CYMBASTELA HOOPERI AND *AMPHIMEDON TERPENENSIS*: WHERE DO THEY REALLY BELONG?

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A sponge sample identified as *Cymbastela hooperi* collected from Kelso Reef, the Great Barrier Reef, Australia, yielded a series of natural products, mainly diterpene isonitriles, which demonstrated significant in vitro antimalarial activity. As a result of these compounds being consumed in a number of bioassays it was considered desirable to have more of them so as to enable further and more detailed biological testing to be undertaken. Subsequently three *Cymbastela* samples (two of *C. concentrica* and one of *C. coralliophila*) and two of *Amphimedon* (*Cymbastela*) *terpenensis* were tested for antimalarial activity and investigated for their natural product content. The results of these investigations provided further evidence that either, *Amphimedon terpenensis* is more appropriately *Cymbastela terpenensis*, or that both *C. hooperi* and *A. terpenensis* belong to an as yet undefined genus and may perhaps be the same species. ❐ *Porifera, Cymbastela, Amphimedon, Acanthella, biological testing, malaria, cytotoxicity, taxonomy, Great Barrier Reef, diterpene isonitriles, marine natural products.*

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Since the discovery by Angerhofer et al. (1992a) of the antimalarial activity of axisonitrile-3 (Fig. 1A) isolated from the sponge *Acanthella klethra*, much of our research activity has focused on finding further marine natural products with this biological activity. These efforts resulted in the identification of other marine derived natural products having selective antimalarial activity (König et al., 1998, Wright et al., 1996), particularly the compounds isolated from *Cymbastela hooperi* (König & Wright, 1995, 1997a; König et al., 1996; Linden et al., 1996; Wright et al., 1996). In order to obtain further amounts of these natural products it was decided to investigate some sponge samples likely to contain this class of compound. In the present paper we provide a discussion of these biologically-guided isolations, the results of chemical analyses, as well as the possible taxonomic implications of these findings.

MATERIALS AND METHODS

General methodology follows Wright et al. (1996). Abbreviations: DCM, dichloromethane; MeOH, methanol; EtOAc, ethyl acetate; VLC, vacuum liquid chromatography; HPLC, high performance liquid chromatography; TLC, thin layer chromatography; GC, gas chromatography; GC-MS, gas chromatography coupled mass spectrometry; ^{1}H NMR, proton detected nuclear magnetic resonance spectroscopy.

MATERIAL. All sponges were collected using SCUBA from the Great Barrier Reef, in the vicinity of Lizard Island between 11-30m depth. All specimens were frozen, then freeze dried. Five samples of 3 sponge species were: *Cymbastela coralliophila* Hooper & Bergquist, 1992 (Demospongiae, Halichondrida, Axinellidae) (specimen CTA); *C. concentrica*, Lendenfeld, 1887 (Demospongiae, Halichondrida, Axinellidae) (specimens CTD and CTE); *Amphimedon terpenensis*, Fromont, 1993 (Demospongiae, Haplosclerida, Niphatidae) (specimens CTB and CTC).

EXTRACTION AND ISOLATION. Initially, a small piece (~5g of freeze dried tissue) from each sample was exhaustively extracted with DCM followed by MeOH. A portion of the resultant extracts was then sent for antimalarial and cytotoxicity testing (~2mg). ^{1}H NMR and TLC investigations of these extracts were also made. On the basis of the results obtained from the biological testing and the ^{1}H NMR and TLC investigations, specimens CTA, CTC and CTD were subsequently selected for bulk extraction and fractionation.

TABLE 1. Antimalarial activity, towards clones D6 and W2 of *Plasmodium falciparum*, of the dichloromethane (D) and methanol (M) extracts from 5 sponge samples. SI = the ratio of the KB cell cytotoxicity to the *Plasmodium flaciparum* toxicity. * Extract was non-toxic only towards KB cells, in other cell lines it was at least 100 times more toxic.

