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Chemical defence in sponges

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Abstract - Many sponges are known to synthesize secondary metabolites showing a great diversity of structures and of biological activities. Furthermore, the same sponge species, although sessile, exposed to predation and apparently devoid of mechanical or physical defence, suffer negligible predation and are seldom invaded by epizoic organisms. By putting these two observations together, a cause-effect relationship has been repeatedly suggested. Our purpose is to review the existing data and the arguments having led to this proposal. Recent structural studies and biological tests performed in our laboratory will serve to illustrate the complexity of the chemically mediated interactions of sponges with their environment.

INTRODUCTION

Every organism interacts with a variety of other organisms during the course of its life-time. Important types of interaction are the predator-prey and the internal or external parasite-host interactions which impose a constant chance of extinction of prey populations. Therefore, any adaptation of the prey capable of reducing the impact of the predatory pressure would be favored, and thus, it is no wonder that many organisms have evolved elaborate antipredator systems. The diversity of these defensive mechanisms is as great as the variety of predators. This is well illustrated by the various defensive strategies found amongst the marine invertebrates (ref. 1 to 3).

For example, many of these benthic animals have spines or thickened skeletons to discourage predators. The sea-urchin *Diadema* has long slender spines with reverse barbs. Gastropods living in areas of heavy crab predation often have thickened shells, a thickened outer lip, a low spine and small or narrow apertures to deter intruders (ref. 2). Most of the Cnidaria have developed specialized poisonous stinging cells, the nematocysts, that constitute a very effective defence mechanism. Hermatypic corals put much energy into the production of a protective compact calcareous exoskeleton, whereas some scallops have evolved violent escape behavior when confronted by predators. When disturbed, the sea-anemones quickly contract their tentacles and diminish their exposed surface. This list could be enlarged almost indefinitely.

Next to species having evolved mechanical or behavioral defensive adaptations, there is a great number of marine invertebrates that produce or sequester bioactive secondary metabolites (ref. 4 to 6). It is now generally admitted that most of these compounds serve to discourage attack of mobile predators, to kill potential fouling or contaminating organisms, or perhaps to prevent competitors from intruding into the living space of sessile species. The defensive role of a few of these secondary metabolites has been demonstrated, but before extending systematically this assertion we agree with Faulkner (ref. 6) that many more of them should be assayed for their effectiveness. Considering the great number of indexed species of marine invertebrates ($\sim$ 200,000), only a small fraction of them has been examined until now for their ability to produce or sequester defensive compounds, and some classes of organisms have been studied more intensively than others. Our present view of the distribution of chemical defence in these animals is therefore undoubtedly distorted. Nevertheless, it is apparent that chemical defence is very unevenly distributed.

A similar situation prevails in the terrestrial arthropods and it has been pointed out by Pasteels et al. (ref. 7) that phylogeny alone does not determine the distribution of chemical defence within these latter organisms, but that ecological conditions may be a more important factor. To quote the authors : "chemical defence should be positively correlated with the probability of discovery by predators and negatively with the existence of alternative defence mechanisms".

In our opinion this prediction could be easily extended to the marine invertebrates. Thus within the molluscs, presence of chemical defence is mainly restricted to the shell-less

molluscs, while amongst the Cnidaria it is limited to the octocorals which are almost devoid of stinging cells and which have no protective exoskeleton. Many species of other groups of marine invertebrates e.g. the sponges, the tunicates, the starfishes, the sea-cucumbers or the bryozoans which are also known to be devoid of obvious mechanical or behavioral defence mechanisms, are toxic and/or unpalatable for many predators. The distribution of chemical defence is often patchy within these groups. Hence, not all sponges or octocorals contain bioactive secondary metabolites. Moreover, it is amongst the aposematic sessile or slow moving species, living largely exposed to the view of mobile predators such as fishes, that the percentage of ichthyotoxic species is the highest, especially in the tropics where a heavier predatory pressure of fishes is assumed to exist in comparison with more temperate regions (ref. 8 and 9). Of course, such a generalization based on indirect or scarce information should be considered as heuristic prediction needing either confirmation or amendment.

