A PRELIMINARY ASSESSMENT OF 'SPACE WARS' AS A DETERMINING FACTOR IN THE PRODUCTION OF NOVEL BIOACTIVE INDOLES BY *IOTROCHOTA* SP.

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Iotrochota sp. from Salamander Reef, North Queensland, has yielded a plethora of at least ten bioactive indoles including mono-, di- and non-brominated variants. Metabolite composition varies within and between individual sponges, and competition for hard substrate was suspected as a determining factor in this variability. To provide a preliminary assessment of this, profiles of six identified indoles were compared between tissue samples categorised according to neighbour contact and growth thickness. Five of these compounds contained either two indole moieties (indolyl) or one indole and one benzene ring (benzoyl). The sixth indole, by virtue of its structure, was identified as the putative precursor of the other compounds. There were no significant differences between tissue category and abundance of either indolyl or benzoyl product, or their putative precursor. However, two predominant populations of metabolites were identified. Diminished precursor and increased indolyl product occurred in tissue from sponge edges with direct neighbour contact and thick fleshy projections. This relationship was not absolute, and some samples from these tissue categories contained increased precursor and diminished indolyl product. Tissue from thin central sponge areas and edge samples without direct neighbour contact exclusively contained chemistry in the latter group. The quantity of benzoyl product remained constant between tissue categories. These results neither clearly support nor discount the potential role of space competition in determining production of these compounds. The issues involved are more complex than those examined here, and courses for further investigation are suggested. ❐ *Porifera, chemical ecology, indole, bioactivity, competition.*

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Porifera continue to be the most prolific source of marine derived bioactive compounds in published literature (Marinlit, 1998). Numerous authors have sought to attribute sponge secondary metabolites to a role in the source organism, and many have supported correlations between metabolite variability and environmental parameters such as depth, UV light, and chemical defence (Thompson et al., 1987; Kreuter et al., 1992; Pawlik, 1993; Pawlik et al., 1995; Swearingen & Pawlick, 1995). This report presents some preliminary findings on the possible role of competition for space in variability of some novel indoles produced by a north Queensland sponge from the genus *Iotrochota*.

A sample of this species of *Iotrochota*, a black thin encrusting sponge with occasional thick vertical fleshy projections, was initially collected from Salamander Reef, North Queensland in 1988. The sample was part of the Australian Institute of Marine Science biodiversity collection for natural products screening, and was initially erroneously assigned to the genus *Ircinia*. In 1995, a crude extract of this sponge was found to be highly active in a neuronal nitrous oxide synthase inhibition bioassay (authors' unpublished data). Initial fractionation yielded a plethora of novel indoles including mono-, di- and non-brominated variants. Seven of these compounds have been isolated and identified to date (Bowden et al., 1998; this report; and authors' unpublished data), but several still await further attention. The sponge was recollected in 1996 in order to provide sufficient material for follow-up bioassay and structure elucidation. While both samples contained a similar range of novel indoles, the relative abundance of each compound varied substantially between samples.

An understanding of the cause of this variability will become important if one of these

TABLE 1. Characteristics (HPLC, structure and mass) of compounds examined in this study. Key: A= Brominated species showing two isotopes (^{79}Br and ^{81}Br); B= Nonbrominated species.

Peak no.	Figure no.	HPLC chromatophore		Structural class	Ion species [M-1]
		280nm	360nm		
7	4A	Yes	No	Precursor	306.9/308.9 [A]
9	4B	Yes	Yes	Indolyl	402.1 [B]
14	4C	Yes	Yes	Indolyl	356.1 [B]
16	4E	Yes	Yes	Benzoyl	411.0/413.0 [A]
19	4D	Yes	Yes	Indolyl	434.0/436.0 [A]
21	4F	Yes	Yes	Benzoyl	395.0/397.0 [A]

compounds progresses to become a new drug candidate and there is a need to optimise its yield through manipulative culture or selective recollection. Field observations indicated that competition for hard substrate at Salamander Reef was fierce, yet *Iotrochota sp.* remained abundant. Hence 'Space Wars' was suspected to be a potential controlling factor in metabolite variability in this sponge.

This study aims to provide a preliminary assessment of variability in the production by *Iotrochota* sp. of six bioactive novel indoles, with respect to direct neighbour contact and tissue thickness. It also aims to create a basis for further work to develop an understanding of factors that determine indole variability in this sponge.

MATERIALS AND METHODS

Sponge tissue samples were collected from Salamander Reef, 19°10.91'S, 147°03.76'E, a small rocky inshore reef off Cape Cleveland near Townsville, North Queensland, in March 1998. 29 samples from 8 individual sponges where collected from 10-15m depth.

