Pergamon

Tetrahedron, Vol. 52, No. 8, pp. 2727-2734, 1996

0040-4020/96 $15.00 + 0.00

S0040-4020(96)00009-9

Storniamides A-D: Alkaloids from a Patagonian Sponge *Cliona sp.*

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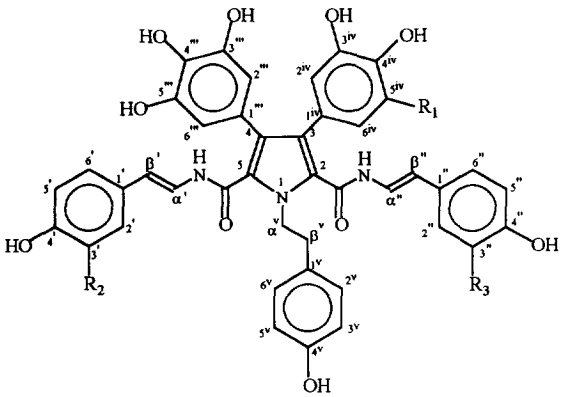
Abstract: Four novel peptide alkaloids, storniamides A-D (1 - 4) were isolated from a sponge *Cliona sp.* collected near San Antonio Oeste, Río Negro, Argentina, and their structures established by spectroscopic methods.

Marine sponges of the genus *Cliona* (family *Clionidae*) are usually burrowing organisms that live on a wide variety of calcareous substrates, like rocks, shells, coral or coralline algae where they bore into the calcareous material and then overgrow it to form a massive colony. This ability to bore into shells makes them a threat to oyster fisheries, as they make the shells fragile and likely to shatter on opening. They also play a significant role in the bioerosion of coral reefs.

Previous studies on the secondary metabolites of *Cliona celata* have shown that this sponge is able to produce linear peptide alkaloids, namely the clionamides[1,2] and celenamides.[3,4] As part of our research project on the study of marine invertebrates from the South Atlantic and Antarctica, we made a collection of a soft, burrowing yellow sponge *Cliona sp.* The crude ethanolic extract of this sponge showed antibiotic activity against Gram positive bacteria. Bioassay-guided fractionation of this extract allowed us to purify and identify the bioactive compounds, among them a new family of peptide alkaloids, the storniamides. In this paper we describe the stuctures of the major compounds, storniamides A-D (**1 - 4**).[5]

Cliona sp. was collected during low tide (-0.5 m) at Punta Verde, near San Antonio Oeste, province of Río Negro, Argentina. This location shows a tidal amplitude of several meters, thus exposing benthic invertebrates to severe environmental stress and strong currents, and at low tide *Cliona sp.* is nearly exposed to the air and direct sunlight. The crude ethanolic extract of *Cliona sp.* (see **Experimental**) was partitioned between hexane and MeOH/H_2O (9:1), and the activity was concentrated in the methanolic layer, which was fractionated by reversed phase flash chromatography. The active compounds were located in the fraction eluted with MeOH/H_2O (6:4). This fraction, which consisted of a complex mixture of aromatic compounds, was subjected to HPLC on reversed phase using acetonitrile/H_2O (3:7) as eluant, allowing the purification of compounds **1-4**.



	R_1	R_2	R_3
Storniamide A (**1**)	OH	H	H
Storniamide B (**2**)	OH	H	OH
Storniamide C (**3**)	H	OH	OH
Storniamide D (**4**)	OH	OH	OH

The molecular formulae of these compounds were similar, as indicated by HRFABMS, the only difference being the number of oxygen atoms. In all cases the storniamides showed 42 carbon, 35 hydrogen and three nitrogen atoms. This, together with their similar features in the ^{1}H NMR spectra, suggested that these compounds had the same carbon skeleton with 27 degrees of unsaturation. The chemical shifts of most of the aromatic protons suggested the presence of a varying number of phenolic hydroxy groups and different substitution patterns in the aromatic rings. The structures of storniamides A-D were established by a variety of 1D and 2D NMR spectroscopic techniques (COSY, NOESY, homonuclear 2DJ, HETCOR and COLOC).

