Axinellins A and B: New Proline-Containing Antiproliferative Cyclopeptides from the Vanuatu Sponge *Axinella carteri*☆

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Two new bioactive cyclopeptides, named axinellins A (**1**) and B (**2**) have been isolated from the marine sponge *Axinella carteri*. Their structure elucidation was based on two-dimensional (2D) NMR (500 MHz) as well as HRFABMS and ESMS/MS data. All amino acid residues derived from axinellins A and B were found to possess L configuration at Cα by HPLC analysis of their FDAA derivatives (Marfey's method). The amino acid sequence of **1** and **2** was established on the basis of tandem mass spectrometry data (ESMS/MS) and on ^{1}H-^{1}H through-space connectivities observed in NOESY and ROESY spectra. Axinellins A (**1**) and B (**2**) exhibited moderate in vitro antitumor activity against human broncopulmonary non-small-cell-lung-carcinoma lines (NSCLC-N6) with IC_{50} values of 3.0 and 7.3 μg/ml, respectively.

Introduction

Naturally occurring cyclopeptides are becoming increasingly important due the their broad spectrum of pharmacological activities[1] and interesting chemical structures[2], often containing novel or unusual amino acid residues[3]. In particular, many examples of antiproliferative natural cyclopeptides have lately been reported, especially from marine sources, suggesting that, in principle, new anticancer agents based on peptidic (or peptidomimetic) structures may be developed. A new emerging class of marine cyclopeptides is represented by proline-rich compounds, usually containing seven or eight amino acid residues, and often occurring as complex mixtures of structurally related derivatives. Examples of this kind of bioactive natural products are axinastatins[4], phakellistatins[5], hymenamides[6], stylostatin 1[7], and others[8]. Interestingly, nearly all these cyclopeptides have been isolated from South Pacific marine organisms.

In the frame of a Marine Science and Technology (MAST-III) project financially supported by European Community and aimed at the discovery of new metabolites with antiviral, antitumoral, and immunomodulant activities, we have recently had the occasion to study the extracts of the marine sponge *Axinella carteri* (Order Halicondrida, Family Axinellidae) collected in the waters off Vanuatu Islands. In this paper we wish to report on the isolation, structure characterization, and bioactivity of two new cyclopeptides, named axinellins A (**1**) and B (**2**) from the above marine sponge.

Isolation

The sponge (0.3 kg of lyophilized powder) was sequentially extracted three times with MeOH at room temperature and the methanol soluble material (70 g) was partitioned according to a modified Kupchan procedure[9] (see Experimental Section) affording four extracts at increasing polarity: *n*-hexane (0.8 g; IC_{50} = 23.4 μg/ml against NSCLC-N6 cancer cell lines), CCl_4 (1.0 g; IC_{50} < 3.3 μg/ml), $CHCl_3$ (2.5g; IC_{50} < 3.3 μg/ml), *n*BuOH (10 g; IC_{50} = 11.8 μg/ml). Preliminary chromatographic and ^{1}H-NMR analyses reaveled that the most active $CHCl_3$ fraction contained substances of peptidic nature, whereas the nearly

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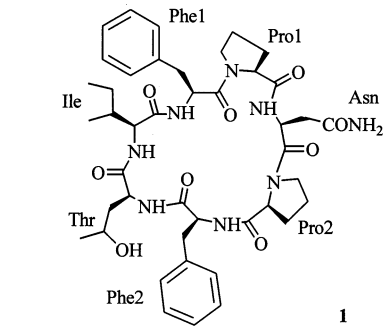

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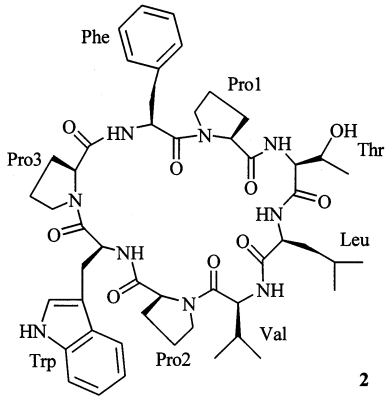

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equally active CCl_4 extract was mainly comprised by complex mixtures of lipids. Therefore, the $CHCl_3$ material was selected for the isolation work. This extract was first subjected to a medium-pressure silica-gel flash chromatography (MPLC) eluting with $CHCl_3$/MeOH mixtures with increasing amounts of MeOH. Fractions eluted with 3−10% of MeOH in $CHCl_3$ were further purified by reverse-phase HPLC on a C-18 Vydac column eluting with MeCN/H_2O (these solvents contained 0.01% and 0.1% of TFA, respectively) to yield pure axinellin A (**1**, 7.9 mg) and axinellin B (**2**, 1.8 mg) along with five known cyclopeptides: stylostatin 1 (27.4 mg)[7], hymenamide C (31.9 mg)[6a], phakellistatin 1 (7.1 mg)[5a], phakellistatin 10 (2.0 mg)[5c] and axinastatin 3 (5.1 mg)[4].

