Phyllanthusols A and B, Cytotoxic Norbisabolane Glycosides from *Phyllanthus acidus* Skeels

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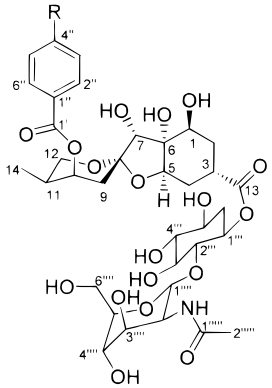
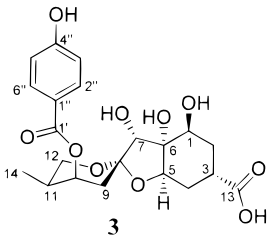
Received March 23, 2000

Roots of *Phyllanthus acidus* Skeels have frequently been used in the rehabilitation program for alcoholic addicts in Thailand. The use of the spirit extract of this plant in treating alcoholism is reported to be very effective against the craving for alcohol. However, many patients have later encountered serious side effect in the form of chronic illness; this has prompted us to explore chemicals in the roots of *P. acidus*. We report here the novel cytotoxic water-soluble norbisabolane glycosides, named phyllanthusol A (**1**) and phyllanthusol B (**2**), isolated from the MeOH extract of the roots of *P. acidus*; these substances might be responsible for the illness of patients who have been treated with the root extract of *P. acidus*.

Phyllanthusol A (**1**) was present in the roots of *P. acidus* at a relatively high concentration of ca. 1 mg/g wet weight (by HPLC analysis). Interpretation of the ^{1}H NMR spectrum of phyllanthusol A (**1**) alone was not straightforward as a result of complex overlapping signals at δ_H 3.25−4.25. Therefore, phyllanthusol A (**1**) was subsequently hydrolyzed by an aqueous solution of NaOH (1 N) to yield the norbisabolane aglycon (**3**) and the saccharide residue (**4**). The ^{1}H NMR spectrum (D_2O)



1, Phyllanthusol A, R = OH
2, Phyllanthusol B, R = H



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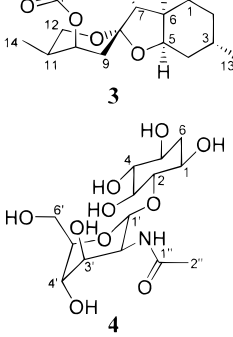

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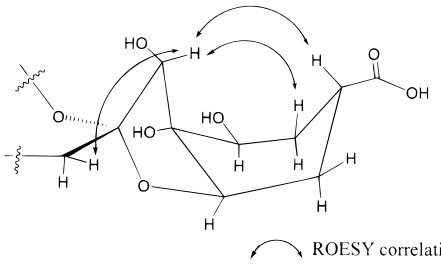

Figure 1. Possible twist boat conformation (in D_2O solution) of **3**.

of the aglycon (**3**) prominently showed the presence of a *para* substituted benzene (δ_H 6.81 and 7.79, both d with *J* value of 8.7 Hz), one methyl (δ_H 0.64, d, 6.9 Hz), one methine with ester linkage (δ_H 5.05, br d, 2.0 Hz), protons on carbons bearing oxygen atoms (δ_H 3.25−4.10) as well as methylene and methine protons (δ_H 1.25−2.24). The ^{13}C NMR spectrum of **3** revealed two carbonyl carbons, an ester (δ_C 168.8) and a carboxylic carbonyl (δ_C 184.6). Extensive analysis of the ^{13}C, DEPT135, ^{1}H−^{1}H COSY, and HMQC spectral data of **3** allowed a complete assignment of protons attached to their respective carbons (Table 1). The TOCSY spectrum of **3** readily confirmed the connectivity from H-1 to H-5, and from H-9 to H-12. The existence of a *p*-hydroxybenzoate in **3**, with an ester linkage at C-10, was established by the HMBC (optimized $^nJ_{HC}$ 4.0 Hz) correlations: H-10 to C-1′; H-2″ and H-6″ to C-1′ and C-4″; and H-3″ and H-5″ to C-1″ and C-4″. The HMBC spectrum of **3** also conclusively demonstrated the correlations of H-10 to C-8 and C-14; H-12 to C-8 and C-10; H-7 to C-1, C-8 and C-9; H-1 to C-6 and C-7; and H-3 to C-1, C-5 and C-13 (the carbonyl of the acid), leading to the structure of a norbisabolane bridged with a spiropyran. On the basis of these ^{1}H−^{1}H COSY, TOCSY, and HMBC spectral data of **3**, the basic chemical structure of **3** was readily established. The ESITOF mass spectrum of **3** deduced a molecular formula of **3** as $C_{21}H_{26}O_{10}$ (observed *m/z* 461.1421 $(M + Na)^+$, Δ −0.3 mmu).

