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GALLOTANNINS OF THE FRESHWATER GREEN ALGA *SPIROGYRA* SP.*

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Key Word Index—*Spirogyra* sp.; Zygnemataceae; HPLC; ^{1}H NMR; ^{13}C NMR; gallotannins; 3-*O*-digalloyl-1,2,6-tri-*O*-galloyl-β-D-glucose.

Abstract—The green alga *Spirogyra* sp. accumulates tetra- through undecagalloylglucosyl gallotannins. The hexa- through undecagalloylglucoses are predominantly based on 1,2,3,4,6-penta-*O*-galloylglucose, whereas the major pentagalloylglucose is 3-*O*-digalloyl-1,2,6-tri-*O*-galloyl-β-D-glucose.

INTRODUCTION

Gallotannins or related galloyl esters of β-D-glucose are known from some 22 families of higher plants [1], but are not known to occur in ferns, lycopods, liverworts, horsetails or mosses. Surprisingly, however, gallotannins have been reported [2–6] to constitute 2.0–6.4% (dry wt) of the freshwater green alga *Spirogyra arcta* (Zygnematales). The *Spirogyra* tannin was found [6] to contain gallic acid and D-glucose in a 5.3:1 molar ratio, with one of the five hydroxyls of the glucose remaining nonesterified and the galloyl depside(s) *meta*-linked.

We have now reinvestigated the tannins [7] of a *Spirogyra* sp. using HPLC, ^{1}H and ^{13}C NMR methods previously applied to gallotannins from *Rhus semialata* [8], *Quercus infectoria* [9] and *Paeonia lactiflora* var. *trichocarpa* [10].

RESULTS

Compounds which react with $FeCl_3$–$K_3Fe(CN)_6$ and which precipitate casein from solution were extracted from frozen *Spirogyra* sp. into aqueous acetone, and were partitioned into ethyl acetate from acidified aqueous solution. ^{13}C NMR of this crude tannin fraction demonstrated the presence of β-glucose galloyl esters and galloyl depsides (see Experimental). A trace of free gallic acid was indicated by HPLC (Experimental).

Normal-phase HPLC separation of the crude tannin fraction yielded well-resolved signals for penta- through decagalloylglucoses, and a trace of presumed undecagalloylglucose(s) (Table 1). Reversed-phase HPLC separated isomers [8–10]: one identifiable tetra-, two or three penta-, three hexa-, five or six hepta-, four or five octa-, and four nonagalloylglucoses. Nine of these were identified by comparing their retention times (Table 2) with those of authentic compounds from Chinese gallotannin [8]. The three major isomers (R_ts 5.74, 13.13 and 18.84 min at 24% MeCN) were not identical with any of the available reference compounds; the latter two are presumably octa- and nonagalloylglucoses.

The major component at 5.74 min (above) was recovered in 0.8% yield from the crude tannin fraction following three HPLC fractionations. Comparison of its ^{13}C NMR spectrum with that of authentic 1,2,3,6-tetra-*O*-galloyl-β-D-glucose (Table 3) located the digalloyl group at C-3, and indicated both *m*- and, to a smaller extent, *p*-depsidic linkages. Methanolysis of this component yielded 1,2,3,6-tetra-*O*-galloyl-glucose and methyl gallate, which were identified by reversed-phase HPLC. Methanolysis of the crude tannin fraction gave primarily 1,2,3,4,6-penta-*O*-galloylglucose and methyl gallate, plus a smaller quantity of 1,2,3,6-tetra-*O*-galloylglucose corresponding to *ca* 16% of the initial gallotannin molecules.

DISCUSSION

These results confirm earlier reports [2–6] that algae of the genus *Spirogyra* may accumulate several percent (dry

Table 1. *Spirogyra* sp. gallotannins resolved by normal-phase HPLC

Compounds	R_t (min)*	% Fresh wt of alga†
Pentagalloylglucoses	5.43	0.008
Hexagalloylglucoses	6.52	0.005
Heptagalloylglucoses	7.81	0.008
Octagalloylglucoses	9.26	0.014
Nonagalloylglucoses	11.02	0.017
Decagalloylglucoses	13.26	0.007
Undecagalloylglucoses (?)	~15.9	*tr*
		0.060

*Nucleosil 50-5, *n*-hexane–MeOH–THF–HCOOH, 55:33:11:1 (oxalic acid 1 g·l^{-1}), 1.8 ml·min^{-1}; 280 nm.
†Normalized from UV 280 nm.

