

Euscaphinin, a New Ellagitannin Dimer from *Euscaphis japonica* (THUNB.) KANITZ

Hajime MAEDA,^{a,b} Yosuke MATSUO,^a Takashi TANAKA,^{*,a} and Isao KOUNO^{*,a}

^a Graduate School of Biomedical Sciences, Nagasaki University; 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan; and

^b Nagasaki Agriculture and Forestry Experiment Station; 3118 Kaidzu, Isahaya, Nagasaki 854-0063, Japan.

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A new ellagitannin dimer named euscaphinin was isolated from the leaves of *Euscaphis japonica* (THUNB.) KANITZ. The structure of this dimer was determined on the basis of spectroscopic and chemical evidence. The resulting structure indicated that it was produced by intermolecular C–O oxidative coupling between the galloyl groups of molecules of 1(β -O-galloyl pedunculagin, the major ellagitannin of the leaves. The ellagitannins were not detected in the bark and wood part of the plant. This is the first example of the isolation of ellagitannins from Staphyleaceae plants.

Key words *Euscaphis japonica*; Staphyleaceae; ellagitannin; pedunculagin; tannin

Recent studies on plant polyphenols have revealed various biological activities for ellagitannins.^{1,2} Distribution of ellagitannins in plants is relatively limited compared with that of condensed tannins (proanthocyanidins).³ In this study, we proposed to find new plant sources of functional polyphenols and examined the leaves, bark and wood of 39 plant samples belonging to 32 genera of 29 families, distributed in the Nagasaki region of Japan, by thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC). Plants belonging to the Elaeocarpaceae,⁴ Myricaceae,⁵ Anacardiaceae,⁶ Juglandaceae,⁷ Stachyuraceae,⁸ and Fagaceae^{9–11} families were shown to contain high concentrations of polyphenols, substances that have already been studied chemically. Unexpectedly, our study disclosed accumulation of ellagitannins in the leaves of *Euscaphis japonica* (THUNB.) KANITZ (Staphyleaceae). *E. japonica* is a deciduous tree distributed in western Japan, and little is known about the phenolic constituents of this plant.^{12–16} Presence of ellagitannins in Staphyleaceae has not yet been reported. This paper describes the isolation and structural determination of two ellagitannins, including a new dimeric ellagitannin.

Results and Discussion

An aqueous acetone extract of the fresh leaves of *E. japonica* was subjected to a combination of Diaion HP20SS, Sephadex LH-20, and Chromatorex ODS column chromatography to furnish two major phenolic compounds. One of the products was identified as 1(β -O-galloyl pedunculagin (1)^{17,18}) by comparisons of ¹H-NMR spectral data, HPLC retention time and UV absorption with those of an authentic sample.

Compound 2 was isolated as a white amorphous powder and produced a dark-blue coloration upon reaction with FeCl₃ and a brown coloration upon reaction with NaNO₂–AcOH reagent,¹⁹ suggesting this compound is an ellagitannin. The *R_f* value on silica gel TLC was much smaller than that of 1 and the matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) MS showed a [M+Na]⁺ peak at *m/z* 1893, indicating that 2 is a dimeric ellagitannin. This was supported by appearance of signals attributable to two sugar anomeric protons [δ 6.16 (1H, d, *J*=8.5 Hz, Glc-1), 6.06 (1H, d, *J*=8.7 Hz, Glc-1')] in the ¹H-NMR spectrum.