The relative stereochemistry of **3** was assigned by analysis of coupling constants and by ROESY correlations. The *J* values (br d, 2.0 Hz) suggested an equatorial orientation of H-10, while that of H-12$_{ax}$ (δ_H 3.68, dd, 11.6 and 11.6 Hz) revealed an axial configuration of H-11. The ROESY spectrum of **3** revealed that H-7, H-3 and H-9 (2H) were on the same face of the skeleton. The doublet with *J* values of 11.6 and 5.8 Hz of H-1 implied an axial orientation of H-1, however, there were no correlations of both H-3 and H-7 to H-1 on the ROESY spectrum of **3**. On the basis of these spectral data, the possible twist boat conformation (in D_2O solution) of **3** was therefore proposed as shown in Figure 1. This conformation supported the proximity of H-3 and H-7 as their spatial correlations could be observed by the ROESY technique and also validated an axial orientation of H-1 with its *J* values ($J_{H\text{-}1,H\text{-}2ax}$ = 11.6 Hz, and $J_{H\text{-}1,H\text{-}2eq}$ = 5.8 Hz). It should be noted that the proposed twist boat conformation is possibly responsible for the small dihedral angles of H-5 to H-4$_{ax}$ and H-4$_{eq}$, as indicated by the *J* values (dd, 3.2 and 3.2 Hz).

A molecular formula of $C_{14}H_{25}O_{10}N$ for the saccharide

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10.1021/jo000439+ CCC: $19.00
Published on Web 08/03/2000

Table 1. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data (in D_2O) of 3 and 4

aglycon **3**			saccharide **4**		
C	δ_C	δ_H, multiplicity, *J* in Hz	C	δ_C	δ_H, multiplicity, *J* in Hz
1	71.5	3.82, dd, 11.6, 5.8	1	67.5	3.50
2	29.1	1.51, ddd, 14.0, 11.0, 11.0	2	85.6	3.34
		1.82	3	74.0	3.20, dd, 9.2, 9.2
3	36.3	2.26, m	4	77.4	3.13, dd, 9.3, 9.3
4	27.5	1.67, ddd, 14.7, 5.5, 4.0	5	68.4	3.38
		1.76	6	36.1	1.31, ddd, 12.1, 12.1, 12.1
5	82.2	4.02,dd, 3.2, 3.2			2.07, ddd, 12.4, 4.7, 4.7
6	76.3		1′	102.0	4.62, d, 8.4
7	74.3	3.72, s	2′	56.1	3.61
8	101.45		3′	74.1	3.43
9	34.7	1.89, dd, 15.1, 2.5	4′	70.1	3.34
		2.05, dd, 15.0, 2.9	5′	76.1	3.34
10	71.7	5.05, br d, 2.0	6′	60.9	3.61
11	32.7	1.94, m			3.78
12	62.0	3.41, dd, 11.3, 4.5	1″	175.2	
		3.68, dd, 11.6, 11.6	2″	22.5	1.91, s
13	184.6				
14	12.3	0.64, d, 6.9			
1′	168.8				
1″	122.0				
2″	132.6	7.79, d, 8.7			
3″	115.7	6.81, d, 8.7			
4″	161.3				
5″	115.7	6.81, d, 8.7			
6″	132.6	7.79, d, 8.7			

