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Stereoselective synthesis of 15- and 16-substituted isosteviol derivatives and their cytotoxic activities

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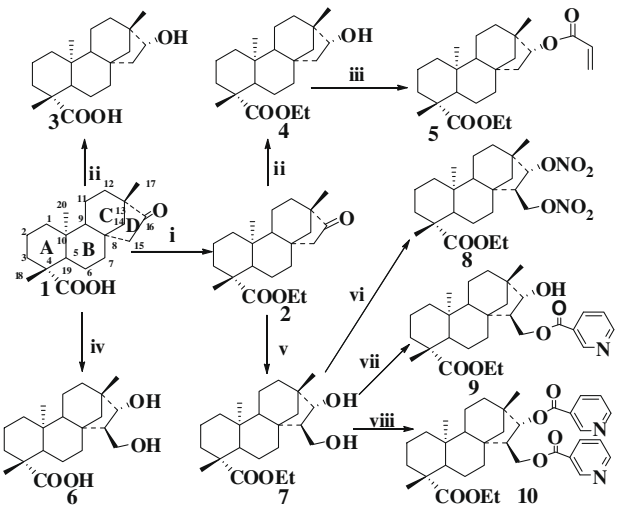
ABSTRACT

By means of functional interconversions in ring D of the tetracyclic diterpene isosteviol (ent-16-ketobeyeran-19-oic acid **1**), various 15- and 16-substituted isosteviol derivatives were stereoselectively prepared. The cytotoxic activities in vitro of these new isosteviol derivatives were investigated, and some of them showed noteworthy activities against B16-F10 melanoma cells.

Isosteviol (ent-16-ketobeyeran-19-oic acid **1**) is a tetracyclic diterpenoid with a beyerane skeleton, obtained by acid hydrolysis of stevioside.[1,2] In recent years, isosteviol derivatives have attracted scientific attention because of their remarkably broad spectrum of biological activities including antihypertension,[3] antihyperglycemia,[4] anti-inflammatory,[5] antioxidation and potential antitumor[6] and other biological activities.[7–14]

As proposed for the Rabdosia diterpenoids,[15] hydroxyl groups may play an important role in binding to some receptors. So hydrophilic functional groups attached to ring D in the isosteviol skeleton may significantly improve the biological properties of these compounds. In addition, it may also provide some possibilities for the construction of heterocycles into isosteviol skeleton. On the other hand, some new heterocyclic compounds containing nitrogen atom have been synthesized and showed antiparkinsonian,[16] antitumor,[17–19] antiandrogenic,[20] antidiabetic[21,22] and antimicrobial activities.[23,24] In view of these reports and in continuation of our previous work[25] in heterocyclic chemistry, some new compounds containing pyrazoline and isoxazolidine ring fused with isosteviol structure have been designed and synthesized for the propose of evaluation of cytotoxic activities against B16-F10 melanoma cells.

Isosteviol derivatives were synthesized. The synthetic routes are outlined in Scheme 1. Initial synthetic efforts were focused on structural modifications at C-15 and C-16 positions. Treatment of isosteviol **1** obtained by acid hydrolysis of stevioside with CH_3CH_2Br and KOH in DMSO afforded the corresponding ethyl ester of isosteviol **2** in 95% yield.[26] Reduction of compound **1** and **2** with $NaBH_4$ in C_2H_5OH at 0 °C afforded *ent*-16β-hydroxybeyeran-19-oic acid **3** and ethyl *ent*-16β-hydroxybeyeran-19-oate **4** in good yield, respectively.[27] The presence of signal at δ_H 3.85 (δ_C 81.3) in



Scheme 1. Reagents and conditions: (i) EtBr, DMSO, KOH, rt, 3 h, 96%; (ii) $NaBH_4$, C_2H_5OH, 0 °C, 1 h, 96%; (iii) DCC/DMAP, acrylic acid, CH_2Cl_2, rt, 12 h, 85%; (iv)HCHO, aq. NaOH, C_2H_5OH, 60 °C, 1 h, 95%; (v) HCHO, C_2H_5ONa, C_2H_5OH, 60 °C, 3 h, 90%; (vi) HNO_3/H_2SO_4, CH_2Cl_2, 80% (vii) 1*eq*. nicotinoyl chloride, Et_3N, rt, 1 h, 81%; (viii) 2*eq*. nicotinoyl chloride, Et_3N, rt, 3 h, 88%.

