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Carbamate derivatives of betulinic acid and betulin with selective cytotoxic activity

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ABSTRACT

Synthesis and antiproliferative activity of eight new derivatives of betulinic acid (**1**) and betulin (**2**) are described. The compounds were tested against fifteen tumor cell lines. The toxicity against normal human fibroblasts and the mode of cell death on lung cancer cell line induced by the most active compounds **9** (bis(ethylcarbamate)betulin) and **11** (3-*O*-ethylcarbamate of 28-*O*-acetylbetulin) was investigated. Caspase 3 activity on lung cancer cell line (A549) was determined for **1**, **5** (3-*O*-ethylcarbamate of betulinic acid), **9** and **11**. All derivatives exerted a dose dependent antiproliferative action at micromolar concentrations toward target tumor cell lines. Treatment of lung cancer cells for 24 h with **9** and **11** induced apoptosis, as observed by the appearance of a typical ladder pattern in the DNA fragmentation assay.

The preclinical development of bioactive natural products and their analogs as chemotherapeutic agents is a major objective of anticancer research programs. Triterpenoids are one of the most important classes of natural products occurring widely in the plant kingdom. The derivatives of triterpenoids have been one of the most interesting areas of research in the past few years vested to their broad range of biological and medicinal properties.[1–6] Betulinic acid and betulin (Fig. 1) belong to the class of pentacyclic lupane-type triterpenes. The birch tree (*Betula* spp., *Betulaceae*) is one of the substantial source for betulin.[7]

Betulinic acid is one of few such compounds that have been shown to possess several medicinal properties including anticancer, antimalarial, antimicrobial and anti-HIV activities.[2] Initially betulinic acid was considered to be melanoma specific[8–10] but recent studies suggested that it shows anticancer activity against a broad spectrum of cancers.[11] Eventhough, the anticancer mechanism is not completely clear and well established, betulinic acid was found to cause cancer cell death by induction of apoptosis through changes in the mitochondrial membrane potential, production of reactive oxygen species, and permeability of transition pore openings. These processes lead to the release of mitochondrial apoptogenic factors, activation of caspases, and DNA fragmentation on neurectodermal tumors.[12–15] Also, betulinic acid inhibits topoisomerase I and can act as possible scaffold for the design of potent topoisomerase inhibitors.[16,17] Moreover, betulinic acid was found to be selective to tumor cells and non toxic to normal non cancerous cells.[18] In combination with cholesterol, the cytotoxicity of betulin is more enhanced, which is not observed with betulinic acid.[19]

Derivatives of betulin were found to show hepatoprotective and anti-HIV activity.[20] Recently, some betulin derivatives containing sulfur showed significant antiinflammatory and immunomodulatory effects.[21]

In the present study, the synthesis and in vitro anticancer activity of eight new derivatives of betulin and betulinic acid on several cell lines is reported. In addition, the mode of cell death with the interplay of caspases along with structure–activity relationship is also investigated. The derivatives **3** and **4** are prepared according to the methods available in literature.[22,23]

The synthetic chemical routes followed in producing the compounds in series **3**–**12** are outlined in Scheme 1 and details for the synthesis and characterization are given in Supplementary data.

