Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 68 (2007) 562–570
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Microbial metabolism of steviol and steviol-16α,17-epoxide

Li-Ming Yang [a,e], Feng-Lin Hsu [b], Shwu-Fen Chang [c,d], Juei-Tang Cheng [f], Ju-Yin Hsu [a], Chung-Yi Hsu [d], Pan-Chun Liu [a], Shwu-Jiuan Lin [a,*]

[a] Department of Medicinal Chemistry, College of Pharmacy, Taipei Medical University, Taipei, Taiwan
[b] Graduate Institute of Pharmacognosy, College of Pharmacy, Taipei Medical University, Taipei, Taiwan
[c] Graduate Institute of Cell and Molecular Biology, Taipei Medical University, Taipei, Taiwan
[d] Graduate Institute of Neuroscience, Taipei Medical University, Taipei, Taiwan
[e] Division of Medicinal Chemistry, National Research Institute of Chinese Medicine, Taipei, Taiwan
[f] Department of Pharmacology, College of Medicine, National Cheng-Kung University, Tainan, Taiwan

Received 6 June 2006; received in revised form 12 August 2006

Abstract

Steviol (**2**) possesses a blood glucose-lowering property. In order to produce potentially more- or less-active, toxic, or inactive metabolites compared to steviol (**2**), its microbial metabolism was investigated. Incubation of **2** with the microorganisms *Bacillus megaterium* ATCC 14581, *Mucor recurvatus* MR 36, and *Aspergillus niger* BCRC 32720 yielded one new metabolite, *ent*-7α,11β,13-trihydroxykaur-16-en-19-oic acid (**7**), together with four known related biotransformation products, *ent*-7α,13-dihydroxykaur-16-en-19-oic acid (**3**), *ent*-13-hydroxykaur-16-en-19-α-D-glucopyranosyl ester (**4**), *ent*-13,16β,17-trihydroxykauran-19-oic acid (**5**), and *ent*-13-hydroxy-7-ketokaur-16-en-19-oic acid (**6**). The preliminary testing of antihyperglycemic effects showed that **5** was more potent than the parent compound (**2**). Thus, the microbial metabolism of steviol-16α,17-epoxide (**8**) with *M. recurvatus* MR 36 was continued to produce higher amounts of **5** for future study of its action mechanism. Preparative-scale fermentation of **8** yielded **5**, *ent*-11α,13,16α,17-tetrahydroxykauran-19-oic acid (**10**), *ent*-1β,17-dihydroxy-16-ketobeyeran-19-oic acid (**11**), and *ent*-7α,17-dihydroxy-16-ketobeyeran-19-oic acid (**13**), together with three new metabolites: *ent*-13,16β-dihydroxykauran-17-acetoxy-19-oic acid (**9**), *ent*-11β,13-dihydroxy-16β,17-epoxykauran-19-oic acid (**12**), and *ent*-11β,13,16β,17-tetrahydroxykauran-19-oic acid (**14**). The structures of the compounds were fully elucidated using 1D and 2D NMR spectroscopic techniques, as well as HRFABMS. In addition, a GRE (glucocorticoid responsive element)-mediated luciferase reporter assay was used to initially screen the compounds **3**–**5**, and **7** as glucocorticoid agonists. Compounds **4**, **5** and **7** showed significant effects.

Keywords: Microbial transformation; Steviol; Steviol-16α,17-epoxide; Diterpenoid

1. Introduction

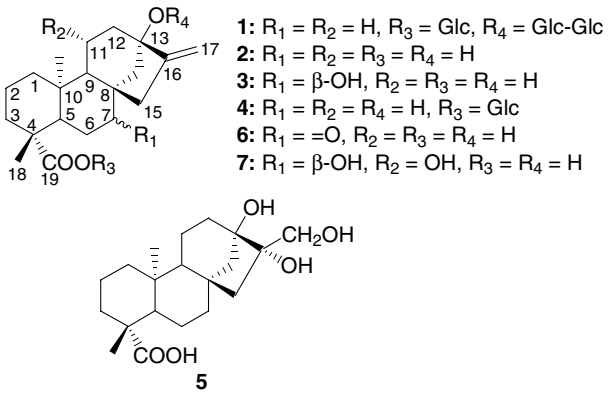
Stevioside (**1**) is the major sweet component isolated from leaves of *Stevia rebaudiana* (Bertoni) Bertoni (Compositae) (Geuns, 2003). Recently, Chen et al. demonstrated that **1** was able to regulate blood glucose levels by enhancing not only insulin secretion, but also insulin utilization in insulin-deficient rats (Chen et al., 2005). Steviol [*ent*-13-hydroxykaur-16-en-19-oic acid] (**2**), an aglycone of stevioside (**1**), is one of the major formed during hydrolysis of (**1**) by intestinal microflora from various animal species, including man (Hutapea et al., 1997; Koyama et al., 2003a,b; Gardana et al., 2003; Geuns et al., 2003). Gardana et al. also demonstrated that only the group of bacteroides was able to degrade stevioside (**1**) (Gardana et al., 2003). Recently, Geuns et al. reported steviol glucuronide as a urinary excretion product after oral intake of stevioside (**1**) by human volunteers (Geuns et al., 2006). Steviol (**2**) has a weak mutagenic activity in the forward mutation assay with *Salmonella typhimurium* TM677 after addition of the

* Corresponding author. Tel.: +886 2 27361661x6133; fax: +886 2 27370903.
E-mail address: shwu-lin@tmu.edu.tw (S.-J. Lin).

0031-9422/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2006.11.021

supernatant 9000 g (S9) fraction (Pezzuto et al., 1985; Matsui et al., 1996; Terai et al., 2002). In contrast, steviol (**2**) decreased glucose production and inhibited oxygen uptake (Yamamoto et al., 1985). Jeppesen et al. indicated that **1** and **2** might have a potential role as antihyperglycemic agents, as they stimulate insulin secretion via direct action on β cells (Jeppesen et al., 2000).

Microorganisms and their enzymes have proven to be versatile biocatalysts, which represent a powerful method for the regio- and stereoselective introduction of hydroxyl groups into organic compounds (Holland and Weber, 2000). The use of selected microorganisms as "models for xenobiotic metabolism" has been successfully exploited in mammalian metabolism studies of many classes of biologically active natural and synthetic compounds (Srisilam and Veeresham, 2003). On the other hand, drug metabolism reactions have traditionally been regarded as detoxication processes. However, many drugs are also biotransformed to pharmacologically active metabolites (Srisilam and Veeresham, 2003). In the past years, the biotransformations of **2** focused on using *Gibberella fujikuroi* and *Rhizopus stolinifer* as biocatalysts (Bearder et al., 1976; Hanson and de Oliveira, 1990; Hanson and White, 1968; Shigematsu et al., 1982). As a part of a program to produce mammalian metabolites and/or novel analogs for biological testings, the bioconversion of **2** by microorganisms was undertaken. By incubation of **2** with *Bacillus megaterium* ATCC 14581, *Mucor recurvatus* MR 36, and *Aspergillus niger* BCRC 32720, metabolites **3**–**7** were isolated (Fig. 1). Preliminary testing of their antihyperglycemic activities showed that *ent*-13,16β,17-trihydroxykauran-19-oic acid (**5**) was more potent than steviol (**2**) (Table 3). The possible pathway for the formation of **5** might be the initial epoxidation of the double bond by cytochrome P450 and then hydrolysis by epoxide hydrolase. Due to **5** obtained from the incubation of **2** with *M. recurvatus*, the preparative-scale fermentation of steviol-16α,17-epoxide (**8**) with *M. recurvatus* MR 36 was continued to produce the expected greater amounts of **5** and six related products (**9**–**14**) (Fig. 2). This report describes the metabolic products produced by culturing **2** with *B. megaterium*, *M. recurvatus*, and *A. niger*, and **8** with *M. recurvatus*, as well as the structural elucidation of the new metabolites **7**, **9**, **12**, and **14**. In addition, a GRE (glucocorticoid responsive element)-mediated luciferase reporter assay was also used to initially screen the compounds **3**–**5**, and **7** as glucocorticoid agonists.



