Isolation of Four New Calyculins from the Marine Sponge *Discodermia calyx*[1]

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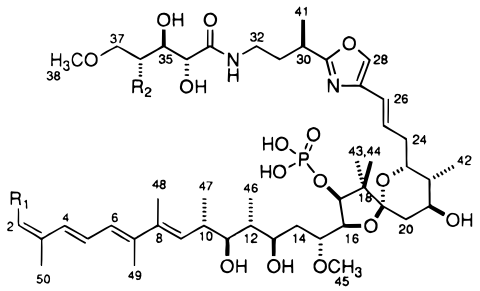
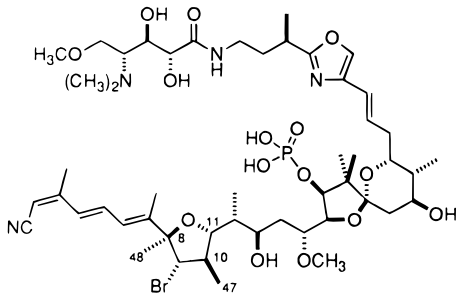
The calyculins, unique polyketides bearing nitrogen and phosphorus functions isolated from the Japanese marine sponge *Discodermia calyx*,[2−5] exhibit a variety of biological activities including antitumor, smooth muscle contractile, and tumor promotion,[6] which are believed to be attributable to inhibition of protein phosphatases 1 and 2A.[7] Therefore, the calyculins are important research reagents in the biological sciences. Eight known calyculins, A(**1**)−H, which differ by a methyl group on C-32 and in the geometry of C-2,3 and C-6,7 olefins,[4] display similar inhibitory activity against protein phosphatase 2A. Further examination of *D. calyx* led to isolation of four more calyculin derivatives. This paper deals with the isolation and structure elucidation of these new metabolites.

Frozen specimens (1.7 kg, wet weight) of the sponge were extracted with EtOH; the extract was partitioned between water and CH_2Cl_2. The organic phase was purified by silica gel and ODS chromatographies followed by ODS-HPLC to afford calyculin J (**2**; 1.9 mg, 1.1×10^{-4} % wet weight), calyculinamide A (**3**; 0.1 mg, 5.8×10^{-6} % wet weight), calyculinamide F (**4**; 0.2 mg, 1.2×10^{-5} % wet weight), and des-*N*-methylcalyculin A (**5**; 0.4 mg, 2.3×10^{-5} % wet weight).

The presence of one bromine atom in calyculin J (**2**) was readily inferred from the 1:1 intensity of the $(M + H)^+$ ion peaks at *m/z* 1088 and 1090 in FABMS. The molecular formula was assigned as $C_{50}H_{80}BrN_4O_{15}P$ on the basis of HRFABMS and NMR data. The ^1H NMR spectrum was similar to that of calyculin A,[3] with diagnostic signals for an oxazole (6.69 ppm), *N*,*N*-dimethylamino (2.07 and 1.99), two *O*-methyl groups (3.72 and 3.03), five singlet *C*-methyls (1.72, 1.57, 1.52, 1.40, and 0.89), and four doublet methyls (1.28, 1.10, 1.04, and 0.78). However, signals in the olefinic region were different; a doublet at δ 6.65 assigned to H9 was missing in calyculin J. Interpretation of the COSY, HOHAHA, and HMQC spectra revealed that structural units C-2 to C-6, C-20 to C-26, C-30 to C-32, and C-34 to C-37 were identical with those of calyculin A. However, C-9 with δ_H 3.78 and δ_C 64.5 was no longer olefinic. These chemical shift values were consistent with a bromomethine. The portion from C-10 to C-17 displayed ^1H and ^{13}C data superimposable on those of calyculin A.[3] An HMBC experiment not only confirmed the partial structures mentioned above, but also revealed that C-8 at δ 84.1 was oxygenated. The molecular formula indicates possible formation of an ether linkage, most likely between C-8 and C-11; this was supported by a downfield shift of C-11 by 2.4 ppm, as compared with the value in **1** and by NOESY data. Coupling constants and NOESY data showed that the relative stereochemistry of portions C-1 to C-7 and C-13 to C-37 in calyculin J was the same as that in calyculin A. ^{13}C NMR chemical shifts of the relevant portions were virtually identical. However, the H-10 to H-11 coupling constants (**1**, $J_{10,11} = 7.6$ Hz; **2**, $J_{10,11} = 5.6$ Hz) were different for calyculins A and J, thereby supporting the presence of an ether linkage. The relative stereochemistry of the tetrahydrofuran ring was assigned on the basis of NOESY cross peaks: H-9/H-11, H-9/Me-47, and H-10/Me-48, which demonstrated that H-9, Me-47, and H-11 are on the same side of the tetrahydrofuran ring, whereas Me-48, the bromine atom on C-9, and H-10 are on the opposite side. Stereochemical relationships between C-11 and C-12 could not be determined from NMR data, although it was likely that C-10, C-11, and C-12 as well as the rest of the chiral carbons in **2** share the same stereochemistry as their counterparts in **1**. Finally, the structure of calyculin J