Structure Elucidation of Axinellin A

Axinellin A (**1**) showed a pseudomolecular ion peak at *m/z* 817.4229 (M + H^+, calculated 817.4248) in the HRFABMS (positive ions) spectrum consistent with a molecular formula $C_{42}H_{56}N_8O_9$, requiring 19 degrees of formal unsaturation. The peptidic nature of this product was suggested by the molecular formula itself, from characteristic IR bands at 3360 and 1645 cm^{-1} (*NH*CO and NH*CO* stretching) and from in-depth analysis of its ^1H- and ^{13}C-

Figure 1. Daughter ion spectra of the singly charged ions at *m/z* 817.2 and at *m/z* 938.2 corresponding to the molecular ions of axinellin A (**A**) and axinellin B (**B**)

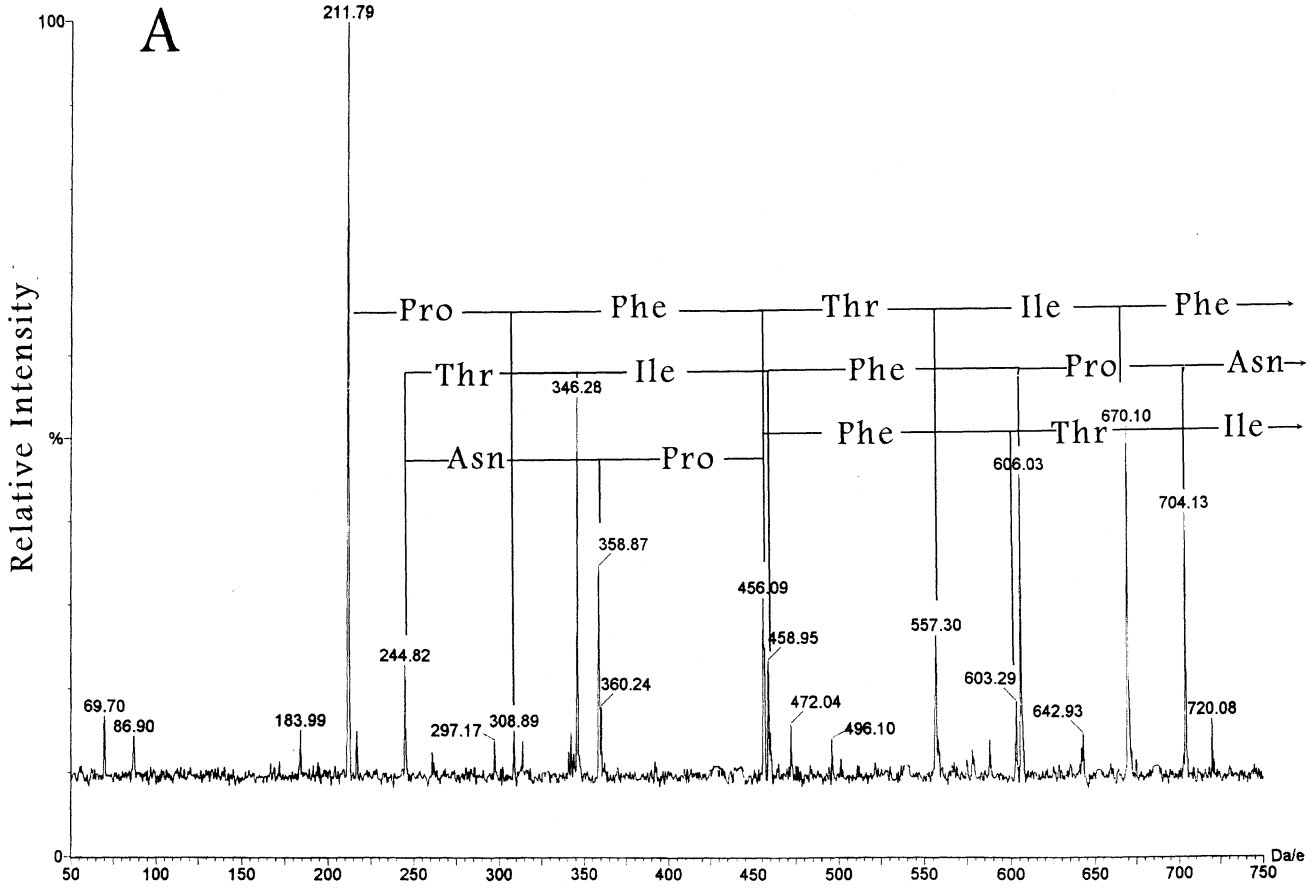
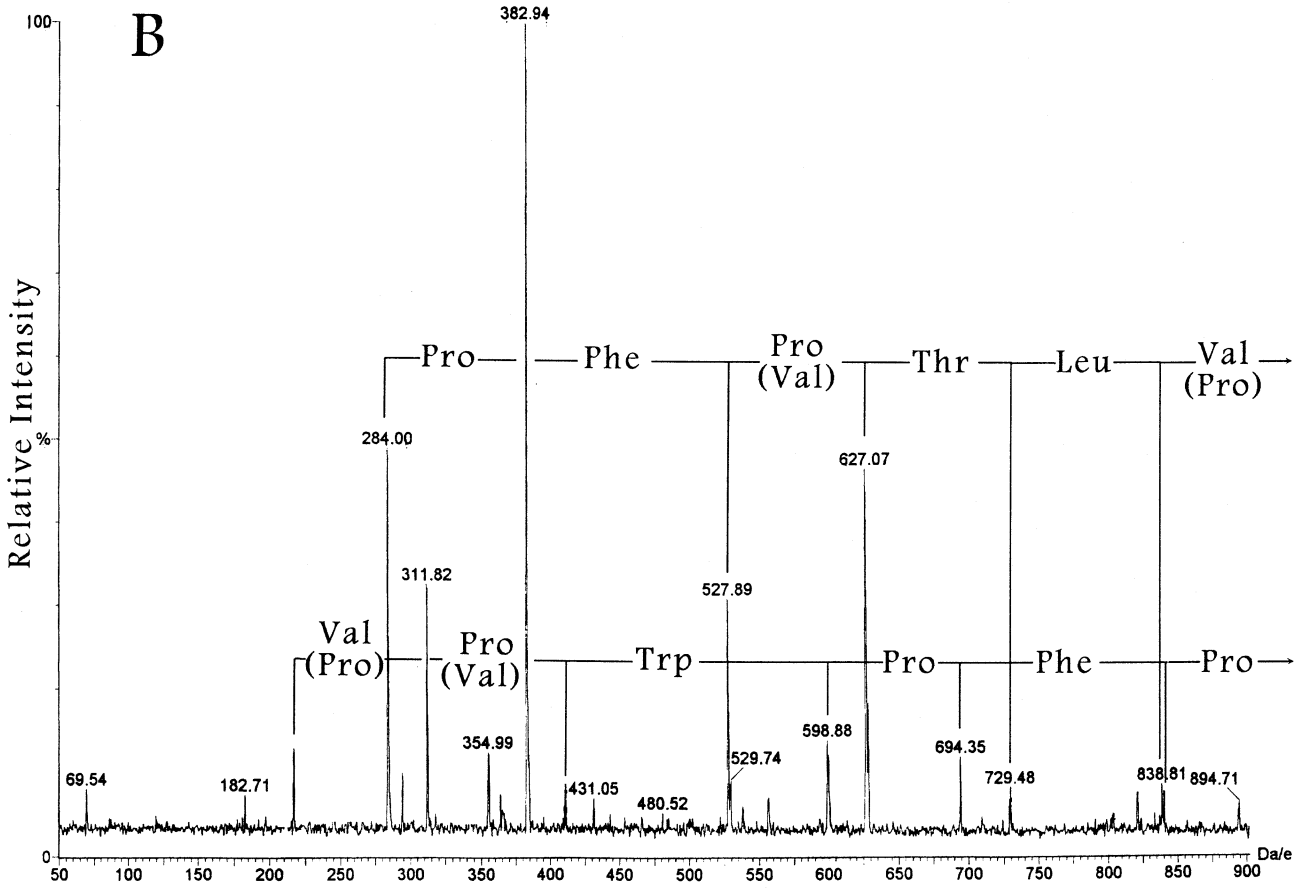


