

TRITERPENOID SAPONINS FROM *HEDYOTIS NUDICAULIS*

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Key Word Index—*Hedyotis nudicaulis*; Rubiaceae; nudicaucins A, B and C; triterpenoid saponin.

Abstract—Three new triterpenoid saponins, nudicaucins A, B and C and a known saponin guaiacin D have been isolated from the *n*-butanol extract of *Hedyotis nudicaulis*. The structures of nudicaucins A, B and C were elucidated to be β -D-glucopyranosyl 3-*O*-[β -D-galactopyranosyl(1 $\rightarrow$ 3) α -L-arabinopyranosyl]-30-norolean-12,20(29)-dien-28-oate, β -D-glucopyranosyl 3-*O*-[β -D-galactopyranosyl(1 $\rightarrow$ 3) α -L-arabinopyranosyl]oleanoate and β -D-glucopyranosyl 3-*O*-[α -L-rhamnopyranosyl(1 $\rightarrow$ 2) α -L-arabinopyranosyl(3 $\rightarrow$ 1) β -D-glucopyranosyl]oleanoate, respectively, on the basis of NMR spectroscopic analysis and comparison of the spectral data with those of known saponins. © 1998 Published by Elsevier Science Ltd. All rights reserved

INTRODUCTION

Hedyotis nudicaulis is used as a health tea for treatment of fever in South-East Asia. The plant is commonly found in various places of South-East Asia but the chemical components of the plant have not been examined yet. In our search for useful drug lead compounds in tropical plants, we have investigated the chemical constituents of the *n*-butanol extract of *H. nudicaulis*, and their biological properties. Various chromatographic purifications of the extract yielded four triterpenoid saponins, three new saponins named nudicaucins A (1), B (3), C (4), and a known compound, guaiacin D (2) which has been isolated from the leaves of *Guaiacum officinale* [1]. In this paper, we describe the isolation and the structure determination of nudicaucins A, B and C. The antibacterial activity of compounds 1, 2, 3 and 4 against *Bacillus subtilis* M45 (a recombination deficient mutant) and its parent strain, *B. subtilis* H17 are also discussed.

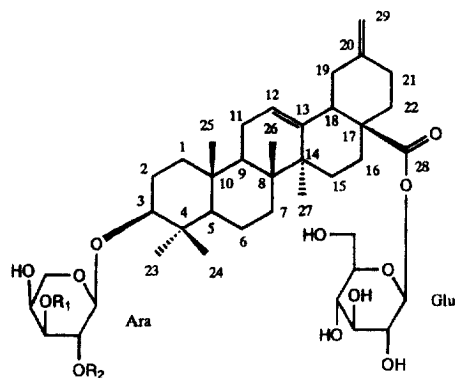
RESULTS AND DISCUSSION

Four saponins (1–4) were obtained as amorphous white powders after a series of chromatographic purifications from the *n*-BuOH extract of *Hedyotis nudicaulis*.

Compound 1 exhibited in the FAB-mass spectrum

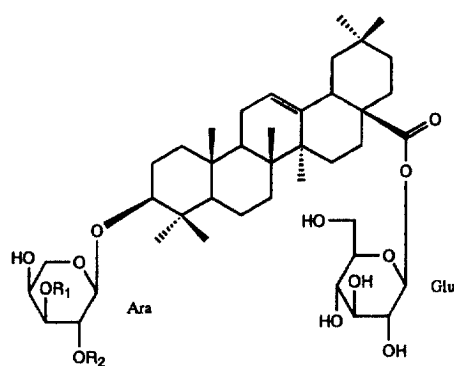
a pseudomolecular ion at m/z 919 $[M + Na]^+$ which shifted to m/z 935 $[M + K]^+$ and m/z 914 $[M + NH_3]^+$ upon addition of KCl or NH_4Cl , respectively. The mass spectrum also showed significant ion peaks at m/z 423 and 436 which allowed assignment of 30-norolean-12,20(29)-dien-28-oic acid for the aglycone structure of compound 1 [1]. The 1H NMR and ^{13}C NMR spectra of compound 1 supported this aglycone structure and, in addition, indicated the presence of three sugars: two hexoses and one pentose (anomeric protons: δ_H 4.73, 5.27 and 6.25, anomeric carbons: δ_C 95.8, 106.8 and 107.2, in pyridine- d_5) (Tables 1 and 2). The ^{13}C NMR spectrum of 1 was similar to that of guaiacin A [2], β -D-glucopyranosyl 3-*O*-[β -D-glucopyranosyl(1 $\rightarrow$ 3) α -L-arabinopyranosyl]-30-norolean-12,20(29)-dien-28-oate (Tables 1 and 2). However, the chemical shifts of the 3-*O*-terminal hexopyranosyl sugar of 1 (δ 106.2, 73.0, 74.7, 70.3, 76.8 and 62.6) were significantly different from the corresponding chemical shifts of the β -D-glucopyranosyl moiety of guaiacin A (δ_C 107.1, 75.3, 77.9, 71.1, 77.9 and 62.4). The observed 1H - 1H coupling constants of this sugar moiety ($\delta_{H1-H2} = 7.5$ Hz, $\delta_{H2-H3} = 9.8$ Hz, $\delta_{H3-H4} = 3.3$ Hz, and $\delta_{H4-H5} = ca$ 0 Hz) strongly suggested a galactopyranose structure. Extensive analyses of the 2D NMR spectra, including the HMBC spectrum, gave a conclusive sequence for the 3-*O*-diglycoside moiety to be β -D-galactopyranosyl (1 $\rightarrow$ 3)- α -L-arabinopyranose. Furthermore, anomeric protons of the α -arabinose in the sequence exhibited a correlation with the C-3 (δ_C 88.6) of the aglycone moiety. The anomeric proton of the other sugar, β -D-glucose (δ_H 6.25 and δ_C

