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Daphcalycine, a Novel Heptacycle Fused Ring System Alkaloid from *Daphniphyllum calycinum*

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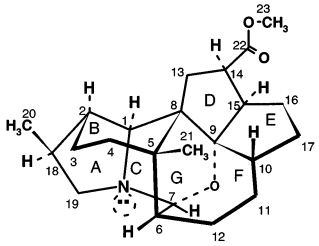
A novel *Daphniphyllum* alkaloid, daphcalycine (**1**), was isolated together with known daphnicyclidin D (**2**) from the stem bark of *Daphniphyllum calycinum*. The highly condensed polycyclic structure, established by spectral analysis, possessed an unusual framework: a central quinuclidine like tricycle produced by fusion of a piperidine, a tetrahydropyran, and an oxazine ring in turn condensed to surrounding three penta-, one hexa-, and one hepta-membered rings. The relative configuration of 11 carbon stereocenters of **1** was elucidated on the basis of NOESY.

Introduction

Daphniphyllum alkaloids possess highly complex polycyclic structures.[1] Nearly 40 alkaloids have been isolated from oriental trees of the genus *Daphniphyllum*, Daphniphyllaceae. The remarkably varied structures were classified into six main types of nitrogen heterocyclic skeletons. Radioactive tracer experiments revealed that these alkaloids are generated from six molecules of mevalonic acid via a squalene-like intermediate.[2] The biogenetic transformations between the six structural groups are well-demonstrated.[1] Heathcock and co-workers developed an exceptionally efficient biomimetic total synthesis of several polycyclic alkaloids.[3−8] Recently, Kobayashi, Morita, and co-workers reported a number of additional new structural types of *Daphniphyllum* alkaloids.[9−15] Previous studies on *D. calycinum* reported the presence of alkaloids[16] and isolation of antioxidant flavonoid glycosides.[17]

In the couse of survey of Vietnamese plants, we investigated the stem bark of *Daphniphyllum calycinum* and isolated a quite intriguing alkaloid, daphcalycine (**1**): seven saturated fused rings surrounding an oxazine ring skeleton with 11 chiral carbon centers, together with known daphnicyclidin D (**2**).[13] We describe herein isolation and structure elucidation of alkaloid **1** and the relative configuration of eleven chiral carbon atoms and of nitrogen atom with localized electron lone pair.



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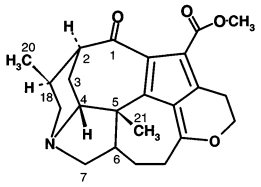

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Results and Discussion

Stem bark of *D. calycinum* was extracted with MeOH, and the extract was partitioned between 3% HCl and EtOAc. The aqueous layer basified by ammonia was extracted with EtOAc. The combined alkaloid containing fractions were subjected to repeated chromatography on

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TABLE 1. ^{1}H and ^{13}C NMR Data for Daphcalycine 1

C no.	δ_H in pyridine-d_5, mutiplicity (J in Hz)	δ_C	HMBC, C → H	δ_H in $CDCl_3$	δ_C in $CDCl_3$
1	4.15 d (7.4)	61.10	3b, 7, 13a,b, 19a	3.68 d	60.40
2	2.23 m	37.57	CH_3-20, 3a, 4ab, 18a,1	2.11 m	37.68
3a	1.53 m	18.42	1, 18, 2,4, 18	1.57 m	18.52
3b	1.45 m			1.52 m	
4a	1.39 m	43.51	CH_3−21, 18ab	1.45 m	43.83
4b	1.35 m			1.42 m	
5		34.08	1, 7, 21, 3ab, 4ab, 6, 12ab, 13b		34.17
6	1.98 dd (3.3, 7.1)	46.90		1.92 ddd	47.54
7	4.93 d (3.3)	86.18	19b, 12a, 6	4.55 d	86.19
8		47.24	1, 6, 13b, 21		47.38
9		95.33	1, 7, 11, 13a, 15, 16a, 17a, 25a		94.92
10	2.13 m	46.48		2.22 m	46.33
11a	1.56 m	25.15	12ab, 17b	1.65 m	24.99
11b	1.56 m			1.65 m	
12a	1.56 m	24.36	10, 25ab	1.63 m	24.21
12b	1.83 m			1.92 m	
13a	1.84 dd (7.1, 13.3)	29.74	1, 14, 15	1.75 dd	29.44
13b	2.23 t (13.3)			2.11 t	
14	3.49 ddd (7.1, 13.3, 17.6)	44.42	13ab	3.25 ddd	44.39
15	3.09 ddd (2.4, 8.9, 17.6)	49.01	14, 16ab, 17a	2.83 ddd	48.99
16a	1.82 m	28.38	14, 15, 17ab	1.88 m	28.07
16b	0.97 m			0.86 m	
17a	1.64 m	36.37		1.70 m	35.96
17b	1.30 m			1.30 m	
18	2.71 m	36.99		2.52 m	36.93
19a	3.40 t (6.7)	53.97	1, 18, 2, 20	3.16 t	54.28
19b	2.76 dd (6.7, 15.3)			2.66 dd	
20	0.90 d (CH_3) 6.6	12.77	18, 19b	1.03 d	13.21
21	0.99 s (CH_3)	26.13	4ab, 1	1.12 s	26.30
22		174.43	14, 13b, 15, 23		174.91
23	3.68 s (OCH_3)	51.32		3.62 s	51.39

