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Potent vasorelaxant analogs from chemical modification and biotransformation of isosteviol

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ABSTRACT

Isosteviol (**1**) has been reported to exhibit moderate vasorelaxant activity. In order to enhance the bioactivity of this compound, chemical modification of **1** to the dihydro analog, *ent*-16β-hydroxybeyeran-19-oic acid (**2**), was undertaken. Compound **2** was then converted to the corresponding acetate derivative, *ent*-16β-acetoxybeyeran-19-oic acid (**3**). Biotransformation of compounds **1**–**3** by the fungus *Cunninghamella echinulata* NRRL 1386 was investigated and the metabolites **4**–**9** were obtained. The substrates and their metabolites were subjected to in vitro rat aorta relaxant activity evaluation. The metabolite **4**, *ent*-7α-hydroxy-16-ketobeyeran-19-oic acid, exhibited the most highly potent activity, with EC_{50} of 3.46 nM, whereas the parent compound **1** showed relatively low activity (EC_{50} 57.41 nM). A 17-fold increase in vasorelaxant activity of the analog **4** relative to compound **1** is of particular significant. Compound **4** exerted vasorelaxant activity at particularly low concentration and the vasorelaxant profile reached maximum at relatively low concentration, especially when compared with acetylcholine, the positive control.

1. Introduction

Hypertension is one of the most common cardiovascular diseases that can cause coronary disease, myocardial infarction, stroke and sudden death and is the major contributor to cardiac failure and renal insufficiency. There are several classes of anti-hypertensive drugs, diuretics, β-blockers, calcium channel blockers, angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers and vasodilators [1]. These different classes of drugs have both advantages and disadvantages and some of them have adverse side effects [2,3]. It would therefore be beneficial for patients with hypertension if novel antihypertensive agents with lesser side effects are available.

Isosteviol (**1**, Scheme 1), a tetracyclic diterpenoid from the beyerane series, was obtained from acid hydrolysis of stevioside [4], a diterpenoid glycoside from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni. This non-caloric sweetening agent is 300 times sweeter than sucrose [5]. Several studies suggested that the non-sweetening agent **1** possesses a variety of biological activities including reducing blood pressure and cardioprotective effect [6–9], anti-hyperglycemic [10] and potential anti-tumor effects [11]. Previous reports of **1** and stevioside [12–16] on cardiovascular and related effects led us to investigate antihypertensive action of this type of compounds. This class of compounds is of special interest, since stevioside could be obtained from *S. rebaudiana* in large quantity [5,17] and compound **1** in turn was obtained in good yield from this glycoside by acid hydrolysis [4,9]. Moreover, the non-toxic or less toxic nature of this class of diterpenoids has prompted us to investigate antihypertensive activity of compound **1**. It has been reported that this non-sweetening compound exhibited moderate vasorelaxant activity [6], which might not be sufficiently potent for further drug development study. It was therefore of interest to see whether structural modification of **1** would give rise to analog(s) with considerably high vasorelaxant activity. The present work deals with chemical modification and microbial transformation of isosteviol (**1**) to analogs **2**–**9** (Scheme 1), some of which exhibited high vasorelaxant activity.

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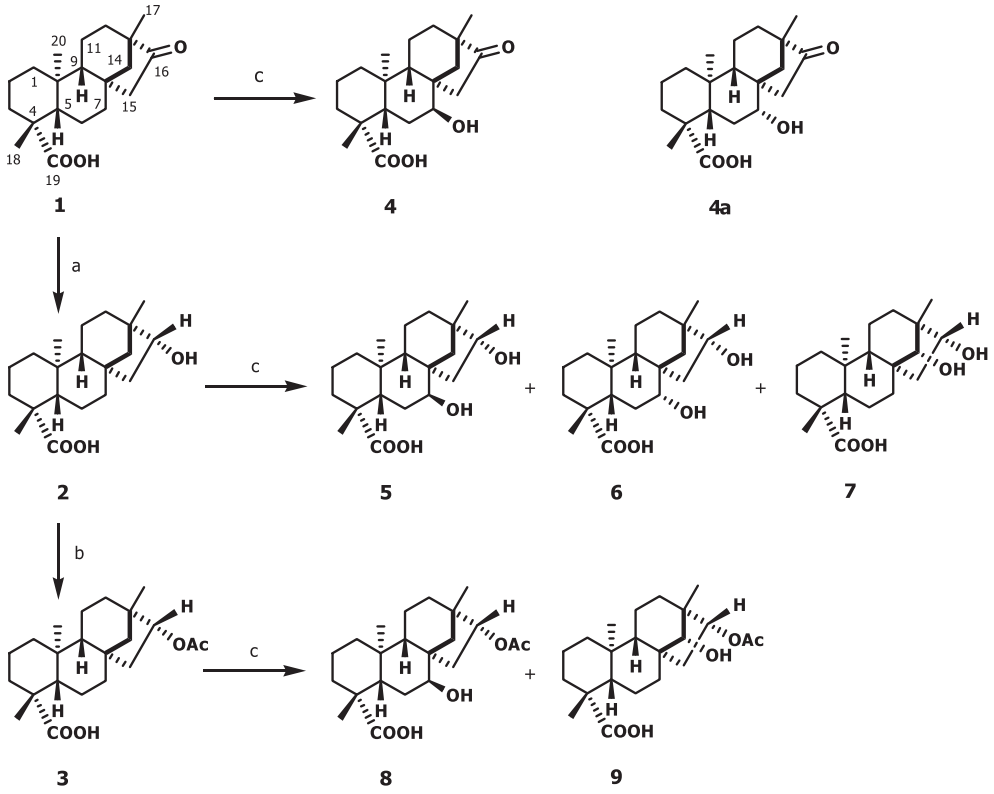

