Phytochemistry 70 (2009) 759–764

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Fungal transformation of isosteviol lactone and its biological evaluation for inhibiting the AP-1 transcription factor

Bo-Hon Chou [a], Li-Ming Yang [a,d], Shwu-Fen Chang [b], Feng-Lin Hsu [c], Chia-Hsin Lo [a,e], Wen-Kuang Lin [a], Li-Hsuan Wang [f], Pan-Chun Liu [a], Shwu-Jiuan Lin [a,*]

[a] Department of Medicinal Chemistry, College of Pharmacy, Taipei Medical University, Taipei 110, Taiwan
[b] Division of Cell and Molecular Biology, Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei 110, Taiwan
[c] Graduate Institute of Pharmacognosy, College of Pharmacy, Taipei Medical University, Taipei 110, Taiwan
[d] Division of Medicinal Chemistry, National Research Institute of Chinese Medicine, Taipei 112, Taiwan
[e] Forensic Science Center of Taipei City Police Department, Taipei 115, Taiwan
[f] Department of Clinical Pharmacy, College of Pharmacy, Taipei Medical University, Taipei 110, Taiwan

ARTICLE INFO

Article history:
Received 25 October 2008
Received in revised form 21 January 2009
Available online 22 April 2009

Keywords:
Microbial transformation
Isosteviol lactone
Diterpenoid
Activator protein-1

ABSTRACT

A number of hydroxylated diterpenoids were obtained from the microbial transformation of isosteviol lactone (4α-carboxy-13α-hydroxy-13,16-*seco*-*ent*-19-norbeyeran-16-oic acid 13,16-lactone) (**2**) with *Mucor recurvatus* MR 36, *Aspergillus niger* BCRC 31130, and *Absidia pseudocylindrospora* ATCC 24169. Incubation of **2** with *M. recurvatus* and *Asp. niger* led to isolation of seven known compounds (**1** and **3**–**8**). Incubation of **2** with *Abs. pseudocylindrospora* produced **5** and six previously unreported compounds (**9**–**14**). The structures of these isolated compounds were deduced by high-field NMR techniques (^{1}H, ^{13}C, DEPT, COSY, NOESY, HSQC, and HMBC), and those of **9** and **11** were further confirmed by X-ray crystallographic analyses. Subsequently, the inhibitory effects on activator protein-1 (AP-1) activation in lipopolysaccharide-stimulated RAW 264.7 macrophages of all of these compounds were evaluated. Compounds **2**–**5**, **8**, **9**, **11**, and **12** exhibited significant inhibitory activity, while **3** was more potent than the reference compound of dexamethasone.

1. Introduction

Activator protein-1 (AP-1) is a group of dimeric factors constituted by members of the Jun and Fos families of DNA-binding proteins (Jochum et al., 2001). Many stimuli induce the binding of AP-1 to the promoter region of various genes that govern cellular processes such as inflammation, proliferation, and apoptosis (Wisdom, 1999). Thus, AP-1 proteins are recognized as regulators of cytokine expression and important modulators in inflammatory diseases, such as rheumatoid arthritis, psoriasis, and psoriatic arthritis (Zenz et al., 2008). Glucocorticoids have long been used as effective immunosuppressive agents in the treatment of conditions involving T-cell- or cytokine-mediated tissue damage (Barnes, 2006). They are also widely used to treat inflammatory and autoimmune disorders. The major anti-inflammatory effects of glucocorticoids appear to largely be due to interactions between the activated glucocorticoid receptor (GR) and proinflammatory transcription factors that mediate the expression of inflammatory genes (Saklatvala, 2002). Many researchers have described a mechanism of GR-mediated transcriptional repression involving the physical interaction of GR and AP-1. This interaction results in glucocorticoid-mediated repression through AP-1-responsive elements (Jonat et al., 1990; Smith et al., 1996; González et al., 2000). Thus, inhibition or modulation of AP-1 activation may represent an attractive target for developing novel therapeutic agents for treating inflammatory-mediated conditions (Tsuchida et al., 2006).

Microbial transformation is an area of great interest for preparing products which are difficult to obtain by conventional chemical methods (Arantes and Hanson, 2007). There is an increasing body of information about use of biocatalysis for selective conversion of synthetic and natural products to intensify either their biological properties or to lead to new biological activities (Buchanan and Reese, 2001; Gładkowski et al., 2007; Venisetty and Ciddi, 2003). Special attention has been paid to filamentous fungi because they are capable of catalyzing regio- and stereoselective hydroxylation of a variety of nonfunctionalized hydrocarbon centers of a great variety of substrates (Lehmann and Stewart, 2001). Isosteviol lactone (**2**), an *ent*-beyerane tetracyclic diterpenoid, was prepared by reacting isosteviol (**1**) with *m*-chloroperbenzoic acid, and its activity on mitochondrial metabolism has been described (Braguini et al., 2003). Ghisalberti (1997) indicated that some highly oxygenated diterpenoids usually have higher levels of biological activity than their less-hydroxylated precursors. Previously, we studied the microbial transformation of **2** and evaluated the transformation products on the androgen response element (Chou et al., 2008).

* Corresponding author. Tel.: +886 2 27361661x6133; fax: +886 2 28264276.
E-mail address: shwu-lin@tmu.edu.tw (S.-J. Lin).

