Microbial Transformations of Isosteviol

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Microbial transformations of the tetracyclic diterpenoid isosteviol (*ent*-16-ketobeyeran-19-oic acid) (**2**) have revealed that isosteviol is metabolized by *Cunninghamella bainieri*, *Actinoplanes* sp., *Mucor recurvatus*, and *Cunninghamella blakesleeana* to yield five new metabolites, *ent*-11α,12α-dihydroxy-16-ketobeyeran-19-oic acid (**5**), *ent*-11α,12α,17-trihydroxy-16-ketobeyeran-19-oic acid (**6**), *ent*-12α,15α-dihydroxy-16-ketobeyeran-19-oic acid (**7**), *ent*-7α,15α-dihydroxy-16- ketobeyeran-19-oic acid (**8**), and *ent*-9α-hydroxy-16-ketobeyeran-19-oic acid (**9**), together with three known metabolites, *ent*-7α-hydroxy-16-ketobeyeran-19-oic acid (**3**), *ent*-7*β*-hydroxy-16-ketobeyeran-19-oic acid (**4**), and *ent*-12α-hydroxy-16-ketobeyeran-19-oic acid (**10**). The structures of these metabolites were established on the basis of HRFABMS and 1D and 2D NMR spectral data. In addition, metabolites **3**−**10** were tested for antihypertensive activity and were found to be less active than the parent compound **2**.

Isosteviol (**2**) is a beyerane diterpene obtained by acid hydrolysis of stevioside (**1**).[1] The biological activities of **2** include the inhibition of rat liver mitochondria functions,[2] decrease of glucose production and inhibition of oxygen uptake in isolated rat renal tubules,[3] and inhibition of D-glucose and D-fructose transport across the cell membrane.[4] In addition, isosteviol lowered blood pressure in spontaneously hypertensive rats through opening of the K^+ channel to inhibit calcium influx.[5]

Utilizing microorganisms for studying the metabolism of natural and synthetic drugs has been documented.[6] Smith and Rosazza postulated the concept of using microorganisms as models for mammalian metabolism of xenobiotics in the early 1970s.[7,8] Biohydroxylation represents a powerful method for the regioselective and stereoselective introduction of hydroxyl groups into organic compounds.[9] In recent years, microbial transformations have been used to introduce hydroxyl groups at difficult positions on diterpenoid compounds.[10,11] Thus, from a chemical view, the diterpene skeleton of isosteviol (**2**) is a suitable substrate for the study of microbiological hydroxylation. As far as we know, only three papers on the microbial transformation of isosteviol have been published,[12−14] in which the hydroxyl group was reported to be introduced at the 7α-, 7*β*-, 12*β*-, 17-, and 1α,7*β*-positions of isosteviol. To evaluate the antihypertensive activity of hydroxylated isosteviol, five new metabolites, **5**−**9**, and three known metabolites, **3**, **4**, and **10** (Figure 1), were prepared by microbial transformation technology. This paper describes the isolation and characterization of these metabolites.

