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Pachyelasides A−D, Novel Molluscicidal Triterpene Saponins from *Pachyelasma Tessmannii*

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This is the first report for secondary metabolites in an African medicinal plant, *Pachyelasma tessmannii* (Leguminosae). Four novel saponins, pachyelasides A−D, were isolated from the methanol extract of the root bark by using recycling HPLC. These compounds showed molluscicidal activity ($LD_{50} \leq 8.0$ μg/mL) against the South American snail, *Biomphalaria glabratus*. The structures were determined on the basis of extensive nuclear magnetic resonance spectroscopic and matrix-assisted laser desorption/ionization time-of-flight mass spectrometric studies.

KEYWORDS: Saponin; pachyelaside; *Pachyelasma tessmannii*; Leguminosae; molluscicide; *Biomphalaria glabratus*

INTRODUCTION

Pachyelasma tessmannii (Leguminosae) is a tree widely distributed in central Africa. The fruit of this plant is used for a traditional folk medicine in order to cure several symptoms, especially diarrhea and abdominal pain (*1*). Detailed phytochemical studies on *P. tessmannii* have not been carried out, although a number of unique biologically active substances have been identified in other species of legume (*2*−*6*). Thus, there has been considerable interest in phytochemical investigations of *P. tessmannii* during our continuing study of biologically active substances from medicinal plants (*7*−*11*). The methanol extract of the root bark of *P. tessmannii* exhibited potent molluscicidal properties against the South American snail, *Biomphalaria glabratus*. By bioassay-guided fractionation, novel saponins, pachyelasides A−D (**1**−**4**), were isolated from the extract (Figure 1). The aim of this study was to achieve their efficient separation by recycling HPLC and structural determination by means of extensive nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) techniques and to determine their molluscicidal activities.

MATERIALS AND METHODS

General Experimental Procedures. Recycling HPLC was performed on a JAI LC-09 instrument (Japan Analytical Industry, Tokyo, Japan). Optical rotation was measured on a SEPA-300 polarimeter (Horiba, Kyoto, Japan) at 25 °C. The IR spectra were obtained on a model 1310 spectrometer (Perkin-Elmer, Boston, MA) with Nujol. Samples were examined by NMR spectroscopy as solutions (10 mg/mL) in pyridine-d_5. Spectra were recorded on a JMN-α400 spectrometer (JEOL, Akishima, Japan). Chemical shifts are given in parts per million (ppm), using the pyridine-d_5 signals (8.71 and 149.9 ppm for ^{1}H and ^{13}C, respectively) as references. Two-dimensonal NMR experiments were carried out on all compounds using pyridine-d_5. On the basis of these NMR analyses, assignment of the ^{1}H and ^{13}C signals were obtained. The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was measured by a Voyager-DE STR mass spectrometer (Applied Biosystems, Foster City, CA).

Plant Material. The methanol extract of the root bark of *P. tessmannii* collected in Cameroon was kindly provided by Professor J. F. Ayafor. A voucher specimen was deposited at the National Herbarium, Yaoundé, Cameroon.

Extraction and Isolation. The air-dried root bark (500 g) was cut into small pieces and extracted with MeOH (500 mL × 3) at ambient temperature for 2 weeks. The MeOH extract (70 g) was evaporated to dryness and suspended in 500 mL of H_2O, and then it was extracted successively with *n*-hexane, diethyl ether, $CHCl_3$, EtOAc, and *n*-BuOH to yield four fractions, *n*-hexane and diethyl ether (3 g), $CHCl_3$ (14 g), EtOAc (10 g), and *n*-BuOH (30 g). The *n*-BuOH fraction was ultrafiltrated using Amicon YM1 membrane filter (1000 MW cutoff). The resulting high-molecular-weight residue (4 g) was dissolved in MeOH, which was subjected to a recycling HPLC on a 500 mm × 25 mm i.d., 5-μm, Asahipack GS-320 poly(vinyl alcohol) resin column with methanol mobile phase at a flow rate of 3 mL/min to give compounds **1** (110 mg), **2** (81 mg), **3** (103 mg), and **4** (17 mg). Detection was done by refractive index. The amount of sample loaded was 500 mg.

Pachyelaside A (1). $[\alpha]^{20}_D$ +37.13° (*c* 0.167, EtOH); IR (Nujol) λ_{max} 3420, 1720, 1630 cm^{-1}; ^{1}H NMR (pyridine-d_5, 400 MHz), see Tables 1 and 2; ^{13}C NMR (pyridine-d_5, 100 MHz), see Tables 3 and 4; high-resolution MALDI-TOF MS *m*/*z* 1273.5980 (calcd for $C_{63}H_{94}O_{25}$-Na, 1273.5976).

