Marine Biology 74, 35–40 (1983)

Marine Biology
International Journal on Life in Oceans and Coastal Waters

Transformation of soft coral (Coelenterata: Octocorallia) terpenes by *Ovula ovum* (Mollusca: Prosobranchia)

J. C. Coll [1], D. M. Tapiolas [1], B. F. Bowden [1], L. Webb [1] and H. Marsh [2]

[1] **Department of Chemistry and Biochemistry, James Cook University of North Queensland; Townsville, Queensland 4811, Australia**

[2] **Department of Zoology, James Cook University of North Queensland; Townsville, Queensland 4811, Australia**

Abstract

The faecal pellets from specimens of the prosobranch mollusc *Ovula ovum* found feeding on the soft coral *Sarcophyton* sp. at Eclipse Island, Palm Island Group (18°46′S; 146°33′E) in November 1980 were analysed. The only terpene present in the faeces, 7,8-deoxysarcophytoxide, differed from the major constituent of the soft coral, sarcophytoxide, suggesting that the latter had been transformed into the former within the cowrie. This transformation is not trivial, and could not be produced simply by acid catalysis. Subsequent analysis of tissues dissected from different regions of *O. ovum* indicates that the transformation is probably effected by enzymes in the digestive diverticula stomach region of the prosobranch. The transformed compound is significantly less toxic to the mosquito fish *Gambusia affinis* Baird and Girard than the ingested compound.

Introduction

Although soft corals (Coelenterata, Octocorallia) are sessile and generally soft and fleshy, they have relatively few predators (Benayahu and Loya, 1977). This has been attributed to the presence of toxic substances in their tissues (Bakus, 1981; Coll *et al.*, 1982 b) and to the protection afforded to certain genera (e.g. *Dendronephthya* = *Spongodes*) by sharp and protruding sclerites (needles of calcite). However, the egg-cowrie *Ovula ovum* Linnaeus, 1758 (Mollusca, Prosobranchia) is frequently reported to eat soft corals (e.g. Laboute and Magnier, 1978, p. 105; Bennett, 1981, p. 130) despite their elaborate defences. Indeed, soft corals of the genus *Sarcophyton* on which the ovulid regularly feeds contain highly toxic terpenoid compounds (Ne'eman *et al.*, 1974).

Recently, we observed a group of three egg-cowries (O'Connor, 1976, p. 5) feeding on a *Sarcophyton* sp. colony. They had created a depression in the surface of the soft coral in which their faecal material collected. This provided a unique opportunity to study the fate of ingested terpenes. This report describes the structures and relative ichthyotoxicities of the terpenes present in the cowrie faeces and the soft coral, and identifies the site within the cowrie where the terpenoid constituents of the soft coral are modified by the ovulid.

Materials and methods

Sample collection

All specimens were collected from the northern edge of the fringing reef of Eclipse Island, Palm Island Group (18°46′S; 146°33′E), in the central region of the Great Barrier Reef, at a depth of 3 m.

On 9th November 1980, we sampled tissue from a soft coral (*Sarcophyton* sp.) and the accumulated faecal material from three resident cowries (*Ovula ovum*). Each sample was drained of sea water and preserved in 70% aqueous ethanol. On 27 February 1981, we sampled two further *Sarcophyton* sp. colonies, and collected the associated cowries. Each specimen was sealed in a plastic bag and preserved by freezing prior to analysis. Two further specimens of *O. ovum* were collected and maintained in a recirculating sea water system without food in order to provide tissues suitable for histology.

Dissection and histological sections of *Ovula ovum*

Each of three frozen *Ovula ovum* was removed from its shell, thawed and dissected under a binocular microscope. The mantle and foot were removed (1 and 2 in Fig. 3), and the digestive tract and attached gonad were divided into 7 further portions (numbered 3 through 9 in Fig. 3) for subsequent organic analysis.

The live *Ovula ovum* were similarly dissected. The radular sac, oesophageal glands and 5 mm^3 cubes of diges-

tive diverticula (midgut gland, digestive gland) and gonad of one specimen, and the stomach of the other were excised, fixed in Baker's formol calcium (Pantin, 1964) at room temperature for 48 h, washed in water, dehydrated in ethanol, cleared in xylene, vacuum-embedded in Paraplast and sectioned on a rotary microtome at 7 μm. One section from each block was stained with Mayer's haemalum and Young's erythrosin, and another with the periodic acid Schiff (PAS) procedure (Cook, 1974). Sections of the digestive diverticula and stomach were also stained with the Gram technique for micro-organisms (Gram, 1884, *in* Cook, 1974).