0031-9422/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2009.03.015

In order to obtain additional hydroxylated compounds, the present investigation was carried out to continue studying the ability of other microorganisms to accomplish structural modifications of **2** and to develop new biological activities. Since tetracyclic diterpenoids possess a formal similarity to steroids (Hanson, 1992) and are still being used to develop immunoinflammatory agents (Chang et al., 2006, 2008), an AP-1-mediated luciferase reporter gene assay was used to evaluate the modified compounds with respect to the parent compound. The production, isolation, and structural characterization as well as the inhibitory effects on AP-1 activation in lipopolysaccharide (LPS)-stimulated macrophages of these metabolites are reported herein. Additionally, the stereochemical characteristics of **9** and **11** were also confirmed by X-ray crystallographic analyses.

2. Results and discussion

In a continuing investigation of the microbial transformation of isosteviol lactone (**2**) (Chou et al., 2008), the bioconversion of **2** with *Mucor recurvatus*, *Aspergillus niger*, and *Absidia pseudocylindrospora* was undertaken and led to isolation of compounds **3**–**14** (Fig. 1). Incubation of **2** with *M. recurvatus* yielded four known compounds of **1** and **3**–**5**, whereas incubation of **2** with *Asp. niger* afforded the five known compounds **1**, **4**, and **6**–**8**. The structures of known metabolites were determined by comparison of the NMR spectroscopic data with values in the literature (de Oliveira and Strapasson, 1996; Chou et al., 2008). On the other hand, incubation of **2** with *Abs. pseudocylindrospora* yielded **5** and six new compounds of **9**–**14**. Compounds **9** and **10** displayed similar ^{1}H and ^{13}C NMR spectra. They also showed quasi-molecular ions $[M+H]^+$ at *m*/*z* 351.2174 and 351.2173, respectively, in the HRESIMS, corresponding to the molecular formula of $C_{20}H_{31}O_5$ (calc. 351.2171). The ^{13}C NMR spectra of **9** and **10** displayed the same resonances for 20 carbons, while the DEPT spectra showed the presence of three methyl, eight methylene, three methine, and six quaternary carbons. The HSQC spectra of **9** and **10** showed new resonances at δ_H 4.01 (δ_C 71.7) and δ_H 3.68 (δ_C 75.8), respectively, in comparison with those of **2**. This indicated that **9** and **10** contain one more oxygen atom than does **2**. Analysis of the HSQC and HMBC spectra of **9** and **10** and their comparison with **2** demonstrated that δ_H 4.01 showed connectivities with δ_C 25.6 (C-17), 42.5 (C-14), 48.8 (C-9), and 82.3 (C-13) in **9**, and δ_H 3.68 had connectivities with δ_C 28.7 (C-11), 82.6 (C-13), and 24.4 (C-17) in **10**. Thus, the additional oxygen present in both molecules was located at C-12. The orientation of the 12-OH follows from the multiplicity of the H-12 signal in the ^{1}H NMR spectrum, which is a broad singlet in **9** and a double-doublet in **10**, indicated that the proton was in an α-orientation in **9** and a β-orientation in **10** (de Oliveira and Strapasson, 1996). The NOESY spectra also showed cross-peaks of δ 4.01 with H-11 and CH_3-17 in **9**; and δ 3.68 with H-9β, and H-11, H-14 in **10**. In addition, an X-ray crystallographic experiment was also carried out to confirm the structure of **9** (Fig. 2). Thus, structures of **9** and **10** were determined to be 4α-carboxy-12β,13α-dihydroxy-13,16-*seco*-*ent*-19-norbeyeran-16-oic acid 13,16-lactone and 4α-carboxy-12α,13α-dihydroxy-13,16-*seco*-*ent*-19-norbeyeran-16-oic acid 13,16-lactone, respectively.

Compound **11** had a quasi-molecular ion at *m*/*z* 351.2178 $[M+H]^+$ (calc. 351.2171) in the HRESIMS, consistent with a molecular formula of $C_{20}H_{31}O_5$. The ^{13}C NMR spectrum displayed resonances for 20 carbons, while the DEPT spectrum showed the presence of three methyl, eight methylene, three methine, and six quaternary carbons. Comparison of the ^{13}C NMR and DEPT spectra with **2** suggested that **11** contains one more oxygen atom than **2**. The HSQC spectrum showed a new proton geminal to an alcohol resonating at δ_H 3.53 (δ_C 77.2). In the HMBC spectrum, δ_C 77.2 exhibited cross-peaks with δ_H 1.24 (H-5β), 2.36-2.45 (H-6 and H-14), and 3.33 (H-15). Thus, hydroxylation had occurred at C-7. The α-orientation of the 7-OH was suggested from the cross-peaks of δ 3.53 (H-7) with H-5β, H-9β, H-6, and H-14 in the NOESY experiment. Moreover, the structure of **11** was confirmed by X-ray crystallographic analysis (Fig. 3). Thus, **11** was established as 4α-carboxy-7α,13α-dihydroxy-13,16-*seco*-*ent*-19-norbeyeran-16-oic acid 13,16-lactone.

