Isolation and Structure Elucidation of Calyculins B, C, and D, Novel Antitumor Metabolites, from the Marine Sponge *Discodermia calyx*[1]

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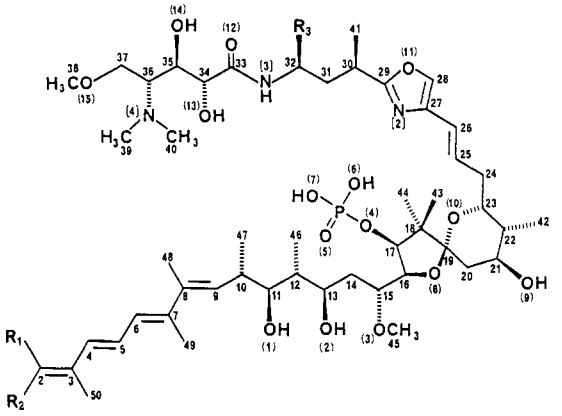
Calyculins B (**2**), C (**3**), and D (**4**) and the previously reported calyculin A (**1**), potent antitumor metabolites, have been isolated from the Japanese sponge *Discodermia calyx*. The structures of **2**–**4** have been determined by spectroscopic analyses including extensive NMR experiments and by comparison of spectral data with those of calyculin A.

Marine sponges are elaborate cytotoxic or antitumor metabolites with highly complex structures, e.g., polyethers,[2] macrolides,[3-5] alkaloids,[6,7] and peptides.[8,9] In the course of our search for antitumor substances from Japanese marine invertebrates, we encountered the marine sponge *Discodermia calyx*,[10] which showed marked activity in the anti-cell-division assay using fertilized starfish eggs[11] and in cytotoxicity tests against P388 and L1210 leukemia cells. From the sponge we isolated four active compounds that we designated calyculins A–D. The structure of the major metabolite, calyculin A, has been determined by X-ray diffraction.[10] Now we report the structure elucidation of the remaining metabolites, calyculins B–D, which are closely related to calyculin A.

The sponge *D. calyx* is a hard orange sponge shaped like a sake cup, as indicated by the species name. *D. calyx* is often covered by epiphytes. It was the most active marine extract tested so far in our starfish egg assay. The sponge (1 kg) collected in the Gulf of Sagami at a depth of 5–15 m was extracted with ethanol and the extract was separated by solvent partitioning and silica gel column chromatography (dichloromethane–methanol). The active fractions were finally purified by HPLC on ODS with MeOH/H_2O (8:2), yielding calyculins A (150 mg), B (20 mg), C (30 mg), and D (5 mg).

The second active compound, calyculin B (**2**), showed spectra similar to those of calyculin A. The molecular formula of $C_{50}H_{81}N_4O_{15}P$, identical with that of calyculin A, was established by an MH^+ ion peak at m/z 1009 in the FAB mass spectrum and by NMR data. The ^1H and ^{13}C NMR as well as homonuclear COSY spectra were virtually superimposable on those of calyculin A except for the signals of the terminal tetraene unit. The structure of this portion was deduced to be 2*E*,4*E* by NOE experiments in C_6D_6: irradiation of the H2 proton signal at δ 4.73 enhanced the H4 signals at δ 5.90, while irradiation of H_3-50 at δ 1.91 enhanced the H5 signals at δ 6.81. These NOE's were not observed for calyculin A, for which mutual NOE's were seen between H2 (δ 4.51) and H_3-50 (δ 1.51). Thus, calyculin B has structure **2**.



1: R_1 = CN, R_2 = H, R_3 = H
2: R_1 = H, R_2 = CN, R_3 = H
3: R_1 = CN, R_2 = H, R_3 = CH_3
4: R_1 = H, R_2 = CN, R_3 = CH_3

Calyculin C (**3**) is also closely related to calyculin A. The MH^+ ion at m/z 1023 in the FAB mass spectrum together with ^{13}C NMR data suggested the molecular formula $C_{51}H_{83}N_4O_{15}P$. The ^1H NMR, homonuclear COSY, and heteronuclear COSY spectra clearly showed the presence of an additional methyl group linked to C-32 of calyculin A (Table I).