1 R_1=CN, R_2=NMe_2
3 R_1=$CONH_2$, R_2=NMe_2
5 R_1=CN, R_2=NHMe



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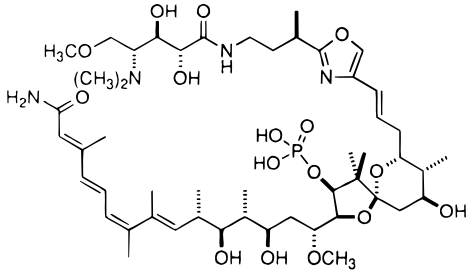

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Table 1. ^{1}H and ^{13}C NMR Data for Compounds 2−5 [C_6D_6, 300 K][a]

cabon No.	2		3		4		5	
	^{13}C ppm	^{1}H ppm (mult, *J* (Hz))	^{13}C ppm	^{1}H ppm (mult, *J* (Hz))	^{13}C ppm	^{1}H ppm (mult, *J* (Hz))	^{13}C ppm	^{1}H ppm (mult, *J* (Hz))
1	117.5	−		−	168.0	−		−
2	96.2	4.41 (s)	118.5	5.08 (s)	120.5	5.42 (s)	96.0	4.44 (s)
3	155.3	−		−	149.6	−	156.0	−
4	129.8	6.92 (d, 15)	130.1	8.63 (d, 15.4)	133.8	6.12 (d, 15)	129.0	7.04 (d, 15)
5	131.8	6.53 (dd, 15, 11)	132.0	7.05 (dd, 15.8, 11)	128.0	7.13	133.9	6.84 (dd, 15, 11.5)
6	122.3	6.76 (d, 11)	126.0	6.58 (d, 11)	125.1	5.98 (d, 10.4)	124.8	6.35 (d, 11.5)
7	147.5	−		−	147.5	−	144.5	−
8	84.1	−		−	135.0	−	136.7	−
9	64.5	3.78 (d, 9.6)	131.5	6.55 (d, 9.6)	131.5	6.19 (d, 9.3)	133.0	6.58 (d, 9.2)
10	45.0	2.32 (dd, 9.6, 5.6)	36.3	2.72 (m)	36.5	2.62 (m)	36.5	2.74 (m)
11	82.5	3.94 (dd, 10.1, 5.6)	81.4	3.67 (dd, 9.2, 2.5)	81.0	3.64 (dd, 9.6, 2.7)	81.5	3.67 (dd, 9.6, 2.3)
12	44.1	2.38 (m)	42.7	1.73 (m)	39.0	1.86 (m)	42.7	1.70 (m)
13	69.1	3.81 (m)	75.5	3.71 (m)	75.6	3.74 (m)	75.5	3.72 (m)
14a	43.3	2.07 (m)	39.0	1.99 (m)		2.04 (m)	39.0	1.94 (t, 12.2)
14b	−	1.67 (m)	−	1.65 (dd, 13.5, 3.5)	−	1.74 (m)	−	1.60 (m)
15	78.0	4.04 (m)	77.5	4.11 (t, 9.6)	77.5	4.14 (m)	77.7	4.07 (m)
16	85.7	4.12 (dd, 9.5, 3.4)	85.6	4.08 (dd, 10, 3.3)	85.8	4.17 (m)	85.6	4.03 (m)
17	81.8	4.28 (dd, 10.6, 3.4)	82.4	4.32 (dd, 10.4, 3.3)	83.0	4.38 (dd, 10.4, 3.5)	82.2	4.28 (m)
18	50.9	−		−	51.0	−	50.0	−
19	109.0	−		−	107.8	−	108.5	−
20a	30.4		30.3	1.59 (m)	30.5	1.57 (m)	30.3	1.55 (m)
20b	−	1.66 (m)	−	1.67 (m)	−	1.60 (m)	−	1.60 (d, 4.6)
21	71.8	3.97 (m)	71.6	3.98 (m)	70.0	3.90 (m)	71.9	3.94 (m)
22	38.5	1.85 (m)	38.5	1.85 (m)	39.8	1.86 (m)	38.6	1.86 (m)
23	68.1	4.67 (d, 12.2)	68.0	4.65 (dt, 12.3, 2.2)	67.2	4.66 (d, 12)	68.0	4.63 (d, 11.2)