Scheme 1. Chemical modification of *ent*-16-ketobeyeran-19-oic acid (isosteviol, **1**) to the dihydro analog **2** and the corresponding acetate **3**, and microbial transformation of compound **1** to the hydroxylated analog **4**, compound **2** to the hydroxylated analogs **5**, **6** and **7**, and compound **3** to the hydroxylated analogs **8** and **9**. a: $NaBH_4$/THF; b: Ac_2O/pyridine; *c*: *Cunninghamella echinulata* NRRL 1386.

2. Chemistry

2.1. Chemical modification

Isosteviol (**1**) was chosen as the parent compound for structural modification to its analogs for vasorelaxant evaluation. We aimed to modify the keto function at the 16-position to the corresponding dihydro analogs. Acid hydrolysis of stevioside yielded **1** in 81%. Reduction of **1** with $NaBH_4$ gave the dihydro analog **2** in 97%. The reduction occurred exclusively from the β-face of the molecule. The presence of carbinol proton at δ 4.10 (br d, $J = 6.7$ Hz) confirmed the conversion of the keto group of **1** into the hydroxyl group in **2**. The spectroscopic (^{1}H NMR and mass spectra) data of **2** were consistent with the reported values [18,19]. Acetylation of compound **2** with acetic anhydride and pyridine gave the corresponding acetate **3** in 76%. The ^{1}H NMR spectrum of **3** showed a three-proton singlet signal at δ 2.02, thus indicating that acetylation has taken placed. A downfield shift of the carbinolic proton at C-16 also confirmed that acetylation has taken placed at the C-16 hydroxyl group.

2.2. Microbial transformation

The limited number of functional groups in isosteviol (**1**) has prevented us from further chemical modification. Microbial transformation is a powerful method for the regioselective and stereoselective introduction of hydroxyl group at un-activated position of beyerane diterpenoids including isosteviol [9,18–24]. Microbial transformation of isosteviol (**1**) with *Cunninghamella echinulata* NRRL 1386 produced the more polar metabolite **4** (see Scheme 1), which showed the $[2M-H]^-$ ion at *m/z* 667 consistent with the molecular formula $C_{20}H_{30}O_4$. Analysis of 1D and 2D NMR spectra revealed the presence of a hydroxyl group, which was located at C-7 by HMBC analysis. The ^{1}H and ^{13}C NMR spectra of **4** were consistent with the reported values of *ent*-7α-hydroxy-16-ketobeyeran-19-oic acid [22–24].

Incubation of **2** with the same fungus yielded three metabolites, **5**–**7**. Compound **5** was identified as *ent*-7α,16β-dihydroxybeyeran-19-oic acid by comparison of the NMR and MS data with literature [18,19]. The conversions of **1** and **2** by *C. echinulata* NRRL 1386 to the corresponding 7β-hydroxylated analogs **4** and **5**, respectively, have revealed the regio and stereoselectivity of the enzyme of this fungus.

Compound **6** displayed the $[M-H]^-$ ion at *m/z* 335.2208 in the HR-TOFMS (ESI$^-$), compatible with a molecular formula $C_{20}H_{32}O_4$. The IR spectrum suggested the presence of hydroxyl groups at 3624 and 3414 cm^{-1} and carboxyl group at 1701 cm^{-1}. The ^{13}C NMR and DEPT spectra revealed the presence of eight CH_2 and seven CH/CH_3 indicating the presence of a proton geminal to a new hydroxyl group at δ 75.7 in the ^{13}C NMR spectrum. The ^{1}H NMR spectrum of **6** revealed a new resonance at δ 3.70 and the HMBC spectra showed correlations with C-5 (δ 54.6), C-6 (δ 32.6), C-8 (δ 49.0), C-14 (δ 51.0) and C-15 (δ 34.3), thus confirming that hydroxylation has taken placed at C-7. In the ^{1}H NMR spectrum, a double doublet ($J = 11.5$ and 3.6 Hz) of H-7 was observed. The large coupling constant of H-7 indicated that it was in the axial position. The large *J* value resulted from axial–axial coupling between H-6ax and H-7. The hydroxyl group therefore adopted the equatorial orientation. This was in agreement with the reported product **4a** obtained from microbial transformation of **1** [21,24]. Furthermore, the orientation of the OH group at C-7 was also confirmed by the ROESY experiments. Thus, H-7 showed cross-peak with H-5 (δ 1.32), H-9 (δ 1.29) and H-14 (δ 1.08 and δ 2.46). On the basis of the spectroscopic data above led to the identification of **6** as *ent*-7β,16β-dihydroxybeyeran-19-oic acid.

