Nitrile-Containing Phenolic Glucosides from the Leaves of Glochidion acuminatum

Yukiko Yamashita-Higuchi, Sachiko Sugimoto, Katsuyoshi Matsunami, Masanori Inagaki, Hideaki Otsuka, and Yoshio Takeda

Graduate School of Biomedical and Health Sciences, Hiroshima University; 1–2–3 Kasumi, Minami-ku, Hiroshima 734–8553, Japan; and Faculty of Pharmacy, Yasuda Women's University; 6–13–1 Yasuhigashi, Asaminami-ku, Hiroshima 731–0153, Japan.

Received September 5, 2014; accepted October 19, 2014

[0x0]δ

From the EtOAc-soluble fraction of a MeOH extract of the leaves of Glochidion acuminatum, six new compounds along with five known ones were isolated. The structures of the new compounds were elucidated to be two gallates, a p-hydroxybenzoate and an (5)-2-(4-hydroxycyclohex-1-en-1-yl)acetate of a nitrile-containing phenolic glucoside, methyl 2-(2-hydroxyphenyl)acetate β-α-glucopyranoside, and (5)-methyl 2-[4-sulfoxycyclohex-1-en-1-yl]acetate on the basis of spectroscopic evidence.

Key words Glochidion acuminatum; Euphorbiaceae; 2-(2,4-dihydroxyphenyl)acetonitrile; gallate; sulfate

In previous works, a novel dimeric butenolide[1] and C-8 compounds[2] were isolated from the 1-BuOH-soluble fraction of a methanol (MeOH) extract of leaves of Glochidion acuminatum Müller-Argoviensis. It is known that the larvae of the Epicephala moth feed on the fruit of G. acuminatum.[3] From the EtOAc-soluble fraction of a MeOH extract of the leaves of G. acuminatum, two gallic acid (1, 2), one p-hydroxybenzoic acid (3), and one (5)-1-(4-hydroxycyclohex-1-en-1-yl)acetic acid ester (4) of a nitrile-containing phenolic glucoside, a methyl 2-(2-hydroxyphenyl)acetate β-α-glucopyranoside (5), and a (5)-methyl 2-[4-sulfoxycyclohex-1-en-1-yl]acetate (6), along with five known compounds, (+)-menisdaurilide (7),[4] (+)-aquiliegiolide (8),[5] (-)-loliolide (9),[6] (+)-epigallocatechin 3-O-gallate (10),[7] and phloretin 4′-O-β-glucopyranoside (11),[8] were isolated (Fig. 1).

Compounds 1–6 were isolated using various separation methods, i.e. normal and reversed-phase silica gel column chromatography, HPLC and droplet counter-current chromatography. The structures of the new compounds were elucidated on the basis of spectroscopic evidence and those of the known compounds were identified by comparison with reported spectral data in the literature.