The sugar was confirmed to be D-glucose by the HPLC analysis of the thiocarbamoyl-thiazolidine derivative of the acid hydrolysis products.²⁰ The chemical shifts (δ 3.7–6.2) and the large coupling constants of the sugar proton signals were similar to those of 1 and indicated that the anomeric positions were β -configurations and that all the hydroxyl groups of the pyranose ring were acylated. In the aromatic region of the spectrum, nine singlets [δ 7.16, 6.66, 6.65, 6.51, 6.50, 6.47, 6.46, 6.33, 6.33] and two mutually coupled doublets [δ 7.30, 6.84 (each d, *J*=1.9 Hz)] were observed. The hetero-nuclear multiple-bond connectivity (HMBC) correlations (Fig. 2) of these aromatic protons indicated that all of the protons are attached to the 3,4,5-trihydroxybenzoyl ester moieties. The ¹³C-NMR spectrum showed signals attributable to ten ester carbon signals, among which eight were observed in the range of 167 to 169 and remaining two appeared upfield (δ 162.4, 165.0). The ester signal at δ 165.0 [dehydrodigalloyl (DHDG) C-7] showed three HMBC corre-

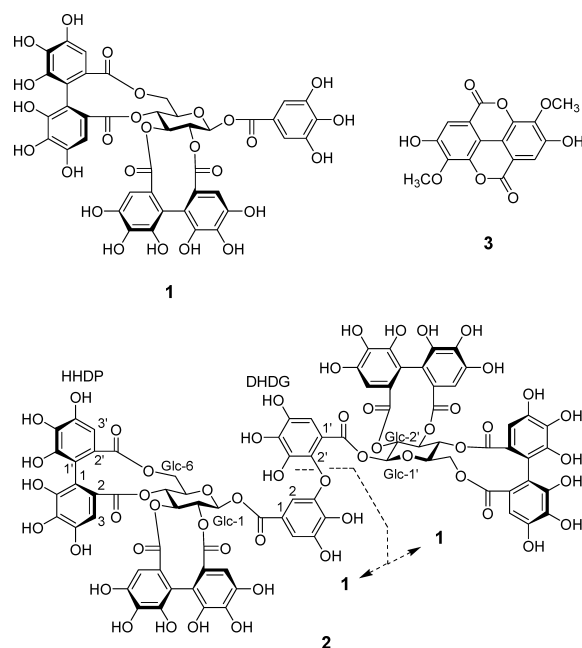


Fig. 1. Structures of Compounds 1, 2, and 3

* To whom correspondence should be addressed. e-mail: t-tanaka@nagasaki-u.ac.jp

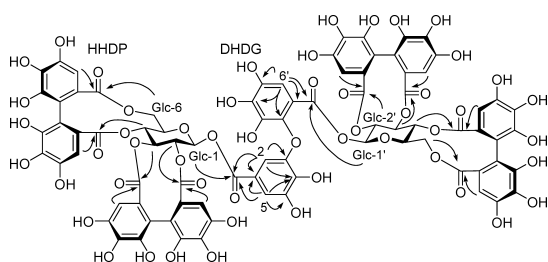


Fig. 2. Selected HMBC Correlations for **2**

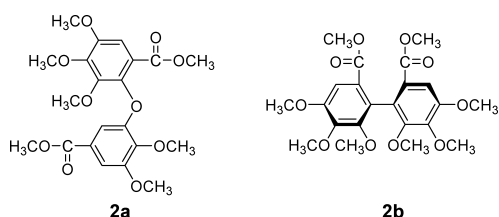


Fig. 3. Structures of **2a** and **2b**

lation peaks with one of the glucose anomeric proton signal [δ 6.16 (Glc-1)], and the *meta*-coupled aromatic doublets at δ 7.30 and 6.84. In turn, the ester carbon signal at δ 162.4 (DHDG C-7') was correlated with the other anomeric proton [δ 6.06 (Glc-1')] and the aromatic singlet at δ 7.16. The remaining eight ester carbons showed correlation peaks with glucose H-2—H-6 protons and the aromatic singlets resonated in the range of δ 6.66—6.33. These aromatic proton signals correlated with the aromatic carbon signals, the chemical shifts of which indicated the presence of four hexahydroxydiphenyl (HHDP) ester groups similar to those of **1**, though complete assignments were difficult due to overlapping signals.

