New Bisabolane-Type Sesquiterpenes from the Aerial Parts of *Lippia dulcis*

Masateru ONO,*[,a] Hiroaki MORINAGA,[a] Chikako MASUOKA,[a] Tsuyoshi IKEDA,[b] Masafumi OKAWA,[c] Junei KINJO,[c] and Toshihiro NOHARA[b]

[a] *School of Agriculture, Kyushu Tokai University; 5435 Minamiaso, Aso, Kumamoto 869–1404, Japan:* [b] *Faculty of Medical and Pharmaceutical Sciences, Kumamoto University; 5–1 Oe-honmachi, Kumamoto 862–0973, Japan: and* [c] *Faculty of Pharmaceutical Sciences, Fukuoka University; 8–19–1 Nanakuma, Jonan-ku, Fukuoka 814–0180, Japan.*

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Two new bisabolane-type sesquiterpenes, lippidulcine A (3) and epilippidulcine A (4), have been isolated from the aerial parts of *Lippia dulcis* TREV. along with five known flavonoids, cirsimaritin (5), salvigenin (6), eupatorin (7), 5-hydroxy-6,7,3′,4′-tetramethoxyflavone (8) and 5,3′-dihydroxy-6,7,4′,5′-tetramethoxyflavone (9), three known phenylethanoid glycosides, decaffeoylverbascoside (10), acteoside (11) and isoacteoside (12), and two known iridoid glucosides, 8-epiloganin (13) and lamiide (14). Their chemical structures have been determined on the basis of spectroscopic data. Among them, 5, 7, and 9 exhibited almost the same activity as that of α-tocopherol, and 10—12 were identified as stronger antioxidants than α-tocopherol using the ferric thiocyanate method.

Key words *Lippia dulcis*; bisabolane-type sesquiterpene; antioxidative activity; flavonoid; phenylethanoid glycoside; iridoid glucoside

Lippia dulcis TREV. is an intensely sweet herb endemic to tropical America and the leaves are used as a traditional medicine for the treatment of coughs and bronchitis.[1] A study on the constituent of the leaves and flowers of this herb was made by Compadre *et al.*,[2] Kaneda *et al.*[3] and Abe *et al.*,[4] and two sweet bisabolane-type sesquiterpenes, (+)-hernandulcin (**1**) and (+)-4β-hydroxyhernandulcin, two non-sweet bisabolane-type sesquiterpenes, (−)-epihernandulcin (**2**) and (+)-anymol, 6-methyl-5-heptene-2-one, and four phenylethanoid glycoside esters, acteoside, isoacteoside, martynoside and diacetylmartynoside were isolated.

In the course of our studies on Verbenaceae plants and natural antioxidants, we examined the constituents of the MeOH extract of the aerial parts of *L. dulcis*. The present paper describes the isolation and structure elucidation of two new bisabolane-type sesquiterpenes (**3**, **4**) along with five known flavonoids (**5**—**9**), three known phenylethanoid glycosides (**10**—**12**) and two known iridoid glucosides (**13**, **14**), as well as the anioxidative activity of eight phenolic compounds (**5**—**12**).

The MeOH extract of the aerial parts of *L. dulcis* was successively subjected to Diaion HP20, silica gel, Sephadex LH20 and Chromatorex ODS column chromatographies as well as HPLC on ODS to afford twelve compounds (**3**—**14**).

Compounds **5**—**14** were identified as cirsimaritin (**5**),[5] salvigenin (**6**),[6] eupatorin (**7**),[7] 5-hydroxy-6,7,3′,4′-tetramethoxyflavone (**8**),[8] 5,3′-dihydroxy-6,7,4′,5′-tetramethoxyflavone (**9**),[9] decaffeoylverbascoside (**10**),[10] acteoside (**11**),[11] isoacteoside (**12**),[12] 8-epiloganin (**13**)[13] and lamiide (**14**),[14] respectively, based on their physical and spectral data.

