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A brominated secondary metabolite synthesized by the cyanobacterial symbiont of a marine sponge and accumulation of the crystalline metabolite in the sponge tissue

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Abstract The dictyoceratid marine sponge *Dysidea herbacea* (Keller, 1889) is common in shallow waters of the tropical Pacific Ocean. Polybrominated biphenyl ethers such as 2-(2′,4′-dibromophenyl)-4,6-dibromophenol (*1*) are characteristic secondary metabolites of some specimens of this sponge and may represent as much as 12% of the dry weight. We have found *1* to be deposited as conspicuous crystals throughout the sponge tissue. The dominant prokaryotic endosymbiont in the mesohyl of the sponge is a filamentous cyanobacterium (*Oscillatoria spongeliae*), although a vacuole-containing, heterotrophic bacterium is also present. The cyanobacteria were separated from the sponge cells and heterotrophic bacteria by flow cytometry. Coupled gas chromatography–mass spectrometry and proton nuclear magnetic-resonance spectroscopy revealed that the major brominated Compound *1* isolated from the intact symbiotic association is found in the cyanobacteria and not in the sponge cells or heterotrophic bacteria. This suggests that the production of the compound is due to the cyanobacterium, and not to the sponge or symbiotic heterotrophic bacteria, as had been suggested earlier.

Introduction

Sponges are a rich source of secondary metabolites (natural products) from marine invertebrates. Virtually all sponges harbor prokaryotic endobionts, and it is often assumed that the latter can synthesize the secondary metabolites isolated from the sponge tissue. This assumption is usually made on the grounds that a metabolite isolated from a sponge is: (1) identical or similar in structure to a metabolite produced by a free-living micro-organism, (2) found in taxonomically-diverse sponges, or (3) found both in the sponge and in some unrelated invertebrate that does not feed on the sponge. Unfortunately, little experimental work has been done to test such proposals, and in most cases a clear symbiotic relationship was never established. Moreover, the term "symbiosis" is probably employed perhaps too freely in the chemical literature because many of the relationships are ill-defined. In this paper, the term *symbiosis* will refer to a consistent (although not necessarily obligate) association between two organisms that is either mutualistic or commensal, but not to parasitism, amensalism, or cases of non-specific endobiosis or epibiosis.

The prokaryotic endobionts of marine sponges comprise heterotrophic bacteria and cyanobacteria (Vacelet and Donadey 1977, Wilkinson 1978a, 1992). Of these, some of the heterotrophic bacteria are consistently isolated from sponges and appear to differ from strains isolated from the surrounding seawater (Wilkinson 1978b, Wilkinson et al. 1981, Santavy 1988). Particular morphological types of prokaryotes have been consistently demonstrated in specific sponges (Vacelet 1975, 1981, Santavy 1985, Larkum et al. 1987). However, most studies of sponge-associated prokaryotes have been severely biased by considering only the small fraction of species that can actually be cultured from natural samples, as has been pointed out by Amann et al. (1991) and Schmidt et al. (1991).

To elucidate how symbiotic micro-organisms contribute to the production of secondary metabolites isolated from sponges and how such metabolites function in the symbiotic relationships, the biosynthetic origin of these metabolites must be ascertained. Some researchers have approached this problem by growing putative symbionts in pure culture and analysing extracts of the culture for metabolites previously obtained from extracts of the sponge from which the micro-organism was isolated (Stierle et al. 1988, Elyakov et al. 1991). However, the putative symbionts are difficult to grow in pure, axenic culture, and even then there is no guarantee that a symbiont in pure culture will produce the secondary metabolites that it would nor-

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mally produce while associated with the sponge. Furthermore, it is possible that exchange of materials (nutrients, biochemical stimuli, or metabolic intermediates) between the symbiotic partners is required for the production of a particular natural product. The use of labelled precursors to determine the biosynthetic origin of a compound in symbiotic associations can be confounded by the possibility that both species participate in the biosynthesis. For relatively slow-growing invertebrates such as sponges, the diverse microbial flora (which probably includes at least some non-specific endobionts) inhabiting the invertebrate's tissues are likely to be the first to take up precursors such as amino acids or sugars; only later are some labelled compounds translocated to the "host" (Wilkinson and Garrone 1980).

It is reasonable to assume that a compound localized *exclusively* in a single cell type is highly likely to have been produced there. This requires that the cell type (e.g. a presumptive symbiont) is easily distinguishable from others and that the natural product is sufficiently distinctive so that convergent biosynthesis or non-specific primary metabolism is unlikely.

We studied the sponge *Dysidea herbacea* and its symbiotic cyanobacterium *Oscillatoria spongeliae* because the secondary metabolites of this and related sponges have been extensively investigated over the past two decades (Faulkner 1984, 1986, 1987, 1988, 1990, 1992) and because this particular association is well-documented. *D. herbacea* (Keller, 1889) (order Dictyoceratida, family Dysideidae) is a shallow-water marine sponge common in the Indo-Pacific (Bergquist 1965, 1980). The filamentous cyanobacterium *O. spongeliae* occurs extracellularly within the sponge's mesohyl in strikingly high abundances (up to 50% of the tissue volume). This association, first described by Schulze (1879), was duly noted by other researchers (Hauck 1879, Feldmann 1933, Bergquist 1965, 1980), culminating in a detailed ultrastructural study of the cyanobacterium published by Berthold et al. (1982). In our own experience, all specimens of *D. herbacea* that we have collected from different Indo-Pacific locations over the past several years contained high levels of a single, morphologically-distinct cyanobacterium similar in appearance at both the light- and electron-microscope level to that described by Berthold et al. (1982).

In the present study, we used flow-cytometry to separate glutaraldehyde-fixed cyanobacterial and sponge cells from a Palauan specimen of *Dysidea herbacea* on the basis of phycoerythrin fluorescence, and obtained a highly enriched (>95% pure) cynobacterial fraction. Chemical analysis by high-field proton nuclear magnetic-resonance (^{1}H-NMR) spectroscopy and coupled gas chromatography–mass spectroscopy (GC–MS) unequivocally showed that the polybrominated biphenyl ether, 2-(2′,4′-dibromophenyl)-4,6-dibromophenol (*1*), which can be isolated from the intact association, was present in the cyanobacterial filaments but not in the sponge cells. With a similar approach, Unson and Faulkner (1993) showed that another natural product, 13-demethylisodysidenin (*2*) (see present Fig. 1), occured only in the cyanobacteria, whereas the accompanying sesquiterpenes herbadysidolide (*3*) and spirodysin (*4*) were limited entirely to the sponge cells (Unson and Faulkner 1993).