Some common structural blocks could be readily identified from the NMR spectra of compounds **1-4**. A tyramine unit was present in the four compounds, as shown by a pair of doublets (J = 8.4 Hz, 2H each) at δ 7.05 and 6.75 and a pair of broad triplets at δ 4.80 and 3.05 (J = 7 Hz, 2H each). This also suggested that the nitrogen atom of this moiety had to be trisubstituted. Another common feature was the presence of two enamide functionalities. The ^{13}C NMR spectrum of **1-4** showed the corresponding carbonyls at δ 160.1, while the ^{1}H NMR spectrum (acetone-d_6/MeOD) indicated the presence of two *trans* disubstituted double bonds as shown by signals at δ 7.30 and 5.70 (d, J = 14 Hz, 2H each). The spectra in DMSO-d_6 showed the signal at δ 7.30 as broads multiplets coupled to the amide protons at δ 8.5, while on addition of D_2O this signal turned into a doublet (J= 14 Hz).

Storniamide A (**1**) was the key for the structure elucidation of this family of compounds since the substitution pattern of the aromatic rings was extremely simple. Although a molecular formula of $C_{42}H_{35}N_3O_{11}$ was established by HRFABMS, the ^{13}C NMR spectrum showed only 19 signals, indicating that the molecule was symmetrical. This fact was supported by the equivalence of both enamide functionalities in the ^{1}H NMR spectrum. Besides the tyramine moiety common to all the storniamides, the ^{1}H NMR spectrum of **1** showed signals corresponding to four additional phenolic rings. Doublets at δ 7.12 (J= 8.1 Hz, 4H) and δ 6.75 (J = 8.1 Hz, 4H), partially superposed to one of the signals of the tyramine moiety, indicated the presence of two equivalent *p*-hydroxyphenyl groups. A singlet at δ 6.25 (4H) showed in the COLOC spectrum correlations to quaternary carbons at δ 146.8 and δ 133.6 and to a protonated carbon at δ 110.6. This ^{13}C signal also showed a direct correlation to the same proton by HETCOR. Taking into account the symmetry of the molecule, these data suggested the structure of a 3,4,5 trihydroxyphenyl group. Integration of the ^{1}H NMR spectrum showed that two of these rings were present. COSY and LCOSY spectra showed that the *p*-hydroxyphenyl groups were

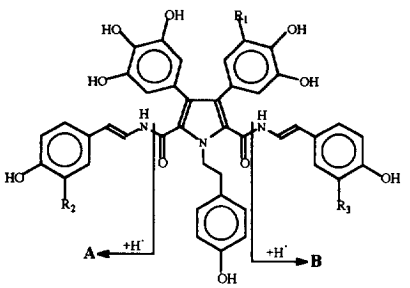
linked to the enamide functionalities, while the trihydroxyphenyl groups had to be attached to quaternary aromatic carbons.

The structural fragments assembled thus far accounted for 24 degrees of unsaturation, including 38 carbons (17 signals of the ^{13}C NMR spectrum), and all the hydrogen, oxygen and nitrogen atoms present in the molecule. Only four quaternary carbon atoms (two pairs of equivalent quaternary carbons) were left to account for the three remaining double bond equivalents. The only possibility for this fragment was a fully substituted pyrrole ring formed with the nitrogen of the tyramine moiety. The molecule adopts the shape of a star with the pyrrole in the center, both enamide carbonyls attached at C-2 and C-5 of the pyrrole and the other two aromatic rings linked at C-3 and C-4. In this way the symmetry of the molecule was easily explained. A different number and substitution pattern of the hydroxy groups on the aromatic rings would account for the structures of the remaining compounds. This structure was confirmed by the correlations observed in the COLOC spectra. The most prominent fragments in the FABMS of **1** were due to two consecutive losses of 135 u, which were attributed to the enamide moieties. This fragmentation behaviour was observed in all the storniamides and was useful in the structure elucidation of the non-symmmetrical compounds (see Figure 1).

