# BETULIN-DERIVED COMPOUNDS AS INHIBITORS OF ALPHAVIRUS REPLICATION

*Leena Pohjala*[†,‡,⊥], *Sami Alakurtti*[§,||], *Tero Ahola*[‡], *Jari Yli-Kauhaluoma*[§], *Päivi Tammela*[†,*]

## Supporting Information

### Experimental details

[†] Centre for Drug Research, Faculty of Pharmacy, P.O. Box 56, FI-00014 University of Helsinki, Finland

[‡] Institute of Biotechnology, P.O. Box 56, FI-00014 University of Helsinki, Finland

[§] Division of Pharmaceutical Chemistry, Faculty of Pharmacy, P.O. Box 56, FI-00014 University of Helsinki, Finland

[⊥] Division of Pharmaceutical Biology, Faculty of Pharmacy, P.O. Box 56, FI-00014 University of Helsinki, Finland

[||] Technical Research Centre of Finland, VTT, P.O. Box 1000, FI-02044 VTT (Espoo), Finland

* Corresponding author: Päivi Tammela, Centre for Drug Research, Faculty of Pharmacy, P.O. Box 56 (Viikinkaari 5 E), FI-00014 University of Helsinki, Finland; phone: +358 9 191 59628, fax: +358 9 191 59138, e-mail: paivi.tammela@helsinki.fi

**Biological assays**

**Cells, viruses and solutions.** Baby hamster kidney BHK-21 cell line used for antiviral studies was purchased from the American Type Culture Collection (ATCC code CCL-10). The cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 8% fetal calf serum (FCS), 2% tryptose-broth phosphate, 1% L-glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin. Human hepatocellular Huh-7 cells were obtained as a gift from Prof. Ralf Bartenschlager (University of Heidelberg, Germany) and used for assaying the effects of betulin derivatives on cell viability. For Huh-7 cultures, DMEM-based medium containing 10% FCS, 1% non-essential amino acids, 1% L-glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin was used. The cultures were kept at 37 °C with 5% $CO_2$ atmosphere and 95% air humidity.

Recombinant SFV containing *Renilla reniformis* luciferase insertion (SFV-Rluc) was produced from the infectious clone SFV-RlucH2, which was a kind gift from Dr. Andres Merits (University of Tartu, Estonia). Respectively, wild type SIN stock was derived from the infectious clone TOTO1101, by linearization, *in vitro* transcription and RNA transfection into BHK cells using electroporation. The initial virus stocks were titrated and amplified in fresh BHK cells at 0.01 PFU (plaque forming units) /cell for 24 h. These working stocks were titrated and used in all the experiments. Minimum essential medium (MEM) supplemented with 0.2% bovine serum albumin and 10 mM HEPES (pH 7.0) was used for all infections.

All betulin derivatives and standard compounds were dissolved in dimethylsulfoxide (DMSO) and diluted in MEM - 0.2% BSA - 10 mM HEPES (pH 7.0) buffer. The final DMSO concentration in the experiments was 0.5%, and all the compounds were added to the cultures simultaneously to the virus inocula, unless otherwise stated in the text.

**Anti-SFV assay.** Inhibition of Semliki Forest virus (SFV) replication was assayed using a miniaturized and automated screening assay, essentially as described in Ref. 1. Briefly, confluent BHK cell cultures in 96-well plates were infected at low infection multiplicity (0.001 PFU/cell) using a recombinant marker virus strain SFV-Rluc containing *Renilla* luciferase insertion in the nonstructural protein coding region of its genome. EnduRen Live Cell Substrate (Promega) was added into cultures simultaneously to SFV-Rluc infection, and the luminometric signal resulting from luciferase reporter gene expression was read at 14 h post infection (Varioskan Flash, Thermo Fisher Scientific, Vantaa, Finland). 25 µM 3'-amino-3'-deoxyadenosine, which was a kind gift from Prof. Seppo Lapinjoki and Prof. Igor Mikhailopulo (University of Kuopio, Finland) was used as a positive control in the assay.

**Anti-SIN assay.** The inhibitory potency of selected betulin derivatives against another alphavirus, Sindbis virus, was assayed by determining viral RNA synthesis rate by radiolabeled uridine incorporation assay. The confluent BHK cell cultures in 35-mm dishes were infected with SIN stock dilution using MOI 0.001 PFU/cell. At 14 h post infection, the cultures were treated with 2 µg/mL actinomycin D to block host cell RNA synthesis, and 10 µCi of [$^3$H]-uridine (PerkinElmer Life Sciences) was added at 15 h post infections. After 1 h incubation at 37 °C, the cultures were washed three times with phosphate-buffered saline and lyzed using 1% sodium dodecyl sulphate (SDS) solution (V = 400 µL/well). The lysates were warmed up to 70 °C for 2 minutes and used for trichloroacetic acid precipitation on glassfiber filters (100 mL of lysate per each filter). The incorporated label was quantitated with scintillation counter (MicroBeta Trilux, PerkinElmer, Turku, Finland). 25 µM 3'-amino-3'-deoxyadenosine was used as a positive control in this assay as well.

**ATP assay.** The intracellular ATP level determination was used as a counter-screen to evaluate the harmful effects of test compounds on mammalian cell viability. DMSO-dissolved compounds were diluted in cell culture medium, and polymyxin B sulphate (7500 IU/mL, Promega) was used as positive control. After 24 h exposure, ATP levels were determined using CellTiter-Glo® Cell Viability assay (Promega) according to the manufacturer's instructions. The plates were first stabilized to room temperature and the assay reagent was added. After 10 min shaking, Varioskan Flash (Thermo Fisher Scientific, Vantaa, Finland) was used for the luminometric readout.

**Data analysis.** In all assays, the results were expressed as surviving fractions, a percentage of remaining luciferase activity, uridine incorporation or intracellular ATP content, compared to the untreated control. For the compounds selected for potency evaluation, antiviral $IC_{50}$ values were determined by fitting the results from dose-response studies into sigmoidal dose-response curves with GraphPad Prism 3.0 software. In all experiments, a minimum of three replicates of each sample was used, and assay quality parameters S/B, S/N and Z' were calculated for each assay plate in order to monitor the data robustness. Formulas to calculate the quality parameters[2] are as follows ($\mu$ = mean, $\sigma$ = standard deviation):

$$\text{signal-to-background S/B} = \mu_{signal}/\mu_{background}$$

$$\text{signal-to-noise S/N} = (\mu_{signal}-\mu_{background})/\sqrt{(\sigma_{signal}^2+\sigma_{background}^2)}$$

$$\text{signal window coefficient Z'} = 1-[(3\sigma_{signal}+3\sigma_{background})/(\mu_{signal}-\mu_{background})]$$

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**Synergism studies.** To study the potential synergistic inhibition of betulin-derived compounds with 3'-amino-3'-deoxyadenosine, the $IC_{50}$ values of three selected betulin derivatives were determined alone and in the presence of 0.5 µM, 2 µM, 5 µM, 20 µM and 50 µM nucleoside. The

$IC_{50}$ values were used to generate isobolograms, as described in Ref. 3. Here, the NE-SW diagonal of the graph, crossing the axes at (0,1) and (1,0), represents the Loewe additivity, i.e. the expected response for the given combination, and bending of the graph below the additivity diagonal is a hallmark of Loewe synergism. To analyze the intensity of synergism in individual concentration combinations, interaction indices (I) were calculated for each normalized surviving fraction resulting from a given combination, using the equation $I = D_1/D_{X1} + D_2/D_{X2}$, where $D_1$ and $D_2$ represent the concentrations of compounds 1 and 2 in the combination, and $D_{X1}$ and $D_{X2}$ represent the concentrations of drug 1 and 2 alone yielding the same response. The I values for the combinations that were calculable (excluding those leading to response in either upper or lower plateau of the dose-response curve) are presented in tables below.

**Table 1.** Interaction indices for (a) betulinic acid **13**, (b) 28-*O*-tetrahydropyranylbetulin **17** and (c) heterocycle **41** in combination with 3'-amino-3'-deoxyadenosine. $D_1$ represents the concentration of each betulin derivative and $D_2$ is the concentration of 3'-amino-3'-deoxyadenosine.

a.

| $D_1$ (μM) | $D_2$ (μM) | response (% luc left) | I |
|---|---|---|---|
| 10 | 0.5 | 67 | 0.96 |
| 50 | 0.5 | 5 | 1.12 |
| 20 | 2 | 65 | 1.27 |
| 50 | 2 | 6 | 1.16 |
| 0.08 | 5 | 45 | 0.28 |
| 0.4 | 5 | 39 | 0.25 |
| 2 | 5 | 49 | 0.35 |
| 10 | 5 | 40 | 0.52 |
| 0.08 | 20 | 22 | 0.52 |
| 2 | 20 | 35 | 0.88 |
| 10 | 20 | 27 | 0.99 |
| 0.08 | 50 | 18 | 1.23 |

b.

| $D_1$ (μM) | $D_2$ (μM) | response (% luc left) | I |
|---|---|---|---|
| 10 | 0.5 | 35 | 0.27 |
| 50 | 0.5 | 9 | 0.51 |
| 2 | 2 | 73 | 0.90 |
| 10 | 2 | 37 | 0.48 |
| 50 | 2 | 5 | 0.54 |
| 0.4 | 5 | 77 | 1.44 |
| 2 | 5 | 37 | 0.24 |
| 10 | 5 | 11 | 0.81 |
| 0.08 | 20 | 36 | 0.91 |
| 0.08 | 50 | 21 | 1.28 |

c.

| $D_1$ (μM) | $D_2$ (μM) | response (% luc left) | I |
|---|---|---|---|
| 2 | 0.5 | 72 | 0.82 |
| 10 | 0.5 | 41 | 0.26 |
| 2 | 2 | 88 | 1.50 |
| 10 | 2 | 28 | 0.25 |
| 0.4 | 5 | 47 | 0.27 |
| 2 | 5 | 23 | 0.18 |
| 0.08 | 20 | 27 | 0.81 |
| 0.4 | 20 | 21 | 0.53 |
| 0.08 | 50 | 13 | 0.94 |

**Administration time experiments.** The effect of administration time on the antiviral effect of betulin-derived compounds was studied in high-multiplicity infections: BHK cell cultures in 96-well plates were infected at 5 PFU/cell of SFV-Rluc. 50 μM betulinic acid **13**, 28-*O*-tetrahydropyranylbetulin **17**, 4-phenyl-1,2,4-triazolidine derivative **41** and 3'-amino-3'-deoxyadenosine were present in cultures either throughout the infection (0-5 h), during virus adsorption only (0-1 h), from 1 h to 5 h or from 2 h to 5 h. The luciferase activity in each sample was determined at 5 h using Renilla Luciferase Assay System (Promega). Briefly, the cultures were washed with PBS (2×10 μL) and lysed with provider's lysis buffer (500 μL), and the luciferase activity of each sample was determined by luminometric readout after mixing 20 μL of lysate with 100 μL of substrate solution. The data was normalized as described above.

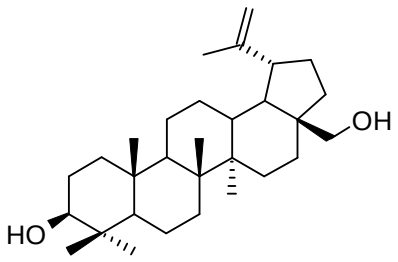
**Guanosine supplementation experiments.** The anti-SFV potency of betulin-derived compounds **13**, **17** and **41** as well as nucleoside analogue 3'-amino-3'-deoxyadenosine (3'-NH-3'-dAdo) and

ribavirin (Sigma-Aldrich) were evaluated in the presence of 50 µg/mL guanosine (Sigma-Aldrich). The experiment was carried out using both low (0.001 PFU/cell) and high (5 PFU/cell) multiplicity infections, where 50 µM betulin-derived compounds, 50 µM 3'-NH-3'-dAdo and 200 µM ribavirin were administered into the BHK cell cultures simultaneously to guanosine and SFV-Rluc virus inoculum. At the time of detection (5 h and 14 h post infection for high and low multiplicity infections, respectively), the cultures were lyzed and the luciferase activity was determined using Renilla Luciferase Assay System (Promega). The results were normalized using infected cultures treated with guanosine only as controls. According to the data obtained, none of the betulin-derived compound or the nucleoside analogue showed attenuated antiviral activity in the presence of guanosine supplementation. In contrast, ribavirin that is known to, among its other effects, act by depleting intracellular GTP pools, lost its anti-SFV activity when combined to guanosine supplementation.

