Bioorganic & Medicinal Chemistry Letters 24 (2014) 1005–1008

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

A-ring modified betulinic acid derivatives as potent cancer preventive agents

Hsin-Yi Hung [a,e], Kyoko Nakagawa-Goto [a,b], Harukuni Tokuda [c], Akira Iida [d], Nobutaka Suzuki [c], Ibrahim D. Bori [a], Keduo Qian [a,*], Kuo-Hsiung Lee [a,e,*]

[a] Natural Products Research Laboratories, Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599-7568, USA
[b] Division of Pharmaceutical Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan
[c] Department of Complementary and Alternative Medicine, Clinical R&D Graduate School of Medicine Science, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-8640, Japan
[d] Faculty of Agriculture, Kinki University, Nara 631-8505, Japan
[e] Chinese Medicine Research and Development Center, China Medical University and Hospital, Taichung 401, Taiwan

ARTICLE INFO

Article history:
Received 2 October 2013
Revised 6 December 2013
Accepted 10 December 2013
Available online 16 December 2013

Keywords:
Betulinic acid
seco A-ring
Chemopreventive
EBV-EA

ABSTRACT

Ten new 3,4-*seco* betulinic acid (BA) derivatives were designed and synthesized. Among them, compounds **7**–**15** exhibited enhanced chemopreventive ability in an in vitro short-term 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induced Epstein–Barr virus early antigen (EBV-EA) activation assay in Raji cells. Specifically, analogs with a free C-28 carboxylic acid, including **7**, **8**, **11**, and **13**, inhibited EBV-EA activation significantly. The most potent compound **8** displayed 100% inhibition at 1×10^3 mol ratio/TPA and 73.4%, 35.9%, and 8.4% inhibition at 5×10^2, 1×10^2, and 1×10 mol ratio/TPA, respectively, comparable with curcumin at high concentration and better than curcumin at low concentration. The potent chemopreventive activity of novel *seco* A-ring BAs (**8** and **11**) was further confirmed in an in vivo mouse skin carcinogenesis assay.

The concept of chemoprevention has grown over the past few decades. Cancer is a multistage process, and the question of how to stop the progression stage of its development has gradually drawn increasing attention.[7] The Epstein–Barr virus early antigen (EBV-EA) activation assay was been established to quickly evaluate chemopreventive activity in vitro,[8] while a two-stage mouse skin carcinogenesis assay can further assess in vivo activity.[9,10] Development of these biological assays has greatly facilitated research on chemopreventive agents.

Betulinic acid (BA, Fig. 1) and its derivatives reportedly possess various pharmacological functions, such as anti-cancer, anti-HIV, anti-inflammatory, anti-malarial, and anti-bacterial effects. Prior modifications of BA have focused mainly on the C-3 hydroxyl, C-28 carboxylic acid, and C-30 allylic positions.[1,2] Recently, we introduced short fatty acids at the C-3 position of BA and the resulting BA analogs demonstrated excellent cancer chemopreventive activity in both EBV-EA activation and two-stage mouse skin carcinogenesis assays.[3] Akihisa et al. also reported that compound **1** with a 3,4-*seco* lanostane structure exhibited inhibitory effects against EBV-EA activation in Raji cells.[4] In addition, some limonoids [e.g. nomilin (**2**)] with an A-ring lactone showed anti-proliferative effects on neuroblastoma cancer cells (SH-SY5Y).[5] The mechanism of action involved apoptosis induction, cancer cell cycle arrest and aneuploidic effects.[5] Furthermore, other studies reported that 3,4-*seco* ursolic acid derivatives induced cell cycle arrest and apoptosis in a human bladder cancer cell line (NTUB1).[6] Based on these discoveries, a group of novel 3,4-*seco* BA analogs **7**–**16** were designed to enhance the chemopreventive activity. Herein, this Letter reports the design, synthesis and biological evaluation of these novel compounds (see Scheme 1).