Compounds 1, [α] D = –45.1, was isolated as an amorphous powder and its elemental composition was determined to be C16H11NO5 by observation of a quasi-molecular ion peak in high-resolution (HR) electrospray-ionization (ESI) MS. The IR spectrum exhibited absorptions due to hydroxy groups (3323 cm−1), a triple bond (2260 cm−1), an ester carboxyl group (1699 cm−1), an aromatic ring (1614 and 1511 cm−1), and phenolic and aliphatic C–O bonds (1239 and 1073 cm−1), respectively, and the presence of the aromatic ring was also supported by the UV absorption band at 278 nm. The 1H-NMR spectral data comprised three aromatic protons [δH 6.51 (dd, J = 8, 2 Hz), 6.69 (d, J = 2 Hz) and 7.12 (d, J = 8 Hz)], coupled in an ABX system, one singlet aromatic signal for two protons (δH 7.08), isolated methylene protons [δH 3.77 (d, J = 11 Hz) and 3.78 (d, J = 11 Hz)] and an anomeric proton (δH 4.87 (d, J = 7 Hz)). The 13C-NMR spectrum exhibited five typical signals [δC 110.4(2) (d), 121.4 (s), 139.9 (s), 146.5(2) (s), and 168.4 (s)] assignable to a gallic acid moiety (Table 1), and acid hydrolysis of 1 liberated D-glucose as a sugar component. Of the remaining eight carbon signals, δC 120.1 (s) was assigned as that of a nitrile functional group with a typical IR absorption band at 2260 cm−1, and six sp2 signals as those of a trisubstituted benzene ring (Table 1). The heteronuclear multiple bond correlation (HMBC) spectroscopy (Fig. 2) between methylene protons (δH 3.77 and 3.78) and the nitrile carbon, C-2 and C-6 [with δH 7.12 (d, J = 8 Hz)], H-5 and C-1 and 3, and H-6 and C-2 and 4, established the structure of the aglycone to be as shown in Fig. 1. This substitution arrangement is rarely found in nature. Further correlations, the anomeric proton with C-2 and H-2′ with the carbonyl carbon of the galloyl moiety, were also observed in the HMBC spectrum (Fig. 2). Mild alkaline hydrolysis of 1 with NaOCH3 in MeOH gave a deacetylated derivative, 2-(2,4-dihydroxyphenyl)acetonitrile 2-O-β-D-glucopyranoside (1a) (=12 in Fig. 1). Therefore, the structure of 1 was elucidated to be 2-(2,4-dihydroxyphenyl)acetonitrile 2-O-β-D-glucopyranoside 6′-O-gallate, as shown in Fig. 1. 2-(2,4-Dihydroxyphenyl)acetonitrile 2-O-β-D-glucopyranoside (1a) was first isolated in 1994 from Ehretia philippinensis, as ehretioside B,[9] while in 2005, Su et al. claimed the first isolation of 12 from the roots of Semiaquilegia adoxoides (Ranunculaceae) as a novel cyanogenic glucoside.[10] However, compound 12, isolated by Su et al. is ehretioside B (1a) and it is not cyanogenic.

Compounds 2, [α] D = –5.12, was isolated as an amorphous powder and its elemental composition was determined to be C12H10NO3S by HR-ESI-MS. The IR and UV spectra were similar to those of 1, and the NMR spectra also showed close resemblance with those of 1. In the 1H-NMR spectrum, two two-proton singlet signals (δH 7.10 and 7.17) were observed in the aromatic region and five sets of signals assignable to a gallic acid moiety were observed in the 13C-NMR spectrum. The elemental composition of 2 coincided with the presence of one more galloyl unit in 2 than in 1. In the NMR spectra, H-3′ was obviously shifted downfield (δH 5.26), and C-3′ shifted downfield by 1.0 ppm, and both C-2′ and C-4′ shifted upfield by 1.5 ppm (Table 1), when these signals were compared with

*To whom correspondence should be addressed. e-mail: hotuska@hiroshima-u.ac.jp; otsuka-h@yasuda-u.ac.jp

© 2015 The Pharmaceutical Society of Japan
those of 1. In the HMBC spectrum, H-3′ showed a significant correlation peak with one of the carbonyl groups in the galloyl moiety and a H-1′–H-2′–H-3′–H contiguous relation was observed on 1H–1H correlation spectroscopy. Therefore, the structure of 2 was elucidated to be 2-(2,4-dihydroxyphenyl)-acetonitrile 2-O-β-D-glucopyranoside 3,6′-O-digallate, as shown in Fig. 1.

Compound 3, [α]D −42.9, was isolated as an amorphous powder and its elemental composition was determined to be C21H21NO9. Its IR spectrum also exhibited a characteristic absorption for a triple bond and its NMR spectra were similar to those of compound 1. Since the aromatic proton signals of the acyl substituent appeared as AB doublets, 7.90 (2H, d, J = 9 Hz, H-2′ and 5′) and 6.82 (2H, d, J = 9 Hz, H-2′ and 5′), together with the presence of a highly deshielded carbon signal at δC 163.6, the galloyl group in 1 must be replaced by p-hydroxybenzoate. Therefore, the structure of 3 was elucidated to be 2-(2,4-dihydroxyphenyl)acetonitrile 2-O-β-D-glucopyranoside 6′-O-p-hydroxybenzoate, as shown in Fig. 1.

