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STUDIES ON THE BIOSYNTHESIS OF DISCORHABDIN B IN THE NEW ZEALAND SPONGE *LATRUNCULIA* SP. B

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ABSTRACT.—The production of discorhabdins in vivo by preparations of the marine sponge *Latrunculia* sp. B has been investigated. Slices of sponge tissue in an artificial medium were shown to effect the incorporation of radioactivity from [U-^{14}C]-L-phenylalanine into discorhabdin B [**2**], the principal cytotoxic metabolite produced by this organism. Additional experiments have shown that synthesis of this discorhabdin does not require metabolic cooperation of symbiotic microorganisms.

The discorhabdins are a novel group of pyrroloquinoline alkaloids, now numbering at least thirteen (discorhabdins A to M),[2] predominantly found in New Zealand marine sponges of the genus *Latrunculia* du Bocage (order Hadromerida; family Spirastrellidae). The first members of the family to be isolated were discorhabdins A [**1**], B [**2**], and C [**3**] (1,2). Each alkaloid is the major pigment present in each of the three species of the genus *Latrunculia* found most abundantly around the New Zealand coast line. Discorhabdin A [**1**] is found in *Latrunculia brevis*, discorhabdin B [**2**] in *Latrunculia* sp. B, and discorhabdin C [**3**] in *Latrunculia* sp. cf. *bocagei*.[3] The discorhabdins, also known as the prianosins (3,4), have been found in the Japanese sponge *Prianos melanos* and more recently discorhabdin A [**1**] has been found, along with the structurally related makaluvamines, in the Fijian sponge *Zyzzya* cf. *marsailis* (5). Interest in these compounds and their biosynthesis is stimulated by the fact that several members of the group possess extremely potent activity against P-388 lymphocytic leukemia cells in vitro (IC_{50} values 30–1,500 ng/ml) and demonstrate panel-selective cytotoxicity in the National Cancer Institute's panel of 60 human tumor cell lines (6–9). Among the various discorhabdins tested by the NCI, the most active appears to be discorhabdin C [**3**]. The biological activity of this compound is currently being investigated further. Several groups have approached the synthesis of the discorhabdin skeleton, and discorhabdin C [**3**] has been synthesized (10–17).

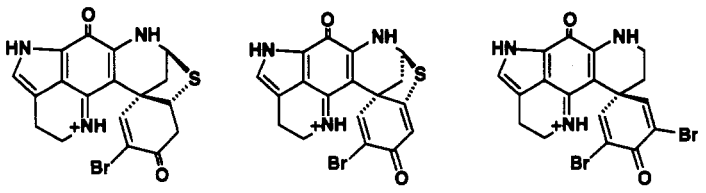
On a structural basis, the discorhabdins are clearly related to makaluvamine alkaloids such as makaluvamines D [**6**] and F [**7**] (5). Based on these structural similarities, it is tempting to propose a biogenetic pathway which interrelates these alkaloids (Scheme 1).

The field of biosynthetic studies on complex metabolites from marine invertebrates is still at a relatively early stage of development. Progress using intact sponges has been complicated by slow

[1]Royal Society Visiting Fellow at the University of Canterbury, July–October, 1993.

[2]The structures of discorhabdins E–M were presented at the New Zealand Institute of Chemistry Conference, Auckland, New Zealand, December 7–10, 1993.

[3]The assignment of these species is currently under review, as are synonomy problems associated generally with the Latrunculidae, particularly the Antarctic fauna (C.N. Battershill, P.R. Bergquist, J.W. Blunt, and M.H.G. Munro, manuscript in preparation).



