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 2007). The present classification of sponges is mainly based on external and internal morphological characters and some life history characteristics (Hooper 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 (Euplacella) biru* de Voogd 2004 (Sulawesi, Indonesia), *Callyspongia (Callyspongia) truncata* (von Lendenfeld, 1887) (Shizuoka, 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 $CHCl_3$. The water-soluble fraction was extracted with *n*-butanol. The organic phase was partitioned between $MeOH/H_2O$ (9:1) and *n*-hexane. The aqueous MeOH layer was then partitioned between $MeOH/H_2O$ (6:4) and $CHCl_3$. Finally the *n*-butanol fraction and $MeOH/H_2O$ (6:4) fraction were combined. All fractions were analyzed by TLC on silica gel with $CHCl_3/MeOH/H_2O$ (7:3:0.5) and different mixtures of $CHCl_3$/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 $CHCl_3/MeOH/H_2O$ (7:3:0.5). The fractions showing spots positive to Draggendorff on TLC were further separated by ODS HPLC (Column: COMOSIL-5C_{18}-ARII 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 Draggendorff were analyzed by ^{1}H 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 R*f* 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. (Poecilosclerida, 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 (haliclonacyclamines/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 $MeOH/H_2O$ (6:4) fractions. We were unable to trace any amphitoxins or 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-alkylpiperidines were concentrated in Chalinidae and Petrosiidae (Andersen *et al.*

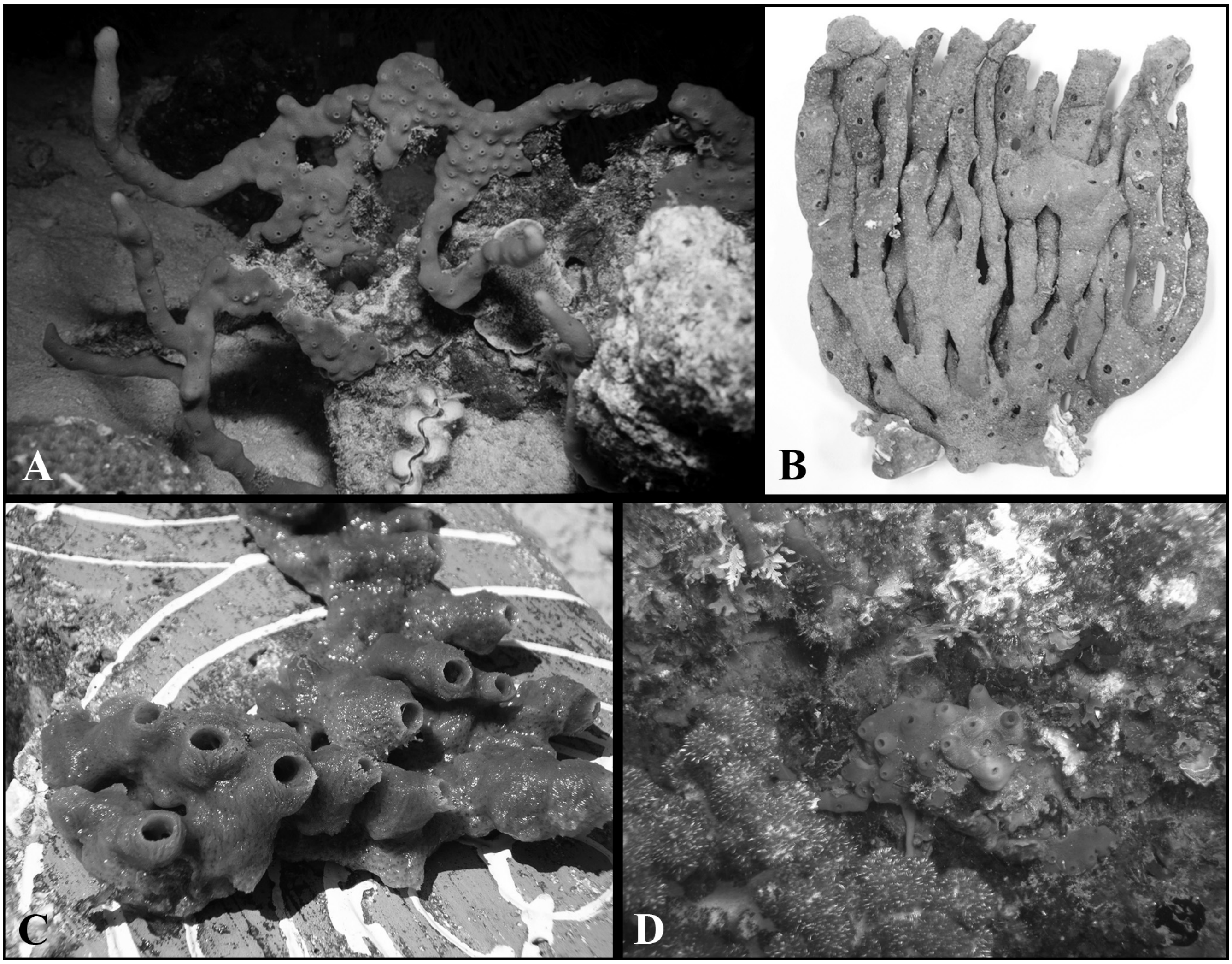


Fig. 1A-D: Sponges examined in this study: **A.** *Callyspongia* (*Euplacella*) *biru* (photo: B. W. Hoeksema), **B.** *Callyspongia* (*Callyspongia*) *truncata* (photo: S. Hoshino), **C.** *Amphimedon* aff. *queenslandica* (photo: L. E. Becking), **D.** *Haliclona* sp. (photo: Y. Nakao).

