Clavosines A−C from the Marine Sponge *Myriastra clavosa*: Potent Cytotoxins and Inhibitors of Protein Phosphatases 1 and 2A

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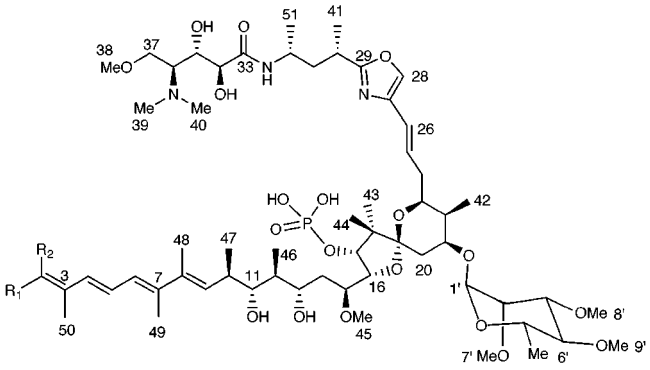
Clavosines A (**1**) and B (**2**), novel metabolites closely related to calyculins and calyculinamides, have been isolated from the marine sponge *Myriastra clavosa*. Clavosine C (**3**) was also isolated and is assumed to be an artifact from clavosine B (**2**). Structures of these compounds were determined by spectroscopic analysis and by comparison of their NMR data with those of the calyculins and calyculinamides. Clavosines A and B were found to be very potent cytotoxins in the National Cancer Institute's screening panel of 60 tumor cell lines and were potent inhibitors of the type 1 and 2A serine/threonine protein phosphatases.

Calyculin A is a novel metabolite[1] isolated initially from the Japanese sponge *Discodermia calyx*.[1] A wide spectrum of bioactivities, such as cytotoxicity against several cell lines,[1,2] in vivo antitumor activity,[2] potent inhibition of protein phosphates 1 and 2A,[3,4] and insecticidal activity[5] have been reported for this compound. Its structure and the relative stereochemistry were determined by X-ray crystallography,[1] and the absolute stereochemistry was established by chemical degradation[6,7] and confirmed by total syntheses.[8] Since the discovery of calyculin A, related compounds, calyculins B−H,[2,3,5] calyculin J,[9] calyculinamides A and F,[9] des-*N*-methylcalyculin A,[9] and dephosphonocalyculin A,[10] have been isolated from the Lithistid sponge *D. calyx*. Calyculins A, B, E, and F and calyculinamides A and B have been reported[11] from the Epipolasid sponge *Lamellomorpha strongylata* as well. All these metabolites are either potent cell-growth inhibitors or protein phosphatases 1 and 2A inhibitors. During our ongoing search for potential anticancer agents from marine organisms,[12] we examined extracts of the sponge *Myriastra clavosa* Ridley 1884 (Order Astrophorida, Family Ancorinidae) because they showed significant cytotoxicity in a screening program. From this sponge, we have isolated three compounds, designated as clavosines A−C (**1**−**3**), which are closely related to the calyculins and calyculinamides. We report here the isolation and structure elucidation of these unusual metabolites.



Clavosine A (**1**): R_1 = H R_2 = $CONH_2$
Clavosine B (**2**): R_1 = $CONH_2$ R_2 = H
Clavosine C (**3**): 6*Z* isomer of **2**

Small amounts of *M. clavosa* were initially collected in 1993 from Chuuk, Federated States of Micronesia. An extract of the specimens exhibited cytotoxicity against

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human breast (MDA-MB435) and human lung (A549) tumor cell lines. We were unable to isolate any pure active compounds from this collection due to scarcity of sample, although ^{1}H NMR clearly indicated the presence of unsaturated, complex compounds. Subsequently, more of the sponge was collected in Palau in 1995. Extracts of these specimens showed cytotoxicity comparable to that of the Chuuk specimens. The MeOH and MeOH/CH_2Cl_2 (1:1) extracts of lyophilized specimens were concentrated and subjected to solvent partitioning[13] to yield hexane, CH_2Cl_2, *n*-BuOH, and H_2O-soluble fractions, the bioactivity being concentrated in the CH_2Cl_2 solubles. A portion of the CH_2Cl_2-soluble materials was therefore chromatographed successively over silica gel open column, C_{18} reversed-phase column, and reversed-phase HPLC to afford clavosines A−C (**1**−**3**), but each pure isolate rapidly equilibrated upon exposure to ambient light to a mixture comprised of all three components. This photochemical isomerization had previously been noted with the calyculins.[3] Repetition of the separation sequence and subsequent handling of samples in a darkened room resulted in purification of **1**−**3**. From a collection of the sponge in Palau in November, 1996, only clavosines A (**1**) and B (**2**) were obtained, care being taken to minimize exposure to light in all phases of the extraction and purification process.

