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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 15 (2005) 4196–4200

Correlation of cytotoxic activity of betulinines and their hydroxy analogues

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Received 26 April 2005; revised 24 June 2005; accepted 27 June 2005
Available online 26 July 2005

Abstract—This research is based on intention to prepare and test 3β-hydroxy and 3β,28-dihydroxy analogues of new pro-apoptotic derivatives (betulinines) using selective hydrolysis procedure and strategic protective groups. The evaluation of cytotoxicity of prepared compounds on several tumor cell lines using an MTT test was our interest. It was found that hydrolysis of acetates in betulinines afforded compounds with higher cytotoxicity in case of 18-lupene-21-ones (e.g., ethyl 3β-hydroxy-21-oxolup-18-en-28-oate), whereas hydrolysis of the 18-lupene-21,22-diones gave less active derivatives.

1. Introduction

A group of new 18-lupene, 18,19-secolupane, des-E lupane, and other highly oxidized terpenes was prepared in our workgroup. High antitumor activity and fast apoptosis (comparable with paclitaxel) on CEM cell line were found among these derivatives, now called betulinines,[1] within our next research. Molecular mechanism of action in the group of betulinines is still unknown, which is therefore now intensively studied. In our more recent study,[2] the cytotoxicity of the two best compounds—3β,28-diacetoxy-18-oxo-19,20,21,29,30-pentanorlupan-22-oic acid and methyl 3β-acetoxy-21,22-dioxolup-18-en-28-oate (**9**)—on other cancer cell lines (e.g., DU145, HT29, K-562, OVCAR-3, and U2OS) was confirmed; both of them dispose of $IC_{50} < 1$, respectively, <5 μmol/L. In contrast to the first known cytotoxic triterpenoide, betulinic acid,[3] the antitumor activity of these derivatives is not limited to the malign melanoma,[4] since similar results were obtained on more lines.[2]

All active compounds prepared here had 3β-hydroxy group substituted with acetate, which is in contrast to the literature,[5] that showed lower biological activity (e.g., antitumor and antimicrobial) in acetylated compounds than in derivatives with free 3β-hydroxy group. In addition, the structure–activity relationship study[3] of betulinic acid derivatives confirmed this fact.

Although the hydrolysis is one of the low-cost derivatization methods, its use is rare. Its use often leads to more polar derivatives with lower molecular weights, which are more suitable for the biological tests. In addition, compounds with a free hydroxy group represent significant derivatization[6] potential for synthesis of new biologically compatible derivatives for testing. In this research, we intended to find out whether all structure–activity relationship correlations, known in case of betulinic acid derivatives are the same in the group of betulinines. These could lead to compounds with a higher cytotoxicity than the starting material. After the hydrolysis of highly active acetates, even more active hydroxy derivatives should be obtained. This fact was not confirmed.

2. Chemical synthesis

Betulin (**1**) was converted to known[7] 21-oxo acid **2** using a five-step procedure[1,8] in total yield of 34%. Methyl ester **3** was obtained in quantitative yield by the reaction of acid **2** with diazomethane in diethylether. Ethyl ester

Keywords: Triterpenes; Hydrolysis; Acetate; Cytotoxicity.
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doi:10.1016/j.bmcl.2005.06.087

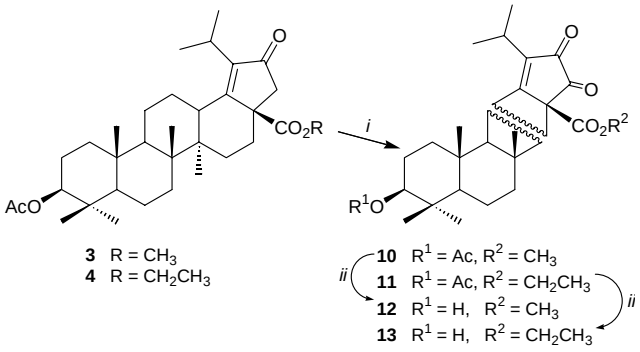
4 was obtained analogously using diazoethane. Benzyl ester **5** was obtained from acid **2** using the alkylation procedure with benzylbromide and DBU in dichloromethane and acetonitrile, which yielded 78% of **2**. We obtained 3β-hydroxy acid **6** by hydrolysis of acetate **2** using potassium hydroxide in refluxing mixture of ethanol and dioxane. In contrast, the 3β-hydroxy derivatives **7**–**9** were prepared from acetates **3**–**5** in good yields using the acidic hydrolytic procedure carried out by concentrated aqueous hydrochloric acid in a mixture of dioxane and methanol at room temperature (Scheme 1).