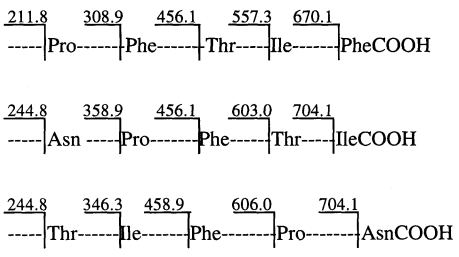
Figure 1 *(Continued)*



NMR spectra. Full ^{1}H- and ^{13}C-NMR assignments for **1** were obtained through interpretation of DQF-COSY, TOCSY, NOESY, ROESY, HMQC, and HMBC data (see Table 1), allowing identification of seven conventional amino acid residues: 2 × Pro, 2 × Phe, Thr, Asn, and Ile. This amino acid composition accounted for 18 out of the 19 degrees of unsaturation, requiring that **1** is a cyclic heptapeptide. Indeed, the cyclic nature of **1** was also evident by the high degree of chemical shift dispersion observed for peptide-bond amide proton signals resonating between δ = 7.8 and 9.8. The high magnetic non-equivalence of such resonances is in fact typically observed in folded proteins or in small cyclopeptides that show a strong conformational preference in solution and it is consistent with the rather small size of the cyclopeptide and high content of Pro residues of axinellin A. The amino acid composition was confirmed by HPLC analysis of the acid hydrolysate of **1** after derivatization with the Marfey reagent[10] (1-fluoro-2,4-dinitrophenyl-5-L-alaninamide, FDAA), allowing the absolute stereochemistry at Cα to be assigned as L configuration for all residues (see Experimental Section).

The amino acid sequence of axinellin A (**1**) was deduced with a combined approach of 2D-NMR and electrospray tandem mass spectrometry techniques (ESMS/MS). In fact, the relatively high content of Pro residues in **1**, lacking the important peptide-bond NH proton NMR signals, makes the determination of the primary structure of the cyclopeptide difficult to obtain with solely the well-established NOESY (or ROESY) approach that relies on through-space dipolar connectivities between the NH of a given residue and the Hα of the residue preceding it in the sequence. In our case, two diagnostic Hα_i-NH$_{i+1}$ NOESY cross-peaks could be observed, namely Hα-Thr (δ_H = 5.22)/NH-Ile (δ_H = 9.16) and Hα-Ile (δ_H = 4.98)/NH-Phe1 (δ_H = 9.84), therefore providing the sequence for the fragment Thr−Ile−Phe1. The complete sequence of axinellin A was deduced on the basis of the results of ESMS/MS experiments. Figure 1A shows the daughter ion spectrum of the singly charged ion at *m/z* 817.2 corresponding to the molecular ion of **1**. The fragmentation process generated a complex pattern of

Scheme 1



fragment ions mainly belonging to the b series[11], although some ions of the a series were also observed. Three related patterns of b fragments could be distinguished in this spectrum leading to the reconstruction of the sequences in Scheme 1.

On the basis of these data, the entire structure of axinellin A could be deduced as cyclo(Pro2Phe2ThrIlePhe1-Pro1Asn). The cyclic nature of this peptide, already proposed for its NMR properties and HRFABMS (see above), was also confirmed by ESMS/MS data. In fact, the expected molecular mass for the linear oligopeptide chain that can be built on the basis of the above sequential fragmentations would be 834.2 a.m.u., 18 mass units higher than that determined by ESMS (816.2 a.m.u). The complexity of this tandem mass spectrum can be explained taking into account that the first fragmentation event caused the opening of the molecule at different peptide bonds, leading to the formation of a mixture of isomeric linear structures. Each isomeric peptide so generated fragmented independently giving rise to its own fragment ions series. It is interesting to note that under these conditions the cleavage of peptide bonds occurs preferentially at Pro residues.

The structure of axinellin A was further confirmed by partial acid hydrolysis (see Experimental Section) followed by HPLC fractionation of the resulting peptide mixture. Individual fractions were manually collected and identified by ESMS analysis. The most prominent peaks were also submitted to ESMS/MS experiments. The major component showed a molecular mass of 835.5 a.m.u. ($M + H^+$) corresponding to the linear form of axinellin A. The daughter ion spectrum of this component yielded a single C-terminal sequence as −ThrIlePhe1Pro1AsnCOOH, thus confirming previous results.

