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BIOTRANSFORMATION OF STEVIOL BY CULTURED CELLS OF *EUCALYPTUS PERRINIANA* AND *COFFEA ARABICA**

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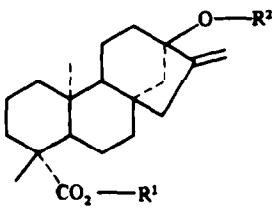
Key Word Index—*Eucalyptus perriniana*; Myrtaceae; *Coffea arabica*; Rubiaceae; cell suspension culture; biotransformation; steviol; rubusoside; steviol 19-β-gentiobiosyl ester; glucosylation.

Abstract—A new biotransformation product, steviol 19-β-gentiobiosyl ester, together with steviol 19-β-glucopyranosyl ester and steviol-13-*O*-β-glucopyranoside 19-β-glucopyranosyl ester (rubusoside), was isolated from *Eucalyptus perriniana* jar fermentor culture following the administration of steviol. Only rubusoside was isolated as a biotransformation product of steviol from *Coffea arabica* cell suspension culture.

INTRODUCTION

Stevioside (**1**) and rebaudioside A (**2**) are the main sweet diterpene glycosides of *Stevia rebaudiana* leaves, and are used as sweeteners in the food industry. Several attempts to produce the sweet compounds using plant tissue culture techniques have been performed [1].

Cultured cells of *Eucalyptus perriniana* characteristically produce eight triterpenes [2]. This cell line also has the ability to glycosylate and hydroxylate (−)-menthol [3], (+)-menthol [4] and 18β-glycyrrhetinic acid [5]. Cultured cells of *Coffea arabica* can produce theobromine and caffeine [6], and convert phenylacetic and 2-phenylpropionic acids to their sucrose esters [7] and 18β-glycyrrhetinic acid to its glucopyranosyl ester [5]. We now report on the isolation and structure elucidation of biotransformation products of steviol (**3**), the aglycone of **1** and **2**, produced by cultured cells of *E. perriniana* and *C. arabica*.



	R^1	R^2
1	Glc	Glc^{2}—Glc
2	Glc	Glc^{2}—Glc, Glc^{3}
3	H	H
4	Glc	H
5	Glc	Glc
6	Glc^{6}—Glc	H

RESULTS AND DISCUSSION

The substrate, steviol (**3**), was obtained by the hydrolysis of crude stevioside (**1**) with crude hesperidinase in citrate–phosphate buffer (pH 4). In preliminary experiments, it was clear that **3** is more toxic to plant cells than mono- and triterpenes, so that low concentrations of the substrate were required. An ethanol solution of **3** (500 mg × 2, three days interval) with glucose (100 g × 1) was administered to the 10 l jar fermentor culture of *E. perriniana* at stationary phase. After final administration, the cells were cultured for a further eight days and harvested. From this biotransformation experiment, products **4**–**6** were isolated. By contrast only **5** was isolated from eight culture flasks of *C. arabica* (administered 200 mg of **3**). On TLC analysis, **4** and **5** were detected in the medium.

The ^{13}C NMR chemical shifts of **3** and its biotransformation products **4**–**6** are shown in Table 1. These data show that **4** contains 26 carbon atoms, i.e. six more than **3**, and that **5** and **6** contains 32 carbon atoms, i.e. 12 more than **3**. Their signals of the steviol moiety were compared with those of **3**, the substrate. In all products, C-19 was shifted to a higher field. On the other hand only in **5** was C-13 shifted to lower field and C-12, C-14 and C-16 to higher field. From these data, sugars are connected to C-19 carboxylic acid in all products and to the C-13 tertiary hydroxyl group in **5**.

Product **4** was isolated as needles, and gave a M_r of 480 on the basis of FAB-MS. As proton signals of acetate (**4a**) assignable to 1′-5′ had relatively large coupling constants (J = 8–9.5 Hz), connected sugar was determined as β-glucose. Thus **4** is steviol 19-β-glucopyranosyl ester.