Pachyelaside B (2). $[\alpha]^{20}_D$ −36.23° (*c* 0.167, EtOH); IR (Nujol) λ_{max} 3420, 1725, 1630 cm^{-1}; ^{1}H NMR (pyridine-d_5, 400 MHz), see

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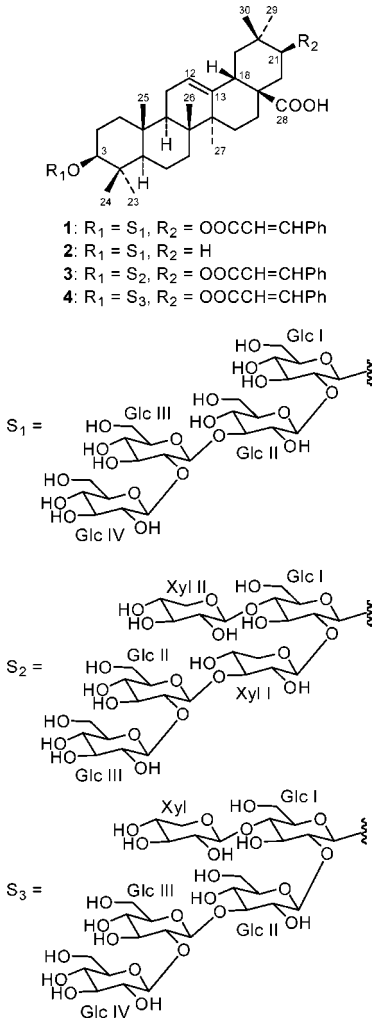

Figure 1. Structures of pachyelasides A–D (**1**–**4**).

Tables 1 and 2; ^{13}C NMR (pyridine-d_5, 100 MHz), see Tables 3 and 4; high-resolution MALDI-TOF MS *m*/*z* 1127.5699 (calcd for $C_{54}H_{88}O_{23}$-Na, 1127.5609).

Pachyelaside C (3). $[\alpha]^{20}_D$ +22.46° (*c* 0.167, EtOH); IR (Nujol) λ_{max} 3420, 1725, 1630 cm^{-1}; ^{1}H NMR (pyridine-d_5, 400 MHz), see Tables 1 and 2; ^{13}C NMR (pyridine-d_5, 100 MHz), see Tables 3 and 4; high-resolution MALDI-TOF MS *m*/*z* 1375.6375 (calcd for $C_{67}H_{100}O_{28}$-Na, 1375.6293).

Pachyelaside D (4). $[\alpha]^{20}_D$ +16.77° (*c* 0.167, EtOH); IR (Nujol) λ_{max} 3425, 1720, 1625 cm^{-1}; ^{1}H NMR (pyridine-d_5, 400 MHz), see Tables 1 and 2; ^{13}C NMR (pyridine-d_5, 100 MHz), see Tables 3 and 4; high-resolution MALDI-TOF MS *m*/*z* 1405.6476 (calcd for $C_{68}H_{102}O_{29}$-Na, 1405.6399).

Acid Hydrolysis. Each saponin (1 mg) was dissolved in MeOH (1 mL) and refluxed for 2 h with 6 N HCl (1 mL) under N_2 atmosphere. The aqueous hydrolysate was neutralized with Na_2CO_3 and then washed with EtOAc (1 mL × 3). The aqueous layer was examined for sugars by normal phase TLC using $CHCl_3$−MeOH−H_2O (7:2:0.5) as the solvent system (*12*). The spots on TLC were visualized by spraying 10% H_2SO_4 followed by heating.

Molluscicidal Assay. The original molluscicidal activity of the crude extract of *P. tessmannii* was found against two species of the African snails, *Lymnaca natalensis* and *Biomphalaria pfeifferi*. However, *B. glabratus*, which is an American snail, was employed for the bioassay-guided fractionation. To develop preliminary information on the toxicity of their saponins to snails, lethal dose 50% (LD_{50}) was determined as described previously (*13*). Snails of uniform sizes were used as far as was possible. The average diameter of the shell was 8 mm. The initial test was carried out by placing two snails in a 100 μg/mL solution of the crude extract dissolved in deionized water. After 24 h, the snails are placed on a Petri dish, light was shone from the bottom, and the heartbeat was checked by a microscope from the top. As the extracts became more concentrated, the solution concentration was diluted, and the time of immersion was shortened.

