Minor nitrogenous sesquiterpenes from the marine sponge *Axinella cannabina*. A hypothesis for the biogenesis of the spiro-axane skeleton[1]

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Summary. Three nitrogenous sesquiterpenoids (**4–6**), possessing an isocyanide, isothiocyanate or formylamino function, have been isolated from the marine sponge *Axinella cannabina*. The proposed structures were based on interpretation of spectral data including 2D-NMR techniques and chemical evidence. A plausible pathway for the biogenesis of these compounds and the co-occurring spiro-axane (**1–3**) metabolites is proposed.
Key words. Isocyanides; sesquiterpenes; biogenesis.

The marine sponge *Axinella cannabina* (Pallas) elaborates a wide variety of biologically interesting sesquiterpenoids generally carrying $-N^+\equiv C^-$, $-N=C=S$ or $-NHCHO$ groups[2], among which spiro-axane representatives are present[3] (**1–3**). For this skeleton which, to the best of our knowledge, is peculiar to this organism, no biogenetical hypothesis has been proposed so far. With the aim of providing insight into the biogenesis of this novel ring system, we are now examining the minor metabolites from *A. cannabina*. In this paper we report the structural elucidations of a further isocyanide-isothiocyanate-formylamino series, where the nitrogenous functions are linked to a carbon atom of the ring junction of a cadinane skeleton. The co-occurrence of these metabolites and those of the spiro-axane type enabled us to suggest a possible biogenetical pathway through which both series could be originated.

Material and methods. Specimens of *A. cannabina* were collected in the Bay of Taranto, near Porto Cesareo, in the Spring 1984. Freshly collected animals (500 g, dry weight after extraction) were extracted by MeOH for 3 days. The organic soluble fraction of the MeOH extract was purified using a silica gel column with eluent solvent systems of increasing polarity: *n*-hexane to Et_2O through C_6H_6. Fractions eluted with *n*-hexane-C_6H_6 (8:2) gave a mixture of isothiocyanates from which pure **5** (38 mg) was obtained by HPLC (ODS-2, 10% H_2O in MeOH). Fractions eluted with *n*-hexane-C_6H_6 (2:8) were further partitioned by HPLC (ODS-2, 5% H_2O in MeOH) to afford **4** (18 mg). Finally compound **6** (5 mg) was isolated from the fractions eluted with Et_2O, which were further chromatographed by HPLC (Li-Chrosorb Si-60, EtOAc). Most of our structural work was carried out with the most abundant **5**.

Aromatization of **5** was performed by heating 20 mg of the compound at 250 °C for 24 h with 10% Pd/C (20 mg). The crude product was chromatographed on silica gel (hexane as eluent) to give the major product: cadalene (**7**) identified by comparison of physical properties (UV, MS and NMR) with those of a reference sample. Conversion of **4** (8 mg) into **5** was carried out by treatment with sulphur at 120 °C for 20 h. After addition of 40–70 °C light petroleum (8 ml) and filtration, the solvent was evaporated and the residue was purified by HPLC (ODS-2, 10% H_2O in MeOH) to give a product (6 mg), which was identical with natural **5** on the basis of their chromatographic and spectral properties.

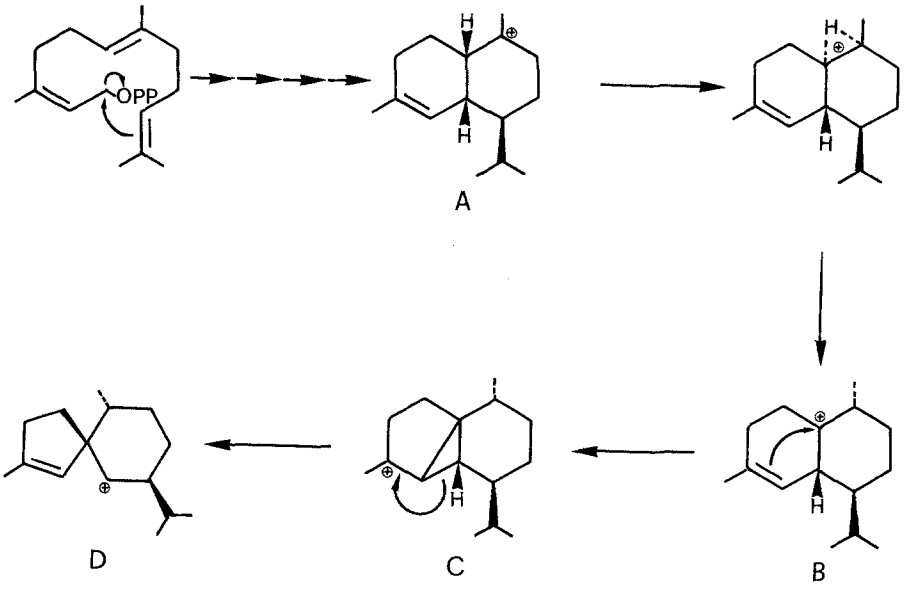
Hydration of **4** (8 mg) to give **6** was performed in anhydrous Et_2O (4 ml) and AcOH (3 ml) at room temperature for 2 h. After washing with 10% aqueous Na_2CO_3 and then with H_2O, the organic phase was taken to dryness by HPLC (Li-Chrosorb Si-60, EtOAc) to give a compound whose spectral and chromatographic properties matched those of natural **6**.