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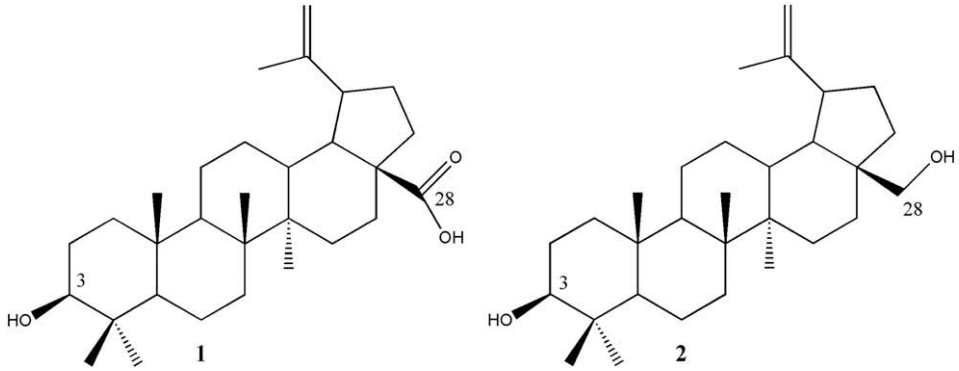
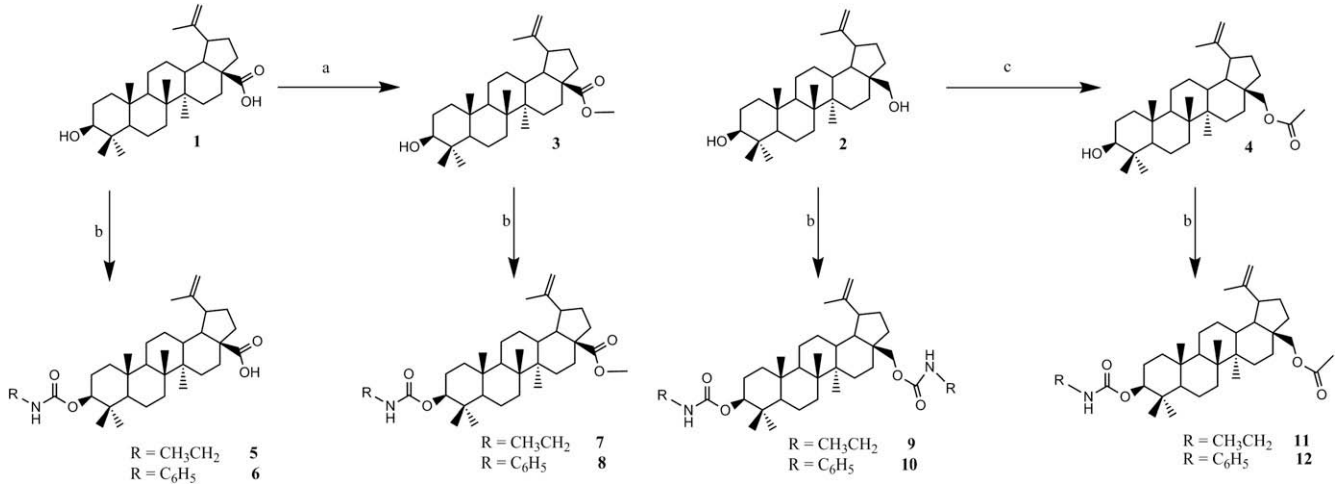


Figure 1. Betulinic acid (**1**) and betulin (**2**).



Scheme 1. Reagent and reaction conditions: (a) CH_2N_2, ether, rt; (b) ethyl/phenyl isocyanate, chloroform, 60 °C, 48 h; (c) acetic anhydride, dichloromethane, rt.

The in vitro cytotoxic activity of the betulin and betulinic acid derivatives on fifteen different tumor cell lines: 8505C and SW1736 (anaplastic thyroid), A253 and FaDu (head and neck), A431 (cervical), A2780 (ovarian), DLD-1, HCT-8, HCT-116, HT-29 and SW480 (colon), MCF-7 (breast), 518A2 (melanoma), A549 (lung) and liposarcoma (connective tissue) was studied by sulforhodamine B colorimetric assay[24] in triplicate. The compounds showed dose dependent antitumoral activity against investigated cell lines. The IC_{50} values are summarized in Table 1.

The most active compound in the panel of derivatives synthesized is compound **11**, and was found to be two to eight times more cytotoxic than betulinic acid on the tumor cell lines used in the study. The best activity of 3-*O*-ethylcarbamate of 28-*O*-acetylbetulin (**11**) was observed on lung cancer cell line (A549) (IC_{50} = 1.69 μM) and on colon cancer cell line (SW480) (IC_{50} = 1.77 μM).

