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by column chromatography (silica gel, hexane–ethyl acetate, 9:1) and afforded compound **3** (11.3 g, 69%) and **4** (4.5 g, 25%) as white solids. Data for **3**: mp 209–212 °C (lit.: 217–219 °C;[27] 210–212 °C[16]); $[\alpha]_D$ +7.4 (*c* 3.4, $CHCl_3$) (lit.: $[\alpha]_D$ +8.5 (*c* 1.58 $CHCl_3$),[16] $[\alpha]_D$ +11.0 (*c* 1.0 $CHCl_3$)[28]); R_f 0.47 (silica gel, hexane–ethyl acetate, 8:2). Data for **4**: mp 217–218 °C (lit.: 220 °C;[29] 218–219 °C[30]); $[\alpha]_D$ +18.5 (*c* 3.7, $CHCl_3$) (lit.: $[\alpha]_D$ +21 (*c* 0.8 $CHCl_3$),[29] $[\alpha]_D$ +22 ($CHCl_3$)[30]); R_f 0.62 (silica gel, hexane–ethyl acetate, 9:1).

5.1.8. 28-*O*-Acetyl-3-*O*-methylbetulin (5)

To a solution of 28-*O*-acetyl-betulin (10.0 g, 20.6 mmol) in dry DMF (100 ml) NaH (60% in mineral oil, 1.0 g, 24.7 mmol) was added in several portions. After gas evolution has ceased methyl iodide (3.5 g, 24.7 mmol) was added and stirred for 5 h at room temperature. The solvent was removed in vacuo and the residue treated with CH_2Cl_2 (200 ml) and H_2O (200 ml). After separation of the phases, the organic layer was dried over Na_2SO_4 and concentrated in vacuo to afford **5** (10.0 g, 97%) as a white solid. An analytical sample was obtained by column chromatography (silica gel, hexane–ethyl acetate, 8:2); mp 163–165 °C; $[\alpha]_D$ +8.7 (*c* 4.1, $CHCl_3$); R_f 0.71 (silica gel, hexane–ethyl acetate, 9:1); IR (KBr): ν = 3562s, 3086w, 2941s, 2868m, 1723m, 1646m, 1452m, 1389m, 1367m, 1346m, 1260s, 1191w, 1127w, 1107m, 1087w, 1044m cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 4.67 (d, 1H, *J* = 2.1 Hz, CH_a (30)), 4.57 (dd, 1H, *J* = 1.7, 1.2 Hz, CH_b (30)), 4.22 (d, 1H, *J* = 11.2 Hz, CH_a (28)), 3.84 (d, 1H, *J* = 11.2 Hz, CH_b (28)), 3.32 (s, 1H, OCH_3), 2.62 (dd, 1H, *J* = 11.6, 4.2 Hz, C*H*OCH$_3$ (3)), 2.43 (ddd, 1H, *J* = 11.2, 10.8, 5.8 Hz, C*H* (19)), 2.05 (s, 3H, Ac), 1.98–1.90 (m, 1H, CH_a (21)), 1.85–1.86 (m, 1H, CH_a (16)), 1.78–1.50 (m, 7H, CH_a (2)+CH_a (22)+CH_a (1)+CH_a (15)+C*H* (13)+CH_a (12)+C*H* (18)), 1.66 (s, 3H, CH_3 (29)), 1.44–1.32 (m, 7H, CH_2 (6)+CH_a (11)+CH_b (2)+CH_2 (7)+CH_b (21)), 1.27–1.10 (m, 3H, CH_b (16)+C*H* (9)+CH_b (11)), 1.07–0.98 (m, 1H, CH_b (22)+CH_b (15)+CH_b (12)), 1.01 (s, 3H, CH_3 (25)), 0.95 (s, 3H, CH_3 (27)), 0.93 (s, 3H, CH_3 (23)), 0.81 (s, 3H, CH_3 (26)), 0.78–0.74 (m, 1H, CH_b (1)), 0.72 (s, 3H, CH_3 (24)), 0.65 (d, 1H, *J* = 9.5 Hz, C*H* (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 171.6 (*C*=O), 150.2 (C20, *C*=CH_2), 109.8 (C30, CH_2=C), 88.6 (C3, *C*HOCH$_3$), 62.8 (C28, CH_2), 57.5 (OCH$_3$), 55.9 (C5, *C*H), 50.4 (C9, *C*H), 49.8 (C18, *C*H), 47.7 (C19, *C*H), 46.3 (C17, $C_{quart.}$), 42.7 (C14, $C_{quart.}$), 41.9 (C8, $C_{quart.}$), 38.8 (C1, CH_2), 38.6 (C4, $C_{quart.}$), 37.6 (C13, *C*H), 37.2 (C10, $C_{quart.}$), 34.6 (C7, CH_2), 34.2 (C22, CH_2), 29.8 (C16, CH_2), 29.6 (C21, CH_2), 28.0 (C23, CH_3), 27.1 (C15, CH_2), 25.2 (C12, CH_2), 22.2 (C2, CH_2), 21.0 (Ac), 20.8 (C11, CH_2), 19.1 (C29, CH_3), 18.2 (C6, CH_2), 16.1 (C24, CH_3), 16.0 (C26, CH_3), 16.0 (C25, CH_3), 14.7 (C27, CH_3) ppm; MS (i.e., 70 eV): *m/z* (%) = 498 (67), 466 (44), 425 (41), 221 (48), 203 (46), 189 (100), 135 (52). Anal. Calcd for $C_{33}H_{54}O_3$ (498.78): C, 79.47; H, 10.91. Found: C, 79.38; H, 11.05.

5.1.9. 3-*O*-Methylbetulin (6)

A solution of **5** (10.0 g, 20.0 mmol) and sodium methoxide (1.08 g, 20 mmol) in THF (200 ml) and MeOH (100 ml) was heated under reflux until the absence of starting material was confirmed by TLC (normally within 24 h). The solvents were removed in vacuo, the residue was suspended in 2 N HCl (100 ml) and extracted with ethyl acetate (2 × 200 ml). The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo to yield **6** (8.7 g, 95%) as a white solid. The crude product was used without further purification; an analytical sample was obtained by column chromatography (silica gel, hexane–ethyl acetate, 8:2); mp 230–233 °C; $[\alpha]_D$ −7.3 (*c* 2.7, $CHCl_3$); R_f 0.45 (silica gel, hexane–ethyl acetate, 8:2); IR (KBr): *v* = 3442m, 3070m, 2939s, 2867m, 1734w, 1643w, 1558w, 1453m, 1390w, 1373m, 1246w, 1181m, 1103m, 1013m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 4.66 (d, 1H, *J* = 2.1 Hz, CH_a (30)), 4.55 (dd, 1H, *J* = 2.1, 1.2 Hz, CH_b (30)), 3.77 (dd, 1H, *J* = 10.8, 1.7 Hz, CH_a (28)), 3.32 (s, 3H, OCH_3), 3.31 (d, 1H, *J* = 10.8 Hz, CH_b (28)), 2.60 (dd, 1H, *J* = 12.0, 4.6 Hz, CHOCH_3 (3)), 2.36 (ddd, 1H, *J* = 10.9, 10.9, 5.8 Hz, CH (19)), 1.97–1.80 (m, 2H, CH_a (21)+CH_a (16)), 1.78–1.55 (m, 6H, CH_a (2)+CH_a (15)+CH_a (1)+CH (13)+CH_a (12)+CH (18)), 1.66 (s, 3H, CH_3 (29)), 1.52–1.32 (m, 6H, CH_2 (6)+CH_a (11)+CH_a (22)+CH_b (2)+CH_2 (7)), 1.27–1.14 (m, 5H, CH (9)+CH_b (22) CH_b (11)+CH_b (21)+CH_b (12)), 1.06–1.00 (m, 1H, CH_b (15)+CH_b (16)), 0.99 (s, 3H, CH_3 (25)), 0.95 (s, 3H, CH_3 (27)), 0.93 (s, 3H, CH_3 (23)), 0.86–0.81 (m, 1H, CH_b (1)), 0.80 (s, 3H, CH_3 (26)), 0.72 (s, 3H, CH_3 (24)), 0.65 (d, 1H, *J* = 9.5 Hz, CH (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 150.5 (C20, *C*=CH_2), 109.6 (C30, *C*H_2=C), 88.6 (C3, *C*HOCH_3), 60.5 (C28, *C*H), 57.5 (OCH_3), 55.8 (C5, *C*H), 50.4 (C9, *C*H), 48.8 (C18, *C*H), 47.8 (C17, $C_{quart.}$), 47.7 (C19, *C*H), 42.7 (C14, $C_{quart.}$), 40.9 (C8, $C_{quart.}$), 38.8 (C1, *C*H_2), 38.6 (C4, $C_{quart.}$), 37.3 (C13, *C*H), 37.2 (C10, $C_{quart.}$), 34.2 (C7, *C*H_2), 34.0 (C16, *C*H_2), 29.6 (C22, *C*H_2), 29.2 (C21, *C*H_2), 28.0 (C23, *C*H_3), 27.0 (C15, *C*H_2), 25.2 (C12, *C*H_2), 22.2 (C2, *C*H_2), 20.9 (C11, *C*H_2), 19.1 (C29, *C*H_3), 18.2 (C6, *C*H_2), 16.1 (C24, *C*H_3), 16.1 (C25, *C*H_3), 16.0 (C26, *C*H_3), 14.7 (C27, *C*H_3) ppm; MS (i.e., 70 eV): *m/z* (%) = 456 (43), 425 (52), 234 (57), 221 (61), 203 (64), 189 (100), 135 (41). Anal. Calcd for $C_{31}H_{52}O_2$ (456.74): C, 81.52; H, 11.48. Found: C, 81.47; H, 11.61.