DEFENCE IN SPONGES

Sponges (phylum PORIFERA) are primitive multicellular invertebrates which have contributed significantly to the biomass of the marine ecosystems since at least the Inferior Cambrian period ($\pm$ 600.10^{-6} years). They have a world-wide distribution and are found in a great diversity of habitats. It is generally assumed that this persistent ecological success is due, not only to well adapted and adaptable reproductive and physiological strategies, but also to effective defence mechanisms.

Physical or behavioral defences are rare amongst sponges. Thus, a few sponges seem to derive protection from the presence of sharp spicules (e.g. *Tetilla*) or of special cortical spicules providing a superficial armour (*Geodia*). Others live in crevices or holes, or are covered by epibiotic organisms which can ensure them an effective camouflage. As a consequence, we may expect that chemical defence should by far be the commonest mode of defence found amongst sponges. Indeed, many of them are toxic, especially in the tropics, to a wide range of organisms or at least unpalatable for most predators (ref. 10). On the other hand, a great number and variety of secondary metabolites have been isolated from sponges (ref. 6, 11 and 12), but few data have yet been published concerning their precise ecological role and the possible cause-effect relationship that may exist between the two observations (ref. 13).

Being sessile microphagic feeders, sponges have to face a large array of dangers : attacks from mobile predators, overgrowing by space competitors, fouling by epizoic organisms and infection by external pathogenic microorganisms. Thus, it is probably not mere chance if many sponges show antibacterial and/or antifungal activities. In the past few years, an increasing number of secondary metabolites responsible for these activities have been isolated and identified (ref. 14 to 17). In addition, some of these antibiotics, as well as several other sponge metabolites, have been shown to possess the ability to kill or to inhibit the growth of other forms of marine life. For example, the sesterterpenes idiadione and 12-deacetyl-12,18-diepiscalaradial are toxic to the starfish *Pisaster giganteus* and to the brine shrimp *Artemia* sp.. Both compounds immobilize the larvae of the red abalone *Haliothis rufescens* (ref. 18). The antimicrobial peptides discodermin B, C and D inhibit the development of starfish embryo (ref. 19), whereas sesquiterpenoid quinones isolated from *Dysidea avara* inhibit cell cleavage of the fertilized eggs from the sea-urchin *Spaerechinus granularis* (ref. 20). These metabolites may be viewed as a protection against external pathogens invasion. Another possible ecological role for these compounds is to act as antifouling agents. Indeed, macroinvertebrate fouling is a complex process thought to involve the establishment of a preliminary microbiological film (ref. 21). Nevertheless, these working hypotheses, although attractive, have yet to be unambiguously demonstrated.

It has also been observed that allelochemical substances maintain the presence of bare zones around sponges, thus serving as an important weapon in interference competition for space amongst coral reef invertebrates (ref. 22). To our knowledge, in only one case has the active compound been isolated and its structure determined. Faulkner et al. (ref. 23) have demonstrated that siphonodictidine, a terpenoguanidine, is responsible for the inhibition of coral growth around the base of the oscular chimney of the burrowing sponge *Siphonodictyon* sp., thus preventing overgrowth of the sponge by the coral polyps.

Results of investigations of the ecology of toxicity in marine sponges from different latitudes indicated that toxicity increased with decreasing latitude (ref. 8 and 10). On the other hand, in marine tropical ecosystems, the most important predators and grazers are fishes. This is not characteristic of cold-water ecosystems, where this role is played predominantly by invertebrates (ref. 2). It has been suggested, by comparing these two situations, that toxicity in non-cryptic sedentary sponges may have evolved *via* natural selection due to high intensities of fish predation (ref. 8 to 10).

In a few cases, it has been reported that the ichthyotoxicity was associated with peculiar secondary metabolites (e.g. the latrunculins from *Latrunculia magnifica* (ref. 24 and 25) ; the sigmosceptrellins from *Sigmosceptrella laevis* (ref. 13) ; cavernosine from *Fasciospongia cavernosa* (ref. 26) ; the strongylophorins from *Strongylophora durissima* (ref. 27)).