Compound **12** showed a quasi-molecular ion peak at *m*/*z* 351.2193 $[M+H]^+$ (calc. 351.2171) in the HRESIMS, corresponding to a molecular formula of $C_{20}H_{31}O_5$. The DEPT spectrum showed the disappearance of one CH_3 signal and the presence of one new CH_2 signal at δ 69.3 in comparison with those of **2**. Analysis of the ^{1}H, ^{13}C NMR, HSQC, and HMBC spectra and comparison with **2** indicated that the resonances of C-13 and C-16 had shifted downfield from δ 79.8 to 83.5 and δ 171.8 to 172.7, respectively. The resonances of C-12 and C-14 had shifted upfield from δ 38.7 to 34.5 and 47.5 to 43.4, respectively, suggesting that an OH might reside at C-17. Additionally, two new protons resonating at δ 3.81 and 3.91 and showing connectivities to C-12 (δ 34.5), C-13 (δ 83.5), and C-14 (δ 43.4) also suggested the presence of an OH at C-17. On the basis of the above evidence, **12** was assigned to be 4α-carboxy-13α,17-dihydroxy-13,16-*seco*-*ent*-19-norbeyeran-16-oic acid 13,16-lactone.

Compound **13** had a quasi-molecular ion peak at *m*/*z* 389.1955 $[M+Na]^+$ in the HRESIMS, corresponding to the molecular formula of $C_{20}H_{30}O_6Na$. The DEPT, HSQC, and HMBC spectra showed the disappearance of one CH_3 and one CH signal, and the presence of

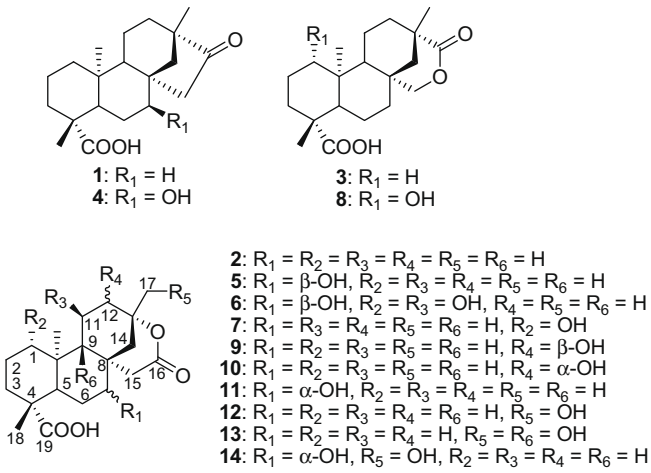

Fig. 1. Structures of compounds **1**–**14**.

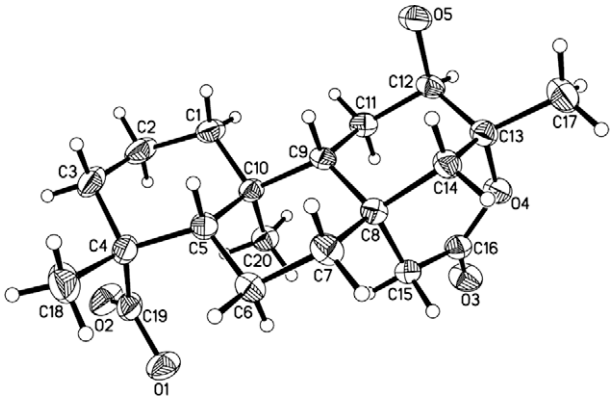

Fig. 2. Perspective drawing of the X-ray structure of **9**.

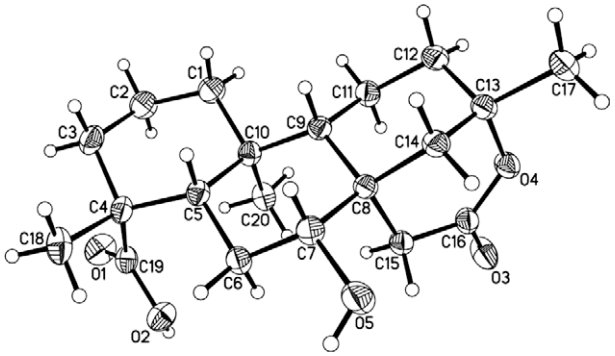

Fig. 3. Perspective drawing of the X-ray structure of **11**.

Table 1
Data of compounds showing significant results on an AP-1-mediated luciferase reporter gene assay[a].

Compounds	Luciferase activity	Compounds	Luciferase activity
2	2.38 ± 0.18	**9**	2.73 ± 0.58
3	1.88 ± 0.39	**11**	3.02 ± 0.71
4	2.69 ± 0.92	**12**	2.58 ± 0.86
5	2.39 ± 0.64	Control	3.95 ± 0.53
7	5.46 ± 1.53	Dex	2.33 ± 0.36
8	2.66 ± 0.99		

[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. Dexamethasone (Dex) was the reference compound as the positive control. Each value is the average of the firefly/*Renilla* luciferase ratio and is presented as the mean ± SEM ($n = 3$). *Significantly different at $p < 0.05$, using Student's *t*-test for paired samples.

one new CH_2 signal at δ 69.4 and one quaternary carbon at δ 77.4 when compared with **2**. In the HMBC spectrum, two new proton resonances at δ 3.86 (*d*, *J* = 11.5 Hz) and 3.94 (*d*, *J* = 11.5 Hz) showing connectivities to C-12 (δ 30.5), C-13 (δ 83.9), and C-14 (δ 38.5) suggested the presence of an OH at C-17. On the other hand, 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 another OH might have been introduced at C-5 or C-9. In the HMBC spectrum, the resonance of δ_C 77.4 showed connectivities with H-7 (δ_H 1.32), H-14 (δ_H 1.67), H-11 (δ_H 1.87), H-12 (δ_H 1.99), H-15 (δ_H 3.75), CH_3-20 (δ_H 1.41), and OH (δ_H 5.57), thus confirming the presence of an OH at C-9. Thus, **13** was assigned to be 4α-carboxy-9β,13α,17-trihydroxy-13,16-*seco*-*ent*-19-norbeyeran-16-oic acid 13,16-lactone.

