Crellastatin A: A Cytotoxic Bis-Steroid Sulfate from the Vanuatu Marine Sponge *Crella* sp.†

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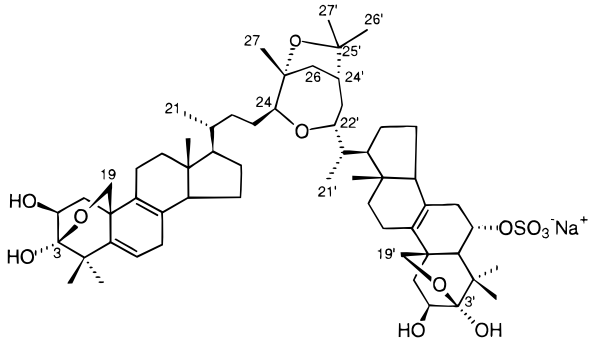
A new nonsymmetric dimeric steroid, crellastatin A (**1**), was isolated from the Vanuatu Island marine sponge *Crella* sp. Structural assignment was accomplished through extensive 2D NMR spectroscopy. The stereochemistry of **1** was established from an analysis of ROESY experiments and from molecular mechanics and dynamics calculations. Crellastatin A (**1**), which possesses an unprecedented connection through the side chains, exhibits in vitro cytotoxic activity against NSCLC-N6 cells (IC_{50} of 1.5 μg/mL).

Introduction

Dimeric and oligomeric steroids represent an emerging class of compounds which has recently attracted attention for their rigid, predictable, and inherently asymmetric architecture. They could have a wide range of potential applications in pharmacology, as well as in molecular recognition and in ion complexion and enzymatic catalysis.[1,2] Despite the increasing interest and the potential biological applications of these molecules, chemical studies have so far been limited to semisynthetic dimeric or oligomeric derivatives with very few reports of naturally occurring bioactive compounds. Among these, the first example of naturally occurring dimeric steroid was the sulfur-containing japindine, isolated from the root-bark of *Chonemorpha macrophylla*.[3] Another example of a natural bis-steroid is cephalostatin 1, a powerful cell growth inhibitor (ED_{50} 10^{-7} to 10^{-9} μg/mL), isolated from the marine worm *Cephalodiscus gilchristi*.[4] Following the discovery of cephalostatin 1 in 1988, Pettit's group has reported the structure of 16 other cephalostatins,[5] all containing the novel structural framework of two polyoxygenated steroidal units joined through a pyrazine ring involving C2 and C3 of each monomeric unit. Related steroidal pyrazine ring dimers, the ritterazines, were reported from the tunicate *Ritterella tokioka*,[6,7] suggesting a common symbiotic origin for both cephalostatins and ritterazines. Two additional examples of dimeric steroids, bistheonellasterone and bisconicasterone, were isolated from *Theonella swinhoei*. They represent Diels Alder adducts of the corresponding 3-keto-4-methylene sterols theonellasterone and conicasterone, respectively.[8−10]

In this paper we report the isolation and structure elucidation of crellastatin A (**1**) from the Vanuatu Islands marine sponge *Crella* sp. This compound, which represents the first example of a unsymmetrical dimeric steroid through the side chain, exhibits cytotoxicity against human cancer NSCLC-N6 cells with an IC_{50} value of 1.5 μg/mL.



crellastatin A (1)

Results and Discussion

The methanolic extract (20 g) of the lyophilized sponge (80 g) was subjected to a modified Kupchan partitioning procedure.[11] The cytotoxic $CHCl_3$ extract (1.6 g, IC_{50} < 3.3 μg/mL on NSCLC-N6 cells) was purified by droplet countercurrent chromatography ($CHCl_3$:MeOH:H_2O 7:13:

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Table 1. ^{1}H and ^{13}C NMR of Crellastatin A (1) (500 MHz, methanol-d_4)