3,4-*seco* BA analogs **7**–**16** were designed and synthesized through oxepanone A-ring intermediates **5** and **6**. A *N*-heptane acetamide side chain was included in our analog design (**9**, **10** and **14**–**16**), because this group enhanced the biological activity of BA in our prior studies.[11] Initially, betulin, a commercially available pentacyclic triterpene, was oxidized with Jones reagent to provide compound **3** with a ketone at C-3 and carboxylic acid at C-28. Baeyer–Villiger reaction of **3** using 3-chloroperbenzoic acid (mCPBA) produced **5** with an oxepanone A-ring. Because prenyl-like groups have played an important role in cancer preventive effects in our prior studies,[3] acid-catalytic lactone ring opening in MeOH was employed to produce the 4-methylene-3-methyl ester **7**. Hydrolysis of **7** gave the corresponding dicarboxylic acid **8**. Hydroboration–oxidation of **7** produced the alcohol **11**, which was esterified using various acid anhydrides, such as 2,2-dimethylsuccinic and acetic

* Corresponding authors. Address: Natural Products Research Laboratories, Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599-7568, USA. Tel.: +1 919 962 0066; fax: +1 919 966 3893 (K.-H. L.); tel.: +1 919 883 5306 (K.Q.).
E-mail addresses: kdqian@unc.edu (K. Qian), khlee@unc.edu (K.-H. Lee).

0960-894X/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmcl.2013.12.041

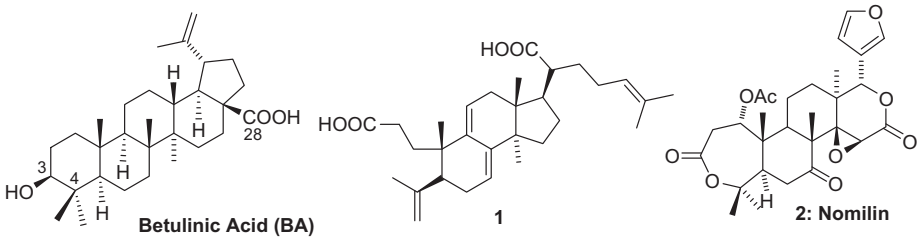
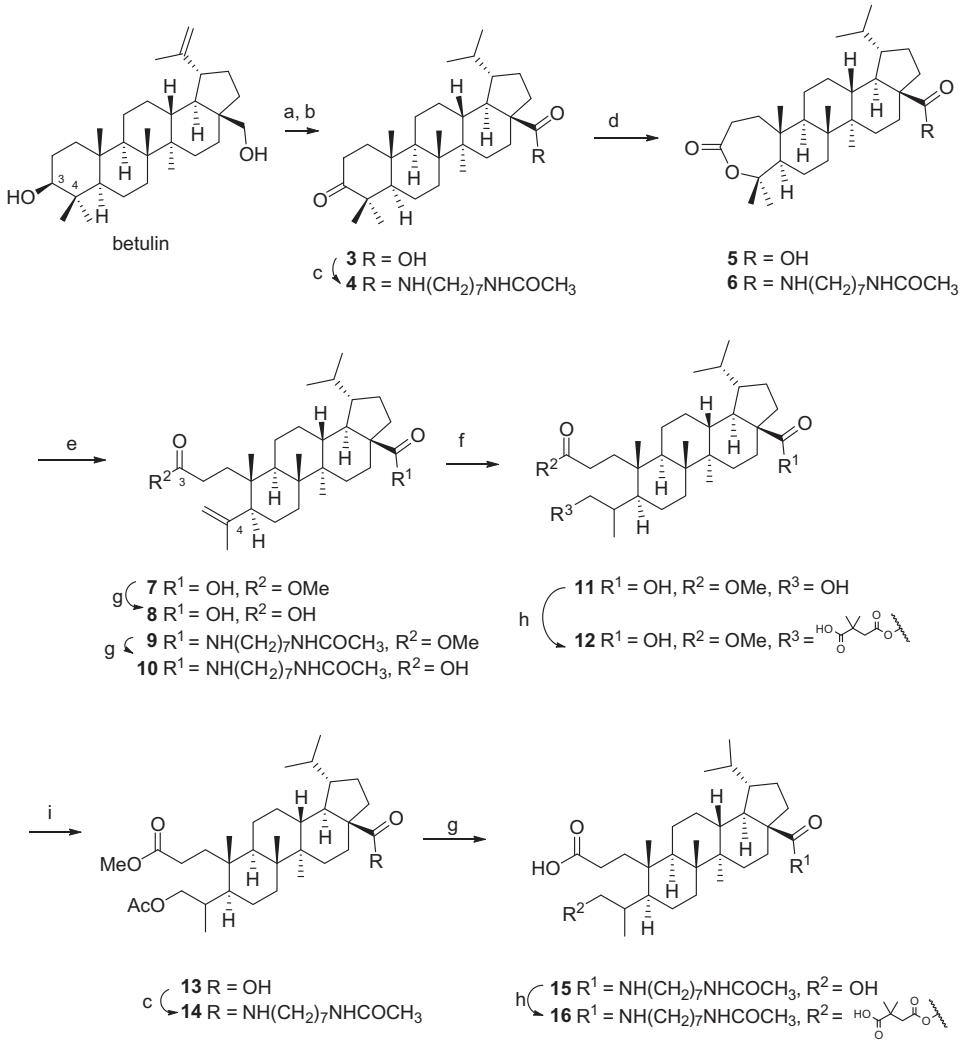