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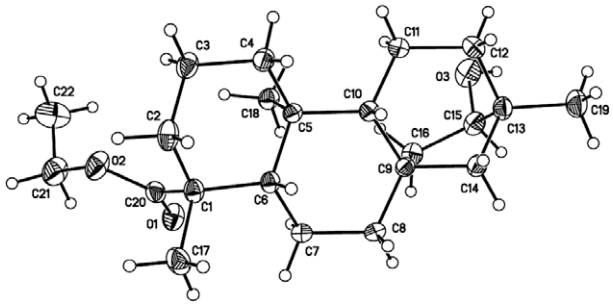


Figure 1. X-ray structure of compound **4**.

the ^{1}H and ^{13}C NMR spectra confirmed the form of hydroxyl group in compound **4**. The stereostructure of compound **4** was confirmed through X-ray crystallographic analysis (Fig. 1).[28] Treatment of ethyl *ent*-16α-hydroxybeyeran-19-oate **4** with acrylic acid in the presence of DCC and DMAP in CH_2Cl_2 furnished ethyl *ent*-16α-acryloxybeyeran-19-oate **5** (85%). *Ent*-16β-hydroxy-15α-hydroxymethylbeyeran-19-oic acid (**6**) and ethyl *ent*-16β-hydroxy-15α-hydroxymethylbeyeran-19-oate (**7**) were stereoselectively synthesized via an one pot Tollens' reaction in good yield (95%, 90%, respectively).[29] The products were characterized by HRMS, IR and NMR, and the stereostructure of compound **6** was confirmed by X-ray crystallographic analysis (Fig. 2).[28] Treatment of 1,3-diol **7** with HNO_3/H_2SO_4 in CH_2Cl_2 gave the corresponding dinitrate product **8** (80%). Meanwhile, compound **9** and **10** were selectively synthesized in Et_3N from compound **7** by controlling the amount of nicotinoyl chloride in good yield (81%, 88%, respectively).

The results obtained above showed that the newly formed hydroxymethyl group at C-15 in compound 6 was always stereoselectively posited on exo position. From the crystal structure of compounds **4** and **6**, we found that the steric hindrance of C-10–CH_3 and ring C may be the reason that substituent at C-15 could not be posited on the endo position. Meanwhile, the newly introduced hydroxy group at C-16 was stereoselectively posited on the endo position because of the steric hindrance effects of C-13–CH_3 and ring C.

Treatment of compound **7** with 4-Methylphenylsulfonyl chloride in pyridine furnished compound **11** (75%), which was further converted to the ring opening product **12** in 96% yield by Grob fragmentation of compound **11** in the presence of NaOH in CH_3CN (Scheme 2).[30] The driving force of this C—C bong cleavage reaction may result from conversion of a C—O bond to C=O bond and oxy anion to stable sulfonate anion. Based on knowledge on characteristics of isosteviol derivatives and compound **7**, the signal at *m*/*z* 383.2560 ($[M + Na]^+$) in the HRMS of compound **12** indicated the molecular formula of $C_{23}H_{36}O_3$. In the ^{1}H and ^{13}C NMR spectra of **12**, additional resonances were observed at δ_H 9.27 (δ_C 205.2), δ_H 5.95 (δ_C 142.5) and δ_H 5.12, 5.08 (δ_C 113.6), suggesting the introduction of an aldehyde group and an vinyl group. The stereostructure of **12** was confirmed by X-ray crystallographic analysis (Fig. 3).[28]

With compound **12** in hand, some efforts were carried out for the structural modification and functional groups conversion at the aldehyde group in order to probe the effect of the newly introduced substituents. In this regard, the corresponding carboxylic acid, amine, alcohol derivatives of compound **12** were synthesized as depicted in Scheme 3. The aldehyde **12** was oxidized with the Jones reagent (8 N) in acetone, resulting in the carboxylic acids **13** (90%). Treatment of compound **12** in presence of hydrogen peroxide and sodium hydroxide in methanol furnished the compound **15** (75%). Reduction of **12** with sodium borohydride in ethanol at 0 °C led to the corresponding alcohol **16** (96%), which was further converted to the tolylsulfonyloxymethyl derivative **17** in 85% yield by esterification of alcohol **16** with 4-methylphenylsulfonyl chloride in pyridine. Introduction of the azido group of compound **17** was carried out by reaction of **16** with sodium azide under basic conditions to azide **18** (80%). Treatment of compound **18** with tri-

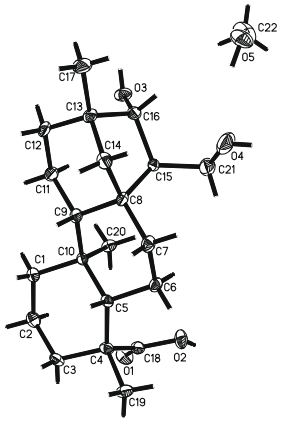


Figure 2. X-ray structure of compound **6**.