5.1.10. 3-*O*-Methylbetulinic aldehyde (7)

Following GP1, **7** (6.8 g, 97%) was obtained from **6** (7.0 g, 15.3 mmol) as a colourless solid; mp 192–194 °C; $[\alpha]_D$ +30.3 (*c* 5.3, $CHCl_3$); R_f 0.80 (silica gel, hexane–ethyl acetate, 9:1); IR (KBr): *v* = 3441m, 3072w, 2944s, 2869m, 1709m, 1646w, 1449m, 1389w, 1373w, 1355w, 1248w, 1181w, 1102m, 1000w cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 9.65 (d, 1H, *J* = 1.7 Hz, C*H*O), 4.74 (d, 1H, *J* = 1.7 Hz, CH_a (30)), 4.61 (dd, 1H, *J* = 2.1, 1.7 Hz, CH_b (30)), 3.32 (s, 1H, OCH_3), 2.84 (ddd, 1H, *J* = 11.2, 11.2, 5.8 Hz, CH (19)), 2.60 (dd, 1H, *J* = 11.6, 4.6 Hz, CHOCH_3 (3)), 2.08–1.96 (m, 2H, CH_a (16)+CH (13)), 1,89–1,82 (m, 1H, CH_a (21)), 1.78–1.65 (m, 5H, CH_a (2)+CH_a (22)+CH_a (12)+CH (18)+CH_a (1)), 1.68 (s, 3H, CH_3 (29)), 1.49–1.12 (m, 13H, CH_2 (6)+CH_b (16)+CH_b (21)+CH_2 (15)+CH_2 (11)+CH_b (2)+CH_2 (7)+CH_b (22)+CH (9)), 1.05–0.99 (m, 1H, CH_b (12)), 0.95 (s, 3H, CH_3 (27)), 0.92 (s, 3H, CH_3 (23)), 0.89 (s, 3H, CH_3 (25)), 0.86–0.82 (m, 1H, CH_b (1)), 0.80 (s, 6H, 2CH_3 (24+26)), 0.64 (d, 1H, *J* = 9.1 Hz, CH (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 206.6 (*C*=O), 149.7 (C20, *C*=CH_2), 110.1 (C30, *C*H_2=C), 88.6 (C3, *C*HOCH_3), 59.3 (C17, $C_{quart.}$), 57.4 (C31, *C*H_3), 55.9 (C5, *C*H), 50.5 (C9, *C*H), 48.1 (C18, *C*H), 47.5 (C19, *C*H), 42.5 (C14, $C_{quart.}$), 40.8 (C8, $C_{quart.}$), 38.8 (C1, *C*H_2), 38.7 (C13, *C*H), 38.6 (C4, $C_{quart.}$), 37.2 (C10, $C_{quart.}$), 34.3 (C7, *C*H_2), 33.2 (C22, *C*H_2), 29.8 (C21, *C*H_2), 29.2 (C16, *C*H_2), 28.8 (C15, *C*H_2), 28.0 (C23, *C*H_3), 25.5 (C12, *C*H_2), 22.2 (C2, *C*H_2), 20.8 (C11, *C*H_2), 19.0 (C29, *C*H_3), 18.1 (C6, *C*H_2), 16.1 (C24+C26, 2*C*H_3), 15.9 (C25, *C*H_3), 14.2 (C27, *C*H_3) ppm; MS (i.e., 70 eV): *m/z* (%) = 454 (74), 425 (31), 221 (63), 189 (100), 135 (43). Anal. Calcd for $C_{31}H_{50}O_2$ (454.73): C, 81.88; H, 11.08. Found: C, 81.66; H, 11.18.

5.1.11. (*R*)-4-[3β-Hydroxy-28-norlup-20(29)-en-17β-yl]-2-methylene-γ-butyrolactone (10)

Following GP2, **10** (0.45 g, 65%) was obtained from betulinic aldehyde (0.60 g, 1.35 mmol) as a colourless solid; mp 253–256 °C; $[\alpha]_D$ −8.0 (*c* 4.8, $CHCl_3$); R_f 0.25 (silica gel, hexane–ethyl acetate, 8:2); IR (KBr): *v* = 3423s, 2941s, 2872m, 1756s, 1640w, 1453w, 1371w, 1284w, 1250w, 1132w, 1108w, 1033w cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 6.18 (dd, 1H, *J* = 2.9, 2.6 Hz, CH_a (34)), 5.58 (dd, 1H, *J* = 2.5, 2.3 Hz, CH_b (34)), 4.89 (dd, 1H, *J* = 7.6, 7.6 Hz, CH (28)), 4.70 (d, 1H, *J* = 2.1 Hz, CH_a (30)), 4.54–4.53 (m, 1H, CH_b (30)), 3.12 (dd, 1H, *J* = 11.4, 4.8 Hz, CHOH (3)), 2.88–2.79 (m, 2H, CH_a (31)+CH (19)), 2.70–2.63 (m, 1H, CH_b (31)), 2.00 (ddd, 1H, *J* = 12.2, 12.2, 3.8 Hz, CH (13)), 1.92–1.82 (m, 1H, CH_a (21)), 1.78–1.65 (m, 2H, CH (18)+CH_a (12)), 1.62 (s, 3H, CH_3 (29)), 1.65–1.42 (m, 7H, CH_a (1)+CH_a (22)+CH_a (16)+CH_2 (2)+CH_a (6)+CH_a (15)), 1.40–1.00 (m, 11H, CH_2 (11)+CH_b (6)+CH_b (16)+CH_2 (7)+CH (9)+CH_b (22)+CH_b (21)+CH_b (12)+CH_b (15)), 0.96 (s, 3H, CH_3 (27)), 0.95 (s, 3H, CH_3 (25)), 0.90 (s, 3H, CH_3 (23)), 0.92–0.88 (m, 1H, CH_b (1)), 0.77 (s, 3H, CH_3 (26)), 0.70 (s, 3H, CH_3 (24)), 0.62 (d, 1H, *J* = 9.5 Hz, CH (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 170.5 (*C*=O), 150.3 (C20, *C*=CH_2), 134.6 (C32, *C*=CH_2), 121.6 (C34, *C*H_2=C), 110.0 (C30, *C*H_2=C), 78.9 (C3, *C*HOH), 78.1 (C28, *C*H), 55.3 (C5, *C*H), 50.3 (C9, *C*H), 49.4 (C18, *C*H), 47.7 (C19, *C*H), 45.5 (C17, $C_{quart.}$), 42.8 (C14, $C_{quart.}$), 40.9 (C8, $C_{quart.}$), 38.8 (C4, $C_{quart.}$), 38.7 (C1, *C*H_2), 37.1 (C10, $C_{quart.}$), 36.9 (C13, *C*H), 34.2 (C7, *C*H_2), 32.9 (C22, *C*H_2), 31.5 (C16, *C*H_2), 31.2 (C21, *C*H_2), 30.4 (C31, *C*H_2), 28.0 (C23, *C*H_3), 27.8 (C15, *C*H_2), 27.3 (C2, *C*H_2), 24.9 (C12, *C*H_2), 20.8 (C11, *C*H_2), 18.8 (C29, *C*H_3), 18.3 (C6, *C*H_2), 16.1 (C26, *C*H_3), 15.9 (C25, *C*H_3), 15.3 (C24, *C*H_3), 15.2 (C27, *C*H_3) ppm; MS (ESI, MeOH): *m/z* = 508.4 (30% $[M+H]^+$), 1039.4 (100% $[2M+Na]^+$). Anal. Calcd for $C_{34}H_{52}O_3$ (508.78): C, 80.26; H, 10.30. Found: C, 80.00; H, 10.45.