The molecular formula of compound **7** was determined as $C_{20}H_{32}O_4$ from its HR-TOFMS (ESI$^+$) at *m/z* 359.2180 $[M+Na]^+$. The

IR spectrum showed the absorption band of the hydroxyl and carboxyl groups at 3452 and 1696 cm^{-1}, respectively. Analysis of the ^{13}C NMR spectrum, compared to that of compound **2**, a DEPT experiment showed eight CH_2 and seven CH/CH_3 for **7** whilst for **2** there are nine CH_2 and six CH/CH_3, respectively. Therefore, one methylene group of **2** was converted into a hydroxymethine group, suggesting **7** to be a hydroxylated product of **2**. Comparison of the ^1H and ^{13}C NMR spectra of **7** with those of compounds **2**, **5** and **6** revealed that an additional OH group in **7** should be located at C-14, which was confirmed by a detailed analysis of the HMBC data. The ^1H NMR chemical shift at δ 3.49 clearly showed connectivities with C-9 (δ 56.1), C-12 (δ 34.2), C-15 (δ 42.3) and C-16 (δ 78.8). The ROESY experiments revealed the correlations between H-14 and H-7ax, H-9 and H-12ax (see Fig. 1). This led to a conclusion that H-14 is in the β-orientation. The structure of **7** was thus established as *ent*-14β,16β-dihydroxybeyeran-19-oic acid. Microbial hydroxylation of dihydroisosteviol (**2**) at the 14-position by *Mortierella isabellina* has been reported [18], but the spectroscopic data of the metabolite are different from those of compound **7** isolated by our group and their NOESY experiments indicated that the structure of the isolated compound was different from compound **7**.

Incubation of the substrate *ent*-16β-acetoxybeyeran-19-oic acid (**3**) with *C. echinulata* NRRL 1386 resulted in the production of two new metabolites, **8** and **9**, which have been identified by spectroscopic techniques. Compound **8** showed the $[M-H]^-$ ion at *m/z* 377.2298 in the HR-TOFMS (ESI^-), corresponding to $C_{22}H_{34}O_5$. The ^1H NMR data were similar to those of the parent compound **3**. The significant different was the presence of the carbinolic proton (H-7) at δ 3.56 which correlated to C-7 (δ 76.5) in the HMQC spectrum. The acetoxyl group was still present as a singlet signal at δ 2.04. The HMBC correlations of H-7 with C-5 (δ 47.2), C-6 (δ 28.8), C-9 (δ 49.7) and C-15 (δ 39.3) confirmed the location of the hydroxyl group. The small coupling constant of H-7 ($W_{1/2} = 6.5$ Hz) of **8** was consistent with the β-orientation of the 7-hydroxyl group. The spectroscopic data (see experimental part) led to the identification of **8** as *ent*-16β-acetoxy-7α-hydroxybeyeran-19-oic acid.

Compound **9** showed the $[M+Na]^+$ ion at *m/z* 401.2298 in the HR-TOFMS (ESI^+), which was in agreement with the molecular formula $C_{22}H_{34}O_5$. The ^1H NMR data of **9** were similar to those of **7**. The significant different was the presence of the acetoxyl group at δ 2.01 and the downfield shift (0.12 ppm) of H-16. The carbinolic proton at δ 3.04 (H-14) showed HMBC correlations with C-9 (δ 54.7), C-12 (δ 33.3), C-15 (δ 38.3) and C-16 (δ 81.1), thus confirming the location of the hydroxyl group at the 14-position. The β-orientation of H-14 was also deduced from the ROESY experiments by the same analogy to that of compound **7**. The structure of **9** was thus established as *ent*-16β-acetoxy-14β-hydroxybeyeran-19-oic acid. The production of the 14α-hydroxy analogs **7** and **9** have indicated that another enzyme of *C. echinulata* NRRL 1386 regioselectively and stereoselectively hydroxylated at the 14-position of the beyerane diterpenoids.

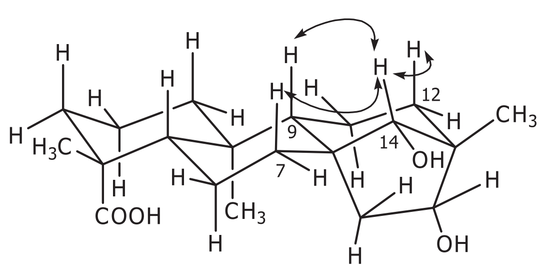

Fig. 1. Selected ROESY correlations of compound **7**.