24a	35.4	2.42 (m)	36.4	2.45 (t, 10)		2.45 (t, 11.6)	36.6	2.25 (m)
24b	−	1.79 (t, 11.8)	−	1.82 (m)	−	1.85 (m)	−	1.84 (m)
25	133.9	7.38 (ddd, 15.5, 10.4, 3.4)	133.1	7.37 (ddd, 16, 11.2, 3.8)	134.0	7.41 (ddd, 14.2, 10, 5.4)	130.0	7.30 (m)
26	116.7	5.85 (d, 15.5)	116.6	5.91 (d, 16)	118.2	5.91 (d, 14.2)	116.8	5.91 (d, 16.9)
27	138.0	−		−	138.2	−		−
28	134.0	6.69 (s)	134.0	6.72 (s)	134.7	6.71 (s)	134.0	6.72 (s)
29	170.2	−		−	170.5	−	172.0	−
30	29.1	3.58 (m)	29.1	3.58 (m)	39.5	3.56 (m)	29.1	3.50 (m)
31a	33.5		34.2	2.28 (t, 10)	34.5	2.20 (m)	34.2	2.25 (m)
31b	−	1.55 (m)	−	1.69 (m)	−	1.60 (m)	−	1.70 (m)
32a	34.7	4.17 (m)	34.8	4.23 (m)		4.11 (m)	34.5	4.24 (m)
32b	−	2.62 (t, 13.7)	−	3.10 (d, 13.1)	−	3.03 (d, 13.9)	−	3.08 (d, 13.4)
33	177.0	−		−		−		−
34	69.8	4.35 (d, 10)	69.0	3.80 (t, 10.5)		3.68 (m)	69.0	3.74 (t, 7.3)
35	74.1	4.78 (d, 10)	74.0	4.72 (d, 10)	74.1	4.61 (d, 10.7)	72.9	4.70 (d, 10)
36	64.5	4,19 (m)	63.8	4.00 (m)	64.0	3.88 (d, 7.3)	58.0	3.95 (m)
37a	65.6	3.61 (dd, 8.7, 13.2)	65.5	3.50 (m)	66.5	3.50 (m)	66.6	3.34 (m)
37b	−	4.01 (m)	−	3.42 (m)	−	3.47 (m)	−	3.47 (m)
38	58.2	3.03 (s)	58.1	2.90 (s)	58.9	2.92 (s)	58.7	2.78 (s)
39	36.5	1.99 (s)	36.5	1.92 (t)	37.0	1.93 (d)		
40	43.3	2.07 (s)	43.2	1.99 (d)	43.5	2.13 (d)	−	−
41	17.1	1.28 (d, 8.9)	17.8	1.35 (d, 6.9)	17.5	1.27 (d, 6.9)	18.0	1.42 (d)
42	11.0	0.78 (d, 7.0)	11.9	0.79 (d, 7.3)	10.8	0.78 (d, 7.3)	11.0	0.79 (d, 7.3)
43	22.5	0.89 (s)	22.3	0.84 (s)	22.5	1.00 (s)	22.1	0.82 (s)
44	18.4	1.57 (s)	18.1	1.55 (s)	18.0	1.58 (s)	18.5	1.52 (s)
45	60.5	3.72 (s)	61.0	3.71 (s)	60.5	3.75 (s)	61.0	3.72 (s)
46	10.6	1.04 (d, 8.9)	12.5	0.49 (d, 6.5)	12.8	0.63 (d, 6.9)	13.0	0.54 (d, 6.9)
47	15.2	1.10 (d, 6.5)	18.3	1.28 (d, 6.9)	18.5	1.30 (d, 6.5)	18.2	1.30 (d, 6.9)
48	27.0	1.52 (s)	13.9	1.71 (s)	15.0	1.62 (s)	14.7	1.76 (s)
49	14.0	1.72 (s)	14.0	2.20 (s)	23.8	1.88 (s)	14.8	2.10 (s)
50	19.0	1.40 (s)	20.7	1.83 (s)	14.0	2.54 (s)	18.0	1.42 (s)
NH	−	7.57 (d, 10.8)	−	8.75 (d, 8.8)	−	8.63 (d, 10.0)	−	8.64 (d, 8.1)
34-OH	−		−	8.15 (d, 9.2)	−	7.90 (d, 14.9)	−	