silica gel column, followed by TLC purification to afford daphcalycine (**1**) (0.003%) and known daphnicyclidin D (**2**) (0.002%).

Daphcalycine (**1**) was an optically active, $[\alpha]_D$ −73°, amorphous solide. The molecular formula, $C_{23}H_{33}NO_3$, was deduced from HRESMS protonated molecular ion $[M + H]^+$ at m/z 372.2503 and NMR spectra. Of eight degrees of unsaturation in this molecular formula, one was attributed to ester carbonyl indicated by ^{13}C NMR spectrum (Table 1), and the absence of double bond bearing sp^2 carbon atom suggested that daphcalycine (**1**) may possess seven saturated rings. The ^{1}H, ^{13}C NMR, and HSQC spectra (pyridine-d_5) revealed the presence of one methoxy, two methyls, eight methylenes, eight sp^3 methines, and three sp^3 quaternary carbons.

The ^{1}H−^{1}H COSY spectrum displayed the spin systems of four structural fragments (Figure 1): one tetramethylene spin system, bearing a methyl group at CH-18 (δ_C 36.99, δ_H 2.71) and an ethyl side chain at CH-2 (δ_C 37.57, δ_H 2.23). ^{13}C shifts of CH-1 and CH_2-19, respectively, at δ_C 61.10, δ_H 4.15 and at δ_C 53.97, δ_H 2.76, 3.40 suggested their vicinal connection to nitrogen atom. This spin system was better resolved in the COSY spectrum analyzed in $CDCl_3$. Hence, a pyrrolidine ring fragment **a** was established. The other isolated methine signals showed clear correlations. Thus, the methine CH-15 at δ_C 49.01, δ_H 3.09 was neighboring CH-14 (δ_C 44.42, δ_H 3.49) and CH_2-16 (δ_C 28.38, δ_H 0.97, 1.82), and in turn, connected respectively to CH_2-13 and CH_2-17. This spin system was involved in the structural fragment **b**. CH-6 (δ_C 46.90, δ_H 1.98) was vicinal to CH-7 and CH_2-12, forming fragment **c**. Final fragment **d** contained CH-10 attached to CH_2-17 and CH_2-11.

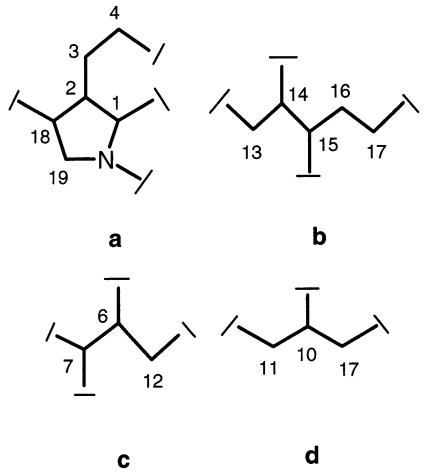

FIGURE 1. Structural fragments of daphcalycine (**1**).

However, the overlapping signals made it uncertain to obtain the connection of some methines and methylenes only from the ^{1}H−^{1}H COSY spectrum. The exhaustive analysis of long-range HMBC correlations (Table 1) confirmed the unambiguous structural fragments **a**−**d**. The fragment **d** was found as junction group between fragments **b** and **c**, forming a decyl chain. The terminal methine CH-7 at δ_C 86.18, δ_H 4.93 may be linked both to nitrogen and oxygen atoms as suggested by their chemical shifts. In fact, HMBC cross-peaks were observed between C-1 and H-19a and H-7 passing through the nitrogen atom of a *N*-decylpyrrolidine group. The HMBC spectrum further provided the connection of eight methines, three quaternary carbons, methyl and carbonyl

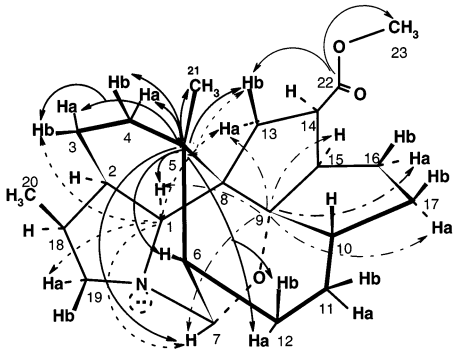

FIGURE 2. Selected long-range HMBC correlations of daphcalycine (**1**).