1: $R_1 = R_2 = H$, R_3 = Glc, R_4 = Glc-Glc
2: $R_1 = R_2 = R_3 = R_4 = H$
3: R_1 = β-OH, $R_2 = R_3 = R_4 = H$
4: $R_1 = R_2 = R_4 = H$, R_3 = Glc
6: R_1 = =O, $R_2 = R_3 = R_4 = H$
7: R_1 = β-OH, R_2 = OH, $R_3 = R_4 = H$

Fig. 1. Structures of stevioside (**1**), steviol (**2**), and compounds **3**–**7**.

2. Results and discussion

2.1. Bioconversion of steviol (2) by B. megaterium ATCC 14581, M. recurvatus MR 36, and A. niger BCRC 32720

Steviol (**2**) was prepared as previously reported (Ogawa et al., 1980) and identified by 1D and 2D NMR spectroscopy, and by HRFABMS. The bioconversion of steviol (**2**) by *B. megaterium* ATCC 14581, *M. recurvatus* MR 36, and *A. niger* BCRC 32720 led to the isolation of metabolites **3**–**7**. Of these, *ent*-7α,13-dihydroxykaur-16-en-19-oic acid (**3**) was previously obtained as a methyl ester from incubation of **2** with *Gibberella fujikuroi* (Hanson and de Oliveira, 1990; Shigematsu et al., 1982), whereas *ent*-13-hydroxykaur-16-en-19-α-D-glucopyranosyl ester (**4**) was isolated as a methyl ester from the biotransformation of **2** by culture with cells of *Eucalyptus perriniana* (Orihara et al., 1991). *ent*-13,16β,17-Trihydroxykauran-19-oic acid (**5**) was also previously obtained from the hydrolysis of suavioside I by crude hesperidinase (Ohtani et al., 1992), and *ent*-13-hydroxy-7-ketokaur-16-en-19-oic acid (**6**) was earlier prepared from the oxidation of methyl *ent*-7α, 13-dihydroxykaur-16-en-19-oate with Jones' reagent (Shigematsu et al., 1982). Metabolite **7** was previously unreported and was obtained as white needles. Its HRFABMS (negative-ion mode) showed an $[M-H]^-$ at *m*/*z* 349.2021, indicating a molecular weight of 350, compatible with a molecular formula of $C_{20}H_{29}O_5$ (calc. 349.2015). The ^{13}C NMR spectrum displayed resonances for 20 carbons, while the DEPT spectrum showed the presence of two methyls, eight methylenes, four methines, and six quaternary carbons. The HMQC spectrum showed new resonances at δ_H 4.78 (δ_C 70.9) and δ_H 3.96 (δ_C 76.9) in comparison with those of **2**. This indicates that metabolite **7** contains two more oxygen atoms than does **2**. In the DEPT spectrum, the resonances of C-6 and C-8 shifted downfield from δ 22.7 to 30.6 ($\Delta\delta$ +7.9) and 41.9 to 46.3 ($\Delta\delta$ +4.4), respectively; the resonances of C-5, C-9, and C-15 shifted upfield from δ 57.1 to 48.4 ($\Delta\delta$ −8.7), 54.4 to 53.0 ($\Delta\delta$ −1.4), and 48.3 to 45.1 ($\Delta\delta$ −3.2), respectively, due to the γ-gauche effect. Thus, γ-gauche interactions observed between the hydroxyl substituent and C-5, C-9, and C-15 confirmed that 7-hydroxylation had occurred (Silva et al., 1999). In the HMBC spectrum, δ_H 3.96 also exhibited cross-peaks with δ_C 47.0 (C-14), 48.4 (C-5), and 53.0 (C-9). Furthermore, the NOESY spectrum also showed cross-peaks of δ 3.96 (H-7) with H-6 (δ 2.41–2.47 and 2.69), H-14β (δ 1.74), and H-15 (δ 2.75–2.79). Accordingly, the hydroxyl group was determined to be in the axial (β) position. The location of the second hydroxyl group at the C-11 position of **7** was deduced by HMBC correlations

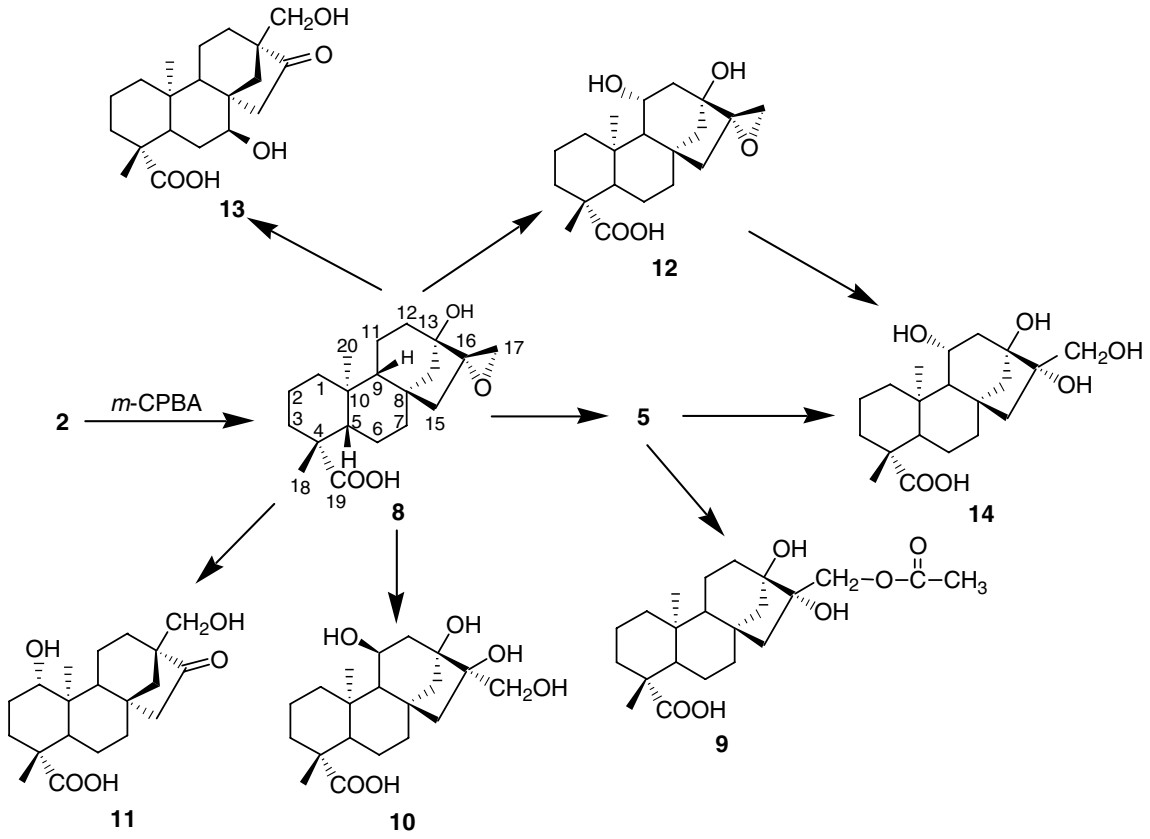


Fig. 2. Microbial transformation of steviol-16α,17-epoxide (**8**) by *Mucor recurvatus*.