Results and Discussion

Isosteviol (**2**) was obtained by hydrolysis of stevioside (**1**) with dilute hydrochloric acid. Proton and carbon assignments of isosteviol (**2**)[12,13] were confirmed by HMBC and HMQC NMR spectral analyses. Incubation of isosteviol (**2**) with *Cunninghamella bainieri* (UI 3065) yielded two major metabolites, which were determined to be *ent*-7α-hydroxy-16-ketobeyeran-19-oic acid (**3**) and *ent*-7*β*-hydroxy-16-ketobeyeran-19-oic acid (**4**) by comparison of the NMR and MS data with the literature.[12−14] The fermentation of isosteviol (**2**) with *Actinoplanes* sp. for 6 days produced two hydroxylated metabolites (**5** and **6**), which were separated by repeated column chromatography. The HRFAB mass spectrum of metabolite **5** gave a molecular ion peak $[M + H]^+$ at *m*/*z* 351.2167, corresponding to the molecular formula $C_{20}H_{31}O_5$. The structure of **5** was determined from its $^1H-^1H$ COSY, NOESY, HMQC, and HMBC NMR spectra. The ^{13}C NMR spectrum of **5** displayed resonances for 20 carbons, while the DEPT spectrum showed the presence of three methyl, seven methylene, four methine, and six quaternary carbons. In the HMQC spectrum, the observation of two signals at δ_H 3.86 (δ_C 76.1) and δ_H 4.01 (δ_C 71.0) indicated that metabolite **5** contains two more oxygen atoms than does isosteviol (**2**). Examination of the $^1H-^1H$ COSY spectrum correlations between two new hydroxyl-bearing methines at δ_H 3.86 (d, $J = 3.5$ Hz) and 4.01 (dd, $J = 10.5$, 3.5 Hz) suggested that they are on vicinal carbons. Comparison of ^1H and ^{13}C NMR spectra of **2** and **5** revealed that the substitutions of hydroxyl groups in metabolite **5** are at C-11 and C-12. This coincides with the ^{13}C NMR signals observed for downfield shifts of C-11 ($\Delta\delta$ +49.9), C-12 ($\Delta\delta$ +38.2), and C-13 ($\Delta\delta$ +6.8) and upfield shifts of C-14 ($\Delta\delta$ −7.3), C-16 ($\Delta\delta$ −2.0), and C-17 ($\Delta\delta$ −2.9), in comparison with those of **2** (Table 1). The location of the two hydroxyl groups was confirmed by detailed analysis of HMBC data. The correlations were between C-9 (δ_C 55.4), and H-11 (δ_H 4.01) and H-12 (δ_H 3.86); C-10 (δ_C 40.2) and H-11 (δ_H 4.01); C-12 (δ_C 76.1) and H-11 (δ_H 4.01); C-11 (δ_C 71.0) and H-12 (δ_H 3.86); C-14 (δ_C 47.5) and H-12 (δ_H 3.86); and C-17 (δ_C 17.9) and H-12 (δ_H 3.86). The relative stereochemistry of the hydroxyl group at C-11, in the equatorial position, was suggested from the cross-peaks between H-11_{ax} (δ_H 4.01) and CH_3-20 (δ_H 1.38) and H-15_{eq} (δ_H 2.91) in the NOESY experiment. A 10.5 Hz coupling constant between C-9 and C-11 protons also established the hydroxyl group at C-11 in the *β*-configuration. Evidence that the other hydroxyl group is in the 12*β*-orientation was disclosed by a NOESY cross-peak

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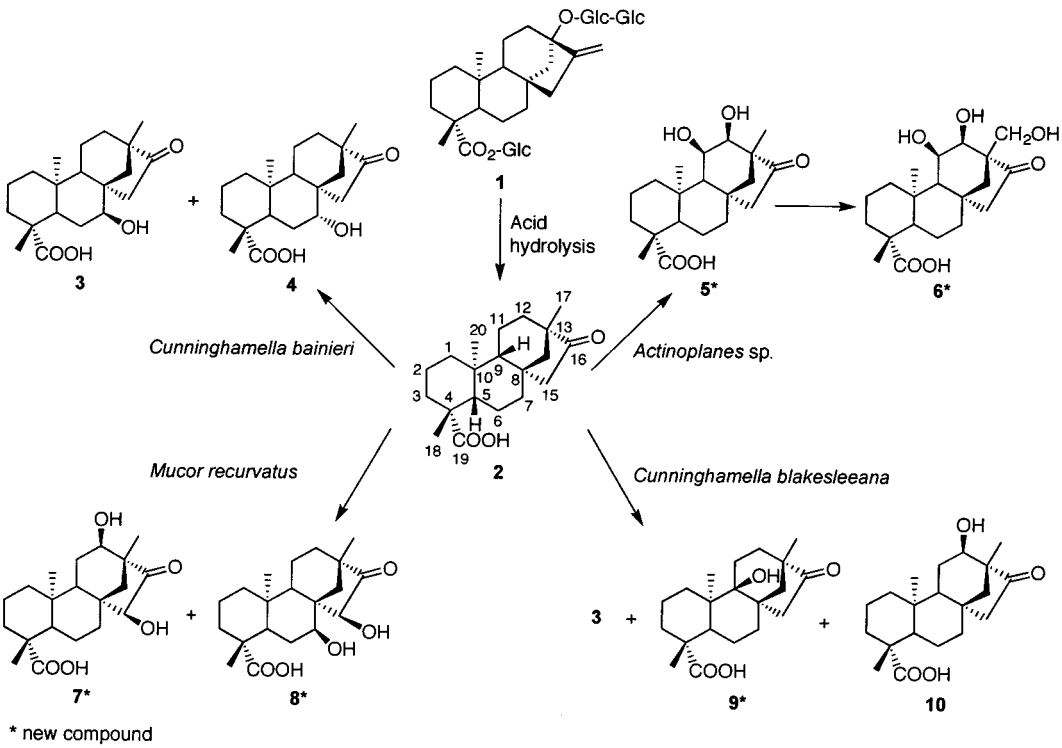

Figure 1. Structures of stevioside (**1**), isosteviol (**2**), and metabolites (**3**−**10**).

