A New Cytotoxic Calyculinamide Derivative, Geometricin A, from the Australian Sponge *Luffariella geometrica*

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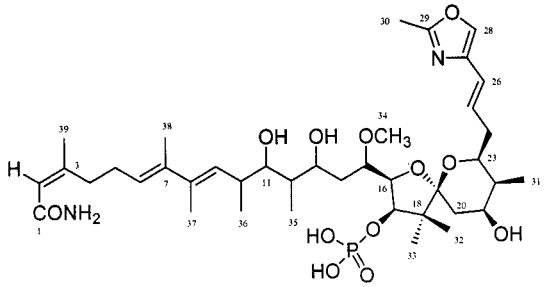
Received October 31, 2001

Geometricin A (**1**), together with the known compounds (7*E*,12*E*,18*S*,20*Z*)-variabilin (**2**), clathryimine A (**3**), tryptophol (**4**), and L-tryptophan, has been isolated from the methanol extract of the Australian sponge *Luffariella geometrica*. The structure of the new compound geometricin A (**1**) was elucidated by employing spectroscopic techniques (NMR, MS, UV, and IR) and by comparison of its NMR data with those of the calyculins and calyculinamides. Geometricin A (**1**) was found to be moderately cytotoxic toward the tumor cell lines HM02 and HEPG2 with GI_{50}'s of 1.7 and 2.8 μg/mL, respectively, and to have antialgal activity (growth inhibition zone 5 mm at the 50 μg level).

Sponges of the genus *Luffariella* are known as a rich source of manoalide-related sesterterpenoids with potent bioactivities.[1−6] Calyculin- and calyculinamide-related compounds, to date, have not been obtained from sponges of the genus *Luffariella*. In 1986, calyculin A was the first compound of this series to be isolated from the Japanese sponge *Discodermia calyx*.[7] Further compounds of this type having a wide spectrum of bioactivities such as cytotoxicity against tumor cell lines and potent inhibition of protein phosphatases 1 and 2A were reported from several sponges of the orders Lithistida and Epipolasida.[8−12]

The current sample of *Luffariella geometrica*, Kirkpatrick 1900, (Irciniidae) was collected at Heron Island's, Wistari Reef, Australia. After extraction with MeOH the extract was evaluated for biological activities. Simultaneous with these assays, investigation of the secondary metabolite chemistry of the sponge sample was started. Chromatographic separation of the MeOH extract yielded one new calyculinamide-related compound (**1**), the known compounds **2**−**4**, and L-tryptophan. The structures of the known compounds (**2**−**4**) and L-tryptophan were determined by comparison of their ^{1}H and ^{13}C NMR spectroscopic data with published values. To date, (7*E*,12*E*,18*S*,20*Z*)-variabilin (**2**) has commonly been encountered in sponges of the genus *Ircinia*,[13] whereas clathryimine A (**3**) has only been found in the Indo-Pacific sponge *Clathria basilana*.[14] In the terrestrial environment tryptophol (**4**) has been isolated from the fungi *Drechslera nodulosum* and *Acremonium lolii*,[15] but not from marine macro-organisms.[16]

