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Cyanobacterial symbiont biosynthesis of chlorinated metabolites from *Dysidea herbacea* (Porifera)

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Abstract. The tropical marine sponge *Dysidea herbacea* contains large numbers of a symbiotic filamentous cyanobacterium identified on the basis of a detailed ultrastructural study as *Oscillatoria spongeliae*. We report the flow-cytometric separation of the symbiont from the sponge cells, and demonstrate by chemical analyses that a unique group of polychlorinated compounds isolated from the whole sponge tissue is limited to the cyanobacterial filaments, whereas the accompanying sesquiterpenoids are found only in the sponge cells. This is the first demonstration that secondary metabolites ascribed to a sponge are localized in prokaryotic symbiont cells.
Key words. Sponge; *Dysidea herbacea*; cyanobacterium; *Oscillatoria spongeliae*; symbiosis, secondary metabolites; flow cytometry.

Marine sponges are a rich source of diverse natural products, many of which are of chemotaxonomic or biomedical interest[1,2]. Prokaryotic endosymbionts, primarily cyanobacteria and heterotrophic eubacteria, are almost ubiquitous in marine sponges[3-5] and in some cases they have been demonstrated to be advantageous to the host[6,7]. We have been interested in determining the role of these symbionts in the production of natural products, as well as the possible functions of metabolites produced by symbionts in maintaining the symbiosis. To do this, it is necessary to determine the biosynthetic source of the metabolites.

Dysidea herbacea (Keller 1889) is a shallow-water dictyoceratid sponge common throughout the Indo-Pacific[8-10]. Its major prokaryotic symbiont, a filamentous non-heterocystous cyanobacterium (blue-green alga) identified on the basis of a detailed ultrastructural study as *Oscillatoria spongeliae* (Schulze 1879)[11], occurs intercellularly in large numbers, up to 20% of the symbiotic association's volume (our own observations). We investigated this well-documented symbiosis[11,12] because the biosynthesis of certain compounds consistently isolated from the *Dysidea herbacea–Oscillatoria spongeliae* association could be ascribed either to the sponge or to the cyanobacterium. Sesquiterpenes (C_{15} compounds) occur throughout the genus *Dysidea* (including species lacking cyanobacteria) as well as related sponges[13], and therefore herbadysidolide **1**[14] and spirodysin **2**[15] (fig. 1), isolated from *D. herbacea*, seemed likely to be true sponge metabolites[16]. On the other hand, a group of unique polychlorinated amino-acid derivatives, which are found at levels of up to 5% dry weight in *D. herbacea* (our own observations), slightly resemble compounds isolated from free-living filamentous cyanobacteria and might be expected to be cyanobacterial metabolites. For example, malyngamide A **3**, isolated from the marine cyanobacterium *Lyngbya majuscula*[17], and mirabimide A **4**, from a terrestrial strain of *Scytonema mirabile*[18], contain a nitrogenous moiety similar to that of dysidin **5** from *D. herbacea*[19] (fig. 1).

For many organisms, proof of biosynthesis is usually obtained by feeding labelled precursors, but in vivo biosynthetic studies are intrinsically complicated for symbiotic assemblages comprising two or more species, each of which may, independently or in combination, be involved in biosynthesis. One strategy to determine the biosynthetic potential of the symbionts has been to isolate putative symbionts in pure culture and examine their secondary metabolites. A report of the successful culture of a heterotrophic bacterium from a sponge describes the subsequent isolation from the culture of diketopiperazines (cyclic dipeptides) previously ascribed to the sponge[20]. However, it is now known that most culturable unicellular marine bacteria produce similar or identical diketopiperazines (J. Trischman, pers. comm.). More recently, Elyakov et al.[21] reported the isolation of a *Vibrio* sp. from two specimens of an unidentified *Dysidea* and showed that the cultured bacterium produced the same polybrominated biphenyl ether **6** (fig. 1) previously obtained from organic extracts of a *Dysidea* sp., although it is not clear whether ether **6** was in fact found in the same sponge specimens from which the bacterium was isolated[21]. The polybrominated biphenyl ethers found in certain *Dysidea* spp. are typically isolated in yields of 2–5% dry weight[22,23]. These high yields would require a significant volume of the alleged symbiont in the sponge tissues if it indeed produced these compounds, which was not demonstrated in this case[21].