residue **4** was deduced by the ESITOF mass spectrum (observed *m*/*z* 368.1556 $(M + H)^+$, Δ +0.7 mmu). The ^{1}H and ^{13}C NMR spectra of **4** showed the presence of an anomeric proton and carbon (δ_H 4.62, d, 8.4 Hz; δ_C 102.0), implying the existence of a sugar unit in **4**. The DEPT135, ^{1}H−^{1}H COSY, and HMQC spectra of **4** allowed the assignment of protons attached to their respective carbons (Table 1). The ^{1}H−^{1}H COSY and TOCSY spectral data of **4** readily established the connectivity from H-1 to H-6, and from H-1′ to H-6′. The *J* values of H-6$_{ax}$ (δ_H 1.31, ddd, 12.1, 12.1 and 12.1 Hz) indicated that H-1 and H-5 were equatorially oriented; all axial configurations of H-2, H-3 and H-4 were assigned on the basis of the *J* values of H-3 (δ_H 3.20, dd, 9.2 and 9.2 Hz) and H-4 (δ_H 3.13, dd, 9.3 and 9.3 Hz). These spectral data therefore led to the identification of the first unit (cyclohexanepentol) in the saccharide residue **4** as *scyllo* quercitol. The connection of *scyllo* quercitol with the sugar moiety was confirmed by the HMBC spectrum of **4**, showing the correlation of H-1′ to C-2 (δ_C 85.6). The chemical shift of C-2′ (δ_C 56.1) indicated that the sugar had an amino substituent at C-2′; the HMBC spectrum of **4** revealed the presence of an acetate attached to the amino at C-2′, showing the correlations of H-2′ and methyl protons (H-2″) to the carbonyl C-1″ (δ_C 175.2). The IR absorption peaks at 1621 and 1550 cm^{-1} also confirmed the amide functionality in **4**. The $J_{H\text{-}1',H\text{-}2'}$ value of 8.4 Hz implied an axial orientation of H-1′ and H-2′, and the ROESY spectrum of **4** demonstrated the correlation of H-1′ to oxymethylene protons (H-6′), suggesting that H-5′ was equatorial. The respective axial, axial, and equatorial configurations of H-1′, H-2′ and H-5′, in combination with comparison of the ^{13}C chemical shift of the sugar unit in **4** with those of known 2-acetamido-2-deoxy-hexoses,[1] conclusively demonstrated that the sugar in **4** was mannosamine-*N*-acetate. On the basis of these spectral data, the saccharide **4** was *scyllo* quercitol-2-*O*-α-mannosamine-*N*-acetate.

The gross chemical structure of phyllanthusol A (**1**) was thus deduced by assembling of the structures of **3** and **4**. The HMBC spectral data of phyllanthusol A (**1**) allowed the connection of **3** and **4**, exhibiting the correlation of H-1‴ to the carbonyl ester carbon C-13. The HRFABMS data of phyllanthusol A (**1**) established its molecular formula as $C_{35}H_{49}O_{19}N$ (observed *m*/*z* 788.2992 $(M + H)^+$, Δ +1.5 mmu). The extensive analyses of the DEPT135, ^{1}H−^{1}H COSY, TOCSY, HMQC, and HMBC spectral data of phyllanthusol A (**1**) allowed complete assignments of protons and carbons in phyllanthusol A (**1**) (Table 2).

Phyllanthusol B (**2**) was the dehydroxy derivative of **1**. The ^{1}H, ^{13}C and DEPT135 spectra of phyllanthusol B (**2**) revealed that the hydroxyl group at C-4″ of **1** was replaced by a proton. A molecular formula $C_{35}H_{49}O_{18}N$ of phyllanthusol B (**2**) was obtained from the HRFABMS (observed *m*/*z* 772.3025 $(M + H)^+$, Δ −0.2 mmu). Extensive analyses of the DEPT135, ^{1}H−^{1}H COSY, TOCSY, HMQC, and HMBC spectra of phyllanthusol B (**2**) again led to complete assignments of phyllanthusol B (**2**) (Table 2).

The norbisabolane skeleton of **3** is rare in nature. Phyllanthusols A (**1**) and B (**2**) possessed the chemical backbone of an aglycon similar to that of the potent antineoplastic phyllanthostatins 1−6, the less polar bisabolane glycosides previously isolated from *P. acuminatus* by Pettit and co-workers.[2] Phyllanthusols A (**1**) and B (**2**) exhibited cytotoxicity against BC (EC_{50} at 4.2 and 4.0 μg/mL for **1** and **2**, respectively) and KB (EC_{50} at 14.6 and 8.9 μg/mL for **1** and **2**, respectively) cell lines, while the aglycon **3** and saccharide **4** showed no cytotoxicity. The presence at a relatively high concentration (ca. 1 mg/g wet weight) of the cytotoxic norbisabolane glycoside **1** in the roots of *P. acidus* is alarming and possibly