		western hemisphere				eastern hemisphere	
no.	δ_C	δ_H	*J* values (Hz)	no.	δ_C	δ_H	*J* values (Hz)
1α	42.8	2.17 overlapped		1′α	44.7	2.04 overlapped	
1β		2.20 overlapped		1′β		1.94 overlapped	
2	68.2	4.08 dd	8.8, 3.3	2′	67.2	4.04 dd	9.7, 4.8
3	99.7			3′	99.5		
4	42.6			4′	40.5		
5	147.8			5′	52.4	1.58 dd	10.5, 1.0
6	115.7	5.60 t	3.1	6′	75.3	4.65 dt	10.5, 5.9
7	30.0	2.65 br s	$W^{1/2}$ 7.8	7′α	35.2	2.04 dd overlapped	
				7′β		2.92 dd	16.5, 5.9
8	130.3			8′	127.7		
9	126.6			9′	131.5		
10	38.4			10′	40.7		
11α	23.4	2.26 overlapped		11′α	23.2	2.17 overlapped	
11β		2.04 overlapped		11′β		2.06 overlapped	
12α	38.2	2.08 overlapped		12′α	38.2	1.42 overlapped	
12β		1.46 overlapped		12′β		2.06 overlapped	
13	42.5			13′	42.6		
14	53.3	2.22 overlapped		14′	53.8	2.24 overlapped	
15α	23.9	1.67 overlapped		15′α	24.9	1.74 overlapped	
15β		1.36 overlapped		15′β		1.32 overlapped	
16α	30.5	2.17 overlapped		16′α	29.5	1.88 overlapped	
16β		1.39 overlapped		16′β		1.47 overlapped	
17	54.5	1.33 overlapped		17′	52.8	1.58 overlapped	
18	11.6	0.72 s		18′	11.2	0.68 s	
19_R	72.8	4.30 d	8.6	$19'_R$	69.8	3.94 dd	8.8, 1.0
19_S		3.66 dd	8.6, 2.7	$19'_S$		3.83 dd	8.8, 1.8
20	36.2	1.66 overlapped		20′	43.8	1.38 overlapped	
21	19.2	1.01 d	6.6	21′	13.2	1.02 d	6.6
22a	36.1	1.45 overlapped		22′	71.4	3.72 dd	11.4, 4.2
22b		1.58 overlapped					
23a	25.1	1.66 overlapped		23′a	34.0	1.32 overlapped	
23b		1.40 overlapped		23′b		2.06 overlapped	
24	85.7	3.22 br t	4.0	24′	45.6	2.17 overlapped	
25	87.9			25′	85.4		
26a	36.4	2.25 overlapped		26′	25.5	1.36 s	
26b		2.07 overlapped					
27	27.1	1.19 s		27′	32.4	1.26 s	
28	25.6	1.22 s		28′	17.7	1.38 s	
29	25.6	1.16 s		29′	28.4	1.19 s	

8, ascending mode) and then by reverse phase HPLC on a μ-Bondapak C-18 column eluting with MeOH:H_2O 68:32 affording 231 mg of crellastatin A (**1**) as a colorless glassy solid.

Crellastatin A (**1**) had a molecular formula of $C_{58}H_{88}O_{12}S$ as established by HRFABMS and NMR data (Tables 1 and 2, 500 MHz, methanol-d_4). The FAB mass spectrum showed a quasimolecular ion $[MSO_3NaNa]^+$ at *m*/*z* 1053 and a base peak at *m*/*z* 933, due to the loss of a $NaHSO_4$ group from the MSO_3NaNa^+ ion. HR mass measurements were performed on the peaks at *m*/*z* 1053 and 933. While the measurements on the quasimolecular ion gave poor results because of the relatively weak intensity of that ion, HR mass measurement on the ion at *m*/*z* 933 established the formula $C_{58}H_{86}O_8Na$ *m*/*z* 933.6154 (Δ +6.6 mmu), thus leading to the molecular formula given above. The presence of a sulfated group in the structure of crellastatin A (**1**) was definitively confirmed by strong IR absorptions at 1200 and 1060 cm^{-1} and by FABMS analysis on the desulfated derivative (see Experimental Section). The ^{1}H NMR spectrum recorded in DMSO-d_6 (Table 3) revealed the presence of four exchangeable signals, assigned to two secondary and two tertiary OH groups. In fact, on acetylation under standard conditions, crellastatin A (**1**) gave a diacetate derivative (see Experimental Section). The low field region of the ^{13}C NMR spectrum revealed the presence of six olefinic carbons (corresponding to a trisubstituted double bond and two tetrasubstituted double bonds), two ketalic/hemiketalic carbons (δ_C 99.7 and 99.5 ppm) and nine signals in the range 87.9−67.2 ppm, assigned to oxygenated carbons. On the basis of DEPT experiments, these were ascribed to two oxygenated quaternary carbons (δ_C 87.9, 85.4 ppm), five oxygenated methines (67.2, 68.2, 71.4, 75.3, 85.7), and two oxygenated methylenes (72.8, 69.8). The three double bonds derived from ^{13}C NMR analysis accounted for only three of the fifteen degrees of unsaturation expected for the molecular formula of the desulfated derivative, thus implying a polycyclic nature for crellastatin A (**1**). At the same time, the existence of several pairs of analogous signals in both ^{1}H and ^{13}C NMR spectra suggested that **1** was a nonsymmetrical dimeric molecule.

COSY coupling networks were interrupted by many quaternary carbons and complicated by poor chemical shift dispersion; furthermore, the high degree of functionalization of the molecule did not allow the straightforward identification the steroidal nature of the isolate. Interpretation of the data from COSY, HOHAHA, HMQC, and HMBC experiments led to the identification of four structural units A, B, C, D (Figure 1) which were eventually combined to give the complete structure of crellastatin A (**1**).

