Pergamon

Tetrahedron, Vol. 53, No. 18, pp. 6565-6572, 1997

0040-4020/97 $17.00 + 0.00

PII: S0040-4020(97)00311-6

Chemistry of Verongida Sponges VIII[1] - Bromocompounds from the Mediterranean Sponges *Aplysina aerophoba* and *Aplysina cavernicola*

Patrizia Ciminiello, Ernesto Fattorusso*, Martino Forino, Silvana Magno

Dipartimento di Chimica delle Sostanze Naturali, Università degli Studi di Napoli Federico II, Via D. Montesano 49, I-80131 Napoli, Italy

Maurizio Pansini

Istituto di Zoologia, Via Balbi 5, 16126 Genova, Italy

Abstract: A detailed analysis of the secondary metabolites of *Aplysina aerophoba* and *Aplysina cavernicola* has been performed. A number of bromotyrosine derivatives have been identified of which two, **14** and **15**, both isolated from *A. cavernicola*, are novel compounds. Their structures have been assigned on the basis of spectroscopic and chemical data. The obtained results indicate a remarkable difference in the secondary metabolism of the two sponges, which eventually confirms the separation of the two Mediterranean *Aplysina* species, in perfect agreement with Vacelet's opinion.

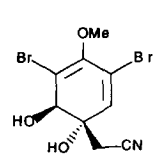
Horny sponges belonging to the genus *Aplysina* Nardo, 1834 (Demospongiae, Aplysinidae) have a single type of pithed spongin fibres, always devoid of foreign inclusions, which form a skeletal net of wide hexagonal meshes. Most of the recorded species are from tropical and temperate areas, but a single species was recorded from the Antarctic.

The first Mediterranean *Aplysina* was described by Schmidt in 1862 and named *aerophoba* after its peculiar color variation - from bright yellow to dark violet and blue - if exposed to air. *A. aerophoba* is a clearly photophilous species thriving even in shallow waters. In 1959 Vacelet described a second Mediterranean species from Corse and Marseille, naming it *cavernicola* because of its preferred habitat, marked by low light intensity, that may be found at the entrance of submerged marine caves or in relatively deep waters.

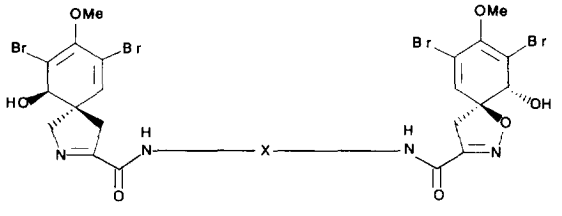
The internal morphology and the skeleton are almost identical in the two species, which differ for a series of not easily detectable characters as shape, color shade of living specimens, ramified processes arising from the sponge surface, chromatic reaction to fixatives etc. According to these rather questionable diagnostic characters, sponge taxonomists have longly debated if the two taxa, showing so different ecological requirements, should be considered as ecotypes or distinct species.

The analysis of the secondary metabolites produced by the two species may offer further elements to cope with the question. Unfortunately, in the past, chemical data on Mediterranean Verongida were not always supported by a sure identification of the studied specimens.

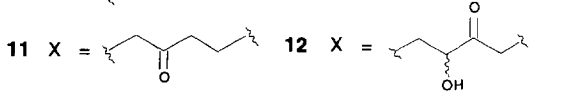
In the seventies, several papers[2-7] reported the isolation of compounds **1-5** from a Mediterranean sponge, indicated as *Aplysina aerophoba*. Successively Cimino et al.[8] hypothesized that the examined sponge was the "related" species *Aplysina cavernicola;* in addition they found a quite different metabolic content in a "true" *A. aerophoba*, which was shown to contain the bromocompounds **1, 4, 6-8**. Recently, Pietra *et al.* isolated from another specimen of a Mediterranean *A. cavernicola,* minute amounts of other compounds[9,10], probably deriving from **4**[11] by ring closure and very small amount of the chlorinated analogue of compound **4**[12].



