Microbial Transformation of Isosteviol Lactone and Evaluation of the Transformation Products on Androgen Response Element

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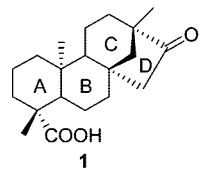
Two filamentous fungi, *Cunninghamella bainieri* ATCC 9244 and *Aspergillus niger* BCRC 32720, were used to investigate the biotransformation of isosteviol lactone (4α-carboxy-13α-hydroxy-13,16-*seco*-*ent*-19-norbeyeran-16-oic acid 13,16-lactone) (**2**), which was derived by reacting isosteviol (*ent*-16-oxobeyeran-19-oic acid) (**1**) with *m*-chloroperbenzoic acid. Incubation of **2** with *C. bainieri* afforded metabolites **3**–**6**, which involved isomerization, hydroxylation, and ring cleavage reactions followed by oxidation and selective *O*-methylation. Incubation of **2** with *A. niger* afforded mono-, di-, and trihydroxylated metabolites **3**, **4**, and **7**–**12**. The structures of **3**–**12** were elucidated on the basis of spectroscopic analyses, and structures **3**, **4**, and **6** were confirmed by X-ray crystallographic studies. Compounds **2**–**6**, **8**–**10**, and **12** were assayed as androgen agonists using an ARE (androgen response element)-mediated luciferase reporter gene assay. Compounds **3**, **6**, and **10** were significantly active, with **6** showing more activity than testosterone.

Androgens, through interactions with the androgen receptor (AR), play a decisive role in sexual differentiation of the male reproductive tract, accessory reproductive organs, and other tissues during fetal development.[1] The AR, an important drug target, is a transcription factor and member of the steroid receptor superfamily.[2] Unliganded AR exists in an inactive form and is localized in the cytoplasm. 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.[3] Steroidal androgens, mainly testosterone and its derivatives, have been used clinically as replacement therapy for androgen deficiency. However, the broader use of steroidal androgens for additional treatments, such as osteoporosis, is limited by undesirable AR-mediated side effects, such as prostatic hypertrophy and hirsutism.[4] Thus, the discovery of novel classes of nonsteroidal agents with all of the beneficial pharmacologic activities of androgens without the unwanted side effects would significantly improve the quality of life of individual patients with various androgen-dependent conditions.[5]

Microbial transformation with whole cells is a currently used system for chemical modification using biocatalytic activities inside living cells. The ability of microorganisms to hydroxylate chemically inaccessible sites is potentially a powerful synthetic technique.[6] Notable attention has been paid to filamentous fungi, since they are capable of catalyzing the regio- and stereoselective hydroxylation of nonfunctionalized hydrocarbon centers of a diverse array of substrates.[7] Isosteviol (**1**), an *ent*-beyerane tetracyclic diterpene with a ketone on the D ring, has been used in biotransformation studies.[8–10] Small modifications to the structure of a compound can modify its biological activities.[11] Transformation of cyclic ketones into lactones by the Baeyer–Villiger reaction has been widely used for synthesis of many natural and other products.[12,13] Many cyclic lactone natural products possess biological activities.[14,15] Thus, isosteviol lactone (**2**)[16,17] has been prepared by reacting **1** with *m*-chloroperbenzoic acid, and its activity on mitochondrial metabolism has been described.[16] The bridged ring skeleton of **2** is a suitable substrate for the study of microbial transformation. However, no report on the microbial transformation of **2** has been described. Thus, in an effort to produce new functionalized analogues with new biological activities, microbial transformation of **2** was carried out. Several microorganisms were screened for their ability to biotransform **2**. *Cunninghamella bainieri* ATCC 9244 and *Aspergillus niger* BCRC 32720 were selected for preparative-scale fermentation because they reproducibly converted **2** into many metabolites. Since tetracyclic diterpenoids possess a formal similarity to steroids,[18] an ARE-mediated luciferase reporter gene assay was used to evaluate **2** and related biotransformed compounds (**3**–**6**, **8**–**10**, and **12**). The present report describes the production, isolation, structure elucidation, and biological activities of these metabolites. The structures of **3**, **4**, and **6** were also confirmed by X-ray crystallographic analyses.



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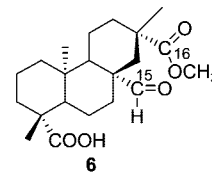

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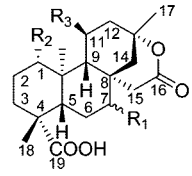
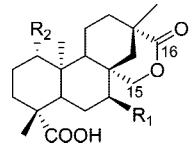


2: $R_1 = R_2 = R_3 = H$
4: R_1 = β-OH, $R_2 = R_3 = H$
7: $R_1 = R_3 = H$, R_2 = OH
9: R_1 = β-OH, R_2 = OH, $R_3 = H$
11: R_1 = =O, R_2 = OH, $R_3 = H$
12: R_1 = β-OH, R_2 = OH, R_3 = OH



3: $R_1 = R_2 = H$
5: R_1 = OH, $R_2 = H$
8: $R_1 = H$, R_2 = OH
10: $R_1 = R_2$ = OH

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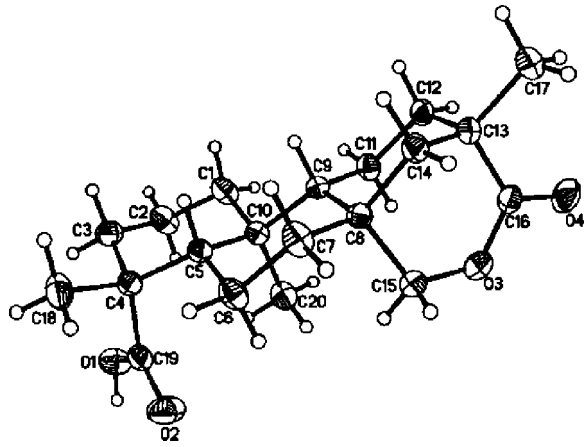

Figure 1. Perspective drawing of the X-ray structure of **3**.