Cyanobacteria are generally difficult to grow in pure, axenic culture[24]. Furthermore, there is no guarantee that a symbiont, particularly if obligate, will produce

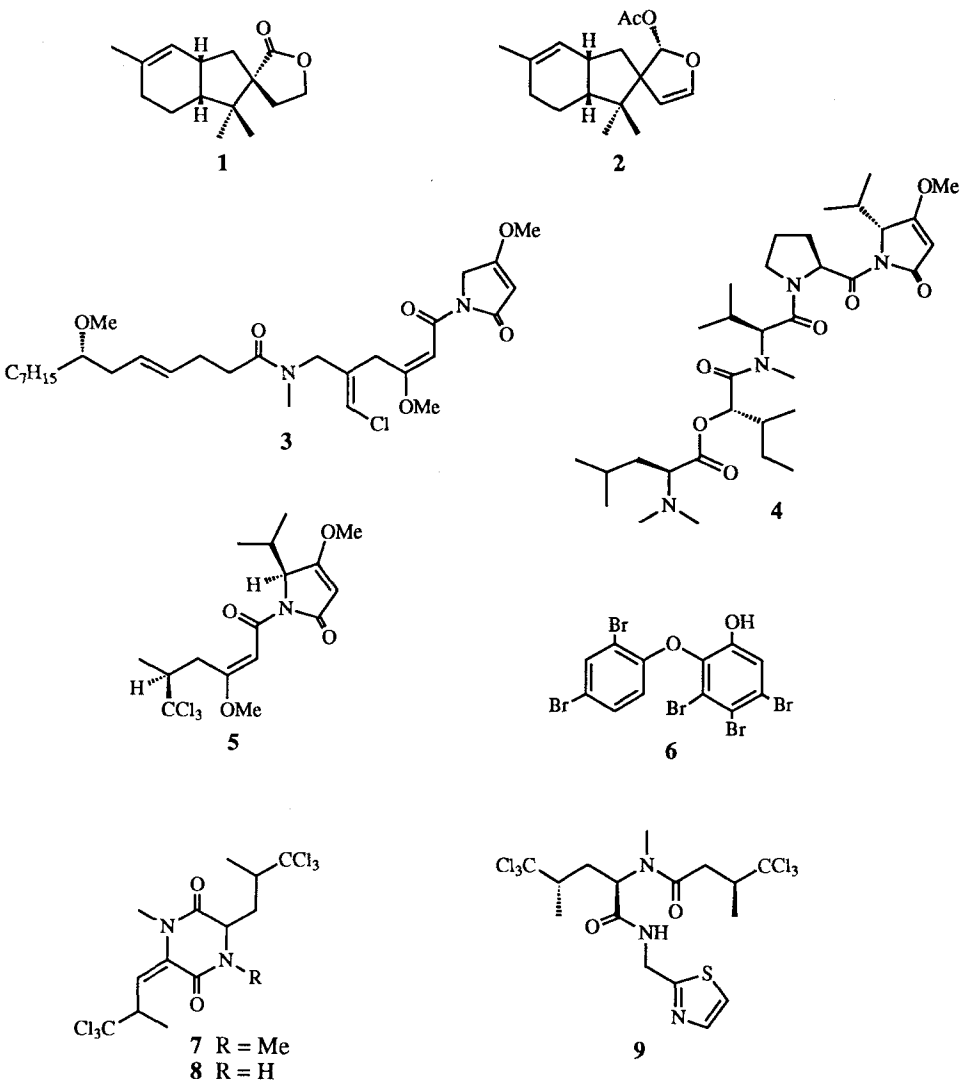

Figure 1. Compounds typical of *D. herbacea* include herbadysidolide (**1**), spirodysin (**2**), dysidin (**5**), 2-(2′,4′-dibromophenoxy)-3,4,5-tribromophenol (**6**), chlorinated diketopiperazines (**7** and **8**), and 13-demethylisodysidenin (**9**). Compounds related to dysidin (**5**) from free-living filamentous cyanobacteria include malyngamide A (**3**) and mirabimide A (**4**).

the same secondary metabolites when grown in culture[24]. An alternative approach to finding the biosynthetic origin of secondary metabolites is based on the cellular localization of selected compounds. The polychlorinated amino-acid derivatives, with their trichloromethyl functionality that has not been found elsewhere in nature, are sufficiently complex to be regarded as biosynthetically unique chemical markers. If they are found only in the cyanobacterial cells, we argue that this would be convincing evidence that they are biosynthesized by the cyanobacterium. We thus chose to attempt to separate the cyanobacterial filaments from the sponge cells (and any associated eubacteria), and perform chemical analyses of the different cell types.