**Chemistry**

Commercially available reagents were used without further purification and all of the solvents were HPLC grade. Anhydrous solvents were purchased from Sigma-Aldrich and used without further drying. All reactions in anhydrous solvents were performed in oven dried glassware under an inert atmosphere of dry argon or nitrogen. Thin layer chromatography (TLC) was performed on E. Merck Silica Gel 60 aluminium packed plates, with visualization accomplished by UV illumination and staining with 5% $H_2SO_4$ in MeOH. The $^1$H NMR spectra were measured on a Varian Mercury-VX 300 MHz or a Chemagnetics CMX 400 MHz spectrometer with chemical shifts reported as parts per million (in $CDCl_3$ at 23 °C, solvent peak at 7.26 ppm as an internal standard or in DMSO-$d_6$ at 23 °C, solvent peak at 2.50 ppm as an internal standard). The $^{13}$C NMR spectra were obtained on a Varian Mercury-VX 75 MHz or a Chemagnetics CMX 100 MHz

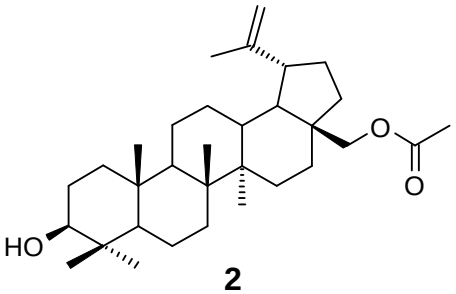
spectrometer with chemical shifts reported as parts per million (in $CDCl_3$ at 23 °C, solvent peak at 77.0 ppm as an internal standard or in DMSO-$d_6$ at 23 °C, solvent peak at 39.50 ppm as an internal standard). All the compounds had a purity of at least 95%. Purities of the intermediates and all tested compounds were determined by HPLC-MS and combustion analysis. HPLC-mass spectra were measured on a Bruker Daltonik Esquire-HPLC spectrometer, with XTerra MS RP18 column (4.6×30 mm, 2.5 µm) or on a JEOL JMS-AX505 (Tokyo, Japan) spectrometer with direct input and electron ionisation (EI). Elemental analyses were performed by Robertson Microlit Laboratories (Madison, NJ, USA). Melting points were obtained with a Sanyo Gallenkamp apparatus without correction. The Fourier transform infrared (FTIR) spectra were recorded on a Bruker Vertex 70 spectrometer with Pike MIRacle diamond crystal or with a Bruker Equinox 55 spectrometer including IRScope II and diamond anvil.




**1**

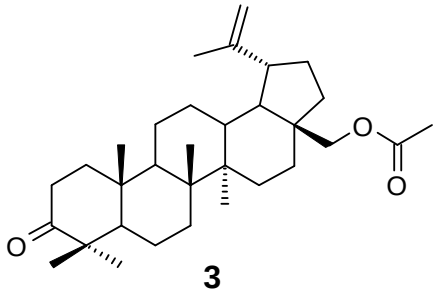
**Betulin (1).** Betulin **1** was isolated (95% purity) from the bark of birch (*Betula* sp.) by extraction and was obtained from UPM Kymmene (Lappeenranta, Finland). The crude betulin was recrystallized from 2-propanol/$H_2O$ azeotrope to give **1** as a white solid.[4] mp 252-253 °C; $R_f$ 0.2 (1:4 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$) δ 0.75 (s, 3H), 0.82 (s, 3H), 0.96 (s, 3H), 0.97 (s, 6H), 1.02 (s, 3H), 1.68 (s, 3H), 2.38 (m, 1H), 3.18 (dd, $J$ = 5.1, 10.8 Hz, 1H), 3.32 (d, $J$ = 10.8 Hz, 1H), 3.79 (d, $J$ = 10.8 Hz, 1H), 4.57 (s, 1H), 4.68 (s, 1H); $^{13}$C NMR (75 MHz, DMSO) δ 14.7, 15.3, 15.9, 16.1, 18.3, 19.1, 20.8, 25.2, 27.0, 27.3, 27.9, 29.1, 29.7, 33.9, 34.2, 37.1, 37.3, 38.7,

38.8, 40.9, 42.7, 47.7, 47.7, 48.7, 50.4, 55.2, 60.5, 79.0, 109.7, 150.4; FTIR (ν, $cm^{-1}$): 879, 1009, 1035, 1232, 1375, 1452, 1739, 2939, 3360; MS (direct, EI+): *m/z* 442; Anal. ($C_{30}H_{50}O_2$) C, H.



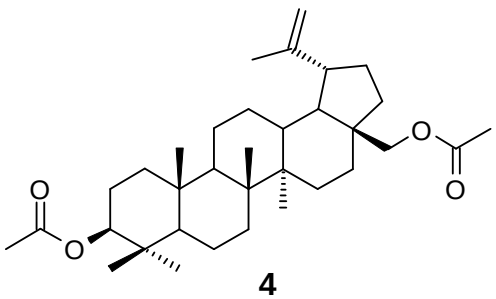

**2**

**28-*O*-Acetylbetulin (2).** To a mixture of **1** (8.0 g, 18 mmol) and 4-(dimethylamino)pyridine (0.8 g, 6.6 mmol) in $CH_2Cl_2$ (72 mL) was added pyridine (12 mL) and acetic anhydride (1.8 mL, 19 mmol). The reaction mixture was stirred at room temperature for 22 h. The organic layer was washed with 10% hydrochloric acid (80 mL), water (80 mL), saturated aqueous $NaHCO_3$ solution (80 mL), water (80 mL) and dried with anhydrous $Na_2SO_4$. Solvent was evaporated *in vacuo*, and the light brown residue was purified by flash chromatography on $SiO_2$ using hexane:EtOAc (85:15) as an eluent to give **7** (3.8 g, 45 %) as a white solid. mp 198-200 °C; $R_f$ 0.3 1:4 (EtOAc:hexanes); $^1$H NMR (300 MHz, $CDCl_3$): δ 0.76 (s, 3H), 0.83 (s, 3H), 0.97 (s, 3H), 0.98 (s, 3H), 1.03 (s, 3H), 1.68 (s, 3H), 2.07 (s, 3H), 2.45 (m, 1H), 3.18 (dd, *J* = 5.7, 12.0, 1H), 3.86 (d, *J* = 12.7, 1H), 4.25 (dd, *J* = 2.0, 12.0, 1H), 4.59 (q, *J* = 1.5, 1H), 4.69 (d, *J* = 2.7, 1H)**;** $^{13}$C NMR (75 MHz, $CDCl_3$): δ 14.7, 15.3, 16.0, 16.1, 18.3, 19.1, 20.8, 21.0, 25.2, 27.0, 27.4, 28.0, 29.5, 29.7, 34.2, 34.5, 37.1, 37.5, 38.7, 38.8, 40.8, 42.7, 46.3, 47.7, 48.7, 50.3, 55.3, 62.8, 78.9, 109.8, 150.1, 171.6; FTIR (ν, $cm^{-1}$): 888, 983, 1043, 1259, 1720, 2940, 3560; MS (direct, EI+): *m/z* 484; Anal. ($C_{32}H_{52}O_3$) C, H.



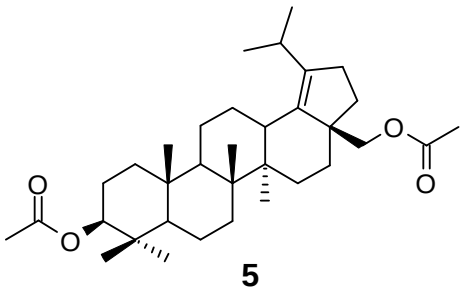
**3**

**28-*O*-Acetyl-3-oxobetulin (3).** A mixture of **2** (590 mg, 1.2 mmol) and pyridinium chlorochromate (1.3 g, 6.1 mmol) in $CH_2Cl_2$ (60 mL) was stirred at room temperature for 24 h.[5] The reaction mixture was diluted with $Et_2O$ (30 mL), stirred for 10 min, and the solids were filtered off. The filtrate was evaporated *in vacuo*, and the crude product was purified by flash chromatography ($Al_2O_3$) using *n*-hexane:EtOAc (2:1) as an eluent to give **3** (330 mg, 57%) as a white solid. mp 77-79 °C; $R_f$ 0.6 (1:4 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$): δ 0.94 (s, 3H), 0.99 (s, 3H), 1.03 (s, 3H), 1.07 (s, 6H), 1.69 (s, 3H), 2.08 (s, 3H), 2.45 (m, 1H), 3.87 (d, *J* = 12.0 Hz, 1H), 4.26 (dd, *J* = 1.7, 12.7 Hz, 1H), 4.60 (q, *J* = 1.6 Hz, 1H), 4.70 (d, *J* = 2.3 Hz, 1H); $^{13}$C NMR (75 MHz, $CDCl_3$): δ 14.8, 14.9, 16.1, 19.4, 19.8, 21.3, 21.5, 25.4, 26.7, 26.8, 27.3, 29.8, 29.9, 33.7, 34.4, 34.8, 37.1, 37.9, 39.8, 41.0, 43.0, 46.5, 47.6, 47.9, 48.9, 50.0, 55.2, 63.0, 110.2, 150.3, 171.9, 218.3; FTIR (ν, $cm^{-1}$): 885, 1034, 1236, 1388, 1461, 1709, 1742, 2950; MS (ESI+): *m/z* 483.4 $[M+1]^+$; Anal. ($C_{32}H_{50}O_3$) C, H: calcd, 79.62, 10.44; found, 78.13, 10.40.



**4**

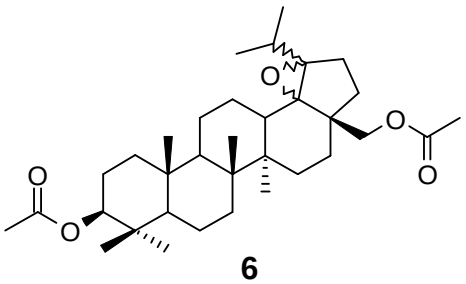
**3,28-Di-*O*-acetylbetulin (4).** To a solution of **1** (15 g, 34 mmol), 4-(dimethylamino)pyridine (0.41 g, 3.4 mmol), and pyridine (25 mL) in $CH_2Cl_2$ (150 mL) was added acetic anhydride (19 mL, 200 mmol). The resulting mixture was stirred at room temperature for 17 h. The reaction mixture was then washed with 10% hydrochloric acid (200 mL), saturated aqueous $NaHCO_3$ solution (400 mL), water (100 mL) and dried with anhydrous $Na_2SO_4$. Removal of the solvent *in vacuo* gave **4** (17 g, 97%) as a white solid. mp 219-220 °C; $R_f$ 0.6 (1:4 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$) δ 0.83 (s, 3H), 0.84 (s, 3H), 0.96 (s, 3H), 1.02 (s, 3H), 1.38 (s, 3H), 1.68 (s, 3H), 2.03 (s, 3H), 2.06 (s, 3H), 2.39-2.48 (m, 1H), 3.84 (d, *J* = 11.0 Hz, 1H), 4.24 (d, *J* = 11.0 Hz, 1H), 4.46 (dd, *J* = 5.8, 10.2 Hz), 4.58 (s, 1H), 4.68 (s, 1H); $^{13}$C NMR (75 MHz, $CDCl_3$) δ 14.6, 16.0, 16.1, 16.4, 18.1, 19.0, 20.7, 21.0, 21.3, 23.6, 25.1, 27.0, 27.9, 29.5, 29.7, 34.1, 34.5, 37.0, 37.5, 37.7, 38.3, 40.8, 42.6, 46.2, 47.6, 48.7, 50.2, 55.3, 62.7, 80.8, 109.8, 150.1, 170.9, 171.6; FTIR (ν, cm$^{-1}$): 1243, 1731, 2951; MS (direct, EI+): *m/z* 526; Anal. ($C_{34}H_{54}O_4$) C, H.