The acyl groups were confirmed by chemical degradation. First, **2** was methylated with CH_2N_2 and the product was hydrolyzed under alkaline conditions. The resulting carboxylic acids were converted to methyl esters with CH_2N_2 to yield dimethyl pentamethyl dehydrodigallic acid (**2a**) and dimethyl (*S*)-4,4',5,5',6,6'-hexamethoxydiphenolate (**2b**) (Fig. 3). The latter showed a negative optical rotation value ($[\alpha]_D -24.0^\circ$); thus the atropisomerism of the biphenyl bond of **2b** was determined to be *S*-configuration.²¹ The aforementioned HMBC correlations indicated that the two ester carbonyls of the dehydrodigalloyl groups were attached to the both the anomeric hydroxyl groups. The HHDP ester groups are believed to bridge over the 2,3-hydroxy and 4,6-hydroxy groups based on the large coupling constants of the sugar proton signals ($J_{2,3}=J_{3,4}=J_{4,5}=9\text{--}10\text{ Hz}$) indicating that the glucose adapted a ${}^4\text{C}_1$ conformation. In addition, the large chemical shift differences ($\Delta\delta > 1.4$) for the glucose methylene protons [δ 5.31, 5.29 (each 1H, dd, $J=6.9, 13.5\text{ Hz}$, Glc-6a, 6a'), 3.82, 3.77 (each 1H, dd, $J=1.4, 13.5\text{ Hz}$, Glc-6b, 6b')] were observed. This spectroscopic phenomenon was commonly observed in the spectrum of ellagitannins having (*S*)-HHDP esters at the C-4 and C-6 hydroxyl groups of the ${}^4\text{C}_1$ -glucopyranose.²² The location of the esters was also supported by the partial hydrolysis of **2** in hot water, which yielded pedunculagin [a mixture of α and β form of 2,3,4,6-bis-(*S*)-HHDP-D-glucose]. On the basis of these spectroscopic and chemical results, the structure of this dimeric el-

lagitannin was assigned to **2**, and named euscaphinin. This tannin was deduced to be produced by intermolecular simple oxidative coupling between the two galloyl groups of **1**. Some dimeric ellagitannins having *m*-dehydrodigalloyl groups were already isolated from Rosaceae,^{23,24} Tamaricaceae,²⁵ Coriariaceae,²⁶ Euphorbiaceae²⁷ and Nymphaeaceae^{28,29} plants. Among them, agrimoniin³⁰ is the only isomer of **2** composed of **1** and its isomer differing only in the anomeric configuration.

The ellagitannins **1** and **2** were only found in the leaves of *E. japonica* and were not detected in the bark and wood. HPLC analysis of the aqueous acetone extract of the bark showed a large peak corresponding to a major phenolic compound, which was isolated and identified to be 3,3'-di-*O*-methyl ellagic acid (**3**).³¹ The ellagitannin **1** was reported to be a selective inhibitor of the β -regulatory subunit of A-kinase³² and an effective autophosphorylation inhibitor of C-kinase.³³ Since **1** was found to be accumulated in the leaves in high concentration (1.28% of fresh leaves), *E. japonica* is expected to be a potent plant source of this biologically active ellagitannin.

Experimental

General UV spectra were obtained with a Jasco V-560 UV/Vis spectrophotometer and optical rotations were measured with a Jasco DIP-370 digital polarimeter. ${}^1\text{H}$ - and ${}^{13}\text{C}$ -NMR spectra were measured in acetone- d_6 at 27°C with a Varian Unity plus 500 spectrometer operating at 500 MHz for ${}^1\text{H}$ and at 125 MHz for ${}^{13}\text{C}$. Coupling constants are expressed in Hz and chemical shifts are given on a δ (ppm) scale. MS were recorded on a Voyager DE-PRO (Applied Biosystems) and a JEOL JMS-700N spectrometer. 2,5-Dihydroxybenzoic acid and glycerol were used as the matrix for MALDI-TOF-MS and FAB-MS measurements, respectively. Column chromatography was performed using Diaion HP20SS (Mitsubishi Chemical), Sephadex LH-20 (25—100 μm ; Sigma), and Chromatorex ODS (100—200 mesh; Fuji Silysia Chemical) columns. TLC was performed on precoated Kieselgel 60 F₂₅₄ plates (0.2 mm thick; Merck) with toluene—ethyl formate—formic acid (1 : 7 : 1, v/v) as a solvent, and spots were detected by UV illumination (254 nm) and by spraying with 2% ethanolic FeCl_3 and 10% sulfuric acid reagent, followed by heating.