Oxidation of 21-oxo esters **3** and **4** gave corresponding 21,22-dioxo derivatives **10** and **11**, according to Ref. 1, where selenium dioxide in the mixture of dioxane and acetic acid at reflux was used. Using the same acidic hydrolysis as above, we got 3β-hydroxy analogues **12** and **13** (yields 80–90%) (Scheme 2).

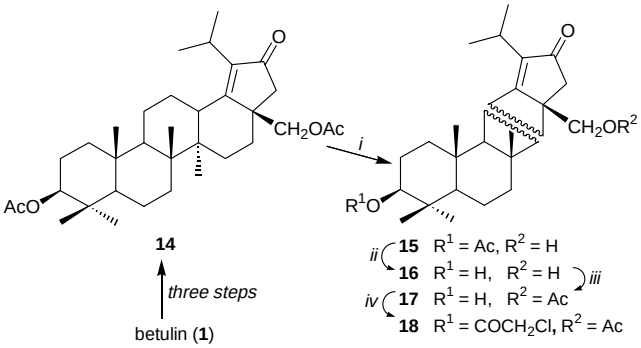
Ketone diacetate **14**[9] was prepared from betulin (**1**) via a known[10] three-step procedure. Customary alkaline hydrolysis with potassium hydroxide gave dihydroxy ketone **16**. The monoacetate **17** was obtained on parcial acetylation of diol **16** in refluxing ethyl acetate in the presence of alumina (Brockmann 1),[11] or alternatively by the treatment analogously with acetic anhydride in the presence of imidazole.[12] Both of the processes gave monoacetate **17** in similar yields (60–70%).

The treatment with alumina takes a very long time, usually more than 10 days to give good conversion of starting material, however, it gave monoacetate **17** in higher purity. Whereas the acetylation with a mixture of acetic anhydride and imidazole runs much faster, it is inevitable to separate resulted monoacetate **17** and diacetate **14** using column chromatography. For a bulk quantity production of monoacetate **17**, it is much better to use the procedure with alumina and for the reactions in semi-preparative quantities, the acetylation with acetic anhydride and imidazole (Scheme 3).

Ketone chloroacetate **18**, prepared according to Scheme 3, was oxidized with selenium dioxide to give diketone

