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TETRAHEDRON

Isolation and Structural Elucidation of the Crellastatins I-M: Cytotoxic Bis-Steroid Derivatives from the Vanuatu Marine Sponge *Crella* sp.

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Abstract. Continued investigation of cytotoxic extracts of the marine sponge *Crella* sp. has resulted in the discovery of crellastatins I-M (**2-6**). Their structures were determined by interpretation of their NMR spectroscopic and FABMS data. These new bis-steroid derivatives exhibited cytotoxic activity against NSCLC cell lines with IC_{50} values in the range of 1-8 μg/ml. © 1999 Elsevier Science Ltd. All rights reserved.

Key words. Marine sponge; *Crella* sp.; Dimeric sterols; Cytotoxic; 2D-NMR.

INTRODUCTION

Marine sponges continue to be a rich source of new secondary metabolites with unusual architecture and remarkable biological activity.[1] Recently, from the sponge *Crella* sp. collected at the Vanuatu islands, we isolated a bis steroid derivative, which we named crellastatin A (**1**).[2] It represents the first example of a dimeric steroid in which the junction between the two monomeric units is obtained through their side chains. Subsequently, we isolated and characterized another seven crellastatins B-H,[3] which differ from the parent one in their hydroxylation pattern in the two tetracyclic cores. All the crellastatin derivatives exhibited good activity against human NSCLC cell lines with IC_{50} in the range 1-5 μg/ml. In order to obtain additional amounts of these compounds for further pharmacological studies, a second larger recollection of *Crella* sp. sponge was performed in June 1997.

RESULTS AND DISCUSSION

Fractionation of the bioactive chloroform and butanol extracts also afforded trace amounts of five more related compounds, crellastatins I-M (**2-6**), together with the previously reported crellastatins A-H. The lyophilized sponge (300 g) was extracted with MeOH, and the combined extracts were fractionated according to the Kupchan partitioning procedure.[4] The bioactive chloroform extract was purified by DCCC (Droplet Counter Current Chromatography) followed by reverse-phase HPLC (65% aqueous MeOH) to give crellastatins

