Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 1707–1712

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

SYNTHESIS OF BETULINIC ACID DERIVATIVES WITH ACTIVITY AGAINST HUMAN MELANOMA

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Received 17 March 1998; accepted 29 May 1998

Abstract: Betulinic acid has been modified at C-3, C-20, and C-28 positions and the toxicity of the derivatives has been evaluated against cultured human melanoma (MEL-2) and human epidermoid carcinoma of the mouth (KB) cell lines. This preliminary investigation demonstrates that simple modifications of the parent structure of betulinic acid can produce potentially important derivatives, which may be developed as antitumor drugs.

Introduction: Betulinic acid (**1**), a pentacyclic triterpene, has recently been reported to possess antitumor activity against cultured human melanoma cells in both in vitro and in vivo models.[1] Betulinic acid was isolated through bioassay-guided fractionation of the stem bark of *Ziziphus mauritiana* Lam. (Rhamnaceae). The active principle, betulinic acid, showed selective cytotoxicity against cultured human melanoma (MEL-2) as opposed to several other cell types including human epidermoid carcinoma of the mouth (KB).[1] Furthermore, the ability of betulinic acid to induce apoptosis in melanoma[1] and other cell types[2] and the favorable therapeutic index from the lack of toxicity towards normal cells[1] suggests betulinic acid is an attractive and promising antitumor agent. The selective toxicity towards malignant melanoma as compared to normal melanocytes was underscored by a study reporting a lack of toxicity towards melanocytes in an in vivo model.[3] Other biological activities reported for betulinic acid include anti-inflammatory activity[4] and inhibition of phorbol ester-induced epidermal ODC accumulation in the mouse ear model with subsequent inhibition of the carcinogenic response in the two-stage mouse skin model.[5] This paper describes the synthesis and biological evaluation of betulinic acid analogs against melanoma (MEL-2) and fibrosarcoma (KB). Betulin (**2**), available in abundance up to 25% dry weight of bark from white birch bark,[6] can be synthetically converted to betulinic acid in a two-step process of high yield[7] (Scheme 1). It was envisioned that a series of simple modifications of the parent structures could produce a number of potentially important derivatives that could improve the selective antitumor activity.

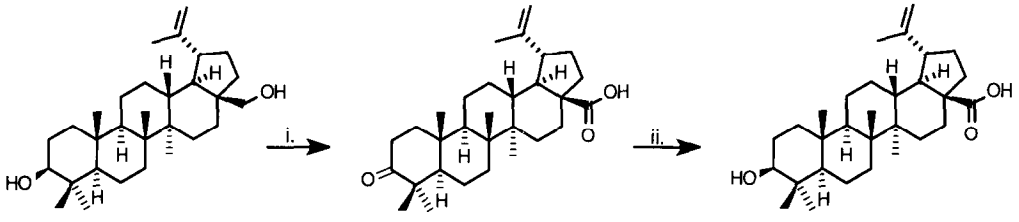
Materials and Procedures for Cytotoxicity Evaluation: Human melanoma cell line, MEL-2, was obtained from the Department of Surgical Oncology, University of Illinois and maintained in minimum essential medium with Hank's salt (Life Technologies) supplemented with 10% fetal bovine serum (FBS, Atlanta

0960-894X/98/$19.00
PII: S0960-894X(98)00295-9

Biologicals) and 1% Penicillin G-Streptomycin-Fungizone (PSF, Life Technologies). Human epidermoid carcinoma of the mouth cell line, KB, was obtained from ATCC and maintained in Dubecco's minimum essential medium (Life Technologies) supplemented with 10% calf serum (Atlanta Biologicals), 1% PSF, and nonessential amino acids (Life Technologies). Both cell lines were incubated in a humidifier, 5% CO_2 atmosphere; however, the MEL-2 cells were cultured in a closed-cap manner due to the low sodium bicarbonate concentration of the media. The day prior to the toxicity assessment, the media was changed to ensure the cells were in logarithmic growth. The cytotoxicity assessment followed the procedure described previously.[8] Briefly, various concentrations of the testing compounds (dissolved in 10 μL of 10% DMSO) were transferred to 96-well plates and 190 μL of cell suspension were added to each well. After incubating the plates for three days at 37° (100% humidity with 5% CO_2 atmosphere), the cellular proteins were precipitated to the plates with trichloroacetic acid and stained with 0.4% sulphorhodamine (SRB). Protein-bound SRB was solubilized with Tris base and read at 515 nm in an ELISA reader. The protein content of the compound-treated cells was compared to that of DMSO solvent control and ED_{50} values were obtained.

