Perplexing distribution of 3-alkylpyridines in haplosclerid sponges

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Abstract: In this study we reviewed the natural product literature for the distribution of 3-alkylpyridines among sponge taxa. In parallel, we traced selected 3-alkylpyridines, amphitoxins, in three haplosclerid genera (Amphimedon, Callyspongia, Haliclona) in order to establish the utility of such compounds as genuine chemotaxonomic markers. We confirmed that this group of compounds had been almost solely extracted from sponges of the order Haplosclerida. Three groups of compounds within the 3-alkylpyridine derivatives were noteworthy, as they appear to be concentrated in restricted taxonomic units within the order Haplosclerida: 1) polymers, 2) cyclic dimers, and 3) bicyclic dimers. There was a concentration of the polymer amphitoxin in the families Niphatidae and Callyspongiidae of the suborder Haplosclerina, and more particularly in the genera Amphimedon and Callyspongia. Our experimental results reconfirmed the presence of amphitoxin in Callyspongia (Euplacella) biru, but we were unable to trace any amphitoxins or 3-alkylpyridines of any kind in Callyspongia (Callyspongia) truncata, Haliclona sp., and Amphimedon aff. queenslandica. Assuming that these classifications are correct, our results diminish the value of both amphitoxins and 3-alkylpyridines as monophyletic markers.

Keywords: 3-alkylpyridines, amphitoxin, chemotaxonomy, Order Haplosclerida, secondary metabolites

Introduction

Sponges have proven to be a magnificent source of numerous highly bioactive compounds with pharmaceutical potential (Faulkner 2000, Sipkema et al. 2005). Since the 1960s natural product chemists have been active in extracting these compounds from sponges, which were collected directly and randomly from the seas. For the past thirty decades several authors have been considering sponge compounds as candidates for additional markers that could potentially be useful in verifying or aiding the standing phylogeny of the Porifera. (e.g. Bergquist and Hartman 1969, van Soest and Braekman 1999, Erpenbeck and van Soest 2002). This classification of sponges is not infrequently disputed amongst sponge taxonomists and in fact the phylogenetic tree has been altered several times over the past decades. The field of chemotaxonomy in its turn has, however, yet to prove its utility.

The bulk of published data on natural products from marine organisms has been archived in a database called MarinLit (Munro and Blunt 2004) that is updated annually. Andersen et al. (1996) and van Soest and Braekman (1999) reviewed the MarinLit database to discuss the utility of secondary metabolites of sponges as systematic tools. One conclusion from these studies was that 3-alkylpyridine derivatives might be useful markers for the Order Haplosclerida. Since the publication of the reviews of Andersen et al. (1996) and van Soest and Braekman (1999), numerous additional compounds have been isolated from sponges. We, therefore, once again reviewed the distribution of 3-alkylpyridines in sponge genera. A well-reported problem with allocating taxonomic relevance to a compound based on the results of literature reviews is the strong bias of natural product laboratories to publish only novel compounds, thereby confounding any conclusions on the absence of compounds in certain taxonomic groups. To overcome this problem we selected particular 3-alkylpyridine derivatives, amphitoxins, and traced their distribution in representatives of three genera to establish whether such compounds could be viewed as genuine chemotaxonomic markers.

Material and methods

Literature review

MarinLit (Munro and Blunt, 2004), “Web of Science” and “scholar.google.com” were reviewed for publications reporting extraction of 3-alkylpyridine derivatives from sponges. Keywords of all known names within this compound
group were submitted in the databases, as well as the names of the genera within the Order Haplosclerida.

Collection, extraction and fractionation

Four tropical reef sponge species were collected at depths ranging from 2-10 m: Callyspongia (Euplocella) biru de Voogd 2004 (Sulawesi, Indonesia), Callyspongia (Callyspongia) truncata (von Lendenfeld, 1887) (Shizouka, Japan), Amphimedon aff. queenslandica Hooper and van Soest 2006 (Okinawa, Japan), Haliclona sp. (Kagoshima, Japan), (see Fig. 1A-D). Voucher specimens have been deposited at the Zoological Museum, University of Amsterdam, the Netherlands (C. (E.) biru collection number POR.15222), and at the National Museum of Natural History Naturalis, Leiden The Netherlands (C. (C.) truncata, A. aff. queenslandica, Haliclona sp. collection numbers respectively: POR.2971, POR.2972, POR.2973). Two individuals of each species were screened for amphitoxins.

Amphitoxins are known to be mainly responsible for the bioactivity of the crude extracts of C. (E.) biru (Dubut 2000 as Callyspongia sp. "blue"; de Voogd et al. 2005). To date no amphitoxins have been reported from C. (C.) truncata, while a large number of bioactive polyacetylenes have been extracted from this species (Nakao et al. 2002).

