Isolation of Echinocystic Acid-3-O-Sulfate, a New Triterpene, from Tetrapleura tetraptera, and Evaluation of the Mutagenic Potential of Molluscicidal Extracts and Isolates

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ISOLATION OF ECHINOCYSTIC ACID-3-*O*-SULFATE, A NEW TRITERPENE, FROM *TETRAPLEURA TETRAPTERA*, AND EVALUATION OF THE MUTAGENIC POTENTIAL OF MOLLUSCICIDAL EXTRACTS AND ISOLATES[1]

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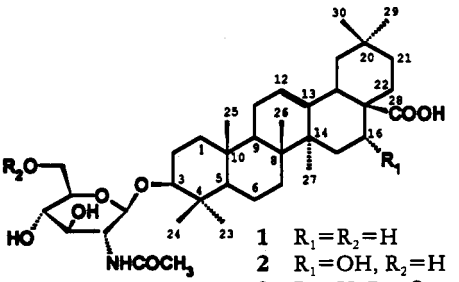
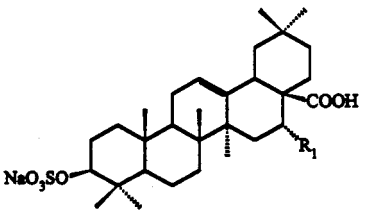
ABSTRACT.—A known triterpene glycoside, 3-*O*-[β-D-glucopyranosyl-(1″-6′)-2′-acetamido-2′-deoxy-β-D-glucopyranosyl]olean-12-en-28-oic acid [**3**], and a new sulfated triterpene, echinocystic acid-3-*O*-sodium sulfate [**4**], have been isolated from the stem bark of *Tetrapleura tetraptera*. Compound **3** was 100% lethal to *Biomphalaria glabrata* at 20 ppm, while **4** was not molluscicidal at the same concentration. In a forward mutation assay utilizing *Salmonella typhimurium* strain TM677, *T. tetraptera* stem bark extracts were found to be mutagenic in the absence of a metabolic activating system (S-9). An MeOH extract of the fruit exhibited weak mutagenic activity only in the presence of S-9. The stem bark isolates, which included aridanin [**1**], 3-*O*-(2′-acetamido-2′-deoxy-β-D-glucopyranosyl)echinocystic acid [**2**], and compounds **3** and **4**, were not mutagenic, either with or without metabolic activation.

Prior to our work on *Tetrapleura tetraptera* (Schum. et Thonn.) Taub. (Leguminosae), four molluscicidal olean-12-ene triterpene glycosides, 3-*O*-(2′-acetamido-2′-deoxy-β-D-glucopyranosyl)olean-12-28-oic acid [**1**] (aridanin), 3-*O*-(2-acetamido-2′-deoxy-β-



1 $R_1=R_2=H$
2 $R_1=OH$, $R_2=H$
3 $R_1=H$, $R_2=$β-D-glucopyranosyl



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[1]Parts of this study were derived from the Ph.D. Thesis of O.N., University of Illinois at Chicago (1992).

glucopyranosyl)-16α-hydroxyolean-12-en-28-oic acid [**2**], 3-*O*-[β-D-glucopyranosyl-(1″-6)-2′-acetamido-2′-deoxy-β-D-glucopyranosyl]olean-12-en-28-oic acid [**3**], and 3-*O*-[β-D-galactopyranosyl-(1″-6)-2′-acetamido-2′-deoxy-β-D-glucopyranosyl]olean-12-en-28-oic acid, were reported from the fruits of this plant (1,2). The isolation of an additional molluscicidal triterpene glycoside, 3-*O*-[β-D-glucopyranosyl-(1″-6′)-β-D-glucopyranosyl]-27-hydroxyolean-12-en-28-oic acid, was reported recently (3) following further investigation of the fruits of *T. tetraptera*. We initially investigated the stem bark of this plant for its molluscicidal activity against *Biomphalaria glabrata*. Consistent with previous work (2), fractionation of the *n*-BuOH-soluble portion of an MeOH extract of the stem bark led to the isolation of two active triterpene glycosides, **1** and **2** (4). In continuation of our study on the stem bark constituents we have isolated an additional known triterpene glycoside **3**, and a new sulfated triterpene, echinocystic acid-3-*O*-sodium sulfate [**4**]. In this paper we report the isolation, structure elucidation, and molluscicidal activity of these compounds. As an additional step in the safety evaluation of *T. tetraptera*, we have also evaluated the mutagenic potential of extracts from the stem bark and fruits, and of compounds 1–4, using *Salmonella typhimurium* strain TM677.