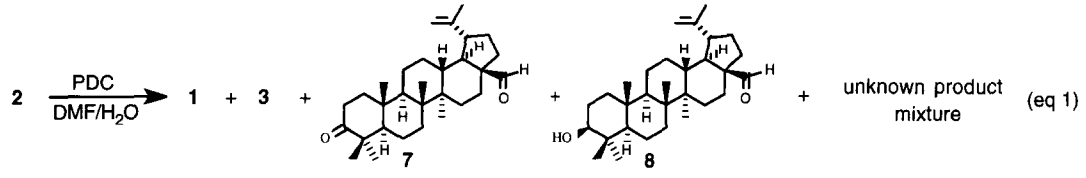
Synthesis Scheme I: Betulin (**2**) was converted to betulonic acid (**3**)[4-7] in over 75% yield by using Jones' oxidation (CrO_3/H_2SO_4/acetone/0 °C).[7,9] Betulonic acid (**3**) was reduced with $NaBH_4$/THF[4] to betulinic acid (**1**) in quantitative yield (> 98%) as a 5/95 mixture of α and β isomers and was recrystallized in hot methanol to afford 75% β isomer as white needles.[7]

Scheme 1

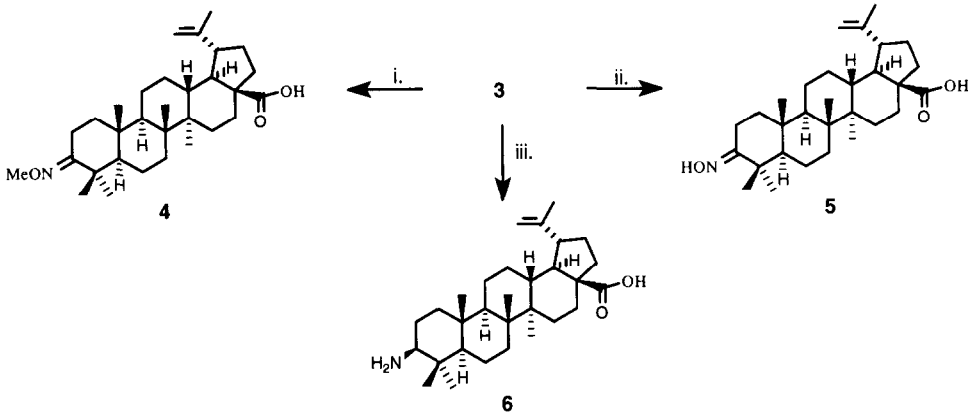


i. Jones' oxidation (CrO_3/H_2SO_4/acetone/0°C) ii. $NaBH_4$/THF

Synthesis Scheme II: Betulonic acid (**3**) was reacted with methoxylamine chloride or hydroxylamine chloride in the presence of NaOAc in ethanol[10,11] to afford the corresponding oximes **4** and **5** in quantitative yield, respectively. Reductive amination[12-14] of betulonic acid (**3**) with ammonium acetate in the presence of $NaBH_3CN$ in methanol successfully introduced the amine functionality at position C-3 to give **6** in 80% yield as an approx. 5/95 mixture of α and β isomers. A selective oxidation[15,16] of betulin (**2**) by PDC in DMF[17] was attempted, but a complex mixture of products that include betulinic acid (**1**, 8%), betulonic acid (**3**, 13%), betulin aldehyde-ketone **7** (20%) and betulin aldehyde-alcohol **8** (7%) was obtained (eq 1).