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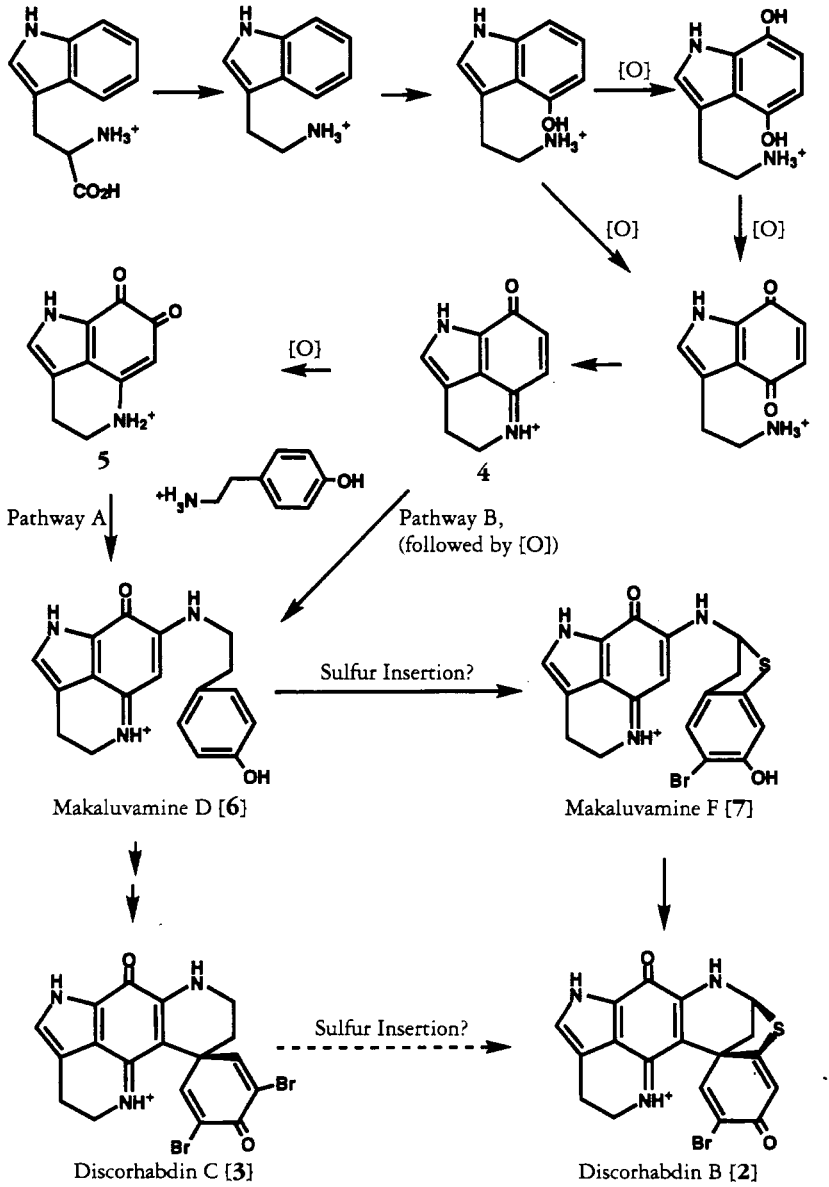
growth rates, low incorporation rates, and the presence of symbiotic microorganisms (18). Sustaining metabolite production in sponges for significant periods under laboratory conditions is not normally straightforward and the diversity



SCHEME 1. Postulated biogenesis of the discorhabdins.

of cell types involved in these organisms limits the potential use of tissue culture methods in exploring biosynthetic pathways (18). In this preliminary investigation, we have examined the production of discorhabdin B [**2**] using slices and unfractionated dispersed cells of *Latrunculia* sp. B and show (*inter alia*) that these preparations retain the biosynthetic ability of the intact organism and that phenylalanine is a precursor of the metabolite.

