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ABSTRACT

The biotransformation of dihydroisosteviol with *Absidia pseudocylindrospora* ATCC 24169, *Streptomyces griseus* ATCC 10137, *Mucor recurvatus* MR36, and *Aspergillus niger* BCRC 31130 yielded 15 metabolites, eight of which were previously unknown. Structures of metabolites were established by 2D NMR techniques and HRMS data, two of which were further corroborated by chemical means, and another via single-crystal X-ray diffraction analysis. Subsequently, two steroidogenic cell lines (Y-1 mouse adrenal tumor and MA-10 mouse Leydig tumor cells) were used in a reverse transcription-PCR analysis to assess the effects of all compounds on steroidogenic gene expressions using forskolin as a positive control. The tested gene expressions included steroidogenic factor-1 (SF-1), steroidogenic acute regulatory protein (StAR), and cytochrome P450 side-chain cleavage (P450scc) enzyme. Gene expression profiles showed that ten of the tested compounds effectively suppressed $P450_{SCC}$ mRNA expression in both Y-1 and MA-10 cells. Several induced SF-1 gene expression and two enhanced StAR gene expression in Y-1 cells. By contrast, in MA-10 cells, one compound effectively suppressed StAR mRNA expression, whereas for others effectively suppressed SF-1 gene expression. The results suggest that analogs of dihydroisosteviol can be potential modulators to alter steroidogenic gene expressions and subsequent enzyme activities.

1. Introduction

Biotransformation involves enzymatic reactions that can often show characteristic regio- and stereoselectivities. Such reactions provide an alternative method for producing novel and useful products that cannot be obtained, or can be obtained only with great difficulty, using conventional chemical approaches (Arantes and Hanson, 2007; Gładkowski et al., 2007; Venisetty and Ciddi, 2003). Enzyme-assisted biotransformation reactions are caused by enzymes inside microorganisms, and frequently include hydroxylation, oxidation, reduction, methylation, acetylation, glycosylation, and isomerization reactions (Liu and Yu, 2010). Dihydroisosteviol (**2**), a diterpenoid with an *ent*-beyerane backbone, can be prepared by reducing isosteviol (**1**) with $NaBH_4$ (Ali et al., 1992). De Oliveira et al. (1999) reported that introducing an OH group to nonhydroxylated diterpenoids might enhance their existing biological properties or lead to new biological capabilities, because the hydroxylation pattern of active compounds might influence, for example, the receptor binding.

The microbial transformation of **2** was previously studied, including evaluation of the transformed products against hypertension and cytotoxicity in $HepG_2$ cells (Lin et al., 2007; Yang et al., 2004). In addition, Dorfman and Nes (1960) reported that inunction of **2** to chicks' combs inhibited the action of injected testosterone. However, **2** did not inhibit testosterone action on the seminal vesicles, prostate, and levator ani muscle of castrated rats. Testosterone is primarily synthesized in testicular Leydig cells in a steroidogenic process that involves cholesterol; the testosterone is then released into circulation (Miller, 1988). Steroidogenesis in Leydig cells is stimulated by luteinizing hormone (LH) binding to its cognate receptor and activating adenylyl cyclase and thereafter the G-protein-mediating signaling pathway. The increased intracellular cAMP triggers phosphorylation cascade of protein kinase A (PKA). In turn, this activates expression of steroidogenic genes, such as genes that encode the steroidogenic acute regulatory protein (StAR) and cytochrome P450-mediated cholesterol side-chain cleavage ($P450_{SCC}$) (Richards, 2001). The StAR protein is a crucial regulator of steroidogenesis, which mediates transport of cholesterol from the outer to the inner mitochondrial membrane (Stocco and Clark, 1996). In males, the rate-limiting enzyme, cytochrome $P450_{SCC}$, oxidizes and converts cholesterol to pregnenolone, which

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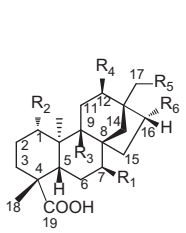
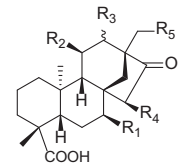
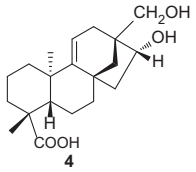
then is transported to the smooth endoplasmic reticulum for the synthesis of testosterone for reproduction (Habert et al., 2001).



	R_1	R_2	R_3	R_4	R_5	R_6
2	H	H	H	H	H	OH
3	OH	H	H	H	OH	OH
6	H	H	OH	H	OH	OH
8	H	H	H	OH	H	OH
9	H	H	H	H	OH	OH
14	H	OH	H	H	H	OH
15	$O(CO)_2OCH_3$	H	H	H	H	OH
16	OH	H	H	H	H	$O(CO)_2OCH_3$
17	OH	H	H	H	H	OH
18	H	H	H	H	H	$O(CO)_2OCH_3$
19	$O(CO)_2OCH_3$	H	H	H	H	$O(CO)_2OCH_3$



	R_1	R_2	R_3	R_4	R_5
1	H	H	H	H	H
5	H	H	α-OH	H	OH
7	H	H	β-OH	H	OH
10	OH	H	H	H	H
11	H	H	β-OH	OH	H
12	OH	H	H	OH	H
13	H	OH	H	OH	H



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Steroidogenic factor-1 (SF-1), an orphan nuclear hormone receptor, is highly expressed in testes and ovaries, and in several other tissues or organs including skin, pituitary, spleen, brain, and adrenal glands (Lin and Achermann, 2008). The main function of SF-1 is to mediate the expression of several gene-encoding enzymes required either for testosterone biosynthesis (in testicular Leydig cells) or for androgen synthesis (in the adrenals). This transcriptional factor is known to be regulated by interacting with numerous other nuclear proteins (Ye et al., 2009). Thus, SF-1 is required for the transcription of steroidogenic genes and cholesterol transport protein, as well as for development and functioning of the entire endocrine system (Hoivik et al., 2010). Accordingly, SF-1 modulators can be exploited to suppress both androgen and gonadal testosterone synthesis in the treatment of prostate cancer and adrenocortical tumors (Doghman et al., 2009). In general, natural products always play a vital role in drug discovery and are useful as template molecules for producing new drugs (Lee, 2010; Venisetty and Ciddi, 2003). Due to some diterpenoids possessing some level of similarity to steroids (Hanson, 1992), the effect of tetracyclic diterpenoids on the androgen response element was previously examined (Chang et al., 2008; Chou et al., 2008). Compound **2** reportedly displays anti-androgenic activity (Dorfman and Nes, 1960). Thus, these findings led to the hypothesis that analogs of **2** must function on potential modulators for steroidogenic gene expressions, and the biotransformation of **2** was thus investigated. Subsequently, the effect of **2** and its analogs on expression of steroidogenic genes, including gene-encoding StAR, P450scc, and SF-1, was analyzed by a reverse transcription-PCR analysis. A bioassay system was developed on the Y-1 mouse adrenal tumor and the MA-10 mouse Leydig tumor cell lines. Forskolin, a potent stimulator of adenylate cyclase, was used as a positive control (Seamon et al., 1981) to increase the intracellular cAMP. This report discusses the isolation, structure elucidation, and bioactivity results for these biotransformed compounds. Additionally, 4 derivatives prepared by chemical means are also reported herein.

2. Results and discussion

Four microorganisms have been shown to consistently convert **2** into several metabolites, based on previous screening results (Yang et al., 2004). These microorganisms are *Absidia pseudocylindrospora*, *Streptomyces griseus*, *Mucor recurvatus*, and *Aspergillus niger*. Incubation of **2** with *Abs. pseudocylindrospora* gave compounds **3**–**6**, whereas *S. griseus* afforded compounds **3** and **7**–**9**. Incubation of **2** with *M. recurvatus* resulted in formation of **10**–**14**, and incubation of **2** with *Asp. niger* afforded compounds **15**–**17**. Characterization of these compounds using NMR spectroscopic and HRMS data established that **5**, **7**, **9**–**12**, and **17** had been previously described (Chang et al., 2006; De Oliveira and Strapasson, 1996; De Oliveira et al., 2008; Hsu et al., 2002; Yang et al., 2004), whereas **3**, **4**, **6**, **8**, and **13**–**16** had not yet been reported.