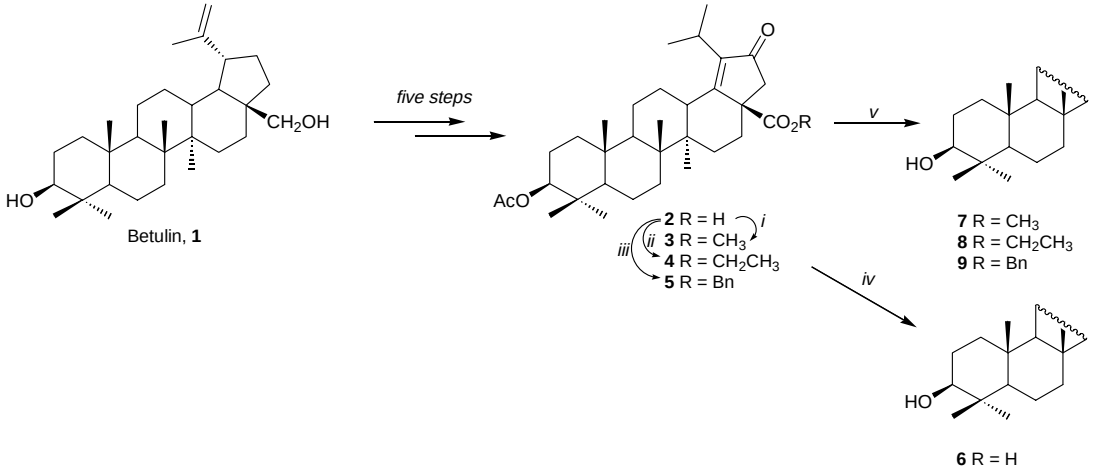


Scheme 2. Reagents and conditions: (i) SeO_2/dioxane, AcOH, reflux and (ii) HCl/dioxane, MeOH, rt.

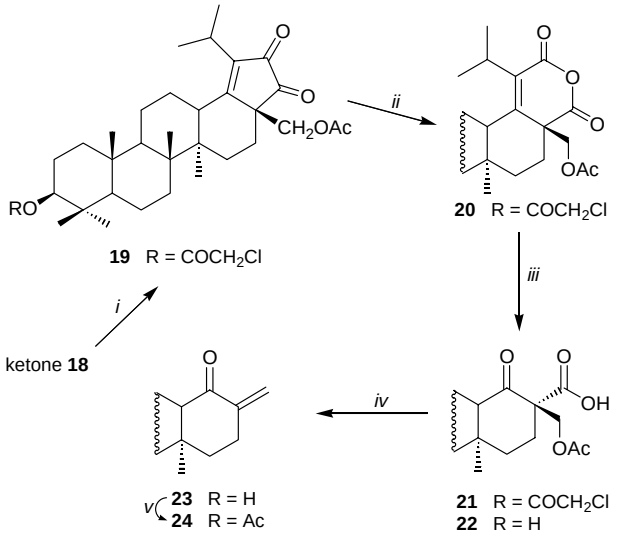


Scheme 3. Reagents and conditions: (i) KOH (1 equiv)/EtOH, PhMe; rt; (ii) KOH (2 equiv)/EtOH, PhMe; reflux; (iii) Ac_2O, imidazole/$CHCl_3$, reflux; (iv) $(CH_2ClCO)_2O$, Py, rt.

chloroacetate **19**, from which the anhydride chloroacetate **20** was obtained using peroxoacetic acid. Oxidation of anhydride **20** with ruthenium tetraoxide gave β-keto-acid **21**. Following experiment with its deprotection using conditions described in Scheme 4 gave methyleneketone **23** as the major product, which was then characterized as known[1] acetate **24**. Desired acid **22** was probably unstable and might have decarboxylated during the deprotection and deacetylation. Methyleneketone **23** was obtained (Scheme 4).



Scheme 1. Reagents and conditions: (i) CH_2N_2/Et_2O, rt; (ii) CH_3CHN_2/Et_2O, rt; (iii) BnBr, DBU/CH_2Cl_2, MeCN, rt; (iv) KOH/EtOH, PhMe, reflux; and (v) HCl/dioxane, MeOH, rt.



Scheme 4. Reagents and conditions: (i) SeO_2/dioxane, AcOH, reflux; (ii) $AcO_2H/CHCl_3$, rt; (iii) RuO_2, $NaIO_4$/EtOAc, H_2O, rt; (iv) *o*-$NH_2C_6H_4NH_2$/EtOH, Py, rt; and (v) Ac_2O, Py, rt.

3. Results and discussion

It was found that classical basic hydrolysis was good in hydrolysis of stable 28-acetoxy-21-oxo derivatives. In contrast to this, hydrolysis of more reactive 21,22-dioxo derivatives with basic conditions gave a complex mixture that was not separated. In case of alkyl 21-oxo-28-oates, the basic conditions of the hydrolysis gave free acids on C-28. Special acidic hydrolysis led to good yields in the case of the hydrolysis of both, the 21,22-dioxo derivatives and alkyl 21-oxo-28-oates. Sometimes both, basic and acidic hydrolysis, could be used (e.g., preparation of **6** and **16**).

