Dactylolide, a New Cytotoxic Macrolide from the Vanuatu Sponge *Dactylospongia* sp.

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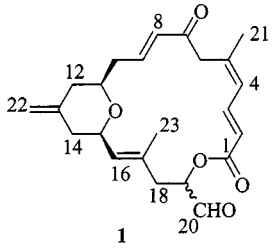
Keywords: Structural elucidation / Macrolides / Antitumor agents / Natural products / NMR spectroscopy

Dactylolide (**1**), a new cytotoxic 20-membered macrolide, was isolated from a marine sponge of the genus *Dactylospongia* collected off the coast of the Vanuatu islands. It co-occurred with other known bioactive macrolides: latrunculin A (**2**), laulimalide (**3**), isolaulimalide (**4**) and with the anthelminthic mycothiazole (**5**). The structure of **1**, which is a minor metabolite, was elucidated by spectroscopy (mainly by 1D/2D NMR and MS techniques). It showed cytotoxic activity against the L1210 and SK-OV-3 tumor cell lines (63% and 40% inhibition at 3.2 μg/mL).

Introduction

Over the past few years macrolides of marine origin have continued to be of interest on account of their wide spectrum of biological and pharmacological properties. Perhaps the best known examples are bryostatin 1[1,2] and halichondrin B,[3] both of which possess perhydropyran rings as prominent structural features. Many novel, bioactive macrolides have been recently isolated from marine systems as phylogenetically diverse as marine bacteria[4] and sponges. Among Porifera, marine sponges of the genus *Theonella*[5−9] have been shown to produce diverse bioactive macrolides, such as misakinolide,[5,6] a dimeric 40-membered lactone showing a potent antitumor activity, and the dimeric lactones swinholides[7−9] which exhibit a high cytotoxic activity that is probably related to their ability to disrupt the actin cytoskeleton by dimerizing actin. In turn, these interesting compounds may be derived from symbiotic blue-green algae. Other pertinent examples of interesting macrolides from sponges are sphinxolides and redispongiolides;[10−12] they represent a new class of cytotoxic macrolides characterized by a very similar 26-membered lactone ring which has proved to be very selective against human tumor cells. In particular, sphinxolides have been proved to cause rapid loss of microfilaments in cultured cells without affecting microtubule organisation.[13]

In the course of our continuing study on bioactive metabolites from sponges, we have examined several marine organisms collected in different geographical areas in the frame of the European project "Bioactive Marine Natural Products in the Field of Antitumoral, Antiviral and Immunomodulant Activity", financially supported by the EU.[14]



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In this paper we report the results of chemical and pharmacological investigation of the Vanuatu sponge *Dactylospongia* sp.

On the basis of preliminary pharmacological screening, the crude extract of this sponge was selected for chemical analysis. In order to isolate the metabolites responsible for the observed bioactivity, we extracted the lyophilized organism with MeOH and subjected the methanolic extract to a modified Kupchan partition procedure, thereby affording four extracts (see Experimental Section). The most active CCl_4 extract was submitted to medium pressure liquid chromatography (MPLC) on silica gel (gradient elution system: *n*-hexane/EtOAc from 100% *n*-hexane to 100% EtOAc) affording mycothiazole,[15] latrunculin A,[16−18] isolaulimalide[19,20] and laulimalide[19,20] as pure compounds. The fractions eluted with 80% *n*-hexane were further purified by C-18 reversed-phase HPLC (MeOH/H_2O, 70:30) to afford **1** as a pure metabolite.