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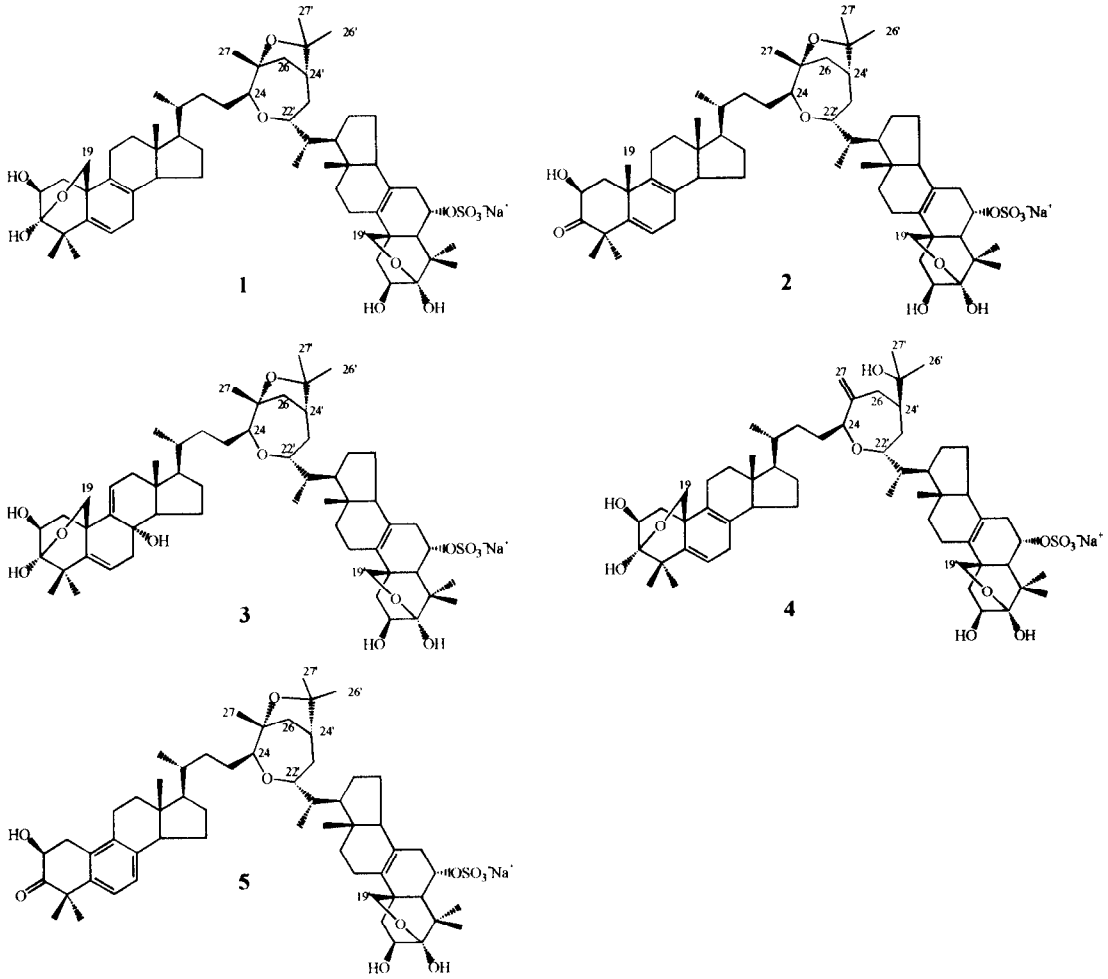
I-L (**2-5**).The more polar disulphated crellastatin M (**6**) was obtained from the butanol extract, cromatographed by DCCC ($CHCl_3$, MeOH, H_2O ascending mode) and reverse-phase HPLC (52% aqueous



MeOH). Structural elucidation of the new crellastatins was carried out by NMR spectroscopic and FABMS data and by comparison with the eight previously isolated crellastatins.

Crellastatin I (**2**): the molecular formula of $C_{58}H_{88}O_{11}S$ was deduced by combined analysis of HRFABMS at *m/z* 917.6252 [base peak $(MSO_3^-Na^+ + Na^+ - NaHSO_4)^+$] (Δ -2.1 mmu) and ^{13}C NMR spectrum. NMR spectroscopic data revealed the presence of a ketone functionality (δ_C 219.3) and of an additional methyl signal (δ_H 1.10, δ_C 26.7). HMBC analysis allowed us to define the 3-keto-19-methyl system in the western hemisphere of crellastatin I (**2**) in a structural situation which parallels that found in crellastatin E, reported previously.[3]

Crellastatin J (**3**) had one more oxygen atom than crellastatin A (**1**) as evidenced by FABMS data, *m/z* 1023, $[M-H]^-$ and ^{13}C NMR data. NMR spectra indicated that the eastern hemisphere of crellastatin J (**3**) was the same as that of crellastatin A (**1**) whereas a perturbation was observed in the tetracyclic core of the western hemisphere. The additional sp^2 proton signal at δ_H 5.69 (br d, J = 6.2) observed in the ^1H-NMR spectrum was found to correlate with an isolated allylic methylene (δ_H 2.35 dd, J = 13.0, 6.2; 2.07 dd, overlapped) in the COSY spectrum. This methylene was found to correlate in the HMQC spectrum with a carbon at δ_C 42.9. The

HMBC correlation between this latter carbon with the angular C-18 methyl protons at δ_H 0.72 as well as the presence in the COSY spectrum of a W-type long range coupling between the angular C-18 methyl protons at δ_H 0.72 and one of the allylic protons at δ_H 2.07 implied the presence of $\Delta^{9,11}$ double bond. The HMBC correlations of H11, $H_2$7 and H14 with an oxygenated nonprotonated carbon at δ_C 83.7, allowed us to place a hydroxyl group at the C8 position, so defining the gross structure of crellastatin J as in **3**. The α-orientation of the 8-OH group was suggested on the basis of ^{1}H-NMR chemical shift of H_3-18 proton signal at δ_H 0.72, substantially unchanged with respect to the value for the 18-Me in crellastatin A.

