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-Juan 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
* Forensic Science Center of Taipei City Police Department, Taipei 115, Taiwan
e Department of Clinical Pharmacy, College of Pharmacy, Taipei Medical University, Taipei 110, Taiwan

A R T I C L E   I N F O

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

A B S T R A C T

A number of hydroxylated diterpenoids were obtained from the microbial transformation of isosteviol lactone (4α-carboxy-13α-hydroxy-13,16-seco-19-norlcyperen-16-oic acid, 13,16-lactone) (2) with Mucor recurratus MR 36, Aspergillus niger BCRC 31130, and Absidia pseudocylindrospora ATCC 24169. Incubation of 2 with M. recurratus 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 (1H, 13C, 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.

© 2009 Elsevier Ltd. All rights reserved.

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 (Tsukuda 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; Gladkowski 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). Chisalberti (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).
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 (Chang et al., 2006, 2008), an AP-1-mediated luciferase reporter gene 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-nobeyeran-16-oic acid 13,16-lactone and 4α-carboxy-12α,13α-dihydroxy-13,16-seco-ent-19-nobeyeran-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 13C 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 13C NMR and DEPT spectra with 2 suggested that 11 contains one more oxygen atom than 2. The HSQC spectrum showed a new proton 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-5δ), 2.36-2.45 (H-6 and H-14), and 3.33 (H-15). Thus, hydration had occurred at C-7. The α-orientation of the 7-OH was suggested from the cross-peaks of δ_{H} 3.53 (H-7) with H-5δ, H-9δ, 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-nobeyeran-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_{32}O_{6}. 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 1H, 13C NMR, HSQC, and HMBC spectra with 2 indicated that the resonances of C-13 and C-16 had shifted downfield from 79.8 to 83.5 and 171.8 to 172.7, respectively. The resonances of C-12 and C-14 had shifted upfield from 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-nobeyeran-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_{32}O_{7}Na. The DEPT, HSQC, and HMBC spectra showed the disappearance of one CH_{3} and one CH signal, and the presence of the multiplicity of the H-12 signal in the 1H 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-9δ, 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-nobeyeran-16-oic acid 13,16-lactone and 4α-carboxy-12α,13α-dihydroxy-13,16-seco-ent-19-nobeyeran-16-oic acid 13,16-lactone, respectively.

Fig. 1. Structures of compounds 1–14.

Fig. 2. Perspective drawing of the X-ray structure of 9.
one new CH₂ 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 (J = 11.5 Hz) and 3.94 (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-9. On the other hand, the lack of a carbonyl–methine resonance in the 1H NMR spectrum when compared with 3.63 (1H, m) contains one more oxygen atom than 2. The location of the OH was confirmed by a detailed analysis of the HMBC data. The chemical shift of δc 77.4 showed connectivities with H-7 (δh 1.32), H-14 (δh 1.67), H-11 (δh 1.87), H-12 (δh 1.99), H-15 (δh 3.75), CH₂-20 (δh 1.41), and OH (δh 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-nobeyeran-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 C₂₀H₂₃O₆. The 1H, 13C NMR, DEPT, and 2D-NMR (COSY, NOESY, HSQC, and HMBC) spectra were similar to those of 12 except for new signals at δc 2.66, 3.63 (1H, m) and δh 77.9. This indicated that 14 contains one more oxygen atom than 12. The orientation of the OH at C-7 was suggested from the cross-peaks of δc 3.63 with H-5 (δh 1.31), H-6 (δh 2.46-2.52), H-9β (δh 1.07-1.14), and H-14 (δh 2.91), whereas no effect was observed between δc 3.63 and 1.16 (CH₂-20) in the NOESY experiment. Thus, 14 was established to be 4α-carboxy-7β,13α,17-trihydroxy-13,16-seco-ent-19-nobeyeran-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-kB 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. 1H, 13C 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 (20–230 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 x 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% H₂SO₄, 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, 4 prisms. The crude residue from fraction 2 (910 mg) over silica gel (720–230 mesh, 5 × 90 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 CH2Cl2–EtOH, CH2Cl2, EtOAc, CH2Cl2, and MeOH with increasing polarity to yield 3, 4, and 5. The crude residue from Abs. pseudocylindrospora (4.7 g) was purified by semi-preparative HPLC (CH2Cl2–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, CH2Cl2, 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 (CH2Cl2–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 (CH2Cl2–isopropanol, 12:1) to give 13 (8 mg) and 14 (16 mg).

4.5.1. Compound 9

White crystals, m.p. > 300 °C; [α]D 25 −76.8 (c 0.5, MeOH); for 1H and 13C NMR spectra, see Tables 2 and 3; HRESIMS m/z 351.2173 [M+H]+ (C20H31O5, calc. 351.2171).

4.5.2. X-ray diffraction analysis of 9

C20H31O5, M 350.44, monoclinic, C2, a 44.8856 (11) Å, b 7.2959 (2) Å, c 11.8141 (3) Å, V 3825.76 (17) Å3; Z 8, Dcalc 1.217 g cm−3, F(0 0 0) 1520, λ (Mo Kα) 0.71073 Å, θ 295(2) K, 15643 reflection collected. Final Goof 0.958, final R indices R1 = 0.0574, wR2 = 0.1375, 478 parameters, μ 0.086 mm−1, R indices based on 8427 reflections with I > 2σ(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; [α]D 25 −76.8 (c 0.5, MeOH); for 1H and 13C NMR spectra, see Tables 2 and 3; HRESIMS m/z 351.2173 [M+H]+ (C20H31O5, calc. 351.2171).

4.5.4. Compound 11

White crystals, m.p. > 300 °C; [α]D 25 −10.4 (c 0.5, MeOH); for 1H and 13C NMR spectra, see Tables 2 and 3; HRESIMS m/z 351.2178 [M+H]+ (C20H31O5, calc. 351.2171).

4.5.5. X-ray diffraction analysis of 11

C20H31O5, M 350.44, orthorhombic, P212121, a 11.1996 (2) Å, b 12.2087 (2) Å, c 13.4538 (2) Å, V 1939.57 (5) Å3; Z 4, Dcalc 1.265 g cm−3, F(0 0 0) 760, λ (Mo Kα) 0.71073 Å, θ 295(2) K, 12063 reflection collected. Final Goof 1.026, final R indices R1 = 0.0443, wR2 = 0.1200, 227 parameters, μ 0.089 mm−1, R indices based on 4156 reflections with I > 2σ(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.a-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; [α]D 25 −7.2 (c 0.5, MeOH); for 1H and 13C NMR spectra, see Tables 2 and 3; HRESIMS m/z 351.2193 [M+H]+ (C20H31O5, calc. 351.2171).

4.5.7. Compound 13

White powder; [α]D 25 −34.0 (c 0.5, MeOH); for 1H and 13C NMR spectra, see Tables 2 and 3; HRESIMS m/z 389.1955 [M+Na]+ (C28H40O5Na, calc. 389.1940).

4.5.8. Compound 14

White powder; [α]D 25 −52.4 (c 0.5, MeOH); for 1H and 13C NMR spectra, see Tables 2 and 3; HRESIMS m/z 367.2132 [M+H]+ (C20H31O5, calc. 367.2121).

4.6. Cell culture, transfection, and reporter gene assays

Twenty-four hours before transfection, about 1 × 105 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-hLuc, 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 DMEM was added to the cells. Cells were harvested 24 h
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 < 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