Clavosine B (**2**), $[\alpha]_D$ −3.2°(*c* 0.62, CH_2Cl_2), was obtained as a white powder. This isomer was the most abundant and was consequently subjected to the most detailed spectroscopic analysis. The molecular formula $C_{60}H_{101}N_4O_{20}P$ for **2** was established by high-resolution FABMS and NMR data (Table 2). The IR spectrum contained bands at 3460, 3300, 3180, 1666, 1640, and 1580 cm^{-1}; UV absorptions at 228 (ϵ 18 572), 328 (ϵ 22 998) nm were consistent with the presence of an oxazole and a highly conjugated system. The ^{1}H and ^{13}C NMR spectra of clavosine B (**2**) were clearly reminiscent of those of the calyculins.[1−3,5] Interpretation of the COSY, RCT-COSY, HMQC, and HMBC spectra established the connectivity of the overall carbon skeleton. The ^{1}H and ^{13}C NMR chemical shift data and ^{1}H coupling in CD_2Cl_2 for H/C-1 to H/C-17 matched closely the data for this identical segment in calyculinamide B[11] and also the data in C_6D_6 for H/C-9 to H/C-17 matched that reported in this solvent for this region of calyculin E.[3] This established the all *E*-configuration for the tetraene moiety, and the relative stereochemistries from C-10 to C-17 are also taken to be the same as in the calyculins and calyculinamides. The chemical shift and coupling data for the segment C-25 to C-40 were essentially coincident with the data for this portion of calyculin C[2] and G[3] and this established the structure and stereochemistry of this major section of the molecule with a 25*E* double bond. The connections surrounding the spiro ketal unit were surmised by analogy with the calyculins and confirmed by the HMBC correlations observed for H-20, H-43, and H-44, see Table 2. The stereochemistry of the tetrahydropyran ring was deduced as follows. The coupling constants (dt, J = 11.3, 4.2 Hz) for H-21 indicated an axial H at C-21 and an equatorial H at C-22 (Figure 1).[14] In contrast, only small coupling constants were observed for H-21 in the calyculins. Furthermore,

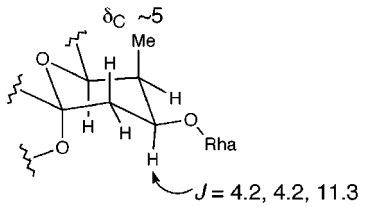

Figure 1.

the unusually upfield ^{13}C NMR chemical shift (δ 4.88 in CD_2Cl_2; δ 5.24 in C_6D_6) for C-42 (Me at C-22) was close to that of a methyl (C-50, δ 5.98) in a similarly substituted tetrahydropyran unit in the phorboxazoles,[14] which represents shielding of about 6 ppm (γ-gauche effect) in comparison with the methyl group at this position in the calyculins,[1−3,5] calyculinamides,[9,11] altohyrtins,[15] spongistatins,[16] and lasonolide A.[17] In addition to the significant change of the chemical shift of C-42, change was also observed for the chemical shifts of C-20 to C-23 due to the C-21 equatorial oxygen substituent. The presence of a trimethoxy rhamnose moiety was evident from the ^{1}H and ^{13}C chemical shifts[18] for this unit, ^{1}H−^{1}H correlations observed in the COSY and RCT-COSY, ^{1}H−^{13}C long-range couplings observed in the HMBC spectrum (Table 2), and the ^{1}H−^{1}H coupling constants (Table 2, C_6D_6 data). Linkage of the rhamnose unit to C-21 was deduced from a HMBC correlation between C-21 and the anomeric proton (H-1′).[19] The absolute configuration of the rhamnose unit was not determined but is assumed to be the common (L) configuration. The α-anomeric assignment is based on the large $^1J_{CH}$ value at the anomeric carbon (167.5 Hz).[18] Consistent with the rhamnose stereochemical assignment, an NOE was observed between H-1′/H-2′ but not between H-1′ and either H-3′ or H-5′. Therefore, structure **2** was assigned to clavosine B.

Clavosine A (**1**) possessed the same molecular formula, $C_{60}H_{101}N_4O_{20}P$, as clavosine B judging from FABMS analysis and NMR data (Table 1). Comparison of the NMR data of clavosine A (**1**) with those of clavosine B

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(19) Determination of the glycosidation site was difficult because the chemical shifts of C-35 and C-21 are virtually identical and no correlation between C-1′ and any protons was observed. However, hydroxyl groups could confidently be placed at C-13 and C-34 on the basis of correlations noted in the COSY spectrum, and hydroxyl groups were also placed at C-11 and C-35 because these singlet OH signals showed HMBC correlations to neighboring carbons, see Table 1, as is reported for calyculin E.[9] Hence the methylated rhamnose residue was located at C-21.