Unit A: Tetracyclic Core of Western Hemisphere. In the HMBC spectra both signals assigned to a highly anisochronous oxygenated methylene (AB system δ_H 4.30 d, J = 8.6 Hz and 3.66 dd, J = 8.6, 2.7 Hz) showed an unusual high number of correlations with carbons at δ_C

Table 2. ROESY and HMBC Data of Crellastatin A 1 (500 MHz, methanol-d_4)

western hemisphere				eastern hemisphere			
no.	δ	ROESY	HMBC	no.	δ	ROESY	HMBC
1α	2.20	H2	C2, C3, C5, C9, C10,	1′α	1.94	H2′	C2′, C3′, C5′, C10′, C15′
1β	2.18	H19$_R$	C19	1′β	2.04	H19′$_R$	C2′, C10′, C19′
2	4.08	H29, H1α	C10	2′	4.04	H1′α, H5′, H29′	C1′
5					1.58	H2′, H29′	C4′ C6′, C7′, C19′
6	5.60	H28, H29, H7	C4, C5, C7, C8, C10, C19	6′	4.65	H7′β, H19′$_S$, H28′	C4′ C5′
7α	2.65		C5, C6, C8, C9	7′	2.04	H14′	C5′, C6′, C8′
7β					2.92	H6′	C5′, C6′, C8′, C9′, C14′
11α	2.26	H19$_R$	C9	11′α	2.17		C9′
11β	2.04		C8, C9	11′β	2.06	H19′$_S$	
12α				12′	1.42	H14′	
12β					2.06	H18′	
14	2.22		C8, C13	14′	2.22	H12′a	
15β	1.36		C14	15β	1.32		C16′
	2.17		C14	16α			
17				17′	1.58		C22′
18	0.72		C12, C13, C14, C17	18′	0.68	H11′β	C12′, C13′, C14′, C17′
19$_R$	4.30	H1β, H11α	C1, C3, C5, C6, C9,	19′$_R$		H1′β	C3′, C5′, C9′, C10′
19$_S$	3.66	H28	C10	19′$_S$		H6′, H11′β, H28′	C5′, C10′
			C1, C3, C5				
20				20′	1.38	H22′	C23′
21	1.01		C17, C20	21′	1.02	H22′, H23′b, H26′	C17′, C20′, C22′
22a	1.45			22′	3.72	H20′, H21′, H22b, H23b, H26a	C17′, C20′, C21′, C24
22b	1.58	H24, H22′	C24				
23a	1.66			23′a	1.32	H22′	C25′, C26
23b	1.40	H22′		23′b	2.06	H21′, H26′	C20′
24	3.22	H22a, H22b, H23a, H23b, H27	C23, C25, C26, C22′	24′	2.17	H26′	C22′, C23′, C25, C26
26a	2.25	H22′	C25, C23′, C24′, C25′	26′	1.36	H21′	C24′, C25′, C27′
26b	2.07	H27, H27′	C24, C25, C27, C23′, C24′, C25′				
27	1.19	H24, H26b, H27′	C24, C25, C26	27′	1.26	H26b, H27	C24′, C25′, C26′
28	1.22	H6, H19$_S$	C3, C4, C5, C29	28′	1.38	H19′$_S$, H6′	C3′, C4′, C5′, C29′
29	1.16	H6, H2	C3, C4, C5, C28	29′	1.19	H5′, H2′	C3′, C4′, C5′, C28′

Table 3. Selected ^{1}H and ^{13}C NMR of Crellastatin A (1) (500 MHz, DMSO-d_6)

western hemisphere				eastern hemisphere			
no.	δ_C	δ_H	J values (Hz)	no.	δ_C	δ_H	J values (Hz)
1α	41.5	1.98 overlapped		1′α	43.7	1.73 overlapped	
1β		1.90 overlapped		1′β		1.82 overlapped	
2	66.8	3.80 m		2′	65.4	3.80 m	
3	98.1			3′	98.1		
4	41.0			4′	38.5		
5	147.2			5′	50.7	1.36 d overlapped	
6	114.3	5.47 t	3.1	6′	71.4	4.27 dt	11.0, 6.6
7	28.4	2.53 br s		7′α	34.4	1.74 dd overlapped	16.9, 1.9
				7′β		2.73 dd	
8	128.4			8′	129.9		
9	125.8			9′	136.7		
10	37.1			10′	38.5		
17	53.7	1.13 overlapped		17′	51.7	1.46 overlapped	
18	11.5	0.59 s		18′	11.2	0.55 s	
19$_R$	71.1	4.13 d	7.43	19′$_R$		3.72 br d	8.6, 1.0
19$_S$		3.44 dd	7.43, 1.5	19′$_S$	68.3	3.59 dd	8.6, 1.3
20	35.1	1.46 overlapped		20′	42.4	1.27 overlapped	
21	18.8	0.90 d	6.6	21′	13.2	0.90 d	6.6
22	34.5			22′	69.5	3.59 overlapped	
23a	24.2	1.47 overlapped		23′a	34.0	1.22 overlapped	
23b		1.20 overlapped		23′b		1.85 overlapped	
24	84.0	3.07 br t	3.7	24′	44.2	2.0 overlapped	
25	85.9			25′	83.1		
26a	35.4	2.0 overlapped		26′	25.3	1.19 s	
26b		1.89 overlapped					
27	27.3	1.05 s		27′	32.5	1.12 s	
28	25.4	1.09 s		28′	17.6	1.17 s	
29	25.5	1.01 s		29′	27.9	1.01 s	
2 OH		4.56 d	5.22	2′OH		4.68 d	4.4
3 OH		5.16 br s		3′OH		4.80 br s	