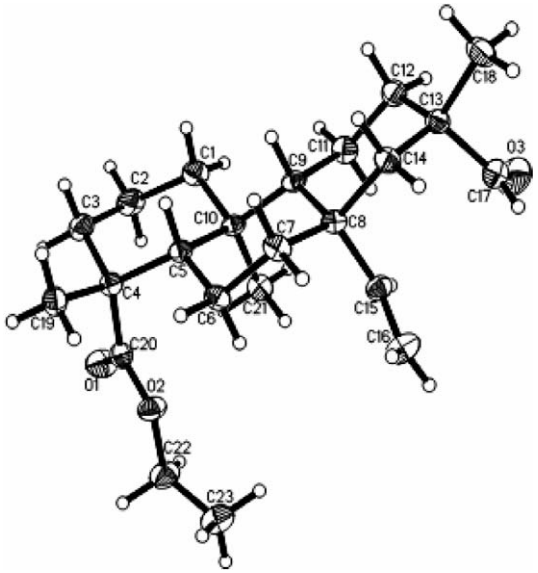
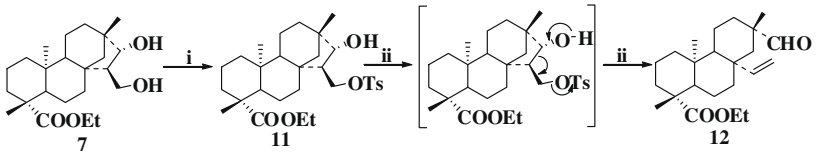
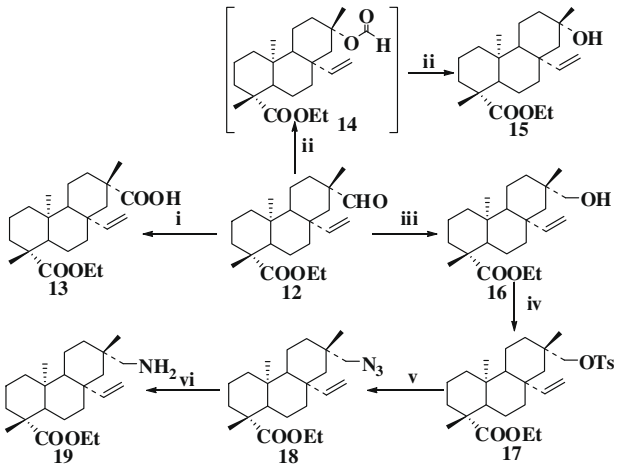


Figure 3. X-ray structure of compound **12**.



Scheme 2. Reagents and conditions: (i) TsCl, Pyr, rt, 18 h, 75%; (ii) NaOH, CH_3CN, rt, 3 h, 96%.



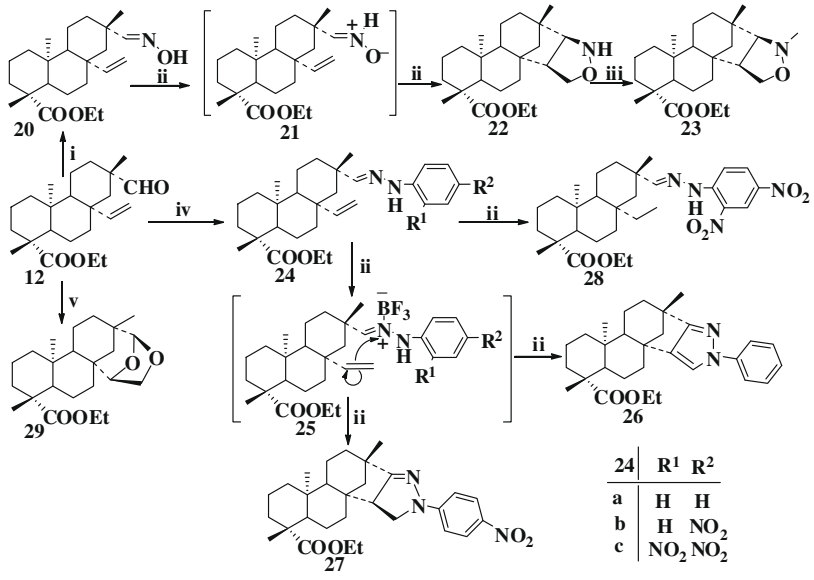
Scheme 3. Reagents and conditions: (i) Jones reagent (8 N), acetone, 0 °C, 2 h, 90%; (ii) CH_3OH, NaOH, H_2O_2, 65 °C, 4 h, 75%; (iii) $NaBH_4$, C_2H_5OH, 0 °C, 10 min, 96%; (iv) TsCl, Pyr, rt, 12 h, 85%; (v) NaN_3, DMF, 80 °C, 3 h, 80%; (vi) PPh_3, H_2O, 65 °C, 3 h, 85%.

