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Daphcalycinosidines A and B, new iridoid-alkaloids from *Daphniphyllum calycinum*

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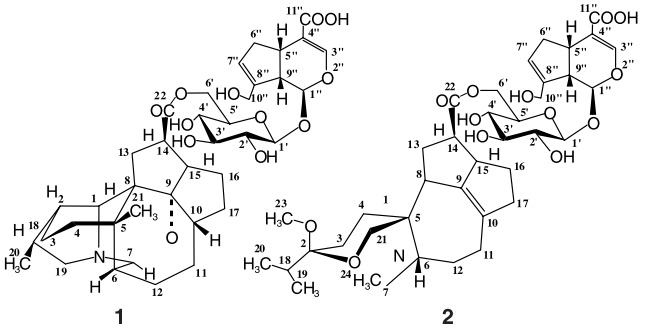
Abstract—Three new alkaloids, daphcalycinosidines A (**1**) and B (**2**) and daphcalycic acid (**3**) have been isolated from the seeds of *Daphniphyllum calycinum*. The structures and relative stereochemistries were determined on the basis of spectral studies including 2D NMR, mass spectrometry and chemical transformations. Structures **1** and **2** are characterized by an iridoid glucoside moiety linked to new *Daphniphyllum* alkaloid moieties.

Daphniphyllum calycinum Benth. (Daphniphyllaceae) is a shrub native to North Vietnam and China, where it is used in folk medicine for wound healing and as an antiinflammatory remedy, but it is also known as a poisonous plant.[1] As part of our search for plant-derived agents possessing cytotoxic activity against tumour cells, we examined *D. calycinum* and previously reported the isolation from its stem bark of the alkaloid, daphcalycine possessing a heptacyclic fused ring system.[2] Recently, Morita et al. described cytotoxic hexacyclic alkaloids from this species.[3]

The *Daphniphyllum* genus has yielded a series of more than 40 alkaloids forming a special group termed *Daphniphyllum* alkaloids possessing unique ring systems: their carbon skeleton is derived from squalene, has diverse and complex polycyclic structures and more than seven skeletal types have been defined.[4]

Investigation of the constituents of the seeds of *D. calycinum* yielded in addition to classical *Daphniphyllum* alkaloids, a new type with an iridoid glucoside moiety. The recent characterization of a similar type of compound from *D. humile*[5] prompts us to describe the new iridoid-alkaloids, daphcalycinosidines A **1** and B **2**, isolated from the seeds of *D. calycinum*, together with daphcalycic acid **3**, a new compound related to daphcalycine[2] and geniposidic acid **4**.[6]

The seeds of *D. calycinum* collected in North Vietnam were ground, defatted with cyclohexane and then extracted with MeOH. The methanolic extract was fractionated by reversed phase (RP2) and silica gel column chromatography, and fractions were finally purified by preparative thin layer chromatography on silica gel to yield daphcalycinosidines A **1**, B **2**, daphcalycic acid **3** and geniposidic acid **4**. Compounds **1**–**3** gave a positive reaction with the Dragendorff reagent.



1 **2**

Keywords: *Daphniphyllum calycinum*; Iridoid-alkaloid; *Daphniphyllum*-alkaloid; Structure determination; NMR.
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Daphcalycinosidine A **1**, was obtained as a colorless solid, mp 134–136 °C (MeOH), which was optically active $[\alpha]_D^{22}$ −16 (*c* 0.6, MeOH). The ESI-TOF spectrum showed the protonated molecular ion $[M+H]^+$ at *m/z* 714 and the HR(+)FABMS established for this ion the molecular formula $C_{38}H_{52}NO_{12}$ (*m/z* 714.3490; calcd: 714.3502). The ^{13}C NMR data (Table 1) depicted 38 carbon signals distributed into 6 sp^2 carbons (two carbonyls at δ_C 176.1 and 175.9 and four ethylenic, two of which were methines) and 32 sp^3 carbons due to 2 methyls, 11 methylenes, 16 methines and 3 quaternary carbons. Of the 14 degrees of unsaturation, 10 were