**5**

**3,28-Di-*O*-acetyllup-18-ene (5).** To a solution of HBr (47%, 250 g), acetic anhydride (100 g) and acetic acid (300 g) (resulting 14% HBr and 35% acetic acid) was added 3,28-di-*O*-acetylbetulin **4** (17 g, 33 mmol) in toluene (200 mL).[6, 7] The reaction mixture was allowed to stand for 3 weeks at room temperature. The mixture was diluted with water (400 mL) and aqueous phase was separated and extracted with toluene (400 mL). Combined organic phases were washed with water (300 mL), saturated aqueous $NaHCO_3$ solution (600 mL) and dried with anhydrous $Na_2SO_4$. Solvent was removed *in vacuo* and resulting crude product was purified by flash chromatography on silica gel

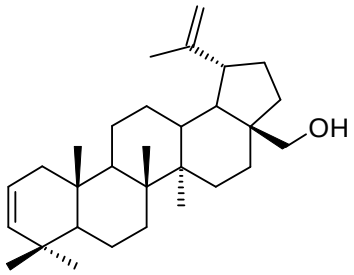
(hexane:EtOAc 8:1) to afford 3,28-di-*O*-acetyllup-18-ene **5** (7.4 g, 42%). mp 202-203 °C; $R_f$ 0.8 (1:4 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$) δ 0.83 (s, 3H), 0.84 (s, 3H), 0.88 (s, 3H), 0.91 (s, 3H), 0.97 (s, 3H), 1.00 (s, 3H), 1.05 (s, 3H), 2.03 (s, 3H), 2.04 (s, 3H), 2.24 (m, 2H), 2.41 (dd, *J* = 12.4 Hz, 1H), 3.13 (m, 1H), 4.00 (d, *J* = 4.4 Hz, 2H), 4.48 (dd, *J* = 6.2, 10.3 Hz, 1H); $^{13}$C NMR (75 MHz, $CDCl_3$) δ 15.5, 16.5, 16.5, 16.8, 18.1, 21.0, 21.3, 21.5, 21.6, 22.1, 23.7, 26.6, 27.9, 28.2, 28.2, 29.3, 32.3, 34.6, 34.9, 37.1, 37.8, 38.6, 40.8, 40.9, 43.2, 51.0, 52.1, 55.5, 66.9, 80.9, 134.0, 143.6, 171.0, 171.4; FTIR (ν, $cm^{-1}$): 982, 1029, 1246, 1372, 1738, 2946; MS (direct, EI+): *m/z* 526. Anal. ($C_{34}H_{54}O_4$) C, H.



**6**

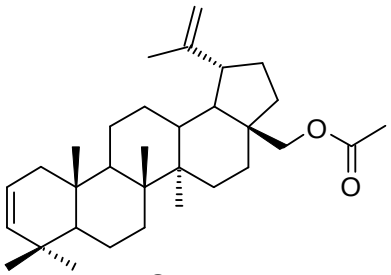
**3,28-Di-*O*-acetyl-18,19-epoxylupane (6).** To a solution of 3,28-di-*O*-acetyllup-18-ene **5** (4.91 g, 9.33 mmol) in $CHCl_3$ (120 mL) was added $Na_2CO_3$ (4.94 g, 46.7 mmol) and *m*-chloroperbenzoic acid (mCPBA 70%, 3.69 g, 14.9 mmol), and the resulting mixture was stirred at room temperature for 2 h.[5] The reaction mixture was washed with water (150 mL), saturated aqueous $NaHSO_3$ solution (150 mL), saturated aqueous $NaHCO_3$ solution (150 mL) and dried with anhydrous $Na_2SO_4$. Solvent was removed *in vacuo*, and the resulting crude product was crystallised from EtOH (175 mL) to give 3,28-di-*O*-acetyl-18,19-epoxylupane **6** (3.31 g, 65%). mp 210-212 °C; $R_f$ 0.7 (1:4 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$) δ 0.81 (s, 3H), 0.86 (s, 3H), 1.00 (s, 3H), 1.02 (s, 3H), 1.03 (s, 3H), 1.04 (s, 3H), 1.07 (s, 3H), 1.08 (s, 3H), 2.02 (s, 6H), 3.85 (d, *J* = 10.7 Hz, 1H), 4.45 (dd, *J* = 7.4, 8.8 Hz, 1H), 4.48 (d, *J* = 10.7 Hz, 1H); $^{13}$C NMR (75 MHz, $CDCl_3$) δ 16.4,

16.4, 16.7, 16.7, 18.1, 18.8, 19.9, 20.9, 21.3, 21.4, 22.6, 23.6, 23.7, 26.3, 26.6, 27.9, 28.5, 29.9, 34.1, 37.1, 37.7, 38.1, 38.5, 41.1, 43.1, 45.6, 51.3, 55.5, 66.7, 75.5, 77.7, 80.8, 170.9, 171.3; FTIR ($\nu$, $cm^{-1}$): 982, 1244, 1732, 2940; MS (direct, EI+): *m/z* 542; Anal. ($C_{34}H_{54}O_5$) C, H.



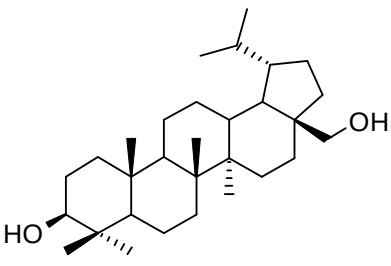

**7**

**3-Deoxy-2,3-didehydrobetulin (7).** To a mixture of **1** (5.00 g, 11.3 mmol), triphenylphosphine (11.9 g, 45.2 mmol), 3,3-dimethylglutarimide (6.38 g, 45.2 mmol) in dry THF (100 mL) was added dropwise 40% diethyl azodicarboxylate solution in PhMe (20.7 mL, 45.2 mmol) at 0 °C.[8] The reaction mixture was allowed to warm to room temperature and stirred for 24 h. The formed precipitate was filtered off, solution was concentrated *in vacuo*, and the crude product was purified by flash chromatography on $SiO_2$ using *n*-hexane:EtOAc (10:1) as an eluent to give **7** (1.47 g, 31%) as a white solid. mp 89-91 °C; $R_f$ 0.2 (1:4 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$) δ 0.87 (s, 3H), 0.88 (s, 3H), 0.95 (s, 3H), 1.00 (s, 6H), 1.06 (s, 3H), 1.69 (s, 3H), 2.45 (m, 1H), 3.35 (d, *J* = 11.1, 1H), 3.82 (d, *J* = 11.1 Hz, 1H), 4.59 (d, *J* = 2.1 Hz, 1H), 4.70 (d, *J* = 2.1 Hz, 1H), 5.38 (m, 2H); $^{13}$C NMR (75 MHz, $CDCl_3$) δ 14.7, 15.6, 16.4, 19.1, 19.5, 21.2, 22.6, 25.3, 27.0, 29.1, 29.7, 31.7, 33.3, 34.0, 34.7, 36.4, 37.4, 41.0, 41.2, 42.7, 47.78, 47.81, 48.7, 49.0, 52.1, 60.5, 109.7, 121.6, 137.9, 150.5; FTIR ($\nu$, $cm^{-1}$): 732, 883, 1025, 1376, 2941; MS (direct, EI+): *m/z* 424; Anal. ($C_{30}H_{48}O$) C, H: calcd, 84.84, 11.39; found, 83.56, 11.32.



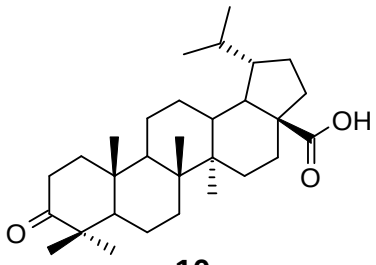

**8**

**3-Deoxy-2,3-didehydro-28-*O*-acetylbetulin (8).** To a mixture of **7** (0.52 g, 1.2 mmol) and 4-(dimethylamino)pyridine (15 mg, 0.12 mmol) in $CH_2Cl_2$ (25 mL) was added pyridine (4 mL) and acetic anhydride (0.23 mL, 2.5 mmol), and the reaction mixture was stirred at room temperature for 22 h. The organic layer was washed with 4% hydrochloric acid (100 mL), water (50 mL), saturated aqueous $NaHCO_3$ solution (50 mL), water (50 mL) and dried with anhydrous $Na_2SO_4$. Solvent was evaporated *in vacuo* to produce **8** (470 mg, 81%) as a white solid. mp 73-75 °C; $R_f$ 1.0 (1:4 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$) δ 0.87 (s, 3H), 0.88 (s, 3H), 0.95 (s, 3H), 1.00 (s, 6H), 1.07 (s, 3H), 1.70 (s, 3H), 2.08 (s, 3H), 2.47 (m, 1H), 3.88 (d, *J* = 10.8 Hz, 1H), 4.26 (d, *J* = 11.1 Hz, 1H), 4.60 (d, *J* = 1.5 Hz, 1H), 4.70 (d, *J* = 1.2 Hz, 1H), 5.38 (m, 2H); $^{13}$C NMR (75 MHz, $CDCl_3$) δ 14.7, 15.7, 16.4, 19.1, 19.5, 21.1, 21.2, 22.6, 25.3, 27.0, 29.6, 29.7, 31.7, 33.3, 34.5, 34.7, 36.3, 37.7, 40.9, 41.2, 42.7, 46.3, 47.7, 48.7, 49.0, 52.1, 62.8, 109.8, 121.6, 137.9, 150.2, 171.6; FTIR (ν, cm$^{-1}$): 732, 885, 1032, 1233, 1741, 2941; MS (direct, EI+): *m/z* 466; Anal. ($C_{32}H_{50}O_2$) C, H: calcd, 82.35, 10.80; found, 79.19, 10.54.




**9**

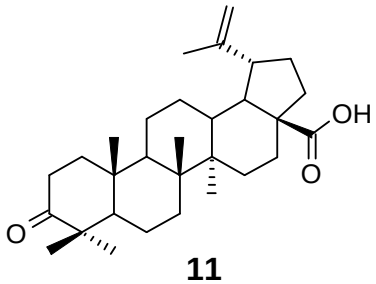
**Dihydrobetulin (9).** To a solution of **1** (2.0 g, 4.5 mmol) in a mixture of THF (40 mL) and MeOH (80 mL) was added 5% Pd on carbon (0.20 g) under $N_2$. $N_2$ atmosphere was replaced with $H_2$, and the reaction mixture was stirred at room temperature for 22 h. The reaction mixture was filtered through a thin layer of Celite, and the resulting yellowish filtrate was evaporated *in vacuo* to afford **9** (2.0 g, 99%) as an off-white solid. mp 265-267 °C; $R_f$ 0.4 (1:4 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$) δ 0.75 (s, 3H), 0.76 (s, 3H), 0.77 (s, 3H), 0.83 (s, 3H), 0.85 (s, 3H), 0.96 (s, 3H), 0.97 (s, 3H), 1.03 (s, 3H), 3.20 (dd, *J* = 11.0, 5.2 Hz, 1H), 3.30 (d, *J* = 11.0 Hz, 1H), 3.77 (d, *J* = 11.0 Hz, 1H); $^{13}$C NMR (75 MHz, $CDCl_3$) δ 14.6, 14.9, 15.4, 16.0, 16.0, 18.3, 20.8, 21.7, 22.9, 26.9, 26.9, 27.4, 28.0, 29.3, 29.5, 34.0, 34.3, 36.8, 37.1, 38.7, 38.9, 40.9, 42.9, 44.5, 47.9, 48.1, 50.0, 55.2, 60.6, 79.0; FTIR (ν, $cm^{-1}$): 1031, 2932, 3374; MS (direct, EI+): *m/z* 444; Anal. ($C_{30}H_{52}O_2$) C, H.




**10**

**Dihydrobetulonic acid (10).** The Jones reagent was prepared by dissolving $Na_2CrO_7$ (2.01 g, 6.75 mmol) to a mixture of $H_2SO_4$ (1.45 mL, 27.0 mmol) and water (10 mL). The freshly prepared Jones reagent was added to a mixture of **9** (1.00 g, 2.25 mmol) in acetone (75 mL), and the reaction mixture was stirred at room temperature for 20 h.[9] MeOH (20 mL) was added, and the resulting mixture stirred for 10 min. Water (40 mL) was added, and organic solvent was evaporated *in vacuo*. Green aqueous phase with a pale precipitate was extracted with ethyl acetate (2×100 mL), and the combined organic phases were washed with water (2×50 mL), dried with anhydrous $Na_2SO_4$ and evaporated *in vacuo*. The crude product was purified by flash chromatography on $SiO_2$ (*n*-

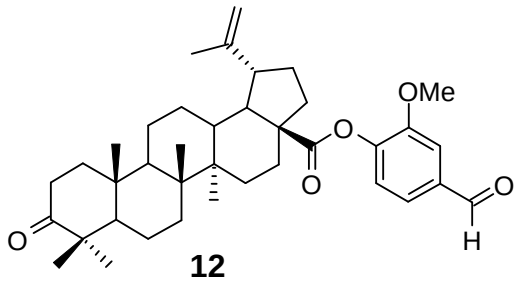
hexane:EtOAc 6:1 → 4:1) to afford **10** (318 mg, 31%). mp 238-239 °C; $R_f$ 0.6 (1:4 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$) δ 0.89 (s, 3H), 0.92 (s, 6H), 0.97 (s, 3H), 1.03 (s, 3H), 2.21 (m, 1H), 2.41 (m, 1H); $^{13}$C NMR (75 MHz, $CDCl_3$) δ 14.4, 14.6, 15.7, 15.8, 19.5, 20.9, 21.3, 22.6, 22.9, 26.5, 26.7, 29.6, 31.9, 33.5, 33.9, 34.0, 36.7, 37.3, 38.2, 39.4, 40.5, 42.5, 44.0, 47.2, 48.5, 49.5, 54.7, 56.7, 182.7, 218.2; FTIR (ν, $cm^{-1}$): 1691, 1711, 2949; MS (direct, EI+): *m/z* 456; Anal. ($C_{30}H_{48}O_3$) C, H.