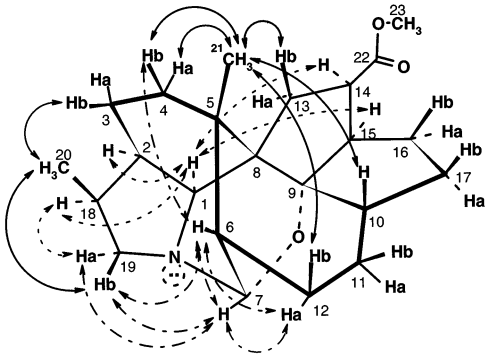

FIGURE 3. Selected NOE interactions of daphcalycine (**1**).

as follows (Figure 2). The quaternary carbon atom (C-5) at δ 34.08 was correlated to methyl-21 and protons of dimethylene at C-3 and C-4 as well as H-1, thus forming a cycle **B** fused to pyrrolidine ring **A**. The correlation of C-5 to H-7, H-6, and CH_2-12 of the fragment **c** made it possible to establish the C-5−C-6 bond, thus producing another ring **C**. The final cross-peak with H-13b circled the macrocycle. The quaternary carbon C-8 at δ 47.24 must be included in six-membered rings **B** and **C** because of its connections with H-1, H-6, H-13b, and CH_3-21, thus defining another 11-membered macrocycle. The methoxy carbonyl group was fixed to C-14 by correlations of C-22 at δ174.43 with H-13b, H-14, H-15, and methoxy-23.

Strongly deshielded sp^3 quaternary carbon atom C-9 at δ 95.33 must be attached to the oxygen atom. The long-range correlations of C-9 with H-1 made it possible to bind C-9 to C-8, and these with H-13a and H-15 suggested the presence of a cyclopentane ring D: C9−C8−C13−C14−C15. The another cross-peaks of C-9 with H-16a and H-17a revealed a second fused cyclopentane ring E. The preceding HMBC correlations indicated a yuzurimine-like skeleton[18] for daphcalycine (**1**). However, C-9 presented one more critical cross-peak with H-7, creating one tetrahydropyran ring G and an oxazine ring: C1−C8−C9−O−C7−N.

In the NOESY spectrum, the methyl-21 protons interacted with H-4a, H-4b, H-10, H-12b, and H-13b on the up-side of the molecule as shown on Figure 3, whereas H-1 on the down-side interacted with H-2, H-14, and H-15. The H-6 was found to be spatially proximal to H-7, H-19b, H-12a and H-4b. The methyl-20 was placed on the up-side because of the correlation with H-3b and H-19b, whereas H-18 interacted with H-1. The relative configuration of the 11 stereocenters was thus established as 1*S**, 2*R**, 5*S**, 6*S**, 7*S**, 8*R**, 9*S**, 10*R**, 14*R**, 15*R**, and 18*S** as well as *S**-nitrogen atom.

To examine the conformation, the energy minimized structure of daphcalycine (**1**) was obtained by molecular mechanics force field simulation calculation[19] *in vacuo* as shown in stereoview (Figure 4). The starting structure was built up with experimental distance restraints based on NOESY (2−4 Å) and following biogenetic consideration. The ring B is in chair conformation; the ring C, involved in quinuclidine-like fused tricycles, is evidently in boat conformation; while corresponding B and C rings in yuzurimine (**v**) are both in chair conformation.[18] The supplementary tetrahydropyran ring G rendered very stable the core of daphcalycine (**1**). The methyl-21 is surrounded closely in distance less than 2.3 Å with H-4a, H-4b, H-10, H-12b, and H-13b on the up-side of the molecule, and H-1 is found at the center of H-2, H-14, H-15, and H-18 on the opposite side at 2.1−2.9 Å.