5.1.12. (*R*)-4-[3β-*tert*-Butyloxy-28-norlup-20(29)-en-17β-yl]-2-methylene-γ-butyrolactone (11)

Compound **11** (0.50 g, 89%) was obtained as a colourless solid from **2** (0.50 g, 1.00 mmol) following GP2; mp 243–245 °C; $[\alpha]_D$ −3.0 (*c* 5.6, $CHCl_3$); R_f 0.57 (silica gel, hexane–ethyl acetate, 8:2); IR (KBr): *v* = 2943s, 2873m, 1765s, 1718m, 1639m, 1465m, 1387m, 1365m, 1283m, 1248m, 1195m, 1132m, 1108m, 1083m, 1020m cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 6.22 (dd, 1H, *J* = 2.9, 2.9 Hz, CH_a (34)), 5.62 (dd, 1H, *J* = 2.5, 2.1 Hz, CH_b (34)), 4.94 (dd, 1H, *J* = 7.9, 7.9 Hz, CH (28)), 4.75 (d, 1H, *J* = 2.1 Hz, CH_a (30)), 4.58–4.54 (m, 1H, CH_b (30)), 2.94 (dd, 1H, *J* = 10.8, 5.4 Hz, CHO^{t}Bu (3)), 2.92–2.80 (m, 2H, CH_a (31)+CH (19)), 2.75–2.67 (m, 1H, CH_b (31)), 2.06 (ddd, 1H, *J* = 12.2, 12.2, 3.8 Hz, CH (13)), 1.98–1.85 (m, 1H, CH_a (21)), 1.79–1.53 (m, 8H, CH_a (12)+CH (18)+CH_a (16)+CH_a (22)+CH_a (1)+CH_2 (2)+CH_a (6)), 1.66 (s, 3H, CH_3 (29)), 1.52–1.10 (m, 11H, CH_a (15)+CH_b (22)+CH_2 (11)+CH_b (6)+CH_2 (7)+CH_b (12)+CH_b (16)+CH (9)+CH_b (21)), 1.16 (s, 9H, *t*-Bu), 1.06–0.99 (m, 1H, CH_b (15)), 1.00 (s, 3H, CH_3 (27)), 0.99 (s, 3H, CH_3 (25)), 0.87 (s, 3H, CH_3 (23)), 0.89–0.82 (m, 1H, CH_b (1)), 0.82 (s, 3H, CH_3 (26)), 0.72 (s, 3H, CH_3 (24)), 0.68 (d, 1H, *J* = 9.5 Hz, CH (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 170.5 (*C*=O), 150.3 (C20, *C*=CH_2), 134.6 (C32, *C*=CH_2), 121.5 (C34, *C*H_2=C), 110.1 (C30, *C*H_2=C), 78.5 (C3, *C*HO^{t}Bu), 78.1 (C28, *C*H), 72.8 (*t*-Bu, *C*), 56.0 (C5, *C*H), 50.5 (C9, *C*H), 49.5 (C17, $C_{quart.}$), 49.4 (C18, *C*H), 47.7 (C19, *C*H), 42.8 (C14, $C_{quart.}$), 40.9 (C8, $C_{quart.}$), 39.0 (C4, $C_{quart.}$), 38.9 (C1, *C*H_2), 37.0 (C10, $C_{quart.}$), 36.9 (C13, *C*H), 34.4 (C7, *C*H_2), 33.0 (C22, *C*H_2), 31.9 (C16, *C*H_2), 31.7 (C21, *C*H_2), 30.4 (C31, *C*H_2), 29.2 (*t*-Bu, *C*H_3), 28.7 (C23, *C*H_3), 27.8 (C15, *C*H_2), 27.2 (C2, *C*H_2), 25.0 (C12, *C*H_2), 20.8 (C11, *C*H_2), 19.0 (C29, *C*H_3), 18.5 (C6, *C*H_2), 16.4 (C26, *C*H_3), 16.2 (C25, *C*H_3), 16.1 (C24, *C*H_3), 15.2 (C27, *C*H_3) ppm; MS (ESI, MeOH): *m/z* = 587.5 (10% $[M+Na]^+$), 1151.5 (100% $[2M+Na]^+$), 1167.3 (70% $[2M+K]^+$). Anal. Calcd for $C_{38}H_{60}O_3$ (564.88): C, 80.80; H, 10.71. Found: C, 80.67; H, 10.92.

5.1.13. (*R*)-4-[3β-Methoxy-28-norlup-20(29)-en-17β-yl]-2-methylene-γ-butyrolactone (12)

Following GP1, **12** (0.50 g, 87%) was obtained from aldehyde **7** (0.50 g, 1.00 mmol) as a colourless solid; mp 219–220 °C; $[\alpha]_D$ 1.7 (*c* 4.28, $CHCl_3$); R_f 0.69 (silica gel, hexane–ethyl acetate, 8:2); IR (KBr): *v* = 3441m, 3070w, 2954s, 2947s, 1757s, 1638w, 1456m, 1388w, 1358w, 1282m, 1250m, 1185m, 1127m, 1105m, 1088m, 1011m cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 6.22 (dd, 1H, *J* = 2.9, 2.5 Hz, CH_a (33)), 5.58 (dd, 1H, *J* = 2.5, 2.5 Hz, CH_b (33)), 4.94 (dd, 1H, *J* = 7.4, 7.9 Hz, C*H* (28)), 4.75 (d, 1H, *J* = 2.1 Hz, CH_a (30)), 4.58 (dd, 1H, *J* = 2.1, 1.7 Hz, CH_b (30)), 3.33 (s, 1H, OCH_3), 2.94–2.83 (m, 2H, CH_a (31)+C*H* (19)), 2.75–2.67 (m, 1H, CH_b (31)), 2.61 (dd, 1H, *J* = 11.6, 4.6 Hz, C*H*OCH$_3$ (3)), 2.06 (ddd, 1H, *J* = 12.0, 12.0, 3.3 Hz, C*H* (13)), 1.97–1.86 (m, 1H, CH_a (21)), 1.80–1.59 (m, 6H, CH_a (2)+C*H* (18)+CH_a (12) +CH_a (1) +CH_a (22)+CH_a (16)), 1.67 (s, 3H, CH_3 (29)), 1.50–1.08 (m, 13H, CH_2 (6)+CH_2 (11)+CH_b (22)+CH_a (15)+CH_b (2)+CH_2 (7)+CH_b (21)+CH_b (12)+CH_b (16)+C*H* (9)), 1.05–1.02 (m, 1H, CH_b (15)), 1.00 (s, 3H, CH_3 (27)), 0.99 (s, 3H, CH_3 (25)), 0.93 (s, 3H, CH_3 (23)), 0.85–0.77 (m, 1H, CH_b (1)), 0.82 (s, 3H, CH_3 (26)), 0.72 (s, 3H, CH_3 (24)), 0.66 (d, 1H, *J* = 9.5 Hz, C*H* (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 170.5 (*C*=O), 150.3 (C20, *C*=CH$_2$), 134.6 (C32, *C*=CH$_2$), 121.6 (C33, C=*C*H$_2$), 109.8 (C30, *C*H$_2$=C), 88.5 (C3, *C*HOCH$_3$), 78.1 (C28, *C*H), 57.5 (O*C*H$_3$), 55.8 (C5, *C*H), 50.3 (C9, *C*H), 49.5 (C17, $C_{quart.}$), 49.4 (C18, *C*H), 47.7 (C19, *C*H), 42.8 (C14, $C_{quart.}$), 41.0 (C8, $C_{quart.}$), 38.8 (C1, *C*H$_2$), 38.5 (C4, $C_{quart.}$), 37.2 (C10, $C_{quart.}$), 36.9 (C13, *C*H), 34.2 (C7, *C*H$_2$), 33.0 (C22, *C*H$_2$), 31.8 (C16, *C*H$_2$), 31.6 (C21, *C*H$_2$), 30.4 (C31, *C*H$_2$), 28.0 (C23, *C*H$_3$), 27.8 (C15, *C*H$_2$), 25.0 (C12, *C*H$_2$), 22.2 (C2, *C*H$_2$), 20.9 (C11, *C*H$_2$), 19.0 (C29, *C*H$_3$), 18.1 (C6, *C*H$_2$), 16.2 (C24, *C*H$_3$), 16.1 (C26, *C*H$_3$), 16.0 (C25, *C*H$_3$), 15.2 (C27, *C*H$_3$) ppm; MS (ESI, MeOH): *m/z* = 523.3 (10% $[M+H]^+$), 540.3 (47% $[M+Na]^+$), 576.8 (75% $[M+Na+MeOH]^+$), 1067.2 (100% $[2M+Na]^+$). Anal. Calcd for $C_{35}H_{54}O_3$ (522.80): C, 80.41; H, 10.41. Found: C, 80.31; H, 10.62.

5.1.14. (*R*)-4-[3β-Acetoxy-28-norlup-20(29)-en-17β-yl]-2-methylene-γ-butyrolactone (13)