Once that the amino acid sequence of axinellin A (**1**) was determined, the last structural feature that remained to be elucidated was the geometry of the peptidic linkages at Pro residues. Generally speaking, isomeric *cis*- and *trans*-Pro forms can be distinguished in solution by careful analysis of ^{13}C-NMR data[12] and/or of the pattern of dipolar couplings in NOESY and ROESY spectra[13]. Indeed, Pook et al.[12] demonstrated, with a series of models containing *cis*- and *trans*-Pro residues, that the chemical shift difference between β- and γ-carbon atoms can be diagnostic for such stereochemical assignment. This difference, directly related to the dihedral angle ψ(Pro), is usually in the range $\Delta\delta$ = 2−10 for *cis*-Pro and $\Delta\delta$ = 0−5 in *trans*-Pro. These ranges suggest that safe assignments can be achieved only in the case of $\Delta\delta_{C\beta\text{-}C\gamma}$ of ca. 8−10 for *cis*-Pro and about $\Delta\delta$ = 0−1 for *trans*-Pro. The ^{1}H-NMR method relies on the observation that *cis*-Pro residues are likely to give Hα-X/Hα-Pro NOE effects with the preceding residue in the sequence (X), whereas an Hα-X/H_2δ-Pro effect is often observed for *trans*-Pro geometries. In our case, NMR data of axinellin A (**1**) pointed to a *cis* geometry for the Phe1−Pro1 peptide bond ($\Delta\delta_{C\beta\text{-}C\gamma}$ = 8.9 and ROESY peak Hα-Phe1/Hα-Pro1) and a *trans* stereochemistry for the Asn−Pro2 linkage ($\Delta\delta_{C\beta\text{-}C\gamma}$ = 5.3 and ROESY peak Hα-Asn/H_2δ-Pro2).

Structure Elucidation of Axinellin B

Axinellin B (**2**) showed a pseudomolecular ion peak at *m/z* 938.5098 ($M + H^+$, calculated 938.5144) in the HRFABMS (positive ions) spectrum consistent with a molecular formula $C_{50}H_{67}N_9O_9$, requiring 22 degrees of formal unsaturations. The cyclopeptidic nature of axinellin B was apparent from IR bands at 3320 and 1650 cm^{-1} (*NH*CO and NH*CO* stretching) and ^{1}H- and ^{13}C-NMR spectra. As in **1**, the amino acid residues were identified by an extensive NMR analysis: DQF-COSY, TOCSY, HMQC, HMBC, and ROESY allowed us to reveal the presence of eight residues: 3 × Pro, Phe, Trp, Val, Thr, and Leu. These residues accounted for 21 degrees of unsaturation out of the 22, requiring that **2** is a cyclopeptide, too. Absolute configuration at Cα for these amino acids (except Trp) were based on the Marfey′s method and we found also in this case that all residues belong to the L-steric series.

Analogously to the preceding case, the molecular ion of the axinellin B at *m/z* 938.2 was fragmented by ESMS/MS giving rise to the daughter ion spectrum shown in Figure 1B. A complex spectrum was recorded due to the contemporaneous fragmentation of isomeric linear peptides following the opening of the cyclic structure as already reported for the axinellin A sample. The interpretation of the MS/MS spectra eventually led to the definition of the entire amino acid sequence: as cyclo[Pro(Val)TrpProPhePro-ThrLeuVal(Pro)]. Again, the cyclic structure was confirmed by measurement of the peptide molecular mass that was 18 a.m.u. lower than that expected from the linear sequence. The correct positioning in the sequence of the adjacent Pro and Val residues was established by the observation of a ROESY correlation peak between the Hα-Val (δ_H = 5.21) and the δ-protons of the subsequent Pro2 (δ_H = 4.26 and 3.82), finally establishing for **2** the structure cyclo[Pro2Trp-Pro3PhePro1ThrLeuVal]. The latter dipolar coupling was also indicative of a *trans* geometry around the Val−Pro2 linkage, in good agreement with the $\Delta\delta_{C\beta\text{-}C\gamma}$ = 0.6. The geometry of the Trp−Pro3 peptide bond could be determined with confidence as *trans* through an intense ROESY effect between Hα-Trp (δ_H = 5.03) and H_2δ-Pro3 (δ_H = 3.66 and 3.90), while the spatial arrangement of the remaining proline-containing segment Phe−Pro1 could not be characterized, due to the absence of both informative NOEs and a diagnostic $\Delta\delta_{C\beta\text{-}C\gamma}$ value for this proline.

Molecular Mechanics and Dynamics Calculations on Axinellins A (1) and B (2)

Molecular mechanics and dynamics calculations were carried out in the CHARMm force field in order to search for the minimum energy conformations of axinellins A and B. The most stable conformations of both cyclopeptides were then visually inspected in order to assess the consistency between the spectroscopic properties, particularly long range NOEs, expected on the basis of these three-dimensional models and the experimental NMR data. A satisfactory agreement between theoretical and experimental data

was observed, giving additional support to our structural and stereochemical assignments.

