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Microbial Transformation of Isosteviol and Bioactivities against the Glucocorticoid/Androgen Response Elements

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Preparative-scale fermentation of isosteviol (*ent*-16-oxobeyeran-19-oic acid) (**1**) with *Mucor recurvatus* MR 36, *Absidia pseudocylindrospora* ATCC 24169, and *Aspergillus niger* BCRC 32720 afforded nine known metabolites (**2**, **3**, **5**–**10**, and **14**) and nine new metabolites (**4**, **11**–**13**, and **15**–**19**). The reactions involved stereoselective introduction of OH groups at positions C-1, -6, -7, -9, -11, -12, -15, and -17 as well as further ketonization at the C-1 and C-7 positions. The structures of the metabolites were established on the basis of 1D and 2D NMR and IR supported by HRFABMS. GRE (glucocorticoid response element)- and ARE (androgen response element)-mediated luciferase reporter gene assays were used to screen for the biological activities of **1** and its metabolites. Compounds **7**, **13**, **16**, and **18** showed significantly enhanced GRE-mediated luciferase activity, but at levels less than that induced by either methylprednisolone or dexamethasone. On the other hand, compounds **3**, **4**, **12**, **13**, **14**, and **18** showed significant effects on ARE-mediated luciferase activity; in particular, **3**, **12**, **14**, and **18** were more active than testosterone.

The glucocorticoid (GC) receptor (GR) and androgen receptor (AR) are members of the nuclear receptor superfamily of ligand-regulated transcription factors.[1] These two steroid hormone receptors play pivotal roles in some of the most fundamental aspects of human physiology. GCs, a class of naturally occurring and synthetic steroid hormones, exert a variety of physiological functions via the GR.[2] Upon binding to cognate ligands, the activated GR, which is a transcription regulator, occupies specific genomic glucocorticoid response elements (GREs) and modulates the transcription of nearby genes. In addition, the activated GC−GR complex may also interact with other transcription regulators, such as activator protein-1 (AP-1) and nuclear factor-κB (NF-κB), and inhibit the gene expression of inflammatory mediators.[3] GCs such as dexamethasone and prednisolone have long been recognized as effective treatments for inflammatory conditions and immunomodulation.[4] However, current GC therapy still possesses unsolved problems such as the GC insensitivity/resistance or serious adverse reactions.[5] Androgens, also termed rejuvenating hormones or male hormones, play crucial roles in several stages of male development and musculoskeletal growth.[6] They act via an interaction with the androgen receptor (AR). After binding to the ligand, the activated AR may be translocated to the nucleus and bind to androgen response elements (AREs) on the target gene that affect development, growth, and regulation of male reproductive functions.[7] However, the broader use of steroidal androgens for additional treatments, such as osteoporosis, is limited by undesirable AR-mediated effects, such as prostatic hypertrophy and hirsutism.[8] Thus, the development of GR agonists that exhibit a reduced incidence or reduced severity of side effects while maintaining potent anti-inflammatory activity, and androgen agonists with full anabolic activity but reduced impacts of undesirable effects and with an important role on endocrine therapies to treat muscle wasting and osteoporosis, is currently a demanding goal.[9,10]

Microorganisms are natural biocatalysts that modify a wide array of substances. In particular, filamentous fungi contain numerous hydroxylating enzymes with broad specificities, which catalyze regio- and stereoselective hydroxylation of nonactivated carbons on a variety of natural and synthetic organic compounds.[11] The regio- and stereoselectivities of the hydroxylation reaction are controlled by cytochrome P-450 enzymes. Bridged ring polycyclic diterpenoids are one class of studied substrates in microbial transformation.[12,13] Isosteviol (**1**) (*ent*-16-oxobeyeran-19-oic acid), an *ent*-beyerane tetracyclic diterpene, is obtained by the acid hydrolysis of stevioside,[14] a constituent of *Stevia rebaudiana* (Bertoni) Bertoni (Compositae), which is a noncaloric sugar substitute used in Japan and Brazil.[15] Several biological activities of **1** have been reported including inhibition of D-glucose and D-fructose transport across the cell membrane in isolated perfused rat liver cells,[16] potent inhibition of tumor promoters,[17] reducing systemic blood pressure and relaxing smooth muscle in blood vessels,[18,19] cardioprotective effect on rats with heart ischemia-reperfusion injury,[20] and reducing plasma glucose levels in a diabetic animal model.[21] Previously, De Oliveira et al. indicated that introducing an OH group in nonhydroxylated diterpenoids might enhance existing properties or lead to new biological activities because the hydroxylation pattern of active compounds might influence their binding to receptors.[12] Ghisalberti also indicated that some highly oxygenated diterpenoids usually have higher levels of biological activity than their less hydroxylated precursors.[22] Thus, as a continuing study to obtain additional hydroxylated compounds for pharmaceutical investigations,[23] the biocatalytic modification of isosteviol (**1**) was carried out. In addition to previous studies on introducing OH groups at 7α-, 7β-, 9β-, 11β-, 12β-, 17-, 11β,12β-, 11β,12β,17-, 12β,15β-, 7β,15β-, and 1α,7β-positions of **1**,[12,23–26] a number of new oxygenated metabolites were prepared. Both GRE- and ARE-mediated luciferase reporter gene assays were used to screen **1** and the biotransformed products as glucocorticoid and/or androgen agonists. In this paper, the isolation, structural characterization, and biological activities of these compounds are presented.