among δ_H 4.78 (H-11) and δ_C 53.0 (C-9), 41.4 (C-10), and 51.5 (C-l2). The α-orientation of the hydroxyl group at C-11 was suggested from the cross-peaks between δ 4.78 (H-11) and δ 2.52–2.53 (2H, H-9β and H-12) and 2.75–2.79 (3H, H-12 and H-15), and by the absence of a cross-peak with CH_3-20 (δ 1.78) in the NOESY spectrum. In a comparison of the ^{1}H NMR spectrum of **7** with that of methyl *ent*-7β,11α,13-trihydroxykaur-16-en-19-oate (Shigematsu et al., 1982), it was found that H-11 was located at δ 4.78 as a doublet triplet ($^3J_{11,12} = 13.0$, $^3J_{9,11} = 6.5$ Hz) in **7**, and H-11α was located at δ 4.48 as a doublet ($J = 6.0$ Hz) in methyl *ent*-7β,11α,13-trihydroxykaur-16-en-19-oate. Thus, the orientation of the hydroxyl group at C-11 was established to be α. On the basis of the above evidence, the structure of **7** was determined to be *ent*-7α,11β,13-trihydroxykaur-16-en-19-oic acid.

2.2. Bioconversion of steviol-16α,17-epoxide (*8*) by M. recurvatus MR 36

Steviol-16α,17-epoxide (**8**) was obtained from steviol (**2**) by reaction with *m*-chloroperbenzoic acid (Pezzuto et al., 1985; Terai et al., 2002). Proton and carbon assignments of **8** were initially confirmed by 1D and 2D NMR, IR, and HRFABMS. Incubation of **8** with *M. recurvatus* for 6 days led to the formation of *ent*-13,16β,17-trihydroxykauran-19-oic acid (**5**), *ent*-11α,13,16α,17-tetrahydroxykauran-19-oic acid (**10**), *ent*-1β,17-dihydroxy-16-ketobeyeran-19-oic acid (**11**), and *ent*-7α,17-dihydroxy-16-ketobeyeran-19-oic acid (**13**) (Chang et al., 2006), along with three unreported compounds (**9**, **12**, and **14**), which were separated by repeated column chromatography using silica gel, MCI-gel CHP 20P, Sephadex LH-20, and Cosmosil 75C_{18}-OPN, respectively. Metabolite **9** had a molecular formula of $C_{22}H_{35}O_6$ as determined from its positive ion HRFABMS $[M+H]^+$ at *m/z* 395.2450 as well as from its ^{13}C NMR spectrum. The IR spectrum also suggested the presence of two carbonyl groups (1726 and 1691 cm^{-1}). The ^{13}C NMR spectrum displayed resonances for 22 carbons, while the DEPT spectrum showed the presence of three methyl, ten methylene, two methine, and seven quaternary carbons. The ^{1}H and ^{13}C NMR spectra were similar to *ent*-13,16β, 17-trihydroxykauran-19-oic acid (**5**) except for new signals at δ_H 2.01 (3H, *s*), and at δ_C 20.9 and 171.3 (C=O), which are typical of an acetate group (Lee et al., 2004). These findings suggested the presence of an acetyl group in the molecule. The placement of the acetoxy group at CH_2OH-17 was deduced from the HMBC spectrum of **9**, which clearly correlated CH_2OH-17 (δ 4.59, 2H, *s*) with the carboxyl carbon of the acetate (δ_C 171.3). On the basis of analysis of the ^{1}H and ^{13}C NMR spectra, together with that of the HMQC and HMBC spectra, **9** was characterized *ent*-13,16β-dihydroxykauran-17-acetoxy-19-oic acid.

Metabolite **12** was isolated as a white powder and displayed a quasi-molecular ion peak $[M+H]^+$ at *m/z*

351.2188, corresponding to a molecular formula of $C_{20}H_{31}O_5$. Its IR spectrum showed the presence of 1694 cm^{-1} (C=O) and 1245 cm^{-1} (epoxide). The ^{13}C NMR spectrum displayed resonances for 20 carbons, while the DEPT spectrum showed the presence of two methyl, eight methylene, four methine, and six quaternary carbons. The HMQC spectrum showed new resonances at δ_H 4.75 (δ_C 69.7) indicating that metabolite **12** contains one more oxygen atom than does **8**. Comparison of the ^1H and ^{13}C NMR spectra of **8** and **12** revealed that the substitution of a hydroxy group in **12** occurred at C-11. The location of the hydroxyl group was confirmed by a detailed analysis of the HMBC data. The chemical shift of δ_H 4.75 showed connectivities with C-8 (δ_C 41.5) and C-10 (δ_C 41.3). The α-orientation of the hydroxyl group at C-11 was suggested from the cross-peaks of δ_H 4.75 with H-9β (δ_H 1.62), H-15 (δ_H 1.97), and H-12 (δ_H 2.47–2.57), whereas no effect was observed between δ 4.75 and 1.67 (CH_3-20) in the NOESY experiment. Thus, **12** was established as *ent*-11β,13-dihydroxy-16β,17-epoxy-kauran-19-oic acid.

Metabolite **14** showed a quasi-molecular ion peak at *m/z* 369.2295 $[M+H]^+$ in the HRFABMS, corresponding to the molecular formula of $C_{20}H_{33}O_6$. Its IR spectrum showed the presence of one carbonyl group (1689 cm^{-1}). The ^1H NMR spectrum showed the absence of an epoxy methylene signal [δ 2.92 (2H, *s*)], but a new AB system at δ 3.33 and 3.37 (each 1H, $^2J_{17,17} = 11.0$ Hz) appeared instead. This thus suggested that the epoxy ring was hydrolyzed to the skeleton of **5**. The ^{13}C NMR and DEPT spectra showed the same multiplicity of carbons as that of *ent*-11α,13,16α,17-tetrahydroxykauran-19-oic acid (**10**). The HMQC spectrum showed a proton geminal to a new alcohol at δ_H 4.10 (δ_C 68.2). In the HMBC spectrum, δ_C 68.2 exhibited cross-peaks with δ_H 1.14 (H-9β), 1.60–1.70 (H-12), and 1.78 (H-12). Thus, hydroxylation occurred at C-11. The α-orientation of the hydroxy group at C-11 was suggested from the cross-peaks of H-11 (δ 4.10) with H-9β (δ 1.14), H-15 (δ 1.47), and H-12 (δ 1.78) in the NOESY experiment. Furthermore, the orientation of 16-hydroxy was established by a NOE correlation of CH_2OH-17 (δ 3.33 and 3.37) with H-15 (δ 1.47) and H-12 (δ 1.78), whereas no effect was observed between CH_2OH-17 (δ 3.33 and 3.37) and H-14 (δ 1.53 and 1.74–1.76). Accordingly, the 16-hydroxy functionality was in the α orientation. On the basis of the above evidence, **14** was established as *ent*-11β,13,16β,17-tetrahydroxykauran-19-oic acid.