Table 1. NMR Spectral Data for Clavosine A (1)

C (mult)[a]	^{13}C (CD_2Cl_2)	^{1}H (CD_2Cl_2, mult, *J* in Hz)	^{1}H (C_6D_6 mult, *J* in Hz)
1(s)	168.3		
2(d)	118.5	5.59 (s)	5.11 (s)
3(s)	148.2		
4(d)	129.4	7.76 (d, 15.6)	8.64 (d, 15.6)
5(d)	132.3	6.95 (dd, 15.6, 10.9)	7.04 (dd, 15.6, 10.4)
6(d)	125.3	6.37 (d, 10.9)	6.63 (d, 10.4)
7(s)	142.3		
8(s)	135.3		
9(d)	131.4	6.22 (d, 9.0)	6.58 (d, 9.0)
10(d)	36.2	2.76 (m)	2.66 (m)
11(d)	81.4	3.54 (dd, 10.0, 2.4)	3.64 (br d, 10.0)
12(d)	42.4	1.44 (m)	1.71 (m)
13(d)	75.7	3.35 (br q, 10)	3.74 (m)
14(t)	38.4	1.75 (dd, 14.2, 11.3); 1.50 (br dd, 14.2, 10.4)	2.02 (m); 1.68 (m)
15(d)	77.8	3.62 (m)	4.07 (dd, 9.9, 9.4)
16(d)	84.9	3.93 (m)	4.15 (dd, 9.9, 3.8)
17(d)	83.1	4.05 (dd, 10.4, 3.8)	4.35 (dd, 10.4, 3.8)
18(s)	50.1		
19(s)	108.6		
20(t)	31.3	1.64 (dd, 12.3, 4.2); 1.59 (dd, 12.3, 11.3)	2.02 (m); 1.81 (m)
21(d)	73.9	4.10 (dt, 11.3, 5.2)	4.51 (dt, 11.8, 4.7)
22(d)	35.0	1.99 (m)	1.96 (m)
23(d)	70.8	3.93 (m)	4.20 (br d, 11.8)
24(t)	36.1	2.52 (br t, 12.8); 1.99 (m)	2.53 (br t, 12.7); 1.80 (m)
25(d)	133.1	6.90 (ddd, 17.5, 10.8, 3.8)	7.38 (ddd, 16.0, 10.9, 3.8)
26(d)	116.5	6.14 (d, 17.5)	5.98 (d, 16.0)
27(s)	137.9		
28(d)	134.0	7.38 (s)	6.89 (s)
29(s)	170.3		
30(d)	29.8	3.27 (m)	3.70 (m)
31(t)	39.8	1.78 (m)	2.18 (br t, 12.7); 1.82 (m)
32(d)	41.4	4.34 (m)	4.66 (m)
33(s)	176.0		
34(d)	69.3	3.62 (m)	3.78 (m)
35(d)	73.7	4.52 (d, 9.9)	4.77 (d, 10.4)
36(d)	64.1	4.02 (br d, 7.6)	3.97 (br d, 5.2)
37(t)	65.8	3.93 (m); 3.73 (dd, 12.3, 2.4)	3.48 (m)
38(q)	59.3	3.41 (s)	2.90 (s)
39(q)	44.7	2.79 (br s)	1.98 (br s)
40(q)	37.8	2.88 (br s)	1.98 (br s)
41(q)	19.1	1.27 (d, 7.1)	1.40 (d, 7.1)
42(q)	48.9	0.84 (d, 7.1)	0.98 (d, 6.6)
43(q)	22.7	0.91 (s)	0.92 (s)
44(q)	17.5	1.21 (s)	1.62 (s)
45(q)	61.0	3.43 (s)	3.78 (s)
46(q)	12.8	0.57 (d, 6.6)	0.48 (d, 6.6)
47(q)	18.0	1.04 (d, 6.6)	1.24 (d, 6.6)
48(q)	13.8	1.86 (s)	1.67 (s)
49(q)	14.2	2.01 (s)	2.17 (s)
50(q)	20.95	2.02 (s)	1.84 (s)
51(q)	20.99	1.31 (d, 6.6)	1.43 (d, 6.1)
1′(d)	94.6	4.94 (br s)	5.19 (br s)
2′(d)	78.1	3.51 (br s)	3.65 (br s)
3′(d)	81.6	3.45 (m)	3.85 (dd, 9.9, 3.4)
4′(d)	82.5	3.02 (t, 9.5)	3.53 (t, 9.9)
5′(d)	68.3	3.58 (m)	4.01 (dq, 9.9, 6.1)
6′(q)	18.3	1.22 (d, 7.1)	1.43 (d, 6.1)
7′(q)	59.1	3.44 (s)	3.35 (s)
8′(q)	57.5	3.43 (s)	3.33 (s)
9′(q)	60.9	3.48 (s)	3.49 (s)
1-CONH2		5.47 (br), 5.23 (br)	4.97 (br); 4.33 (br)
11-OH		6.62 (s)	7.28 (s)
13-OH		6.04 (d, 10)	6.59 (d, 9.0)
33-CONH		7.98 (d, 10.4)	8.52 (d, 9.4)
34-OH		7.54 (br d, 9.5)	8.02 (br d, 10.8)
35-OH		6.03 (s)	6.77 (s)
-OPO(OH)2		13.65 (br); 11.74 (br)	13.75 (br); 12.06 (br)