3. Results and discussion

3.1. Vasorelaxant activity

The modification of isosteviol (**1**) at 16-keto group to its dihydro analog **2** and the corresponding acetate derivative **3** was achieved by chemical reactions. The substrates **1**–**3** were subjected to microbial transformation by *C. echinulata* NRRL 1386. The metabolite **4** was obtained from the bioconversion of **1**. Compound **2** was biotransformed to the metabolites **5**, **6** and **7**. The metabolites **8** and **9** were produced from biotransformation of **3**. The advantages of microbial transformation in this work are hydroxylation has taken placed at the un-activated position and the acetate group in the substrates remained intact. *C. echinulata* NRRL 1386 regioselectively and stereoselectively hydroxylated the beyerane diterpenoids at C-7β and C-14α. The exception was for compound **6** that hydroxylation also occurred on the α-face of C-7. The vasorelaxant activity of these compounds was evaluated using rat aorta rings (see Fig. 2). The parent compound **1** and the dihydro analog **2** showed EC_{50} values of 57.41 and 29.07 nM, respectively (Table 1). The result has indicated that reduction of the C-16 keto group of **1** to the corresponding hydroxyl group resulted in 2-fold increase in activity. Acetylation at the C-16 hydroxyl group of **2** afforded the acetate **3**, with the EC_{50} value of 4.88 nM, which was 6-fold more active than compound **2**, or approximately 12-fold more active than compound **1**. Compound **4**, the biotransformation product of **1**, exhibited the most active vasorelaxant effect, with the EC_{50} value of 3.46 nM, or approximately 17-fold more active than compound **1**. However, compound **2**, upon introduction of a hydroxyl group to the C-7 position to yield the isomeric metabolites **5** and **6**, did not increase the activity of the analogs. The activity in going from the acetate **3** to **8** seemed to follow the same trend. The presence of both the hydroxyl function at the 7-position and the hydroxyl or acetoxyl function at the 16-position decreased the vasorelaxant activity. Introduction of a hydroxyl group to C-14 position of **2** to yield the metabolite **7** resulted in sharp decrease in activity. The similar trend was also observed for the bioconversion of the acetate **3** to the analog **9**. The assay results have indicated that, for

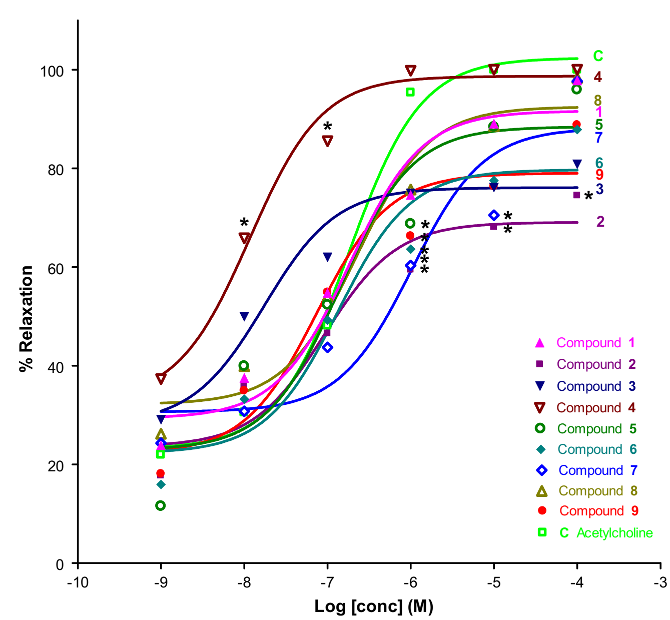

Fig. 2. Effect of various concentrations of isosteviol (**1**) and analogs **2**–**9** (10^{-9} to 10^{-4} M) on the relaxation of phenylephrine (10^{-6} M)-induced contraction in rat aorta. *$p < 0.05$, significant difference compared to control (acetylcholine).

Table 1
Vasorelaxant activity of compounds **1**–**9**.

Compound	EC_{50} (nM)
1	57.41
2	29.07
3	4.88
4	3.46
5	41.75
6	39.79
7	303.40
8	59.71
9	19.53
Acetylcholine[a]	65.00

[a] As acetylcholine iodide.

isosteviol (**1**), introduction of the 7β-hydroxyl group markedly increased the vasorelaxant activity. However, for the dihydro analogs of isosteviol, hydroxylations at C-7 and C-14 decreased the activity. The activity of **4** was particularly significant (see Fig. 2). This compound exerted vasorelaxant activity at particularly low concentration and the vasorelaxant profile reached maximum at relatively low concentration, especially when compared with the positive control acetylcholine. It is interesting to note that this compound was not toxic against the Vero cell, one of the representatives of normal cell line (data not shown).

4. Conclusion

Chemical modification of isosteviol (**1**) at 16-keto group to its dihydro analog **2** and the corresponding acetate derivative **3** resulted in marked increase in vasorelaxant activity of the latter analog. Its EC_{50} value was 4.88 nM, or 12-fold more active than the parent compound **1**. The substrates **1**–**3** were subjected to microbial transformation by *C. echinulata* NRRL 1386 to the metabolites **4**–**9**. The analog **4** exhibited the most active vasorelaxant effect, with the EC_{50} value of 3.46 nM, or approximately 17-fold more active than compound **1**. The highly active compound **4** deserves special attention for antihypertensive drug development.