The selected microorganisms examined herein have the abilities to perform hydroxylation, acetylation, glucosidation, and rearrangement of substrates **2** and **8**. *ent*-7α,13-Dihydroxykaur-16-en-19-oic acid (**3**) and *ent*-13-hydroxy-7-ketokaur-16-en-19-oic acid (**6**) are simple hydroxylated and oxidized metabolites likely formed by activation and insertion of one atom of oxygen into the 7β-position of **2**, and then oxidizing **3** to form ketone **6**. *ent*-7α,11β,13-Trihydroxykaur-16-en-19-oic acid (**7**) was obtained by further hydroxylation of the 11α-position of **3**. *ent*-13-Hydroxykaur-16-en-19-α-D-glucopyranosyl ester (**4**) is a conjugated product formed by insertion of β-D-glucose into the 19-position by *B. megaterium* ATCC 14581. The reaction was also reported to occur on the *ent*-beyerane skeleton in our recent study (Yang et al., 2004). A possible pathway to *ent*-13,16β,17-trihydroxykauran-19-oic acid (**5**) can be obtained by a two-step process involving the initial epoxidation of the 16,17 double bond by cytochrome P450 and subsequent hydrolysis by epoxide hydrolase to form the *trans*-dihydrodiol metabolite. In addition, **2** and its analogs (**3**–**7**) were tested for antihyperglycemic effect in vivo using rats with streptozocin (STZ)-induced diabetes (Hsu et al., 2000). Due to the small amount of isolated metabolites, the preliminary experiments were at a dose of 5 mg/kg by an intraperitoneal (IP) injection to the STZ-diabetic rats. *ent*-13,16β,17-Trihydroxykauran-19-oic acid (**5**) produced a significant reduction in plasma glucose at 60 ($\Delta G = -15.56 \pm 5.06\%$) and 90 min ($\Delta G = -15.71 \pm 6.44\%$). However, steviol (**2**) produced no significant reduction in plasma glucose at 60 ($\Delta G = -2.68 \pm 1.42\%$) or 90 min ($\Delta G = -9.87 \pm 0.19\%$). In contrast, compound **5** produced no significant reduction in plasma glucose at 120 min ($\Delta G = 2.04 \pm 2.14\%$), but **2** produced a significant reduction in plasma glucose at 120 min ($\Delta G = -14.89 \pm 3.88\%$). Koyama et al., utilizing negative ion LC/ESI/MS, analyzed the oxidative metabolites ($[M-H]^-$ at *m/z* 333, 331, and 351) of steviol (**2**) after incubation with both human and rat liver microsomes (Koyama et al., 2003a), and human intestinal microflora (Koyama et al., 2003b). They suggested that cytochrome P450 might be involved in steviol (**2**) oxidation in both human and rat liver microsomes. *ent*-13,16β,17-Trihydroxykauran-19-oic acid (**5**) (negative HRFABMS: *m/z* 351.2173, calc. for $C_{20}H_{31}O_5$, 351.2172) was proposed as being one of the metabolites of steviol (**2**) in the literature (Koyama et al., 2003a). The data obtained herein suggest that the parent compound **2** might be converted into its metabolites to achieve its pharmacological effect. Thus, the onset of action for *ent*-13,16β,17-trihydroxykauran-19-oic acid (**5**) was faster than that of steviol (**2**). The mutagenic effects of **2** and/or its structurally related derivatives, except for **5**, have been studied (Pezzuto et al., 1985; Matsui et al., 1996; Terai et al., 2002). Whether *ent*-13,16β,17-trihydroxykauran-19-oic acid (**5**) is the sole active metabolite of steviol remains to be elucidated. Compound **5** was obtained from the incubation of **2** with *M. recurvatus*. Thus, preparative-scale fermentation of steviol-16α,17-epoxide (**8**) with *M. recurvatus* was continued to produce greater amounts of **5**. The results suggested that the double bond in **2** was converted to the epoxidation product first, and then hydrolyzed to **5** by epoxide hydrolase. Due to tetracyclic diterpenoids possessing a formal similarity to steroids (Hanson, 1992), a GRE (glucocorticoid responsive element)-mediated luciferase reporter assay was also used to initially screen the compounds **3**–**5**, and **7** as glucocorticoid agonists. Compounds **4**, **5**, and **7** showed significant effects, but less active than the reference compounds of methylprednisolone and dexamethasone (Table 4).

3. Conclusion

In conclusion, the metabolism of stevioside (**1**) in mammals has been studied (Hutapea et al., 1997; Koyama et al., 2003a,b; Gardana et al., 2003; Geuns et al., 2003, 2006). However, the metabolism of steviol (**2**) alone in mammals and the actual mutagenic species have not been established. Thus, these isolated metabolites will also be used as reference standards for monitoring mammalian metabolism of steviol (**2**) and for determining further toxicological effects. The larger amounts of *ent*-13,16β,17-trihydroxykauran-19-oic acid (**5**) obtained from biotransformation of steviol-16α,17-epoxide (**8**) with *M. recurvatus* will also provide a technical basis for studying advances in its action mechanism, and associated pharmacological and toxicological effects.

4. Experimental

4.1. General experimental

Melting points were determined using a Yanagimoto micromelting 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, HMQC, and HMBC) spectra were recorded on a Varian Unity Inova 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 FAB mass spectra were recorded using a JEOL JMX-HX 110 and a JMS-700 HRMS spectrometer. IR spectra were performed on a Perkin Elmer spectrum GX/AutoImage microscope FT-IR spectrometer. CC was performed with Sephadex® LH-20 (Pharmacia, Sweden), MCI-gel CHP 20P (75–150 μm, Mitsubishi Chemical, Tokyo, Japan), Cosmosil 75C_{18}-OPN (Nacalai Tesque, Inc., Kyoto, Japan), and Kieselgel silica (70–230 and 230–400 mesh, Merck, Darmstadt, Germany). TLC plates of Merck 1.05554 (Si 60 with F_{254}) were purchased from Merck. Spots for all were detected by spraying with 10% H_2SO_4, followed by heating.

4.2. Substrates

Steviol (**2**) and steviol-16α,17-epoxide (**8**) were prepared as previously reported (Ogawa et al., 1980; Pezzuto et al., 1985; Terai et al., 2002). Signals for the protons and carbons of **2** and **8** were initially assigned by 1D and 2D NMR, IR, and HRFABMS spectroscopic analyses.

4.3. Microorganisms, incubation, and screening procedures

Twenty-seven microorganisms, 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, were used for initial screening of **2**. The following genera (with the number of species screened) were screened for their abilities to metabolize steviol (**2**): *Absidia* (1), *Aspergillus* (3), *Bacillus* (2), *Beauveria* (2), *Cunninghamella* (5), *Gliocladium* (1), *Mortierella* (1), *Mucor* (3), *Mycobacterium* (1), *Nocardia* (4), *Pseudomonas* (2), and *Streptomyces* (2). Stock cultures were maintained on Sabouraud-maltose agar slants or those recommended by the ATCC and BCRC, stored at 4 °C, and subcultured periodically. The fermentation protocol for screening was identical to that previously described (Yang et al., 2004) using a medium of the following composition: dextrose (20 g), yeast extract (5 g), NaCl (5 g), K_2HPO_4 (5 g), soybean flour (5 g), and distilled H_2O (1 L); the pH of the final mixture was adjusted to 7.0 with 6 N HC1 before sterilization by autoclaving at 121 °C for 15 min. For screening, cultures were grown in 25 mL of medium in 125-mL Erlenmeyer flasks equipped with stainless steel caps. Substrate **2** was added to each flask 24 h later as 100-μL dimethylformamide (DMF) aliquots each containing 10 mg of substrate in solution to give a final concentration of 0.4 mg/mL of culture. Cultures were incubated with shaking on a rotatory shaker, operating at 250 rpm and 28 °C for a maximum period of 14 days with sampling and TLC monitoring at 3-day intervals. Samples of culture broth (4 mL) were acidified with 6 N HC1, and extracted with EtOAc-*n*-butanol (9:1, 1 mL) The organic layer was spotted onto a TLC plate with CH_2Cl_2–MeOH (9:1) as the solvent system. Culture controls and substrate control were both run. Five metabolites were reproducibly produced by *B. megaterium* ATCC 14581, *M. recurvatus* MR 36, and *A. niger* BCRC 32720.