Sample	Species	IC_{50} (ng/ml)	Clone D6		Clone W2	
		KB cells	IC_{50} (ng/ml)	SI	IC_{50} (ng/ml)	SI
CTA (D)	*C. coralliophila*	>20,000	>10,000	-	>10,000	-
CTA (M)	*C. coralliophila*	>20,000	5150	>3.9	6380	>3.1
CTB (D)	*A. terpenensis*	>20,000	4820	>4.1	>10,000	-
CTB (M)	*A. terpenensis*	>20,000	3240	>6.2	9680	2.1
CTC (D)*	*A. terpenensis*	>20,000	<41	>490	<41	>490
CTC (M)	*A. terpenensis*	>20,000	540	>37	5250	>3.8
CTD (D)	*C. concentrica*	>20,000	1360	>14.7	>10,000	-
CTD (M)	*C. concentrica*	>20,000	2730	>7.3	1360	>14.7
CTE (D)	*C. concentrica*	>20,000	>10,000	-	>10,000	-
CTE (M)	*C. concentrica*	>20,000	8470	>2.4	5700	>3.5

1) Specimen CTA: Freeze-dried tissue (108.4g) was exhaustively extracted with DCM (2L) and MeOH (2L) to yield 15.9g (14.7%) of DCM soluble material, and 11.3g (10.4%) of MeOH/H_2O solubles. VLC separation of the DCM solubles over silica, employing gradient elution from hexane to acetone to MeOH, yielded 15 fractions each of approximately 90ml. Fractions 3-14 were predominantly compounds depicted in Figure 1C-D. The remaining fractions and the MeOH/H_2O solubles were ubiquitous lipids and a number of other sterols.

2) Specimen CTC: Freeze-dried tissue (38.8g) was exhaustively extracted with DCM (1.5L) and MeOH (2L) to yield 3.0g (7.7%) of DCM soluble material. VLC separation of the DCM solubles over silica, employing gradient elution from hexane to EtOAc to MeOH, yielded 12 fractions each of approximately 100ml. Fractions 1-5 were found by GC-MS analysis to contain compounds depicted in Figure 1E-Q. Fractions 6 and 7 were essentially the pure compound shown in Figure 1B. Fractions 10 and 11 were found to contain the compounds depicted in Figures 1R-X and 2A. The remaining fractions and the MeOH/H_2O solubles were ubiquitous lipids and a number of sterols.

3) Specimen CTD: Freeze-dried tissue (57.5g) was exhaustively extracted with DCM (2L) and MeOH (2L) to yield 600mg (1.1%) of DCM soluble material. VLC separation of the DCM solubles over silica, employing gradient elution from hexane to ethyl acetate (EtOAc) to MeOH, yielded 13 fractions, each of approximately 80ml. All fractions and the MeOH/H_2O solubles were ubiquitous lipids and a number of sterols. One of the major components of fractions 8-12 was a sterol of the type represented by the compound shown in Figure 2A.

GC SEPARATIONS. GC analyses were done according to methods previously described (Witte et al., 1993). From each of VLC fractions 1 and 2, obtained from the DCM extract of sponge specimen CTC approximately 1mg of material was taken and analysed my GC-MS. The results of these analyses indicated VLC fraction 1 to contain the compounds depicted in Figures 1E-H, and VLC fraction 2 to contain the compounds depicted in Figures 1I-Q.

BIOLOGICAL TESTING. The antimalarial (antimalarial activity is defined as the ability of some substance, pure or mixture, to inhibit the growth of, or be lethal to, one or other strains of *Plasmodium falciparum*) and cytotoxicity testing was undertaken as previously described (Angerhofer et al., 1992b, Likhitwitayawuid et al., 1993).

RESULTS

A small piece (~5g of dry tissue) from each of 5 sponge samples thought likely to contain diterpene isonitriles of the type represented by diisocyanoadociane (Fig. 1B), was exhaustively extracted with DCM, followed by MeOH, and the resultant extracts sent for antimalarial activity assessment. Out of the 10 extracts only 2 were found to have significant activity, the DCM and MeOH extracts of sample CTC (*A. terpenensis*) (Table 1). The only other extracts to show some promise in terms of their activity and selectivity were the DCM and MeOH extracts of sample CTD (*C. concentrica*), and to a lesser extent the