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1 : R₁ = Gal R₂ = H

2 : R₁ = Glu R₂ = Rha



3 : R₁ = Gal R₂ = H

4 : R₁ = Glu R₂ = Rha

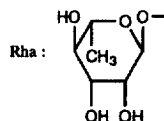
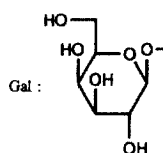


Table 1. ¹³C NMR chemical shifts of the aglycone moieties of compounds 1–4

Compound	1	1§	2	3	4
1	39.9	38.7	40.2	39.9	40.1
2	27.1	26.2	27.2	28.6	27.2
3	90.5	88.6	89.7	90.5	89.7
4	40.3	39.5	40.4	40.3	40.4
5	57.1	55.8	57.2	57.1	57.2
6	19.4	18.4	19.3	19.4	19.3
7	34.0	33.0	34.0	33.2	33.1
8	40.8	39.8	40.8	40.8	40.7
9	—	47.9	—	48.1	48.0
10	37.9	36.9	38.0	37.9	37.9
11	24.2	23.7	24.2	24.1	24.0
12	124.4	123.2	124.4	123.9	123.8
13	144.3	143.3	144.3	144.9	144.8
14	42.9	42.0	42.9	42.7	42.6
15	28.9	28.1	28.9	28.9	28.9
16	24.6	23.5	24.6	24.0	24.0
17	—	47.2	—	47.3	47.2
18	—	47.6	—	47.6	47.2
19	42.6	41.6	42.6	42.9	42.9
20	149.5	148.4	149.5	31.6	31.5
21	30.9	30.0	30.9	34.9	34.9
22	38.5	37.5	38.5	34.0	33.9
23	28.5	28.1	28.6	28.6	28.6
24	17.1	16.9	17.3	17.0	17.2
25	16.1	15.5	16.1	16.1	16.1
26	17.8	17.4	17.8	17.8	17.7
27	26.4	26.0	26.4	26.3	26.3
28	177.3	175.7	177.4	178.1	178.1
29	107.4	107.2	107.4	33.5	33.5
30	—	—	—	24.6	24.5

§Pyridine-ds.

Table 2. ¹³C NMR chemical shifts of the sugar moieties of compounds 1–4

	1	1§	2	3	4
Ara-1	106.2	107.2	105.2	106.2	105.1
2	72.2	71.8	75.3	72.2	75.2
3	84.0	84.1	82.2	84.0	82.2
4	69.8	69.2	68.6	69.8	68.5
5	66.6	66.9	64.7	66.6	64.6
Glu-1	—	—	104.3	—	104.3
2	—	—	73.9	—	73.9
3	—	—	78.0	—	78.0
4	—	—	71.1	—	71.1
5	—	—	78.0	—	78.0
6	—	—	62.4	—	62.4
Gal-1	107.1	106.8	—	107.1	—
2	73.9	73.1	—	73.0	—
3	74.7	75.1	—	74.7	—
4	70.3	70.1	—	70.3	—
5	76.8	77.0	—	76.8	—
6	62.6	62.3	—	62.6	—
Rha-1	—	—	102.0	—	102.0
2	—	—	72.1	—	72.1
3	—	—	72.1	—	72.1
4	—	—	73.9	—	73.8
5	—	—	70.3	—	70.3
6	—	—	18.0	—	18.0
28-Glu-1	95.8	95.8	95.8	95.8	95.7
2	73.0	74.0	75.0	74.0	75.0
3	78.3	78.7	78.3	78.4	78.3
4	71.1	71.1	71.2	71.2	71.2
5	78.7	79.2	78.7	78.8	78.7
6	62.4	62.2	62.4	62.5	62.4