The relative configuration of the C32-methyl group was deduced from NMR experiments. The conformation of the relevant portion of calyculin A in solution was determined through NMR experiments. First, we examined the effect of D_2O on the amide proton signal at δ 8.72 (dd, J = 10.5, 1.0 Hz) in C_6D_6 solution, which resulted in no significant exchange of the signal even after 2 weeks.[12] We also observed that no broadening occurred for the amide

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Table I. NMR Spectral Data for Calyculins A (1), B (2), C (3), and D (4)

	^{13}C		^{1}H				
C	1[a]	3[b]	1[c,e]	2[c]	2[d]	3[c]	4[c]
1	117.3 s	116.9 s					
2	94.6 d	95.4 d	4.51 s	4.73	5.16	4.52	4.73
3	156.3 s	155.0 s					
4	128.2 d	128.4 d	7.10 d	5.90	6.30	7.10	5.90
5	133.8 d	133.5 d	6.92 dd	6.81	6.93	6.94	6.81
6	123.7 d	123.9 d	6.42 d	6.31	6.23	6.40	6.30
7	134.7 s	134.8 s					
8	144.5 s	144.4 s					
9	132.4 d	132.9 d	6.65 d	6.70	6.22	6.66	6.71
10	35.8 d	36.1 d	2.79 m	2.80	2.75	2.74	2.76
11	80.7 d	81.0 d	3.72 dd	3.73	3.56	3.68	3.68
12	42.1 d	42.3 d	1.75 m	1.75	1.44	1.69	1.69
13	74.9 d	75.3 d	3.79 m	3.79	3.44	3.71	3.71
14	38.3 t	38.8 t	1.64 m, 2.01 m	1.64, 2.01	1.54, 1.79	1.64, 2.01	1.65, 2.01
15	77.3 d	77.3 d	4.15 t	4.15	3.75	4.12	4.12
16	84.8 d	85.1 d	4.09 dd	4.09	3.95	4.10	4.10
17	81.9 d	82.2 d	4.34 dd	4.31	4.06	4.30	4.30
18	50.1 s	50.2 s					
19	108.7 s	108.9 s					
20	29.6 t	30.0 t	1.62 m, 1.72 m	1.62, 1.71	1.58, 1.79	1.62, 1.70	1.62, 1.70
21	71.4 d	71.4 d	4.00 br m	4.00	3.84	4.00	4.01
22	37.7 d	38.3 d	1.88 m	1.88	1.70	1.88	1.88
23	67.1 d	67.6 d	4.68 m	4.68	4.31	4.68	4.68
24	35.9 t	36.0 t	1.89 m, 2.50 br t	1.89, 2.49	1.95, 2.46	1.86, 2.50	1.86, 2.50
25	132.6 d	133.0 d	7.38 m	7.38	6.94	7.38	7.38
26	116.1 d	116.4 d	5.97 dd	5.96	6.10	5.98 br d	5.98
27	137.3 s	137.8 s					
28	133.8 d	133.6 d	6.79 s	6.79	7.31	6.81	6.80
29	169.8 s	170.1 s					
30	28.4 d	29.8 d	3.62 m	3.62	3.20	3.67	3.67
31	33.6 t	40.0 t	1.77 m, 2.31 m	1.77, 2.30	1.76, 1.91	1.84, 2.18	1.84, 2.18
32	34.4 t	41.2 d	3.15 m, 4.29 m	3.15, 4.29	3.02, 4.03	4.70	4.70
32Me		20.5 q				1.47 d	1.46 d
33	176.2 s	176.0 s					
34	73.4 d	73.7 d	4.76 d	4.77	4.48	4.78	4.77
35	68.9 d	69.1 d	3.81 m	3.81	3.59	3.80	3.80
36	63.7 d	63.5 d	4.04 m	4.00	4.03	3.96	3.96
37	65.6 t	65.3 t	3.47 m	3.47	3.69 dd, 3.94 dd	3.47	3.47
38	58.9 q	69.1 q	3.76 s	3.76	3.40	3.78	3.78
39	44.3 q	58.1 q	2.93 s	2.85	2.87[f]	2.92	2.85
40	37.4 q	58.1 q	2.93 s	2.85	2.79[f]	2.92	2.85
41	17.5 q	18.8 q	1.40 d	1.46	1.31	1.42	1.43
42	10.8 q	10.8 q	0.83 d	0.83	0.86	0.84	0.84
43	17.5 q	17.9 q	1.58 s	1.59	1.23	1.59	1.60
44	22.4 q	22.4 q	0.88 s	0.88	0.90	0.89	0.88
45	60.9 q	69.1 q	3.76 s	3.76	3.49	3.78	3.78
46	12.7 q	12.5 q	0.57 d	0.57	0.58	0.54	0.56
47	17.9 q	18.2 q	1.34 d	1.36	1.03	1.30	1.33
48	13.7 q	13.5 q	1.80 s	1.90	1.82	1.77	1.87
49	13.9 q	13.9 q	2.17 s	2.16	2.02	2.16	2.14
50	19.2 q	18.5 q	1.51 s	1.91	2.17	1.51	1.91
NH			8.72 dd	8.76	8.22	8.47 d	8.48 d
OH			13.89 br s, 12.05 br s, 8.21 br d,[g] 7.26 s, 6.86 s, 6.71 d,[h] 4.40 br s[i]	13.80, 12.18, 8.22, 7.33, 6.85, 6.75, 4.33	13.76, 12.13, 7.69, 6.54, 6.15, 6.04, 5.28	13.87, 12.16, 8.08, 7.27, 6.95, 6.62, 4.44	13.78, 12.15, 8.05, 7.29, 6.91, 6.64, 4.42