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Table 2. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR Spectral Data (in D_2O/CD_3OD, 1:2) of Phyllanthusol A (1) and Phyllanthusol B (2)

	phyllanthusol A (**1**)		phyllanthusol B (**2**)	
C	δ_C	δ_H, multiplicity, J in Hz	δ_C	δ_H, multiplicity, J in Hz
1	71.5	3.91, dd, 10.9, 5.4	71.7	3.97, dd, 10.8, 5.7
2	28.2	1.62, ddd, 14.4, 10.2, 10.2	28.0	1.63
	2.14			2.14
3	34.4	2.61, m	34.4	2.59, m
4	28.2	1.90	28.0	1.98
		2.03		2.11
5	81.9	4.14	81.9	4.22
6	76.6		76.6	-
7	75.6	3.86, s	75.1	3.75, s
8	102.3		102.2	
9	35.9	2.14	35.5	2.18
		2.21, dd, 15.0, 2.9		2.30
10	71.9	5.27, br d, 2.0	72.7	5.35, br s
11	33.8	2.14, m	33.4	2.22
12	62.9	3.66	62.8	3.71
		4.05, dd, 11.5, 11.5		4.11, dd, 11.5, 11.5
13	177.2		177.6	
14	12.9	0.91, d, 6.9	12.9	0.97, d, 6.7
1′	168.8		169.0	
1″	121.3		131.4	
2″	133.2	8.00, d, 8.7	130.7	8.20, d, 7.7
3″	117.1	6.87, d, 8.7	129.7	7.65, dd, 7.7, 7.7
4″	165.6		134.8	7.81, t, 7.4
5″	117.1	6.87, d, 8.7	129.7	7.65, dd, 7.7, 7.7
6″	133.2	8.00, d, 8.7	130.7	8.20, d, 7.7
1‴	70.8	4.76[d]	70.5[b]	4.78[d]
2‴	83.7	3.61	83.2	3.56
3‴	76.0[a]	3.35	75.5[c]	3.40
4‴	78.1	3.35	78.0	3.34
5‴	69.2	3.48	68.8	3.60
6‴	35.3	1.51, ddd, 12.1, 12.1, 12.1	35.0	1.46, ddd, 12.0, 12.0, 12.0
		2.14		2.14
1⁗	103.0	4.52,d, 8.4	102.7	4.55, d, 7.8
2⁗	57.7	3.57	57.2	3.56
3⁗	75.9[a]	3.35	75.2[c]	3.40
4⁗	71.0	3.41	70.7[b]	3.49
5⁗	77.0	2.91, m	76.7	3.02
6⁗	62.0	3.57	61.7	3.60
		3.66		3.71
1⁗′	175.1		175.4	
2⁗′	23.2	2.08, s	23.3	2.11, s

[a–c] May be interchangeable in the same column. [d] Signals overlapped with H_2O peak and were assigned by the HMQC spectral data.

responsible for the chronic illness of those who drank the root extracts of this plant.

Experimental Section

General. The ^{1}H, ^{13}C, ^{1}H−^{1}H COSY,[3] TOCSY,[4] ROESY,[5] and DEPT 135[6] NMR spectra were recorded on a Bruker DRX400, operating at 400 MHz for proton and 100 MHz for carbon. Proton-detected heteronuclear correlations were measured using HMQC[7] (optimized for $^1J_{HC}$ = 145 Hz) and HMBC[8] (optimized for $^nJ_{HC}$ = 4.0 Hz) pulse sequences. The ESITOF mass spectra were obtained from a Micromass LCT mass spectrometer, while the HRFABMS spectra were from Quattro Ultima mass spectrometer. The IR spectra and optical rotations were measured on a Perkin-Elmer 2000 spectrometer and Jasco DIP370 polarimeter, respectively.

Extraction and Isolation. Roots of *P. acidus* were collected from Nakhon Sawan Province, Thailand. Ground dried roots (2 kg) of *P. acidus* were macerated in MeOH for 3 days at room temperature, giving 200 g of the crude extract. A portion of the crude extract (100 g) was subjected to MPLC, using a C_{18} reversed phase column and a gradient elution of H_2O (100%) to H_2O−MeOH (50:50). Fractions containing phyllanthusols A (**1**) and B (**2**) were further purified by preparative HPLC, using a C_{18} reversed phase column (Prep Nova-Pak, 40 mm × 100 mm) and H_2O−MeOH (50:50) as a mobile phase, to yield phyllanthusols A (**1**) (2.5 g) and B (**2**) (32.4 mg). Amounts of **1** and **2** in fresh roots of *P. acidus* were analyzed by analytical HPLC, using a C_{18} reversed phase column and H_2O−MeOH (50:50) as eluent. At the flow rate of 1 mL/min (UV detector set at 254 nm), compounds **1** and **2** were eluted with the retention times of 10.2 and 12.6 min, respectively (by co-injection with the standard samples). The amount of **1** was found to be ca. 1 mg/g wet weight of the roots, while **2** was present as a trace.

