A New Cytotoxic Diterpene with the Dolabellane Skeleton from the Marine Sponge *Sigmosceptrella quadrilobata*

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A new diterpene with the dolabellane skeleton **1** has been isolated from the sponge of the Indian Ocean, *Sigmosceptrella quadrilobata*. Its structure elucidation, including absolute stereochemistry, was performed with the aid of MS, NMR, and CD data and by molecular modeling. Compound **1** is moderately cytotoxic against four tumor cell lines.

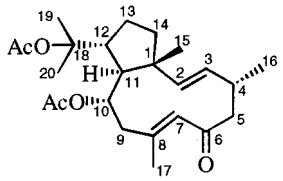
Introduction

Diterpenes with the dolabellane skeleton have mainly been found in marine organisms, particularly in octocorals[1−5] and in brown algae belonging to the family Dictyotacee,[6−10] as well as in the digestive gland of their predators, sea hares.[11−13] The skeleton name itself comes from the sea hare *Dolabella californica*, which was the source of the first-found dolabellane diterpene.[11] In contrast, until now no such diterpene has been isolated from Porifera. Interest in these compounds has been increased by reports on their biological properties which include cytotoxic,[3,10] molluscicidal,[14] and antifungal[14] activities.

As part of our continuing investigation on bioactive metabolites from sponges, we have analyzed the lipophilic extract of *Sigmosceptrella quadrilobata*, a marine sponge belonging to the order Hadromerida collected along the coasts of Mayotte, Comoros Islands. The chemistry of *Sigmosceptrella* sponges has not been extensively investigated so far, and only some norsesterterpenes have been reported from sponges of this genus.[15,16] In this paper we report the isolation from *S. quadrilobata* and the structure determination of a novel cytotoxic diterpene **1** based on the dolabellane skeleton.

Results and Discussion

Specimens of *Sigmosceptrella quadrilobata* were extracted in sequence with MeOH and $CHCl_3$. The extract was subjected to chromatography on an RP-18 column and then on an SiO_2 column, yielding a fraction containing compound **1**. Purification by HPLC on an RP-18 column and subsequently on an SiO_2 column gave 4.4 mg of pure **1**.



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Scheme 1

The high-resolution EI mass spectrum of compound **1** displayed a weak molecular ion peak at *m/z* 404.2541, accounting for the molecular formula $C_{24}H_{36}O_5$ (calcd. 404.2563). Two more intense fragment peaks at *m/z* 344.2340 ($C_{22}H_{32}O_3$, calcd. 344.2351) and 284.2110 ($C_{20}H_{28}O$, calcd. 284.2140), originating from consecutive losses of one and two acetic acid molecules, indicated the presence of two acetoxy groups in compound **1**. This was confirmed by the presence of two methyl singlets at δ = 1.93 and 2.07 in the ^{1}H-NMR spectrum, and two ester carbonyl signals at δ = 170.2 and 169.2 in the ^{13}C-NMR spectrum. The latter spectrum also showed signals for one disubstituted and one trisubstituted double bond [δ = 146.3 (C, C-8), 138.2 (CH, C-2), 132.2 (CH, C-3), 131.3 (CH, C-7)], and one oxo group [δ = 205.5 (C, C-6)]. The corresponding protons could be identified from an HMQC experiment as a doublet at δ = 5.01 (2-H), a double doublet at δ = 5.55 (3-H), and a broad singlet at δ = 6.06 (7-H), respectively. All these functionalities accounted for all the oxygen atoms in the molecule and, considering that the molecular formula implies seven degrees of unsaturation, indicated that **1** is a bicyclic compound.

The carbon atoms bearing the two acetoxy groups were readily recognized in the ^{13}C-NMR spectrum as the sole signals in the midfield region, namely from a quaternary carbon atom at δ = 84.8 (C-18) and from a methine carbon atom at δ = 71.9 (C-10). The 10-H proton resonated as a double doublet at δ = 4.82 as shown by the HMQC experiment.

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The terpenic nature of **1** was suggested by the presence of five further methyl signals in the proton spectrum, one doublet at δ = 1.04 and four singlets at δ = 0.87, 1.39, 1.62, and 2.02. The vinylic nature of the last methyl group, suggested by its chemical shift, was confirmed by the COSY spectrum, which showed it to be long-range-coupled with 7-H, and therefore linked to C-8.

Spin-spin coupling information from the COSY spectrum was also useful to identify two spin systems, including most of the protons in compound **1**. Further allylic coupling of 7-H with 9a-H and/or 9b-H (both resonating at δ = 2.30) identified these protons, which were coupled with 10-H (resonating at δ = 4.82). Further analysis of the COSY spectrum allowed the identification, in sequence, of two methine [δ = 1.65 (11-H), 3.15 (12-H)] and two methylene groups [δ = 1.96 (13a-H), 1.47 (13b-H), 1.50 (14a-H, 14b-H)]. This completed the first spin system.

