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# PREPARATION OF AMINO ACID CONJUGATES OF BETULINIC ACID WITH ACTIVITY AGAINST HUMAN MELANOMA

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**Abstract**: Betulinic acid has been coupled with a series of amino acids at C-28 carboxylic acid position 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. A number of amino acid conjugates of betulinic acid showed improved water solubility as well as selective cytotoxicity. This investigation demonstrates that amino acid conjugates of betulinic acid can produce potentially important derivatives, which may be developed as antitumor agents. 

**Introduction**: For the past four decades the incidence of melanoma has been increasing at a rate higher than that of any other type of cancer.[1,2] For patients with metastatic melanoma, DTIC (5-(3,3-dimethyl-1-triazenyl)-1*H*-imidazole-4-carboxamide) has been the most efficacious single chemotherapeutic agent, and combination therapy with other synthetic and recombinant agents has been used for treatment of human melanoma.[3] But, a durable complete response is uncommon and greater toxicity is often observed.[4,5] Thus, current treatment for patients with metastatic melanoma are unsatisfactory. During a drug discovery effort from natural resources for potential anticancer activity agents, 3β-hydroxy-lup-20(29)-ene-28-oic acid, betulinic acid (**1**), has been isolated from *Ziziphus mauritiana* Lam. (Rhamnaceae), that displayed a selective antitumor activity against cultured human melanoma cells.[6] Unfortunately, betulinic acid (**1**) suffers a low water solubility, resulting in inefficient biological efficacy. It was envisioned that a series of amino acid conjugates of the parent compound, betulinic acid (**1**), could resolve the water solubility problem while producing a number of potentially important derivatives, which may be developed as antitumor agents. Syntheses of amino acid conjugates of betulinic acid related compounds have been demonstrated in efforts to develop potential antiviral agents.[7,8] This paper describes the synthesis of amino acid conjugates of betulinic acid and their biological evaluation against melanoma (MEL-2) and fibrosarcoma (KB) along with their water solubility evaluation.

**Preparation of Glycine Methyl Ester Conjugate of Betulinic Acid, A General Procedure**:[9] To a $CH_2Cl_2$ solution (10 mL) containing betulinic acid[6,10] (**1**, 100 mg, 0.22 mmol), glycine methyl ester (50 mg), 1,3-dicyclohexylcarbodiimide (DCC, 54 mg, 0.26 mmol) and dimethylaminopyridine (DMAP, 15 mg, 0.12 mmol) was added at room temperature and stirred overnight under $N_2$. After the completion of reaction 50 mL of EtOAc and 50 mL of $H_2O$ was added. The organic layer was separated, dried ($MgSO_4$), filtered, and the solvent was

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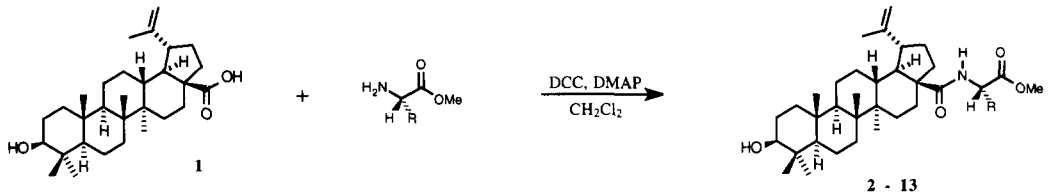
removed under vacuum. The residue was chromatographed over silica gel using pet ether/EtOAc (2/1) to give the glycine methyl ester conjugate of betulinic acid, (**4**, 60-75 mg). Structure of the compound was confirmed using $^{1}H$ NMR and $^{13}C$ NMR.

**Hydrolysis of Glycine Methyl Ester Conjugate of Betulinic Acid, A General Method**:[11] To a THF/$H_2O$ (4/1) solution (5 mL) containing glycine methyl ester conjugate of betulinic acid (**4**, 10 mg), LiOH (2 mg) was added at room temperature and stirred for 4 h under $N_2$. After the completion of reaction, 50 mL of EtOAc and 50 mL of $H_2O$ was added. The organic layer was separated, dried ($MgSO_4$), filtered, and the solvent was removed under vacuum to give a residue. The glycine conjugate of betulinic acid, **16**, was isolated using HPLC (C-18 ODS semipreparative column, 20 mm x 250 mm, YMC, gradient $CH_3CN/H_2O$ system, flow rate: 5 mL/min).

**Scheme 1**




1

2 - 13

**2** and **14**. R = $CH_2C_6H_5$; (phenylalanine)
**3** and **15**. R = $CH_2(C_6H_4(4\text{-}OH))$; (tyrosine)
**4** and **16**. R = H; (glycine)
**5** and **17**. R = $CH_2CH_2SCH_3$; (methionine)
**6** and **18**. R = $CH_2$(3-indole); (tryptophan)
**7** and **19**. R = $CH(CH_3)CH_2CH_3$; (isoleucine)
**8**. R = $CH_2CO_2Me$; (methyl aspartate). **20**. R = $CH_2CO_2H$; (aspartic acid)
**9** and **21**. R = $CH_2CH(CH_3)_2$; (leucine)
**10**. R = $CH_2CH_2CO_2Me$; (methyl glutamate). **22**. R = $CH_2CH_2CO_2H$; (glutamic acid).
**11** and **23**. R = $CH_3$; (alanine)
**12**. R = $NCH_2CH_2CH_2CHCO_2Me$; (methyl proline). **24**. R = $NCH_2CH_2CH_2CHCO_2H$; (proline).
**13** and **25**. R = $CH(CH_3)_2$; (valine)

14 - 25

**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 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.[12,13] Briefly, various concentrations of the testing compounds (dissolved in 10 mL of 10% DMSO) were transferred to 96-well plates and 190 mL of cell suspension were added to each well. After incubating the plates for three days at 37 °C (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 using 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 (Table 1).