Figure 1. structures of betulinic acid, 3,4-seco lanostane compound and nomolin.

anhydrides, to provide **12** and acetate **13**, respectively. For the preparation of *N*-heptane acetamide analogs, the related carboxylic acids **3** and **13** were treated with oxalyl chloride and heptane-1,7-diamine followed by acetic anhydride to obtain **4** and **14**, respectively. Analogs **6**, **9** and **10** were prepared from **4** through the same procedure as for the preparation of **5**, **7** and **8**. The methyl ester and acetate on **14** were hydrolyzed to produce **15**, which was treated with dimethylsuccinic anhydride to provide **16**.

Compounds **6**–**16** were evaluated in an in vitro EBV-EA inhibition assay and the results are shown in Table 1. Bevirimat and BA were also evaluated and curcumin was used as a positive control. As seen in the results, 3,4-*seco* BA analogs showed substantial chemopreventive activity. Four compounds **7**, **8**, **11**, and **13** significantly inhibited EBV-EA activation, showing 100% inhibition at the highest tested concentration. All four compounds contained a C-28 carboxylic acid and showed better activity than corresponding compounds with a C-28-*N*-heptane acetamide (compare **7**/**9**, **8**/**10**, **13**/**14**). Three additional compounds with a C-28-*N*-heptane acetamide (compare **6**, **15**, **16**) were also less active. The most active compound **8** also contained a C-3 carboxylic acid and C-4 methylene in addition to the C-28 carboxylic group. It displayed 100% inhibition at 1×10^3 mol ratio/TPA, and 73.4%,



Scheme 1. Synthesis of 3,4-*seco* betulinic acid derivatives. Reagents and conditions: (a) Jone's oxidation, (b) H_2,THF,Pd/C, HOAc, (c) (1) $(CO)_2Cl_2$, $NH_2(CH_2)_7NH_2$, CH_2Cl_2, (2) Ac_2O, DMAP, pyridine, r.t. (d) mCPBA, CH_2Cl_2, (e) H_2SO_4, MeOH, (f) (1) BH_3SMe_2,(2) NaOH, H_2O_2, (g) 4N NaOH/THF and MeOH, (h) 2,2-dimethylsuccinic anhydride, DMAP, pyridine, 160 °C, (i) Ac_2O, DMAP, pyridine, r.t.

