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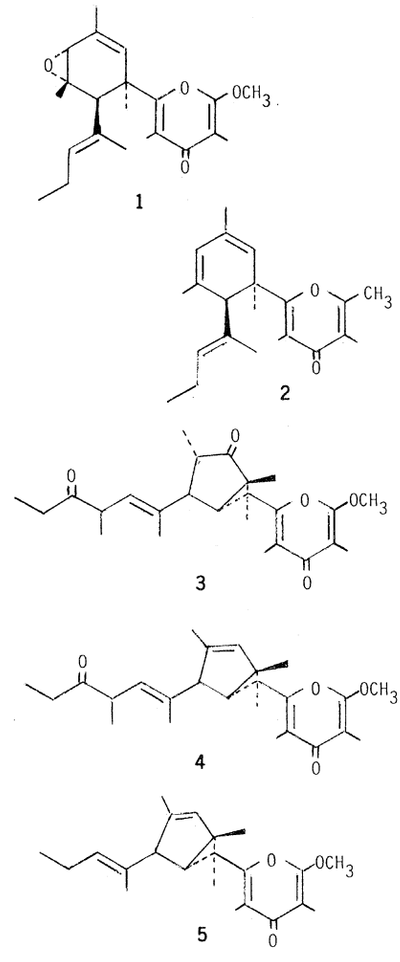
Photosynthetic Marine Mollusks: In vivo ^{14}C Incorporation into Metabolites of the Sacoglossan *Placobranchus ocellatus*

Abstract. *In seawater enriched with carbon-14-labeled sodium hydrogen carbonate the sacoglossan* Placobranchus ocellatus *when exposed to light incorporates carbon-14 at a rate 50-fold of that for animals kept in the dark. 9,10-Deoxytridachione, a secondary metabolite of the mollusk, undergoes a photorearrangement to photodeoxytridachione in vivo.*

The marine mollusk *Placobranchus ocellatus* (Van Hasselt) (phylum Mollusca, class Gastropoda, subclass Opisthobranchia, order Sacoglossa) is one of a unique group of mollusks that assimilate photosynthetically viable chloroplasts from siphonous algae upon which they graze (*1*). We report the results of our ^{14}C-labeling experiments, which indicate that secondary metabolites isolated from *P. ocellatus* are biosynthesized by the mollusk-chloroplast symbiotic pair and that these metabolites are interconverted through an unprecedented in vivo photoisomerization. These biosynthetic experiments are a result of our recent research into the constituents of the sacoglossans *Tridachiella diomedea* and *Tridachia crispata* (*2–4*).

Trench *et al.* (*5*) had previously investigated the primary metabolism of *T. diomedea*, *T. crispata* and *P. ocellatus* by radioactive isotope labeling techniques. They demonstrated that organic carbon fixed during photosynthesis is transmitted to chloroplast-free animal tissue. The greatest concentration of ^{14}C occurs in the digestive diverticula and the pedal mucus gland (*1*). Chromatographic analysis indicated the presence of labeled carbon in a wide variety of primary metabolites, including lipids, amino acids, and primarily mono- and oligosaccharides.

During research into the secondary metabolites of sacoglossan mollusks, we isolated tridachione (**1**) and 9,10-deoxytridachione (**2**) from the ether-soluble oil of *T. diomedea* collected in the Gulf of California (*2*), as well as crispatone (**3**) and crispatene (**4**) from *T. crispata* collected in the Caribbean (*3*). The structural relation between these two carbon skeletons was demonstrated by the in vitro photochemical isomerization of **2** to photodeoxytridachione (**5**) (*4*). The interconversion in vitro of these skeletons suggested to us that this transformation occurs in vivo and represents an unprecedented biosynthetic step. Furthermore, the well-documented photosynthetic capability of the mollusk-chloroplast symbiotic pair suggested that these secondary metabolites are biosynthesized in situ by the symbiotic pair.



To confirm our hypothesis, we required a sacoglossan that contained both carbon skeletons, thus facilitating labeling experiments. We investigated *P. ocellatus*, which we collected at Ala Moana beach, Oahu, Hawaii. Silica-gel chromatography of the acetone extract of *P. ocellatus* yielded a single ultraviolet-absorbing band (eluant, a mixture of ether and hexane, 50 : 50). Reversed phase chromatography with an octadecylsilane-coated support (eluant, a mixture of methanol and water, 80 : 20) resolved the band into three isomeric components. The two major components were readily identified by spectral analysis (^1H and ^{13}C nuclear magnetic resonance) and comparison with authentic samples as 9,10-deoxytridachione (**2**) and photodeoxytridachione (**5**). The minor component (**X**) appears to be an epimer of **2**, but the amount of available material was too small for complete characterization.

We designed our initial labeling experiments to show that these metabolites are produced in situ under photosynthesis conditions. Specimens of *P. ocellatus* were placed in aerated Millipore-filtered seawater (4 ml per animal) enriched with ^{14}C-labeled sodium hydrogen carbonate at 20 μCi/ml. Four animals ("light" animals) were incubated in sunlight at 25°C for 8 hours, then transferred to the dark for an additional 18 hours. Two animals ("dark" controls) were maintained in the dark for 26 hours. The four light animals were killed and extracted with methanol. The combined extracts were partitioned between ether and 20 percent aqueous NaCl. The resulting ether layer was dried over $MgSO_4$ and evaporated, yielding an oil. The dark animals were treated similarly. The light animals yielded 12 mg of oil, the dark control yielded 4 mg. Each extract was diluted with methanol to 1 ml; a 10-μl portion of the light animal extract had 18,020 count/min above background, while a 30-μl portion of the dark control extract showed 351 count/min, which is less than 2 percent incorporation compared with the light animals. All samples were counted to a 95 percent confidence level (*6*). Identical portions were chromatographed on an Eastman Kodak silica gel thin-layer chromatograph (TLC) sheet (elution with diethyl ether) and the individual bands were assayed (Table 1). Material

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Table 1. TLC analysis of ^{14}C-labeled extract of incubated *P. ocellatus*, on Eastman Kodak silica gel plates. Abbreviations: A, origin; B, green pigments; C, pyrones; and D, yellow pigments.

