Modified Monoterpenes from Biotransformation of (−)-Isopiperitenone by Suspension Cell Culture of *Mentha piperita*

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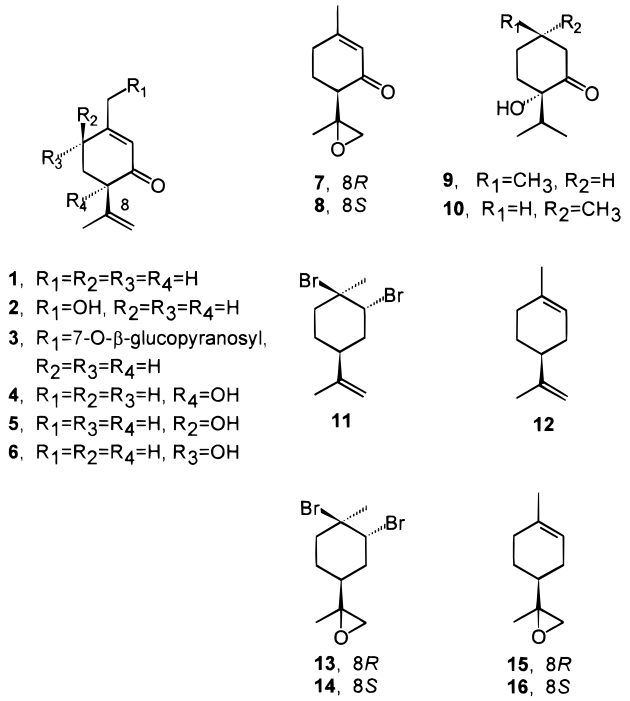
The biotransformation of (−)-(4*R*)-isopiperitenone (**1**) by suspension cell culture of *Mentha piperita* yielded three new hydroxylated derivatives, **4**−**6**, and two new epoxidized derivatives, **7** and **8**. (−)-7-Hydroxyisopiperitenone (**2**) and its glucoside were previously isolated from the culture. The structures of **4**−**8** were elucidated using spectral methods, and their absolute stereochemistry was established by NMR experiments and correlation with compounds of known configuration.

Several studies have been reported to produce essential oils by plant cell cultures.[1,2] In the case of *Mentha piperita* L. (Lamiaceae), there have been attempts to produce major components of peppermint oils through biotransformations of biosynthetic intermediates.[3−5] However, feeding of (−)-isopiperitenone (**1**), an intermediate at the branching point leading either to menthol or piperitone in *M. piperita*, to the cell culture of the plant results in 7-hydroxylation[6,7] and subsequent glucosylation of the hydroxylated terpene[8] to yield **2** and **3** with some stimulated conversion into the desired products.[6]

There has been considerable interest in producing novel structures from natural products through biotransformation using plant cell culture.[9] The above finding with *Mentha* cells indicates that the cells possess the capacity to transform exogenously fed monoterpenes. This paper reports the isolation and identification of three new hydroxylated (**4**−**6**) and two epoxidized compounds (**7** and **8**) from cell culture of *M. piperita* fed with **1**. These compounds account for a significant portion of the total biotransformation products.



1, R_1=R_2=R_3=R_4=H
2, R_1=OH, R_2=R_3=R_4=H
3, R_1=7-O-β-glucopyranosyl, R_2=R_3=R_4=H
4, R_1=R_2=R_3=H, R_4=OH
5, R_1=R_3=R_4=H, R_2=OH
6, R_1=R_2=R_4=H, R_3=OH

7, 8*R*
8, 8*S*

9, R_1=CH_3, R_2=H
10, R_1=H, R_2=CH_3

11

12

13, 8*R*
14, 8*S*

15, 8*R*
16, 8*S*

Results and Discussion

Compounds **1** and **4** showed the same base peak at *m*/*z* 82, which was assigned to fragment $[C_5H_6O]^+$ derived from retro-Diels−Alder reaction of the cyclohexenone ring. The ^{1}H and ^{13}C NMR spectra of **4** were similar to those of **1** and showed peaks corresponding to 14 protons and 10 carbons. The disappearance of the signal at 3.04, corresponding to H-4 of **1**, and the appearance of a signal at 3.78 revealed that a hydroxyl group had been introduced at C-4. This conclusion was supported by the appearance of a new carbon signal at 77.14 in the ^{13}C NMR spectra.