Following GP1, **13** (0.60 g, 80%) was obtained from 3-*O*-acetylbetulinaldehyde **9** (0.65 g, 1.35 mmol) as a colourless solid; mp 245–247 °C; $[\alpha]_D$ −5.1 (*c* 3.3, $CHCl_3$); R_f 0.50 (silica gel, hexane–ethyl acetate, 8:2); IR (KBr): *v* = 2941s, 2873m, 1762s, 1730s, 1466m, 1374m, 1335w, 1284m, 1247s, 1195m, 1132m, 1108m, 1028m cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 6.23 (dd, 1H, *J* = 2.9, 2.9 Hz, CH_a (34)), 5.62 (dd, 1H, *J* = 2.5, 2.1 Hz, CH_b (34)), 4.94 (dd, 1H, *J* = 7.9, 7.9 Hz, C*H* (28)), 4.75 (d, 1H, *J* = 2.1 Hz, CH_a (30)), 4.58–4.54 (m, 1H, CH_b (30)), 4.45 (dd, 1H, *J* = 10.8, 5.4 Hz, C*H*OAc (3)), 2.94–2.83 (m, 2H, CH_a (31)+C*H* (19)), 2.76–2.67 (m, 1H, CH_b (31)), 2.02 (s, 3H, Ac), 2.06 (ddd, 1H, *J* = 12.2, 12.2, 3.8 Hz, C*H* (13)), 1.96–1.86 (m, 1H, CH_a (21)), 1.79–1.55 (m, 8H, CH_a (12)+C*H* (18)+CH_a (1)+CH_a (16)+CH_a (22)+CH_2 (2)+CH_a (6)), 1.53 (s, 3H, CH_3 (29)), 1.53–1.10 (m, 11H, CH_a (15)+CH_b (22)+CH_2 (11)+CH_b (6)+CH_2 (7)+CH_b (12)+CH_b (16)+C*H* (9)+CH_b (21)) 1.08–0.92 (m, 1H, CH_b (15)), 1.00 (s, 6H, CH_3 (27)+CH_3 (25)), 0.84 (s, 3H, CH_3 (23)), 0.92–0.88 (m, 1H, CH_b (1)), 0.83 (s, 3H, CH_3 (26)), 0.82 (s, 3H, CH_3 (24)), 0.78 (d, 1H, *J* = 9.5 Hz, C*H* (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 170.8 (*C*=O), 170.4 (*C*=O), 150.2 (C20, *C*=CH$_2$), 134.7 (C32, *C*=CH$_2$), 121.5 (C34, *C*H$_2$=C), 110.1 (C30, *C*H$_2$=C), 80.9 (C3, *C*HOAc), 78.1 (C28, *C*H), 55.3 (C5, *C*H), 50.3 (C9, *C*H), 49.7 (C17, $C_{quart.}$), 49.6 (C18, *C*H), 47.8 (C19, *C*H), 43.0 (C14, $C_{quart.}$), 41.1 (C8, $C_{quart.}$), 38.4 (C1, *C*H$_2$), 37.8 (C4, $C_{quart.}$), 37.1 (C10, $C_{quart.}$), 37.0 (C13, *C*H), 34.3 (C7, *C*H$_2$), 33.1 (C22, *C*H$_2$), 31.9 (C16, *C*H$_2$), 31.7 (C21, *C*H$_2$), 30.5 (C31, *C*H$_2$), 28.0 (C23, *C*H$_3$), 27.9 (C15, *C*H$_2$), 25.1 (C12, *C*H$_2$), 23.8 (C2, *C*H$_2$), 20.9 (C11, *C*H$_2$), 21.3 (Ac), 18.8 (C29, *C*H$_3$), 18.2 (C6, *C*H$_2$), 16.5 (C26, *C*H$_3$), 16.3 (C25, *C*H$_3$), 16.2 (C24, *C*H$_3$), 15.3 (C27, *C*H$_3$) ppm; MS (ESI, MeOH): *m/z* = 551.2 (30% $[M+H]^+$), 1123.4 (100% $[2M+Na]^+$). Anal. Calcd for $C_{36}H_{54}O_4$ (550.81): C, 78.50; H, 9.88. Found: C, 78.41; H, 10.11.

5.1.15. (*R*)-4-[3β-Hydroxy-28-norlup-20(29)-en-17β-yl]-γ-butyrolactone (14)

Following GP3, **14** (0.32 g, 47%) was obtained from aldehyde **1** (0.60 g, 1.36 mmol) as a colourless solid; mp 190–192 °C; $[\alpha]_D$ −9.6 (*c* 4.2, $CHCl_3$); R_f 0.17 (silica gel, hexane–ethyl acetate, 8:2); IR (KBr): *v* = 3432s, 2942s, 2872m, 2221w, 1767s, 1641w, 1454w, 1376w, 1352m, 1224m, 1191s, 1142m, 1109w, 1033w cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 4.90 (dd, 1H, *J* = 6.6, 10.0 Hz, C*H* (28)), 4.73 (d, 1H, *J* = 2.1 Hz, CH_a (30)), 4.54 (dd, 1H, *J* = 2.1, 1.6 Hz, CH_b (30)), 3.16 (dd, 1H, *J* = 11.4, 4.8 Hz, C*H*OH (3)), 2.79 (ddd, 1H, *J* = 12.0, 10.4, 6.6 Hz, C*H* (19)), 2.53–2.46 (m, 2H, CH_2 (32)), 2.21–2.11 (m, 1H, CH_a (31)), 2.03 (ddd, 1H, *J* = 12.2, 12.2, 3.8 Hz, C*H* (13)), 2.00–1.88 (m, 2H, CH_b (31)+CH_a (21)), 1.76–1.62 (m, 5H, C*H* (18)+CH_a (12)+CH_a (16)+CH_a (1)+CH_a (22)), 1.66 (s, 3H, CH_3 (29)), 1.61–1.36 (m, 9H, CH_2 (2)+CH_2 (6)+CH_a (11)+CH_a (15)+CH_b (22)+CH_b (16)+CH_a (7)), 1.36–1.09 (m, 5H, CH_b (7)+CH_b (21)+CH_b (11)+C*H* (9)+CH_b (12)), 1.06–1.01 (m, 1H, CH_b (15)), 1.01 (s, 3H, CH_3 (27)), 1.00 (s, 3H, CH_3 (25)), 0.95 (s, 3H, CH_3 (23)), 0.91–0.85 (m, 1H, CH_b (1)), 0.82 (s, 3H, CH_3 (26)), 0.75 (s, 3H, CH_3 (24)), 0.66 (d, 1H, *J* = 9.5 Hz, C*H* (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.5 (*C*=O), 150.2 (C20, *C*=CH$_2$), 110.0 (C30, *C*H$_2$=C), 81.3 (C28, *C*H), 78.9 (C3, *C*HOH), 55.3 (C5, *C*H), 50.3 (C9, *C*H), 49.4 (C18, *C*H), 49.2 (C17, $C_{quart.}$), 47.8 (C19, *C*H), 42.8 (C14, $C_{quart.}$), 40.9 (C8, $C_{quart.}$), 38.8 (C4, $C_{quart.}$), 38.7 (C1, *C*H$_2$), 37.1 (C10, $C_{quart.}$), 36.9 (C13, *C*H), 34.2 (C7, *C*H$_2$), 32.7 (C22, *C*H$_2$), 32.1 (C16, *C*H$_2$), 31.6 (C21, *C*H$_2$), 28.6 (C32, *C*H$_2$), 28.0 (C23, *C*H$_3$), 27.8 (C15, *C*H$_2$), 27.3 (C2, *C*H$_2$), 24.9 (C12, *C*H$_2$), 24.5 (C31, *C*H$_2$), 20.8 (C11, *C*H$_2$), 18.8 (C29, *C*H$_3$), 18.2 (C6, *C*H$_2$), 16.2 (C26, *C*H$_3$), 16.1 (C25, *C*H$_3$), 15.3 (C24, *C*H$_3$), 15.2 (C27, *C*H$_3$) ppm; MS (ESI, MeOH): *m/z* = 497.4 (20% $[M+H]^+$), 1015.4 (100% $[2M+Na]^+$). Anal. Calcd for $C_{33}H_{52}O_3$ (496.76): C, 79.79; H, 10.55. Found: C, 79.62; H, 10.73.

5.1.16. (*R*)-4-[3β-Methoxy-28-norlup-20(29)-en-17β-yl]-γ-butyrolactone (15)

Following GP3, **15** (0.38 g, 56%) was obtained from **7** (0.60 g, 1.32 mmol) as a colourless solid; mp 231–234 °C; $[\alpha]_D$ −0.6 (*c* 3.84, $CHCl_3$); R_f 0.55 (silica gel, hexane–ethyl acetate, 8:2); IR (KBr): *v* = 3441m, 2943s, 1777m, 1639w, 1456m, 1454m, 1385m, 1356m, 1183m, 1140w, 1100m, 1020w cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 4.90 (dd, 1H, *J* = 6.2, 10.0 Hz, C*H* (28)), 4.74 (d, 1H, *J* = 2.1 Hz, CH_a (30)), 4.57 (dd, 1H, *J* = 1.7, 1.2 Hz, CH_b (30)), 3.33 (s, 1H, OCH_3), 2.83–2.74 (m, 1H, C*H* (19)), 2.61 (dd, 1H, *J* = 11.6, 4.2 Hz, C*H*OCH$_3$ (3)), 2.53–2.46 (m, 2H, CH_2 (32)), 2.20–2.11 (m, 1H, CH_a (31)), 2.04 (ddd, 1H, *J* = 12.0, 12.0, 3.3 Hz, C*H* (13)), 2.00–1.88 (m, 2H, CH_b (31)+CH_a (21)), 1.80–1.60 (m, 6H, CH_a (2)+CH_a (12) +C*H* (18)+ CH_a (1)+CH_a (16)+CH_a (22)), 1.67 (s, 3H, CH_3 (29)), 1.51–1.20 (m, 12H, CH_2 (6)+CH_2 (11)+CH_b (22)+CH_a (15)+CH_b (2)+CH_2 (7)+CH_b (21)+CH_b (12)+ C*H* (9)), 1.19–1.12 (m, 1*H*, CH_b (16)), 1.05–0.99 (m, 1H, CH_b (15)), 1.00 (s, 6H, 2 × CH_3 (25+27)), 0.93 (s, 3H, CH_3 (23)), 0.86–0.77 (m, 1H, CH_b (1)), 0.82 (s, 3H, CH_3 (26)), 0.73 (s, 3H, CH_3 (24)), 0.66 (d, 1H, *J* = 9.5 Hz, C*H* (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.5 (*C*=O), 150.4 (C20, *C*=CH$_2$), 110.1 (C30, *C*H$_2$=C), 88.6 (C3, *C*HOCH$_3$), 81.3 (C28, *C*H), 57.5 (OCH$_3$), 55.8 (C5, *C*H), 50.4 (C9, *C*H), 49.4 (C18, *C*H), 49.2 (C17, $C_{quart.}$), 47.9 (C19, *C*H), 42.8 (C14, $C_{quart.}$), 41.0 (C8, $C_{quart.}$), 38.8 (C1, *C*H$_2$), 38.6 (C4, $C_{quart.}$), 37.2 (C10, $C_{quart.}$), 37.0 (C13, *C*H), 34.3 (C7, *C*H$_2$), 32.7 (C22, *C*H$_2$), 32.1 (C16, *C*H$_2$), 31.6 (C21, *C*H$_2$), 28.6 (C32, *C*H$_2$), 28.0 (C23, *C*H$_3$), 27.8 (C15, *C*H$_2$), 25.0 (C12, *C*H$_2$), 24.5 (C31, *C*H$_2$), 22.2 (C2, *C*H$_2$), 20.9 (C11, *C*H$_2$), 19.0 (C29, *C*H$_3$), 18.1 (C6, *C*H$_2$), 16.2 (C24, *C*H$_3$), 16.1 (C26, *C*H$_3$), 16.1 (C25, *C*H$_3$), 15.2 (C27, *C*H$_3$) ppm; MS (ESI, MeOH): *m/z* = 511.3 (5% $[M+H]^+$), 528.4 (47% $[M+Na]^+$), 565.0 (75% $[M+Na+MeOH]^+$), 1043.4 (100%

