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一、中文摘要

Isosteviol 是由酸水解 stevioside 所得到的產物，具有潛在的降血糖及降血壓效果。經由 *Cunninghamella elegans*，*Cunninghamella blakesleeana* 及 *Cunninghamella bainieri* 進行微生物轉換後，新的氫氧基被立體專一的引入 isosteviol 的 7α-，7β-，9β-，12β- 的位置。分離得到的代謝物經由 1D, 2D NMR 及 HRFABMS 的光譜分析以決定其構造。

關鍵詞：Isosteviol, 微生物轉換, 二萜類

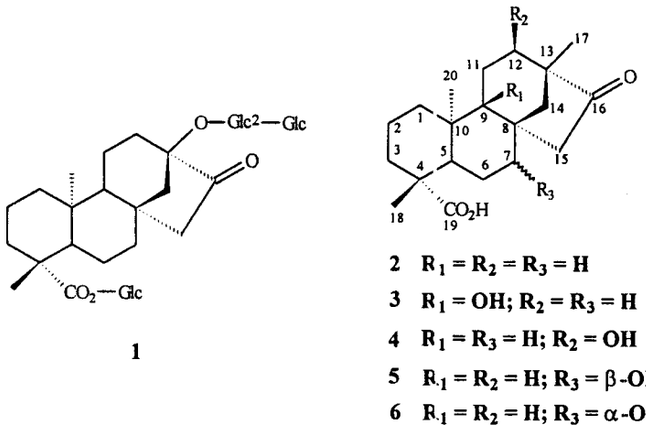
Abstract

Microbial transformation of isosteviol, using *Cunninghamella blakesleeana*, *Cunninghamella elegans* and *Cunninghamella bainieri*, resulted in the isolation of four microbial metabolites that were characterized on the basis of 1D and 2D NMR spectroscopy, and HRFABMS. The metabolites were identified as *ent*-7α-hydroxy-16-ketobeyeran-19-oic acid, *ent*-9α-hydroxy-16-ketobeyeran-19-oic acid, *ent*-12α-hydroxy-16-keto-beyeran-19-oic acid, and *ent*-7β-hydroxy-16-ketobeyeran-19-oic acid. Among them, 9β-hydroxyl derivative is being reported for the first time.

Keywords: Isosteviol; Microbial hydroxylation; *Cunninghamella*.

二、緣由與目的

Stevioside (**1**) is the main diterpene glycosides of *Stevia rebaudiana* leaves, and is used as a sweetener in the food industry. *Stevia rebaudiana* leaves have physiologic and therapeutic effects including cardiotonic,[1] contraceptive[2] and hypoglycemic properties.[3] Isosteviol (**2**) with a beyerane skeleton was obtained by acid hydrolysis of the stevioside.[4] Bracht et al. demonstrated that isosteviol was an inhibitor on rat liver mitochondria functions.[5] It also decreased glucose production, inhibited oxygen uptake in isolated rat renal tubules[6] and inhibited D-glucose and D-fructose transport across the cell membrane.[7] Recently, isosteviol was thought to be a potential hypoglycemic compound with streptozotocin-treated rats,[8a] and lowered the blood pressure in spontaneously hypertensive rats through opening the K^+ channel to inhibit calcium influx.[8b] Thus, isosteviol analogues would be of interest to exploit its potential activities and to establish structure activity relationships. However, the branched-chain nature of isoprenoid backbone provides a block to many conventional chemical manipulations. In the past years, microbial transformations have been widely employed in the preparation of difficult to synthesize analogues of steroids[9] and diterpenoids.[10] Smith and Rosazza have postulated the concept of using microorganisms as models for mammalian metabolism of xenobiotics in the early 1970s.[11-12] The fungal systems have been proposed as models for mammalian drug metabolism, and the advantages of using such a microbial model include low cost, ease of handling, and scale-up capability as reviewed by Davis and Abourashed et al.[13-14] Further, the zygomycete fungi of the genus *Cunninghamella* have been shown to have the ability to metabolize xenobiotics with various structures features to produce metabolites similar to those found in mammalians.[13, 15-18] As far as we know, only three papers on the microbial transformations of isosteviol have been published. The hydroxyl group was introduced at 7α-, 7β-, 12β-, 17- and 1α,7β-positions of isosteviol.[19-21] Thus, the genus *Cunninghamella* was utilized for microbial