Table 1. Selected ^{1}H NMR of crellastatins I-M (**2-6**, 500 MHz, methanol-d_4)

n°	**2**	**3**	**4**	**5**	**6**
1	2.70	2.90	2.20	3.56 dd (15.6, 6.8)	1.94
	1.85	1.62	2.13	2.60	2.39 t
2	4.55 dd (6.6, 9.6)	3.97 dd (9.8, 2.2)	4.02 dd	4.77 dd (6.8, 12.3)	4.24 dd (4.8, 9.6)
5					1.84 d (12.0)
6	5.84 t (3.1)	5.46 dd (5.4, 2.4)	5.61 t (2.8)	6.98* d (8.1)	4.69 m
7	2.65	2.28 brs	2.65 brs W½ 7.8	7.19* d (8.1)	2.94
					2.18
11		5.69 d (6.2)		2.78	
18	0.73 s	0.72 s	0.73 s	0.57 s	0.67 s
19	1.10 s	4.41 d (8.0)	4.33 d (8.0)		3.90 br d (8.8)
		3.61 dd (8.0, 3.0)	3.66 dd (2.4, 8.0)		3.82 dd (1.6, 8.8)
24	3.22 t (4.4)	3.29 m	4.02 dd	3.22 br t (3.8)	3.12 br d (6.4)
26			1.93	2.24	2.42
			2.68	2.06	1.58
27	1.19 s	1.19 s	5.01 s	1.20	0.97 s
			5.04 s		
28	1.35 s	1.22 s	1.23 s	1.55 s	1.38 s
29	1.33 s	1.15 s	1.13 s	1.36 s	1.23 s
n°	**2**	**3**	**4**	**5**	**6**
1'	2.05	2.09	2.0	1.82	1.96
	2.02	2.0		1.98	2.18
2'	4.05 dd (5.1, 10.3)	4.04 dd (9.8, 5.2)	4.05 dd (5.6, 8.8)	3.98 dd (4.8, 9.9)	4.30 dd (5.6, 9.6)
5'	1.59 d (11.0)	1.71 d (10.5)	1.64 d (10.4)	1.88	1.81 d (12.0)
6'	4.65 dt (5.1, 11.0)	4.68 dt (9.8, 5.8)	4.68 dt (5.5, 10.4)	4.57 dd (4.9, 11.0)	4.68 m
7'	2.92 dd (5.1, 16.2)	2.95 dd	2.94 dd (5.6, 17.6)	2.80 dd (15.0, 3.6)	2.94
	2.05	2.08	2.08	2.05	2.06
18'	0.67 s	0.66 s	0.65 s	0.65 s	0.64 s
19'	3.94 dd (1.0, 8.8)	3.93 brd (9.0)	3.93 dd (1.6, 9.6)	3.90 dd (1.4, 9.3)	3.90 d (8.8)
	3.83 dd (1.5, 8.8)	3.83 dd (9.0, 1.8)	3.84 dd (0.8, 9.6)	3.78 dd (1.6, 9.3)	3.82 dd (1.6, 8.6)
22'	3.73	3.77 dd (11.6, 4.6)	3.48 m	3.76 dd (10.0, 2.6)	3.84 dd (3.2, 11.2)
23'		2.07	1.55	1.30	1.34
		1.37	2.0	2.04	1.81
24'	2.17	2.17	1.52	2.15	2.10
26'	1.36 s	1.35	1.19 s	1.38	4.98 s
27'	1.25 s	1.25	1.19 s	1.26	1.74 s
28'	1.36 s	1.38 s	1.38s	1.32 s	1.38 s
29'	1.17 s	1.20 s	1.20 s	1.09 s	1.30 s

*These signals are interchangeable

[a] ^{1}H assignments aided by COSY and HMQC experiments.