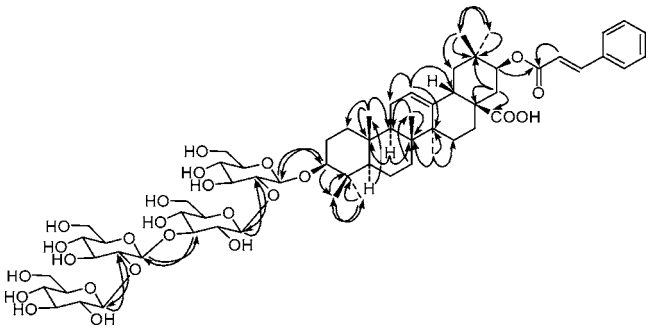

Figure 2. Significant HMBC correlations of **1**.

RESULTS AND DISCUSSION

Separation of the methanol extract of *P. tessmannii* into *n*-hexane, diethyl ether, $CHCl_3$, EtOAc, *n*-BuOH, and water-soluble portions indicated the active constituents were mainly in the *n*-BuOH portion. Biological activity was monitored as previously described (*13*). Due to its polar nature, the bioactive *n*-BuOH extract seemed ideally suited for further separation by recycling HPLC as has been previously applied to the resolution of several saponin mixtures (*14*, *15*). The *n*-BuOH fraction was initially partitioned using ultrafiltration (1000 MW cutoff). The resulting high-molecular-weight residue was subjected to recycling HPLC using a multi-mode column. After six consecutive cycles, the four triterpene saponins, pachyelasides A−D (**1**−**4**), were completely resolved. Polar compounds of such a high molecular weight are often especially difficult to separate. The combination of ultrafiltration and recycling HPLC resulted in the isolation of four saponins by a straightforward procedure with neither derivatization nor degradation.

Pachyelaside A (**1**) was isolated as an amorphous solid. The molecular formula was established as $C_{63}H_{94}O_{25}$ by high-resolution MALDI-TOF MS. The IR spectrum of **1** revealed broadened absorption at 1720 cm^{-1}, which indicated the presence of a carbonyl moiety. The observation of four anomeric signals (δ_H 4.83, 5.46, 5.04, 5.19; δ_C 104.7, 103.6, 103.5, 106.8) in the ^{1}H and ^{13}C NMR spectra suggested that this compound possessed four sugar moieties. Interestingly, a structural moiety consisted of seven methine carbons (δ_C 119.1, 128.4, 129.4, 130.4, 144.7) and two quaternary carbons (δ_C 134.8, 166.4) were detected by ^{13}C NMR and distortionless enhancement by polarization transfer (DEPT) experiments. By the inspection of heteronuclear multiple bond coherence (HMBC) spectrum, as well as coupling constants in the ^{1}H NMR spectrum (Figure 2), this structure was deduced as a *trans*-cinnamoyl moiety. The remaining structure contained seven methyls (δ_H 0.77, 0.92, 1.04, 1.05, 1.18, 1.23, 1.28; δ_C 15.2, 16.7, 17.1, 18.4, 25.7, 28.0, 28.7), one trisubstituted olefin (δ_H 5.47; δ_C 123.7, 143.3), and one carboxylic acid (δ_C 178.4).

The ^{1}H and ^{13}C NMR assignments for **1** were performed by double quantum filter correlation spectroscopy (DQF-COSY), heteronuclear single quantum coherence (HSQC), HMBC, homonuclear Hartman−Hahn (HOHAHA), and nuclear Overhauser effect spectroscopy (NOESY) experiments. An oxymethine proton at 3.26 ppm (H-3) possessed cross-peaks to diastereotopic methylene protons at 1.76 and 2.16 ppm (H-2) in the DQF-COSY spectrum. These protons were further coupled to methylene protons at 0.82 and 1.36 ppm (H-1). A quaternary

Table 1. ^{1}H NMR Data for the Aglycone Moieties of **1**, **2**, **3**, and **4** in Pyridine-d_5