Table 1. ^1H NMR Data for Compounds **1** - **4** (200.13 MHz, acetone-d_6 / CD_3OD (9:1))[6]

	1	**2**	**3**	**4**
H-2'	7.12 (d, J = 8.1[c])	7.13 (d, J = 8.1)	6.80 (d, J = 1.8)	6.82 (d, J = 1.8)
H-3'	6.75 (d, J = 8.1)	6.76 (d, J = 8.1)	---	---
H-5'	6.75 (d, J = 8.1)	6.76 (d, J = 8.1)	6.71 (d, J = 7.6)	6.72 (d, J = 7.6)
H-6'	7.12 (d, J = 8.1)	7.13 (d, J = 8.1)	6.61 (dd, J = 7.6; 1.8)	6.61 (dd, J = 7.6; 1.8)
H-α'	7.33 (d, J = 14)	7.34 (d, J = 14)	7.29 (d, J = 14)	7.28 (d, J = 14)
H-β'	5.74 (d, J = 14)	5.73 (d, J = 14)	5.71 (d, J = 14)	5.70 (d, J = 14)
H-2"	7.12 (d, J = 8.1)	6.81 (d, J = 1.8)	6.80 (d, J = 1.8)	6.82 (d, J = 1.8)
H-3"	6.75 (d, J = 8.1)	---	---	---
H-5"	6.75 (d, J = 8.1)	6.72 (d, J = 7.6)	6.71 (d, J = 7.6)	6.72 (d, J = 7.6)
H-6"	7.12 (d, J = 8.1)	6.61 (dd, J = 7.6; 1.8)	6.61 (dd, J = 7.6; 1.8)	6.61 (dd, J = 7.6; 1.8)
H-α"	7.33 (d, J = 14)	7.31 (d, J = 14)	7.29 (d, J = 14)	7.28 (d, J = 14)
H-β"	5.74 (d, J = 14)	5.67 (d, J = 14)	5.69 (d, J = 14)	5.70 (d, J = 14)
H-2"',6"'	6.26 (s)	6.27 (s)	6.27 (s)	6.27 (s)
H-2^{iv}	6.26 (s)	6.27 (s)	6.67 (d, J = 2)	6.27 (s)
H-5^{iv}	---	---	6.77 (d, J = 8)	---
H-6^{iv}	6.26 (s)	6.27 (s)	6.57 (dd, J = 8; 2)	6.27 (s)
H-2^{v},6^{v}	7.05 (d, J = 8.4)	7.04 (d, J = 8.4)	7.05 (d, J = 8.4)	7.06 (d, J = 8.4)
H-3^{v},5^{v}	6.75 (d, J = 8.4)	6.75 (d, J = 8.4)	6.75 (d, J = 8.4)	6.75 (d, J = 8.4)
H-α^{v}	4.80 (bt, J = 7)	4.78 (bt, J = 7)	4.77 (bt, J = 7)	4.80 (bt, J = 7)
H-β^{v}	3.05 (bt, J = 7)	3.02 (bt, J = 7)	3.03 (bt, J = 7)	3.04 (bt, J = 7)

[c]Coupling constants (*J*) are given in Hz.



	$(M+H)^+$	$(M+H-A)^+$	$(M+H-A-B)^+$
1	758	623	488
2	774	639, 623	488
3	774	623	472
4	790	639	488

Figure 1. Fragmentation pattern of compounds **1**- **4** by FABMS

Storniamide B (**2**) had a molecular formula $C_{42}H_{35}N_3O_{12}$ established by HRFABMS and, as shown by ^1H NMR spectroscopy, was a non symmetrical molecule. The substitution pattern of the aromatic rings was established by COSY and homonuclear 2D-J resolved spectra. In this way, the following structural fragments were determined: a *p*-hydroxyphenyl, a 3,4-dihydroxyphenyl and two 3,4,5-trihydroxyphenyl groups similar to those of **1**. Interpretation of the fragmentation pattern of the FABMS (loss of 135 and 151 u fragments), as well as the correlations observed in the COSY and LCOSY spectra indicated that the *p*-hydroxyphenyl and 3,4-dihyroxyphenyl groups were attached to the enamide double bonds. As in the case of **1**, the two trihydroxyphenyl rings were located at positions 3 and 4 of the central pyrrole.