The ^{1}H, ^{13}C NMR, DEPT, and the HSQC spectra of compounds **3**, **4**, and **6**, in contrast with those of **2**, showed the disappearance of the CH_3-17 group at δ_H 1.06 (δ_C 25.7) and the presence of a new $-CH_2OH$ group. The $-CH_2OH$ group resonated as a pair of doublets at δ_H 3.87 (*d*, *J* = 10.5 Hz) and 3.97 (*d*, *J* = 10.5 Hz) (δ_C 69.1) in **3**; at δ_H 3.94 (*d*, *J* = 10.5 Hz) and 4.00 (*d*, *J* = 10.5 Hz) (δ_C 68.9) in **4**; and at δ_H 3.89 (*d*, *J* = 10.5 Hz) and 3.95 (*d*, *J* = 10.5 Hz) (δ_C 69.1) in **6**. These findings suggested that the methyl group had been oxidized during incubation. Thus, **3**, **4**, and **6** possessed the basic backbone of *ent*-16β,17-dihydroxy-beyeran-19-oic acid (**9**) (De Oliveira et al., 2008). Compound **3** exhibited a quasi-molecular ion peak at *m*/*z* 351.2169 $[M-H]^-$ in the HRTOFMS analysis. This implied that the molecular formula of **3** was $C_{20}H_{32}O_5$. The ^{1}H NMR, DEPT and HSQC spectra, compared to those of **9**, showed the disappearance of one CH_2 resonance and the presence of one new CH resonance at δ_C 77.1 (δ_H 3.98). In the HMBC spectrum, the new resonance at δ_H 3.98 showed connectivities with C-6 (δ_C 31.3), C-8 (δ_C 48.1), C-9 (δ_C 51.9), and C-14 (δ_C 47.7), confirming the additional OH group at C-7. The orientation of 7-OH was assigned as β-oriented because of the presence of cross-peaks of H-7 with H-6 (δ 2.51–2.56), H-14α (δ 1.95–2.08), and H-15 (δ 2.30–2.41) in the NOESY spectrum. The C-7 hydrogen resonated as a broad singlet at δ 3.98, indicating that the proton was α-oriented (De Oliveira and Strapasson, 1996). On the basis of these findings, **3** was established as *ent*-7α,16β,17-trihydroxy-beyeran-19-oic acid.

Compound **4** displayed a quasi-molecular ion peak at *m*/*z* 333.2065 $[M-H]^-$ in the HRTOFMS analysis, corresponding to the molecular formula $C_{20}H_{30}O_4$. The ^{1}H, ^{13}C NMR, and HSQC spectra were similar to those of **9**, apart from a new CH resonance at δ_C 117.5 (δ_H 5.69) and one quaternary carbon at δ 156.4. Examination of the HSQC and HMBC spectra of **4** indicated that the new resonance at δ_H 5.69 had connectivities with each of C-8, 10, 12, and 13 (δ_C 43.0, 40.8, 31.9, and 48.6, respectively). The spectra also showed connectivities between CH_3-20 (δ_H 1.49)/H-14 (δ_H 1.87)/H-12 and H-15 (δ_H 2.48–2.51)/H-12 (δ_H 2.91) with δ_C 156.4. These data implied that a double bond was located between C-9 and C-11. Moreover, single-crystal X-ray crystallographic analysis confirmed that **4** was *ent*-16β,17-dihydroxy-beyer-9(11)-en-19-oic acid (Fig. 1).

Compound **6** displayed a quasi-molecular ion peak at *m*/*z* 351.2173 $[M-H]^-$ in the HRESIMS data, corresponding to the molecular formula of $C_{20}H_{32}O_5$. The DEPT and ^{13}C NMR spectroscopic data showed one new carbon resonance at δ 78.5, compared with **9**. The lack of a carbinol-methine resonance in the ^{1}H NMR spectrum and the disappearance of one tertiary carbon in the DEPT spectrum suggested that the OH group might have been introduced at C-5 or C-9. In the ^{13}C NMR spectrum, C-11 had shifted from δ_C 21.0 to 27.1, and CH_3-20 had shifted from δ_C 13.9 to 17.9, compared with **2**. These findings suggested that an OH group might reside at C-9. In the HMBC spectrum, the new resonance at δ_C 78.5 showed connectivities with H-7 and H-14 (δ_H 1.44–1.48), H-11 (δ_H 1.85), H-12 and H-15 (δ_H 2.08–2.20), H-7 and H-12 (δ_H 2.22–2.29), H-14 and H-15 (δ_H 2.61–2.65), and H-11 (δ_H 2.73), confirming the presence of an OH group at C-9. Based on these findings, **6** was established as *ent*-9α,16β,17-trihydroxy-beyeran-19-oic acid.

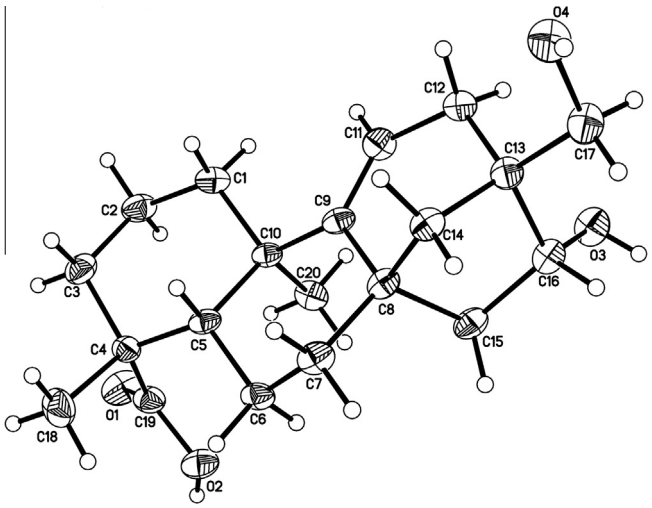

Fig. 1. ORTEP drawing of the X-ray structure of **4**.

Compound **8** exhibited a quasimolecular ion peak at *m*/*z* 335.2227 $[M-H]^-$ in the HRESIMS analysis, corresponding to the molecular formula $C_{20}H_{32}O_4$. The ^{13}C NMR spectrum of **8** exhibited resonances for 20 carbons, whereas the DEPT spectrum showed the disappearance of one CH_2 resonance and the presence of one new CH resonance at δ 71.6. These findings suggested that **8** might be a monohydroxylated product of **2**. Detailed analysis of the DEPT, HSQC, and HMBC spectra of **8** and comparison with **2** established that the new CH resonance at δ_H 4.53 had connectivities with the signals at δ_C 23.4 (CH_3-17), 50.1 (C-14), 52.2 (C-9), and 47.9 (C-13). Thus, the OH group was located at C-12. Additionally, evidence for the equatorial position of H-12 included not only the NOE correlations to H-11 and CH_3-17, but also the multiplicity of the H-12 resonance in the ^1H NMR spectrum showing a singlet resonance (De Oliveira et al., 1999). Accordingly, the 12β-OH was established. On the basis of the above evidence, **8** was characterized as *ent*-12α,16β-dihydroxy-beyeran-19-oic acid.