Materials and methods

Collection

The specimen of *Dysidea herbacea* (Keller, 1889) (Collection No. 93-165), a 3 to 5 mm thick, greyish-green encrusting sponge growing in convoluted upright folds, was collected by hand at −1 m adjacent to the Hotel Nikko Palau boat ramp, Koror, Republic of Palau, Caroline Islands, on 23 January 1993. The sponge was removed from its substrate (coral sand and rubble), and the fresh tissue was stored frozen (except for the portions fixed for microscopy or for cell separation, see subsections "Microscopy" and "Cell separation") until extraction. Voucher samples are maintained in this laboratory (Scripps Institution of Oceanography Benthic Invertebrate Collection No. P1143).

Reagents

All solvents used were mass-spectral grade (Fisher Optima) or freshly distilled from reagent grade before use. Glutaraldehyde used for microscopy was Grade I, Sigma Chemical Co. (St. Louis, Missouri). Deuterated chloroform was purchased from Isotec, Inc. (Miamisburg, Ohio). All other reagents used were reagent grade. Calcium/magnesium-free artificial seawater (CMF-ASW) consists of 6.75 g NaCl, 0.25 g Na_2SO_4, 0.20 g KCl, and 0.050 g $NaHCO_3$ made up to 250 ml with freshly doubly-distilled water with the pH adjusted to 7.4 with 1.0 *M* NaOH.

Instrumentation

^{1}H-NMR spectra were obtained on a Varian 500 MHz spectrometer. ^{13}C-NMR spectra were obtained on a Bruker WP200 spectrometer operating at 50 MHz. Samples were dissolved in deuterated chloroform.

Gas chromatographic separation was done using a Hewlett Packard 5890 Series II gas chromatograph fitted with an Alltech AT35 capillary column (35% phenyl/65% methyl cross-linked silicon, 22 m long, 0.2 mm i.d., 0.22 µm film thickness) and coupled to a Hewlett Packard 5988A mass spectrometer. Gas chromatography was performed using 7 psi of helium at 33 cm min^{-1} linear velocity. The oven was temperature-programmed for 0.5 min at 35 °C, then ramped at 10 C° min^{-1} to 250 °C, and then ramped at 2 C° min^{-1} to 300 °C. Injections were made in the splitless mode with an injector port temperature of 180 °C. Transfer-line temperature was 280 °C; ion-source temperature was 180 °C. For direct insertion-probe samples, the temperature of the sample probe was increased from 25 to 325 °C at 10 C° min^{-1}. For all MS experiments the ionization voltage was 70 eV, with detection in the positive ion mode and mass spectral scan range set to 50–550 *m/z*.

Extraction of whole-sponge tissue and purification of secondary metabolites

Part of the sponge (3.4 g dry weight) was extracted with methanol (2×50 ml) and then with a 1:1 mixture of dichloromethane-methanol (3×50 ml) at room temperature. The organic extracts were combined, the solvent removed under reduced pressure, and the resulting aqueous suspension (30 ml) was triturated with dichloromethane (3×30 ml). The dichloromethane-soluble material was dried over sodium sulfate and the solvent removed under reduced pressure. This crude extract (303 mg) was chromatographed on a Merck silica gel 60 (70 to 230 mesh ASTM) flash column (3 cm diam, 11 cm length) eluted with a solvent gradient from 100% hexanes to 100% ethyl acetate in 10% steps. Fractions that were similar in composition as shown by thin-layer chromatography were combined. The fraction eluted by hexanes/EtOAc (9:1) contained the single major polybrominated secondary metabolite 2-(2′,4′-dibromophenyl)-4,6-dibro-

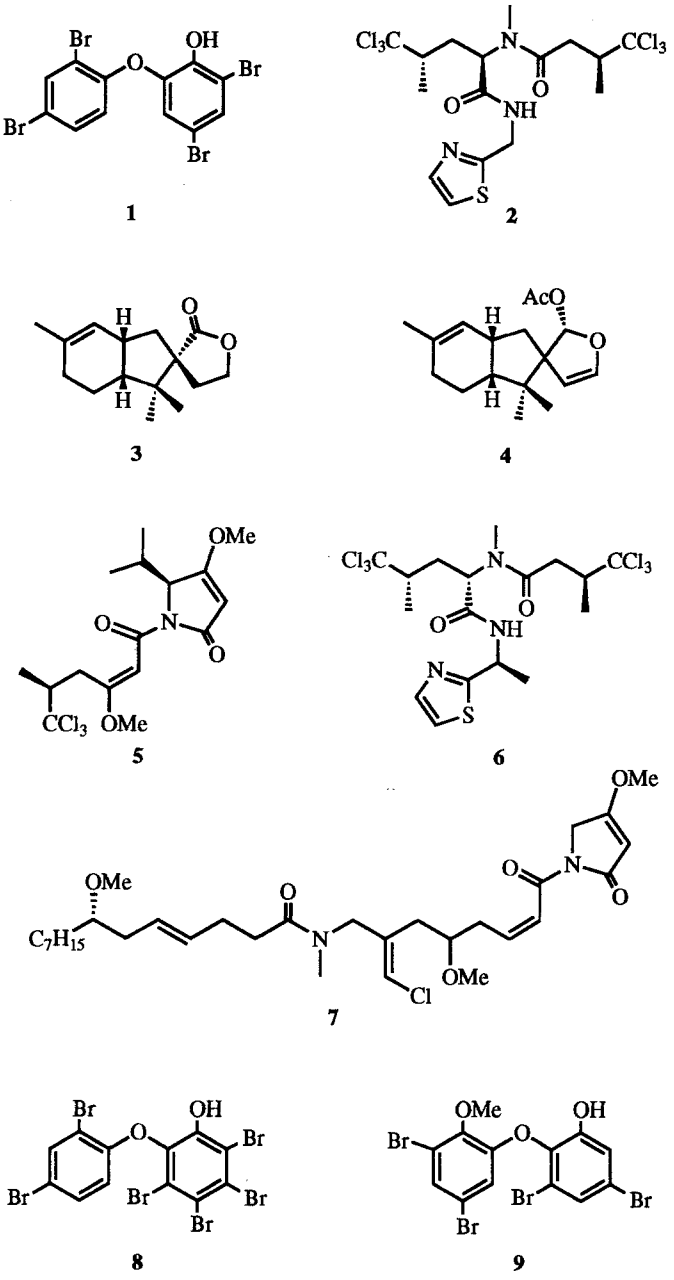

Fig. 1 Structures of compounds isolated from sponges, *Dysidea herbacea* (Compounds 1–6) or *Dysidea* spp. (Compounds 8–9), or from cyanobacterium *Lyngbya majuscula* (Compound 7)

