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Cyercenes, novel pyrones from the ascoglossan mollusc *Cyerce cristallina*. Tissue distribution, biosynthesis and possible involvement in defense and regenerative processes

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Abstract. The extraordinary regenerative phenomena in the marine ascoglossan mollusc *Cyerce cristallina* are described here for the first time. The possible correlation between cyercenes, pyrones recently isolated from the mollusc regenerating dorsal appendages (cerata), and the processes of chemical defense and regeneration in the mollusc, was investigated by studying the tissue distribution, biological activity and biogenesis of cyercenes. Differences in distribution between the *C. cristallina* mantle, cerata and mucous secretion were found. Cyercenes showed activity in the *Hydra vulgaris* regeneration assay and the mosquito fish ichthyotoxicity assay. The de novo biosynthesis of cyercenes from propionic acid was demonstrated by means of in vivo adsorption experiments with radiolabeled propionate.
Key words. Pyrones; polypropionates; marine molluscs; regeneration; growth factors; ichthyotoxins; marine toxins; *Hydra*.

Marine gastropod molluscs have provided many attractive models for the study of marine biochemistry, physiology, neurobiology and ecology[1] and, from this point of view, the species belonging to the ascoglossan genus *Cyerce* are no less interesting. Ecological studies conducted on the Australian species *Cyerce nigricans* have shown, for example, that the live mollusc is repellent to coral reef fish, as is its organic crude extract[2], although a recent chemical study conducted on the same species did not succeed in correlating the substances isolated from the mollusc mantle with this chemical deterrence[3]. Morphologically very similar to *C. nigricans* is the Mediterranean species *Cyerce cristallina* (Trinchese, 1881), whose body volume is mainly due to the presence of aposematically colored dorsal appendices (cerata)[4]. When the animal is attacked by predators, the cerata are detached from the mantle and exhibit prolonged contractions while secreting large amounts of a supposedly toxic mucous secretion (Perrone[4], and unpublished observations). In the laboratory, this typical defensive behavior, known as autotomy, can be induced either by pinching the cerata with pliers or by slightly raising the temperature of the seawater containing the mollusc (unpublished observations). After the occurrence of the autotomic process, the mollusc provides a striking example of regeneration by completely reproducing the cerata within only 7–10 days (fig. 1); to the best of our knowledge, this phenomenon, which is probably very unusual for such a complex organism, has not been reported before.

With the aim of characterizing some of the chemical signals which are, at least in part, responsible for either the chemical defense or the regenerative processes of *C. cristallina*, we very recently conducted a chemical study on the mollusc cerata and isolated the seven novel py-