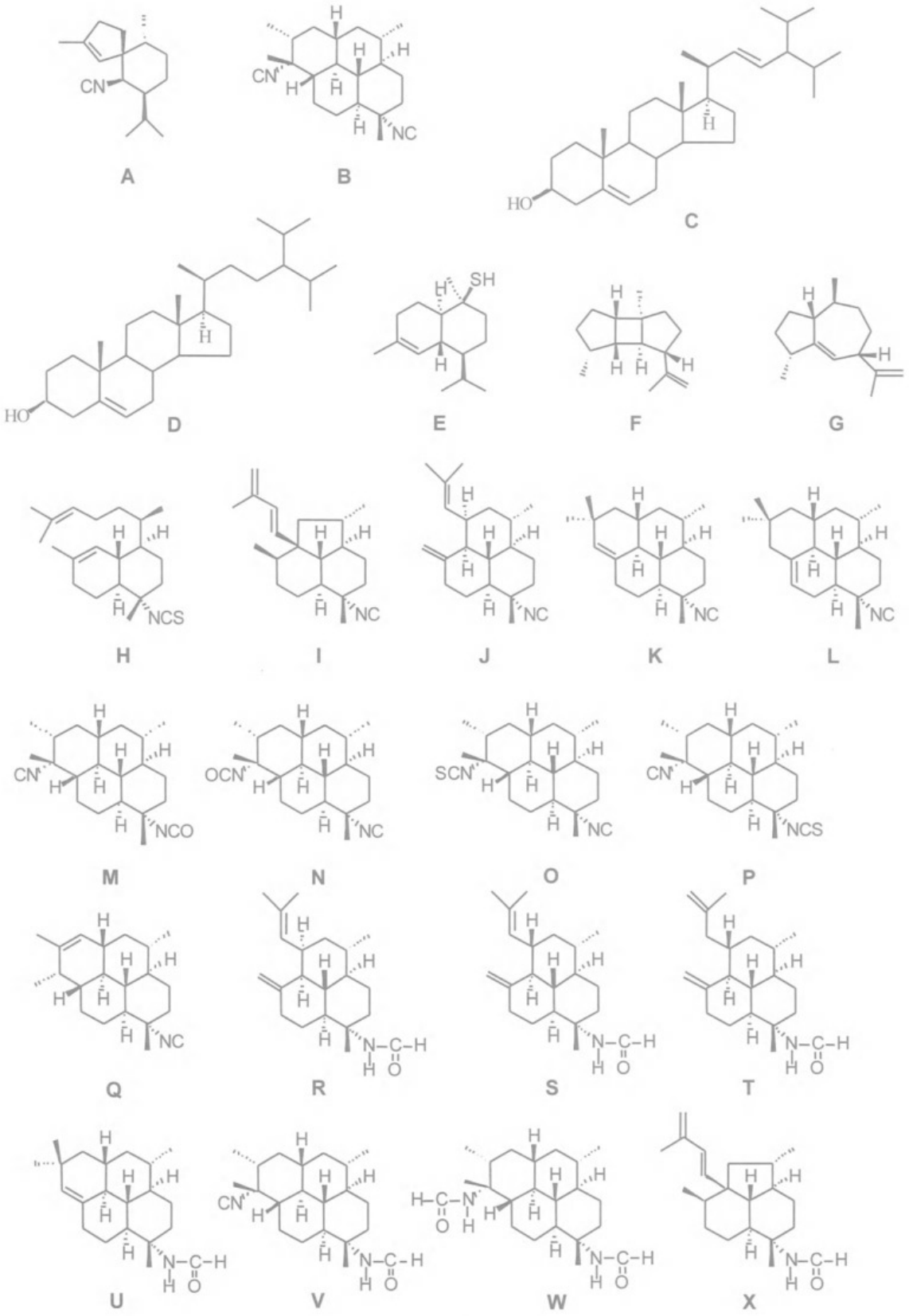

FIG. 1. A-X, chemical structures of secondary metabolites derived mainly from sponges of the genera *Cymbastela* and *Amphimedon* (refer to text for further information).