Compound	R_F	Radioactivity (count/min)	Percent incorporation
D	0.75	3392	19
C	0.45	3746	21
B	0.20	1019	6
A	0.00	7618	42

remaining at the origin of the TLC sheet accounts for the bulk of the incorporation, which is not surprising since most of the strongly polar primary metabolites would not migrate under these chromatographic conditions. The band associated with the pyrones (**2**, **X**, **5**) represented the next highest incorporation of 21 percent. Chromatography of a 10-μl portion of the light animal extract on octadecylsilane reversed phase resolved the pyrones. Relative percentages of incorporation into each pyrone are shown in Table 2.

In a second experiment we demonstrated that the biosynthesis of photodeoxytridachione (**5**) proceeds via a photochemical rearrangement of 9,10-deoxytridachione (**2**). Four animals were placed in Millipore-filtered seawater (4 ml per animal) containing ^{14}C-labeled sodium hydrogen carbonate at 20 μCi/ml. All four animals were incubated in sunlight at 25°C for 30 minutes. After incubation, the animals were rinsed thoroughly with fresh seawater to remove labeled carbon. Two animals (light pulse) were returned to sunlight for 8 hours. The remaining two (dark pulse) were maintained in the dark for 8 hours. This experimental procedure was repeated with four new animals with 1-hour incubation. The animals were killed and extracted as before. Assuming that biosynthesis of photodeoxytridachione (**5**) proceeds via a photorearrangement of 9,10-deoxytridachione (**2**), photodeoxytridachione from the light animals should show greater incorporation than the dark animals, but overall incorporation into the pyrones should remain constant. Incorporation into the pyrones represented 6 to 7 percent of the total incorporation in each of the four samples (Table 2). In the dark pulse samples 9,10-deoxytridachione (**2**) accounted for most of the incorporation into the pyrones (> 40 percent), with incorporation into photodeoxytridachione (**5**) significantly lower. However, in the light pulse sample the situation is reversed and incorporation into photodeoxytridachione (**5**) (50 percent) is considerably higher than into 9,10-deoxytridachione (**2**) (32 percent) (*7*). We believe that our labeling experiments demonstrate an in vivo photoconversion of **2** to **5**. We also observed that **2** isomerizes to **5** in sunlight (62 percent in 7 hours, with benzene as solvent). This suggests that the in vivo process is a nonenzymatic reaction that occurs when the amount of ultraviolet radiation penetrating the dorsal surface of the animal exceeds the absorption limits of the pyrones present.

The origin of secondary metabolites isolated from marine organisms that are involved in symbiotic associations has been a long-standing question. Ciereszko and Karns (*8*) speculated that zooxanthellae, which are symbiotic with Caribbean gorgonians, participate in the biosynthesis of secondary metabolites isolated from these coelenterates. Unfortunately, labeling experiments to test this hypothesis were not performed. We have now demonstrated that secondary metabolites isolated from *P. ocellatus* are produced in situ by a symbiotic pair. Trench *et al.* (*5*) identified the green alga *Caulerpa sertularoides* as the source of the chloroplasts of *T. crispata*. This alga and related species have been examined, and several metabolites have been isolated (*9*); none of these are biogenetically or structurally related to the *Tridachia* metabolites, which would indicate that the *Tridachia* constituents are molluscan rather than transmitted algal metabolites.

A common biosynthetic pathway for the *Tridachia* and *Tridachiella* metabolites has been proposed on the basis of the in vitro photochemical interconversion of the two skeletons (*4*). We have now confirmed this hypothesis by showing that light-catalyzed interconversion also occurs in vivo.

With respect to the biological function of these metabolites, Trench *et al.* indicated that the digestive diverticula and the pedal mucus gland are the major receptors of photosynthetically fixed carbon (*1*). Our results indicate that the greatest incorporation of ^{14}C occurs in the sugars and pyrones **2**, **X**, **5**. There is an obvious correlation between incorporation of ^{14}C into sugars and translocation of ^{14}C to the pedal mucus gland, which is the site of the production of mucus. However, it is difficult to rationalize the high incorporation into the pyrones. The one structural feature common to all of the secondary metabolites is an α-methoxy-γ-pyrone ring, which absorbs ultraviolet light (*10*). We believe

Table 2. Percentage of radioactivity in each of the three pyrones; A, 26 hours; B, 0.5 hour (dark pulse); C, 1.0 hour (dark pulse); D, 0.5 hour (light pulse); and E, 1.0 hour (light pulse).

Pyrone	Percent radioactivity in conditions				
	A	B	C	D	E
2	55	42	47	31	32
X	16	23	21	15	18
5	29	35	32	54	50

that these metabolites are biosynthesized and translocated to the tissue of the digestive diverticula where they serve as sunscreens. The digestive diverticula flank the dorsal surface of the animal (*11*). We believe this represents a biochemical adaptation of these mollusks to their environment. All three sacoglossans live on sandy reef flats in shallow lagoons, where they are exposed to sunlight during the day, and burrow into the sand at night. Ultraviolet light is rapidly attenuated by seawater, but significant amounts penetrate as deep as 3 m (*12*).

CHRIS IRELAND
PAUL J. SCHEUER

Department of Chemistry,
University of Hawaii at Manoa,
Honolulu 96822

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