SYNTHESIS, STRUCTURE, AND CYTOTOXIC ACTIVITIES OF A NOVEL LACTAM OF THE DITERPENOID ISOSTEVIOL

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A novel compound lactam of 15β -hydroxymethylisosteviol ethyl ester **3** has been synthesized and structurally characterized by IR, NMR, and HR-MS. Its X-ray crystallographic analysis revealed that the nitrogen is attached to C-13 instead of C-15. The reaction mechanism was discussed, and the title compound was further evaluated against HCT-116, HGC-27, and JEKO-1 cells by the MTT assay. The results demonstrated that compound **3** exhibited better cytotoxic activities than its corresponding precursor isosteviol.

Keywords: isosteviol, synthesis, cytotoxic activity.

Isosteviol (*ent*-16-ketobeyeran-19-oic acid, **1**), a metabolite of stevioside originally isolated from the natural *Stevia rebaudiana* Bertoni, is a tetracyclic diterpenoid with the beyerane skeleton [1, 2]. In recent years, isosteviol as well as its derivatives have been shown to exhibit a wide range of biological activities, such as antihyperglycemic [3], antihypertension [4], and antimicrobial [5] activities, as well as anticancer activity [6]. In particular, when some bioactive structural fragments, such as exo-methylene cyclopentanone, amino-alcohol, and so on, were bridged onto the isosteviol skeleton, these hybridized isosteviol derivatives can display promising anticancer activities [7, 8]. The acylamino group is an important structural fragment of many pharmaceutical molecules [9–11]. Introduction of the acylamino group to terpenoid compounds can enhance their existing biological properties or lead to new biological capabilities [12]. However, few reports have focused on the relationship of amide-fused isosteviol derivative was designed and synthesized by a ring-expansion reaction (Scheme 1), and its antitumor activity was also evaluated in order to screen out highly active lead compounds with application values.



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TABLE 1. Comparison of Rearrangement Products of Compound 2 Under Different Catalysts (refluxed, 15 h)

Yield	Catalyst–solvent		
	BF ₃ ·OEt ₂ -toluene	H ₂ SO ₄ -acetone	
3	81%	33%	
4–5	8% (4–5, 3:1)	54% (4–5, 3:1)	
6	7%	8%	

TABLE 2. Cytotoxic Activities of Compound 3 and Isosteviol in vitro, (IC₅₀, µM)^{a,b}

Compound	HCT-116	HGC-27	JEKO-1
1	102.39 ± 2.38	118.58 ± 2.84	132.18 ± 3.13
3	34.28 ± 1.16	46.73 ± 2.66	59.93 ± 2.01
Cisplatin	3.91 ± 0.26	2.03 ± 0.10	2.74 ± 0.24

^a Values are the mean of triplicate of three independent experiments; $^{b} \pm$ SD.



Fig. 1. Molecular structure of compound 3.

Compound 2 has been synthesized in our previous work [13]. Under acid condition, compound 2 underwent Beckmann rearrangement and fragmentation (Table 1). Treatment of compound 2 with BF₃·OEt₂ in refluxing toluene can result in a Beckmann rearrangement, yielding the D-ring expanded lactam 3 in 81% yield. In its IR and NMR spectra, the broad absorption band at v 3371 cm⁻¹ is assigned for N-H, whose proton signal is displayed as a singlet peak at $\delta_{\rm H}$ 6.29 ppm. The stretching vibration of C=O, whose carbon signal appears at $\delta_{\rm C}$ 177.03 ppm, is observed as a strong band at v 1644 cm⁻¹. In accord with the molecular formula $C_{23}H_{37}NO_4$, there was an [M + Na]⁺ peak at *m/z* 414.2622 in the HR-MS. The stereo-structure of compound 3 was further confirmed by X-ray crystallographic analysis (Fig. 1), indicating the nitrogen is attached to C-13 instead of C-15. When H_2SO_4 was used as catalyst, Beckmann fragmentation occurred readily to afford the olefinic nitriles 4 and 5 with a yield of 54%, which could not be separated by the chromatography method due to the isomers having very similar physical properties and polarities. Their IR spectrum shows the weak absorption band of -CN group at v 2230 cm⁻¹. In their ¹H NMR spectra, resonances are distinctly observed at $\delta_{\rm H}$ 5.53, 5.15 ppm, demonstrating the formation of the CH=C group. The peak integral area at $\delta_{\rm H}$ 5.53 and 5.15 ppm is 1 and 0.37, respectively, which indicates a 3:1 mixture of olefinic nitriles 4 and 5. Moreover, the by-product 6 was also characterized by IR, NMR, and HR-ESI-MS. According to some related literature [14, 15], the mechanism of the rearrangement and fragmentation reaction can be tentatively shown in Fig. 2.