The stereochemistry of C-4 in **4** was deduced to be *S* by comparison with optical rotation values of 4-hydroxymenthones as follows. Compound **4** was reduced to a mixture of 4-hydroxymenthones. The mixture, with a specific optical rotation value of +104°, contained (1*R*)- and (1*S*)-4-hydroxymenthone with an unknown configuration at C-4. The optical rotation sign of (1*S*,4*S*)- and (1*R*,4*S*)-4-hydroxymenthone is positive and that of (1*S*,4*R*)- and (1*R*,4*R*)-4-hydroxymenthone is negative.[10−12] Therefore, the reduced mixture with positive optical rotation must be composed of (1*R*,4*S*)- and (1*S*,4*S*)-4-hydroxymenthone (**9** and **10**), confirming the configuration of compound **4** at C-4 as *S*.

Compound **5** showed the H-2 proton signal at 5.88 and the base peak, due to retro-Diels−Alder reaction, at *m*/*z* 98, suggesting that a hydroxyl group was present at C-6. In the ^{1}H−^{1}H COSY spectrum, the doublet of doublets at 3.05 (J = 13.6, 4.5 Hz) assigned to H-4 was coupled with the H-5 signals at 2.14 and 2.31. These two H-5 signals were assigned to H-5_{ax} and H-5_{eq}, respectively, based on analysis of coupling with the axial H-4. The C-6 stereochemistry in **5** could be easily determined if the H-6 signal had been resolved into multiplets.

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Nevertheless, the stereochemistry at C-6 was determined to be *R* on the basis of an ^{1}H NMR coupling pattern analysis at H-5 as follows. The H-5_{ax} signal had three large coupling constants, one (J = 12.4 Hz) due to the geminal coupling, the other (J = 13.6 Hz) due to coupling with H-4, so the last (J = 10.2 Hz) must then be due to coupling with H-6. The coupling constant between H-5_{ax} and H-6 was in the range expected for axial−axial proton coupling. Assuming axial orientation of H-4 due to preference of propenyl side chain toward equatorial, we concluded that H-6 was axial. Therefore, the absolute configuration at C-6 was *R*. This conclusion was supported by molecular modeling calculations by PCMODEL for Windows (Serena Software, IN) which predicted $J_{H-5_{ax}-H-4}$ to be 12.3 Hz and $J_{H-5_{ax}-H-6}$ 11.1 Hz.

Spectral data of **6** were very similar to **5** and indicated that **6** was a stereoisomer of **5**. The H-6 proton signal of **6** appeared at 4.35 (br s) and was shown to couple with H-5 in ^{1}H−^{1}H COSY. Here again, the coupling between H-6 and H-5 was determined indirectly as in the case of compound **5**. Assuming axial orientation of H-4, the coupling constant of 9.1 Hz could be assigned to axial−axial coupling between H-4 and H-5_{ax}. Starting from this, the coupling constant of 4.2 Hz then must be due to axial−equatorial coupling between H-5_{ax} and H-6. Therefore, the configuration of the hydroxyl group at C-6 was concluded to be *S*. Molecular mechanics calculations gave 2.81 Hz for $J_{H-5_{ax}-H-6}$, but the *S* configuration was still indicated from this value.

HREIMS of both **7** and **8** indicated molecular formulas of $C_{10}H_{14}O_2$. The IR spectra of compounds **7** and **8** showed an asymmetric C−O−C stretching peak instead of the characteristic O−H stretching peak, which suggested the presence of an oxirane ring. In the ^{1}H NMR spectrum, disappearance of the signal for H-9 and the presence of the signal for H-2 at 5.87 revealed that an oxirane ring had been introduced at C-8 and C-9. The H-9 signals were split into doublets (J = 4.5−4.6 Hz) typical for methylene protons of a terminal oxirane ring. Thus, compounds **7** and **8** were determined to be 8,9-epoxyisopiperitenone isomers.