HRFABMS indicated a molecular formula $C_{42}H_{35}N_3O_{12}$ for storniamide C (**3**), thus indicating that **2** and **3** were isomers. As in the case of **2**, interpretation of COSY and homonuclear 2D-J resolved spectra allowed the identification of three 3,4-dihydroxyphenyl groups (two of them equivalent) and just one 3,4,5-trihydroxyphenyl moiety. The fragmentation pattern of the FABMS and the COSY spectrum showed that the two equivalent 3,4-dihydroxyphenyl groups were attached to the enamide double bonds (two losses of 151 u), thus indicating that the remaining 3,4-dihydroxyphenyl and 3,4,5-trihydroxyphenyl groups were linked to the central pyrrole ring. As in the case of the other compounds, this structure was confirmed by the correlations observed in the COLOC spectra (see Figure 2).

Storniamide D (**4**) had a molecular formula $C_{42}H_{35}N_3O_{13}$ established by HRFABMS. In this case, the NMR spectra indicated that the molecule was symmetrical as in the case of **1**. ^{13}C NMR spectroscopy showed only 19 signals, while the pattern of aromatic signals in ^1H NMR was simpler than in the case of **2** and **3**. Both enamide moieties were equivalent, and besides the common tyramine group, two other classes of aromatic rings were present. Analysis of the 2D spectra showed that these fragments were two pairs of equivalent 3,4-dihydroxyphenyl and 3,4,5-trihydroxyphenyl groups. The fragmentation pattern observed by FABMS (two losses of 151 u) and the COSY spectrum established that the 3,4-dihydroxyphenyl groups were linked to the enamide double bonds, while the trihydroxyphenyl groups were attached to the pyrrole. In this way the structure, as well as the symmetry of **4** was ascertained.

Table 2. ^{13}C NMR Data for Compounds **1** - **4** (50.1 MHz, acetone-d_6 / CD_3OD (9:1))

	1	**2**	**3**	**4**
C-2	127.9	127.9	128.0	128.1
C-3	127.2	127.6	127.8	127.7
C-4	127.2	127.6	127.3	127.7
C-5	127.9	127.9	128.0	128.1
C-1'	128.9	128.8	129.5	129.4
C-2'	127.8	127.7	112.9	113.2
C-3'	116.5	116.6	146.4	146.3
C-4'	157.5	157.5	145.6	145.5
C-5'	116.5	116.6	116.5	116.7
C-6'	127.8	127.7	118.8	119.1
C-α'	121.0	120.1	120.9	120.8
C-β'	114.4	114.4	114.9	115.4
C'=O	160.1	160.0	160.3	160.4
C-1"	128.9	129.6	129.5	129.4
C-2"	127.8	112.9	112.9	113.2
C-3"	116.5	146.4	146.4	146.3
C-4"	157.5	145.5	145.6	145.5
C-5"	116.5	116.4	116.5	116.7
C-6"	127.8	118.8	118.8	119.1
C-α"	121.0	120.1	120.9	120.8
C-β"	114.4	114.6	114.7	115.4
C"=O	160.1	160.1	160.1	160.4
C-1'''	125.6	125.6	125.6	125.6
C-2''', C-6'''	110.6	110.6	110.6	110.7
C-3''', C-5'''	146.8	146.8	146.9	146.8
C-4'''	133.6	133.6	133.7	133.7
C-1^{iv}	125.6	125.6	126.2	125.6
C-2^{iv}	110.6	110.6	118.5	110.7
C-3^{iv}	146.8	146.8	146.1	146.8
C-4^{iv}	133.6	133.6	145.8	133.7
C-5^{iv}	146.8	146.8	112.8	146.8
C-6^{iv}	110.6	110.6	123.0	110.7
C-1^{v}	130.4	130.4	130.4	130.4
C-2^{v},C-6^{v}	131.1	131.1	131.1	131.2
C-3^{v},C-5^{v}	116.5	116.2	116.2	116.2
C-4^{v}	157.2	157.2	157.2	156.9
C-α^{v}	49.1	49.2	49.0	49.1
C-β^{v}	38.6	38.6	38.5	38.7

[a]Assignments are based on the results of the following 2D NMR spectra: HETCOR, long range-optimized HETCOR (J_{C-H}: 7 Hz), and COLOC (J_{C-H}: 8 and 10 Hz)