Results and Discussion

Substrate **2** was prepared as follows: isosteviol (**1**) was reacted with *m*-chloroperbenzoic acid to give a mixture of keto lactones. The reaction mixture was subjected to column chromatography over silica gel to afford **2** and **3**. Compound **2** was described previously,[17] and the ^{1}H and ^{13}C assignments were confirmed by 1D and 2D NMR. Compound **3** was previously unreported. The HRFABMS of **3** displayed a quasi-molecular ion $[M + H]^+$ at *m/z* 335.2218 ($C_{20}H_{31}O_4$). Analysis of the DEPT and HSQC spectra and comparison with **1** and **2** illustrated that most of the carbon signals had similar chemical shifts, except for those of C-13, C-15, C-16, and C-17.[8] In the HMBC spectrum, the resonance at δ 1.23 (17-CH_3) exhibited cross-peaks with δ 40.2 (C-13), 47.9 (C-14), and 176.1 (C═O). Thus, this suggested that one carbonyl group was connected to C-13. On the other hand, the resonance at δ 3.85 exhibited cross-peaks with δ 34.8 (C-8), 40.6 (C-7), 47.9 (C-14), 56.0 (C-9), and 176.1 (C═O). The resonance at δ 4.83 exhibited cross-peaks with δ 34.8 (C-8), 47.9 (C-14), 56.0 (C-9), and 176.1 (C═O). This suggested that the signals at δ 3.85 and 4.83 were due to the protons at C-15. Comparison of the ^{1}H and ^{13}C NMR spectra of **2** and **3** suggested that the two compounds were regioisomers. Single-crystal X-ray crystallographic studies were used to distinguish between the structures of **2** and **3** (Figure 1), and the structure of **3** was established as 4α-carboxy-15α-hydroxy-15,16-*seco-ent*-19-norbeyeran-16-oic acid 15,16-lactone.

Twenty-five selected microbial cultures were used to identify organisms capable of metabolizing **2**. *Cunninghamella bainieri* ATCC 9244 and *Aspergillus niger* BCRC 32720 were selected as biocatalysts for scaled-up biotransformations. Preparative-scale fermentation of **2** with *C. bainieri* produced metabolites **3**–**6**. Metabolites **4** and **5** possessed the same molecular formula ($C_{20}H_{30}O_5$) as determined from their positive ion HRFABMS and from their ^{13}C NMR spectra. Comparison of the ^{13}C NMR and DEPT spectra of **4** and **5** with those of **2** and **3** found that **4** and **5** exhibited features similar to those of **2** and **3**, respectively. Analyses of the ^{13}C NMR, DEPT, and the HSQC spectra of **4** and **5** indicated the presence of one carboxylic carbonyl, another carbonyl group, eight CH_2, three CH (one oxymethine), three CH_3, and four quaternary carbons. These data suggested that they contained one more oxygen atom than **2** and **3**. Analyses of the HSQC and HMBC spectra of **4** and **5** revealed that the new proton resonance at δ 3.72 showed connectivities with C-5 (δ 47.3), C-9 (δ 49.2), and C-15 (δ 38.2) in **4**; the new resonance at δ_H 3.72 showed connectivities with C-5 (δ 47.6), C-9 (δ 49.5), and C-15 (δ 77.0) in **5**. Thus, the additional oxygen present in both molecules occurred at C-7. The orientation of the OH group at C-7 followed from the multiplicity of the H-7 signal in both ^{1}H NMR spectra, a broad singlet in **4** and **5**, indicating that H-7 was α-oriented.[10] An X-ray crystallographic experiment was carried out to confirm the structure

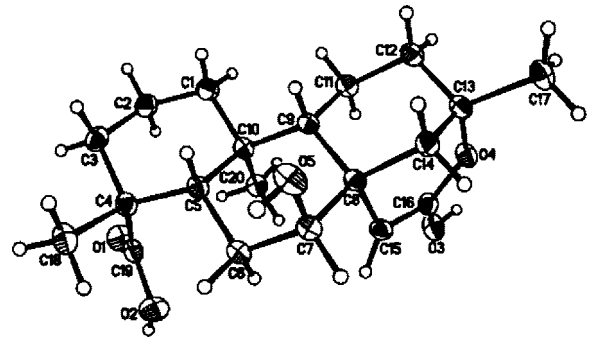

Figure 2. Perspective drawing of the X-ray structure of **4**.

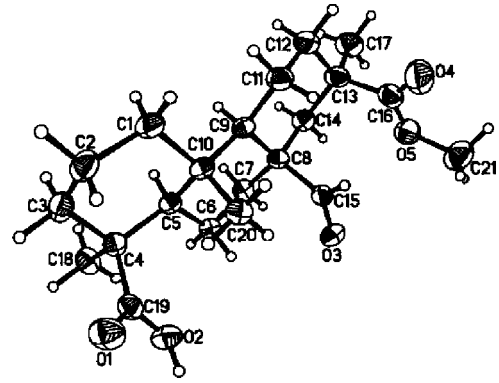

Figure 3. Perspective drawing of the X-ray structure of **6**.

of **4** (Figure 2). Thus, **4** and **5** were characterized as 4α-carboxy-7β,13α-dihydroxy-13,16-*seco-ent*-19-norbeyeran-16-oic acid 13,16-lactone and 4α-carboxy-7β,15α-dihydroxy-15,16-*seco-ent*-19-norbeyeran-16-oic acid 15,16-lactone, respectively.