**Water Solubility Evaluation**: The water solubility of the derivatives cytotoxic to MEL-2 was evaluated by dissolving 2 mg of the sample to 200 μL of DMSO and diluting 20 μL aliquots of the DMSO solution to 5x, 10x, 20x, 30x, 50x, and 100x with distilled water.

**Table 1**. Cytotoxicity profile of amino acid conjugates of betulinic acid against MEL-2 and KB cells lines.

| | methyl ester | | | | free acid | | |
|---|---|---|---|---|---|---|---|
| Compound number | MEL-2 ($ED_{50}$ = μg/mL) | KB ($ED_{50}$ = μg/mL) | water solubility | Compound number | MEL-2 ($ED_{50}$ = μg/mL) | KB ($ED_{50}$ = μg/mL) | water solubility |
| | | | | **1** | 4.2 | > 20 | << 5x |
| **2** | 9.0 | > 20 | 5x | **14** | > 20 | > 20 | - |
| **3** | > 20 | > 20 | - | **15** | > 20 | > 20 | - |
| **4** | 10.2 | > 20 | 50x | **16** | 4.2 | > 20 | 100x |
| **5** | 12.8 | > 20 | 5x | **17** | 9.0 | > 20 | 5x |
| **6** | > 20 | > 20 | - | **18** | 8.6 | > 20 | 10x |
| **7** | > 20 | > 20 | - | **19** | > 20 | > 20 | - |
| **8** | > 20 | > 20 | - | **20** | > 20 | > 20 | - |
| **9** | 6.2 | > 20 | 5x | **21** | 9.0 | > 20 | 10x |
| **10** | 15.3 | > 20 | 20x | **22** | > 20 | > 20 | - |
| **11** | 3.5 | > 20 | 30x | **23** | 1.5 | 4.6 | 50x |
| **12** | > 20 | > 20 | - | **24** | 13.1 | > 20 | 20x |
| **13** | 2.1 | > 20 | 5x | **25** | 9.0 | 9.0 | 20x |

Note: The assay was performed twice in triplicates.
The solubility was tested on the compounds that showed cytotoxicity against MEL-2.

**Discussion:** Cytotoxicity evaluation of both methyl ester and free acid of amino acid conjugates of betulinic acid against MEL-2 and KB cell lines were performed (Table 1). Among the derivatives, only free acid of alanine (**23**) and valine (**25**) conjugates showed toxicity against KB ($ED_{50}$ = 4.6 and 9.0 μg/mL, respectively). Methyl ester of alanine (**11**) and valine (**13**) conjugates and free acid of glycine (**16**) conjugate showed toxicity against MEL-2 comparable to betulinic acid (**1**) ($ED_{50}$ = 3.5, 2.1, 4.2, and 4.2 μg/mL, respectively). Free acid of alanine (**23**) conjugate showed the best toxicity profile ($ED_{50}$ = 1.5 μg/mL) against MEL-2; however, it also showed toxicity against KB ($ED_{50}$ = 4.6 μg/mL). While methyl ester of glycine (**4**), methionine (**5**), tryptophan (**6**), alanine (**11**), and proline (**12**) conjugates showed improved cytotoxicity against MEL-2 when converted to the corresponding free acid conjugates (**16**, **17**, **18**, **23**, and **24**, respectively, and $ED_{50}$ = from 10.2 to 4.2 μg/mL, from 12.9 to 9.0 μg/mL, from > 20 to 8.6 μg/mL, from 3.5 to 1.5 μg/mL, and from > 20 to 13.1 μg/mL, respectively), methyl ester of phenylalanine (**2**), leucine (**9**), glutamic acid (**10**), and valine (**13**) conjugates showed the loss of cytotoxicity against MEL-2 when converted to the corresponding free acid conjugates (**14**, **21**, **22**, and **25**, respectively, and $ED_{50}$ = from 9.0 to > 20 μg/mL, from 6.2 to 9.0 μg/mL, from 15.3 to >20 μg/mL, from 2.1 to 9.0 μg/mL, respectively). The methyl ester of tyrosine (**3**), tryptophan (**6**), isoleucine (**7**), aspartic acid (**8**) and

proline (**12**) conjugates, along with the free acid of phenylalanine (**14**), tyrosine (**15**), isoleucine (**19**), aspartic acid (**20**), and glutamic acid (**22**) conjugates, showed the total loss of cytotoxicity against MEL-2 ($ED_{50}$ > 20 μg/mL). An interesting observation was that the derivatives cytotoxic to MEL-2 retained selective cytotoxicity of the parent compound, betulinic acid (**1**), except for the free acid of alanine (**23**) and valine (**25**) conjugates.

The water solubility of the derivatives that showed cytotoxicity against MEL-2 was evaluated (Table 1). These derivatives demonstrated remarkably improved water solubility than that of the parent compound, betulinic acid. Free acid of glycine (**16**) conjugate showed the best solubility profile, resulting in a clear solution at 100x dilution. Free acid of alanine (**23**) conjugate gave clear solution down to 50x dilution. The derivatives **11**, **13**, **16**, and **23** showed improved water solubility as well as selective cytotoxicity.

This investigation demonstrated that modification of the parent structure of betulinic acid as amino acid conjugates can produce a number of potentially important derivatives with improved selective toxicity and water solubility. However, results from a more extensive investigation using a greater number of derivatives are needed for structure activity relationship (SAR) study for the design and alternative synthesis of more effective betulinic acid derived antitumor agents.

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