The configuration of daphcalycine (**1**) may also be deduced by inspection of the biogenesis. From a biogenetic point of view, it is admitted that the yuzurimine-type alkaloids **vi** derive from alkaloids of homosecodaphniphylline (**ii**) group (Scheme 1)[1,20] arisen from an imine **i.**[7,8] The key intermediate, N,7-9,10-diene **iii**, should be generated by fragmentation of C-7−C-10 bond of the alkaloid **ii**.[1,7,8] The several step oxidations may convert the alkaloid **iii** to yuzurimine (**vi**).[1,2] By another biogenetic path, an attack at C-7 by a hydroxyl radical may provoke ring closure between a nitrogen radical and C-19 to produce the pyrrolidine A-ring and therefore 7-hydroxyyuzurimine (**iv**). Then, the 7-oxy radical should bind to nearest C-9, saturating the 9,10 double bond to create finally the oxazine ring of daphcalycine (**1**). The alternative path to yuzurimine (**vi**) via daphnezomine G (**v**), derived also from the imine **i** by linking C-1 and C-2 to the pyrrolidine A-ring, was postulated by Kobayashi and Morita.[10] They further proposed plausible biogenetic conversion of yuzurimine (**vi**) into daphnicyclidin D (**2**).[13] Daphcalycine (**1**) may be also produced from a daphnezomine G-type (**v**) intermediate by oxidation of C-7. The biogenesis of very reactive *Daphniphyllum* alkaloids thus demonstrate that the absolute configuration of C-20 and C-21 is preserved over the multiple-step transformations to be found on the same upper-side as drawn in Scheme 1.

Since the absolute configurations of methyl homosecodaphniphyllate (**ii**), [1,20] yuzurimine (**vi**),[18] and daphnicyclidin D (**2**)[13] were previously determined, daphcalycine (**1**) may possess the same absolute configuration of C-20 and C-21. However, this point calls for further experimental confirmation. Daphcalycine (**1**) was thus constructed by a surprising polycycle fused framework: central quinuclidine like tricycle produced by fusion of a piperidine, a tetrahydropyran and an oxazine was again

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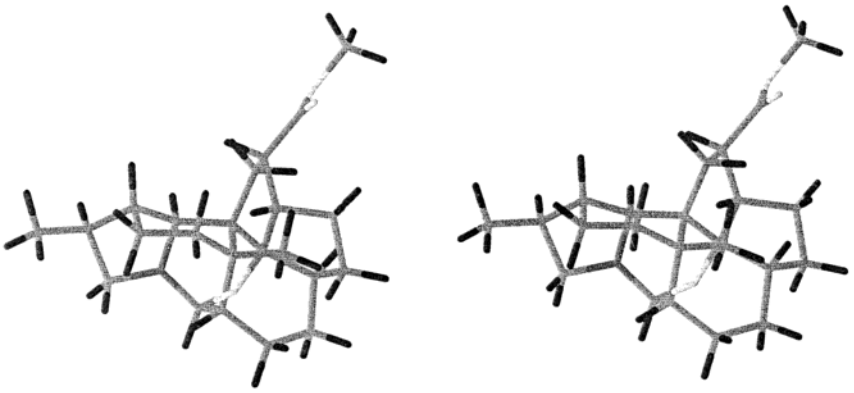
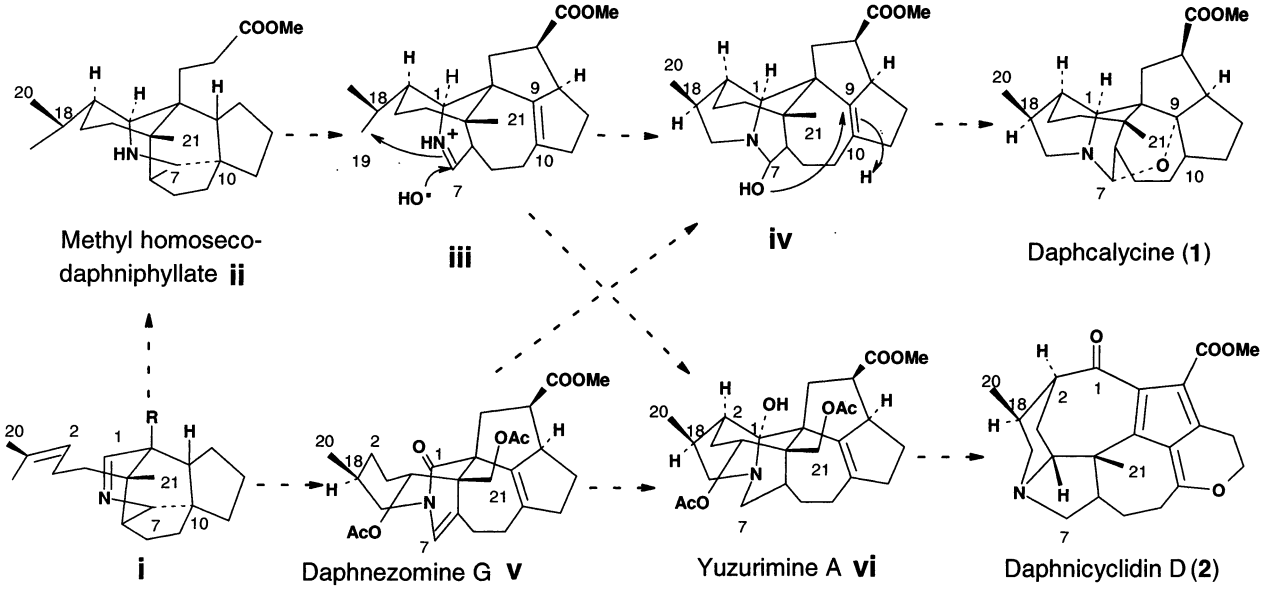


