# Polypropionates from the Mediterranean Mollusk Elysia timida

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# POLYPROPIONATES FROM THE MEDITERRANEAN MOLLUSK *ELYSIA TIMIDA*

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ABSTRACT.—The Mediterranean sacoglossan *Elysia timida* contains, along with the already known photodeoxytridachione [**2**] and 9,10-deoxytridachione [**3**], two new related polypropionates, 15-norphotodeoxytridachione [**5**] and *iso*-9,10-deoxytridachione [**6**]. The presence of all these propionates in the mucous secretion of the mollusc suggests a potential defensive role of these metabolites toward attacks of predators, which is supported by their ichthyotoxicity in a mosquito fish bioassay.

Opisthobranchs, which are marine mollusks scarcely protected by the shell, are very interesting models to study marine ecology of benthic organisms (1–3). Opisthobranchs belonging to the order Sacoglossa display a complete evolutionary series from shelled (conchoid) molluscs with a large or reduced shell to shell-less (aconchoid) species characterized either by lateral parapodia or by dorsal cerata (4).

While continuing our studies on the chemical ecology of Mediterranean opisthobranchs (2), we have recently studied the defensive strategy of the conchoid *Oxynoe olivacea* (5) and that of other two conchoid sacoglossans (*Lobiger serradifalci* and *Cylindrobulla fragilis*), which feed upon the same green alga, *Caulerpa prolifera* (6). All these mollusks are able to sequester and to biomodify the main algal metabolite, caulerpenyne (7). Studies have been also directed to the aconchoid sacoglossans which are classified (4) in two main super-families: Polybranchioidea and Elysioidea. All studied Polybranchioidea species contain some α- and γ-pyrones formally constructed from five propionate units (8–12), whereas the Elysioidea *Thuridilla hopei* contains some diterpenoids, e.g., thuridillin-C [**1**] (13), closely related to algal metabolites (14,15). In this paper, we report the chemical study of the Mediterranean Elysioidea, *Elysia timida* Risso. *E. timida* is a very small sacoglossan which lives closely associated with the alga *Acetabularia acetabulum* (16). The mollusk was collected by hand from Italian (Augusta and Sorrento) and Spanish (Murcia) coasts.

The $Et_2O$-soluble fraction from the $Me_2CO$ extract of both Italian and Spanish populations displayed the same pattern of secondary metabolites. In particular, tlc ($SiO_2$ petroleum ether-$Et_2O$, 1:1) analysis showed, as main component, a single uv-absorbing spot ($R_f$ 0.3). After column chromatography over Si gel, the uv-sensitive faction was analyzed by hplc [Spherisorb S5 ODS2, MeOH-$H_2O$, (72:28)], which yielded a profile characterized by four peaks. The same relative ratio among the four components was observed in the crude extracts from all *E. timida* populations and also in the extract of the mucous secretion of the mollusk. The fractions 1–4 were separated by prep. hplc of the uv-sensitive mixture isolated from the extract of 714 specimens, affording 1.8, 8.6, 2.6, and 6.6 mg, respectively.

The $^1$H-nmr spectrum of fraction 4

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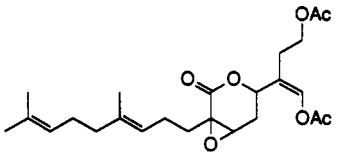

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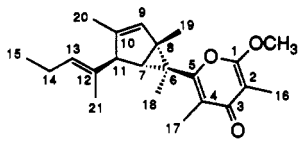

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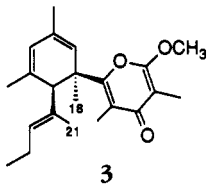

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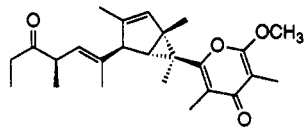

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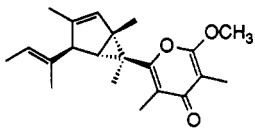

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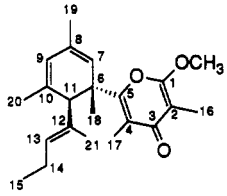

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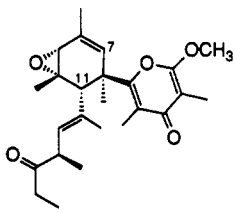

