

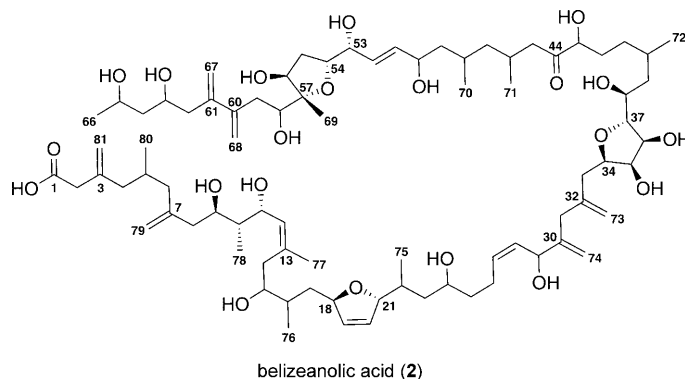
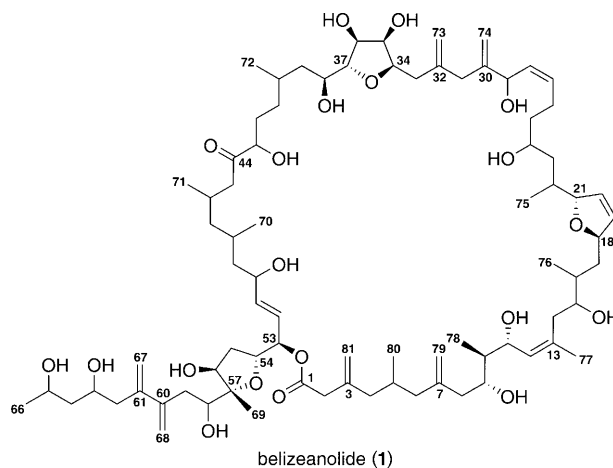
Belizeanolide, a Cytotoxic Macrolide from the Dinoflagellate *Prorocentrum belizeanum***

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Marine dinoflagellates produce many of the most active and complex secondary metabolites found in nature.^[1] In fact, some of these molecules have had an extraordinary impact upon different areas of life science such as human health, seafood control analysis, pharmacology, natural product chemistry, and the economics of the fishery industry, thus promoting new developments in all these areas. In particular, the dinoflagellates of the genus *Prorocentrum*, recognized as co-responsible of the diarrhetic shellfish poisoning (DSP) syndrome, are known to produce several unique bioactive secondary metabolites with a broad diversity of skeletons, including “linear” polycyclic compounds and macrolides.^[2–6]

Herein, we report on the isolation, structure determination, partial relative stereochemistry assignment, and biological activity of the first member of a new class of macrolides, belizeanolide (**1**), which is produced by the marine dinoflagellate *Prorocentrum belizeanum*, together with its open form belizeanolic acid (**2**).

Belizeanolide and belizeanolic acid were isolated as optically active white amorphous solids ($[\alpha]_{\text{D}}^{25} = -9.2 \text{ deg cm}^3 \text{ g}^{-1} \text{ dm}^{-1}$ ($c = 0.0013 \text{ g cm}^{-3}$, in methanol) and $[\alpha]_{\text{D}}^{25} = -5.2 \text{ deg cm}^3 \text{ g}^{-1} \text{ dm}^{-1}$ ($c = 0.0016 \text{ g cm}^{-3}$, in methanol), respectively) from the culture media of the dinoflagellate. The clarified culture media was slowly passed through a polyaromatic adsorbent resin that was successively extracted with methanol. By using this procedure, 1.52 g of crude extract was obtained from 750 L of culture. Next the extract was successively purified by several chromatographic steps to



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yield 6.7 mg of belizeanolide (**1**) and 44.7 mg of belizeanolic acid (**2**). Finally, the purity of the isolated compounds was evaluated by using tandem LC/MS experiments, resulting in a single-peak total ion current (TIC) chromatogram for each sample.

The positive MALDI-TOF mass spectra of belizeanolide showed pseudomolecular ions at m/z 1447.891 and 1463.932, corresponding to the sodium and potassium adducts. The exact monoisotopic molecular weight was determined to be 1424.931 Da, and subsequently the molecular formula $\text{C}_{81}\text{H}_{132}\text{O}_{20}$ was confirmed after a structural analysis based on NMR spectroscopy. The IR spectrum suggested the presence of a lactone moiety (1711 cm^{-1}) together with characteristic bands at 3359 cm^{-1} (hydroxy) and 1059 cm^{-1} (C–O stretching).