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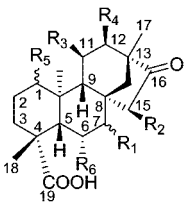
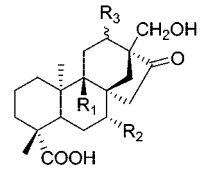


1: $R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = H$
2: $R_1 = \beta$-OH, $R_2 = R_3 = R_4 = R_5 = R_6 = H$
3: $R_1 = \alpha$-OH, $R_2 = R_3 = R_4 = R_5 = R_6 = H$
4: $R_1 = R_3 = R_4 = R_5 = R_6 = H$, $R_2 = OH$
5: $R_1 = R_3 = R_5 = R_6 = H$, $R_2 = R_4 = OH$
6: $R_1 = \beta$-OH, $R_2 = OH$, $R_3 = R_4 = R_5 = R_6 = H$
11: $R_1 = \alpha$-OH, $R_4 = OH$, $R_2 = R_3 = R_5 = R_6 = H$
13: $R_1 = R_2 = R_3 = R_4 = R_6 = H$, $R_5 = \alpha$-OH
14: $R_1 = \beta$-OH, $R_5 = \alpha$-OH, $R_2 = R_3 = R_4 = R_6 = H$
15: $R_1 = =O$, $R_3 = OH$, $R_2 = R_4 = R_5 = R_6 = H$
16: $R_1 = =O$, $R_5 = \alpha$-OH, $R_2 = R_3 = R_4 = R_6 = H$
17: $R_1 = \beta$-OH, $R_5 = =O$, $R_2 = R_3 = R_4 = R_6 = H$
18: $R_1 = \beta$-OH, $R_3 = OH$, $R_2 = R_4 = R_5 = R_6 = H$
19: $R_1 = \beta$-OH, $R_5 = \alpha$-OH, $R_6 = OH$, $R_2 = R_3 = R_4 = H$



7: $R_1 = OH$, $R_2 = R_3 = H$
8: $R_1 = R_2 = H$, $R_3 = \beta$-OH
9: $R_1 = R_2 = H$, $R_3 = \alpha$-OH
10: $R_1 = R_3 = H$, $R_2 = OH$
12: $R_1 = H$, $R_2 = OH$, $R_3 = \beta$-OH

Results and Discussion

Previous screening showed that many microorganisms are capable of reproducibly converting isosteviol (**1**) into many metabolites.[23] Three filamentous fungi, *Mucor recurvatus* MR 36, *Absidia pseudocylindrospora* ATCC 24169, and *Aspergillus niger* BCRC 32720, were selected for detailed studies of the biotransformation of **1**. Metabolites **2**–**6**, **7**–**12**, and **13**–**19** were respectively isolated from the fermentation broths of *M. recurvatus*, *Abs. pseudocylindrospora*, and *Asp. niger*. Among them, **4**, **11**–**13**, and **15**–**19** were identified as new metabolites. The nine known metabolites were **2**, **3**, **5**−**10**, and **14**, as determined by comparison of the TLC and NMR data with authentic samples isolated from previous microbial metabolism studies.[12,23,27,28] The nine new compounds were structurally identified on the basis of their IR, ^{1}H and ^{13}C NMR, COSY, HSQC, HMBC, NOESY, and HRFABMS results.

Incubation of isosteviol (**1**) with *M. recurvatus* produced four known metabolites, **2**, **3**, **5**, and **6**,[23] along with one new metabolite, **4**. Metabolite **4** was obtained as a white powder. Its molecular formula was deduced as $C_{20}H_{31}O_4$ by high-resolution FABMS [M + H]$^+$ at *m*/*z* 335.2208 and ^{13}C NMR and DEPT spectra. Its IR spectrum showed the presence of two carbonyl groups (1697 and 1726 cm^{-1}). The DEPT and HSQC spectra, compared to that of **1**, displayed new resonances at δ_H 4.39 and δ_C 74.5, suggesting **4** to be a hydroxylated product of **1**. Comparison of the ^{1}H and ^{13}C NMR spectra of **1** and **4** revealed that an additional OH group was present in **4** at C-15. The location of the OH group was confirmed by a detailed analysis of the HMBC data. The chemical shift of δ_H 4.39 clearly showed connectivities with C-9 (δ 56.5), C-13 (δ 47.8), and C-14 (δ 51.4). In the NOESY experiment, the correlation of δ 4.39 with CH_3-20 (δ 1.07) indicated that 15-OH was β-oriented. Thus, **4** was established as *ent*-15α-hydroxy-16-oxobeyeran-19-oic acid.

Preparative-scale fermentation of **1** by *Abs. pseudocylindrospora* produced **7**–**12**. Metabolites **7**–**10** were obtained from our previous incubation of steviol-16α,17-epoxide with *Streptomyces griseus* and *Cunninghamella bainieri*.[27] The HRFAB mass spectrum of **11** gave a quasi-molecular ion [M + H]$^+$ peak at *m*/*z* 351.2172, consistent with a molecular formula of $C_{20}H_{31}O_5$. This was corroborated by the ^{13}C and DEPT NMR spectra, which displayed 20 resonances including one carboxylic carbonyl and one carbonyl group, and seven methylene, four methine, three methyl, and four quaternary carbons. In the HSQC spectrum, the observation of resonances at δ_H 3.84 (δ_C 74.7) and 3.97 (δ_C 70.5) indicated that **11** had two more oxygen atoms than **1**. Comparison of ^{1}H and ^{13}C NMR spectra of **1** and **11** indicated the presence of OH groups in **11** at C-7 and C-12. In the DEPT spectrum, the resonance of C-6 had shifted downfield from δ 22.4 to 32.3, and the resonances of C-5 and C-9 had shifted upfield, from δ 56.9 to 54.0 and from δ 54.7 to 48.9, respectively. In the HMBC spectrum, δ 3.84 exhibited cross-peaks with δ 40.4 (C-15) and 42.9 (C-14). Thus, hydroxylation occurred at C-7. The α-orientation of the OH group at C-7 follows from the multiplicity of the C-7 signal in the ^{1}H NMR spectrum, which is a double-doublet in **11**.[26] The OH group at C-12 was indicated in the ^{1}H NMR by the dramatic shift of the CH_3-17 signal from δ 1.09 in **1** to δ 1.36 in **11**. It was also deduced by HMBC correlations of δ_C 70.5 with δ_H 1.36 (CH_3-17) and 2.34–2.47 (H-14). This coincided in the ^{13}C NMR with downfield shifts of C-11 ($\Delta\delta$ +9.2 ppm), C-12 ($\Delta\delta$ +33.1 ppm), and C-13 ($\Delta\delta$ +6.1 ppm) and upfield shifts of C-9 ($\Delta\delta$ −5.8 ppm), C-14 ($\Delta\delta$ −11.3 ppm), and CH_3-17 ($\Delta\delta$ −2.2 ppm), in comparison with those of **1** (Table 3). The 12β-OH was disclosed by a NOESY cross-peak between H-12$_{eq}$ (δ_H 3.97) and CH_3-17. The multiplicity of the H-12 signal in the ^{1}H NMR spectrum, a broad singlet, indicated that it was equatorial or α-oriented.[12] Thus, **11** was established to be *ent*-7β,12α-dihydroxy-16-oxobeyeran-19-oic acid.