Latrunculin A and laulimalide are two further examples of biologically active macrolides; in fact, apart from their primary effect to protect the manufacturing sponges against predators, latrunculin A has been shown to exert a strong reversible effect on microfilament organisation in cultured mouse neuroblastoma cells,[21] while laulimalide showed a taxol-like activity on tubulin systems.[22]

Results and Discussion

The determination of the molecular formula of compound **1** was not a trivial task due to a singular behaviour

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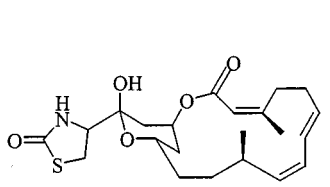

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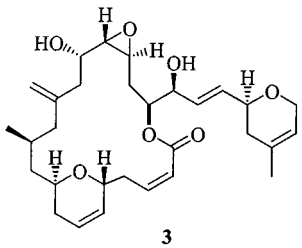

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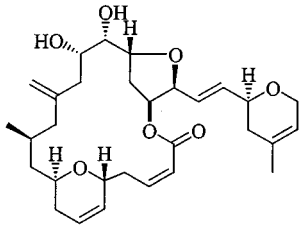

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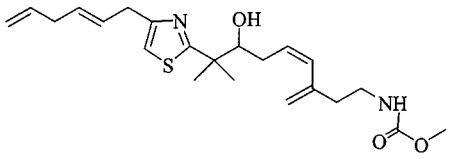

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of this product under mass spectrometry conditions. In fact, the electrospray mass spectrum (ESIMS) of compound **1** dissolved in MeOH showed three prominent peaks at *m/z* = 417, 439 and 855. The two ion peaks at *m/z* = 439 and 417 suggested that a species with an apparent molecular weight of 416 was being observed both as its proton (*m/z* = 417) and sodium (*m/z* = 439) adducts. This range of mass for compound **1** seemed also appropriate and in good agreement with NMR spectroscopic data that were indicative of a relatively unfunctionalized C_{23} carbon skeleton. The molecular formula of compound **1** was revealed as $C_{23}H_{28}O_5$ by HRESIMS data (see Experimental Section). The origin of the peak at *m/z* = 855 was not immediately understood, but its high mass value was indicative of a molecular cluster species, probably consisting of two simpler species, as indicated by the observation that 855 = 439 + 416. In order to investigate the nature of this cluster, and to firmly establish the molecular weight of **1**, we performed ESIMS/MS experiments on the peaks at *m/z* = 417, 439, and 855. These results clearly indicated that both the ions at *m/z* = 417 [M + MeOH + H]$^+$ and at *m/z* = 439 [M + MeOH + Na]$^+$ readily lost 32 a.m.u. (a molecule of MeOH) yielding stable ions at *m/z* = 385 and 407, respectively. Additional evidence confirming our hypothesis came from the ESIMS/MS spectrum in CD_3OD solution, where a pseudomolecular ion peak at *m/z* = 443 [M + CD_3OD + Na]$^+$ lost 36 a.m.u,. generating the peak at *m/z* = 407 [M + Na]$^+$, as expected. On the basis of this data we could establish that compound **1** had a molecular weight of 384 a.m.u. ($C_{23}H_{28}O_5$). In MeOH solution, compound **1** quantitatively incorporated one molecule of MeOH giving rise to the species at *m/z* = 417 [M + MeOH + H]$^+$ and 439 [M + MeOH + Na]$^+$. The higher mass ion at *m/z* = 855 gave rise in the fragmentation process to a peak at *m/z* = 439 with a net loss of 416 a.m.u. This conclusion was strongly corroborated by the presence in our molecule of an aldehyde function (see below), with an electronegative atom at the α-carbon promoting nucleophilic attack of MeOH onto the carbonyl functionality to generate a hemiacetal. With this in mind, the peak at *m/z* = 855 can be explained by a cluster formed by two molecules in their hemiacetal forms and incorporating one sodium ion [2M + 2MeOH + Na]$^+$.

Interpretation of the ^{1}H and ^{13}C NMR spectral data of **1** (Table 1) indicated the presence of 23 carbon signals: one conjugated ester carbonyl (C1: δ = 166.0), an aldehyde function (C20: δ = 199.7), an α,β-unsaturated ketone (C7: δ = 197.0), one *exo*-methylene (C13: δ = 143.4; C22: δ = 109.0), two disubstituted (C2−C3; C8−C9) and two trisubstituted (C4−C5; C16−C17, C19: δ = 75.5) double bonds, three oxymethines (C11: δ = 76.7; C15: δ = 76.1; C19: δ = 75.5), five sp^3 methylenes (C6: δ = 44.8; C10: δ = 40.1; C12: δ = 40.8; C14: δ = 40.4; C18: δ = 39.6) and two methyls on sp^2 carbons (C21: δ = 23.6; C23: δ = 16.2).