[2M+Na]$^+$). Anal. Calcd for $C_{34}H_{54}O_3$ (510.79): C, 79.95; H, 10.66. Found: C, 79.77; H, 10.81.

5.1.17. (*R*)-4-[3β-Acetoxy-28-norlup-20(29)-en-17β-yl]-γ-butyrolactone (16)

Following GP3, **16** (0.35 g, 52%) was obtained from **9** (0.60 g, 1.24 mmol) as a colourless solid; mp 242–246 °C; $[\alpha]_D$ −4.9 (*c* 5.4, $CHCl_3$); R_f 0.39 (silica gel, hexane–ethyl acetate, 8:2); IR (KBr): ν = 2944s, 2872m, 1766s, 1735s, 1641m, 1459m, 1393m, 1369m, 1250s, 1187m, 1142w, 1073w, 1019m cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 4.90 (dd, 1H, *J* = 6.6, 10.0 Hz, C*H* (28)), 4.73 (d, 1H, *J* = 2.1 Hz, CH_a (30)), 4.57 (dd, 1H, *J* = 2.1, 1.6 Hz, CH_b (30)), 4.45 (dd, 1H, *J* = 11.4, 4.8 Hz, C*H*OAc (3)), 2.77 (ddd, 1H, *J* = 12.0, 10.4, 6.6 Hz, C*H* (19)), 2.51–2.45 (m, 2H, CH_2 (32)), 2.19–2.11 (m, 1H, CH_a (31)), 2.01 (s, 3H, Ac), 2.08–1.86 (m, 3H, C*H* (13)+CH_b (31)+CH_a (21)), 1.76–1.52 (m, 7H, C*H* (18)+CH_a (12)+CH_a (16)+CH_a (1)+CH_a (22)+CH_2 (2)), 1.66 (s, 3H, CH_3 (29)), 1.51–1.09 (m, 12H, CH_2 (6)+CH_2 (11)+CH_b (22)+CH_a (15)+CH_2 (7)+CH_b (21)+C*H* (9)+CH_b (12)+CH_b (16)), 1.02–0.95 (m, 1H, CH_b (15)), 1.00 (s, 3H, CH_3 (25)), 0.99 (s, 3H, CH_3 (27)), 0.84 (s, 3H, CH_3 (23)), 0.91–0.80 (m, 1H, CH_b (1)), 0.82 (s, 3H, CH_3 (26)), 0.81 (s, 3H, CH_3 (24)), 0.78 (d, 1H, *J* = 9.5 Hz, C*H* (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.4 (*C*=O), 171.0 (*C*=O), 150.3 (C20, *C*=CH$_2$), 110.1 (C30, *C*H$_2$=C), 81.3 (C28, *C*H), 80.8 (C3, *C*HOAc), 55.3 (C5, *C*H), 50.2 (C9, *C*H), 49.4 (C18, *C*H), 49.2 (C17, $C_{quart.}$), 47.8 (C19, *C*H), 42.8 (C14, $C_{quart.}$), 40.9 (C8, $C_{quart.}$), 38.3 (C1, *C*H$_2$), 37.8 (C4, $C_{quart.}$), 37.0 (C10, $C_{quart.}$), 36.9 (C13, *C*H), 34.1 (C7, *C*H$_2$), 32.7 (C22, *C*H$_2$), 32.1 (C16, *C*H$_2$), 31.6 (C21, *C*H$_2$), 28.5 (C32, *C*H$_2$), 27.9 (C23, *C*H$_3$), 27.8 (C15, *C*H$_2$), 24.8 (C12, *C*H$_2$), 24.5 (C31, *C*H$_2$), 23.6 (C2, *C*H$_2$), 21.2 (Ac, *C*H$_3$), 20.8 (C11, *C*H$_2$), 18.8 (C29, *C*H$_3$), 18.2 (C6, *C*H$_2$), 16.4 (C24, *C*H$_3$), 16.2 (C25, *C*H$_3$), 16.1 (C26, *C*H$_3$), 15.1 (C27, *C*H$_3$) ppm; MS (ESI, MeOH): *m/z* = 538.4 (30% [M+H]$^+$), 557.4 (100% [M+Na]$^+$). Anal. Calcd for $C_{35}H_{54}O_4$ (538.80): C, 78.02; H, 10.10. Found: C, 77.83; H, 10.21.

5.1.18. Methyl (28*S*)-3-[3β,28-dihydroxy-lup-20(29)-en-28-yl]-propiolate (17)

Following GP4, **17** (0.36 g, 67%) was obtained from betulinic aldehyde **1** (0.47 g, 1.03 mmol) as a colourless solid; mp 139–142 °C; $[\alpha]_D$ +5.8 (*c* 4.3, $CHCl_3$); R_f 0.28 (silica gel, hexane–ethyl acetate, 8:2); IR (KBr): ν = 3423br, 2943s, 2870m, 2231m, 1717s, 1640w, 1455m, 1435m, 1377m, 1252s, 1139w, 1108w, 1070w, 1033m cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.00 (s, 1H, C*H* (28)), 4.67 (br s, 1H, CH_a (30)), 4.56 (br s, 1H, CH_b (30), 3.76 (s, 3H, OCH_3), 3.18 (dd, 1H, *J* = 11.4, 4.8 Hz, C*H*OH (3)), 2.86 (ddd, 1H, *J* = 11.2, 10.4, 7.0 Hz, C*H* (19)), 2.07–1.85 (m, 4H, CH_a (16)+CH_a (21)+CH_a (22)+C*H* (13)), 1.78–1.45 (m, 7H, C*H* (18)+CH_a (12)+CH_a (1)+CH_2 (2)+CH_a (6)+CH_a (15)), 1.66 (s, 3H, CH_3 (29)), 1.45–1.09 (m, 10H, CH_b (6)+CH_b (21)+CH_b (22)+CH_b (16)+CH_2 (7)+C*H* (9)+CH_2 (11)+CH_b (12)), 1.05–0.98 (m, 1H, CH_b (15)), 1.01 (s, 3H, CH_3 (27)), 0.99 (s, 3H, CH_3 (25)), 0.95 (s, 3H, CH_3 (23)), 0.81 (s, 3H, CH_3 (26)), 0.75 (s, 3H, CH_3 (24)), 0.68 (d, 1H, *J* = 9.5 Hz, C*H* (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 153.8 (*C*=O), 150.6 (C20, *C*=CH$_2$), 109.7 (C30, *C*H$_2$=C), 88.3 (C31, *C*≡C), 78.9 (C3, *C*HOH), 77.8 (C32, *C*≡C), 66.0 (C28, *C*H), 55.3 (C5, *C*H), 52.7 (OMe), 51.0 (C17, $C_{quart.}$), 50.2 (C9, *C*H), 49.0 (C18, *C*H), 48.5 (C19, *C*H), 43.0 (C14, $C_{quart.}$), 40.9 (C8, $C_{quart.}$), 38.8 (C1, *C*H$_2$), 38.7 (C4, $C_{quart.}$), 37.4 (C13, *C*H), 37.1 (C10, $C_{quart.}$), 34.2 (C7+C16, 2 × *C*H$_2$), 34.0 (C22, *C*H$_2$), 32.2 (C21, *C*H$_2$), 28.0 (C23, *C*H$_3$), 27.8 (C15, *C*H$_2$), 27.4 (C2, *C*H$_2$), 25.0 (C12, *C*H$_2$), 20.8 (C11, *C*H$_2$), 18.8 (C29, *C*H$_3$), 18.2 (C6, *C*H$_2$), 16.1 (C26, *C*H$_3$), 16.0 (C25, *C*H$_3$), 15.3 (C24, *C*H$_3$), 15.1 (C27, *C*H$_3$) ppm; MS (ESI, MeOH): *m/z* = 524.4 (10% [M+H]$^+$), 1071.4 (100% [2M+Na]$^+$). Anal. Calcd for $C_{34}H_{52}O_4$ (524.77): C, 77.82; H, 9.99. Found: C, 77.67; H, 10.17.