4.4. Preparative transformations of steviol (2) by B. megaterium ATCC 14581, M. recurvatus MR 36, and A. niger BCRC 32720

Using 24-h-old stage II cultures, a solution of **2** (500 mg dissolved in 5 mL DMF) was evenly distributed among 50 flasks containing stage II cultures. Substrate-containing cultures were incubated for 144 h. Extraction as previously described (Yang et al., 2004) produced 892 mg of brown oil, 927 mg of yellow oil, and 857 mg of green oil after respective bioconversions with *B. megaterium*, *M. recurvatus*, and *A. niger*. The crude residue from *B. megaterium* (892 mg) was subjected to silica gel CC (70–230 mesh, 3 × 50 cm). In total, 10 fractions were eluted with mixtures of CH_2Cl_2–MeOH (600 mL each of 25:1, 15:1, and 10:1). The elutes were monitored using TLC. The fractions were combined on the basis of similar TLC profiles. With further chromatography of the fractions 3 and 4 (410 mg) over silica gel (230–400 mesh, 1 × 30 cm) eluted with CH_2Cl_2–MeOH (20:1), 255 mg of **2** was recovered. Fractions 6 and 7 (90 mg) were chromatographed over silica gel (230–400 mesh, 0.8 × 20 cm) eluted with CH_2Cl_2–MeOH (20:1) to give *ent*-7α,13-dihydroxykaur-16-en-19-oic acid (**3**). After recrystallization with CH_3OH, 21 mg (4.0%) of **3**

was obtained. Fractions 8 and 9 (140 mg) were chromatographed over silica gel (230–400 mesh, 1 × 30 cm) eluted with CH_2Cl_2–MeOH (10:1) to give *ent*-13-hydroxykaur-16-en-19-α-D-glucopyranosyl ester (**4**). After recrystallization with CH_3OH, 67 mg (8.9%) of **4** was obtained. The crude residue from *M. recurvatus* (927 mg) was subjected to column chromatography over silica gel (70–230 mesh, 3 × 50 cm). In total, 12 fractions were eluted with mixtures of CH_2Cl_2–MeOH (600 mL each of 25:1, 15:1, and 10:1). With further chromatography of fractions 3 and 4 (450 mg) over silica gel (230–400 mesh, 1 × 30 cm), 289 mg of **2** was recovered. Fractions 5 and 6 (90 mg) were chromatographed over silica gel (230–400 mesh, 0.8 × 20 cm) and eluted with CH_2Cl_2–MeOH (20:1), and 37 mg (6.96%) of *ent*-7α,13-dihydroxykaur-16-en-19-oic acid (**3**) was obtained. Fractions 8–10 (126 mg) were chromatographed over silica gel (230–400 mesh, 1 × 20 cm) and eluted with CH_2Cl_2–MeOH (15:1), and 10 mg (1.79%) of *ent*-13,16β,17-trihydroxykauran-19-oic acid (**5**) was obtained. The crude residue from *A. niger* (857 mg) was

Table 1
^{1}H NMR chemical shifts for compounds **2**, **7**–**9**, **12**, and **14** (C_5D_5N, δ values in ppm)[a,b]

