SAPONINS FROM ROOT CULTURES OF *PHYTOLACCA ACINOSA*

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Abstract—Twelve triterpene saponins were detected (by TLC) in root cultures of *Phytolacca acinosa*. Seven were isolated in 0.5–1 g quantities and identified by TLC, ^{13}C, ^{1}H NMR and mass spectra. Among these, esculentoside B, S, A and H, and phytolaccoside F have been detected previously in roots or leaves of *P. acinosa* and/or *P. americana*: phytolaccoside F (3-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranosyl-(1 → 2)-β-D-xylopyranosyl-phytolaccagenic acid) has been identified for the first time in *P. acinosa*. Esculentoside L₁ (3-*O*-β-D-glucopyranosyl-(1 → 2)-β-D-xylopyranosyl-28-*O*-β-D-glucopyranosyl-phytolaccagenic acid) is an isomer of the earlier described esculentoside L, having an altered linkage of the sugar moiety. Esculentoside R has been isolated for the first time. It has been identified as a bidesmoside of phytolaccoside F with an additional glucose, ester glycosidic linked with C-28.

INTRODUCTION

Over a dozen triterpene saponins have been isolated and identified from roots of *Phytolacca* species. Roots from *P. acinosa* Roxb. are an especially rich source of triterpene saponins as documented by Yi and coworkers [1–4] and others [6–8]. Although some of these compounds have been found in large amounts in the roots of intact plants, their further isolation and identification would be facilitated by the use of an *in vitro* culture system, which would allow plant material to be obtained in large amounts in a seasonally independent way under constant conditions and free from any interferring pests and pesticides.

Cell cultures of *P. americana* [9], *P. acinosa* and other *Phytolacca* species [10, 11] have been described and the existence of saponins in them has been reported [10]. Hairy root cultures [e.g. 12–14] and conventional root cultures [e.g. 15–17] have been successfully employed for the analysis and production of many different plant secondary metabolites but so far no reports exist on triterpene saponin formation by either kind of root culture. (The term 'conventional root culture' is used here for all types of untransformed root cultures including excised or adventitious root cultures and is henceforth referred to as 'root culture'.) The formation of monoterpenoids by root cultures of *Mentha* ssp. has recently been published [17]. Hairy root cultures of *P. acinosa* were established in the laboratory of one of us (W.S.) [11], but were not analysed phytochemically. Here we report on the isolation and identification of the triterpene saponins occurring in root cultures of *P. acinosa*.

RESULTS AND DISCUSSION

Root cultures

Successful initiation and continuous culturing of root cultures not only depends on the species and plant source used, but also on the culture conditions employed [18]. In the case of *P. acinosa*, root initiation from leaf explants was possible on hormone-free culture media of White [19], and Linsmaier and Skoog [20]. Once initiated, most of the isolated root clones were able to grow continuously on either media without addition of phytohormones. The selected root clone (c1) consisted mainly of a white, root-hair free, 1–2 mm thick main axis root and some lateral roots. Growth took place predominantly on the apices of the main axis root and the lateral roots. Root clone c1 grew in liquid LS-H medium as judged by dry weight determinations, with a lag phase of 10 days, followed by an active growth phase of 25 days with a mean doubling time of 3 days, and then proceeding to a stationary phase after a total of 35 days when growth slowed down and the roots turned increasingly brown. No sign of degeneration was observed even after 10 culture passages at least with respect to morphology and growth performance. For the phytochemical analysis, root clone c1 was cultured in 295 shake flasks with a total culture volume of 14.75 l of LS-H medium and harvested in the late phase of active growth after 28 days. The mass of harvested roots, 1.20 kg fr. wt (235 g dry wt), indicated that the carbon source (sucrose) had been converted to root material with a remarkably high efficiency (yield coefficient $Yx/s = 0.53$).

Isolation and identification of triterpene saponins

The freeze-dried and pulverized root cultures were extracted with methanol. After evaporation of the solvent, the remaining residue was taken up in water and extracted exhaustively with *n*-butanol. The butanolic extract was subjected to flash silica gel chromatography and subsequently to flash RP 18 silica gel chromatography and/or preparative TLC.

From acid hydrolysis of the pure glycosides, it was determined that they were all based on the two genins phytolaccagenin and phytolaccagenic acid. The number of sugars could be determined from the ^{13}C NMR and mass spectral data, respectively. Interestingly, the sugar units attached to the C-3 hydroxyl group of phytolaccagenin have a 1 → 4 linkage, whereas those bound to the C-3 hydroxyl group of phytolaccagenic acid are 1 → 2 linked.