5. Experimental

5.1. General

Melting points were determined with an Electrothermal melting point apparatus and are uncorrected. Optical rotations were measured on a JASCO-1020 polarimeter. IR spectra were obtained using a Bruker Tensor 27 FT-IR spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE 400 FT-NMR spectrometer, operating at 400 (^{1}H) and 100 (^{13}C) MHz. For the spectra taken in $CDCl_3$ and C_5D_5N, the residual nondeuterated solvent signals at δ 7.24 and 8.71, and the solvent signals at δ 77.0 and 149.90 were used as references for ^{1}H and ^{13}C NMR spectra, respectively. ESMS and ES-TOFMS spectra were measured with a Finnigan LC-Q and a Bruker micrOTOF mass spectrometer. Column chromatography was carried out using Merck silica gel 60 (<0.063 mm) and Pharmacia Sephadex LH-20. For TLC, Merck precoated silica gel 60 F_{254} plates were used. Spots on TLC were detected under UV light and by spraying with anisaldehyde–H_2SO_4 reagent followed by heating.

5.2. Microorganism, media and culture conditions

The stock culture of *C. echinulata* NRRL 1386 was maintained on a potato dextrose agar slant. Erlenmeyer flasks (250 ml), each containing 100 ml of liquid medium consisting of 0.1% peptone, 0.1% yeast extract, 0.1% beef extract and 0.5% glucose were inoculated with freshly obtained *C. echinulata* cultured from the agar slant on a rotary shaker at 200 rpm for 72 h [25].

5.3. Chemical modification of isosteviol (*1*)

5.3.1. Acid hydrolysis of stevioside to isosteviol (*1*)

Stevioside (1.0 g) was hydrolyzed with 20% H_2SO_4 (10 ml) at 60–70 °C for 2 h. The mixture was extracted with CH_2Cl_2 and the crude extract was subjected to column chromatography eluting with CH_2Cl_2–MeOH (98:2) to yield **1** [26] (320.4 mg, 81%). Spectroscopic (^{1}H NMR and ESMS) data were in agreement with the structure and the ^{1}H NMR data were consistent with the reported values [26].

5.3.2. Synthesis of ent-16β-hydroxybeyeran-19-oic acid (*2*)

To a solution of compound **1** (150 mg) in THF (2 ml) was added $NaBH_4$ (50 mg). After stirring at ambient temperature for 1 h, water was added and the solution was extracted with EtOAc. The EtOAc layer was washed with water, dried over anhydrous Na_2SO_4 and the solvent was evaporated in vacuo. The crude product was purified by column chromatography using CH_2Cl_2–MeOH (95:5) to give **2** (137 mg). Spectroscopic (^{1}H NMR and ESMS) data were in agreement with the structure and the NMR data were consistent with the reported values [18–20].

5.3.3. Acetylation of ent-16β-hydroxybeyeran-19-oic acid (*2*)

Acetic anhydride (0.5 ml) was slowly added to a solution of **2** (150 mg) in pyridine (1 ml). The progress of the reaction was followed by TLC while the mixture was kept stirring for 2 h. The reaction was worked up with water and extracted with EtOAc. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4 and the solvent evaporated in vacuo. The residue was subjected to column chromatography eluting with CH_2Cl_2–MeOH (98:2) to yield *ent*-16β-acetoxybeyeran-19-oic acid (**3**) (130 mg).

*5.3.3.1. ent-16β-Acetoxybeyeran-19-oic acid (**3**).* Colorless solid (from methanol), m.p. 196–198 °C; α_D^{31} − 56.7 (c = 0.50 in MeOH); IR (KBr) ν_{max} cm^{-1}: 3267, 2949, 2846, 1728, 1705, 1455, 1372, 1278, 1214, 1150, 1033, 976, 819; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.78 (3H, s, H-20), 0.81 (1H, partially superimposed signal, H-1), 0.87 (3H, s, H-17), 0.94 (1H, partially superimposed signal, H-3ax), 0.97 (1H, obscured signal, H-9), 1.01 (1H, br d, J = 13.8 Hz, H-5), 1.04 (1H, d, J = 12.1 Hz, H-12), 1.19 (3H, s, H-18), 1.25 (1H, partially superimposed signal, H-12), 1.30 (1H, br d, J = ca 12 Hz, H-7), 1.32 (1H, d, J = 12.1 Hz, H-14), 1.37 (1H, br d, J = 13.1 Hz, H-2), 1.50 (1H, obscured signal, H-7), 1.53 (2 × 1H, m, 2 × H-11), 1.64 (1H, br d, J = 13.8 Hz, H-6), 1.68 (1H, obscured signal, H-1), 1.74 (1H, obscured signal, H-12), 1.80 (1H, obscured signal, H-6), 1.82 (2 × 1H, m, 2 × H-15), 1.86 (1H, obscured signal, H-2), 2.02 (3H, s, OAc), 2.11 (1H, br d, J = 13.1 Hz, H-3eq), 4.70 (1H, dd, J = 9.9, 4.6 Hz, H-16); ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.3 (C-20), 18.8 (C-2), 20.2 (C-11), 21.1 (CH_3-acetate), 21.5 (C-6), 24.8 (C-17), 29.0 (C-18), 34.5 (C-12), 37.7 (C-3), 38.1 (C-10), 39.8 (C-1), 40.6 (C-15), 41.4 (C-7, C-13), 42.2 (C-8), 43.6 (C-4), 54.7 (C-14), 55.7 (C-9), 57.0 (C-5), 81.7 (C-16), 171.4 (CO-acetate), 184.1 (C-19); ESMS (−ve): m/z (% rel. abund.) 723 [2M−H]$^-$ (100); HR-TOFMS (ESI$^-$): m/z 361.2383 [M−H]$^-$; calcd for $C_{22}H_{34}O_4$–H, 361.2373.