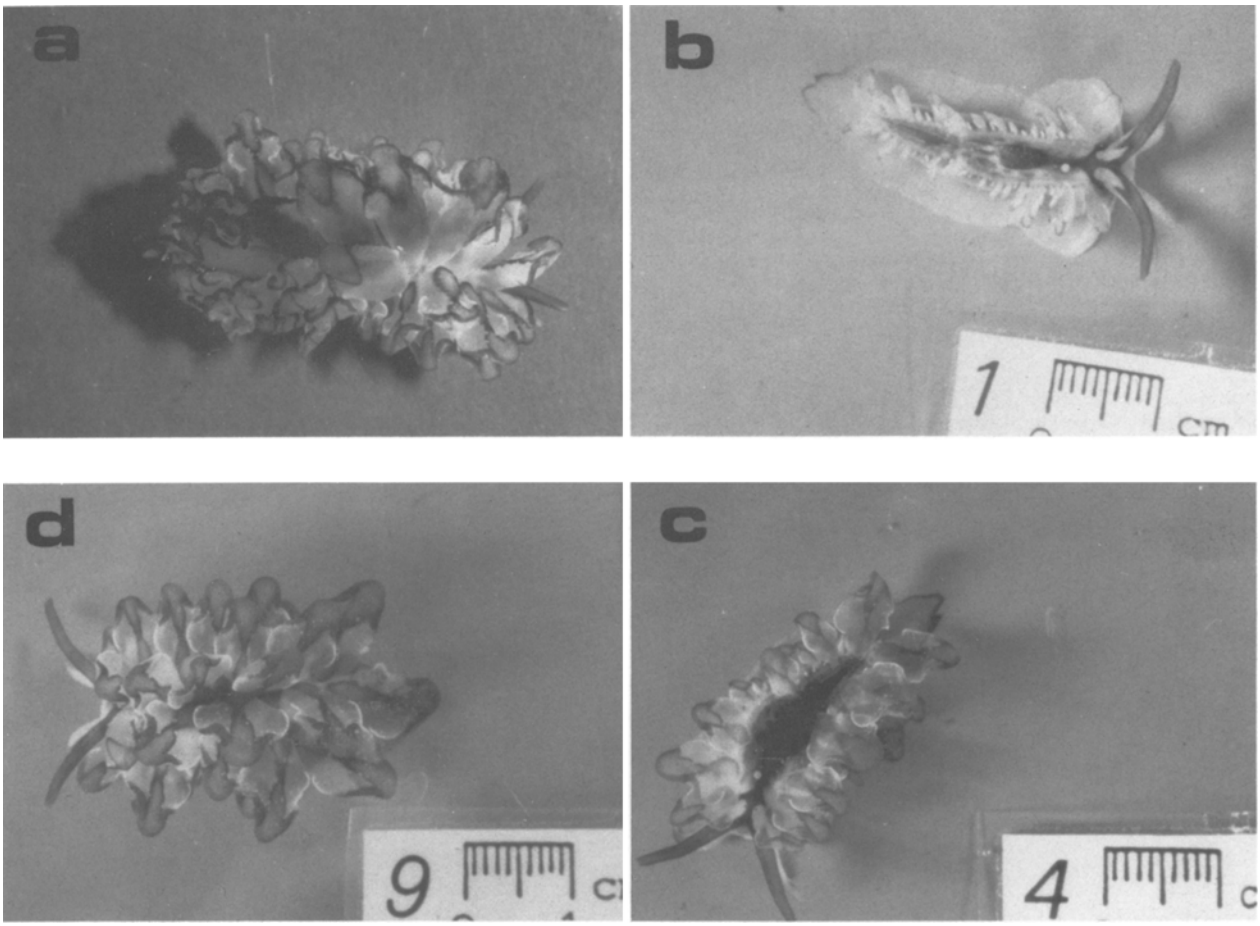

Figure 1. *C. cristallina* before (*a*), immediately after (*b*), 4 days after (*c*) and 9 days after (*d*) the autotomic process.

rones, named cyercenes, shown in figure 2[5]. In the present study we describe the results of a new investigation, conducted in order to define the tissue distribution, biological activity and biogenesis of cyercenes, and to establish whether a correlation exists between these metabolites and the interesting biological phenomena exhibited by *Cyerce cristallina*.

Materials and methods

The molluscs were caught in the Bay of Naples, where they can be found hidden underneath stones throughout the year, though in very limited numbers. Cyercenes were extracted, purified and characterized as described elsewhere[5]. Comparative analyses of the extracts from mantle, cerata, mucus and digestive gland (hepatopancreas) from the same specimens were carried out by loading the extracts directly onto a reverse phase HPLC column (Spherisorb ODS-2, 4.5 × 250 mm, i.d. = 5 μm) eluted with a 40-min gradient from 60 to 75% methanol in water, flow rate = 1 ml/min, UV absorbance being monitored at 285 nm. Although the total amounts of cyercenes in *C. cristallina* varied from specimen to specimen, their tissue distribution was always reproducible.

Ichthyotoxicity assays were conducted in seawater on the mosquito fish *Gambusia affinis* as described in Gunthorpe and Cameron[6], using the toxicity ranking defined in Coll et al.[7]. Six specimens were used for each test. Assays for regenerative activity were carried out on the freshwater coelenterate *Hydra vulgaris* as described in De Petrocellis et al.[8]. Briefly, hydra of the same size and from buds detached on the same days were decapitated, suspended in the assay buffer ($NaHCO_3$, 1 mM, $CaCl_2$, 1 mM) and treated for 24 h with the substance to be tested. Hydra were then washed and left to regenerate for 8–10 days in the assay buffer. At least 30 specimens were used for each test. In both assays, parallel control experiments were conducted with the same amount of solvent used to dissolve the substances to be tested (respectively 0.5 ml acetone and 2 μl methanol/ml buffer). Statistical analysis in the regeneration assay was carried out using the χ^2 test.