Table 1. ^{13}C NMR Assignments for Isosteviol (**2**) and Metabolites **5**−**9** (C_5D_5N, δ values in ppm)

carbon	2	5	6	7	8	9
1	40.6	42.9	42.9	40.3	40.6	32.9
2	20.1	20.4	20.4	20.0	20.2	20.3
3	39.1	38.9	38.9	38.8	39.1	39.0
4	44.4	44.6	44.6	44.2	43.9	44.6
5	57.5	57.9	57.9	57.8	47.9	48.9
6	22.9	22.7	22.8	22.7	31.1	23.2
7	42.2	43.1	43.2	34.1	68.5	37.5
8	40.1	40.8	40.9	44.3	47.5	49.1
9	55.2	55.4	56.3	51.8	51.2	76.6
10	38.8	40.2	40.3	39.0	39.2	44.4
11	21.1	71.0	71.0	29.9	20.5	27.1
12	37.9	76.1	73.7	71.8	38.6	34.2
13	49.1	55.9	62.0	54.5	48.3	45.4
14	54.8	47.5	42.3	44.7	48.5	48.6
15	49.1	49.5	50.5	74.4	75.4	51.0
16	221.5	219.5	218.8	221.1	223.1	221.0
17	20.8	17.9	62.5	18.8	21.2	20.8
18	29.9	30.2	30.2	29.9	29.7	30.2
19	180.5	180.6	180.6	180.5	180.9	180.8
20	14.1	14.2	14.2	14.1	14.1	17.4

between H-12_{eq} and CH_3-17 and by the absence of cross-peaks with H-9_{ax} and H-14_{ax}. Thus, **5** was established to be *ent*-11α,12α-dihydroxy-16-ketobeyeran-19-oic acid.

Metabolite **6** was isolated as an amorphous solid. It contains three additional oxygen atoms, as deduced from its molecular ion peak at *m*/*z* 367.2110 $[M + H]^+$ (calcd for $C_{20}H_{31}O_6$, 367.2120) and ^{13}C NMR. The most striking difference between **6** and **5** is the lack of the CH_3-17 signal in the ^1H and ^{13}C NMR spectra of **6**, which is replaced by a CH_2OH group (δ_H 4.32 and 4.43, d, J = 10.5 Hz, each 1 H/δ_C 62.5). Due to hydroxylation of the CH_3-17 group, H-12α and H-14 of **6** exhibit downfield shifts from δ 3.86 to 4.11 and from δ 1.28 to 2.11, respectively, in comparison with the corresponding protons in **5**. The hydroxyl group was confirmed to be at C-17 by the HMBC spectrum, displaying cross-peaks between CH_2OH (δ_H 4.32, 4.43) and C-12 (δ 73.7), C-13 (δ 62.0), C-14 (δ 42.3), and C-16 (δ 218.8). Thus, the ^{13}C NMR spectral data, compared to that of **2**, showed downfield shifts of C-11 ($\Delta\delta$ +49.9), C-12 ($\Delta\delta$ +35.8), C-17 ($\Delta\delta$ +41.7), and C-13 ($\Delta\delta$ +12.9) and upfield shifts of C-14 ($\Delta\delta$ −12.5) and C-16 ($\Delta\delta$ −2.7) (Table 1). Comparison of $^1H-^1H$ COSY, NOESY, DEPT, HMQC, and HMBC data with those of **5** indicates that metabolite **6** is *ent*-11α,12α,17-trihydroxy-16-ketobeyeran-19-oic acid and probably forms by hydroxylation of **2** to produce *ent*-11α,-12α-dihydroxy-16-ketobeyeran-19-oic acid (**5**) followed by hydroxylation at CH_3-17 to yield **6**.