Despite the relatively large number of proton and carbon atoms in this molecule, the NMR spectra showed relatively

good signal dispersion and therefore were rich in structural information. HSQC analysis revealed the existence of 10 methyl groups, 21 aliphatic methylene units, 6 olefinic exocyclic methylene units, 7 olefinic methine units, and 27 methine units (including 20 oxymethine groups). Additionally, one ketone group, one carboxylic ester group, and eight quaternary carbon centers (seven of them belonging to double bonds) were identified from both the ^{13}C NMR and HMBC spectra.

Extensive analyses of the 2D NMR spectra of belizeanolide, particularly based on COSY, TOCSY, HSQC, HSQC-TOCSY, and HMBC experiments resulted in the elucidation of seven discrete ^1H , ^1H spin systems: I (H4–H6), II (H8–H12), III (H14–H29), IV (H33–H43), V (H45–H56), VI (H58–H59), and VII (H62–H66) (Figure 1).^[7] Connectivity among the above fragments was established by using ^1H , ^{13}C long-range correlations extracted from HMBC experiments.

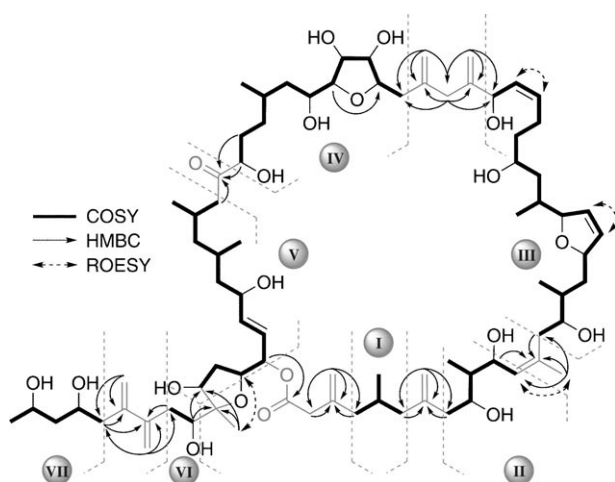


Figure 1. Selected NMR-derived correlations observed for belizeanolide (1). The ^1H , ^1H spin systems are numbered from I to VII.

The presence of six pendant vinyl methylene groups (as a repeating structural motif) accounts for six out of the eight quaternary carbon centers in the molecule. The overlap of signals caused by this common subunit turned out to be problematic during the structure elucidation. To overcome such difficulty semiselective HMBC experiments were acquired, which gave higher spectral resolution. As a result, connectivities arising from HMBC correlations were clearly assigned. Moreover, to the best of our knowledge, the existence of two consecutive exocyclic vinyl methylene units in the side chain turned out to be a new motif in the field of natural products. Such a substructure was confirmed by using HMBC correlations and its existence is consistent with the UV/Vis spectrum that shows an absorption maximum at 224 nm.

Thus the whole carbon skeleton of belizeanolide was assigned, leaving only the positions of the hydroxy groups and the ether linkages to be identified (Table 1). Deuterium-induced isotope shifts (upfield), as observed by the chemical shift differences between two ^{13}C NMR experiments recorded in CD_3OD and CD_3OH , led to the identification of hydroxy-

bearing carbon atoms. In this way, significant chemical shift differences ($\Delta\delta = 0.03$ – 0.12 ppm) were observed for most of the oxymethine groups with the exception of C18, C21, C34, C37, C53, C54, and C57 that were superimposed within $\Delta\delta = 0.03$ ppm. At C53, the macrocycle is closed through an ester linkage and was determined by observation of an HMBC correlation between H53 and the carboxylic carbon atom at C1. In addition, the isolation of the open form of belizeanolide (1), belizeanolic acid (2), allowed us to compare the chemical shifts of both metabolites to further support the previous proposal. This result together with the number of unsaturations derived from the molecular formula and the structural features described above suggested the presence of three ether rings. Therefore, three five-membered ether rings were constructed, based on the relative positions of the non-hydroxy-oxygen-bearing carbon atoms. These ether linkages were confirmed by dipolar correlations and/or HMBC scalar correlations. In addition, a large long-range H,H homoallylic coupling value ($J = 9.0$ Hz), which is characteristic of *trans*-2,5-dihydrofurans, is clearly observed between H18 and H21.^[8] The double bonds at C12–C13, C19–C20, and C27–C28 were determined to have the *Z* geometry on the basis of NOE cross-correlations and $^3J_{\text{H,H}}$ values ($^3J_{\text{H19,H20}} = 6$ Hz, $^3J_{\text{H27,H28}} = 9$ Hz). On the other hand, the olefin at C51–C52 showed a $^3J_{\text{H51,H52}}$ value of 15 Hz, which is characteristic of double bonds with an *E* configuration.