Fig. 2. Possible reaction mechanism.

Firstly, compound **2** was attacked by acid to furnish the intermediate X with positive charge on the oxygen atom due to the electron deficiency of acid. In the next stage, intramolecular migration of C-13 (with its pair of electrons) *anti* to the departing hydroxyl function occurred intermediate Y with the electron-deficient carbon atom formed by partial ionization of the oxygen-nitrogen bond as the migration terminus. The resulting imine derivatives may then rearrange to the lactam **3**. Meanwhile, a competitive process was proceeding as fragmentation to furnish the intermediate Z with a stable carbonium ion. As the H-12 and H-14 went, the corresponding fission products **4** and **5** were generated with olefin-nitrile character. The formation of compound **6** from intramolecular cyclization of the intermediate Z further proves the rationality of the reaction mechanism.

The title compound **3** and isosteviol $1([\alpha]_D^{20}-110.2^{\circ} (c \ 1.0, CH_2Cl_2))$ were further utilized to investigate their cytotoxic activities against three human cancer cell lines: colon carcinoma (HCT-116), gastric carcinoma (HGC-27), mantle cell lymphoma (JEKO-1). Cisplatin, employed in the treatment of human cancers as one of the most effective chemotherapeutic drugs, was used as the positive control. The *in vitro* antiproliferative activities were evaluated by the standard MTT assay. The IC₅₀, summarized in Table 2, expresses the inhibitory activity of tumor cell growth. The obtained results showed that the IC₅₀ values of compounds 1 and 3 against HCT-116 were lower than HGC-27 and JEKO-1, which demonstrated that HCT-116 was the most sensitive cell line to the tested compounds. More importantly, the title compound 3 exhibited better cytotoxic activities than its corresponding precursor 1, which indicated that the introduction of the lactam subunit could enhance the antitumor activities.

EXPERIMENTAL

General. All chemicals were used as received unless otherwise noted. Reagent grade solvents were redistilled prior to use. Chromatography was performed on silica gel (100–200 mesh). All melting points were measured with a Beijing Keyi XT5 apparatus, and the temperature was not corrected. Optical rotations were determined on a INESA WZZ-2B polarimeter. The IR spectra were recorded as KBr pellets on a Thermo Nicolet (IR200) spectrometer. ¹H and ¹³C NMR spectra were collected with a Bruker DPX-400 spectrometer at 400 and 100 MHz, respectively, with TMS as internal standard. High-resolution mass spectra (HR-MS) were obtained with a Waters Micromass Q-Tof MicroTM instrument by the ESI technique.

Synthesis of Compound 2. The solution of stevioside in 10% H₂SO₄ solution was stirred for 6 h at 60°C. The precipitate was filtered and crystallized with acetone to give the isosteviol 1. Isosteviol 1, KOH, and CH₃CH₂Br were added in DMSO. After stirring the solution for 5 h at 40°C, a precipitate came out as excess water was added. The precipitate was filtered and crystallized with ethyl acetate to give the ethyl *ent*-16-oxobeyeran-19-oate. To a solution of Na in ethanol, 37%

formaldehyde aqueous solution and ethyl *ent*-16-oxobeyeran-19-oate were added. After stirring for 8 h at 55°C, the mixture was concentrated under vacuum. Excess water was added, and a white solid separated out. The precipitate was filtered and crystallized with ethyl acetate to give the ethyl *ent*-15 α -hydroxymethyl-16 β -hydroxybeyeran-19-oate. *ent*-15 α -Hydroxymethyl-16 β -hydroxybeyeran-19-oate was dissolved in CH₂Cl₂ and then TCC/Silica gel was added. After stirring for 0.5 h at room temperature, the reaction mixture was filtered and washed with CH₂Cl₂. Finally, the combined organic phase was washed, dried, concentrated, and purified by column chromatography on silica to give the ethyl *ent*-15 α -hydroxymethyl-16-oxobeyeran-19-oate and hydroxylamine hydrochloride in C₂H₅OH was stirred in the presence of NaHCO₃ under refluxing condition for 3 h; then the reaction mixture was concentrated under vacuum to give target compound **2**.