A chloroacetate group was used successfully as a protective group for the 3β-hydroxy group. It was good for the preparation and stability; however, the hydroxy acid **22** could not be supplied in this way. To sum up, the 3β-chloroacetoxy derivatives prepared were interesting for their biological properties.

Within this study, a structure–activity relationship of betulinines and their hydroxy derivatives was studied. This led to two results. First, a comparison of activity of 3β-acetoxy-21-ones **2**, **3**, **4**, **5** and their corresponding 3β-hydroxy analogs **6**, **7**, **8**, **9** showed that the cytotoxicity increased 2–10 times (on CEM cell line) after the hydrolysis. In contrast, the cytotoxicity decreased 3–6 times (on CEM cell line) in the case of the hydrolysis of 3β-acetate of 21,22-diones **10**, **11**. In a group of 21-oxo-17-hydroxymethyl derivatives **14**–**18**, only the compounds with one free and one acetylated hydroxy group **15** and **17** were active; meanwhile, diacetate **14** and diol **16** were found inactive. *β*-Ketoacid **21** had the highest activity ($IC_{50} < 1$ μmol/L on three tumor cell lines) among the 3β-chloroacetoxy derivatives. In addition, the cytotoxicity of methyleneketone **24** on three tumor lines should be mentioned, which is in contrast with the activity on T-lymphoblastic leukemia cell line, where the compound **24** was inactive[1] (Table 1).

4. Conclusion

We found that hydrolysis of acetyl groups in betulinines afford compounds with higher cytotoxicity; however,

Table 1. Cytotoxic activity of compounds **1**–**21** and **24** against CEM, K562, HT 29, PC-3, and SK MEL2 cells

Compound	IC_{50} (μmol/L[a])					
	CEM	K562	K562-Tax	HT-29	PC-3	SK-MEL2
1	>250	>250	>250	>250	>250	>250
2	>250	>250	>250	149	117	128
3	44	34	96	149	246	165
4	102	59	206	224	244	216
5	250	245	250	250	250	250
6	29	30	34	33	40	160
7	26	38	36	38	38	80
8	18	17	19	13	33	80
9	26	27	28	21	37	156
10	16	29	36	33	34	87
11	38	59	130	93	87	148
12	44	35	46	54	72	138
13	210	239	85	176	165	140
14	>250	>250	>250	>250	>250	>250
15	28	37	35	33	39	84
16	>250	>250	>250	>250	>250	>250
17	250	39	59	93	163	85
18	44	110	97	110	242	229
19	242	231	>250	>250	208	170
20	162	181	220	216	250	121
21	1	0.2	0.4	3.6	2.3	1.3
24	>250	0.4	1.1	129	143	2.1

Value >250 μmol/L means that compound is not active.
[a] The lowest concentration that kills 50% of tumor cells.

this is limited only to 18-lupene-21-ones (**7**, **9**). On the other hand, hydrolysis of 18-lupene-21,22-diones gave hydroxy derivatives of lower activity. Two very active compounds were obtained, methyleneketone **24** and β-ketoacid **21**[13] ($IC_{50} < 1$ μmol/L), even though the first of them was inactive on CEM line.

In this research, the 3β-hydroxy analogs from 3β-acetates and the 3β,28-diols from 3β,28-diacetoxy derivatives of betulinines were prepared using selective hydrolysis methods. Hydroxy derivatives prepared here are attractive materials for a preparation of new cytotoxic derivatives using acylation, esterification, glycosidation, and other reactions. Easily obtainable betulin (**1**), whose presence in birch bark is up to 30%, was used as the starting compound. In the case of commercial[10] use of betulinines, the availability of betulin as a major compound of waste in paper factories in northern countries (e.g., Sweden) makes its use cost-effective, easy, and ecological friendly.

5. Materials and methods

5.1. Chemicals

Ruthenium (IV) oxide, selenium dioxide, chloroacetic anhydride, sodium mateperiodate, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), and SDS were purchased from Sigma–Aldrich, s.r.o., Prague, Czech republic.