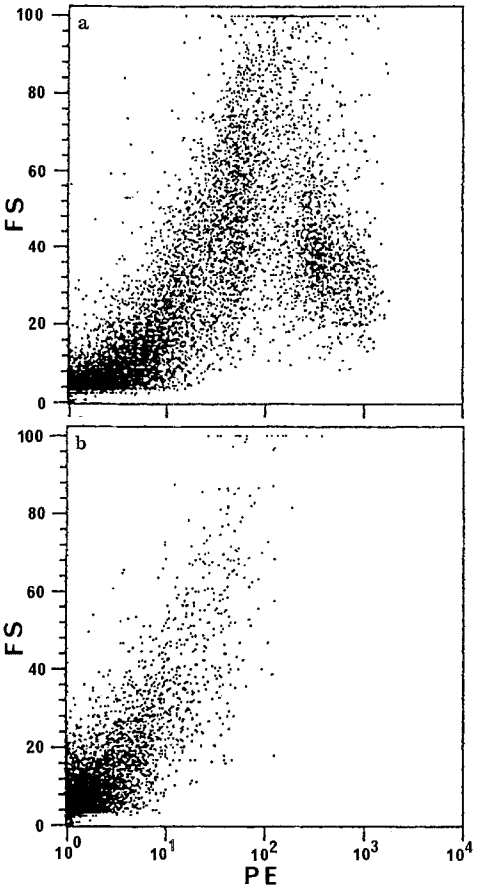

Fig. 2 *Dysidea herbacea.* Scatter plot of forward-scattered (FS) light versus phycoerythrin fluroescence (PE) of glutaraldehyde-fixed cells (a) before sorting, showing distinct populations, and (b) after sorting, showing "negatives" (low-fluorescence cells). Purity of "positives" (high-fluorescence cells) was checked by light microscopy

mophenol (*1*) (Fig. 1), the structure of which was determined by direct-insertion probe electron-impact (70 eV, positive-ion detection) mass spectrometry, and ^{1}H and ^{13}C nuclear magnetic-resonance spectroscopy. Other fractions contained mostly sterols and fats, and no chlorinated amino-acid derivatives or sesquiterpenes were detected. The purified Secondary Metabolite *1* was then used as the reference standard for the chemical analyses.

Microscopy

Small blocks of tissue of 2 to 5 mm on each side were cut from the sponge and fixed immediately in 2.5% glutaraldehyde in 0.45 µm-filtered natural seawater. Fixed material was stored for 12 d, refrigerated, in the dark. The blocks were washed twice in CMF-ASW and examined by light microscopy using transmitted light or polarized light. For transmission electron microscopy, some blocks were washed in CMF-ASW (3×10 min), post-fixed in a 1% solution of OsO_4 in CMF-ASW (30 min at 26 °C), washed in CMF-ASW (2×20 min), dehydrated through an ethanol series (20 min per change from 50% ethanol-water in 10% steps to absolute ethanol) followed by propylene oxide (3×10 min), and then infiltrated with and embedded in Spurr's resin (Sigma). Ultra-thin (silver or gold) sections were cut with glass knives (or diamond knives when in relatively sand-free areas of the tissue). These were stained with Reynold's lead citrate (6 min at 26 °C) and 7% aqueous uranium acetate (1 h at 26 °C).

Scanning electron microscopy was done on a Cambridge Instruments Stereoscan 360 SEM fitted with a Link Analytical QX2000 energy-dispersive X-ray detector. Some blocks were soaked overnight in either 5% w/v aqueous ammonium formate or double-distilled water and then rapidly frozen by immersion in liquid N_2-chilled *n*-pentane (−130 °C). The frozen tissue was transferred to Parafilm cylinders (4 mm diam) which were filled with liquid N_2-chilled *n*-pentane. The cylinders were sealed by crimping both ends, rapidly frozen by immersion in liquid N_2, and then fractured by a firm tap with a pre-chilled razor blade. The fractured pieces of tissue were removed from the *n*-pentane and freeze-dried, mounted on graphite stubs, and carbon-coated. Small blocks of the unfixed, frozen tissue were thawed either in cold CMF-ASW or in 3.7% formaldehyde in CMF-ASW and examined in the same way by light microscopy or SEM. X-ray micro-analysis was done on carbon-coated material mounted on carbon stubs.