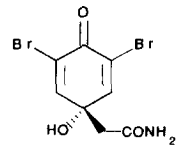
1



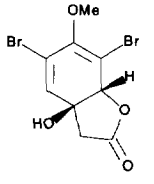
2 X = **3** X =



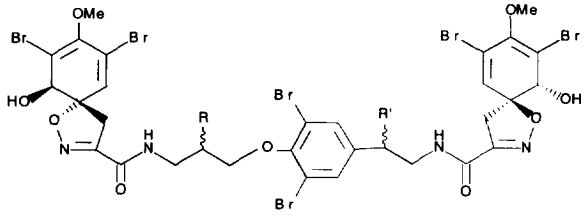
11 X = **12** X =



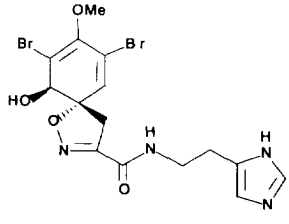
4



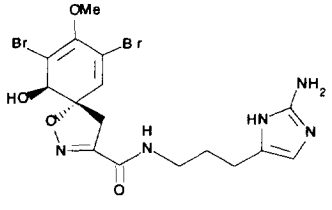
5



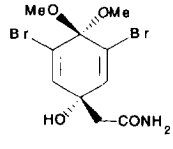
6 R = OH; R' = OH
13 R = H; R'= H



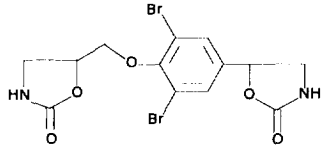
7



8



9



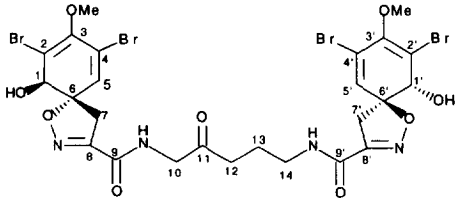
10

Aiming to clarify the problem of the Mediterranean *Verongida* and chiefly to ascertain the existence or not of two different species, we have now re-examined their secondary metabolism.

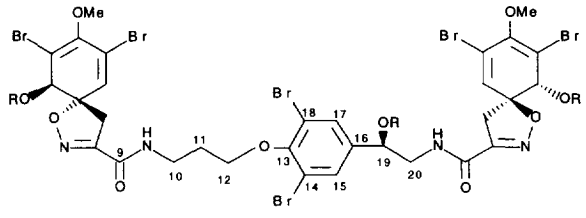
In order to avoid the difficulty in the identification, the material we analyzed was purposely collected from cliffs and caves of Capo Caccia (North-western Sardinia) and all the specimens were identified *in vivo* (before fixation). Examining fresh material we were able to separate the collected specimens in two groups

which actually conformed to the peculiar characteristics reported in the literature for the two Mediterranean species. Since they were sympatric, the recorded differences in the secondary metabolite content are necessarily related to a specific difference, and may not be attributed to environmental or geographical variations.

The obtained results on their metabolic composition are reported in the present paper, which also describes the structure determination of two novel bromocompounds (oxohomoaerothionin, **14** and 11-hydroxyfistularin-3, **15**), present in *A. cavernicola*.



14



15 R = H

16 R = *(R)*-MTPA

17 R = *(S)*-MTPA

The isolation procedures for both specimens were the same; the animals, frozen after collection, were extracted sequentially with methanol-toluene 3:1 and chloroform. The combined extracts were partitioned between water and ethyl acetate and successively butanol. The material soluble in the organic solvents was repeatedly chromatographed on silica gel columns using solvents of increasing polarity to give the pure products reported in Table 1. The previously described compounds **1-3** and **5-13** were identified by comparison of their spectral data with those reported in the literature. It is to be noted that, as frequently reported for a number of Verongida sponges, compound **4** has not been isolated as such, but as its ketal derivative, **9**, formed during the extraction procedure.

Table 1. Percentages of Halogenated Metabolites in Specimens of *A. aerophoba* and *A. cavernicola*.