[a] Multiplicity in agreement with HMQC.

(**2**) suggested that **1** was closely related to **2**, the only difference being *Z* geometry for the C-2−C-3 double bond instead of *E* as in **2**. This was evident from a downfield shift of C-50 (δ 20.95 instead of 13.4) and an upfield shift of C-4 (δ 129.4 in place of 135.2) in **1** when compared to **2**. The NMR data for this region of the molecule was nearly identical to that for the same portion of calyculinamide A which has this same geometry.[11]

Table 2. NMR Spectral Data for Clavosine B (2)

	CD_2Cl_2		C_6D_6		
C (mult)[a]	^{13}C	^1H (mult, *J* in Hz)	^{13}C	^1H (mult, *J* in Hz)	HMBC (C No.)
1(s)	168.8		168.1		
2(d)	120.2	5.74 (br s)	120.8	5.41 (br s)	1, 4, 50
3(s)	150.2		149.7		
4(d)	135.2	6.29 (d, 15.1)	135.5	6.28 (d, 15.1)	2, 6, 50
5(d)	131.3	6.97 (dd, 15.1, 11.4)	131.1	7.07 (dd, 15.1, 11.3)	3
6(d)	124.4	6.29 (d, 11.4)	124.5	6.49 (d, 11.3)	4, 8, 49
7(s)	142.1		142.4		
8(s)	135.1		134.9		
9(d)	131.5	6.23 (d, 9.0)	132.0	6.66 (d, 9.0)	7, 48
10(d)	36.2	2.75 (m)	36.4	2.71 (m)	
11(d)	81.4	3.54 (m)	81.3	3.67 (br d, 11.8)	9
12(d)	42.4	1.44 (m)	42.5	1.70 (m)	
13(d)	75.7	3.35 (br q, 10)	75.6	3.75 (m)	
14(t)	38.4	1.75 (m); 1.50 (br dd, 14.3, 10)	39.1	2.02 (m); 1.68 (br dd, 14.6, 9.4)	13, 15
15(d)	77.8	3.62 (m)	77.7	4.07 (dd, 9.9, 9.4)	16, 45
16(d)	84.8	3.94 (m)	85.5	4.15 (dd, 9.9, 3.3)	
17(d)	83.1	4.05 (dd, 10.4, 3.8)	83.4	4.31 (dd, 10.4, 3.5)	
18(s)	50.1		50.1		
19(s)	108.6		108.8		
20(t)	31.3	1.64 (dd, 12.3, 5.2); 1.59 (dd, 12.3, 11.8)	32.0	1.99 (m); 1.82 (br t, 12.3)	19, 21
21(d)	73.9	4.10 (dt, 11.8, 4.7)	74.1	4.50 (dt, 11.3, 4.2)	
22(d)	35.0	1.99 (m)	35.3	1.95 (m)	
23(d)	70.8	3.95 (br d, 10.9)	71.2	4.19 (br d, 12.2)	
24(t)	36.1	2.52 (br t, 13.0); 1.99 (m)	36.2	2.53 (br t, 12.7); 1.82 (m)	
25(d)	133.1	6.90 (ddd, 14.2, 10.7, 4.2)	133.3	7.37 (ddd, 16.0, 10.8, 3.7)	
26(d)	116.5	6.14 (d, 14.2)	116.7	5.98 (d, 16.0)	24, 27
27(s)	137.9		138.2		
28(d)	134.0	7.38(s)	133.9	6.83 (s)	27, 29
29(s)	170.3		170.5		
30(d)	29.8	3.27 (m)	30.2	3.70 (m)	29
31(t)	39.8	1.78 (m)	40.3	2.18 (br t, 13.2); 1.82 (br t, 12.7)	30, 32, 41
