Tannins and Related Compounds. LVIII. 1) Novel Gallotannins Possessing an α-Glucose Core from Nuphar japonicum DC.

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Together with 6-O- and 2,3,4,6-tetra-O-galloylglucoses, two unusual gallotannins possessing an α-glucopyranose core have been isolated from the rhizomes of Nuphar japonicum DC. (Nymphaeaceae), and their structures have been established as 1,2,4-tri-O-galloyl-α-D-glucose (3) and 1,2,3,4,6-penta-O-galloyl-α-D-glucose (6) on the basis of chemical and spectroscopic evidence.

Keywords—Nuphar japonicum; Nymphaeaceae; hydrolyzable tannin; gallotannin; α-glucose; gallic acid; HPLC

The rhizomes of Nuphar japonicum DC. (Nymphaeaceae) have been used, mixed with other crude drugs in most cases, as a tonic and a diuretic, and also to treat bleeding, a blood-stasis syndrome and menstrual disorder. Because of these pharmacological activities, this crude drug is regarded in Japan as important especially for women pre- and post-partum. With regard to the constituents of this drug, many investigators have focused their attention on analysis of alkaloids, and have isolated several novel sesquiterpene alkaloids, such as nupharidine, nupharamine, etc. 3) Our earlier brief report described the presence of hydrolyzable tannins in this drug and showed them to contain an unusual α-glucose core. 4) In this paper, we wish to present details of the isolation and structural elucidation of the component gallotannins.

A preliminary examination of the ethyl acetate-soluble portion of the aqueous acetone extract by normal-phase high-performance liquid chromatography (HPLC) 5) showed the presence of three major gallotannins corresponding to tri-, tetra- and pentagalloylglucoses (Fig. 1). The large-scale extraction of the freeze-dried material with aqueous acetone gave an extract which was chromatographed on Sephadex LH-20 with water containing increasing amounts of ethanol to yield individual fractions containing mono-, tri-, tetra- and pentagalloylglucoses. The mono- and trigallate fractions were each almost homogeneous on reverse-phase HPLC, 5) and were further purified by Sephadex LH-20 and MCI-gel CHP 20P chromatography.

Fig. 1. HPLC of Tannins in Nupharis Rhizoma
raphies to furnish compounds 1 and 3, respectively. On the other hand, the tetra- and pentagallate fractions were shown to be complex mixtures by reverse-phase HPLC. Repeated chromatography of these fractions yielded compounds 2 and 6, and several ellagitannins.

Detailed examinations of the proton nuclear magnetic resonance (1H-NMR) spectra of the mono- and tetragallates (1 and 2) led us to conclude that 1 and 2 are identical with 6-O- and 2,3,4,6-tetra-O-galloylglucoses which were previously isolated from commercial rhubarb and the underground part of Sanguisorba officinalis L. respectively.

The major gallotannin 3 formed colorless fine needles when treated with water. Acid hydrolysis (1 N H2SO4) afforded gallic acid and glucose. The presence of three gallic acid ester groups in 3 was confirmed by observation of three two-proton aromatic singlets at δ 7.10, 7.19 and 7.20 in its 1H-NMR spectrum, and also by field desorption mass spectrometry (FD-MS) (M+ : m/z 752) of the nonamethyl ether (3a) prepared from 3 by methylation with dimethyl sulfate and potassium carbonate in dry acetone.

The 1H-NMR spectrum of 3 showed, in association with the three galloyl peaks, three lowfield signals due to glucose methine protons geminal to the galloyloxy group at δ 6.59 (d, J = 4 Hz), 5.29 (t, J = 9 Hz) and 5.19 (dd, J = 4, 9 Hz). Based on the coupling modes of these signals, the two signals at δ 6.59 and 5.19 were readily assignable to the C(1) and C(2) protons, respectively. The assignment of the remaining triplet was achieved by spin-decoupling techniques. On irradiation at the frequency (δ 5.19) of the C(2) proton, an upfield triplet signal (δ 4.57) changed into a doublet (the C(1) proton signal at δ 6.59 likewise changed to a singlet), thus indicating that this upfield triplet is due to the C(3) proton. Next, irradiation of this C(3) proton caused a change of the triplet at δ 5.29, as well as the C(2) proton signal. These results