Since eight out of ten formal unsaturations required by the molecular formula $C_{23}H_{28}O_5$ were thus accounted for, compound **1** was inferred to contain two rings (one lactone and one ether ring). The ^{1}H-^{1}H COSY spectrum of **1** clearly showed the proton connectivities for the four spin systems: H2−H4, H8−H12, H14−H16, H18−H20. These fragments, identified also on the basis of HSQC data,[23] were connected with the remaining functional groups to build up the macrolide skeleton **1** through the correlations observed in the HMBC[24] and ROESY[25] spectra (Figure 1).

In particular, the correlation H2/C1 observed in the HMBC spectrum allowed us to place the ester carbonyl (δ = 166.0) adjacent to the disubstituted double bond C2−C3. The isolated methylene CH_2-6 (δ = 3.94, 3.23) showed HMBC connectivities with C4 (δ = 125.7), the sp^2 quaternary carbon at δ = 144.4 (C5), the methyl group at δ = 23.6 (C21) and the carbonyl at δ = 197.0 (C7), thus placing it between the carbonyl C7 and the sp^2 carbon C5, which, in turn, had to be connected to C4 and to the methyl C21. The carbonyl C7 was revealed to be connected to the spin system H8−H12 by the HMBC correlations H8/C7 and H9/C7. The HMBC correlations $H_2$12/C22, $H_2$12/C13, $H_2$14/C22 and $H_2$14/C13 placed the exomethylene group, made up of C13 (δ = 143.4) and C22 (δ = 109.0), between CH_2-12 and CH_2-14 and extended the carbon framework

Table 1. NMR spectroscopic data for **1** ($CDCl_3$, 600 MHz)

	^{1}H, m, J (Hz)	^{13}C	HMBC	ROESY
1		166.0		
2	5.96, d, 15.1	120.0	C1; C4	$H6_A$; $H6_B$
3	7.62, dd, 15.1; 11.6	140.7	C1; C2; C4; C5	$H6_A$; $H6_B$
4	6.15, d, 11.6	125.7	C2	H21
5		144.4		
$\mathbf{6_A}$	3.94, d, 14.5	44.8	C4; C5; C7; C21	H2; H3; H9; H21
$\mathbf{6_B}$	3.23, d, 14.5		C4; C5; C7; C21	H2; H3; H9; H21
7		197.0		
8	6.0, d, 16.0	131.3	C7	$H10_A$; $H10_B$
9	6.84, ddd, 5.9; 8.5; 16.0	146.8	C7	$H6_A$; $H6_B$
$\mathbf{10_A}$	2.35, m	40.1	C8; C9	H8;
$\mathbf{10_B}$	2.28, m		C8; C9	H8; H11
11	3.31, dddd, 12.3; 9.6; 2.6; 2.6	76.7		$H10_B$; $H12_A$; H15
$\mathbf{12_A}$	2.16, m	40.8	C13; C22	H11; H22
$\mathbf{12_B}$	1.95, t, 12.3		C13; C22	H22
13		143.4		
$\mathbf{14_A}$	2.10, m	40.4	C13; C22	H15; H16; H22
$\mathbf{14_B}$	1.95, m		C13; C22	H16; H22
15	3.96, ddd, 10.9; 8.3, 2.6	76.1		H11; $H14_A$; H23
16	5.24, d, 8.3	130.8	C18; C23	$H14_A$; $H14_B$; $H18_B$
17		131.3		
$\mathbf{18_A}$	2.53, brd, 14.0	39.6	C23	H23
$\mathbf{18_B}$	2.31, brd, 14.0		C23	H16
19	5.31, dd, 11.2; 2.2	75.5	C1; C20	H20; H23
20	9.66, s	199.7	C19	H19
21	1.85, s	23.6	C4; C5; C6	H4; $H6_A$; $H6_B$
22	4.73, brs	109.0	C14; C12	$H12_A$; $H12_B$; $H14_A$; $H14_B$
23	1.70, s	16.2	C17; C18	H15; $H18_A$; H19