Interestingly, the phenylcarbamate derivatives **6**, **8**, **10** and **12** were found to be inactive or seem to show activity only at higher concentrations whereas in most of the cases the ethylcarbamate derivatives **5**, **7**, **9** and **11** show higher activity than betulinic acid on the investigated tumor cell lines. A modification of secondary hydroxyl group at C3 into ethylcarbamate seems to be the reason for higher activity. On the other hand, modification into a phenyl carbamate yields less cytotoxic derivatives. One reason for low activity of the phenyl carbamates can be due the straight and rigid phenyl groups and the possible interactions with the π-system of the phenyl ring lead to a much more bulky molecule compared to the ethyl carbamates, thereby with a lower ability to penetrate the cell membrane, as observed by Drag-Zalesinska et al.[25]

Interestingly, modification of C28 carboxylic acid group of betulinic acid (**1**) into a methyl ester (**3**) lead to a fall in activity but a modification of C3 hydroxy group of **1** or **3** into ethylcarbamate has yielded derivatives **5** and **7**, respectively, where compound **5** was found to be less cytotoxic than betulinic acid in most of the cases (except on lung cancer A549, ovarian cancer A2780, anaplastic thyroid cancer SW1736) and compound **7** was twofold more toxic than **5** on most of the investigated cell lines.

The derivatization of C3 secondary hydroxyl group of 28-*O*-acetylbetulin (**4**) and both primary and secondary hydroxyl groups of betulin (**2**) into ethylcarbamate has yielded highly cytotoxic derivatives **11** and **9**, respectively. Introduction of imidazole scaffold into the lupane skeleton has yielded similar toxicities[26] as the active new carbamate derivatives reported in this work.

Another important parameter to evaluate for an anticancer drug is its stability in solution. The in vitro stability was determined by HPLC after incubation at 37 °C of compound **9** in phosphate buffer (pH 7.2). It was found that compound was quite stable, and no decomposition products were observed even after 96 h incubation time in phosphate buffer.

The most active derivatives, **9** and **11**, were screened for their selectivity towards tumor cells and summarized in Table 2. The compounds were tested on human fibroblasts (WWO70327). The IC_{50} of betulinic acid, **1**, on fibroblasts was found to be 20.81 μM and for **9** and **11**, >100 and 24.35 μM, respectively. Thus the

Table 1
IC_{50} (μM)[a] for the 96 h of activity of investigated compounds on tumor cell lines determined by SRB colorimetric assay

	1	3	4	5	6	7	8	9	10	11	12
518A2	8.13	28.75	15.84	17.84	51.49	16.72	>100	8.18	>100	5.12	>100
8505C	7.26	23.67	14.74	17.56	39.55	7.22	>100	9.39	>100	5.05	>100
A253	9.18	18.51	12.87	17.28	34.66	16.69	>100	9.71	>100	4.88	>100
A431	12.60	26.99	12.19	15.20	26.71	14.80	91.45	6.08	>100	5.00	>100
A549	11.10	20.63	14.37	5.55	22.36	2.32	>100	2.91	>100	1.69	>100
A2780	11.07	29.31	11.74	7.41	24.82	8.35	69.52	4.37	80.62	4.28	84.23
DLD-1	11.87	45.86	13.12	17.00	26.32	18.94	>100	8.32	>100	6.93	>100
FaDu	10.19	20.33	13.95	13.82	24.25	5.53	>100	7.88	>100	4.62	>100
HCT-8	13.10	17.25	17.98	16.74	25.18	14.98	>100	3.91	>100	3.99	>100
HCT-116	10.80	20.47	10.71	15.20	21.40	13.46	>100	3.84	>100	4.30	>100
HT-29	13.93	30.72	10.96	16.74	31.63	7.22	68.96	6.20	69.24	5.07	76.44
Liposarcoma	12.07	23.26	15.54	17.93	51.85	6.85	>100	12.03	>100	5.35	>100
MCF-7	12.27	24.39	11.38	17.37	47.88	7.03	>100	17.70	>100	5.98	>100
SW480	6.48	17.92	13.68	16.56	41.08	6.67	>100	2.77	>100	1.77	>100
SW1736	13.09	32.10	11.95	5.24	18.76	2.35	>100	3.60	>100	3.15	>100

[a] Values are mean of three experiments, standard deviation values are less than 10% in all cases.