42.8 (C1), 99.7 (C3), 147.8 (C5), 115.7 (C6), 126.6 (C9), 38.4 (C10). This indicated the presence of a tricyclic system in which the CH_2O methylene occupies a bridge position. These data correspond to a structural feature, that can be accommodated in the A−B ring framework of a steroidal or triterpenoid skeleton, in which the angular $CH_3$19 has been oxydized to a hydroxymethylene and is involved in a hemiketal linkage to carbon 3, as

Table 4. ROESY and HMBC Data of Crellastatin A (1) (500 MHz, DMSO-d_6)

western hemisphere				eastern hemisphere			
no.	δ	ROESY	HMBC	no.	δ	ROESY	HMBC
1α	1.98	H2	C2, C3, C5, C9, C10, C19	1′α	1.73	H2′, 2′OH	C2′, C3′, C5′, C10′, C15′
1β	1.90	H19$_R$, 2OH		1′β	1.82	H19′$_R$, 3'OH	C2′, C10′, C19′
2	3.80	H29, H1α, 3OH	C10	2′	3.80	H1′α, H5′, H29′, 3′OH	C1′
5					1.36	H2′, H29′	C4′, C6′, C7′, C19′
6	5.47	H28, H29, H7	C4, C5, C7, C8, C10, C19	6′	4.27	H7′β, H19′$_S$, H28′	C4′, C5′
7α	2.53		C5, C6, C8, C9	7′	1.74	H14′	C5′, C6′, C8′
7β					2.73	H6′	C5′, C6′, C8′, C9′, C14′
17	1.13			17′	1.46	H20′	C22′
18	0.59		C12, C13, C14, C17	18′	0.55		C12′, C13′, C14′, C17′
19$_R$	4.13	H1β, H11α, 2OH	C1, C3, C5, C6, C9	19′$_R$	3.72	H1′β	C3′, C5′, C9′, C10′
19$_S$	3.44	H28	C1, C3, C5	19′$_S$	3.59	H28′, H6′	C5′, C10′
20				20′	1.27		C23′
21	0.90		C17, C20	21′	0.90	H23′b, H26′	C17′, C20′, C22′
22a	1.58	H24		22′	3.59	H23a	C17′, C20′, C21′, C24
22b							
23a	1.47	H22′, H24		23′a	1.22	H22′	C25′
23b	1.20	H24		23′b	1.85	H21′, H26′	C20′
24	3.07	H27,H23a, H23b	C23, C25, C26, C22′	24′	2.0	H26′	C22′, C23′, C25, C26
26a	2.0		C25, C23′, C24′, C25′	26′	1.19	H24′, H23′b	C24′, C25′, C27′
26b	1.89		C24, C25, C27, C23′, C24′, C25′				
27	1.05	H24, H26b	C24, C25, C26	27′	1.12	H26b, H24′	C24′, C25′, C26′
28	1.09	H6, H19$_R$, 3OH	C3, C4, C5, C29	28′	1.17	H19'$_R$, H6′, 3′OH	C3′, C4′, C5′, C29′
29	1.01	H6, H2, 3OH	C3, C4, C5, C28	29′	1.01	H5′, H2′, 3′OH	C3′, C4′, C5′, C28′
2OH	4.58	H1α, H1β, H19$_R$, 3OH		2′OH	4.68	H1′α, H1′β	C3′
3OH	5.16	2OH, H28, H29		3′OH	4.80	H2′, H28', H29′	

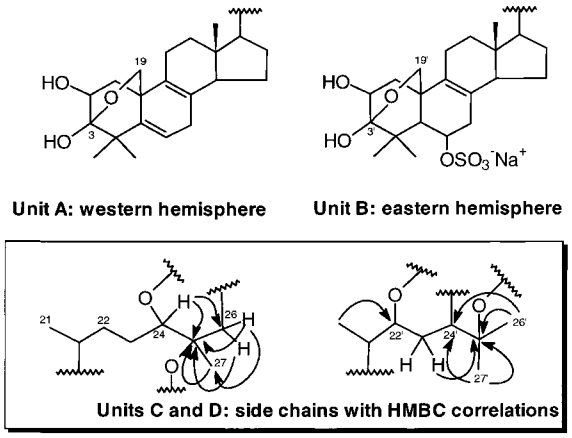

Figure 1. Units of crellastatin A (1).