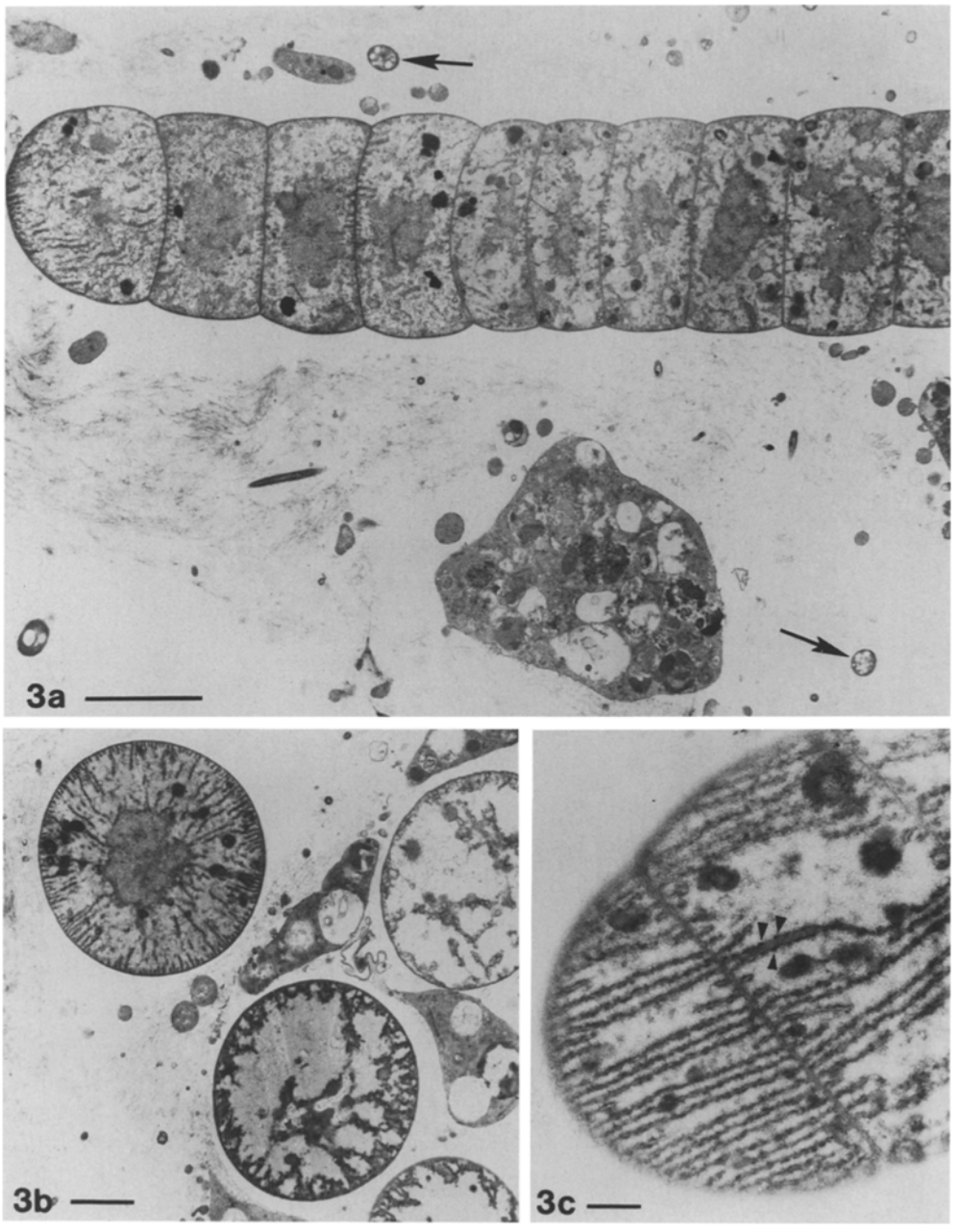

Fig. 3 *Dysidea herbacea.* (a) Longitudinal section of cyanobacterial filament in sponge mesohyl (magnification 4100×; scale bar=4 µm); (b) cross-section, cyanobacteria (magnification 4500×; scale bar=2 µm); (c) detail of thylakoids (arrowed) and cell inclusions (magnification 15 000×; scale bar=500 nm)

Cell separation

A subsample (5 ml) of fresh tissue from the same specimen of *Dysidea herbacea* was cut into small pieces and put through 300 µm Nitex mesh in order to separate most cellular material from the spongin skeleton. This coarsely dissociated cellular material was filtered by vacuum onto 1.2 µm nylon filters (Micron Separations, Inc., Westborough, Massachusetts) and fixed immediately by placing the filters in 2.5% glutaraldehyde in 0.45 µm-filtered natural seawater. The fixed material was stored in the dark at 4 °C for 17 d. The cellular material was removed from the filters by gentle aspiration with a pipette, passed through a 40 µm Nitex filter, and then centrifuged for 30 min at 1000 ×*g* to give a pellet (≃0.5 ml sedimented volume) that was then resuspended in 10 ml cold CMF-ASW. Flow cytometric analysis and sorting were performed using a Becton-Dickinson FACStar Plus cell-sorter equipped with an argon laser (excitation wavelength 488 nm) and a 575 nm bandpass filter. The cells were analysed for forward light scatter, 90-degree side-scatter, and both chlorophyll fluorescence and phycoerythrin fluorescence. Phycoerythrin fluorescence was chosen for the sorting parameter as initial analysis had shown the fluorescence of the intact algal filaments to be from 1 to 2 orders of magnitude greater than that of the sponge cells (Fig. 2a). The fixed cells were sorted into "positive" particles (high fluorescence) and "negative" particles (low fluorescence). Sorting was monitored by periodic examination of the two populations by light microscopy.

A subsample of the post-sorted negatives was checked for purity by re-analysis through the flow cytometer; because of their relatively low numbers, the positives were examined for purity by light microscopy only. Approximately half of each of the two sorted fractions was extracted twice with methanol and thrice with a 1:1 mixture of methanol-dichloromethane; the rest of the sorted cells was reserved for microscopy. The organic extracts of the positives and of the negatives were analysed by ^{1}H-NMR and GC–MS using Pure Compound *1* isolated from the whole tissue as the reference standard for both chromatographic behaviour (retention time) and mass spectra.

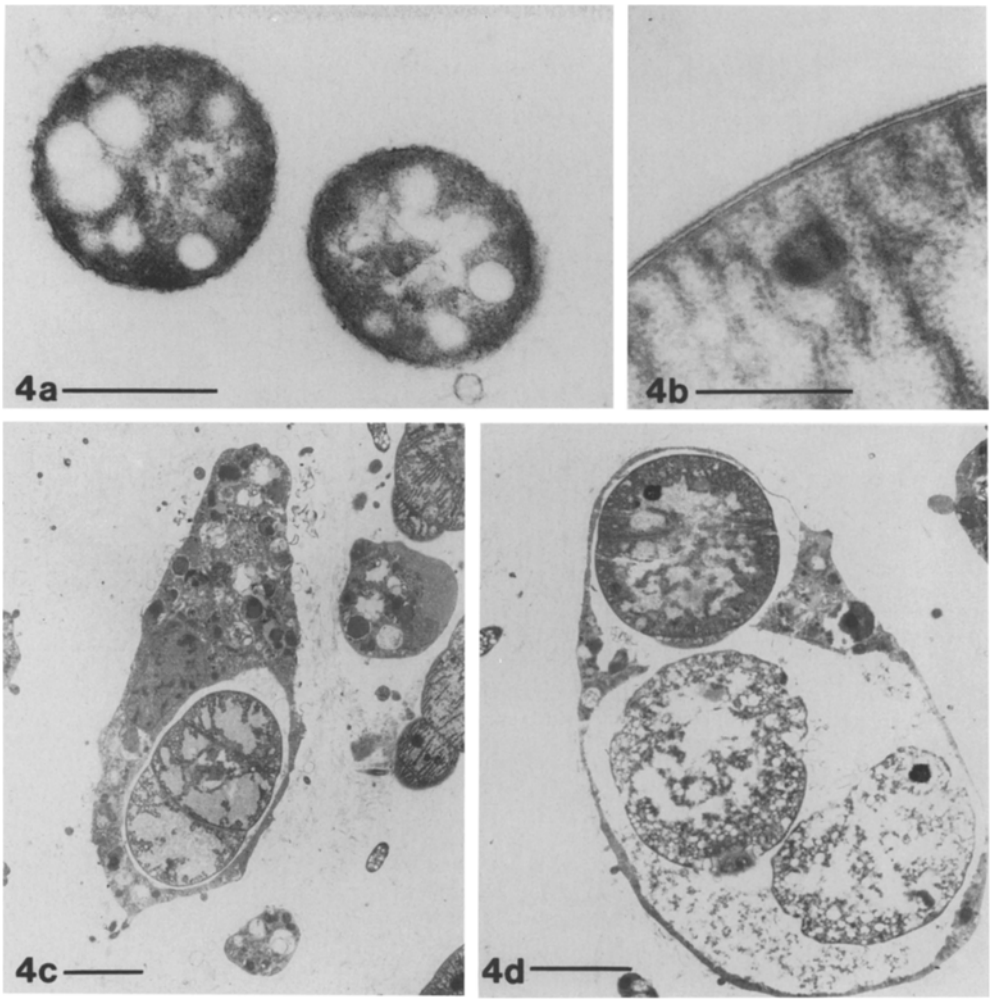

Fig. 4 *Dysidea herbacea.* (a) Eubacteria in sponge mesohyl (magnification 41 000×; scale bar=500 nm); (b) detail of cyanobacterial cell wall (magnification 41 000×; scale bar=500 nm); (c) cyanobacterium ingested by sponge cell (magnification 2600×; scale bar= 4 μm); (d) cyanobacteria ingested by sponge cell (magnification 3400×; scale bar=4 μm)