Compound 4, [α]D −61.8, was isolated as an amorphous powder and its elemental composition was C22H27NO9. Spectroscopic evidence revealed that compound 4 was a similar compound to 1–3, the acyl substituent being different. As revealed by the NMR spectroscopic data, the acyl moiety comprised one trisubstituted double bond, and four methylenes, one oxygenated methine carbon and a carbonyl carbon. Judging from this elemental composition, one degree of unsaturation must be satisfied by the formation of a ring system in the acyl framework. The above evidence substantiates that the structure of the acyl group was 2-(4-hydroxycyclohex-1-en-1-yl)acetate, which is found as an acyl moiety in various compounds, isolated from the 1-BuOH-soluble fraction of the same MeOH extract of the title plant. Since the absolute configuration at the 4-position of the methyl 2-(4-hydroxycyclohex-1-en-1-yl)acetate, which is found as an acyl moiety in various compounds, isolated from the 1-BuOH-soluble fraction of the same MeOH extract of the title plant, was determined by the modified Mosher’s method, it must have the same S configuration. Therefore, the structure of 4 was elucidated to be 2-(2,4-dihydroxyphenyl)acetonitrile 2-O-β-D-glucopyranoside 6′-O-(S)-2-(4-hydroxycyclohex-1-en-1-yl)acetate, as shown in Fig. 1.

Compound 5, [α]D −38.0, was isolated as an amorphous powder and its elemental composition was determined to be C13H20O6. In the 13C-NMR spectrum, six typical signals were...
assigned as those of β-glucopyranoside, and the aglycone portion comprised a disubstituted benzene ring, and methyl-ene and carbomethoxy carbons. The four aromatic protons were arranged in sequence and thus the structure of 5 was elucidated to be methyl 2-(2-hydroxyphenyl) acetate β-D-glucopyranoside, as shown in Fig. 1. From the 1-BuOH soluble fraction, a metal salt of 2-(2-hydroxyphenyl) acetic acid β-D-glucopyranoside was isolated, and its metal free form was also expected as a new compound. However, the metal-free form was first isolated by Zhang et al. from the roots of Phyl-lanthus emblica and its methyl ester (5) may be an artifact formed during the isolation procedures.

Compound 6, \([\alpha]_{D}^{26} +3.5\), was isolated as an amorphous powder and its elemental composition was determined to be \(\text{C} \, \text{H}_9 \, \text{O}_6 \, \text{S}\) by HR-ESI-MS in a negative-ion mode. NMR spectra were similar to those of the acyl moiety of 4 with a signal of methoxy group. In Table 1, the 13C-NMR data for 6 are listed in the double prime section for comparison with those of the acyl region of 4. The 4-position was obviously shifted downfield (\(\Delta \delta +10.7\)), and the 3- and 5-positions upfield (\(\Delta \delta -2.4\) and \(-2.6\), respectively). Therefore, the structure of 6 was expected to be the sulfuric acid ester of a methyl 2-(4-hydroxycyclohex-1-en-1-yl) acetate, as shown in Fig. 1. The absolute configuration at the 4-position was also expected to be \(S\) from the co-occurring compounds in G. acuminatum, although the optical rotation sign was opposite to that of \((S)\)-methyl 2-(4-hydroxycyclohex-1-en-1-yl)acetate in MeOH, because sulfation had taken place on the hydroxy group, which is a crucial position for induction of optical activity.

Experimental

General Experimental Procedures Optical rotations were measured on a JASCO P-1030 digital polarimeter. IR and UV spectra were measured on Horiba FT-710 and JASCO V-520 UV/Vis spectrophotometers, respectively. 1H- and 13C-NMR spectra were taken on a JEOL JNM α-400 at 400MHz and 100MHz, respectively, or a Bruker Avance III at 600MHz and 150MHz, respectively, with tetramethylsilane.
as an internal standard. HR-ESI-MS was performed with an Applied Biosystems QSTAR XL NanoSpray™ System.

Silica gel column chromatography (CC) was performed on silica gel 60 (E. Merck, Darmstadt, Germany), and for octadeyl silica (ODS) open CC Cosmosil 75C18-OPN (Nacalai Tesque, Kyoto, Japan) was used. The droplet counter-current chromatograph (DCCC) (DCCC-300 supplemented with extra 200 columns, Tokyo Rikakikai, Tokyo, Japan) was equipped with 500 glass columns (Φ=2 mm, L=40 cm), the lower and upper layers of a solvent mixture of CHCl₃, MeOH–H₂O–1-ProH (9:12:8:2) being used as the stationary and mobile phases, respectively. Five-gram fractions were collected and numbered according to their order of elution with the mobile phase. HPLC (JASCO PU-980) was performed on an ODS column (Inertsil; GL Science, Tokyo, Japan; Φ=6 mm, L=250 mm, 1.6 mL/min), and the eluate was monitored with a UV detector (JASCO UV-975) at 254 nm, and a refractive index monitor (JASCO RI-930).