Table 1. ^1H (400.13 MHz) and ^{13}C (75.43 MHz) NMR data for daphcalycinosidines A (**1**) and B (**2**) (CD_3OD; d: doublet, h: hept; m: multiplet; p: pent; s: singlet)

C no	**1**			**2**		
	δ_C	δ_H multiplicity, *J* (Hz)	HMBC with H	δ_C	δ_H multiplicity, *J* (Hz)	HMBC with H
1a	62.3	3.81 d 7.4	7-13a/b-19a	57.5	3.12 d 13.0	7a-13a/b-24
1b	—	—		—	3.08 d 13.0	
2	38.6	2.34 m	1-4a/b-18-19a-20	102.3	—	18-19-20-21a/b-23
3a	19.2	1.71 m	1-2-4a/b-18	23.0	1.75 ddd 14.0; 4.5; 4.5	4b-18
3b	—	1.63 m		—	1.43 ddd 14.0; 4.5; 4.5	
4a	44.6	1.56 m	2-3a/b-21	23.3	1.98 ddd 13.7; 4.5; 4.0	21a/b
4b	—	1.56 m		—	1.73 m	
5	35.0	—	1-4a/b-7-12a/b-13a-21	37.2	—	1-3b-7a-13a-21b
6	47.0	2.69 br dd 7.5; 7.5	7-12a/b	33.6	2.55 m	7b-21a
7a	87.6	4.72 br s	1-12a/b-19b	55.3	3.48 dd 13.5; 3.5	1-24
7b	—	4.72 br s		—	3.39 dd 13.5; 5.5	
8	48.2	—	1-4a/b-13a/b-21	46.8	—	13a/b
9	97.0	—	1-7-13b-15-17a	143.3	—	1-11b-14-16a-17b
10	47.8	2.32 p 8.5	11a/b-12a-17b	138.3	—	16a-17b
11a	26.0	1.77 m	10-12a/b-17a/b	26.4	1.78 m	10-12a/b-17a/b
11b	—	1.61 m		—	2.33 m	
12a	25.2	2.05 ddd 13.1; 9.3; 9.3	7-11a/b	26.6	2.48 m	7b
12b	—	1.71 m		—	2.35 m	
13a	30.3	2.17 dd 13.2; 12.5	1-14-15	39.8	2.76 dd 15.5; 3.1	
13b	—	1.85 dd 13.2; 7.0		—	1.75 dd 15.5; 9.0	
14	45.4	3.37 ddd 12.5; 11.0; 7.0	13a/b-15-16a/b	43.2	2.99 ddd 10.0; 9.0; 3.0	13a/b
15	50.0	2.83 ddd 11.0; 9.5; 9.5	14-16a/b-17a/b	55.7	3.62 m	16a-17b
16a	29.4	1.90 m	14-15-17a/b	29.4	1.90 ddd 10.8; 6.6; 2.4	17b
16b	—	0.97 dddd 12.8; 12.8; 7.9; 6.9		—	1.40 ddd 10.8; 8.4; 2.2	
17a	37.2	1.78 m	10-11b-16a/b	43.9	2.65 m	
17b	—	1.30 m		—	2.38 m	
18	38.1	2.52 m	2-19a/b-20	32.5	2.08 h 7.0	19a/b-20
19a	54.7	3.16 dd 9.4; 7.9	1-2-18-20	17.6	0.94 d 7.0	18-20
19b	—	2.85 dd 9.4; 9.4		—	—	
20	13.0	1.11 d 6.9	18-19b	16.7	0.86 d 7.0	18-19
21a	26.8	1.22 s	1-4a/b	63.3	4.09 d 12.5	1-4a/b
21b	—	—		—	3.77 dd 12.5; 2.7	
22	175.9	—	6′a/b-13a-14	175.7	—	6′a/b-13a/b-14
23	—	—		47.0	3.20 s	
24	—	—	—	45.9	2.84 s	1-7b
1′	100.2	4.70 d 7.9	1″-2′	100.0	4.71 d 7.9	1″-2′-3′-6′b
2′	74.9	3.24 dd 9.1; 7.9	1′-3′-4′	74.8	3.23 dd 9.2; 7.9	1′-3′-4′
3′	77.6	3.42 dd 9.1; 8.5	1′-2′-4′-5′	77.6	3.40 dd 9.2; 9.0	1′-2′-4′
4′	71.6	3.33 dd 9.7; 8.5	3′-5′-6′a/b	71.6	3.30 dd 9.0; 9.0	3′-5′-6′a
5′	75.6	3.46 ddd 9.7; 6.0; 2.0	1′-4′-6′a/b	75.8	3.48 ddd 9.0; 6.4; 2.0	1′-3′-4′-6′a/b
6′a	64.6	4.45 dd 11.9; 2.0	4′-5′	64.5	4.48 dd 12.0; 2.0	4′
6′b	—	4.16 dd 11.9; 6.0		—	4.14 dd 12.0; 6.4	
1″	98.1	4.94 d 7.9	1′-5″-9″	98.2	5.00 d 8.0	1′-3″-5″-9″
3″	148.8	7.25 d 1.3	1″-5″	151.8	7.43 br d 1.2	1″-5″
4″	118.9	—	5″-6″b-9″	114.5	—	3″-5″
5″	38.0	3.24 ddd 8.0; 8.0; 8.0	1″-3″-6″-7″-9″	37.3	3.20 ddd 8.0; 8.0; 8.0	1″-3″-7″-9″
6″a	40.3	2.88 ddd 15.5; 8.0; 8.0	5″-7″-9″	40.1	2.86 ddd 15.0; 8.0; 8.0	5″-7″-9″
6″b	—	2.03 ddd 15.5; 8.0; 8.0		—	2.06 ddd 15.0; 8.0; 8.0	
7″	128.7	5.77 s	6″a/b-9″-10″a/b	128.9	5.80 s	6″b-9″-10″a/b
8″	145.2	—	1″-6″a/b-7″-9″-10″a/b	145.0	—	1″-6″b-7″-9″-10″
9″	47.0	2.69 dd 7.5; 7.5	1″-5″-6″a -7″-10″a/b	46.9	2.70 m	1″-5″-7″
10″a	61.7	4.25 dd 12.3; 1.1	7″	61.6	4.26 dd 13.7; 1.0	7″
10″b	—	4.20 dd 12.3; 1.7		—	4.24 dd 13.7; 1.8	
11″	176.1	—	3″-5″	172.0	—	3″-5″