Crellastatin K (**4**) was isomeric with crellastatin A (**1**) (HRFABMS data, *m/z* 933.6210 [base peak (MSO_3^- Na^+ + Na^+ - $NaHSO_4$)$^+$] (Δ -1.0 mmu). Inspection of NMR spectra clearly indicated that this compound shared the same tetracyclic nuclei as **1**. However the ^{1}H-NMR spectrum revealed a modification in the oxabicyclic system of the side chains. The absence of one methyl singlet as well as the appearance of two additional proton signals at δ_H 5.04 and 5.01 was observed, suggesting the presence of an exomethylene functionality in the

molecule. In fact, in the HMQC spectrum, these two olefinic protons were found to be correlate to a sp^2 methylene carbon at δ_C 112.5. The H24' (δ_H 1.52, δ_C 54.0) proton was correlated with the H_2-26 methylene protons at δ_H 2.68-1.93 which were found downfield shifted with respect to crellastatin A (δ_H 2.25-2.07). In the western hemisphere the proton spin system H20-H24 was assigned by COSY analysis. The diagnostic downfield shift of 0.80 ppm for H24 (δ_H 4.02 vs 3.22 in **1**) together with the allylic couplings observed in the COSY spectrum between the exomethylene proton signal at δ_H 5.04 and both H24 and H26, indicated the presence of a C25-27 double bond. The presence of the above functionality suggested an alternative junction between the two side chains implying the cleavage of the C25-C25' ether bridge. The chemical shift of the methyl bearing C25' carbon at δ_C 73.4, as determined from the HMBC correlation H_3-26'-C25', was indicative of the presence of a free hydroxyl group at this position in agreement with FABMS data.

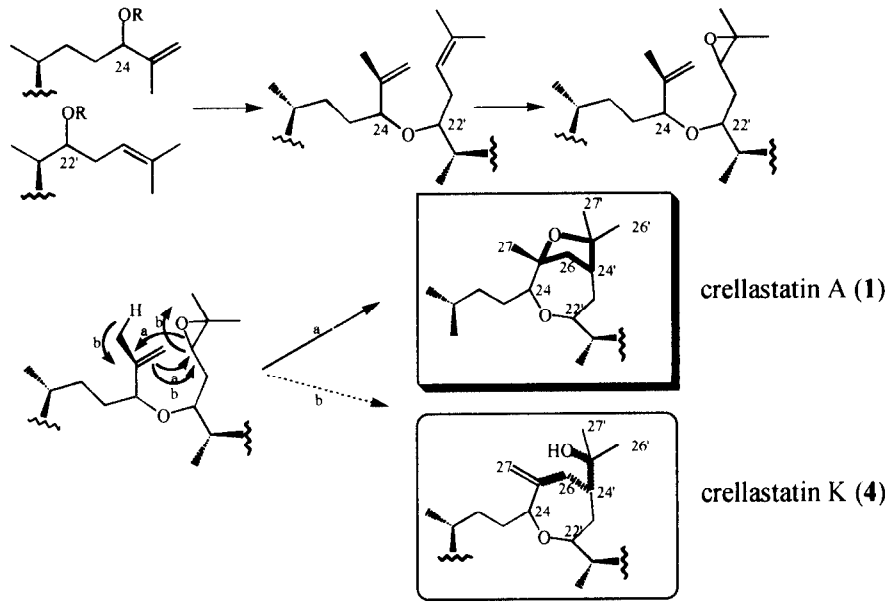
Table 2. ^{13}C NMR of western hemisphere of Crellastatins I-M (**2-6**) (125 MHz, methanol-d_4)