A reasonable premise, following from the putative biogenetic scheme outlined in Scheme 1, is that the amino acids tryptophan and phenylalanine (via tryptamine and tyramine) are precursors of the discorhabdin skeleton. Tryptamine, after appropriate functionalization and oxidation, can be considered to be the direct precursor of the pyrroloiminoquinone backbone characteristic of the damirone [**5**] (5,19), makaluvamine [**6**, **7**] (5), and discorhabdin [**1–3**] (1,2) skeletons (see Scheme 1). It is suggested that incorporation of tyramine, or a functionalized tyramine derivative, would lead directly to makaluvamine D [**6**], or a closely related structure. At this stage of the investigation, uncertainty associated with the precise mechanism of this step is revealed by the alternative pathways proposed in Scheme 1. This incorporation could arise by way of a nucleotide triphosphate-mediated amination (20) of a damirone-like molecule **5** (pathway A), or by direct Michael addition to the electronically-favored iminoquinone **4** followed by a 2-electron oxidation (pathway B). Likewise, the timing of the insertion of *S* can not be commented on at this early stage of our investigation of the biosynthesis of the discorhabdins. The cooccurrence of discorhabdin A [**1**] with the makaluvamines (5) and our recent finding of makaluvamines D and F [**6** and **7**] as minor components in work on *Latrunculia apicalis* from Antarctica (21) and of **6** from *Latrunculia brevis* from Kaikoura (22), are strong evidence in favor of a biosynthetic network such as that suggested in Scheme 1.

To determine the capacity of sponge cells of the producing organism to effect biosynthesis of the skeleton under culture conditions, we chose to use the incorporation of one of these amino acids as a probe. In view of the diversity of secondary metabolite composition, implying perhaps a different balance in enzymatic capabilities, which occurs in marine sponges (albeit of the same apparent gross phenotype), we determined the discorhabdin concentrations in two different specimens of the same species from geographically distinct locations some 300 km apart. This was preliminary to evaluation of their ability to produce discorhabdins under effectively artificial conditions. The metabolite content of each of the sponges used was analyzed by fractionation of $MeOH/CH_2Cl_2$ extracts of homogenized tissue samples. Initial fractionation was effected using C_{18} cartridge columns which were eluted with H_2O, then MeOH. The discorhabdins, which were concentrated in the MeOH fractions, were separated by prep. reversed-phase hplc on cyanopropyl silica and the isolated metabolites identified by comparison of their physical (uv, ^{1}H-nmr) and chromatographic (analytical hplc on C_{18} and CN columns) properties with those of authentic samples. Gratifyingly, the chosen specimens showed very similar discorhabdin compositions. In each case, discorhabdin B [**2**] proved to be the major metabolite (>90%), accompanied by significantly lower and variable proportions of the related discorhabdins A [**1**] (1), L, and M.

Evaluation of the ability of *Latrunculia* sp. B tissue to produce discorhabdin B [**2**] under culture conditions was carried out as follows: thin slices of the sponge were incubated with [U-^{14}C]-L-phenylalanine in M199 synthetic medium. After a period of 24 h the total discorhabdin B content was separated from the tissue matrix and culture medium as described

TABLE 1. Incorporation of [U-^{14}C]-L-Phenylalanine into Discorhabdin B [**2**].

Experiment	Time (h)	[U-^{14}C]-L-Phenylalanine fed (dpm)	^{14}C-Incorporation into discorhabdin B [**2**] (dpm)	% Incorporation into discorhabdin B [**2**]
1. No antibiotic treatment .	24	4.3×10^7	7.4×10^4	0.18%
2. Antibiotic pre-treatment .	24	4.3×10^7	1.4×10^5	0.32%
3. Antibiotic incubation . . .	24	4.3×10^7	1.1×10^5	0.26%
4. Control[a]	24	4.3×10^7	$<10\times10^3$	<0.02%

[a]Boiled, non-antibiotic treated slices.

above, and then carefully purified to constant specific activity by sequential chromatography on two different analytical reversed-phase supports. The extent of *de novo* synthesis was evaluated by determination of radiochemical incorporation into the purified metabolite. The results (shown in Table 1) clearly indicate that ^{14}C from [U-^{14}C]-L-phenylalanine is incorporated into discorhabdin B [**2**] by the tissue preparation, albeit at a relatively low efficiency (ca. 0.3%).