Two other new oxidized metabolites of isosteviol **2** obtained from biotransformation by *Mucor recurvatus* are **7** and **8**. The molecular formula of **7** was deduced to be $C_{20}H_{29}O_5$ from negative HRFABMS (*m*/*z* 349.2024) and ^{13}C NMR. The ^1H NMR spectrum of **7** displays two downfield signals for oxygen-bearing methine protons at δ 4.03 and 4.62. The DEPT spectrum shows the disappearance of two CH_2 signals relative to **2** and the presence of two new CH signals at δ 71.8 and δ 74.4, confirming that **7** is a dihydroxylated metabolite of **2**. The proton signal of CH_3-17 is dramatically shifted from δ 1.05 in **2** to δ 1.45 in **7** in the ^1H NMR. In the HMBC spectrum, one of the new proton signals at δ 4.03 showed three-bond connectivities to carbons 9, 14, and 17, suggesting the location of one of the hydroxyl groups at C-12. Moreover, HMQC, HMBC, and COSY data revealed that the resonances of carbons 11 and 13 are shifted significantly downfield to δ 29.9 ($\Delta\delta$ +8.8) and 54.5 ($\Delta\delta$ +5.4), respectively; the carbons at 9, 14, and 17 exhibit upfield shifts from δ 55.2 to 51.8 ($\Delta\delta$ −3.4), 54.8 to 44.7 ($\Delta\delta$ −10.1), and 20.8 to 18.8 ($\Delta\delta$ −2.0), respectively, in comparison with those of **2**. This evidence confirms that the hydroxyl group is at C-12. The configuration of the hydroxyl group at C-12 was established to be β on the basis of NOESY data. The NOESY experiment displayed NOE correlations between H-12_{eq} and H-11 and CH_3-17 and displayed no NOE correlations between H-12_{eq} and H-9_{ax} and H-14_{ax}. Accordingly, the β-orientation of 12-OH was established. Examination of the HMBC spectrum allows us to assign the other newly introduced hydroxyl group at

C-15. In the HMBC spectrum, cross-peaks are observed between δ_H 4.62 (H-15) and δ_C 44.7 (C-14), 51.8 (C-9), 54.5 (C-13), and 221.1 (C-16). In addition, the NOESY spectrum defines the β-orientation of the hydroxyl group at C-15 as correlations between H-15 (δ 4.62) and the α-orientation of H-11 (δ 1.54) and CH_3-20 (δ 1.12). On the basis of all these observations, metabolite **7** is characterized as *ent*-12α,15α-dihydroxy-16-ketobeyeran-19-oic acid.

Metabolite **8** shows a $[M + H]^+$ ion at *m/z* 351.2172 in the HRFABMS, corresponding to the molecular formula $C_{20}H_{31}O_5$. Examination of DEPT and HMQC spectra of **8** indicate that a dihydroxylation has occurred. The HMQC spectrum, compared to that of **2**, showed new resonances at δ_H 5.07 and 4.53, and δ_C 68.5 and 75.4, respectively. In the DEPT spectrum, the resonance of C-6 has shifted downfield from δ 22.9 to 31.1, and the resonances of C-5 and C-9 have shifted upfield, from δ 57.5 to 47.9 and from δ 55.2 to 51.2, respectively. In the HMBC spectrum, δ_H 5.07 exhibits cross-peaks with δ_C 47.9 (C-5) and 51.2 (C-9). Therefore, hydroxylation occurs at C-7, to which the resonance at δ 68.5 is attributed. The stereochemistry at C-7 follows from the multiplicity of the H-7 signal in the ^{1}H NMR spectrum, which is a broad singlet, indicating that the proton is in the equatorial (α) position.[14] Furthermore, the NOESY spectrum also shows cross-peaks between δ 5.07 (H-7α) and H-6 (δ 2.63 and 2.79) and H-14 (δ 2.46). Irradiation of the signal at δ 5.07 led to positive NOE enhancements at H-6 (δ 2.63, 5.9% and δ 2.79, 6.3%) and H-14 (δ 2.46, 2.7%). Accordingly, the hydroxyl group is in the axial (β) position. The location of the second hydroxyl group at the C-15 position of **8** was deduced by HMBC correlations between δ_H 4.53 (H-15) and δ_C 48.5 (C-14), 51.2 (C-9), and 223.1 (C-16). The β-orientation of 15-OH was established by a NOESY experiment, in which H-15 (δ 4.53) exhibits a significant correlation with CH_3-20. Further, irradiation of the signal (δ 1.19) of α-orientated CH_3-20 produces a 9.9% NOE enhancement of a singlet signal at δ 4.53 (H-15), which must be α-orientated; accordingly the relatively stereochemistry of 15-OH is confirmed as a β-configuration. On the basis of the above evidence, the structure of **8** is determined to be *ent*-7α,15α-dihydroxy-16-ketobeyeran-19-oic acid.