	1	**2**	**3**	**4**
position	δ_H (mult., *J* in Hz)	δ_H (mult., *J* in Hz)	δ_H (mult., *J* in Hz)	δ_H (mult., *J* in Hz)
1	0.82 (m), 1.36 (m)	0.79 (m), 1.34 (m)	0.83 (m), 1.36 (m)	0.81 (m), 1.35 (m)
2	1.76 (m), 2.16 (m)	1.74 (m), 2.15 (m)	1.75 (m), 2.16 (m)	1.76 (m), 2.16 (m)
3	3.26 (dd, 11.8, 4.4)	3.34 (dd, 11.8, 4.4)	3.26 (dd, 11.8, 4.4)	3.26 (dd, 11.8, 4.4)
5	0.61 (d, 11.2)	0.68 (d, 11.7)	0.60 (d, 11.2)	0.62 (d, 11.7)
6	1.21 (m)	1.19 (m)	1.21 (m)	1.20 (m)
7	1.37 (m)	1.41 (m)	1.38 (m)	1.37 (m)
9	1.56 (t, 8.8)	1.54 (m)	1.56 (t, 8.8)	1.56 (t, 8.8)
11	1.85 (m)	1.81 (m)	1.84 (m)	1.84 (m)
12	5.47 (m)	5.34 (m)	5.47 (m)	5.47 (m)
15	1.11 (m)	1.10 (m)	1.10 (m)	1.10 (m)
16	2.09 (m)	1.85 (m)	2.09 (m)	2.08 (m)
18	3.36 (dd, 13.7, 3.9)	3.09 (dd, 13.2, 3.9)	3.36 (dd, 13.7, 3.9)	3.35 (dd, 13.2, 3.9)
19	1.42 (dd, 13.7, 3.9), 2.04 (t, 13.7)	1.14 (m), 2.04 (m)	1.41 (m), 2.02 (m)	1.43 (m), 2.04 (m)
21	5.31 (dd, 11.7, 4.4)	1.35 (m), 1.93 (m)	5.31 (dd, 11.2, 4.4)	5.31 (dd, 11.7, 4.4)
22	2.11 (dd, 13.2, 11.7), 2.27 (dd, 13.2, 4.4)	1.04 (m), 1.72 (m)	2.10 (dd, 13.2, 11.7), 2.26 (dd, 13.2, 4.4)	2.11 (dd, 13.2, 11.7), 2.27 (dd, 13.2, 4.4)
23	1.28 (s)	1.27 (s)	1.27 (s)	1.28 (s)
24	1.05 (s)	1.07 (s)	1.05 (s)	1.06 (s)
25	0.77 (s)	0.78 (s)	0.76 (s)	0.77 (s)
26	0.92 (s)	0.83 (s)	0.92 (s)	0.92 (s)
27	1.23 (s)	1.17 (s)	1.21 (s)	1.22 (s)
29	1.04 (s)	0.86 (s)	1.04 (s)	1.04 (s)
30	1.18 (s)	0.83 (s)	1.18 (s)	1.17 (s)
2′	6.78 (d, 16.1)		6.78 (d, 16.1)	6.78 (d, 16.1)
3′	7.95 (d, 16.1)		7.95 (d, 16.1)	7.95 (d, 16.1)
5′, 9′	7.63 (dd, 8.5, 2.4)		7.63 (dd, 8.5, 2.4)	7.63 (dd, 8.5, 2.4)
6′, 8′	7.32 (m)		7.32 (m)	7.32 (m)
7′	7.32 (m)		7.32 (m)	7.32 (m)

Table 2. ^{1}H NMR Data for the Sugar Moieties of **1**, **2**, **3**, and **4** in Pyridine-d_5