Conclusion

Two new bioactive prolin-containing cyclopeptides named axinellins A (**1**) and B (**2**) have been isolated from the marine sponge *Axinella carteri*. They exhibited moderate in vitro antitumor activity against human bronchopulmonary non-small-cell-lung-carcinoma lines (NSCLC-N6) with IC_{50} values of 3.0 and 7.3 μg/ml, respectively. These molecules are structurally related to several other proline-rich antitumor cyclopeptides recently reported from marine sponges, such as axinastatins[4], phakellistatins[5], hymenamides[6], and stylostatin 1[7]. Interestingly, despite these derivatives have been isolated from different species, they can be regarded, in a sense, like a family. In fact, they display a remarkable analogy in their amino acid content and, to some extent, also in their sequences. These molecules are generally cyclohepta- or cyclooctapeptides, characterized by the presence of two or three prolines, an array of apolar residues, such as Leu, Ile, Val, and one or two aromatic residues like Trp, Phe, or Tyr. Only Thr and Asn/Asp or Gln/Glu residues are encountered with high frequency in amino acids. In addition, almost all members of this class possess a Pro−Phe(Tyr) segment in their structures. These similarities suggest that there might be a model

Table 1. ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) chemical shifts, NOESY, ROESY, HMBC correlations of **1**

	Axinellin A ([D$_5$]pyridine)				
aa	δ_H[a]	δ_C	**NOESY**[b]	**ROESY**[b]	**HMBC**[c]
Phe 1					
α	4.62 dd (4.0, 10.0)	55.2	NH	Hα (**Pro 1**)	
β	3.20 dd (4.4, 12.0), 3.31 t (12.0)	38.5	H5/H9, H6/H8, NH		Cα, C4, C5/C9
C1		136.3			
C2/C6	7.12	129.8			
C3/C5	7.11	128.9			
C4	7.13	127.7			
CO		170.4			
NH	9.84 br. s		Hα, Hβ, Hα (**Ile**)		
Pro 1					
α	3.46 d (8.4)	61.1		Hα (**Phe 1**)	CO
β	2.25, 1.25	31.4			
γ	1.41, 1.48.	22.5			
δ	3.55, 3.55	47.1			
CO		171.7			
Asn					
α	5.35 ddd (3.2,8.4,13.6)	49.1	NH	Hδ (**Pro 2**)	
β	3.04 dd (3.2, 13.6) , 4.16 t (13.6)	39.0	NH		
$CONH_2$	8.54 br. s, 8.53 br. s				
CO					
NH	9.81 br. d		Hα, Hβ		
Pro 2					
α	4.49 dd (4.0, 9.6)	62.0			Cγ
β	1.45, 1.63	29.8			
γ	1.47, 1.47	24.5			
δ	3.95 dd(5.4,15.2), 4.28 dd(6.4,15.2)	48.0		Hα (**Asn**)	
CO		172.1			
Phe 2					
α	5.19 ddd(3.2,8.8,14.4)	56.7	NH		CO
β	3.66 dd (3.2, 14.4) 3.41t (14.4)		NH		Cα, C4, C5/C9
C1		139.4			
C2/C6	7.46 d (7.6)	129.8			Cβ, C7
C3/C5	7.20 t (7.6)	128.6			C6, C8
C4	7.17	126.7			
CO		173.2			
NH	8.60 d (8.8)		Hα, Hβ (3.56)		
Thr					
α	5.22 d (9.6)	58.4	NH, NH (**Ile**)		CO
β	4.56 br. q (6.42)	67.7			
γ	1.46 d (6.42)	21.6			Cα, Cβ
CO		169.8			
NH	7.94 d (9.6)		Hα		
Ile					
α	4.98 t (8.4)	56.9	NH		
β	2.12	39.2			
βMe	1.29 d (6.82)	15.4			Cα, Cβ, Cγ
γ	1.83, 1.41	25.7			
δ	0.81 t (7.63)	11.4			Cβ, Cγ
CO		172.2			
NH	9.16 d (8.4)		Hα, Hα (**Thr**)		

[a] Coupling costants are in parentheses and given in Hz. ^{1}H assignments aided by COSY and TOCSY (τ_m = 100 ms) experiments. − [b] τ_m = 400 ms (T = 300 K). − [c] HMBC optimized for $^{2,3}J_{CH}$ = 10.0 Hz.

for rationalizing the relationship between their structures and their biological activities, even if the biomolecular target of these compounds is currently not known. Studies, aiming to the definition of a possible common pharmacophore for these molecules are presently under way in our laboratories.

This contribution is part of the Marine Science and Technology (MAST-III) European project named *Bioactive Marine Natural Products in the Field of Antitumoral, Antiviral and Immunomodulant Activity*. Therefore we thank the *European Community* for financial support (contract MAS3-CT95-0032). NMR and FAB-MS spectra were recorded at C.R.I.A.S. *Centro Interdipartimentale di Analisi Strumentale*, Faculty of Pharmacy, University of Naples "Federico II". Tandem ESMS/MS spectra were recorded at *Centro Internazionale di Servizi di Spettrometria di Massa* of Naples.