DCM and MeOH extracts of sample CTB (*A. terpenensis*) (Table 1). On the basis of results presented in Table 1, TLC and ^{1}H NMR examinations of the extracts, detailed investigations were made of samples CTA (*C. coralliophila*), CTC (*A. terpenensis*) and CTD (*C. concentrica*). For this purpose DCM and MeOH extracts were prepared from bulk material of each of the 3 samples to identify the major components present.

SAMPLE CTA (*C. CORALLIOPHILA*). The DCM extract of this sample (CTA, *C. coralliophila*) was found to contain predominantly lipids and 2 sterols (Fig. 1C-D), previously isolated from *Pseudaxinyssa* sp. The latter sample was collected from several mid-shelf reefs on the Great Barrier Reef, by Hofheinz & Oesterhelt (1979). These 2 sterols have also been isolated from another *Pseudaxinyssa* sp. collected from a reef fringing Pelorus Island on the Great Barrier Reef (König, G. M. & Wright, A. D., unpublished data). An interesting observation concerning these compounds (Fig. 1C-D) is, that they always seem to occur as a 1:1 mixture which is essentially inseparable, even by GC (Bergquist et al., 1980). The MeOH extract was composed of ubiquitous lipids and a number of other sterols (Bergquist et al., 1980).

SAMPLE CTC (*A. TERPENENSIS*). Chromatographic and ^{1}H NMR analyses indicated the MeOH extract of CTC (*A. terpenensis*) to contain many of the components to be found in the DCM extract. The MeOH extract was therefore partitioned between water and DCM and the resulting DCM solubles combined with the DCM extract. These DCM solubles were fractionated as outlined in the experimental section. ^{1}H NMR analysis of the resultant fractions indicated a similarity in composition to those produced by the fractionation of the DCM solubles obtained from the previously investigated *C. hooperi* (König et al., 1996; König & Wright, 1997b). Based on this observation GC-MS investigations of selected VLC fractions were undertaken. These analyses indicated the sample to contain compounds shown in Figures 1E-Q, and thus, to be almost identical in secondary metabolite content to *C. hooperi* (König et al., 1996; König & Wright, 1997b). This finding also explained the observed antimalarial activity of its DCM extract. As a result of these studies it was also observed that VLC fraction 11 contained a number of resonances in the 8.0-8.3ppm region of the proton NMR spectrum. Comparison of this ^{1}H NMR spectrum with an equivalent VLC fraction from *C. hooperi* (König et al., 1996; König & Wright, 1997b; Wright et al., 1996) showed the two VLC fractions to be almost identical. Purification of the main components from both VLC fraction 11s has resulted in the identification of 7 diterpene formamide derivatives (Fig. 1R-X), a number of which are new natural products, and a mixture of peroxide containing sterols of the type represented by Figure 2A; the detailed results of this investigation will be presented elsewhere (König et al., in preparation).

SAMPLE CTD (*C. CONCENTRICA*). Both the DCM and MeOH extracts of this sample (CTD, *C. concentrica*) were found to be complex mixtures of ubiquitous lipids and sterols. In VLC fractions 8-12, made from the DCM solubles of CTD, sterols of the type represented by Figure 2A were abundant.

TLC and ^{1}H NMR of the extracts of the two remaining sponge samples, CTB (*A. terpenensis*), and CTE (*C. concentrica*), clearly showed that specimen CTB is very similar in all respects to CTC (*A. terpenensis*) and that sample CTE shows the greatest similarity to samples CTA and CTD, particularly with respect to their ^{1}H NMR spectra. The reduced activity of the DCM and MeOH extracts of CTB when compared to the activity of the equivalent extracts of CTC, appears to be due to the relatively large amounts of lipids present in the extracts of CTB.

DISCUSSION

Of the 3 species of *Cymbastela* investigated none were shown to contain terpenoids substituted with isonitrile based functionalities. This is in direct contrast to results obtained for *C. hooperi* (König & Wright, 1995, 1997a; König et al., 1996; Linden et al., 1996; Wright et al., 1996). In this respect it is of interest to note that *C. hooperi* is also morphologically distinguished from other *Cymbastela* species (Van Soest et al., 1996). The investigation of the two samples of *A. terpenensis* (CTB and CTC), however, led to the identification of secondary metabolites identical, or closely related, to those obtained from *C. hooperi*.