Mass spectral analysis of compound **1** indicated it to have the molecular formula $C_{39}H_{63}N_2O_{12}P$ and showed it to have 11 elements of unsaturation and one phosphate group. Its ^{13}C NMR data contained a total of 39 resonances for 10 methyl, five methylene, and 16 methine groups and eight quarternary carbons. These data also revealed the presence of seven double bonds (5 × C=C; 1 × C=N; 1 × CO). One degree of unsaturation could be assigned to the phosphate group; **1** was thus tricyclic. After assignment of all protons to their directly bonded carbon atoms via a ^{1}H−^{13}C 2D NMR shift correlated measurement (HMQC), it was possible to deduce major fragments of the molecule from the results of a ^{1}H−^{1}H COSY measurement. Thus, analysis of the COSY spectrum of **1** gave evidence for connectivities from CH-9 to CH-17 and showed that H-10 and H-12 further coupled with H_3-36 and H_3-35, respectively. A second major molecular fragment from C-20 to C-26 was established on the basis of couplings observed between H-26 and H-25, H-25 and H_2-24, H_2-24 and H-23, H-23 and H-22, and H-22 and H-21, which in turn coupled with H_2-20. In addition, H_2-4 coupled to H_2-5, which further coupled to H-6. As H-17 appeared as a doublet of doublets, even though in the COSY spectrum only a coupling to H-16 was observed, the phosphate group clearly had to be connected to C-17 ($^3J_{P-H}$: 10.4 Hz). A long-range ^{1}H−^{13}C 2D NMR shift correlated measurement (HMBC; see Table 1) permitted the planar structure of **1** to be further elaborated. Thus, long-range CH correlations observed between C-1 and H-2, between C-3 and H-2, H_2-4, and H_3-39, between C-7 and H_2-5, H_3-37, and H_3-38, and between C-8 and H-10, H_3-37,



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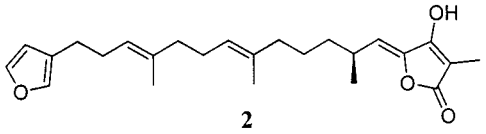

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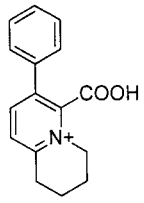

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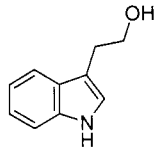

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10.1021/np010544u CCC: $22.00
Published on Web 05/24/2002

Table 1. ^{1}H NMR and ^{13}C NMR Spectral Data for Compound **1**[a]

carbon	^{13}C[b] (δ in ppm)	^{1}H[b] (δ ppm, mult., J in Hz)	HMBC[d]
1	171.8 (s)[c]		2
2	119.4 (d)	5.78 (1H, brs)	4, 39
3	156.3 (s)		2, 4, 5, 39
4	33.7 (t)	2.76 (2H, t, J = 7.6)	2, 5
5	28.3 (t)	2.36 (2H, ddd, J = 7.6, 7.6, 7.6)	4, 6
6	125.7 (d)	5.56 (1H, t, J = 7.6)	4, 5, 38
7	138.3 (s)		5, 9, 37, 38
8	136.9 (s)		6, 10, 37, 38
9	127.6 (d)	5.70 (1H, d, J = 9.5)	11, 36, 37
10	36.8 (d)	2.75 (1H, m)	9, 11, 36
11	79.4 (d)	3.44 (1H, dd, J = 2.2, 9.5)	9, 12, 35, 36
12	44.2 (d)	1.66 (1H, m)	11, 13, 35
13	72.0 (d)	3.87 (1H, dd, J = 5.8, 10.7)	11, 14, 15, 35
14	35.9 (t)	1.49 (1H, brdd, J = 9.5, 14.3) 1.92 (1H, m)	13, 15, 16
15	80.5 (d)	3.82 (1H, t, J = 9.5)	14, 16, 34
16	85.7 (d)	4.10 (1H, dd, J = 4.3, 9.5)	14, 15, 17
17	84.2 (d)	4.42 (1H, dd, J = 4.3, 10.4)	16, 32, 33
18	51.2 (s)		17, 32, 33
19	110.0 (s)		16, 17, 20, 32, 33
20	33.2 (t)	1.57 (1H, m), 1.62 (1H, m)	22
21	69.5 (d)	4.16 (1H, dt, J = 11.4, 4.9)	20, 23, 31
22	39.4 (d)	1.90 (1H, m)	20, 31
23	72.9 (d)	4.00 (1H, ddd, J = 2.1, 3.7, 10.4)	22, 24, 25, 31
24	37.2 (t)	2.12 (1H, ddd, J = 3.7, 10.1, 14.0) 2.53 (1H, m)	23, 25, 26
25	132.7 (d)	6.77 (1H, ddd, J = 4.6, 10.1, 15.3)	23, 24, 26
26	119.4 (d)	6.26 (1H, d, J = 15.3)	24
27	139.7 (s)		25, 26, 28
28	136.0 (d)	7.76 (1H, s)	26
29	164.2 (s)		28, 30
30	13.5 (q)	2.50 (3H, s)	
31	4.5 (q)	0.90 (3H, J = 7.0)	21, 23
32	18.2 (q)	1.30 (3H, s)	33
33	23.2 (q)	1.03 (3H, s)	17, 32
34	60.9 (q)	3.52 (3H, s)	15
35	12.0 (q)	0.77 (3H, d, J = 7.0)	11, 13
36	18.9 (q)	1.06 (3H, d, J = 7.0)	9, 10, 11
37	14.3 (q)	1.81 (3H, s)	6, 9
38	14.4 (q)	1.83 (3H, s)	6, 9
39	25.1 (q)	1.92 (3H, d, J = 1.2)	2, 4
$CONH_2$		5.00 (2H, brs)	
OH		6.60 (1H, brs)	
		7.38 (1H, brs)	