Preparative-scale biotransformation of **2** by *Cunninghamella blakesleeana* (ATCC 8688a) produced metabolites **3**, **9**, and **10**. The structures of metabolites **3** and **10** were determined by 1D, 2D NMR and HRFABMS, and by comparison with previous data.[13,14] HRFABMS data of **9** show a molecular ion at *m/z* 335.2206 $[M + H]^+$, which is consistent with the molecular formula of $C_{20}H_{31}O_4$. The ^{13}C NMR spectrum displays one signal at δ 76.6. However, by comparison with **2**, the lack of the carbinol-methine signal and disappearance of one tertiary carbon in the ^{1}H NMR and DEPT spectra of **9** suggest that the new hydroxyl group was introduced at C-5 or C-9. In the ^{13}C NMR spectrum, C-11 is shifted from δ 21.1 in **2** to δ 27.1 in **9**. CH_3-20 is also shifted significantly downfield to δ_C 17.4 ($\Delta\delta$ +3.3) and δ_H1.29 ($\Delta\delta$ +0.31) in NMR spectra vs **2**, suggesting that a hydroxyl group might be at C-9. HMQC and HMBC spectral analyses show that the signal at δ 76.6 is three-bond correlated with protons 7, 12, 14, 15, and a CH_3-20 group, and two-bond correlated with proton 11, thus confirming the location of the hydroxyl group at C-9. On the basis of the spectral evidence, metabolite **9** is assigned the structure *ent*-9α-hydroxy-16-ketobeyeran-19-oic acid.

The species of *Cunninghamella bainieri* and *C. blakesleeana* have the ability to functionalize isosteviol (**2**) at the C-7 position. This is a common occurrence for other microbes with kaurane and beyerane skeletons.[10,13,14] Hydroxylation at the 9β-position is obtained for the first time on a beyerane skeleton. Previously, two hydroxyl groups on isosteviol have been reported only at the 1α- and 7β-positions.[14] In this study, several hydroxylated isosteviol analogues were prepared by hydroxylation at 11β,12β-, 11β,12β,17- (by *Actinoplanes* sp.), 12β,15β-, 7β,15β- (by *Mucor revurvatus*), 7α-, 7β- (by *Cunninghamella bainieri*), and 7β-, 9β-, and 12β- (by *Cunninghamella blakesleeana*). Furthermore, isolated metabolites were evaluated for antihypertensive effects using deoxycorticosterone acetate (DOCA)-salt-induced hypertensive rats. Due to the small amount of isolated metabolites, the preliminary experiments were at a dose of 4.8 mg/kg. Metabolite **9**, with the hydroxyl group at the 9β-position, produced a reduction in blood pressure (Δ −7.0 ± 5.3 mmHg), but it was less active than the parent compound **2** (Δ −12.0 ± 5.2 mmHg). These isolated metabolites will also be used as reference standards for monitoring our continuing studies on the mammalian metabolism of isosteviol.

Experimental Section

General Experimental Procedures. Melting points were determined using a Yanagimoto micromelting point apparatus and are uncorrected. Optical rotations were determined on JASCO DIP-140 and DIP-1020 digital polarimeters. ^{1}H and ^{13}C NMR were run on Bruker AM-300 and AM-500 spectrometers in C_5D_5N solution, using the corresponding solvent as internal standard. NMR experiments included ^{1}H−^{1}H COSY, DEPT, NOE, NOESY, HMQC, and HMBC. Coupling constants (*J* values) are given in hertz. Multiplicities of all carbon signals were verified through DEPT experiments. Low- and high-resolution FAB mass spectra were obtained using a JEOL JMX-HX 110 spectrometer.

Acid Hydrolysis of Stevioside. Stevioside **1** (1.0 g) was dissolved in H_2O and then treated with 2 N HCl (20 mL). The reaction mixture was refluxed for 2 h and filtered to give a solid. After recrystallization with Me_2CO, 320 mg of isosteviol (**2**) was obtained as prisms.