Position	**2**	**7**	**8**	**9**	**12**	**14**[c]
1	1.65–1.90[d] (*m*)	3.63 (*br d*, $^3J_{1,2} = 13.6$)	1.81–2.02[d] (*m*)	1.81–1.84[d] (*m*)	3.58 (*m*)	2.83 (*d*, $^3J_{1,2} = 13.5$)
	0.85 (*td*, $^3J_{1,2} = 11.5$, $^2J_{1,1} = 4.0$),	1.52–1.59[d] (*m*)	0.86 (*m*)	0.85 (*td*, $^3J_{1,2} = 12.5$, $^2J_{1,1} = 4.0$)	1.29 (*dd*, $^3J_{1,2} = 14.0$, $^2J_{1,1} = 3.5$)	0.83 (*td*, $^3J_{1,2} = 13.5$, $^2J_{1,1} = 4.5$)
2	2.12–2.30[d] (*m*)	2.41–2.47[d] (*m*)	2.18–2.27[d] (*m*)	2.12–2.29[d] (*m*)	2.41 (*m*)	1.74–1.76[d] (*m*)
	1.48–1.57[d] (*m*)	1.52–1.59[d] (*m*)	1.50–1.56[d] (*m*)	1.47 (*m*)	1.54 (*br d*, $^3J_{1,2} = 13.5$)	1.19–1.28[d] (*m*)
3	2.46 (*d*, $^3J_{2,3} = 13.0$)	2.54–2.56[d] (*m*)	2.46 (*d*, $^3J_{2,3} = 12.7$)	2.44 (*d*, $^3J_{2,3} = 13.0$)	2.47–2.57[d] (*m*)	1.95 (*d*, $^3J_{2,3} = 13.0$)
	1.03–1.10[d] (*m*)	1.26 (*td*, $^3J_{2,3} = 13.2$, $^2J_{3,3} = 4.0$)	1.03–1.08[d] (*m*)	1.02 (*m*)	1.14 (*td*, $^3J_{2,3} = 13.5$, $^2J_{3,3} = 4.5$)	0.89 (*td*, $^3J_{2,3} = 13.0$, $^2J_{3,3} = 4.0$).
5β	1.03–1.10[d] (*m*)	2.54–2.56[d] (*m*)	1.03–1.08[d] (*m*)	1.07 (*d*, $^3J_{5,6} = 10.5$)	1.24 (*d*, $^3J_{5,6} = 10.5$)	0.99 (*br d*, $^3J_{5,6} = 11.0$)
6	2.12–2.30[d] (*m*)	2.69 (*t*, $^3J_{5,6} = {}^3J_{6,7} = 13.5$)	2.18–2.27[d] (*m*)	2.12–2.29[d] (*m*)	2.21–2.29[d] (*m*)	1.60–1.70[d] (*m*)
	1.97–2.08[d] (*m*)	2.41–2.47[d] (*m*)	1.81–2.02[d] (*m*)	1.94–1.99[d] (*m*)	2.02 (*m*)	
7	1.48–1.57[d] (*m*)	α3.96 (*br s*)	1.50–1.56[d] (*m*)	1.69 (*d*, $^3J_{6,7} = 13.5$)	1.71 (*br d*, $^3J_{6,7} = 13.0$)	1.42 (*br d*, $^3J_{6,7} = 12.5$)
	1.43 (*td*, $^3J_{6,7} = 13.5$, $^2J_{7,7} = 3.5$)		1.42 (*t*, $^3J_{6,7} = 10.6$)	1.42 (*dd*, $^3J_{6,7} = 13.5$, $^2J_{7,7} = 3.5$)	1.44 (*td*, $^3J_{6,7} = 13.0$, $^2J_{7,7} = 2.5$)	1.19–1.28[d] (*m*)
9β	1.0 (*d*, $^3J_{9,11} = 8.0$)	2.52–2.53[d] (*m*)	0.96 (*d*, $^3J_{9,11} = 7.3$)	0.96 (*d*, $^3J_{9,11} = 6.5$)	1.62 (*d*, $^3J_{9,11} = 8.0$)	1.14 (*d*, $^3J_{9,11} = 7.5$)
11	1.65–1.90[d] (*m*)		1.81–2.02[d] (*m*)	1.75 (2H, *m*)		
		β4.78 (*dt*, $^3j_{11,12} = 13.0$, $^2J_{9,11} = 6.5$)	1.63–1.75[d] (*m*)		β4.75 (*dt*, $^3J_{11,12} = 12.0$, $^2J_{9,11} = 7.0$)	β4.10 (*dt*, $^3J_{11,12} = 11.5$, $^2J_{9,11} = 7.5$)
12	1.97–2.08[d] (*m*)	2.75–2.79[d] (*m*)	1.81–2.02[d] (*m*)	1.94–1.99[d] (*m*)	2.47–2.57[d] (*m*)	1.78 (*m*)
	1.65–1.90[d] (*m*)	2.52–2.53[d] (*m*)	1.63–1.75[d] (*m*)			1.60–1.70[d] (*m*)
14	2.33 (*dd*, $^3J_{14,15} = 11.0$, $^2J_{14,14} = 2.0$)	2.63 (*d*, $^3J_{14,15} = 10.8$)	2.37 (*d*, $^3J_{14,15} = 10.7$)	2.12–2.29[d] (*m*)	2.47–2.57[d] (*m*)	1.74–1.76[d] (*m*)
	1.48–1.57[d] (*m*)	1.74 (*d*, $^3J_{14,15} = 10.8$)	1.63–1.75[d] (*m*)		2.21–2.29[d] (*m*)	1.53 (*dd*, $^3J_{14,15} = 10.5$, $^2J_{14,14} = 1.5$)
15	2.12–2.30[d] (*m*)	2.75–2.79[d] (*m*)	1.79 (2H, *s*)	1.94–1.99[d] (*m*) 1.81–1.84[d] (*m*)	1.97 (2H, *m*)	1.47 (*d*, $^3J_{14,15} = 14.0$) 1.19–1.28[d] (*m*)
17	5.45 (*d*, $^2J_{17,17} = 1.0$)	5.49 (*s*)	2.92 (2H, *s*)	4.59 (2H, *s*)	4.03 (1H, *d*, $^2J_{17,17} = 11.5$)	3.37 (*d*, $^2J_{17,17} = 11.0$)
	5.01 (*d*, $^2J_{17,17} = 1.0$)	4.99 (*s*)			4.05 (1H, *d*, $^2J_{17,17} = 11.5$)	3.33 (*d*, $^2J_{17,17} = 11.0$)
18-CH_3	1.34 (*s*)	1.44 (*s*)	1.33 (*s*)	1.32 (*s*)	1.34 (*s*)	1.06 (*s*)
20-CH_3	1.18 (*s*)	1.78 (*s*)	1.18 (*s*)	1.16 (*s*)	1.67 (*s*)	1.02 (*s*)
CH_3CO				2.01 (*s*)		

[a] Attributes based on DEPT, HMQC, and HMBC.
[b] Signal multiplicity and coupling constants (Hz) are in parantheses.
[c] In DMOS-d_6.
[d] Overlapping signals.

Table 2
^{13}C NMR chemical shifts for compounds **2**, **7**–**9**, **12**, and **14** (C_5D_5N, δ values in ppm)[a]

Position	2	7	8	9	12	14[b]
1	41.1	43.3	41.1	40.9	43.0	41.9
2	19.9	20.3	19.8	19.8	20.0	18.8
3	38.7	30.1	38.7	38.6	38.8	37.7
4	44.0	43.9	43.9	43.9	44.1	43.0
5	57.1	48.4	57.0	56.8	58.2	57.1
6	22.7	30.6	22.6	22.8	22.7	21.8
7	42.0	76.9	41.9	42.7	43.9	43.1
8	41.9	46.3	41.6	41.7	41.5	40.5
9	54.4	53.0	54.2	55.1	58.6	57.6
10	39.9	41.4	39.8	39.8	41.3	40.2
11	20.9	70.9	19.9	20.1	69.7	68.2
12	40.8	51.5	36.7	34.1	44.7	43.6
13	79.9	78.7	75.1	80.2	79.5	77.9
14	47.6	47.0	46.4	44.5	45.2	43.7
15	48.3	45.1	47.8	52.3	52.4	50.4
16	157.8	158.2	65.3	77.1	78.5	77.8
17	103.0	102.6	48.2	68.0	51.1	63.8
18	29.4	29.9	29.4	29.3	29.9	29.2
19	180.2	180.9	180.2	180.1	180.3	178.8
20	16.0	16.8	16.2	15.8	16.6	15.8
$COCH_3$				20.9		
$COCH_3$				171.3		

[a] Attributes based on DEPT, HMBC, and HMQC.
[b] In DMSO-d_6.

Table 3
Effects of plasma glucose lowering activity of compounds **1**–**7** in STZ-induced diabetic rats

Compound	Plasma glucose lowering activity (%)[a]		
	60 min	90 min	120 min
1	−5.27 ± 8.81	−5.60 ± 3.65	−9.06 ± 6.55
2	−2.68 ± 1.42	−9.87 ± 0.19	−14.89 ± 3.88
3	14.75 ± 0.04	12.55 ± 0.49	8.21 ± 7.33
4	−9.76 ± 10.01	−7.50 ± 1.00	4.50 ± 2.98
5	−15.56 ± 5.06	−15.71 ± 6.44	2.04 ± 2.14
6	11.29 ± 6.27	6.52 ± 7.55	2.40 ± 8.76
7	−9.26 ± 3.35	−6.37 ± 1.39	5.46 ± 4.22

[a] Values (mean ± SEM) ($n = 4$) are obtained from one group of experimental animals.

Table 4
Data for compounds **3**–**5**, and **7** on a gluconcorticoid receptor-mediated assay

Compound	Luciferase activity	Compound	Luciferase activity
MP	2.05 ± 0.297[a]	**4**	1.62 ± 0.201[a]
Dex	1.99 ± 0.218[a]	**5**	1.41 ± 0.151[a]
3	1.23 ± 0.044	**7**	1.53 ± 0.209[a]

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

subjected to column chromatography over silica gel (70–230 mesh, 3 × 50 cm). In total, 15 fractions were eluted with mixtures of CH_2Cl_2–MeOH (600 mL each of 25:1, 20:1, and 15:1). With further chromatography of fractions 3–8 (560 mg) over silica gel (230–400 mesh, 1 × 30 cm) eluted with CH_2Cl_2–MeOH (100 mL each of 30:1, 20:1, and 10:1) in five fractions (fractions 1–5) was obtained with **2** (195 mg, 39.0%) recovered from fraction 3. After recrystallization of fractions 1 and 4 with EtOAc and MeOH, *ent*-7α-13-hydroxy-7-ketokaur-16-en-19-oic acid (**6**) (12 mg, 2.3%) and *ent*-7α,13-dihydroxy-kaur-16-en-19-oic acid (**3**) (11 mg, 2.1%) were obtained, respectively. Fractions 10–12 (85 mg) were applied to over silica gel column (230–400 mesh, 0.8 × 20 cm) eluted with CH_2Cl_2–MeOH (10:1) to obtain *ent*-7α,11β,13-trihydroxykaur-16-en-19-oic acid (**7**). After recrystallization with $(CH_3)_2CO$, 15 mg (2.7%) of **7** was obtained as white needles.