5.4. Biotransformation by C. echinulata NRRL 1386

5.4.1. Incubation of isosteviol (*1*)

The stock culture of *C. echinulata* NRRL 1386 was maintained on a potato dextrose agar slant. Twenty Erlenmeyer flask (250 ml), each containing 100 ml of liquid medium consisting of 0.1% peptone, 0.1% yeast extract, 0.1% beef extract, and 0.5% glucose, were inoculated with freshly obtained *C. echinulata* cultured from the

agar slant on a rotary shaker at 200 rpm. After cultivation at ambient temperature for 72 h, the substrate solution (100 mg of substrate dissolved in 2.0 ml DMSO and 0.2 ml Tween 80) was prepared [25]. Equal volume of the substrate solution was added to each flask and the incubation continued for 3 days. Culture control consisted of fermentation blank in which *C. echinulata* was grown under identical condition but without substrate. After 3 days the culture was filtered and the broth was extracted with EtOAc, washed with water and the solvent was evaporated in vacuo. The crude extract (210.8 mg) was subjected to column chromatography eluting with CH_2Cl_2 and CH_2Cl_2–MeOH with increasing amount of the more polar solvent, to give **4** (45.7 mg, 54% based on the unrecovered starting material) and the starting material **1** (20 mg). Compound **4** was the biotransformation product of isosteviol (**1**) by several fungi and the NMR spectroscopic data of our compound were consistent with the reported values [9,22–24].

*5.4.1.1. ent-7α-Hydroxy-16-ketobeyeran-19-oic acid (**4**).* Colorless solid (from methanol), m.p. 230–232 °C; α_D^{29} − 90.6 (c = 0.06 in MeOH); IR (KBr) ν_{max} cm^{-1}: 3451, 3128, 2960, 2918, 2851, 1713, 1453, 1371, 1337, 1260, 1241, 1182, 1149, 1118, 1050, 952, 887, 856, 783; ESMS (−ve): m/z (% rel. abund.) 667 $[2M-H]^-$ (100).

*5.4.2. Incubation of ent-16β-hydroxybeyeran-19-oic acid (**2**)*

Compound **2** (100 mg) was subjected to microbial transformation in similar manner as that of compound **1**. The crude extract (195.7 mg) was purified by column chromatography on silica gel with a stepwise elution with CH_2Cl_2 and CH_2Cl_2–MeOH (2:0.1). The fractions containing the products were further purified by Sephadex LH-20 and crystallized with MeOH to afford the metabolites **5**, **6** and **7** (31.3 mg, 19.5 mg, 17.7 mg, respectively). The yields of **5**, **6** and **7** were 30, 19 and 17%, respectively. The metabolite **5** was identified as *ent*-7α,16β-dihydroxybeyeran-19-oic acid by comparison of the spectroscopic data with those of the reported values [18,19]. Compounds **6** and **7** were new metabolites.

*5.4.2.1. ent-7α,16β-Dihydroxybeyeran-19-oic acid (**5**).* White solid; (from methanol); m.p. 228–230 °C; α_D^{31} − 23.5 (c = 0.10 in MeOH); IR (KBr): ν_{max} cm^{-1} 3476, 3412, 2978, 2952, 2889, 2845, 1701, 1456, 1386, 1214, 1155, 1061, 1020; ESMS (−ve): m/z (% rel. abund.) 671 $[2M-H]^-$ (100).