32(d)	41.4	4.35 (m)	41.5	4.67 (m)	
33(s)	176.0		176.4		
34(d)	69.3	3.62 (m)	69.3	3.78 (m)	33, 35
35(d)	73.7	4.51 (d, 9.9)	74.0	4.74 (d, 9.9)	33, 34, 36, 37
36(d)	64.2	4.02 (br d, 7.6)	63.9	3.92 (dd, 7.6, 3.3)	37
37(t)	65.7	3.92 (dd, 12.3, 8.5); 3.73 (dd, 12.3, 2.5)	65.6	3.43 (m)	36
38(q)	59.3	3.41 (s)	58.5	2.84 (s)	37
39(q)	44.7	2.79 (br s)	43.4	1.93 (br s)	36, 40
40(q)	37.8	2.88 (br s)	36.8	1.95 (br s)	36, 39
41(q)	19.1	1.27 (d, 7.0)	19.3	1.40 (d, 7.0)	29, 30, 31
42(q)	4.88	0.84 (d, 6.6)	5.24	0.98 (d, 6.5)	21, 22, 23
43(q)	22.7	0.91 (s)	22.9	0.91 (s)	17, 18, 19, 44
44(q)	17.5	1.21 (s)	18.2	1.60 (s)	17, 18, 19, 43
45(q)	61.0	3.43 (s)	61.0	3.79 (s)	15
46(q)	12.8	0.57 (d, 6.6)	12.9	0.52 (d, 6.5)	11, 12, 13
47(q)	17.9	1.04 (d, 7.1)	18.5	1.29 (d, 7.0)	9, 10, 11
48(q)	13.7	1.85 (s)	13.8	1.87 (s)	7, 8, 9
49(q)	14.2	2.01 (s)	14.3	2.16 (s)	6, 7, 8
50(q)	13.4	2.31 (s)	13.5	2.60 (s)	2, 3, 4
51(q)	21.0	1.30 (d, 6.5)	20.9	1.43 (d, 6.5)	31, 32
1′(d)	94.7	4.94 (br s)	95.5	5.19 (br s)	21, 3′, 5′
2′(d)	78.1	3.50 (br s)	78.4	3.65 (br s)	3′, 7′
3′(d)	81.6	3.45 (m)	82.7	3.84 (dd, 9.4, 3.3)	4′, 8′
4′(d)	82.5	3.02 (t, 9.0)	82.9	3.52 (t, 9.4)	3′, 5′, 9′
5′(d)	68.3	3.58 (m)	69.1	4.02 (dq, 9.4, 6.6)	
6′(q)	18.3	1.22 (d, 6.6)	18.2	1.43 (d, 6.6)	4′, 5′
7′(q)	59.1	3.44 (s)	59.0	3.35 (s)	2′
8′(q)	57.5	3.43 (s)	57.3	3.33 (s)	3′
9′(q)	60.9	3.48 (s)	60.8	3.49 (s)	4′
1-CONH2		5.49 (br), 5.31 (br)		4.96 (br), 4.44 (br)	
11-OH		6.59 (s)		7.35 (s)	10, 11
13-OH		6.07 (d, 9.5)		6.66 (d, 9.0)	13
33-CONH		7.98 (d, 10.4)		8.53 (d, 10.9)	33
34-OH		7.54 (br d, 11.8)		8.01 (br d, 11.8)	
35-OH		6.03 (s)		6.78 (s)	34, 35, 36
-OPO(OH)2		13.62 (br); 11.74 (br)		13.62 (br); 12.03 (br)	

[a] Multiplicity in agreement with HMQC.

The molecular formula of clavosine C (**3**), $C_{60}H_{101}N_4O_{20}P$, deduced from FABMS and NMR data (Table 3), was also identical to that of **1** and **2**. The UV and IR spectra of **3** were very similar to those of compounds **1** and **2**. The ^1H and ^{13}C NMR spectral data of clavosine C (**3**) were assigned by detailed analysis of the COSY, RCT-COSY,

Table 3. NMR Spectral Data for Clavosine C (3)