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.

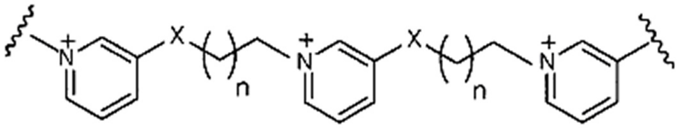
Three groups of compounds within the 3-alkylpyridines were noteworthy in that they have structures with presumed similar biogenetic pathways and appear to be concentrated in small taxonomic units (see Table 1A-C). Based on the common presence of these specific compounds, the close relationship between 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

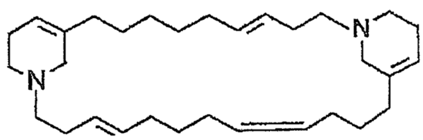
Table 1A-C: Three compound groups that appear to be concentrated in restricted taxonomic units (compound figures reproduced from Andersen *et al.* 1996): **A.** polymers, **B.** cyclic dimers, **C.** bicyclic dimers.

A. Polymers



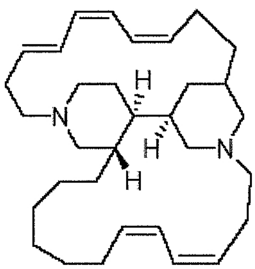
Family	Species	Compound	Location	Identification	Reference
Callyspongidae	*Callyspongia* (*Euplacella*) *biru*	Amphitoxin	Indonesia	de Voogd	de Voogd *et al*. 2005
Callyspongidae	*Callyspongia* (*Cladochalina*) *fibrosa*	Halitoxin	Micronesia	van Soest	Davies-Coleman *et al*. 1993
Callyspongidae	*Callyspongia ridleyi*	Halitoxin	*unknown*	*unknown*	Scott *et al*. 2000
Niphatidae	*Amphimedon compressa*	Halitoxin	Caribbean	Hartman	Schmitz *et al*. 1978
Niphatidae	*Amphimedon erina*	Halitoxin	Caribbean	Hartman	Schmitz *et al*. 1978
Niphatidae	*Amphimedon viridis*	Halitoxin	Caribbean	Hartman	Schmitz *et al*. 1978
Niphatidae	*Amphimedon viridis*	Halitoxin	Brazil	Hajdu	Berlinck *et al*. 1996
Niphatidae	*Amphimedon viridis*	Hali/Amphitoxin	Red Sea	*unknown*	Kelman *et al*. 2001
Niphatidae	*Amphimedon compressa*	Amphitoxin	Bahamas	Genoa Museum	Albrizio *et al*. 1995
Niphatidae	*Amphimedon paraviridis*	Amphitoxin	Indonesia	de Voogd	de Voogd *et al* 2005
Chalinidae	*Haliclona* (*Rhizoniera*) *sarai*	Halitoxin	Adriatic Sea	Vacelet	Sepčić *et al*. 1997
Chondropsidae	*Batzella* sp.	Halitoxin	Madagascar	Diaz	Segraves and Crews 2005

B. Cyclic dimers



Family	Species	Compound	Location	Identification	Reference
Chalinidae	*Haliclona* sp.	Haliclamines	Japan	Watanabe	Fusetani *et al*. 1989
Chalinidae	*Haliclona* (*Rhizoniera*) *viscosa*	Haliclamines	Arctic	de Weerdt	Volk *et al*. 2004
Chalinidae	*Haliclona* sp.	Cyclostellettamines	Japan	van Soest	Fusetani *et al*. 1994
Chalinidae	*Haliclona* (*Rhizoniera*) *viscosa*	Cyclostellettamines	Arctic	de Weerdt	Volk and Köck 2004
Niphatidae	*Pachychalina* sp.	Cyclostellettamines	Brazil	Hajdu	de Oliviera *et al*. 2004
Petrosiidae	*Xestospongia* sp.	Cyclostellettamines	Japan	van Soest	Oku *et al*. 2004

C. Bicyclic dimers



Family	Species	Compound	Location	Identification	Reference
Niphatidae	*Amphimedon* sp.	Tetradehydrohalicyclamine	Japan	van Soest	Matsunaga *et al*. 2004
Niphatidae	*Amphimedon* sp.	22-hydroxyhalicyclamine	Japan	van Soest	Matsunaga *et al*. 2004
Niphatidae	*Amphimedon* sp.	Tetrahydrohalicyclamine	Japan	van Soest	Matsunaga *et al*. 2004
Callyspongiidae	*Arenosclera braziliensis*	Arenosclerins	Brazil	Hajdu	Torres *et al*. 2002
Callyspongiidae	*Arenosclera braziliensis*	Haliclonacyclamines	Brazil	Hajdu	Torres *et al*. 2002
Chalinidae	*Haliclona* sp.	Haliclonacyclamines	Australia	Hooper	Clark *et al*. 1998, Charan *et al*. 1996
Chalinidae	*Haliclona* sp.	Halicyclamine	Indonesia	*unknown*	Jaspars *et al*. 1994
Petrosiidae	*Xestospongia* sp.	Halicyclamine	Indonesia	Diaz	Harrison *et al*. 1996
Halichondriidae	*Halichondria* sp.	Halichondramine	Eritrea	van Soest	Chill *et al*. 2002

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 definate 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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