Materials and methods

General experimental procedures. All solvents used were mass-spectral grade (Fisher Optima) or freshly distilled from reagent grade. Column chromatography was carried out using Merck silica gel 60 (70-230 mesh ASTM). ^{1}H-NMR spectra were obtained on a Varian 500 MHz spectrometer; samples were dissolved in deuterated chloroform (Isotec). High-resolution mass spectrometry was carried out at the Mass Spectrometry Facility, University of California at Riverside. Gas chromatographic separation was done using a Hewlett Packard 5890 Series II gas chromatograph fitted with an Alltech AT35 capillary column (25 m long, 0.2 mm i.d., 0.22 μm film thickness) and programmed for a temperature gradient from 140 °C to 310 °C at 4 °C min^{-1}, followed by analysis with a Hewlett Packard 5988A mass spectrometer with the mass spectral scan range set to 50–550 m/z. Light and fluorescence microscopy was done on an Olympus microscope. Electron microscopy was done on a Hitachi H500 scanning and transmission electron microscope.

Collection. The specimen of *Dysidea herbacea*, a 2-mm-thick, greenish-grey sponge, was collected by hand at −4 m at Heron Island in the southern Great Barrier Reef, Australia. The sponge was transported in seawater to the laboratory where it was carefully removed from its substrate (dead *Acropora* sp. and coral rock) and the coralline rhodophyte (*Jania* sp.) which it encrusted. Examination of the fresh sponge by light microscopy showed it to match the distinguishing characters described for the species by Bergquist[10]; the surface is finely conulose, the collagen fibres are concentrically stratified and heavily cored with sand grains, and the matrix densely filled with a filamentous cyanobacterium.

Extraction of whole tissue and purification of secondary metabolites. Part of the sponge (80 ml fresh volume) was extracted sequentially with methanol and dichloromethane at room temperature. The organic extracts were combined, the solvent removed under reduced pressure, and the resulting aqueous suspension triturated with dichloromethane. The dichloromethane-soluble material was dried over sodium sulfate and the solvent removed. This crude extract (525 mg) was chromatographed on a silica gel flash column eluted with a solvent gradient from 100% hexanes to 100% ethyl acetate in 10% steps. Fractions that contained the metabolites of interest were further purified by HPLC on a silica gel column (Whatman Partisil 10, 1 cm × 45 cm) with mixtures of hexanes/ethyl acetate. The structures of the purified compounds spirodysin **2**, 13-demethylisodysidenin **9**, herbadysidolide **1**, and diketopiperazines **7** and **8** (fig. 1) were determined primarily by ^{1}H- and ^{13}C-NMR spectroscopy and high-resolution mass spectrometry. The purified secondary metabolites were then used as reference standards for the chemical analyses.

Cell separation study. A subsample (10 ml) of fresh tissue from the same *D. herbacea* specimen was cut into 5 mm × 5 mm pieces and squeezed into cold calcium/magnesium-free artificial seawater (CMF-ASW, pH 7.4). The resulting cell suspension was centrifuged for 2 min at 800 × g to give a pellet which was fixed immediately in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4) that was 0.3 M in NaCl. Fixed samples were stored at 4 °C in the dark. The fixed, dissociated cells were examined by fluorescence microscopy, confirming retention of native phycoerythrin fluorescence in the cyanobacterial filaments. 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 glutaraldehyde-fixed cells (0.2 ml sedimented volume) were resuspended in CMF-ASW, passed through a 43-μm nylon filter, and analyzed 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 showed the fluorescence of the intact algal filaments to be two orders of magnitude greater than that of the sponge cells. The fixed cells were sorted into 'positive' particles (high fluorescence) and 'negative' particles (low fluorescence) (fig. 2). Sorting was monitored by periodic examination of the two populations by light microscopy, and subsamples of the post-sorted cells were checked for purity by re-analysis through the flow cytometer. The sorted cells were immediately centrifuged 5 min at 300 × g. About one-fourth of each of the resulting cell pellets were reserved for microscopy, and the rest of the pellets individually extracted as described above. For light microscopy, samples of the separated cells were mounted in GelMount (Fisher Scientific) and photographed with colour film (Kodak Ektachrome P800/1600) using both light field and fluorescence (blue excitation at 450–490 nm, 515 nm barrier filter). Samples of the separated cells were also dehydrated through an ethanol series and embedded in Spurr's resin (Sigma Chemical Co.) for electron microscopy. The organic extracts of the whole sponge tissue, the positives, and the negatives were analyzed by ^{1}H-NMR (fig. 3) and