Metabolite **12** contained three oxygen atoms more than **1**, as deduced from its molecular ion peak at *m*/*z* 367.2136 ($C_{20}H_{31}O_6$) and ^{13}C NMR. The most striking difference between **12** and **11** was that the CH_3-17 signal in the ^{1}H and ^{13}C NMR spectra of **12** was replaced by a C*H*$_2$OH group (δ_H 4.37 and 4.40, d, J = 10.8 Hz, each 1H/δ_C 63.5). Due to hydroxylation of the CH_3-17 group, H-12 and H-14 of **12** exhibited downfield shifts from δ 3.97 to 4.26 and from δ 2.34−2.47 (2H) to 2.49 (dd, J = 11.2, 3.2 Hz) and 3.06 (d, J = 11.2 Hz), respectively, in comparison with the corresponding protons in **11**. The OH group at C-17 was confirmed by the HMBC cross-peaks of C*H*$_2$OH (δ_H 4.37, 4.40) with C-12 (δ 69.1), C-13 (δ 60.3), C-14 (δ 38.1), and C-16 (δ 219.3). The ^{13}C NMR data, compared to that of **1**, also showed downfield shifts of C-11 ($\Delta\delta$ +9.1 ppm), C-12 ($\Delta\delta$ +31.7 ppm), C-17 ($\Delta\delta$ +43.3 ppm), and C-13 ($\Delta\delta$ +11.8 ppm) and upfield shifts of C-14 ($\Delta\delta$ −16.1 ppm) and C-16 ($\Delta\delta$ −1.4 ppm) (Table 3). Comparisons of ^{1}H−^{1}H COSY, NOESY, DEPT, HSQC, and HMBC data with those of **11** indicated that **12** is *ent*-7β,12α,17-trihydroxy-16-oxobeyeran-19-oic acid.

Eight oxidized metabolites obtained from the microbial transformation of isosteviol (**1**) by *Asp. niger* BCRC 32720 were **2** and **13**–**19**. Metabolites **2** and **14** were previously isolated from the biotransformation of **1** with *M. recurvatus* and *Asp. niger* CMI 17454.[12,23] Metabolites **13** and **15**–**19** are previously unreported. The HRFABMS of **13** showed an [M + H]$^+$ at *m*/*z* 335.2234 ($C_{20}H_{31}O_4$). The IR spectrum showed carbonyl absorptions at 1691 and 1731 cm^{-1}. The ^{1}H NMR spectrum, when compared to **1**, showed a new peak at δ_H 3.64 integrating one proton, indicating hydroxylation at a methylene carbon. This was confirmed by analysis of the ^{13}C NMR and DEPT spectra, which showed eight methylenes, instead of nine, and an extra methine resonance. The resonance of C-2 shifted downfield from δ 19.6 to 30.7 and the resonances of C-3 and C-20 shifted upfield from δ 38.5 to 36.6 and δ 13.6 to 9.7, respectively, in comparison with **1**. The HSQC and HMBC spectra revealed that the new resonance at δ_H 3.64 showed connectivities with δ_C 9.7 (CH_3-20), 30.7 (C-2), 44.6 (C-10), and 55.6 (C-9), confirming the presence of an OH at C-1. The α-orientation of 1-OH was deduced from the cross-peaks of H-1 with H-5, H-3, and H-2 in the NOESY experiment. The proton resonance at δ 3.64 was a double-doublet (J = 11.2, 4.8 Hz) due to coupling with the protons of C-2, indicating that the proton was axial in orientation.[12] Thus, **13** was determined to be *ent*-1β-hydroxy-16-oxobeyeran-19-oic acid.

Metabolites **15**–**17** displayed quasi-molecular ions [M + H]$^+$ at *m*/*z* 349.2022, 349.2015, and 349.2011 ($C_{20}H_{29}O_5$). Their IR spectra suggested the presence of one carboxyl carbonyl absorption (1731, 1744, and 1736 cm^{-1} for **15**–**17**, respectively) and two carbonyl absorptions (1698 and 1651, 1701 and 1657, and 1711 and 1674 cm^{-1} for **15**–**17**, respectively). The ^{13}C NMR and DEPT spectra of **15**–**17** revealed the presence of three methyl, seven methylene, three methine, one carboxylic carbonyl, and two carbonyl groups,

Table 1. ^{1}H NMR Chemical Shifts of Metabolites **4**, **11**–**13**, and **15** (C_5D_5N, δ values in ppm)[a]

position	**4**	**11**	**12**	**13**	**15**
1	1.57–1.66, m[b]	1.58–1.67, m[b]	1.62–1.70, m[b]	β 3.64, dd (11.2, 4.8)	3.28, br d (14.4)
	0.91, td (13.2, 4.0)	0.91, td (13.2, 3.6)	0.91, td (13.2, 4.0)		1.33–1.42, m
2	2.12–2.22, m[b]	2.12, m	2.16, m	2.55, qd (13.6, 3.6)	2.24, m
	1.47, br d (14.8)	1.34–1.43, m[b]	1.38–1.45, m[b]	1.93, m	1.47–1.54, m
3	2.46, br d (13.6)	2.34–2.47, m[b]	2.38–2.47, m[b]	2.43, dt (13.2, 3.6)	2.45, br d (13.2)
	1.11–1.24, m[b]	1.05, m	1.01–1.09, m	1.22, dd (13.6, 4.0)	1.03–1.12, m
5	1.26–1.37, m[b]	1.34–1.43, m[b]	1.38–1.45, m[b]	1.14, dd (12.2, 2.0)	1.69–1.82, m[b]
6	2.40, br d (11.2)	2.57–2.63, m[b]	2.60, m	2.14, m	3.43, t (14.0)
	2.12–2.22, m[b]	2.34–2.47, m[b]	2.38–2.47, m[b]	2.04, m	3.04, dd (14.0, 2.0)
7	2.73, dt (13.6, 3.2)	β 3.84, dd (12.0, 4.0)	β 3.88, dd (12.4, 4.0)	1.51–1.63, m[b]	
	1.11–1.24, m[b]			1.38–1.49, m[b]	
9	1.26–1.37, m[b]	1.89, dd (12.0, 4.0)	1.98–2.03, m[b]	1.51–1.63, m[b]	1.88, br d (10.0)
11	1.57–1.66, m[b]	1.97, m	1.98–2.03, m[b]	3.11, m	α 4.34, td (10.4, 5.6)
	1.11–1.24, m[b]	1.58–1.67, m[b]	1.62–1.70, m[b]	1.51–1.63, m[b]	
12	1.57–1.66, m[b]	α 3.97, br s	α 4.26, br s	1.51–1.63, m[b]	2.10–2.17, m[b]
	1.40, m			1.38–1.49, m[b]	1.69–1.82, m[b]
14	2.08, dd (11.2, 2.4)	2.34–2.47, m[b]	3.06, d (11.2)	1.38–1.49, m[b]	2.62, dd (12.0, 3.2)
	1.26–1.37, m[b]		2.49, dd (11.2, 3.2)		1.69–1.82, m[b]
15	α 4.39, s	3.10, d (18.4)	3.19, d (18.0)	2.75, dd (18.4, 3.2)	2.96, dd (18.4, 3.6)
		2.57–2.63, m[b]	2.62, dd (18.0, 3.2)	1.79, d (18.4)	2.10–2.17, m[b]
17	1.09, s	1.36, s	4.40, d (10.8)	1.01, s	1.10, s
			4.37, d (10.8)		
18-CH_3	1.37, s	1.32, s	1.34, s	1.35, s	1.30, s
20-CH_3	1.07, s	1.03, s	1.05, s	1.27, s	1.49, s