[a] 125 MHz; $CDCl_3$ as internal reference = 77.0 ppm. [b] 125 MHz; C_6D_6 as internal reference = 127.7 ppm. [c] 500 MHz; residual C_6H_6 as internal reference = 7.20 ppm. [d] 500 MHz; residual $CHCl_3$ as internal reference = 7.25 ppm. [e] Coupling constants in hertz: 4,5 = 15.0; 5,6 = 12.0; 9,10 = 10.0; 10,47 = 7.0; 12,46 = 7.0; 13,(2)OH = 10.0; 15,16 = 12.0; 16,17 = 5.0; 22,42 = 7.0; 25,26 = 16.5; 30,41 = 7.0; 32,[3]NH = 1.0; 32′,[3]NH = 10.5; 34,35 = 10.0; 35,(14)OH = 10.0. Most of these coupling constants are essentially the same for other calyculins. [f] Assignments may be interchanged. [g] Assigned as (14)O–H. [h] Assigned as (2)O–H. [i] Assigned as (9)O–H.

proton signal when the spectrum was measured at elevated temperatures (304–350 K), although a small change in the chemical shift (Δ 0.18 ppm)[13] was observed. These findings indicate that the amide proton is involved in strong hydrogen bonding. In fact, X-ray diffraction studies indicate that calyculin A possesses four intramolecular short contacts between O1 and N3, O5 and O2, O7 and N4, and O6 and N2, which lock the portion from C30 through C33 in a folded chair-like conformation in the solid state. The conformation of this portion in C_6D_6 was elucidated by analysis of vicinal coupling constants and NOE experiments. A series of decoupling experiments disclosed these coupling constants: $J_{30,31eq}$ = 13.0 Hz, $J_{30,31ax}$ = 1.0, $J_{31eq,31ax}$ = 12.5, $J_{31eq,32eq}$ = 1.0, $J_{31eq,32ax}$ = 2.0, $J_{31ax,32eq}$ = 1.0, $J_{31ax,32ax}$ = 13.0, $J_{32eq,NH}$ = 1.0, and $J_{32ax,NH}$ = 10.5. NOE's were observed for signals at δ 3.62 (H30), δ 3.15 ($H_{eq}32$), and δ 8.72 (N3-H) when the signal at δ 2.31 ($H_{ax}31$) was irradiated. It was also observed that irradiation of the H_3-41 methyl protons at δ 1.40 enhanced the $H_{ax}32$ proton signals at δ 4.29. This suggested that these protons were in a relation similar to a 1,3-diaxial position. Thus, the con-

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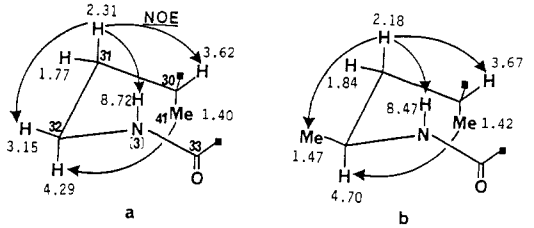
Table II. Biological Activities of Compounds 1-4[a]

	A. pectinifera	H. pulcherrimus	L1210
1	2×10^{-2}	1×10^{-2}	7.4×10^{-4}
2	2×10^{-2}	1×10^{-2}	8.8×10^{-4}
3	2×10^{-2}	5×10^{-3}	8.6×10^{-4}
4	5×10^{-2}	5×10^{-3}	1.5×10^{-3}

[a] Values represent IC_{50} (μg/mL).

formation of this section of calyculin A (part structure a) appears to be the same in benzene solution as in the crystalline state.