The δ values for multiplets were measured on the 2D spectra.

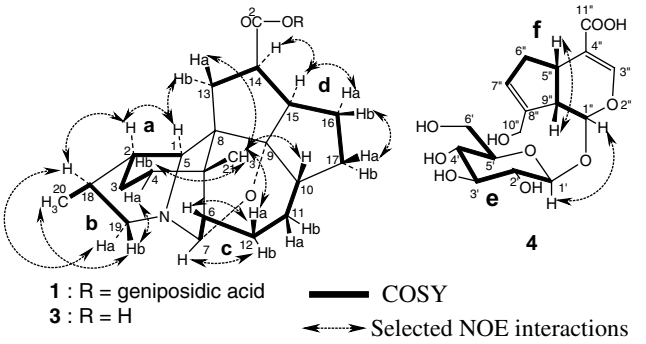

Figure 1. Selected 2D NMR correlations (COSY and NOESY) for daphcalycinosidine A (**1**, R = geniposidic acid), daphcalycic acid (**3**, R = H) and geniposidic acid (**4**).

assigned to 10 rings. The chemical shifts of the methine carbons at δ_C 100.2 and 98.1 suggested they were hemiketals. Methylenes at δ_C 61.7 and 64.6 were linked to an oxygen and that at 54.7 to a nitrogen. The chemical shifts of the methine at δ_C 62.3 suggested it bore a nitrogen atom and that at δ_C 87.6 a nitrogen and an oxygen. The quaternary carbon at δ_C 97.0 was linked to an oxygen.