Organic analyses

Frozen samples of soft coral and portions 1–9 (Fig. 3) of the cowrie were freeze-dried. The alcohol was removed from the preserved samples under reduced pressure prior to freeze-drying. The residue from each freeze-dried sample was extracted exhaustively with dichloromethane at room temperature and the solvent removed using a Büchi rotary evaporator at $< 30\,^{\circ}C$. The resulting organic extract was analysed initially by thin-layer chromatography (TLC), using plastic sheets coated with silica gel Type 60 containing a UV indicator (F254 Merck) as stationary phase and a mixture of diethyl ether:hexane (3:7) as mobile phase. Individual components were made to appear as coloured spots by use of a spray reagent consisting of vanillin (10 mg) in concentrated sulphuric acid (20 ml). The sprayed TLC sheets were warmed on a hot plate to develop their full colour intensity.

In order to ascertain the distribution of individual terpenoid constituents in various parts of the cowrie, semi-quantitative TLC examination was carried out as follows. Equal quantities (25 mg) of homogenised freeze-dried sections of the dissected cowrie were suspended in dichloromethane (1 ml) and allowed to stand at room temperature for 15 min. Aliquots (5 μl) of each solution were applied to the base of a TLC plate, which was run and developed as previously described. A sample of the extract of the soft coral associated with the specific cowrie was added to the TLC plate for comparison purposes (numbered 10 in Fig. 3).

Isolation of individual components from the extracts was achieved by rapid chromatography on silica gel (Type 60, Merck, for TLC), and compounds were identified and characterised by melting point and polarimetric determinations, infrared, ultraviolet ^{1}H- and ^{13}C-NMR spectroscopy and mass spectrometry using the apparatuses detailed in an earlier publication (Bowden *et al.*, 1980).

Assessment of relative ichthyotoxicities of terpenes

The common mosquito fish *Gambusia affinis* Baird and Girard which has been used successfully in previous toxicity studies (Cornman, 1968; Ne'eman *et al.*, 1974) was used to determine relative ichthyotoxicity. Specimens were collected from a local creek approximately 24 h prior to testing, and held in a large aquarium. The test aquarium was a rectangular Perspex structure subdivided into 5 sets of two replicate compartments; each held a volume of approximately 400 ml. Divisions between the watertight compartments were translucent to visually isolate specimens. Four small fish (100 to 300 g) were placed in each compartment with 200 ml of test solution and mortality counts made each hour for 12 h. For each group of four replicated test concentrations of a terpene (5, 10, 20 and 30 ppm), there was a control without terpene. The test solutions were prepared by dilutions of individual stock solutions containing sarcophytoxide and 7,8-deoxysarcophytoxide, each at 0.2 mg ml^{-1}.

Results

Field observations of *Ovula ovum* feeding on a *Sarcophyton* sp.

In November 1980, we observed a *Sacrophyton* sp. colony being eaten by three *Ovula ovum*. The soft coral tissue had been extensively eroded by their grazing, and there was a build up of faecal material in the resulting recess (Fig. 1 a). In February 1981, there were no cowries on this colony, which had been grazed down to the supporting substrate (*Pavona cactus*, the dominant scleractinian coral in the region) (Fig. 1 b). Two neighbouring colonies within 2 m of the first colony were now inhabited by cowries which were steadily consuming their tissues.

Organic analyses

Freeze-dried specimens of a *Sarcophyton* sp. colony (1.2 g) and the accumulated faecal material (4.48 g) from the three associated *Ovula ovum* afforded 0.6 and 0.2 g of organic extract, respectively. The principal terpenoid component in the soft coral extract was the known diterpene sarcophytoxide (340 mg) $[\alpha]_D$-156° (Fig. 2), as confirmed by TLC, ^{1}H-NMR, and polarimetric comparisons with an authentic specimen (Tursch, 1976; Bowden *et al.*, 1978).