phenylphosphine in water at 65 °C afforded amine derivative **19** (85%).

The presence of the formyl group and the vinyl moiety in the compound **12** makes the molecule suitable for condensation and subsequent 1,3-dipolar cycloaddition to give fused heteroatom-containing frameworks via intramolecular sequences.[31] In this regard, a series of novel compounds containing pyrazole, pyrazoline and isoxazolidine ring fused with isosteviol structure were stereoselectively synthesized via 1,3-dipolar cycloaddition as shown in Scheme 4. Oximation of the aldehyde **12** with hydroxylamine hydrochloride in presence of sodium bicarbonate in ethanol gave only one of the two possible geometric isomers of the corresponding aldoxime **20** (90%). The exact configuration could not be determined from the NMR spectra of the compound, but the formation of the more stable (E)-oxime is assumed. The aldoxime **20** was catalytically tautomerized by $BF_3{\cdot}OEt_2$ in boiling toluene into its nitrone form **21**, which then intramolecularly cyclized to produce the condensed isoxazolidine derivative **22** in high yield (96%).[32] The structure of the product **22** was confirmed unambiguously by the disappearance of the signals due to the aldoxime groups in the IR, ^1H and ^{13}C NMR spectra, the presence of isoxazolidine ring signal at δ_H 3.81, 3.72, 3.30, 2.90 (δ_C 72.7, 59.9, 57.6), and the NOESY spectrum indicated the α orientation of the protons at C-15 and C-16. Treatment of compound **22** with iodomethane in presence of sodium hydride in DMF at 50 °C afforded *N*-methylated isoxazolidine derivative **23** (85%).

The reaction of the aldehyde **12** with phenylhydrazine in ethanol at 10 °C yielded the corresponding phenylhydrazone **24a**, which readily cyclized after purification in the presence of a catalytic amount of $BF_3{\cdot}OEt_2$ to afford pyrazole **26** (84%).[32,33] The condensation of aldehyde **12** was also carried out with 4-nitrophenylhydrazine in ethanol at 10 °C to gave 4-nitrophenylhydrazone **24b**, and $BF_3{\cdot}OEt_2$-induced cycloaddition of the 4-nitrophenylhydrazone **24b** was also accomplished to give stereoselectively a single pyrazoline derivative **27** in 75% yield.[32,33] The presence of signal at δ_H 4.06, 3.54, 3.25, (δ_C 174.7) in the ^1H and ^{13}C NMR spectra confirmed the form of pyrazoline ring, and the NOESY spectrum indicated the α orientation of the protons at C-15. The 2,4-dinitrophenylhydrazone **24c** was obtained from aldehyde **12** with 2,4-dinitrophenylhydrazine in ethanol at 10 °C, but 2,4-dinitrophenylhydrazone **24c** containing two electron-withdrawing nitro groups exhibited great stability against both thermal and Lewis acid-catalyzed cycloaddition. Unexpectedly, reductive product **28** was obtained. The disappearance of the signals due to the vinyl group and presence of ethyl group signals in the IR, ^1H and ^{13}C NMR confirmed the structure. Treatment of compound **12** with sodium periodate and sodium bromide in acetic acid at 90 °C afforded acetal product **29** (84%).[34] The structure of compound **29** was confirmed by IR, ^1H, ^{13}C NMR and HRMS, and the NOESY spectrum indicated α orientation of the protons at C-15 and C-16.