Table 1
EBV-EA inhibition ability[a] of betulinic acid derivatives

Compd	Percentage EBV-EA positive cells Compound concentration (mol ratio/TPA[b])				
	1000	500	100	10	IC_{50}[d]
6	15.3 ± 0.4 (60)[c]	61.4 ± 1.4	83.2 ± 2.1	100 ± 0.3	523
7	0.0 ± 0.5 (70)	29.1 ± 1.5	66.9 ± 2.5	94.2 ± 0.4	311
8	0.0 ± 0.4 (70)	26.6 ± 1.5	65.1 ± 2.4	91.6 ± 0.5	295
9	7.1 ± 0.6 (60)	54.4 ± 1.6	77.2 ± 2.5	100 ± 0.3	479
10	6.7 ± 0.5 (60)	50.2 ± 1.5	76.3 ± 2.4	100 ± 0.2	460
11	0.0 ± 0.4 (70)	31.6 ± 1.3	68.1 ± 2.3	96.3 ± 0.4	323
12	3.7 ± 0.5 (70)	36.4 ± 1.6	71.3 ± 2.5	100 ± 0.4	386
13	0.0 ± 0.5 (70)	33.8 ± 1.5	68.8 ± 2.3	98.2 ± 0.5	335
14	7.8 ± 0.6 (60)	56.0±1.6	79.4 ± 2.3	100 ± 0.4	490
15	6.6 ± 0.5 (60)	52.1 ± 1.4	77.5 ± 2.5	100 ± 0.4	471
16	11.3 ± 0.4 (60)	57.2 ± 1.6	81.3 ± 2.3	100 ± 0.2	501
BA	7.9 ± 0.4 (60)	37.2 ± 1.2	75.1 ± 2.3	100 ± 0.6	403
Bevirimat	6.1 ± 0.3 (60)	36.1 ± 1.3	74.2 ± 2.2	96.5 ± 0.9	396
Curcumin[e]	0.0 ± 0.2 (60)	22.8 ± 1.2	81.7 ± 2.5	100 ± 0.5	341

[a] For experimental details, see Ref. 13.
[b] TPA concentration is 20 ng/mL (32 pmol/mL).
[c] Relative ratio of EBV-EA activation with respect to the control without test compound (100%). Values in parentheses are viability percentages of Raji cells.
[d] The molar ratio of compound, relative to TPA, required to inhibit 50% of control activated with 32 pmol TPA.
[e] Positive control.

35.9% and 8.4% inhibition at 5×10^2, 1×10^2, and 1×10 mol ratio/TPA, respectively, with an IC_{50} value of 295 mol ratio/TPA. Even at the lowest concentration (1×10 mol ratio/TPA), **8** exhibited greater inhibitory activity than curcumin, a known cancer chemopreventive agent. This result may be because the C-5 isopropenyl group is similar to a prenyl group, which has been an effective modification in cancer prevention research.[3] Considering the C-3 substituent, compound **8** with a C-3 carboxylic acid was slightly more active than **7** with a C-3 methyl ester. Finally, regarding C-4 substituents, analog **7** with a C-4 methylene was slightly more potent than **11** and **13** with hydroxymethyl and acetoxymethyl, respectively.