Compounds **3** and **4** were obtained as a colorless oil. In the FAB-MS, **3** and **4** exhibited an $[M+H]^+$ ion peak at *m/z* 253; the high-resolution (HR) FAB-MS indicated the molecular formula of both compounds to be $C_{15}H_{24}O_3$. The ^{1}H-NMR spectra of **3** and **4** showed signals due to four tertiary methyl groups (δ 1.98, 1.27×2, 1.19 in **3**; δ 1.97, 1.25×2, 1.16 in **4**) and three olefinic protons [δ 5.84 (q like, *J*=1.0 Hz), 5.76 (ddd, *J*=6.0, 8.0, 15.5 Hz), 5.67 (d, 15.5 Hz) in **3**; δ 5.81 (q like, *J*=1.0 Hz), 5.69 (ddd, *J*=6.5, 7.5, 15.5 Hz), 5.60 (d, *J*=15.5 Hz) in **4**], two of which were *trans*-coupling each other. The ^{13}C-NMR spectra of **3** and **4** showed 15 carbon signals, including one carbonyl carbon (δ 205.9 in **3**; δ 204.4 in **4**), four olefinic carbons (δ 166.8, 142.6, 127.8, 123.0 in **3**; δ 166.2, 142.4, 127.9, 123.5 in **4**), and two oxygenated quaternary carbons (δ 75.8, 71.2 in **3**; δ 75.5, 71.2 in **4**). These ^{1}H- and ^{13}C-NMR signals were assigned with the aid of ^{1}H–^{1}H correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond correlation (HMBC) and nuclear Overhauser and exchange spectroscopy (NOESY) spectra; **3** and **4** were determined to possess the same planar structure as illustrated in Fig. 1. Kinghorn and his coworkers[2,3] reported that the ^{13}C-NMR chemical shifts for C-6, C-2′, and C-8′ in **1** and **2** showed the difference owing to the intramolecular steric interactions of the compounds. On the other hand, Mori and Kato[15] established the absolute configuration of **1** and **2** to be 6*S*, 1′*S* and 6*R*, 1′*S*, respectively, by means of the syntheses of **1**, **2** and their epimers. The chemical shifts (in $CDCl_3$) of C-6 (δ 52.0 in **1**; δ 55.3 in **2**; δ 51.9 in **3**; δ 54.6 in **4**),[2,3] C-2′ (δ 40.1 in **1**; δ 37.1 in **2**; δ 43.1 in **3**; δ 40.7 in **4**),[2,3] and C-8′ (δ 23.6 in **1**; δ 25.4 in **2**; δ 23.8 in **3**; δ 26.2 in **4**)[2,3] of **3** and **4** were similar to those of **1** and **2**, respectively. Furthermore, the sign and the value of the specific rotations of **3** ($[\alpha]_D$ +123.6°) and **4** ($[\alpha]_D$ −118.4°) were imposable on those of **1** ($[\alpha]_D$ +126°)[15] and **2** ($[\alpha]_D$ −133°),[15] respectively. On the basis of the above findings, the structures of **3** and **4** were established as shown in Fig. 2, and **3** and **4** were tentatively named lippidulcine A and epilippidulcine A, respectively.

The antioxidative activity of the phenolic compounds (**5**—**12**) was evaluated using linoleic acid as the substrate by the ferric thiocyanate method,[16] and the activity was compared with the standard natural antioxidant, α-tocopherol and the standard synthetic antioxidant, 3-*tert*-butyl-4-hydroxyanisole (BHA), each at a 0.5 mM concentration. Among them, **5**, **7** and **9** showed almost the same activity as that of α-toco-

* To whom correspondence should be addressed. e-mail: mono@ktmail.ktokai-u.ac.jp

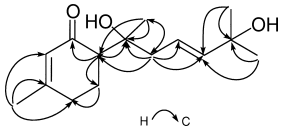

Fig. 1. ^{1}H–^{13}C Long-Range Correlations Observed for **3** and **4** in the HMBC Spectra (in CD_3OD, 500 MHz)

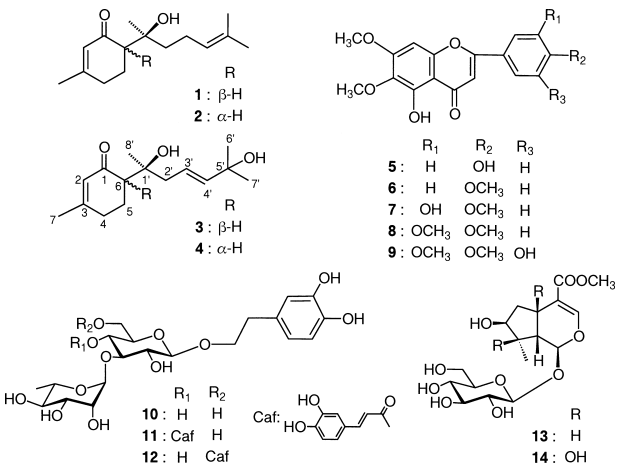

Fig. 2. Structures of **1**—**14**

pherol, and **10**—**12** exhibited even more potent antioxidative activity than α-tocopherol. The activities of **5**, **7** and **9** were presumed to come from phenolic hydroxyl groups in the B-ring of flavonoid skeleton and the catechyl groups of **10**—**12** were considered to be important for the activity.

To the best of our knowledge, the isolation of flavonoid and iridoid is described here for the first time from *L. dulcis*.

Experimental

All the instruments and the materials used were the same as cited in the previous report[17] unless otherwise specified.