[a] Assignments based on DEPT, HMQC, and HMBC. Signal multiplicity and coupling constants (Hz) are in parentheses. [b] Overlapping signals.

Table 2. ^{1}H NMR Chemical Shifts of Metabolites **16**–**19** (C_5D_5N, δ values in ppm)[a]

position	**16**	**17**	**18**	**19**
1	β 3.66, dd (11.2, 4.8)		3.28, br d (14.0)	β 3.70, m
			1.54–1.63, m[b]	
2	2.54, qd (13.6, 3.2)	3.47, td (13.6, 5.6)	2.37, m	2.02, m
	1.94–1.98, m[b]	2.20–2.28, m[b]	1.54–1.63, m[b]	1.79, m
3	2.42, dt (13.2, 3.2)	2.70–2.76, m[b]	2.45–2.60, m[b]	2.43, td (14.0, 4.4)
	1.18, td (13.6, 3.6)	1.41–1.57, m[b]	1.23, td (13.2, 4.0)	1.48, dt (13.6, 4.4)
5	1.55–1.66, m[b]	2.70–2.76, m[b]	2.45–2.60, m[b]	1.84, d (7.2)
6	3.57, t (14.4)	2.51, br t (13.0)	2.45–2.60, m[b]	β 5.19, dd (7.2, 4.8)
	3.02, dd (14.8, 2.8)	2.38, m		
7		α 3.89, br s	α 3.92, s	α 4.59, br s
9	1.94–1.98, m[b]	2.80, dd (12.4, 4.0)	2.23, d (10.4)	1.55–1.59, m[b]
11	3.13, m	2.20–2.28, m[b]	α 4.29, m	2.32, m
	1.77, qd (13.2, 5.2)	1.28, m		1.64–1.74, m[b]
12	1.55–1.66, m[b]	1.41–1.57, m[b]	2.16, m	1.55–1.59, m[b]
	1.34, m		1.82, t (11.2)	1.36, m
14	2.66, dd (12.0, 2.8)	2.14, dd (12.0, 3.6)	2.27, dd (12.0, 3.2)	2.69, dd (12.0, 1.6)
	1.55–1.66, m[b]	1.74, dd (12.0, 2.4)	1.74, dd (12.0, 2.8)	1.64–1.74, m[b]
15	2.83, dd (18.0, 3.2)	2.62, dd (18.4, 3.6)	2.86, dd (18.8, 3.6)	2.79, dd (18.4, 4.0)
	2.16, d (18.0)	1.93, d (18.4)	1.95, d (18.4)	2.50, d (18.4)
17	1.04, s	1.02, s	1.10, s	1.10, s
18-CH_3	1.27, s	1.34, s	1.44, s	1.30, s
20-CH_3	1.41, s	1.39, s	1.41, s	1.20, s

[a] Assignments based on DEPT, HMQC, and HMBC. Signal multiplicity and coupling constants (Hz) are in parentheses. [b] Overlapping signals.

and four quaternary carbons. The DEPT and HSQC spectra of **15**–**17** showed the disappearance of two CH_2 signals and the presence of a proton geminal to a new alcohol at δ_H 4.34 (δ_C 67.4), 3.66 (δ_C 79.9), and 3.89 (δ_C 74.6), respectively, and one new carbonyl group at δ_C 211.7, 212.4, and 215.2, respectively. Thus, this indicated that an OH and a ketone functionality were formed in **15**–**17**. Comparison of the HSQC and HMBC spectra of **15** with **1** revealed that the new resonance at δ_H 4.34 exhibited correlations with δ_C 40.4 (C-10) and 61.1 (C-9). In the COSY spectrum, δ_H 4.34 exhibited cross-peaks with H-12 (δ_H 1.69−1.82 and 2.10−2.17) and H-9β (δ_H 1.88). Accordingly, hydroxylation occurred at C-11. In the NOESY spectrum, 11-OH was assigned to a β-orientation due to the presence of cross-peaks of H-11 with CH_3-20, H-12, and H-15α. The new carbonyl group at the C-7 position was deduced by HMBC correlations of δ_C 211.7 with δ_H1.69−1.82 (H-5β and H-14), 2.10−2.17 (H-15), 3.04 (H-6), and 3.43 (H-6). Thus, **15** was established to be *ent*-11α-hydroxy-7,16-dioxobeyeran-19-oic acid.