Although the stereostructure of six-membered rings is routinely determined by the use of standard NMR spectroscopy, the problem is far more complex for the five-membered rings existing in 1 and 2—as a classical analysis based solely on $^3J_{\text{H,H}}$ measurements is considered to be of little use. Therefore, we decided to measure $^3J_{\text{H,H}}$ values from ^1H DQF-COSY, or zero-quantum suppressed ZCOSY spectra, together with heteronuclear coupling constants ($^2J_{\text{C,H}}$) from HSQC-HECADE and HSQC-TOCSY-IPAP spectra in combination with ROESY experiments (Figure 2).^[9–11]

In fact, the relative configuration of the C34–C37 ring was first and foremost solved by measurement of homo- and heteronuclear coupling constants. Our data imply *cis* relationships for the H34/H35 and H35/H36 proton pairs, but a *trans* conformation for the H36/H37 pair. The relative configuration of C38 was also determined by using the configuration analysis based on coupling constants.^[12] In addition, the existence of dipolar correlations between H34 and H36 confirmed the previous observations. On the other hand, the relative configurations of the three chiral centers present in the ring at C54–C57 were initially established with ROESY analysis, as clear cross-correlations were observed between the methyl group on the quaternary carbon center at C57, H54, and H55. The configuration of C56 was confirmed by the measurement of homo- and heteronuclear coupling constants with the proton and carbon atoms at C54 and C55. The relative configuration of C53 was established by the measurement of $^2J_{\text{C,H}}$ values as well as by the observation of an NOE effect between H52 and H55. The relations between the C9 and C11 stereocenters were also established by using analysis based on coupling constants. Nevertheless, it was not possible to unambiguously define the relative configurations of the remaining chiral centers in belizeanolide from spec-

Table 1: ^1H and ^{13}C NMR data for belizeanolide (**1**) in CD_3OD .