Table 2
Selectivity index[a] of **1**, **9** and **11** towards tumor cells

	1	9	11
518A2	2.56	>12.22	4.76
8505C	2.87	>10.65	4.82
A253	2.27	>10.30	4.99
A431	1.65	>16.45	4.87
A549	1.87	>34.36	14.41
A2780	1.88	>22.88	5.69
DLD-1	1.75	>12.02	3.52
FaDu	2.04	>12.69	5.39
HCT-8	1.59	>25.58	6.20
HCT-116	1.93	>26.04	5.67
HT-29	1.49	>16.13	4.80
Liposarcoma	1.72	>8.31	4.55
MCF-7	1.70	>5.65	4.07
SW480	3.21	>36.10	13.76
SW1736	1.59	>26.32	7.73

[a] Selectivity index (IC_{50} on human fibrobalsts/IC_{50} on corresponding tumor cell line).

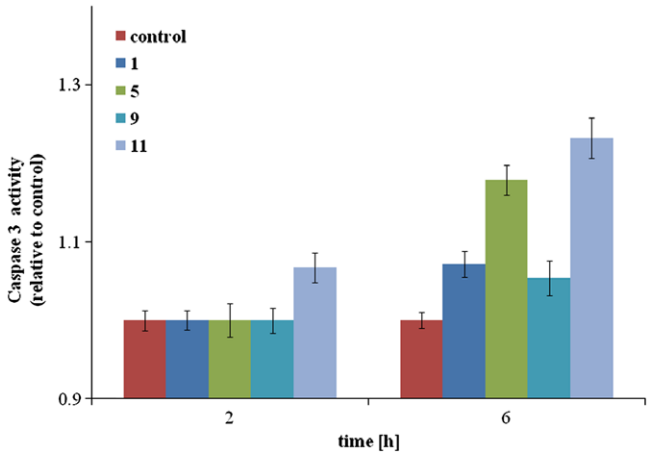


Figure 2. Activation of caspase 3 on A549 lung cancer cell line treated for 2 and 6 h with **1**, **5**, **9** and **11**. Values are mean of three experiments.

compounds, **9** and **11** were less toxic on normal fibroblasts than on the investigated tumor cell lines and more selective to cancer cells than betulinic acid. It was observed that **11** was 3–14 times more toxic to tumor cells, for example, 14 times more selective towards lung cancer cell line (A549) and colon cancer cell line (SW480). Furthermore, the compound **9** did not show any cytotoxic effect on human fibroblasts even at 100 μM concentration (Table 2). Similar to compound **11**, compound **9** was found to be more than 34 and 36 times more selective towards A549 and SW480 cell lines. In addition, the human fibroblasts (WWO70327) showed better tolerance to compound **9** and **11** in comparison with betulinic acid in all cases.

Furthermore, cell death induced by the new compounds was mediated by apoptosis. One critical hallmark of apoptosis is activation of caspases, in particular effector-caspases-3/-7, which are responsible for the degradation process that eventually leads to the typical features of apoptosis. We have chosen lung cancer cell line A549 to investigate general activation of caspases using substrate cleavage assay. After exposure to equitoxic IC_{90} concentrations of **1**, **5**, **9** and **11**, cells were sampled 2 and 6 h for cleavage of typical effector-caspase substrate DEVD. When treated with compounds **5**, **9**, **11** and betulinic acid, **1**, for 2 h it was observed that caspase activation was seen for compound **11** only. After 6 h greater caspase activation was observed for compounds **5** and **11** in contrast to **1** and **9** (Fig. 2).

In addition, apoptotic cell death was confirmed by analysis of typical DNA fragmentation. Floating cells from lung cancer cell line A549 treated with the IC_{90} concentrations of **9** and **11** were collected and extracted DNA was analyzed by DNA gel electrophoresis (see Supplementary data Fig. S1). Occurrence of typical DNA ladders was observed in both cases.

In conclusion, we have described the synthesis of new carbamate derivatives of betulinic acid and their in vitro anticancer activity on fifteen tumor cell lines. Compounds **9** and **11** were found to be most cytotoxic among the derivatives synthesized and they seem to induce apoptosis with the involvement of caspases on lung tumor cell line. Moreover compounds **9** and **11** exhibit a good degree of selectivity towards tumor cells than non tumor cells. The present investigation demonstrates that simple modifications of the parent structure of betulinic acid or betulin can produce a number of highly potent 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 are needed for structure–activity relationship (SAR) study for the design and ultimate synthesis of a more effective betulinic acid and betulin derived antitumor agents.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.04.004.

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