<table>
<thead>
<tr>
<th>Glucose moiety</th>
<th>3</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-1 (d, J = 4)</td>
<td>6.59</td>
<td>6.04 (d, J = 8)</td>
<td>6.75 (d, J = 3)</td>
<td>6.35 (d, J = 8)</td>
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<tr>
<td>H-2 (dd, J = 4, 9)</td>
<td>5.19</td>
<td>5.21 (t, J = 9)</td>
<td>5.50 (dd, J = 3, 9)</td>
<td>5.62 (t, J = 9)</td>
</tr>
<tr>
<td>H-3 (t, J = 9)</td>
<td>4.57</td>
<td>4.32 (t, J = 9)</td>
<td>6.21 (t, J = 9)</td>
<td>6.03 (t, J = 9)</td>
</tr>
<tr>
<td>H-4 (t, J = 9)</td>
<td>5.29</td>
<td>5.35 (t, J = 9)</td>
<td>5.80 (t, J = 9)</td>
<td>5.66 (t, J = 9)</td>
</tr>
<tr>
<td>H-5 (dt-like, J = 4, 9)</td>
<td>4.18</td>
<td>3.92 (m)</td>
<td>4.52 (m)</td>
<td>4.60 (m)</td>
</tr>
<tr>
<td>H-6 (2H, d, J = 4)</td>
<td>4.66</td>
<td>3.68 (2H, m)</td>
<td>4.40 (dd, J = 3, 12)</td>
<td>4.50 (2H, m)</td>
</tr>
<tr>
<td>Galloyl moiety</td>
<td>7.10, 7.19, 7.20</td>
<td>7.09 (4H, s)</td>
<td>7.00, 7.01, 7.08, 7.20</td>
<td>6.97, 7.02, 7.06, 7.12</td>
</tr>
<tr>
<td>(each 2H, s)</td>
<td>7.16 (2H, s)</td>
<td>7.28 (each 2H, s)</td>
<td>7.18 (each 2H, s)</td>
<td>7.18 (each 2H, s)</td>
</tr>
</tbody>
</table>

a) Spectra were measured in acetone-d6 at 100 MHz. b) J values are expressed in Hz. c) Compound 5: 1,2,4-tri-O-galloyl-β-D-glucose.

Table 1. 1H-NMR Data for Compounds 3, 5, 6 and 7 (δ Values)
indicated that the gallic acid ester groups are located at the C(1), C(2) and C(4) positions in the glucose moiety.

Since it is clear from the $^1$H-NMR data (Table I) that the glucopyranose ring adopts $^4C_1$ conformation, the coupling constant ($J=4$ Hz) of the above-mentioned anomeric proton signal clearly indicated that the mode of linkage at the anomeric center is $\alpha$ [cf. $\beta$-form (5), C(1)-H, $J=8$ Hz], thus permitting the assignment of the structure for this compound as 1,2,4-tri-O-galloyl-$\alpha$-d-glucose.

In a previous communication, we proposed the structure 4 for this trigalloylglucose, based on the fact that methanolysis of the permethyl ether prepared by two steps of methylation, first with dimethyl sulfate and potassium carbonate in dry acetone and then with silver oxide and methyl iodide in dimethyl formamide (the Kuhn method), afforded methyl 3,4-di-O-methylglucoside. Careful re-examination of these reactions showed that on prolonged heating in the first methylation step, the galloyl group originally located at the glucose C(4) position migrates to both the neighboring C(6) and C(3) positions, yielding 1,2,6- and 1,2,3-tri-O-trimethylgalloyl-$\alpha$-d-glucoses. The production of 1,2,6-tri-O-trimethylgalloyl-$\alpha$-d-glucose had thus led us to assign the wrong structure 4.

The gallotannin 6 failed to crystallize, and was obtained as an off-white amorphous powder. The $^1$H-NMR spectrum showed the presence of five galloyl and well-defined glucose proton signals (Table I). The large coupling constants in the glucose C(2)-C(5) proton signals indicated that the ring adopts the $^4C_1$ conformation, while the small one ($J=3$ Hz) of the C(1) proton signal showed the anomeric center to have $\alpha$-configuration. Comparison of the $^{13}$C-chemical shift ($\delta90.2$) of the C(1) atom in 6 with that ($\delta93.3$) in the $\beta$-anomer (7) also confirmed the mode of the linkage to be $\alpha$. The lowfield shifts of all of the glucose protons, combined with the fact that the infrared (IR) spectrum of the pentadecamethyl ether of 6 displayed no hydroxyl absorption band, indicated that all of the glucose hydroxyls are acylated. On the basis of these chemical and spectroscopic findings, 6 was characterized as...
1,2,3,4,6-penta-O-galloyl-α-D-glucose.