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Table 2. *Spirogyra* gallotannins identified by comparison with reference compounds during reversed-phase HPLC

Compound	% MeCN	R_t (min)
1,2,3,6-Tetra-*O*-galloylglucose	20	5.60
1,2,3,4,5-Penta-*O*-galloylglucose	20	9.46
3-*O*-Digalloyl-1,2,4,6-tetra-*O*-galloylglucose	24	6.84
2-*O*-Digalloyl-1,3,4,6-tetra-*O*-galloylglucose	24	7.49
4-*O*-Digalloyl-1,2,3,6-tetra-*O*-galloylglucose	24	8.11
2,3-Di-*O*-digalloyl-1,4,6-tri-*O*-galloylglucose	24	9.11
3-*O*-Trigalloyl-1,2,4,6-tetra-*O*-galloylglucose	24	9.74
3,4-Di-*O*-digalloyl-1,2,6-tri-*O*-galloylglucose	24	10.29
2,4-Di-*O*-digalloyl-1,3,6-tri-*O*-galloylglucose	24	10.78

*Nucleosil $5C_{18}$, 20% or 24% MeCN in H_2O (oxalic acid 1 g·l^{-1}), 1.2 ml·min^{-1}; 280 nm.

Table 3. ^{13}C NMR chemical shifts of glucosyl carbons in the major pentagalloylglucose isomer of *Spirogyra* sp.

	C-1	C-2	C-3	C-4	C-5	C-6
Spirogyra pentagalloylglucose	93.5	71.9	*meta* 76.4 *para* 76.5	69.2	76.1	63.7
1,2,3,6-Tetra-*O*-galloyl-β-D-glucose	93.4	71.7	75.9	69.3	76.0	63.6

Me_2CO-d_6, δ (ppm) downfield from TMS.

wt) of gallotannins which contain both (mono)galloyl and *m*-digalloyl groups esterified to (typically) four of the five D-glucosyl hydroxyls. Our studies indicate an average of 7.86 galloyl units per glucose (M_r 1375) in contrast to the earlier estimate of 5.3 per glucose. The major pentagalloylglucose is 3-*O*-digalloyl-1,2,6-tri-*O*-galloyl-β-D-glucose; 1,2,3,4,6-penta-*O*-galloylglucose is only a minor constituent. The presence of ^{1}H and ^{13}C NMR signals for both *m*- and *p*-linked terminal depsidic galloyl groups probably indicates bond migration [8].

Because the *ca* 16% 1,2,3,6-tetra-*O*-galloylglucose released upon methanolysis of the crude tannin fraction is almost entirely accounted for by 3-*O*-digalloyl-1,2,6-tri-*O*-galloylglucose (*ca* 13%), most of the hexa- through decagalloylglucoses must be based on a core of 1,2,3,4,6-penta-*O*-galloylglucose. The very low level of free 1,2,3,4,6-penta-*O*-galloylglucose in extracts from *Spirogyra* sp. indicates that the extraction and chromatographic conditions did not degrade naturally occurring depside bonds.

Using cytological criteria, Pickett-Heaps [11] divided the green algae into two groups, and suggested that the 'phragmoplast' group (including the Zygnematales) is more directly related to higher plants than is the 'phycoplast' group. The distribution of glycolate oxidase and dehydrogenase activities among green algae [12] provided the first biochemical support for Pickett-Heaps's proposal. To the extent that gallotannins are absent from 'phycoplast' green algae, their occurrence in *Spirogyra* spp. lends additional support to a common ancestry of higher plants and 'phragmoplast' green algae.

EXPERIMENTAL

Spirogyra sp. was collected 24 June (600 g), 26 July (1100 g) and 4 August 1983 (183 g fr. wt) from a shallow pond at Mt. St. Vincent University, Halifax, N.S., washed briefly with tap water, and separated from occasional admixed *Rhizoclonium* sp. before being frozen for later use (voucher specimen: NRCC 10356). A crude tannin fraction (0.15% fr. wt, *ca* 1.5% dry wt) was prepared by extraction with 85% Me_2CO under reflux, evaporation *in vacuo*, redissolution in water (filter GF/C, Whatman), acidification (pH ≈ 2, HCl) and partitioning into EtOAc.