Sample	Collection	Depth	Compound %[a]													
			1	**2**	**3**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
V.aerophoba	CapoCaccia Sardinia	-10 m	1.03	-	-	-	1.91	0.05	0.11	0.10	0.09	-	-	-	-	-
V.cavernicola	CapoCaccia Sardinia	-10 m	1.12	3.65	0.52	0.12	0.25	-	-	0.37	-	0.45	0.04	0.21	0.08	0.05

[a] To dry weight of the specimens after extraction.

The molecular formula of $C_{25}H_{26}Br_4N_4O_9$ for the novel compound **14** was suggested by the negative ion FABMS spectrum, which shows a 1:4:6:4:1 quintet for the molecular ion centered at *m/z* 845 $[M-H]^-$; it was confirmed by the ^{13}C NMR spectrum and DEPT experiment.

The UV absorption at λ_{max} 284 (ε 10,500) and 232 (ε 19,000) indicated the presence of a cyclohexadienyl function, while the IR bands showed the presence of alcohol (3450 cm^{-1}) and α-iminoamide (1665 cm^{-1}) functions. An inspection of ^1H, ^{13}C and ^1H-^1H COSY NMR spectra suggested that the above functionalities were included into two 1-hydroxy-2,4-dibromo-3-methoxy-8-carbamoyl spirocyclohexadienyl

isoxazole part structures not symmetrically arranged in the molecule. This is a structural feature commonly found in brominated metabolites of *Verongida*, such as **2** or **6**, for example.

In the light of the above observations it remained still unidentified the central part of the molecule, which must be composed by a ketonic carbonyl and four methylene groups as indicated by the molecular formula, ^{13}C NMR spectrum (δ 206.2, 24.1, 37.6, 39.6 and 49.7) and IR absorption at 1715 cm^{-1}. The sequential arrangement of the above groups, assigned as reported in formula, follows from the lack of symmetry of the molecule; it was supported by ^{1}H-^{1}H COSY spectrum, which delineated the C12-C14 segment. It is to be noted that in the ^{1}H NMR spectrum in CD_3OD the signal of H_2-10 resonates as a singlet slowly disappearing as a result of a deuterium exchange with the solvent.

The whole of the above data allowed assignment of the structure and the relative stereochemistry of **14**. The absolute stereochemistry of the chiral centers of spirocyclohexadiene groups was suggested by CD spectrum [(MeOH) $[\vartheta]_{285}$ +52,000; $[\vartheta]_{250}$ +60,200], which is analogous to that reported for aerothionin[13], whose absolute stereochemistry was established by X-RAY studies as 1*R*, 1'*R*, 6*S* and 6'*S*.

Table 2. ^{13}C and ^{1}H Assignment for Compound 14 and 15 (CD_3OD)[a]

	14		15	
Carbon	δ_C, mult.	δ_H (mult.*J*/Hz)	δ_C, mult.	δ_H (mult.*J*/Hz)
1, 1'	75.50 d	4.16 (s) 4.14 (s)	75.42 d	4.12 (s) 4.11 (s)
2, 2'	122.80 s		122.74 s	
3, 3'	149.31 s		149.28 s	
4, 4'	114.15 s		114.16 s	
5, 5'	131.32 d 132.25 d	6.47 (s) 6.46 (s)	132.24 d 132.16 d	6.46 (s) 6.44 (s)
6, 6'	92.63 s 92.36 s		92.48 s	
7a, 7'a	40.21 t 40.04 t	3.15 (d,18) 3.13 (d,18)	40.13 t 40.04 t	3.14 (d,18) 3.09 (d,18)
7b, 7'b		3.82 (d,18) 3.80 (d,18)		3.81 (d,18) 3.79 (d,18)
8, 8'	155.29 s 154.93 s		155.27 s 155.12 s	
9, 9'	161.81 s		161.68 s 161.55 s	
10	49.17 t	4.12 (s)	37.94 t	3.62 (t. 6.5)
11	206.18 s		30.59 t	2.15 (t, 6.5)
12	37.57 t	2.60 (t, 7)	71.61 t	4.10 (t, 6.5)
13	24.11 t	1.88 (dt,7,7)	153.56 s	
14	39.61 t	3.32 (t,7)	118.99 s	
15			131.69 d	7.64 (s)
16			143.06 s	
17			131.69 d	7.64 (s)
18			118.99 s	
19			72.18 d	4.79 (dd, 4.5, 7.5)
20			47.63 t	3.51[b] 3.46[b]
OMe	60.38 q	3.75 s	60.35 q	3.76 (s)