FIGURE 4. Stereoview of energy minimized structure of daphcalycine (**1**).

SCHEME 1. Biogenetic Pathway Proposed for Daphcalycine (1)



condensed by surrounding three penta-, one hexa-, and one hepta-membered rings.

Daphcalycine (**1**) and daphnicyclidin D (**2**) displayed moderate cytotoxicity against human nasopharynx carcinoma KB cells with IC_{50} values of 13 and 7 μg/mL (4.6 μg/mL[13]).

Experimental Section

General Information. ^1H and ^{13}C NMR spectra were recorded at 400 and 300 MHz spectrometers in $CDCl_3$ and pyridine-d_5. Chemical shifts are referenced to residual $CHCl_3$ δ_H 7.26, pyridine δ_H 7.19, $CDCl_3$ δ_C 77.0, and pyridine-d_5 δ_C 123.5. NOESY spectra were recorded with 1.5 s relaxation delay, 500 ms mixing time, and apodization with a shifted sine bell and baseline corrections.

Material. The stem bark of *D. calycinum* (Daphniphyllaceae) was harvested in north Vietnam in 1999 and identified by Mr. A. Gramain, CNRS, Hanoi. Voucher specimen was deposited in the herbarium of CNRS, Hanoi.

Isolation of Daphcalycine (1) and Daphnicyclidin D (2). Powdered stem bark of *D. calycinum* (1.6 kg), defatted with cyclohexane, was extracted with MeOH. The extract was then partitioned between 5% HCl and EtOAc. The aqueous layer made alkaline with ammonia was extracted with EtOAc. The combined alkaloid-containing extracts were subjected to repeated chromatography on a silica gel column, followed by TLC purification (CH_2Cl_2/MeOH, 9:1) to afford daphcalycine (**1**) (45 mg, 0.003%) and known daphnicyclidin D (**2**) (30 mg, 0.002%). **Daphcalycine (1):** $C_{23}H_{33}NO_3$; amorphous; $[\alpha]_D$ −73° (*c* 0.8, MeOH); UV (MeOH) λ_{max} 210 nm (ϵ 2700); HRESMS $[M + H]^+$ *m*/*z* 372.2503, Δ −3.5 mmu for $C_{23}H_{34}NO_3$. **Daphnicyclidin D (2):** amorphous; $[\alpha]_D$ −49° (*c* 0.2, MeOH).

MMFF Simulation Calculation. Conformational search was performed by molecular mechanics force field using HyperChem software packages (Hypercube, INC. 419 Phillip Street, Waterloo, Ontario N2L 3X2, Canada). The structure was first built with steric and distance constraints based on the NOESY (2−4 Å). The energy minimization was carried out using a geometry optimization algorithm with conjugate gradient iterations to reach the root-mean-square of the gradients 0.001kcal (Å·mol)$^{-1}$. Then the structure was submitted to molecular dynamics at 1000 K. The lower energy minimum structure was again calculated by geometry optimization to obtain the energy minimized structure.

Determination of Biological Activity. The cytotoxicity assays were carried out in 96-well microtiter plates in triplicate against human nasopharynx carcinoma KB cell lines (10^4 cells/mL). Cell growth was estimated by colorimetric measurement of stained living cells by neutral red. Optical density was determined at 540 nm after 72 h of incubation. IC_{50} value is the concentration of compound inhibiting to 50% of the living cells.

Acknowledgment. We thank Mr. Anthony Gramain, CNRS Hanoi, for plant material supply and Region Ils de France for financial support of NMR and mass spectrometers.

Supporting Information Available: 1D and 2D NMR spectra of compound **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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