Each sponge was extracted with MeOH (500 ml x 3) and the extract was partitioned between water and CHCl₃. The water-soluble fraction was extracted with n-butanol. The organic phase was partitioned between MeOH/H₂O (9:1) and n-hexane. The aqueous MeOH layer was then partitioned between MeOH/H₂O (6:4) and CHCl₃. Finally the n-butanol fraction and MeOH/H₂O (6:4) fraction were combined. All fractions were analyzed by TLC on silica gel with CHCl₃/MeOH/H₂O (7:3:0.5) and different mixtures of CHCl₃/MeOH. Tertiary or quaternary bases were visualized by spraying with Dragendorff reagent. The fractions exhibiting orange/red spots were subsequently fractionated by ODS flash chromatography using stepwise elution of aqueous MeOH (0 % to 100 % MeOH), followed by elution with CHCl₃/MeOH/H₂O (7:3:0.5). The fractions showing spots positive to Dragendorff on TLC were further separated by ODS HPLC (Column: COMOSIL-5C₁₈-ARH ø 10 x 250 mm; flow rate 2 ml/min.; UV 266 nm) using gradient elution from 35 % MeCN/0.05 % TFA to 80 % MeCN/0.05 % TFA. The peaks positive to Dragendorff were analyzed by ¹H NMR spectroscopy to examine if typical proton signals for amphitoxin were present.

As C.(E.) biru has previously been shown to contain amphitoxins (Dubut 2000, de Voogd et al. 2005), the above procedure was first performed on this species to obtain a standard reference for amphitoxin. After a standard was obtained, the extracts of the other sponges (after solvent partitioning and ODS flash) were first checked for the presence of a similar compound by comparing Rf values on TLC with that of amphitoxin, before subjected to HPLC.

Results

Literature review

We identified 49 records reporting extraction of 3-alkylpyridine alkaloids from sponges. The majority of the records were identified to genus-level and belonged to the Order Haplosclerida. There were three records of 3-alkylpyridines extracted from sponges of other orders: Theonella swinhoei (Lithistida, from Kobayashi et al. 1989), Batzella sp. (Pociloesclerida, from Segraves and Crews 2005), and Halichondria sp. (Halichondrida, from Chill et al. 2002).

Within the Order Haplosclerida, 3-alkylpyridines have been recorded from the 5 of the 6 main marine haplosclerid families, but have not been recorded from all genera belonging to these families. In the suborder Haplosclerina the families represented were Callyspongiidae (2 genera, 5 species), Chalinidae (2 genera, 3 species), Niphatidae (3 genera, 6 species), and in the suborder Petrosina the families were Petrosiidae (2 genera, 3 species), and Phloedictyidae (1 genus, 1 species).

Three groups of compounds appeared to be concentrated in small taxonomic units (see Table 1A-C):

1. Polymers (hali/amphitoxins)
2. Cyclic dimers (cyclostelletamines/haliclamines)
3. Bicyclic dimers (haliconacyclamines/halicyclamines/arenosclerins)

Extraction and fractionation

Amphitoxin was successfully extracted from the C. (E.) biru specimens, and was present in the 20-100% elution by ODS chromatography of the combined n-butanol and MeOH/H₂O (6:4) fractions. We were unable to trace any amphitoxins of 3-alkylpyridines from the extracts of the C. (C.) truncata, Haliclona sp. or A. aff. queenslandica sp. specimens.

Discussion

We confirmed that 3-alkylpyridines have almost exclusively been isolated from sponges of the order Haplosclerida, after having made the necessary amendments on sponge taxonomy and nomenclature. There are, however, three reports of 3-alkylpyridines from sponges of other orders: Theonella swinhoei is a probable case of mislaid labels or undetected haplosclerid overgrowth (cf. van Soest and Braekman 1999), and Batzella sp. and Halichondria sp. remain unresolved for the time being. It must be noted, that these genera share a single diactinal spicule type with Haplosclerida, making it possible that they have been misidentified Haplosclerida.

In our view the use of more specific compound structures/groups, rather than the relatively broad nominator “3-alkylpyridine derivatives”, ought to be the next step in supporting classifications based on compounds.

In previous reviews linear 3-alkylpiperidines appeared to be concentrated in the families Callyspongiidae and Niphatidae, while cyclic 3-alkylpiiperidines were concentrated in Chalinidae and Petrosiidae (Andersen et al.
1996, van Soest and Braekman 1999). Records of extracted compounds published since that review (notably the cyclic 3-alkylpiperidines found in *Amphimedon* sp. by Matsunaga et al. 2004), however, indicate that a taxonomic distinction based on the presence of cyclic or linear 3-alkylpiperidine derivatives can most likely no longer be upheld.

Three groups of compounds within the 3-alkylpyridines were noteworthy in that they have structures with presumed similar biogenetic pathways and appear to be concentrated in small taxonomic units (see Table 1A-C). Based on the common presence of these specific compounds, the close relationship between Callyspongiidae, Chalinidae, Niphatidae, and Petrosiidae is confirmed. Previous works suggested that the closely related polymers halitoxin and amphitoxin might be markers for the family Niphatidae. Our review showed a concentration of these types of compounds not only in Niphatidae, but also in Callyspongiidae, and more particularly in the genera *Amphimedon* and *Callyspongia* (Table 1A).