In the HMBC spectrum of **16**, the new resonance at δ_H 3.66 showed connectivities with δ_C 9.2 (CH_3-20), 44.5 (C-10), and 56.4 (C-9). This suggested that an OH had been introduced at C-1. The COSY spectrum showed that the methine proton at C-1 (δ_H 3.66) resonated as a double-doublet (J = 11.2, 4.8 Hz) due to coupling with protons of the neighboring C-2, indicating that the proton was axial in orientation.[12] The α-orientation of 1-OH was also deduced from the cross-peaks of H-1 with β-orientation of H-3, H-5, H-2, and H-11 in the NOESY experiment. The new carbonyl group resonating at δ_C 212.4 was at C-7, as deduced by HMBC correlations among δ 212.4 and δ_H 2.16 (H-15), 3.02 (H-6), and 3.57 (H-6). Thus, **16** was characterized as being *ent*-1β-hydroxy-7,16-dioxobeyeran-19-oic acid.

The HMBC correlations of **17** were observed between the new proton resonance at δ 3.89 and δ 41.0 (C-9), 44.3 (C-8), and 48.6 (C-5). On the other hand, the new carbon resonance at δ 74.6 showed connectivities with δ 2.38 (H-6), 1.93 (H-15), 2.70–2.76 (H-5β), and 1.74 (H-14). Thus, this indicated that an OH was at

Table 3. ^{13}C NMR Chemical Shifts of **1**, **4**, **11–13**, and **15–19** (C_5D_5N, δ values in ppm)[a]

position	1	4	11	12	13	15	16	17	18	19
1	40.0	40.1	39.6	39.7	81.0	42.2	79.9	215.2	42.7	71.2
2	19.6	19.7	19.4	19.5	30.7	19.8	30.5	36.7	20.1	30.9
3	38.5	38.6	38.3	38.4	36.6	38.2	36.1	39.4	38.8	26.5
4	43.8	43.9	43.6	43.7	43.5	44.3	43.6	43.5	43.9	42.5
5	56.9	57.3	54.0	54.2	56.0	54.7	53.0	48.6	47.5	53.5
6	22.4	22.3	32.3	32.4	22.4	39.7	39.8	30.7	30.4	85.3
7	41.6	34.0	74.7	74.8	42.3	211.7	212.4	74.6	76.3	74.5
8	39.5	43.1	46.8	47.0	40.3	52.8	52.3	44.3	45.2	43.5
9	54.7	56.5	48.9	49.7	55.6	61.1	56.4	41.0	55.3	56.0
10	38.3	38.7	38.1	38.3	44.6	40.4	44.5	52.4	40.2	39.8
11	20.6	20.2	29.8	29.7	24.2	67.4	23.8	22.9	68.0	22.6
12	37.4	38.0	70.5	69.1	38.0	48.6	37.4	37.4	49.2	37.0
13	48.5	47.8	54.6	60.3	48.7	49.3	49.0	48.9	49.0	50.6
14	54.2	51.4	42.9	38.1	54.4	45.6	46.5	50.9	51.0	47.4
15	48.5	74.5	40.4	41.5	49.0	46.2	46.3	48.2	48.1	50.3
16	220.7	222.4	220.3	219.3	221.1	216.3	217.6	220.4	219.5	219.1
17	20.2	20.6	18.0	63.5	20.2	19.6	20.1	20.3	19.9	20.2
18	29.4	29.4	29.2	29.2	29.2	29.1	28.4	27.9	29.8	24.4
19	179.9	180.1	179.9	179.9	179.9	179.6	179.2	179.9	180.7	182.4
20	13.6	14.0	13.3	13.4	9.7	13.9	9.2	13.6	14.2	13.7

[a] Assignments based on DEPT, HMBC, and HMQC.

C-7. Due to the multiplicity of H-7 exhibited as a broad singlet, the 7β-OH was established.[12] The introduction of a new keto functionality at C-1 was deduced by HMBC correlations among δ_C 215.2 and δ_H 1.39 (CH_3-20), 1.41−1.57 (H-3), 2.20−2.28 (H-2), 2.70−2.76 (H-3), 2.80 (H-9), and 3.47 (H-2). Thus, **17** was established to be *ent*-7α-hydroxy-1,16-dioxobeyeran-19-oic acid.

Metabolite **18** had a molecular formula of $C_{20}H_{31}O_5$ as determined from its HRFABMS $[M + H]^+$ at *m/z* 351.2183 as well as from its ^{13}C NMR and DEPT spectra. The IR spectrum showed two carbonyl groups (1735 and 1719 cm^{-1}). Its ^1H NMR spectrum, when compared to that of **1**, showed two new resonances at δ 3.92 and 4.29. The DEPT spectrum showed the disappearance of two CH_2 resonances relative to **1** and the presence of two new CHs resonating at δ 68.0 and 76.3, confirming that **18** is a dihydroxylated metabolite of **1**. The DEPT and HSQC spectra found that the resonance of C-6 had shifted downfield from δ 22.4 to 30.4, and the resonance of C-5 had shifted upfield from δ 56.9 to 47.5 by comparison with **1**. In the HMBC spectrum, δ_H 3.92 exhibited cross-peaks with δ_C 47.5 (C-5) and 55.3 (C-9). Thus, hydroxylation had occurred at C-7. The β-orientation of 7-OH was suggested from the cross-peaks of H-7 with H-14 and H-15 in the NOESY spectrum. The broad singlet at δ_H 3.92 also confirmed that H-7 was α-oriented.[12] The second OH at C-11 was deduced by HMBC correlations of δ_H 4.29 with δ_C 40.2 (C-10) and 55.3 (C-9); the carbon resonating at δ 68.0 showed cross-peaks with δ_H 1.82 (H-12), 2.16 (H-12), and 2.23 (H-9β). The β-OH at C-11 was suggested from the cross-peaks of δ_H 4.29 with an α-orientation of CH_3-20, H-12, and H-15 in the NOESY spectrum. Thus, **18** was determined to be *ent*-7α,11α-dihydroxy-16-oxobeyeran-19-oic acid.