Metabolite **6** displayed $[M + H]^+$ at *m/z* 365.2338 (HRESIMS), corresponding to the molecular formula $C_{21}H_{33}O_5$, consistent with its ^{13}C NMR and DEPT spectra. The ^{1}H and ^{13}C NMR and DEPT data indicated 21 carbons attributable to six quaternary carbons (including two carbonyl carbons), eight CH_2, four CH_3 (including one methoxy carbon), and three CH (including a typical aldehyde methine) carbons. The ^{13}C NMR and DEPT data showed that C-13 and C-16 resonated at δ 41.9 and 177.3, respectively, suggesting that **6** possessed the same basic skeleton as **3**. Comparison of the HSQC and HMBC data of **6** and **3** revealed that no additional OH group had been introduced. The new aldehyde methine resonance at δ 9.98 showed HMBC connectivities with δ 206.1, 37.3 (C-7), and 50.0 (C-8). The carbon resonance at δ177.3 showed connectivities with OCH_3 (δ 3.56), CH_3-17 (δ 1.10), H-14 (δ 1.12−1.25 and 2.31), and H-12 (δ 1.12−1.25). No connectivity was found between δ 177.3 and 206.1, suggesting that the bond between C-15 and C-16 had been cleaved. In the NOESY experiment, the resonance at δ 9.98 exhibited cross-peaks with α-orientation of CH_3-20, H-14, H-7, and $-OCH_3$. Accordingly, the carbon resonances at δ 206.1 and 177.3 were C-15 and C-16, respectively. The structure of **6** was confirmed by X-ray crystallographic analysis (Figure 3). Thus, **6** was established to be 15,16-dioxo-16-methoxy-15,16-*seco-ent*-beyeran-19-oic acid.

Incubation of **2** with *A. niger* resulted in the isolation of **3**, **4**, and **7**–**12**. The HRESIMS of **7** and **8** displayed quasi-molecular ions $[M + H]^+$ at *m/z* 351.2161 and 351.2174, which indicated the molecular formula of $C_{20}H_{31}O_5$ in combination with the ^{1}H and ^{13}C NMR data. In the ^{1}H and ^{13}C NMR spectra of **7** and **8**, additional resonances were observed at δ_H 3.63 (δ_C 81.1) and 3.65 (δ_C 80.6), respectively, suggesting the introduction of an OH group in both molecules. The HMBC spectra of **7** and **8** showed correlations of δ_C 81.1 with CH_3-20 (δ1.37), H-2 (δ 1.93–1.98 and 2.55), H-3 (δ 2.42), and H-5 (δ 1.08) in **7** and δ_C 80.6 with CH_3-

20 (δ 1.39), H-2 (δ 1.95–2.00 and 2.60), H-3 (δ 2.43), and H-5 (δ 1.12) in **8**, suggesting that an OH group had been introduced at C-1 in both molecules. In the COSY spectra of **7** and **8**, the methine proton at C-1 (δ 3.63 and 3.65) resonated as a double-doublet (J = 11.2, 4.8 Hz in **7**; J = 11.6, 4.8 Hz in **8**) due to coupling with the protons of the neighboring C-2. This indicated that the proton was β-oriented.[10] Thus, **7** and **8** were established as 4α-carboxy-1α,13α-dihydroxy-13,16-*seco-ent*-19-norbeyeran-16-oic acid 13,16-lactone and 4α-carboxy-1α,15α-dihydroxy-15,16-*seco-ent*-19-norbeyeran-16-oic acid 15,16-lactone, respectively.

Metabolites **9** and **10** possessed the molecular formula $C_{20}H_{30}O_6$ (HRESIMS). The ^{13}C NMR and DEPT spectra of **9** and **10** possessed features common to those of **2** and **3**, except for the disappearance of two CH_2 signals and the presence of two new CH resonances at δ 81.4 and 74.7 in **9** and at δ 81.0 and 72.5 in **10**. This suggested that **9** and **10** were dihydroxylated metabolites of **2** and **3**, respectively. In the HMBC spectra of **9** and **10**, the proton resonance at δ 3.83 showed connectivities with CH_3-20 (δ 10.3), C-2 (δ 30.8), C-10 (δ 44.3), and C-9 (δ 50.5) in **9**; the proton resonance at δ 3.84 showed connectivities with CH_3-20 (δ 10.4), C-2 (δ 30.9), C-10 (δ 44.4), and C-9 (δ 51.0) in **10**. Thus, one of the OH groups was at C-1 in both molecules. The 1α-OH was established by NOE correlations of δ 3.83 with H-9β, H-2, and H-5β in **9** and δ 3.84 with H-9β, H-2, and H-5β in **10**. The location of the second OH group at C-7 was deduced by the 2D NMR data. In the HMBC spectra, the proton resonance at δ 3.71 showed connectivities with C-15 (δ 38.8), C-8 (δ 40.5), C-5 (δ 45.9), and C-9 (δ 50.5) in **9**, and the proton resonance at δ 3.72 showed connectivities with C-8 (δ 39.8), C-14 (δ 43.3), C-5 (δ 46.0), C-9 (δ 51.0), and C-15 (δ 77.3) in **10**. These indicated that the OH group was at C-7 in both molecules. The H-7 of **9** and **10** resonated as a broad singlet at δ 3.71 and 3.72, respectively, indicating that the proton was α-oriented.[10] Thus, **9** and **10** were characterized as 4α-carboxy-1α,7β,13α-trihydroxy-13,16-*seco-ent*-19-norbeyeran-16-oic acid 13,16-lactone and 4α-carboxy-1α,7β,15α-trihydroxy-15,16-*seco-ent*-19-norbeyeran-16-oic acid 15,16-lactone, respectively.

The molecular formula of metabolite **11** was determined to be $C_{20}H_{28}O_6$ by negative HRESIMS. Analysis of the ^{13}C NMR and DEPT data revealed the presence of three CH_3, seven CH_3, three CH, three carbonyl carbons, and four quaternary carbons. The one characteristic carbonyl group resonated at δ 170.1 and one quaternary carbon resonated at δ 80.2, suggesting that **11** possessed the same skeleton as **2**. Detailed examination of the HSQC and HMBC spectra revealed that the new carbon resonance at δ 79.4 showed a correlation with δ 0.99 (CH_3-20). The new proton resonance at δ 3.18 showed correlations with CH_3-20 (δ 9.2), C-5 (δ 53.4), and C-9 (δ 56.5). Accordingly, the OH group was at C-1. The 1α-OH was established from the cross-peak of H-1 with H-5β in the NOESY experiment. The position of the ketone group at C-7 was deduced from long-range $^1H-^{13}C$ correlations of the carbon resonance at δ 211.7 with H-6 (δ 2.33) and H-15 (δ 2.21). Thus, **11** was characterized as 4α-carboxy-1α,13α-dihydroxy-7-oxo-13,16-*seco-ent*-19-norbeyeran-16-oic acid 13,16-lactone.