[a] CD_3OD, 500 MHz; exchangeable protons in CD_3OH. [b] Assignments are based on extensive 1D and 2D NMR measurements (HMBC, HMQC, COSY). [c] Implied multiplicities determined by DEPT (C = s; CH = d; CH_2 = t; CH_3 = q). [d] Numbers refer to proton resonances.

and H_3-38 clearly delineated all so far unaccounted C−C bonds concerning the C-1 to C-17 part of the molecule. The linkage between the methoxyl group (C-34) and C-15 was proven by the long-range CH correlation between C-15 and H_3-34. ^{13}C NMR chemical shifts for carbons C-27, C-28, and C-29 together with a singlet proton resonance at δ 7.76 characterized an oxazole ring. Long-range CH correlations between C-29 and H_3-30 indicated C-30 to bond to C-29, while HMBC correlations between C-27 and both H-26 and H-25 confirmed a bond between C-26 and C-27, which thus connects the oxazole ring to the rest of the molecule. The HMBC data also showed the methyl groups CH_3-32 and CH_3-33 both to bond to C-18, and CH_3-31 to bond to C-22. ^{1}H−^{13}C HMBC couplings between C-18 and H-17 and between C-19 and H_3-32, H_3-33, and H_2-20 established the connectivities between C-17 and C-18, between C-18 and C-19, and between C-19 and C-20. Still remaining to be assigned were five oxygen atoms and three protons, accountable for three hydroxyl groups and two ethers, possibly as rings. In the molecule there were five oxygenated methines still lacking oxygen assignment (C-11, C-13, C-16, C-21, and C-23). C-19 had to be connected to two oxygen atoms because of its low-field ^{13}C NMR chemical shift (δ 110.0). The low-field chemical shift of C-16 (δ 85.7) together with a long-range CH correlation between C-19 and H-16 indicated C-16, C-17, C-18, and C-19 together with an oxygen atom to form a tetrahydrofuran ring. Comparison of our spectral data with literature data[11] revealed the oxygen at C-23 also to be linked to C-19 to form a six-membered ketal ring, leaving the three hydroxyl groups to be positioned at C-11, C-13, and C-21.