[a] Assignment based on DEPT, COSY, HetCOSY and COLOC experiments.
[b] Further coupled AB system, J_{AB} 13.5 Hz , J_{AX} 7.5 Hz, J_{BX} 4.5 Hz.

The remaining novel compound **15** showed in the negative FABMS spectrum a cluster of seven ions centered at *m/z* 1097 for the pseudomolecular ion, indicative of the presence of six bromine atoms. Combined

analysis of the FABMS, ^{13}C NMR and DEPT spectra (31 carbons, 26 attached protons) and ^{1}H NMR spectrum (5 D_2O exchangeable protons in CD_3COCD_3) suggested the molecular formula $C_{31}H_{30}Br_6N_4O_{10}$, implying sixteen degrees of unsaturation.

A comparison of overall IR, UV and ^{1}H and ^{13}C NMR data between fistularin-3[14] and 11,19-dideoxyfistularin-3[15], isolated from the same source and **15** allowed to delineate all the cyclic part structures of this last compound corresponding to a 1-alkoxy-2,6-dibromo-4-alkylbenzene and two 1-hydroxy-2,4-dibromo-3-methoxy-8-carbamoylspirocyclohexadienylisoxazoles.

The remaining part of the molecule comprises the segments linking the terminal spirocyclohexadienyl moieties to the central substituted benzene. Their structures were ascertained on the basis of ^{1}H NMR spectrum in CD_3COCD_3 and ^{1}H-^{1}H connectivities observed in a homonuclear shift correlated 2D experiment (COSY) which evidenced the isolated spin systems corresponding to the linear segments NH-C10-C11-C12 [(δ 7,73 (NH-9a', t, *J* 6 Hz), 3.60 (H_2-10, ddd, *J* 6, 6, 6 Hz,) 2.14 (H_2-11, t, *J* 6 Hz,)., and 4.10 (H_2-12, t, *J* 6 Hz,), and C19-C20-NH [δ 4.90 (H-19, m), 3.58 (H_2-20, m), and 7.76 (NH-9a, t, *J* 6 Hz).

The above data established the gross structure of compound **15** together with the relative stereochemistry of the terminal cyclic moieties. Further support was obtained from the HMQC experiment, which allowed to assign the carbon resonances of the molecule.

The absolute stereochemistry of the spirocyclohexadienylisoxazole-moieties, analogously to compound **14**, was deduced from the CD spectrum: [(MeOH) $[\vartheta]_{285}$ +56,500; $[\vartheta]_{250}$ +65,200], which was superimposable on that reported for aerothionin[13].

The configuration of the chiral center at C19 has been assigned through application of the modified Mosher method suggested by Kakisawa[16]. Compound **15** was treated with *R*(-) and *S*(+) α-methoxy-α-(trifluoromethyl)phenylacetyl (MTPA) chloride in pyridine solution at room temperature for 2h to give esters **16** and **17**. The stereochemical determination was based on the chemical shift difference of the protons at C20 [(*R*)-MTPA ester: H_2-20 δ 3.74; (*S*)-MTPA ester: H_2-20 δ 3.71 Δδ -0.03] The obtained results suggested the *S* configuration at C19.

The results reported in the present paper indicate a remarkable difference in the secondary metabolism of the two sponges. Apart from some variations in the profile of minor metabolites, as well as in the relative percentages of compounds co-occurring in the two sponges, the most evident difference consists in aerothionin, **2**, which is by far the major metabolite in *A. cavernicola*, while it is completely absent among the *A. aerophoba* metabolites.