Similar experiments were carried out for calyculin C. The amide proton signal of calyculin C (δ 8.47 d, J = 10.5 Hz) also was not affected upon addition of D_2O and change of temperature. Coupling constants for the protons from C30 to N3 were obtained by extensive decoupling experiments: δ 8.47 (d, J = 10.5 Hz, NH), 4.70 (ddd, J = 13.0, 10.5, 2.0 Hz, $H_{ax}32$), 2.18 (ddd, J = 13.0, 13.0, 1.5 Hz, $H_{ax}31$), 1.84 (ddd, J = 13.0, 12.5, 2.0 Hz, $H_{eq}31$), 3.67 (dd, J = 12.5, 1.5 Hz, H30), suggesting the same conformation as that of calyculin A. Furthermore, irradiation of the $H_{ax}31$ proton (δ 2.18) enhanced the signals for H30 (δ 3.67), $H_{ax}32$ (δ 1.47), and NH (δ 8.47). An NOE was observed for $H_{ax}32$ signals (δ 4.70) upon irradiation of the H_3-41 methyl protons (δ 1.42). Thus, the relative configuration of C32 was concluded to be R^* (part structure b).

The fourth active constituent, calyculin D, is closely related to the other calyculins. The molecular formula of $C_{51}H_{83}N_4O_{15}P$ was established by an FAB mass spectral ion at m/z 1023 (MH^+) and ^{13}C NMR signals, which indicated that calyculin D was an isomer of calyculin C. The ^1H NMR spectra (1-D and H-H COSY) were indistinguishable from those of calyculin C except for the terminal tetraene portion. The structure of this moiety was secured by NOE experiments performed on calyculins C and D. The irradiation of the H_3-50 protons at δ 1.51 in calyculin C enhanced signals at δ 4.52 (H2) and δ 6.94 (H5), while an NOE was seen for the signal at δ 6.81 (H5) upon irradiation of H_3-50 protons (δ 1.91) in calyculin D. Thus, the geometry of $\Delta^{2,3}$ double bond of calyculin D is E.

Calyculins A-D showed significant activity in the starfish *Asterina pectinifera* and sea urchin *Hemicentrotus pulcherrimus* egg assays as shown in Table II. They were also highly cytotoxic against L1210 leukemia cells. Further, calyculin A exhibits antitumor activity against Ehrlich and P388 leukemia in mice (T/C 245.8 and 144.4%, respecitvely, at 15 μg/kg). It inhibited uptake of [^{3}H]thymidine, -uridine, and -leucine in L1210 cells, thus suggesting that the primary target of calyculin A is a system other than macromolecular synthesis.

Experimental Section

UV spectra were recorded on a Hitachi 330 spectrophotometer. IR spectra were taken on a JASCO A-202 infrared spectrophotometer. FAB mass spectra were measured on a JEOL JMS-DX 303 mass spectrometer using glycerol as a matrix. ^1H, ^{13}C, and ^{31}P NMR spectra were measured on a Bruker AM 500 NMR spectrometer. Optical rotations were determined on a JASCO DIP-4 polarimeter. The melting point was obtained on a Yanagimoto melting point apparatus and is uncorrected.

Isolation. Specimens of *D. calyx* were collected off the Izu Peninsula by SCUBA (-5 to -15 m). The animals were frozen immediately, transferred to our laboratory, and kept frozen at -20 °C unit processed. After the epiphytes were removed, the frozen sponge (1 kg) was homogenized and extracted with ethanol (3 × 5 L). The combined extracts were concentrated and partitioned between dichloromethane and water. The dichloromethane-soluble materials (2.2 g) were subjected to low pressure column chromatography on silica gel (BW-300, Fuji Devison) in a dichloromethane–methanol system. The active fractions were purified by HPLC on Cosmosil $5C_{18}$ ODS (Nakarai) with $MeOH/H_2O$ (8:2) to yield four active substances, designated calyculin A (1, 0.015% wet animal), B (2, 0.002%), C (3, 0.003%) and D (4, 0.005%) as colorless solids.