Metabolite **19** showed a quasi-molecular ion $[M + H]^+$ at *m/z* 367.2115 corresponding to the molecular formula $C_{20}H_{31}O_6$. It was the most polar compound in this study. The ^{13}C NMR spectrum displayed resonances for 20 carbons, while the DEPT spectrum showed the presence of three methyl, six methylene, five methine, and six quaternary carbons. The HMQC spectrum showed new resonances at δ_H 3.70 (δ_C 71.2), 4.59 (δ_C 74.5), and 5.19 (δ_C 85.3), indicating that **19** contained three more oxygen atoms than **1**. The COSY spectrum correlations between the two new OH-bearing methines at δ_H 4.59 (br s) and 5.19 (dd, $J = 7.2$, 4.8 Hz) suggested that they were on vicinal carbons. Comparison of the ^1H and ^{13}C NMR spectra of **19** and **1** revealed that the new OH groups in **19** were at C-6 and C-7. They were confirmed by a detailed analysis of the HMBC data. Correlations of δ_H 4.59 with δ_C 43.5 (C-8), 47.4 (C-14), 50.3 (C-15), and 85.3 and of δ_H 5.19 with δ_C 39.8 (C-10), 53.5 (C-5), and 74.5 were observed. The α-OH at C-6 was suggested from the cross-peaks between H-6 (δ_H 5.19) and the β-orientations of CH_3-18 and H-5 in the NOESY spectrum. The other OH in the 7β-orientation was disclosed by NOESY cross-peaks between H-7 (δ_H 4.59) and the α-orientations of CH_3-20 and H-15 in the NOESY spectrum. Examination of the HMBC spectrum allowed us to assign the third newly introduced OH group to C-1. In the HMBC spectrum, cross-peaks were observed of δ_H 3.70 with δ_C 13.7 (CH_3-20), 26.5 (C-3), 39.8 (C-10), and 56.0 (C-9). The 1α-OH was suggested from the cross-peaks of H-1 with β-orientation of H-9, H-5, H-2, and H-11 in the NOESY spectrum. Thus, **19** was characterized as *ent*-1β,6β,7α-trihydroxy-16-oxobeyeran-19-oic acid.

Table 4. Data of Compounds Showing Significant Results on a Glucocorticoid Receptor-Mediated Assay[a]

compd	luciferase activity	compd	luciferase activity
MP	2.25 ± 1.25	**13**	1.69 ± 0.58
Dex	2.56 ± 1.34	**16**	2.07 ± 1.13
7	1.71 ± 0.75	**18**	1.60 ± 0.40

[a] The concentration of each test compound was 10 μM. All firefly luciferase activities were normalized to *Renilla* luciferase activity. The data are expressed as multiples of luciferase activity compared to the no-treatment (control) group, which was designated 1.0. Methylprednisolone (MP) and dexamethasone (Dex) are the reference compounds. Each value is the average of the firefly/*Renilla* luciferase ratio and are presented as the mean ± SEM ($n = 3$). Significantly different equals $p < 0.05$, using Student's *t*-test for paired samples.

In a continuing search for potential novel glucocorticoid agonists,[27,29] isosteviol (**1**) and its metabolites, **2–4** and **6–18**, were screened using a GRE-mediated luciferase reporter gene assay. Compounds **7**, **13**, **16**, and **18** were active, but less active than methylprednisolone and dexamethasone (Table 4). In addition, an ARE-mediated luciferase reporter gene assay was also used to screen for novel potential androgen agonists that may provide for the treatment of androgen insufficiency. Compounds **3**, **4**, **12**, **13**, **14**, and **18** were significantly active; in particular, **3**, **12**, **14**, and **18** were more active than testosterone (Table 5). Compounds **13** and **18**, which possessed OH groups at the 1α- and 7β,11β-positions, respectively, appeared to be able to activate both GRE- and ARE-mediated genomic effects. Further ligand–receptor interactions will be processed to unravel this point. In addition, "nongenomic" actions of several steroids through their receptors or other membrane receptors have recently been demonstrated in different cell systems, and their different activities were examined.[30,31] Whether similar nongenomic activities exerted by these compounds exist was not revealed in our current study.

As the results show, three selected filamentous fungi have the abilities to regio- and stereoselectively hydroxylate rings A,

Table 5. Data of Compounds Showing Significant Results on an Androgen Receptor-Mediated Assay[a]

compd	luciferase activity	compd	luciferase activity
Tes	2.08 ± 0.62	**13**	2.06 ± 0.57
3	2.46 ± 1.64	**14**	2.72 ± 1.58
4	1.96 ± 0.84	**18**	2.17 ± 0.96
12	2.50 ± 0.76		

[a] The concentration of each test compound was 10 μM. All firefly luciferase activities were normalized to *Renilla* luciferase activity. The data were expressed as multiples of luciferase activity compared to the no-treatment (control) group, which was designated 1.0. Testosterone (Tes) is the reference compound. Each value is the average of the firefly/*Renilla* luciferase ratio and presented as the mean ± SEM ($n = 3$). Significantly different equals $p < 0.05$, using Student's *t*-test for paired samples.

B, C, and D of isosteviol (**1**). Among 18 isolated metabolites, **4**, **11**–**13**, and **15**–**19** have not previously been reported. Comparison of the substrate specificity of these three selected fungi suggests that they possess different characteristics of reaction selectivity. *M. recuvatus* MR 36 performs only mono- and dihydroxylation on **1**; *Abs. pseudocylindrospora* ATCC 24169 is able to specifically hydroxylate **1** at C-17, while, *Asp. niger* has the ability to dihydroxylate **1** at the 1α,7β- and 7β,11β-positions followed by oxidation of the 1α- or 7β-OH, respectively, to yield the metabolites **15**–**17**. The dihydroxylation that occurred at the 1α,7β-positions of **1** by *Asp. niger* has previously been reported.[12,28] However, the subsequent oxidation of an OH group to a ketone group has not previously been described.

Experimental Section

General Experimental Procedures. Melting points were determined with a Yanagimoto micromelting point apparatus and are uncorrected. Optical rotations were determined on a JASCO DIP-1020 digital polarimeter. IR spectra were obtained using a Perkin-Elmer spectrum GX/AutoImage microscope FT-IR spectrometer. Nuclear magnetic resonance (NMR) spectra including ^{1}H, ^{13}C NMR, DEPT, and 2D NMR were recorded on a Bruker Avance-400 spectrometer. Chemical shifts are reported in parts per million (ppm) with respect to the corresponding solvent as the internal standard, and coupling constants (*J*) are expressed in hertz (Hz). Low- and high-resolution FAB mass spectra were recorded using a JMS-700 HRMS spectrometer. HPLC separations were performed on a Hitachi L-6200 HPLC instrument with a Si 60 preparative column (250 × 10 mm, 5 μm, at a flow rate of 3 mL/min) (Merck, Darmstadt, Germany), monitored by a Shodex RI-71 RI detector. Column chromatography was performed with Kieselgel silica (70–230 and 230–400 mesh, Merck). TLC analyses were performed on precoated silica gel 60 F_{254} plates (Merck). Spots for all compounds were detected by spraying with 10% H_2SO_4, followed by heating.