The *n*-BuOH-soluble portion of the MeOH extract of the stem bark was fractionated by cc to afford aridanin [**1**]. Further purification of the more polar fractions led to the isolation of compounds **2–4**. Compounds **1** and **2** were identified as described previously (4). The structures of compounds **3** and **4** were determined by analysis of their ir, ms, ^{1}H- and ^{13}C-nmr spectra, and in the case of **3**, by comparison of its spectral data with those reported previously by Maillard *et al.* (2). The eims and fabms of compound **4** did not give any molecular ion, but fabms gave a fragment ion at *m/z* 551. In addition to two bands at 3468 cm^{-1} (OH) and 1674 cm^{-1} (COOH), the ir spectrum of **4** showed a strong broad band at 1251–1200 cm^{-1} (-SO_3Na). ^{1}H- and ^{13}C-nmr spectra of **4** clearly showed that the compound was a triterpene of the olean-12-ene group (5), and the resonances were very close to those of the aglycone moiety of compound **2** (2,4). Six singlets due to seven methyl groups were observed in the upfield region of the ^{1}H-nmr spectra, whereas in the downfield area were two broad singlets, at δ 5.29 and 4.46, and two signals at δ 3.94 and 3.00. Based on ^{1}H- ^{13}C HETCOR spectral analysis, the two most downfield proton signals at δ 5.29 and 4.46 were assigned to H-12 and H-16, respectively, and the signals at δ 3.94 and 3.00 were assigned to H-3 and H-18, respectively. ^{13}C nmr showed signals due to thirty carbons, which included a carboxyl carbon signal at δ 181.13 (C-28), olefinic carbons at δ 123.24 (C-12) and 144.98 (C-13), and two carbinolic carbon signals at δ 75.15 and 87.65 which were assigned to C-16 and C-3, respectively. These assignments were confirmed by selective INEPT (6) results, when the signals of C-1 (δ 39.44), C-23 (δ 28.79), and C-24 (δ 16.97) were enhanced after the irradiation of H-3 at δ 3.94 ($^3J_{CH}$=6 Hz). Likewise, the irradiation of H-18 at δ 3.00 ($^3J_{CH}$=6 Hz) enhanced the signal of C-16 (δ 75.15) in addition to those of C-13 (δ 144.98), C-14 (δ 42.54), C-17 (δ 49.46), C-20 (δ (31.35), and C-28 (δ 181.13). In turn, when H-16 (δ 4.46) was irradiated ($^3J_{CH}$=5 Hz), the signals of C-13, C-14, and C-18 (δ 41.96) were enhanced, confirming the C-16 signal assignment. Examination of the H-3 and C-3 chemical shifts (δ 3.94 and 87.65, respectively) revealed that the hydroxyl group at C-3 was substituted. However, both ^{1}H and ^{13}C nmr did not reveal any signals due to a sugar moiety or any other substituent. Since fabms showed a fragment peak at *m/z* 551, it was possible that C-3 was substituted with an inorganic substituent. Indeed, qualitative element indentification of **4**, by energy-dispersive X-ray spectrometry, revealed the presence of sodium and sulfur. Thus the fragment ion at *m/z* 551 resulted from the loss of a sodium atom ([M−Na]$^-$). The eims gave the highest mass at *m/z* 436 [M−138]$^+$, which resulted

from the rapid loss of water molecule (18 amu) and the C-3 substituent (120 amu). The loss of 120 amu is consistent with the loss of a fragment composed of sodium and the sulfate group. From this it could be deduced that C-3 was substituted with a sodium sulfate moiety, as evidenced by the downfield shift of H-3 (+0.6 ppm) and C-3 (+ 9 ppm) when compared with other olean-12-enes having a free hydroxyl group at C-3 (7,8). The chemical shift of C-16 at 75.15 ppm indicated that the 16-hydroxyl group was α-oriented. Furthermore, in a ^{1}H-^{1}H NOESY experiment H-16 was correlated with H-15β, which in turn was correlated with the β-oriented Me-26, indicating that the 16-OH was α-oriented. The substituent at C-3 was determined to be β-oriented based on the correlation observed between H-3 and the α-oriented Me-23. Thus, the structure of this new compound was established as echinocystic acid-3-*O*-sodium sulfate [**4**].