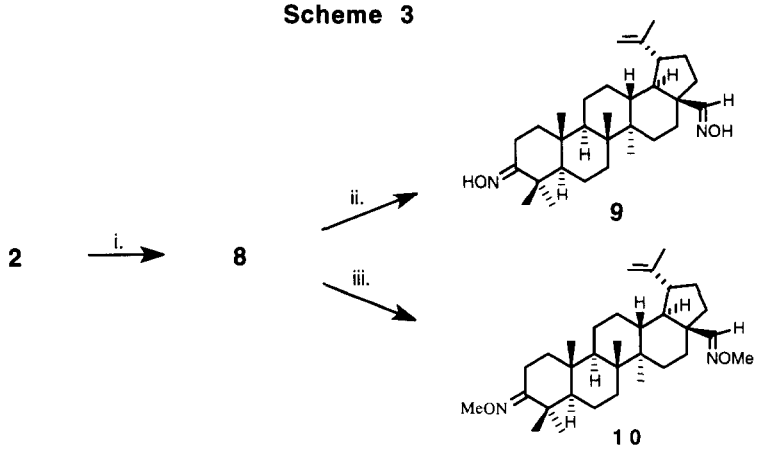
Scheme 2



i. $MeONH_3Cl/NaOAc/EtOH$; ii. $HONH_3Cl/NaOAc/EtOH$; iii. $NH_4OAc/NaBH_3CN/MeOH$.

Synthesis Scheme III: PDC oxidation[17] of betulin (**2**) in CH_2Cl_2 rendered the betulin aldehyde-ketone **7** in excellent yield (87%). The betulin aldehyde-ketone **7** was subjected to hydroxylamine chloride and methoxylamine chloride condensation in the presence of NaOAc in ethanol[10,11] to afford the corresponding oximes **9** and **10** in quantitative yield, respectively.

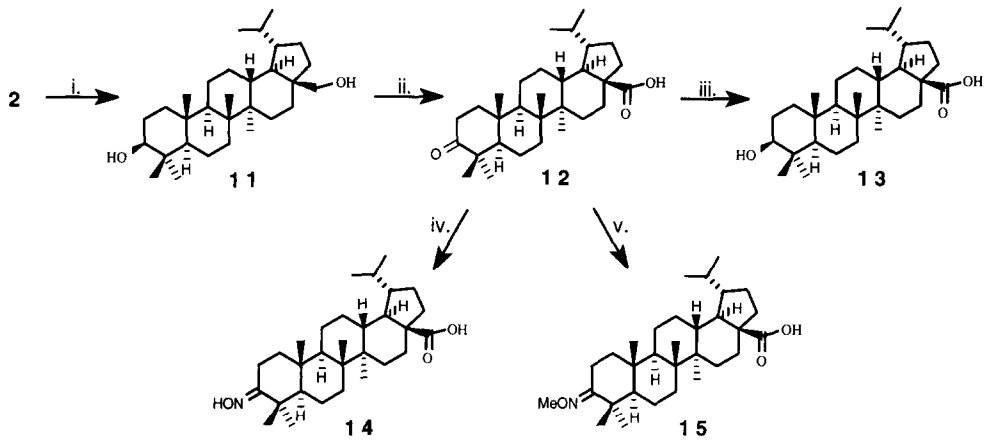
Scheme 3



i. PDC/CH_2Cl_2; ii. $HONH_3Cl/NaOAc/EtOH$; iii. $MeONH_3Cl/NaOAc/EtOH$

Synthesis Scheme IV: Betulin (**2**) was subjected to hydrogenation[18-21] by Pd/C in ethanol/acetic acid (50/50) to afford dihydrobetulin **11** in 65% yield which was oxidized (CrO_3/H_2SO_4/acetone/0 °C)[7,9] to the corresponding dihydrobetulonic acid (**12**) in 80% yield. $NaBH_4$ reduction[4] of dihydrobetulonic acid (**12**) produced the dihydrobetulinic acid (**13**) in a quantitative yield as a 5/95 mixture of α and β isomers, which was recrystallized in hot methanol to give the β-isomer as white needles. The dihydrobetulonic acid **12** was converted to the corresponding hydroxyloxime **14** and methoxyloxime **15** in excellent yield by condensing with hydroxylamine chloride and methoxylamine chloride in the presence of NaOAc in ethanol, respectively.

Scheme 4



i. H_2/Pd-C/EtOH-HOAc; ii. CrO_3/H_2SO_4/acetone/0 °C; iii. $NaBH_4$/THF; iv. $HONH_3Cl$/NaOAc/EtOH; v. $MeONH_3Cl$/NaOAc/EtOH.