§Pyridine-ds.

95.8), showed correlation with the carbonyl carbon of the aglycone (C-28, δ_C 175.7), indicating that this glucose bound to the carbonyl carbon through an ester linkage. Further comparison of the ^{13}C NMR spectrum of **1** with that of guaiacin D (**2** cited below) fully supported the structure of nudicaucin A to be β -D-glucopyranosyl 3-*O*-[β -D-galactopyranosyl(1 $\rightarrow$ 3)- α -L-arabinopyranosyl]-30-norolean-12,20(29)-dien-28-oate (Tables 1 and 2). The absolute configuration of the carbohydrates was provided based on the facts that only sole absolute forms of these sugars have been identified in the terpenoid saponins from plant origins and that guaiacin D (**2**) was isolated as a co-product from this plant.

The assignments of the carbon signals at C-21 and C-22 for the 30-norolean-12,20(29)-dien-28-oate are ambiguous in several references [1, 3]. The HMBSC spectrum of compound **1** gave unambiguous assignments for the signals of C-21 and C-22 (Table 1).

Compound **2** showed a pseudomolecular ion at m/z 1065 $[\text{M}+\text{Na}]^+$ which shifted to m/z 1081 upon addition of KCl. The ^1H NMR and ^{13}C NMR spectra suggested compound **2** to be a tetraglycoside of 30-norolean-12,20(29)-dien-28-oic acid. The ^{13}C NMR spectrum was consistent in all respects with that of guaiacin D, a constituent in the leaves of *Guaiacum officinale* [1] (Tables 1 and 2).

The FAB-mass spectrum of compound **3** showed the $[\text{M}+\text{Na}]^+$ ion at m/z 935, 16 mass units higher than that of **1**. Unlike compounds **1** and **2**, the ^1H NMR and ^{13}C NMR spectra of **3** exhibited seven singlet methyls and seven methyl carbon signals in the aglycone moiety and the presence of three sugars (anomeric carbons δ_C 95.8, 106.2 and 107.1, anomeric protons δ_H 5.36, 4.50 and 4.27) (Tables 1 and 2). These data, coupled with its mass spectral data, suggested an olean-12-ene-28-oic acid type saponin for **3** (Tables 1 and 2). All of the J values of the three anomeric protons in the ^1H NMR spectrum (8.1, 7.5 and 7.3 Hz) indicated β -glycosidic linkages. Furthermore, the spectra closely resembled those reported for guaiacin B [4], β -D-glucopyranosyl 3-*O*-[β -glucopyranosyl(1 $\rightarrow$ 3)- α -L-arabinopyranosyl]oleanate, and those of nudicaucin A (**1**) (Table 1). The ^1H NMR and ^{13}C NMR patterns of the sugar moieties were identical with those of **1** (Table 2). The structure of nudicaucin B (**3**) was thus determined to be β -D-glucopyranosyl 3-*O*-[β -D-galactopyranosyl(1 $\rightarrow$ 3)- α -L-arabinopyranosyl]oleanate.

Compound **4**, the fourth saponin, showed in the FAB-mass spectrum a pseudomolecular ion peak at m/z 1081 $[\text{M}+\text{Na}]^+$. A prominent fragment ion at m/z 439 indicated oleanic acid for the aglycone part of **4**. All the ^1H NMR and ^{13}C NMR signals of its aglycone moiety were consistent with the corresponding signals of **3**, while the signals of the sugar part and their splittings were the same as those of **2** (Tables 1 and 2). Thus, the structure of nudicaucin C (**4**) was determined to be β -D-glucopyranosyl 3-*O*-[α -

L-rhamnopyranosyl(1 $\rightarrow$ 2)- α -L-arabinopyranosyl(3 $\rightarrow$ 1)- β -D-glucopyranosyl]oleanate.