**11**

**Betulonic acid (11).** To a solution of **1** (50.0 g, 113 mmol) in acetone (1500 mL) was added freshly prepared Jones reagent [$K_2Cr_2O_7$, (66.5 g, 226 mmol) and $H_2SO_4$ (60 mL) in water (500 mL)] during 1 h in an ice bath. The reaction mixture was allowed to warm to room temperature and stirring was continued for 21 h.[5,10] First, MeOH (700 mL) was added and then water (1000 mL) to the reaction mixture. Precipitate was filtered off and washed with water (500 mL). The crude product was dried in a vacuum oven, dissolved to $Et_2O$ (600 mL) and washed with water (300 mL), 7.5% hydrochloric acid (200 mL), water (200 mL), saturated aqueous $NaHCO_3$ solution (200 mL) and water (200 mL). Two thirds of $Et_2O$ was removed *in vacuo*, and the residue was treated with aqueous 10% NaOH solution (75 mL). Precipitate was filtered off by suction, and dried in a vacuum oven. Precipitate was dissolved to boiling MeOH (100 mL), and acetic acid (10 mL) was added. The product was precipitated by adding water (200 mL) and then filtered by suction, washed with water (300 mL) and dried in vacuum oven to afford **11** (22.3 g, 44%) as a white solid. mp 230-235 °C; $R_f$ 0.4 (1:4 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$) δ 0.93 (s, 3H), 0.98 (s, 3H), 0.99 (s,

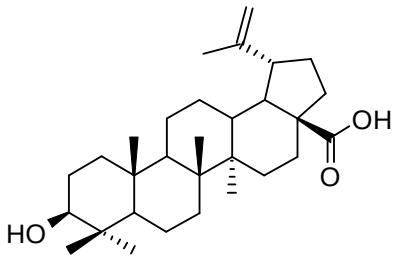
3H), 1.02 (s, 6H), 1.07 (s, 3H), 1.70 (s, 3H), 1.96 (m, 3H), 2.27 (m, 2H), 2.44 (m, 2H), 3.01 (m, 1H), 4.62 (s, 1H), 4.74 (s, 1H); $^{13}$C NMR (75 MHz, $CDCl_3$) δ 14.6, 15.8, 15.9, 19.3, 19.6, 21.0, 21.3, 25.5, 26.6, 29.6, 30.5, 32.1, 33.6, 34.1, 36.9, 37.0, 38.5, 39.6, 40.6, 42.5, 46.9, 47.3, 49.1, 49.8, 54.9, 56.3, 109.7, 150.3, 181.9, 218.4; FTIR (ν, $cm^{-1}$): 883, 1692, 2944; MS (ESI+): *m/z* 455.3 $[M+1]^+$; Anal. ($C_{30}H_{46}O_3$) C, H: calcd, 79.25, 10.20; found, 78.32, 10.16.




**12**

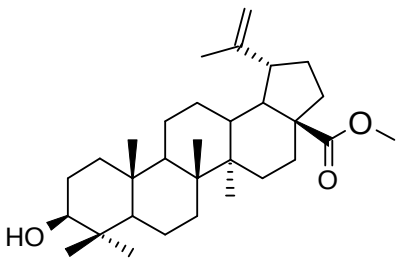
**Vanillinyl betulonate (12).** To a solution of **11** (5.0 g, 11 mmol) in dry $CH_2Cl_2$ (120 mL) was added oxalyl chloride (3.0 g, 23 mmol). The reaction mixture was stirred at room temperature for 6 h. Solvent was removed *in vacuo*. Residue was treated with dry $CH_2Cl_2$ (30 mL) and solvent was removed *in vacuo* three times giving betulonoyl chloride (5.2 g, 99%) as yellowish crystals. A mixture of the freshly prepared betulonoyl chloride (1.5 g, 3.2 mmol), vanillin (0.48 g, 3.2 mmol) and DMAP (0.39 g, 3.2 mmol) in dry pyridine (20 mL) was stirred at 40 ºC for 21 h. The formed precipitate was filtered off, and the filtrate was evaporated to dryness *in vacuo* to yield a dark viscous crude product, which was purified by flash chromatography on $SiO_2$ (9:1 → 6:1 *n*-hexane:EtOAc) to give **12** (0.37 g, 20%) as white crystals. mp 133-135 °C; $R_f$ 0.3 (1:6 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$) δ 0.93 (s, 3H), 1.03 (s, 3H), 1.03 (s, 3H), 1.26 (s, 3H), 1.55 (s, 3H), 1.71 (s, 3H), 3.03 (dt, *J* = 11.1, 4.8 Hz, 1H), 3.89 (s, 3H), 4.62 (s, 1H), 4.73 (s, 1H), 7.14 (d, *J* = 7.8 Hz, 1H), 7.49 (d, *J* = 7.8 Hz, 1H), 7.50 (s, 1H), 9.96 (s, 1H); $^{13}$C NMR (300 MHz, $CDCl_3$) δ 14.6, 15.9, 15.9, 19.3, 19.6, 21.0, 21.4, 25.5, 26.6, 29.6, 30.4, 31.9, 33.7, 34.1, 36.9, 37.0, 38.3, 39.6, 40.7, 42.5, 46.7, 47.3, 49.3, 49.9, 54.9, 55.9, 57.1, 109.8, 110.7, 123.5, 124.8, 135.1, 145.2,

150.2, 152.3, 173.4, 191.1, 218.1; FTIR (ν, $cm^{-1}$): 1076, 1274, 1642, 1756, 2949; MS (ESI+): *m/z* 589.4 $[M+1]^+$; Anal. ($C_{38}H_{52}O_5$) C, H: calcd, 77.51, 8.90; found, 77.60, 9.04.



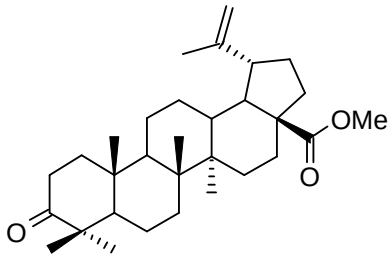

**13**

**Betulinic acid (13).** To a solution of betulonic acid **13** (10.0 g, 22.1 mmol) in 2-propanol (400 mL) was added $NaBH_4$ (1.76 g, 44.2 mmol) during 10 min, and the reaction mixture was stirred at room temperature for 2.5 h.[10] 10% Hydrochloric acid (600 mL) was added, and the precipitated product was filtered by suction, washed with water (200 mL) and dried in a vacuum oven. The crude product was crystallized from EtOH to give **13** (8.25 g, 82 %) as white crystals. mp 288-290 °C; $R_f$ 0.3 (1:4 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$) δ 0.75 (s, 3H), 0.82 (s, 3H), 0.93 (s, 3H), 0.96 (s, 6H), 0.97 (s, 3H), 1.69 (s, 3H), 1.97 (m, 2H), 2.28 (m, 2H), 3.01 (m, 1H), 3.19 (dd, *J* = 5.5, 10.7 Hz, 1H), 4.60 (s, 1H), 4.74 (s, 1H); $^{13}$C NMR (75 MHz, DMSO) δ 14.4, 15.7, 15.8, 16.0, 18.0, 19.0, 20.5, 25.1, 27.2, 28.1, 29.2, 30.1, 31.7, 34.0, 36.4, 36.7, 37.6, 38.3, 38.5, 40.3, 42.0, 46.6, 48.6, 50.0, 54.9, 55.4, 76.8, 109.7, 150.3, 177.3; FTIR (ν, $cm^{-1}$): 884, 1034, 1236, 1689, 2942; MS (direct, EI+): *m/z* 456; Anal. ($C_{30}H_{48}O_3$) C, H.




**14**

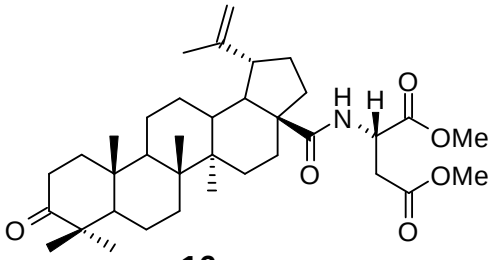
**Methyl betulinate (14).** To a solution of **13** (0.10 g, 0.22 mmol) in a mixture of MeOH (2.0 mL) and PhMe (3.0 mL) was added 2 M solution of (trimethylsilyl)diazomethane in $Et_2O$ (0.17 mL, 0.33 mmol), and the reaction mixture was stirred at room temperature for 40 min. Solvent was evaporated to give **14** (92 mg, 89%) as a white solid. mp 218-220 °C; $R_f$ 0.5 (1:4 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$) δ 0.75 (s, 3H), 0.82 (s, 3H), 0.91 (s, 3H), 0.96 (s, 3H), 1.56 (s, 3H), 1.68 (s, 3H), 1.88 (m, 4H), 2.21 (m, 3H), 3.00 (m, 1H), 3.18 (m, 1H), 3.67 (s, 3H), 4.60 (s, 1H), 4.73 (d, $J$ = 2.2 Hz, 1H); $^{13}$C NMR (75 MHz, $CDCl_3$): δ 14.7, 15.3, 15.9, 16.1, 18.3, 19.3, 20.8, 25.5, 27.4, 27.9, 29.6, 30.6, 32.1, 34.3, 36.9, 37.1, 38.2, 38.7, 38.8, 40.6, 42.3, 46.9, 49.4, 50.5, 54.2, 55.3, 56.5, 78.9, 109.5, 150.5, 176.6; FTIR (ν, $cm^{-1}$): 879, 1047, 1167, 1707, 2942, 3536; MS (direct, EI+): *m/z* 470; Anal. ($C_{31}H_{50}O_3$) C, H: calcd, 79.10, 10.71; found, 76.95, 10.77.




**15**

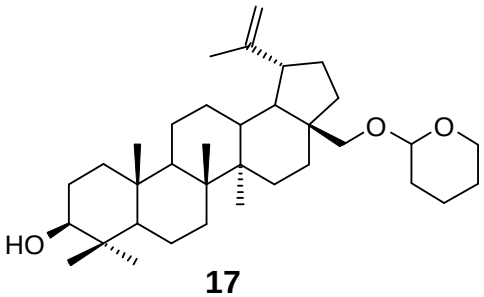
**Methyl betulonate (15).** To a solution of **11** (0.10 g, 0.22 mmol) in a mixture of MeOH (1.0 mL) and PhMe (1.5 mL) was added 2 M solution of (trimethylsilyl)diazomethane in $Et_2O$ (0.17 mL, 0.33 mmol) and the reaction mixture was stirred at room temperature for 40 min. Solvent was evaporated to afford **15** (68 mg, 66%) as a yellow solid. mp 97-99 °C; $R_f$ 0.7 (1:4 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$) δ 0.92 (s, 3H), 0.95 (s, 3H), 0.97 (s, 3H), 1.02 (s, 3H), 1.07 (s, 3H), 1.69 (s, 3H), 1.90 (m, 4H), 2.24 (m, 3H), 2.43 (m, 3H), 3.01 (m, 1H), 3.67 (s, 3H), 4.60 (s, 1H), 4.74 (d, $J$ = 1.7 Hz, 1H); $^{13}$C NMR (75 MHz, $CDCl_3$) δ 14.6, 15.7, 15.9, 19.4, 19.6, 21.0, 21.4, 25.5, 26.6, 29.6, 30.5, 32.1, 33.6, 34.1, 36.9, 36.9, 38.3, 39.6, 40.6, 42.4, 46.9, 47.3, 49.3, 49.9, 51.3, 54.9, 56.5,

109.6, 150.5, 176.6, 218.1; FTIR (ν, $cm^{-1}$): 882, 1139, 1155, 1452, 1706, 1726, 2946; MS (ESI+): *m/z* 469.3 $[M+1]^+$; Anal. ($C_{31}H_{48}O_3$) C, H: calcd, 79.44, 10.32; found, 77.50, 10.16.