shown in Figure 1. In addition, two methyl singlets, resonating at δ 1.22 and 1.16 in the ^{1}H NMR spectrum and determined as geminal by the HMBC correlations C28/H29 and C29/H28, exhibited correlations with carbons at δ_C 99.7 (C3), 147.8 (C5), and 42.6 (C4), thus indicating their location at the biogenetic position 4 of a Δ^5 steroidal skeleton. Although the C19 methylene was flanked by one oxygen and one quaternary carbon, one of the two methylene protons at δ 3.66 (*pro-S* H19) exhibited a W-type long-range coupling (J = 2.7 Hz) with one of the C1 methylene protons (H1α, δ_H 2.17). This long-range correlation was indicative of the folding of the A ring in a rigid boat conformation. Both C1 methylene protons were in turn correlated in the COSY and HOHAHA spectra with a methine resonating a δ 4.08 (H2, dd, J = 8.8, 3.3 Hz) bearing a free hydroxyl group (O*H*, δ 4.58, d, J = 5.22 Hz, DMSO-d_6) as derived by COSY cross-peaks in DMSO-d_6 between the OH doublet signal and the H2 signal resonating at δ 3.80 (H2, m). This location of the OH group was further confirmed by a $^3J_{H-C}$ HMBC (DMSO-d_6) correlation of the O*H* signal with the hemiketal carbon at δ_C 99.7 ppm (C3). Several attempts aimed at the preparation of the methyl ketal at C3 by treatment with MeOH/HCl caused severe degradation of crellastatin A (**1**). However, the presence of a hemiketalic OH at position-3 was definitively confirmed on the basis of diagnostic cross-peaks 3-O*H*/H2, 3-O*H*/Me28, and 3-O*H*/Me29 present in a ROESY spectrum performed in DMSO-d_6 (Table 4).

The H-6 olefinic proton (δ_H 5.60 ppm, t, J = 3.1 Hz) was coupled to an isolated bis-allylic methylene as judged on chemical shift considerations (δ_H 2.65, br s, δ_C 30.0 ppm) which in turn showed HMBC correlations with two quaternary sp^2 carbons (δ_C 130.3 C8, 126.6 C9 ppm) assigned to a Δ^8 double bond. In the HOHAHA spectrum, the H$_2$-7 proton signal was found to be correlated, as a consequence of the homoallylic coupling to H$_2$-11, with two methylene groups (H-11$_{\alpha,\beta}$: δ_H 2.26, 2.04 ppm; H-12$_{\alpha,\beta}$: δ_H, 2.08, 1.46 ppm) assigned to H$_2$-11 and H$_2$-12 on the basis of diagnostic ^{13}C chemical shifts [δ_C 23.4 (C11), 38.2 (C12)]. Finally, a comparative analysis of the remaining data in the two-dimensional NMR spectra (COSY, HMQC, and HMBC) allowed us to assign all the resonances of the western hemisphere tetracyclic centers (Tables 1 and 2).

Unit B: Tetracyclic Core of the Eastern Hemisphere. In addition to the signals previously described for the tetracyclic unit A, the NMR spectra showed the presence of a set of analogous signals that led to the identification of a closely related tetracyclic core. Moreover, the analysis of COSY, HMQC, HMBC spectra indicated that this unit B differs from the western one by the presence of an oxygenated functionality at the C6′ position which replaces the Δ^5 double bond. In this case, both C19′ methylene protons displayed W-type long range couplings, namely *pro-S* H19′ (δ_H 3.83, dd, J = 8.8, 1.9 Hz) with H1′α and *pro-R* H19′ (dd, J = 8.8, 1.0 Hz) with H5′ proton. This indicated an A/B trans ring junction. A sulfated group was located at the C6′ position by consideration of the chemical shift in comparison with

the ^{1}H NMR of the desulfated derivative **1a** (δ 4.65 vs 4.10; **1** vs **1a**) obtained by solvolysis of **1** in a 1:1 dioxane−pyridine mixture at 140 °C (3 h) (see Experimental Section).

It should be noted that compounds containing a 3α-hydroxy-3,19 epoxy functionality (i.e. lantanilic[12] and camarilic acids[13]) were previously reported as natural compounds from the aerial parts of *Lantana camara*, and also recently described as synthetic intermediates.[14] Comparison of the spectral data of the tetracyclic cores of **1** with those reported in the literature for molecules bearing this unusual functionality definitively confirmed our structural assignments.