Results

Specimen 93-165 of *Dysidea herbacea* matched the distinguishing characters described for the species by Bergquist (1980): its surface was micro-conulose, the collagen fibres were concentrically stratified, all fibres were heavily cored with sand grains, and the mesohyl was densely filled with a filamentous cyanobacterium which closely resembles *Oscillatoria spongeliae* (Schulze), as described by Berthold et al. (1982). The cyanobacteria, which were extracellular in the mesohyl, were from 4 to 24 cells in length and had no detectable sheath (Fig. 3 a). Individual cells ranged from 5.9 to 8.5 μm (mean=7.8 μm, n=20) in width and from 2.8 to 5.1 μm (mean=4.0 μm, n=20) in length (Fig. 3 a). The cytoplasm contained dense inclusions (300 to 500 nm), some of which appeared solid (probably equivalent to polyhedral bodies) and some of which had an electron-lucent core (Fig 3 a, b). No "stellar bodies" as reported by Berthold et al. were seen. Thylakoid membranes were arranged radially around the perimeter of the cell when viewed in cross-section (Fig. 3 b, upper left). Most cells contained a large central area of granular, thylakoid-free cytoplasm (Fig. 3 b, upper left). The phycobilisomes on the thylakoid membranes were clearly seen (Fig. 3 c, arrowheads). No heterocysts were observed. Some filaments (presumably older cells) contained few thylakoids or other organelles (Fig. 3 a; 3 b lower right). The terminal cells of each filament were hemispherical, and definite constrictions were seen at the cross-wall between cells (Fig. 3 a). The cell wall (Fig. 4 b) was typical of cyanobacteria. A small percentage of filaments appeared to be dividing by necridia, as described by Berthold et al.

Relatively few eubacteria were observed in the sponge tissue (Fig. 3 a, arrows), and all appeared to represent a single morphological type (Fig. 4 a). The most distinctive structures of the bacterial cytoplasm were vacuoles up to several hundred nanometers in diameter. Most eubacteria-sized profiles in the mesohyl were actually cross-sections of sponge cell processes. The cyanobacterial filaments were occasionally seen within sponge archaeocytes, where they were apparently being digested (Fig. 4 c, d).

A single tetrabrominated compound was isolated as the major metabolite (comprising 67.3% of the total organic extract or 6.0% of the sponge dry weight) from the whole-sponge tissue. Its structure was determined by comparison of its spectral data with those in the literature to be 2-(2′,4′-dibromophenyl)-4,6-dibromophenol (*1*) (Fig. 1), which

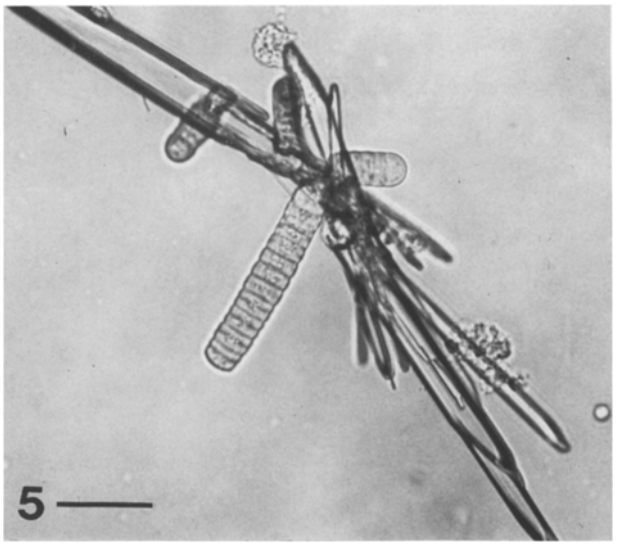

Fig. 5 *Dysidea herbacea.* Polybrominated diphenyl ether crystals with adhering cells, from glutaraldehyde-fixed dissociated tissue (magnification 650×; scale bar=20 μm)

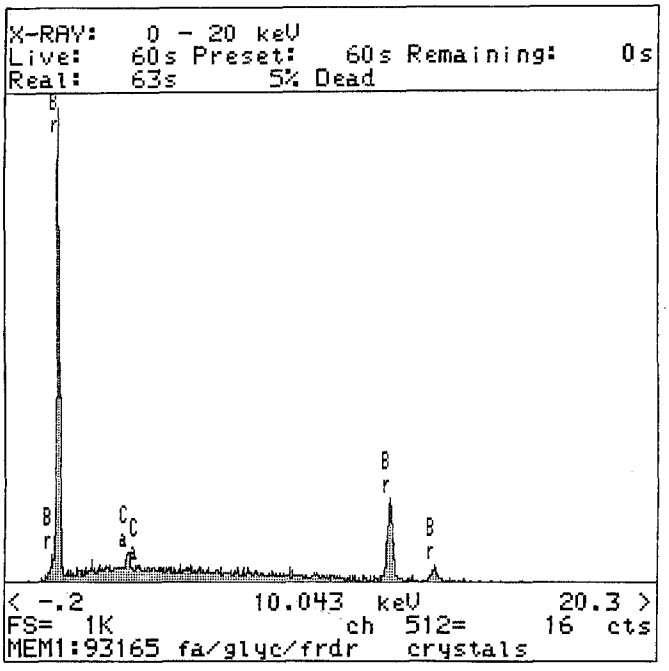

Fig. 6 *Dysidea herbacea.* Energy-dispersive X-ray spectrum of isolated crystalline material. Vertical axis is number of X-ray counts (full scale=1000), horizontal axis is X-ray energy in keV

had previously been reported from other Palauan specimens of *Dysidea herbacea* (Sharma and Vig 1972, Carté and Faulkner 1981).