Plant Material Leaves of *G. acuminatum Müller Argoviensis* (Euphorbiaceae) were collected in Okinawa, Japan, in August 1995, and a voucher specimen was deposited in the Herbarium of the Department of Pharmacognosy, Graduate School of Biomedical Sciences, Hiroshima University (95-GA-Okinawa-0801). The plant was identified by Dr. Taka-kazu Shinzato of Subtropical Field Science Center, Faculty of Agriculture, University of the Ryukyus.

**Extraction and Isolation** The air-dried leaves of *G. acuminatum* (4.76 kg) were extracted three times with MeOH (30 L). The MeOH extract was concentrated to 3.0 L, and then 150 mL of H₂O was added to make a 95% aqueous solution. This solution was washed with 3.0 L of n-hexane and then the methanolic layer was concentrated to a viscous gum (823 g).

The gummy residue was suspended in 3.0 L of H₂O, and then MeOH (3 L) and MeOH–H₂O (1:1, 1 L). The MeOH extract was concentrated to 3.0 L and then 1.6 mL/min), and the eluate was monitored with a UV detector (JASCO UV-975) at 254 nm, and a refractive index monitor (JASCO RI-930).

The residue (2.53 g) in fractions 14–21 was separated by ODS CC (Φ=2 cm, L=40 cm) with a linear gradient [MeOH–H₂O (1:1, 1 L)→MeOH (L=24.5 cm)] with a linear gradient [MeOH–H₂O (1:1, 1 L)→MeOH (1 L)→CHCl₃ (1 L)], 10-g fractions being collected. The residue (2.14 g) was finally purified by HPLC (MeOH–H₂O, 1:4) to yield 46.0 mg of 7 and 31.3 mg of 8 from the peaks at 6.0 min and 7.0 min, respectively. The residue (2.52 g out of 4.52 g in fractions 22–31 was separated by ODS CC (Φ=4.0 cm, L=26.0 cm) with linear gradient [MeOH–H₂O (1:1, 1 L)→MeOH (1 L)→CHCl₃ (1 L)], 10-g fractions being collected. The residue (1.64 g in fractions 6–15 was purified by silica gel CC (Φ=2.0 cm, L=23.0 cm) with a linear gradient from CHCl₃ (1 L) to CHCl₃–MeOH (9:1, 1 L), 15-g fractions were collected, to give 606 mg of 7 in fractions 4–27.

The residue (52.5 g in fractions 30–34 obtained on the first silica gel CC was repeatedly subjected to silica gel (750 g) CC with stepwise increases in the MeOH content in CHCl₃ (CHCl₃ 3 L, CHCl₃–MeOH [19:1 (200 mL), 9:1 (200 mL), 17:3 (200 mL), 4:1 (3 L), 3:1 (3 L), 7:3 (3 L), and 3:2 (3 L)]) and CHCl₃–MeOH–H₂O (60:40:3, 3.12 L), 500-mL fractions being collected. The residue (6.3 mg out of 7.51 g in fractions 19–27 was separated by ODS CC (Φ=2.0 cm, L=22.5 cm) with 10% stepwise increases in the MeOH content in H₂O from 10% MeOH to 100% MeOH (400 mL each), 10-g fractions were collected. The residue (220 mg in fractions 125–138 was separated by silica gel CC (40 g) with stepwise increases in the MeOH content in CHCl₃ (CHCl₃ 200 mL, CHCl₃–MeOH [19:1 (200 mL), 9:1 (200 mL), 17:3, (200 mL), 4:1 (200 mL), 3:1 (200 mL), and 7:3 (200 mL)], 10-g fractions being collected. The residue (32.5 mg) in fractions 111–131 was purified by HPLC [MeOH–H₂O (1:1, 1 L) to give 8.8 mg of 5 from the peak at 12.0 min. The residue (42.3 mg in fractions 132–143 was purified by HPLC [MeOH–H₂O (95:1 L), 2.0 mL/min] to give 4.9 mg of 4 and 16.2 mg of 3 from the peaks at 12.2 min and 13.4 min, respectively. The residue (19.9 mg in fractions 144–159 obtained on the second silica gel CC was separated by HPLC [MeOH–H₂O (9:11, 2.0 mL/min] to give 2.7 mg of 11 from the peak at 35.4 min.