Plant Material The leaves of *E. japonica* were collected in May 2007, and a voucher specimen was deposited at the Nagasaki Agriculture and Forestry Experiment Station, Japan.

Extraction and Separation The fresh leaves of *E. japonica* (500 g) were crushed with acetone— H_2O (4 : 1, v/v, 1 l) in a Warring blender and filtered. The plant debris remaining on the filter paper was further extracted with the same solvent at room temperature two times. The three extracts were combined and acetone was removed by evaporation under reduced pressure (*ca.* 40°C). The resulting dark green precipitates, consisting mainly of chlorophylls and waxes, were removed by filtration. The filtrate was subjected to Diaion HP20SS column chromatography (4.0 cm i.d. \times 30 cm) with water containing increasing proportions of MeOH (10%, 20%, 30%, 40%, 50%, 70%, 100%) to give three fractions containing polyphenols; fr. 1 (3.2 g), fr. 2 (12.8 g), and fr. 3 (5.45 g). The fr. 2, which showed dark blue coloration with 2% ethanolic FeCl_3 reagent, was further separated by Sephadex LH-20 column chromatography (4.0 cm i.d. \times 25 cm) with aqueous MeOH— H_2O —acetone (3 : 2 : 0, 4 : 1 : 0, 1 : 0 : 0, 18 : 1 : 1, 8 : 1 : 1, 3 : 1 : 1, and then 0 : 1 : 1, v/v/v, each 300 ml) to yield 1(β)-*O*-galloyl pedunculagin (**1**) (6.41 g, eluted with MeOH— H_2O —acetone, 4 : 1 : 0 and 1 : 0 : 0) and fraction 2-2 (1.05 g, eluted with MeOH— H_2O —acetone, 8 : 1 : 1 and 3 : 1 : 1) which mainly contained euscaphinin. Fraction 2-2 was successively applied to a Sephadex LH-20 column (3.0 cm i.d. \times 20 cm) with EtOH— H_2O —acetone (1 : 0 : 0, 9 : 1 : 0, 4 : 1 : 0, 3 : 4 : 0, 27 : 18 : 5, 12 : 8 : 5 and then 6 : 4 : 5, v/v/v, each 200 ml, **2** was eluted with 27 : 18 : 5 and 12 : 8 : 5) and a Chromatorex ODS column (3.0 cm i.d. \times 20 cm) with water containing increasing proportions of MeOH (5—40%, 5% stepwise elution) to give euscaphinin (**2**) (0.30 g, eluted with 30% MeOH).

Separation of 3,3'-Di-*O*-methyl Ellagic Acid from Bark Fresh bark of *E. japonica* (500 g) was extracted 3 times with 80% acetone at room temperature, and the resulting extract was concentrated under reduced pressure.

After removal of insoluble precipitates by filtration, the filtrate was separated by Diaion HP20SS column chromatography (0–100% MeOH, 10% stepwise elution), to give 4 fractions. The last fraction yielded colorless needles (26 mg) from MeOH, which were identified to be 3,3'-di-*O*-methyl ellagic acid (**3**) by comparison of the ¹H- and ¹³C-NMR spectral data with previously published values.³¹⁾