Since galactose has rarely been found as a sugar constituent of the oleanic acid type saponins [5], the constituent sugars of **1** and **2** were further confirmed by gas chromatographic analysis [6, 7]. Compounds **1** and **2** were hydrolyzed with 0.5 N HCl at 100°C and the sugars liberated were trimethylsilylated with *N*-trimethylsilylimidazole. Upon development in gas chromatography, the trimethylsilyl sugars were identified by a direct comparison of their retention times with those of the reference trimethylsilyl sugars. Retention times of the sugars obtained from compound **1** were consistent with those of glucose, arabinose and galactose, whereas those from compound **2** were in accord with those of glucose, arabinose and rhamnose. Their peak area intensities were consistent with the expected molar ratios of the sugars.

The above four saponins showed weak antibacterial activity against *Bacillus subtilis* M45 and H17. In this test, the activity of tetraglycoside saponins **2** and **4** were stronger than those of triglycoside saponins **1** and **3**.

EXPERIMENTAL

General

Mps were determined with a Yanaco MP-500 micro melting point apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were recorded on a JEOL EX-400 FTNMR spectrometer in CD_3OD or pyridine- d_5 solutions. FABMS spectra were recorded on JEOL JMS DX-303 spectrometer. Optical rotations were measured with JASCO DIP-370 polarimeter. TLC was performed on RP-18 F₂₅₄ (Merck) with 75% MeOH (System A) and Kieselgel G (Merck) with CHCl_3 :MeOH, 2:1 (System B).

Plant material

Hedyotis nudicaulis was collected in September, 1994 at Serdang Vegetation sandy area, Selangor, Malaysia, and voucher specimens are deposited in Institut Teknologi MARA and UPM, Malaysia. Identification of the plant specimen was performed by Prof. Ruth Kiew of the Biology Department, UPM, Malaysia.

Extraction and isolation

The collected whole plants were dried (weight, 750 g) and extract successively with petrol, CH_2Cl_2 , EtOAc and *n*-BuOH. The BuOH extract was concentrated under red. pres. to yield 4.12 g of crude extract. A part of the extract (2.18 g) was chromatographed on a column of Sephadex LH-20 developing with MeOH. The early eluted fractions were combined and concentrated, and the residue was chromatographed on reversed phase silica (C_{18}) with 80% MeOH. A mix-

ture of compounds **1** and **2** was eluted first (168 mg) followed by a mixture of compounds **3** and **4** (70 mg). Separation of these mixtures was performed by silica gel chromatography with CHCl_3 -MeOH (8:2, for the former mixture and 7:3, for the latter mixture) to yield pure compounds **1** (31 mg), **2** (62 mg), **3** (8 mg) and **4** (3 mg) after final purification with Sephadex LH-20 CC. A portion of compound **1** was crystallized from MeOH as white plates.

Nudicaucin A (1). White plates. mp 291.5–293° (dec.). TLC: R_f 0.25 (system A) and 0.15 (system B). $[\alpha]_D + 71.3^\circ$ (c 0.44, MeOH). $^1\text{H NMR}$ (CD_3OD): δ 0.79 (s, Me), 0.83 (s, Me), 0.94 (s, Me), 1.04 (s, Me), 1.17 (s, Me), 4.28 (d, $J = 7.3$ Hz, Ara-1), 4.50 (d, $J = 7.5$ Hz, Gal-1), 4.61 (br s, H-29), 5.32 (br t, $J = \text{ca } 1.0$ Hz, H-12), 5.36 (d, $J = 8.0$ Hz, 28-Glu-1).

Guaiacin D (2). Mp 254–258° (dec.). TLC: R_f 0.25 (system A) and 0.04 (system B). $[\alpha]_D + 22.8^\circ$ (c 0.41, MeOH). $^1\text{H NMR}$ (CD_3OD): δ 0.80 (s, Me), 0.85 (s, Me), 0.95 (s, Me), 1.02 (s, Me), 1.19 (s, Me), 1.21 (d, $J = 6.2$ Hz, Rha-6-Me), 4.49 (d, $J = 7.6$ Hz, Glu-1), 4.51 (d, $J = 7.2$ Hz, Ara-1), 4.61 (br s, H-29), 5.21 (d, $J = 1.2$ Hz, Rha-1), 5.32 (t, H-12), 5.36 (d, $J = 8.0$ Hz, 28-Glu-1).