Substrate. Isosteviol (**1**) was prepared as described previously.[23]

Microorganisms. Fungal cultures of *Mucor recurvatus* MR36, *Absidia pseudocylindrospora* ATCC 24169, and *Aspergillus niger* BCRC 32720 were obtained from the Division of Medicinal and Natural Products Chemistry, College of Pharmacy, University of Iowa, Iowa City, IA, and Bioresources Collection and Research Center, Hsinchu, Taiwan. These fungi were maintained on Sabouraud-maltose and Czapek's agar slants and stored in a refrigerator at 4 °C.

Preparation of Medium. Fungal fermentations were carried out in a medium as described previously.[29] The pH of the medium was adjusted to 7.0 with 6 N HCl before sterilization by autoclaving at 121 °C for 15 min.

Biotransformation of Isosteviol (1) by *M. recurvatus* MR 36. *Mucor recurvatus* was grown according to the usual fermentation procedure.[29] Using 24-h-old stage II cultures, 1000 mg of **1**, dissolved in 10 mL of DMF, was evenly distributed among one hundred 125 mL stainless-steel-capped flasks containing stage II cultures. Substrate-containing cultures were incubated for 144 h. The incubation mixtures were pooled and acidified with 6 N HCl and then filtered to remove the cells. The filtrate was extracted three times with equal volumes of EtOAc−*n*-butanol (9:1). Cells were washed with acetone, filtered, evaporated, and extracted with EtOAc. The extracts were evaporated to dryness under reduced pressure. The combined crude residues from the filtrate and cells (4.6 g) were subjected to column chromatography (CC) over silica gel. In total, eight fractions (A–H) were eluted with mixtures of CH_2Cl_2−CH_3OH (600 mL each of 25:1, 15:1, 10:1, 8:1, and 6:1). With further chromatography of fraction B (530 mg) over silica gel eluted with CH_2Cl_2−CH_3OH (20:1), 227 mg of **1** was recovered. Fraction D (652 mg) was divided into three subfractions (D_1−D_3) using column chromatography over silica gel eluted with a gradient of CH_3OH in CH_2Cl_2. Fractions D_2 (224 mg) and D_3 (127 mg) gave **2** (53 mg), **3** (6 mg), and **4** (5 mg) with repeated normal-phase HPLC (CH_2Cl_2−CH_3OH, 20:1). Fraction E (250 mg) was chromatographed over silica gel eluted with CH_2Cl_2−CH_3OH (20:1) to give three subfractions (E_1−E_3). Further purification of fraction E_2 (30 mg) using normal-phase HPLC (CH_2Cl_2−CH_3OH, 17:1) gave 5 mg of **5**. Fraction F (239 mg) was subjected to CC over silica gel and eluted with CH_2Cl_2−CH_3OH (20:1) to give subfractions F_1−F_3. Fraction F_1 (30 mg) gave 6 mg of **6** using repeated normal-phase HPLC (CH_2Cl_2−CH_3OH, 15:1).

Biotransformation of Isosteviol (1) by *Abs. pseudocylindrospora* ATCC 24169. *Absidia pseudocylindrospora* bioconversion was conducted as with *M. recurvatus*. The preparative-scale reaction was terminated after 144 h following 1000 mg of substrate addition. Extraction as described above afforded 5.9 g of black oil. The oil was subjected to CC over silica gel. In total, seven fractions (A−G) were eluted with mixtures of CH_2Cl_2−CH_3OH (600 mL each of 30:1, 20:1, 15:1, 12:1, and 6:1). Further chromatography of fraction B (630 mg) over silica gel eluted with CH_2Cl_2−CH_3OH (20:1) gave 350 mg of **1**. Fraction C (1409 mg) was chromatographed over silica gel to give four subfractions (C_1−C_4). Fraction C_2 (389 mg), purified by using normal-phase HPLC (CH_2Cl_2−CH_3OH, 20:1), gave **7** (28 mg) and **8** (138 mg). Further chromatography of fraction D (578 mg) over silica gel eluted with CH_2Cl_2−CH_3OH (20:1) gave three subfractions (D_1−D_3). Fraction D_3 (153 mg), purified using normal-phase HPLC (CH_2Cl_2−CH_3OH, 15:1), yielded **9** (17 mg) and **11** (40 mg). Fraction E (456 mg) was chromatographed over silica gel eluted with CH_2Cl_2−CH_3OH (20:1) to afford three subfractions (E_1−E_3). Further purification of fraction E_2 (98 mg) using normal-phase HPLC (CH_2Cl_2−CH_3OH, 15:1) yielded 23 mg of **10** and 18 mg of **12**.

Biotransformation of Isosteviol (1) by *Asp. niger* BCRC 32720. *Aspergillus niger* bioconversion was conducted as with the *M. recurvatus* preparative-scale reaction and terminated after 144 h following 1000 mg of substrate addition. The extraction described above produced 5.1 g of black oil. The oil was subjected to CC over silica gel. Eight fractions (A−H) were obtained by eluting with mixtures of CH_2Cl_2−CH_3OH (600 mL each of 25:1, 20:1, 15:1, 12:1, 8:1, and 6:1). Further chromatography of fraction C (560 mg) over silica gel eluted with CH_2Cl_2−CH_3OH (30:1) gave 350 mg of **1**. Fraction D (250 mg) was chromatographed over silica gel eluted with CH_2Cl_2−CH_3OH (20:1) to yield five subfractions (D_1−D_5). Further chromatography of fractions D_2 (67 mg) and D_3 (45 mg), respectively, using normal-phase HPLC (CH_2Cl_2−CH_3OH−*n*-hexane, 9:1:3) yielded **2** (20 mg), **13** (9 mg), **15** (9 mg), and **19** (6 mg). Fraction E (231 mg) was chromatographed over silica gel eluted with CH_2Cl_2−CH_3OH (20:1) to yield four subfractions (E_1−E_4). Further purification of fraction E_3 (112 mg) using normal-phase HPLC (CH_2Cl_2−CH_3OH−*n*-hexane, 9:1:5) yielded **16** (17 mg), **17** (9 mg), and **18** (9 mg). Fraction F (197 mg) was chromatographed over silica gel eluted with CH_2Cl_2−CH_3OH (15:1) to yield four subfractions (F_1−F_4). Further purification of fraction F_2 (45 mg) using normal-phase HPLC (CH_2Cl_2−CH_3OH−*n*-hexane, 9:1.3:5) yielded 6 mg of **14**.