Position	δ_{C} [ppm]	δ_{H} [ppm], J [Hz]	Position	δ_{C} [ppm]	δ_{H} [ppm], J [Hz]
1	171.1 C		42	30.7 CH_2	1.71, <i>m</i> ; 1.63, <i>m</i>
2	41.3 CH_2	3.00, <i>s</i>	43	77.0 CH	3.94, <i>dd</i> (9.4, 7.1)
3	141.4 C		44	213.4 C	
4	44.3 CH_2	2.03, <i>m</i> ; 1.84, <i>m</i>	45	45.5 CH_2	2.40, <i>dd</i> (7.3, 14.1); 2.29, <i>dd</i> (6.8, 14.1)
5	28.2 CH	1.76, <i>m</i>	46	25.9 CH	2.04, <i>ddd</i> (6.8, 7.0, 7.3)
6	43.5 CH_2	2.07, <i>m</i> ; 1.67, <i>m</i>	47	44.8 CH_2	1.04, <i>m</i>
7	144.7 C		48	26.2 CH	1.65, <i>m</i>
8	41.2 CH_2	2.11, <i>m</i>	49	45.0 CH_2	1.36, <i>m</i> ; 1.09, <i>m</i>
9	70.1 CH	3.81, <i>dd</i> (8.2, 10.8)	50	68.9 CH	4.06, <i>dd</i> (7.1, 9.8)
10	42.9 CH	1.45, <i>ddd</i> (6.5, 6.9, 10.8)	51	138.5 CH	5.71, <i>dd</i> (7.1, 15.4)
11	70.9 CH	4.29, <i>dd</i> (6.5, 8.5)	52	123.7 CH	5.54, <i>dd</i> (5.9, 15.4)
12	129.0 CH	5.19, <i>d</i> (8.5)	53	77.2 CH	5.09, <i>dd</i> (5.9, 12.3)
13	135.4 C		54	77.6 CH	4.11, <i>ddd</i> (7.5, 9.1, 12.3)
14	43.4 CH_2	2.05, <i>dd</i> (7.8, 14.2); 2.03, <i>dd</i> (5.4, 14.2)	55	36.4 CH_2	1.88, <i>ddd</i> (8.1, 9.1, 14.1); 1.80, <i>ddd</i> (3.7, 7.5, 14.1)
15	71.6 CH	3.69, <i>ddd</i> (5.4, 7.8, 10.7)	56	73.3 CH	4.21, <i>dd</i> (3.7, 8.1)
16	34.6 CH	1.66, <i>m</i>	57	88.4 C	
17	39.8 CH_2	1.63, <i>m</i> ; 1.34, <i>m</i>	58	73.6 CH	3.40, <i>dd</i> (4.2, 10.2)
18	84.4 CH	4.84, <i>dddd</i> (3.4, 9.0, 9.3, 9.3)	59	36.0 CH_2	2.70, <i>dd</i> (4.2, 14.3); 2.08, <i>dd</i> (10.1, 14.3)
19	127.2 CH	5.76, <i>dd</i> (3.4, 6.0)	60	143.9 C	
20	131.1 CH	5.85, <i>dd</i> (3.4, 6.0)	61	144.2 C	
21	89.9 CH	4.67, <i>ddd</i> (3.4, 9.0, 11.4)	62	42.9 CH_2	2.44, <i>dd</i> (4.7, 14.1); 2.26, <i>dd</i> (8.3, 14.1)
22	35.3 CH	1.74, <i>m</i>	63	68.6 CH	3.84, <i>ddd</i> (4.7, 6.7, 8.3)
23	40.4 CH_2	1.49, <i>m</i> ; 1.20, <i>m</i>	64	45.1 CH_2	1.49, <i>m</i>
24	68.8 CH	3.59, <i>m</i>	65	66.2 CH	3.86, <i>dd</i> (9.4, 6.2)
25	36.9 CH_2	1.44, <i>m</i> ; 1.35, <i>m</i>	66	22.3 CH_3	1.07, <i>d</i> (6.2)
26	23.9 CH_2	2.20, <i>dddd</i> (5.4, 7.6, 10.6, 14.4); 2.05, <i>dddd</i> (7.0, 8.6, 11.0, 14.4)	67	114.6 CH_2	5.14, <i>s</i> ; 4.97, <i>s</i>
27	131.5 CH	5.46, <i>dd</i> (7.0, 7.6, 9.6)	68	113.9 CH_2	5.10, <i>s</i> ; 5.02, <i>s</i>
28	130.9 CH	5.27, <i>dd</i> (7.8, 9.6)	69	14.0 CH_3	1.07, <i>s</i>
29	68.7 CH	4.80, <i>d</i> (7.8)	70	18.5 CH_3	0.81, <i>d</i> (7.2)
30	149.1 C		71	18.8 CH_3	0.79, <i>d</i> (7.0)
31	40.0 CH_2	2.82, <i>d</i> (14.7); 2.71, <i>d</i> (14.7)	72	19.4 CH_3	0.86, <i>d</i> (7.3)
32	144.3 C		73	113.1 CH_2	4.92, <i>s</i> ; 4.80, <i>s</i>
33	35.1 CH_2	2.32, <i>dd</i> (6.4, 14.3); 2.27, <i>dd</i> (6.8, 14.3)	74	110.2 CH_2	5.11, <i>s</i> ; 4.82, <i>s</i>
34	79.8 CH	4.02, <i>ddd</i> (6.4, 6.8, 8.9)	75	15.5 CH_3	0.81, <i>d</i> (7.1)
35	72.7 CH	3.86, <i>dd</i> (4.7, 8.9)	76	13.1 CH_3	0.84, <i>d</i> (7.0)
36	73.0 CH	4.12, <i>dd</i> (4.7, 9.3)	77	16.0 CH_3	1.65, <i>s</i>
37	83.2 CH	3.53, <i>dd</i> (4.5, 9.3)	78	7.5 CH_3	0.90, <i>d</i> (6.9)
38	68.8 CH	3.59, <i>m</i>	79	112.7 CH_2	4.76, <i>s</i> ; 4.70, <i>s</i>
39	41.0 CH_2	1.37, <i>m</i> ; 1.33, <i>m</i>	80	18.8 CH_3	0.76, <i>d</i> (6.8)
40	29.2 CH	1.59, <i>m</i>	81	114.6 CH_2	4.88, <i>s</i> ; 4.82, <i>s</i>
41	31.3 CH_2	1.40, <i>m</i> ; 1.12, <i>m</i>			

troscopic analysis—owing to chemical shift degeneracy. These stereochemical relationships are currently under investigation.