*5.4.2.2. ent-7β,16β-Dihydroxybeyeran-19-oic acid (**6**).* Colorless solid (from methanol); m.p. 226–228 °C; α_D^{29} − 51.0 (c = 0.39 in MeOH); IR (KBr): ν_{max} cm^{-1} 3624, 3414, 3250, 2937, 2869, 2846, 1701, 1650, 1455, 1260, 1193, 1069; ^{1}H NMR (400 MHz, C_5D_5N): δ 0.95 (1H, ddd, J = 13.0, 12.8, 3.3 Hz, H-1ax), 1.08 (1H, d, J = 11.7 Hz, H-14a), 1.10 (1H, obscured signal, H-3), 1.13 (3H, s, H-17), 1.22 (3H, s, H-20), 1.29 (1H, obscured signal, H-9), 1.32 (1H, obscured signal, H-5), 1.35 (3H, s, H-18), 1.49 (1H, partially superimposed signal, H-12), 1.50 (1H, br d, J = ca 14 Hz, H-2), 1.72 (1H, obscured signal, H-11), 1.78 (1H, br d, J = 13.0 Hz, H-1eq), 1.93 (1H, br d, J = 12.0 Hz, H-15a), 2.23 (1H, obscured signal, H-11), 2.26 (1H, obscured signal, H-2), 2.27 (1H, obscured signal, H-12), 2.40 (1H, obscured signal, H-6ax), 2.46 (1H, br d, J = 11.7 Hz, H-14b), 2.47 (1H, br d, J = 13.0 Hz, H-3), 2.57 (1H, br d, J = 11.5 Hz, H-6eq), 3.00 (1H, dd, J = 12.0, 10.6 Hz, H-15b), 3.70 (1H, dd, J = 11.5, 3.6 Hz, H-7), 4.25 (1H, dd, J = 10.6, 4.2 Hz, H-16); ^{13}C NMR (100 MHz, C_5D_5N): δ 14.0 (C-20), 19.7 (C-2), 20.8 (C-11), 25.9 (C-17), 29.5 (C-18), 32.6 (C-6), 34.3 (C-15), 34.6 (C-12), 38.6 (C-10), 38.9 (C-3), 40.3 (C-1), 42.2 (C-13), 43.8 (C-4), 49.0 (C-8), 51.0 (C-14), 54.6 (C-5), 55.9 (C-9), 75.7 (C-7), 80.0 (C-16), 180.3 (C-19); ESMS (−ve): m/z (% rel. abund.) 671 $[2M-H]^-$ (100); HR-TOFMS (ESI^-): m/z 335.2208 $[M-H]^-$; calcd for $C_{20}H_{32}O_4$–H, 335.2217.

*5.4.2.3. ent-14β,16β-Dihydroxybeyeran-19-oic acid (**7**).* Colorless solid (from methanol); m.p. 215–218 °C; α_D^{29} − 29.9 (c = 0.32 in MeOH); IR (KBr): ν_{max} cm^{-1} 3452, 3411, 2927, 1696, 1462, 1260, 1155, 1055, 990; ^{1}H NMR (400 MHz, C_5D_5N) δ 0.97 (1H, ddd, J = 12.4, 12.2, 4.2 Hz, H-1ax), 1.09 (1H, obscured signal, H-3), 1.13 (1H, obscured signal, H-5), 1.24 (3H, s, H-20), 1.27 (1H, dd, J = 10.4, 6.3 Hz, H-9), 1.34 (3H, s, H-18), 1.39 (1H, m, H-12ax), 1.44 (3H, s, H-17), 1.47 (1H, m, H-2), 1.64 (2 × 1H, m, 2 × H-11), 1.68 (1H, obscured signal, H-7ax), 1.81 (1H, br d, J = 12.4 Hz, H-1eq), 2.18 (2 × 1H, m, 2 × H-6); 2.24 (1H, partially superimposed signal, H-2); 2.30 (1H, dd, J = 12.6, 5.3 Hz, H-12eq), 2.36 (2H, d, J = 7.1 Hz, 2 × H-15), 2.44 (1H, m, H-3), 2.46 (1H, m, H-7eq), 3.49 (1H, s, H-14), 4.86 (1H, t, J = 7.1 Hz, H-16); ^{13}C NMR (100 MHz, C_5D_5N): δ 14.2 (C-20), 19.9 (C-2), 20.7 (C-11), 21.0 (C-17), 22.3 (C-6), 29.6 (C-18), 34.2 (C-12), 37.5 (C-7), 38.8 (C-3, C-10), 40.8 (C-1), 42.3 (C-15), 44.0 (C-4), 47.4 (C-13), 47.6 (C-8), 56.1 (C-9), 57.1 (C-5), 78.8 (C-16), 91.8 (C-14), 180.4 (C-19); ESMS (−ve): m/z (% rel. abund.) 335 $[M-H]^-$ (100); HR-TOFMS (ESI^+): m/z 359.2180 $[M+Na]^+$; calcd for $C_{20}H_{32}O_4$+Na, 359.2192.

*5.4.3. Incubation of ent-16β-acetoxybeyeran-19-oic acid (**3**)*

Microbial transformation of compound **3** (100 mg) was carried out in similar manner to that of compound **1**. The crude extract (240.7 mg) was subjected to column chromatography eluting with CH_2Cl_2 and CH_2Cl_2–MeOH with increasing amount of the more polar solvent from CH_2Cl_2 to CH_2Cl_2–MeOH (5:0.1) to give the metabolites **8** and **9** (35.8 and 18.3 mg, respectively). The yields of **8** and **9** were 34 and 18%, respectively.