Interestingly, all these sponges are tropical species living well exposed on the slope of coral reefs. Of course, this again does not imply that all the secondary metabolites isolated so far from exposed sponges have necessarily evolved as a defensive mechanism against fish. Moreover, ichthyotoxicity is not the only chemical strategy able to protect sponges against heavy fish predation. Deterrence may also be effective and *a priori* it is not evident how a particular metabolite will be operative. The following examples are illustrative of the complex and subtle chemically mediated interactions between sponges and their environment.

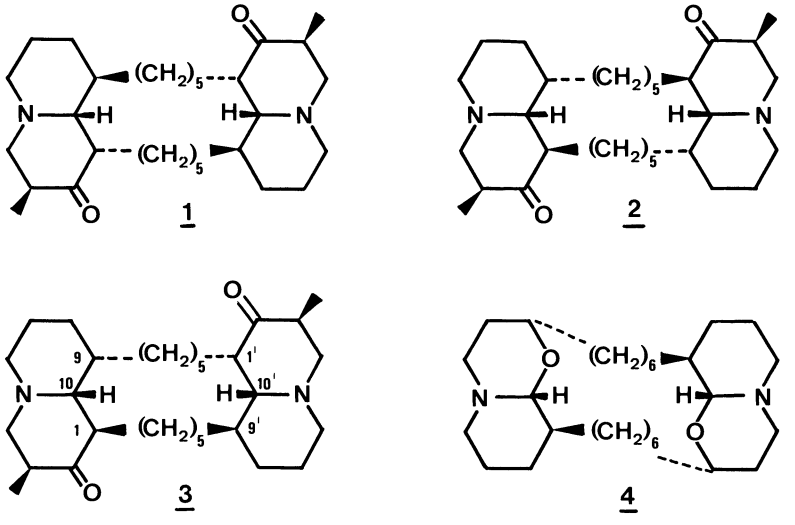
PETROSIA SERIATA

P. seriata is a great dark brown sponge common on the reefs of the North Coast of Papua-New Guinea. At Laing Island, it lives well exposed at the foot of the outer slope of the reef at depth varying from 20 to 35 m. The dichloromethane extract of sun-dried specimens of the sponge is toxic to the fish *Lebistes reticulatus* (LD 20 mg/l).

In recent papers (ref. 28 and 29) we reported that this toxicity is associated with an alkaloidic fraction which contains at least eight different alkaloids. The major compound of this complex mixture, named petrosine (1), could be isolated as a crystalline compound. Its structure was established by X-ray diffraction analysis (ref. 28). It is a bis-quinolizidine having a C_2 symmetry and containing a sixteen-membered ring. Two further ichthyotoxic stereoisomers of 1, petrosin-A (2) and petrosin-B (3) could be isolated after repetitive alumina column chromatographies. Comparison of their spectral properties with those of 1 indicated that the three derivatives differ only by their configuration at the junctions of the quinolizidines with the sixteen-membered ring. The determination of the configuration at these ring-junctions was based on the values of the coupling constants J_{1-10}, $J_{1'-10'}$, J_{9-10} and $J_{9'-10'}$ measured by 2D- ^1H NMR (ref. 29).

Recently, Nakagawa et al. (ref. 30) have isolated from the Australian sponge *Xestospongia exigua*, a series of compounds, the xestospongins (e.g. structure 4 for xestospongin A), whose structures are related to those of the petrosins. The ^{13}C NMR spectra of crude samples of some of the remaining unknown derivatives of the *Petrosia* alkaloidic fraction, show characteristic signals around 95 ppm attributable to CH groups, bonded to two heteroatoms, similar to the HC-10 and HC-10' of the xestospongins. This suggests that 1-oxa-quinolizidine alkaloids are also present in our sponge.

The results of the ichthyotoxicity and feeding inhibition tests (see figure 1) show clearly that the petrosins may be effective in preventing heavy fish predation on *Petrosia seriata*. It is interesting to note that the concentration at which the deterrence by the petrosins becomes effective corresponds to the concentration of the total alkaloid content of the sponge. This indicates that the effectiveness of the feeding inhibition depends rather on the concentration of the total alkaloidic mixture, than on that of one particular component of the mixture. Although devoid of alkaloids, the methanolic extract is also deterrent, thus suggesting that other antifeeding compounds are present in *P. seriata*.