Product **6** was isolated as needles, and had a M_r of 642, 162 more than **4**. In the ^{13}C NMR spectrum, the steviol moiety of **6** was similar to **4**, so that two sugars were connected to C-19 carboxylic acid. From the proton NMR analysis of acetate, both sugars are β-glucose. As

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Table 1. ^{13}C NMR spectral data of compounds **3**–**6** (pyridine-d_5) and their acetates ($CDCl_3$) (in parentheses) (at 75 MHz)

	3	4	4a	5	5a	6	6a
1	41.2	41.0	(40.4[a])	40.9	(40.5)	40.9	(40.4[a])
2	20.1	19.7	(19.0)	19.6	(19.1)	19.7	(19.1)
3	38.8	38.6	(37.8)	38.5	(37.7)	38.5	(37.8)
4	44.0	44.3	(44.1)	44.2	(44.2)	44.3	(44.1)
5	57.1	57.6	(56.9)	57.5	(56.9)	57.6	(57.0)
6	22.8	22.4	(21.6)	22.3	(21.5)	22.4	(21.6)
7	42.1	42.1	(41.2)	41.9	(41.2)	42.1	(41.3)
8	42.1	42.0	(41.6)	42.6	(42.3)	42.0	(41.8)
9	54.4	54.4	(53.7)	54.1	(53.5)	54.4	(53.8)
10	40.0	40.0	(39.4)	40.0	(39.4)	40.0	(39.4)
11	21.0	21.0	(20.4)	20.8	(20.3)	21.0	(20.4)
12	41.0	41.0	(39.2[a])	37.4	(37.5)	40.9	(39.7[a])
13	79.9	79.9	(80.2)	86.1	(86.4)	80.0	(79.8)
14	47.6	47.5	(47.2)	44.7	(43.4)	47.5	(47.0)
15	48.3	48.3	(47.4)	47.9	(47.5)	48.2	(47.4)
16	157.9	157.9	(156.0)	154.7	(151.7)	157.9	(155.3)
17	103.1	103.0	(103.0)	104.6	(105.1)	103.0	(103.0)
18	29.5	28.7	(28.7)	28.5	(28.8)	28.8	(29.0)
19	180.2	177.0	(175.6)	177.1	(175.3)	177.1	(175.1)
20	16.1	15.9	(15.7)	15.8	(16.1)	16.0	(16.1)
1′		96.0	(91.2)	96.1	(91.1)	95.9	(91.1)
2′		74.2	(70.2)	74.2	(70.4)	73.9	(70.3)
3′		79.3	(73.1)	79.3	(73.0)	79.2[a]	(72.9)
4′		71.3	(68.1)	71.2	(68.0)	71.7[b]	(68.6)
5′		79.5	(72.4)	79.5	(72.4)	78.6[a]	(74.8)
6′		62.3	(61.7)	62.2	(61.5)	69.8	(67.5)
1″						105.4	(100.7)
2″						75.3	(71.1)
3″						78.3[a]	(73.0)
4″						71.3[b]	(68.4)
5″						78.5[a]	(71.7)
6″						62.8	(61.8)
1‴				99.9	(95.9)		
2‴				75.7	(71.6)		
3‴				79.0	(73.0)		
4‴				72.5	(68.7)		
5‴				78.2	(71.5)		
6‴				63.2	(62.4)		

[a,b] Assignments may be reversed in each vertical column.

^{13}C chemical shifts of C-6′ was observed at δ 69.8, shifted to lower field than common glucose, connection of glucoses was that second glucose connected to C-6′ in the first glucose. Thus **6** is steviol 19-β-gentiobiosyl ester.

Product **5** was isolated as amorphous solid and had a M_r of 642 as well as **6**. As previously mentioned, two sugars were connected to 13-hydroxyl and 19-carboxylic acid groups. Connected sugars were determined as β-glucose by the analysis of proton NMR of its acetate, so that **5** is steviol-13-*O*-β-glucopyranoside 19-β-glucopyranosyl ester.