Compounds **3** and **4** were evaluated for molluscicidal activity against the schistosome-transmitting snail *B. glabrata*. Compound **3** exhibited 100% mortality at 20 ppm, while **4** was inactive at this concentration. However, **3** was less active when compared with compounds **1** and **2**, which gave the same response at 1.25 ppm and 15 ppm, respectively (2).

The stem bark and fruits of *T. tetraptera* are widely used in ethnomedicine (9,10). Additionally, there is a potential for the use of the fruit as a molluscicide in the future. Previous studies have demonstrated that fruit extracts were not toxic to domestic animals and some aquatic forms (11,12). However, the chronic effects of these extracts have not been studied. Thus, the mutagenic potential of the stem bark and fruit extracts and the isolates **1**–**4** from the stem bark was studied as an additional step in the safety evaluation studies of *T. tetraptera*. A forward mutation assay was carried out using *S. typhimurium* strain TM677, according to a procedure described previously (13–15). The results shown in Table 1 indicate that stem bark extracts were mutagenic, in a concentration-dependent manner, in the absence of a metabolic activating system (S-9). A dose-dependent bactericidal effect was also observed. However, no significant mutagenic or bactericidal activity was observed in the presence of metabolic activation. The results imply that the stem bark of *T. tetraptera* contains a compound (or combination of compounds) which is a direct-acting mutagen. Fruit extracts did not exhibit any significant mutagenic activity in the absence of metabolic activation. However, slight dose-dependent mutagenic and bactericidal effects were observed for the MeOH extract of the fruit, in the presence of the metabolic activating system. Consequently, there is a possibility that the fruits have a mutagen which requires metabolic activation. None of the isolates **1**, **2**, **3**, or **4** exhibited any significant mutagenic activity in the absence of the metabolic activating system (Table 2). Furthermore, neither isolates **1**, **2**, or **4**

TABLE 1. Evaluation of the Mutagenic Potential[a] of *Tetrapleura tetraptera* Extracts in the Absence (−S-9) and Presence (+S-9) of a Metabolic Activating System.

Concentration (mg/ml)	Extract							
	Bark −S-9	(MeOH) +S-9	Bark −S-9	(H_2O) +S-9	Fruit −S-9	(MeOH) +S-9	Fruit −S-9	(H_2O) +S-9
0.0	8.5 (100)	3.7 (100)	11.2 (100)	2.2 (100)	8.5 (100)	3.7 (100)	11.2 (100)	2.2 (100)
0.625	10.1 (100)	6.9 (100)	9.7 (100)	4.1 (100)	10.6 (100)	4.5 (100)	12.3 (98)	6.2 (100)
1.25	9.1 (86)	8.6 (100)	9.8 (96)	5.6 (100)	10.0 (100)	5.3 (100)	12.7 (81)	4.8 (100)
2.5	11.3 (50)	8.8 (100)	9.9 (100)	7.2 (100)	9.5 (100)	6.0 (88)	11.8 (82)	5.2 (100)
5.0	18.1 (24)	9.0 (100)	11.0 (68)	5.9 (100)	9.2 (100)	9.3 (56)	10.5 (95)	5.5 (100)
10.0	40.6 (16)	8.9 (100)	28.8 (29)	7.4 (50)	11.5 (90)	11.1 (35)	10.3 (94)	6.7 (100)

[a]The results are expressed as mutant fraction ($\times 10^5$). Numbers in parentheses indicate percent survival relative to controls (solvent only).

TABLE 2. Evaluation of the Mutagenic Potential[a] of the Isolates from *T. tetraptera* Stem Bark in the Absence (−S-9) and Presence (+S-9) of a Metabolic Activating System.

Concentration (mg/ml)	Compound[b]				
	1		2		3[c]
	−S-9	+S-9	−S-9	+S-9	−S-9
0.0	4.2 (100)	2.2 (100)	4.2 (100)	2.2 (100)	4.2 (100)
0.3125	4.5 (89)	2.5 (100)	4.9 (100)	3.2 (100)	4.1 (100)
0.625	5.6 (98)	2.9 (100)	5.4 (100)	3.1 (100)	4.2 (100)
1.25	4.1 (100)	3.2 (100)	5.4 (100)	2.6 (200)	4.0 (100)
2.5	3.3 (100)	2.8 (100)	4.7 (100)	2.9 (100)	3.1 (100)
5.0	4.2 (100)	4.0 (100)	4.7 (100)	4.6 (100)	5.0 (100)

[a]The results are expressed as mutant fraction ($\times 10^5$). Numbers in parentheses indicate bacterial percent relative to controls (solvent only).