n°	2	3	4	5	6
1	45.5	44.6	44.4	*	44.4
2	70.6	68.5	68.0	71.6	67.1
3	219.3	99.7	99.9	216.5	99.4
4	49.1	42.8	42.5	50.0	40.4
5	147.7	148.1	147.6	142.0	50.7
6	120.4	115.9	116.0	*	75.9
7	30.0	31.0	30.8	*	35.5
8	130.3	83.7	130.0	*	128.1
9	132.5	138.8	127.4	*	133.5
10	39.4	39.2	38.1	*	41.0
11	23.5	126.5	23.4	*	22.5
12	38.3	42.9	37.8	38.5	38.1
13	42.9	44.9	42.8	43.0	41.9
14	53.8	52.9	53.3	52.8	52.9
15	24.2	22.2	24.5	24.0	24.5
16	30.5	30.4	30.8	*	30.7
17	54.6	57.6	56.0	53.5	53.8
18	11.6	13.2	11.5	11.5	11.5
19	26.7	74.9	72.5	*	71.0
20	36.4	36.2	36.7	36.5	36.1
21	19.3	18.7	19.2	19.3	19.1
22	36.4	35.1	36.2	36.3	36.1
23	24.9	25.0	25.5	*	25.2
24	85.7	84.8	80.4	86.0	84.8
25	88.4	87.6	148.0	88.0	54.4
26	36.5	36.6	37.0	36.2	38.3
27	27.3	27.8	112.5	27.1	24.0
28	29.5	25.0	24.9	30.0	17.4
29	28.6	26.2	25.3	25.5	28.0

*Not determined due to the paucity of compound **5**.

The finding of crellastatin K (**4**), characterised by an alternative linkage between the side chains, suggested likely pathways for the formation of both the dioxabicyclo system of crellastatin A and the alternative oxetane system of crellastatin K. As depicted in scheme 1, the two possible precursors of crellastatin A might be 3,19-epoxy-6α-sulfoxy-cholesta-8,24-dien-2β,3α,22-triol for the eastern hemisphere and 3,19 epoxy-cholesta-5,8(9),25 trien-2β,3α,24-triol for the western hemisphere. After the formation of the 24-22' ether bridge through the condensation of the appropriate oxygenated functions, epoxidation of the Δ^{24} double bond in theside chain of the western hemisphere could afford the electrophilic site which might be attacked by the Δ^{25}double bond present in the other side chain. The concerted attack by the nucleophilic epoxy oxygen anion on

the incipient carbocation at C25 would afford the dioxabicyclo system of crellastatin A (pathway a in scheme 1). The exomethylene functionality present in crellastatin K may arise from an alternative stabilisation of the above mentioned C25 carbocation through elimination of the α-proton on C26 (pathway b in scheme 1).



Scheme 1. Proposed biosynthetic pathway of crellastatin A (**1**) and K (**4**)

Crellastatin L (**5**) is a very minor component of the chloroform extract of *Crella* sp.. The NMR spectroscopic signals attributed to the eastern hemisphere and to the side chain units in crellastatin A (**1**) appeared in the spectra of crellastatin L, indicating that these portions of the molecular skeleton remained unchanged. The ^{1}H-NMR spectrum displayed two mutually coupled one proton doublets at δ_H 6.98 (d, J =8.1) and 7.19 (d, J =8.1) suggesting the presence of a tetrasubstituted aromatic ring. Though a good quality HMBC spectrum was not obtained due to the paucity of material, an aromatic ring B was hypothesised in the left hemisphere mainly on the basis of chemical shift considerations. In the DQF-COSY the H2 signal at δ_H 4.77 (dd, J =12.3, 6.8) was found to correlate with a methylene group resonating at δ_H 3.56 (dd, J=15.6, 6.8) and 2.60 ppm. HMBC analysis, with crosspeaks between Me-28 and Me-29, at δ_H 1.55 and 1.36 respectively, with a carbon signal at δ_C 216.5 in close structural analogy with crellastatin E allowed us to place a ketone at the C3 position. The low field chemical shifts the H_3-28, H_3-29 and H_2-1, as well as the upfield shift of Me-18 proton signal (s, δ_H 0.57) were strongly indicative of the presence of an anisotropic aromatic B ring. The proposed structure is in agreement with the molecular formula $C_{57}H_{84}O_{11}S$ deduced from HRFABMS data [*m/z* 901.5933, $(MSO_3^-Na^+ + Na^+ - NaHSO_4)^+$]. The aromatic system found in crellastatin L might arise from the parent crellastatin A by oxidation of the 19-hydroxymethylene group to a formyl group followed by its elimination in a process similar to the formation of the aromatic A nucleus in the oestrogens. To the best of our knowledge, crellastatin L represents the first example of a marine steroidal system with an aromatic B ring.