	CD_2Cl_2		C_6D_6		
C (mult)[a]	^{13}C	^1H (mult, *J* in Hz)	^{13}C	^1H (mult, *J* in Hz)	HMBC (C No.)
1(s)	169.0		b		
2(d)	119.7	5.66 (s)	120.4	5.47 (s)	1, 4
3(s)	150.1		149.5		
4(d)	132.6	6.08 (d, 15.1)	133.4	6.18 (d, 15.2)	6, 50
5(d)	133.1	6.84 (dd, 15.1, 10.7)	133.4	7.20[c]	
6(d)	124.9	5.87 (d, 10.7)	125.5	6.03 (d, 11.0)	5
7(s)	148.8		147.6		
8(s)	133.6		133.5		
9(d)	131.4	5.68 (d, 10.3)	131.8	6.16 (d, 8.3)	7, 11, 48
10(d)	35.4	2.67 (m)	35.6	2.63 (m)	
11(d)	81.4	3.52 (m)	81.2	3.67 (m)	
12(d)	41.9	1.56 (m)	42.3	1.88 (m)	
13(d)	75.2	3.38 (m)	75.3	3.83 (m)	
14(t)	38.5	1.75 (m); 1.50 (br dd, 13.5, 9.2)	38.9	2.10 (m); 1.82 (m)	
15(d)	77.6	3.62 (dd, 9.9, 9.5)	77.8	4.14 (dd, 9.8, 9.5)	45
16(d)	84.9	3.91 (m)	85.6	4.28 (m)	
17(d)	83.1	4.09 (m)	82.9	4.43 (dd, 10.3, 3.7)	
18(s)	50.1		50.1		
19(s)	108.7		108.9		
20(t)	31.4	1.65 (dd, 11.9, 4.7); 1.58 (t, 11.9)	32.0	2.08 (m); 1.88 (m)	21
21(d)	74.0	4.09 (m)	74.1	4.57 (dt, 11.8, 4.3)	
22(d)	35.0	1.99 (m)	35.3	2.00 (m)	
23(d)	70.8	3.94 (m)	71.2	4.25 (br d, 11.5)	
24(t)	36.1	2.51 (br t, 12.5); 1.99 (m)	36.3	2.58 (m); 1.86 (m)	
25(d)	133.1	6.89 (ddd, 15.9, 10.3, 4.2)	132.8	7.43 (ddd, 14.1, 10.6, 3.7)	
26(d)	116.5	6.13 (dd, 15.9, 2.0)	116.6	6.03 (d, 14.1)	27
27(s)	137.8		138.1		
28(d)	134.0	7.37 (s)	133.8	6.85 (s)	27, 29
29(s)	170.2		170.4		
30(d)	29.7	3.25 (m)	29.8	3.72 (m)	
31(t)	39.5	1.74 (m)	39.9	2.20 (m); 1.80 (m)	
32(d)	41.4	4.25 (m)	41.5	4.60 (m)	
33(s)	176.1		176.5		
34(d)	69.2	3.48 (m)	69.5	3.74 (m)	
35(d)	73.9	4.37 (d, 10.3)	74.3	4.69 (d, 10.1)	34
36(d)	64.1	3.90 (m)	63.7	3.94 (dd, 6.6, 4.9)	37
37(t)	66.0	3.91 (m); 3.67 (d, 10.3)	66.2	3.53 (m)	36
38(q)	59.3	3.42 (s)	58.5	2.99 (s)	37
39(q)	44.8	2.76 (br s)	44.0	2.04 (br s)	
40(q)	37.7	2.86 (br s)	36.8	2.19 (br s)	
41(q)	19.1	1.22 (d, 6.4)	19.2	1.36 (d, 7.2)	29, 30, 31
42(q)	48.8	0.84 (d, 6.8)	52.4	1.03 (d, 6.6)	21, 22, 23
43(q)	22.6	0.92 (s)	22.9	1.10 (s)	17, 18, 19, 44
44(q)	17.5	1.21 (s)	18.2	1.68 (s)	17, 18, 19, 43
45(q)	61.0	3.43 (s)	60.9	3.85 (s)	15
46(q)	12.9	0.64 (d, 6.7)	13.0	0.71 (d, 6.6)	11, 12, 13
47(q)	18.4	1.11 (d, 7.1)	18.7	1.33 (d, 6.9)	9, 10, 11
48(q)	15.3	1.71 (s)	15.2	1.67 (s)	7, 8, 9
49(q)	23.3	1.88 (s)	23.6	1.95 (s)	6, 7, 8
50(q)	13.8	2.18 (s)	14.4	2.58 (s)	2, 3, 4
51(q)	20.9	1.18 (d, 6.4)	20.9	1.37 (d, 6.1)	31, 32
1′(d)	94.7	4.94 (br s)	95.5	5.24 (br s)	21, 3′, 5′
2′(d)	78.1	3.45 (m)	78.5	3.70 (br s)	7′
3′(d)	81.5	3.44 (m)	82.7	3.88 (dd, 9.2, 3.2)	8′
4′(d)	82.5	3.02 (t, 9.2)	82.7	3.56 (dd, 9.5, 9.2)	9′
5′(d)	68.3	3.57 (dd, 9.2, 6.4)	69.0	4.05 (dq, 9.5, 6.3)	
6′(q)	18.0	1.22 (d, 6.4)	18.2	1.46 (d, 6.3)	4′, 5′
7′(q)	59.1	3.44 (s)	58.9	3.40 (s)	2′
8′(q)	57.5	3.43 (s)	57.3	3.38 (s)	3′
9′(q)	60.9	3.49 (s)	60.8	3.54 (s)	4′
1-CONH2		5.20 (br)		5.50 (br); 5.05 (br)	
11-OH		6.60 (s)		6.98 (br s)	10
13-OH		6.01 (d, 10.3)		6.65 (d, 9.5)	
33-CONH		7.85 (d, 10.3)		8.40 (d, 10.5)	33
34-OH		7.37 (br d, 11.9)		7.84 (br d, 11.8)	
35-OH		6.09 (s)		6.83 (s)	36
-OPO(OH)2		13.70 (br); 11.75 (br)		13.60 (br); 12.10 (br)	