Some of the glycosides, namely esculentoside A (3), B (1), H (6) and L (5) had already been isolated from the roots of *P. acinosa* [2–4], and esculentoside A, B and H had been found in *P. americana* [5–7]. Compounds 3, 1 and 6 were compared by TLC with authentic samples obtained from Y.-H. Yi, and found to be identical. Our spectral data for 3, 1 and 6 were in full agreement with

those described in the literature. The other known compounds were phytolaccoside F (4) from *P. americana* [21] and esculentoside S (2) [5]. Esculentoside S was one of the main components in the root cultures, although it could be isolated from the leaves in small quantities. All the spectral and chromatographic data of 2 were identical with those of authentic material [5].

The ^{13}C NMR and $[\alpha]_D$ data of phytolaccoside F (4) were identical with those reported for the same compound by Kang and Woo [21]. This is the first time that this compound has been found in *P. acinosa*. The sugar chain in all previously isolated glycosides from *P. acinosa* consists of glucose and xylose. Phytolaccoside F (4) (3-O-[α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranosyl-(1 → 2)-β-D-xylopyranosyl]-phytolaccagenic acid) is the first rhamnose-containing glycoside observed in *P. acinosa*.

Acid hydrolysis of 5 yielded phytolaccagenic acid, xylose and glucose. The ^{13}C NMR spectrum (Table 2) showed the presence of three sugars, one of them a glycosidic linked ester. This led to the conclusion that 5 could be esculentoside L as identified by Yi [3]. However, a comparison of the ^{13}C NMR data revealed a difference in the xylose linkage. The signal at $\delta 83.9$ for C-2 in our spectrum was not present in Yi's spectrum of esculento-
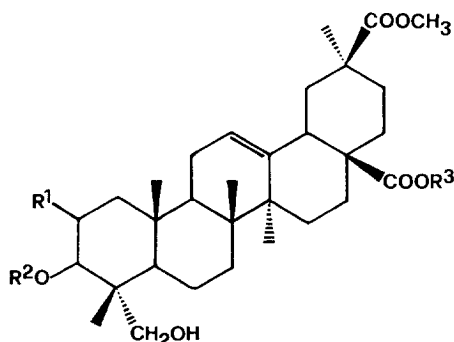
	R^1	R^2	R^3	substance
1	OH	xyl	H	esculentoside B phytolaccoside B phytolaccasaponin G
2	OH	xyl	glc	esculentoside S
3	OH	xyl $\xrightarrow{4}$ glc	H	esculentoside A phytolaccoside A phytolaccasaponin E
4	H	xyl $\xrightarrow{2}$ glc $\xrightarrow{2}$ rha	H	phytolaccoside F
5	H	xyl $\xrightarrow{2}$ glc	glc	esculentoside L$_1$
6	OH	xyl $\xrightarrow{4}$ glc	glc	esculentoside H
7	H	xyl $\xrightarrow{2}$ glc $\xrightarrow{2}$ rha	glc	esculentoside R
8	OH	H	H	phytolaccagenin
9	H	H	H	phytolaccagenine acid

glc: β-D-glucopyranosyl.
rha: α-L-rhamnopyranosyl.
xyl: β-D-xylopyranosyl.

Table 1. ^{1}H NMR spectral data of 1–9 (200 MHz, δ in pyridine-d_5)

	Me s	Me s	Me s	Me s	Me s	Me s	C-12 m	C-1 d Xyl or Glc	C-1 d Xyl or Glc	C-1 s Rha	C-1 d Ester	C-6 d Rha
1	1.08	1.22	1.29	1.37	1.59	3.66	5.63	5.10 (6.9)	—	—	—	—
2	1.19	1.19	1.24	1.38	1.62	3.61	5.59	5.09 (7.1)	—	—	6.34 (7.6)	—
3	1.08	1.23	1.29	1.37	1.59	3.67	5.65	5.03 (7.7)	5.09 (7.8)	—	—	—
4	0.88	1.00	1.02	1.22	1.22	3.65	5.61	5.16 (6.0)	5.87 (6.6)	6.34	—	1.84 (6.1)
5	0.96	1.11	1.14	1.19	1.19	3.61	5.58	5.06 (6.3)	5.42 (7.3)	—	6.35 (7.6)	—
6	1.20	1.20	1.25	1.37	1.62	3.61	5.59	5.03 (7.5)	5.09 (7.8)	—	6.35 (7.3)	—
7	0.92	1.03	1.12	1.19	1.19	3.60	5.57	5.16 (6.0)	5.88 (6.4)	6.35	6.33 (6.2)	1.83 (6.0)
8	1.11	1.22	1.28	1.38	1.61	3.66	5.66	—	—	—	—	—
9	0.97	1.05	1.06	1.22	1.25	3.66	5.64	—	—	—	—	—