	1	**2**	**3**	**4**
position	δ_H (mult., *J* in Hz)	δ_H (mult., *J* in Hz)	δ_H (mult., *J* in Hz)	δ_H (mult., *J* in Hz)
	Glc I	Glc I	Glc I	Glc I
1″	4.83 (d, 7.4)	4.82 (d, 7.3)	4.79 (d, 7.2)	4.77 (d, 7.8)
2″	4.24 (dd, 8.8, 7.4)	4.24 (m)	4.26 (m)	4.35 (dd, 9.3, 7.8)
3″	4.27 (t, 8.8)	4.26 (t, 8.8)	4.28 (t, 8.6)	4.27 (t, 9.3)
4″	4.10 (m)	4.08 (m)	4.22 (m)	4.14 (m)
5″	3.84 (ddd, 8.8, 5.4, 2.4)	3.84 (ddd, 8.8, 5.4, 2.4)	3.76 (td, 9.0, 3.0)	3.74 (td, 9.3, 3.0)
6″	4.30 (m), 4.50 (m)	4.30 (m), 4.45 (m)	4.40 (m), 4.48 (m)	4.36 (m), 4.48 (m)
	Glc II	Glc II	Xyl I	Glc II
1‴	5.46 (d, 7.8)	5.47 (d, 7.8)	5.55 (d, 8.0)	5.64 (d, 7.8)
2‴	4.20 (dd, 8.8, 7.8)	4.20 (dd, 9.3, 7.8)	4.14 (m)	4.14 (m)
3‴	3.89 (t, 8.8)	3.90 (t, 9.3)	3.89 (t, 8.8)	3.93 (dd, 10.2, 8.3)
4‴	4.10 (m)	4.10 (m)	4.10 (m)	4.05 (m)
5‴	3.75 (ddd, 9.3, 3.9, 2.4)	3.76 (ddd, 9.3, 3.9, 2.4)	3.56 (dd, 11.2, 9.6), 4.28 (m)	3.80 (m)
6‴	4.28 (m), 4.33 (m)	4.28 (m), 4.35 (m)		4.21 (m), 4.36 (m)
	Glc III	Glc III	Glc II	Glc III
1⁗	5.04 (d, 7.8)	5.05 (d, 7.8)	5.13 (d, 8.0)	5.09 (d, 7.8)
2⁗	4.02 (t, 7.8)	4.02 (dd, 8.8, 7.8)	4.12 (m)	4.07 (dd, 8.3, 7.8)
3⁗	4.26 (t, 7.8)	4.27 (m)	4.31 (m)	4.28 (m)
4⁗	4.09 (m)	4.09 (m)	4.11 (m)	4.07 (m)
5⁗	3.97 (m)	3.98 (m)	4.01 (m)	3.94 (m)
6⁗	4.19 (m), 4.50 (m)	4.20 (m), 4.53 (m)	4.20 (m), 4.55 (m)	4.19 (m), 4.55 (m)
	Glc IV	Glc IV	Glc III	Glc IV
1′′′′′	5.19 (d, 7.8)	5.19 (d, 7.8)	5.26 (d, 7.6)	5.24 (d, 7.8)
2′′′′′	4.07 (m)	4.07 (m)	4.12 (m)	4.09 (m)
3′′′′′	4.15 (m)	4.16 (t, 9.3)	4.18 (m)	4.16 (m)
4′′′′′	4.10 (m)	4.11 (t, 9.3)	4.11 (m)	4.11 (m)
5′′′′′	3.94 (m)	3.94 (m)	3.94 (m)	3.93 (m)
6′′′′′	4.39 (m), 4.50 (m)	4.31 (m), 4.48 (m)	4.36 (m), 4.50 (m)	4.35 (m), 4.50 (m)
			Xyl II	Xyl
1′′′′′′			5.06 (d, 7.7)	5.01 (d, 7.3)
2′′′′′′			3.99 (dd, 8.8, 7.7)	3.99 (dd, 8.2, 7.3)
3′′′′′′			4.10 (d, 8.8)	4.06 (m)
4′′′′′′			4.17 (m)	4.14 (m)
5′′′′′′			3.66 (t, 10.0), 4.28 (m)	3.61 (t, 10.7), 4.24 (m)

Table 3. ^{13}C NMR Data for the Aglycone Moieties of **1**, **2**, **3**, and **4** in Pyridine-d_5

	1	**2**	**3**	**4**
position	δ_C (mult.)	δ_C (mult.)	δ_C (mult.)	δ_C (mult.)
1	38.5 (t)	38.4 (t)	38.4 (t)	38.5 (t)
2	26.3 (t)	26.3 (t)	26.3 (t)	26.3 (t)
3	88.7 (d)	88.7 (d)	88.7 (d)	88.6 (d)
4	39.2 (s)	39.2 (s)	39.2 (s)	39.2 (s)
5	55.6 (d)	55.5 (d)	55.5 (d)	55.6 (d)
6	18.2 (t)	18.2 (t)	18.2 (t)	18.2 (t)
7	33.0 (t)	33.0 (t)	33.0 (t)	33.0 (t)
8	39.4 (s)	39.5 (s)	39.4 (s)	39.4 (s)
9	47.7 (d)	47.6 (d)	47.7 (d)	47.7 (d)
10	36.7 (s)	36.7 (s)	36.7 (s)	36.8 (s)
11	23.6 (t)	23.0 (t)	23.6 (t)	23.6 (t)
12	123.7 (d)	123.7 (d)	123.7 (d)	123.7 (d)
13	143.3 (s)	143.6 (s)	143.3 (s)	143.4 (s)
14	41.9 (s)	41.8 (s)	41.9 (s)	41.9 (s)
15	28.0 (t)	27.7 (t)	27.9 (t)	28.0 (t)
16	24.7 (t)	23.5 (t)	24.7 (t)	24.7 (t)
17	48.2 (s)	47.1 (s)	48.2 (s)	48.0 (s)
18	41.2 (d)	41.7 (d)	41.2 (d)	41.2 (d)
19	46.7 (t)	45.7 (t)	46.7 (t)	46.5 (t)
20	35.4 (s)	30.6 (s)	35.4 (s)	35.4 (s)
21	75.7 (d)	33.7 (d)	75.7 (d)	75.7 (d)
22	37.3 (t)	32.7 (t)	37.3 (t)	37.3 (t)
23	28.0 (q)	28.0 (q)	28.0 (q)	28.0 (q)
24	16.7 (q)	16.7 (q)	16.7 (q)	16.8 (q)
25	15.2 (q)	15.2 (q)	15.2 (q)	15.2 (q)
26	17.1 (q)	17.3 (q)	17.1 (q)	17.1 (q)
27	25.7 (q)	25.7 (q)	25.7 (q)	25.5 (q)
28	178.4 (s)	175.6 (s)	178.4 (s)	178.4 (s)
29	28.7 (q)	32.9 (q)	28.7 (q)	28.7 (q)
30	18.4 (q)	23.5 (q)	18.4 (q)	18.4 (q)
1′	166.4 (s)		166.4 (s)	166.3 (s)
2′	119.1 (d)		119.0 (d)	119.1 (d)
3′	144.7 (d)		144.7 (d)	144.7 (d)
4′	134.8 (s)		134.7 (s)	134.8 (s)
5′, 9′	128.4 (d)		128.4 (d)	128.5 (d)
6′, 8′	129.4 (d)		129.4 (d)	129.4 (d)
7′	130.4 (d)		130.4 (d)	130.4 (d)