In our opinion, aerothionin, easily detectable on the basis of its spectral properties, could be usefully employed as chemical marker for the distinction of the two taxa.

Futhermore, our results confirm the suggestion of Cimino *et al.*[8] that the sponge analyzed at beginning of 1970's and reported as A. *aerophoba*, was actually *A. cavernicola.*

The secondary metabolism eventually confirms the separation of the two Mediterranean *Aplysina* species, in perfect agreement with Vacelet's opinion[17]. However *A. aerophoba* and *A. cavernicola* are very close to one another and their specific separation may be the result of a progressive isolation process due to their remarkably different ecological requirements[18].

EXPERIMENTAL SECTION

General methods. ^1H and ^{13}C NMR spectra were determined on a Brüker AMX-500 spectrometer and the solvent was used as an internal standard (CD_3OD: ^1H δ 3.34, ^{13}C δ 49.0; CD_3COCD_3: ^1H δ 2.05). Methyl, methylene and methine carbons were distinguished by a DEPT experiment. FABMS were obtained in glycerol matrix on a VC Prospec Fisons mass spectrometer. FT-IR spectra were recorded on a Brüker IFS-48 spectrophotometer using a KBr matrix. UV spectra were performed on a Beckman DU70 spectrometer in aqueous solution. The CD spectra were recorded in MeOH solution on a JASCO J710 spectropolarimeter. Medium pressure liquid chromatographies (MPLC) were performed on a Büchi 861 apparatus using a SiO_2 (230-400) mesh. High performance liquid chromatographies (HPLC) were performed on a Varian 2510 apparatus equipped with an RI-3 index detector, using Hibar columns.

Collection and extraction. Fresh sponges *A. aerophoba* (34 g, dry weight after extraction) and *A. cavernicola* (20 g, dry weight after extraction) were collected from cliffs and caves of Capo Caccia (North-western Sardinia), at a depth of about 10 meters, and identified by Prof. M. Pansini, University of Genova, Italy. Reference specimens are deposited at the Istituto di Zoologia dell'Università di Genova (Italy).

The initial procedure employed for the extraction and fractionation of the lipid mixture was common to both sponges and was performed as follows.

The homogenized materials were separately extracted with MeOH/toluene (3x1 liter) and subsequently with $CHCl_3$ (3x1 liter) at room temperature.

The combined MeOH/toluene solutions, after filtration, were concentrated *in vacuo* to give an aqueous suspension which was extracted with EtOAc and subsequently with *n*-BuOH.

The combined EtOAc, $CHCl_3$ and BuOH extracts were chromatographed by MPLC on an SiO_2 column using a solvent gradient system from *n*-hexane to EtOAc and then to MeOH.

Selected fractions were combined on the basis of TLC analyses and chromatographed on HPLC.

Isolation of bromocompounds from A. aerophoba.

Isolation of 1. Fractions eluted with *n*-hexane/EtOAc 1:1 (575 mg) were purified by HPLC using a Hibar LiChrospher Si 60 (10 x 250 mm) column with EtOAc/$CHCl_3$ 1:1 as eluent giving 350 mg of pure aeroplysinin-1, **1** identified by comparison of its spectral properties with those reported in literature[2,5].

Isolation of 4, 9, and 10. Evaporation of fractions eluted with 100% EtOAc afforded a mixture (820 mg) which was purified by HPLC using a Hibar LiChrospher Si 60 (10 x 250 mm) column with a mobile phase of EtOAc/$CHCl_3$ 9:1 to obtain compounds **6**[14] (650 mg), **9**[19] (35 mg), and **10**[20] (30 mg), identified by comparison of their spectral properties with those reported in the literature.