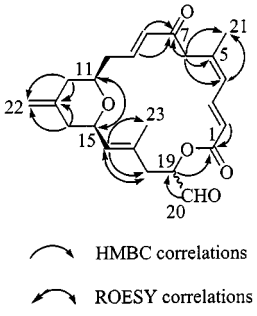

Figure 1. Key HMBC and ROESY correlations for **1**

connectivity to C16 (δ = 130.8). A strong ROESY cross-peak was observed between H11 and H15, thus suggesting that the oxymethines at C11 and C15 were linked through an oxygen to form a tetrahydropyran ring, and that H11 and H15 were both axially oriented. This observation is supported by the coupling constants [H11: δ = 3.31 (dddd, J = 12.3, 9.6, 2.6, 2.6 Hz); H15: δ = 3.96 (ddd, J = 10.9, 8.3, 2.6 Hz)]. The connection of units C14−C16 and C18−C20 through the remaining sp^2 quaternary carbon (C17) bearing a methyl group was substantiated by HMBC correlations H16/C23, H16/C18 as well as a ROESY effect between H16 and $H18_B$, thus giving information also on the *E* geometry of the C16−C17 double bond. The aldehyde function identified by the ^{13}C signal at δ = 199.7 and by the proton signal at δ = 9.66, was located at the oxygenated C19 by the HMBC correlation H20/C19. Furthermore, the proton-carbon ^2J value of 50 Hz between H20/C19, observed in the HMBC spectra, is extremely diagnostic for the presence of an aldehyde function.[26] Further evidence came from the analysis of the ^{1}H NMR spectrum performed in CD_3OD, in which the proton signal at δ = 9.66 disappeared in accordance with the formation of a hemiacetal functionality at C20 as already described. Finally, the C19 oxymethine proton, resonating at a relatively low field (δ = 5.31) showed an HMBC correlation with the ester carbonyl (δ = 166.0, C1), thus closing a 20-membered macrocyclic lactone ring and establishing the complete carbon framework of **1**. The configuration of the C2−C3 and C8−C9 double bonds was deduced to be *E* by ^{1}H NMR analysis of coupling constants ($J_{H2\text{-}H3}$ = 15.1 Hz, $J_{H8\text{-}H9}$ = 16.0 Hz), whereas the geometry of the C4−C5 and C16−C17 double bonds was established to be *Z* and *E*, respectively, on the basis of key ROESY correlations observed for H4/H_3-21 and H16/$H18_B$.

Experimental Section

General: NMR spectra: Bruker Avance DRX 600; HR-ESIMS: VG AutoSpec mass spectrometer; ESIMS: LCQ Finnigan mass spectrometer; $[\alpha]_D$: Perkin−Elmer 161 polarimeter; UV: Beckman DU 640 spectrophotometer.

Biological Material: A sample of *Dactylospongia* sp. was collected off the Vanuatu Islands and taxonominally identified by Dr. John Hooper (Museum of Queenland, Brisbane, Australia) and registered in this Museum under the acquisition number 306876.

Isolation: The organism (lyophilized material, 353 g) was extracted exhaustively with MeOH (4 × 1.5 L) at room temperature. The methanolic extract, filtered through paper and concentrated under reduced pressure gave a red brown oil. The oily residue was successively extracted using a modified Kupchan partition procedure: the extract was dissolved in 0.7 L of a mixture of MeOH/H_2O con-