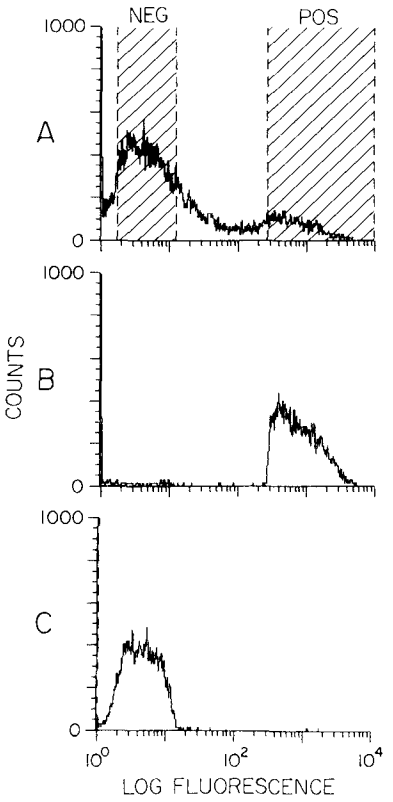

Figure 2. Flow cytometric analyses of (A) the cell population prior to sorting, (B) the post-sorted 'positive' population, and (C) the post-sorted 'negative' population. The histogram shows the distribution of particles using phycoerythrin fluorescence as a sorting parameter; shaded areas indicate the selected 'positive' or 'negative' populations.

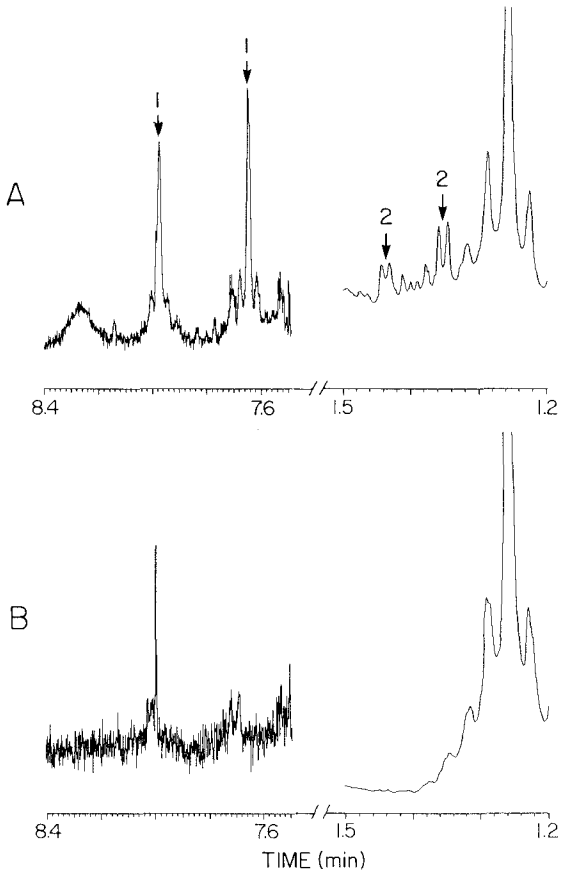

Figure 3. 500 MHz ^{1}H-NMR spectra in $CDCl_3$ of (A) total organic extract of the 'positive' cell population, and (B) total organic extract of the 'negative' cell population. Arrows indicate proton signals of (1) the thiazole moiety and (2) methyl doublets of 13-demethylisodysidenin **9**.

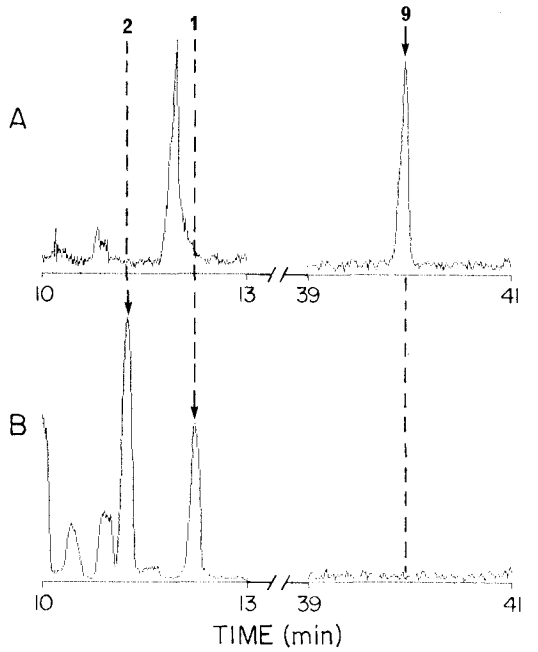

Figure 4. Total ion current profiles from the gas chromatography analyses of (A) total organic extract of the 'positive' cell population, and (B) total organic extract of the 'negative' cell population. Pure compounds isolated from the whole tissue were used as external standards (not shown) for both retention times and mass spectra. Arrows indicate the peaks from herbadysidolide (**1**), spirodysin (**2**), and 13-demethylisodysidenin (**9**).