The cytotoxic activities of these compounds were then evaluated against B16-F10 melanoma cells lines as described in Table 1.[35] Introduction of hydroxyl and hydroxymethyl group into isosteviol precursor results in higher cytotoxicities (**2** vs **4** and **2** vs **7**), and ring opened derivatives containing hydroxyl, amine and oxime group were more potent than ring opened product **12** (**15, 16, 19, 20** vs **12**). But isosteviol derivative containing carboxy group were inactive (**3** vs **4**, **6** vs **7** and **12** vs **13**). In addition, the



24	R^1	R^2
a	H	H
b	H	NO_2
c	NO_2	NO_2

Scheme 4. Reagents and conditions: (i) $HONH_3Cl$, $NaHCO_3$, C_2H_5OH, 60 °C, 2 h, 97%; (ii) toluene, $BF_3{\cdot}OEt_2$, 80 °C, 1 h, 84–96%; (iii) DMF, NaH, CH_3I, 50 °C, 2 h, 85%; (iv) for **24a**: C_2H_5OH, acetic acid, phenylhydrazine, 10 °C, 3 h, 95%; for **24b**: C_2H_5OH, acetic acid, 4-nitrophenylhydrazine, 10 °C, 2 h, 85%; for **24c**: C_2H_5OH, acetic acid, 2,4-dinitrophenylhydrazine 10 °C, 2 h, 81%; (v) $NaIO_4$, NaBr, acetic acid, 90 °C, 3 h, 84%.

Table 1
Cytotoxic activities of isosteviol derivatives against B16-F10 melanoma cells

Compound	B16-F10	Compound	B16-F10	Compound	B16-F10
1	NI[a]	**8**	NI[a]	19	34.3[c]
2	NI[a]	**9**	25[b]	**20**	27.5[b]
3	NI[a]	**10**	22[b]	**22**	15[b]
4	58[b]	**12**	NI[a]	**23**	NI[a]
5	NI[a]	**13**	NI[a]	**26**	19[b]
6	NI[a]	**15**	26[b]	**27**	21[b]
7	68[b]	**16**	24[b]	**29**	NI[a]

[a] No inhibition at 100 μM.
[b] IC_{50} (μM).
[c] Inhibition (%) determined at 100 μM concentration of compound.

protons of hydroxyl group and amine group were necessary in potency (**4** vs **5, 7** vs **8** and **22** vs **23**). The isoxazolidine derivative **22** (IC_{50} = 15 μM), pyrazole **26** (IC_{50} = 19 μM) and pyrazoline derivative **27** (IC_{50} = 21 μM) exhibited more inhibitory potency against B16-F10 melanoma cells, indicating that D-ring fused heterocyclic analogues might deserve some attention for further cytotoxic activities design.

In summary, a novel series of 15- and 16-substituted isosteviol derivatives were first prepared, especially some new compounds containing pyrazoline and isoxazolidine ring fused with isosteviol structure were stereoselectively synthesized from compound **7** via Grob fragmentation and subsequent intramolecular 1,3-dipolar cycloaddition. The cytotoxic activities in vitro of these new isosteviol derivatives were investigated, and some of them showed noteworthy activities against B16-F10 melanoma cells. Further research and drug development on isosteviol derivatives for cytotoxicity test are ongoing in our laboratory and the results will be reported in due course.

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35. In vitro cytotoxicity study: B16-F10 melanoma cells (purchased from Nanjing KeyGen Biotech. Co. Ltd, China), was cultured in RPMI-1640 medium (GIBCO Co. Grand Island, NY) supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin (Sigma Chemical Co., St. Louis, MO) at 37 °C in humidified air atmosphere of 5% CO_2 (Binder, CB150, Germany). Cell cytotoxicity was assessed by MTT assay. Briefly, cells were plated into 96-well-plate (1×10^4cells/well). The next day compound at various concentrations diluted in culture medium was added (200 μL/well) to the wells. 48 h later 20 μL MTT (Sigma Chemical Co. St. Louis, MO) (0.5 mg/mL MTT in PBS) was added and cells were incubated for a further 4 h. Two hundred microliters of DMSO was added to each culture to dissolve the reduced MTT crystals. The MTT-formazan product dissolved in DMSO was estimated by measuring absorbance at 570 nm with a microplate reader (Biotech, Power Wave, CA). Then the inhibitory percentage of each compound at various concentrations was calculated, and the IC_{50} value was determined.