Metabolite **12** had a molecular formula of $C_{20}H_{30}O_7$. Analysis of its ^{13}C NMR spectrum with the aid of DEPT revealed the presence of signals for five CH, six CH_2, three CH_3, four quaternary carbon atoms, and two carbonyl carbons. The characteristic one carbonyl group and one quaternary carbon resonated at δ 171.1 and 80.6, respectively, suggesting that **12** also possessed the same skeleton as **2**. The ^1H NMR spectrum of **12** displayed three new CH resonances at δ 3.67 (br s), 4.01 (dd, J = 11.2, 5.2 Hz), and 4.36 (ddd, J = 10.4, 10.4, 5.2 Hz), characteristic of protons geminal to OH groups. The ^{13}C NMR spectrum showed three downfield methine carbon resonances at δ 64.3, 75.3, and 77.9. The HMBC correlations of δ_H 3.67 with C-15 (δ 39.2), C-8 (δ 41.4), C-14 (δ 43.2), C-5 (δ 45.7), and C-9 (δ 57.2) indicated the presence of one OH group at C-7. COSY correlations between δ 4.01 and H-2 (δ 2.06) and HMBC correlations of δ_H 4.01 with CH_3-20 (δ 12.0), C-2 (δ 29.8), C-5 (δ 45.7), and C-9 (δ 57.2) indicated the position of one OH group at C-1. The third new proton resonance at δ 4.36 showed correlations with C-8 (δ 41.4), C-9 (δ 57.2), and C-12 (δ 49.3), indicating the presence of one OH group at C-11. The orientations of these OH groups at C-1, C-7, and C-11 were assigned as α, β, and β, respectively, on the basis of the NOESY experiment. NOESY correlations of H-1 with H-2 and H-9β suggested that H-1 was β-oriented, and H-7 resonated as a broad singlet at δ 3.67, suggesting that the proton was α-oriented.[10] NOESY correlations of H-11 with an α-orientation of CH_3-20 and H-15 suggested that H-11 was α-oriented.[19] Thus, **12** was established as 4α-carboxy-1α,7β,11β,13α-tetrahydroxy-13,16-*seco-ent*-19-norbeyeran-16-oic acid 13,16-lactone.

The two selected filamentous fungi possess the ability to perform regio- and stereoselective hydroxylation, oxidation, and methylation. *A. niger* possesses the abilities not only to isomerize the lactone ring but also to cause regio- and stereoselective hydroxylation at the 1α-, 7β-, and 11β-positions in this study. Compounds **2**−**6**, **8**−**10**, and **12** were examined for their activity in the ARE assay to search for novel potential androgen agonists that may be useful for treating androgen insufficiency. Compounds **3**, **6**, and **10** were significantly active; in particular, **6** was more active than testosterone (Table 4). The results presented herein provide preliminary information on the potential use of microbial metabolites as androgen agonists. It also demonstrates the use of microbial transformation techniques as a useful tool for preparing new derivatives of diterpenoids that are inaccessible by chemical means.

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 recorded on a Perkin-Elmer spectrum GX/AutoImage microscope FT-IR spectrometer. NMR spectroscopic data were recorded on a Bruker Avance-400 spectrometer. Mass spectra were recorded on a JMS-700 HRMS spectrometer and a VG platform electrospray mass spectrometer. X-ray single-crystal diffraction was measured on a Siemens SMART CCD XRD. Column chromatography (CC) was performed with MCI-gel CHP 20P (75−150 μm, Mitsubishi Chemical, Tokyo, Japan), Cosmosil 75C_{18}-OPN (Nacalai Tesque, Kyoto, Japan), and Kieselgel silica (70−230 and 230−400 mesh, Merck, Darmstadt, Germany). Spots for all compounds were detected by spraying with 10% H_2SO_4, followed by heating.

Substrate. Isosteviol (**1**) was obtained as previously reported.[8] Compounds **2** and **3** were prepared as described previously[16] and obtained in 34% and 0.16% yields, respectively.

Compound 3: cubic crystals (EtOAc); mp 249−251 °C; $[\alpha]^{25}_D$ +39.6 (*c* 0.5, CH_3OH); IR (KBr) V_{max} 3429 (OH), 1665, 1607 (C=O) cm^{-1}; ^1H and ^{13}C NMR, see Tables 1 and 3; HRFABMS *m/z* 335.2218 $[M + H]^+$ (calcd for $C_{20}H_{31}O_4$, 335.2222).

X-ray Crystallographic Data for 3: $C_{20}H_{30}O_4$, M 334.44, orthorhombic, $P2_12_12_1$, a 7.47470(10) Å, b 11.0181(2) Å, c 21.0792(4) Å, V 1736.02(5) $Å^3$; Z 4, D_{calcd} 1.280 g cm^{-3}, F(000) 728, λ(Mo Kα) 0.71073 Å, T 295(2) K, 11 717 reflections collected. Final GooF 1.036, final R indices R_1 0.0447, wR_2 0.1162, 218 parameters, μ 0.087 mm^{-1}, R indices based on 3975 reflections with $I > 2\sigma(I)$, absorption corrections applied. Complete crystallographic data of **3** have been deposited in the Cambridge Crystallographic Data Centre (CCDC 660698).[20]

Microorganisms and Culture Conditions. All cultures were obtained from the Division of Medicinal and Natural Products Chemistry, College of Pharmacy, University of Iowa, Iowa City, IA, and the Bioresources Collection and Research Center, Hsinchu, Taiwan. Twenty-five microorganisms were used for the preliminary screening including 12 genera (number of species): *Absidia* (one), *Amycolata* (one), *Aspergillus* (four), *Bacillus* (two), *Cunninghamella* (five), *Beauveria* (one), *Curvularia* (one), *Cylindrocarpon* (one), *Mortierella* (one), *Mucor* (three), *Nocardia* (three), and *Streptomyces* (two). The fermentation protocol for screening was identical to that described previously.[8] Culture controls and substrate control were both run. Ten metabolites were reproducibly produced by *C. bainieri* ATCC 9244 and *A. niger* BCRC 32720.