Literature relating to the secondary metabolite chemistry of sponges from *Amphimedon* and *Cymbastela* shows sponges from the former to have received the most attention. In the 40 or so publications on *Amphimedon* the compounds which are typically reported are: various classes of alkaloids (e.g. Fig. 2B-F; Chehade et al., 1997; Kobayashi et al., 1994a; Kobayashi et al., 1994b; Schmitz et al., 1983; Tsuda et al., 1994), long

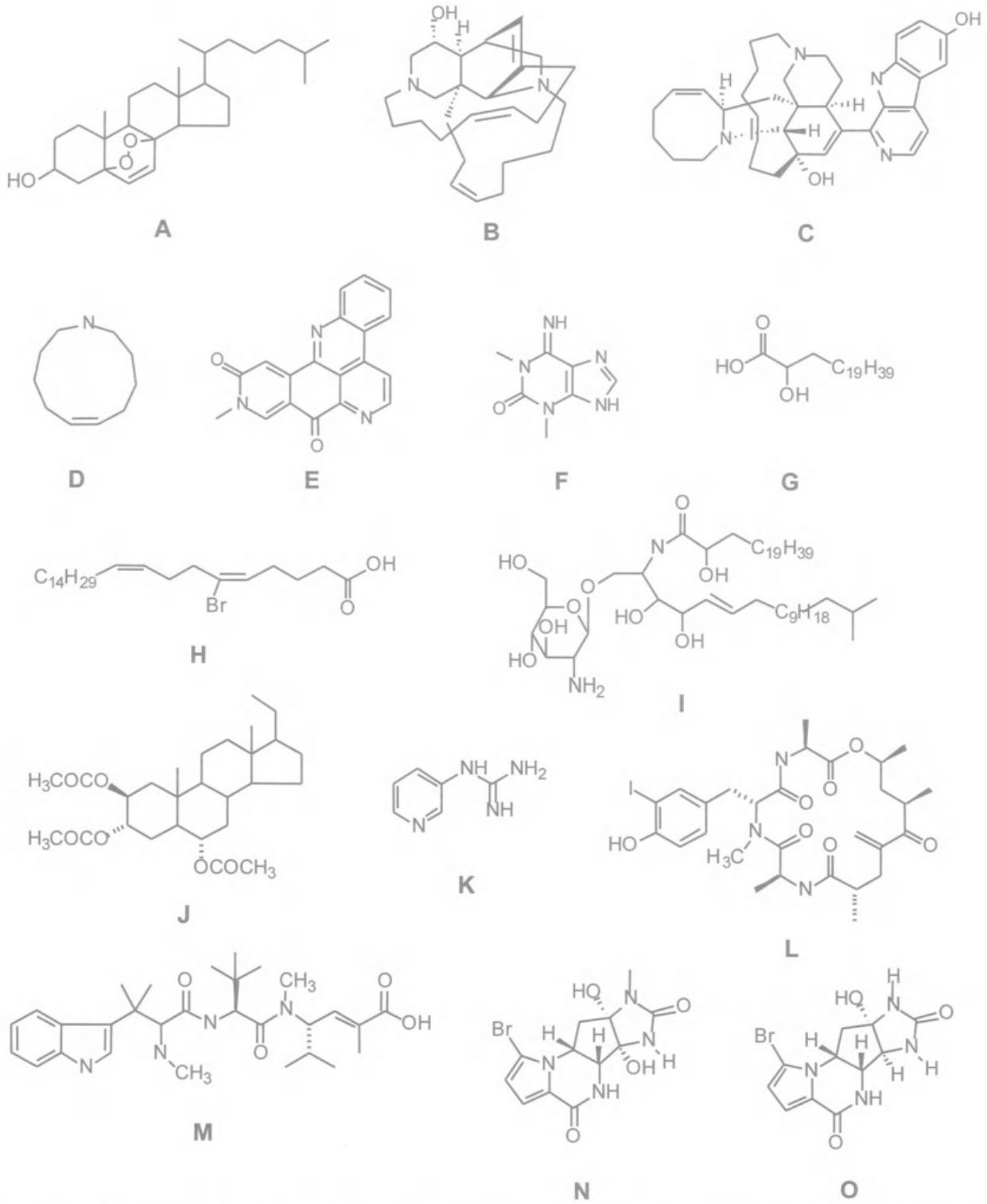

FIG. 2. A-O, chemical structures of secondary metabolites derived mainly from sponges of the genera *Cymbastela* and *Amphimedon* (refer to text for further information).