[b]Compound **4** was neither mutagenic nor bactericidal in the absence (at 0.058, 0.58, and 5.8 mg/ml) and in the presence (at 0.5, 5.0, and 50.0 mg/ml) of S-9.

[c]Due to insufficient amount, compound **3** was tested only in the absence of metabolic activation.

exhibited any significant mutagenic activity in the presence of the metabolic activating system (**3** could not be tested due to a shortage of material). This indicates that the mutagenic activity of the stem bark, and possibly the fruit, could be due to compounds other than those obtained in our studies.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Nmr spectra were obtained in pyridine-d_4 or CD_3OD with TMS as an internal standard. ^{1}H-nmr spectra were recorded with a Varian XL-300 spectrometer operating at 300 MHz. ^{13}C-nmr spectra were obtained with Varian XL-300 (75.4 MHz) and Nicolet NMC-360 (90.8 MHz) spectrometers. The eims and fabms were obtained with Varian MAT-112S and Finnigan MT-90 spectrometers, respectively. Ir spectra were recorded with a Nicolet MX-1 FT-IR spectrophotometer. An energy-dispersive X-ray spectrum for qualitative element analysis was obtained using a scanning electron microscope model JSM-35 (JEOL Ltd, Tokyo, Japan). Column chromatography was performed on Si gel 60 (70–230 mesh, E. Merck, Darmstadt, Germany). All solvents were spectral grade or redistilled before use.

PLANT MATERIAL.—The stem bark and fruits of *T. tetraptera* were collected in June 1987, in Mampong-Akwapim, Ghana, and identified by the staff of the Centre for Scientific Research in Plant Medicine, Mampong-Akwapim, Ghana. A voucher specimen is deposited in the John G. Searle Herbarium of the Field Museum of Natural History, Chicago, Illinois.

EXTRACTION AND ISOLATION.—The ground stem bark of *T. tetraptera* (3.5 kg) was extracted exhaustively and successively with petroleum ether, $CHCl_3$ and MeOH, at room temperature. After drying, the MeOH extract (310 g) was partitioned between H_2O (2 liters) and *n*-BuOH (5 liter). The *n*-BuOH-soluble residue (130 g) was fractionated further by vlc on Si gel (450 g). The column was eluted with mixtures of solvents of increasing polarity, starting with petroleum ether-$CHCl_3$ (1:1→0:100), then $CHCl_3$-MeOH (98:2→20:80), and ending with an MeOH wash. A total of 56 fractions (1 liter each) were collected and concentrated to a small volume (ca. 25 ml each). Aridanin [**1**] (877 mg) was obtained from fraction 34 following concentration, and by purification of the remainder of fraction 34 and fractions 35–38 (4.5 g) by vlc and flash chromatography on Si gel, using a $CHCl_3$/MeOH gradient. Fractions 35–38 yielded another compound, **2** (287 mg), which was crystallized from MeOH/Me_2CO. The combined fractions 39–46 (16.2 g) were purified by repeated vlc on Si gel (tlc grade, 200 g), using $CHCl_3$-MeOH-H_2O (80:20:5) as the eluent, to afford two white powdered compounds, **3** (90 mg) and **4** (195 mg).

Compound **4**.—White powder (MeOH/H_2O): mp 194–197°; ir (KBr) ν max (cm^{-1}) 3468 (OH), 2964 (CH, aliphatic), 1674 (COOH), 1471, 1386, 1251–1200, 1066, 965 ^{1}H nmr (CD_3OD, 300 MHz) δ 5.29 (1H, br s, H-12), 4.46 (1H, br s, H-16), 3.94 (1H, dd, J=11.8, 4.4 Hz, H-3)., 3.00 (1H, dd, J=14.1, 3.8 Hz, H-18, 1.38 (3H, s, Me-27), 1.04 (3H, s, Me-23), 0.97 (6H, s, Me-25 and Me-30), 0.88 (3H, s, Me-29), 0.83 (3H, s, Me-24), 0.80 (3H, s, Me-26); ^{13}C nmr (CD_3OD, 90.8 MHz) δ 181.13 (s, C-28), 144.98 (s, C-