Table 1. ^{1}H- and ^{13}C-NMR data of compound **1** in $CDCl_3$[a]

Position		δC [mult.]	δ_H [mult., *J* (Hz)]
1		47.8 (C)	
2		138.2 (CH)	5.01(d, 16.2)
3		132.2 (CH)	5.55 (dd, 16.2, 6.4)
4		34.2 (CH)	2.57 (m)
5	a	51.2 (CH_2)	2.72 (dd, 11.3, 3.4)
	b		2.33(dd, 11.3, 3.4)
6		202.5 (C)	
7		131.3 (CH)	6.07 (s)
8		146.3 (C)	
9	a	43.9 (CH_2)	2.33[b]
	b		2.31[b]
10		71.9 (CH)	4.82 (dd, 10.2, 2.9)
11		53.9 (CH)	1.65[b]
12		44.9 (CH)	3.15 (ddd, 10.5, 10.5, 4.5)
13	a	26.3 (CH_2)	1.96[b]
	b		1.47[b]
14	a	39.2 (CH_2)	1.50[b]
	b		1.50[b]
15		17.5 (CH_3)	0.87 (s)
16		18.4 (CH_3)	1.04 (d, 7.1)
17		21.5 (CH_3)	2.02 (s)
18		84.8 (C)	
19		26.7 (CH_3)[c]	1.62 (s)[d]
20		23.3 (CH_3)[c]	1.39 (s)[d]
10-Ac		22.7 (CH_3)	1.93 (s)
		169.9 (C)	
19-Ac		21.1 (CH_3)	2.07 (s)
		170.2 (C)	

[a] Assignment aided by COSY, ROESY, and HMQC experiments. – [b] Overlapped by other signals. – [c,d] Resonances with the same superscript may be reversed.

The olefinic protons [δ = 5.01 (2-H) and 5.55 (3-H)] were mutually coupled, and their large (16.2 Hz) coupling constant was indicative of a *trans* double bond. The 3-H proton was also coupled with a methine proton [δ = 2.57 (4-H)], in turn coupled with the methyl protons (d, 15-H_3) and two methylene protons [δ = 2.72, 2.33 (5-H_2)], which concluded the second spin system.

The connectivity of the above part structures with each other and with the remaining unassigned carbon atoms (three methyl groups, one oxo group, and two quaternary carbon atoms) was achieved through the analysis of the HMBC spectrum. The linkage of the ketone carbon atom (C-6) with C-5 and C-7 was demonstrated by its HMBC correlation peak with 5b-H and 7-H. The presence of an enone functionality was confirmed by the absorption maximum at λ_{max} = 243 nm in the UV spectrum. The long-range correlation of the methyl singlet at δ = 0.87 (15-H_3) with the C-2, C-11, C-14 signals, and the quaternary carbon atom signal at δ = 47.8 (C-1) established the linkage of C-1 with C-2, C-11, C-14, and C-15, thus joining the two partial structures into the bicyclic skeleton required by the molecular formula. Finally, the presence of an acetoxyisopropyl group at position 12 was demonstrated by the long-range ^{1}H-^{13}C coupling of the two remaining methyl singlets at δ = 1.39 and 1.62 (19-H_3 and 20-H_3) with C-12 and the oxygen-bearing quaternary carbon atom signal at δ = 84.8 (C-18). The many additional ^{1}H-^{13}C couplings detected with the HMBC spectrum, all in accordance with the proposed structure, are depicted in Figure 1.

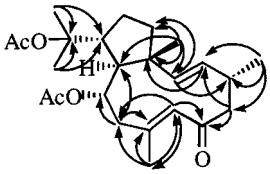

Figure 1. $^{1}H \rightarrow ^{13}C$ HMBC correlations detected for compound **1**

Having established the constitution of the terpene **1**, we focused our attention on its stereochemistry. The *E* configuration of the double bond at position 7 was evidenced by the NOE enhancement (3.2%) of 7-H signal by saturation of the protons at C-9. The *trans* junction between the two rings was deduced by a long-range coupling evidenced by the COSY spectrum between 11-H and the methyl protons 15-H_3, indicative of a W coupling. This is only possible when the dihedral angle 11-H/C-11/C-1/C-15 is near 180°, i.e. in case of *trans* junction. In addition, the NOE observed for 10-H and 12-H by irradiation of 15-H_3 (see Table 2) revealed that the substituents at C-10 and C-12 lay on the opposite side of the molecule relative to the angular methyl group C-15.