Table 1. ^{1}H NMR Chemical Shifts of **2** and Metabolites **3–7** (C_5D_5N, δ values in ppm)[a]

position	**2**	**3**	**4**	**5**	**6**	**7**
1	1.62, br d (12.8) 0.76–0.85, m[b]	1.63–1.66, m[b] 0.82, td (13.2, 4.0)	1.58–1.72, m[b] 0.98, td (12.8, 3.6)	1.72–1.78, m[b] 1.01, td (13.2, 4.0)	1.61–1.69, m[b] 0.80–0.90, m[b]	β3.63, dd (11.2, 4.8)
2	2.14–2.25, m[b] 1.39–1.53, m[b]	2.18, m 1.39–1.50, m[b]	2.17–2.37, m[b] 1.50–1.54, m[b]	2.20–2.34, m[b] 1.45–1.56, m[b]	2.09–2.18, m[b] 1.49, m	2.55, qd (13.6, 3.6) 1.93–1.98, m[b]
3	2.40, d (13.2) 1.01–1.10, m[b]	2.39, br d (13.2) 1.09–1.18, m[b]	2.43–2.50, m[b] 1.08, m	2.45, br d (13.2) 1.18, m	2.38, br d (13.2) 1.00–1.09, m[b]	2.42, dt (13.2, 3.2) 1.19–1.28, m[b]
5	1.01–1.10, m[b]	1.09–1.18, m[b]	2.17–2.37, m[b]	2.20–2.34, m[b]	1.12–1.25, m[b]	1.08, dd (12.2, 2.0)
6	2.14–2.25, m[b] 1.92, m	1.89–2.02, m[b]	2.43–2.50, m[b] 2.17–2.37, m[b]	2.37, m 2.20–2.34, m[b]	2.09–2.18, m[b] 1.92, m	2.25, m 1.93–1.98, m[b]
7	1.39–1.53, m[b] 1.15–1.23, m[b]	1.39–1.50, m[b] 1.09–1.18, m[b]	α3.72, br s	α3.72, br s	2.45, dt (12.8, 3.2) 0.80–0.90, m[b]	1.42–1.53, m[b] 1.19–1.28, m[b]
9	0.76–0.85, m[b]	0.91, dd (12.8, 3.6)	1.58–1.72, m[b]	1.72–1.78, m[b]	1.00–1.09, m[b]	1.19–1.28, m[b]
11	1.39–1.53, m[b] 1.15–1.23, m[b]	1.54, m 1.39–1.50, m[b]	1.58–1.72, m[b] 1.35–1.43, m[b]	1.65, br d (18.0) 1.45–1.56, m[b]	2.02, dd (13.2, 2.8) 1.61–1.69, m[b]	3.30–3.37, m[b] 1.60, m
12	1.82, m 1.33, m	1.89–2.02, m[b] 1.26, m	1.83, br d (12.8) 1.35–1.43, m[b]	1.93, br d (12.4) 1.31, m	2.55, m 1.12–1.25, m[b]	1.84, m 1.42–1.53, m[b]
14	1.39–1.53, m[b] 1.28, m	1.63–1.66, m[b] 1.09–1.18, m	2.17–2.37, m[b] 1.50–1.54, m[b]	2.11, dd (13.2, 2.4) 1.72–1.78, m[b]	2.31, dd (13.6, 2.4) 1.12–1.25, m[b]	1.42–1.53, m[b] 1.39, m
15	3.20, dd (18.4, 2.4) 2.06, br d (18.4)	4.83, dd (12.0, 2.8) 3.85, dd (12.0, 0.8)	3.23, d (18.0) 2.17–2.37, m[b]	4.88, dd (12.0, 2.4) 3.97, d (12.0)	9.98, s	3.30–3.37, m[b] 2.10, d (18.4)
16-OCH_3					3.56, s	
17-CH_3	1.25, s	1.23, s	1.29, s	1.28, s	1.10, s	1.23, s
18-CH_3	1.32, s	1.31, s	1.38, s	1.38, s	1.29, s	1.33, s
20-CH_3	1.04, s	1.06, s	1.13, s	1.16, s	0.88, s	1.37, s

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

Table 2. ^{1}H NMR Chemical Shifts of Metabolites **8–12** (C_5D_5N, δ values in ppm)[a]