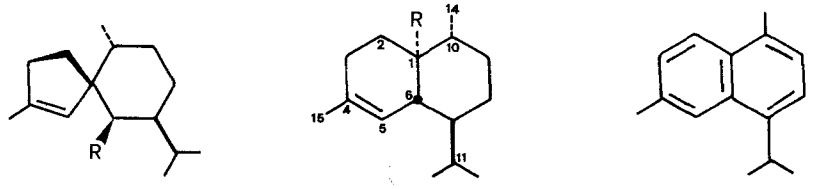
Results and discussion. Isothiocyanate **5** is an optically active ($[\alpha]_D = -91.17°$) colorless oil, which had a molecular formula

^{13}C- and ^1H-NMR data for isonitrile **4** and isothiocyanate **5**

∂C 4[a]	∂C 5[a]	Assignment	∂H 4[b]	∂H 5[b]	J (Hz)
69.34	69.14	1			4: 2ax-2eq = 12.5; 2ax-3ax = 13.0;
		2ax	1.12 (ddd)	1.18 (ddd)	2ax-3eq = 6.0; 2eq-3ax = 6.0;
32.68	31.75				
		2eq	1.93 (dd)	1.99 (dd)	2eq-3eq = very small; 3ax-3eq = 17.0;
		3ax	2.18 (ddd)	2.10 (ddd)	5–6 = very small; 6–7 = 10.5;
27.55	27.37				
		3eq	1.77 (dd)	1.76 (dd)	7–8eq = 4.0; 7–8ax = 12.0; 7–11 = 4.0;
134.53	134.61	4			8ax-8eq = 12.5; 10–14 = 6.5
118.54	118.57	5	5.31 (bs)	5.32 (bs)	11–12 = 6.5; 11–13 = 6.5.
45.92	46.89	6	1.69 (bd)	1.73 (bd)	
39.28	40.69	7	*	1.34 (dddd)	5: 2ax-2eq = 12.5; 2ax–3ax = 12.5;
		8ax	0.89 (dddd)	0.85 (dddd)	2ax-3eq = 6.0; 2eq-3ax = 6.0;
23.84	23.85				
		8eq	1.51 (dddd)	1.50 (dddd)	2eq-3eq = very small; 3ax-3eq = 17.0;
		9ax	*	1.32 (dddd)	5–6 = very small; 6–7 = 10.5;
30.95	30.42				
		9eq	*	1.39 (dddd)	7–8eq = 4.0; 7–8ax = 12.0; 7–11 = 4.0;
40.38	40.80	10	1.12 (m)	1.10 (m)	8ax-8eq = 12.5; 8eq-9eq = 4.0;
26.09	26.04	11	2.00 (m)	1.96 (m)	8eq-9ax = 4.0; 8ax-9eq = 4.5;
15.02	14.94	12	0.63 (d)	0.62 (d)	8ax-9ax = 12.5; 9ax-9eq = 12.5;
21.10	21.11	13	0.81 (d)	0.80 (d)	9ax-10 = 12.5; 9eq-10 = 4.0;
15.41	15.84	14	0.85 (d)	0.82 (d)	10–14 = 6.5; 11–12 = 6.5; 11–13 = 6.5.
23.44	23.84	15	1.60 (bs)	1.61 (bs)	
156.21	154.43	16			

Spectra have been recorded on a Bruker WM-500 spectrometer in C_6D_6 : $CDCl_3$ (1:1) solution; this solvent system was chosen to minimize overlap of ^1H-NMR resonances. [a]Assignments based on ^{13}C-^1H-shift correlated 2D-NMR spectroscopy via ^1J couplings, which showed the inter-relation of all the protonated carbons with the pertinent proton(s). The shift correlation with polarization transfer via J-coupling experiments were performed using a Bruker microprogramm adjusting the fixed delays D_3 and D_4 to give maximum polarization for $J_{C-H} = 135$ Hz; [b]Assignments based on extensive spin-spin decoupling and decoupling difference experiments; *submerged by other signals in the region 1.35-1.47 ∂.