1

2 $R_1 = R_2 = R_3 = H$

3 $R_1 = OH$; $R_2 = R_3 = H$

4 $R_1 = R_3 = H$; $R_2 = OH$

5 $R_1 = R_2 = H$; $R_3 = \beta$-OH

6 $R_1 = R_2 = H$; $R_3 = \alpha$-OH

Figure 1. Structures of compound **1-6**

transformation of isosteviol (**2**) to yield new hydroxylated compounds. Sufficient quantities of metabolites could then be isolated and used for pharmacological, toxicological, and analytical testing in mammalian metabolism studies. This report describes the preparation of isosteviol analogues using microbial transformation technology and the detailed NMR data of isolated metabolites is described herein.

三、結果與討論

Isosteviol (**2**) was obtained by hydrolysis of stevioside (**1**) with dilute hydrochloric acid. The structure of isosteviol (**2**) was identified by ^{1}H-, ^{13}C NMR and X-ray crystallography. Preparative scale biotrans-formation of **2** by *Cunninghamella elegans* (ATCC 8688a) afforded metabolite **3**, **4**, and **5**. HRFABMS data of **3** showed the exact molecular weight at *m/z* 335.2206 $[M + H]^+$, which was consistent with the molecular formula of $C_{20}H_{31}O_4$. The ^{13}C NMR spectrum displayed one signal at δ 76.6. However, the lake of the carbinol-methine signal in the ^{1}H NMR spectrum suggested that the hydroxyl group should be introduced at one of tertiary carbons. In ^{13}C NMR spectrum, the chemical shift of C-11 was shifted from δ 21.1 to 27.1. The 20-methyl group was also shifted significantly downfield in both carbon and proton NMR spectra to δ 17.4 (Δδ + 3.3 ppm vs. **2**) and δ 1.29 (Δδ + 0.3 ppm vs. **2**), suggesting a hydroxyl group might be at C-9. HMQC and HMBC spectral analyses showed that the signal at δ 76.6 was three-bond correlated with carbons 7, 12, 14, 15, and 20-methyl group, and two-bond correlated with carbon 11, thus confirming the location of the hydroxyl group at C-9. On the basis of spectral analyses, metabolite **3** was assigned the structure of *ent*-9α-hydroxy-16-keto-beyeran-19-oic acid. The second metabolite was obtained as white needles. The molecular formula was deduced as $C_{20}H_{31}O_4$ from a $[M + H]^+$ peak observed at *m/z* 335.2224 in the HRFABMS and its ^{13}C NMR data. One of methylene signal substituted with a methine signal in DEPT experiment and one singlet peak with one proton appeared at δ 3.93 in ^{1}H NMR spectrum indicating hydroxyl group was introduced at methylene carbon with axial (β) position. The resonance of carbons 11 and 13 was significantly downfield shifted to δ 30.4 (Δδ + 9.3 ppm vs. **2**) and 55.6 (Δδ + 6.5 ppm vs. **2**), respectively. On the other hand, the carbons at 9, 14, and 17 were exhibited upfield shifts from δ 55.2 to 50.4 (Δδ - 4.8 ppm vs **2**), δ 54.7 to 48.3 (Δδ - 6.4 ppm vs. **2**), and δ 20.8 to 18.5 (Δδ - 2.3 ppm vs. **2**), respectively. HMBC correlation's of the position-12 methine proton at δ 3.93 showed three-bond connectivities to carbons 14, 15,