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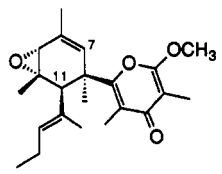

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displayed resonances that were identical to those reported for photodeoxytridachione [**2**], a polypropionate isolated from *Placobranchus ocellatus* (17) and obtained (18) by photochemical rearrangement of 9,10-deoxytridachione [**3**], a metabolite from *Tridachiella diomedea* [(−) enantiomer] (18) and *Elysia chlorotica* [(+) enantiomer] (19). Compound **2** was previously only partially characterized (18), mainly by comparison of some $^1$H-nmr resonances with those of crispatene [**4**], a related metabolite from *Tridachia crispata*. The previous papers lacked $^{13}$C-nmr data for **2** and, generally, proton and carbon nmr values reported in the literature for related compounds (18–20) were assigned without the application of the recently developed 2D nmr techniques. As a result of this, more complete assignments should be useful. All $^1$H- and $^{13}$C-nmr resonances have now been assigned (Table 1) confirming, also by comparison with the nmr data of **5**, the suggested structure.

The chemical structure of the main metabolite from *E. timida* (fraction 2; 15-norphotodeoxytridachione [**5**]) is closely related to that of **2**, displaying an unique difference in the length of the side-chain, containing only four carbons. The uv and ir spectra of **5** were similar to those of **2**; the elemental composition ($C_{21}H_{28}O_3$), obtained by hreims, suggested that **5** is a lower homolog of **2**. The $^1$H-nmr spectrum of **5** was almost identical to that of **2**, lacking the signals at δ 0.97 ($H_3$-15) and 2.04 ($H_2$-14), which were replaced by a broad doublet at δ 1.63 (3H, $J$=6.7 Hz, H-14) coupled with an olefinic broad quartet at δ 5.37 (1H, $J$=6.7 Hz, H-13). 2D Nmr experiments ($^1$H-$^1$H COSY, $^1$H-

TABLE 1. $^1$H- and $^{13}$C-Nmr Data[a] of Compounds **2**, **5**, and **6**.