By means of HRFABMS data [1087.5463, $(M-H)^-$] the molecular formula of crellastatin M (**6**) was determined as $C_{58}H_{88}O_{15}S_2$. Comparing the NMR spectra of crellastatin M and F it was evident that the two 6-

sulphated tetracyclic units of crellastatin M were unchanged with respect to the parent crellastatin F,[3] whereas a substantial modification of the side chains had occurred in crellastatin M. The COSY spectrum allowed us to define the spin system H22'-H26. The oxygenated methine H22' (δ_H 3.84, dd, J= 11.2, 3.2) was coupled to a

Table 3. ^{13}C NMR of eastern hemisphere of Crellastatins I-M (**2-6**) (125 MHz, methanol-d_4)

n°	2	3	4	5	6
1'	44.8	44.4	44.0	45.9	44.5
2'	66.9	67.5	66.9	67.5	67.3
3'	99.8	100.0	99.9	99.8	99.4
4'	40.9	40.9	40.6	40.5	40.4
5'	52.8	52.2	52.2	52.0	51.4
6'	73.3	75.9	75.4	74.8	75.9
7'	35.5	35.4	35.1	35.4	35.6
8'	127.9	127.6	128.4	127.9	126.3
9'	131.8	132.0	131.3	131.8	130.9
10'	40.8	40.8	41.0	*	40.9
11'	23.5	23.5	23.2	*	22.7
12'	38.5	38.4	37.8	38.2	38.1
13'	42.6	43.4	42.8	42.5	42.2
14'	53.4	54.0	53.3	54.0	52.9
15'	25.0	25.0	24.9	*	25.0
16'	29.7	29.2	29.6	*	29.5
17'	52.8	52.9	53.1	53.5	52.8
18'	11.2	11.6	11.5	11.5	11.5
19'	69.7	70.1	69.6	70.0	70.0
20'	44.1	44.0	49.7	43.9	43.6
21'	13.63	13.2	13.3	13.2	13.0
22'	71.6	71.3	75.8	71.4	72.6
23'	34.0	34.3	34.0	*	34.5
24'	45.9	45.8	54.0	46.4	45.5
25'	85.7	85.5	73.4	85.4	145.7
26'	25.6	25.7	26.8	25.5	132.5
27'	32.5	32.5	26.8	32.5	13.9
28'	18.1	17.9	17.4	17.7	17.4
29'	28.6	28.2	27.9	28.5	27.9

*Not determined due to the paucity of compound **5**.