and 17, thus confirming the location of the hydroxyl group at C-12. Further, an NOESY experiment did not display the significant NOE correlations among H-12 and H-9, and H-14. Accordingly, the β-configuration of 12-OH was confirmed. Based on the above evidences, the structure of **4** was *ent*-12α-hydroxy-16-ketobeyeran-19-oic acid. The third metabolite was identified as *ent*-7α-hydroxy-16-ketobeyeran-19-oic acid. HRFABMS also displayed a molecular ion at *m/z* 335.2231 $[M + H]^+$ for $C_{20}H_{31}O_4$, suggesting the presence of an additional oxygen in the molecular. New signals at δ_H 3.95 and δ_C 76.0 also indicated that a hydroxylation had occurred. HMQC and HMBC analyses suggested the presence of a C-7 hydroxyl group in the metabolite structure. The axial (β) hydroxylation was identified by the downfield shifts of C-6 (Δδ + 8.4 ppm vs. **2**) and C-8 (Δδ + 4.9 ppm vs. **2**) and upfield shifts of C-5 (Δδ - 9.4 ppm vs. **2**), C-9 (Δδ - 5.5 ppm vs. **2**) and C-14 (Δδ - 3.4 ppm vs. **2**) due to γ-gauche effect.[22] This was also deduced from a broad singlet signal of H-7α, which indicated the β stereochemistry of the newly introduced hydroxyl group at C-7. The identity was finally confirmed by X-ray crystallography.

The fermentation of isosteviol (**2**) with *Cunninghamella elegans* (ATCC 9245) yielded one major metabolite, which identified as *ent*-7α-hydroxy-16-ketobeyeran-19-oic acid due to its ^1H- and ^{13}C NMR showing the same spectra as metabolite **5**. Two metabolites were yielded by incubation of isosteviol **2** with *Cunninghamella bainieri* (UI 3065). One metabolite was *ent*-7α-hydroxy-16-ketobeyeran-19-oic acid (**5**), and the other metabolite **6** was identified as *ent*-7β-hydroxy-16-ketobeyeran-19-oic acid. HRFABMS of **6** displayed a molecular ion at *m/z* 335.2218 $[M + H]^+$, which corresponded to the molecular formula $C_{20}H_{31}O_4$. The ^1H NMR spectrum of **6** displayed a downfield signal at δ 3.73 suggesting a hydroxyl group was in the molecule structure. HMBC and HMQC spectral analyses suggested that the C-7 was the most likely position of hydroxylation of the ring system. HMBC correlations of the position-7 proton at δ 3.73 showed three-bond connectivities to carbons C-9, C-14, and C-15, and two-bond connectivities with carbons C-6 and C-8. Hydroxylation at C-7 position was apparent from the downfield shift of the resonance assigned to C-6 (Δδ + 10.0 ppm vs. **2**) and C-8 (Δδ + 8.7 ppm vs. **2**), and the γ-gauche shielding experienced by C-5 (Δδ - 3.0 ppm vs. **2**), C-9 (Δδ - 0.9 ppm vs. **2**) and C-14 (Δδ - 4.7 ppm vs. **2**). The new hydroxyl group was at C-7 in an *ent*-7β orientation, since its proton was displayed at δ 3.73 as double doublet pattern (J = 5.5, 15.0 Hz). Thus, *Cunninghamella bainieri* (UI 3065) was able to convert isosteviol **2** to its two 7-hydroxyl isomers.

The genus *Cunninghamella* has the ability to highly functionalize isosteviol **2** at C-7 position. This is a common occurrence for other microbes with kaurenes and beyerenes skeletons. The 7α-, 7β-, and 12β-hydroxyisosteviol have been reported previously by 1D NMR.[19-21] We now report their detailed spectroscopic data by 2D NMR techniques, HRFABMS and/or X-ray crystallography. Although 9β-hydroxylation product has been observed for kaurane skeleton,[23] it is the first time to obtain for beyerenes. These metabolites will be used as analytical standards for mammalian metabolic studies of isosteviol.

四、計畫成果自評

By using microbial transformation technology, isosteviol was transformed into four metabolites by *Cunninghamella*. The new hydroxyl group was stereospecifically introduced at 7α–, 7β–, 9β–, and 12β–position. The result will be submitted to the Journal for publication later. On the other hand, *Cunninghamella* has the enzymes similar to the mammalian cytochrome P450. Thus, these metabolites will provide us to do pharmacological and analytical testing in

mammalian metabolism studies. In addition to this result, we now are still working at other microorganisms. Some new metabolites have been isolated. The preliminary spectroscopic analyses show that two or three new hydroxyl groups are on the rings. It also will be reported later. These results will provide us to do other microbial transforamtions of natural products.

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