The molecular formula of **13** was determined to be $C_{20}H_{30}O_5$ from its HRESI mass spectrum analysis (*m*/*z* 349.2028 $[M-H]^-$) and ^1H and ^{13}C NMR spectroscopic data. Examination of the ^{13}C NMR spectrum of **13**, including DEPT, sorted 20 resonances into one carboxylic carbonyl, one carbonyl group, four CH, seven CH_2, three CH_3, and four quaternary carbons. Compared with **2**, the lack of H-16β and C-16 signals in the ^1H and ^{13}C NMR spectra (which were replaced by a carbonyl group at δ_C 221.5) suggested that **13** possessed the same basic backbone as isosteviol (**1**). In the HSQC spectrum, the observation of resonances at δ_H 4.26 (δ_C 67.8) and δ_H 4.63 (δ_C 75.1) indicated that **13** possessed two more oxygen atoms than **1**. In the HMBC spectrum, the signals for H-12 (δ_H 1.93 and 2.22–2.26)/H-9 (δ_H 1.74) exhibited cross-peaks with δ_C 67.8, suggesting an OH group at C-11. In addition, HMBC correlations of δ_H 4.63 with C-7 (δ_C 35.4), and of H-9 (δ_H 1.74)/H-14 (δ_H 1.57–1.60)/H-7 (δ_H 1.29) with δ_C 75.1, enabled the replacement of the second OH group at C-15. The orientations of both OH groups at C-11 and C-15 were assigned to be β, based on a NOESY experiment. NOESY correlations for H-11 were observed for CH_3-20 and H-12. The C-15 hydrogen showed cross-peaks with CH_3-20, H-6, and H-11α. Thus, **13** was characterized as *ent*-11α,15α-dihydroxy-16-oxobeyeran-19-oic acid.

Compound **14** obtained the molecular formula $C_{20}H_{32}O_4$, as deduced from HRESIMS and NMR spectroscopic data. The ^{13}C NMR spectra of **14** displayed resonances for 20 carbons, whereas the DEPT spectrum showed the disappearance of one CH_2 resonance and the presence of one new CH resonance at δ_C 82.0. These findings suggested that **14** might be a monohydroxylated product of **2**. Detailed analysis of the HSQC and HMBC spectra of **14**, and comparisons with **2**, indicated that δ_H 3.74 showed connectivities with each of C-5, 3, 2, and CH_3-20 (δ_C 57.0, 37.4, 31.4, and 10.3, respectively). Thus, the OH group was located at C-1. The α-orientation of 1-OH was established from the NOE correlations of H-1 with H-5 (δ 1.20) and H-9 (δ 1.55). The multiplicity of the H-1 resonance in the ^1H NMR spectrum exhibiting a doublet of doublets (J = 11.0, 4.5 Hz) confirmed that 1-OH was α-oriented (De Oliveira et al., 1999). Based on these observations, **14** was identified as *ent*-1β,16β-dihydroxy-beyeran-19-oic acid.

Compounds **15** and **16**, respectively, displayed quasi-molecular ion peaks at *m*/*z* 421.2237 $[M-H]^-$ and at 421.2248 $[M-H]^-$ (calcd for $C_{23}H_{33}O_7$, 421.2226) in the HRESIMS data. This finding implied a molecular formula of $C_{23}H_{34}O_7$. Their ^{13}C NMR spectra consisted of 23 resolved signals, in agreement with the molecular formula. The multiplicity of the individual resonances determined by DEPT experiments indicated four CH, eight CH_2, three CH_3, one $-OCH_3$, and seven quaternary carbons, including one carboxylic carbonyl (δ_C 180.3 and 181.0, respectively) and a pair of carbonyls (δ_C 158.7/160.2 and 159.0/159.6, respectively). Comparison of the overall NMR spectroscopic data with **2** established that compounds **15** and **16**, respectively, exhibited significant variations in the ^1H and ^{13}C NMR chemical shifts for H-7/C-7 and H-16/C-16. The DEPT and HSQC spectra of **15** and **16** showed the disappearance of one CH_2 resonance and the presence of new resonances at δ_H 5.29 (δ_C 84.4) and δ_H 3.87 (δ_C 76.3), respectively, compared with **2**. Analysis of the HSQC and HMBC spectra of **15** and **16** established that the new resonance at δ_H 5.29 in **15** had connectivities with δ_C 49.3 (C-5) and 52.0 (C-9), whereas the new resonance at δ_H 3.87 in **16** showed connectivities with δ_C 48.1 (C-8) and 50.7 (C-9). Inferred from these observations was that the substituent in both compounds was at C-7. In addition, in **15**, two new carbonyls and one new methoxy group resonated at δ_C 158.7/160.2 and δ_C 53.8/δ_H 3.86 (3H, *s*), respectively. Meanwhile, the new signal at δ_H 3.86 ($-OCH_3$) showed an HMBC correlation with δ_C 160.2. Similarly, in **16**, two new carbonyls and one new methoxy group resonated at δ_C 159.0/159.6 and δ_C 53.7/δ_H 3.78 (3H, *s*), respectively. Meanwhile, the new signal at δ_H 3.78 ($-OCH_3$) showed an HMBC correlation with δ_C 159.6. These findings suggested the existence of a methyloxalyl moiety in both compounds. The ^1H and ^{13}C NMR spectra of **17** (*ent*-7α,16β-dihydroxy-beyeran-19-oic acid) was next compared with compound **16** where it was noted that the resonances of H-7/C-7 in **16** were similar to **17** (δ_H 3.87 vs 3.92/δ_C 76.3 vs 76.6), suggesting an OH group at C-7. However, the resonances of H-7/C-7 in **15** showed a downfield shift from δ_H 3.92 to 5.29/δ_C 76.6 to 84.4, implying that a methyloxalyl moiety might be linked to 7-OH. By contrast, the resonances of H-16β/C-16 in **16** were respectively downfield shifted from δ_H 4.10 to 5.09/δ_C 79.8 to 85.0, implying a methyloxalyl moiety with an ether linkage at 16α-OH. The orientation of the substituent group at C-7 followed from the multiplicity of the H-7 resonating as a singlet signal, indicating that H-7 was α-oriented in both compounds (De Oliveira et al., 1999). However, H-7α in **15** and H-16β in **16** did not show correlations with, respectively, δ_C 158.7 and 159.0 in the HMBC spectra. To clarify this structural ambiguity through chemical means, compound **17** was reacted with methyloxalyl chloride to afford two monomethyloxalyl derivatives (**15S** and **16S**) and one dimethyloxalyl derivative (**19**). All ^{13}C NMR and DEPT spectral chemical shifts for transformed products and synthetic compounds (**15S** and **16S**) were congruent. Both the ^1H NMR and the HRESIMS data (*m*/*z* 445.2216 $[M+Na]^+$ and 421.2233 $[M-H]^-$), as well as the TLC R_f-values were comparable for each of **15** and **16**. In the HMBC spectrum of **15S**, the resonance at δ_H 5.29 (H-7α) showed connectivities with δ_C 42.8 (C-15), 46.1 (C-8), 49.3 (C-5), 52.0 (C-9), and 158.7, whereas δ_H 3.86 (3H, *s*) showed connectivity with δ_C

160.2. In the HMBC spectrum of **16S**, the resonance at δ_H 5.09 (H-16β) showed connectivities with δ_C 25.6 (C-17), 35.2 (C-12), 40.0 (C-15), 42.5 (C-13), 48.2 (C-8), and 159.0, whereas δ_H 3.78 (3H, *s*) showed connectivity with δ_C 159.6. Accordingly, the methyloxalyl moiety was attached at 7β-OH in **15** and at 16α-OH in **16**. Based on these findings, **15** and **16** were established as *ent*-16β-hydroxy-7α-*O*-methyloxalyl-beyeran-19-oic acid and *ent*-7α-hydroxy-16β-*O*-methyloxalyl-beyeran-19-oic acid, respectively.

Compound **18** was an unreported compound obtained from reacting **2** with methyloxalyl chloride. The interpretation of its HRESIMS data coupled with the DEPT and ^{13}C NMR data yielded a molecular formula of $C_{23}H_{34}O_6$ (*m*/*z* 429.2258 [M+Na]$^+$). The resonances of H-16β/C-16 were respectively downfield shifted from δ_H 4.09 to 5.03/δ_C 79.8 to 85.1, compared with **2**. In the HMBC spectrum, the signal at δ_H 5.03 showed connectivities with δ_C 25.4 (C-17), 35.1 (C-12), 41.1 (C-15), 42.5 (C-13), 43.1 (C-8), and 158.9, whereas δ_H 3.76 (3H, *s*) showed connectivity with δ_C 159.2. Thus, **18** was characterized as *ent*-16β-*O*-methyloxalyl-beyeran-19-oic acid, which was prepared for biological testing in this study.