Experimental Section

General Experimental Procedures: NMR spectra: Bruker AMX-500 (^{1}H at 500 MHz, ^{13}C at 125 MHz), δ (ppm), *J* in Hz, spectra referred to [D_5]pyridine as internal standard. Standard pulse sequences were employed for DQF-COSY. Phase-sensitive ROESY spectra were measured with a mixing time of 400 ms, while HMQC and HMBC were optimised for $^1J_{C\text{-}H}$ = 135 Hz and $^{2,3}J_{C\text{-}H}$ = 10 Hz, respectively. HRFABMS [in glycerol; Cs^+ ions bombardment] were obtained with VG AUTOSPEC mass spectrometer; optical rotations were measured with a Perkin-Elmer 141 polarimeter; reverse-phase HPLC, C_{18} Vydac column (300 × 7.8 mm i.d.; flow rate 5 ml min^{-1}) Waters Model 6000 A or 512 pump equipped with U6K injector and an UV detector.

Isolation: The organism (lyophilized material, 300 g) was extracted with MeOH (3 × 2.5 l) at room temperature. The methanolic extracts were filtered and concentrated under reduced pressure and successively extracted using a modified Kupchan partition as follows: the methanolic extract was dissolved in 1 l of a mixture of MeOH/H_2O containing 10% of H_2O and partitioned against 1 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 concetrated to remove MeOH and then extracted with *n*BuOH. The crude chloroform (2.53 g) extract was chromatographated by MPLC on a silica gel column (2 × 150 g) using a solvent gradient from $CHCl_3$/MeOH (97:3) to $CHCl_3$/MeOH (90:10). MPLC fractions were further purified by HPLC on a semipreparative (7.8 × 300 mm) Vydac-C18 column (flow rate 4 ml/min) eluting with MeCN/H_2O mixtures to afford pure compounds **1** and **2**. The purity of each compound was judged to be > 90% by HPLC and ^{1}H NMR.

Axinellin A (**1**): White amorphous solid, $[\alpha]_D$ = −98.2 (*c* = 0.003 MeOH). − IR (KBr) $\tilde{\nu}_{max}$ = 3390, 2940, 2900, 1640, 1520, 1430 cm^{-1}. − HRFABMS; *m/z*: 817.4229 (M^+; calculated for $C_{42}H_{57}O_9N_8$, 817.4248, Δmmu = 1.9); t_R = 21.6 min: linear gradient elution, H_2O (0.1% TFA)/MeCN (0.01% TFA) (75:25 to 50:50 in 40 min). − ^{1}H and ^{13}C NMR see text and Table 1.

Table 2. ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) chemical shifts of the axinellina B (**2**)

Axinellin B ([D_5]pyridine)							
aa	δ_H[a]	δ_C	ROESY[b]	aa	δ_H[a]	δ_C	ROESY[b]
Phe				**Val**			
α	4.64 m	57.9		α	5.21	57.3	Hδ (**Pro 2**)
β	4.39 t (4.29), 4.10	25.3		β	2.62 m	29.8	
C1		138.1		βMe-1	1.30 d (6.3)	18.8	
C2/C6	7.48 d (7.4)	129.6		βMe-2	1.24	20.1	
C3/C5	7.41 t (7.4)	128.8		CO			
C4	7.30 t (7.4)	127.2		NH	7.80		
CO				**Pro 2**			
NH	8.94 d (5.9)			α	5.08	61.3	
Pro 1				β	2.16, 1.98	25.8	
α	4.56 t (7.0)	60.8		γ	1.87, 1.97	25.2	
β	1.91, 1.81	29.2		δ	4.26, 3.82	49.3	Hα (**Val**)
γ	1.52, 1.40	24.6		CO			
δ	3.47, 3.47	47.8		**Trp**			
CO				α	5.03	53.6	Hδ (**Pro 3**)
Thr				β	3.17, 3.23	36.0	
α	5.40	56.9		NH-1	11.78		
β	4.50	69.2		C2	7.48 s	121.8	
γ	1.65	19.9		C3		112.1	
CO				C4		128.7	
NH	8.18			C5	7.86 d (7.8)	119.2	
Leu				C6	7.15 t (7.8)	119.2	
α	4.13	54.6		C7	7.24 t (7.8)	122.3	
β	2.85, 2.34	37.3		C8	7.54 d (7.8)	112.7	
γ	1.87	29.0		C9		137.6	
γMe-1	0.96	21.2		CO			
γMe-2	0.99	23.7		NH	8.10		
CO				**Pro 3**			
NH	10.70			α	4.26	61.7	
				β	1.74, 1.53	25.0	
				γ	1.98, 2.16	30.4	
				δ	3.66, 3.90	48.0	Hα (**Trp**)
				CO			

[a] Coupling costants are in parentheses and given in Hz. ^{1}H assignments aided by COSY and TOCSY (τ_m = 100 ms) experiments. − [b] τ_m = 400 ms (*T* = 300 K).