Metabolite 4: white powder; $[\alpha]^{25}_D$ −88.0 (*c* 0.65, CH_3OH); IR (KBr) ν_{max} cm^{-1} 3393 (OH), 1726 (C=O), 1697 (C=O); ^{1}H and ^{13}C NMR, see Tables 1 and 3; LRFABMS *m/z* 335 $[M + H]^+$; HRFABMS *m/z* 335.2208 (calcd for $C_{20}H_{31}O_4$, 335.2222).

Metabolite 11: white powder; $[\alpha]^{25}_D$ −70.9 (*c* 0.95, CH_3OH); IR (KBr) ν_{max} 3418 (OH), 1721 (C=O), 1701 (C=O) cm^{-1}; ^{1}H and ^{13}C NMR, see Tables 1 and 3; LRFABMS *m/z* 351 $[M + H]^+$; HRFABMS *m/z* 351.2172 (calcd for $C_{20}H_{31}O_5$, 351.2171).

Metabolite 12: colorless, flaky crystals (acetone); mp 143–145 °C; $[\alpha]^{25}_D$ −69.3 (*c* 0.97, CH_3OH); IR (KBr) ν_{max} 3388 (OH), 1721 (C=O), 1666 (C=O) cm^{-1}; ^{1}H and ^{13}C NMR, see Tables 1 and 3; LRFABMS *m/z* 367 $[M + H]^+$; HRFABMS *m/z* 367.2136 (calcd for $C_{20}H_{31}O_6$, 367.2121).

Metabolite 13: needles (acetone); mp 133–135 °C; $[\alpha]^{25}_{D}$ −39.2 (*c* 0.5, CH_3OH); IR (KBr) ν_{max} 3453 (OH), 1731 (C=O), 1691 (C=O) cm^{-1}; ^1H and ^{13}C NMR, see Tables 1 and 3; LRFABMS *m/z* 335 $[M + H]^+$; HRFABMS *m/z* 335.2234 (calcd for $C_{20}H_{31}O_4$, 335.2222).

Metabolite 15: white powder; $[\alpha]^{25}_{D}$ −20.0 (*c* 0.7, CH_3OH); IR (KBr) ν_{max} cm^{-1} 3498 (OH), 1731 (C=O), 1698 (C=O), 1651 (C=O); ^1H and ^{13}C NMR, see Tables 1 and 3; LRFABMS *m/z* 349 $[M + H]^+$; HRFABMS *m/z* 349.2022 (calcd for $C_{20}H_{29}O_5$, 349.2015).

Metabolite 16: white powder; $[\alpha]^{25}_{D}$ −40.7 (*c* 0.55, C_5H_5N); IR (KBr) ν_{max} 3367 (OH), 1744 (C=O), 1701 (C=O), 1657 (C=O) cm^{-1}; ^1H and ^{13}C NMR, see Tables 2 and 3; LRFABMS *m/z* 349 $[M + H]^+$; HRFABMS *m/z* 349.2015 (calcd for $C_{20}H_{29}O_5$, 349.2015).

Metabolite 17: white powder; $[\alpha]^{25}_{D}$ −103.7 (*c* 0.6, CH_3OH); IR (KBr) ν_{max} 3410 (OH), 1736 (C=O), 1711 (C=O), 1674 (C=O) cm^{-1}; ^1H and ^{13}C NMR, see Tables 2 and 3; LRFABMS *m/z* 349 $[M + H]^+$; HRFABMS *m/z* 349.2011 (calcd for $C_{20}H_{29}O_5$, 349.2015).

Metabolite 18: white powder; $[\alpha]^{25}_{D}$ −7.0 (*c* 0.6, CH_3OH); IR (KBr) ν_{max} 3316 (OH), 1735 (C=O), 1719 (C=O) cm^{-1}; ^1H and ^{13}C NMR, see Tables 2 and 3; LRFABMS *m/z* 351 $[M + H]^+$; HRFABMS *m/z* 351.2183 (calcd for $C_{20}H_{31}O_5$, 351.2171).

Metabolite 19: colorless oil; $[\alpha]^{25}_{D}$ −27.2 (*c* 1.35, CH_3OH); IR (KBr) ν_{max} 3428 (OH), 1752 (C=O), 1735 (C=O) cm^{-1}; ^1H and ^{13}C NMR, see Tables 2 and 3; LRFABMS *m/z* 367 $[M + H]^+$; HRFABMS *m/z* 367.2115 (calcd for $C_{20}H_{31}O_6$, 367.2121).

GRE-Mediated Luciferase Reporter Gene Assay. Experiments were performed as described previously.[27]

ARE-Mediated Luciferase Reporter Gene Assay. Twenty-four hours before transfection, about 1×10^5 mouse RAW 264.7 macrophage cells per well were seeded in 96-well white plates. The pARE-Luc plasmid (Panomics Inc., Fremont, CA.) and an internal control plasmid, p*hRL*-TK, were transfected into RAW 264.7 cells using the lipofectamine plus agent (Invitrogen, San Diego, CA) according to the manufacturer's instructions. At 24 h post-transfection, final concentrations of 10 μM of each test compound including the reference compound, testosterone (Sigma, St. Louis, MO), in DMSO were added to the cells. Cells were harvested 24 h after treatment, and the reporter activity of firefly luciferase expressed from pARE-Luc and *Renilla* luciferase from p*hRL*-TK were assayed in a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA) using the dual-luciferase reporter assay system (Promega, Madison, WI).

Statistical Analysis. Data were from at least three individual experiments. The averages of the firefly/*Renilla* luciferase ratios were analyzed by two-tailed Student's *t*-test for paired samples, with a value of $p < 0.05$ accepted as being statistically significant.

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