Compound **19** was a reaction product and displayed a quasi-molecular ion peak at *m*/*z* 531.2194 [M+Na]$^+$ in the HRESIMS data, corresponding to the molecular formula $C_{26}H_{36}O_{10}$. The ^{13}C NMR spectrum showed signals for 26 carbons, which were differentiated by DEPT as follows: one carboxylic carbonyl, four carbonyl, four

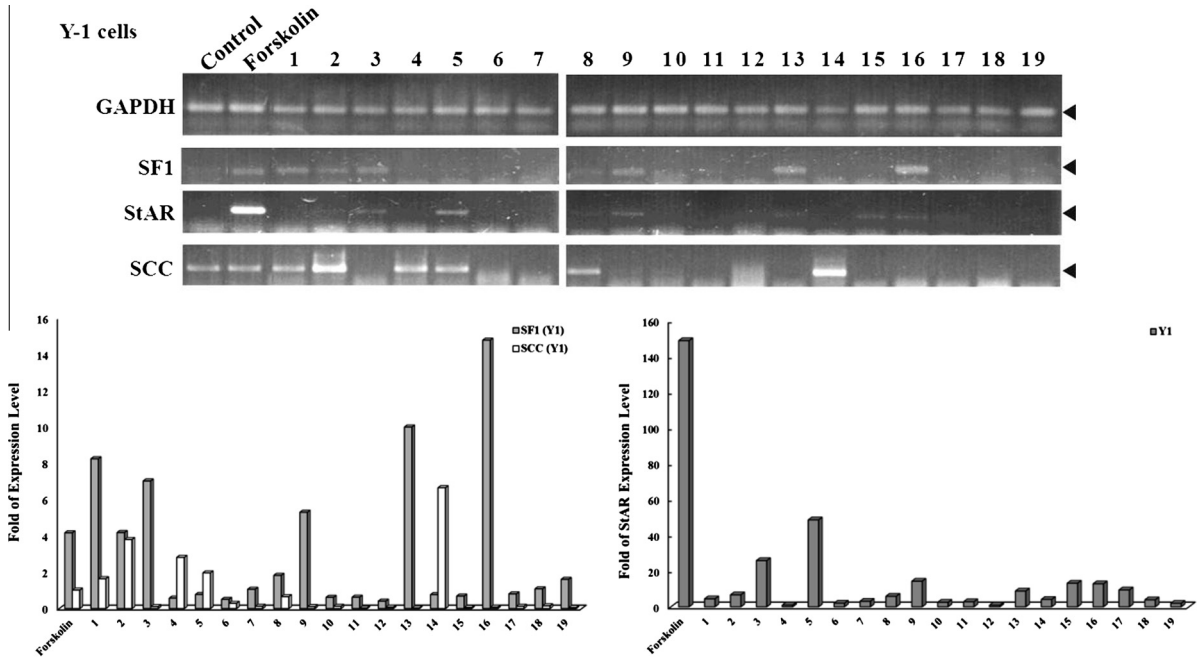

Fig. 2. Effects of compounds **1**–**19** on expression of steroidogenic genes in the Y-1 mouse adrenal tumor cell line.

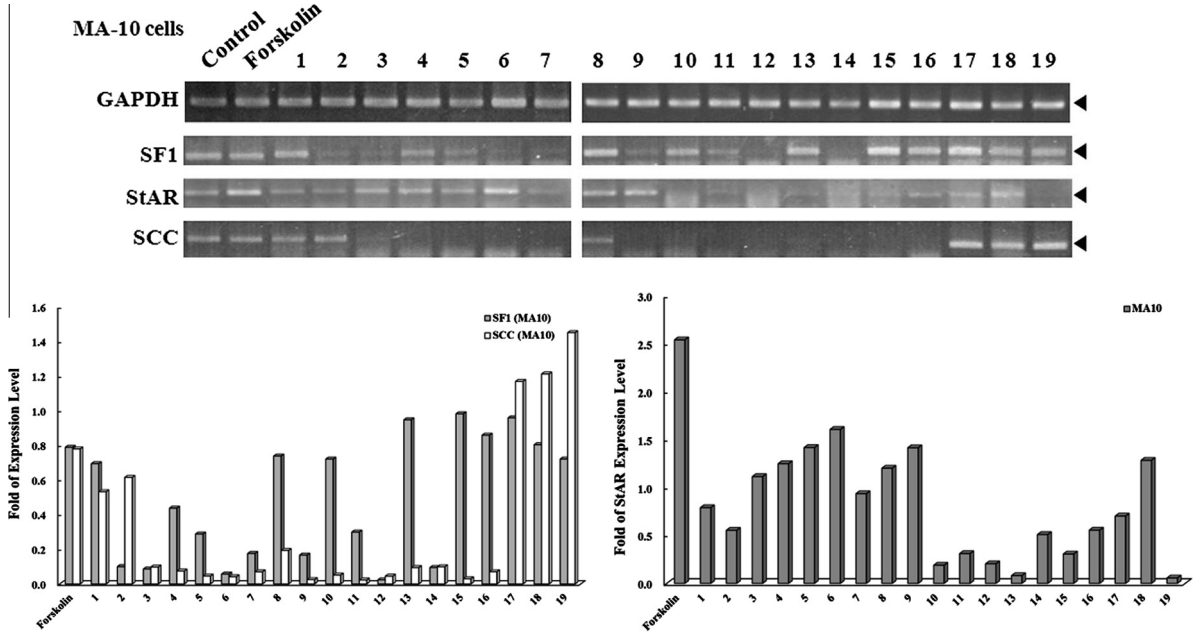

Fig. 3. Effects of compounds **1**–**19** on expression of steroidogenic genes in the MA-10 mouse Leydig tumor cell line.

CH, eight CH_2, three CH_3, two $-OCH_3$, and four quaternary carbons. Analysis of HSQC and HMBC spectra and by comparison with **17** established that δ_H 5.25 showed connectivities with δ_C 27.5 (C-6), 39.4 (C-15), 46.2 (C-8), 49.0 (C-5), 51.4 (C-9), and 158.5, whereas δ_H 3.77 (3H, *s*) showed a correlation with δ_C 159.3. Accordingly, the methyloxalyl moiety was attached to 7β-OH. By contrast, δ_H 5.05 showed connectivities with δ_C 25.2 (C-17), 34.6 (C-12), 39.4 (C-15), 42.4 (C-13), 46.2 (C-8), and 158.8, whereas δ_H 3.85 (3H, *s*) showed connectivity with δ_C 159.9. Thus, a second methyloxalyl moiety was attached to 16α-OH. On the basis of the spectroscopic data, **19** was characterized as *ent*-7α,16β-di-*O*-methyloxalyl-beyeran-19-oic acid.

Subsequently, compounds **1**–**19** were examined for their effects on the expression of steroidogenic genes, including StAR, P450scc, and SF-1. The reverse transcription-PCR analysis was conducted in both Y-1 mouse adrenal tumor and MA-10 mouse Leydig tumor cell lines. Gene expression profiles showed that **3**, **6**, **7**, **9**–**13**, **15**, and **16** effectively suppressed expression of $P450_{SCC}$ mRNA in both Y-1 and MA-10 cells (Figs. 2 and 3). In Y-1 cells, compounds **1**–**3**, **9**, **13**, and **16** induced SF-1 gene expression, whereas **3** and **5** enhanced StAR gene expression (Fig. 2). By contrast, in MA-10 cells, compound **19** effectively suppressed the StAR gene expression, whereas **2**, **3**, **6**, **12**, and **14** effectively suppressed the expression of SF-1 gene (Fig. 3).