Compound **4** was synthesized from **3** and glucose derivatives [8]. Compound **5** was firstly isolated from *Rubus chingii* [9] (*R. suavissimus*, corrected in following paper [10]) as a sweet compound, and named rubusoside. However, **6** is a new compound.

Biotransformation yields from **3** to **4**, **5** and **6** were calculated as 9.7, 10.6 and 21%, respectively. In biotransformation experiment with cultured cells of *C. arabica*, only product **5** (yield, 7.5%) was isolated and product **4** was detected by TLC analysis. In coffee cells **3** may be glucosylated at C-19 firstly then at C-13. All these products have a sweet taste, but they are not so sweet as stevioside.

EXPERIMENTAL

Mps: uncorr. ^1H NMR: 300 MHz, setting $CHCl_3$ signal at δ_H 7.26 and the lowest pyridine (pyridine-d_4) signal at δ_H 8.60 ppm. ^{13}C NMR: 75 MHz, setting $CDCl_3$ signal at δ_C 77.0 and the lowest pyridine-d_5 signal at δ_C 150.0 ppm.

Cell lines. Cultured cells of *Eucalyptus perriniana* used in this investigation were derived from young stems of this plant in 1980 and maintained on BA1 agar medium [Murashige and Skoog (MS) medium [11] supplemented with sucrose (30 g l^{-1}), agar (9 g l^{-1}) and 6-benzylaminopurine (1 mg l^{-1})], as previously reported [2]. Cultured cells of *Coffea arabica* were derived from seed segments in 1982 and subcultured on DK agar medium

[MS medium supplemented with sucrose (30 g l^{-1}), agar (9 g l^{-1}), 2,4-dichlorophenoxyacetic acid (1 mg l^{-1}) and kinetin (0.1 mg l^{-1})], as previously reported [6].

Enzymatic hydrolysis of crude stevioside. Stevioside (**1**) (10 g) and crude hesperidinase (1 g) were dissolved in citrate–Pi buffer (pH 4.0, 1.5 l), and then added EtOH (0.5 l). These mixt. was kept at 37° with shaking for 7 days. Two portions of **1** (5 g × 2) were added to the reaction mixt. every 7 days after the crude steviol (**3**) was filtered off. The reaction mixt. was kept at 37° for a further 2 weeks and the crude **3** was filtered off. These crude steviols were combined and recrystallized from MeOH to yield needles (5.225 g, yield 66%).

Steviol (**3**). Mp 204–205°; $[\alpha]_D^{21}$ −65° ($CHCl_3$; *c* 1.0); IR ν_{max}^{KBr} cm^{-1}: 3460 (OH), 3275 (OH), 2800–3000 (CH_2), 1650 (CO_2H); ^1H NMR (pyridine-d_5): δ 1.07 (3H, *s*, Me), 1.23 (3H, *s*, Me), 4.90 (1H, *br s*, H_a-17), 5.35 (1H, *br s*, H_b-17).

Feeding experiment to the jar fermentor culture of E. perriniana. Cell suspension culture were initiated from static cultured cells in a 1 l conical flask containing 250 ml medium, and cultured on a reciprocal shaker (90 strokes min^{-1}) for 3 weeks at 25° in the dark. The cells and the medium in 8 flasks were inoculated into a 10 l jar fermentor (Takasugi Seisakusho, Japan) containing 8 l BA1 medium, and cultured for 50 days setting temp. at 25°, aeration ratio at 0.125 VVM and agitation speed at 50 rpm. When the culture was achieved to the stationary phase, glucose (100 g/400 ml H_2O, autoclaved) and **3** (500 mg/20 ml EtOH, through membrane filter) were administered, and 3 days later additional **3** (500 mg/20 ml EtOH) was administered. After 8 days culture, the cells and the medium were sepd by filtration. The medium was passed through Diaion HP20 column and the column was washed with H_2O and eluted with MeOH. The MeOH eluate was concd and partitioned between H_2O and *n*-BuOH. The BuOH layer was concd to obtain medium-BuOH Fr. (0.43 g). The cells (fr. wt 2563 g) was extracted (× 2) with MeOH and MeOH extract was partitioned between EtOAc and H_2O. The H_2O layer was further extracted (× 2) with *n*-BuOH and *n*-BuOH layer was concd to obtain cell-BuOH Fr. (17.59 g). The biotransformation products of **3** could be detected on TLC from medium-BuOH Fr. and cell-BuOH Fr. visualized by exposing I_2 vapor. These frs were chromatographed on silica gel and further purified by HPLC [column; Senshu Pak ODS-4301-N, solvent; MeOH-H_2O (80:20)]. From medium-BuOH Fr. products **4** (93.1 mg) and **5** (52.5 mg), from a part of cell-BuOH Fr. (7.975 g) products **4** (24.6 mg), **5** (73.4 mg) and **6** (193.1 mg) were obtained.