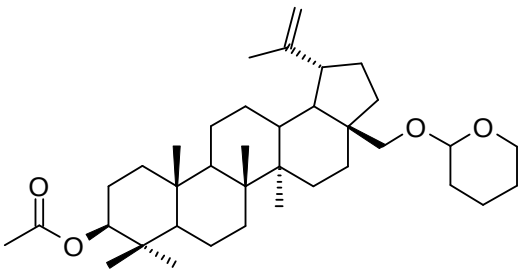

**16**

**L-Aspartyl amide of betulonic acid (16).** To a solution of **11** (4.0 g, 8.8 mmol) in dry $CH_2Cl_2$ (100 mL) was added oxalyl chloride (1.6 mL, 19 mmol).[11] The reaction mixture was stirred at room temperature for 22 h. Solvent was removed *in vacuo*, and the residue was washed with $Et_2O$ (40 mL) to give betulonoyl chloride (3.5 g, 85%). To a solution of freshly prepared betulonoyl chloride (2.0 g, 4.2 mmol) and L-aspartic acid dimethyl ester hydrochloride (1.1 g, 5.5 mmol) in dry $CH_2Cl_2$ (80 mL) was added triethylamine (1.5 mL, 11 mmol), and the reaction mixture was stirred at room temperature for 19 h. The reaction mixture was diluted with $CH_2Cl_2$ (70 mL), washed with 5% hydrochloric acid (2×50 mL), water (100 mL) and dried with anhydrous $Na_2SO_4$. Solvent was removed *in vacuo*, and the crude product was purified by flash chromatography on $SiO_2$ (*n*-hexane:EtOAc 8:1 → 2:1) to give **16** (1.1 g, 42%) as white crystals. mp 94-96 °C; $R_f$ 0.2 (1:4 EtOAc:*n*-hexane); $^1$H NMR (400 MHz, $CDCl_3$) δ 0.91 (s, 3H), 0.94 (s, 3H), 0.97 (s, 3H), 1.01 (s, 3H), 1.06 (s, 3H), 1.67 (s, 3H), 2.43 (m, 3H), 2.90 (dd, *J* = 4.4, 17.2 Hz, 1H), 3.04 (dd, *J* = 4.4, 17.2 Hz, 1H), 3.08 (m, 1H), 3.69 (s, 3H), 3.76 (s, 3H), 4.59 (s, 1H), 4.73 (d, *J* = 1.6 Hz, 1H), 4.79 (m, 1H), 6.60 (d, *J* = 7.6 Hz, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 15.9, 19.4, 19.6, 21.0, 21.4, 25.6, 26.6, 29.2, 30.7, 33.5, 33.7, 34.1, 35.9, 36.9, 37.8, 38.0, 39.6, 37.8, 38.0, 39.6, 40.6, 42.5, 46.6, 47.3, 48.4, 49.9, 52.0, 52.8, 54.9, 55.7, 109.4, 150.7, 171.4, 171.7, 175.9, 218.2; FTIR (ν, $cm^{-1}$): 881, 1168, 1200, 1739, 2948. MS (ESI+): *m/z* 598.4 $[M+1]^+$; Anal. ($C_{36}H_{55}NO_6$) C, H, N.




**17**

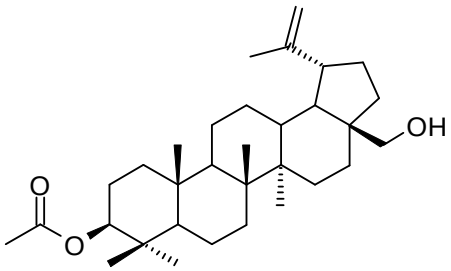
**28-*O*-Tetrahydropyranylbetulin (17).** To a mixture of **1** (30 g, 68 mmol) and pyridine *p*-toluenesulfonate (3.0 g, 12 mmol) in $CH_2Cl_2$ (1000 mL) was added 3,4-dihydro-2*H*-pyran (8.4 mL, 92 mmol), and the reaction mixture was stirred at room temperature for 2 d.[10] The organic layer was washed with saturated aqueous $NaHCO_3$ solution (2×200 mL), water (2×200 mL) and dried with anhydrous $Na_2SO_4$. Solvent was evaporated *in vacuo*, and the crude product was purified by flash chromatography on $SiO_2$ (*n*-hexane:EtOAc 8:1 → 2:1) to give **17** (7.7 g, 30%) as a yellowish solid. mp 111-113 °C; $R_f$ 0.4 (1:4 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$): δ 0.76 (s, 3H), 0.82 (s, 3H), 0.97 (s, 3H), 1.01 (s, 3H), 1.03 (s, 3H), 1.68 (s, 3H), 2.42 (m, 1H), 2.98 (d, *J* = 9.3 Hz, 0.5H), 3.18 (dd, *J* = 5.1, 10.9 Hz, 1H), 3.37 (d, *J* = 9.6 Hz 0.5H), 3.51 (d, *J* = 9.6 Hz, 0.5 H), 3.52 (m, 1H), 3.84 (m, 1H), 3.92 (d, *J* = 9.9 Hz, 0.5H), 4.55 (m, 1H), 4.56 (s, 1H), 4.67 (s, 1H); FTIR (ν, $cm^{-1}$): 881, 981, 1027, 1118, 2940; MS (direct, EI+): *m/z* 526; Anal. ($C_{35}H_{58}O_3$) C, H.




**18**

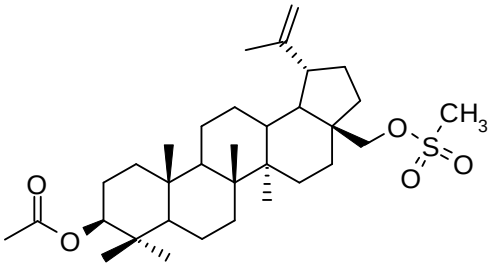
**3-*O*-Acetyl-28-*O*-tetrahydropyranylbetulin (18).** To a solution of **17** (5.0 g, 9.5 mmol), 4-(dimethylamino)pyridine (0.12 g, 0.95 mmol) and pyridine (10 mL) in $CH_2Cl_2$ (50 mL) was added acetic anhydride (5.4 mL, 57 mmol).[10] The resulting mixture was stirred at room temperature for 20

h. The reaction mixture was diluted with $CH_2Cl_2$ (50 mL) and washed with 2 M hydrochloric acid (3×100 mL), saturated aqueous $NaHCO_3$ solution (4×100 mL), water (100 mL) and dried with anhydrous $Na_2SO_4$. Removal of the solvent *in vacuo* gave **18** (5.2 g, 95%) as an off-white solid. mp 249-250 °C; $R_f$ 0.9 (1:4 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$): δ 0.82 (s, 3H), 0.83 (s, 3H), 0.95 (s, 3H), 1.00 (s, 3H), 1.02 (s, 3H), 1.67 (s, 3H), 2.02 (s, 3H), 2.21 (s, 2H), 2.43 (m, 1H), 2.96 (d, *J* = 9.6 Hz, 0.5H), 3.35 (d, *J* = 9.3 Hz 0.5H), 3.49 (d, *J* = 9.6 Hz, 0.5 H), 3.51 (m, 1H), 3.84 (m, 1H), 3.91 (d, *J* = 9.3 Hz, 0.5H), 4.46 (m, 1H), 4.55 (m, 1H), 4.56 (s, 1H), 4.66 (s, 1H); FTIR (ν, $cm^{-1}$): 981, 130, 1244, 1733, 2943; MS (direct, EI+): *m/z* 568; Anal. ($C_{37}H_{60}O_4$) C, H.



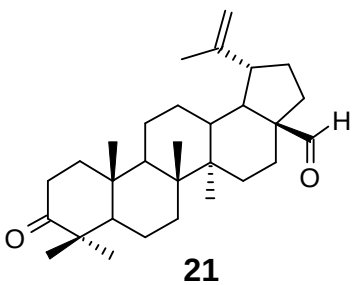

**19**

**3-*O*-Acetylbetulin (19).** A mixture of **18** (3.00 g, 5.27 mmol) and pyridine *p*-toluenesulfonate (265 mg, 1.06 mmol) in MeOH (100 mL) was stirred at room temperature for 2 weeks.[10] Saturated aqueous $NaHCO_3$ solution (100 mL) was added, and the formed white precipitate was dissolved to ethyl acetate (300 mL). Organic layer was separated, washed with water (2×400 mL), dried with anhydrous $Na_2SO_4$ and evaporated to give **19** (2.40 g, 94%) as a white solid. mp 238-240 °C; $R_f$ 0.4 (1:4 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$) δ 0.83 (s, 3H), 0.84 (s, 6H), 0.97 (s, 3H), 1.01 (s, 3H), 1.68 (s, 3H), 2.03 (s, 3H), 2.38 (m, 1H), 3.32 (d, *J* = 10.7 Hz, 1H), 3.79 (d, *J* = 11.0 Hz, 1H), 4.46 (m, 1H), 4.58 (s, 1H), 4.67 (d, *J* = 1.9 Hz, 1H); $^{13}$C NMR (75 MHz, $CDCl_3$) δ 14.7, 15.9, 16.1, 16.5, 18.1, 19.0, 20.8, 21.3, 23.7, 25.1, 27.0, 27.9, 29.1, 29.7, 33.9, 34.1, 37.0, 37.2, 37.8, 38.3, 40.9, 42.7, 47.7, 47.8, 48.7, 50.3, 55.3, 60.5, 80.9, 109.7, 150.4, 171.0; FTIR (ν, $cm^{-1}$): 887, 979, 1025, 1245, 1731, 2942, 3380; MS (direct, EI+): *m/z* 484; Anal. ($C_{32}H_{52}O_3$) C, H.



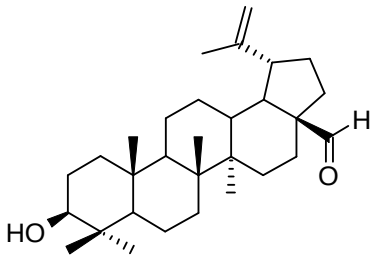
**20**

**3-*O*-Acetyl-28-*O*-mesylbetulin (20).** A mixture of **19** (1.00 g, 2.06 mmol) in $CH_2Cl_2$ (20 mL) was cooled to 0 °C and triethylamine (0.43 mL, 3.09 mmol) and $CH_3SO_2Cl$ (0.19 mL, 2.47 mmol) were added dropwise. Reaction mixture was stirred at 0 °C for 2 h and the diluted with $CH_2Cl_2$ (20 mL). Water (40 mL) was added and separated water phase was extracted with EtOAc (3×40 mL). Organic phases were combined, washed with water (80 mL), dried with anhydrous $Na_2SO_4$ and evaporated to give **20** (1.51 g, 99%) as an off-white solid. mp 124-126 °C; $R_f$ 0.7 (1:4 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$) δ 0.83 (s, 3H), 0.84 (s, 6H), 0.97 (s, 3H), 1.03 (s, 3H), 1.68 (s, 3H), 2.03 (s, 3H), 2.38 (m, 1H), 3.02 (s, 3H), 3.94 (d, *J* = 9.3 Hz, 1H), 4.40 (d, *J* = 9.6 Hz, 1H), 3.16 (dd, *J* = 5.6, 10.6 Hz, 1H), 4.60 (s, 1H), 4.68 (s, 1H); $^{13}$C NMR (75 MHz, $CDCl_3$) δ 14.7, 16.0, 16.1, 16.5, 18.1, 19.0, 20.7, 21.3, 23.6, 25.1, 26.7, 27.9, 29.1, 29.3, 34.0, 34.1, 37.0, 37.1, 37.8, 38.3, 40.9, 42.7, 46.7, 47.7, 48.7, 50.2, 55.3, 68.6, 77.2, 80.8, 110.2, 149.5, 171.0; FTIR (ν, $cm^{-1}$): 843, 954, 1029, 1176, 1247, 1361, 1731, 2945; MS (direct, EI+): *m/z* 562; Anal. ($C_{33}H_{54}O_5S$) C, H: calcd, 70.42, 9.67; found, 69.67, 9.36.