3. Conclusions

Fifteen compounds were obtained from the biotransformation of **2**. Among them, **3**, **4**, **6**, **8**, and **13**–**16** had not been reported prior to this study. The formation of **4** might be due to the introduction of an OH group into **2** at the C-9 position, followed by loss of water to form a 9,11-double bond. The specific hydroxylation of **2** at C-17 was observed during incubation with either *Abs. pseudocylindrospora* or *M. recurvatus* in this study. The main reaction detected in *M. recurvatus* was oxidation of 16α-OH into 16-oxo and subsequent stereospecific hydroxylation at B, C, and D rings. The formation of metabolites **15** and **16** were probably artificial fermentation products. A previous study reported that *Asp. niger* produced oxalic acid through the Krebs cycle by forming oxalate through splitting oxalacetate (Cleland and Johnson, 1956). Thus, the formation of methyloxalyl derivatives in *Asp. niger* likely entails initial stereoselective hydroxylation at the 7β position (**17**), followed by introduction of the oxalyl group at 7β-OH and 16α-OH, respectively, to produce the intermediates of 7β-*O*-oxaly and 16α-*O*-oxalyl derivatives. The two intermediates then performed specific methylation at oxalyl moiety to obtain **15** and **16**.

A study on the chick's comb by Dorfman and Nes (1960) showed that inunction with **2** inhibited the action of injected testosterone; however, **2** did not affect testosterone action in the seminal vesicles, prostate, and levator ani muscle of the castrated rat. The results herein showed that **2** did not display any significant effect on steroidogenic gene expression, apart from inducing SF-1 in Y-1 adrenal tumor cells and suppressing SF-1 in MA-10 Leydig tumor cells. Further investigation is required to clarify the underlying molecular mechanism of the anti-androgenic effect of **2**. In addition, introducing OH groups at the 7β-, 7β,15β-, 11β,15β-, 12β,15β-, and 12β,17-positions of isosteviol (**1**) could suppress $P450_{SCC}$ mRNA expression in both Y-1 and MA-10 cells. Compounds **15** and **16**, which possessed a methyloxalyl moiety with an ether linkage at 7β-OH and 16α-OH of **17**, respectively, appeared to be able to reduce the level of $P450_{SCC}$ mRNA in both Y-1 and MA-10 cells. By contrast, in Y-1 cells, **16** displayed a significant induction of SF-1 mRNA, whereas **15** did not (Fig. 2). In MA-10 cells, **17** and **18** with β-OH and α-methyloxalyl groups substituted at C-7 and C-16 of **2**, respectively, did not influence $P450_{SCC}$ mRNA expression. Similarly, **19** possessing methyloxalyl group with an ether linkage at both 7β-OH and 16α-OH of **17** did not show an effect on $P450_{SCC}$ mRNA expression, but it did effectively suppress StAR mRNA expression (Fig. 3). Thus, these observations suggest that the methyloxalyl moiety substitution at the 7β-OH or 16α-OH of **17** may be essential for reducing the level of the $P450_{SCC}$ mRNA in MA-10 cells. Additionally, compounds **12** and **14**, which possessed OH groups at the 7β,15β- and 1α-positions of **1** and **2**, respectively, suppressed SF-1, StAR, and $P450_{SCC}$ mRNA expressions in MA-10 cells, suggesting a potential for the application of them to treat hormone-dependent cancers. The results presented here suggest that analogs of **2** may be potential modulators to alter steroidogenic gene expressions and subsequent enzyme activities. Thus, they may be able to assist in controlling synthesis and metabolism of steroid hormones. Further mechanistic studies are required for a better understanding of their actions and the differences of their effects on steroidogenic gene expression.

4. Experimental

4.1. General

Optical rotations were measured using a JASCO DIP-1020 digital polarimeter. Spectra for ^1H (500 MHz), ^{13}C (125 MHz), and 2D NMR were acquired using on a Bruker Avance 500 spectrometer using the corresponding solvent as the internal standard. Mass spectra were recorded on a VG platform electrospray mass spectrometer, a Shimadzu LCMS-IT-TOF mass spectrometer, and a Bruker maXis impact QTOF mass spectrometer. Column chromatography (CC) was performed using Kieselgel silica (70–230 and 230–400 mesh; Merck, Darmstadt, Germany), MCI-gel CHP 20P (75–150 μm; Mitsubishi Chemical, Tokyo, Japan), and Cosmosil $75C_{18}$-OPN (Nacalai Tesque, Kyoto, Japan). Single-crystal X-ray diffraction was measured using a Siemens SMART CCD XRD.

4.2. Substrate **2**

Dihydroisosteviol (**2**) was prepared as described previously (Ali et al., 1992).

4.3. Microorganisms and preparation of medium

Abs. pseudocylindrospora, *S. griseus*, *M. recurvatus*, and *Asp. niger* 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. They were maintained on Sabouraud-maltose agar slants or those recommended by the ATCC and BCRC, and stored in a refrigerator at 4 °C. The incubation medium consisted of dextrose (20 g), yeast (5 g), NaCl (5 g), K_2HPO_4 (5 g), soybean flour (5 g), and distilled H_2O (1 L). The pH of the medium was adjusted to 7.0 with 6 N HCl before sterilization by autoclaving at 121 °C for 15 min.

4.4. Preparative-scale transformations of **2** by Abs. pseudocylindrospora ATCC 24169, S. griseus ATCC 10137, M. recurvatus MR36, and Asp. niger BCRC 31130

Abs. pseudocylindrospora, *S. griseus*, *M. recurvatus*, and *Asp. niger* were cultivated by a previously described two-stage fermentation protocol (Hsu et al., 2002). Culture controls and substrate control were both run. Using 24-h-old stage II cultures, a solution of **2** (1.00 g dissolved in 10 ml 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 and then extracted as previously described (Hsu et al., 2002). Four

crude residues of brown oils weighing 4.45 g, 5.87 g, 4.41 g, and 5.85 g, respectively, were obtained after bioconversions with *Abs. pseudocylindrospora*, *S. griseus*, *M. recurvatus*, and *Asp. niger*. The crude residue from *Abs. pseudocylindrospora* (4.45 g) was subjected to silica gel CC. Ten fractions (1–10) were obtained by elution with various mixtures of CH_2Cl_2-CH_3OH (500 ml each of 20:1, 18:1, 15:1, 10:1, and 5:1, respectively). Fraction 2 (1.02 g) was subjected to further silica gel CC eluted with CH_2Cl_2-CH_3OH (20:1) and **2** (614 mg) was recovered. Fraction 4 (290 mg) was applied to a silica gel column (CH_2Cl_2-CH_3OH with increasing polarity) to yield subfractions 4.1 and 4.2. With further chromatography of fraction 4.2 (60 mg) over MCI-gel CHP 20P, eluted with CH_3OH-H_2O (1:4) and increasing amounts of CH_3OH, compounds **4** (29 mg) and **5** (6 mg) were obtained. Fraction 8 (220 mg) further purified over silica gel (CH_2Cl_2-CH_3OH with increasing polarity) to yield three subfractions: 8.1–8.3. With further chromatography of fraction 8.2 (47 mg) over Cosmosil 75 C_{18}-OPN, eluted with CH_3OH-H_2O (1:4) and increasing amounts of CH_3OH , **6** (27 mg) was obtained. Fraction 9 (91 mg) was subjected to silica gel CC (CH_2Cl_2-CH_3OH with increasing polarity) to yield three subfractions: 9.1–9.3. With further purification of fraction 9.2 (38 mg) over MCI-gel CHP20P, eluted with CH_3OH-H_2O (1:4) and increasing amounts of CH_3OH, **3** (25 mg) was obtained.