When the glutaraldehyde-fixed dissociated cells were examined under the light microscope, abundant, fine, needle-like objects were observed, singly and in clusters, often with sponge cells and cyanobacterial filaments adhering to them (Fig. 5). These were not artifacts of glutaraldehyde fixation, as they were observed also in frozen, unfixed tissue that had been either: (1) thawed and observed under the light microscope, or (2) thawed, rinsed briefly in distilled water, freeze-dried without dehydration through organic solvents, and observed with the scanning electron microscope. The needles ranged from about 50 to over 300 μm in length; the thickest observed was 16 μm in width. Under cross-polarized light, these needles appeared brightly refractive, as is characteristic of crystalline material. The occasional exogenous siliceous spicule in the preparations did not refract polarized light. In the fixed tissue blocks, these crystals were detected throughout the sponge tissue, but were more abundant in the ectosomal layer where they were arranged in feather-like patterns. Crystals were insoluble in water, but dissolved easily in methanol, ethanol, dichloromethane, and toluene, indicating that they were organic rather than inorganic. To test whether the crystals might be composed of the brominated metabolite *1*, samples of the crystals were air-dried onto a glass cover-slip, carbon-coated, and their elemental X-ray spectrum was observed (Fig. 6). The X-ray spectra showed an extremely high bromine content, indicating that the crystalline material within the sponge's tissues was probably Compound *1*.

Examination by light microscopy of the fixed cell preparation showed that it consisted primarily of sponge cells and cyanobacterial filaments, with relatively few heterotrophic bacteria. The absence of crystalline material in the fixed cell population was confirmed by examining the preparation under cross-polarized light. Flow-cytometric sorting of the fixed cells resulted in ~5×10^5 "positive" particles and ~10^8 "negative" particles. Re-analysis of the sorted negatives through the flow cytometer (Fig. 2b) indicated that they were >99% pure (i.e., almost no intact cyanobacterial filaments were present; a few broken or lysed, low-fluorescence filaments were observed), and light microscopy showed them to consist primarily of sponge cells, with relatively few heterotrophic bacteria. The sorted positives was confirmed by light microscopy to be an almost pure (>95%) preparation of intact, brightly-fluorescent cyanobacterial filaments, with a small number of broken filaments.

Under the GC–MS conditions described above, Compound *1* had a retention time of 28.5 min, and the appropriate molecular ion cluster was observed (Figs. 7a and 8a). GC–MS analysis of the organic extracts of the sorted cell fractions showed that the extract of the positives contained relatively few components compared to the extract of the negatives (Fig. 7b). Compound *1* was identified by retention time (Fig. 7b) and mass spectra (Fig. 8b) only in the positives; none could be detected in the negatives (Figs. 7c and 8c) by examination of all mass spectra taken

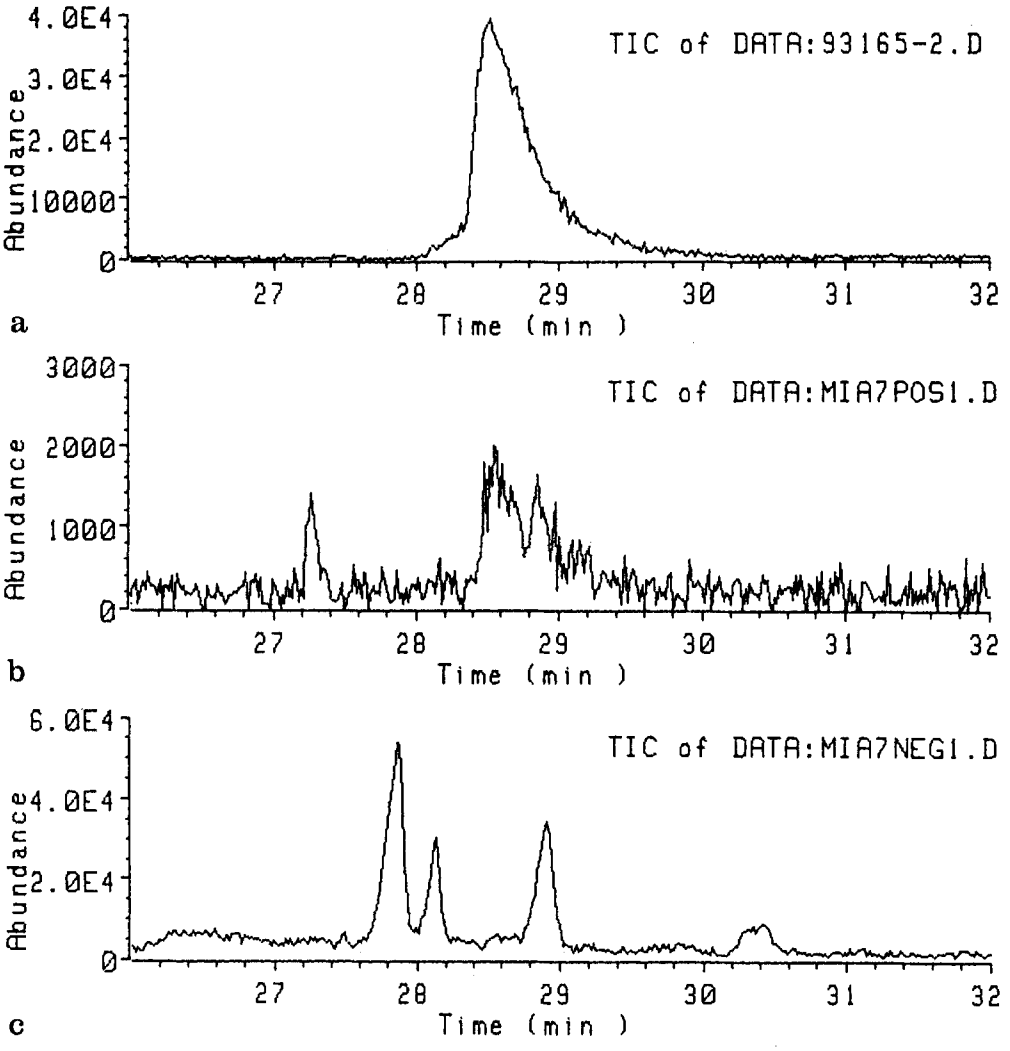

Fig. 7 *Dysidea herbacea.* Total ion gas-chromatographic traces between 26 and 32 min of pure standard *1* (a), and of organic extracts from the flow-cytometrically sorted "positive" (i.e., cyanobacteria) (b), and "negative" (i.e., sponge cells, bacteria) (c) cell fractions

between 27 and 30 min retention times. ^{1}H-NMR spectroscopy supported these findings: only a very slight trace of Compound *1* could be detected in the negatives (Fig. 9a), but it was conspicuously present in the positives (Fig. 9b, arrows).