Small biopsies of sponge tissue (approx $1cm^2$) were taken and assigned one of four categories according to the degree of direct neighbour contact and tissue thickness, as follows: 1) Edge Interaction (edge of sponge in direct contact with a neighbouring organism); 2) Edge No Interaction (edge of sponge without direct neighbour contact); 3) Centre Thin (centre of sponge, no fleshy projection); and 4) Centre Thick (thick fleshy projection in the centre of sponge).

Tissue samples were freeze dried, and 80mg (dry weight) of tissue was extracted in 5ml of a solvent solution made up of equal parts dichloromethane and methanol, with sonication for 80mins. Extract was carefully decanted into clean vials, dried, then redissolved in 1ml methanol for High Performance Liquid Chromatography (HPLC) analysis. Where less than 80mg tissue was available for extraction, solvent quantities were adjusted to achieve the same extraction concentration.

Extracts were analysed for brominated indoles of interest using HPLC with an Alltima C18 column (250x4.6mm, Alltech Australia). A linear gradient from 60-100% of methanol in water was used. UV spectra were recorded with a Shimadzu MXA diode array and absorbance monitored at 280 and 360nm. Major components of HPLC peaks were then characterised by negative-ion electrospray mass-spectrometry to confirm that common compounds could be identified between different sponge extracts.

Areas under HPLC peaks were then used as a measure of relative amount of each fraction, for comparison between samples. These estimates were not suitable to compare quantities of different fractions within individuals, as a full analysis of extinction-coefficients of each compound was not undertaken.

One-way analyses of variance (ANOVA) with $\alpha=0.05$ was used to compare HPLC peak areas of each fraction group of interest, between sample categories. Four samples from each of the four tissue categories were selected from the available sample pool. Samples were independently selected in this way for analysis of each fraction group of interest. Where a result was non-significant, the detectable effect size (standard deviation between means) with 80% power was calculated according to Cohen (1977) and expressed as a percentage of the overall mean.

TABLE 2. Power analysis results for non-significant ANOVAs on HPLC peak areas between tissue categories.

Compound Type	Grand Mean (arbitrary units)	Mean Square Within Groups (from ANOVA)	Standard Deviation between detectably different means	
			(Arbitrary units)	(% of grand mean)
Precursor	42621802	2.37143E+14	14937464.6	35
Indolyl Product	3707935	3.94114E+13	6089514.452	164
Benzoyl Product	14993786	1.08893E+13	3200897.12	21