position	**8**	**9**	**10**	**11**[c]	**12**
1	β3.65, dd (11.6, 4.8)	β3.83, dd (11.6, 4.8)	β3.84, dd (10.8, 4.4)	β3.18–3.28, m[b]	β4.01, dd (11.2, 5.2)
2	2.60, m 1.95–2.00, m[b]	2.59–2.65, m[b] 1.95, m	2.58, m 1.86–2.00, m[b]	1.82–1.98, m[b] 1.32–1.49, m[b]	2.63–2.76, m[b] 2.06, m
3	2.43, m 1.21–1.29, m[b]	2.48, m 1.33, m	2.46, d (13.2) 1.33–1.45, m[b]	1.82–1.98, m[b] 0.88, m	2.39–2.46, m[b] 1.27, dd (13.6, 4.0)
5	1.12, m	2.33–2.39, m[b]	2.30–2.40, m[b]	1.19–1.22, m[b]	2.39–2.46, m[b]
6	2.11, m 1.95–2.00, m[b]	2.59–2.65, m[b] 2.33–2.39, m[b]	2.30–2.40, m[b]	3.18–3.28, m[b] 2.33, dd (14.0, 1.6)	2.63–2.76, m[b] 2.39–2.46, m[b]
7	1.41–1.49, m[b] 1.21–1.29, m[b]	α3.71, br s	α3.72, br s		α3.67, br s
9	1.36, m	2.06, dd (12.8, 2.8)	2.13–2.25, m[b]	1.32–1.49, m[b]	2.17, d (10.4)
11	3.26, m 1.85, m	3.36–3.43, m[b] 1.71, m	3.35, d (15.6) 1.86–2.00, m[b]	2.81–2.87, m[b] 1.19–1.22, m[b]	α4.36, ddd (10.4, 10.4, 5.2)
12	1.95, m 1.41–1.49, m[b]	1.86, m 1.49–1.56, m[b]	1.86–2.00, m[b] 1.49, m	1.69, br d (11.6) 1.32–1.49, m[b]	2.63–2.76, m[b] 1.89, dd (14.0, 3.6)
14	1.70, dd (12.8, 2.8) 1.21–1.29, m[b]	2.33–2.39, m[b] 1.49–1.56, m[b]	2.13–2.25, m[b] 1.75, d (12.4)	1.82–1.98, m[b] 1.55, dd (14.2, 1.6)	2.50, dd (14.0, 2.4) 1.50, dd (14.0, 3.2)
15	4.95, dd (12.0, 2.8) 3.93, d (12.0)	3.36–3.43, m[b] 2.21, d (18.4)	4.98, d (11.6) 4.02, d (11.6)	2.81–2.87, m[b] 2.21, d (18.4)	3.45, dd (18.4, 2.0) 2.21, d (18.4)
17-CH_3	1.23, s	1.28, s	1.27, s	1.28, s	1.34, s
18-CH_3	1.34, s	1.39, s	1.38, s	0.97, s	1.40, s
20-CH_3	1.39, s	1.48, s	1.48, s	0.99, s	1.57, s

[a] Assignments based on DEPT, HSQC, and HMBC. Signal multiplicity and coupling constants (Hz) are in parentheses. [b] Overlapping signals. [c] In DMSO-d_6.

Table 3. ^{13}C NMR Chemical Shifts of Compounds **2**−**12** (C_5D_5N, δ values in ppm)[a]

position	2	3	4	5	6	7	8	9	10	11[b]	12
1	40.1	40.1	40.0	40.0	39.3	81.1	80.6	81.4	81.0	79.4	77.9
2	19.5	19.6	19.5	19.6	19.8	30.7	30.8	30.8	30.9	29.6	29.8
3	38.5	38.5	38.5	38.5	38.5	36.7	36.7	36.9	36.9	35.5	36.5
4	43.8	43.8	43.4	43.4	43.7	43.6	43.6	43.2	43.2	42.7	43.5
5	57.1	57.3	47.3	47.6	56.6	55.9	56.0	45.9	46.0	53.4	45.7
6	20.2	21.0	28.4	29.5	21.3	20.2	21.1	28.4	29.5	37.8	28.4
7	43.6	40.6	74.4	72.0	37.3	44.0	41.3	74.7	72.5	211.7	75.3
8	35.1	34.8	39.7	39.1	50.0	35.9	35.5	40.5	39.8	47.9	41.4
9	55.6	56.0	49.2	49.5	57.2	56.7	57.3	50.5	51.0	56.5	57.2
10	38.1	38.2	38.0	38.1	38.1	44.4	44.5	44.3	44.4	43.2	46.6
11	18.8	20.9	18.6	20.7	18.5	22.0	24.3	21.9	24.2	20.6	64.3
12	38.7	39.9	38.4	39.6	36.3	39.2	40.4	39.1	40.3	37.7	49.3
13	79.8	40.2	80.7	39.9	41.9	79.8	40.0	80.7	39.8	80.2	80.6
14	47.5	47.9	42.5	42.9	48.9	47.7	48.1	43.1	43.3	38.8	43.2
15	39.1	78.1	38.2	77.0	206.1	39.6	78.5	38.8	77.3	34.5	39.2
16	171.8	176.1	171.8	176.3	177.3	172.1	176.4	172.1	176.6	170.1	171.1
17	28.4	25.4	28.5	25.6	29.4	28.4	25.5	28.7	25.8	27.9	28.2
18	29.2	29.2	29.0	29.0	29.0	29.1	29.1	29.0	29.0	28.4	29.4
19	179.7	179.7	180.1	180.2	179.7	179.9	179.6	180.1	180.1	178.6	180.3
20	13.8	13.8	13.6	13.6	13.7	10.4	10.4	10.3	10.4	9.2	12.0
16-OCH_3					51.6						

[a] Assignments based on DEPT, HMBC, and HSQC. [b] In DMSO-d_6.

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

compd	luciferase activity	compd	luciferase activity
Tes	2.08 ± 0.62	**6**	2.17 ± 1.45
3	1.73 ± 0.54	**10**	2.04 ± 1.03

[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.

Preparative-Scale Fermentation of 2 by *C. bainieri* ATCC 9244. *C. bainieri* was grown according to the usual fermentation procedure.[8] Using 24-h-old stage II cultures, 1 g of **2**, 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. Extraction as previously described[19] produced 2.8 g of brown oil. The oil was subjected to CC over silica gel. In total, three fractions (A−C) were obtained from eluting with a mixture of CH_2Cl_2−EtOH (10:1) and EtOH. Fraction A (1.1 g) was further fractionated on a silica gel column using *n*-hexane−EtOAc to give six crude fractions (A-1−A-6). Fractions A-4 (520 mg), A-5 (10 mg), and A-6 (9 mg) were recrystallized with *n*-hexane−EtOAc (2:1) respectively to give **4** (500 mg), **5** (5 mg), and **6** (6 mg). Fraction A-3 (450 mg) was recrystallized with *n*-hexane−EtOAc (2:1), and 406 mg of **2** was recovered. The mother liquid of A-3 was evaporated in vacuum and then subjected to CC over silica gel eluted with *n*-hexane−EtOAc (2:1) to give **3** (3 mg).