Synthesis of Compounds 3–6. To a solution of compound 2 (3.9 g, 10 mmol) in toluene (50 mL), 48% BF₃·OEt₂ (1 mL) was added dropwise, and the mixture was refluxed for 15 h under nitrogen atmosphere. After completion of the reaction, water (100 mL) was added to the mixture, which was next neutralized with NaHCO₃, and the organic phase was separated and dried over Na₂SO₄. After evaporation *in vacuo*, the crude product was purified by column chromatography to give compounds 3–6.

Compound 3, mp 109.7–111.8°C; $[\alpha]_D^{20}$ –39.8° (*c* 1.0, CH₂Cl₂). IR (KBr, v, cm⁻¹): 3371 (NH), 1720 (COO), 1644 (CONH). ¹H NMR (400 MHz, CDCl₃, δ , ppm, J/Hz): 6.29 (1H, s, NH), 4.13–4.08 (2H, m, 19-(O)OCH₂), 4.02 (1H, dd, J = 10.0, 4.2, CH₂-15), 3.67 (1H, t, J = 9.4, H-15), 2.81 (1H, dd, J = 8.0, 4.0, CH₂-15), 2.16 (1H, d, J = 13.6, H-3), 1.90–0.85 (17H, m, isosteviol skeleton), 1.26 (3H, t, J = 7.2, 19-(O)OCH₂CH₃), 1.18 (3H, s, H-18), 0.99 (3H, s, H-17), 0.81 (3H, s, H-20). ¹³C NMR (100 MHz, CDCl₃, δ , ppm): 177.30 (C, C-19), 177.03 (C, C-16), 62.13 (CH₂, 19-(O)OCH₂), 60.39 (C, C-13), 59.31 (CH₂, 15-CH₂), 57.65 (CH, C-5), 51.45 (CH, C-15), 47.04 (CH, C-9), 44.52 (CH₂, C-14), 43.65 (C, C-4), 40.11 (CH₂, C-1), 40.00 (CH₂, C-12), 38.53 (C, C-8), 38.29 (C, C-10), 37.84 (CH₂, C-3), 36.81 (CH₂, C-7), 28.71 (CH₃, C-18), 28.56 (CH₃, C-17), 20.10 (CH₂, C-6), 18.87 (CH₂, C-2), 17.75 (CH₂, C-11), 14.19 (CH₃, C-20), 14.19 (CH₃, 19-(O)OCH₂CH₃). HR-ESI-MS *m*/z 414.2622 [M + Na]⁺ (calcd for C₂₃H₃₇NO₄Na, 414.2620).

Compounds 4–5, IR (KBr, v, cm⁻¹): 3373 (OH), 2230 (CN), 1720 (COO), 1677 (C=C). ¹H NMR (400 MHz, CDCl₃, δ , ppm, J/Hz): 5.53 (1H, s, H-12, for 4), 5.15 (0.37H, s, H-14, for 5), 4.16–4.06 (3H, m, 19-(O)OCH₂, for 4 and 5), 3.86–3.76 (3H, m, H-15 and CH₂-15, for 4 and 5), 3.39 (0.47H, dd, J = 8.9, 4.9, CH₂-15, for 5), 3.13 (1H, dd, J = 8.2, 4.4, CH₂-15, for 4), 2.48–0.86 (23H, m, isosteviol skeleton, for 4 and 5), 1.69 (1H, s, H-17, for 5), 1.67 (3H, s, H-17, for 4), 1.27 (4H, t, J = 7.2, 19-(O)OCH₂CH₃, for 4 and 5), 1.17 (3H, s, H-18, for 4), 1.16 (1H, s, H-18, for 5), 0.91 (3H, s, H-20, for 4), 0.88 (1H, s, H-20, for 5). ¹³C NMR (100 MHz, CDCl₃, δ , ppm) for 4: 177.01 (C, C-19), 127.34 (C, C-13), 123.26 (C, CN), 122.55 (CH, C-12), 61.28 (CH₂, 19-(O)OCH₂), 60.21 (CH₂, CH₂-15), 57.78 (CH, C-5), 54.35 (CH, C-9), 43.51 (C, C-4), 39.76 (CH₂, C-1), 39.62 (CH₂, C-14), 37.83 (CH₂, C-3), 37.70 (CH, C-15), 37.17 (C, C-10), 36.91 (CH₂, C-7), 29.71 (CH₃, C-18), 28.81 (C, C-8), 23.21 (CH₃, C-17), 22.86 (CH₂, C-11), 20.02 (CH₂, C-6), 19.00 (CH₂, C-2), 14.15 (CH₃, C-20), 13.79 (CH₃, 19-(O)OCH₂CH₃). HR-ESI-MS *m/z* 396.2519 [M + Na]⁺ (calcd for C₂₃H₃₅NO₃Na, 396.2515).