	Dragendorff Spot test	% of the sponge dry weight	Ichthyotoxic activity (LD mg/l)[a]	Antifeeding activity[b]
Petrosia seriata				
Dichloromethane extract	+	4.6%	+ (20)	+
Methanol extract	-	17.5%	- (>50)	+
Total alkaloidic fraction	+	2.0%	+ (10)	+
Petrosin (1)	+	0.5%	+ (10)	+[c]
Petrosin-A (2)	+	0.04%	+ (10)	+[c]
Petrosin-B (3)	+	0.02%	+ (10)	+[c]
Carteriospongia foliascens				
Dichloromethane extract	-	2.5%	+ (20)	-
Methanol extract	-	3.7%	- (>50)	-
Total terpenic fraction	-	1.1%	+ (5)	-
5	-	-	- (>40)	NT
6	-	Major compound	+ (5)	-[d]
7	-	-	+ (20)	NT
8	-	-	- (>40)	NT
9	-	-	+ (40)	NT
10	-	-	+ (5)	NT
Axinella damicornis				
Methanol extract	NT	25.0%	- (>50)	+
Non polar fraction	NT	3.5%	- (>50)	-
Polar fraction	NT	21.0%	- (>50)	+
23	NT	1.5%	- (>50)	-

NT = not tested
[a] The procedure is described in ref. 13. ( ) dose required to kill the fishes.
[b] The procedure is described in ref. 31. The antifeeding activity was measured at concentrations equal to that of the compound or the fraction in the sponge.
[c] (+) at concentrations $\geqslant$ 2% and (-) at concentrations $<$ 2%
[d] (-) at concentrations $\leqslant$ 2.5%.

Figure 1 : Ichthyotoxic and antifeeding activities.

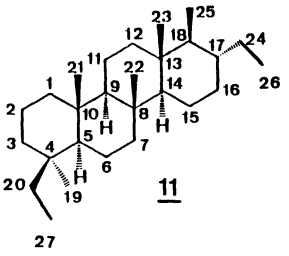
CARTERIOSPONGIA FOLIASCENS

C. foliascens is also a common, non-cryptic sponge living on the outer slope of the reef surrounding Laing Island (Papua-New Guinea). Again, we observed that the dichloromethane extract of sun-dried specimens of this sponge is toxic to *Lebistes reticulatus* (LD : 20 mg/l). This time, the toxicity was associated with a complex neutral fraction from which six tetracyclic terpenoids (5 to 10) could be separated (ref. 32). Their spectral properties clearly suggested that they belong to the 20,24-dimethylscalarane series (11), and more precisely, that they are 12α-acetoxy-20,24-dimethylscalarane derivatives differing in the substitution pattern of ring D.

The acid 5 was purified as its methyl ester (12) prepared by treatment of crude 5 with an ethereal solution of CH_2N_2. The structure of 12 first proposed on the basis of its spectral properties, was confirmed by X-ray diffraction analysis (ref. 33). The ethyl group at C-4 was found to be axial (β).

Several other 19 (or 20), 24-dimethylscalarane derivatives have already been isolated from three *Carteriospongia* (ref. 34 to 36) and one *Dysidea* species (ref. 37). The relative configuration at C-4 has been established without ambiguity by X-ray diffraction analysis for two of these derivatives, namely 13 isolated from a Fijian *Carteriospongia* sp. (ref. 36) and 14 isolated from an Australian sample of *Carteriospongia foliascens* (ref. 34). In 13, the C-4 ethyl group is axial (β) as in 5. In contrast, the C-4 ethyl group is equatorial (α) for 14. In all other C_{27} derivatives described, the position of the ethyl group is either undetermined (ref. 37) or claimed to be equatorial only by analogy with the structure of 14 (ref. 34 and 35). This confusing situation, together with the fact that we had to determine the configuration at C-4 of our derivatives, prompted us to devise a practical method for solving this problem.