Typically, sponge tissue is not homogeneous and it frequently comprises numbers of eukaryotic cell types coexistent with, or symbiotic with, a variety of microorganisms. This poses a peculiar biosynthetic problem, that is, whether the biosynthesis of a particular metabolite is a property of the sponge cells themselves, is exclusively the product of a "parasitic" or commensal microorganism, or requires metabolic cooperation of symbiotic organisms in its production. To address this problem we repeated the above experiment with tissue slices of *Latrunculia* sp. B, which were (a) presoaked in a broad-spectrum antibiotic mixture prior to incubation with [U-^{14}C]-L-phenylalanine, and (b) presoaked and then incubated in the presence of broad-spectrum antibiotics during the incorporation experiment. ^{14}C-Incorporation from [U-^{14}C]-L-phenylalanine into discorhabdin B [**2**] under both conditions was similar to that obtained with tissue slices which had not been subjected to any antibiotic treatment (Table 1) indicating that, at least under culture conditions with an adequate supply of primary nutrients, the biosynthesis of discorhabdin B in *Latrunculia* sp. B is a property of the eukaryotic sponge cells alone and does not require the participation of symbiotic bacteria.

This successful initial biosynthetic study of the origins of the discorhabdin skeleton has established several important facts. The most obvious are that L-phenylalanine is a precursor of the discorhabdin skeleton and that the alkaloid is most probably a product of the sponge cells themselves rather than that of a symbiotic organism. The opportunity to use tissue slices as opposed to separated cells is another finding from this study.

Future work will examine the incorporation of more advanced intermediates in an attempt to answer the involvement, or not, of a damirone [**5**] in the pathway and the possible timing and insertion point for the introduction of sulfur.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Hplc was carried out on a Phillips PU4100 instrument equipped with a photodiode array detector (Model PU4120), and nmr spectra were obtained using a Varian Unity 300 spectrometer with samples prepared in MeOH-d_4. Scintillation counting was carried out in a LKB scintillation cocktail (HiSafe 3) using a Wallac Counter (Model 1410). Cartridge columns were purchased from Baker and hplc columns from Alltech. Chromatography solvents were obtained from BDH (HiPerSolv) and M-199 marine medium from Sigma (St. Louis, MO). [U-^{14}C]-L-phenylalanine (474 mCi/mM) was purchased from Amersham Life Science, UK. Discorhabdin samples were available from earlier work (21–23). Quantification of individual discorhabdin concentrations was based on integration of hplc

peak areas over the range 195–390 nm vs. authentic samples.

ANIMAL MATERIAL.—*Latrunculia* sp. B samples were collected from Lyall Bay, Wellington at a depth of 15 m and from Rhino Horn, Kaikoura, New Zealand. *Latrunculia* sp. B (order Hadromerida; family Spirastrellidae) is an undescribed species distinct from the other New Zealand species of this genus, *L. brevis*. *Latrunculia* sp. B is a massive, spherically shaped sponge and is found typically below 10 m depth in turbid, oceanic waters. Spiculation is similar to *L. brevis* with discorhabs displaying three whorls, but tend to be somewhat more elongate in *L.* sp. B. This genus is currently under taxonomic review.[3] The sponge is distinguished from other *Latrunculia* spp. by a brown, rather than green, coloration, which is consistent internally. Surface papillae appear as large uneven craters scattered haphazardly over the surface of the sponge. However, individuals may be seen which have completely smooth surfaces, suggesting some mobility of surface structure. The species is represented by voucher specimen 85K01-01 held at the University of Canterbury Marine Chemistry Group's museum. All collected sponge samples were transported on ice in aerated sea water and maintained in a salt water aquarium prior to the experiments. Surprisingly, the *Latrunculia* sp. B samples were very resilient and could be maintained under aquarium conditions for long periods (2–3 weeks) without any deterioration in condition. The *Latrunculia* samples were identified to a species level by evaluation of morphological characteristics and microscopic examination of spicule samples. Voucher samples of the Kaikoura collection (94K24-01) are held at the University of Canterbury Marine Group's museum and specimens from Lyall Bay held in New Zealand Oceanographic Institute Museum. The taxonomic identifications were by one of us (C.N.B.).