Isolation of 7-8. Fractions emerging from the MPLC column with EtOAc/MeOH 8:2 (138 mg) were purified by HPLC on a LiChroprep RP 18 column (10 x 250 mm) using a linear gradient from 100% H_2O containing 0.2% of trifluoroacetic acid to 100% CH_3CN containing 0.2% of trifluoroacetic acid in 20 minutes thus affording pure aerophobin-1, **7** (18 mg) and aerophobin-2, **8** (39 mg), identified comparing their spectral properties with those reported in the literature[8].

Isolation of bromocompounds from A. cavernicola.

Isolation of 1, 5, 13 and 15. Fractions eluted with *n*-hexane/EtOAc 1:1 (575 mg) were purified by HPLC using a Hibar LiChrospher Si 60 (10 x 250 mm) column with *n*-hexane/EtOAc 1:1 as eluent giving 25 mg of aeroplysinin-2, **5**, 350 mg of aeroplysinin-1, **1**, 42 mg of 11,19-dideoxyfistularin-3, **13** and 11 mg of 11-deoxyfistularin-3, **15**.

Compounds **1**[2,5], **5**[7], and **13**[15] were identified by comparison of their spectral properties with those reported in literature.

Compound 15. UV (MeOH) λ_{max} 284 (ε 11,000), 232 (ε 19,000); IR (KBr matrix) ν_{max} 3450, 1715 and 1665 cm^{-1}; CD (MeOH) $[\vartheta]_{285}$ +56,500; $[\vartheta]_{250}$ +65,200; FABMS: $[M-H]^-$ 1091, 1093, 1095, 1097, 1099, 1101, 1103; ^1H and ^{13}C data in CD_3OD are reported in table 2; ^1H NMR in CD_3COCD_3 (selected signals): δ 7.76 and 7.73 (1H each, NH-9a and NH-9a' respectively, t, *J* 6 Hz), 4.90 (1H, m, H-19), 4.10 (2H, t, *J* 6 Hz, H_2-12), 3.60 (2H, ddd, *J* 6, 6, 6 Hz, H_2-10), 3.58 (2H, m, H_2-20), 2.14 (2H, t, *J* 6 Hz, H_2-11).

Isolation of 2, 3, 6, 9, 11, 12 and 14. Evaporation of the fractions eluted with 100% EtOAc gave 1.2 g of a mixture of bromotyrosines. The mixture was separated by HPLC on a silica gel column (10 x 250) using $CHCl_3$/EtOAc 3:7 as eluent, thus obtaining compounds **2**[3,6,12] (730 mg), **3**[4,6] (104 mg), **9**[19] (75 mg), **6**[14] (50 mg), **11**[21] (90 mg) and **12**[22] (8 mg) identified comparing their spectral properties with those reported in the literature, and 16 mg of oxohomoaerothionin, **14**.

Compound 14: UV (MeOH) λ_{max} 284 (ε 10,500), 232 (ε 19,000); IR (KBr matrix) ν_{max} 3450, 1715, 1665 cm^{-1}; CD (MeOH) $[\vartheta]_{285}$ +52,000; $[\vartheta]_{250}$ +60,200; FABMS: $[M-H]^-$ 841, 843, 845, 847, 849; ^1H and ^{13}C data in CD_3OD are reported in table 2.

Synthesis of the (R)- and (S)-MTPA esters of 15.

To compound **15** (2.5 mg) in 200 μl of anhydrous pyridine, 5 μl of (*R*)-MTPA chloride [MTPA= α–methoxy-α-(trifluoromethyl)phenylacetyl] were added, and the mixture was allowed to react at room temperature. After 2h, 5 ml of H_2O and solid K_2CO_3 were added, and the solution was extracted with $CHCl_3$ (5 ml). The organic phase, after evaporation of the solvent, yielded the (*R*)-MTPA triesters **16**. The use of (*S*)-MTPA chloride in the same procedure led to the (*S*)-MTPA triesters **17**.

19S-Hydroxyfistularin-3-(R)-MTPA triester, 16. ^1H NMR (CD_3OD): δ 6.61 and 6.55 (1H each, s, H-5 and H-5'), 6.12 (1H, m, H-19), 6.06 and 5.94 (1H each, s, H-1 and H-1'), 3.74 (2H, m, H_2-20).