The only terpenoid constituent isolated from the cowrie faeces was 7,8-deoxysarcophytoxide (33 mg) (Fig. 2), which was identified on the basis of its ^{1}H NMR spectrum and by direct TLC comparison with a sample prepared by Zn-Cu couple reduction of sarcophytoxide (Bowden *et al.*, 1979). This represents the first isolation of *7,8-deoxysarcophytoxide* from a natural source, and its full characterisation is reported: It was an oil, $[\alpha]_D$ 226.1° (*c*, 0.4); ^{1}H NMR ($CDCl_3$): δ1.60, 2 methyls; 1.64, 1.70, 2 methyls; 4.5, *d*, J 4 Hz (16-CH_2); 4.94, *dt* J 10, 4 Hz, H2; 5.0, *m*, 1H; 5.1, *d*, J 10 Hz, H3; 5.5, m, 1H; additional proton resonances (integral: 12 protons) were evident between δ1.7 to 2.5, but these were not interpretable at 100 MHz. ^{13}C NMR: 140.2 (*s*), 135.3 (*s*), 133.8 (*s*), 133.1

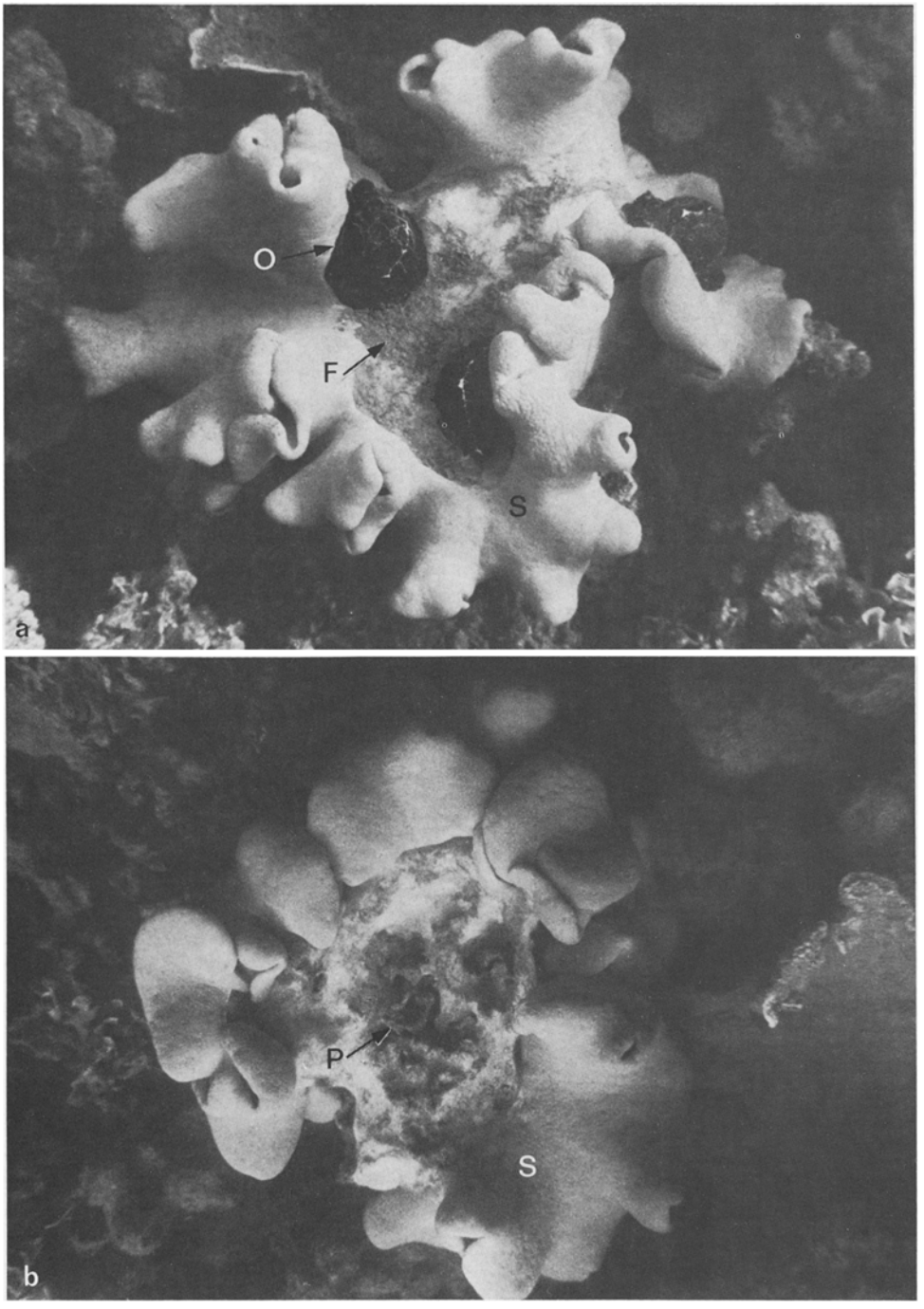
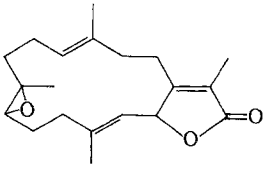
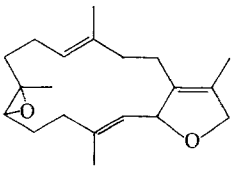