[a] ^{13}C Chemical shifts were determined on the basis of the HMQC and HMBC spectra.

was confirmed by chemical transformation from calyculin A. Treatment of calyculin A with *N*-bromosuccinimide[8] furnished a major product whose ^{1}H NMR spectrum and optical rotation were identical with those of **2**. Therefore, the absolute stereochemistry of calyculin J was assigned as shown. Formation of calyculin J was rationalized as follows. Conformation of calyculin A in solution was almost identical with that in the solid state, which was disclosed by X-ray crystallography.[4] With respect to the C-10/C-11 bond, C-9 and C-12 are in a gauche relationship reflecting the steric repulsion of Me-46 and Me-45. The electrophilic bromine atom attacks the $\Delta^{8(9)}$ olefin from the less hindered side, i.e., the face opposite C-11, to form an intermediate bromonium ion, which is opened by the attack of 11-OH, preferentially forming a tetrahydrofuran ring.

The second new compound **3** exhibited a pseudomolecular ion 18 units larger than **1**.[9] HRFABMS revealed that compound **3** was larger by the elements of H_2O than calyculin A, suggesting a hydrated **1**. The ^{1}H NMR spectrum of **3** was superimposable on that of **1** except for H-2 and H-4 signals, both of which experienced considerable downfield shifts. The C-2 signal, whose chemical shift value was determined by the HMQC spectrum, was shifted downfield by 24 ppm. Although further information such as ^{13}C NMR or HMBC data was not obtained due to the paucity of material, compound **3** was likely to have a terminal amide instead of the nitrile in **1**. Therefore, attempts were made to hydrate the nitrile group in calyculin A without affecting other parts

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(9) Compound **3** was isolated as the major compornent of a 4:1 mixture of double bond isomers. The structure of the minor constituent was not studied further. All attempts at separating the mixture using a variety of mobile phases and stationary phases in HPLC were unsuccessful.

of the molecule. Hydrolysis with KOH/*t*-BuOH[10] or with 2-mercaptoethanol[11] afforded complex products, whereas treatment of **1** with H_2O_2 in 25% NH_4OH[12] furnished a product with the molecular weight of 1043, which was 16 units larger than **3**, thus indicating that in addition to hydration of the nitrile the tertiary amine was oxidized to an amine oxide. Therefore, the amine oxide was reduced with PPh_3[13] and separated by HPLC to yield a compound which exhibited a ^{1}H NMR spectrum identical with that of **3**. Compound **3** was named calyculinamide A.