| Carbon | Compound | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **2** | | | | **5** | | | | | **6** | | | | |
| | δ $^{13}$C | m[b] | δ $^1$H | m (*J*, Hz) | δ $^{13}$C[c] | m[b] | δ $^1$H[c] | m (*J*, Hz) | Hs correlated[d] | δ $^{13}$C[c] | m[b] | δ $^1$H[c] | m (*J*, Hz) | Hs correlated[d] |
| 1 ..... | 162.31 | s | | | 162.28 | s | | | $H_3$-16, OMe | 161.58 | s | | | $H_3$-16, OMe |
| 2 ..... | 99.47 | s | | | 99.47 | s | | | $H_3$-16 | 98.73 | s | | | $H_3$-16 |
| 3 ..... | 181.67 | s | | | 181.61 | s | | | $H_3$-16, $H_3$-17 | 181.90 | s | | | $H_3$-16, $H_3$-17 |
| 4 ..... | 120.39 | s | | | 120.38 | s | | | $H_3$-17 | 118.80 | s | | | $H_3$-17 |
| 5 ..... | 160.52 | s | | | 160.48 | s | | | H-7, $H_3$-17, $H_3$-18 | 163.46 | s | | | H-11, $H_3$-17, $H_3$-18 |
| 6 ..... | 40.69 | s | | | 40.67 | s | | | H-9, H-11, $H_3$-18, $H_3$-19 | 45.10 | s | | | H-11, $H_3$-18 |
| 7 ..... | 36.77 | d | 1.42 | b s | 36.67 | d | 1.41 | b s | $H_3$-18, $H_3$-19 | 124.36 | d | 5.14 | b s | H-11, $H_3$-18, $H_3$-19 |
| 8 ..... | 31.77 | s | | | 31.79 | s | | | H-11, $H_3$-18, $H_3$-19, $H_3$-20 | 131.72 | s | | | $H_3$-19 |
| 9 ..... | 128.61[e] | d | 5.32 | b s | 128.63 | d | 5.33 | s | H-11, $H_3$-19, $H_3$-20 | 123.72 | d | 5.61 | b d (1.3) | H-7, H-11, $H_3$-19, $H_3$-20 |
| 10 ..... | 144.04 | s | | | 144.04 | s | | | H-9, H-11, $H_3$-20 | 136.95 | s | | | H-11, $H_3$-20 |
| 11 ..... | 58.34 | d | 2.73 | b s | 58.39 | d | 2.75 | b s | H-7, H-9, H-13, $H_3$-20, $H_3$-21 | 56.41 | d | 3.18 | s | H-9, H-13, $H_3$-18, $H_3$-20, $H_3$-21 |
| 12 ..... | 133.98 | s | | | 136.51 | s | | | H-7, H-11, $H_3$-14, $H_3$-21 | 131.40 | s | | | H-11, $H_2$-14, $H_3$-18, $H_3$-21 |
| 13 ..... | 128.79[e] | d | 5.29 | dt (7.0, 1.1) | 120.68 | d | 5.37 | b q (6.7) | H-11, $H_3$-14, $H_3$-21 | 133.10 | d | 5.25 | b t (7.1) | H-11, $H_2$-14, $H_3$-15, $H_3$-21 |
| 14 ..... | 21.20 | t | 2.04 | bdq (7.5, 7.0) | 13.45 | q | 1.63 | b d (6.7) | H-13 | 21.26 | t | 2.01 | dq (7.5, 7.1) | H-13, $H_3$-15 |
| 15 ..... | 14.28 | q | 0.97 | t (7.5) | | | | | | 13.96 | q | 0.93 | t (7.5) | H-13, $H_2$-14 |
| 16 ..... | 6.84 | q | 1.84 | s | 6.83 | q | 1.84 | s | | 6.89 | q | 1.83 | s | |
| 17 ..... | 10.77 | q | 1.96 | s | 10.74 | q | 1.97 | s | $H_3$-16 | 11.39 | q | 2.08 | s | |
| 18 ..... | 13.05 | q | 1.10 | s | 13.05 | q | 1.10 | s | | 22.47 | q | 1.30 | s | H-7, H-11 |
| 19 ..... | 17.05 | q | 1.19 | s | 17.08 | q | 1.20 | s | H-7 | 20.96 | q | 1.76 | d (1.6) | H-7 |
| 20 ..... | 13.67 | q | 1.57 | b s | 13.69 | q | 1.56 | s | H-9 | 21.70 | q | 1.66 | b s | H-9, H-11 |
| 21 ..... | 12.74 | q | 1.48 | b d (0.5) | 12.64 | q | 1.49 | b s | H-11, H-13 | 13.96 | q | 1.53 | d (0.6) | H-11, H-13 |
| OMe ... | 55.27 | q | 3.96 | s | 55.27 | q | 3.96 | s | | 55.37 | q | 3.92 | s | |

[a]Bruker AM500; $CDCl_3$. Values are reported in ppm referenced to $CHCl_3$ (δ 7.26 for proton and δ 77.0 for carbon).
[b]By DEPT sequence.
[c]Assignments were aided by $^1$H-$^1$H COSY, $^1$H-$^{13}$C HETCOR and HMBC ($J$=10 Hz) experiments.
[d]By HMBC experiment.
[e]May be reversed.

$^{13}$C HECTOR, HMBC) confirmed the proposed structure and allowed the complete assignment of all $^{1}$H- and $^{13}$C-nmr resonances (Table 1).