19S-Hydroxyfistularin-3-(S)-MTPA triester, 17. ^1H NMR (CD_3OD): δ 6.54 and 6.51 (1H each, s, H-5 and H-5'), 6.03 (1H, m, H-19), 5.95 and 5.87 (1H each, s, H-1 and H-1'), 3.71 (2H, m, H_2-20).

ACKNOWLEDGMENTS

This work is a result of a research supported by CNR (CONTR: 95.1304.CT03) and by M.U.R.S.T. (40 and 60%), Rome, Italy. NMR, FABMS and FTIR spectra were performed at "Centro di Ricerca Interdipartimentale di Analisi Strumentale", Università di Napoli "Federico II". The assistance of the staff is gratefully appreciated.

REFERENCES

1. Part VII: Ciminiello, P.; Dell'Aversano, C.; Fattorusso, E.; Magno, S.; Pansini, M. *Tetrahedron*, 1996, **52**, 9863.
2. Fattorusso, E.; Minale, L.; Sodano, G. *Chemical Communications*, 1970, 751.
3. Fattorusso, E.; Minale, L.; Sodano, G. *Chemical Communications*, 1970, 752.
4. Fattorusso, E.; Minale, L.; Moody, K.; Sodano, G.; Thomson R. H. *Gazz. Chim. It.*, 1971, **101**, 61.
5. Fattorusso, E.; Minale, L.; Sodano, G. *J.C.S. Perkin I*, 1972, 16.
6. Moody, K.; Thomson, R.H.; Fattorusso, E.; Minale L. *J.C..S. Perkin I*, 1972, 18.
7. Minale, L.; Sodano, G.;Chan W. R.; Chen A. M.; *J.C.S. Chem Com.*, 1972, 674.
8. Cimino, G.; De Rosa, S.; De Stefano, S.; Self, R.; Sodano, G. *Tetrahedron Letters*, 1983, **24**, 3029.
9. D'Ambrosio, M.; Guerriero, A.; Traldi, P.; Pietra, F. *Tetrahedron Letters*, 1982, **23**, 4403.
10. D'Ambrosio, M.; Mealli, C.; Guerriero, A.; Pietra, F. *Helvetica Chimica Acta*, 1985, **68**, 1453.
11. Sharma, G.M.; Burkholder, P.R. *Tetrahedron Lett.*, 1967, 4147.
12. D'Ambrosio, M.; Guerriero, A.; Pietra, F. *Helvetica Chimica Acta*, 1984, **67**, 1484.
13. McMillan, J.A.; Paul, I.C.; Goo, Y.M.; Rinehart, K.L. *Tetrahedron Lett.*, 1981, **22**, 39.
14. Gopichand, T.; Schmits, F.J. *Tetrahedron Lett.*, 1979, 3921.
15. Kernan, M.R.; Cambie, R.C. *J. Nat. Prod.*, 1990, **53**, 615.
16. Ohtani, I.; Kusumi, T.; Kashman, Y.; Kakisawa, H. *J.Am.Chem.Soc.*, 1991, **113**, 4092.
17. Vacelet J. *Recl. Trav. St. Mar. Endoume*, 1959, **16**, 39.
18. Pansini, M. *Rivista Italiana di Biologia Marina*, 1996, **4**, in press.
19. Sharma, G.M.; Vig, B.; Burkholder, P.R. *J. Org. Chem.*, 1970, **55**, 2823.
20. Borders, D.B.; Morton, G.O.; Wetzel, E.R. *Tetrahedron Lett.*, 1974, 2709.
21. Acosta, A.L.; Rodriguez, A.D. *J. Nat. Prod.*, 1992, **55**, 1007.
22. Ciminiello, P.; Costantino, V.; Fattorusso, E.; Magno, S.; Mangoni, A.; Pansini, M. . *J. Nat. Prod.*, 1994, **57**, 705.

(*Received in UK* 11 *March* 1997; *accepted* 20 *March* 1997)