Table 4. ^{13}C NMR Data for the Sugar Moieties of **1**, **2**, **3**, and **4** in Pyridine-d_5

	1	**2**	**3**	**4**
position	δ_C (mult.)	δ_C (mult.)	δ_C (mult.)	δ_C (mult.)
	Glc I	Glc I	Glc I	Glc I
1″	104.7 (d)	104.7 (d)	104.6 (d)	104.7 (d)
2″	82.5 (d)	82.4 (d)	80.0 (d)	79.8 (d)
3″	78.3 (d)	78.3 (d)	76.7 (d)	76.8 (d)
4″	70.9 (d)	70.0 (d)	80.7 (d)	80.7 (d)
5″	77.75 (d)	77.76 (d)	76.4 (d)	76.0 (d)
6″	62.7 (t)	62.7 (t)	61.9 (t)	61.9 (t)
	Glc II	Glc II	Xyl I	Glc II
1‴	103.6 (d)	103.6 (d)	103.5 (d)	102.5 (d)
2‴	74.7 (d)	74.6 (d)	74.3 (d)	74.6 (d)
3‴	90.6 (d)	90.6 (d)	90.7 (d)	91.0 (d)
4‴	71.2 (d)	70.9 (d)	71.4 (d)	69.8 (d)
5‴	77.5 (d)	77.5 (d)	66.3 (t)	76.1 (d)
6‴	62.1 (t)	62.1 (t)		62.2 (t)
	Glc III	Glc III	Glc II	Glc III
1⁗	103.5 (d)	103.5 (d)	103.6 (d)	103.7 (d)
2⁗	85.5 (d)	85.5 (d)	85.8 (d)	85.8 (d)
3⁗	77.4 (d)	77.4 (d)	77.7 (d)	77.7 (d)
4⁗	71.2 (d)	71.3 (d)	69.6 (d)	70.8 (d)
5⁗	78.2 (d)	78.2 (d)	78.4 (d)	78.5 (d)
6⁗	62.1 (t)	61.9 (t)	62.3 (t)	62.4 (t)
	Glc IV	Glc IV	Glc III	Glc IV
1⁗′	106.8 (d)	106.8 (d)	107.0 (d)	107.0 (d)
2⁗′	76.1 (d)	76.1 (d)	76.2 (d)	76.4 (d)
3⁗′	77.81 (d)	77.80 (d)	78.0 (d)	78.0 (d)
4⁗′	69.1 (d)	69.1 (d)	70.8 (d)	71.2 (d)
5⁗′	78.9 (d)	78.9 (d)	78.9 (d)	79.1 (d)
6⁗′	62.0 (t)	62.1 (t)	62.0 (t)	62.3 (t)
			Xyl II	Xyl
1⁗″			105.5 (d)	105.5 (d)
2⁗″			75.0 (d)	74.9 (d)
3⁗″			78.3 (d)	78.3 (d)
4⁗″			70.9 (d)	70.8 (d)
5⁗″			67.4 (t)	67.4 (t)