5.2. Cell lines

Cell lines CEM, HT29, K562, PC-3, and SK MEL2 were purchased from the American Tissue Culture Collection (ATTC). Paclitaxel-resistant subline of K562 cells (K-562-Tax) was prepared and characterized in our laboratories.[14] The human T-lymphoblastic leukemia cell line, CEM, was used for routine screening of compounds.

The cells were maintained in Nunc/Corning 80 cm^2 plastic tissue culture flasks and cultured in cell culture medium (DMEM/RPMI 1640 with 5 g/L glucose, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10% fetal calf serum, and $NaHCO_3$).

5.3. Cytotoxic MTT assay

Cell suspensions were prepared and diluted according to the particular cell type and the expected target cell density (2500–30,000 cells/well based on cell growth characteristics). Cells were added by pipette (80 μL) into 96-well microtiter plates. Inoculates were allowed a preincubation period of 24 h at 37 °C and 5% CO_2 for stabilization. Fourfold dilutions, in 20 μL aliquots, of the intended test concentration were added at time 0 to the microtiter plate wells. All test compound (dissolved in 10 μL of 10% DMSO) concentrations were examined in duplicate. Incubation of the cells with the test compounds lasted for 72 h at 37 °C in a 5% CO_2 atmosphere at 100% humidity. At the end of the incubation period, the cells were assayed using MTT. Aliquots (10 μL) of the MTT stock solution were pipetted into each well and incubated for a further 1–4 h. After this incubation period, formazan produced was dissolved by the addition of 100 μL/well of 10% aq SDS (pH 5.5), followed by a further incubation at 37 °C overnight. The optical density (OD) was measured at 540 nm with a Labsystem iEMS Reader MF. Tumor cell survival (IC) was calculated using the following equation: IC = (ODdrug-exposed well/mean ODcontrol wells) × 100%. The IC_{50} value, the drug concentration lethal to 50% of the tumor cells, was calculated from appropriate dose–response curves.

Acknowledgments

This study was supported in part by the Ministry of Education of the Czech Republic (MSM 6198959216), which paid for instrumental equipment and the Czech Science Foundation (203/03/D152), from which the chemicals were paid, and by MPO project (FT-TA/027) from which the transportation costs of birch bark from Sweden and its extraction were paid. Biological testing was supported by the Czech Science Foundation (301/03/1570). We are grateful to Iva Tislerova for measurement of NMR spectra, Stanislav Hilgard for measurement of IR spectra, and Martin Sticha for measurement of MS. Special thanks to Bohunka Sperlichova for measurement of optical rotations.

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1721 b, 1239 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 0.87, 0.88, 0.90, 0.92, 1.15 (each 3H, s, $5 \times CH_3$), 2.04 (3H, s, $COCH_3$), 2.20 (2H, m, $2 \times$ H-16), 2.88 (1H, dd, J = 11.9, 3.7 Hz, H-13β), 4.03 (1H, d, J = 14.7 Hz, $COCH_aCl$), 4.08 (1H, d, J = 14.7 Hz, $COCH_bCl$), 4.42 (1H, d, J = 11.1 Hz, H-28a), 4.57 (1H, dd, J = 11.0, 5.5 Hz, H-3α), 4.73 (1H, d, J = 11.1 Hz, H-28b). ^{13}C NMR (100 MHz, $CDCl_3$): δ 215.6, 173.3, 170.4, 167.1, 83.0, 66.3, 57.8, 55.4, 50.5, 49.9, 47.0, 41.2, 41.1, 38.5, 38.0, 37.1, 33.9, 29.2, 27.9, 26.9, 23.4, 21.7, 20.6, 19.6, 18.0, 16.7, 16.4, 16.2, 16.1. MS, *m/z* (%): [for $C_{29}H_{43}ClO_7$, M^+ 538], 434 ($[M-104]^+$, 1), 340 (1), 325 (1), 310 (1), 298 (1), 283 (1), 230 (1), 216 (15), 204 (46), 189 (100).

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