1 $R-\overset{\oplus}{N}\equiv\overset{\ominus}{C}$

2 $R-N=C=S$

3 $R-\underset{H}{N}-\underset{H}{C}=O$

4 $R-\overset{\oplus}{N}\equiv\overset{\ominus}{C}$

5 $R-N=C=S$

6 $R-\underset{H}{N}-\underset{H}{C}=O$

7

$C_{16}H_{25}NS$ [from HRMS on the parent ion at M^+ 263.1703; $C_{16}H_{25}NS$ requires 263.1709 and ^{13}C-NMR data (table)]. Mass spectrum shows an intense peak at m/z 204 indicating a facile loss of HNCS. The presence of the isothiocyanate function was further substantiated by an IR absorption at ν_{max} 2100 cm^{-1}.

^{1}H- and ^{13}C-NMR spectra (see table) indicated that **5** possesses a bicyclic skeleton containing a trisubstituted double bond, three secondary Me groups and an olefinic methyl, the isothiocyanate function being linked to a tertiary carbon atom. The nature of the carbocyclic skeleton was established by aromatization of **5** which afforded cadalene (**7**).

Data which made it possible confidently to assign the position of the double bond and of the –N=C=S group in **5** were obtained by further consideration of both ^{1}H-and ^{13}C-NMR features, including extensive spin decoupling experiments and two dimensional ^{13}C-^{1}H-shift correlated spectroscopy, which allowed the chemical shifts, multiplicities and coupling constants for all the protons and chemical shifts for all the carbon atoms to be readily defined. Assignments are given in the table.

^{1}H-NMR data also provided key evidence for the relative stereochemistry of C-6, C-7 and C-10, through consideration of the J values of 6-H with 7-H and of 10-H with the adjacent methylene protons. The chirality at C-1 was established on the corresponding isocyanide **4**, as described below.

Isocyanide **4**, $[\alpha]_D = -65.70°$ (c = 1 in $CHCl_3$), ν_{max} 2135 cm^{-1}, had the molecular formula $C_{16}H_{25}N$ [from HRMS at 231.1983, $C_{16}H_{25}N$ requires 231.1988 and ^{13}C-NMR (see table)].

^{1}H- and ^{13}C-NMR features reported in the table, strongly reminiscent of those of **5**, led to formula **4** as the most likely for this compound. Final proof of the correctness of this structure and definition of part of the relative stereochemistry was provided by conversion of **4** into **5** by treatment with sulphur.

The relative stereochemistry at C-1 in **4**, and consequently in **5**, was established by ^{1}H-NMR spectra of **4** performed in the presence of varying amounts of $Eu(fod)_3$. The *pseudo*-axial proton linked to C-3 and the *pseudo*-equatorial proton linked to C-2 suffered strong europium shifts thus indicating their *cis*-relationship with the $-N^+\equiv C^-$ group. On the other hand 6-H must be *trans* to this function since its signal (δ 1.69) is much less affected by addition of the shift reagent.

Finally the most polar compound **6**, present in very small amounts and isolated as an oil, had the molecular formula

$C_{16}H_{27}NO$ (HRMS on the parent ion at M^+ 249.2090, $C_{16}H_{27}NO$ requires 249.2094). ^{1}H-NMR spectrum contained signals at δ 8.14 (1 H, d, J = 13.0 Hz) and 3.63 (1 H, broad) indicative of a HCONH- group, and at δ 5.36 (1 H, bs) attributable to the vinylic proton of a trisubstituted double bond. Resonances due to three secondary [δ 0.72, 0.92 and 0.98 (3 H each, d's, J = 7.0 Hz)] and one vinylic methyl [δ 1.69 (3 H, bs)] are also present. These data coupled with the presence in the IR spectrum of a band at ν_{max} 3440 cm^{-1}, suggested the compound could be the formamide corresponding to **4** and **5**. This was established by way of its synthesis, starting from isonitrile **4**. The ^{1}H-NMR spectrum shows that compound **6**, which thus differs from the other formylamino sesquiterpenes which are generally mixtures of the two rotational isomers of the formamide group, exists almost exclusively as the *trans* isomer, as indicated by the J value (13 Hz) of the signal at δ 8.14.

The structural similarity (including relative stereochemistry at C-7 and C-10) between the series **1–3** and **4–6** and their co-occurrence in the same sponge point to the biogenetic pathway reported in the scheme. The carbonium ion A, through a hydride shift, could generate the key intermediate B. The genesis of compounds **4–6** requires only introduction of the nitrogenous functions on carbonium ion B, whereas its rearrangement, through the cyclopropane-containing ion C, could account for the production of the spiro-axane ion D which is positively charged, just at the carbon atom carrying the functionalities in **1–3**.