Compounds **3** and **4** had the same R_f values on silica gel TLC, eluted closely in reversed phase HPLC, and had the same molecular formula. The ^{1}H NMR spectra of these two compounds were similar, except for signals of the terminal tetraene portion, thus indicating that they are isomers differing in the geometry of one or more double bonds. ^{13}C Chemical shift values of C-48 (15.0 ppm), C-49 (23.8 ppm), and C-50 (14.0 ppm) in **4** indicated 2*E*,4*E*,6*Z*-geometry, while the HMBC spectrum placed a carbonyl group (δ 168.0) at C-1. Therefore, compound **4** was calyculinamide F, the C-1 amide of calyculin F.

The last compound **5** had a molecular formula of $C_{49}H_{79}N_4O_{15}P$ as determined by HRFABMS and NMR data, differing from calyculin A by loss of CH_2. Interpretation of the COSY spectrum measured in benzene-d_6 led to the carbon framework identical with that of calyculin A, except for the lack of both *N*,*N*-dimethyl signals. The C-36 was shifted downfield by 6 ppm, consistent with *N*-monomethylation of the nitrogen on this carbon.[14] Although the *N*-methyl signal was not observed in benzene-d_6 due to broadening, it appeared at δ_H 2.76/δ_C 30.2 in CD_3OD; thus **5** is des-*N*-methylcalyculin A.

Compounds **1**−**5** inhibited protein phosphatase 2A with IC_{50} values of 0.5, 75, 0.5, 1.2, and 49 nM, respectively. Conversion of the terminal nitrile to the amide did not affect the activity as observed in compounds **3** and **4**, whereas presence of a tetrahydrofuran ring (compound **2**) or loss of a methyl group on the nitrogen at C-36 (compound **5**) resulted in reduction of activity. As expected from the similar potency observed for calyculins A−H and decahydrocalyculin derivatives,[15] which are saturated derivatives of calyculin A differing in the stereochemistry at C-3, C-7, and C-8, the phosphate ester hydrogen bonded to the hydroxyl group on C-13, the nitrogen on C-36, and the nitrogen in the oxazole appear to play a crucial role in enzyme inhibition. A C-11,C-13-isopropylidene derivative of **1** was three orders of magnitude less active than calyculin A.[15] With the crystal structure of the catalytic subunit of protein phosphatase 1 and microcystin-LR in hand,[16] it is possible to study the mode of binding of calyculins to protein phophatases.

Experimental Section

Ultraviolet spectra were measured on a Hitachi 330 spectrophotometer. Optical rotations were determined with a JASCO DIP-1000 polarimeter. FAB mass spectra were measured with a JEOL SX-102 mass spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM-A600 NMR spectrometer.

Isolation. The sponge *D. calyx* was collected by SCUBA at depths of 15−20 m off the Izu Peninsula. Specimens were immediately frozen and kept frozen at −20 °C until processed. After epibionts were removed, the frozen sponge (1.7 kg) was homogenized and extracted with ethanol (3 × 3 L). The combined extracts were concentrated and partitioned between CH_2Cl_2 and H_2O. The organic phase (5.2 g) was subjected to silica gel flash chromatography with CH_2Cl_2/MeOH solvent pairs. Fractions were monitored by silica gel TLC ($CHCl_3$−MeOH, 9:1). The CH_2Cl_2/MeOH (95:5) fraction was subjected to ODS flash chromatography with aqueous methanol. The 80% and 100% MeOH eluates were further fractionated by silica gel column chromatography, eluting with mixtures of $CHCl_3$/MeOH. Final purification of the new calyculins was accomplished by ODS-HPLC with 82% MeOH and then with 70% MeCN to yield **2** (1.9 mg), **3** (0.1 mg), **4** (0.2 mg), and **5** (0.4 mg).