[a] Multiplicity in agreement with HMQC. [b] Not observed. [c] Immersed in the solvent (C_6D_6) peak.

HMQC, and HMBC spectra and were also very similar to those of **2** except for the conjugated tetraene moiety. The ^{13}C chemical shift for C-49 (δ 23.3) strongly suggested that clavosine C is a 6*Z* geometrical isomer of clavosine B (**2**). This was confirmed by NOE correlations of the tetraene portion in the NOESY spectrum of **3** in C_6D_6: H-2/H-4, H-4/H-6, H-6/H-49, and H-5/H-48. The NMR data for H/C-1 to H/C-10 in **3** coincided well with the

corresponding data of calyculinamide F[9] which has the same terminal tetraene amide. Therefore, structure **3** was assigned to clavosine C.

Although the absolute configuration of **1**−**3** have not been determined, we assume they are the same as those established for the calyculins since the optical rotations are all negative, albeit considerably smaller, than those of the calyculins.

Compounds **1**−**3** have many structural features in common with the calyculins[1−3,5] and calyculinamides[9,11] but differ from them in having a methylated rhamnose at C-21 instead of a hydroxyl and a 21*S* configuration in place of a 21*R*. It has been noted[20] that except for calyculins A and C, the predominant components of the Japanese sponge *D. calyx*, the remaining calyculins may be artifacts generated during isolation. We believe that clavosine C (**3**), the minor component of the sponge *M. clavosa*, is also an artifact due to photochemical isomerization of the terminal tetraene unit, because reversed-phase HPLC of the freshly prepared extracts of deep or shallow water specimens collected in 1996 and prepared shortly after collection showed only two peaks which correspond to clavosines A and B.

The yield of **1** and **2** (∼ 1:1) was approximately five times greater from specimens collected in deep water (−25 to −40 M) than in shallow water (−3 M) in Palau in the 1996 collection. Sponges from deep water were gray colored while those from shallow water were reddish-pink. To our knowledge, this is the first report of metabolites isolated from the sponge *M. clavosa*.

Clavosines A (**1**) and B (**2**) were tested in the National Cancer Institute's screening panel of 60 tumor cell lines[21] and were found to be very potent. The mean graph midpoint data for all cell lines was as follows: **1**, $\log_{10}$ GI_{50} −10.90 (0.01, 0.34); $\log_{10}$ TGI −10.52 (0.39, 4.00); $\log_{10}$ LC_{50} −9.80 (1.10, 4.00); **2**, $\log_{10}$ GI_{50} −10.79 (0.11, 2.64); $\log_{10}$ TGI −10.28 (0.62, 4.00); $\log_{10}$ LC_{50} −9.28 (1.62, 4.00) M (Δ, range).

Clavosines A and B were assayed for their ability to inhibit the purified catalytic subunit of native protein phosphatase-1 (PP-1c) from rabbit skeletal muscle, human recombinant PP-1cγ expressed in and purified from *E. coli*, and the catalytic subunit of protein phosphatase 2A (PP-2Ac) from bovine heart. IC_{50} values for the clavosines were: PP-1cγ, **1** = 0.5 nM; **2** = 13 nM; native PP-1c, **1** = 0.25 nM; **2** = 1.0 nM; PP-2Ac, **1** = 0.6 nM; **2** = 1.2 nM.

Experimental Section

General Experimental Procedures. All proton NMR spectra were measured at 500 MHz and carbon-13 NMR spectra at 125 MHz.

