Monoterpene Glucosides from Ziziphora clinopodioides (Labiatae)

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Three new monoterpene glucosides, ziziphoroside A (I), B (2), and C (3), together with fifteen known compounds were isolated from the whole herb of Z. clinopodioides. The structures of new compounds were determined primarily from 1D-, 2D-NMR and circular dichroism (CD) spectroscopic analyses. The isolated compounds were evaluated for their inhibitory activity against nitric oxide (NO) production by lipopolysaccharide (LPS) and interferon (IFN)-γ activated macrophages, RAW 264.7. Shizonepetoside A (8) and flavones (11, 12, 13) showed potent inhibitory activity against NO production.

Key words Labiatae; Ziziphora clinopodioides; monoterpene glucoside; nitric oxide; macrophage

Ziziphora clinopodioides (Labiatae) is a folk medicine which has been used to treat fevers and headaches in Xinjiang, China. Although there have been some reports on the constituents of this plant, those studies have been mainly focused on the essential oil of Z. clinopodioides. In this paper, we describe the isolation and structure elucidation of three new monoterpene glucosides, named ziziphorosides A (1), B (2), and C (3), along with fifteen known compounds. Inhibitory effect of isolated compounds on nitric oxide (NO) production stimulated by lipopolysaccharide (LPS) and interferon (IFN)-γ in RAW 264.7 cells is also described.

Results and Discussion

From an ethanolic extract of whole herbs of Z. clinopodioides, eighteen compounds (1—18) were isolated. The structures of the known compounds were determined to be benzylalcohol glucoside (4), phenethylalcohol glucoside (5), shizonepetoside C (6), erigeside B (7), shizonepetoside A (8), piceine (9), 9-O-glucopyranosyl-p-menthan-3-one (10), apigenin (11), luteolin (12), diosmetin (13), ursolic acid (14), oleanolic acid (15), maslinic acid (16), ethyl caffeate (17) and benzoic acid (18) by comparing their spectroscopic data with those reported in the literature (Fig. 1).

Ziziphoroside A (1) was obtained as a colorless powder. In positive ion high resolution (HR) FAB-MS, a quasi-molecular ion peak at m/z 331.1757 (Calcd 331.1757) [M+H]+, which was consistent with a molecular formula of C16H27O7, was observed. The 1H-NMR and correlation spectroscopy (COSY) spectra exhibited the presence of two tertiary methyl groups [δ 1.41, 1.44 (each 3H, s)], a secondary methyl group [δ 0.98 (3H, d, J = 5.7 Hz)], a methine–methylene–methine–methylene (C-5–C-6–C-1–C-2) sequence, and an anomeric proton [δ 4.31 (1H, d, J = 7.5 Hz)]. The 13C-NMR, distortionless enhancement by polarization transfer (DEPT), and heteronuclear multiple quantum coherence (HMQC) spectra revealed the presence of a carbonyl group (δ 200.1), a vinylmethine group [δ 7.29 (1H, dd, J = 5.7Hz), δ 147.1] and a hexose (δ 61.7, 70.5, 74.1, 76.7, 77.0, 97.6). The heteronuclear multiple bond correlation (HMBC) spectrum exhibited the long-range correlations, as shown in Fig. 2, between the following proton and carbon

Fig. 1. Structures of Compounds 1—18

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In positive ion HR-FAB-MS, a quasi-molecular ion peak at \( m/z \) 353.1577 (Calcd 353.1577) \([\text{M} + \text{Na}]^+\), which was consistent with a molecular formula of \( \text{C}_{16}\text{H}_{26}\text{O}_{7}\text{Na} \), was observed. The spectral data and the degree of unsaturation indicated that \( \text{Ziziphoroside C} \) should be a \( \alpha,\beta \)-unsaturated ketone\(^{10}\). The stereochemistry at C-1 was investigated by analysis of the HMBC spectrum. The Cotton effect at \( \delta \) 2.11, (t, \( J = 13.5\) Hz) in \( \text{Ziziphoroside} \text{ B} \) and the absolute configuration of \( \text{Ziziphoroside A} \) was investigated by measurement of circular dichroism (CD) spectrum. The Cotton effect of CD spectrum was \( \Delta_{254} \) 6.7 and \( \Delta_{234} \) +0.5. The observed Cotton led to the \( 1R \) absolute configuration based on the helicity rule for \( \alpha,\beta \)-unsaturated ketone\(^{10}\) (Fig. 3).