Fig. 1. (a) View of 3 *Ovula ovum* (O) eating soft coral *Sarcophyton* sp. (S) (9 November 1980); note build up of faecal material (F). (b) Same *Sarcophyton* sp. (S) colony, showing *Pavona cactus* substrate (P) (27 February 1981). (Photos by B. Willis)



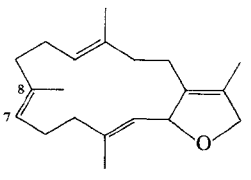
Sarcophine
(Sn)

1.0



Sarcophytoxide
(Sx)

0.5



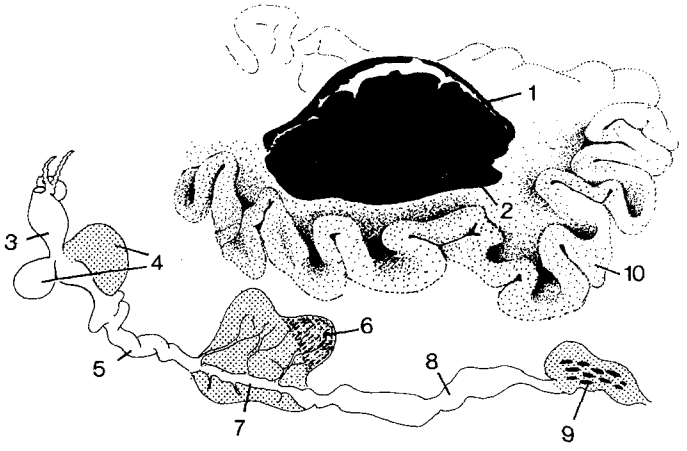
7, 8–Deoxysarcophytoxide
(Deo–Sx)

0.1

Fig. 2. Structure names, abbreviations, and ichthyotoxicities of terpenes identified in this study relative to sarcophine = 1.0

(*s*), 127.0 (*s*), 125.6 (*d*), 125.4 (*d*), 123.9 (*d*), 83.9 (*t*), 74.9 (*d*), 40.3 (*t*), 39.1 (*t*), 37.1 (*t*), 25.8 (*t*), 24.7 (*t*), 23.4 (*t*), 15.5 (*q*), 14.9 (*q*), 14.7 (*q*), 10.1 (*q*); Mass spectrum [Found: $M^{\cdot+}$ 286.24; $C_{20}H_{30}O$ requires 286.23]: m/e 286 (50%), 271 (13), 203 (27), 201 (14), 149 (100), 135 (100), 121 (40), 93 (50), 81 (65), 67 (70), 55 (75), 43 (90).

Extraction of the freeze-dried coral (150 g) afforded a 17% organic extract (26 g), which was composed of a considerable quantity of lipid (large polymethylene peak at δ1.25 in the ^{1}H NMR spectrum) and two known diterpenes – sarcophine (0.1%, 0.15 g) and sarcophytoxide (3%, 4.5 g) (Fig. 2). Each compound had identical chromatographic and spectroscopic properties with those reported elsewhere (Bowden *et al.*, 1980).