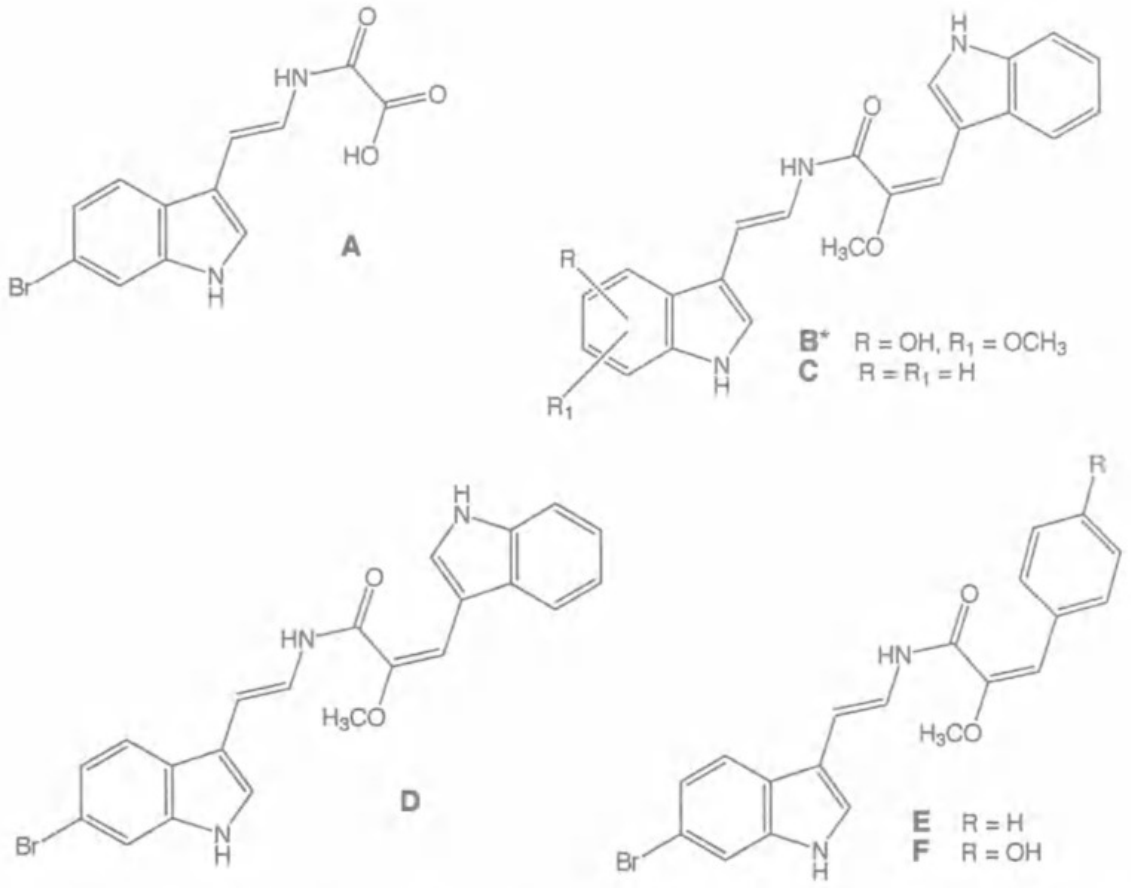

FIG. 1. Structure of the six indoles considered in this study: A, Putative precursor (HPLC Peak 7); B – D, Indolyl product (HPLC Peaks 9,14,19); E – F, Benzoyl product (HPLC peaks 16,21). * Assignment of substituent position not established

RESULTS

Six compounds of interest were separated using the HPLC system described above. Fourier Transform Mass Spectrometry and NMR confirmed that these indoles were the major components of the peaks listed and characterised in Table 1 and depicted by the structures shown in Figure 1. These data suggest that the low molecular weight indole in peak 7 (Fig. 1A) is the precursor of the more complex compounds in the other five peaks. These 'product' indoles fall into two structural classes based on whether the addition to the peak 7 core contains another indole (Fig. 1B-D) (= indolyl product) or a benzene group (Fig. 1E-F) (= benzoyl product).

ANOVAs on HPLC peak areas for precursor (peak 7), indolyl product (sum of peaks 9, 14 and 19) and benzoyl product (sum of peaks 16 and 21) found no significant difference in the amount of precursor or product present in tissue samples from the different tissue categories, with α=0.05. However, with 80% power, these non-significant tests were only capable of detecting differences between groups with a standard deviation between their means of 35% of the overall mean (precursor), 164% of the overall mean (indolyl product) and 21% of the overall mean (benzoyl product) (Table 2).

On the basis of HPLC, the 29 tissue sample extracts fell into two distinct groups. Figure 2 depicts a typical chromatograph of each group. When compared to Group 1, Group 2 contained more of peak 7 (Precursor) and less of peaks 9, 14 and 19 (indolyl product), while the quantity of peaks 16 and 21 (benzoyl product) were fairly consistent between the two groups. These relationships are summarised and quantified further in Figure 3.

Table 3 presents group membership with respect to tissue category. Only four tissue samples from three individuals had Group 1 chemistry (more indolyl product, less precursor). Two of these came from edges of direct interaction, and two

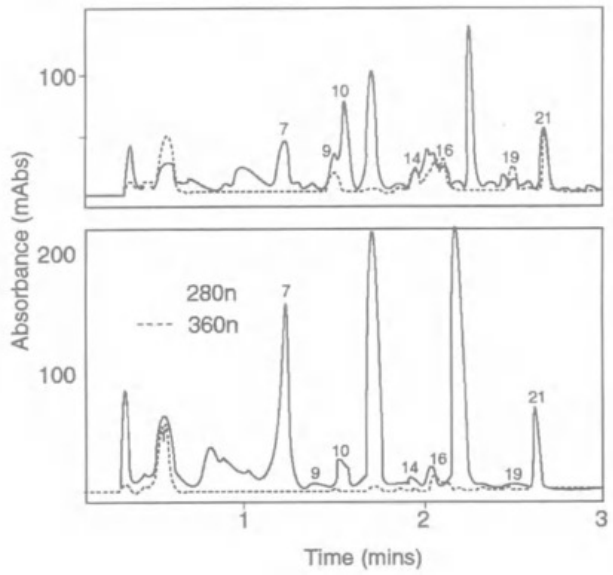

FIG. 2. Typical chromatograms of group 1 and group 2 samples. Peaks numbered for compounds considered in this study.

came from thick fleshy projections. While there were other samples from these categories with Group 2 chemistry (less indolyl product and more precursor), samples from either central sponge tissue or edge sites without direct interaction exclusively belonged to Group 2. Most samples analysed (25 out of 29) belonged to Group 2, and only two of the ten individuals examined contributed samples to both groups.

TABLE 3: Tissue categories sampled with respect to chemistry group membership.