Finally, spectral analysis of fraction 3 revealed close analogies with 9,10-deoxytridachione [**3**], suggesting an epimerization at one of the chiral centers of **3**. The new compound, *iso*-9,10-deoxytridachione [**6**] possesses the same elemental composition, $C_{22}H_{30}O_3$, as **3** and similar uv ($\lambda$ max 253 nm, $\epsilon$ 20,200) and ir ($\nu$ max 1659 and 1612 $cm^{-1}$) absorptions, due to the presence of an $\alpha$-methoxy-$\beta,\beta'$-dimethyl-$\gamma$-pyrone system. The $^{1}$H-nmr spectrum of **6** confirmed this analogy, displaying resonances assignable to the same side-chain and also signals attributable to two vinyl methyls ($\delta$ 1.76 and 1.66), two olefinic protons ($\delta$ 5.61 and 5.14), a bis-allylic proton ($\delta$ 3.18) and a tertiary methyl ($\delta$ 1.30) that, by analogy with **3**, supported the presence of a pentasubstituted cyclohexadiene ring. However, some diagnostic differences were observed for the protons in the side-chain, and also for H-11 ($\delta$ 3.18 in **6**, $\delta$ 2.71 in **3**) and H-7 ($\delta$ 5.14 in **6**, $\delta$ 5.56 in **3**). These differences might be justified by a different orientation of the substituents at C-6 and C-11. Because of this, we suggest that **6** is an epimer of **3**, either at C-6 or at C-11, displaying the methyl at C-6 and the alkyl chain at C-11 with a *cis*-oriented stereochemistry. Irradiation of H-11 ($\delta$ 3.18) resulted in an nOe of H-13 ($\delta$ 5.25), while no effect was observed on $H_3$-18 ($\delta$ 1.30), supporting a trans- orientation of the methyl at C-6 and H-11. Our assignment is also supported by $^{1}$H-nmr data reported in the literature (20) for tridachiapyrone-C [**7**] ($\delta$ 5.50 for H-7, $\delta$ 3.40 for H-11) and for tridachione [**8**] ($\delta$ 6.10 for H-7, $\delta$ 2.91 for H-11). The $^{13}$C-nmr spectrum of **6** displayed almost all resonances closely related to those of **3** (18), with the exception of those assigned to C-6 ($\delta$ 45.10 for **6**, $\delta$ 47.6 for **3**) and C-11($\delta$ 56.41 for **6**, $\delta$ 59.6 for **3**). However, the $^{13}$C-nmr assignments for C-18 ($\delta$ 13.8) and C-21 ($\delta$ 22.3) reported for **3** (20) are not in agreement with our data, but this difference disappears by inverting the $^{13}$C-nmr values previously assigned to C-18 and C-21. All $^{1}$H- and $^{13}$C-nmr resonances (Table 1) for **6** were connected by a series of bi-dimensional experiments ($^{1}$H-$^{1}$H COSY, $^{1}$H-$^{13}$C HETCOR, HMBC). In particular, some highly diagnostic HMBC correlations were observed for C-11 (H-9, H-13, $H_3$-18, $H_3$-20 and $H_3$-21) and for C-6 (H-11 and $H_3$-18), thus confirming the suggested relative stereochemistry of **6**.

Surprisingly, the metabolic pattern of *E. timida* is not related to that of *Thuridilla hopei* (13), whereas there is a strong analogy between the metabolism of the Mediterranean *E. timida* and those of some Pacific Elysioidea sacoglossans, in particular that of *P. ocellatus*, where along with 9,10-deoxytridachione [**3**] and photodeoxytridachione [**2**], a third metabolite, suggested as a probable epimer of **3**, was recovered from the hplc purification in an amount too small for complete characterization (17). By analogy, minor amounts of **3** were revealed by $^{1}$H-nmr, among the components of the first hplc fraction. Furthermore, 15-nor-derivatives of **8** and **3** were also found among the minor metabolites of *T. diomedea* (21).

The photorearrangement of the cyclohexadiene ring of **3** to the bicyclohexene ring system of **2** has been rigorously demonstrated (18). The isomerization of **3** to **2** in sunlight has also been reported (17). *P. ocellatus* is able to biosynthetize de novo both propionates, **2** and **3**, from $NaH^{14}CO_3$. The incorporation of labeled carbon, when the animals are exposed to light, has a rate fiftyfold of that of animals kept in the dark, where the incorporation into photodeoxytridachione [**2**] was significantly lower (17). We have analyzed populations of *E. timida* collected from different places (Augusta and Sorrento, Italy and Murcia, Spain), in

different months (from June to November). We have also maintained aquarium populations either in the dark or in the light. The extracts of all samples displayed the same hplc profile. Finally, biosynthetic experiments in vivo with [1-$^{14}$C]-sodium propionate and [1-$^{14}$C]-NaOAc were carried out in order to demonstrate the origin de novo of metabolites **2**, **5**, and **6**. Unfortunately, all the experiments led to a very poor incorporation and results were not conclusive. However, samples of the alga *A. acetabulum*, the prey of *E. timida*, were extracted with $Me_2CO$, and the $Et_2O$-soluble fractions were analyzed by hplc, but no trace of polypropionates was detected.