Euscaphinin (**2**): White amorphous powder. $[\alpha]_D^{30}$ 40.5° ($c=0.2$, acetone-H₂O, 1:1, v/v). UV $\lambda_{\max}^{\text{EtOH}}$ (log ϵ): 261 (sh) (4.35). IR ν_{\max} cm⁻¹: 3445, 1733, 1615, 1515, 1446. MALDI-TOF-MS m/z : 1893 [M+Na]⁺. *Anal.* Calcd for C₈₂H₅₄O₅₂·5H₂O: C, 50.22; H, 3.29. Found: C, 50.11; H, 3.21. ¹H-NMR (acetone-*d*₆) δ : 7.30 [1H, d, $J=1.9$ Hz, dehydrodigalloyl (DHDG)-6], 7.16 (1H, s, DHDG-6'), 6.84 (1H, d, $J=1.9$ Hz, DHDG-2), 6.66, 6.65, 6.51, 6.50, 6.47, 6.46, 6.33, 6.33 [each 1H, s, hexahydroxydiphenoyl (HHDP)-3, 3'], 6.16 (1H, d, $J=8.5$ Hz, Glc-1), 6.06 (1H, d, $J=8.7$ Hz, Glc-1'), 5.41, 5.38 (each 1H, dd, $J=9.2$, 10.0 Hz, Glc-3, 3'), 5.31, 5.29 (each 1H, dd, $J=6.9$, 13.5 Hz, Glc-6a, 6a'), 5.14, 5.13 (each 1H, t, $J=10.0$ Hz, Glc-4, 4'), 5.13 (1H, dd, $J=8.5$, 9.2 Hz, Glc-2), 5.11 (1H, dd, $J=8.7$, 9.2 Hz, Glc-2'), 4.45, 4.41 (each 1H, ddd, $J=1.4$, 6.9, 9.9 Hz, Glc-4, 4'), 3.82, 3.77 (each 1H, dd, $J=1.4$, 13.5 Hz, Glc-6b, 6b'). ¹³C-NMR (acetone-*d*₆) δ : 169.2, 169.1 (COO attached to Glc-3, 3'), 168.5, 168.4 (COO attached to Glc-4, 4'), 167.9 (2C) (COO attached to Glc-6, 6'), 167.8, 167.7 (COO attached to Glc-2, 2'), 164.96 (DHDG-7), 162.4 (DHDG-7'), 148.2 (DHDG-3), 146.6 (DHDG-5), 145.2 (2C), 145.1 (2C), 145.1 (2C), 145.0 (2C), 144.5 (6C), 144.5 (2C) (HHDP-4, 6, 4', 6'), 143.4, 141.1, 137.7 (DHHDG-2', 4', 5'), 141.6 (DHDG-4), 141.1 (DHHDG-3'), 136.6 (2C), 136.5, 136.5, 136.4, 136.4, 136.2, 136.1 (HHDP-5, 5'), 126.3 (4C), 125.9 (2C), 125.8, 125.7 (HHDP-2, 2'), 119.3 (DHDG-1), 116.0, 115.9, 115.7 (2C), 114.9 (2C), 114.2, 114.1 (HHDP-1, 1'), 113.3 (DHDG-1'), 112.2 (DHDG-6), 109.6 (DHDG-6'), 108.3, 108.2 (HHDP-6, 6'), 108.1 (DHHDG-2), 107.5 (2C), 107.3, 107.2, 107.1 (2C) (HHDP-6, 6'), 92.2 (Glc-1), 91.9 (Glc-1'), 77.2, 77.1 (Glc-3, 3'), 75.8, 75.7 (Glc-2, 2'), 73.4, 73.3 (Glc-5, 5'), 69.0 (2C) (Glc-4, 4'), 62.9 (2C) (Glc-6, 6').