Side Chains (units C and D). Once the ^{1}H and ^{13}C resonances relative to the two tetracyclic cores were assigned, the remaining 16 carbon signals (5 methyls, 4 methylenes, 5 methines, and 2 quaternary carbons) were suggestive of two highly functionalized steroidal side chains. To satisfy the formal unsaturations required by the molecular formula, the units C and D had to be arranged in a bicyclic structural framework. We will discuss in the order the structural determination of each side chain and their pertinence to the two relative tetracyclic units, and we will leave to the end the analysis of the spectral data leading to the junction of the two subunits that defines the complete planar structure of crellastatin A (**1**). By following the coupling network of unit C starting from the C21 methyl at δ_H 1.01, δ_C 19.2 ppm, using ^{1}H−^{1}H−COSY, HOHAHA, and HMBC correlations, a C24 oxygenated carbon (δ_H 3.22, δ_C 85.7) was determined. This latter was proved to be linked to an oxygenated quaternary carbon (C25, δ_C 87.9) bearing a methyl (C27, δ_H 1.19, δ_C 27.1 ppm) and an unfunctionalized methylene (C26, δ_H 2.07, 2.25, δ_C 36.4) on the basis of the following diagnostic HMBC correlations: H24−C25, H24−C26, H27−C24, and H26−C25, H26−C27 (Table 2 and Figure 1). Although in the remaining side chain (unit D) no H20′/H22′ (δ_H 3.72, dd J = 11.4, 4.2 Hz) COSY cross-peak was observed, a C22′ oxygenated functionality (δ_C 71.4 ppm) could be deduced by the diagnostic shielding of Me21′ (δ_C 13.2 vs 18.7 in cholesterol)[15] and by the HMBC cross-peaks C21′/H22′ and H21′/C22′. Connectivities from C22′ to C27′ were inferred from HOHAHA and HMBC analysis (Figure 1). In particular, tracing the HOHAHA slice starting from the isolated H22′ resonance, signals originating from the coherence transfer through the H22′−H24′ spin system could be observed, including the $H_2$26 from the other side chain unit. The linkage of the C24′ methine to an oxygenated carbon (C25′, δ_C 85.4 ppm) bearing two geminal methyl (C26′ and C27′) was inferred by the HMBC cross-peaks C24′/H26′, C24′/H27′, C25′/H26′, C24′/H27′. Sequential three-bond HMBC correlations from H22′ to C21′, H21′ to C17′, H18′ to C17′, H18′ to C14′, H7′β to C14′ connected the C22′ oxygenated side chain (unit D) to the 6-sulfated tetracyclic unit B, thus defining the eastern hemisphere as despited in Figure 2.

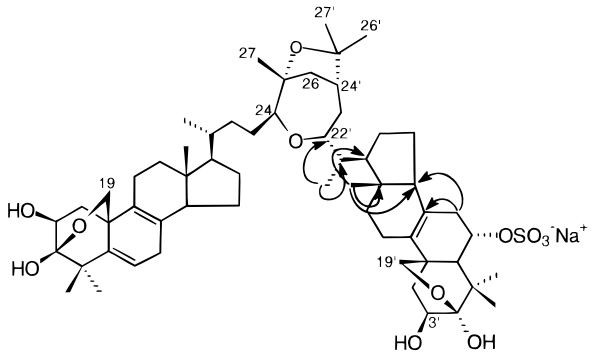

Figure 2. Sequential three-bond HMBC correlations defining the eastern hemisphere of crellastatin A (1).

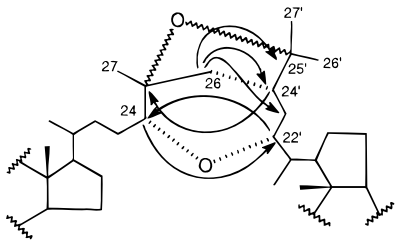

Figure 3. Connection of two side chains via HMBC correlations.

Assembly of Two Halves. The unprecedented linkage between C24′ and C26 was first deduced, as stated before, during the C21′−C24′ HOHAHA analysis and then confirmed on the basis of key HMBC correlations involving the protons and carbons of the two side chains: C24′/H26, C23′/H26, C25/H24′, C25′/H26 (see Table 2 and Figure 3). An ether bridge between C24 and C22′ was derived on the basis of a consideration of the ^{13}C chemical shifts and HMBC correlations C22′/H24 and C24/H22′. The last structural feature that defines the complete planar structure of crellastatin A (**1**), the ether function connecting the two oxygenated quaternary carbons C25−C25′, was inferred on the basis of the chemical shifts of C25 and C25′ (δ_C 87.9 and δ_C 85.4, respectively vs 71.0 reported for 25-OH steroids[16]) and from the further formal unsaturation required by the established molecular formula $C_{58}H_{88}O_{12}S$.

Stereochemistry. The relative stereochemistry of each steroidal unit in **1** was determined by ROESY data together with the values of the coupling constants. A ROESY correlation between H1α and H2 and between 2-O*H* and 19 methylene (DMSO-d_6, in Table 3) indicated a β disposition for the OH on C2 in the western side of **1**.