13), 123.44 (d, C-12), 87.65 (d, C-3), 75.15 (d, C-16), 57.19 (d, C-5), 49.46 (s, C-17), 48.00 (d, C-9), 47.60 (t, C-19), 42.54 (s, C-14), 41.96 (d, C-18), 40.54 (s, C-8), 39.61 (s, C-4), 39.44 (t, C-1), 37.96 (s, C-10), 36.49 (t, C-15), 36.13 (t, C-21), 34.17 (t, C-7), 33.43 (q, C-29), 32.65 (t, C-22), 31.35 (s, C-20), 28.79 (q, C-23), 27.27 (q, C-27), 25.13 (t, C-2), 24.94 (q, C-30), 24.42 (t, C-11), 19.45 (t, C-6), 17.72 (q, C-26), 16.97 (q, C-24), 16.05 (q, C-25); fabms (negative ion mode) *m/z* $[M-Na]^-$ 551 (calcd for $C_{30}H_{47}O_7NaS$, 574); eims (70 eV) *m/z* (% rel. int.) $[M-H_2O-NaHSO_4]^+$ 436 (12), 421 (9), 392 (6), 375 (4), 347 (3), 299 (6), 246 (34), 203 (38), 190 (53), 131 (30), 81 (44), 41 (100). Elemental analysis calcd for $C_{30}H_{45}O_7SNa_3$, C 58.23, H 7.34, S 5.17; found C 58.21, H 7.34, S 5.17. Energy-dispersive X-ray spectrometry qualitative elemental analysis indicated presence of sodium (Na) and sulfur (S).

MOLLUSCICIDAL ASSAY.—The snails *B. glabrata* (albino Puerto Rican strain) were originally obtained through the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland. Since 1981, the snails have been maintained in dechlorinated, aged tap H_2O in the laboratory of one of us (T.O.H.). Those used in the assay had an average shell diameter of 12 mm. Tests were performed in duplicate, using five snails for each test, at an H_2O temperature of 21–22°. Extracts and compounds were initially dissolved in MeOH, then desired dilutions were made with dechlorinated tap H_2O so that the final MeOH concentration in test solutions did not exceed 1% v/v. The snails were exposed to the test compound by immersion in the solutions for a 24-h period; then they were rinsed three times with dechlorinated tap H_2O and left to recover in the same for a further 24 h, when deaths were recorded. Death was ascertained by examining immobilized snails under a dissecting microscope for the absence of heartbeat. Containers with solvent only (1% MeOH in dechlorinated tap H_2O) were used as negative controls.

PREPARATION OF EXTRACTS FOR MUTAGENICITY ASSAYS.—Dried, ground stem bark and fruits (50 g each) were extracted with either boiling H_2O or MeOH (2×200 ml each). The solvents were removed under reduced pressure to afford the bark extracts designated as BW (H_2O extract, 2.36 g) and BM (MeOH extract, 3.66 g), and the fruit extracts designated as FW (H_2O extract, 7.96 g) and FM (MeOH extract, 9.14 g).

MUTAGENICITY ASSAYS.—*S. typhimurium* strain TM677, carrying the "R-factor" plasmid pKM101, was used for the assessment of mutagenic activity. The forward mutation assays were performed according to a procedure described previously (13–15). The assays were conducted in the presence or absence of a 9000×g supernatant fraction (S-9) from the livers of Aroclor 1254-pretreated rats. Briefly, duplicate 0.98 ml reaction mixtures containing 1.0 mg of $NADP^+$, 1.0 mg of glucose-6-phosphate, 0.8 units of glucose-6-phosphate dehydrogenase, 0.67 mg of $MgCl_2$, the S-9 fraction, and approximately 7×10^6 bacteria, in logarithmic phase, were prepared in minimal essential medium (MEM). When metabolic activation was not required, only bacteria and MEM were mixed. After addition of the test substance, dissolved in 20 µl of DMSO, the mixtures were incubated at 37° for 2 h, after which the reaction mixture was quenched by addition of 4 ml phosphate-buffered saline. The bacteria were recovered by centrifugation, re-suspended, diluted as appropriate, and plated, in triplicate, in the presence or absence of 8-azaguanine. Plates were scored after 48–60 h growth period at 37°, and the mutant fraction was expressed as the average number of colonies observed on plates containing 8-azaguanine divided by the average number of colonies on plates not containing 8-azaguanine, after correcting for dilution factors. The latter value was used to define the percentage of bacteria surviving the treatment, relative to a control in which only an equivalent amount of solvent (DMSO) had been added. The spontaneous mutant fraction for this assay in our laboratory is $7.4\pm5\times10^{-5}$ (n=145). A compound is said to cause significant mutation if the induced mutant fraction is greater than or equal to two times the spontaneous mutant fraction.

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