GC-MS (fig. 4) using the pure compounds isolated from the whole tissue as reference standards for both chromatographic behaviour (retention times) and mass spectra. In all cases individual components of the organic extracts were identified by both retention time and mass spectra.

Results and discussion

Five major secondary metabolites were isolated from the whole tissue. These were the major known sesquiterpene spirodysin **2**[15] (24.7% by weight of the total crude extract, or 0.245% of the tissue's dry weight), the major chlorinated metabolite 13-demethylisodysidenin **9** (9.4% of the total crude extract; this was previously reported as both a natural product with the incorrect absolute configuration[25], and as a synthetic compound[26]), the known sesquiterpene herbadysidolide **1**[14] (5.4% of the total crude extract), the known diketopiperazine **7**[27] (1.5% of the total crude extract), and a new diketopiperazine **8** (1.0% of the total crude extract). NMR and mass spectra of the previously reported compounds were consistent with published data. The structural elucidation data for diketopiperazine **8** will be reported elsewhere.

The flow cytometric sorting resulted in $\sim 2 \times 10^6$ 'positives' and $\sim 10 \times 10^6$ 'negatives'. Examination by light, fluorescence, and electron microscopy showed that the 'positives' consisted of >95% intact cyanobacterial filaments; conversely, the 'negatives' were primarily sponge cells, with <1% segments of cyanobacterial filaments.

Proton NMR signals due to the chlorinated metabolites (mostly 13-demethylisodysidenin **9**) were detected only in the organic extracts of the whole sponge tissue and of the 'positive' (i.e., cyanobacterial) cells, indicating that these compounds are localized within the cyanobacterial filaments. No sesquiterpene ^{1}H signals were clearly recognizable in either the 'positive' or 'negative' extracts. However, GC-MS analysis unambiguously showed that the sesquiterpenes **1** and **2** were present only in the organic extracts of the whole sponge tissue and of the 'negative' (i.e., sponge) cells, and also confirmed that the chlorinated metabolites were limited solely to the cyanobacterial filaments. GC retention times and mass spectra of identifiable peaks in the extracts were essentially identical (within 3 s and >95% of all major fragment ions, respectively) to those of the pure compounds used as reference standards.

These are the first experimental data to show that the polychlorinated metabolites ascribed to *Dysidea herbacea* are localized in the symbiont *Oscillatoria spongeliae*. This implies that the chlorinated compounds are

produced by the cyanobacterium, as has often been suggested[22,23,28,29], since the complete translocation of metabolites subsequent to their synthesis must be considered extremely unlikely. This, the first demonstration that marine natural products ascribed to a sponge are localized in symbiont cells, was made possible when we were able to separate sponge and symbiont cells after fixation with glutaraldehyde and still recover the natural products they contained. It had been the dogma among chemists that fixation also 'fixed' the natural products. We believe that this research lays the groundwork for many interesting applications of the use of cell separation techniques in natural products chemistry.

The *D. herbacea* specimens that contain polychlorinated metabolites always harbour large populations of *O. spongeliae*, and in general are thinly encrusting sponges growing in well-lighted areas (our own observations). As well as providing a relatively protected habitat for the cyanobacterium, the sponge's flattened morphology also maximizes exposure to light. Preliminary studies show that *O. spongeliae* translocates some photosynthetically-fixed organic carbon to the host sponge (R. Hinde, pers. comm.), an obvious benefit in nutrient-poor tropical waters, although the symbiotic association apparently does not fix nitrogen[30]. Even though many marine natural products possess some biological or pharmacological activity, their in vivo biological functions are often not understood, particularly in the case of symbiosis. It seems reasonable to propose that the production of secondary metabolites by a symbiont would benefit a host if the chemicals deter potential predators and/or competitors. The polychlorinated metabolites are not anti-microbial, but strongly deter fish-feeding (our unpublished data), and possibly confer increased fitness to the sponge-cyanobacterial symbiosis. Whether they play alternate roles in maintaining the symbiosis is not yet known.

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