Calyculin A (1):[14] colorless needles from a mixture of hexane, ether, and acetone; mp 247-249 °C; $[\alpha]_D$ -60° (c 0.1, EtOH); UV (EtOH) 342 (ϵ 19000), 230 nm (12000); IR (film) 3500, 3300, 3150, 3050, 2950, 2900, 2250, 1640, 1580, 1530, 1470, 1370, 1260, 1100, 1060, 1010, 950 cm^{-1}; ^1H, ^{13}C, and ^{31}P NMR (see Table I and ref 10); FABMS, m/z 1009 $(M + H)^+$, 1007 $(M - H)^-$.

Calyculin B (2): colorless solid; $[\alpha]_D$ -61° (c 0.05, EtOH); UV (EtOH) 341 (ϵ 25000), 230 nm (18000); IR (film) 3500, 3300, 3150, 3050, 2950, 2900, 2250, 1640, 1580, 1530, 1470, 1380, 1100, 1060, 1010, 960 cm^{-1}; ^{13}C NMR ($CDCl_3$) δ 176.3 s, 169.9 s, 156.9 s, 144.5 s, 137.3 s, 134.7 s, 133.8 d, 133.4 d, 132.7 d, 132.2 d, 130.9 d, 123.4 d, 117.1 s, 116.1 d, 108.8 s, 96.2 d, 84.9 d, 82.0 d, 80.7 d, 77.3 d, 75.0 d, 73.4 d, 71.5 d, 69.2 d, 67.2 d, 65.6 t, 63.8 d, 61.0 q, 59.0 q, 50.2 s, 44.4 q, 42.2 d, 38.5 t, 37.8 d, 37.5 q, 35.9 t, 35.9 d, 34.5 t, 33.7 t, 29.7 t, 28.5 d, 22.5 q, 17.8 q, 17.5 q, 14.0 q, 13.7 q, 12.8 q, 10.9 q; FABMS, m/z 1009 $(M + H)^+$.

Calyculin C (3): colorless solid; $[\alpha]_D$ -65° (c 0.05, EtOH); UV (EtOH) 340 (ϵ 19000), 230 nm (15000); IR (film) 3500, 3300, 3150, 3050, 2950, 2900, 2250, 1640, 1570, 1530, 1470, 1370, 1110, 1060, 1010, 960 cm^{-1}; ^1H and ^{13}C NMR (see Table I); FABMS, m/z 1023 $(M + H)^+$.

Calyculin D (4): colorless solid; $[\alpha]_D$ -41° (c 0.05, EtOH); UV (EtOH) 340 (ϵ 12000), 230 nm (10000); IR (film) 3500, 3300, 3150, 3050, 2950, 2900, 2250, 1640, 1570, 1530, 1470, 1380, 1220, 1110, 1060, 1010, 960 cm^{-1}; ^1H NMR (see Table I); ^{13}C NMR (C_6D_6) δ 176.9 s, 170.8 s, 156.5 s, 144.9 s, 138.6 d, 135.3 s, 134.5 d, 133.9 s 133.7 d, 133.5 d, 131.8 d, 124.4 d, 117.2 d, 109.8 s, 106.8 s, 97.7 d, 85.9 d, 83.0 d, 81.7 d, 78.1 d, 76.0 d, 74.5 d, 72.2 d, 69.9 q, 68.5 q, 66.1 d, 64.5 t, 64.3 d, 61.5 d, 58.9 q, 51.0 s, 43.2 d, 42.0 d, 40.9 t, 39.1 t, 37.4 d, 37.0 t, 36.8 d, 30.7 d, 29.4 t, 23.2 q, 21.4 q, 19.6 q, 18.9 q, 18.7 q, 16.6 q, 14.7 q, 14.3 q, 13.4 q, 11.6 q; FABMS, m/z 1023 $(M + H)^+$.

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Registry No. 1, 101932-71-2; 2, 107537-44-0; 3, 107537-45-1; 4, 107447-09-6.

(14) The optical rotation, UV extinction coefficients, and yield reported for calyculin A in the previous communication were incorrect.