EXTRACTION AND ISOLATION.—The discorhabdin content of each of the sponges used in the work was isolated by fractionation of MeOH-CH_2Cl_2 (1:1, 20 ml) extracts of homogenized tissue samples (1–2 g). Extracts were evaporated onto Celite (ca. 200 mg) and applied to C_{18} cartridge columns (1×2 cm) which were eluted with H_2O (20 ml) and MeOH (10 ml). The concentrated MeOH fraction was fractionated by prep. hplc (5 μm, CNpropyl, 10 mm×20 cm) using MeOH-H_2O (0.05% TFA) (1:1) as eluent. The isolated discorhabdins were identified on the basis of comparison of their R_fs by analytical hplc and of their uv and ^{1}H-nmr spectra with those of authentic samples. In each case, the major discorhabdin present was discorhabdin B [**2**] (ca. 2 mg/g of wet tissue), accompanied by smaller, variable amounts of discorhabdins A [**1**], L and M[2] (<10% in total).

BIOSYNTHETIC PRODUCTION OF DISCORHABDINS.—Blocks of sponge tissue from samples of *Latrunculia* sp. B (94K24-01) (approximately 1×2 cm and 2.5 g wet wt) were transferred to sterile calcium/magnesium-free artificial sea water (cmf solution) and cut into thin strips (1–2 mm thick) with a sterile scalpel. The slices were presoaked for 1 h in either (a) sterile cmf solution (10 ml) or (b) sterile cmf solution (10 ml) containing an antibiotic mixture (1 ml) [gentamycin sulfate (0.25 mg/ml), kanamycin monosulfate (0.25 mg/ml), penicillin G (0.3 mg/ml), streptomycin (0.25 mg/ml), and nystatin (13 μg/ml)]. The tissue strips were then transferred to M199 medium diluted with cmf solution (1:1, 10 ml) in sterile 50-ml Erlenmeyer flasks (Experiments 1 and 2, Table 1). In a third experiment (Experiment 3, Table 1), strips of tissue pre-incubated under condition (b) above were shaken in M199 medium diluted with cmf solution (1:1, 10 ml) containing the antibiotic mixture at 25% of the pre-soak conditions. In the control experiment (Experiment 4, Table 1) strips of tissue in sterile cmf solution (10 ml) were heated at 100° for 10 min, then cooled to 15° in an ice bath immediately prior to incubation. The tissue strips were transferred to M199 medium diluted with cmf solution (1:1, 10 ml) in a sterile 50-ml Erlenmeyer flask. A solution of [U-^{14}C]-L-phenylalanine (50 μl, 10 μCi) was added to each flask. Flasks were shaken on an orbital shaker (100 rpm) at 15° for defined periods (see Table 1).

After incubation, the slices, dispersed cells, and cell debris were separated from the medium by centrifugation (2000 rpm, 5 min), extracted with MeOH-CH_2Cl_2 (2×5 ml, 1:1), and the extract concentrated by evaporation under a stream of N_2 and subjected to preliminary fractionation on C_{18} cartridges as above. The culture medium in each case was fractionated by application to a C_{18} cartridge column (1×2 cm), which was eluted with H_2O (10 ml), then MeOH (10 ml). The MeOH eluates were combined and concentrated by evaporation under a stream of N_2. Discorhabdin B [**2**] was isolated and purified from this extract by sequential hplc on CNpropyl and C_{18} prep. hplc columns as noted above. Final purification (to constant specific activity) was achieved by repetitive hplc of an aliquot of the isolated discorhabdin B [**2**] on a CNpropyl analytical hplc column.

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