Animal Material. The sponge was collected in Palau in November 1995 at Pelih's and West Bainer at −33 to −50 m (22-PA-95) and in 1996 at Big Dropoff at −27 to −40 m (1-PA-96) and at Helen's Reef, Southwest Islands at −3 m (2-PA-96). Samples were frozen shortly after collection. In life the sponge is 5−13 mm diameter, consistently spherical, with an apical oscular depression; dark olive green, frequently with a purplish tinge. Individuals are free living, often attached to seagrass or inorganic surfaces by long protruding visible spicules. The sponge is *Myriastra clavosa* (Ridley 1884) (Order Astrophorida, Family Ancorinidae). A voucher specimen has been deposited in The Natural History Museum, London (BMNH 1998.3.5.1) and the University of Oklahoma (54-T-93).

Extraction and Isolation. The frozen specimens from the 1995 Palau collection (22-PA-95) (480 g wet wt, 41 g dry wt after extraction) were lyophilized and then extracted sequentially with MeOH and MeOH/CH_2Cl_2 (1:1), twice each. The combined extracts were concentrated in vacuo and partitioned using the procedure as reported previously[13] to give three organic-soluble fractions: hexane (1.95 g), CH_2Cl_2 (1.43 g), and *n*-BuOH (1.54 g), and a water-soluble fraction (12.24 g). A portion of the CH_2Cl_2-soluble materials (0.61 g) was subjected to chromatography over silica gel using gradient elution (5% acetone in CH_2Cl_2 to acetone to MeOH). A fraction which was eluted by acetone and contained calyculin-like compounds was further fractionated on a C_{18} reversed-phase open column with 20% H_2O in MeOH followed by 15% H_2O in MeOH to give a mixture of clavosines A−C. This mixture was resolved by reversed-phase HPLC employing 18% H_2O in MeOH as eluent to give pure clavosine A (**1**) (4.2 mg, 1.68×10^{-2}% of dry specimens), clavosine B (**2**) (5.1 mg, 2.04×10^{-2}%), clavosine C (**3**) (1.8 mg, 7.19×10^{-3}%). All operations *following* extraction and partitioning were performed in the dark and/or in glass covered with aluminum foil. For the 1996 collection, even the extraction and solvent partitioning were conducted in a darkened room.

Clavosine A (1): white powder; $[\alpha]_D$ −5.0° (*c* 0.36, CH_2Cl_2); UV (MeOH) 228 (ϵ 20 925), 332 (ϵ 23 270) nm; IR (neat) 3460, 3350, 3180, 1665, 1645, 1585, 1535 cm^{-1}; ^1H and ^{13}C NMR (see Table 2); FABMS *m*/*z* 1251 $(M + Na)^+$; 1229 $(M + H)^+$.

Clavosine B (2): white powder; $[\alpha]_D$ −3.2° (*c* 0.62, CH_2Cl_2); UV (MeOH) 228 (ϵ 18 572), 328 (ϵ 22 998) nm; IR (neat) 3460, 3300, 3180, 1666, 1640, 1580, 1530 cm^{-1}; ^1H and ^{13}C NMR (see Table 1); FABMS *m*/*z* 1251 $(M + Na)^+$; 1229 $(M + H)^+$; high-resolution FABMS found *m*/*z* 1229.6838 $(M + H)^+$; $C_{60}H_{102}N_4O_{20}P$ requires 1229.6829.

Clavosine C (3): white powder; $[\alpha]_D$ −31.7° (*c* 0.12, CH_2Cl_2); UV (MeOH) 228 (ϵ 20 300), 318 (ϵ 19 957) nm; IR (neat) 3470, 3300, 1660, 1635, 1580, 1530 cm^{-1}; ^1H and ^{13}C NMR (see Table 3), FABMS *m*/*z* 1251 $(M + Na)^+$; 1229 $(M + H)^+$, 1228 $(M)^+$.

PP-1c assays. Protein phosphatase inhibition was assayed using ^{32}P-radiolabeled phosphorylase *a* as substrate, as previously described.[22] Assays (final volume 30 μL) contained 50 mM Tris HCl, 0.1 mM EDTA, 25 mM 2-mercaptoethanol, 0.8 mM $MnCl_2$, 1 mg/mL bovine serum albumin, 3.75 mM caffeine, 10 μM ^{32}P-radiolabeled phosphorylase *a*, calyculin A or clavosines A/B, and native/recombinant PP-1c and PP-2Ac as noted. Assays with native PP-1c did not contain $MnCl_2$ or EDTA. All reactions were performed in duplicate. Calyculin A was obtained from Calbiochem, native PP-1c from Upstate Biotechnology, recombinant human PP-1cγ was expressed in and purified from *E. coli*,[23] and PP-2Ac was purified from bovine cardiac tissue.[24]

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra for clavosine A−C (**1**−**3**) (11 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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