Coupling constants (J in Hz) in parentheses.

side L. A signal at δ79.3 for C-4 of xylose indicated glycosidation at this C atom. However, our compound gave a signal at δ71.0 owing to a free hydroxyl group in this position. A downfield shift of 9.7 ppm resulting in δ83.9 was attributed to glycosidation at C-2 of xylose. The ^{1}H NMR spectrum in pyridine-d_5 (Table 1) contained five tertiary methyl signals at δ0.96 (s, 3H), 1.11 (s, 3H), 1.14 (s, 3H) and 1.19 (s, 6H) and a singlet for one O-methyl at δ3.61, a multiple^t for the olefinic proton of C-12 at δ5.58 and three anomeric protons at δ5.06 (d, J = 6.3 Hz, 1H), 5.42 (d, J = 7.3 Hz, 1H) and 6.35 (d, J = 7.6 Hz, 1H). These coupling constants established β-configuration for the xylose and glucose moieties. In the ^{1}H NMR spectrum the anomeric proton signals of esculentoside L appear at δ4.94, 5.02 and 6.27 [3]. We conclude that the present molecule is the same as esculentoside L with the exception that the sugar chain at the C-3 hydroxyl group has a 1 → 2 linkage instead of 1 → 4. We propose for 5 the name esculentoside L_1 (3-O-[β-D-glucopyranosyl-(1 → 2)-β-D-xylopyranosyl-28-β-D-glucopyranosyl]-phytolaccagenic acid).

The most polar of the seven isolated substances, esculentoside R, a bidesmosidic glycoside with the molecular formula $C_{54}H_{86}O_{24}$ (= 1118), is new. Acidic hydrolysis gave phytolaccagenic acid as the aglycone and glucose, xylose and rhamnose as the sugar moieties. From the ^{13}C NMR spectrum (Table 2) of 7, it was clear that esculentoside R contained four sugar residues. One of them with the anomeric C at δ95.8 was typical for an ester glycosidic linked sugar. This agreed with the doublet for the anomeric proton at δ6.33 in the ^{1}H NMR spectrum. The other sugars showed the same shifts for all the ^{13}C atoms as those described by Kang and Woo [21] for phytolaccoside F (4). The ^{1}H NMR spectrum in pyridine-d_5 showed five tertiary methyl groups at δ0.92 (s, 3H), 1.03 (s, 3H), 1.12 (s, 3H) and 1.19 (s, 6H), a secondary

methyl signal at δ1.83 (d, J = 6.0 Hz, 3H), a singlet of one O-methyl at δ3.60, the olefinic H at C-12, δ5.57 (m, 1H) and four anomeric protons at δ5.16 (d, J = 6.0 Hz, 1H), 5.88 (d, J = 6.4 Hz, 1H) and 6.33 (d, J = 6.2 Hz, 1H) indicating β-configurations for xylose and glucose and δ6.35 (br s, 1H) indicating the α-configuration for rhamnose (Table 1). The terminal glucose at C-28 could be removed with β-glucosidase and the resulting glycoside was identical with phytolaccoside F (4). Compound 7 could not be methylated with diazomethane, consequently, there was no free carboxyl group at C-28. The mass spectrum (FAB neg.) exhibited $[M]^+$ at m/z 1118 (2) together with m/z 1117 $[M - 1]^-$ (5). The main fragment ions were: m/z 956 $[M - Glc]^-$ (22), 955 $[(M - 1) - Glc]^-$ (12), 809 $[(M - 1) - Glc - Rha]^-$ (18), 648 $[M - 2Glc - Rha]^-$ (9), 647 $[(M - 1) - 2Glc - Rha]^-$ (25), 516 $[M - 2Glc - Rha - Xyl]^-$ (6) (phytolaccagenic acid) and 515 $[(M - 1) - 2Glc - Rha - Xyl]^-$ (14). Acetylation of 7 yielded a product with 13 acetoxy groups, as expected. These observations led to the conclusion that esculentoside R (7) was a bidesmoside of phytolaccoside F (4) bearing an ester glycosidic linked glucose at C-28.

The root cultures of *P. acinosa* accumulated triterpene saponins in amounts comparable to those found in organs of intact plants, without any further optimization of the culture conditions or selection of high producing root clones. The total content of triterpene saponins, defined by their extractability with n-butanol, was 6.6% of the dry mass as calculated from the product yields in the Experimental section, whereas the seven isolated and identified triterpene saponins constituted 3.7% of the dry mass. Production per volume of culture medium amounted to $1.5 \, g \, l^{-1}$ for all triterpene saponins, and to $0.12 \, g \, l^{-1}$ for the most prominent compound, esculentoside B, excluding compounds released to the culture medium.