Microorganisms. All cultures were obtained from the Division of Medicinal and Natural Products Chemistry, College of Pharmacy, University of Iowa, Iowa City, IA. Thirty microorganisms were used for the preliminary screening as follows: *Absidia pseudocylindrospora* ATCC 24169, *Actinoplanes* sp. ATCC 53771, *Amycolata autotrophica* ATCC 35203, *Amycolatopsis mediterran* ATCC 21271, *Aspergillus aliaceus* UI 315, *Aspergillus niger* ATCC 10581, *Bacillus cereus* UI 1477, *Bacillus megaterium* ATCC 14581, *Beauveria sulfurescens* ATCC 7159, *Curvularia lunata* NRRL 2178, *Cunninghamella blakesleeana* ATCC 8688a, *Cunninghamella bainieri* ATCC 9244, *Cunninghamella elegans* ATCC 9245, *Cylindrocarpon radicicola* ATCC 11011, *Gliocladium deliquescens* NRRL 1086, *Mortierella isabellina* ATCC 38063, *Mucor mucedo* ATCC 20094, *Mucor recurvatus* MR 36, *Mucor mucedo* UC 4605, *Mycorbacterium fortuitum* AM 53378, *Nocardia aurantia* ATCC 12674, *Nocardia butanica* ATCC 21197, *Nocardia restricta* ATCC 14887, *Nocardia* sp. ATCC 5646, *Penicillum notatum* ATCC 36740, *Pseudomonas putida* ATCC 33015, *Pseudomonas testosteroni* ATCC 11996, *Streptomyces griseolus* ATCC 11796, *Streptomyces griseus* ATCC 10137, and *Streptomyces griseus* ATCC 13273.

Cultures and Fermentation Procedures. Stock cultures were maintained on Sabouraud-maltose agar slants or those recommended by the ATCC and were stored at 4 °C. All preliminary screening experiments were performed by a two-stage fermentation procedure in a medium consisting of dextrose (20 g), yeast (5 g), NaCl (5 g), K_2HPO_4 (5 g), soybean flour (5 g), and 1 L of distilled water. The medium was adjusted to pH 7.0 before sterilization by autoclaving at 121 °C for 15 min. Incubations were initiated by suspending the surface growth from slants in 5 mL of sterile medium and using the suspensions to inoculate stage I cultures. Cultures were

incubated with shaking on a rotatory shaker (HOTECH 706R), operating at 250 rpm and 28 °C. After 72 h of incubation in the above-described medium, 5 mL of stage I culture was used as inoculum for stage II culture. Isosteviol **2** was added to each flask 24 h later as 100 μL dimethylformamide (DMF) aliquots each containing 10 mg of isosteviol in solution to give a final concentration of 0.4 mg/mL of culture. Culture controls consisted of fermentation blanks in which microorganisms were grown under identical conditions but without substrate. Substrate controls consisted of sterile medium containing the same amount of substrate and incubated under the same conditions. The fermentations were sampled for TLC analysis at 24, 72, and 144 h. Samples of 4 mL of culture broth were acidified with 6 N HCl and extracted with 1 mL of EtOAc−butanol (9:1). The organic layer was spotted onto a TLC plate with $CHCl_3$−MeOH (10:1) as the solvent system. Eight metabolites were reproducibly produced by *Cunninghamella bainieri*, *Actinoplanes* sp., *Mucor recurvatus*, and *Cunninghamella blakesleeana* and displayed TLC R_f values at 0.42 (**3**), 0.40 (**4**), 0.37 (**5**), 0.27 (**6**), 0.41 (**7**), 0.36 (**8**), 0.48 (**9**), and 0.43 (**10**) as compared to 0.75 (**2**).

Microbial Metabolism of Isosteviol (2) by *C. bainieri* (UI 3065). *Cunninghamella bainieri* (UI 3065) was grown in twenty-five 125 mL stainless steel-capped DeLong flasks, each containing 25 mL of medium. A total of 250 mg of isosteviol **2** (in 2.5 mL of dimethylformamide) was evenly distributed among 25 flasks containing stage II cultures. The cultures were incubated on a rotator shaker (250 rpm) at 28 °C for 6 days. The incubation mixture was pooled and acidified with 6 N HCl and then filtered to remove cells. The filtrate was extracted three times with equal volumes of EtOAc−butanol (9:1). The combined extracts were dried over anhydrous Na_2SO_4, and the solvent was evaporated to give 918 mg of a brown residue. The crude residue was purified by chromatography over a silica gel column using $CHCl_3$−CH_3OH (30:1) as the eluent. After recrystallization with acetone, 67 mg of **3** (25.5%) and 37 mg of **4** (14.1%) were obtained as white prisms and white needles, respectively.