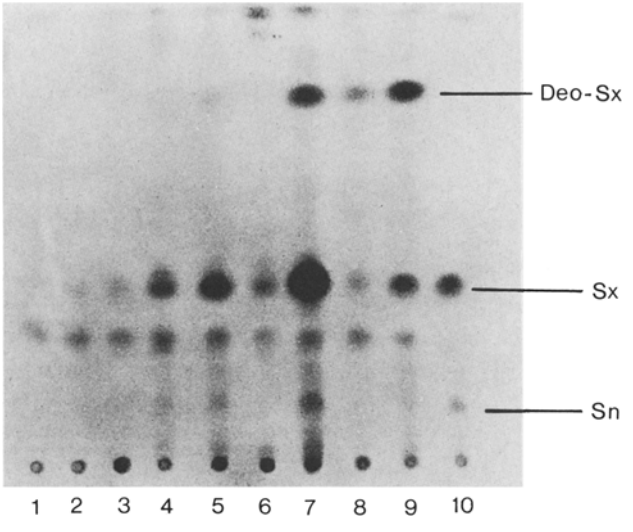


Fig. 3. *Ovula ovum* (1–9) and soft coral *Sarcophyton* sp. (10). Schematic diagram of digestive tract and associated viscera of *O. ovum* and results of semiquantitative TLC study. 1: mantle; 2: foot; 3: proboscis; 4: foregut glands; 5: foregut; 6: gonad; 7: digestive diverticula, associated gonad and stomach region; 8: intestine; 9: faecal pellets (internal); 10: soft coral. (Drawing by S. La Barre)

Anatomy of the digestive tract of *Ovula ovum*

The digestive tract anatomy of *Ovula ovum* is illustrated in Fig. 3. The identification of the associated glands was confirmed histologically. The histology of the digestive diverticula indicates that their structure is typical for a prosobranch as described by Hyman (1967, pp 231–233). The digestive diverticula consist of numerous blind-ending tubules which communicate with the stomach by a system of branched ducts. Two cell types similar to those described by Owen (1966, pp 78–80) were present in the tubules. Occasional Gram-positive bacteria were detected in the stomach.

TLC examination of various dissected portions of the ovulid

The results of the semiquantitative TLC study of the distribution of terpenes in the various portions of the cowrie are given in Fig. 3.

Ichthyotoxicity of terpenes to *Gambusia affinis*

At 20 ppm, sarcophytoxide killed all the test fish (8 out of 8) in 45 min; at 10 ppm, 75% of the fish were dead (6/8) after 3 hr; at 5 ppm, 25% of the fish were dead (2/8) after 7 hr. All controls were alive and active after 7 h.

At 30 ppm, 7,8-deoxysarcophytoxide produced narcotisation in the test fish (i.e., lack of response to overshadowing by a dark object, lack of general activity; disorientation) and at 7 h only one fish was dead. Lower concentrations produced no deleterious effects, i.e., behaviour was indistinguishable from controls. All controls were healthy and active after 7 h. Insufficient material was available to permit a study of concentrations greater than 30 ppm.

Discussion

Detailed chemical analysis of faecal material from three *Ovula ovum* feeding on a soft coral showed that while the predominant terpene present in the soft coral was sarcophytoxide, none of this compound was present in the cowrie faeces. This was unexpected, since previous chemical investigation of *Sinularia grayi* and an *O. ovum* feeding on it had revealed the same terpene in the tissues of each (Bowden *et al.*, 1978). The major terpene in the faecal material was 7,8-deoxysarcophytoxide. The chemical transformation which had occurred in the ovulid was not trivial, and involved a reductive elimination reaction. This can best be achieved chemically using a Zn-Cu couple (Herin and Tursch, 1976), and could not occur by catalysis under the acidic conditions present in the digestive diverticula (mid-gut or digestive gland) of the mollusc as found in *Aplysia californica* by Stallard and Faulkner (1974). We thus assume that the overall transformation

was enzyme-mediated, and this was consistent with the high degree of stereospecificity of the reaction producing a product of high enantiomeric purity, and the formation of only the 7*E*-trisubstituted double bond in the product (methyl resonances all upfield of 15.5 ppm in the ^{13}C NMR spectrum) (Johnson and Jankowski, 1972, p. 497).

Semiquantitative TLC analysis of each region of the digestive tract of *Ovula ovum* (Fig. 3) showed that the terpenoid constituents present in the ovulid were localised in the digestive diverticula/stomach region. Furthermore, there was none of the transformed metabolite in the mantle, foot or any region of the digestive tract before the stomach (Fig. 3). It was clearly formed in the digestive diverticula or in the stomach into which they open. The mechanism by which the terpene accumulates in this region is not known.