Discussion

Natural products reported from *Dysidea herbacea* may be classified into three groups: terpenoids, polychlorinated amino-acids, and polybrominated biphenyl ethers. Terpenoids occur in great variety in marine sponges and are of some use in taxonomy (reviewed by Bergquist and Wells 1983). The Dysideidae, including several species of the genus *Dysidea*, usually yield bi- or tricyclic sesquiterpenes (C_{15} compounds) with various carbon skeletons and frequently possessing a furan functionality; typical examples are the furanoid sesquiterpenes, herbadysidolide (*3*) (Charles et al. 1978a) and spirodysin (*4*) (Kazlauskas et al. 1978b) (present Fig. 1). Other types of terpenoids from *Dysidea* species include meroterpenes (compounds of mixed biosynthesis) (Minale et al. 1974, Hirsch et al. 1991), diterpenes (C_{20}) (Walker and Faulkner 1981, Carmely et al. 1988), a C_{21} furanoterpene (Kashman and Zviely 1980), a triterpene (C_{30}) hydroquinone sulfate (Fusetani et al. 1987), and a monoterpene (C_{10}) (Mancini et al. 1987). The terpenes of *D. herbacea* have been assumed to be true sponge metabolites because of their occurrence throughout the dictyoceratids and related sponges (Minale 1978, Bergquist and Wells 1983), and this has recently been confirmed (Unson and Faulkner 1993).

The polychlorinated compounds reported from *Dysidea herbacea* are exemplified by dysidin (*5*) (Hofheinz and Oberhänsli 1977) and dysidenin (*6*) (Kazlauskas et al. 1977). The former resembles malyngamide A (*7*) from the free-living filamentous cyanobacterium *Lyngbya majuscula* (Fig. 1) (Cardellina et al. 1979). The polybrominated biphenyl ethers found in some specimens of *D. herbacea* are typified by 2-(2′,4′-dibromophenoxy)-3,4,5,6-tetrabromophenol (*8*) (Utkina et al. 1987, Salvá and Faulkner 1990) (present Fig. 1). Very similar brominated compounds have been reported from a green alga *Cladophora*

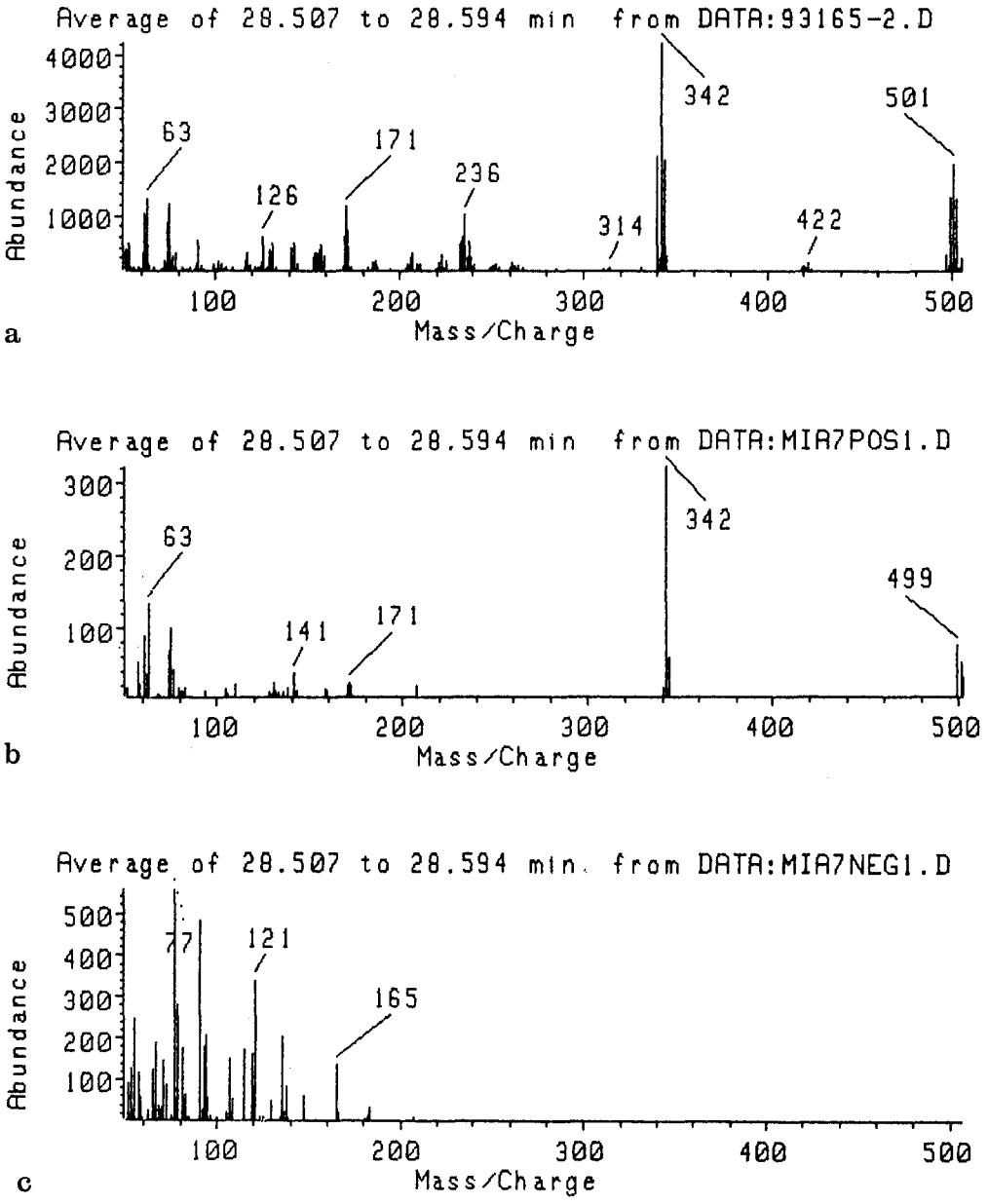

Fig. 8 *Dysidea herbacea.* Mass spectra averaged over 28.507 to 28.594 min of gas-chromatographic analyses of pure standard *1* (a), and of organic extracts from the flow-cytometrically sorted "positive" (i.e., cyanobacteria) (b), and "negative" (i.e., sponge cells, bacteria) (c) cell fractions

fascicularis and a sea hare *Aplysia dactylomela* grazing on it (Kuniyoshi et al. 1985), from an unidentified sponge in the family Callyspongidae (Capon et al. 1981), and from acorn worms (Hemichordata, Enteropneusta) (Ashworth and Cormier 1967, Higa and Sakemi 1983). To the best of our knowledge, no biphenyl ethers have yet been reported from free-living cyanobacteria. It has often been suggested that the halogenated compounds of *D. herbacea* are of "symbiont" biosynthesis (Kazlauskas et al. 1977, Charles et al. 1978b, Cardellina et al. 1979, Carté and Faulkner 1981, Norton et al. 1981, Braekman et al. 1990), and this has been confirmed by Unson and Faulkner (1993) and by the present study.