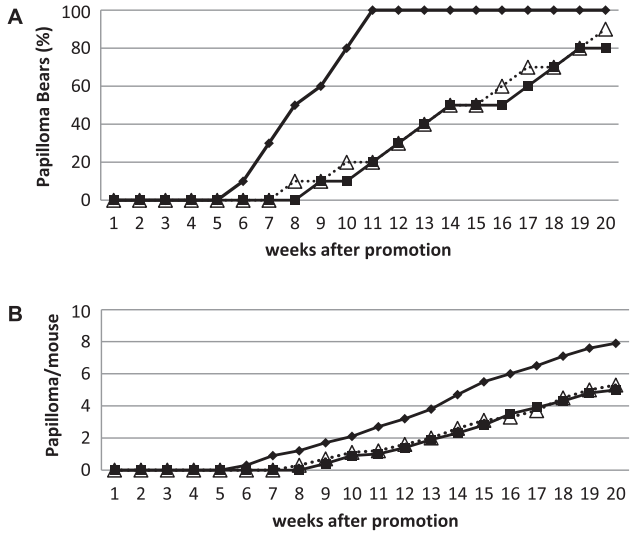


Figure 2. Inhibitory effects of compounds **8** and **11** on DMBA-TPA mouse skin carcinogenesis. Tumor formation in all mice was initiated with DMBA (390 nmol) and promoted with TPA (1.7 nmol) twice weekly beginning 1 week after initiation. (A) Papilloma percentage in mice. (B) Average number of papillomas/mouse. (♦) Control TPA alone; (△) TPA + compound **11** (85 nmol); (■) TPA + compound **8** (85 nmol). After 20 weeks of promotion, a significant difference in the number of papillomas/mouse between the treated groups and the control group was evident ($p < 0.05$).

Two of the most active compounds, **8** and **11**, were further evaluated in a mouse skin carcinogenesis assay (Fig. 1). Mouse skin papillomas were induced by DMBA and promoted by TPA. Inhibitory effects of **8** and **11** were monitored and determined over 20 weeks by both the percentages of papilloma-bearing mice (Fig. 2A) and the average numbers of papillomas/mouse (Fig. 2B). Although skin papillomas still appeared, their occurrence was delayed for three weeks in both treated groups compared with the control group. At weeks 8 and 11, 50% and 100% of mice in the positive control group bore papillomas, while only 0–10% and 10–20% of mice in the **8**- and **11**-treated groups displayed papillomas. The average numbers of papillomas/mouse at week 8 and 11 were 1.2 and 2.7 for the positive control group, but only 0 and 0.4, respectively, for the **8**-treated group. The in vivo results are quite promising and consistent with the in vitro data.

The structure–activity relationship (SAR) trends are summarized as follows. The 3,4-*seco* structural feature can significantly increase chemopreventive activity. A C-3 carboxylic acid is better than a methyl ester. A C-4 methylene is better than hydroxymethyl or acetoxymethyl groups. A C-28 carboxylic acid is considerably better than *N*-heptane acetamide. Among all new compound, **7**, **8**, **11**, and **13** significantly inhibited TPA induced EBV-EA activation.

In this study, new BA derivatives were designed, synthesized and evaluated for cancer chemopreventive activity. Most of the newly synthesized compounds showed significant cancer prevention effects. In an in vitro EBA-EA assay, **8** was the most potent derivative with comparable inhibitory ability to curcumin, a known chemopreventive agent, at high concentration and better inhibitory ability at low concentration. Compounds **8** and **11** delayed occurrence of papillomas in an in vivo mouse skin carcinogenesis assay. These results provided convincing evidence that 3,4-*seco* modification can greatly enhance the chemopreventive activity of BA analogs.

Acknowledgments

This work was supported in part by NIH Grant CA177584-01 from the National Cancer Institute and AI-33066-22 from the National Institute of Allergy and Infectious Diseases awarded to K.-H. Lee.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.12.041.