Axinellin B (**2**): White amorphous solid, $[\alpha]_D = +50.0$ (c = 0.001, MeOH). – IR (KBr) $\tilde{\nu}_{max}$ = 3320, 2950, 2860, 1645, 1540, 1440 cm^{-1}. – HRFABMS; *m/z*: 938.5098 (MH^+; calculated for $C_{50}H_{68}O_9N_9$, 938.5144, Δmmu = 4.6); t_R = 34.7 min: linear gradient elution, H_2O (0.1% TFA)/MeCN (0.01% TFA) (75:25 to 50:50 in 40 min). – ^1H and ^{13}C NMR see text and Table 2.

Molecular Modeling Calculations: All calculations were made with an SGI Personal IRIS 35G computer using the force field CHARMm (QUANTA 4.0 software package). The lowest energy conformations were searched by performing a high temperature molecular dynamics simulation (HTMDS) followed by energy minimization[14]. By means of a molecular dynamics simulation of 100 ps at 1000 K using the Verlet algorithm, 250 conformations were obtained. All the conformations were then subjected to an energy minimization (700 steps, conjugated gradient algorithm). Inspection of minimized structures provided the lowest energy conformations.

Hydrolysis and Derivatization of **1** *and* **2** *(Marfey's Procedure)*[10]: Two 100-μg samples of **1** and **2** were dissolved in HCl (6 N, 0.5 ml) under nitrogen in two sealed tubes at 130°C for 16 h. After concentration, the two residual hydolysates were suspended in 100 μl of water and treated with 250 μl of a solution of FDAA (1%) in acetone and 300 μl of a solution 1 M of $NaHCO_3$, and then heated at 50°C for 1 h. HPLC analysis (Vydac C18, analytical coloumn; linear gradient elution, H_2O (0.1% TFA)/MeCN (0.01% TFA) (9:1 to 1:1 in 45 min); UV detection at 340 nm) of FDAA-derivatized hydrolysates in conjunction with similarly derivatized amino acid standards established the stereochemistry of the constituent amino acids (except tryptophan). All amino acids were found to be L.

Partial Acid Hydrolysis: Axinellin A (**1**) was incubated with 100 μl of 1.2 M HCl at 110°C for 1 h. The hydrolysis was stopped by diluting with 3 volumes of cold water and the peptide mixture was fractionated by a reverse-phase HPLC on a Vydac C18 column (218TP54, 250 mm × 4.6 mm, 5 μm, 300 Å pore size). The elution system consisted of aqueous 0.1% TFA (eluent A) and 95% aqueous MeCN containing 0.07% TFA (eluent B); peptides were eluted by means of a linear gradient of eluent B (from 5% to 60% in 30 min), at a flow rate of 1 ml/min. The elution profile was monitored by UV at 220 nm and fractions were manually collected and lyophilized.

Electrospray (ESMS) Mass Spectrometric Analyses: Electrospray mass spectrometric (ESMS) and electrospray tandem mass spectrometric (ESMS/MS) analyses were performed by using a BioQ triple quadrupole mass spectrometer (Micromass, Manchester, UK). Aliquots of the peptide solution were injected in the ion source at a flow rate of 5 μl/min and scanning was performed from *m/z* 300 to *m/z* 1000 at 10 s/scan, using a cone voltage of 40 V. Fragmentation experiments were carried out using argon as collision gas and a collision energy of 10–30 V. Data were acquired and elaborated using the MassLynx program. Mass calibration was performed by means of the multiply charged ions from a separate injection of horse heart myoglobin (Sigma; average molecular mass 16951.5 a.m.u.); all ES masses are reported as average mass.

Cytotoxic Assays: Experiments were performed in 96 wells microtiter plates ($2 \cdot 10^5$ NSCLC-N6 cells/ml). Cell growth was estimated by a colorimetric assay based on the conversion of tertrazolium dye (MTT) to a blue formazan product using live mitochondria[15]. Eight determinations were performed for each concentration. Control growth was estimated for 16 determinations. Optical density at 570 nm corresponding to solubilized formazan was read for each well on Titertek Multiskan MKII. The IC_{50} values found by this procedure for axinellin A (**1**) and B (**2**) were 3.0 and 7.3 μg/ml, respectively.

☆ Dedicated to the memory of Professor *Luigi Minale*.

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