In the ^{13}C NMR spectrum of 12 the signal at 24.5 ppm (t) is attributable to the methylene at C-20 (ref. 38). Since in this compound the ethyl group is axial, it is expected that in the epimeric compound at C-4, this carbon atom will absorb at a much lower field. An analogy is given by the scalarane derivatives (e.g. 15) in which the chemical shift of the axial and equatorial methyl group is about 21 and 33 ppm respectively (ref. 39). The chemical shift of



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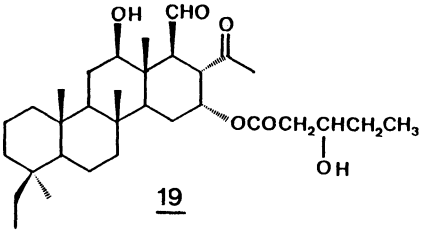

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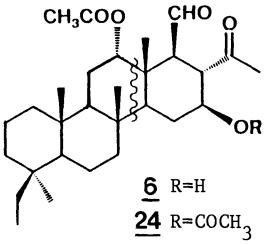
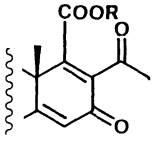
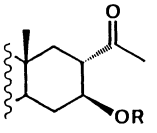


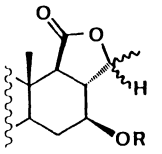
6 R=H
24 R=$COCH_3$

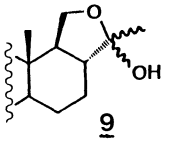


5 R=H
12 R=$COCH_3$



7 R=H
20 R=$COCH_3$





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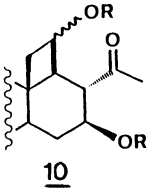

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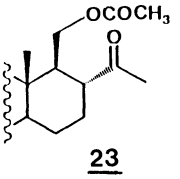

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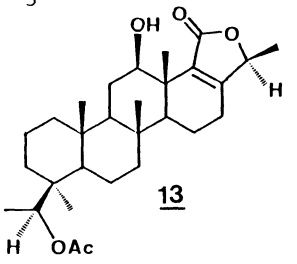
8 R=H
22 R=$COCH_3$



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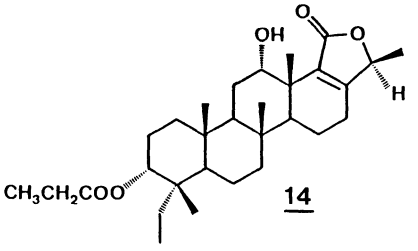

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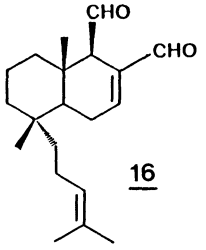

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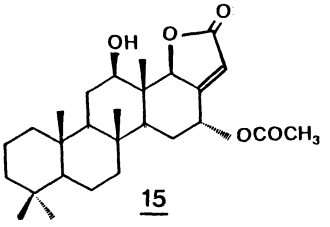

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the ethyl methylene would thus be of diagnostic value for the determination of the configuration at C-4 in the C_{27} tetracyclic terpenes. Suitable derivatives having the 4α-ethyl configuration are not readily available for comparison purposes. The only structurally related compound reported in the literature is the diterpene 16, isolated from the liverwort *Trichocolecpsis sacculata*, in which the pertinent methylene absorbs at 37.3 ppm (ref. 40).

This prompted us to synthesize the model compound 18, which was prepared from dehydroabietic acid methyl ester (17) using the sequence of classical reactions described in figure 2. In compound 18 the ethyl methylene absorbs at 36.4 ppm. Hence, the chemical shift of this carbon atom may be used to assign the configuration at C-4 of the 19(or20),24-dimethylscalarane derivatives. Application of this observation led us to assign the 4-axial position (β)