The residue (4.66 g out of 6.78 g in fractions 28–33 obtained on the second silica gel CC was separated by ODS CC (Φ=4.0 cm, L=24.5 cm) with a linear gradient [MeOH–H₂O (1:1, 1 L)→MeOH (1 L)→CHCl₃ (1 L)], 10-g fractions being collected. The residue (200 mg in fractions 27–48 was purified by silica gel CC (35 g) with stepwise increases in the MeOH content in CHCl₃ (CHCl₃ 200 mL, CHCl₃–MeOH [19:1 (200 mL), 9:1 (200 mL), 17:3, (200 mL), 4:1 (200 mL), 3:1 (200 mL), and 7:3 (200 mL)], 10-g fractions being collected, to give 5306 mg of 6 in fractions 177–200. The residue (387 mg in fractions 83–99 was subjected to DCCC to give 11.4 mg of 10 in fractions 13–17 and 210 mg of 1 in fractions 18–24. The residue (5.90 g in fractions 34–42 obtained on the second silica gel CC was purified by ODS CC (Φ=4.0 cm, L=24.5 cm) with a linear gradient [MeOH–H₂O (1:1, 1 L)→MeOH (1 L)→CHCl₃ (1 L)], 10-g fractions were collected. The residue (40.3 mg out of 130 mg in fractions 141–150 was purified by HPLC (MeOH–H₂O, 1:1) to give 24.3 mg of 2 from the peak at 34.0 min.

**Compound 1:** Off-white amorphous powder; [α]₂⁰° = −45.1 (c=0.49, MeOH); IR νmax (film) cm⁻¹: 3323, 2945, 2260, 1699, 1614, 1511, 1293, 1073, 1038; UV λmax (MeOH) nm (log ε): 278 (4.05), 216 (4.37); 1H-NMR (400 MHz; CD₃OD) δ: 7.12 (1H, d, J=8 Hz, H-6), 7.08 (2H, s, H-2" and 6"), 6.69 (1H, d, J=2 Hz, H-3), 6.51 (1H, dd, J=8 Hz, H-5), 4.87 (1H, d,
**Mild Alkaline Hydrolysis of 1** Compound 1 (16mg) was treated in 1 mL of 0.1 M CH₃ONa in MeOH for 3 h at 35°C. The reaction mixture was diluted with 4 mL of H₂O and then neutralized with Amberlite IR-120B (H⁺). The aqueous layer was extracted with CHCl₃ (4 mL) and then evaporated to dryness. The residue was dissolved in MeOH and the precipitate formed was removed by filtration to leave 10mg of 1a (12). An expected compound, methyl gallate, was not present in the CHCl₃ layer. Probably it was decomposed in the basic media.

The NMR spectroscopic data for 1a for pyridine-d₅ were identical with those of ehretioside B (12), and those for CD₃OD with those of 12 isolated from S.adoxoides (Table 1).

**Sugar Analysis** About 1.0mg aliquots of compounds 1-5 were hydrolyzed with 1m HCl (0.1mL) at 90°C for 2h. The reaction mixtures were then each washed with an equal amount of EtOAc and the aqueous layers were analyzed by HPLC, which gave a peak for α-glucose at 15.2min with positive optical rotation signs. [Optical rotation detector: JASCO OR-2090Plus, column: Shodex Asahipak NH2P-50; φ=4.5mm, L=25cm, solvent: CH₃CN-H₂O (4:1), 1mL/min]. Peaks were identified by co-chromatography with authentic α-glucose.

Acknowledgments

The authors are grateful for access to the superconducting NMR instrument (JEOL JNM alpha-400) and an Applied Biosystem QSTAR XL system ESI (Nano Spray)-MS at the Analysis Center of Life Science of the Graduate School of Biomedical Sciences, Hiroshima University. This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Nos. 22590006, 23590130, and 25860078), the Japan Society for the Promotion of Science, and the Ministry of Health, Labour and Welfare. Thanks are also due to the Research Foundation for Pharmaceutical Sciences and the Takeda Science Foundation for the financial support.

Conflict of Interest

The authors declare no conflict of interest.

References