It is possible that the enzymic transformation occurs during the passage of 7,8-deoxysarcophytoxide through the digestive diverticula/stomach to the intestine for incorporation into the faecal pellets. From the TLC study (Fig. 3), the predominant terpene in the digestive diverticula/stomach region is sarcophytoxide, whereas the 7,8-deoxycompound predominates in the faecal material. Although amounts of sarcophytoxide were visible in the TLC of the internal faecal pellets, this may have been due to contamination during dissection and isolation. No sarcophytoxide was detected in the faecal pellets collected in the field. Thus, considerable selectivity is demonstrated in the distribution of terpenes in the various regions of the cowrie's digestive tract, which is further evidence for the involvement of enzymes in the transformation of sarcophytoxide.

Reductive transformations are often associated with the action of bacteria (Doelle, 1969, pp 256–306). By contrast, most animals use oxidative mechanisms to detoxify ingested compounds which do not have dietary value (Lehninger, 1975, p. 502). No significant bacterial presence was detected by microscopic examination or by Gram staining in the regions of the mollusc where the transformation occurred. Although it is possible that the transformation may have been caused by symbiotic bacteria (many of which are known to be Gram-negative) in the stomach of *Ovula ovum,* we consider that the transformation was effected by enzymes produced in the tubules of the digestive diverticula.

The results of the ichthyotoxicity studies suggest that the transformation may represent a detoxification. Sarcophine is known to have an LD_{50} of 3 mg l^{-1} against *Gambusia affinis* (Ne'eman *et al.,* 1974). We have found that sarcophytoxide is only about one-half as toxic as sarcophine while 7,8-deoxysarcophytoxide is only one-fifth as toxic as sarcophytoxide towards *G. affinis.* Clearly, the ingestion of significant quantities of sarcophytoxide did not adversely affect the cowries, and so detoxification was necessary only in the absorptive regions of the digestive tract, i.e., the digestive diverticula and intestine.

On the basis of detailed chemical analysis of the organic extracts of three *Sarcophyton* sp. colonies, each contained essentially the same range of terpenes in approximately the same proportions. *Ovula ovum* appears to show a feeding preference for this species, although there were a number of other soft corals (e.g. species of *Sinularia, Lobophytum* and *Nephthea*) in the vicinity. Sarcophine and sarcophytoxide are both highly toxic terpenes (Ne'eman *et al.,* 1974) and it is possible that the ovulids have specialised on this otherwise protected food source – being able to assimilate these highly toxic compounds without ill effects. Precedence for this type of coevolution exists in the terrestrial environment. Thus, *Chrysolina* sp. beetles can detoxify hypericin from the poisonous plant *Hypericum* sp., giving them access to a food source virtually untouched by other herbivores. This toxin acts as a kairomone (Brown *et al.,* 1970), attracting the beetles to the plant and stimulating the feeding response (Whittaker and Feeny, 1971), Diterpenes from gorgonian corals have been shown to remove the cilia from the larvae of the mollusc *Phestilla sibogae,* causing them to settle on the host coral. Hadfield and Ciereszko (1978) suggested that terpenes may play a role in larval attraction and immobilisation of molluscs including *O. ovum.* Since sarcophine and sarcophytoxide have been detected in sea water surrounding *Sarcophyton* sp. colonies (Coll *et al.,* 1982 a), it is possible that these terpenes act as attractants for *O. ovum.*

Acknowledgements. Special thanks are due to R. and B. Willis for collection and photography of certain specimens. Mr. L. Windsor prepared the sections for histology. The manuscript benefitted from comments by D. J. Faulkner, R. P. Kenny, D. Yellowlees and P. W. Sammarco. We acknowledge the financial support of the Australian Research Grants Committee, the Australian Marine Sciences and Technologies Council, and the University Special Research Grants.