4.4.1. *ent*-7α,11β,13-Trihydroxykaur-16-en-19-oic acid (7)

M.p. 237–239 °C [$(CH_3)_2CO$]; $[\alpha]_D^{25}$ −19.6 (*c*0.5, MeOH); For ^1H and ^{13}C NMR spectra, see Tables 1 and 2; LRFABMS *m/z* 349 $[M-H]^-$; HRFABMS *m/z* 349.2021 ($C_{20}H_{29}O_5$, calc. 349.2015).

4.5. Preparative transformations of steviol-16α,17-epoxide (8) by Mucor recurvatus MR 36

Mucor recurvatus was grown according to the usual fermentation procedure, and stage II fermentations were conducted in one hundred 125-mL stainless-steel-capped flasks. Compound **8** (1.0 g) was next dissolved in DMF (10 mL), with aliquots of equal volume evenly distributed among the 24-h-old stage II cultures. After 144 h, the incubation mixtures were pooled and acidified with 6 N HCl and then filtered to remove the cells. The filtrate was extracted with EtOAc-*n*-butanol (9:1, 3×). Cells were washed with acetone, filtered, evaporated, and extracted with EtOAc. The extracts were evaporated to dryness under reduced pressure. The combined crude residues from the filtrate and cells (4.0 g) were subjected to silica gel CC (70–230 mesh, 3 × 50 cm). In total, 30 fractions were eluted with mixtures of CH_2Cl_2–MeOH (600 mL each of 30:1, 20:1, 15:1, 12:1, and 6:1). The elutes were monitored using TLC. The fractions were combined on the basis of similar TLC profiles. With further chromatography of fractions 4–7 (430 mg) over silica gel (230–400 mesh, 1 × 30 cm) eluted with CH_2Cl_2–MeOH (20:1), **8** (145 mg, 14.5%) was recovered. Fraction 11 (50 mg) was applied to a MCI-gel CHP 20P (0.8 × 20 cm) column eluted with MeOH–H_2O (7:3) to yield *ent*-13,16β-dihydroxykauran-17-acetoxy-19-oic acid (**9**) (10 mg, 0.85%). Further chromatography of fractions 13 and 14 (67 mg) over silica gel (230–400 mesh, 0.8 × 30 cm) eluted with hexane–EtOAc (1:2) yielded *ent*-11α,13,16α,17-tetrahydroxykauran-19-oic acid (**10**). After recrystallization with EtOAc, 40 mg (3.63%) of **10** was obtained. Fractions 15–17 (110 mg) were subjected to a MCI-gel CHP 20P (0.6 × 25 cm) eluted with MeOH–H_2O

(65:35) to yield of *ent*-1β,17-dihydroxy-16-ketobeyeran-19-oic acid (**11**) (8 mg, 0.76%). Fraction 18 (260 mg) was applied to a Sephadex LH-20 (1.5 × 30 cm) column eluted with $CHCl_3$–MeOH (15:1 to 7:1) to obtain fractions A and B, and of *ent*-11β,13-dihydroxy-16β,17-epoxykauran-19-oic acid (**12**) (12 mg 1.15%). Fraction B (80 mg) was further subjected to MCI-gel CHP 20P (0.8 × 20 cm) CC eluted with MeOH–H_2O (6:4) to yield *ent*-7α,17-dihydroxy-16-ketobeyeran-19-oic acid (**13**) (35 mg, 3.34%). Fraction 20 (270 mg) was separated on a Sephadex LH-20 column eluted with MeOH–H_2O (80% to 60% MeOH) to obtain fractions a–c. Fraction b was recrystallized with EtOAc–MeOH, and 195 mg (18.5%) of **5** was obtained as needles. Fractions 23–28 (45 mg) were applied to a Cosmosil C_{18}-OPN column eluted with MeOH–H_2O (4:6) to yield *ent*-11β,13,16β,17-tetrahydroxykauran-19-oic acid (**14**). After recrystallization with $(CH_3)_2CO$, **14** (8 mg, 0.73%) was yielded. Using CH_2Cl_2–MeOH (9:1) as solvent system, TLC R_f values of seven metabolites (**5** and **8**–**14**) were obtained: 0.32 (**5**), 0.52 (**9**), 0.50 (**10**), 0.50 (**11**), 0.40 (**12**), 0.45 (**13**), and 0.07 (**14**) compared to 0.65 of **8**.

4.5.1. ent-13,16β-Dihydroxykauran-17-acetoxy-19-oic acid (9)

White powder; $[\alpha]_D^{25}$ −91.2 (*c*0.45, MeOH); IR (KBr): ν_{max} cm^{-1} 3412, 1726 (C=O), 1691 (C=O); for ^1H and ^{13}C NMR spectra, see Tables 1 and 2; LRFABMS *m/z* 395 $[M+H]^+$; HRFABMS *m/z* 395.2450 ($C_{22}H_{35}O_6$, calc. 395.2434).

4.5.2. ent-11β,13-Dihydroxy-16β,17-epoxykauran-19-oic acid (12)

White powder; $[\alpha]_D^{25}$ −49.3 (*c*1.2, MeOH); IR (KBr) ν_{max} cm^{-1} 3409, 1694 (C=O), 1245 (epoxide); for ^1H and ^{13}C NMR spectra, see Tables 1 and 2; LRFABMS *m/z* 351 $[M+H]^+$; HRFABMS *m/z* 351.2188 $[M+H]^+$ ($C_{20}H_{31}O_5$, calc. 351.2172).

4.5.3. ent-11β,13,16β,17-Tetrahydroxykauran-19-oic acid (14)

White needles, m.p. >300°C (acetone); $[\alpha]_D^{25}$ −45.2 (*c* 1.0 MeOH); IR (KBr) ν_{max} cm^{-1} 3379, 1689 (C=O); for ^1H and ^{13}C NMR spectra, see Tables 1 and 2; LRFABMS *m/z* 369 $[M+H]^+$; HRFABMS *m/z* 369.2295 $[M+H]^+$ ($C_{20}H_{33}O_6$, calc. 369.2277).

4.6. Antihyperglycemic testing

The preliminary antihyperglycemic activity was evaluated using rats with streptozocin (STZ)-induced diabetes, and blood glucose was determined as described previously (Hsu et al., 2000). Metformin ($\Delta G = -18.4 \pm 3.1\%$ at a dose of 100 mg/kg) was used as a positive control. Results were calculated as the percentage decrease in the initial value according to the formula: $[(G_t - G_i)/G_i \times 100\%]$ where G_i is the initial value and G_t is the value after receiving the compound.

4.7. Cell culture, transfection, and reporter gene assays

Twenty-four hours before transfection, about 1×10^5 mouse Raw 264.7 macrophage cells per well were seeded in 96-well white plates. The pGR-Luc plasmid and an internal control plasmid, the pGL-*hRluc*, were transfected into Raw 264.7 cells using the lipofectamine plus agent (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. At 24 h post-transfection, final concentrations of 10 μM of each test compound including the reference compounds, methylprednisolone and dexamethasone (Sigma, St. Louis, MO, USA), in DMSO were added to the cells. Cells were harvested 24 h after treatment, and the reporter activity of firefly luciferase expressed from pGR-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).