Compound **14** displayed a quasi-molecular ion peak at *m*/*z* 367.2132 $[M+H]^+$ (calc. 367.2121) in the HRESIMS, corresponding to the molecular formula of $C_{20}H_{31}O_6$. The ^{1}H, ^{13}C NMR, and HSQC spectra were similar to those of **12** except for new signals at δ_H 3.63 (1H, *m*) and δ_C 77.9. This indicated that **14** contains one more oxygen atom than **12**. The location of the OH was confirmed by a detailed analysis of the HMBC data. The chemical shift of δ_H 3.63 showed connectivities with C-5 (δ_C 54.8), C-8 (δ_C 41.5), C-14 (δ_C 39.5), and C-15 (δ_C 32.4). Thus, hydroxylation had occurred at C-7. An α-orientation of the OH at C-7 was suggested from the cross-peaks of δ_H 3.63 with H-5 (δ_H 1.31), H-6 (δ_H 2.46-2.52), H-9β (δ_H 1.07-1.14), and H-14 (δ_H 2.91), whereas no effect was observed between δ_H 3.63 and 1.16 (CH_3-20) in the NOESY experiment. Thus, **14** was established to be 4α-carboxy-7α,13α,17-trihydroxy-13,16-*seco*-*ent*-19-norbeyeran-16-oic acid 13,16-lactone.

Subsequently, the inhibitory effects on AP-1 activation in LPS-stimulated RAW 264.7 macrophages of all of these compounds were evaluated to search for potential novel immunoinflammatory agents. Results of evaluation of inhibitory effects using an AP-1-mediated luciferase reporter gene assay established that compounds **2**–**5**, **8**, **9**, **11**, and **12** showed significant suppression of the expression of AP-1 target genes, with **3** exhibiting greater inhibition than the reference compound of dexamethasone (Table 1).

3. Conclusions

Thirteen compounds, **1** and **3**–**14**, were obtained from the preparative-scale fermentation of **2** with *M. recurvatus* MR 36, *Asp. niger* BCRC 31130, and *Abs. pseudocylindrospora* ATCC 24169. Compounds **9**–**14** have not previously been reported. The results indicated that these three selected fungal strains also possess the characteristics of reaction selectivity on the *ent*-beyerane skeleton as we previously reported (Chang et al., 2008; Chou et al., 2008). The reactions involved not only isomerization of the lactone ring but also regio- and stereoselective hydroxylation. In addition, results showed that **2**–**5**, **8**, **9**, **11**, and **12** exhibited significant inhibitory activities toward AP-1 activation. In particular, **3** exhibited greater inhibition than dexamethasone. Further work is ongoing to investigate **3** functioning on other transcriptional factor-regulated pathways, such as NF-κB and Sp-1. This investigation also demonstrates that biohydroxylation represents a powerful tool for the regio- and stereoselective introduction of hydroxyl groups into organic compounds.

4. Experimental

4.1. General

Melting points were determined using a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were determined on a JASCO DIP-1020 digital polarimeter. ^{1}H, ^{13}C NMR, DEPT, and 2D-NMR (COSY, NOESY, HSQC, and HMBC) spectra were recorded on a Bruker Avance-500 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 in Hertz (Hz). Low- and high-resolution ESI mass spectra were recorded using a VG Platform Electrospray ESI/MS spectrometer. X-ray single-crystal diffraction was measured on a Siemens SMART CCD XRD. Column chromatography (CC) was performed with Kieselgel silica (70–230 and 230–400 mesh, Merck, Darmstadt, Germany). HPLC was performed on a Hitachi L-2130 apparatus equipped with a refractometer detector (L-2490). Purification by means of HPLC was conducted using a Betasil Silica-100 column (250 × 10 mm, 5 μm, at a flow rate of 2 mL/min) (Thermo Scientific, Waltham, MA, USA). TLC plates (Si 60 with F254) were purchased from Merck (Darmstadt, Germany). All spots were detected by spraying with 10% H_2SO_4, followed by heating.

4.2. Substrate *2*

Isosteviol lactone (**2**) was prepared as previously reported (Braguini et al., 2003).

4.3. Microorganisms

Fungal cultures of *M. recurvatus* MR 36, *Asp. niger* BCRC 31130, and *Abs. pseudocylindrospora* ATCC 24169 were obtained from the Division of Medicinal and Natural Products Chemistry, College of Pharmacy, University of Iowa, Iowa City, IA, USA, 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.

4.4. Preparation of medium

Fungal fermentations were carried out in medium as described previously (Chang et al., 2008). The pH of the medium was adjusted to 7.0 with 6 N HCl before sterilization by autoclaving at 121 °C for 15 min.