The geometry of the double bonds Δ^2, Δ^6, and Δ^8 were all assigned on the basis of the chemical shifts for CH_3-39, CH_3-38, and CH_3-37, respectively. Thus, the downfield ^{13}C NMR resonance of CH_3-39 (δ 25.1) established the *Z* geometry of Δ^2, whereas resonances for CH_3-37 and CH_3-38 (δ 14.3 and 14.4, respectively) showed both Δ^6 and Δ^8 to have the *E* geometry. The size of the coupling constant $J_{H-25,H-26}$ = 15.3 Hz established the geometry of Δ^{25} also as *E*. The relative stereochemistry of the six-membered ring was deduced from 2D ^{1}H−^{1}H NOESY correlations and by considering ^{1}H−^{1}H coupling constants. The axial orientation of H-21 and H-23 and the equatorial orientation of H-22 were evident from the coupling constants (see Table 1). NOESY cross-peaks between H-21 and H-23 and between H_3-31 and H_2-24 together with the unusual upfield chemical shift of C-31 established H-21, H-22, and H-23 all to be on the same side of the molecule.[9] Intense ROESY cross-peaks between H_3-33 and H-16, H-17, and H_2-20 established the relative stereochemistry for C-19 to be as shown in **1**. The relative stereochemistry for the centers C-10, C-11, C-12, C-13, and C-15 were not established. The absolute configuration, which for calyculin A was established by chemical degradation[17] and stereoselective synthesis,[18−20] was also not determined here. For **1**, the trivial name geometricin A is proposed. Geometricin A (**1**) is closely related to the calyculins and calyculinamides,[7−12] but it differs concerning the substitution at C-29 and lacks the Δ^4 double bond.

Calyculin derivatives have been reported to be extremely cytotoxic toward several cell lines.[8−10] We investigated the cytotoxic effects of geometricin A against HM02 and HepG2 cell lines and found growth inhibition of these tumor cells with 50% growth inhibition (GI_{50}) values of 1.7 and 2.8 μg/mL, respectively, and total growth inhibition (TGI) values of 3.2 and 8.8 μg/mL, respectively. These activities must be judged as moderate when compared with the activities of calyculin derivatives reported previously.

The antimicrobial and antialgal activities of geometricin A were investigated in agar diffusion tests (50 μg/disk) and showed geometricin A to have moderate effects against the green microalga *Chlorella fusca* (growth inhibition zone: 5 mm) and the fungus *Microbotryum violaceum* (growth inhibition zone: 5 mm).

Experimental Section

General Experimental Procedures. HPLC was carried out using a Merck-Hitachi system consisting of a L-6200A pump, a L-4500 A photodiode array detector, and a D-6000 A interface, together with a Knauer K-2300 differential refractometer as detector. ^{1}H (1D, 2D COSY, 2D NOESY, 2D ROESY) and ^{13}C (1D, DEPT 135, 2D HMQC, 2D HMBC) NMR spectra were recorded on a Bruker Avance 500 DMX spectrometer in CD_3OD and CD_3OH. Spectra were referenced to residual solvent signals with resonances at $\delta_{H/C}$ 3.35/49.0 (CD_3OD). UV and IR spectra were obtained employing Perkin-Elmer Lambda 40 and Perkin-Elmer Spectrum BX instru-

ments, respectively. Optical rotations were measured on a Jasco DIP 140 polarimeter. HREIMS were recorded on a Kratos MS 50 spectrometer. All other experimental details were as previously reported.[21]

Animal Material. The sponge sample was collected in July 1998 at Heron Island's, Wistari Reef, the Great Barrier Reef, Australia, from a depth of 20 m and stored in EtOH at −20 °C until workup. The sponge was identified by Dr. J. N. A. Hooper, Queensland Museum, South Brisbane, Australia. A voucher specimen has been deposited at the Institute for Pharmaceutical Biology, Bonn, voucher number HER-43.