Plant Material The plant of *Lippia dulcis* Trev. was purchased from Tamagawa Engei Co., Ltd., a commercial outlet of herbs, in Yamanashi prefecture, Japan, and identified by Professor Toshihiro Nohara, Faculty of Medical and Pharmaceutical Sciences, Kumamoto University. The aerial parts were collected in July 2003 on the campus of Kyushu Tokai University, Kumamoto prefecture. A voucher specimen is deposited in the Laboratory of Natural Products Chemistry, School of Agriculture of that university.

Extraction and Isolation The cut fresh aerial parts of *L. dulcis* (234 g) were extracted with MeOH (1500 ml) for 7 days at room temperature (this procedure was repeated three times), and the solvent was removed under reduced pressure to give a syrup (30.9 g). The MeOH extract was chromatographed over Diaion HP20 (H_2O, 40% MeOH, 60% MeOH, 90% MeOH, MeOH, acetone) to give fractions (frs.) 1—6. Fraction 2 (9.80 g) was subjected to silica gel column chromatography [$CHCl_3$–MeOH–H_2O (14 : 2 : 0.1, 10 : 2 : 0.1, 8 : 2 : 0.2, 7 : 3 : 0.5, 6 : 4 : 1, 0 : 1 : 0)] to give frs. 7—12. Chromatography of fr. 10 (4422 mg) over Sephadex LH20 (MeOH) furnished fr. 13 and fr. 14. Fraction 13 (1765 mg) was subjected to silica gel column chromatography [hexane–acetone (1 : 0, 9 : 1, 5 : 1, 3 : 1, 2 : 1, 1 : 1, 1 : 5, 0 : 1)] to give frs. 15—18. HPLC [column, YMC-Pack SIL-06, YMC Co., 250 mm×20 mm i.d.; solvent, $CHCl_3$–MeOH–H_2O (8 : 2 : 0.2)] of fr. 16 (289 mg) afforded **11** (33 mg) and **13** (13 mg). Fraction 17 (434 mg) was successively subjected to silica gel column chromatography [$CHCl_3$–MeOH–H_2O (15 : 1 : 0, 14 : 2 : 0.1, 10 : 2 : 0.1, 8 : 2 : 0.2, 0 : 1 : 0)] and HPLC on COSMOSIL 5C18 AR-II [Nacalai Tesque, Inc., 250 mm×20 mm i.d.; solvent, 20% MeOH)] to give **10** (33 mg). Fraction 18 (914 mg) was successively subjected to silica gel [$CHCl_3$–MeOH–H_2O (14 : 2 : 0.1, 10 : 2 : 0.1, 8 : 2 : 0.2, 7 : 3 : 0.5, 0 : 1 : 0)] and Chromatorex ODS (30% MeOH, MeOH) column chromatographies, and HPLC under the same conditions as for fr. 16 to afford **14** (7 mg). Fraction 11 (3263 mg) was successively subjected to silica gel [$CHCl_3$–MeOH–H_2O (7 : 3 : 0.5, 6 : 4 : 1, 0 : 1 : 0)] and Chromatorex ODS (30% MeOH, 40% MeOH, 50% MeOH, MeOH) column chromatographies, and HPLC (35% MeOH) under the similar conditions to fr. 17 to give **12** (12 mg). Fraction 4 (2.30 g) was chromatographed over silica gel [hexane–acetone (20 : 1, 5 : 1, 3 : 1, 2 : 1, 1 : 1, 0 : 1)] to furnish frs. 19—23. Chromatography of fr. 21 (247 mg) over silica gel [hexane–acetone (5 : 1, 3 : 1, 2 : 1, 1 : 1, 0 : 1)] gave frs. 24—28. Similar HPLC (55% MeOH) of fr. 26 (67 mg) to fr. 17 afforded **3** (5 mg) and frs. 29—31. Fraction 22 (226 mg) was subjected to Chromatorex ODS column chromatography (60% MeOH, 65% MeOH, 70% MeOH, MeOH) to give **6** (16 mg) and frs. 32—35. Fraction 33 (53 mg) and fr. 34 (28 mg) were each subjected to HPLC (fr. 33, 55% MeOH; fr. 34, 60% MeOH) under the similar conditions to fr. 17 to afford **4** (5 mg), fr. 36 and fr. 37 from fr. 33, and **5** (5 mg), **7** (12 mg) and **9** (6 mg) from fr. 34. Fraction 5 (2.0 g) was chromatographed over silica gel [hexane–acetone (5 : 1, 3 : 1, 2 : 1, 1 : 1, 0 : 1)] to furnish **6** (5 mg), **8** (26 mg) and frs. 38—42.