In conclusion, Mediterranean *E. timida* displays a metabolic pattern very similar to that of Pacific *P. ocellatus*. The relative ratio among the metabolites is the same in populations from distinct geographical areas and collected in different seasons and does not depend on the level of exposure to light. The biological role of the polypropionates may be linked to the protection of the mollusk. This is supported by the presence of the polypropionates in the mucous secretion of *E. timida*. In fact, the $Me_2CO$ extract of the mucus, without any purification, displayed the same hplc profile of the uv-absorbing fraction from *E. timida*. In addition, the compounds **2**, **5**, and **6** were toxic at 5 ppm in an ichthyotoxicity assay against the mosquito fish *Gambusia affinis* (22,23).

## EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Si gel chromatography was performed using precoated Merck $F_{254}$ plates and Merck Kieselgel 60 powder. Hplc purifications were carried out on a Waters chromatograph equipped with an uv detector. The ir spectra were taken on a Nicolet FT 5D×B spectrometer. The uv spectra were recorded on a Varian DMS 90 spectrophotometer. Optical rotations were measured on a Jasco DIP 370 polarimeter. $^1$H- and $^{13}$C-nmr spectra were recorded on a Bruker AM 500 spectrometer; chemical shifts are reported in parts per million referenced to $CHCl_3$ as internal standard (δ 7.26 for proton and δ 77.0 for carbon). Mass spectra were obtained on Kratos MS50 and VG TRIO 2000 instruments.

BIOLOGICAL MATERIAL.—A population of 100 *E. timida* specimens (median size 1 cm) was collected by scuba-diving, in June 1990, in Massalubrense, Conca Azzurra, near Sorrento (Italy). Another collection (100 specimens) was made from the coast of Augusta (Sicily, Italy), during September of 1990. Finally, 714 *E. timida* specimens were collected at Mazarrón Bay, near Murcia (Spain) from October to November of 1991. Voucher specimens are available at the Departamento de Biología Animal y Ecología de Murcia. Three Spanish collections of *E. timida* (169, 217, and 42 specimens) were maintained in an aquarium in the dark for 24 h, 48 h and 10 days, respectively. Before being killed and extracted in the usual way, the three groups of animals were molested in order to obtain the mucous secretions, which were extracted with $Me_2CO$ and analyzed by hplc. Two other populations from Murcia (216 and 70 individuals) were exposed to light in an aquarium for 24 h and 10 days, respectively. These animals were also disturbed to obtain the mucous secretions, before being killed and extracted.

ISOLATION PROCEDURES.—In a typical procedure, 216 individuals were extracted with $Me_2CO$ (30 ml×4). The combined extracts were evaporated under reduced pressure to give 380 mg of crude material. The $Et_2O$-soluble fraction was chromatographed on a $SiO_2$ column using petroleum ether with increasing amounts of $Et_2O$ as eluent. The uv-absorbing fraction (10.0 mg, $R_f$ 0.4 in 1:1 petroleum ether-$Et_2O$) was further analyzed by hplc [Spherisorb S5 ODS2, MeOH-$H_2O$ (72:28), flow 1.4 ml/min, monitored at 254 nm, revealing the presence of four peaks. All *E. timida* populations were extracted with $Me_2CO$ and the extracts were purified as described above. The combined uv-sensitive fractions (30.5 mg) from the different Spanish collections (714 individuals) were submitted to prep. hplc purification [Spherisorb S5 ODS2, i.d. 10 mm, MeOH-$H_2O$ (8:2), flow 3 ml/min] giving 4 fractions: fr 1 (1.8 mg, an unresolved mixture of propionate-derived metabolites), fr. 2 (8.6 mg, **5**), fr. 3 (2.6 mg, **6**) and fr. 4 (6.6 mg, **2**). The mucous secretions obtained from the animals exposed to different conditions of light were similarly extracted with $Me_2CO$ and analyzed, without any purification, by hplc, revealing identical chromatographic profiles.