Feeding experiment to the shake flask culture of C. arabica. Cell suspension culture was initiated in DK liquid medium as well as *E. perriniana*. An EtOH soln (12.5 mg ml^{-1}, 2 ml to each flask) of **3** was administered and cultures returned to the shaker for a further 7 days. The cells were harvested through nylon mesh and the medium was extracted with *n*-BuOH. The cells were treated as well as the extraction procedures of *E. perriniana* cells. From the cell-BuOH Fr. (0.66 g) of 8 culture flasks (200 mg of **3** was administered) product **5** (22.6 mg) was isolated. Products **4** and **5** were also detected on TLC analysis of medium-BuOH Fr.

Steviol 19-β-glucopyranosyl ester (**4**). Needles, mp 190–192° (MeOH); $[\alpha]_D^{28}$ −57° (MeOH; *c* 1.0); IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 2850–2950 (CH_2), 1730 (CO_2-Glc); FAB-MS *m/z* 503 [M + Na]$^+$; ^1H NMR (pyridine-d_5): δ 1.18 (6H, *s*, Me), 3.90 (1H, *ddd*, *J* = 9.0, 4.0, 2.5 Hz, H-5′), 4.07 (1H, *dd*, *J* = 9.0, 8.0 Hz, H-2′), 4.13 (1H, *dd*, *J* = 9.0, 9.0 Hz, H-3′), 4.22 (1H, *dd*, *J* = 9.0, 9.0 Hz, H-4′), 4.26 (1H, *dd*, *J* = 12.0, 4.0 Hz, H-6′$_a$), 4.32 (1H, *dd*, *J* = 12.0, 2.5 Hz, H-6′$_b$), 4.87 (1H, *br s*, H_a-17), 5.32 (1H, *br s*, H_b-17), 6.10 (1H, *d*, *J* = 8.0 Hz, H-1′).

Steviol-13-O-*β-glucopyranoside 19-β-glucopyranosyl ester* (**5**). Amorphous solid; $[\alpha]_D^{28}$ −45° (MeOH; *c* 1.0); IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 2850–2930 (CH_2), 1730 (CO_2-Glc); FAB-MS *m/z* 665 [M + Na]$^+$; ^1H NMR (pyridine-d_5): δ 1.11 (3H, *s*, Me), 1.13 (3H, *s*, Me), 3.80–3.92 (2H, *m*, H-5′, H-5‴), 3.92–4.00 (2H, *m*, H-2‴, H-4‴), 4.02–4.21 (5H, *m*, H-2′, H-3′, H-4′, H-3‴, H-6‴$_a$), 4.25 (1H, *dd*, *J* = 12.0, 4.0 Hz, H-6′$_a$), 4.32 (1H, *br d*, *J* = 12.0 Hz, H-6′$_b$), 4.49 (1H, *br d*, *J* = 11 Hz, H-6″$_b$), 4.87 (1H, *br s*, H_a-17), 5.01 (1H, *d*, *J* = 8.0 Hz, H-1‴), 5.41 (1H, *br s*, H_b-17), 6.02 (1H, *d*, *J* = 8.0 Hz, H-1′).