Analysis of the ^1H–^1H COSY and HSQC spectra allowed the six spin systems (**a**–**f**) marked in bold line on Figure 1, to be defined in addition to isolated carbons: **a** (C-1 to C-4), **b** (C-18 to C-20), **c** (C-7 to C-10 via C-6 and C-12), **d** (C-13 to C-17), **e** (C-1′ to C-6′) and **f** (C-9″ to C-5″ and C-1″, and C-6″ to C-5″ and C-7″). The proton spin system **e**, together with the chemical shift values of the related carbons (δ_C 100.2, 74.9, 77.6, 71.6, 75.6 and 64.6) was typical of a glucose moiety. The anomeric proton at δ_H 4.70 (H-1′) of this moiety had a large coupling ($J = 7.9$ Hz) indicating that it was β-glucose. HMBC correlations allowed the characterization of a de-*O*-methylgenipin structure unit: protons H-1″, H-3″, CH_2-6″, H-7″ and H-9″ were connected to C-5″ (Table 1 and Fig. 1). The methylene protons at δ_H 4.20 and 4.25 (CH_2-10″) gave cross-peaks with C-7″, C-8″ and C-9″. Finally, the carbonyl at δ_C 176.1 was correlated with both protons H-3″ and H-5″. From these data was deduced the presence of a de-*O*-methyl genipin moiety. In the NOESY spectrum, H-9″ showed only strong correlation with H-5″, suggesting they were *cis* disposed, and thus that the relative configuration was the same as genipin. The anomeric proton (H-1′) of the glucose moiety showed HMBC correlations with C-1″ of the de-*O*-methyl genipin moiety and reciprocally the proton at δ_H 4.94 (H-1″) was correlated to C-1′: this linkage formed a geniposidic acid substructure.

The second substructure of **1** was identified as de-*O*-methyldaphcalycine or daphcalycic acid: HMBC correlations were observed between the carbon at δ_C 97.0 (C-9) and protons C*H*-13b, H-15, C*H*-17a and also with the methine protons at δ_H 3.81 (H-1) and 4.72 (H-7), this latter correlation being through the oxygen atom. The methine protons H-7 gave HMBC correlations through C-6 to protons at C-12 and through the nitrogen atom to protons at C-19. Correlations for H-1, CH_2-4, CH_2-13 and CH_3-21 to C-5 and C-8, and for H-7 and CH_2-12 to C-5 allowed identification of a cyclohexane ring substituted at C-5 by the methyl singlet (C-21). The carbonyl at δ_C 175.9 was identified as C-22 as it correlated with CH_2-13 and H-14, and was linked to the glucose at C-6′ of the geniposidic acid, because strong HMBC cross-peaks were apparent with CH_2-6′ protons. The NOESY spectrum confirmed the identity of the alkaloid moiety, including the relative stereochemistry, with that of daphcalycine: H-1 gave strong cross-peaks with H-2, H-13b, H-14 and H-15. The other following correlations were also depicted: H-14 with H-15; CH_3-21 with H-4b, H-10, H-12a and H-13a; H-7 with H-12b; H-18 with H-19a, and CH_3-20 with H-19b, which was in turn correlated with H-4a. This latter correlation suggested an equilibrium between the chair and boat conformations for the cyclohexane ring bearing CH_2-4. Main fragmentations observed in the ESI-TOF MS–MS of **1** were at m/z 520 and 502 corresponding to the cleavage at ether bonds between de-*O*-methylgenipin and glucose and at m/z 358 and 340 corresponding to that of the ester bond between the glucose and daphcalycic acid moieties. Saponification of **1** (1 mg) with aqueous KOH (1%) at room temperature for 2 h afforded, after acidification and purification by TLC, daphcalycic acid (0.3 mg) and geniposidic acid (0.2 mg) identical with **3** and **4**, respectively.