Finally, the proposed structure of belizeanolide (**1**) was further supported by positive ion ESI tandem mass spectrometry studies. Positive mode experiments on a solution of **1** in 2 mM ammonium acetate buffer showed a $[M+\text{Na}]^+$ ion (m/z 1447.8) that was selected as a precursor ion. Characteristic product ions at m/z 1430.6, 1404.7, 1277.7, and 1123.7 were observed. Furthermore, MS/MS analysis of these ions yielded several fragments that would result from the proposed structure.

The *in vitro* antiproliferative activity was assessed in ovarian (A2780), lung (SW1573), breast (HBL100, T47D), and colon (WiDr) human solid tumor cells by using the NCI protocol with minor modifications.^[13] The GI_{50} (μM) values for **1** were 3.28 ± 0.45 (A2780), 3.23 ± 0.45 (SW1573), 3.23 ± 0.38

(HBL100), 3.16 ± 0.40 (T47D), and 4.58 ± 0.40 (WiDr). However, the open compound belizeanolic acid is ten times more potent than belizeanolide. The GI_{50} (μM) values for **2** were 0.26 ± 0.09 (A2780), 0.31 ± 0.06 (SW1573), 0.32 ± 0.04 (HBL100), 0.40 ± 0.09 (T47D), and 0.41 ± 0.04 (WiDr). The results of the biological activity studies showed no selectivity between cell lines. This is an interesting result, as standard and investigational anticancer drugs indicate that colon cancer cells are more drug resistant than ovarian cancer cells.^[14]

Belizeanolide was isolated from a benthic marine dinoflagellate belonging to the genus *Prorocentrum* that has shown to be one of the genera with a broader diversity in the production of bioactive metabolites in structural terms.^[15] Belizeanolide represents the first member of an unprecedented class of polyunsaturated and polyhydroxylated macro- lides. Although it shares some structural features with other

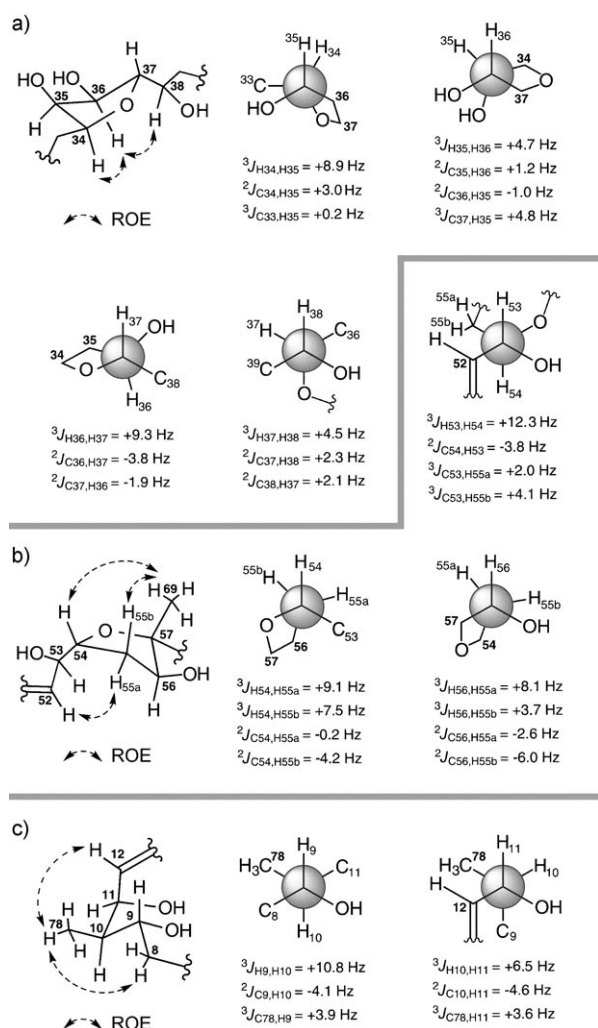


Figure 2. Relative configurations determined for a) the C34–C38 fragment, b) the C53–C57 fragment, and c) the C9–C11 fragment.

polyhydroxy polyenes produced by marine dinoflagellates, it has a backbone with 66 carbon atoms that includes a unique 54-membered lactone containing two furan-type rings, therefore making it the third largest known macrolide.^[16] The acyclic portion of the molecule incorporates an additional tetrahydrofuran ring and a novel diene unit, which is unprecedented in the field of natural products. Also, the dihydrofuran moiety found in this molecule has only been reported once for a secondary metabolite.^[17] However,

despite all of the uncommon structural features present in this molecule, its structure is consistent with polyketide biosynthesis. The biological activity and its novel structural features set belizeanolide apart from other known dinoflagellate metabolites.^[18]

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