**21**

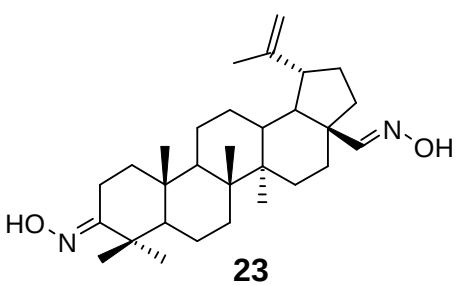
**Betulonic aldehyde (21).** A mixture of **1** (3.00 g, 6.78 mmol) and pyridinium chlorochromate (8.76 g, 40.7 mmol) in $CH_2Cl_2$ (400 mL) was stirred at room temperature for 1 h.[6,7] The reaction mixture was diluted with $Et_2O$ (100 mL), stirred for 10 min, and filtered through a thin layer of $Al_2O_3$. The filtrate was washed with water (100 mL), 5% hydrochloric acid (100 mL), water (100 mL) and dried with anhydrous $Na_2SO_4$. Solvent was evaporated *in vacuo*, and the residue was crystallized from mixture of *n*-hexane (30 mL) and ethyl acetate (3 mL) to give **21** (2.43 g, 82%) as a white solid. mp 150-152 °C; $R_f$ 0.7 (1:4 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$): δ 0.92 (s, 3H), 0.94 (s, 3H), 0.97 (s, 3H), 1.01 (s, 3H), 1.06 (s, 3H), 1.69 (s, 3H), 2.43 (m, 2H), 2.86 (m, 1H), 4.62 (s, 1H), 4.75 (s, 1H), 9.66 (s, 1H); $^{13}$C NMR (75 MHz, $CDCl_3$): δ 14.1, 15.6, 15.9, 18.9, 19.5, 21.0, 21.2, 25.4, 26.5, 28.7, 29.1, 29.8, 33.1, 33.5, 34.1, 36.8, 38.7, 39.6, 40.7, 42.5, 47.3, 47.4, 47.9, 49.7, 54.9, 59.2, 110.2, 149.6, 206.5, 218.0; FTIR (ν, $cm^{-1}$): 872, 1703, 2940; MS (ESI+): *m/z* 439.4 $[M+1]^+$; Anal. ($C_{30}H_{46}O_2$) C, H.



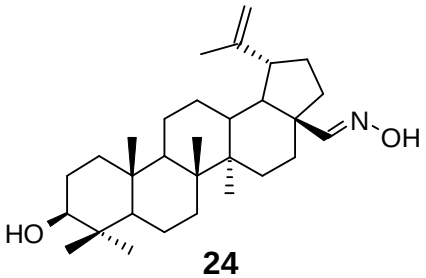

**22**

**Betulin aldehyde (22).** A mixture of **1** (8.0 g, 18 mmol) and pyridinium chlorochromate (7.0 g, 33 mmol) in $CH_2Cl_2$ (800 mL) was stirred at room temperature for 40 min.[12, 13] The reaction mixture was diluted with $Et_2O$ (200 mL), stirred for 10 min and filtered through a thin layer of $Al_2O_3$. Solvent was evaporated *in vacuo*, and a sample of the crude product (2.0 g) was purified by flash chromatography on $Al_2O_3$ (*n*-hexane:EtOAc 5:1) to give relatively unstable **22** (0.36 g, 18%) as a white solid. mp 242-244 °C; $R_f$ 0.4 (1:4 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$) δ 0.75 (s, 3H), 0.82 (s, 3H), 0.91 (s, 3H), 0.96 (s, 3H), 0.97 (s, 3H), 1.69 (s, 3H), 2.86 (m, 1H), 3.18 (dd, *J* = 4.9, 10.7 Hz, 1H), 4.63 (s, 1H), 4.75 (s, 1H); $^{13}$C NMR (75 MHz, $CDCl_3$) δ 14.3, 15.3, 15.9, 16.1,

18.3, 19.0, 20.7, 25.5, 27.4, 28.0, 28.8, 29.2, 29.8, 33.2, 34.3, 37.2, 38.7, 38.7, 38.8, 40.8, 42.5, 47.5, 48.0, 50.4, 55.3, 59.3, 79.0, 110.2, 149.7, 206.7; FTIR (KBr) (ν, $cm^{-1}$): 732, 885, 1034, 1378, 1454, 1708, 2945, 3385; MS (direct, EI+): *m/z* 440; Anal. ($C_{30}H_{48}O_2$) C, H: calcd, 81.76, 10.98; found, 79.43, 10.99.




**23**



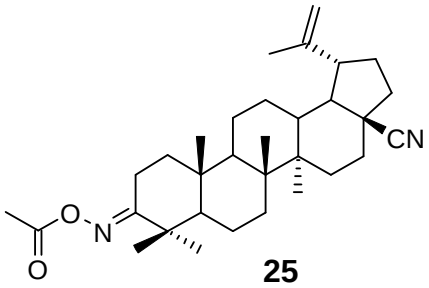

**24**

**Betulin-3,28-dioxime (23) and betulin-28-oxime (24).** A mixture of **1** (8.0 g, 18 mmol) and pyridinium chlorochromate (7.0 g, 33 mmol) in $CH_2Cl_2$ (800 mL) was stirred at room temperature for 40 min. The reaction mixture was diluted with $Et_2O$ (200 mL), stirred for 10 min and filtered through a thin layer of $Al_2O_3$. Solvent was evaporated *in vacuo* to give a 3:1 mixture of **21** and **22**. To this mixture (6.0 g) in pyridine (40 mL) and EtOH (120 mL) was added hydroxylamine hydrochloride (10 g, 140 mmol), and the reaction mixture was refluxed for 18 h.[14] Solvent was evaporated *in vacuo*, and the residue treated with water (160 mL) and filtrated. The precipitate was dried in desiccator to give a mixture of dioxime **23** and mono-oxime **24** (5.7 g). A sample of the crude product (2.9 g) was purified by flash chromatography on $SiO_2$ (*n*-hexane:EtOAc 8:1 → 1:2) to give **23** (0.32 g, 10%) and **24** (1.0 g, 33%) as white solids.

Betulin-3,28-dioxime **23**. mp 242-244 °C; $R_f$ 0.4 (1:4 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$): δ 0.76 (s, 3H), 0.82 (s, 3H), 0.97 (s, 3H), 0.98 (s, 3H), 1.38 (s, 3H), 1.69 (s, 3H), 2.50 (m, 1H), 3.20 (dd , *J* = 11.0, 4.7, 1H), 4.61 (s, 1H), 4.72 (s, 1H), 7.58 (s, 1H); $^{13}$C NMR (75 MHz, $CDCl_3$): δ 14.2, 14.7, 15.4, 16.0, 16.1, 18.3, 19.1, 20.8, 25.2, 27.3, 27.9, 28.0, 29.7, 32.4, 34.3, 37.0,

37.1, 38.7, 38.8, 40.9, 42.8, 47.9, 49.4, 49.9, 50.4, 55.3, 79.0, 110.2, 149.6, 156.6; FTIR (ν, $cm^{-1}$): 881, 921, 1387, 1453, 2936; MS (direct, EI+): *m/z* 468; Anal. ($C_{30}H_{48}N_2O_2$) C, H, N.

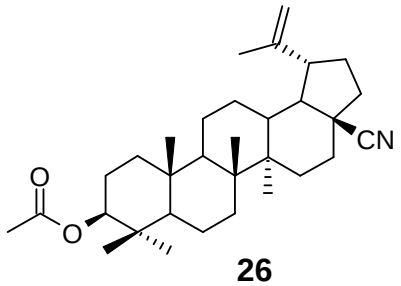
Betulin-28-oxime **24**. mp 192-194 °C; $R_f$ 0.3 (1:4 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$) δ 0.92 (s, 3H), 0.97 (s, 3H), 1.01 (s, 3H), 1.13 (s, 3H), 1.24 (s, 3H), 1.69 (s, 3H), 2.46 (m, 2H), 2.98 (dt, 1H), 4.61 (s, 1H), 4.72 (s, 1H), 7.55 (s, 1H); $^{13}$C NMR (75 MHz, $CDCl_3$) δ 14.6, 15.8, 16.0, 17.2, 19.0, 19.1, 21.1, 22.9, 25.2, 27.3, 27.8, 29.7, 32.3, 33.9, 36.9, 37.2, 38.6, 38.7, 40.2, 40.9, 42.9, 47.9, 49.3, 49.7, 50.0, 55.5, 110.1, 149.8, 155.6, 167.7; FTIR (ν, $cm^{-1}$): 880, 917, 1029, 1374, 1450 2943, 3348; MS (ESI+): *m/z* 456.4 $[M+1]^+$; Anal. ($C_{30}H_{49}NO_2$) C, H, N: calcd, 79.07, 10.84, 3.07; found, 78.88, 11.57, 2.92.




**25**

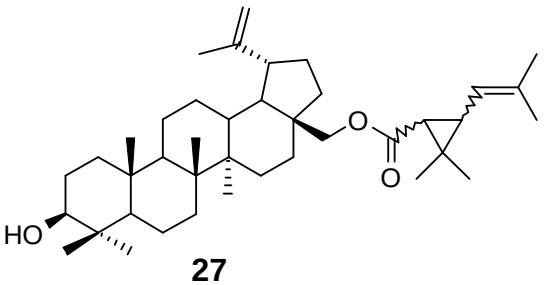
**3-Acetoxymebetulinyl-28-nitrile (25).** Betulin-3,28-dioxime **23** (0.10 g, 0.21 mmol) was heated at 120 °C in acetic anhydride (2.5 mL) for 2 h.[14] The reaction mixture was diluted with water (25 mL), and the formed precipitate was filtered. The precipitate was dissolved in chloroform (15 mL), washed with water (20 mL), saturated aqueous $NaHCO_3$ solution (2×20 mL), water (20 mL) and dried with anhydrous $Na_2SO_4$. Solvent was evaporated *in vacuo*, and the crude product was purified by flash chromatography on $SiO_2$ (*n*-hexane:EtOAc 8:1 → 3:1) to give **25** (50 mg, 46%) as a white solid. mp 226-227 °C; $R_f$ 0.8 (1:4 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$) δ 0.82 (s, 3H), 0.83 (s, 3H), 0.85 (s, 3H), 0.93 (s, 3H), 1.06 (s, 3H), 1.67 (s, 3H), 2.03 (s, 3H), 2.63 (m, 1H), 4.45 (m, 1H), 4.64 (s, 1H), 4.75 (s, 1H); $^{13}$C NMR (75 MHz, $CDCl_3$) δ 14.8, 15.9, 16.2, 16.4, 18.1, 19.3, 20.6, 21.3, 23.6, 24.9, 27.9, 29.0, 29.5, 31.0, 34.3, 35.8, 37.0, 37.7, 38.3, 40.6, 41.1, 42.2, 48.5,

49.0, 49.1, 50.3, 55.3, 80.8, 110.9, 123.4, 148.1, 171.0; FTIR (ν, $cm^{-1}$): 886, 981, 1029, 1245, 1729, 2936; MS (direct, EI+): *m/z* 492; Anal. ($C_{32}H_{48}N_2O_2$) C, H, N: calcd, 78.00, 9.82, 5.69; found, 75.05, 9.57, 4.55.