**Ziziphoroside B** (2) was obtained as a colorless powder. In positive ion HR-FAB-MS, a quasi-molecular ion peak at \( m/z \) 353.1577 (Calcd 353.1577) \([\text{M} + \text{Na}]^+\), which was consistent with a molecular formula of \( \text{C}_{16}\text{H}_{25}\text{O}_{7} \text{Na} \), was observed. The \( ^1\text{H}-\text{NMR} \) and \( ^{13}\text{C}-\text{NMR} \), COSY, HMOC and HMBC spectra were very similar to those of \( \text{Ziziphoroside A} \). Thus, the structure of \( \text{Ziziphoroside C} \) was consistent with a molecular formula of \( \text{C}_{16}\text{H}_{25}\text{O}_{7} \text{Na} \), which was consistent with a molecular formula of \( \text{C}_{16}\text{H}_{25}\text{O}_{7} \text{Na} \), was observed. The \( ^1\text{H}-\text{NMR} \) and COSY spectra of \( \text{Ziziphoroside C} \) showed the presence of two vinyl methyl groups [\( \delta \) 1.98 (3H, s), \( \delta \) 2.08 (3H, s)], a methylene–methylene sequence [\( \delta \) 2.40 (2H, t, \( J = 6.2\) Hz), 2.77 (2H, m)], oxymethylene protons [\( \delta \) 4.39 (2H, d, \( J = 2.4\) Hz)], a vinyl proton [\( \delta \) 5.88 (1H, s)] and an anomeric proton [\( \delta \) 4.24 (1H, d, \( J = 7.8\) Hz)] (Table 1). The \( ^{13}\text{C}-\text{NMR} \) spectrum exhibited six signals due to two methyl carbons (\( \delta \) 19.2, 24.0), two methylene carbons (\( \delta \) 29.0, 33.5), an oxymethylene carbon (\( \delta \) 70.0), a vinyl carbon (\( \delta \) 129.1), three tertiary carbons (\( \delta \) 134.4, 141.7, 164.8), a carbonyl carbon (\( \delta \) 194.5) and a sugar moiety (Table 1). These spectral data and the degree of unsaturation indicated that \( \text{Ziziphoroside C} \) should be a monoterpenoid glucoside. The connectivities of these partial structures and the functional groups were investigated by analysis of the HMBC spectrum. As shown in Fig. 5, HMBC correlations due to long-range coupling were observed between the following proton and carbon signals: H-7 and C-1, C-2, C-6; H-2, H-3, C-3; H-5 and C-3, C-4, C-8; H-9 and C-4, C-8, H-10 and C-4, C-8; and H-1’ and C-8. The sugar was identified as \( \alpha \)-glucose by both TLC and DEAE-Sepharose column chromatography (Table 1). The plane of \( \text{Ziziphoroside C} \) was represented as shown in Fig. 3.
The 9-hydroxy menthone type derivatives and their glucosides have been rarely isolated from natural products. In this time, it is interesting that their absolute structure of shizonepata tenuifolia 6 was revealed as 1R configuration. The 9-hydroxy menthone type deriversives and their glucosides have been rarely isolated Schizonepata tenuifolia 6 in natural products. In this time, it is interesting that their derivatives isolated from Z. clinopodioides.