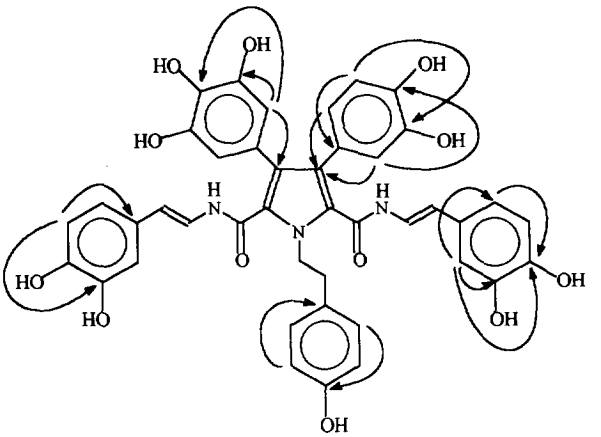
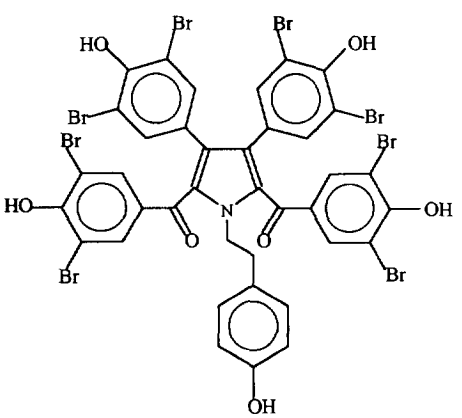


Figure 2. Long range ^{1}H - ^{13}C correlations observed for Storniamide C (**3**).

Storniamides A-D showed antibiotic activity against Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis* and *Micrococcus luteus*) at 50 μg / disk. These compounds did not show activity against Gram-negative bacteria or *Candida albicans*.

As for the biogenesis of the storniamides, it seems clear that these compounds are of peptide origin. Five tyrosine units take part in the building of the structural framework while subsequent hydroxylation in the aromatic rings gives rise to the different compounds. Several recently isolated compounds from marine invertebrates show structures related to the storniamides, all of them having a central pyrrole ring. The lukianols[7] isolated from an unidentified tunicate lack the two enamide functionalities together with their corresponding aromatic rings. More recently, policytone A (**5**) and policytrins A and B,[8] were isolated from the South African tunicate *Policytor sp.* and are polybrominated aromatic compounds which resemble the storniamides but lack the two enamide groups.



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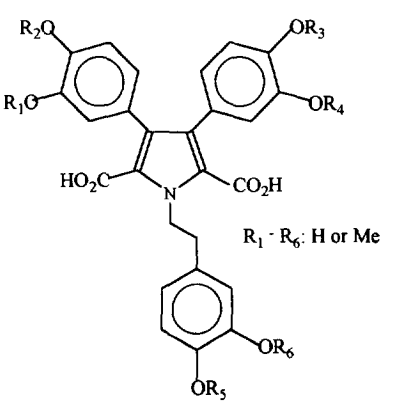

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Other examples of structurally related compounds that show additional rings, are purpurone,[9] a sponge metabolite, as well as the lamellarins,[10,13] isolated from a variety of organisms. Interestingly, Kashman et al.[8] propose a biosynthetic precursor (**6**) for polycitone A and the lamellarins which could also serve as the precursor of the storniamides and lukianols. We have not been able to detect such a compound, but several minor metabolites from this sponge are currently under study in our laboratory and will bring new light upon the origin and bioactivity of this interesting family of new compounds.

EXPERIMENTAL SECTION

General. UV spectra were recorded on a Hewlett-Packard 8451A Diode Array Spectrophotometer; IR on a Nicolet Magna-FT-IR 550 spectrometer; FABMS (matrix: glycerol, Cs 3.5 kv) on a VG-ZAB-SEQ hybrid mass spectrometer. HRFABMS were acquired on a VG Autospec Q spectrometer; NMR experiments were recorded on a Bruker AC 200 spectrometer using TMS as internal standard. 2D NMR experiments were performed using standard pulse sequences. HPLC was performed using a Thermo Separations pump and UV detector, a Shodex RI detector and a YMC Rp-18 (20 x 250 mm) column.