Microbial Transformation of Isosteviol (2) by *Actinoplanes* sp. (ATCC 53771). *Actinoplanes* sp. bioconversion was conducted as with *C. bainieri* preparative-scale reactions and was terminated after 144 h following 250 mg of substrate addition. Extraction as described above produced 560 mg of brown oil. The crude residue was purified by chromatography over a silica gel column using $CHCl_3$−MeOH (10:1 and 9:1). After recrystallization with acetone, 26 mg of **5** (5.8%) and 8 mg of **6** (1.7%) were obtained.

***ent*-11α,12α-Dihydroxy-16-ketobeyeran-19-oic acid (5):** prisms (acetone); mp 221−223 °C; $[\alpha]^{25}_D$ +17.7° (*c* 0.5, MeOH); ^{1}H NMR (C_5D_5N, 500 MHz) δ 1.11 (1H, m, H-3), 1.24−1.28 (3H, m, H-1, H-5, H-14), 1.33 (3H, s, CH_3-20), 1.36 (3H, s, CH_3-17), 1.38 (3H, s, CH_3-18), 1.49−1.56 (2H, m, H-2, H-7), 1.66 (1H, m, H-7), 1.82 (1H, d, J = 10.5 Hz, H-9β), 1.84 (1H, d, J = 18.0 Hz, H-15), 1.85−1.88 (2H, m, H-6), 2.29 (1H, m, H-2), 2.45−2.49 (2H, m, H-3, H-14), 2.91 (1H, dd, J = 18.0, 3.5 Hz, H-15), 3.18 (1H, br d, J = 13.5 Hz, H-1), 3.86 (1H, d, J = 3.5 Hz, H-12α), 4.01 (1H, dd, J = 10.5, 3.5 Hz, H-11α); ^{13}C NMR (C_5D_5N, 125 MHz), see Table 1; FABMS *m/z* 351 $[M + H]^+$; HRFABMS *m/z* 351.2167 (calcd for $C_{20}H_{31}O_5$, 351.2171).

***ent*-11α,12α,17-Trihydroxy-16-ketobeyeran-19-oic acid (6):** amorphous powder (acetone); mp 134−136 °C; $[\alpha]^{25}_D$ +34.6° (*c* 0.5, MeOH); ^{1}H NMR (C_5D_5N, 500 MHz) δ 1.09 (1H, m, H-3), 1.26−1.32 (2H, m, H-1, H-5), 1.34 (3H, s, CH_3-20), 1.37 (3H, s, CH_3-18), 1.50 (1H, br d, J = 14.5 Hz, H-2), 1.61 (1H, m, H-7), 1.72 (1H, d, J = 13.0 Hz, H-7), 1.93 (1H, d, J = 18.5 Hz, H-15), 1.94 (1H, d, J = 10.5 Hz, H-9β), 2.09−2.11 (3H, m, H-6, H-14), 2.29 (1H, d, J = 13.5 Hz, H-2), 2.45 (1H, d, J = 12.0 Hz, H-3), 2.55 (1H, dd, J = 11.5, 3.5 Hz, H-14), 2.97 (1H, dd, J = 18.5, 3.5 Hz, H-15), 3.19 (1H, d, J = 14.0 Hz, H-1), 4.06 (1H, dd, J = 10.5, 3.5 Hz, H-11α), 4.11 (1H, d, J = 3.5 Hz, H-12α), 4.32, 4.43 (each 1H, d, J = 10.5 Hz, CH_2-OH); ^{13}C NMR (C_5D_5N, 125 MHz), see Table 1; FABMS *m/z* 367 $[M + H]^+$; HRFABMS *m/z* 367.2110 (calcd for $C_{20}H_{31}O_6$, 367.2120).

Microbial Metabolism of Isosteviol (2) by *Mucor recurvatus* (MR 36). The fermentation procedure was conducted as described for *C. bainieri* except that the incubation reaction was terminated 24 h after substrate addition. Biotransformation of **2** (500 mg) produced 1.1 g of brown oil, which was subjected to silica gel column chromatography using the eluent $CHCl_3$−CH_3OH (10:1 and 9:1) to give 9 mg of **7** (4.0%) and 5 mg of **8** (2.2%).