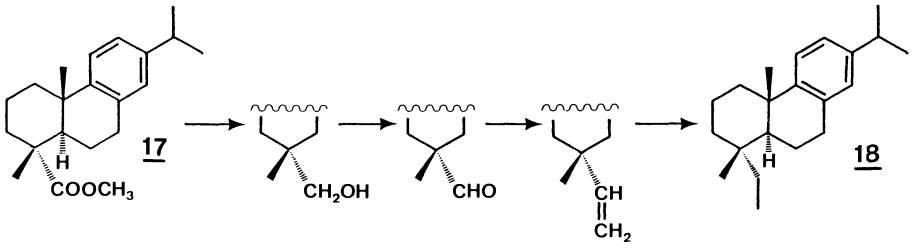


Figure 2 : Hemisynthesis of the model compound 18 from dehydroabietic acid methyl ester (17).

to the ethyl group of all our derivatives, as well as to correct the structure of foliaspongin (19) (ref. 35) (see figure 3).

Compound	7	10	12	16	18	19	22	23	24
δCH_2CH_3	24.6	24.6	24.7	37.3	36.4	24.5	24.5	24.6	24.5

Figure 3 : Comparison of the ethyl methylene chemical shifts.

The spectral properties of our second derivative are compatible with structure 6. This structure is identical, except for the configuration at C-4, to that of one of the derivatives isolated by Hofheinz and Daly from an Australian specimen of *C. foliascens* (ref. 34). Direct comparison of their ^1H NMR spectra showed nevertheless that they were identical (unfortunately, the comparison could not be made at the level of the ^{13}C NMR spectra). This infers that the configuration at C-4 for the derivative of the Australian sample should be reversed. Moreover, it is more than likely that this will also be the case for the other C_{27} derivatives isolated from the same sponge, but for which detailed spectroscopic data are not available (ref. 34).

The ^1H and ^{13}C NMR spectra of 7 and its acetylated derivative 20 are very similar to those of 6 except that the signals due to the aldehyde group at C-18 are missing, thus suggesting that in 7 and 20 the aldehyde group had been replaced by an hydrogen atom. This was proved by transforming 6 into 20 using the reaction scheme described in figure 4. Acetylation of 6 followed by oxidation of the aldehyde function by potassium permanganate in acetone yielded the acid 21 (yield : 60%). This acid was then treated with oxalyl chloride in pyridine to give the corresponding acyl chloride. The latter was decarboxylated (yield = 40%) using the radical decarboxylation reaction described by Barton et al. (ref. 41). The C_{26} derivative isolated is identical in all respects with compound 20.

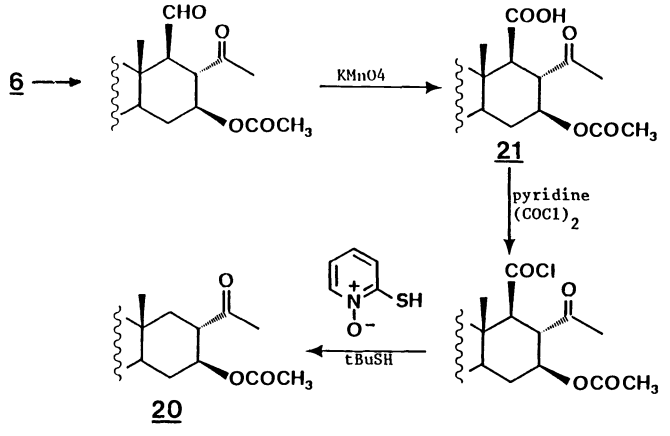
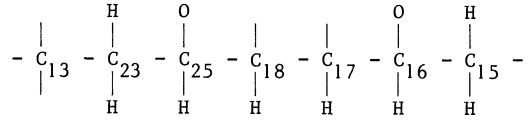


Figure 4 : Chemical correlation between 6 and 20.

The structure of the next two derivatives (8 and 9) were deduced mainly from their spectral data and extensive ^1H NMR double irradiation experiments performed on the acetate 22 and the keto ester 23 obtained by acetylation of 8 and 9 respectively.

The structure of the last derivative (10) is by far the most interesting. Indeed, only three singlets attributable to angular tertiary methyl groups could be seen in its ^1H NMR spectrum. This observation, coupled to the fact that in its ^{13}C NMR spectrum the quartet attributable to the H_3C-23 present in the other derivatives, is replaced by a triplet (δ 37.3), implies that in 10, C-23 is not only bonded to C-13 but also to another carbon atom of the skeleton.