5.1.19. Methyl (28*S*)-3-[3β-methoxy-28-hydroxy-lup-20(29)-en-28-yl]-propiolate (18)

Following GP4, **18** (0.40 g, 72%) was obtained from **7** (0.47 g, 1.03 mmol) as a colourless solid; mp 235–237 °C; $[\alpha]_D$ +21.7 (*c* 3.52, $CHCl_3$); R_f 0.67 (silica gel, hexane–ethyl acetate, 8:2); IR (KBr): ν = 3518 m, 3078w, 2955s, 2842m, 2228m, 1693s, 1649m, 1454m, 1433m, 1391m, 1376m, 1357w, 1244m, 1180m, 1098m, 1064m, 1041m, 1017w cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.99 (s, 1H, C*H* (28)), 4.68 (d, 1H, *J* = 2.1 Hz, CH_a (30)), 4.57 (dd, 1H, *J* = 2.1, 1.7 Hz, CH_b (30)), 3.77 (s, 1H, OCH_3), 3.33 (s, 1H, OCH_3), 2.86 (ddd, 1H, *J* = 11.2, 10.8, 5.8 Hz, C*H* (19)), 2.61 (dd, 1H, *J* = 11.6, 4.2 Hz, C*H*OCH$_3$ (3)), 2.06–1.84 (m, 4H, CH_a (16)+CH_a (21)+CH_a (22)+C*H* (13)), 1.79–1.67 (m, 4H, CH_a (2)+C*H* (18)+CH_a (12) +CH_a (1)), 1.66 (s, 3H, CH_3 (29)), 1.57–1.23 (m, 12H, CH_a (15)+CH_2 (6)+CH_2 (11)+CH_b (22)+ CH_b (2)+CH_2 (7)+CH_b (21)+ CH_b (16)+C*H* (9)), 1.19–1.11 (m, 1H, CH_b (12)), 1.05–1.02 (m, 1H, CH_b (15)), 1.01 (s, 3H, CH_3 (25)), 0.98 (s, 3H, CH_3 (27)), 0.94 (s, 3H, CH_3 (23)), 0.85–0.77 (m, 1H, CH_b (1)), 0.82 (s, 3H, CH_3 (26)), 0.73 (s, 3H, CH_3 (24)), 0.66 (d, 1H, *J* = 9.1 Hz, C*H* (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 153.8 (*C*=O), 150.7 (C20, *C*=CH$_2$), 109.8 (C30, *C*H$_2$=C), 88.6 (C3, *C*HOCH$_3$), 88.3 (C31, *C*≡C), 77.8 (C32, *C*≡C), 66.0 (C28, *C*H), 57.5 (OCH_3), 55.8 (C5, *C*H), 52.7 (OCH_3), 51.0 (C17, $C_{quart.}$), 50.3 (C9, *C*H), 49.0 (C18, *C*H), 48.5 (C19, *C*H), 43.0 (C14, $C_{quart.}$), 40.9 (C8, $C_{quart.}$), 38.8 (C1, *C*H$_2$), 38.6 (C4, $C_{quart.}$), 37.4 (C13, *C*H), 37.1 (C10, $C_{quart.}$), 34.2 (C7+C16, 2 × *C*H$_2$), 34.0 (C22, *C*H$_2$), 32.3 (C21, *C*H$_2$), 28.0 (C23, *C*H$_3$), 27.8 (C15, *C*H$_2$), 25.1 (C12, *C*H$_2$), 22.2 (C2, *C*H$_2$), 20.8 (C11, *C*H$_2$), 18.8 (C29, *C*H$_3$), 18.1 (C6, *C*H$_2$), 16.2 (C24, *C*H$_3$), 16.1 (C26, *C*H$_3$), 16.0 (C25, *C*H$_3$), 15.1 (C27, *C*H$_3$) ppm; MS (i.e., 70 eV): *m/z* (%) = 538 (12), 425 (94), 393 (62), 221 (38), 203 (57), 189 (100), 135 (46). Anal. Calcd for $C_{35}H_{54}O_4$ (538.80): C, 78.02; H, 10.10. Found: C, 77.86; H, 10.24.

5.1.20. Methyl (28*S*)-3-[3β-acetoxy-28-hydroxy-lup-20(29)-en-28-yl]-propiolate (19)

Following GP4, **19** (0.43 g, 73%) was obtained from **9** (0.50 g, 1.03 mmol) as a colourless solid; mp 210–212 °C; $[\alpha]_D$ +18.5 (*c* 4.5, $CHCl_3$); R_f 0.55 (silica gel, hexane–ethyl acetate, 8:2); IR (KBr): ν = 3475s, 2955s, 2870 m, 2228m, 1713s, 1638w, 1455m, 1431m, 1380m, 1250s, 1155w, 1107w, 1066m, 1049m, 1033m cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 4.98 (s, 1H, C*H* (28)), 4.67 (br s, 1H, CH_a (30)), 4.56 (br s, 1H, CH_b (30)), 4.44 (dd, 1H, *J* = 11.4, 4.8 Hz, C*H*OAc (3)), 3.76 (s, 3H, OCH_3), 2.85 (ddd, 1H, *J* = 11.2, 10.4, 7.0 Hz, C*H* (19)), 2.04–1.80 (m, 4H, CH_a (16)+CH_a (21)+CH_a (22)+C*H* (13)), 2.02 (s, 3H, Ac), 1.65 (s, 3H, CH_3 (29)), 1.78–1.45 (m, 8H, C*H* (18)+CH_a (12)+CH_a (1)+CH_2 (2)+CH_a (6)+CH_a (15)+CH_a (11)), 1.45–1.09 (m, 10H, CH_b (6)+CH_b (21)+CH_b (22)+CH_b (16)+CH_2 (7)+C*H* (9)+CH_b (11)+CH_b (12)+CH_b (15)), 1.04–0.86 (m, 1H, CH_b (1)), 1.00 (s, 3H, CH_3 (25)), 0.97 (s, 3H, CH_3 (27)), 0.83 (s, 6H, CH_3 (26)), 0.82 (s, 6H, CH_3 (23)), 0.81 (s, 3H, CH_3 (24)), 0.77 (d, 1H, *J* = 9.5 Hz, C*H* (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 171.0 (*C*=O), 153.8 (*C*=O), 150.6 (C20, *C*=CH$_2$), 109.8 (C30, *C*H$_2$=C), 88.3 (C31, *C*≡C), 80.9 (C3, *C*HOAc), 77.7 (C32, *C*≡C), 66.0 (C28, *C*H), 55.3 (C5, *C*H), 52.8 (OMe), 51.0 (C17, $C_{quart.}$), 50.3 (C9, *C*H), 49.0 (C18, *C*H), 48.6 (C19, *C*H), 43.0 (C14, $C_{quart.}$), 40.9 (C8, $C_{quart.}$), 38.3 (C1, *C*H$_2$), 37.8 (C4, $C_{quart.}$), 37.4 (C13, *C*H), 37.1 (C10, $C_{quart.}$), 34.2 (C7, *C*H$_2$), 34.1 (C16, *C*H$_2$), 34.0 (C22, *C*H$_2$), 32.2 (C21, *C*H$_2$), 27.9 (C23, *C*H$_3$), 27.8 (C15, *C*H$_2$), 25.0 (C12, *C*H$_2$), 23.7 (C2, *C*H$_2$), 21.3 (Ac, *C*H$_3$), 20.8 (C11, *C*H$_2$), 18.8 (C29, *C*H$_3$), 18.1 (C6, *C*H$_2$), 16.5 (C24, *C*H$_3$), 16.0 (C25+C26, 2x*C*H$_3$), 15.1 (C27, *C*H$_3$) ppm; MS (i.e., 70 eV):

m/z (%) = 566 (12), 453 (100), 393 (61), 203 (19), 189 (24). Anal. Calcd for $C_{36}H_{54}O_5$ (566.81): C, 76.28; H, 9.60. Found: C, 76.17; H, 9.75.