Compound 4: cubic crystals (*n*-hexane−EtOAc); mp 289−290 °C; $[\alpha]^{25}_D$ +30.8 (*c* 1.0, CH_3OH); IR (KBr) ν_{max} 3395 (OH), 1719, 1683 (C=O) cm^{-1}; ^1H and ^{13}C NMR, see Tables 1 and 3; HRFABMS *m/z* 351.2177 $[M + H]^+$ (calcd for $C_{20}H_{31}O_5$, 351.2171).

X-ray Crystallographic Data for 4: $C_{20}H_{30}O_5$, M 350.44, orthorhombic, $P2_12_12_1$, a 7.7583(2) Å, b 14.2010(3) Å, c 15.9128(3) Å; V 1753.20(7) $Å^3$; Z 4, D_{calcd} 1.328 g cm^{-3}, F(000) 760, λ(Mo Kα) 0.71073 Å, T 295(2) K, 10 056 reflection collected. Final GooF 1.043, final R indices R_1 0.0485, wR_2 0.1290, 227 parameters, μ 0.094 mm^{-1}, R indices based on 4009 reflections with $I > 2\sigma(I)$ absorption corrections applied. Complete crystallographic data of **4** have been deposited in the Cambridge Crystallographic Data Centre (CCDC 660699).[20]

Compound 5: white powder; $[\alpha]^{25}_D$ +20.4 (*c* 0.5, CH_3OH); IR (KBr) ν_{max} 3495 (OH), 1708, 1689 (C=O) cm^{-1}; ^1H and ^{13}C NMR, see Tables 1 and 3; HRFABMS *m/z* 351.2166 $[M + H]^+$ (calcd for $C_{20}H_{31}O_5$, 351.2171).

Compound 6: acicular crystals (*n*-hexane−EtOAc); mp 247−249 °C; $[\alpha]^{25}_D$ +26.2 (*c* 0.5, CH_3OH); IR (KBr) ν_{max} 1722, 1683 (C=O) cm^{-1}; ^1H and ^{13}C NMR, see Tables 1 and 3; HRESIMS *m/z* 365.2338 $[M + H]^+$ (calcd for $C_{21}H_{33}O_5$, 365.2328).

X-ray Crystallographic Data for 6: $C_{21}H_{32}O_5$, M 364.47, monoclinic, $P2_12_12_1$, a 11.6522(3) Å, b 14.4030(3) Å, c 12.4585(3) Å, V 1985.28(8) $Å^3$; Z 4, D_{calcd} 1.219 g cm^{-3}, F(000) 792, λ(Mo Kα) 0.71073 Å, T 295(2) K, 12 683 reflection collected. Final GooF 1.103, final R indices R_1 0.0628, wR_2 0.1563, 470 parameters, μ 0.085 mm^{-1}, R indices based on 8181 reflections with $I > 2\sigma(I)$ absorption corrections applied. Complete crystallographic data of **6** have been deposited in the Cambridge Crystallographic Data Centre (CCDC 660700).[20]

Preparative-Scale Fermentation of 2 by *A. niger* BCRC 32720. *A. niger* was grown according to the usual fermentation procedure,[8] and stage II fermentations were conducted as with the *C. bainieri* preparative-scale reaction and terminated after 144 h following the addition of **2** (1 g). Extraction as previously described[19] produced 3.1 g of black oil. The oil was subjected to CC over silica gel. In total, four fractions (A−D) were eluted with mixtures of CH_2Cl_2−CH_3OH. Further chromatography of fraction D (0.9 g) over silica gel eluted with *n*-hexane−EtOAc yielded four fractions (D-1−D-4). Fraction D-2 (236 mg) was chromatographed over silica gel eluted with CH_2Cl_2−CH_3OH (15:1), and this yielded 4 mg of **4**. Fraction D-3 (80 mg) was chromatographed over silica gel eluted with CH_2Cl_2−CH_3OH (20:1) to give three fractions (D-3a−D-3c). Fraction D-3b (25 mg) was applied to an MCI-gel CHP 20P column eluted with CH_3OH−H_2O (80:20) to yield **8** (4 mg). Fraction D-4 (95 mg) was chromatographed over silica gel eluted with CH_2Cl_2−CH_3OH (8:1) to give three subfractions (D-4a−D-4c). Fraction D-4c (24 mg) was chromatographed over Cosmosil 75C_8-OPN eluted with CH_3OH−H_2O (25:75), and **9** (10 mg) was obtained. Fraction E (400 mg) was chromatographed over silica gel eluted with *n*-hexane−EtOAc, and three fractions (E-1−E-3) were obtained. Fractions E-2 (263 mg) and E-3 (20 mg) were respectively recrystallized with *n*-hexane−EtOAc (2:1) to give **2** (200 mg) and **3** (5 mg). Fraction F (400 mg) was chromatographed over silica gel eluted with *n*-hexane−EtOAc to afford two fractions (F-1 and F-2). Chromatography of fraction F-1 (207 mg) over silica gel eluted with CH_2Cl_2−CH_3OH (7:1) yielded **9** (56 mg) and **10** (14 mg). Fraction F-2 (56 mg) was chromatographed over MCI-CHP 20 eluted with CH_3OH−H_2O (80:20) to yield **12** (14 mg). Fraction G (190 mg) was applied to Cosmosil 75C_8-OPN eluted with CH_3OH−H_2O (25:75) to give two fractions (G-1 and G-2). Chromatography of fractions G-1 (151 mg) and G-2 (11 mg) over MCI-CHP 20 eluted with CH_3OH−H_2O (80:20) yielded **7** (60 mg) and **11** (6 mg), respectively.

Compound 7: white powder; $[\alpha]^{25}_D$ +21.6 (*c* 0.5, CH_3OH); IR (KBr) ν_{max} 3426 (OH), 1722, 1688 (C=O) cm^{-1}; ^1H and ^{13}C NMR, see Tables 1 and 3; HRESIMS *m/z* 351.2161 $[M + H]^+$ (calcd for $C_{20}H_{31}O_5$, 351.2171).