chain fatty acid derivatives (e.g. Fig. 2G-H; Carballeira & Lopez, 1989; Garson et al., 1994), glycosphingolipids (e.g. Fig. 2I; Hirsh & Kashman, 1989) and some isonitrile containing diterpenes (e.g. Fig. 1B, J, K; Fookes et al., 1988; Kazlauskas et al., 1980; König & Wright, 1995). As there have only been about 11 reports on the secondary metabolite chemistry of sponges from

Cymbastela it is possible to show most of the isolates in this contribution. From *C. coralliophila* steroids of the type represented by Figure 2J were isolated (Makarieva et al., 1995). Pyraxinine (Fig. 2K), a novel alkaloid was isolated from *C. cantharella* (Mourabit et al., 1997), while from two unidentified species of *Cymbastela* two peptides (Fig. 2L-M; Coleman et al., 1995) and two pentacyclic bromopyrroles (agelastatins C and D; Fig. 2N-O, respectively; Hong et al., 1998) were obtained. The present authors have also published a number of works about secondary metabolites from *C. hooperi* (König & Wright, 1995, 1997a; König et al., 1996; Linden et al., 1996; Wright et al., 1996) and the typical metabolites described are mainly isonitrile containing diterpenes (e.g. Fig. 1B, I-Q).

When the literature is considered for *Cymbastela* and *Amphimedon* it is evident that there is currently no class of secondary metabolite one might designate as being 'characteristic' for one or the other of these genera. What is evident, however, is that in both genera only one species produces isonitrile containing diterpenes; *C. hooperi* and *A. terpenensis*.

Two observations can be made: *A. terpenensis* is positioned in an order (Haplosclerida) and family (Niphatidae) where no other sponges are known to produce secondary metabolites that have isonitrile or similar functionalities, and *C. hooperi* is located in an order (Axinellida or Halichondrida; see Van Soest, 1996) and family (Axinellidae) that are known to contain sponges that produce isonitrile containing secondary metabolites. It is clear that '*A. terpenensis*' does not fit in *Amphimedon* as currently defined (Bergquist, Fromont, Hooper, Van Soest, pers. comm.), nor is it clearly a haplosclerid, it is possibly an axinellid close to *Cymbastela*, as suggested by Van Soest et al. (1996), but it's life characteristics do not conform well with the other species of *Cymbastela* (e.g. growth form, texture, mucus production, surface features and amount of spongin to spicule ratio). *Cymbastela* as defined by Hooper & Bergquist is a fairly homogeneous genus, and '*A. terpenensis*' clearly disrupts that homogeneity.

CONCLUSIONS

These observations and the fact that *C. hooperi* and a specimen of *A. terpenensis* have been shown to have almost identical secondary metabolite chemistry, lead to three possible conclusions concerning their current taxonomic classification. 1) *A. terpenensis* belongs to *Cymbastela*, as proposed by Van Soest et al. (1996); 2) both *A. terpenensis* and *C. hooperi* belong to another, possibly new genus located in the family Axinellidae; 3) they are the same species with *hooperi* representing an unusual morphotype of *terpenensis*. The results of the current work indicate that the taxonomic classification of *C. hooperi* and *A. terpenensis* needs to be clarified, particularly since sponges belonging to both of these species produce so many interesting and biologically active compounds. This study also serves to further highlight the significance of secondary metabolite chemistry as an important taxonomic tool. It is also hoped that continued investigations into the biologically active secondary metabolites produced by both of these sponge species will eventually lead to the development of an agent suitable for the treatment of malaria and/or some other disease.