4.5. Biotransformation of isosteviol lactone (**2**) by M. recurvatus, Asp. niger, and Abs. pseudocylindrospora

Using 24-h-old stage II cultures, a solution of **2** (1.06 g dissolved in 10.6 mL DMF) was evenly distributed among 50 flasks containing stage II cultures. Substrate-containing cultures were incubated for 144 h. Extraction as previously described (Chang et al., 2008) produced 4.2 g of brown oil, 3.1 g of black oil, and 4.7 g of brown oil after respective bioconversions with *M. recurvatus*, *Asp. niger*, and *Abs. pseudocylindrospora*. The crude residue from *M. recurvatus* (4.2 g) was subjected to CC over silica gel (70–230 mesh, 5 × 90 cm). In total, four fractions (1–4) were obtained by eluting with mixtures of CH_2Cl_2–EtOH (600 mL each of 20:1, 15:1, and 10:1). The elutes were monitored using TLC. The fractions were combined on the basis of similar TLC profiles. Further chromatography of fraction 2 (910 mg) over silica gel (230–400 mesh, 3 × 50 cm) eluted with *n*-hexane–EtOAc (1:1) yielded three fractions (2-1 to 2-3), and 760 mg of **2** was recovered from fraction 2-1. Fractions 2-2 (9 mg) and 2-3 (90 mg) were recrystallized with EtOAc to give 4.5 mg of **3** and 80 mg of **4** as white prisms and white needles, respectively. Fraction 3 (602 mg) was applied to a silica gel column (230–400 mesh, 3 × 55 cm) eluted with *n*-hexane–EtOAc (1:2) to give three fractions (3-1 to 3-3). Fraction 3-1 (570 mg) was recrystallized with *n*-hexane–EtOAc (1:2) to give **1** (66 mg). The mother liquid of fraction 3-1 was evaporated *in vacuum* and then subjected to CC over silica gel eluted with *n*-hexane–EtOAc (1:2) to give **3** (490 mg). After recrystallization of fraction 3-3 (15 mg) with EtOAc, 5 mg of **5** was obtained as white prisms. The crude residue from *Asp. niger* (3.1 g) was subjected to CC over silica gel (70–230 mesh, 4 × 60 cm). In total, 5 fractions (1–5) were obtained by eluting with mixtures of CH_2Cl_2–MeOH (500 mL each of 20:1, 15:1, and 10:1). With further chromatography of fraction 2 (360 mg) over silica gel (230–400 mesh, 3 × 55 cm) eluted with *n*-hexane–EtOAc (150 mL each of 3:1, 2:1, and 1:1), **1** (60 mg) was obtained. After recrystallization of fractions 3 and 4 with EtOAc and MeOH, **7** (17 mg) and **5** (21 mg) were respectively obtained. The mother liquid of fraction 4 (650 mg) was evaporated *in vacuum* and then applied to a silica gel column (230–400 mesh, 2 × 55 cm) eluted with *n*-hexane–EtOAc (2:1) to yield **3**. After recrystallization with EtOAc–MeOH, 500 mg of **3** was obtained as white crystals. Fraction 5 (32 mg) was purified by semi-preparative HPLC (CH_2Cl_2–isopropanol, 12:1) to give **8** (10 mg) and **9** (6 mg). The crude residue from *Abs. pseudocylindrospora* (4.7 g) was purified by CC over silica gel using mixtures of *n*-hexane, EtOAc, CH_2Cl_2, and MeOH with increasing polarity to obtain five fractions (1–5). Fraction 3 (321 mg) was subjected to CC over silica gel eluted with *n*-hexane–EtOAc to yield a white solid (279 mg). The white solid was further purified by HPLC on a semi-preparative column (CH_2Cl_2–isopropanol, 12:1) to give **6** (80 mg), **9** (90 mg), **10** (8 mg), **11** (68 mg), and **12** (2.5 mg). Fraction 4 (90 mg) was subjected to repeated semi-preparative HPLC separation (CH_2Cl_2–isopropanol, 12:1) to give **13** (8 mg) and **14** (16 mg).

4.5.1. Compound **9**

White crystals, m.p. > 300 °C; $[\alpha]_{25}^{D}$ −34.8 (*c* 0.5, MeOH); for ^1H and ^{13}C NMR spectra, see Tables 2 and 3; HRESIMS *m*/*z* 351.2174 $[M+H]^+$ ($C_{20}H_{31}O_5$, calc. 351.2171).

4.5.2. X-ray diffraction analysis of **9**

$C_{20}H_{30}O_5$, *M* 350.44, monoclinic, *C*2, *a* 44.8056 (11) Å, *b* 7.2959 (2) Å, *c* 11.8141 (3) Å, *V* 3825.76 (17) Å^3; *Z* 8, D_{calc} 1.217 g cm^{-3}, *F*(0 0 0) 1520, λ (Mo Kα) 0.71073 Å, *T* 295(2) K, 15643 reflection collected. Final GooF 0.958, final *R* indices R_1 0.0574, wR_2 0.1375, 478 parameters, μ 0.086 mm^{-1}, *R* indices based on 8427 reflections with $I > 2\sigma(I)$ absorption corrections applied. Complete crystallographic data of **9** were deposited in the Cambridge Crystallographic Data Centre (CCDC 706276). 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).

4.5.3. Compound **10**

White powder; $[\alpha]_{25}^{D}$ −76.8 (*c* 0.5, MeOH); for ^1H and ^{13}C NMR spectra, see Tables 2 and 3; HRESIMS *m*/*z* 351.2173 $[M+H]^+$ ($C_{20}H_{31}O_5$, calc. 351.2171).