*5.4.3.1. ent-16β-Acetoxy-7α-hydroxybeyeran-19-oic acid (**8**).* Colorless solid (from methanol); m.p. 220–221 °C; α_D^{29} − 38.9 (c = 0.32 in MeOH); IR (KBr): ν_{max} cm^{-1} 3505, 2992, 2944, 1718, 1444, 1372, 1253, 1059; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.79 (3H, s, H-20), 0.92 (3H, s, H-17), 0.97 (1H, partially superimposed signal, H-1), 1.05 (1H, ddd, J = 13.0, 12.0, 4.3 Hz, H-3ax), 1.20 (3H, s, H-18), 1.23 (1H, partially superimposed signal, H-12), 1.34 (2 × 1H, s, 2 × H-14), 1.41 (2 × 1H, obscured signal, H-2 and H-9), 1.58 (2 × 1H, m, 2 × H-11), 1.66 (1H, obscured signal, H-5), 1.70 (1H, obscured signal, H-1), 1.76 (2 × 1H, obscured signal, 2 × H-15), 1.77 (1H, obscured signal, H-12), 1.85 (1H, obscured signal, H-2), 1.91 (2 × 1H, m, 2 × H-6), 2.04 (3H, s, OAc), 2.14 (1H, br d, J = 13.0 Hz, H-3eq), 3.56 (1H, br s, $W_{½}$ = 6.5 Hz, H-7), 4.70 (1H, dd, J = 10.2, 4.4 Hz, H-16); ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.1 (C-20), 18.8 (C-2), 19.8 (C-11) 21.1 (CH_3-acetate), 24.9 (C-17), 28.8 (C-6, C-18), 34.3 (C-12), 37.6 (C-3), 38.0 (C-10), 39.3 (C-15), 39.5 (C-1), 41.5 (C-13), 43.1 (C-4), 46.6 (C-8), 47.2 (C-5), 49.7 (C-9), 50.2 (C-14), 76.5 (C-7), 81.1 (C-16), 171.4 (CO-acetate), 183.1 (C-19); ESMS (−ve): m/z (% rel. abund.) 755 $[2M-H]^-$ (100); HR-TOFMS (ESI^-): m/z 377.2298 $[M-H]^-$; calcd for $C_{22}H_{34}O_5$–H, 377.2322.

*5.4.3.2. ent-16β-Acetoxy-14β-hydroxybeyeran-19-oic acid (**9**).* Colorless solid (from methanol); m.p. 218–220 °C; α_D^{29} − 43.5 (c = 0.32 in MeOH); IR (KBr): ν_{max} cm^{-1} 3509, 3205, 2949, 2880, 1718, 1456, 1373, 1253, 1000; ^{1}H NMR ($CDCl_3$) δ 0.77 (3H, s, H-20), 0.82 (1H, ddd, J = 13.3, 13.0, 3.9 Hz, H-1), 0.94 (3H, s, H-17), 0.95 (1H, partially superimposed signal, H-3), 1.03 (1H, dd, J = 10.4, 6.3 Hz, H-9), 1.15 (3H, s, H-18), 1.20 (1H, partially superimposed signal, H-5), 1.25 (1H, obscured signal, H-12), 1.28 (1H, obscured signal, H-7), 1.35 (1H, br d, J = 13.3 Hz, H-2), 1.47 (2 × 1H, m, 2 × H-11), 1.58 (1H, br d, J = 13.0 Hz, H-6), 1.72 (2 × 1H, obscured signal, H-1 and H-12), 1.79 (1H, obscured signal, H-15a), 1.82 (2 × 1H, partially superimposed signal, H-2 and H-6), 1.86 (1H, obscured signal, H-7), 2.01 (3H, s, OAc), 2.03 (1H, partially superimposed signal, H-15b), 2.08 (1H, br d, J = 13.3 Hz, H-3), 3.04 (1H, s, H-14), 4.98 (1H, dd, J = 10.3, 3.9 Hz, H-16); ^{13}C NMR (C_5D_5N, 100 MHz): δ 13.3 (C-20), 18.8 (C-2), 19.4 (C-17), 19.6 (C-11), 20.8 (C-6), 21.0 (CH_3-

acetate), 28.9 (C-18), 33.3 (C-12), 35.5 (C-7), 37.7 (C-3), 38.1 (C-10), 38.3 (C-15), 40.0 (C-1), 43.3 (C-4), 46.0 (C-13), 46.4 (C-8), 54.7 (C-9), 56.3 (C-5), 81.1 (C-16), 90.8 (C-14), 171.4 (CO-acetate), 183.0 (C-19); ESMS (−ve): *m/z* (% rel. abund.) 755 $[2M-H]^-$ (100); HR-TOFMS (ESI^+): *m/z* 401.2298 $[M+Na]^+$; calcd for $C_{22}H_{34}O_5$+Na, 401.2304.

5.5. Vasorelaxant assay

5.5.1. Animals

Male Sprague-Dawley rats (age 8 weeks) were obtained from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. The animals were housed under a 12:12-h light–dark cycle and maintained at 24 ± 1 °C. Animal feed and water was supplied *ad libitum*.

5.5.2. Smooth muscle tone determination

Aortic rings 4 mm in length were cut and immediately placed in 100% oxygen-saturated HEPES-buffer physiological salt solution (HPSS: 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 11 mM glucose, pH 7.4). The ring was equilibrated for 2 h with several changes of HPSS. The changes in isometric force were recorded on a computer system using the Chart version 7 PowerLab ADInstruments (2009) program. The resting tension was pre-contracted with phenylephrine (PE). The % relaxation of the aortic rings against log concentration of the compounds at 10^{-9} to 10^{-4} M is presented in Fig. 2. The EC_{50} of compounds **1**–**9** is presented in Table 1.

5.5.3. Statistical analysis

Statistical analysis was performed with one way analysis of variance (ANOVA). The differences were considered statistically significant when compared to normal control at $P < 0.05$.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.01.022.

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