taining 10% of H_2O and partitioned against 0.7 L of *n*-hexane. The water content (% v/v) of the methanolic fraction was adjusted to 20% and 40% and partitioned against 1 L of CCl_4 and 1 L of $CHCl_3$, respectively. The aqueous phase was concentrated to remove MeOH and then extracted with *n*-butyl alcohol (0.5 L). The CCl_4 soluble material (4.9 g) was chromatographed by medium pressure liquid chromatography (MPLC) on a silica gel column (230−400 mesh) using a gradient elution system *n*-hexane/EtOAc from 100% *n*-hexane to 100% EtOAc (30 mL/fraction). The collected fractions were controlled by TLC on silica gel (Merck, kieselgel F_{254}, 0.25 mm) and revealed by spraying with $Ce(SO_4)_2$ in sulfuric acid solution. Homogeneous fractions were pooled into 19 groups. MPLC fractions 51−56 contained mycothiazole (432.7 mg), fractions 81−87 contained latrunculin A (165.1 mg), fractions 122−130 contained isolaulimalide (191.0 mg) and fractions 131−139 contained laulimalide (55.0 mg) as pure compounds. The fractions 67−80 eluted with 80% *n*-hexane were further purified by HPLC on an analytical C-18 Simmetry column and eluted with MeOH/H_2O (70:30) to afford compound **1** (5.5 mg) as pure metabolite. The $CHCl_3$ extract (4.5 g), chromatographed by MPLC using the conditions mentioned above, gave mycothiazole (59.7 mg), latrunculin A (138.4 mg), isolaulimalide (60.0 mg) and laulimalide (209.3 mg) as pure compounds.

Dactylolide (1): White amorphous solid. $[\alpha]_D$ = +30.0 (*c* = 1.0, MeOH. − UV: λ_{max} (ε) = 317 (800), 266 (16000), 222 nm (11000). − ESIMS (MeOH): *m/z* = 855 (21) $[2M + 2MeOH + Na]^+$, 439 (100) $[M + MeOH + Na]^+$, 417 (55) $[M + MeOH + H]^+$. − ESIMS (CD_3OD): *m/z* = 443 (41) $[M + CD_3OD + Na]^+$, 407 (100) $[M + Na]^+$. − HR-ESIMS: *m/z* = 439.210670 $[M + MeOH + Na]^+$ ($C_{24}H_{32}O_6Na$ requires 439.209659). − For NMR spectroscopic data see Table 1.

Cytotoxicity Assay: A defined number of tumor cells were placed in microtiterplates (100μL) and incubated at 37 °C/5% CO_2 for 24 hours. The samples were dissolved in DMSO (333 times more concentrated than the highest test concentration) and 50 μL of the solutions/concentrations or 50 μL pure DMSO (controls) were pipetted to the cell cultures. Then, the cell cultures were incubated with the samples for an additional 45 hours. After this incubation time, 75 μL XTT-solution was added to the culture and incubation continued for 3 hours at 37 °C/5% CO_2. After that, the extinction of all cell cultures was measured at 490 nm and compared to the extinction of the corresponding control cultures. The assay was run on a screening robot (Biomek 2000, Beckman), according to the above protocol.[27] The inhibition percentages found for **1** by this procedure were 63% and 40% on the L1210 (lymphatic leukaemia of mice) and SK-OV-3 (carcinoma of the ovaries) tumour cell lines, at 3.2 μg/mL, respectively.

Acknowledgments

The authors thank the IRD diving team of Noumèa (New Caledonia) and the Government of Vanuatu through the Fisheries Department for allowing the collection of biological material. The authors also thank Dr. Gerhard Noessner (ASTA MEDICA, Frankfurt, Germany) for biological assays, performed within the frame of the EU project MAST III. Mass spectral data were kindly provided by C.R.I.A.S. "Centro Ricerche Interdipartimentale di Analisi Strumentale", Facoltà di Farmacia, Università di Napoli "Federico II", Italy. HRESIMS spectra were obtained at the Centro Internazionale di Servizi di Spettrometria di Massa (Napoli), which is gratefully acknowledged. Financial support was provided by the European Union through the project MAST III "*Bioactive Marine Natural Products in the Field of Antitumoral, Antiviral and Immunomodulant Activity*" (contract MAS3-CT95−0032), MURST (Ministero dell'Università e della Ricerca Scientifica e Tecnologica) PRIN 97, CNR (Consiglio Nazionale delle Ricerche) and Università di Salerno, Italy.

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Received July 7, 2000
[O00343]