4.5.4. Compound **11**

White crystals, m.p. > 300 °C; $[\alpha]_{25}^{D}$ −10.4 (*c* 0.5, MeOH); for ^1H and ^{13}C NMR spectra, see Tables 2 and 3; HRESIMS *m*/*z* 351.2178 $[M+H]^+$ ($C_{20}H_{31}O_5$, calc. 351.2171).

4.5.5. X-ray diffraction analysis of **11**

$C_{20}H_{30}O_5$, *M* 350.44, orthorhombic, $P2_12_12_1$, *a* 11.1996 (2) Å, *b* 12.2087 (2) Å, *c* 13.4538 (2) Å, *V* 1839.57 (5) Å^3; *Z* 4, D_{calc} 1.265 g cm^{-3}, *F*(0 0 0) 760, λ (Mo Kα) 0.71073 Å, *T* 295(2) K, 12063 reflection collected. Final GooF 1.026, final *R* indices R_1 0.0443, wR_2 0.1200, 227 parameters, μ 0.089 mm^{-1}, *R* indices based on 4156 reflections with $I > 2\sigma(I)$ absorption corrections applied. Complete crystallographic data of **11** were deposited in the Cambridge Crystallographic Data Centre (CCDC 706277). 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).

4.5.6. Compound **12**

White powder; $[\alpha]_{25}^{D}$ −7.2 (*c* 0.5, MeOH); for ^1H and ^{13}C NMR spectra, see Tables 2 and 3; HRESIMS *m*/*z* 351.2193 $[M+H]^+$ ($C_{20}H_{31}O_5$, calc. 351.2171).

4.5.7. Compound **13**

White powder; $[\alpha]_{25}^{D}$ −34.0 (*c* 0.5, MeOH); for ^1H and ^{13}C NMR spectra, see Tables 2 and 3; HRESIMS *m*/*z* 389.1955 $[M+Na]^+$ ($C_{20}H_{30}O_6Na$, calc. 389.1940).

4.5.8. Compound **14**

White powder; $[\alpha]_{25}^{D}$ −52.4 (*c* 0.5, MeOH); for ^1H and ^{13}C NMR spectra, see Tables 2 and 3; HRESIMS *m*/*z* 367.2132 $[M+H]^+$ ($C_{20}H_{31}O_6$, calc. 367.2121).

4.6. Cell culture, transfection, and reporter gene assays

Twenty-four hours before transfection, about 1 × 10^5 mouse RAW 264.7 macrophages per well were seeded in 96-well white plates. The reporter plasmid, pAP-1-Luc plasmid, and an internal control plasmid, pGL-*hRluc*, were transfected into RAW 264.7 cells using lipofectamine plus (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. At 48 h post-transfection, lipopolysaccharide (LPS) from *Escherichia coli* (serotype 0111:B4) (Sigma, St. Louis, MO, USA) at a final concentration of 100 ng/mL was added to the transfected cells for 6 h. After LPS stimulation, a final concentration of 10 μM of each test compound including the reference compound, dexamethasone (Sigma, St. Louis, MO, USA), in DMSO was added to the cells. Cells were harvested 24 h

Table 2
^{1}H NMR chemical shifts of compounds **9**–**14** (C_5D_5N, δ values in ppm)[a,b].