Literature cited

Bakus, G. J.: Chemical defence mechanisms on the Great Barrier Reef, Australia. Science, N.Y. *211,* 497–499 (1981)

Benayahu, Y. and Y. Loya: Space partitioning by stony corals, soft corals and benthic algae on the coral reefs of the northern Gulf of Eilat (Red Sea). Helgoländer wiss. Meeresunters. *30,* 362–382 (1977)

Bennett, I.: The Great Barrier Reef, 184 pp. Sydney: Lansdowne 1981

Bowden, B. F., J. C. Coll, W. Hicks, R. Kazlauskas and S. J. Mitchell: Studies of Australian soft corals. X. The isolation of epoxyisoneocembrene-A from *Sinularia grayi* and isoneocembrene-A from *Sarcophyton ehrenbergi.* Aust. J. Chem. *31,* 2707–2712 (1978)

Bowden, B. F., J. C. Coll and S. J. Mitchell: Studies of Australian soft corals. XVIII. Further cembranoid diterpenes from soft corals of the genus *Sarcophyton.* Aust. J. Chem. *33,* 879–884 (1980)

Bowden, B. F., J. C. Coll, S. J. Mitchell and G. J. Stokie: Studies of Australian soft corals. XI. Two new cembranoid diterpenes from a *Sarcophyton* species. Aust. J. Chem. *32,* 653–659 (1979)

Brown, W. L., Jr., T. Eisner and R. H. Whittaker: Allomones and kairomones: transspecific chemical messengers. BioSci. *20,* 21–22 (1970)

Coll, J. C., B. F. Bowden, D. M. Tapiolas and W. C. Dunlap: *In situ* isolation of allelochemicals released from soft corals (Coelenterata, Octocorallia): a totally submersible sampling apparatus. J. exp. mar. Biol. Ecol. *60,* 293–299 (1982 a)

Coll, J. C., S. La Barre, P. W. Sammarco, W. T. Williams and G. J. Bakus: Chemical defences in soft corals (Coelenterata: Alcyonacea) of the Great Barrier Reef: a study of comparative toxicities. Mar. Ecol. Prog. Ser. *8,* 271–278 (1982 b)

Cook, H. C.: Manual of histological demonstration techniques, 314 pp. London: Butterworths 1974

Cornman, I.: Dregs from the sea: heterotargeting. *In:* Drugs from the sea, pp 177–185. Ed. by H. D. Freudenthal. Washington: Marine Technology Society 1968

Doelle, H. W.: Bacterial metabolism, 486 pp. New York: Academic Press 1969

Hadfield, M. G. and L. S. Ciereszko: Action of cembranolides derived from octocorals on larvae of the nudibranch *Phestilla sibogae. In:* Drugs and food from the sea, myth or reality? pp 145–150. Ed. by P. N. Kaul and C. J. Sindermann. Norman: The University of Oklahoma Press 1978

Herin, M. and B. Tursch: Chemical studies of marine invertebrates. XXIV. Minor cembrane diterpenes from the soft coral *Sinularia flexibilis* (Coelenterata, Octocorallia). Bull. Soc. chim. Belg. *85,* 707–719 (1976)

Hyman, L. H.: The invertebrates: volume VI. Mollusca 1, 792 pp. New York: McGraw-Hill 1967

Johnson, L. R. F. and W. C. Jankowski: Carbon-13 NMR spectra, 500 pp. New York: John Wiley & Sons 1972

Laboute, P. et Y. Magnier: Guide sons-marin de Nouvelle-Caledonie, 160 pp. Papeete: Les Editions du Pacifique 1978

Lehninger, A. C.: Biochemistry, Ed. 2. 1104 pp. New York: Worth Publishers, Inc. 1975

Ne'eman, I., L. Fishelson and Y. Kashman: Sarcophine – a new toxin from the soft coral *Sarcophyton glaucum* (Alcyonaria). Toxicon *12,* 593–598 (1974)

O'Connor, M.: Reef poems, 59 pp. Brisbane: University of Queensland Press 1976

Owen, G.: Digestion. *In:* Physiology of Mollusca, Vol. 2. pp 53–96. Ed. by K. M. Wilbur and C. M. Yonge. New York: Academic Press 1966

Pantin, C. F. A.: Notes on microscopial technique for zoologists, 77 pp. Cambridge: University Press 1964

Stallard, M. O. and D. J. Faulkner: Chemical constituents of the digestive gland of the sea hare *Aplysia californica.* II. Chemical transformations. Comp. Biochem. Physiol. *49 B,* 37–41 (1974)

Tursch, B.: Some recent developments in the chemistry of alcyonaceans. Pure appl. Chem. *48,* 1–6 (1976)

Whittaker, R. H. and P. P. Feeny: Allelochemicals: chemical interactions between species. Science, N.Y. *171,* 757–770 (1971)

Date of final manuscript acceptance: December 7, 1982.
Communicated by G. F. Humphrey, Sydney