References and notes

1. Qian, K.; Kuo, R. Y.; Chen, C. H.; Huang, L.; Morris-Natschke, S. L.; Lee, K. H. *J. Med. Chem.* **2010**, *53*, 3133.
2. Qian, K.; Yu, D.; Chen, C. H.; Huang, L.; Morris-Natschke, S. L.; Nitz, T. J.; Salzwedel, K.; Reddick, M.; Allaway, G. P.; Lee, K. H. *J. Med. Chem.* **2009**, *52*, 3248.
3. (a) Nakagawa-Goto, K.; Yamada, K.; Taniguchi, M.; Tokuda, H.; Lee, K. H. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 3378; (b) Suzuki, M.; Nakagawa-Goto, K.; Nakamura, S.; Tokuda, H.; Morris-Natschke, S. L.; Kozuka, M.; Nishino, H.; Lee, K. H. *Pharm. Biol.* **2006**, *44*, 178; (c) Itoigawa, M.; Ito, C.; Tokuda, H.; Enjo, F.; Nishino, H.; Furukawa, H. *Cancer Lett.* **2004**, *214*, 1654.
4. Akihisa, T.; Nakamura, Y.; Tokuda, H.; Uchiyama, E.; Suzuki, T.; Kimura, Y.; Uchikura, K.; Nishino, H. *J. Nat. Prod.* **2007**, *70*, 948.
5. Manners, G. D. *J. Agric. Food Chem.* **2007**, *55*, 8285.
6. Tu, H. Y.; Huang, A. M.; Wei, B. L.; Gan, K. H.; Hour, T. C.; Yang, S. C.; Pu, Y. S.; Lin, C. N. *Bioorg. Med. Chem.* **2009**, *17*, 7265.
7. Gravitz, L. *Nature* **2011**, *471*, S5.
8. Ito, Y.; Yanase, S.; Fujita, J.; Harayama, T.; Takashima, M.; Imanaka, H. *Cancer Lett.* **1981**, *13*, 29.
9. Konoshima, T.; Takasaki, M.; Tatsumoto, T.; Kozuka, M.; Kasai, R.; Tanaka, O.; Nie, R. L.; Tokuda, H.; Nishino, H.; Iwashima, A. *Biol. Pharm. Bull.* **1994**, *17*, 668.

10. Itoigawa, M.; Ito, C.; Ju-ichi, M.; Nobukuni, T.; Ichiishi, E.; Tokuda, H.; Nishino, H.; Furukawa, H. *Cancer Lett.* **2002**, *176*, 25.
11. Huang, L.; Ho, P.; Lee, K. H.; Chen, C. H. *Bioorg. Med. Chem.* **2006**, *14*, 2279.
12. *In vitro EBV-EA activation experiments:* EBV-EA positive serum from a patient with nasopharyngeal carcinoma (NPC) was a gift from Professor H. Hattori, Department of Otorhinolaryngology, Kobe University. The EBV genome carrying lymphoblastoid cells (Raji cells derived from Burkitt's lymphoma) were cultured in 10% fetal bovine serum (FBS) in RPMI-1640 medium (Sigma R8758, USA). Spontaneous activation of EBV-EA in our subline of Raji cells was less than 0.1%. The inhibition of EBV-EA activation was assayed using Raji cells (virus non-producer type) as described below. The cells were incubated at 37 °C for 48 h in 1 mL of medium containing *n*-butyric acid (4 mM), TPA [32 pM = 20 ng in 2 μL dimethyl sulfoxide (DMSO)] and various amounts of the test compounds dissolved in 2 μL of DMSO. Smears were made from the cell suspension. The EBV-EA inducing cells were stained by the means of an indirect immunofluorescence technique. In each assay, at least 500 cells were counted, and the number of stained cells (positive cells) was recorded. Triplicate assays were performed for each compound. The average EBV-EA induction of the test compound was expressed as a ratio relative to the control experiment (100%), which was carried out with *n*-butyric acid (4 mM) plus TPA (32 pM). EBV-EA induction was ordinarily around 35%. The viability of treated Raji cells was assayed by the Trypan blue staining method. The cell viability of the TPA positive control was greater than 80%. Therefore, only compounds that induced less than 80% (% of control) of the EBV-active cells (those with a cell viability of more than 60%) were considered able to inhibit the activation caused by promoter substances. Student's *t*-test was used for all statistical analysis.