The stereochemistry of **7** and **8** was identical at C-4 but opposite at C-8. The chiral center at C-8, located on a freely rotating side chain, rendered it impossible to determine the stereochemistry at C-8 through an NOE experiment. The stereochemistry of **7** and **8** was thus determined by comparing the optical rotation values and NMR data between the isolated products and a synthetic sample of the known configuration. 1,2-Dibromolimonene (**11**) was synthesized by the low-temperature monobromination of (−)-limonene (**12**). (1*R*,2*R*,4*S*)-Dibromolimonene (**11**) was epoxidized with *m*-CPBA to yield a mixture of two epoxides **13** and **14**. One of the dibromoepoxides (**13**) was debrominated with zinc powder to give an 8,9-epoxy-*p*-menth-1-ene (**15**). The physicochemical data for **15** were identical to those of (4*R*,8*S*)-8,9-epoxy-*p*-menth-1-ene,[13] except for the sign of optical rotation. From these results, the absolute configuration of **15** was clearly identified as (4*S*,8*R*). Compound **15** was oxidized with CrO_3 and *t*-BuOOH[14] to yield (4*R*,8*R*)-epoxyisopiperitenone, whose physicochemical properties were identical to those of the biotransformed compound **7**. Therefore, the absolute configuration of compound **7** was finally determined to be (4*R*,8*R*).

The stereochemistry of **8** was determined in the same manner as **7**. The physicochemical properties of **16** were identical to those of (4*R*,8*R*)-8,9-epoxy-*p*-menth-1-ene,[13] with the opposite sign of optical rotation. Therefore, compound **16** was identified as (4*S*,8*S*)-8,9-epoxy-*p*-menth-1-ene. Compound **16** was then oxidized to yield (4*R*,8*S*)-epoxyisopiperitenone, whose physicochemical properties were identical to those of compound **8**.

Nicotiana cell culture is the most extensively studied plant cell biotransformation system. *Nicotiana* cell cultures possess catalytic capacity for reduction,[15] hydroxylation,[16] and epoxidation[17] of monoterpenes. Biotransformation of piperitone into oxygenated products such as 4-hydroxypiperitone via hydroxylation using cell suspension cultures of *Catharanthus roseus* has also been reported.[18] The report, however, lacks information on absolute stereochemistry of the products. The present paper describes the complete structural elucidation of products as well as demonstrating the versatile metabolic capability of *Mentha* cells.

There have been few reports on the nature of the enzymes responsible for biotransformation reactions except for a report describing evidence for the involvement of cytochrome P-450 monooxygenase.[18] Thus, it is also possible that the described bioconversion by *M. piperita* cell culture was catalyzed by a cytochrome P-450 monooxigenase.

Experimental Section

General Experimental Procedures. Optical rotations were measured on an Autopol polarimeter. UV−vis spectra were obtained on a HP8452A diode-array spectrophotometer. The IR spectra were measured on a Magna 550 infrared spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded with a JEOL LA400 FT NMR at 400 and 100 MHz, respectively, in $CDCl_3$ using TMS as an internal standard. EIMS spectra were obtained with a JEOL AX505WA mass spectrometer. Silica gel 60 (Merck, 230−400 mesh) was used for CC.

Plant Cell Culture and Biotransformation Procedure. Suspension culture of *M. piperita*, established in Lin and Staba medium[20] with 2,4-D (2 mg/L), was a gift from Professor H. J. Lee, Department of Food Science and Technology, Seoul National University. The culture was incubated in a 250 mL Erlenmeyer flask containing 100 mL of the medium on a rotary shaker (120 rpm) at 27 °C with a 16−8 h light−dark cycle. The culture was subcultured monthly with the initial inoculum of 1 g fresh cell per flask. A total of 800 mg of (−)-isopiperitenone (**1**) was administered to 40 flasks containing 3-week-old suspension cells, and these were subsequently incubated for 48 h prior to isolation of monoterpenes.