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New phytotoxic butenolides produced by *Seiridium cardinale*, the pathogen of cypress canker disease[1]

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Summary. Two new butenolides, seiridin and *iso*-seiridin, were isolated from culture filtrates of *Seiridium cardinale*, the pathogen of cypress canker, a destructive disease of *Cupressus* and related *Coniferae*. These metabolites were characterized as 3-methyl-4-(2-hydroxyheptyl)-2(5H)-furanone and its 4-(3-hydroxyheptyl) isomer, respectively. Chlorotic and necrotic symptoms were produced on leaves of either host or non-host test plants by absorption of 0.3 mg/ml solutions of either compound. These also showed antibacterial activity.

Key words. Seiridium cardinale; cypress canker; seiridin; *iso*-seiridin; butenolides; phytotoxins; antibiotics.

Since its first introduction in Europe[2], the canker caused by the imperfect fungus *Seiridium cardinale* (Wag.) Sutt. et Gibs. *(Coelomycetes)* has become the major disease of the Mediterranean cypress (*Cupressus sempervirens* L.) and other species of *Cupressaceae*. This cypress canker, which had been previously reported in the U.S.A.[3] and later in other parts of the world, is a destructive disease that kills the affected trees. Over the last two decades, heavy losses have been caused in Italy and in other Mediterranean countries to the nursery industry, the cypress plantations used for afforestation and wind-breaks, and to ornamental cypresses[4,5].

The foliage of infected twigs and branches first shows a diffuse yellowing and later turns brown or reddish as the die-back progresses. Cankers develop on the live bark of branches and stem around the sites of infection of the pathogen. A cardinal-red discoloration and a necrotic browning of the infected bark tissues occur, and a flow of resin exudes from cracks formed on these cankers. Extensive infection leads to the dying of branches and eventually of the whole tree.

The nature and appearance of the damage caused by *S. cardinale* to its host suggest that necrotic toxins are produced in the infected tissues and are possibly involved in pathogenesis.

Early work[6,7,8] provided preliminary information on the toxicity of culture filtrates of *S. cardinale* on test plants and on the molecular weight of the toxins involved. In a previous paper[9], the in vitro production of phytotoxins by the pathogen under various cultural conditions, and the first attempts to separate the active substances from culture filtrates, were reported.

This paper briefly reports on the production, isolation, structure determination and biological activity of two new phytotoxins from culture filtrates of *S. cardinale*. Details of this study and further information will be presented elsewhere.

The single-spore strain of *S. cardinale* used in this study was isolated from an infected cypress tree *(C. sempervirens)* in Italy and was grown in tubes of Czapek-corn meal agar. Czapek's medium with the addition of 2% corn meal was dispensed in 1 l Roux flasks (150 ml/flask) and used for stationary cultures. Each flask was seeded with 2 ml of a suspension of the homogenate of two 15-day-old culture tubes in 50 ml sterile water. The flasks were incubated at 23 °C for 30 days in the dark.

After removal of the mycelial mat, the culture filtrate was adjusted to pH 4 with 0.1 N HCl and subsequently extracted 4 times with one fourth its volume of *t*-butyl-methyl-ether. The pooled organic extract was evaporated under reduced pressure to give an oily residue, which was fractionated on a silica gel column using chloroform – *iso*-propanol (9:1, v:v) as an eluent system. The fractions were tested for toxicity (see later) and examined by thin layer chromatography (TLC) using various solvent systems. The TLC plates developed with petroleum ether (b.p. 40–70 °C) – acetone (6:4, v:v) showed that the phytotoxic activity was associated with two substances having R_f values of 0.51 and 0.56. The fractions containing these substances were combined and evaporated under reduced pressure. Purification of the residue on a silica gel column, followed by TLC of the phytotoxic fractions (both the column and plates were run with the petroleum ether – acetone mixture mentioned above), afforded two pure oily substances which were named seiridin (**1**) (49.5 mg/l) and *iso*-seiridin (**2**) (17.4 mg/ml).

Seiridin had a molecular formula $C_{12}H_{20}O_3$ as shown by high resolution mass spectral data, m/z 212.1413 (calc. 212.1413), and an optical rotation $[\alpha]_D^{25} = -4.80°$ (c = 1.48 $CHCl_3$). IR and UV absorption frequencies were typical for a $\Delta^{\alpha,\beta}$ butenolide [2(5H) furanone])[10].

These findings were confirmed and further extended by NMR