*Fraction 1 (***[3]** *as main component).*—Selected $^1$H-nmr data ($CDCl_3$): δ 5.67 (1H, s, H-9), 5.58 (1H, s, H-7), 3.98 (3H, s, OMe), 2.72 (1H, s, H-11), 2.05 (3H, s, H-17), 1.83 (3H, s, H-16), 1.78 (3H, b s, H-20), 1.72 (3H, s, H-19), 1.43 (3H, s, H-21), 0.70 (3H, t, $J$=7.5 Hz, H-15). (Assignments according to ref. 20).

*Photodeoxytridachione* [**2**].—$[\alpha]^{25}D$ +14.4° ($CHCl_3$, $c$=0.63); ir $\nu$ max (liquid film) 1662 and 1601 $cm^{-1}$, uv $\lambda$ max (MeOH) 255 ($\epsilon$ 13,700) nm; $^1H$ and $^{13}C$ nmr, see Table 1); hreims *m/z* 342.2207 ($[M]^+$, $C_{22}H_{30}O_3$ requires 342.2195); eims *m/z* 342 (5.5), 327 (21), 295 (9), 267 (20), 239 (27), 199 (53), 155 (base peak), 91 (97).

*15-Norphotodeoxytridachione* [**5**].—$[\alpha]^{25}D$ +26.5° ($CHCl_3$, $c$=0.46); ir $\nu$ max (liquid film) 1663, 1621, and 1601 $cm^{-1}$; uv $\lambda$ max (MeOH) 254 ($\epsilon$ 15,500) nm; $^1H$ and $^{13}C$ nmr, see Table 1; hreims *m/z* 328.2054 ($[M]^+$, $C_{21}H_{28}O_3$ requires 328.2038); eims *m/z* 328 (6.4), 314 (18), 281 (9), 253 (26), 225 (33), 199 (50), 185 (82), 155 (97), 91 (base peak).

iso-*9,10-Deoxytridachione* [**6**].—$[\alpha]^{25}D$ +5.9° ($CHCl_3$, $c$=0.1); ir $\nu$ max (liquid film) 1659 and 1612 $cm^{-1}$; uv $\lambda$ max (MeOH) 253 ($\epsilon$ 20,200) nm; $^1H$- and $^{13}C$ nmr, see Table 1; hreims *m/z* 342.2180 $[M]^+$, $C_{22}H_{30}O_3$ requires 342.2195); eims *m/z* 342 (18), 327 (10), 267 (10), 239 (17), 199 (40), 168 (50), 155 (60), 91 (72), 83 (82), 42 (base peak).

INCORPORATION EXPERIMENTS WITH $^{14}C$-LABELED PRECURSORS.—In each incorporation experiment, specimens of *E. timida* were placed in aerated sea water and the $^{14}C$-labeled precursors, dissolved in EtOH, were added directly to the aquarium $H_2O$. After various times (see below), the animals were killed, frozen at −20°, then extracted in the usual way.

In the first experiment, 100 individuals were maintained exposed to the light for 7 days in 1 liter of sea water containing a total of 50 μCi of sodium[1-$^{14}C$]propionate. In the second experiment, a total of 50 μCi of sodium[1-$^{14}C$]propionate was administered to 100 specimens placed in 200 ml of sea water exposed to the light; the experiment was interrupted after 3 days. In the third experiment, 37 specimens were exposed to the light for 7 days in 370 ml of sea water, where 18.5 μCi of sodium[1-$^{14}C$]acetate had been added. At the same time, three other experiments were carried out under the above described conditions, but maintaining the animals in the dark.

All the crude organic extracts of the animals from each experiment were found to be radioactive. The fraction containing the propionate-derived metabolites was also radioactive, but this activity, however, decreased rapidly after Si-gel and hplc purifications of the compounds, and for each experiment very poor incorporation in pure compounds was observed.

ICHTHYOTOXICITY BIOASSAYS.—Ichthyotoxicity assays were conducted using a mosquito fish, *G. affinis* (Baird and Girard), as described by Gunthorpe and Cameron (22). Compounds **2**, **5**, and **6** were assayed at 10, 5, and 1 ppm by dissolving the appropriate amount in 0.5 ml of $Me_2CO$. Control tests were carried out in conjunction with each test run. The toxicity ranking was defined according to Coll *et al.* (23).

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