Methylation and Methanolysis of 2 A solution of **2** (50 mg) in MeOH (3 ml) was mixed with an ethereal solution of CH₂N₂ at 4 °C for 15 h. After evaporation of the solvent, the residue was dissolved in 5% aqueous NaOH–MeOH (1:2, v/v) and heated at 80 °C for 1 h. After removal of MeOH by evaporation, the aqueous solution was acidified by addition of 2 M HCl and partitioned with Et₂O. The Et₂O layer was dried with Na₂SO₄ and mixed with an Et₂O solution of CH₂N₂ at 4 °C for 10 h. After evaporation, the residue was separated by silica gel chromatography with toluene–acetone (98:2–92:8) to give dimethyl pentamethyl dehydrodigalate (**2a**) (7.8 mg),²⁸⁾ and dimethyl (*S*)-4,4',5,5',6,6'-hexamethoxydiphenolate (29.9 mg), $[\alpha]_D -24.0^\circ$ ($c=0.3$, CHCl₃).²¹⁾

Partial Hydrolysis of 2 A solution of **2** (2 mg) in water (1.0 ml) was heated at 100 °C in a screw-capped vial for 2 h. The mixture was analyzed by HPLC [Cosmosil 5C₁₈ AR II (250×4.6 mm i.d.); using a gradient elution of CH₃CN in 50 mM H₃PO₄ from 4–30% in 39 min and 30–75% in 15 min; column temperature, 35 °C; flow rate, 0.8 ml/min; detection with a Jasco MD-910 photodiode array detector. The t_R and UV absorptions of the two peaks at 13.26 min and 17.11 min coincided with those of the α - and β -anomers of pedunculagin.

Determination of Aldose Configuration A solution of **2** (5 mg) in 1 M HCl (0.2 ml) was heated at 90–100 °C in a screw-capped vial for 10 h. The mixture was neutralized by addition of Amberlite IRA400 (OH⁻ form) and filtered. The filtrate was dried *in vacuo*, dissolved in 0.2 ml of pyridine containing L-cysteine methyl ester (10 mg/ml) and reacted at 60 °C for 1 h. To this mixture a solution (0.2 ml) of *o*-tolyisothiocyanate in pyridine (10 mg/ml) was added and the solution was heated at 60 °C for 1 h. The final mixture was directly analyzed by HPLC [Cosmosil 5C₁₈ AR II (250×4.6 mm i.d., Nacalal Tesque Inc.); 25% CH₃CN in 50 mM H₃PO₄; flow rate, 0.8 ml/min; detection, 250 nm]. The t_R of the peak at 17.48 min coincided with that of D-glucose.