Steviol 19-β-gentiobiosyl ester (**6**). Needles, mp 262–263° (MeOH); $[\alpha]_D^{28}$ −43° (pyridine; *c* 0.33); IR ν_{max}^{KBr} cm^{-1}: 3390 (OH), 2830–2950 (CH_2), 1730 (CO_2-Glc); FAB-MS *m/z* 665 [M + Na]$^+$; ^1H NMR (pyridine-d_5): δ 1.18 (3H, *s*, Me), 1.19 (3H, *s*, Me), 3.77 (1H, *m*, H-5″), 3.87 (1H, *dd*, *J* = 8.0, 8.0 Hz, H-2″), 3.96–4.14 (6H, *m*, H-2′, H-3′, H-4′, H-5′, H-3″, H-4″), 4.19 (1H, *dd*, *J* = 11.5, 5.0 Hz, H-6′$_a$), 4.23 (1H, *dd*, *J* = 11.5, 5.0 Hz, H-6″$_a$), 4.36 (1H, *br d*, *J* = 11.5 Hz, H-6″$_b$), 4.58 (1H, *br d*, *J* = 11.5 Hz, H-6′$_b$), 4.87 (1H, *br s*, H_a-17), 4.91 (1H, *d*, *J* = 8.0 Hz, H-1″), 5.32 (1H, *br s*, H_b-17), 6.01 (1H, *d*, *J* = 8.0 Hz, H-1′).

Steviol 19-β-glucopyranosyl ester tetraacetate (**4a**). Amorphous solid; ^1H NMR ($CDCl_3$): δ 0.82 (3H, *s*, Me), 1.17 (3H, *s*, Me), 2.00 (3H, *s*, Ac), 2.03 (3H, *s*, Ac), 2.04 (3H, *s*, Ac), 2.07 (3H, *s*, Ac), 3.81 (1H, *ddd*, *J* = 9.5, 5.0, 2.5 Hz, H-5′), 4.06 (1H, *dd*, *J* = 12.5, 2.5 Hz, H-6′$_a$), 4.29 (1H, *dd*, *J* = 12.5, 5.0 Hz, H-6′$_b$), 4.80 (1H, *br s*, H_a-17), 4.97 (1H, *br s*, H_b-17), 5.12 (1H, *dd*, *J* = 9.5, 9.0 Hz, H-4′), 5.20 (1H, *dd*, *J* = 9.0, 8.0 Hz, H-2′), 5.25 (1H, *dd*, *J* = 9.0, 9.0 Hz, H-3′), 5.73 (1H, *d*, *J* = 8.0 Hz, H-1′).

Steviol-13-O-*β-glucopyranoside 19-β-glucopyranosyl ester octaacetate* (**5a**). Amorphous solid; ^1H NMR ($CDCl_3$): δ 0.80 (3H, *s*, Me), 1.16 (3H, *s*, Me), 1.99 (6H, *s*, Ac), 2.01 (3H, *s*, Ac), 2.03 (9H, *s*, Ac), 2.08 (6H, *s*, Ac), 3.75 (1H, *ddd*, *J* = 10.0, 5.5, 2.5 Hz, H-5‴), 3.82 (1H, *ddd*, *J* = 9.5, 4.5, 2.5 Hz, H-5′), 4.11 (1H, *dd*, *J* = 12.0, 2.5 Hz, H-6″$_a$), 4.13 (1H, *dd*, *J* = 12.0, 2.5 Hz, H-6′$_a$), 4.18 (1H, *dd*, *J* = 12.0, 5.5 Hz, H-6″$_b$), 4.28 (1H, *dd*, *J* = 12.0, 4.5 Hz, H-6′$_b$), 4.77 (1H, *d*, *J* = 8.0 Hz, H-1‴), 4.84 (1H, *br s*, H_a-17), 5.00 (1H, *dd*, *J* = 9.0, 8.0 Hz, H-2‴), 5.01 (1H, *br s*, H_b-17), 5.02 (1H, *dd*, *J* = 10.0, 9.0 Hz, H-4‴), 5.15 (1H, *dd*, *J* = 9.5, 9.0 Hz, H-4′), 5.18 (1H, *dd*, *J* = 9.0, 8.0 Hz, H-2′), 5.21 (1H, *dd*, *J* = 9.0, 9.0 Hz, H-3‴), 5.25 (1H, *dd*, *J* = 9.0, 9.0 Hz, H-3′), 5.75 (1H, *d*, *J* = 8.0 Hz, H-1′).