This is the first report on the isolation of gallotannins possessing an α-glucopyranose core from a natural source. It is interesting from the viewpoint of plant physiology that in contrast to ubiquitous plant glucosides which invariably contain a β-linkage, the gallotannins in the rhizomes of *Nuphar japonicum* are based on the configurationally less unstable α-glucose core as long as an acyl group is attached to the anomeric center. We have also isolated the accompanying monomeric, dimeric and trimeric ellagitannins which similarly contain the α-glucose core, and their structures will be reported elsewhere.

**Experimental**

All melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were taken with a JASCO DIP-4 digital polarimeter. IR spectra were obtained with a JASCO DS-301 spectrophotometer. FD-MS were measured with a JEOL JMS-DX 300 instrument using glycerol as the matrix at the accelerating voltage of 2 kV and emitter current of 24–25 mA. 1H- and 13C-NMR spectra were recorded on JEOL PS-100 and JEOL FX-100 spectrometers, respectively, with tetramethylsilane as an internal standard, and chemical shifts are given in δ (ppm). The abbreviations used are as follows: s, singlet; d, doublet; t, triplet; m, multiplet, dd, double doublet. Column chromatography was carried out on Sephadex LH-20 (25—100 μm, Pharmacia Fine Chemical Co., Ltd.) and MCI-gel CHP 20P (75—150 μm, Mitsubishi Chemical Industries, Ltd.). Thin-layer chromatography (TLC) was conducted on precoated Kieselgel 60 F254 plates (0.2 mm thick, Merck) with benzene–ethyl formate–formic acid (1 : 1 or 2 : 1 : 1) for free phenolics and with benzene–acetone (3 : 1 or 5 : 1) for methyl ethers, and on Avicel SF cellulose plates (Funakoshi) with 2% acetic acid. Spots on TLC were detected under ultraviolet (UV) light or by spraying the plates with 1% ferric chloride solution. HPLC was performed on a Hitachi model 638 liquid chromatograph equipped with a Hitachi variable-wavelength spectrophotometric detector. A Nucleosil 50-10 (Macherey-Nagel) column (3 mm i.d. × 300 mm, glass) was used for normal-phase HPLC, and the mobile phase was prepared by dissolving 1.0 g of oxalic acid in 1 l of n-hexane–methanol–tetrahydrofuran (THF)–formic acid (55 : 33 : 11 : 1). A Nucleosil 5 C18 column (4 mm i.d. × 250 mm) was used for reverse-phase HPLC, and the mobile phase was prepared by dissolving 2.0 g of oxalic acid in 1 l of acetonitrile–water (21 : 79).

**HPLC Analysis of Gallotannins**—A finely powdered commercial sample (5 g) of *Nuphar japonicum* was extracted with 50% aqueous acetone (20 ml) at room temperature for 2 h. After removal of insoluble materials by centrifugation, the supernatant was treated with saturated aqueous sodium chloride solution (20 ml) and extracted twice with ethyl acetate (20 ml each). The ethyl acetate-soluble portion was analyzed by HPLC.

**Isolation of Gallotannins**—The freeze-dried rhizomes (4.3 kg) of *N. japonicum*, which were collected near Sapporo in Hokkaido during the autumn, were extracted three times with 80% aqueous acetone. Concentration of the extracts under reduced pressure afforded brown dark precipitates, which were removed by filtration. The filtrate was chromatographed on a Sephadex LH-20 column. Elution with water containing increasing amounts of ethanol yielded fractions consisting of mono-, tri-, tetra- and pentagallates. The mono- and trigallate fractions were separately purified by Sephadex LH-20 chromatography with water and MCI-gel CHP 20P chromatography with a mixture of water and methanol to yield compounds 1 (140 mg) and 3 (990 mg), respectively. The tetragallate fraction was repeatedly chromatographed over Sephadex LH-20 with ethanol and MCI-gel CHP 20P with water–methanol to give compound 2 (252 mg). Similar chromatographic separation of the pentagallate fraction yielded compound 6 (180 mg) and several ellagitannins. The mono- and tetragallates (1 and 2) were shown to be identical with 6-0- and accompanying monomeric, dimeric and trimeric ellagitannins which similarly contain the α-glucose core, and their structures will be reported elsewhere.

**Compound 3**—Colorless fine needles (H2O), mp 208—210 °C, [α]D21 +73.5 ° (c = 1.6, acetone). Anal. Caled for C27H24O18·2H2O·C: 47.86; H, 4.16. Found: C, 47.58; H, 4.38. UV λmax nm (E): 278 (21200). 13C-NMR (acetone-d6): 61.9 (glc. C-6), 70.3, 72.1, 73.7, 74.3 (glc. C-2, C-3, C-4, C-5), 90.4 (glc. C-1), 110.3 (galloyl C-2, C-6), 120.8, 121.0, 121.3 (galloyl C-1), 139.2, 139.5 (galloyl C-5), 145.9, 146.1, 146.2 (galloyl C-3, C-5), 165.0, 166.4 (-COO-).