methylene group at δ_H 1.81-1.34, which in turn was coupled to a methine at δ_H 2.10. This in turn was coupled with one (δ_H 1.58) of two diastereotopic methylene signals at δ_H 1.58-2.42, and also showed an allylic coupling with an sp^2 proton singlet signal at δ_H 4.98. This olefinic proton also showed an allylic coupling with a methyl singlet signal at δ_H 1.74. The complete definition of the oxabicyclic system in crellastatin M (**6**) as shown in fig. 1 was derived from careful analysis of the ^1H-^{13}C long range couplings. An ether bridge was placed between H22' and H24 as in all crellastatin derivatives so far isolated, on the basis of H22'/C24 cross peaks. The location of a methyl bearing C25 quaternary carbon (δ_C 54.4) between the oxygenate carbon C24 and the secondary carbon C26 was inferred from the following HMBC correlations: $H_3$27/C25, $H_3$27/C24, $H_3$27/C26. The carbon-carbon linkage involving C25 and C26' carbons was derived from the $H_3$27/C26' HMBC correlation. The new rigid framework proposed for crellastatin M, was further substantiated by FABMS data, by the chemical shift values relative to all the nuclei of the side chain subunit as well as by the W-type long range coupling observed between H26 at δ_H 2.42 and H23' at δ_H 1.34. The relative stereochemistry around the oxabicyclic system of crellastatin M was defined on the basis of key dipolar couplings observed in the ROESY spectrum. The *trans* junction C24/C22' was inferred from the ROESY crosspeak H22'/$H_2$23. Furthermore, the crosspeaks H26'/H24 and H24'/H22' defined their mutual *cis* relationship. Assuming the natural steroidal

configuration at C20 and C20' and the C22'R configuration by analogy with crellastatin A, the absolute stereochemistry of the oxabicyclic system of crellastatin M was defined as shown in fig. 1.

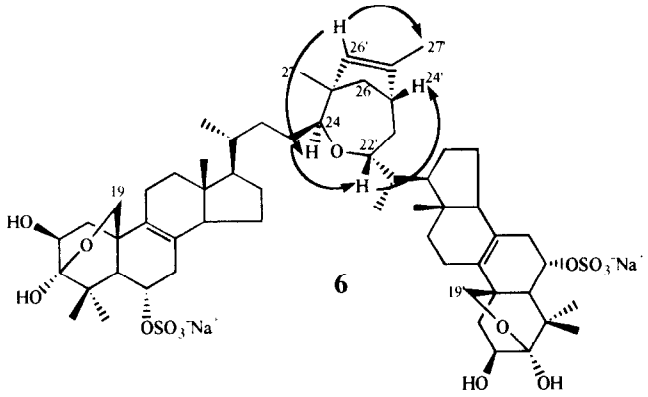


Figure 1. Crellastatin M (**6**) with selected ROESY effects.

The new crellastatins were evaluated for their in vitro cytotoxic activity against the NSCLC human cell lines and the calculated IC_{50} (μg/ml) values are as follows: crellastatin I (**2**) 1.9 μg/ml, crellastatin J (**3**) 7.6 μg/ml, crellastatin K (**4**) 3.7 μg/ml, crellastatin L (**5**) 2.9 μg/ml, crellastatin M (**6**) 1.1 μg/ml.

EXPERIMENTAL SECTION

General Experimental Procedures. For general procedures see M.V. D'Auria *et al.*[2]

Isolation. The sponge was collected at Luganville (Santo) in Vanuatu in June 1997 and identified as *Crella* sp. (family Crellidae, order Poecilosclerida) by Dr.John Hooper (Queensland Museum, Brisbane, Australia). The voucher specimen was deposited at the Queensland museum in Brisbane (accession number G306956). Lyophilised animals (300 g) were extracted in the same manner described in the previous papers [2,3] to obtain 75 g of a brown amorphous solid successively extracted using a modified Kupchan[4] partition. The $CHCl_3$ (8.2 g) extract was fractionated by DCCC ($CHCl_3$:MeOH:H_2O 7:13:8, ascending mode) and then by reverse phase HPLC μ-Bondapak C-18 chromatography eluting with MeOH:H_2O 68:32 to afford crellastatin A (**1**) (3800 mg, 1% dry weight of animal), as a colourless glassy solid, crellastatin B (4.5 mg), crellastatin C (450 mg), crellastatin D (24 mg), crellastatin E (10 mg), crellastatin I (12 mg), crellastatin J (6.2 mg), crellastatin K (8.2 mg), crellastatin L (5.0 mg). The BuOH (6 g) extract was fractionated by DCCC ($CHCl_3$:MeOH:H_2O 7:13:8, ascending mode) and then by reverse phase HPLC μ-Bondapak C-18 chromatography eluting with MeOH:H_2O 68:32 to afford crellastatin F (420 mg), crellastatin G (40 mg) and crellastatin H (3.2 mg), crellastatin M (3.5 mg).