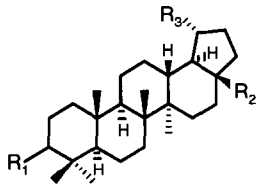
As a preliminary study in the developing betulinic acid (**1**) as an antitumor agent against human melanoma, a series of betulinic acid derivatives **3-18** were prepared and tested against human melanoma cell line MEL-2, in order to obtain a greater understanding of the structural requirements for the biological effect (Table 1). The cytotoxicity data of the derivatives against nonmelanoma cell line KB indicates the retention of selective melanoma index or the introduction of general toxic effects (Table 1).

Discussion: The introduction of the methoxyl oxime at position C-3 (**4**, **10**, and **15**) resulted in a loss of cytotoxicity in MEL-2. The loss of toxic effect with **18** suggested a size limitation at position C-3. The other C-3 modified compounds, except oxime **15**, showed comparable biological activity towards MEL-2. However, the loss of melanoma-selective cytotoxicity of compounds **3**, **4**, and **5** with respect to betulinic acid (**1**) suggests that an extensive investigation is needed to have a better understanding of the structural requirements at position C-3 for biological effects.

The nearly identical selective cytotoxicity of the hydrogenated compounds **12** and **14** conferred with that of nonhydrogenated compounds **3** and **5**, respectively, suggests the unimportance of hydrogenation of position C-17 side chain for biological activity. However, the loss of antimelanoma activity of **13** and **15** when compared to betulinic acid (**1**) and **4**, respectively, indicates the functionality at this position should not be overlooked in the synthesis of other analogs.

The hydrogen bonding capability and/or acidity at position C-28 may also be important in the expression of biological effects in MEL-2 as demonstrated by the decrease in toxicity of compounds **2**, **7**, **16**, **17**, and **18**. These results indicate that a free carboxylic acid group at position C-28 is important for the expression of biological activity. Hydroxyloxime **9** improved general toxic effects but showed the loss of melanoma selectivity with respect to betulinic acid (**1**). It appears that a combination of the size, nucleophilicity, and strength of hydrogen bond formation is responsible for the biological effects of betulinic acid derivatives.

Table 1. Cytotoxicity of Betulinic Acid Derivatives



Compound	R_1	R_2	R_3	ED_{50} [μg/mL] MEL-2	KB
1	HO	COOH		1.2	>20
2	HO	CH_2OH		>20	>20
3	O	COOH		0.9	2.5
4	CH_3O·N	COOH		8.3	4.3
5	HO·N	COOH		2.4	>20
6*	H_2N	COOH		1.3	
7	O	CHO		7.4	12.9
9	HO·N	CHNOH		2.2	3.3
10	CH_3O·N	$CHNOCH_3$		>20	20
12	O	COOH		0.7	>20
13	HO	COOH		5.8	>20
14	HO·N	COOH		2.2	>20
15	CH_3O·N	COOH		>20	>20
16[22]	HO	$COOCH_3$		8.3	11.8
17	HO	CH_3		17.6	>20
18[23]	PhCOO	CH_3		>20	>20

* note: KB was not tested.

The above investigation demonstrates that simple modifications of the parent structure of betulinic acid can produce a number of potentially important derivatives, which may improve the selective toxicity profile or introduce general toxic effects. However, results from a more extensive investigation using a greater number of derivatives is needed for structure activity relationship (SAR) study for the design and ultimate synthesis of a more effective betulinic acid-derived antitumor agent.

Acknowledgment. We thank Dr. K. Zaw of the NMR Laboratory of the Department of Pharmacognosy, College of Pharmacy, University of Illinois at Chicago (UIC), for his technical assistance in obtaining the NMR data and Mr. R. Dvorak, Department of Medicinal Chemistry and Pharmacognosy, for obtaining the mass spectral data. This research was supported by UIC Faculty Start-Up Fund and Program for Collaborative Research in the Pharmaceutical Sciences (PCRPS).

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22. Compound **16** was prepared from betulinic acid by treating with azomethine in diethyl ether at rt.
23. Compound **18** was prepared from lupeol by refluxing with benzoyl chloride in pyridine over night.