Position	9	10	11	12	13	14
1	1.55-1.72[c] (*m*)	1.66 (*d*, *J* = 12.4)	1.64 (*d*, *J* = 12.4)	1.69 (*br d*, *J* = 13.5)	2.17 (*m*)	1.62-1.73[c] (*m*)
	0.89 (*td*, *J* = 13.2, 4.0)	0.82 (*td*, *J* = 13.2, 4.0)	0.80 (*td*, *J* = 13.2, 4.4)	0.85 (*td*, *J* = 13.0, 3.5)	1.51 (*d*, *J* = 12.0)	0.87 (*td*, *J* = 13.0, 3.5)
2	2.10-2.21[c] (*m*)	2.08-2.18[c] (*m*)	2.16 (*qt*, *J* = 13.2, 3.6)	2.14-2.26[c] (*m*)	2.24-2.44[c] (*m*)	2.23 (*m*)
	1.38 (*m*)	1.37-1.45[c] (*m*)	1.43 (*m*)	1.48-1.64[c] (*m*)	1.56 (*d*, *J* = 13.5)	1.49-1.57[c] (*m*)
3	2.36 (*d*, *J* = 12.8)	2.39 (*d*, *J* = 13.2)	2.36-2.45[c] (*m*)	2.46 (*d*, *J* = 13.0)	2.24-2.44[c] (*m*)	2.46-2.52[c] (*m*)
	0.99 (*td*, *J* = 13.2, 4.0)	0.99-1.06[c] (*m*)	1.02 (*dd*, *J* = 13.2, 4.0)	1.06-1.08[c] (*m*)	1.12 (*td*, *J* = 13.0, 4.0)	1.07-1.14[c] (*m*)
5β	1.10 (*m*)	0.99-1.06[c] (*m*)	1.24 (*m*)	1.06-1.08[c] (*m*)	2.24-2.44[c] (*m*)	1.31 (*m*)
6	2.10-2.21[c] (*m*)	2.08-2.18[c] (*m*)	2.36-2.45[c] (*m*)	2.14-2.26[c] (*m*)	2.24-2.44[c] (*m*)	2.46-2.52[c] (*m*)
	1.92 (*m*)	1.96 (*m*)		1.95-1.98[c] (*m*)	2.06 (*d*, *J* = 14.0)	
7	1.49 (*m*)	1.51-1.56[c] (*m*)	β 3.53 (*dd*, *J* = 8.4, 6.8)	1.48-1.64[c] (*m*)	2.21 (*dd*, *J* = 13.5, 3.0)	β 3.63 (*m*)
	1.25 (*dd*, *J* = 13.6, 3.6)	1.16 (*td*, *J* = 13.4, 3.6)		1.24-1.32[c] (*m*)	1.32 (*m*)	
9β	1.55-1.72[c] (*m*)	0.99-1.06[c] (*m*)	0.92 (*dd*, *J* = 11.6, 3.6)	0.96 (*dd*, *J* = 12.5, 2.5)	*OH* 5.57 (*s*)	1.07-1.14[c] (*m*)
11	1.83 (*dt*, *J* = 14.0, 2.4)	2.08-2.18[c] (*m*)	1.56 (*m*)	1.48-1.64[c] (*m*)	1.87 (2H, *m*)	1.62-1.73[c] (*m*)
	1.55-1.72[c] (*m*)	1.51-1.56[c] (*m*)	1.32-1.39[c] (*m*)	1.24-1.32[c] (*m*)		1.49-1.57[c] (*m*)
12	α 4.01 (*br s*)	β 3.68 (*dd*, *J* = 11.2, 4.8)	1.85 (*m*)	2.03 (*d*, *J* = 13.0)	2.24-2.44[c] (*m*)	2.09 (*d*, *J* = 13.0)
			1.32-1.39[c] (*m*)	1.48-1.64[c] (*m*)	1.99 (*d*, *J* = 13.0)	1.62-1.73[c] (*m*)
14	2.01 (*dd*, *J* = 13.6, 2.8)	1.51-1.56[c] (*m*)	2.36-2.45[c] (*m*)	1.95-1.98[c] (*m*)	2.71 (*dd*, *J* = 13.0, 2.5)	2.91 (*dd*, *J* = 13.5, 2.0)
	1.32 (*m*)	1.37-1.45[c] (*m*)	1.32-1.39[c] (*m*)	1.48-1.64[c] (*m*)	1.67 (*d*, *J* = 13.0)	1.49-1.57[c] (*m*)
15		3.25 (*dd*, *J* = 18.4, 3.5)	3.33 (*d*, *J* = 18.8)	3.30 (*dd*, *J* = 18.5, 2.0)	3.75 (*dd*, *J* = 18.5, 2.5)	3.47 (*d*, *J* = 18.5)
	2.10-2.21[c] (*m*)	2.08-2.18[c] (*m*)	2.99 (*dd*, *J* = 18.8, 2.4)	2.14-2.26[c] (*m*)	2.24-2.44[c] (*m*)	3.12 (*d*, *J* = 18.5)
17	1.52 (*s*)	1.58 (*s*)	1.28 (*s*)	3.91 (*d*, *J* = 11.5)	3.94 (*d*, *J* = 11.5)	3.95 (*d*, *J* = 11.5)
				3.81 (*d*, *J* = 11.5)	3.86 (*d*, *J* = 11.5)	3.84 (*d*, *J* = 11.5)
18-CH_3	1.30 (*s*)	1.32 (*s*)	1.31 (*s*)	1.36 (*s*)	1.37 (*s*)	1.36 (*s*)
20-CH_3	1.07 (*s*)	1.03 (*s*)	1.09 (*s*)	1.10 (*s*)	1.41 (*s*)	1.16 (*s*)

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

Table 3
^{13}C NMR chemical shifts of compounds **9**–**14** (C_5D_5N, δ values in ppm)[a].

No.	9	10	11	12	13	14
1	39.8	40.0	39.9	40.6	33.1	40.5
2	19.5	19.5	19.5	20.1	20.2	20.1
3	38.4	38.4	38.3	39.0	39.0	38.9
4	43.8	43.8	43.6	44.3	44.6	44.2
5	57.2	56.8	54.2	57.6	49.5	54.8
6	20.2	20.1	30.1	20.7	20.8	30.7
7	43.4	42.9	77.2	44.3	38.5	77.9
8	35.2	35.2	41.2	35.4	39.9	41.5
9	48.8	54.7	54.6	56.8	77.4	55.9
10	37.7	38.1	38.2	38.7	44.2	38.8
11	26.9	28.7	18.6	18.9	24.3	18.7
12	71.7	75.8	38.5	34.5	30.5	34.4
13	82.3	82.6	79.4	83.5	83.9	83.2
14	42.5	46.7	43.6	43.4	38.5	39.5
15	39.4	39.3	31.5	40.0	42.3	32.4
16	172.0	171.9	173.4	172.7	172.9	174.3
17	25.6	24.4	28.5	69.3	69.4	69.5
18	29.2	29.2	29.1	29.7	30.0	29.7
19	179.8	179.7	179.7	180.4	180.8	180.3
20	14.1	14.0	13.9	14.4	17.3	14.5

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

after treatment, and the reporter activities of firefly luciferase expressed from pAP-1-Luc and *Renilla* luciferase from pGL-*hRluc* were assayed in a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA, USA) using the dual-luciferase reporter assay system (Promega, Madison, WI, USA).