Collection and Extraction. The sponge *Cliona sp.* (11.5 kg) was collected (-0.5 m) at Punta Verde, near San Antonio Oeste, province of Rio negro, Argentina and immediately frozen. The sponge was classified by Dr Patricia Bergquist (Dept. of Zoology, Univ. of Auckland) The frozen sponge was extracted exhaustively with ethanol at room temperature, and the extact was evaporated under reduced pressure. The residue was triturated with methanol and filtered. Evaporation of the filtrate afforded 23 g of a greenish syrup. The crude extract was fractionated by reversed phase flash chromatography using a H_2O/MeOH gradient as eluant. The fraction eluted with MeOH/H_2O (6:4) was further separated by HPLC (see ***General***) using H_2O/acetonitrile (7:3) as eluant, to yield pure compounds **1** (35 mg), **2** (55 mg), **3** (115 mg) and **4** (95 mg).

Storniamide A (1): yellow oil; UV λ_{max} (MeOH) 208 nm (ε 25500), 286 (11300), 334 (11500); IR (KBr) 3500-3300, 1669, 1619, 1512, 1234, 956, 849 cm^{-1}; ^1H NMR data see Table 1; ^{13}C NMR data see Table 2; HRFABMS obsd. *m/z* 758.2360 $(M+H)^+$,$C_{42}H_{36}N_3O_{11}$ requires 758.2350. FABMS: 758 (45), 623 (30), 488 (60).

Storniamide B (2): yellow oil; UV λ_{max} (MeOH) 210 nm (ε 24500), 282 (10200), 334 (10800); IR (KBr) 3500-3300, 1640, 1619, 1505, 1448, 1248, 949, 835 cm^{-1}; ^1H NMR data see Table 1; ^{13}C NMR data see Table 2; HRFABMS obsd. *m/z* 774.2279 $(M+H)^+$,$C_{42}H_{36}N_3O_{12}$ requires 774.2299. FABMS: 774 (60), 639 (30), 624 (18), 488 (42).

Storniamide C (3): yellow oil; UV λ_{max} (MeOH) 214 nm (ε 27000), 284 (11100), 342 (14300); IR (KBr) 3500-3300, 1655, 1612, 1519, 1284, 949, 835 cm^{-1}; ^1H NMR data see Table 1; ^{13}C NMR data see Table 2; HRFABMS obsd. *m/z* 774.2322 $(M+H)^+$,$C_{42}H_{36}N_3O_{12}$ requires 774.2299. FABMS: 774 (55), 623 (37), 472 (24).

Storniamide D (4): yellow oil; UV λ_{max} (MeOH) 212 nm (ε 29500), 284 (11500), 338 (12400); IR (KBr) 3500-3300, 1653, 1611, 1511, 1254, 948, 839 cm^{-1}; ^1H NMR data see Table 1; ^{13}C NMR data see Table 2; HRFABMS obsd. *m/z* 790.2236 $(M+H)^+$,$C_{42}H_{36}N_3O_{13}$ requires 790.2248. FABMS: 790 (42), 639 (15), 488 (9).

ACKNOWLEDGEMENTS

We thank Dr. Gabriela Cabrera, Lic. Alejandro Roccatagliata and Mrs. Stella Maris Neira for their help during sponge collection. We are indebted to Dr. E. Di Giacomo, Dr. E. Morsan and all the staff of the "Instituto de Biología Marina y Pesquera Alte. Storni", San Antonio Oeste, for their help and the kind permission to use their facilities. We are also grateful to Dr. Nick Ordsmith (VG Organics, Manchester) for the HRFAB spectra, to LANAIS-EMAR for FABMS and UMYMFOR for NMR spectra, to Dr. Alicia Rossi and Lic. Marcelo Galas (Instituto Malbrán) for the bioassays, and the Universidad de Buenos Aires for an undergraduate fellowship to one of us (MFRB). This work was partially supported by grants from CONICET and Fundación Antorchas.

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(*Received in USA* 13 *September* 1995; *revised* 18 *December* 1995; *accepted* 19 *December* 1995)