***ent*-12α,15α-Dihydroxy-16-ketobeyeran-19-oic acid (7)**: brown oil; $[\alpha]^{25}_D$ −42.3° (*c* 1.04, MeOH); ^{1}H NMR (C_5D_5N, 500 MHz) δ 0.98 (1H, td, J = 13.0, 3.5 Hz, H-1), 1.07 (1H, dd, J = 13.5, 4.0 Hz, H-3), 1.12 (3H, s, CH_3-20), 1.27−1.38 (2H, m, H-5, H-7), 1.40 (3H, s, CH_3-18), 1.45 (3H, s, CH_3-17), 1.54 (1H, td, J = 14.0, 3.5 Hz, H-11), 1.70 (1H, d, J = 13.0 Hz, H-1), 2.00−2.07 (3H, m, H-9, H-11, H-14), 2.20−2.23 (2H, m, H-2, H-6), 2.41−2.48 (3H, m, H-3, H-6, H-14), 2.85 (1H, br d, J = 13.5 Hz, H-7), 4.03 (1H, s, H-12α), 4.62 (1H, s, H-15α); ^{13}C NMR (C_5D_5N, 125 MHz), see Table 1; FABMS *m/z* 349 $[M - H]^-$; HRFABMS *m/z* 349.2024 (calcd for $C_{20}H_{29}O_5$, 349.2015).

***ent*-7α,15α-Dihydroxy-16-ketobeyeran-19-oic acid (8)**: oil; $[\alpha]^{25}_D$ −58.2° (*c* 0.67, MeOH); ^{1}H NMR (C_5D_5N, 500 MHz) δ 1.12 (1H, m, H-1), 1.17 (3H, s, CH_3-17), 1.19 (3H, s, CH_3-20), 1.20−1.37 (3H, m, H-3, H-11, H-12), 1.48 (3H, s, 18-CH_3), 1.49 (1H, m, H-2), 1.69−1.75 (3H, m, H-1, H-11, H-12), 2.08−2.15 (2H, m, H-9, H-14), 2.26 (1H, m, H-2), 2.46−2.56 (3H, m, H-3, H-5, H-14), 2.63 (1H, m, H-6), 2.79 (1H, m, H-6), 4.53 (1H, br s, H-15α), 5.07 (1H, br s, H-7α); ^{13}C NMR (C_5D_5N, 125 MHz), see Table 1; FABMS *m/z* 351 $[M + H]^+$; HRFABMS *m/z* 351.2172 (calcd for $C_{20}H_{31}O_5$, 351.2171).

Microbial Metabolism of Isosteviol (2) by *C. blakesleeana* (ATCC 8688a). Isosteviol (**2**, 250 mg), dissolved in 2.5 mL of DMF, was evenly distributed among 25 flasks containing stage II cultures. Fermentation was stopped after 6 days. The incubation mixtures were acidified with 6 N HCl and then filtered to remove cells. The filtrate was extracted three times with equal volumes of EtOAc−butanol (9:1). The combined extracts were dried over anhydrous Na_2SO_4, and the solvent was evaporated to give 573 mg of a brown residue. The crude residue was chromatographed on silica gel. Elution with $CHCl_3$−CH_3OH (40:1) yielded 12 mg of **3** (4.6%). Elution with $CHCl_3$−CH_3OH (30:1) produced 22 mg of **9** (8.4%) and 74 mg of **10** (28.2%).

***ent*-9α-Hydroxy-16-ketobeyeran-19-oic acid (9):** white needles (acetone); mp 246−248 °C; $[\alpha]^{25}_D$ −87.6° (*c* 0.5, CH_3OH); ^{1}H NMR (500 MHz, pyridine-d_5) δ 1.09 (3H, s, CH_3-17), 1.15 (1H, dd, J = 13.0, 4.0 Hz, H-3), 1.19 (1H, dd, J = 11.5, 2.5 Hz, H-14), 1.29 (3H, s, CH_3-20), 1.39 (3H, s, CH_3-18), 1.45−1.52 (3H, m, H-1, H-7, H-12), 1.58 (1H, m, H-2), 1.75 (2H, dd, J = 9.5, 3.5 Hz, H-11), 1.98 (1H, m, H-12), 2.02 (1H, d, J = 18.5 Hz, H-15), 2.15−2.34 (6H, m, H-1, H-2, H-5, H-6, H-7), 2.43 (1H, d, J = 13.0 Hz, H-3), 2.77 (1H, dd, J = 11.5, 4.0 Hz, H-14), 3.00 (1H, dd, J = 18.5, 4.0 Hz, H-15); ^{13}C NMR (C_5D_5N, 125 MHz), see Table 1; FABMS *m/z* 335 $[M + H]^+$; HRFABMS *m/z* 335.2206 (calcd for $C_{20}H_{31}O_4$, 335.2222).

Antihypertensive Testing. The antihypertensive activity was determined as described previously.[16−19]

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