4.6.1. Statistical analysis

Data are from at least three individual experiments. Average of the firefly/*Renilla* luciferase ratios were analyzed by two-tailed Student's *t*-test for paired samples. Significance was accepted when p was <0.05.

Acknowledgments

We thank Dr. John P.N. Rosazza, Division of Medicinal and Natural Products Chemistry, College of Pharmacy, University of Iowa, Iowa City, IA, for kindly providing the bacterial strains. We also thank Mr. Yi-Hung Liu, Instrumentation Center of National Taiwan University, for conducting of the X-ray crystallography. This research was supported by grants, NSC96-2320-B-038-013 and NSC97-2320-B-038-009, from the National Science Council of Taiwan.

References

Arantes, S.F., Hanson, J.R., 2007. The biotransformation of sesquiterpenoids by *Mucor plumbeus*. Curr. Org. Chem. 11, 657–663.
Barnes, P.J., 2006. Corticosteroid effects on cell signaling. Eur. Respir. J. 27, 413–426.
Braguini, W.L., Biazon, M.A., de Oliveira, B.H., Carnieri, E.G.S., Rocha, M.E.M., de Oliveira, M.B.M., 2003. Activity of isosteviol lactone on mitochondrial metabolism. Toxicol. Lett. 143, 83–92.
Buchanan, G.O., Reese, P.B., 2001. Biotransformation of diterpenes and diterpene derivatives by *Beauveria bassiana* ATCC 7159. Phytochemistry 56, 141151.
Chang, S.F., Yang, L.M., Hsu, F.L., Hsu, J.Y., Liaw, J.H., Lin, S.J., 2006. Transformation of steviol-16α,17-epoxide by *Streptomyces griseus* and *Cunninghamella bainieri*. J. Nat. Prod. 69, 1450–1455.
Chang, S.F., Yang, L.M., Lo, C.H., Liaw, J.H., Wang, L.H., Lin, S.J., 2008. Microbial transformation of isosteviol and bioactivities against the glucocorticoid/androgen response elements. J. Nat. Prod. 71, 87–92.
Chou, B.H., Yang, L.M., Chang, S.F., Hsu, F.L., Lo, J.H., Liaw, J.H., Liu, P.C., Lin, S.J., 2008. Microbial transformation of isosteviol lactone and evaluation of the transformation products on androgen response element. J. Nat. Prod. 71, 602–607.
de Oliveira, B.H., Strapasson, R.A., 1996. Biotransformation of isosteviol by *Fusarium verticilloides*. Phytochemistry 43, 393–395.
Ghisalberti, E.L., 1997. The biological activity of naturally occurring kaurane diterpenes. Fitoterapia 68, 303–325.
Gładkowski, W., Grabarczyk, M., Wińska, K., Ratuś, B., Białońska, A., Ciunik, Z., Wawrzeńczyk, C., 2007. Lactone 26 [1]: selective microbial epoxidation of unsaturated bicyclic γ-lactones with the alkylsubstituted cyclohexane system. J. Mol. Catal. B: Enzy. 49, 79–87.
González, M.V., Jiménez, B., Berciano, M.T., González-Sancho, J.M., Caelles, C., Lafarga, M., Muñoz, A., 2000. Glucocorticoids antagonize AP-1 by inhibiting the activation/phosphorylation of JNK without affecting its subcellular distribution. J. Cell Biol. 150, 1199–1207.
Hanson, J.R., 1992. The microbiological transformation of diterpenoids. Nat. Prod. Rep. 9, 139–151.
Jochum, W., Passegus, E., Wagner, E.F., 2001. AP-1 in mouse development and tumorigenesis. Oncogene 20, 2401–2412.
Jonat, C., Rahmsdorf, H.J., Park, K.K., Cato, A.C.B., Gebel, S., Ponta, H., Herrlich, P., 1990. Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. Cell 62, 1189–1204.

Lehmann, L.R., Stewart, J.D., 2001. Filamentous fungi: potentially useful catalysts for the biohydroxylations of non-activated carbon centers. Curr. Org. Chem. 5, 439–470.
Saklatvala, J., 2002. Glucocorticoids: do we know how they work? Arthritis Res. 4, 146–150.
Smith, M., Burke, Z., Carter, D., 1996. Tonic suppression of adrenal AP-1 activity by glucocorticoids. Mol. Cell. Endocr. 122, 151–158.
Tsuchida, K., Chaki, H., Takakura, T., Kotsubo, H., Tanaka, T., Aikawa, Y., Shiozawa, S., Hirono, S., 2006. Discovery of nonpeptide small-molecule AP-1 inhibitors: lead hopping based on a three-dimensional pharmacophore model. J. Med. Chem. 49, 80–91.
Venisetty, R.K., Ciddi, V., 2003. Application of microbial biotransformation for the new drug discovery using natural drugs as substrates. Curr. Pharm. Biotechnol. 4, 153–167.
Wisdom, R., 1999. AP-1: one switch for many signals. Exp. Cell Res. 253, 180–185.
Zenz, R., Eferl, R., Scheinecker, C., Redlich, K., Smolen, J., Schonthaler, H.B., Kenner, L., Tschachler, E., Wagner, E.F., 2008. Activator protein 1 (Fos/Jun) functions in inflammatory bone and skin disease. Arthritis Res. Ther. 10. doi:10.1186/ar2338.