Extraction and Isolation. The cells were filtered through filter paper and carefully washed with distilled water. The filtrate was extracted with CH_2Cl_2 (500 mL × 3). The CH_2Cl_2 layer was dried with anhydrous $MgSO_4$ and concentrated in vacuo. The oily residue was chromatographed a on silica gel column (2 × 50 cm, hexane−diethyl ether 4:1) and further purified by HPLC. The HPLC conditions for isolation of monoterpenes were as follows: column, LiChrosorb Si60 (Mer-

ck); 10 mm × 25 cm, solvent; hexane−diethyl ether 1:1, flow rate; 3.0 mL/min, UV detector; at 240 nm. The major products **2** and **3** have been described elsewhere.[7,8]

(+)-(4*S*)-4-Hydroxyisopiperitenone (4): colorless oil (7 mg); $[\alpha]^{20}_D$ +136° (*c* 0.05, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 236 (4.3) nm; IR (neat) ν_{max} 3475, 2930, 1670 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.84 (3H, s, H-10), 1.95 (1H, m, H-5a), 1.96 (3H, s, H-7), 2.30 (2H, m, H-6), 2.33 (1H, m, H-5b), 3.78 (1H, s, OH-4), 4.69 (1H, m, H-9a), 4.94 (1H, m, H-9b), 5.98 (1H, m, H-2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 18.09 (C-10), 24.22 (C-7), 29.97 (C-6), 32.62 (C-5), 77.14 (C-4), 113.49 (C-9), 124.42 (C-2), 144.36 (C-8), 164.22 (C-1), 201.16 (C-3); EIMS *m/z* $[M]^+$ 166 (5), $[M - H_2O]^+$ 148 (59), 133 (32), 105 (46), 97 (22), 82 ($[C_5H_6O]^+$, 100), 69 (16); HREIMS *m/z* 166.0974 (calcd for $C_{10}H_{14}O_2$ 166.0994).

(−)-(4*R*,6*R*)-6-Hydroxyisopiperitenone (5): colorless oil (8 mg); $[\alpha]^{20}_D$ −7.2° (*c* 0.08, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 231 (3.9) nm; IR (neat) ν_{max} 3310, 2940, 1640 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.74 (3H, s, H-10), 2.06 (3H, s, H-7), 2.14 (1H, ddd, J = 12.4, 13.6, 10.2 Hz, H-5_{ax}), 2.31 (1H, ddd, J = 12.4, 4.5, 4.7 Hz, H-5_{eq}), 2.46 (1H, br s, −OH), 3.05 (1H, dd, J = 13.6, 4.5 Hz, H-4), 4.51 (1H, br. s, H-6), 4.81 (1H, m, H-9a), 4.98 (1H, m, H-9b), 5.88 (1H, m, H-2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 19.67 (C-7), 19.94 (C-10), 37.94 (C-5), 53.88 (C-4), 69.74 (C-6), 114.49 (C-9), 126.82 (C-2), 143.00 (C-8), 163.82 (C-1), 198.21 (C-3); EIMS *m/z* $[M]^+$ 166 (8), $[M - H_2O]^+$ 148 (27), 133 (16), 105 (22), 98 ($[C_5H_6O_2]^+$, 100), 69 (46); HREIMS *m/z* 166.0990 (calcd for $C_{10}H_{14}O_2$ 166.0994).

(+)-(4*R*,6*S*)-6-Hydroxyisopiperitenone (6): white powder (8 mg); $[\alpha]^{20}_D$ +17.4° (*c* 0.08, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 231 (3.9) nm; IR (KBr) ν_{max} 3410, 2920, 1660 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.77 (3H, s, H-10), 2.05 (3H, s, H-7) 2.39 (1H, ddd, J = 13.6, 9.1, 4.2 Hz, H-5_{ax}), 2.10 (1H, ddd, J = 13.6, 4.8, 5.6 Hz, H-5_{eq}), 3.30 (1H, dd, J = 9.1, 4.8 Hz, H-4), 4.35 (1H, br s, H-6), 4.76 (1H, m, H-9a), 4.96 (1H, m, H-9b), 5.88 (1H, m, H-2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.96 (C-7), 21.08 (C-10), 36.11 (C-5), 50.01 (C-4), 67.28 (C-6), 113.73 (C-9), 127.29 (C-2), 142.42 (C-8), 159.82 (C-1), 198.81 (C-3); EIMS *m/z* $[M]^+$ 166 (10), $[M - H_2O]^+$ 148 (58), 133 (35), 105 (47), 98 ($C_5H_6O_2^+$, 100), 69 (46): HREIMS *m/z* 166.0996 (calcd for $C_{10}H_{14}O_2$ 166.0994).