Analytical HPLC was performed (except as noted) on a Hitachi 638 chromatograph with detection at 280 nm; for other details see Tables 1 and 2 and [8]. Preparative HPLC was done using a Milton-Roy pump, Pharmacia UV-2 monitor, and 250 × 8 mm columns of Fuji-gel RQ-3 and RQ-2 (both: MeCN–H_2O–HOAc) and Nucleosil $10C_{18}$ (Macherey–Nagel) (MeCN–H_2O 1:4, with oxalic acid 1 g·l^{-1}). ^{1}H NMR spectra were recorded at 200 MHz (JMN FX-200) in Me_2CO-d_6 with TMS as reference. ^{13}C NMR spectra were recorded at 20.1 (Varian FT-80) and 50.1 MHz (JMN FX-200) in Me_2CO-d_6 with TMS as reference. Optical rotation was measured using a Perkin–Elmer 243 digital polarimeter. Methanolysis was carried out in 0.05 M acetate buffer (pH 5.5)–MeOH (1:2 v/v) for 24 hr at room temp [10].

Crude tannin fraction. ^{13}C NMR (Me_2CO-d_6): δ_c 164.2–166.5 (8 signals resolved: galloyl ester), 151.4 and 151.1 (C-3,5 of proximal unit of *p*-digalloyl groups), 143.8–147.0 (9 signals: galloyl C-3,5; C-3,5 of proximal unit of *m*-digalloyl), 139.0–139.8 (5 signals: galloyl C-4; C-4 of proximal unit of *m*-digalloyl), *ca* 132.5 (1–2 signals: C-4 of proximal unit of *p*-digalloyl), *ca* 128.0

(1–2 signals: C-1 of proximal unit of *p*-digalloyl), 119.6–121.1 (6–7 signals: galloyl C-1; C-1 of proximal unit of *m*-digalloyl), 118.1 and 117.5 (C-2 of proximal unit of *m*-digalloyl), 114.9 (C-6 of proximal unit of *m*-digalloyl), 110.1–110.6 (2–3 signals resolved: galloyl C-2,6; C-2,6 of proximal unit of *p*-digalloyl), 93.1 (*β*-glucose C-1), *ca* 75.8 (broad, minor), 73.8 (*β*-glucose C-3, C-5), 72.0 (*β*-glucose C-2), 69.2 (*β*-glucose C-4), *ca* 62.8 (broad; *β*-glucose C-6). Signals from *p*-depsidically linked galloyl units were in every case of minor abundance.

Free gallic acid was detected in extracts by HPLC (Gilson/Apple system) using a Brownlee RP-8 column (18–23% MeCN in 2% aq. HCOOH) and UV 280 nm. 3-*O*-Digalloyl-1,2,6-tri-*O*-galloyl-*β*-D-glucose (recovery after three preparative-HPLC purifications: 12 mg) was obtained as a white amorphous solid, $[\alpha]_D^{31} = +60.0°$ (*c* 0.53, Me_2CO). ^1H NMR [3-*O*-(*m*-digalloyl)]: *δ* 7.40 and 7.33 (each 1H, *d*, *J* = 2.0 Hz; H-2 and H-6 of proximal unit of 3-digalloyl), 7.25 (2H, *s*, H-2,6 of terminal unit of 3-digalloyl), 7.17 (2H, *s*, H-2,6 of 2-galloyl), 7.10 (2H, *s*, H-2,6 of 1-galloyl), 7.015 (2H, *s*, H-2,6 of 6-galloyl), 6.19 (1H, *d*, *J* = 7.8 Hz, glucosyl H-1); 3-*O*-(*p*-digalloyl): *δ* 7.24 (2H, *s*, H-2,6 of terminal unit of 3-digalloyl), 7.18 (2H, *s*, H-2,6 of 2-galloyl), 7.15 (2H, *s*, H-2,6 of proximal unit of 3-digalloyl), 7.10 (2H, *s*, H-2,6 of 1-galloyl), 7.020 (2H, *s*, H-2,6 of 6-galloyl), 6.25 (1H, *d*, *J* ≈ 7 Hz); both isomers: *ca* 5.71 (1H, *m*, H-3s), 5.49 (1H, *dd*, spacings *ca* 8 Hz, H-2s), 4.59 (1H, *br s*, H-6s), 4.17 and 4.15 (each 1H, *br s*; H-4s and H-5s). ^{13}C NMR: glucosyl carbons, see Table 3; other signals *δ* 166.7, 165.85, 165.79, 165.06, 165.00, 151.2, 147.1, 146.1–145.9, 144.2, 139.8, 139.63, 139.25, 139.0, 132.6, 128.5, 121.6, 120.88, 120.77, 120.68, 120.1, 117.7, 114.7, 110.75, 110.66, 110.34, 110.17, 110.05 (assignments as above for crude tannin fraction).

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