Table 2. NOE difference data of compound **1**

Saturated proton	Enhanced protons (% enhancement)
2-H	11-H (1.6), 16-H_3 (0.4)
3-H	4-H (2.1), 15-H_3 (0.3)
7-H	5a-H (2.4), 9a-H (2.8), 10-H (7.0)
10-H	7-H (6.9), 15-H_3 (0.9)
15-H_3	2-H (0.7), 3-H (2.4), 10-H (3.1), 12-H (1.6)
16-H_3	2-H (4.4), 4-H (4.5)

At this stage what remained to be established was the relative configuration at C-4. A strong dipolar coupling was detected between 16-H_3 and 2-H; however, on account of the flexibility of the 11-membered ring, interpretation of this experimental data required some knowledge about the preferred conformation of compound **1**.

To this purpose, we performed a conformational study of the two possible epimers at C-4 of compound **1** (referred to

in the following discussion as 4α-Me for the configuration of C-4 like in structure **1**, and 4β-Me for the opposite C-4 configuration) in the CHARMm force field. An important aid to this study came from some intense NOE enhancements observed between four couples of protons which are topologically far apart, namely 3-H and 15-H_3, 7-H and 10-H, 10-H and 15-H_3, and 2-H and 11-H (see Table 2). These data, indicating that protons of each pair are close in space, were included in the calculation as distance constraints (see Experimental Section for details).

A set of 100 initial structures for each epimer was generated using the High Temperature Molecular Dynamics Simulation (HTMDS) technique;[17] the structures were then subjected to energy minimization. In the subsequent analysis, we considered only the conformations within 5 kcal/mol from the lowest energy structure (19 for the 4α-Me and 15 for the 4β-Me epimer), which are significantly populated at room temperature. In 12 of the 19 low-energy structures of the 4α-Me epimer (including the lowest energy structure, Figure 2) the distance between 16-H_3 (the mean position of the three hydrogen atoms was considered) and 2-H was less than 2.70 Å. In contrast, in the 15 low-energy structures of the 4β-Me epimer the distance between 16-H_3 and 2-H was in the range 4.11−4.43 Å and could not account for the NOE observed between these protons. Therefore, the configuration at C-4 was assigned as indicated in structure **1**.

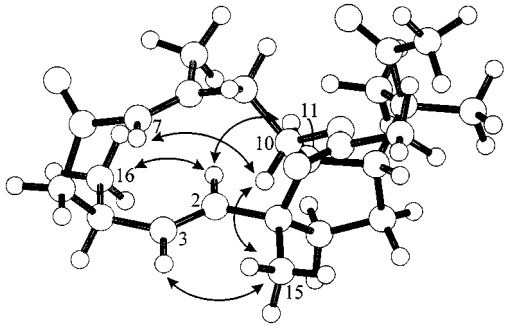

Figure 2. Lowest energy conformation in the CHARMm force field of compound **1**; the NOEs used for molecular modeling are denoted by arrows

This conformational study, in combination with a CD measurement, was also useful for the assignment of the absolute configuration of compound **1**. The CD spectrum showed a negative π-π* Cotton effect at λ_{max} = 243 nm ($\Delta\varepsilon$ = −9.1). It has been shown that in skewed enones in the cisoid conformation, the sign of the π-π* Cotton effect is related to the helicity of the inherently chiral chromophore, a negatively skewed chromophore showing a negative Cotton effect.[18] Since in all the low-energy conformations of the enantiomer depicted in structure **1** the dihedral angle C-8/C-7/C-6/O-6 is negative, this absolute configuration and not the opposite one is to be assigned to the diterpene from *S. quadrilobata*.

Compound **1** was tested in vitro for cytotoxic activity on GM7373 (bovine endothelial), J774 (murine monocyte/macrophage), WEHI 164 (murine fibrosarcoma), and P338 (murine leukemia) cells. It inhibited the growth of all cells lines evaluated at 72 h: the IC_{50} for the various cell lines were 15.8 μg/mL, 17.2 μg/mL, 14.7 μg/mL, and 7.7 μg/mL, respectively. These preliminary results show that compound **1** is particularly, but moderately active on leukemia cells P338.