5.1.21. (*R*)-4-[3β-Hydroxy-28-norlup-20(29)-en-17β-yl]-2-butenolide (20)

Following GP5, **20** (0.17 g, 90%) was obtained from **17** (0.20 g, 0.38 mmol) as a colourless solid; mp 106–109 °C; $[\alpha]_D$ +13.5 (*c* 2.5, $CHCl_3$); R_f 0.36 (silica gel, hexane–ethyl acetate, 8:2); IR (KBr): ν = 3446m, 2942m, 2870m, 1753m, 1706m, 1640m, 1454m, 1377m, 1210m, 1098m, 1035m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.53 (dd, 1H, *J* = 5.8, 1.1 Hz, C*H* (31)), 6.19 (dd, 1H, *J* = 5.8, 2.1 Hz, C*H* (32)), 5.51 (br s, 1H, C*H* (28)), 4.76 (d, 1H, *J* = 1.5 Hz, CH_a (30)), 4.59 (dd, 1H, *J* = 2.2, 1.5 Hz, CH_b (30)), 3.18 (dd, 1H, *J* = 11.5, 4.8 Hz, C*H*OH (3)), 2.90 (ddd, 1H, *J* = 11.6, 10.7, 6.1 Hz, C*H* (19)), 2.12 (ddd, 1H, *J* = 12.1, 11.8, 3.4 Hz, C*H* (13)), 1.89–1.64 (m, 6H, CH_a (21)+CH_a (16)+CH_a (12)+C*H* (18)+CH_a (1)+CH_a (15)), 1.67 (s, 3H, CH_3 (29)), 1.61–1.05 (m, 15H, CH_2 (2)+CH_2 (6)+CH_b (16)+CH_2 (11)+CH_2 (7)+CH_b (21)+CH_2 (22)+C*H* (9)+CH_b (12)+CH_b (15)), 1.03 (s, 6H, 2 × CH_3 (25+27)), 0.96 (s, 3H, CH_3 (23)), 0.95–0.90 (m, 1H, CH_b (1)), 0.83 (s, 3H, CH_3 (26)), 0.75 (s, 3H, CH_3 (24)), 0.68 (dd, 1H, *J* = 11.6, 2.1 Hz, C*H* (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 173.2 (*C*=O), 155.4 (C31, *C*H=CH), 150.0 (C20, *C*=CH_2), 122.2 (C32, *C*H=CH), 110.2 (C30, *C*H_2=C), 84.7 (C28, *C*H), 78.9 (C3, *C*HOH), 55.3 (C5, *C*H), 50.3 (C9, *C*H), 49.8 (C17, $C_{quart.}$), 49.7 (C18, *C*H), 47.7 (C19, *C*H), 43.0 (C14, $C_{quart.}$), 40.9 (C8, $C_{quart.}$), 38.8 (C1, *C*H_2), 38.7 (C4, $C_{quart.}$), 37.3 (C13, *C*H), 37.1 (C10, $C_{quart.}$), 34.3 (C7, *C*H_2), 33.5 (C16, *C*H_2), 32.3 (C22, *C*H_2), 31.9 (C21, *C*H_2), 28.0 (C23, *C*H_3), 27.8 (C15, *C*H_2), 27.4 (C2, *C*H_2), 25.0 (C12, *C*H_2), 20.8 (C11, *C*H_2), 19.1 (C29, *C*H_3), 18.2 (C6, *C*H_2), 16.2 (C26, *C*H_3), 16.1 (C25, *C*H_3), 15.3 (C24, *C*H_3), 15.2 (C27, *C*H_3) ppm; MS (ESI, MeOH): *m/z* = 549.3 (50% $[M+Na+MeOH]^+$), 1011.4 (100% $[2M+Na]^+$), 1043.4 (50% $[2M+Na+MeOH]^+$). Anal. Calcd for $C_{33}H_{50}O_3$ (494.75): C, 80.11; H, 10.19. Found: 80.02; H, 10.23.

5.1.22. (*R*)-4-[3β-Methoxy-28-norlup-20(29)-en-17β-yl]-2-butenolide (21)

Following GP5, **21** (0.17 g, 92%) was obtained from **18** (0.20 g, 0.37 mmol) as a colourless solid; mp 259–261 °C; $[\alpha]_D$ +39.2 (*c* 2.3, $CHCl_3$); R_f 0.73 (silica gel, hexane–ethyl acetate, 8:2); IR (KBr): ν = 3482 m, 3074w, 2943s, 1703m, 1641w, 1439m, 1408w, 1391m, 1375m, 1231m, 1179m, 1100m, 1035w, 1019m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.53 (dd, 1H, *J* = 5.8, 1.3 Hz, C*H* (31)), 6.19 (dd, 1H, *J* = 5.8, 2.1 Hz, C*H* (32)), 5.51 (br s, 1H, C*H* (28)), 4.76 (d, 1H, *J* = 2.0 Hz, CH_a (30)), 4.60 (dd, 1H, *J* = 2.2, 1.3 Hz, CH_b (30)), 3.34 (s, 1H, C*H*OCH_3 (3)), 2.91 (ddd, 1H, *J* = 11.2, 10.4, 7.0 Hz, C*H* (19)), 2.12 (ddd, 1H, *J* = 12.2, 11.7, 3.6 Hz, C*H* (13)), 1.90–1.63 (m, 6H, CH_a (21)+CH_a (16)+C*H* (18)+CH_a (2)+CH_a (12)+CH_a (1)), 1.67 (s, 3H, CH_3 (29)), 1.57–1.05 (m, 15H, CH_2 (6)+CH_2 (11)+CH_b (2)+CH_2 (7)+CH_2 (22)+CH_b (21)+C*H* (9)+CH_b (12)+CH_2 (15)+CH_b (16)), 1.03 (s, 6H, 2 × CH_3 (25+27)), 0.94 (s, 3H, CH_3 (26)), 0.85–0.80 (m, 1H, CH_b (1)), 0.83 (s, 3H, CH_3 (23)), 0.74 (s, 3H, CH_3 (24)), 0.67 (dd, 1H, *J* = 11.6, 2.2 Hz, C*H* (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 173.2 (*C*=O), 155.4 (C31, *C*H=CH), 150.0 (C20, *C*=CH_2), 122.2 (C32, *C*H=CH), 110.2 (C30, *C*H_2=C), 88.5 (C3, *C*HOCH_3), 84.7 (C28, *C*H), 57.5 (OCH_3), 55.8 (C5, *C*H), 50.3 (C9, *C*H), 49.8 (C17, $C_{quart.}$), 49.7 (C18, *C*H), 47.7 (C19, *C*H), 43.0 (C14, $C_{quart.}$), 41.0 (C8, $C_{quart.}$), 38.8 (C1, *C*H_2), 38.5 (C4, $C_{quart.}$), 37.3 (C13, *C*H), 37.2 (C10, $C_{quart.}$), 34.3 (C7, *C*H_2), 33.5 (C16, *C*H_2), 32.3 (C22, *C*H_2), 31.9 (C21, *C*H_2), 28.0 (C23, *C*H_3), 27.8 (C15, *C*H_2), 25.0 (C12, *C*H_2), 22.2 (C2, *C*H_2), 20.8 (C11, *C*H_2), 19.1 (C29, *C*H_3), 18.1 (C6, *C*H_2), 16.2 (C24, *C*H_3), 16.1 (C25, *C*H_3), 16.0 (C26, *C*H_3), 15.2 (C27, *C*H_3) ppm; MS (ESI, MeOH): *m/z* = 563.4 (100% $[M+Na+MeOH]^+$), 1071.2 (80% $[2M+Na+MeOH]^+$). Anal. Calcd for $C_{34}H_{52}O_3$ (508.78): C, 80.26; H, 10.30. Found: C, 80.06; H, 10.45.

5.1.23. (*R*)-4-[3β-Acetoxy-28-norlup-20(29)-en-17β-yl]-2-butenolide (22)

Following GP5, **22** (0.17 g, 90%) was obtained from **19** (0.20 g, 0.35 mmol) as a colourless solid; mp 273–275 °C; $[\alpha]_D$ +0.4 (*c* 4.6, $CHCl_3$); R_f 0.42 (silica gel, hexane–ethyl acetate, 8:2); IR (KBr): ν = 3063w, 2952s, 2875 m, 1735s, 1639w, 1596w, 1466m, 1392m, 1370m, 1250s, 1190w, 1166m, 1095m, 1032m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.53 (dd, 1H, *J* = 5.6, 1.5 Hz, C*H* (31)), 6.18 (dd, 1H, *J* = 5.6, 2.2 Hz, C*H* (32)), 5.50 (s, 1H, C*H* (28)), 4.75 (br s, 1H, CH_a (30)), 4.60 (br s, 1H, CH_b (30)), 4.46 (dd, 1H, *J* = 11.4, 4.8 Hz, C*H*OAc (3)), 2.90 (ddd, 1H, *J* = 11.2, 10.4, 7.0 Hz, C*H* (19)), 2.12 (ddd, 1H, *J* = 12.3, 11.9, 3.7 Hz, C*H* (13)), 1.86–1.72 (m, 4H, CH_a (16)+CH_a (21)+C*H* (18)+CH_a (12)), 2.02 (s, 3H, Ac), 1.67 (s, 3H, CH_3 (29)), 1.70–1.48 (m, 6H, CH_a (1)+CH_2 (2)+CH_a (6)+CH_a (15)+CH_a (11)), 1.47–1.09 (m, 10H, CH_b (6)+CH_2 (7)+CH_b (21)+CH_2 (22)+CH_b (16)+C*H* (9)+CH_b (11)+CH_b (12)+CH_b (15)), 1.04–0.95 (m, 1H, CH_b (1)), 1.03 (s, 3H, CH_3 (25)), 1.02 (s, 3H, CH_3 (27)), 0.85 (s, 3H, CH_3 (26)), 0.84 (s, 3H, CH_3 (23)), 0.83 (s, 3H, CH_3 (24)), 0.79 (d, 1H, *J* = 9.5 Hz, C*H* (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 173.2 (*C*=O), 170.9 (*C*=O), 155.3 (C31, *C*H=CH), 150.0 (C20, *C*=CH_2), 122.2 (C32, *C*H=CH), 110.2 (C30, *C*H_2=C), 84.7 (C28, *C*H), 80.7 (C3, *C*HOAc), 55.3 (C5, *C*H), 50.2 (C9, *C*H), 49.8 (C17, $C_{quart.}$), 49.7 (C18, *C*H), 47.7 (C19, *C*H), 43.0 (C14, $C_{quart.}$), 40.9 (C8, $C_{quart.}$), 38.3 (C1, *C*H_2), 37.8 (C4, $C_{quart.}$), 37.2 (C13, *C*H), 37.0 (C10, $C_{quart.}$), 34.2 (C7, *C*H_2), 33.5 (C16, *C*H_2), 32.3 (C22, *C*H_2), 31.8 (C21, *C*H_2), 27.9 (C23, *C*H_3), 27.8 (C15, *C*H_2), 24.9 (C12, *C*H_2), 23.7 (C2, *C*H_2), 21.2 (Ac, *C*H_3), 20.8 (C11, *C*H_2), 19.1 (C29, *C*H_3), 18.1 (C6, *C*H_2), 16.4 (C24, *C*H_3), 16.2 (C25, *C*H_3), 16.1 (C26, *C*H_3), 15.2 (C27, *C*H_3) ppm; MS (ESI, MeOH): *m/z* = 537.3 (30% $[M+Na]^+$), 1095.3 (100% $[2M+Na]^+$). Anal. Calcd for $C_{35}H_{52}O_4$ (536.79): C, 78.31; H, 9.76. Found: C, 78.20; H, 9.97.