A. Strauss *et al.*

Table 2. ^{13}C NMR spectral data of **5**, **7** and **9** (50.3 MHz, δ in pyridine d_5)

C	5	7	9
1	38.9	38.8	38.8
2	26.1	26.0	27.7
3	82.2	83.4	73.5
4	43.2	43.5	42.9
5	48.2	48.2	48.7
6	18.3	18.3	18.6
7	32.9	32.9	33.0
8	39.9	39.9	39.8
9	48.0	48.2	48.2
10	36.9	36.9	37.2
11	23.6	23.8	23.9
12	123.7	123.8	123.7
13	143.8	143.8	144.5
14	42.1	42.1	42.1
15	28.3	28.3	28.4
16	23.8	23.6	23.9
17	46.5	46.5	46.2
18	44.0	43.2	43.4
19	42.4	42.4	42.1
20	43.6	44.0	44.2
21	30.0	30.6	30.8
22	34.0	34.0	34.5
23	64.8	65.4	68.0
24	13.5	13.4	13.1
25	16.2	16.1	16.2
26	17.5	17.5	17.5
27	26.1	26.0	26.1
28	176.1	176.9	179.8
29	28.2	28.3	28.4
30	176.9	177.0	177.2
31(OMe)	51.7	51.7	51.7
C-3-Xyl 1	106.1	104.6	
2	83.9	83.4	
3	78.1	77.8	
4	71.0	71.0	
5	66.7	66.6	
Glc 1	104.7	102.6^a	
2	76.8	79.9	
3	78.9	78.9	
4	71.5	72.0	
5	78.2	78.9	
6	62.6	62.7	
Rha 1		102.5^a	
2		72.2^b	
3		72.6^b	
4		74.2	
5		70.0	
6		18.9	
C-28-Glc 1	95.8	95.8	
2	74.2	74.2	
3	79.3	79.4	
4	70.8	71.0	
5	78.2	78.9	
6	61.9	61.9	

^{a,b}Assignment may be reversed.

The spectrum of triterpene saponins detected in the root cultures of *P. acinosa* was somewhat different to that found earlier [1–3, 7, 8, 21] in roots or leaves [5] of *P. acinosa* plants.

Out of the seven major triterpene saponins present in root cultures, three (esculentoside A, B and H) are also common to plant roots [1–3], one (esculentoside L_1) is a positional isomer of esculentoside L isolated earlier from roots of *P. acinosa* [3], one (esculentoside S) has been described before only in *P. acinosa* leaves [5], one (phytolaccoside F) is reported to occur only in *P. americana* [7] and one (esculentoside R) represents a new triterpene saponin.

EXPERIMENTAL

NMR: ^{1}H and ^{13}C, 200 and 50.3 MHz, respectively, in pyridine-d_5 with TMS as int. standard; TLC: silica gel GUV_{254}, 0.5 mm thickness for prep. TLC; CC: silica gel and RP 18 silica gel for flash chromatography; mps: uncorr.

Root culture initiation. Fresh, young leaves from *P. acinosa* plants, grown in the Botanical Garden in Brueglingen near Basel, were washed in tap water, dipped for 5 sec in EtOH, surface sterilized by shaking them gently for 5 min in a soln of 3% (w/v) NaOCl + 0.05% (w/v) Tween 80 and subsequently washed twice in H_2O. The underside surface of the sterilized leaves were cut with a scalpel several times across the midrib and placed upside-down on plates containing phytohormone-free Linsmaier and Skoog medium (LS-H) [20] solidified with $8 \, g\,l^{-1}$ agar. The plates were incubated at room temp. at 1000 lux and were regularly checked for root formation. Roots emerging at the midrib after 4–6 weeks were cut off after 8 weeks, transferred to fresh plates with LS-H medium and incubated at room temp. in the dark. After 21 days, ca 1 cm long pieces of the continuously growing root clones were cut off and suspended in 20 ml liquid LS-H medium in 100 ml conical flasks and incubated for 14 days on a rotary shaker shaking at 110 rpm at 26° in dim light. The best growing root clone (c1) was selected from among 23 clones for further analysis.

Root cultures. Stock cultures of root clone c1 were maintained by subculturing at intervals of 14 days by inoculation of 50 ml LS-H medium in 200 ml conical flasks with 4 root pieces of ca 1 cm length from the previous culture passage and incubation on a rotary shaker at 100 rpm and 26° in dim light. For production of roots for phytochemical analysis, the root cultures were inoculated and incubated as mentioned above but the incubation time before harvest was extended to 4 weeks. Roots were harvested by filtration, washed with H_2O frozen to −20°, lyophilized and stored at −18° in an air-tight container.