Experimental Section

General: Optical rotation: Perkin−Elmer 192, in $CHCl_3$. − UV: Beckman DU-70, in *n*-hexane. − CD: Jasco J-720, in *n*-hexane. − EI MS: VG Prospec-Autospec (Fisons), 70 eV. − ^{1}H and ^{13}C NMR: Bruker AMX-500, in $CDCl_3$; chemical shifts are referenced to the residual solvent signal (δ_H = 7.26, δ_C = 77.0). Homonuclear ^{1}H connectivities were determined by COSY experiments. The reverse multiple-quantum heteronuclear correlation (HMQC) spectra were recorded by using a pulse sequence with a BIRD pulse 0.5 s before each scan to suppress the signal originating from protons not directly bound to ^{13}C; the inter-pulse delays were adjusted for an average $^1J_{CH}$ of 142 Hz. The gradient-enhanced multiple-bond heteronuclear correlation (HMBC) experiment was optimized for a $^3J_{CH}$ of 8.3 Hz.

Collection, Extraction and Isolation Procedure: Specimens of *Sigmosceptrella quadrilobata* were collected at Prevoyante Reef, Lagoon of Mayotte, Comoros Island, northwest of Madagascar by scuba at a depth of 10−15 m during April 1996 and identified by J. Vacelet, Station Marine d'Endoume, Centre d'Océanologie de Marseille, France. A voucher sample has been deposited at the Station Marine d'Endoume. Freshly collected animals (250 g of dry weight after extraction) were homogenized and extracted with $CHCl_3$/MeOH 2:1 (5 × 200 mL) at room temperature; the combined extracts were partitioned between H_2O and EtOAc. The organic layer (5.3 g) was concentrated in vacuo and then chromatographed on a column packed with RP-18 silica gel eluting with MeOH/H_2O (from 9:1 to 1:9), MeOH/$CHCl_3$ (9:1), and $CHCl_3$. The MeOH/$CHCl_3$ fraction (422.5 mg) was further chromatographed on an SiO_2 column and a fraction eluted with *n*-hexane/EtOAc (6:4) afforded a mixture containing compound **1** (191.4 mg). Finally, pure **1** (4.4 mg) was obtained by sequential HPLC using a LiChrospher RP-18 column with a mobile phase MeOH/H_2O (75:25) and then using a Phenomenex Luna Silica (250 × 4.60 mm) column with a mobile phase *n*-hexane/EtOAc (8:2).

Compound 1: Amorphous solid; $[\alpha]_D^{25}$ = −36 (*c* = 0.22, $CHCl_3$). − UV (*n*-hexane): λ_{max} = 243 nm (ε = 7600). − CD (*n*-hexane) 243 nm ($\Delta\varepsilon$ = −9.1). − HRMS: *m/z* (calcd. mass, relative intensity) = 404.2531 ($C_{24}H_{36}O_5$: 404.2563, 0.5), 344.2340 ($C_{22}H_{32}O_3$: 344.2351, 4), 302 (7), 284.2110 ($C_{20}H_{28}O$: 284.2140, 45), 269 (16), 241 (17), 188 (37), 173 (100). − ^{1}H- and ^{13}C-NMR data are shown in Table 1.

Molecular Modeling: Molecular modeling studies were performed using the Quanta/CHARMm 4.0 program. All the simulations were performed in vacuo, and the presence of the solvent (chloroform) was approximated using a dielectric constant of 2. Distances between the dipolarly coupled 3-H and 15-H_3, 7-H and 10-H, 10-H and 15-H_3, and 2-H and 11-H were constrained between 1.90 and 3.50 Å using a parabolic potential with a force constant of 25 kcal mol^{-1}Å^{-1} (dynamics simulation) or 100 kcal mol^{-1}Å^{-1} (minimization) outside this range. Molecular dynamics simulations were performed at 1500 K; they involved a heating period of 3 ps, followed by a 5-ps equilibration period, and then 1 ns of dynamics simulation. The coordinates produced by the simulation were saved

every 10 ps, giving 100 structures. Each of them was subjected to energy minimization, using the conjugated gradient protocol.

Cytotoxicity Assay: GM7373, J774, WEHI 164, and P338 (1×10^4 cells) were plated on 96-well plates in 50 μL and allowed to adhere at 37°C in 5% CO_2/95% air for 2 h. Thereafter, the medium was replaced with 50 μL of fresh medium, and 50 μL of 1:4 v/v serial dilutions of the test compound **1** were added and the cells were incubated for 72 h. The cell viability was assessed through the MTT conversion assay.[19] Briefly, after incubation, 25 μL of MTT (5 mg/mL) were added to each well, and the cells were incubated for additional 3 h. After this time, the cells were lyzed and the dark blue crystals solubilized with 100 μL of a solution containing 50% v/v SDS with an adjusted pH of 4.5.[20] The optical density (OD) of each well was measured with a microplate spectrophotometer equipped with a 620-nm filter. The viability of each cell line in response to treatment with compound **1** was calculated as% dead cells = 100 − (OD treated/OD control) × 100. The results are expressed as IC_{50} (the concentration that inhibited the cell growth by 50%).

Acknowledgments

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