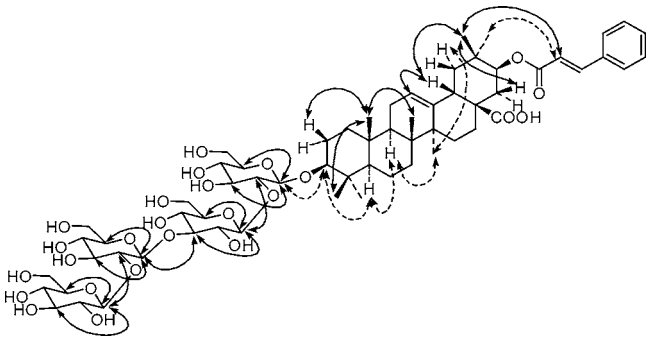

Figure 3. Significant NOE correlations of **1**. Solid and broken lines with arrows on the aglycone indicate NOE of the β- and α-face, respectively.

carbon at 39.2 ppm (C-4) should be assigned to the neighbor for C-3 by observing several HMBC correlations. In turn, a quaternary carbon at 36.7 ppm was assigned to C-10. Consequently, this sequence was expected to come from the A ring structure of the aglycone. Glycosylation at H-3 was supported by an observed NOE (Figure 3) and HMBC correlations between the H-3 in the aglycone and the anomeric position (C″-1) in the sugar moiety. The signal of the oxymethine at the H-3 showed small and large coupling constants (4.4 and 11.8 Hz) in the ^{1}H NMR spectrum, indicating that the orientation of alkoxy moiety was β.

A methine proton at 0.61 ppm (H-5) was correlated to a methylene at 1.37 ppm (H-7) via a methylene at 1.21 ppm (H-6) in the HOHAHA spectrum. These signals established the B ring with the aid of HMBC experiments. A large coupling constant (11.7 Hz) at H-5 and a NOE between H-3 and H-5 proved to be the A−B *cis*-fused ring in this structure. The olefinic methine proton at 5.47 ppm (H-12) was linked to the methine proton at 1.56 ppm (H-9) through methylene protons at 1.85 ppm (H-11) in the HOHAHA spectrum. Several HMBC correlations between H-9 and the A ring indicated that this sequence was assigned to the C ring. The α-orientation of the H-9 was clarified by a key NOE observed between the H-5 and H-9.

A methyl proton at 1.23 ppm (H-27) and the olefinic proton at 5.47 ppm (H-12) were correlated to a quaternary carbon at 41.9 ppm (C-14) in the HMBC spectrum. Four methylene protons at 1.11 and 2.09 ppm (H-15 and H-16) coupled with each other in the DQF-COSY spectrum made up the D ring structure because a HMBC correlation was observed between H-27 and C-15. An NOE between H-27 and H-9 was consistent with the α-orientation of H-27 methyl, as shown in Figure 3. The olefinic proton at 5.47 ppm (H-12) possessed a long-range correlation to a methine carbon at 41.2 ppm (C-18) in the HMBC spectrum. Hence, a proton sequence with methylenes at 1.42 and 2.04 ppm and a methine at 3.36 ppm were assigned to H-18 and H-19, respectively. HMBC experiments provided an observation of a long-range connection between a methylene proton at 2.11 ppm (H-22β) and a carbon (C-28) of the carboxylic acid. The methylene protons (H-22) were coupled to an oxymethine proton at 5.31 ppm (H-21) by a DQF-COSY experiment. These signals allowed constriction of the E ring structure in part. The cinnamoyl moiety was attached on the

oxymethine at H-21 because of the observed HMBC correlation as shown in Figure 2. Small and large coupling constants (4.4 and 11.7 Hz) at the H-21 and the downfield shift of C-28 (*16*, *17*) suggested that this acyloxy moiety was attached in a β-orientation. Consequently, the aglycone of **1** was identified as β-amyrin with the *trans*-cinnamoyloxy moiety at C-21.

A sugar unit (Glc I) that possessed HMBC and NOE correlations from H-3 position at the aglycone was assigned as a β-glucoside because oxymethine protons in the sugar showed coupling constants around 8.0 Hz. A deshielded oxymethine carbon at 82.5 ppm (C-2″) in Glc I indicated the presence of an ether linkage at C-2″. As a matter of fact, HMBC and NOE correlations were observed between H-2″ and H-1‴ positions. The second sugar residue (Glc II) is also a β-glucoside, which was confirmed by the oxymethine coupling constants (7.8−9.3 Hz). The glycosylated position was defined as C-3‴ (δ_C 90.6) due to its downfield shift and cross-peaks observed in the HMBC and NOESY spectra (*18*). An oxymethine proton at 4.02 ppm (H-2⁗) having a HMBC correlation with an anomeric carbon at 106.8 ppm (C-1⁗) clarified that the structure of disaccharide, Glc III and IV, was 2-*O*-(β-glucopyranosyl)-β-glucopyranose. The signal of C-2⁗ (δ_C 85.5) was deshielded compared to that of C-2″, which may be responsible for the unhindered structure surrounding this oxymethine (*18*). After acidic hydrolysis, glucose was the only sugar found in the reaction mixture by TLC analysis, which was consistent with the tetrasaccharide structure of **1**. The NMR assignments were listed in Tables 1−4.