Lippidulcine A (**3**): A colorless oil. $[\alpha]_D^{31}$ +123.6° (c=0.1, $CHCl_3$). Positive FAB-MS *m/z*: 253 $[M+H]^+$. HR positive FAB-MS *m/z*: 275.1615 $[M+Na]^+$ (Calcd for $C_{15}H_{24}O_3Na$: 275.1623). ^{1}H-NMR (in CD_3OD, 500 MHz) δ: 5.84 (1H, q like, J=1.0 Hz, H-2), 5.76 (1H, ddd, J=6.0, 8.0, 15.5 Hz, H-3′), 5.67 (1H, d, J=15.5 Hz, H-4′), 2.45 (1H, dd, J=4.5, 13.5 Hz, H-6), *ca.* 2.39 (1H, Ha-4), *ca.* 2.38 (1H, Hb-4), *ca.* 2.23 (1H, Ha-2′), *ca.* 2.21 (1H, Hb-2′), 2.08 (1H, m, Ha-5), 1.98 (3H, s, H_3-7), 1.71 (1H, m, Hb-5), 1.27 (3H, s, H_3-6′), 1.27 (3H, s, H_3-7′), 1.19 (3H, s, H_3-8′). ^{13}C-NMR (in CD_3OD, 125 MHz) δ: 205.9 (C-1), 166.8 (C-3), 142.6 (C-4′), 127.8 (C-2), 123.0 (C-3′), 75.8 (C-1′), 71.2 (C-5′), 53.1 (C-6), 43.7 (C-2′), 32.1 (C-4), 30.0 (C-6′), 29.9 (C-7′), 25.8 (C-5), 24.6 (C-8′), 24.1 (C-7).

Epilippidulcine A (**4**): A colorless oil. $[\alpha]_D^{31}$ −118.4° (c=0.5, $CHCl_3$). Positive FAB-MS *m/z*: 253 $[M+H]^+$. HR positive FAB-MS *m/z*: 275.1626 $[M+Na]^+$ (Calcd for $C_{15}H_{24}O_3Na$: 275.1623). ^{1}H-NMR (in CD_3OD, 500 MHz) δ: 5.81 (1H, q like, J=1.0 Hz, H-2), 5.69 (1H, ddd, J=6.5, 7.5, 15.5 Hz, H-3′), 5.60 (1H, d, J=15.5 Hz, H-4′), 2.44 (1H, dd, J=6.5, 14.0 Hz, Ha-2′), *ca.* 2.41 (1H, Ha-4), *ca.* 2.41 (1H, Hb-4), *ca.* 2.41 (1H, H-6), 2.31 (1H, J=7.5, 14.0 Hz, Hb-2′), 2.18 (1H, dddd, J=4.5, 4.5, 4.5, 13.0 Hz, Ha-5), 1.97 (3H, s, H_3-7), 1.84 (1H, m, Hb-5), 1.25 (3H, s, H_3-6′), 1.25 (3H, s, H_3-7′), 1.16 (3H, s, H_3-8′). ^{13}C-NMR (in CD_3OD, 125 MHz) δ: 204.4 (C-1), 166.2 (C-3), 142.4 (C-4′), 127.9 (C-2), 123.5 (C-3′), 75.5 (C-1′), 71.2 (C-5′), 55.0 (C-6), 43.4 (C-2′), 32.4 (C-4), 29.8 (C-6′), 29.8 (C-7′), 25.5 (C-5), 25.0 (C-8′), 24.0 (C-7).

Assay of Antioxidative Activity Antioxidative activity of the test sample was assayed using the ferric thiocyanate method.[16] A mixture of 2.51% linoleic acid EtOH solution (0.80 ml), 0.05 M phosphate buffer (pH 7.0, 1.60 ml), EtOH (0.60 ml) and H_2O (0.80 ml) was added to 10 mM EtOH solution (0.20 ml) of each sample in a vial with a cap and placed in darkness at 40 °C to accelerate oxidation. After the 5th day of incubation, this assay solution (0.05 ml) was diluted with 75% EtOH (4.85 ml), which was followed by adding 30% ammonium thiocyanate (0.05 ml). Precisely 3 min after the addition of 0.02 M ferrous chloride in 3.5% hydrochloric acid (0.05 ml) to the reaction mixture, the absorbance of the red color developed was measured at 500 nm. The control sample was prepared in the same manner by mixing all the same chemicals and ingredients and by excluding the test compounds. α-Tocopherol and BHA were used as standard samples. The final concentration of each sample tested was 0.5 mM. The value of control represents 100% lipid peroxidation, and the peroxidation values of BHA, α-tocopherol, **5**, **6**, **7**, **8**, **9**, **10**, **11** and **12** were 4.5, 14.7, 14.3, 100, 11.7, 77.9, 11.4, 6.6, 5.9 and 7.2%, respectively.

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