4.7.1. Statistical analysis

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

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. This study was supported through grants from the National Science Council of the Republic of China (NSC94-2320-B-038-030, NSC94-2320-B-077-005, and NSC94-3112-B-038-004).

References

Bearder, J.R., Frydman, V.M., Gaskin, P., MacMillan, J., Wels, C.M., 1976. Fungal products. Part XVI. Conversion of isosteviol and steviol acetate into gibberellin analogues by mutant B1-4la of *Gibberella fujikuroi* and the preparation of [^{3}H]gibberellin A_{20}. J. Chem. Soc. Perkin I, 173–183.

Chang, S.F., Yang, L.M., Hsu, F.L., Hsu, J.Y., Liaw, J.H., Lin, S.J., 2006. Transformation of steviol-16a, 17-epoxide by *Streptomyces griseus* and *Cunninghamella bainieri*. J. Nat. Prod. 69, 1450–1455.

Chen, T.S., Chen, S.C., Chen, P., Chu, Y.L., Yang, H.Y., Cheng, J.T., 2005. Mechanism of the hypoglycemic effect of stevioside, a glycoside of *Stevia rebaudiana*. Planta Med. 71, 108–113.

Gardana, C., Simonetti, P., Canzi, E., Zanchi, R., Pietta, P., 2003. Metabolism of stevioside and rebaudioside A from *Stevia rebaudiana* extracts by human microflora. J. Agric. Food Chem. 51, 6618–6622.

Geuns, J.M.C., 2003. Stevioside. Phytochemistry 64, 913–921.

Geuns, J.M.C., Augustijns, P., Mols, R., Buyse, J.G., Driessen, B., 2003. Metabolism of stevioside in pigs and intestinal absorption characteristics of stevioside, rebaudioside A and steviol. Food Chem. Toxicol. 41, 1599–1607.

Geuns, J.M.C., Buyse, J., Vankeirsbilck, A., Temme, E.H.M., Compernolle, F., Toppet, S., 2006. Identification of steviol glucuronide in human urine. J. Agric. Food Chem. 54, 2794–2798.

Hanson, J.R., de Oliveira, B.H., 1990. The microbiological transformation of steviol derivatives by *Rhizopus stolinifer* and *Gibberella fujikuroi*. Phytochemistry 29, 3805–3807.

Hanson, J.R., White, A.F., 1968. The transformation of steviol by *Gibberella fujikuroi*. Tetrahedron 24, 6291–6293.

Hanson, J.R., 1992. The microbiological transformation of diterpenoids. Nat. Prod. Rep. 9, 139–151.

Holland, H.L., Weber, H.K., 2000. Enzymatic hydroxylation reactions. Curr. Opin. Biotechnol. 11, 547–553.

Hsu, F.L., Chen, Y.C., Cheng, J.T., 2000. Caffeic acid as active principle from the fruit of *Xanthium strumarium* to lower plasma glucose in diabetic rats. Planta Med. 66, 228–230.

Hutapea, A.M., Toskulkao, C., Buddhasukh, D., Wilairat, P., Glinsukon, T., 1997. Digestion of stevioside, a natural sweetener by various digestive enzymes. J. Clin. Biochem. Nutr. 23, 177–186.

Jeppesen, P.B., Gregersen, S., Poulsen, C.R., Hermansen, K., 2000. Stevioside acts directly on pancreatic b cells to secrete insulin: actions independent of cyclic adenosine monophosphate and adenosine triphosphate-sensitive K^+-channel activity. Metabolism 49, 208–214.

Koyama, E., Sakai, N., Kitazawa, K., Izawa, O., Kakegawa, K., Fujino, A., Ui, M., 2003a. Absorption and metabolism of glycosidic sweeteners of stevia mixture and their aglycone, steviol, in rats and humans. Food Chem. Toxicol. 41, 875–883.

Koyama, E., Kitazawa, K., Ohori, Y., Izawa, O., Kakegawa, K., Fujino, A., Ui, M.I., 2003b. In vitro metabolism of the glycosidic sweeteners, stevia mixture and enzymatically modified stevia in human intestinal microflora. Food Chem. Toxicol. 41, 359–374.

Lee, H.S., Ahn, J.W., Lee, Y.H., Rhom, J.R., Shin, J.H., 2004. New sesterterpenes from the Antarctic sponge *Suberities* sp. J. Nat. Prod. 67, 672–674.

Matsui, M., Matsui, K., Kawasaki, Y., Oda, Y., Noguchi, T., Kitagawa, Y., Sawada, M., Hayashi, M., Nohmi, T., Yoshihira, K., Ishidate Jr., M., Sofuni, T., 1996. Evaluation of the genotoxicity of stevioside and steviol using six in vitro and one in vivo mutagenicity assays. Mutagenesis 11, 573–579.

Ogawa, T., Nozaki, M., Matsui, M., 1980. Total synthesis of stevioside. Tetrahedron 36, 2641–2648.

Ohtani, K., Aikawa, Y., Kasai, R., Chou, W.H., Yamasaki, K., Tanaka, O., 1992. Minor diterpene glycosides from sweet leaves of *Rubus suavissimus*. Phytochemistry 31, 1553–1559.

Orihara, Y., Saiki, K., Furuya, T., 1991. Biotransformation of steviol by cultured cells of *Eucalyptu perriniana* and *Coffea arabica*. Phytochemistry 30, 3989–3992.

Pezzuto, J.M., Compadre, C.M., Swanson, S.M., Nanayakkara, P.D., Kinghorn, A.D., 1985. Metabolically activated steviol, the aglycone of stevioside, is mutagenic. Proc. Natl. Acad. Sci. USA 82, 2478–2482.

Shigematsu, Y., Murofushi, N., Takahashi, N., 1982. Structures of the metabolites from steviol methyl ester by *Gibberella fujikuroi*. Agric. Biol. Chem. 46, 2313–2318.

Silva, E.A., Takahashi, J.A., Boaventura, M.A.D., Oliveira, A.B., 1999. The biotransformation of *ent*-kaur-16-en-19-oic acid by *Rhizopus stolonifer*. Phytochemistry 52, 397–400.

Srisilam, K., Veeresham, C., 2003. Biotransformation of drugs by microbial cultures for predicting mammalian drug metabolism. Biotechnol. Adv. 21, 3–39.

Terai, T., Ren, H., Mori, G., Yamaguchi, Y., Hayashi, T., 2002. Mutagenicity of steviol and its oxidative derivatives in *Salmonella typhimurium* TM677. Chem. Pharm. Bull. 50, 1007–1010.

Yamamoto, N.S., Kelmer, A.M., Bracht, E.L., Ishii, F.S., Kemmelmeier, F.S., Alvarez, M., Bracht, A., 1985. Effect of steviol and its structural analogues on glucose production and oxygen uptake in rat renal tubules. Experientia 41, 55–57.

Yang, L.M., Hsu, F.L., Cheng, J.T., Chang, C.H., Liu, P.C., Lin, S.J., 2004. Hydroxylation and glucosidation of *ent*-16b-hydroxybeyeran-19-oic acid by *Bacillus megaterium* and *Aspergillus niger*. Planta Med. 70, 359–363.