**Methylation of 3**—a) A mixture of 3 (150 mg), dimethyl sulfate (1.5 ml) and anhydrous potassium carbonate (1.8 g) in dry acetone (30 ml) was heated under reflux for 2.5 h. After removal of the inorganic precipitates by filtration, the filtrate was concentrated to dryness under reduced pressure. The residue was chromatographed over silica gel, and elution with benzene–acetone (3 : 1) afforded the nonamethyl ether (3a) as long as an acyl group is attached to the anomeric center. We have also isolated the accompanying monomeric, dimeric and trimeric ellagitannins which similarly contain the α-glucose core, and their structures will be reported elsewhere.
(3a) (121 mg), 1,2,3-tri-O-trimethylgalloyl-α-D-glucose (32 mg) as a white amorphous powder, $[\alpha]_D^{20} + 151.8^\circ$ ($c = 0.85$, CHCl$_3$). $^1$H-NMR (CDCl$_3$): 3.63—3.96 (OCH$_3$), 5.47 (1H, dd, $J = 4$, 9 Hz, glc. 2-H), 5.91 (1H, t-like, $J = 9$ Hz, glc. 3-H), 6.68 (1H, d, $J = 4$ Hz, glc. 1-H), 7.07, 7.22, 7.36 (each 2H, s, galloyl H). FD-MS $m/z$: 752 (M$^+$). And 1,2,6-tri-O-trimethylgalloyl-α-D-glucose (33 mg) as a white amorphous powder, $[\alpha]_D^{20} + 62.0^\circ$ ($c = 0.8$, CHCl$_3$). $^1$H-NMR (CDCl$_3$): 3.68-3.96 (OCH$_3$), 4.42 (1H, dd, $J = 2$, 12 Hz, glc. 6-H), 4.94 (1H, dd, $J = 3$, 12 Hz, glc. 6-H), 5.26 (1H, dd, $J = 4$, 9 Hz, glc. 2-H), 6.60 (1H, d, $J = 4$ Hz, glc. 1-H), 7.12, 7.28, 7.29 (each 2H, s, galloyl H). FD-MS $m/z$: 752.

Acid Hydrolysis of 3 —— A solution of 3 (10 mg) in 1 N H$_2$SO$_4$ (1 ml) was heated at 90°C for 2 h. After cooling, the reaction mixture was extracted with ethyl acetate. TLC examination of the ethyl acetate layer showed the presence of gallic acid. The aqueous layer was neutralized with barium carbonate, and analyzed by cellulose TLC [solvent, n-BuOH–pyridine–H$_2$O (6:2:1); detection, aniline–hydrogen-phthalate reagent]. A spot corresponding to glucose was detected.

Compound 6 —— An off-white amorphous powder, $[\alpha]_D^{20} + 134.5^\circ$ ($c = 0.5$, acetone). Anal Calcd for $C_{41}H_{32}O_{26}$·5H$_2$O: C, 47.78; H, 4.11. Found: C, 47.88; H, 4.11. $^{13}$C-NMR (acetone-d$_6$): 62.7 (glc. C-6), 68.9 (glc. C-4), 71.0, 71.3 (x 2) (glc. C-2, C-3, C-5), 90.2 (glc. C-1), 110.1 (galloyl C-2, C-6), 119.5, 119.8, 120.7 (galloyl C-1), 139.2, 139.6, 139.9 (galloyl C-5), 145.9, 146.2 (galloyl C-3, C-5), 165.4, 166.2, 166.3, 166.9 (x 2) (−COO−).

Methylation of 6 —— A solution of 6 (25 mg) in methanol was treated five times with ethereal diazomethane. The reaction product was purified by silica gel chromatography with benzene–acetone (9:1) to yield the pentadecamethyl ether (6a) as a white amorphous powder (20 mg), $[\alpha]_D^{20} + 67.3^\circ$ ($c = 0.18$, acetone). IR ν$_{\text{max}}$ cm$^{-1}$: 1725 (−COO−), 1585, 1500 (C = O), no OH band. Electron impact mass spectrometry (EI-MS) $m/z$: 1150 (M$^+$), 406, 195.

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References and Notes
2) Present address: Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan.
9) The gallotannin 5 has been isolated from the leaves of Cercidiphyllum japonicum Sieb. et Zucc., and details of its isolation will be reported elsewhere.