The crude residue (5.87 g) obtained from the liquid culture of *S. griseus* was subjected to silica gel CC eluted with a CH_2Cl_2-CH_3OH gradient system (500 ml each of 20:1, 18:1, 15:1, 10:1, and 5:1, respectively) to yield 12 fractions (1–12). Fraction 6 (1.12 g) was applied to a silica gel column, eluted with CH_2Cl_2-CH_3OH (20:1) to recover **2** (847 mg). Fraction 7 (86 mg) was subjected to silica gel CC, eluted with CH_2Cl_2-$(CH_3)_2CO$ (4:1), and yielded subfractions 7.1 and 7.2. Fraction 7.1 (20 mg) was further applied to CC over MCI-gel CHP 20P, eluted with CH_3OH-H_2O (1:4) and increasing amounts of CH_3OH, to afford **7** (12 mg). Fraction 9 (194 mg) was applied to a silica gel column, eluted with CH_2Cl_2–$(CH_3)_2CO$ (4:1) to yield four subfractions: 9.1–9.4. With further chromatography of fractions 9.2 (21 mg) and 9.3 (27 mg) over Cosmosil 75 C_{18}-OPN, eluted with CH_3OH-H_2O (1:4) and increasing amounts of CH_3OH, **8** (6 mg) and **9** (15 mg) were obtained. Fraction 12 (81 mg) was applied to a silica gel column, eluted with CH_2Cl_2–$(CH_3)_2CO$ (4:1) to afford subfractions 12.1 and 12.2. Fraction 12.2 (10 mg) was purified over MCI-gel CHP 20P, eluted with CH_3OH-H_2O (1:4) and increasing amounts of CH_3OH, to yield **3** (3 mg).

The crude residue from *M. recurvatus* (4.41 g) was subjected to silica gel CC. In total, eight fractions (1–8) were obtained by elution with various mixtures of CH_2Cl_2–CH_3OH (600 ml each of 20:1, 18:1, 15:1, 10:1, and 5:1, respectively). Further chromatography of fraction 2 (956 mg) over silica gel eluted with CH_2Cl_2-CH_3OH (20:1) afforded **2** (423 mg). Fraction 4 (216 mg) was then subjected to silica gel CC, eluted with CH_2Cl_2–CH_3OH (15:1) to afford three subfractions: 4.1–4.3. Fraction 4.2 (61 mg) was applied to a Cosmosil C_{18}-OPN column, eluted with CH_3OH-H_2O (1:4) and increasing amounts of CH_3OH, to yield **10** (32 mg). Fraction 5 (196 mg) was subjected to silica gel CC (CH_2Cl_2–CH_3OH with increasing polarity) to give three subfractions: 5.1–5.3. Fraction 5.1 (31 mg) was applied to CC over MCI-gel CHP 20P, eluted with CH_3OH-H_2O (3:7) and increasing amounts of CH_3OH to yield **11** (17 mg). Fractions 5.2 (81 mg) and 5.3 (15 mg) were applied to Cosmosil C_{18}-OPN column, eluted with CH_3OH-H_2O (2:3) and increasing amounts of CH_3OH, to yield **12** (69 mg) and **13** (5 mg), respectively. Fraction 7 (146 mg) was further purified over silica gel, eluted with CH_2Cl_2–CH_3OH (15:1) with increasing amounts of CH_3OH, to yield subfractions 7.1 and 7.2. Fraction 7.1 (25 mg) was subjected to CC over MCI-gel CHP 20P eluted with CH_3OH-H_2O (7:3) to yield **14** (13 mg).

The crude residue from *Asp. niger* (5.85 g) was subjected to silica gel CC. In total, eight fractions (1–8) were obtained by elution with various mixtures of CH_2Cl_2–CH_3OH (500 ml each of 20:1, 18:1, 15:1, 10:1, and 5:1, respectively). Further chromatography of fraction 3 (1.02 g) over silica gel, eluted with CH_2Cl_2-CH_3OH (20:1) yielded four subfractions: 3.1–3.4. Fraction 3.2 was recrystallized with CH_3OH and **2** (892 mg) was recovered. Further chromatography of fraction 5 (137 mg) over silica gel, eluted with CH_2Cl_2–CH_3OH (15:1) yielded three subfractions: 5.1–5.3. Fractions 5.1 (26 mg) and 5.3 (18 mg) were applied to Cosmosil $75C_{1.8}$-OPN columns eluted with CH_3OH–H_2O (13:7), which yielded **15** (5 mg) and **16** (3 mg), respectively. Fraction 6 (175 mg) was further purified by silica gel CC, eluted with CH_2Cl_2–CH_3OH (15:1) with increasing amounts of CH_3OH, to yield subfractions 6.1 and 6.2. Fraction 6.2 (27 mg) was subjected to CC over MCI-gel CHP 20P eluted with CH_3OH-H_2O (7:3), to yield **17** (20 mg).

4.4.1. Compound **3**

White powder; $[\alpha]_D^{25}$ - 18.8 (*c* 0.5, CH_3OH). For ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRTOFMS *m*/*z* 351.2169 $[M-H]^-$ (calcd for $C_{20}H_{31}O_5$, 351.2171).

4.4.2. Compound **4**

White powder; $[\alpha]_D^{25}$ −69.4 (*c* 0.5, CH_3OH). For ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRTOFMS *m*/*z* 333.2065 $[M-H]^-$ (calcd for $C_{20}H_{29}O_4$, 333.2066).

4.4.3. X-ray crystallographic data for **4**

Crystals of **4** were grown by slow evaporation from a mixture of pyridine–CH_3OH-H_2O. A well-shaped crystal of **4** with dimensions of 0.20 × 0.15 × 0.10 mm^3 was selected for X-ray analysis. The data obtained for the crystal were as follows: $C_{20}H_{30}O_4$, *M* 334.44, orthorhombic, $P2_12_12_1$, *a* 7.3140 (4) Å, *b* 10.8789 (7) Å, *c* 22.9393 (14) Å, *V* 1825.24 (19) $Å^3$; *Z* 4, D_{calcd} 1.217 g cm^{-3}, *F*(000) 728, λ (Mo Kα) 0.71073 Å, *T* 295(2) K, 6351 reflections collected. Final GooF 0.554, final *R* indices R_1 0.0421, wR_2 0.0947, 218 parameters, μ 0.083 mm^{-1}, *R* indices based on 4412 reflections with $I > 2\sigma(I)$ absorption corrections applied. Complete crystallographic data of **4** were deposited in the Cambridge Crystallographic Data Centre (CCDC 898187). These data can be obtained free of charge via http://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).

4.4.4. Compound **6**

White powder; $[\alpha]_D^{25}$ −35.6 (*c* 0.5, CH_3OH). For ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRESIMS *m*/*z* 351.2173 $[M-H]^-$ (calcd for $C_{20}H_{31}O_5$, 351.2171).

4.4.5. Compound **8**

White powder; $[\alpha]_D^{25}$ −77.8 (*c* 0.5, CH_3OH). For ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRESIMS *m*/*z* 335.2227 $[M-H]^-$ (calcd for $C_{20}H_{31}O_4$, 335.2222).

4.4.6. Compound **13**

White powder; $[\alpha]_D^{25}$ −65.4 (*c* 0.5, CH_3OH). For ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRESIMS *m*/*z* 349.2028 $[M-H]^-$ (calcd for $C_{20}H_{29}O_5$, 349.2015).

4.4.7. Compound **14**

White powder; $[\alpha]_D^{25}$ −45.6 (*c* 0.5, CH_3OH). For ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRESIMS *m*/*z* 335.2229 $[M-H]^-$ (calcd for $C_{20}H_{31}O_4$, 335.2222).

4.4.8. Compound **15**

White powder; $[\alpha]_D^{25}$ −11.4 (*c* 0.5, CH_3OH). For ^1H and ^{13}C NMR spectroscopic data, see Tables 2 and 3; HRESIMS *m*/*z* 421.2237 $[M-H]^-$ (calcd for $C_{23}H_{33}O_7$, 421.2226).

Table 1
^{1}H NMR spectroscopic data (δ_H)[a] of compounds **3**, **4**, **6**, **8**, **13**, and **14** in pyridine-d_5 (J in Hz).