Compound 6, mp 128.6–129.4°C. IR (KBr, v, cm⁻¹): 2236 (CN), 1719 (COO), 1092 (C–O–C). ¹H NMR (400 MHz, CDCl₃, δ , ppm, J/Hz): 4.39 (1H, t, J = 12.1, H-15), 4.15–4.07 (2H, m, 19-(O)OCH₂), 3.89 (1H, dd, J = 11.8, 6.0, H-16), 2.83 (1H, dd, J = 12.5, 6.0, H-16), 2.49–0.86 (17H, m, isosteviol skeleton), 2.16 (1H, d, J = 13.6, H-3), 1.29 (3H, t, J = 7.2, 19-(O)OCH₂CH₃), 1.19 (3H, s, H-18), 1.11 (3H, s, H-17), 0.99 (3H, s, H-20). ¹³C NMR (100 MHz, CDCl₃, δ , ppm): 177.27 (C, C-19), 121.21 (C, CN), 71.32 (C, C-13), 65.13 (CH₂, C-16), 60.40 (CH₂, 19-(O)OCH₂), 57.93 (CH, C-5), 57.61 (CH, C-9), 53.83 (CH₂, C-14), 44.27 (C, C-4), 43.69 (CH₂, C-1), 40.54 (CH₂, C-12), 38.89 (C, C-10), 38.09 (CH₂, C-3), 37.60 (CH₂, C-7), 37.37 (CH, C-15), 36.82 (C, C-8), 29.58 (CH₃, C-18), 28.48 (CH₃, C-17), 21.92 (CH₂, C-6), 20.63 (CH₂, C-11), 19.19 (CH₂, C-2), 14.23 (CH₃, C-20), 13.92 (CH₃, 19-(O)OCH₂CH₃). HR-ESI-MS *m/z* 396.2515 [M + Na]⁺ (calcd for C₂₃H₃₅NO₃Na, 396.2515).

X-Ray Crystallographic Analysis of Compound 3. Colorless crystals of the title compound were grown at room temperature from ethanol by the slow evaporation technique. X-ray diffraction data of the title compound were collected on a Bruker SMART APEXII CCD area-detector diffractometer with Mo Ka ($\lambda = 0.71073$ Å) radiation at 300(2) K. The collected data were reduced using the program SAINT. The structure was solved by direct methods and refined by the full-matrix least-squares method on all F² data using the SHELX-97 (Sheldrick, 2008) and SHELXL-97 (Sheldrick, 2008) programs, respectively. Crystallographic data (excluding structure factors) have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication numbers CCDC 1951641 (compound 3). Copies of the data can be obtained, free of charge, on applications to CCDC, 12 Union Road, Cambridge CB2 1EZ. UK (fax: +44 (0) 1223 336033 or e-mail: deposit@ccdc.cam.ac.uk or http://www. ccdc.cam.ac.uk).

Cytotoxicity Assay *in vitro*. HCT-116, HGC-27, and JEKO-1 cells were cultured in RPMI-1640 medium (GIBCO: 31800-022) supplemented with 10% FBS, 100 U/mL of penicillin, and 100 mg/mL of streptomycin at 37°C in a 5% CO_2 humidified atmosphere. Cell cytotoxicity was assayed by the MTT method. Briefly, cells were seeded in 96-well tissue culture plates. After 24 h incubation at 37°C and 5% CO_2 , the culture medium was removed and replaced with fresh medium containing the studied compounds in different concentrations to the wells, and the cells were incubated for another 72 h. Afterwards, the MTT (MP: 102227) solution (0.5 mg/mL) was added and the whole incubated for an additional 4 h; 200 microliters of DMSO was added to each well to dissolve the reduced MTT crystals. The optical density of each well was measured at 492/630 nm with an enzyme immunoassay instrument (TECAN: Infinite 200 Pro). Then the inhibitory percentage of cell proliferation of each compound at various concentrations was calculated, and the IC₅₀ value was determined.

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