Extraction and Isolation. After removal of the preservation EtOH, the sponge (wet wt: 0.48 kg) was extracted with MeOH (3 × 0.5 L), followed by CH_2Cl_2 (3 × 0.5 L). The MeOH extract and the EtOH solution were evaporated to dryness, combined, and partitioned between MeOH−H_2O (1:1, 0.2 L) and CH_2Cl_2 (0.2 L). The MeOH−H_2O fraction was evaporated to dryness to yield 7.8 g of a salty yellow extract. This extract was fractionated by vacuum liquid chromatography (VLC) over Polygoprep 60-50 C_{18} material (Macherey-Nagel) using gradient elution from H_2O (100%) to MeOH (100%), to yield 11 fractions. ^{1}H NMR investigations of these fractions indicated VLC fractions 5, 8, 9, and 10 to be of further interest because of a large number of notable aromatic resonances. All these fractions were desalinated using solid-phase extraction (Bakerbond SPE C_{18}) and then fractionated by normal- (column: Knauer Si Eurospher-100, 250 × 8 mm, 5 μm) and reversed-phase (RP) HPLC (column: Phenomenex Aqua C_{18}, 250 × 4.6 mm, 5 μm). Fraction 5 (RP, MeOH−H_2O (1:9), 1.0 mL/min) gave 2.7 mg of L-tryptophan ($[\alpha]_D^{23}$ −31.4°; *c* 0.23, H_2O)[22] as a white powder. HPLC separation of fraction 8 (MeOH−H_2O (3:7), 1.0 mL/min) yielded compound **3** (2.5 mg) as a colorless oil. Fraction 9 was rechromatographed over C_{18} RP HPLC (MeOH−H_2O (1:1), 1.0 mL/min) to yield compounds **1** (3.5 mg) and **4** (1.8 mg), both as colorless solids. HPLC separation (NP Si, CH_2Cl_2−MeOH (7:13), 1.5 mL/min) of fraction 10 yielded semipure compound **2** and another 4 mg of compound **1**. Further purification of semipure **2** by RP HPLC (MeOH−H_2O (7:3), 1.0 mL/min) afforded 15.9 mg of this substance ($[\alpha]_D^{23}$ −34.8°; *c* 1.33, $CHCl_3$)[23] as a colorless oil.

Geometricin A (1): colorless solid (7.5 mg, 0.002%); $[\alpha]_D^{23}$ −36.3° (*c* 0.29, MeOH); UV (MeOH) λ_{max} 206 nm (ϵ 16 280), 221 nm (ϵ 16 370); IR (ATR) ν_{max} 3340, 3211, 2941, 2902, 2830, 1665, 1446, 1097, 1023, 991 cm^{-1}; ^{1}H and ^{13}C NMR data (see Table 1); FABMS *m*/*z* 805 $(M + Na)^+$, 783 $(M + H)^+$; EIMS *m*/*z* (rel int) 684 (15), 666 (60), 648 (33), 99 (100); HREIMS *m*/*z* 684.4356 (calcd for $C_{39}H_{60}N_2O_8$ $[M - H_3PO_4]^+$, 684.4349).

Biological Assays. Activity of geometricin A was tested in agar diffusion assays against the bacteria *Bacillus megaterium* and *Escherichia coli*, the fungi *Microbotryum violaceum*, *Eurotium repens*, and *Mycotypha microsporum*, and the green microalga *Chlorella fusca*.[24] ELISA-based enzyme inhibition assays against HIV-1 reverse transcriptase and tyrosine kinase ($p56^{lck}$) were performed as previously described.[25] Cytotoxicity tests against the cell lines HM02 (stomach carcinoma) and HepG2 (liver carcinoma) followed the standards of the NCI.[26]

Acknowledgment. We thank Dr. J. N. A. Hooper, Queensland Museum Brisbane, Queensland, Australia, for sponge taxonomy. We are grateful to Dr. W. Beil, Institute for Pharmacology, Medical University of Hannover, for performing the tumor cell line tests and Dr. G. Eckhardt, Institute for Chemistry, University of Bonn, for making all MS measurements. Thanks go also to Mr. C. Dreikorn, Institute for Pharmaceutical Biology, University of Bonn, for performing the enzyme and agar diffusion assays. Financial support is gratefully acknowledged from the Deutsche Forschungsgemeinschaft (Ko-902/2-4) and the Fond der Chemischen Industrie (No. 164 357).

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NP010544U