**26**

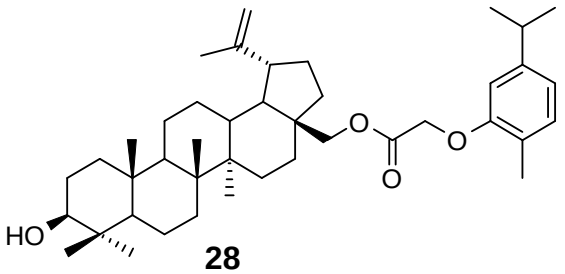
**3-*O*-Acetylbetulinyl-28-nitrile (26).** Betulin-28-oxime **24** (100 mg, 0.22 mmol) was heated at 120 °C in acetic anhydride (2.5 mL) for 2 h.[14] The reaction mixture was diluted with water (25 mL), and the formed precipitate was filtered off. The precipitate was dissolved in chloroform (15 mL), washed with water (20 mL), saturated aqueous $NaHCO_3$ solution (2×20 mL), water (20 mL) and dried with anhydrous $Na_2SO_4$. Solvent was evaporated *in vacuo*, and the crude product was purified by flash chromatography on $SiO_2$ (*n*-hexane:EtOAc 4:1) to give **26** (37 mg, 34%) as a white solid. mp 77-79 °C; $R_f$ 0.6 (1:4 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$) δ 0.92 (s, 3H), 0.93 (s, 3H), 1.09 (s, 3H), 1.11 (s, 3H), 1.22 (s, 3H), 1.67 (s, 3H), 2.16 (s, 3H), 2.38 (m, 1H), 2.64 (m, 1H), 2.84 (m, 1H), 4.64 (s, 1H), 4.75 (s, 1H); $^{13}$C NMR (75 MHz, $CDCl_3$) δ 14.7, 15.8, 16.0, 18.9, 19.3, 19.5, 20.0, 21.0, 22.6, 23.8, 24.9, 27.2, 28.4, 29.0, 30.9, 33.8, 35.7, 37.1, 39.0, 40.6, 41.156, 41.247, 42.3, 48.5, 48.968, 49.002, 50.0, 55.4, 110.9, 123.4, 146.0, 174.7; FTIR (ν, $cm^{-1}$): 883, 920, 1214, 1367, 1451, 1765, 2941; MS (direct, EI+): *m/z* 479; Anal. ($C_{32}H_{49}NO_2$) C, H, N.




**27**

**28-*O*-Chrysanthemoylbetulin (27).** To a solution of ethyl chrysanthemate (9.1 g, 47 mmol) in a mixture of THF:MeOH (1:2, 720 mL) was slowly added aqueous 2 M NaOH solution (190 mL). The resulting mixture was heated at 80 °C for 4 h. The solvent was evaporated *in vacuo*, and the crude product was dissolved in water (600 mL) and washed with $Et_2O$ (3×100 mL). The aqueous phase was acidified with 6 M hydrochloric acid (ca. 70 mL) and extracted with $Et_2O$ (5×200 mL). The combined organic phases were washed with water (2×50 mL), saturated aqueous NaCl solution (2×50 mL) and dried with anhydrous $Na_2SO_4$. The solvent was removed *in vacuo* to give pure chrysanthemic acid (7.2 g, 91%). To a solution of chrysanthemic acid (1.0 g, 5.9 mmol) in dry $CH_2Cl_2$ (30 mL) was added oxalyl chloride (1.5 g, 12 mmol). The mixture was stirred at room temperature for 6 h. The solvent was evaporated *in vacuo*, and the residue was dissolved in dry $CH_2Cl_2$ (15 mL) and re-evaporated. This procedure was repeated three times to give the crude chrysanthemoyl chloride (0.90 g, 81%) as a greenish liquid. Dry pyridine (25 mL) was added to a mixture of **1** (0.39 g, 0.87 mmol), chrysanthemoyl chloride (0.21 g, 1.1 mmol) and DMAP (0.11 g, 0.88 mmol). The reaction mixture was stirred at 40 °C for 48 h. The reaction mixture was diluted with ethyl acetate (100 mL), and the organic phase was washed with 5% hydrochloric acid (3×140 mL), water (3×240 mL) and dried with anhydrous $Na_2SO_4$. The solvent was evaporated, and the residue was refluxed in cyclohexane (50 mL). The precipitate was filtered off, and the solvent was evaporated to yield **27** (0.30 g, 63%) as a white solid. mp 67-70 °C; $R_f$ 0.3 (1:6 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$) δ 0.6-2.2 (m), 2.40 (m), 3.18 (dd, *J* = 5.2, 10.7 Hz 1H), 3.88 (d, *J* = 10.7 Hz, 1H), 4.26 (d, *J* = 11.0 Hz, 1H), 4.58 (s, 1H), 4.67 (s, 1H), 4.6 (d, 2H), 4.90 (d, *J* = 7.7 Hz,

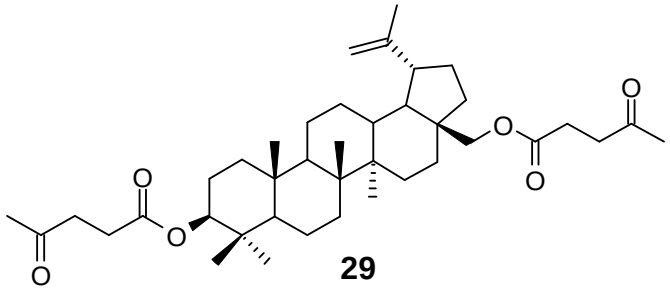
2/3 H), 5.35 (m, 1/3 H); FTIR (KBr) (ν, $cm^{-1}$): 1158, 1376, 1450, 1721, 2942; MS (direct, EI+): *m/z* 592; Anal. ($C_{40}H_{64}O_3$) C, H: calcd, 81.03, 10.88; found, 77.69, 10.53.




**28**

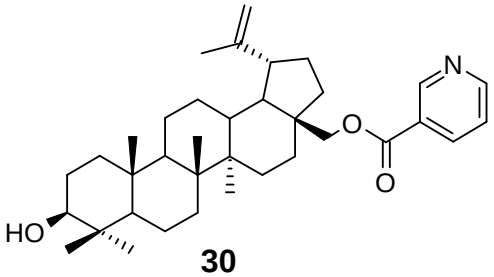
**Betulinyl 28-carboxymethoxycarvacrolate (28).** Sodium hydroxide pellets (2.7 g, 67 mmol) were dissolved in distilled water (12 mL), and the resulting solution was added to a mixture of carvacrol (5.0 g, 33 mmol), chloroacetic acid (3.2 g, 33 mmol) and water (50 mL).[15] The resulting mixture was refluxed at 120 °C for 3 h. After cooling to room temperature the mixture was acidified with concentrated HCl (approx. 10 mL) and extracted with $Et_2O$ (4×40 mL). The ethereal layer was washed with water (3×40 mL) and dried with anhydrous $Na_2SO_4$. Removal of the solvent *in vacuo* gave pure carvacryloxyacetic acid (3.2 g, 45%) as white crystals. To a mixture of **1** (3.2 g, 7.2 mmol) and carvacryloxyacetic acid (1.5 g, 7.2 mmol) in hot (100 °C) PhMe (80 mL), titanium(IV) isopropoxide (0.40 mL, 1.4 mmol) was added through the septum, and the mixture was refluxed with stirring for 6 h using the Dean-Stark apparatus. The mixture was allowed to cool to room temperature and the formed precipitate was filtered off, and the organic phase was washed with saturated aqueous $NaHCO_3$ solution (2×50 mL), dried with anhydrous $Na_2SO_4$, and the solvent was removed *in vacuo*. The crude product was dissolved in refluxing mixture of cyclohexane-PhMe (3:1) and cooled to 4 °C overnight. The precipitate was filtered off, and the solvent was removed *in vacuo* to give **28** (2.5 g, 55 %) as reddish brown solid. mp 93-95 °C; $R_f$ 0.4 (1:6 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$) δ 0.76, 0.82, 0.97, 1.02, 1.20, 1.23, 1.67 (s, 3H), 2.25 (s, 3H), 2.42 (m, 1H), 2.83 (m, 1H), 3.18 (dd, *J* = 4.9, 10.7 Hz, 1H), 3.96 (d, J = 10.7 Hz, 1H), 4.39 (d, *J* = 9.9 Hz, 1H), 4.59 (s, 1H), 4.65 (s, 1H), 4.68 (s, 2H), 6.57 (s, 1H), 6.76 (dd, *J* = 1.4, 7.4 Hz, 1H), 7.07 (d, *J* =

7.7 Hz, 1H); $^{13}$C NMR (75 MHz, $CDCl_3$) δ 14.7, 15.3, 15.8, 16.0, 16.1, 18.2, 19.1, 20.7, 21.7, 24.1, 25.1, 27.0, 27.4, 28.0, 29.4, 29.5, 34.0, 34.1, 34.4, 37.1, 37.6, 38.7, 38.8, 40.8, 42.7, 46.4, 47.7, 48.8, 50.3, 55.3, 63.5, 65.6, 78.9, 109,3, 109.9, 119,1, 125,3, 130,8, 147,8, 150,0, 155,9, 169,8; FTIR (KBr) (ν, $cm^{-1}$): 3450, 2945, 2870, 1759, 1734, 1512, 1455, 1418, 1287, 1199, 1176, 1134; MS (direct, EI+): *m/z* 632; Anal. ($C_{42}H_{64}O_4$) C, H: calcd, 79.70, 10.19; found, 76.77, 9.14.



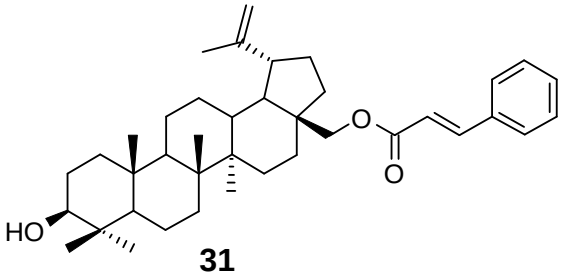

**29**

**3,28-Di-*O*-levulinoylbetulin (29).** To a mixture of **1** (4.0 g, 9.0 mmol) and levulinic acid (1.8 g, 16 mmol) in PhMe (110 mL) was added *p*-toluenesulfonic acid (0.085 g, 0.45 mmol), and the reaction mixture was refluxed at 175 ºC for 23 h using the Dean-Stark apparatus. The reaction mixture was washed with saturated aqueous $NaHCO_3$ solution (3 × 100 mL), water (100 mL), and dried with anhydrous $Na_2SO_4$. Solvent was removed *in vacuo* to give the crude product. It was purified by flash chromatography on $SiO_2$ (*n*-hexane:EtOAc 8:1 → 1:3) to give **29** (1.3 g, 23%) as off-white crystals. mp 95-97 °C; $R_f$ 0.5 (1:3 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$) δ 0.84 (s, 3H), 0.96 (s, 3H), 1.02 (s, 3H), 1.68 (s, 3H), 2.18 (s, 3H), 2.19 (s, 3H), 2.43 (m, 1H), 2.59 (m, 2H), 2.75 (m, 2H), 3.85 (d, *J* = 10.8 Hz, 1H), 4.47 (dd, *J* = 9.0, 7.2 Hz, 1H), 4.58 (s, 1H), 4.67 (s, 1H); $^{13}$C NMR (300 MHz, $CDCl_3$) δ 14.7, 16.0, 16.1, 16.5, 18.1, 19.1, 20.8, 23.6, 25.1, 27.0, 27.9, 28.0, 28.4, 29.5, 29.7, 29.9, 29.9, 34.1, 34.5, 37.0, 37.5, 37.8, 38.0, 38.1, 38.3, 40.8, 42.7, 46.4, 47.7, 48.8, 50.2, 55.4, 62.9, 81.2, 109.8, 150.1, 172.5, 173.1, 206.6, 206.7; FTIR (ν, $cm^{-1}$): 1024, 1157, 1641, 1742, 2935; MS (direct, EI+): *m/z* 638; Anal. ($C_{40}H_{62}O_6$) C, H.