Considering the eastern side of crellastatin A (**1**), a diagnostic ROESY cross-peak between H5′ and H2′ established a β orientation for the OH at C2′. The H6′ in this portion was assigned as axial on the basis of a typical coupling pattern (dt, J = 10.5, 5.9 Hz) with the two adjacent protons (H5′ and H7′) and the ROESY cross-peaks with the *pro-R* H19′ proton and with the C28′ methyl protons.

Stereochemistry of the Side Chains. The relative stereochemistry of the bicyclic system of crellastatin A (**1**), originating from the junction of the two side chains, was deduced by the analysis of the ROESY spectra (Table

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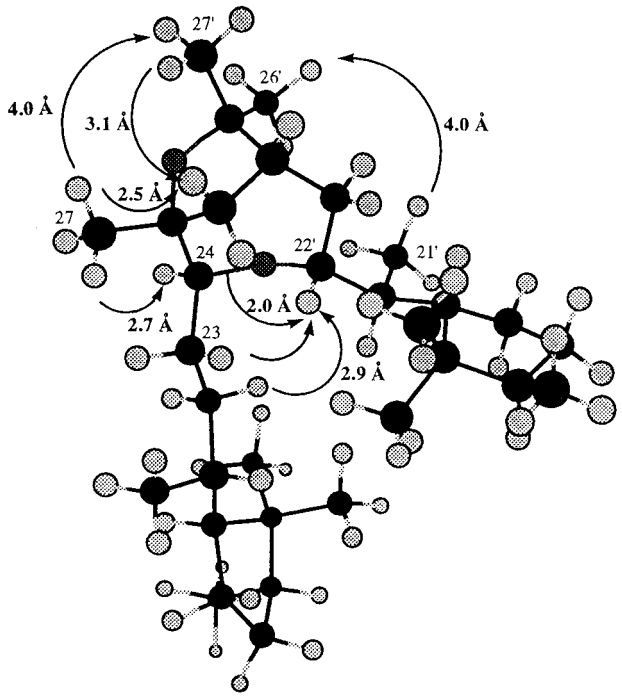

Figure 4. Selected ROESY effects of crellastatin A (**1**) displayed on computer-assisted energy minimization modeling for side chain.

2 and Figure 4). In particular, a strong ROESY cross-peak between H22′ and one of the $H_2$23 methylene protons suggested a trans stereochemistry between H22′ and H24, while a ROESY cross-peak between H22′ and one of H26 proton signals was indicative of a cis relative disposition of H22′ and the C26 methylene bridge. An intense ROESY cross-peak was also observed between the apparently remote H_3-21′ and H_3-26′ protons. Once the relative stereochemistry around the dioxabicyclic system of crellastatin A (**1**) was defined, and assuming the natural steroidal configurations at C-20 and C-20′, a molecular mechanics and dynamics calculation was conducted on the two possible stereoisomers with opposite configurations at C-22′. Only in the lowest energy conformation calculated for the C-22′ *R* stereoisomer are the two methyl groups C-21′ and C-26′ disposed close enough (4 Å) to explain the observed ROESY cross-peak H_3 21′/$H_3$26′. In the lowest energy conformation of the alternative C-22′ *S* stereoisomer, the two methyl groups were found to be too remote (6 Å) to display a significative NOE effect. Thus, the absolute stereochemistry of the bioxabicyclo system of crellastatin A (**1**) was defined as 24*S*,25*R*,22′*R*,24′*R*, and is shown in Figure 4.

Conclusion

Crellastatin A (**1**) represents a new type of bis-nonsymmetric steroid with a particularly unusual dioxabicyclo system joining the side chains, never found before in natural compounds, and thus posing an intriguing question as to its biosynthesis.

Crellastatin A (**1**) was cytotoxic in the in vitro tests on NSCLC-N6 mother cell lines (IC_{50} 1.5 μg/mL) as well as on its clones, C65 (IC_{50} 4.4 μg/mL), C92 (IC_{50} 6.3 μg/mL) and C98 (IC_{50} 9.2 μg/mL). The weaker activities exhibited by the corresponding 2,2′-diacetylated (IC_{50} 9.1 μg/mL) and desulfated (IC_{50} 9.3 μg/mL) derivatives indicated the importance of the hydroxyl and the sulfate groups for cytotoxic activity of crellastatin A. Furthermore, crellastatin A (**1**) caused, in a flow cytometry assays performed on the NSCLC-N6-C98 clone, an accumulation of cells in the G1 phase of the cell cycle with a concomitant decrease in the fraction of cells in the S and G2M phases (dose of 18 μg/mL: 65.7% in G1, 24.9% in S and 9.3% in G2M phases).

Experimental Section

General Procedures. General procedures can be found elsewhere.[17] Mass spectra were provided by the CRIAS "Centro Interdipartimentale di Analisi Strumentale", Faculty of Pharmacy, University of Naples. HRFABMS was provided by M-SCAN S.A., Switzerland, and carried out on M-Scan's VG Analytical ZAB 2SE high field mass spectrometer. A cesium ion gun was used to generate ions for the acquired mass spectra which were recorded with an instrument resolution of 8000 for the HR measurements.