Compound **3**, a colorless solid, mp 124–125 °C (MeOH), was optically active $[\alpha]_D^{22}$ −33 (c 0.3, MeOH) and had a quasimolecular ion $[M+H]^+$ at m/z 358 in the ESI-TOFMS. By HR(+)FABMS the molecular formula $C_{22}H_{32}NO_3$ was determined for $[M+H]^+$ (m/z obsd: 358.2377, calcd: 358.2384). The ^{13}C NMR spectrum depicted signals for 22 carbons: 2 methyls, 8 methylenes, 8 methines and 4 quaternary carbons among which one sp^2 at 182.0 was assigned to a carboxylic acid group.[7] Examination of the ^1H–^1H COSY, HSQC and HMBC spectra allowed the determination of the same structure as described above for the alkaloid moiety of **1**: this structure corresponded to that of the de-*O*-methyl derivative of daphcalycine. NOESY correlations in **3** indicated the same relative configuration as daphcalycine and the alkaloid moiety of **1**: this new compound **3** was named daphcalycic acid.

Compound **4** was a microcrystalline solid, mp 138–140 °C (MeOH) and was optically active $[\alpha]_D^{22}$ +3 (c 0.1, MeOH). The ESI-TOF mass spectrum showed the cationic quasimolecular ion $[M+Na]^+$ at m/z 397, which was in agreement, together with NMR data, with the molecular formula $C_{16}H_{22}O_{10}$ ($M = 374$) for **4**. Analysis of the 2D NMR spectra allowed the identification of **4** as geniposidic acid.[6,8]

Daphcalycinosidine B, **2** formed colorless crystals, mp 201–202 °C (MeOH), and was optically active $[\alpha]_D^{22}$ +6 (c 0.3, MeOH). The molecular formula $C_{40}H_{58}NO_{13}$ was determined for $[M+H]^+$ from its HR(+)FABMS where the protonated quasimolecular ion $[M+H]^+$ was observed at m/z 760.3917 (calcd: 760.3909). The ^{13}C NMR spectrum depicted 40 carbons and of the 13 degrees of unsaturation of the molecule, five were

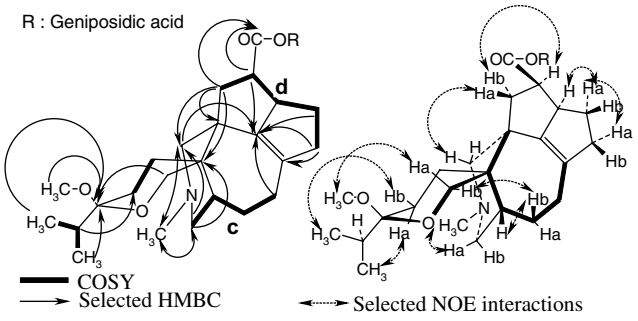

Figure 2. Selected 2D NMR correlations (left: COSY and HMBC, right: NOESY) for the alkaloid moiety of daphcalycinosidine B (**2**).

assigned on the basis of this spectrum to two carbonyls (δ_C 175.7 and 172.0) and three double bonds (Table 1). Many spectral similarities with compound **1** were apparent from the ^1H, ^{13}C and ^1H–^1H COSY spectra, especially spin systems **e** and **f**, characteristic of the geniposidic acid moiety, which were identical.