Position	**3**	**4**	**6**	**8**	**13**	**14**
1	1.86–1.90[b] (*m*)	2.09–2.18[b] (*m*)	2.33–2.41[b] (*m*)	1.84–1.94[b] (*m*)	3.33 (*d*, 13.5)	β 3.74 (*dd*, 11.0, 4.5)
	1.16 (*td*, 13.0, 3.5)	1.44 (*m*)	1.61–1.66[b] (*m*)	1.03 (*td*, 13.5, 3.5)	1.47–1.50[b] (*m*)	
2	2.30–2.41[b] (*m*)	2.30–2.38[b] (*m*)	2.33–2.41[b] (*m*)	2.25 (*m*)	2.38 (*m*)	2.66 (*m*)
	1.52 (*br d*, 13.5)	1.58–1.64[b] (*m*)	1.61–1.66[b] (*m*)	1.45 (*m*)	1.57–1.60[b] (*m*)	1.99 (*dd*, 13.0, 4.0)
3	2.51–2.56[b] (*m*)	2.48–2.51[b] (*m*)	2.47 (*d*, 12.0)	2.47–2.54[b] (*m*)	2.51–2.56[b] (*m*)	2.45–2.52[b] (*m*)
	1.22 (*td*, 13.0, 4.0)	1.09 (*td*, 13.5, 4.0)	1.22 (*m*)	1.10 (*dd*, 13.0, 3.5)	1.23 (*m*)	1.33 (*dd*, 13.5, 4.0)
5	2.30–2.41[b] (*m*)	1.35 (*dd*, 12.0, 1.5)	2.33–2.41[b] (*m*)	1.21 (*d*, 11.5)	1.47–1.50[b] (*m*)	1.20 (*br d*, 11.0)
6	2.51–2.56[b] (*m*)	2.30–2.38[b] (*m*)	2.33–2.41[b] (*m*)	2.18 (*m*)	2.51–2.56[b] (*m*)	2.24–2.32[b] (*m*)
		2.09–2.18[b] (*m*)	2.08–2.20[b] (*m*)	2.05–2.13[b] (*m*)	2.22–2.26[b] (*m*)	2.12 (*dd*, 13.5, 1.5)
7	α 3.98 (*br s*)	1.78–1.80[b] (*m*)	2.22–2.29[b] (*m*)	1.68 (*d*, 13.0)	2.85 (*d*, 13.0)	1.67 (*dt*, 13.0, 3.0)
		1.58–1.64[b] (*m*)	1.44–1.48[b] (*m*)	1.56 (*td*, 13.0, 3.5)	1.29 (*m*)	1.41–1.49[b] (*m*)
9	1.95–2.08[b] (*m*)			1.76 (*dd*, 13.5, 4.0)	1.74 (*d*, 10.5)	1.55 (*dd*, 12.5, 4.5)
11	2.30–2.41[b] (*m*)	5.69 (*t*, 3.5)	2.73 (*td*, 13.5, 6.0)	2.47–2.54[b] (*m*)	α 4.26 (*m*)	3.18 (*m*)
	1.86–1.90[b] (*m*)		1.85 (*dd*, 14.0, 5.0)	2.05–2.13[b] (*m*)		2.45–2.52[b] (*m*)
12	2.30–2.41[b] (*m*)	2.91 (*dd*, 17.5, 3.5)	2.22–2.29[b] (*m*)	α 4.53 (*s*)	2.22–2.26[b] (*m*)	2.24–2.32[b] (*m*)
	1.64 (*td*, 12.0, 5.5)	2.48–2.51[b] (*m*)	2.08–2.20[b] (*m*)		1.93 (*m*)	1.41–1.49[b] (*m*)
14	1.95–2.08[b] (*m*)	1.87 (*d*, 11.0)	2.61–2.65[b] (*m*)	2.05–2.13[b] (*m*)	2.12 (*dd*, 11.5, 2.5)	1.41–1.49[b] (*m*)
		1.78–1.80[b] (*m*)	1.44–1.48[b] (*m*)	1.16 (*d*, 11.0)	1.57–1.60[b] (*m*)	1.27 (*dd*, 11.5, 2.5)
15	2.30–2.41[b] (*m*)	2.48–2.51[b] (*m*)	2.61–2.65[b] (*m*)	2.34 (*m*)	α 4.63 (*s*)	2.38 (*m*)
	1.95–2.08[b] (*m*)	2.09–2.18[b] (*m*)	2.08–2.20[b] (*m*)	1.84–1.94[b] (*m*)		1.91 (*dd*, 11.0, 13.5)
16	4.74 (*dd*, 10.5, 4.5)	4.85 (*dd*, 10.0, 2.5)	4.73 (*dd*, 10.5, 3.0)	4.33 (*dd*, 11.0, 4.0)		4.16 (*dd*, 11.0, 4.5)
17	3.97 (*d*, 10.5)	4.00 (*d*, 10.5)	3.95 (*d*, 10.5)	1.49 (*s*)	1.17 (*s*)	1.11 (*s*)
	3.87 (*d*, 10.5)	3.94 (*d*, 10.5)	3.89 (*d*, 10.5)			
18-CH_3	1.47 (*s*)	1.40 (*s*)	1.41 (*s*)	1.39 (*s*)	1.46 (*s*)	1.41 (*s*)
20-CH_3	1.32 (*s*)	1.49 (*s*)	1.57 (*s*)	1.25 (*s*)	1.47 (*s*)	1.50 (*s*)

[a] Assignments based on DEPT, HSQC, and HMBC.
[b] Overlapping signals.

Table 2
^{13}C NMR spectroscopic data (δ_C)[a] of compounds **2**–**4**, **6**, **8**, **13**–**16**, **18**, and **19** in pyridine-d_5.

No.	**2**	**3**	**4**	**6**	**8**	**13**	**14**	**15**	**16**	**18**	**19**
1	40.5	41.0	39.4	33.1	40.8	43.2	82.0	40.4	40.7	40.6	40.1
2	19.8	20.3	20.7	20.4	20.2	20.7	31.4	20.0	20.2	20.0	19.8
3	38.8	39.4	39.0	39.2	39.3	39.3	37.4	38.9	39.2	39.1	38.7
4	44.0	44.2	44.8	44.6	44.5	44.9	43.9	43.8	44.0	44.3	43.6
5	57.4	48.2	55.4	49.3	57.9	58.5	57.0	49.3	47.9	57.4	49.0
6	22.7	31.3	23.0	23.4	23.2	22.9	23.2	27.7	31.0	22.8	27.5
7	42.5	77.1	41.2	38.4	42.7	35.4	43.7	84.4	76.3	42.1	83.4
8	42.6	48.1	43.0	48.1	43.7	45.3	44.2	46.1	48.1	43.1	46.2
9	56.5	51.9	156.4	78.5	52.2	63.0	57.8	52.0	50.7	56.1	51.4
10	38.7	39.3	40.8	44.7	38.8	41.4	45.4	38.8	39.0	38.9	38.7
11	21.0	20.9	117.5	27.1	30.4	67.8	25.0	20.9	20.8	21.0	20.5
12	34.8	30.8	31.9	27.5	71.6	50.3	35.8	34.6	35.2	35.1	34.6
13	42.6	48.4	48.6	48.3	47.9	48.7	43.1	42.8	42.5	42.5	42.4
14	55.9	47.7	48.3	44.6	50.1	51.8	56.7	51.1	51.9	55.1	50.2
15	44.0	43.2	53.0	46.2	44.0	75.1	44.9	42.9	40.0	41.1	39.4
16	79.8	75.9	76.6	75.4	80.8	221.5	80.4	79.3	85.0	85.1	83.9
17	25.7	69.1	68.9	69.1	23.4	20.5	26.1	25.9	25.6	25.4	25.2
18	29.6	30.0	29.9	30.3	30.0	30.6	29.9	29.7	29.8	29.9	29.6
19	180.3	181.3	180.7	181.2	180.8	181.1	180.6	180.3	181.0	180.5	180.3
20	13.9	14.4	24.4	17.9	14.4	15.2	10.3	14.1	14.0	14.0	13.7
21								158.7	159.0	158.9	158.5
22								160.2	159.6	159.2	159.3
23-OCH_3								53.8	53.7	53.6	53.7
24											158.8
25											159.9
26-OCH_3											53.8

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

4.4.9. Compound **16**

White powder; $[\alpha]_D^{25}$ −17.2 (*c* 0.5, CH_3OH). For ^{1}H and ^{13}C NMR spectroscopic data, see Tables 2 and 3; HRESIMS *m*/*z* 421.2248 $[M-H]^-$ (calcd for $C_{23}H_{33}O_7$, 421.2226).