Extraction of saponins. Freeze-dried and pulverized root culture material (140 g) was extracted (×3) at room temp. for 4.5 hr with 2 l MeOH each time. The solvent was removed *in vacuo* yielding 41 g of extract. This residue was suspended in H_2O and the glycosides were exhaustively extracted with *n*-BuOH satd with H_2O. Evapn of the solvent gave 9.2 g of residue. It was fraction-

ated on a column with flash silica gel under N_2 pressure $(0.5 \, kg \, cm^{-2})$ with $CHCl_3$–MeOH–H_2O (40:10:1) and (28:12:1) for the more polar compounds, yielding as crude products: 1.103 g 1, 2.276 g of a mixt. of 2 and 3, 478 mg 4, 351 mg 5, 555 mg 6 and 479 mg 7. Further purification was carried out using flash silica gel CC, flash RP 18 silica gel CC and/or prep. TLC.

Identification. The substances were identified by spectral comparison with published data and when available with authentic material. The pure glycosides were hydrolysed with 2 N HCl at 100° for 2 hr. After filtration of the pptd genin, the remaining aq. soln was neutralized, the H_2O evapd *in vacuo* and the sugars extracted with pyridine and identified by comparison with authentic samples by means of TLC (MeOH–$CHCl_3$–Me_2CO–conc NH_3 (10:5:6:4)).

Since 1–4 and 6 are known compounds and their spectral data are published, they are not described here: 1 [1, 6, 7, 22], 2 [5], 3 [1, 6, 8, 22], 4 [21] and 6 [2].

Esculentoside L_1: (5). 351 mg crude 5 was flash chromatographed with EtOAc–iso-PrOH–H_2O (90:10:3), than further purified on a RP 18 flash silica gel column with MeOH–H_2O (13:7) and finally on prep. RP 18 TLC (0.25 mm thick) with MeOH–H_2O (13:7) in quantities of 16–20 mg per plate. The silica gel was extracted with MeOH to yield 48 mg 5. Crystallization and recrystallization from iso-PrOH–ether yielded 19 mg, mp 215–220°. Molecular formula $C_{48}H_{76}O_{20}$ 972. $[\alpha]_D^{21}$ + 20.8° (MeOH; c 0.3). No methyl-ester was formed with CH_2N_2.

Acidic hydrolysis of 5 (5 mg) gave 3 mg phytolaccagenic acid, glucose and xylose which were compared with authentic material.

Esculentoside R (7). Crude 7 (479 mg) was crystallized from iso-PrOH:(284 mg). CC on RP 18 Flash silica gel with MeOH–H_2O (7:3) and again on the same sorbent with MeOH–H_2O (1:1) yielded 95 mg 7. Crystallization and recrystallization from iso-PrOH–petrol gave 49 mg, mp 212–216°. Molecular formula: $C_{54}H_{86}O_{24}$ 1118. $[\alpha]_D^{22}$ + 4.9° (MeOH; c 0.4).

Compound 7 (25 mg) was hydrolysed with acid giving 9 mg phytolaccagenic acid, identified by comparison with authentic material (TLC, ^{1}H, ^{13}C NMR, MS). From the aq. soln xylose, glucose and rhamnose were detected on TLC by comparison with authentic material.

Compound 7 (25 mg) was dissolved in 0.2 ml H_2O, 1 mg β-glucosidase was added and the mixt. kept for 24 hr at 37°. Glycoside (16 mg) was extracted with $CHCl_3$–EtOH (4:1) and shown to be identical with phytolaccoside F (TLC, ^{1}H, ^{13}C NMR). From the aq. soln the sugar was shown by TLC to be glucose.

Compound 7 (5 mg) was acetylated in the usual manner and gave 8 mg of a non-crystalline acetate. ^{1}H NMR (pyridine-d_5): 5 tert. Me groups: δ 0.98 (s, 3H), 1.03 (s, 3H), 1.06 (s, 3H), 1.17 (s, 3H), 1.23 (s, 3H), 1.58 (d, J = 6.2 Hz, CH_3-rha) and 13 acetoxy groups: 2.01 (s, 6H), 2.02 (s, 3H), 2.03 (s, 3H), 2.05 (s, 3H), 2.06 (s, 6H), 2.13 (s, 3H), 2.16 (s, 6H), 2.23 (s, 6H), 2.25 (s, 3H), 3.70 (s, OMe).

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