Pachyelaside B (**2**) was isolated as an amorphous solid. The molecular formula was established as $C_{54}H_{88}O_{23}$ by high-resolution MALDI-TOF MS. The IR spectrum of **2** revealed absorption at 1725 cm^{-1}, which indicated the presence of carbonyl moiety. The spectroscopic comparison of the ^{1}H and ^{13}C NMR spectra suggested that the structure of **2** was similar to that of **1**. However, neither the signals from the cinnamoyl moiety nor the oxymethine in the E ring were detected in the NMR spectrum of **2**. C-21 was alternatively identified as a methylene by DQF-COSY and HSQC experiments. Hence, the structure of this saponin was elucidated as a β-amyrin-containing tetrasaccharide. The negative optical rotation values reflected on the absence of a cinnamoyl moiety in the structure of **2**.

Pachyelaside C (**3**) was isolated as an amorphous solid. The molecular formula was established as $C_{67}H_{100}O_{28}$ by high-resolution MALDI-TOF MS. The presence of a carbonyl moiety was suggested by the IR spectrum (1725 cm^{-1}). It should be mentioned that two pairs of triplet oxymethylene protons (δ_H 3.56, 3.66) were observed in the HSQC spectrum. These resonances were identified as the H-5 positions in pentopyranose moieties. The sugar chain branched into two at Glc I, which was indicated by downfield shifted oxymethine carbons at 80.0 and 80.7 ppm (C-2″ and C-4″) (*18-20*). The oxymethine protons (δ_H 4.22, 4.26) were correlated to anomeric carbons at 105.5 and 103.5 ppm, respectively, in the HMBC spectrum. Moreover, the proton resonances of corresponding anomeric protons were individually connected to those of triplet oximethine protons at 3.56 and 3.66 ppm in the HOHAHA spectrum. Hence, it was considered that the xylose moieties were attached to C-2″ and C-4″ positions on Glc I. Also, xylose was detected in its acid hydrolysate by TLC analysis. One of the xylose units, Xyl I, contained an ether linkage with Glc II, which was indicated by an HMBC experiment. The glucosylation at C-2⁗ was confirmed by ^{13}C NMR, NOESY, and HMBC experiments, and the observations led to the elucidation of the structure of **3**.

Pachyelaside D (**4**) was isolated as an amorphous solid. The molecular formula was established as $C_{68}H_{102}O_{29}$ by high-resolution MALDI-TOF MS. The presence of a carbonyl moiety was suggested by the IR spectrum (1720 cm^{-1}). The ^{1}H and ^{13}C NMR spectra of **3** and **4** showed a number of similarities. The resonances for five anomeric protons (δ_H 4.77, 5.01, 5.09, 5.24, 5.64) revealed in the ^{1}H NMR spectrum, indicating that **3** possessed a pentasaccharide unit in the structure, whereas the aglycone was identical with that of **1**. Remarkably, one of these sugars could be deduced as a xylose according to the observation of a triplet signal (10.7 Hz) at 3.61 ppm in the ^{1}H NMR spectrum. Although ^{1}H NMR signals of Glc I H-4″, Glc II H-2‴, and Xyl H-4⁗ were overlapped, ^{13}C NMR signals on Glc I and Xyl were considerably similar to those of **3**, suggesting the attached position of the xylose was C-4″ in Glc I. Consequently, the structure of **4** was determined as that of **1** with a xylose moiety.

The molluscicidal activity of **1**−**4** were LD_{50} = 2, 2, 2, and 8 μg/mL, respectively, within 24 h against *B. glabratus*. In conclusion, the four new saponins have been effectively isolated from the root bark of *P. tessmannii* using recycling HPLC and this is the first example of phytochemical investigations on this plant. The structure of cinnamoyl saponins has been reported by several research groups (*16*, *17*). However, saponins **3** and **4** possess the most complex sugar chains in such structures. Further study for the identification of unique phytochemicals is underway, using *P. tessmannii* as a curious plant source.

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