Calyculin J (2): yellow solid; $[\alpha]^{20}_D$ −10° (*c* 0.08, MeOH); UV λ_{max} (EtOH) 228 (ϵ 11000), 291 (14000), 304 (20000), 318 nm (19000); HRFABMS [(M + H)$^+$ obsd *m*/*z* 1089.4590, Δ −0.9 mmu]; ^{1}H and ^{13}C NMR data, see Table 1.

Calyculinamide A (3): colorless solid; $[\alpha]^{20}_D$ −18° (*c* 0.005, MeOH); UV λ_{max} (EtOH) 229 (ϵ 15000), 238 (13000), 332 nm (16000); HRFABMS [(M + Na)$^+$ obsd *m*/*z* 1049.5378, Δ −6.1 mmu]; ^{1}H and ^{13}C NMR data, see Table 1.

Calyculinamide F (4): colorless solid; $[\alpha]^{20}_D$ −23° (*c* 0.01, MeOH); UV λ_{max} (EtOH) 228 (ϵ 11000), 236 (10000), 315 nm (14000); HRFABMS [(M + H)$^+$ obsd *m*/*z* 1027.5698, Δ +7.8 mmu]; ^{1}H and ^{13}C NMR data, see Table 1.

Des-*N*-methylcalyculin A (5): colorless solid; $[\alpha]^{20}_D$ −18° (*c* 0.01, MeOH); UV λ_{max} (EtOH) 220 (ϵ 9000), 340 nm (8000); HRFABMS [(M + Na)$^+$ obsd *m*/*z* 1017.5255, Δ +7.8 mmu]; ^{1}H and ^{13}C NMR data, see Table 1.

Preparation of Calyculin J from Calyculin A. To a solution of calyculin A (1 mg) in THF was added *N*-bromosuccinimide (10 μL of a 0.1 M solution in THF), and the mixture was stirred at rt for 90 min. The reaction mixture was separated by HPLC (ODS column, 82%MeOH) to afford calyculin J (0.4 mg): yellow solid; $[\alpha]^{20}_D$ −11° (*c* 0.27, MeOH); UV λ_{max}(EtOH) 225 (ϵ 12000), 305 (18000), 319 nm (17000); HRFABMS [(M + H)$^+$ obsd *m*/*z* 1089.4609, Δ +1.0 mmu].

Preparation of Calyculinamide A from Calyculin A. Calyculin A (2 mg) was treated with a mixture of H_2O_2 (20 μL) and 25% NH_4OH (100 μL), and the mixture was stirred at rt for 15 h. The reaction mixture was evaporated to dryness to yield a white powder which was subjected to ODS column chromatography (0.5 × 3 cm) with 40% MeOH (2 mL) and MeOH (2 mL). The MeOH eluate was dissolved in MeOH and reduced with Ph_3P (3 mg) at room temperature for 5 days. The mixture was dried and separated by SiO_2 column chromatography with CH_3Cl, CH_3Cl/MeOH(95:5), CH_3Cl/MeOH (9:1), and MeOH, followed by HPLC on an ODS column with 70% MeCN to obtain calyculinamide A (0.8 mg): colorless solid; $[\alpha]^{20}_D$ −25° (*c* 0.04, MeOH); UV λ_{max} (EtOH) 228 (ϵ 11000), 236 (10000), 336 nm (16000); HRFABMS [(M + H)$^+$ obsd *m*/*z* 1027.5632, Δ +1.2 mmu].

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Supporting Information Available: Spectra for compounds **2**−**5** (15 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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