(−)-(4*R*,8*R*)-8,9-Epoxyisopiperitenone (7): colorless oil (10 mg); $[\alpha]^{20}_D$ −110.0° (*c* 0.70, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 235 (4.1) nm; IR (neat) ν_{max} 2930, 1670, 1200 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.26 (3H, s, H-10), 1.97 (3H, s, H-7), 2.01 (1H, m, H-4), 2.04 (1H, m, H-5a), 2.24 (1H, m, H-5b), 2.37 (2H, m, H-6), 2.73 (1H, d, J = 4.5 Hz, H-9a), 2.82 (1H, d, J = 4.5 Hz, H-9b), 5.87 (1H, m, H-2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 17.15 (C-10), 24.08 (C-7), 24.78 (C-5), 30.81 (C-6), 53.44 (C-4), 55.77 (C-9), 56.57 (C-8), 126.45 (C-2), 162.40 (C-1), 197.82 (C-3); EIMS *m/z* $[M]^+$ 166 (19), $[M - H_2O]^+$ 148 (19), 136 (24), 121 (30), 108 (28), 93 (30), 82 ($[C_5H_6O]^+$, 100), 67 (13); HREIMS *m/z* 166.0984 (calcd for $C_{10}H_{14}O_2$ 166.0994).

(−)-(4*R*,8*S*)-8,9-Epoxyisopiperitenone (8): colorless oil (4 mg); $[\alpha]^{20}_D$ −41.2° (*c* 0.20, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 234 (4.1) nm; IR (neat) ν_{max} 2930, 1670, 1200 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.44 (3H, s, H-10), 1.74 (1H, m, H-5a), 1.95 (3H, s, H-7), 2.08 (1H, m, H-5b), 2.35 (2H, m, H-6), 2.37 (1H, m, H-4), 2.53 (1H, d, J = 4.6 Hz, H-9a), 2.59 (1H, d, J = 4.6 Hz, H-9b), 5.87 (1H, m, H-2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.31 (C-10), 24.12 (C-7), 24.75 (C-5), 30.67 (C-6), 50.89 (C-4), 50.92 (C-9), 56.40 (C-8), 126.84 (C-2), 162.00 (C-1), 198.40 (C-3); EIMS *m/z* $[M]^+$ 166 (19), $[M - H_2O]^+$ 148 (19), 136 (24), 121 (30), 108 (27), 93 (28), 82 ($[C_5H_6O]^+$, 100), 67 (12); HREIMS *m/z* 166.0990 (calcd for $C_{10}H_{14}O_2$ 166.0994).

Conversion of 4 into 4-Hydroxymenthones 9 and 10. (+)-(4*S*)-4-Hydroxyisopiperitenone (**4**) (3 mg) was dissolved in 2 mL of hexane, and 10 mg of Pd/C was added. The suspension was stirred for 12 h under 1 atm of H_2. The suspension was filtered, and the filtrate was evaporated. The residue contained a mixture of (1*R*,4*S*)-4-hydroxymenthone (**9**) and (1*S*,4*S*)-4-hydroxymenthone (**10**). The structures of **9** and **10** were identified by careful comparison of NMR data with reported values.[10−12]

Conversion of (−)-(4*S*)-Limonene (12) into (4*S*,8*R*)- and (4*S*,8*S*)-Epoxy-*p*-menth-1-ene (15 and 16). Compounds **15** and **16** were prepared according to the known procedure starting from **12**.[13] ^1H and ^{13}C NMR data of the intermediates (**11**, **13**−**16**) were identical to those of the enantiomers[13] except for the sign of optical rotation.

(1*R*,2*R*,4*S*)-1,2-Dibromo-*p*-menth-8-ene (11): $[\alpha]^{20}_D$ −57.0° (*c* 0.2, CH_2Cl_2).

(1*R*,2*R*,4*S*,8*R*)-1,2-Dibromo-8,9-epoxy-*p*-menthane (13): $[\alpha]^{20}_D$ −61.7° (*c* 0.6, CH_2Cl_2).