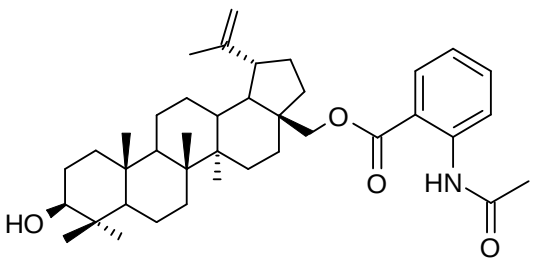

**30**

**28-*O*-Nicotinoylbetulin (30).** To a mixture of **1** (1.0 g, 2.3 mmol), nicotinic acid (0.29 g, 2.4 mmol) and 4-(dimethylamino)pyridine (15 mg, 0.12 mmol) in $CH_2Cl_2$ (30 mL) was added *N*,*N*′-dicyclohexylcarbodiimide (0.49 g, 2.4 mmol) in $CH_2Cl_2$ (30 mL). The reaction mixture was stirred at room temperature for 23 h and diluted with ethyl acetate (250 mL), washed with water (100 mL) and dried with anhydrous $Na_2SO_4$. Solvent was evaporated *in vacuo*, and the crude product was purified by flash chromatography on $SiO_2$ (*n*-hexane:EtOAc 10:1 → 2:1) to give **30** (0.39 g, 31%) as a white solid. mp 114-116 °C; $R_f$ 0.5 (1:1 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$) δ 0.76 (s, 3H), 0.83 (s, 3H), 0.96 (s, 3H), 1.00 (s, 3H), 1.06 (s, 3H), 1.70 (s, 3H), 2.51 (m, 1H), 4.13 (d, *J* = 12.6 Hz, 1H), 4.56 (d, *J* = 10.4 Hz, 1H), 4.61 (s, 1H), 4.72 (s, 1H), 7.42 (dd, *J* = 4.9, 7.7 Hz, 1H), 8.32 (d, *J* = 8.0 Hz, 1H), 8.78 (d, *J* = 3.3 Hz, 1H), 9.24 (s, 1H); $^{13}$C NMR (75 MHz, $CDCl_3$) δ 14.8, 15.3, 16.0, 16.1, 18.3, 19.1, 20.8, 25.2, 27.1, 27.4, 28.0, 29.6, 29.9, 34.0, 34.2, 34.6, 37.1, 37.7, 38.7, 38.9, 40.9, 42.8, 46.7, 47.7, 48.9, 50.4, 55.3, 63.8, 78.9, 110.0, 123.4, 137.2, 149.9, 150.7, 153.2, 165.5; FTIR (ν, $cm^{-1}$): 701, 740, 1025, 1109, 1285, 1723, 3381; MS (ESI+): *m/z* 548.4 $[M+1]^+$; Anal. ($C_{36}H_{53}NO_3$) C, H, N.



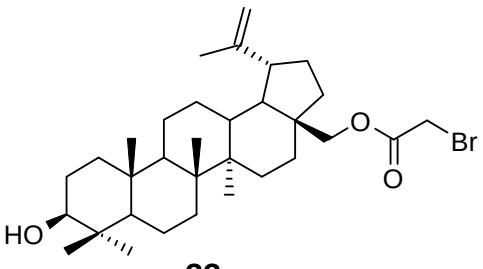

**31**

**28-*O*-Cinnamoylbetulin (31)**. A mixture of cinnamic acid (2.7 g, 18 mmol) and thionyl chloride (13 mL) was stirred at 40 °C for 20 h. Excess thionyl chloride was removed by evaporation, and the residue was dissolved three times to $CH_2Cl_2$ (40 mL) and evaporated to dryness to give the crude cinnamoyl chloride (3.0 g, 18 mmol) that was used in the next reaction step without further purification. A mixture of **1** (2.5 g, 5.7 mmol), 4-(dimethylamino)pyridine (0.83 g, 6.8 mmol) and freshly prepared cinnamoyl chloride (1.1 g, 6.8 mmol) in dry pyridine (40 mL) was stirred at 40 °C for 22 h. The reaction mixture was diluted with PhMe (160 mL), washed with 5% hydrochloric acid (4×100 mL), water (2×100 mL) and dried with anhydrous $Na_2SO_4$. Solvent was evaporated *in vacuo* and the resulting crude product was crystallized from a mixture of cyclohexane-PhMe (5:1) to give **31** (0.67 g, 21%) as a white solid. mp 190-191 °C; $R_f$ 0.4 (1:6 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$) δ 0.77 (s, 3H), 0.83 (s, 3H), 0.97 (s, 3H), 1.00 (s, 3H), 1.06 (s, 3H), 1.70 (s, 3H), 2.50 (m, 1H), 3.19 (dd, *J* = 5.5, 11.0 Hz, 1H), 3.99 (d, *J* = 11.0 Hz, 1H), 4.42 (d, *J* = 11.0 Hz, 1H), 4.60 (s, 1H), 4.72 (s, 1H), 5.38 (m, 2H), 6.47 (d, *J* = 15.9 Hz, 1H), 7.21 (dd, *J* = 7.4, 21.7 Hz, 1H), 7.39 (dd, *J* = 3.0, 6.3 Hz, 2H), 7.53 (dd, *J* = 2.5, 5.8 Hz, 2H), 7.69 (d, *J* = 15.9 Hz, 1H); $^{13}$C NMR (75 MHz, $CDCl_3$) δ 14.8, 15.3, 16.0, 16.1, 18.3, 19.1, 20.8, 25.2, 27.1, 27.4, 28.0, 29.6, 29.8, 34.2, 34.6, 37.1, 37.6, 38.7, 38.8, 40.9, 42.7, 46.5, 47.7, 48.8, 50.3, 55.3, 62.8, 78.9, 109.9, 118.2, 128.0, 128.8, 130.2, 134.4, 144.6, 150.2, 167.5; FTIR (ν, $cm^{-1}$): 682, 766, 975, 1017, 1190, 1279, 1692, 2932, 3557; MS (direct, EI+): *m/z* 572; Anal. ($C_{39}H_{56}O_3$) C, H.




**32**

**28-*O*-(*N*-Acetylanthraniloyl)betulin (32).** A mixture of *N*-acetylanthranilic acid (4.5 g, 25 mmol) and oxalyl chloride (21 mL, 250 mmol) was stirred at room temperature for 3 d. Excess oxalyl chloride was removed by evaporation, and the residue was dissolved to $CH_2Cl_2$ (50 mL) and evaporated to dryness twice to give the crude *N*-acetylanthraniloyl chloride (4.8 g, 24 mmol) that was used in the next reaction step without further purification. A mixture of **1** (2.5 g, 5.6 mmol), 4-(dimethylamino)pyridine (0.69 g, 5.6 mmol) and freshly prepared *N*-acetylanthraniloyl chloride (1.3 g, 6.8 mmol) in dry pyridine (40 mL) was stirred at 40 °C for 40 h. The reaction mixture was diluted with ethyl acetate (120 mL), washed with 5% hydrochloric acid (3×100 mL), water (2×100 mL) and dried with anhydrous $Na_2SO_4$. Solvent was evaporated *in vacuo* and the resulting crude product was purified by flash chromatography on $SiO_2$ (*n*-hexane:EtOAc 20:1 → 1:1) to give **32** (0.85 g, 25%) as a white solid. mp 201-203 °C; $R_f$ 0.3 (1:2 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, DMSO-$d_6$) δ 0.65 (s, 3H), 0.77 (s, 3H), 0.87 (s, 3H), 0.95 (s, 3H), 1.01 (s, 3H), 1.66 (s, 3H), 1.98 (s, 3H), 2.97 (t, *J* = 8.2 Hz, 1H), 4.02 (d, *J* = 10.2 Hz, 1H), 4.51 (d, *J* = 10.2 Hz, 1H), 4.58 (s, 1H), 4.72 (s, 1H), 7.25 (t, *J* = 7.7 Hz, 1H), 7.66 (t, *J* = 7.4 Hz, 1H), 8.06 (d, *J* = 7.7 Hz, 1H), 8.61 (d, *J* = 8.2 Hz, 1H); $^{13}$C NMR (75 MHz, $CDCl_3$) δ 14.1, 14.5, 15.7, 15.8, 15.9, 17.9, 18.8, 20.3, 24.7, 26.6, 27.2, 28.1, 29.0, 33.7, 33.9, 36.7, 37.1, 38.2, 38.5, 40.4, 42.3, 46.1, 47.0, 48.3, 49.7, 54.8, 64.9, 76.8, 110.0, 117.2, 119.6, 124.0, 131.4, 134.2, 139.3, 149.8, 154.2, 159.8, 169.3; FTIR (ν, cm$^{-1}$): 770, 789, 967, 1041, 1143, 1274, 1455, 1528, 1589, 1698, 2941, 3477; Anal. ($C_{39}H_{57}NO_4$) C, H: calcd, 77.57, 9.51, 2.32; found, 71.87, 8.66, 1.87.




**33**

**28-*O*-Bromoacetylbetulin (33).** A mixture of betulin **1** (1.0 g, 2.3 mmol) and *t*-BuOK (2.5 g, 23 mmol) in dry THF (50 mL) was heated to 75 °C and methyl bromoacetate (3.5 g, 23 mmol) was added. After 10 min reaction mixture was poured to water (300 mL). Formed precipitate was removed by suction, dried in vacuum oven and crude product was purified by flash chromatography on $SiO_2$ (*n*-hexane:EtOAc 8:1 → 2:1) to give **33** (0.20 g, 16%) as an off-white solid. mp 154-156 °C; $R_f$ 0.6 (1:4 EtOAc:*n*-hexane); $^1$H NMR (300 MHz, $CDCl_3$) δ 0.73 (s, 3H), 0.80 (s, 3H), 0.94 (s, 3H), 0.95 (s, 3H), 1.01 (s, 3H), 1.66 (s, 3H), 2.40 (m, 1H), 3.16 (dd, *J* = 5.2, 10.4 Hz, 1H), 3.84 (s, 2H), 3.93 (d, *J* = 11.0 Hz, 1H), 4.35 (d, *J* = 10.4 Hz, 1H), 4.57 (s, 1H), 4.67 (s, 1H); $^{13}$C NMR (75 MHz, $CDCl_3$) δ 14.7, 15.3, 15.9, 16.0, 18.2, 19.0, 20.7, 25.1, 25.9, 26.9, 27.3, 27.9, 29.4, 29.5, 34.1, 34.3, 37.0, 37.5, 38.6, 38.8, 40.8, 42.6, 46.5, 47.6, 48.7, 50.2, 55.2, 64.7, 78.8, 109.9, 149.8, 167.6. FTIR (ν, $cm^{-1}$): 884, 983, 1109, 1289, 1388, 1455, 1730, 2870, 2941, 3572; MS (direct, EI+): *m/z* 564; Anal. ($C_{32}H_{51}BrO_3$) C, H.

REFERENCES


(1) Pohjala, L.; Barai, V. N.; Azhayev, A.; Lapinjoki, S.; Ahola, T. *Antivir. Res.* **2008,** *78(2)*, 215-222.

(2) Zhang, J.H.; Chun, T.D.Y.; Oldenburg. K.R. *J. Biomol. Screen.* **1999**, *4*, 67-73.

(3) Greco, W. R.; Bravo, G.; Parsons, J. C. *Pharmacol. Rev.* **1995,** *47*, 331–385.

(4) Eckerman, C.; Ekman, R. *Pap. Puu* **1985**, *67*, 100-106.

(5) Huang, F-Y.; Chung, B. Y.; Bentley, M. D.; Alford, A. R. *J. Agric. Food Chem.* **1995**, *43*, 2513-2516.

(6) Suokas, E.; Hase, T. *Acta Chem. Scand. B* **1975**, *29*, 139-140.

(7) Suokas, E.; Hase, T. *Acta Chem. Scand. B* **1977**, *31*, 182-184.

(8) Sun, I-C.; Wang, H-K.; Kashiwada, Y.; Shen, J-K.; Cosentino, L. M.; Chen, C-H.; Yang, L-M.; Lee, K-H. *J. Med. Chem.* **1998**, *41*, 4648-4657.

(9) Kim, D. S. H. L.; Chen, Z.; Nguyen, T.; Pezzuto, J. M.; Qiu, S.; Lu, Z-Z. *Synth. Commun.* **1997**, *27*, 1607-1612.

(10) Flekhter, O. B.; Nigmatullina, L. R.; Baltina, L. A.; Karachurina, L. T.; Galin, F. Z.; Zarudii, F. S.; Tolstikov, G. A.; Boreko, E. I.; Pavlova, N. I.; Nikolaeva, S. N.; Savinova, O. V. *Pharm. Chem. J.* **2002**, *36*, 484-487.

(11) Petrenko N. I.; Elantseva N. V.; Petukhova V. Z.; Shakirov M. M.; Shults E. E.; Tolstikov G. A. *Chem. Nat. Comp.* **2002**, *38*, 331-339.

(12) Corey E. J.; Suggs, J. W. *Tetrahedron Lett.* **1975**, 2647-2650.

(13) Komissarova, N. G.; Belenkova, N. G.; Spirikhin, L. V.; Shitikova, O. V.; Yunusov, M. S. *Chem. Nat. Comp.* **2002**, *38*, 58-61.

(14) Flekhter, O. B.; Ashavina, O. Y.; Boreko, E. I.; Karachurina, L. T.; Pavlova, N. I.; Kabalnova, N. N.; Savinova, O. V.; Galin, F. Z.; Nikolaeva, S. N.; Zarudii, F. S.; Baltina, L. A.; Tolstikov, G. A. *Pharm. Chem. J.* **2002**, *36*, 303-306.

(15) Leffler, M. T.; Calkins, A. E. *Org. Synth.* **1943**, *23*, 52.