NMR Spectroscopy. NMR spectra were measured at 500 MHz (^{1}H) and 125 MHz (^{13}C). ^{1}H NMR and ^{13}C NMR are referenced to CD_3OD solvent signals at 3.34 and 49.0 ppm, respectively. Multiplicities of ^{13}C spectra were assigned by DEPT experiments. Standard pulse sequences were employed for DEPT and magnitude COSY. Phase sensitive ROESY spectra were measured on a 600 MHz with a mixing time t_m of 400 ms, while HMQC and HMBC were optimized for $^1J_{C-H}$ = 135 Hz and $^{2,3}J_{C-H}$ = 8 and 10 Hz, respectively.

Molecular Mechanics Calculations. Molecular mechanics and dynamics calculations were carried out on a SGI Personal IRIS 35G computer using the force field CHARMm (QUANTA 4.0 software package). The lowest energy conformations for each diastereomer were searched by performing a high-temperature molecular dynamics simulation (HTMDS) followed by energy minimization.[18] By means of a molecular dynamics simulation of 50 ps at 1000 K using the Verlet algorithm, 200 conformations of each diastereomer were achieved. All the conformations were then subjected to an energy minimization (500 steps, conjugated gradient algorithm). Inspection of the minimized structures provided the lowest energy conformations of each diasteromer.

Isolation. The sponge was collected at Santo (Lunganville) in the Vanuatu Islands in June 1996 and identified as *Crella* sp. (family Crellidae, order Poecilosclerida) by John Hooper (Museum of Queensland, Brisbane, Australia). The voucher specimen R1697 was deposited at ORSTOM Centre in Noumèa. Lyophilized animals (80 g) were extracted with MeOH (3 × 2 L) and filtered. The extracts were combined, concentrated to give 20 g of a brown amorphous solid, and successively extracted using a modified Kupchan partition as follows. The methanol extract (20 g) was dissolved in a mixture of MeOH:H_2O containing 10% H_2O and partitioned against *n*-hexane. The water content (% v/v) of the MeOH extract was adjusted to 20% and 40% and partitioned against CCl_4 and $CHCl_3$, respectively. The aqueous phase was concentrated to remove MeOH and then extracted with *n*-BuOH. The $CHCl_3$ (1.6 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 **1** (231 mg, 0.29% dry weight of animal), as a colorless glassy solid.

Data of Crellastatin A (1): $C_{58}H_{88}O_{12}S$ amorphous powder: $[\alpha]_D$ +55° (*c* 1, MeOH); UV (MeOH) λ_{max} 208 nm (ϵ 7000); IR (KBr): 3420, 2924, 1651, 1540, 1458, 1200, 1060 cm^{-1}. HRFABMS (positive mode): *m/z* 1053.6 $(MSO_3^-Na^+ + Na)^+$, 933.6154 ($MSO_3^-Na^+ + Na - NaHSO_4$, base peak, $C_{58}H_{86}O_8$-Na, Δ +6.6 mmu); FABMS $(M - H)^-$:1007.

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Acetylation of Crellastatin A (1): A solution of **1** (5 mg) in Ac_2O and pyridine was stirred at room temperature for 12 h. The solvent was evaporated under reduced pressure afforded a 2,2′-diacetylated derivative (3 mg). The crude product was directly subjected to FABMS and NMR analysis. [FABMS 1091 $(M - H)^-$; selected ^1H NMR δ_H 5.22 (H2′, dd J = 8.8, 3.3 Hz), 5.18 (H2, dd J = 9.7, 4.8 Hz)]. Due to the steric hindrance, the tertiary hemiketalic OH groups were resistant under the acetylation condition.

Solvolysis of Crellastatin A (1) To Give 1a. A solution of **1** (15 mg) in dioxane (5 mL) and pyridine (5 mL) was heated at 130 °C for 2 h in a stoppered reaction vial. The reaction mixture was cooled and then evaporated under reduced pressure. Purification by reverse phase HPLC on a C-18 μ-Bondapak column eluting with MeOH:H_2O 90:10 afforded pure **1a** (3 mg). [FABMS 1011 $(M - H)^-$; selected ^1H NMR δ_H 1.39 (H5′, d, overlapped) 3.90 (H6′, dd, overlapped), 1.88 (H7′α, dd, overlapped), 2.42 (H7′β, dd, overlapped), 3.91 (H19′α, d, J = 8.8 Hz), 3.79 (H19′β, dd, J = 8.8, 1.8 Hz)].

Determination of Biological Activity. Cytotoxic and flow cytometry assays: see Zampella et al.[19]

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Supporting Information Available: ^1H and ^{13}C NMR, COSY, HOHAHA, ROESY HMQC, and HMBC spectra (methanol-d_4 and DMSO-d_6) of crellastatin (**1**) and expansions of COSY, HOHAHA, HMBC, and ROESY spectra (29 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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