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References

- 1) Yokozawa T., Chen C. P., Tanaka T., Kitani K., *Biochem. Pharmacol.*, **63**, 853–858 (2002).
- 2) Okuda T., Yoshida T., Hatano T., *Planta Med.*, **55**, 117–122 (1989).
- 3) Okuda T., Yoshida T., Hatano T., *Phytochemistry*, **55**, 513–529 (2000).
- 4) Tanaka T., Nonaka G., Nishioka I., Miyahara K., Kawasaki T., *J. Chem. Soc., Perkin Trans. 1*, **1986**, 369–376 (1986).
- 5) Nonaka G., Muta M., Nishioka I., *Phytochemistry*, **22**, 237–241 (1983).
- 6) Yokozawa T., Fujioka K., Oura H., Tanaka T., Nonaka G., Nishioka I., *Phytother. Res.*, **9**, 327–330 (1995).
- 7) Tanaka T., Kirihara S., Nonaka G., Nishioka I., *Chem. Pharm. Bull.*, **41**, 1708–1716 (1993).
- 8) Hatano T., Yazaki K., Okonogi A., Okuda T., *Chem. Pharm. Bull.*, **39**, 1689–1693 (1991).
- 9) Tanaka T., Ueda N., Shinohara H., Nonaka G., Fujioka T., Mihashi K., Kouno I., *Chem. Pharm. Bull.*, **44**, 2236–2242 (1996).
- 10) Nonaka G., Sakai T., Mihashi K., Nishioka I., *Chem. Pharm. Bull.*, **39**, 884–888 (1991).
- 11) Ishimaru K., Ishimatsu M., Nonaka G., Mihashi K., Iwase Y., Nishioka I., *Chem. Pharm. Bull.*, **36**, 3319–3327 (1988).
- 12) Takahashi K., Kawaguchi S., Nishimura K., Kubota K., Tanabe Y., Takani M., *Chem. Pharm. Bull.*, **22**, 650–653 (1974).
- 13) Takeda Y., Okada Y., Masuda T., Hirata E., Shinzato T., Takushi A., Yu Q., Otsuka H., *Chem. Pharm. Bull.*, **48**, 752–754 (2000).
- 14) Takeda Y., Okada Y., Masuda T., Hirata E., Takushi A., Otsuka H., *Phytochemistry*, **49**, 2565–2568 (1998).
- 15) Konishi T., Otani T., Kiyosawa S., Fujiwara Y., *Chem. Pharm. Bull.*, **44**, 863–864 (1996).
- 16) Lee M. K., Jeon H. Y., Lee K. Y., Kim S. H., Ma C. J., Sung S. H., Lee H.-S., Park M. J., Kim Y. C., *Planta Med.*, **73**, 782–786 (2007).
- 17) Okuda T., Yoshida T., Ashida M., Yazaki K., *J. Chem. Soc. Perkin Trans. 1*, **1983**, 1765–1772 (1983).
- 18) Tanaka T., Nonaka G., Nishioka I., *J. Chem. Res. M*, **1985**, 2001–2029 (1985).
- 19) Bate-Smith E. C., *Phytochemistry*, **11**, 1153–1156 (1972).
- 20) Tanaka T., Nakashima T., Ueda T., Tomii K., Kouno I., *Chem. Pharm. Bull.*, **55**, 899–901 (2007).
- 21) Ikeya Y., Taguchi H., Yosioka I., Kobayashi H., *Chem. Pharm. Bull.*, **27**, 1383–1394 (1979).
- 22) Gupta R. K., Al-Shafi A. M. K., Layden K., Haslam E., *J. Chem. Soc. Perkin Trans. 1*, **1982**, 2525–2534 (1982).
- 23) Okuda T., Yoshida T., Hatano T., *Prog. Chem. Org. Nat. Prod.*, **66**, 1–117 (1995).
- 24) Yoshida T., Tanaka K., Chen X.-M., Okuda T., *Chem. Pharm. Bull.*, **37**, 920–924 (1989).
- 25) Ahmed A., Memon M., Yoshida T., Okuda T., *Chem. Pharm. Bull.*, **42**, 254–264 (1994).
- 26) Hatano T., Yoshihara R., Hattori S., Yoshizaki M., Shingu T., Okuda T., *Chem. Pharm. Bull.*, **40**, 1703–1710 (1992).
- 27) Lee S.-H., Tanaka T., Nonaka G., Nishioka I., *Chem. Pharm. Bull.*, **39**, 630–638 (1991).
- 28) Ishimatsu M., Tanaka T., Nonaka G., Nishioka I., Nishizawa M., Yamagishi T., *Chem. Pharm. Bull.*, **37**, 1735–1743 (1989).
- 29) Ishimatsu M., Tanaka T., Nonaka G., Nishioka I., Nishizawa M., Yamagishi T., *Chem. Pharm. Bull.*, **37**, 129–134 (1989).
- 30) Okuda T., Yoshida T., Kuwahara M., Memon M. U., Shingu T., *Chem. Pharm. Bull.*, **32**, 2165–2173 (1984).
- 31) Khac D. D., Tran-Van S., Campos A. M., Lallemand J. Y., Fetizon M., *Phytochemistry*, **29**, 251–256 (1972).
- 32) Kosuge S., Maekawa T., Saito C., Tanaka T., Kouno I., Ohtsuki K., *J. Biochem. (Tokyo)*, **129**, 403–409 (2001).
- 33) Ueno T., Miyanaga T., Kawakami F., Okano M., Tanaka T., Ohtsuki K., *Biol. Pharm. Bull.*, **25**, 1401–1404 (2002).