This became evident after extensive double irradiation experiments which clearly established the sequence :

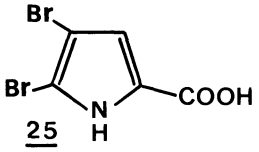


The long-range coupling constant values (J=5 and 0Hz) observed between H-18 and the C-23 methylene protons agree with a cyclobutane ring (ref. 42). To our knowledge such a cyclobutane ring, constructed from an angular methyl group, is unusual in natural substances.

From the results reported in figure 1 it appears that the toxicity of *C. foliascens* against *L. reticulatus* is restricted to the terpene mixture and is unevenly distributed amongst the latter's different constituents. Of course, such a distribution is only characteristic for the test organism used and a completely different response could have been obtained if another species had been utilized. It is a general feature of chemical defense that the biologically active fraction is remarkably complex. This chemical complexity is still poorly understood. This could be the reflection of a synergistic effect or a way for the sponge, as suggested for insect chemical defense (ref. 7), to avoid easy counteradaptation by predators and/or to hit a wider range of competitors, but there is no evidence to support these hypotheses. Contrary to the toxic alkaloids from *P. seriata* which also show antifeeding activity, the toxic tetracyclic terpenes from *C. foliascens* do not inhibit feeding of *L. reticulatus*.

AXINELLA DAMICORNIS

A. damicornis is a yellow-orange sponge of medium size living on hard bottoms along the coast of the Mediterranean Sea. Like the two previously discussed sponges, it apparently does not suffer heavy fish predation. The total methanolic extract of this sponge is non-toxic for *L. reticulatus*, but it is clearly unpalatable (see figure 1). This activity is restricted to the polar fraction of the methanolic extract. The major compound of the active fraction, is the dibromopyrrole 25 (ref. 43). Although several derivatives of bromopyrrole-2-carboxylic acid have been isolated from *Agelas* species (ref. 44 to 47), 4,5-dibromopyrrole-2-carboxylic acid itself had been found only, as a minor constituent, in *Agelas oroïdes* (ref. 45) and in an unidentified sponge collected off the Marshall Islands (ref. 48).



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The amino acid 25 is neither toxic nor antifeedant and is thus an example of a major secondary metabolite present in an exposed sponge which does not seem to be involved in the chemical defence mechanism of the producing organism against fishes. Nothing is known yet about the identity of the compounds rendering the sponge unpalatable to *L. reticulatus*.

CONCLUSION

During the last few years, there has been an intensive search for new pharmacologically active compounds and as a consequence an increasing interest in the chemistry of marine organisms in general and of sponges in particular. This has led to the discovery of a great, and still increasing, number of novel and interesting secondary metabolites. However, much less is known about the biological activities and ecological role of these compounds. Our recent work is in keeping with the current trends to bridge the gap between these two aspects.

P. seriata and *C. foliascens* are further examples of sponges that produce ichthyotoxic compounds able to efficiently participate in their defence strategy. The way these compounds operate is nevertheless slightly different. Whereas the petrosins are both ichthyotoxic and deterrent, the tetracyclic terpenes are only toxic. However, these results must be interpreted with caution, since they were obtained on one test animal only (*L. reticulatus*). Obviously, *in situ* and large-scale comparative studies on the activity of these defensive compounds on an array of marine predators and competitors are badly needed. In this context, it is interesting to emphasize a recent observation, namely that the above-mentioned ichthyotoxic compounds are also cytotoxic for dissociated cells of the freshwater sponge *Ephydatia fluviatilis* and inhibit their aggregation (ref. 49). This suggests that these derivatives could be multipurpose defensive agents whose broad spectrum of activities may have been selected for by the diversity of predators and competitors.

4,5-Dibromopyrrole carboxylic acid (25), the major compound of the deterrent fraction of *A. damicornis* is neither ichthyotoxic nor deterrent, nor cytotoxic (ref. 49) and thus seems not to be involved in a defence mechanism. Its role, if any, remains an open question as is the nature of the compound(s) responsible for the deterrence of *A. damicornis*.

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