Nitrite Assay It has been demonstrated that macrophages treated with LPS and/or IFN-γ increase secretion of several cytokines. LPS and IFN-γ responsive pathways in macrophages are distinct and independent, and these two inducers can cooperate with each other in achieving full macrophage activation. Low NO concentrations play an important physiological role as a defense in the immune system, whereas large amounts of NO in macrophages contribute to numerous pathological processes. In atherosclerotic lesions, inflammatory processes increase NO production in macrophages, resulting in vascular damage. Furthermore, excess NO induces oxidation of LDLs within the arterial wall and cause atherosclerosis. Therefore, suppression of excessive NO production should be important for prevention of atherosclerosis.

Compounds 1—18 were examined with respect to their inhibition of NO production stimulated by LPS and IFN-γ in RAW 264.7 cells. In the assay, aminoguanidine (IC_{50} 17.5 μM), which has been reported to have inhibitory effects on NO production in LPS activated RAW 264.7 macrophages via the down-regulation of inducible nitric oxide synthase (iNOS), was used as a positive control. As shown in Fig. 6, shizonepata tenuifolia A (8), apigenin (11), luteolin (12) and diosmetin (13) showed potent inhibitory effects on NO production. While 8 (IC_{50} 26.6 μM) exhibited inhibitory activity, compound 2 having similar structure showed weak effect, even at the concentration of 50 μM. The results indicated that the stereochemical relationship between C-1 and C-4 is important on inhibition of NO production.

Experimental General {1H} and {13C}-NMR spectra were measured on a JEOL JNM Lambda-400, 500 or JEOL ECA-600 spectrometer in MeOH-d_4, DMSO-d_6, or pyridine-d_5 containing TMS as an internal standard. IR spectra were recorded on a Shimadzu UV-160 spectrophotometer. MS spectra were obtained with a JASCO J-600 spectrophotometer. Optical rotations were measured on a Shimadzu UV-160 spectrophotometer. CD spectra were recorded with a JASCO J-600 spectrophotometer. Column chromatography was carried out on silica gel (Wakogel C-200) and Diaion HP-20 (Nippon Rensui). HPLC was conducted with a Spectra Physics SP 8800 and Sensyu SSC-3160 pump equipped with either ERC-7520 (ERMA)-RI or HITACHI L-400-UV detectors.

Table 1. {1H-NMR (600MHz) and {13C-NMR (150MHz) Data for 1, 2 and 3 (MeOH-d_4)}

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Sugar moiety

| 1'  |              |               |              |               |              |               |
| 2'  | 4.31 (d, 7.5)| 97.6          | 4.48 (d, 7.3)| 104.0         | 4.24 (d, 7.8)| 103.1         |
| 3'  | 77.0         |               | 77.8         |               | 77.0         |               |
| 4'  | 3.08–3.28(4H)| 70.5          | 3.26–3.41(4H)| 71.2          | 3.30–3.89(4H)| 70.7          |
| 5'  | 76.7         |               | 78.1         |               | 77.1         |               |
| 6'  | 3.54 (dd, 12.0, 5.4)| 61.7  | 3.66 (dd, 12.0, 4.6)| 62.5  | 3.66 (dd, 12.0, 5.8)| 62.9  |
| 7'  | 3.72 (dd, 12.0, 2.1)| 61.7  | 3.85 (dd, 12.0, 2.1)| 62.5  | 3.89 (dd, 12.0, 1.7)| 62.9  |

The values in parentheses represent the coupling constants in Hz. The δ values are in ppm downfield from TMS. a) was overlapped.
Plant Material

Z. clinopodioides was collected in Xinjiang Province, China and identified by Mr. Shen Jin Gui, Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. An authentic specimen of this plant was deposited in the above Institute and the Laboratory of Medicinal Chemistry, College of Pharmacy, Nihon University (ZC-001).