Crellastatin I (**2**): $C_{58}H_{88}O_{11}S$ amorphous powder. $[\alpha]_D^{20}$ +40.7 (*c* 0.005, MeOH); IR (KBr): ν_{max} 3410, 2920, 1710, 1200, 1060 cm^{-1}. δ_H (500 MHz, MeOH) in Table 1; δ_C (125 MHz, MeOH) in Table 2; HRMS (FAB positive): *m/z* 1037.6 ($MSO_3^-Na^+ + Na)^+$, base peak ($MSO_3^-Na^+ + Na^+ - NaHSO_4)^+$, found 917.6252. $C_{58}H_{86}O_7Na$ requires 917.6271; FABMS (negative ion mode) *m/z* 991 $[M-H]^-$.

Crellastatin J (**3**): $C_{58}H_{88}O_{13}S$ amorphous powder.[6] δ_H (500 MHz, MeOH) in Table 1; δ_C (125 MHz, MeOH) in Table 2; FABMS (negative ion mode) *m/z* 1023 $[M-H]^-$.

Crellastatin K (**4**): $C_{58}H_{88}O_{12}S$ amorphous powder. $[\alpha]_D^{20}$ +22.3 (*c* 0.002, MeOH); IR (KBr): ν_{max} 3410, 2920, 1630, 1200, 1060 cm^{-1}.δ_H (500 MHz, MeOH) in Table 1; δ_C (125 MHz, MeOH) in Table 2; HRMS (FAB

positive): *m z* 1053.6 $(MSO_3^-Na^+ + Na^+)^+$, base peak $(MSO_3^-Na^+ + Na^+ - NaHSO_4)^+$, found 933.6210. $C_{58}H_{86}O_8Na$ requires 933.6220; FABMS (negative ion mode) *m z* 1007 $[M-H]^-$.

Crellastatin L (**5**): $C_{57}H_{84}O_{11}S$ amorphous powder. $[\alpha]_D^{20}$ +6.11 (*c* 0.003, MeOH); IR (KBr): ν_{max} 3410, 2920, 1640, 1720, 1200, 1060 cm^{-1}. δ_H (500 MHz, MeOH) in Table 1; δ_C (125 MHz, MeOH) in Table 2; HRMS (FAB positive): *m z* 1021.5 $(MSO_3^-Na^+ + Na)^+$, base peak $(MSO_3^-Na^+ + Na^+ - NaHSO_4)^+$, found 901.5933. $C_{57}H_{82}O_7Na$ requires 901.5958; FABMS (negative ion mode) *m z* 975 $[M-H]^-$.

Crellastatin M (**6**): $C_{58}H_{88}O_{15}S_2$ amorphous powder. $[\alpha]_D^{20}$ +48.0 (*c* 0.001, MeOH); IR (KBr): ν_{max} 3410, 2920, 1200, 1060 cm^{-1}.δ_H (500 MHz, MeOH) in Table 1; δ_C (125 MHz, MeOH) in Table 2; HRMS (FAB negative): $[M-H]^-$, found 1087.5463. $C_{58}H_{87}O_{15}S_2$ requires 1087.5486.

Cytotoxic assays. Experiments are performed in 96 wells microtiter plates ($2x10^5$ cells/ml). Cell growth is estimated by colorimetric assay based on the conversion of tetrazolium dye (MTT) to a blue formazan product using live mitichondria.[5] Eight determinations are performed for each concentration. Control growth is estimated for 16 determinations. Optical density at 570 nm corresponding to solubilized formazan is read for each well on Titertek Multiskan MKII.

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6. On standing in the NMR tube, crellastatin J (**3**) substantially decomposed, so the determination of its optical activity, IR absorptions and HRFABMS was not possible.