Nudicaucin B (3). Mp 256–259° (dec.). TLC: R_f 0.17 (system A) and 0.15 (system B). $[\alpha]_D + 34.5^\circ$ (c 0.22, MeOH). $^1\text{H NMR}$ (CD_3OD): δ 0.80 (s, Me), 0.84 (s, Me), 0.91 (s, Me), 0.93 (s, Me), 0.95 (s, Me), 1.14 (s, Me), 4.27 (d, $J = 7.3$ Hz, Ara-1), 4.50 (d, $J = 7.5$ Hz, Gal-1), 5.36 (d, $J = 8.1$ Hz, 28-Glu-1), 5.25 (t, H-12).

Nudicaucin C (4). Mp 257–260° (dec.). TLC: R_f 0.17 (system A) and 0.04 (system B). $[\alpha]_D - 4.9^\circ$ (c 0.41, MeOH). $^1\text{H NMR}$ (CD_3OD): δ 0.79 (s, Me), 0.85 (s, Me), 0.91 (s, Me), 0.93 (s, Me), 0.94 (s, Me), 1.01 (s, Me), 1.15 (s, Me), 1.21 (d, $J = 6.2$ Hz, Rha-6-Me), 4.48 (d, $J = 7.7$ Hz, Glu-1), 4.49 (d, $J = 7.6$ Hz, Ara-1), 5.20 (br s, Rha-1), 5.25 (t, H-12), 5.37 (d, $J = 8.0$ Hz, 28-glu-1).

Acid hydrolysis and identification of sugars

Test compound (ca 5 mg) was heated with a mixture of 0.5 N HCl (0.5 ml) and EtOH (0.2 ml) at 100° for 1.5 h. The precipitated aglycone was collected by filtration and the filtrate was concentrated under red. pres. below 40°C. The residue was dissolved in EtOH (2 ml) and the soln was dried up again. This was repeated 3 $\times$ until the soln became neutral. The resulting residue was then treated with *N*-trimethylsilylimidazole (0.3 ml) for 1 h. After stopping the reaction by addition of H_2O (2 ml), the trimethylsilylated sugars were extracted 2 $\times$ with *n*-hex-

ane (2 ml each). The combined *n*-hexane was washed with H_2O (1 ml) and subjected to GC for determination of the sugars. GC conditions: column, Shimadzu GC-7A ($3\phi \times 2.1$ m); column temp., 170°; injection part temp., 300°; carrier gas, N_2 (30 ml min $^{-1}$). The identification of sugars was performed by comparison of their retention times with those of authentic sugars. Retention time (min): glucose, 17.8 and 11.7; galactose, 10.0 and 11.7; arabinose, 3.85; rhamnose, 4.05.

Bioassay

Antibacterial activity was determined by the paper disc assay against *Bacillus subtilis* M45 (recombination deficient mutant, rec $^-$) and H17 (wild, rec $^+$) strains. They were preincubated in nutrient broth, and the overnight culture was diluted with nutrient agar at around 40° which was cooled to solidify. Each sample was dissolved in MeOH (5 mg ml $^{-1}$) and 40 μ l of the soln was absorbed on an 8 mm paper disc. The dried discs were placed on the agar plate which was incubated at 30°. Diameters of the inhibition zones were calculated after 20 h. Inhibition zone: **2** and **4**, 14–16 mm; **1** and **3**, 10–11 mm.

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REFERENCES

1. Ahmad, V. U., Perveen, S. and Bano, S., *Phytochem.*, 1990, **29**, 3287.
2. Ahmad, V. U., Perveen, S. and Bano, S., *Planta Med.*, 1989, **55**, 307.
3. Ikuta, A. and Itokawa, H., *Phytochem.*, 1988, **27**, 3809.
4. Wang, H.-B., Yu, D.-Q., Liang, X.-T., Watanabe, N., Tamai, M. and Omura, S., *Planta Med.*, 1989, **55**, 303.
5. Nagao, T., Iwase, Y. and Otake, H., *Chem. Pharm. Bull.*, 1993, **41**, 1562.
6. Lemmich, E., Cornett, C., Furu, P., Jorstian, C. L., Knudsen, A. D., Olsen, C. E., Salih, A. and Thilborg, S. T., *Phytochem.*, 1995, **39**, 63.
7. Castro, V. H., Ramirez, E., Mora, G. A., Iwase, Y., Nagao, T., Okabe, H., Matsunaga, H., Katano, M. and Mori, M., *Chem. Pharm. Bull.*, 1997, **45**, 349.