4.5. Preparation of **15S**, **16S**, and **19**

Compounds **15S** and **16S** were prepared according to previously described procedures (Pettit et al., 2007) Briefly, methyl oxalyl chloride (40 μl, 0.321 mmole) and a catalytic amount of 4-(dimethylaminopyridine) (DMAP) was added to a solution of **17** (100 mg, 0.298 mmole) in pyridine (1 ml). The reaction mixture was stirred at room temperature for 1 h. Ice water was then added and the solvent was evaporated *in vacuo* to afford a residue (167 mg). The residue was purified by CC over silica gel eluted with CH_2Cl_2–CH_3OH (20:1) and increasing CH_3OH, to yield **15S** (20 mg), **16S** (5 mg), and **19** (57 mg).

Compound **18** was prepared as described above. The yield for the formation of **18** was 60%.

Table 3
^{1}H NMR spectroscopic data (δ_H)[a] of compounds **15**, **16**, **18**, and **19** in pyridine-d_5 (J in Hz).

Position	15	16	18	19
1	1.75 (*d*, 13.0) 1.00 (*m*)	1.78 (*br d*, 12.5) 1.08 (*m*)	1.69–1.79[b] (*m*) 0.91 (*td*, 13.0, 4.0)	1.68 (*br d*, 12.0) 0.93 (*m*)
2	2.11–2.25[b] (*m*) 1.46 (*m*)	2.21–2.32[b] (*m*) 1.50 (*br d*, 13.0)	2.23 (*m*) 1.48–1.56[b] (*m*)	2.17 (*m*) 1.41–1.46[b] (*m*)
3	2.48 (*m*) 1.09 (*td*, 13.5, 4.0)	2.50 (*d*, 12.5) 1.18 (*td*, 13.5, 4.0)	2.46 (*d*, 13.0) 1.04–1.12[b] (*m*)	2.40–2.44[b] (*m*) 1.05 (*m*)
5	1.90–2.02[b] (*m*)	2.21–2.32[b] (*m*)	1.04–1.12[b] (*m*)	1.85 (*br d*, 12.8)
6	2.60 (*m*) 2.52 (*m*)	2.41 (*m*)	2.00–2.08[b] (*m*)	2.55 (*br d*, 15.2) 2.40–2.44[b] (*m*)
7	α 5.29 (*br s*)	α 3.87 (*br s*)	1.48–1.56[b] (*m*) 1.39 (*m*)	α 5.25 (*s*)
9	1.67–1.70[b] (*m*)	1.81–1.89[b] (*m*)	1.04–1.12[b] (*m*)	1.60–1.65[b] (*m*)
11	2.11–2.25[b] (*m*) 1.67–1.70[b] (*m*)	1.69 (*m*) 1.81–1.89[b] (*m*)	1.69–1.79[b] (*m*) 1.61 (*m*)	1.78 (*m*) 1.60–1.65[b] (*m*)
12	2.11–2.25[b] (*m*) 1.24 (*m*)	1.98–2.06[b] (*m*) 1.33 (*m*)	2.00–2.08[b] (*m*) 1.25–1.33[b] (*m*)	1.98 (*br d*, 12.8) 1.24 (*m*)
14	1.58 (*dd*, 12.0, 2.5) 1.40 (*m*)	1.81–1.89[b] (*m*) 1.64 (*m*)	1.25–1.33[b] (*m*) 1.04–1.12[b] (*m*)	1.55 (*br d*, 12.0) 1.41–1.46[b] (*m*)
15	2.11–2.25[b] (*m*) 1.90–2.02[b] (*m*)	2.09 (*dt*, 14.0, 3.0) 1.98–2.06[b] (*m*)	2.00–2.08[b] (*m*) 1.92 (*dd*, 14.5, 10.5)	2.08 (2H, *m*)
16	4.10 (*dd*, 10.5, 4.0)	5.09 (*br s*)	5.03 (*dd*, 10.5, 4.0)	5.05 (*dd*, 9.5, 5.0)
17	1.04 (*s*)	1.02 (*s*)	0.98 (*s*)	0.94 (*s*)
18-CH_3	1.34 (*s*)	1.43 (*s*)	1.37 (*s*)	1.30 (*s*)
20-CH_3	1.18 (*s*)	1.10 (*s*)	1.00 (*s*)	0.99 (*s*)
OCH_3	3.86 (*s*)	3.78 (*s*)	3.76 (*s*)	3.77 (*s*) 3.85 (*s*)

[a] Assignments based on DEPT, HSQC, and HMBC.
[b] Overlapping signals.

Table 4
Primers used for PCR.

Primer	Sequences	Product size
GAPDH	(F) 5′-GGG CCA AAA GGG TCA TCA TC-3′ (R) 5′-ATG ACC TTG CCC ACA GCC TT-3′	316 bp (R)
SF-1	(F) 5′-TCT CTA ACC GCA CCA TCA AG-3′ (R) 5′-TCG ACA ATG GAG ATA AAG GTC-3′	216 bp
StAR	(F) 5′-ATG TTC CTC GCT ACG TTC AA-3′ (R) 5′-TGA CAT TTG GGT TTC ACT CT-3′	451 bp
SCC	(F) 5′-CGC TCA GTG CTG GTC AAA G-3′ (R) 5′-GGT TGA GCA TGG GGA CAC T-3′	714 bp

4.5.1. Compound ***15S***

White powder; HRESIMS m/z 445.2216 $[M+Na]^+$ (calcd for $C_{23}H_{34}O_7Na$, 445.2202).

4.5.2. Compound ***16S***

White powder; HRESIMS m/z 421.2223 $[M-H]^-$ (calcd for $C_{23}H_{33}O_7$, 421.2226).

4.5.3. Compound ***18***

White powder; $[\alpha]_D^{26}$ −47.5 (c 0.9, CH_3OH). For ^{1}H and ^{13}C NMR spectroscopic data, see Tables 2 and 3; HRESIMS m/z 429.2258 $[M+Na]^+$ (calcd for $C_{23}H_{34}O_6Na$, 429.2253).

4.5.4. Compound ***19***

White powder; $[\alpha]_D^{26}$ +10.1 (c 1.0, CH_3OH). For ^{1}H and ^{13}C NMR spectroscopic data, see Tables 2 and 3; HRESIMS m/z 531.2194 $[M+Na]^+$ (calcd for $C_{26}H_{36}O_{10}Na$, 531.2206).

4.6. Detection of steroidogenic gene expressions

Cells were maintained at 37 °C in a humidified environment containing 95% air and 5% CO_2 for all the experiments. The Y-1 mouse adrenal tumor and MA-10 mouse Leydig tumor cell lines were maintained in Dulbecco's modified Eagle's medium-Ham's F12 (1:1) medium supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 mg/ml of streptomycin. The day before treatment, cells were plated at a density of 2×10^4 cells in 35-mm tissue culture dishes. The effect of each compound was then tested on the transcription of steroidogenic gene expressions. Forskolin (a cAMP agonist) was added to a final 10 μM concentration of cells as a control. Test compounds were added to culture medium at 10 μM concentration, and each mixture was incubated for 24 h. The effects of the tested compounds on steroidogenesis were analyzed in both adrenal and testicular Leydig cells. First, total RNA was extracted from treated cells using TRIZOL® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (2.5 μg) was reverse-transcribed with SuperScriptTM II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA) primed with oligo-dT (10 μM), as described previously (Chou et al., 2011). The amount of cDNA was quantified by PCR, with each reaction performed for a 50-μl volume. The sequences of the primers are listed in Table 4. The PCR protocol used an initial denaturing step at 94 °C for 5 min, followed by 40 cycles at 94 °C for 30 s; 55 °C for 30 s; and 72 °C for 30 s. Samples were analyzed on a 1.5% agarose gel.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2013.07.015.

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