Compound 8: white powder; $[\alpha]^{25}_{D}$ +23.6 (*c* 0.5, CH_3OH); IR (KBr) ν_{max} 3470 (OH), 1715, 1698 (C=O) cm^{-1}; ^1H and ^{13}C NMR, see Tables 2 and 3; HRESIMS *m/z* 351.2174 $[M + H]^+$ (calcd for $C_{20}H_{31}O_5$, 351.2171).

Compound 9: cubic crystals (EtOAc); mp 274−276 °C; $[\alpha]^{25}_{D}$ +17.2 (*c* 0.5, CH_3OH); IR (KBr) ν_{max} 3470 (OH), 1717, 1697 (C=O) cm^{-1}; ^1H and ^{13}C NMR, see Tables 2 and 3; HRESIMS *m/z* 367.2139 $[M + H]^+$ (calcd for $C_{20}H_{31}O_6$, 367.2121).

Compound 10: white powder; $[\alpha]^{25}_{D}$ +40.8 (*c* 0.5, CH_3OH); IR (KBr) ν_{max} 3537, 3409 (OH), 1712, 1694 (C=O) cm^{-1}; ^1H and ^{13}C NMR, see Tables 2 and 3; HRESIMS *m/z* 367.2144 $[M + H]^+$ (calcd for $C_{20}H_{31}O_6$, 367.2121).

Compound 11: white powder; $[\alpha]^{25}_{D}$ +30.3 (*c* 0.5, CH_3OH); IR (KBr) ν_{max} 3420 (OH), 1704 (C=O) cm^{-1}; ^1H and ^{13}C NMR, see Tables 2 and 3; HRESIMS *m/z* 363.1806 $[M - H]^-$ (calcd for $C_{20}H_{27}O_6$, 363.1808).

Compound 12: white powder; $[\alpha]^{25}_{D}$ +43.2 (*c* 0.5, CH_3OH); IR (KBr) ν_{max} 3498 (OH), 1707, 1687 (C=O) cm^{-1}; ^1H and ^{13}C NMR, see Tables 2 and 3; HRESIMS *m/z* 383.2077 $[M + H]^+$ (calcd for $C_{20}H_{31}O_7$, 383.2070).

Transfection Procedures and Reporter Gene Assays. Experiments were carried out following procedures described previously.[9]

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Supporting Information Available: X-ray structure and crystallographic data in CIF format of **2** are available via the Internet at http://pubs.acs.org.

References and Notes

(1) Zhi, L.; Martinborough, E. *Annu. Rep. Med. Chem.* **2001**, *36*, 169–180.
(2) Gao, W. Q.; Dalton, J. T. *Drug Discovery Today* **2007**, *12*, 241–248.
(3) Lee, D. K.; Chang, C. S. *J. Steroid Biochem. Mol. Biol.* **2003**, *84*, 41–49.
(4) Chen, J. Y.; Hwang, D. J.; Bohl, C. E.; Miller, D. D.; Dalton, J. T. *J. Pharmacol. Exp. Ther.* **2005**, *312*, 546–553.
(5) Chen, J.; Kim, J. H.; Dalton, J. T. *Mol. Interv.* **2005**, *5*, 173–188.
(6) Holland, H. L.; Weber, H. K. *Curr. Opin. Biotechnol.* **2000**, *11*, 547–553.
(7) Lehmann, L. R.; Stewart, J. D. *Curr. Org. Chem.* **2001**, *5*, 439–470.
(8) Hsu, F. L.; Hou, C. C.; Yang, L. M.; Cheng, J. T.; Chi, T. C.; Liu, P. C.; Lin, S. J. *J. Nat. Prod.* **2002**, *65*, 273–277.
(9) Chang, S. F.; Yang, L. M.; Lo, C. H.; Liaw, J. H.; Wang, L. H.; Lin, S. J. *J. Nat. Prod.* **2008**, *71*, 87–92.
(10) De Oliveira, B. H.; dos Santos, M. C.; Leal, P. C. *Phytochemistry* **1999**, *51*, 737–741.
(11) Leuenberger, H. G. W. *Pure Appl. Chem.* **1990**, *62*, 753–768.
(12) Ciceri, P.; Joachim, F. W.; de Souza, M. C. F.; Lehmann, M. *J. Braz. Chem. Soc.* **1998**, *9*, 409–414.
(13) Butkus, E.; Stonius, S. *J. Chem. Soc., Perkin Trans 1* **2001**, 1885–1888.
(14) Nobilee, E.; Aniol, M.; Wawrzeńczyk, C. *Tetrahedron* **1994**, *50*, 10339–10344.
(15) Jada, S. R.; Subur, G. S.; Matthews, C.; Hamzah, A. S.; Lajis, N. H.; Saad, M. S.; Stevens, M. F. G.; Stanslas, J. *Phytochemistry* **2007**, *68*, 904–912.
(16) Braguini, W. L.; Biazon, M. A.; de Oliveira, B. H.; Carnieri, E. G. S.; Rocha, M. E. M.; de Oliveira, M. B. M. *Toxicol. Lett.* **2003**, *143*, 83–92.
(17) Militsina, O. I.; Kovyljaeva, G. I.; Bakaleynik, G. A.; Strobykina, I. Y.; Kataev, V. E.; Alfonsov, V. A.; Musin, R. Z.; Beskrovny, D. V.; Litvinov, I. A. *Mendeleev Commun.* **2005**, *1*, 27–29.
(18) Hanson, J. R. *Nat. Prod. Rep.* **1992**, *9*, 139–151.
(19) Chang, S. F.; Yang, L. M.; Hsu, F. L.; Hsu, J. Y.; Liaw, J. H.; Lin, S. J. *J. Nat. Prod.* **2006**, *69*, 1450–1455.
(20) CCDC 660698, 660699, and 660700 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge viahttp://www.ccdc.cam.ac.uk/conts/retrieving.html, or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 1223 336 033; e-mail: data_request@ccdc.cam.ac.uk).

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