Our laboratory work reconfirmed the presence of amphitoxin in *C. (E.) biru*, but we were unable to trace any amphitoxins from *C. (C.) truncata*, nor from the *Haliclona* sp. and *A. aff. queenslandica*. In fact, the situation is rather more complex as we did not extract 3-alkylpyridines of any kind from these three specimens. The sponge specimens that did not contain 3-alkylpyridines were collected from Japan, but we do not suspect this is a case of geographic variation of compound distribution as these compounds have been isolated from Japanese sponges before (Fusetani et al. 1989, 1994, Matsunaga et al. 2004). Causes of these results may lie in the frequently reported high natural variation in compound production, which in turn may be due to physical and environmental variation (e.g. Thacker et al. 1998, de Voogd et al. 2004, de Voogd 2007) or to symbionts that may be the true producers of the compounds (e.g. Jadulco et al. 2002, Becerro and Paul 2004).

Sponges generally harbor vast amounts of symbionts, among which there are not only the specialists, which may...
Table 1A-C: Three compound groups that appear to be concentrated in restricted taxonomic units (compound figures reproduced from Andersen et al. 1996): A. polymers, B. cyclic dimers, C. bicyclic dimers.

### A. Polymers

<table>
<thead>
<tr>
<th>Family</th>
<th>Species</th>
<th>Compound</th>
<th>Location</th>
<th>Identification</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Callyspongidae</td>
<td>Callyspongia (Euplacella) biru</td>
<td>Amphitoxin</td>
<td>Indonesia</td>
<td>de Voogd</td>
<td>de Voogd et al. 2005</td>
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<tr>
<td>Callyspongidae</td>
<td>Callyspongia (Cladochalina) fibrosa</td>
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<td>Micronesia†</td>
<td>van Soest</td>
<td>Davies-Coleman et al. 1993</td>
</tr>
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<td>Callyspongia ridleyi</td>
<td>Halitoxin</td>
<td>unknown</td>
<td>unknown</td>
<td>Scott et al. 2000</td>
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<td>Amphimedon compressa</td>
<td>Halitoxin</td>
<td>Caribbean</td>
<td>Hartman</td>
<td>Schmitz et al. 1978</td>
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<td>Scott et al. 2000</td>
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<td>Brazil</td>
<td>Hajdu</td>
<td>Berlinck et al. 1996</td>
</tr>
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<td>Amphimedon viridis</td>
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<td>de Voogd et al. 2005</td>
</tr>
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<td>Chalinidae</td>
<td>Haliclona (Rhizoniera) sarai</td>
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<td>Adriatic Sea</td>
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<td>Chondropsidae</td>
<td>Batella sp.</td>
<td>Halitoxin</td>
<td>Madagascar</td>
<td>Diaz</td>
<td>Segraves and Crews 2005</td>
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### B. Cyclic dimers

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<th>Family</th>
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<th>Compound</th>
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<th>Identification</th>
<th>Reference</th>
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<td>Haliclona (Rhizoniera) viscosa</td>
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<td>de Weerdt</td>
<td>Volk and Köck 2004</td>
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<td>Brazil</td>
<td>Hajdu</td>
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<td>Petrosiidae</td>
<td>Xestospongia sp.</td>
<td>Cyclostellettamines</td>
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### C. Bicyclic dimers

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<th>Identification</th>
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<td>Niphatidae</td>
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<td>Japan</td>
<td>van Soest</td>
<td>Matsunaga et al. 2004</td>
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<td>Japan</td>
<td>van Soest</td>
<td>Matsunaga et al. 2004</td>
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<td>Callyspongidae</td>
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<td>Petrosiidae</td>
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<td>Eritrea</td>
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<td>Chil et al. 2002</td>
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</table>
have co-evolved with the host sponge, but also the generalist microbes which can be found in a wide array of sponges and some of which are even widely present in seawater (Lee et al. 2001, Taylor et al. 2004). When identical or very similar compounds are found in species from different orders or genera, microbial producers are suspected (e.g. bacteria and fungi). If these generalist symbionts play any role in the production of the toxic compounds extracted from the sponges, this could mystify the classifications based on chemotaxonomy. Erpenbeck and van Soest (2007) provide a full review of the various pitfalls in the use of compounds in chemotaxonomy. They illustrate that the major problems are not only related to the great natural variation in compound production and ambiguous origins of compounds, but also the unknown homology of compounds, misidentifications of sponge species and the lack of reporting by natural products chemists of presence/absence of known compounds.

To conclude, our results diminish the value of both 3-alkylpyridines and amphitoxins as monophyletic markers assuming that the classifications are correct. The classification of haplosclerid families and genera is presently under siege from molecular studies (e.g. McCormack et al. 2002, Nichols 2005). Thus, this study may prove of some value in the ongoing debate on the classification of the poriferan phylum. Any definite conclusions of the utility of 3-alkylpyridines as chemotaxonomic markers would, however, be presumptuous before a more comprehensive systematic study is performed, particularly of genera that have not been examined previously, and the presence in the other orders is unequivocally ruled out.

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