Two specimens of an Eastern Samoan *Dysidea* sp. that contained the bromophenol *9* yielded two strains of an unidentified *Vibrio* sp. (Elyakov et al. 1991, Voinov et al. 1991). The cultures appeared to be axenic by microscopic examination (V. V. Mikhailov personal communication, G. B. Elyakov and V. V. Mikhailov personal communication). GC–MS analyses showed that the butanol extracts of liquid cultures of one of these strains contained traces of *9*, the same compound isolated from the sponge (Elyakov et al. 1991, Voinov et al. 1991). However, there was no evidence that the *Vibrio* strain was consistently associated with *Dysidea* spp. that produce brominated biphenyl ethers, nor were attempts made to quantify the relative abundance of the *Vibrio* sp. in the sponge (V. V. Mikhailov personal communication). The present results show that the brominated biphenyl ethers found in *D. herbacea* are in fact associated with the cyanobacterium and not with the sponge cells or heterotrophic bacteria, and are therefore probably biosynthesized by the cyanobacterium.

Although the polybrominated biphenyl ethers from *Dysidea herbacea* were first described over two decades

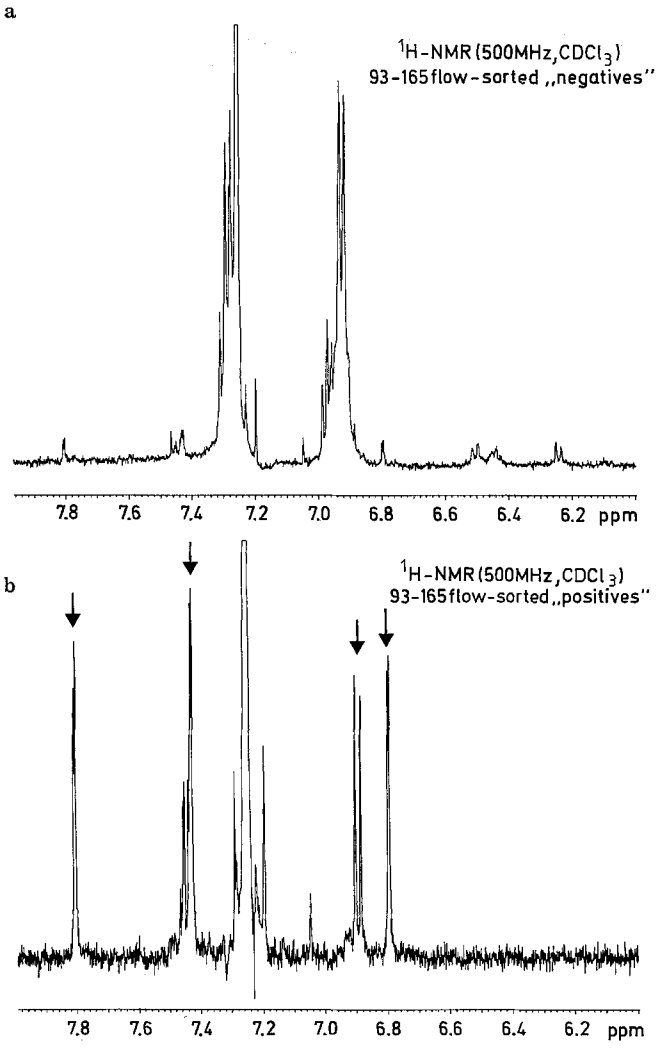

Fig. 9 *Dysidea herbacea.* H-NMR spectra ($CDCl_3$) of organic extracts of flow-cytometrically sorted "positives" (i.e., cyanobacteria) (a), and "negatives" (sponge cells, bacteria) (b). Arrows indicate signals due to aromatic protons of Compound 1

ago, the present study is the first observation that these metabolites can be present as crystalline material within the sponge tissues. This is not unexpected, as the brominated phenols are characteristically found in remarkably high levels – up to 12% of the sponge's dry weight – in *D. herbacea*. It is likely that the polybrominated metabolites are produced by the cyanobacteria and are excreted into the surrounding aqueous milieu in which they are not soluble, where they therefore crystallize. The great bulk of the brominated metabolites is present as crystalline material in the sponge mesohyl, with only a relatively small amount in the cyanobacteria. Since the brominated biphenyl ethers are soluble in organic solvents, tissue samples (fresh, frozen, or aldehyde-fixed) which have been stored in alcohol would show no traces of the crystals. It is possible that the compounds are less toxic to the sponge–cyanobacterial association when in crystalline form.

Most halogenated phenols are strongly antimicrobial. Standard disc assays show that the polybrominated biphenyl ethers from *Dysidea herbacea* are active against both Gram-negative and Gram-positive eubacteria, as well as test strains of the unicellular marine cyanobacterium *Synechococcus* sp. Compound *1* inhibited growth of *Staphylococcus aureus* at 5 μg per disk, *Escherichia coli* at 5 μg per disk, *Bacillus subtilis* at 1 μg per disk, *Vibrio harveyi* at 10 μg per disk, *Synechococcus* Strain PCC 7002 at 10 μg per disk, and *Synechococcus* Strain WH 7803 at 0.1 μg per disk, but did not inhibit growth of the yeasts *Candida albicans* or *Saccharomyces cerevisiae* at 250 μg per disk. This apparent general toxicity to prokaryotes may be the reason why few other eubacteria were observed in the sponge tissue, although at least one case is known of a bacterium (a *Sphingomonas* sp.) that is able to completely catabolize monohalogenated diphenyl ethers (Schmidt et al. 1992). Presumably the *Dysidea herbacea–Oscillatoria spongeliae* association is resistant to the polybrominated compounds.

In both our own experience and in the literature (Kazlauskas et al. 1978a, Dunlop et al. 1982), specimens of *Dysidea herbacea* generally contain either polychlorinated metabolites or polybrominated metabolites, but not both; those specimens containing polychlorinated metabolites usually contain sesquiterpenes, whereas those containing polybrominated metabolites do not. (In the one case where we found all three types of metabolites, the sample proved to be an accidentally mixed collection.) Unfortunately, unequivocal identification of species of *Dysidea* is difficult if not impossible on gross morphological grounds (Bergquist 1965, 1980), and taxonomic identification of specimens of "*Dysidea*" reported in the natural products literature may be suspect because of *Dysidea*'s superficial similarities to other foliose dictyoceratid genera such as *Phyllospongia* and *Carteriospongia* (both in the family Spongiidae) (J. Vacelet personal communication, P. R. Bergquist personal communication). Our findings agree with the previously published observation (Kazlauskas et al. 1978a, Dunlop et al. 1982) that specimens of *D. herbacea* contain either polychlorinated or polybrominated compounds, but not both. The results we present here and in an earlier paper (Unson and Faulkner 1993) indicate that there are possibly two or more "chemotypes" of the symbiotic cyanobacterium, which could account for the chemical variability of the natural products isolated from the sponge. However, no genetic studies have yet been done on the sponge or the cyanobacterium.

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