Growth Type	Group 1	Group 2
Edge Interaction	2	10
Centre Thick	2	2
Edge No Interaction	0	6
Centre Thin	0	7

DISCUSSION

This work does not clearly support a direct relationship between neighbour interaction and indole chemistry in *Iotrochota* sp.. However, significance tests had moderate to low resolution at 80% power, and aspects of the distribution of samples containing Group 1 and Group 2 indole chemistry are consistent with an hypothesis of space competition influence. These are discussed below with respect to appropriate future directions for work in this area, and are not represented as definitive conclusions.

Morphological strategies are important to sessile benthic invertebrates in their struggle for substrate (Jackson, 1979; Hoppe, 1988; Vicente, 1990; Becerro et al., 1994). Becerro et al. (1994) suggested that another thin encrusting sponge, *Crambe crambe*, employed directional growth to either avoid stronger or confront weaker space competitors. Jackson (1979) suggested that vertical growth is another non-confrontational strategy in space competition. Whereas *Iotrochota* sp. is generally a thin encrusting sponge,

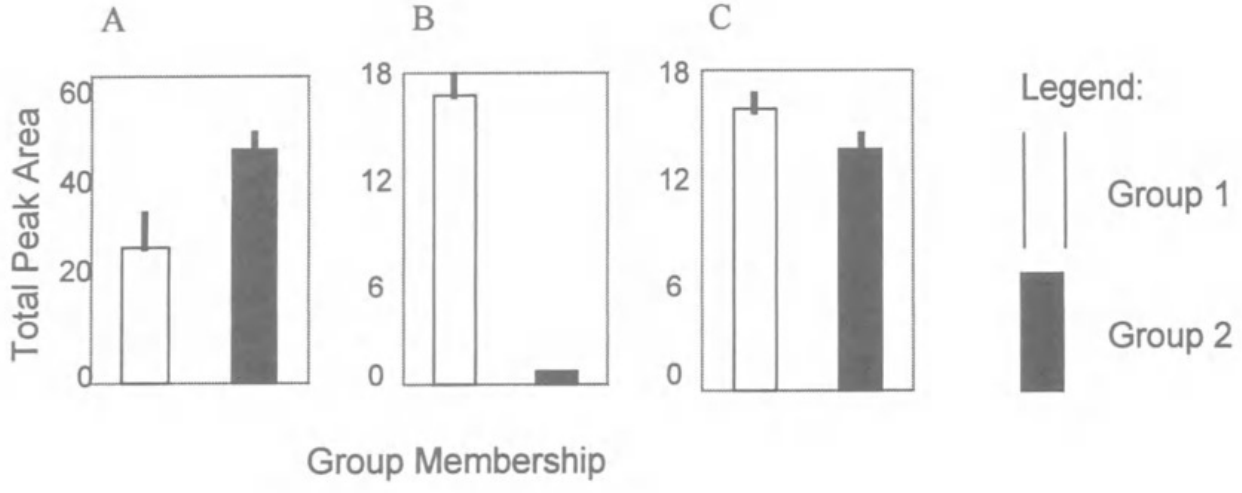

FIG. 3. Relative amounts (area under HPLC peak) of A. Precursor, B. Indolyl product and C. Benzoyl product in the two sample groups.

individuals typically sport several, thick, fleshy, vertical projections. A possible interpretation of this growth form is that the sponge utilises both confrontational and non-confrontational strategies to compete for hard substrate, whereas vertical growth in this otherwise thinly encrusting species may be a product of, or avoidance from encounters with superior space competitors at their outer growth margins.

It is therefore possible that samples containing Group 1 chemistry (i.e. more indolyl product, less precursor) had assumed a space competition strategy, either through direct confrontation at their margins, or non-confrontational vertical growth. However, this trend was not consistent, where both samples at the margins of neighbour contact (i.e. confrontational samples), and thick fleshy projections (i.e. non-confrontational samples), were included in chemistry Group 2. Further investigations into patterns of indole chemistry, which address growth form with respect to different neighbour interactions and the nature of these interactions, are essential to develop appropriate hypotheses.

Allelochemical interactions do not necessarily require direct contact between two individuals (Porter & Targett, 1988, Turon et al., 1996), and any non-contact interaction would be dependant on water flow. Thus, future work should also account for contact, distance and direction data (the latter with respect to currents). This species is amenable to transplantation (authors' unpublished data) and thus a candidate for controlled manipulative experimentation.

Patterns of variability in the other indole compounds known to occur in this *Iotrochota* sp. (authors' unpublished data), may also be important in understanding total metabolite variability in this species. More than 40 additional compounds which can be identified tentatively as indoles on the basis of mass spectrometry evidence, await characterisation, structural elucidation, and quantification. Also, the putative precursor-product relationship proposed in this work needs to be confirmed before any strong assertions about the invocation of a secondary metabolite from its precursor can be attributed to ecological factors.

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