Steviol 19-β-gentiobiosyl ester heptaacetate (**6a**). Amorphous solid; ^1H NMR ($CDCl_3$): δ 0.87 (3H, *s*, Me), 1.17 (3H, *s*, Me), 1.99 (6H, *s*, Ac), 2.01 (3H, *s*, Ac), 2.02 (6H, *s*, Ac), 2.09 (3H, *s*, Ac), 2.14 (3H, *s*, Ac), 3.60–3.69 (2H, *m*, H-6′$_a$, H-5″), 3.75–3.83 (2H, *m*, H-5′, H-6′$_b$), 4.11 (1H, *dd*, *J* = 12.0, 2.5 Hz, H-6″$_a$), 4.24 (1H, *dd*, *J* = 12.0, 4.5 Hz, H-6″$_b$), 4.53 (1H, *d*, *J* = 8.0 Hz, H-1″), 4.81 (1H, *br s*, H_a-17), 4.93 (1H, *dd*, *J* = 9.5, 9.0 Hz, H-4′), 4.96 (1H, *dd*, *J* = 9.0, 8.0 Hz, H-2″), 4.98 (1H, *br s*, H_b-17), 5.04 (1H, *dd*, *J* = 9.5, 9.0 Hz, H-4″), 5.14 (1H, *dd*, *J* = 9.0, 9.0 Hz, H-3″), 5.15 (1H, *dd*, *J* = 9.0, 8.0 Hz, H-2′), 5.22 (1H, *dd*, *J* = 9.0, 9.0 Hz, H-3′), 5.68 (1H, *d*, *J* = 8.0 Hz, H-1′).

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REFERENCES

1. Handro, W. and Ferreira, C. M. (1989) in *Biotechnology in Agriculture and Forestry* Vol. 7 *Medicinal and Aromatic Plants II* (Bajaj, Y. P. S., ed.), p. 468. Springer, Berlin.
2. Furuya, T., Orihara, Y. and Hayashi, C. (1987) *Phytochemistry* **26**, 715.
3. Furuya, T., Orihara, Y. and Miyatake, H. (1989) *J. Chem. Soc., Perkin Trans. I* 1711.
4. Orihara, Y., Miyatake, H. and Furuya, T. (1991) *Phytochemistry* **30**, 1843.

5. Orihara, Y. and Furuya, T. (1990) *Phytochemistry* **29**, 3123.
6. Furuya, T., Orihara, Y., Koge, K. and Tsuda, Y. (1989) *Plant Tiss. Cult. Letters* **6**, 148.
7. Furuya, T., Ushiyama, M., Asada, Y., Yoshikawa, T. and Orihara, Y. (1988) *Phytochemistry* **27**, 803.
8. Kaneda, N., Kasai, R., Yamasaki, K. and Tanaka, O. (1977) *Chem. Pharm. Bull.* **25**, 2466.
9. Tanaka, T., Kohda, H., Tanaka, O., Chen, F.-H., Chou, W.-H. and Leu, J.-L. (1981) *Agric. Biol. Chem.* **45**, 2165.
10. Darise, M., Mizutani, K., Kasai, R., Tanaka, O., Kitahata, S., Okada, S., Ogawa, S., Murakami, F. and Chen, F.-H. (1984) *Agric. Biol. Chem.* **48**, 2483.
11. Murashige, T. and Skoog, F. (1962) *Physiol. Plant.* **15**, 473.