Cytotoxicity Assay. The cytoxicity of the isolated compounds was evaluated, employing the colorimetric method as described by Skehan and co-workers.[9] Ellipticine was used as the reference substance, exhibiting the activity toward BC and KB cell lines, both with the EC_{50} of 0.3 μg/mL.

Hydrolysis of Phyllanthusol A (1). Phyllanthusol A (**1**) (1.1

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g) was dissolved in an aqueous solution of NaOH (1 N) and warmed for 1 h at 60 °C. After workup with a dilute HCl solution, the reaction mixture was purified with MPLC on a C_{18} reversed phase column, eluted with H_2O−MeOH (50:50), yielding the aglycon **3** (439.2 mg) and saccharide **4** (100.2 mg).

Phyllanthusol A (1). White amorphous solid; mp 187−191 °C; $[\alpha]^{28}_D$ −7.4° (*c* 1.82, H_2O); UV (H_2O) λ_{max} (log ϵ) 202 (4.2), 257 (4.1) nm; IR (Nujol) ν_{max} 3350, 1688, 1607, 1460, 1376, 1278, 1116, 1073, 1029 cm^{-1}; HRFABMS *m*/*z* 788.2992 $(M + H)^+$, calcd for $C_{35}H_{50}O_{19}N$, 788.2977; ^1H and ^{13}C NMR see Table 2.

Phyllanthusol B (2). White amorphous solid; mp 183−185 °C; $[\alpha]^{28}_D$ −18.4° (*c* 0.77, H_2O); UV (H_2O) λ_{max} (log ϵ) 200 (4.1), 231 (4.0) nm; IR (Nujol) ν_{max} 3372, 1722, 1642, 1607, 1514, 1460, 1376, 1278, 1167, 1073, 1029 cm^{-1}; HRFABMS *m*/*z* 772.3025 $(M + H)^+$, calcd for $C_{35}H_{50}O_{18}N$, 772.3027; ^1H and ^{13}C NMR see Table 2.

Aglycon (3). White amorphous solid; mp 265−268 °C; $[\alpha]^{28}_D$ +7.2° (*c* 1.93, H_2O); UV (H_2O) λ_{max} (log ϵ) 208 (3.9), 253 (3.9) nm; IR (Nujol) ν_{max} 3359, 1731, 1608, 1569, 1510, 1461, 1376, 1258, 1115, 1078, 1016 cm^{-1}; ESITOF MS *m*/*z* 461.1421 $(M + Na)^+$, calcd for $C_{21}H_{26}O_{10}Na$, 461.1424; ^1H and ^{13}C NMR see Table 1.

Saccharide (4). White amorphous solid; mp 194−196 °C; $[\alpha]^{28}_D$ −19.2° (*c* 0.74, H_2O); UV (H_2O) λ_{max} (log ϵ) 202 (3.1) nm; IR (Nujol) ν_{max} 3460, 3361, 2562, 2533, 2490, 1621, 1608, 1550, 1461, 1377, 1118, 1073 cm^{-1}; ESITOF MS *m*/*z* 368.1556 $(M + H)^+$, calcd for $C_{14}H_{26}O_{10}N$, 368.1549; ^1H and ^{13}C NMR see Table 1.

Acknowledgment. We are indebted to the Biodiversity Research and Training Program (BRT) and BIOTEC/NSTDA for financial support. Y.T. thanks the National Science and Technology Development Agency (NSTDA) for the Senior Research Fellowship Award.

Supporting Information Available: ^1H and ^{13}C NMR spectra of compounds **1**−**4**; $^1H-^1H$ COSY, TOCSY, HMQC, and HMBC spectra of compounds **1** and **2**; and $^1H-^1H$ COSY, ROESY, HMQC, and HMBC spectra of compounds **3** and **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

JO000439+