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THE REPLACEMENT OF NATURAL HARD SUBSTRATA BY ARTIFICIAL SUBSTRATA: ITS EFFECTS ON SPONGES AND ASCIDIANS. *Memoirs of the Queensland Museum 44: 288. 1999:-* Subtidal reefs around coastal cities such as Sydney are composed of a variety of natural and artificial substrata. Commonly these are natural rocky reefs, breakwalls, seawalls and pier pilings. These types of hard substrata differ in their structure. Most natural hard substrata consist of horizontal surfaces; most surfaces on artificial hard substrata are vertical. Therefore, replacing natural hard substrata with artificial hard substrata is likely to change the surface of substrata from predominantly horizontal to mostly vertical. To understand and predict the potential effects of these changes on the assemblage of sponges and ascidians it is important to determine their distribution on horizontal and vertical surfaces.

The few ecological studies on the distribution of algae and invertebrates on horizontal and vertical surfaces have reported that there are more sponges and ascidians on vertical than on horizontal surfaces. It has not been tested whether these patterns exist in the temperate waters around Sydney. Furthermore, of the studies that have examined the effects of horizontal and vertical surfaces on the distribution of sponges and ascidians, none has experimentally tested the factors that cause these distributions.

Here, I present results of my tests of the hypothesis that sponges and ascidians are more abundant on vertical than horizontal surfaces in the shallow subtidal zone around Sydney. I will also discuss future manipulative experiments to determine which factors are important in creating these distributions. ❐ *Porifera, Ascidiacea, distribution, hard substrata, shallow subtidal, habitat.*

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CONVERGENCE IN THE TIME-SPACE CONTINUUM: A PREDATOR-PREY INTERACTION. *Memoirs of the Queensland Museum 44: 288. 1999:-* Community structure is influenced by many biotic and abiotic factors. Predation is a key structuring mechanism for some marine communities. Prey abundances may fluctuate with strength of predator recruitment and persistence, except in cases where some of the prey population has a refuge in space or time from predation. Consistent, moderate predation levels on a predicably available prey resource should lead to stable community structure with relatively small fluctuations in predator and prey population densities. Conversely, prey species lacking a refuge from predation are subject to major population fluctuations commensurate with strength of predator recruitment and abundance.

The sponge *Halichondria panicea* is patchily distributed in the rocky intertidal on the south shore of Kachemak Bay, southcentral Alaska, and in certain locations is the spatial dominant. At one site approximately 55m in horizontal length, *H. panicea* has dominated the mid-intertidal for at least 10 years, with low densities of potential molluscan predators such as *Archidoris montereyensis*, *Katherina tunicata*, and *Diadora aspera* present. Percent cover estimates of primary space occupiers at the site were collected from 10 0.25m^2 permanent quadrats established in August 1994. *H. panicea* averaged 53.4% +/-9.9% cover through August 1996. Other major cover categories were algae, 14.6% +/-6.4%, and open rock, 26.1% +/-10.2%. Visits to the site in early spring of 1997 revealed that the sponge colonies overwintered with few indications of major mortality events. No percent cover data were collected at that time.

Total numbers of the nudibranch *Archidoris montereyensis*, which is a specialist predator on *H. panicea*, present at the site were recorded and ranged from 12-42 from 1994-1996. In the spring of 1997, strong recruitment resulted in an average population of 151 *A. montereyensis* on site from May to July. Percent cover of *H. panicea* declined from visual estimates of 40% in May to 15% in July. By August 1997, when the 10 permanent quadrats and 10 haphazardly placed quadrats were measured, essentially no sponge could be found at the study site. After July, the abundance of nudibranchs declined to 32 individuals commensurate with sponge reduction. By September, only one small sponge colony and 7 predatory nudibranchs were present at the site. Even though *H. panicea* is abundant in the region and potential recruits should be numerous, as of April 1998, the site once dominated by *H. panicea* is predominantly open rock with some recruitment of annual macroalgae occurring. The predator-prey relationship of *A. montereyensis* and *H. panicea* is an example of a chase through space and time with convergence resulting in extreme population fluctuations and an unstable community. ❐ *Porifera, predation, nudibranch, intertidal, predator/prey interaction, community structure, Alaska, recruitment.*

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