Extraction and Isolation

The dried whole herbs of Z. clinopodioides (0.7 kg) were extracted with 70% EtOH (5 L) under ultrasonication. The 70% EtOH extract was concentrated in vacuo to give extracts (108 g). The crude extracts were chromatographed on a Diaion HP-20 column eluted successively with stepwise gradients of 30% MeOH (6.5 L), 70% MeOH (8 L), MeOH (7 L) and acetone (5.5 L), and then each eluate was concentrated in vacuo to afford five fractions [30% MeOH-1 (fr. A, 12.84 g), 30% MeOH-2 (fr. B, 52.81 g), 70% MeOH (fr. C, 17.78 g), MeOH (fr. D, 9.45 g), and acetone (fr. E, 17.07 g)]. Fr. C (15.4 g) was chromatographed on a silica gel column eluted successively with solvents of increasing polarity [CHCl3, MeOH and H2O (1000 mL), 0:100 (1000 mL), EtOAc:MeOH, 10:1 (1000 mL), 4:1 (900 mL), 2:1 (800 mL), 1:1 (1000 mL), 0:100 (1000 mL)]. The 70% EtOH extract was concentrated to give extracts (108 g). The crude extracts (0.7 kg) were extracted with 70% EtOH (5 L) (fr. A, 12.84 g), 30% MeOH (fr. B, 52.81 g), 70% MeOH (fr. C, 12.84 g), MeOH (fr. D, 9.45 g), and acetone (fr. E, 17.07 g) (fr. C, 15.4 g). Fr. C (15.4 g) was chromatographed on a silica gel column eluted successively with solvents of increasing polarity [CHCl3, MeOH and H2O (1000 mL), 0:100 (1000 mL), EtOAc:MeOH, 10:1 (1000 mL), 4:1 (900 mL), 2:1 (800 mL), 1:1 (1000 mL), 0:100 (1000 mL)]. The crude extracts were subjected to GLC analysis to identify the derivatives of Z. clinopodioides. 

GC Analysis of Sugars in 1, 2 and 3

Compounds 1-3 (0.5 mg) were heated in 1 mL HCl (1 mL) and 1,4-dioxane (0.2 mL) at 100°C for 3 h. After cooling, the reaction mixture was neutralized by individually passing each through an ion-exchange resin (Amberlite IRA 400, OH− form) column. The filtrate was transferred to a Sep-Pak C18 cartridge and eluted with H2O and MeOH. The H2O eluate was concentrated and the residue was treated with z-cysteine methyl ester hydrochloride (4 mg) in pyridine (0.2 mL) at 60°C for 1 h. After the reaction, the solution was treated with TMS-14 (150 μL), hexamethyldisilazane and trimethylchlorosilamine in pyridine, TOKYO KASEI Co., Ltd., Tokyo, Japan) at 40°C for 10 min. The reaction mixture was then subjected to GLC analysis to identify the derivatives of d-glucose from 1, 2 and 3. GLC conditions: column, OV 1701, 50 m × 0.25 mm (i.d.), 0.25 μm; detector, FID; injector temperature, 250°C; detector temperature, 280°C; column temperature, 200°C for 2 min and then 5°C/min up to 260°C; He carrier, 26.7 cm/s; d-glucose 10.8 min.

NO Production in Activated Macrophage-Like Cell Line, RAW 264.7

The macrophage-like cell line, RAW 264.7, was obtained from American Type Culture Collection. The cells were cultured in Ham’s F12 medium with 10% fetal bovine serum (FBS) (SAFC Biosciences) at 37°C under a humidified 5% CO2 atmosphere. The RAW 264.7 cells were seeded at 1.2×10^6 cells/mL onto 96-well plates (Sumitomo Bakelite, MS-8096R, Tokyo) and then incubated at 37°C for 2 h. A test sample was then added to the culture simultaneously with both LPS (100 ng/mL) and recombinant mouse IFN-γ (0.33 ng/mL), and the cells were incubated at 37°C, typically for 16 h. The amount of nitrite in the culture supernatants was measured by the Griess assay. A dose–response curve was plotted for each compound, and the concentration giving 50% inhibition (IC50) was calculated. Cell viability was measured using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay method.
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