5.1.24. (3*S*,4*R*)-4-[3β-Hydroxy-28-norlup-20(29)-en-17β-yl]-3-nitromethyl-γ-butyrolactone (23)

To a solution of **22** (0.1 g, 0.2 mmol) in nitromethane (3 ml) DBU (6 mg, 0.04 mmol) was added and stirred at room temperature. When TLC revealed the absence of starting material, the solution was concentrated in vacuo and the residue purified by column chromatography (silica gel, hexane–ethyl acetate, 8:2). Compound **23** (0.1 g, 88%) was obtained as a colourless solid; mp 306–309 °C; $[\alpha]_D$ −0.5 (*c* 4.1, $CHCl_3$); R_f 0.17 (silica gel, hexane–ethyl acetate, 8:2); IR (KBr): ν = 2942s, 2870m, 1772s, 1725s, 1641w, 1555s, 1467w, 1378m, 1250s, 1178m, 1031m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 4.71 (d, 1H, *J* = 3.9 Hz, C*H* (28)), 4.68 (d, 1H, *J* = 1.9 Hz, CH_a (30)), 4.58 (dd, 1H, *J* = 1.9, 1.5 Hz, CH_b (30)), 4.47 (dd, 1H, *J* = 12.9, 5.6 Hz, NO_2CH_a), 4.47–4.41 (m, 2H, C*H*OAc (3)+NO_2CH_b), 3.14–3.06 (m, 1H, C*H* (31)), 2.84 (dd, 1H, *J* = 18.8, 10.5 Hz, CH_a (32)), 2.80 (ddd, 1H, *J* = 11.2, 10.4, 7.0 Hz, C*H* (19)), 2.43 (dd, 1H, *J* = 18.8, 4.6 Hz, CH_b (32)), 1.97 (s, 3H, Ac), 1.96 (ddd, 1H, *J* = 12.3, 11.9, 3.7 Hz, C*H* (13)), 1.82–1.70 (m, 2H, CH_a (21)+C*H* (18)), 1.61 (s, 3H, CH_3 (29)), 1.65–1.51 (m, 7H, CH_a (22)+CH_a (12)+CH_a (1)+CH_a (16)+CH_2 (2)+CH_a (15)), 1.47–1.07 (m, 11H, CH_b (22)+CH_2 (6)+CH_2 (11)+CH_2 (7)+CH_b (21)+CH_b (16)+C*H* (9)+CH_b (12)), 1.05–1.00 (ddd, 1H, *J* = 14.4, 4.0, 2.5 Hz, CH_b (15)), 0.95–0.87 (m, 1H, CH_b (1)), 0.96 (s, 3H, CH_3 (25)), 0.95 (s, 3H, CH_3 (27)), 0.80 (s, 3H, CH_3 (26)), 0.78 (s, 3H, CH_3 (23)), 0.77 (*s*, 3H, CH_3 (24)), 0.72 (d, 1H, *J* = 9.5 Hz, C*H* (5)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 174.1 (*C*=O), 170.9 (*C*=O), 149.9 (C20, *C*=CH_2), 110.3 (C30, *C*H_2=C), 82.2 (C28, *C*H), 80.7 (C3, *C*HOAc), 78.1 (C16, *C*H_2NO_2), 55.3 (C5, *C*H), 50.4 (C17, $C_{quart.}$), 50.1 (C9, *C*H), 49.8 (C18, *C*H), 47.1 (C19,

CH), 43.0 (C14, $C_{quart.}$), 41.0 (C8, $C_{quart.}$), 38.3 (C1, CH_2), 37.8 (C4, $C_{quart.}$), 37.0 (C10, $C_{quart.}$), 36.7 (C13, CH), 35.8 (C31, CH), 34.1 (C7, CH_2), 33.4 (C16, CH_2), 32.6 (C32, CH_2), 32.5 (C22, CH_2), 31.4 (C21, CH_2), 27.9 (C23, CH_3), 27.7 (C15, CH_2), 25.0 (C12, CH_2), 23.6 (C2, CH_2), 21.3 (Ac, CH_3), 20.7 (C11, CH_2), 19.1 (C29, CH_3), 18.0 (C6, CH_2), 16.4 (C24, CH_3), 16.1 (C25+C26, $2 \times CH_3$), 15.1 (C27, CH_3) ppm; MS (ESI, MeOH): $m/z = 537.3$ (30% $[M+Na]^+$), 1095.3 (100% $[2M+Na]^+$). Anal. Calcd for $C_{36}H_{55}NO_6$ (597.83): C, 72.33; H, 9.27; N, 2.34. Found: C, 72.21; H, 9.41; N, 2.29.

5.2. Biology

5.2.1. Cell lines and culture conditions

The cell lines 518A2, 8505C, A253, A2780, A431, A549, DLD-1, FaDu, HCT-116, HCT-8, HT-29, LIPO, MCF-7, SW1736 and SW480 were included in this study. Cultures were maintained as monolayer in RPMI 1640 (PAA Laboratories, Pasching, Germany) supplemented with 10% heat inactivated fetal bovine serum (Biochrom AG, Berlin, Germany) and penicillin/streptomycin (PAA Laboratories) at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

5.2.2. Cytotoxicity assay

The cytotoxic activities of our compounds were evaluated using the sulforhodamine-B (SRB) (Sigma–Aldrich) microculture colorimetric assay. In short, exponentially growing cells were seeded into 96-well plates on day 0 at the appropriate cell densities to prevent confluence of the cells during the period of experiment. After 24 h, the cells were treated with serial dilutions of the compounds (0–30 μM) for 96 h. The final concentration of DMSO or DMF as a solvent never exceeded 0.5%, which was shown to be non-toxic to the cells. The percentages of surviving cells compared to untreated controls were determined 96 h after the beginning of drug exposure. After 96 h of treatment, the supernatant medium from the 96 well plates was discarded and the cells were fixed with 10% TCA. For a thorough fixation, the plates were allowed to stand at 4 °C. After fixation, the cells were washed in a strip washer for four times with water using alternate dispensing and aspiration procedures. The plates were dyed with 100 μl of 0.4% SRB (sulforhodamine-B) for about 20 min. After dying, the plates were washed with 1% acetic acid to remove the dye and allowed to air dry overnight. Then 100 μl of 10 mM Tris base solution were added to each well and absorbance was measured at 570 nm using a 96 well plate reader (Tecan Spectra, Crailsheim, Germany). The IC_{50} was calculated from the semi-logarithmic dose–response curves.

5.2.3. Dye exclusion test

Apoptotic cell death was analyzed by trypan-blue dye (Sigma–Aldrich, Germany) on A431 and A2780 cell lines. The cell culture flasks with 70–80% confluence were treated with IC 90 doses of the compounds for 24 h. After treatment, the supernatant medium with floating cells was collected and centrifuged to collect the dead and apoptotic cells. The cell pellet was suspended in serum free media. Equal amounts of cell suspension and trypan-blue were mixed and analyzed by microscope. The viable cells exclude the dye and are colorless and cells whose cell membrane was destroyed are turning into blue. When there were more colorless cells than colored, death is characterized as apoptotic.

5.2.4. DNA fragmentation assay

Determination of apoptotic cell death was performed by DNA gel electrophoresis. Briefly, A431 and A2780 were treated with respective IC 90 doses of the compounds for 24 h. Floating cells (induced by drug exposure) were collected, washed with PBS and lysed with lysis buffer (100 mM Tris–HCL pH 8.0; 20 mM EDTA; 0.8% SDS) (all from Sigma Aldrich). Then they are treated with RNAse A at 37 °C for 2 h and proteinase K at 50 °C (both from Roche Diagnostics chemical company, Mannheim, Germany). DNA laddering was observed by running the samples on 2% agarose gel followed by ethidium bromide (Sigma–Aldrich) staining.

Acknowledgments

We like to thank Dr. Ralph Kluge for numerous ESI-MS measurements and Dr. Dieter Ströhl for the NMR measurements. The cell lines were kindly provided by Dr. Thomas Müller (Dept. of Haematology/Oncology, Univ. Halle).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.02.042.

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