(1*R*,2*R*,4*S*,8*S*)-1,2-Dibromo-8,9-epoxy-*p*-menthane (14): $[\alpha]^{20}_D$ −50.8° (*c* 0.34, CH_2Cl_2).

(4*S*,8*R*)-8,9-Epoxy-*p*-menth-1-ene (15): $[\alpha]^{20}_D$ −83.6° (*c* 0.47, CH_2Cl_2).

(4*S*,8*S*)-8,9-Epoxy-*p*-menth-1-ene (16): $[\alpha]^{20}_D$ −77.7° (*c* 0.14, CH_2Cl_2).

Conversion of 15 and 16 into 8,9-Epoxyisopiperitenones (7 and 8). *t*-BuOOH (90%, 1.3 mL) was added to a solution of CrO_3 (5 mg) in CH_2Cl_2 (5 mL). The epoxide (152 mg, **15** or **16**) in CH_2Cl_2 (1 mL) was then added dropwise and stirred for 2 h at room temperature. The solution was successively washed with saturated aqueous $NaHCO_3$ and brine and then finally dried over $MgSO_4$ and evaporated. The product was separated using preparative TLC (hexane−diethyl ether 5:1; multiple developments). Synthetic 8,9-epoxyisopiperitenones (**7** and **8**) showed the same physicochemical properties as the isolates from cell culture.

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References and Notes

(1) Hirata, T.; Murakami, S.; Ogihara, K.; Suga, T. *Phytochemistry* **1990**, *29*, 493−495.
(2) Kato, A.; Kawazoe, S.; Iijima, M.; Shimizu, Y. *J. Ferment. Technol.* **1976**, *54*, 82−87.
(3) Aviv, D.; Galun, E. *Planta Med.* **1978**, *33*, 70−77.
(4) Aviv, D.; Krochmal, E.; Dantes, A.; Galun, E. *Planta Med.* **1981**, *42*, 236−243.
(5) Galun, E.; Aviv, D.; Dantes, A.; Freeman, A. *Planta Med.* **1985**, *51*, 511−514.
(6) Park, S.-H.; Chae, Y.-A.; Lee, H. J.; Kim, S.-U. *J. Korean Agric. Chem. Soc.* **1993**, *36*, 358−363.

(7) Park, S.-H.; Chae, Y.-A.; Lee, H. J.; Lim, Y.-H.; Kim, S.-U. *Planta Med*. **1994**, *60*, 374−375.
(8) Park, S. H.; Kim, K. S.; Suzuki, Y.; Kim, S. U. *Phytochemistry* **1997**, *44*, 623−626.
(9) Barnthorpe, D. V. *Nat. Prod. Rep.* **1994**, 303−328.
(10) Suga, T.; Shishibori, T.; Matsuura, T. *J. Org. Chem*. **1967**, *32*, 965−969.
(11) Kaiser, R.; Lamparsky, D.; Schudel, P. *J. Agric. Food Chem*. **1975**, *23*, 943−950.
(12) Suga, T.; Hirata, T.; Hamada, H.; Murakami, S. *Phytochemistry* **1988**, *27*, 1041−1044.
(13) Carman, R. M.; Garner, A. C.; Klika, K. D. *Aust. J. Chem*. **1993**, *46*, 233−238.
(14) Muzart, J. *Tetrahedron Lett.* **1987**, *28*, 4665−4668.
(15) Suga, T.; Tang, Y.-X. *J. Nat. Prod.* **1993**, *56*, 1406−1409.
(16) Suga, T.; Hirata, T.; Hamada, H.; Murakami, S. *Phytochemistry* **1988**, *27*, 1041−1044.
(17) Hirata, T.; Hamada, H.; Aoki, T.; Suga, T. *Phytochemistry* **1987**, *21*, 2209−2212.
(18) Hamada, H.; Fuchikami, Y.; Ikematsu, Y.; Hirata, T.; Williams, H. J.; Scott, A. I. *Phytochemistry* **1994**, *37*, 1037−1038.
(19) Tang, Y.-X.; Suga, T. *Phytochemistry* **1994**, *37*, 731−735.
(20) Lin, M. L.; Staba, E. J. *Lloydia* **1961**, *24*, 139−146.

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