The main differences between **2** and **1** were the presence of a methoxyl (δ_H 3.20), a *N*-methyl (δ_H 2.84), an isopropyl (δ_H 0.86 d, 0.94 d and 2.08 h) instead of a methyl doublet, an additional tetrasubstituted double bond (δ_C 138.3 and 143.3) and an additional ketal carbon (δ_C 102.3); the singlet methyl-21 in **1** was changed into an oxymethylene (δ_C 63.3). The **c** and **d** spin systems of the alkaloid moiety of **1** were present and the structure of this part resulted from the observation in the HMBC spectrum of correlations between C-8 (δ_C 46.8) and CH_2-13, between C-5 (δ_C 37.2) and CH_2-1, C*H*-3b, CH_2-7, CH_2-13 and CH_2-21. The methoxyl group (δ_H 3.20) was linked to C-2 from HMBC correlations observed with C-2 (102.3); C-2 was in turn correlated to CH_3-19, CH_3-20, CH_2-21 and CH-18 (Table 1 and Fig. 2). The *N*-methyl-24 (δ_C 45.9) was correlated to CH_2-1 and CH_2-7. Finally, the quaternary sp^2 carbon at δ_C 143.3 (C-9) was correlated to CH_2-1, CH_2-11, CH_2-16, CH_2-17 and CH-14, whereas that at δ_C 138.3 (C-10) was correlated to CH_2-16. The carbonyl at δ_C 175.7 (C-22), assigned from its cross-peaks with protons at C-13 and C-14, was correlated to CH_2-6′ through an oxygen atom and C-1′ was correlated to H-1″ and reciprocally C-1″ to H-1′, allowing the linkage of the three substructures as shown in structure **2**. The relative stereochemistry of the alkaloid moiety resulted from analysis of the NOESY spectrum. Strong NOEs were observed between H-13b and H-14, H-14 and H-15, H-15 and H-16a, H-16a and H-17a, which indicated all these protons to be on the same side of the bi-pentacyclic ring system. Another series of NOEs was observed between H-13a and H-21a, H-21a and OCH_3-23, and between H-21b and H-12b, H-12b and H-6, H-6 and H-7b, CH_3-19 and H-3a and finally between CH_3-20 and H-3b, indicating the relative stereochemistry for the two spiro-heterocyclic rings to be as indicated in Figure 2.

Daphcalycinosidines A, **1** and B, **2** and daphcalycic acid, **3** were not found to be significantly cytotoxic against human nasopharynx carcinoma KB cells ($IC_{50} > 10\,\mu g/mL$). Daphnezomine P, which has structural similarities with compound **2** has been shown to be moderately cytotoxic against murine lymphoma L1210 cells.[5]

Acknowledgements

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7. NMR data for daphcalycic acid (**3**) (CD_3OD): ^{13}C (75.43 MHz) (δ): 62.2 (1), 38.9 (2), 19.3 (3), 44.9 (4), 35.1 (5), 48.1 (6), 87.6 (7), 48.5 (8), 96.7 (9), 47.8 (10), 26.3 (11), 25.3 (12), 31.8 (13), 48.7 (14), 50.4 (15), 29.3 (16), 37.4 (17), 38.3 (18), 54.8 (19), 13.2 (20), 26.9 (21), 182.0 (22). ^1H (400.13 MHz): 3.80 (d, *J* 7.2 Hz, H-1), 2.28 (brddd, *J* 7.5, 7.5, 7.2 Hz, H-2), 1.73 (dddd, *J* 15.0, 15.0, 15.0, 7.5 Hz, H-3a), 1.60 (dddd, *J* 15.0, 3.5, 3.5, 2.0 Hz, H-3b), 1.57 (m, H-4a), 1.51 (m, H-4b), 2.07 (m, H-6), 4.56 (s, H-7), 2.37 (m, H-10), 1.73 (m, H-11a), 1.63 (m, H-11b), 2.05 (m, H-12a), 1.68 (m, H-12b), 2.15 (dd, *J* 13.2, 12.8 Hz, H-13a), 1.74 (dd, *J* 13.2, 9.0 Hz, H-13b), 3.15 (ddd, *J* 12.8, 11.3, 7.0 Hz, H-14), 2.81 (ddd, *J* 12.8, 12.0, 7.5 Hz, H-15), 1.90 (m, H-16a), 1.28 (m, H-16b), 1.77 (m, H-17a), 1.28 (m, H-17b), 2.48 (dddq, *J* 11.9, 7.7, 7.5, 7.0 Hz, H-18), 3.02 (dd, *J* 8.9, 7.7 Hz, H-19a), 2.78 (dd, *J* 11.9, 8.9 Hz, H-19b), 1.10 (d, *J* 7.0 Hz, H-20), 1.20 (s, H-21).
8. NMR data for geniposidic acid (**4**) (DMSO-d_6): ^{13}C (75.43 MHz) (δ): 95.7 (1), 146.4 (3), 117.0 (4), 36.4 (5), 38.7 (6), 125.5 (7), 144.7 (8), 46.1 (9), 59.7 (10), 169.5 (11), 98.4 (1′), 73.4 (2′), 77.2 (3′), 70.0 (4′), 76.7 (5′), 60.9 (6′).