In vivo incorporation experiments with [^{14}C]-propionic acid (Amersham, 20 Ci/mmol, labeled on C-2) were performed by dissolving 25 μCi of the labeled precursor in 1.5 l of seawater containing four 'naked' (without cerata) specimens of *C. cristallina*. The molluscs were left to

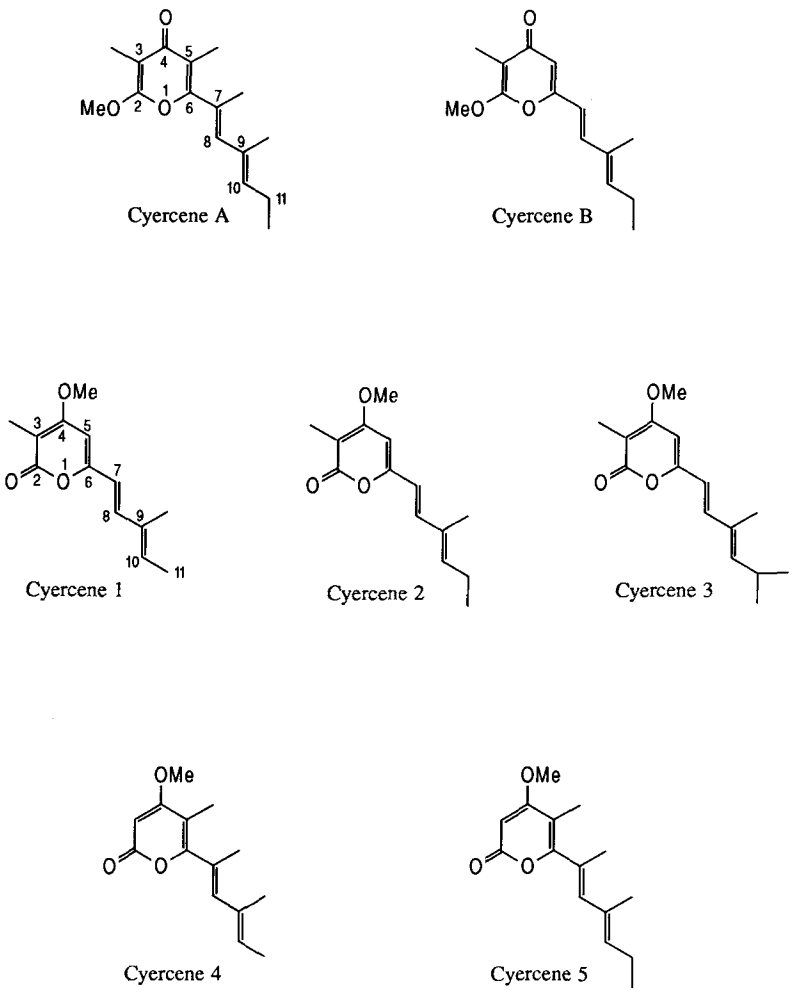

Figure 2. The structures of cyercenes.

absorb the labeled propionate for 5 days, during which their cerata regenerated up to at least 50% of their original volume. Detachment of these dorsal appendages was then induced and the cerata, together with the mucus contained in them, were extracted as previously described [5]. Aliquots (1/3) of the extract (total incorporation 30000 cpm) were submitted to either HPLC, performed as described above, or semipreparative thin layer chromatography (TLC) on a silica gel plate developed with petroleum ether/diethyl ether 1/1. After HPLC, 1 ml fractions were collected, 10 ml of scintillation liquid added to each, and β-emission counted ($\sim$ 80% efficiency). After TLC, bands at Rf = 0.05 intervals were scraped off the plate and suspended in 2 ml diethyl-ether; 8 ml of scintillation liquid was added, and the radioactivity counted in the β-counter ($\sim$ 65% efficiency).

Results and discussion

HPLC analysis showed differences in chemical composition between acetone extracts of *C. cristallina* mantle, cerata and digestive gland, and the ethereal extract of the mollusc mucous secretion (fig. 3). While no cyercene was present in the extract of the digestive gland, only cyercenes 1, 2 and 3 were found in the mantle. The mucus contained all cyercenes except cyercene A. This metabolite was only present in the cerata, which contained all seven compounds.

The presence of cyercene A only in the regenerating tissue of *C. cristallina*, the cerata, seemed to suggest the possible involvement of this metabolite in the quick regenerative mechanisms exhibited by the mollusc. We therefore decided to test this hypothesis by assaying the activity of the most abundant cyercenes in stimulating regeneration. Since it was not possible to obtain enough specimens of *C. cristallina* for a statistical analysis of the effect of cyercenes on this mollusc to be made, we used the hydra head and tentacle regeneration assay. This is a simple model which has provided positive responses with several cell growth and differentiation factors such as diacylglycerols, phorbol esters, 5-azacytidine and hydra activator [8–10], and in which the regeneration speed (normally 8 days) is comparable to that of *C. cristallina* cerata (7–10 days).

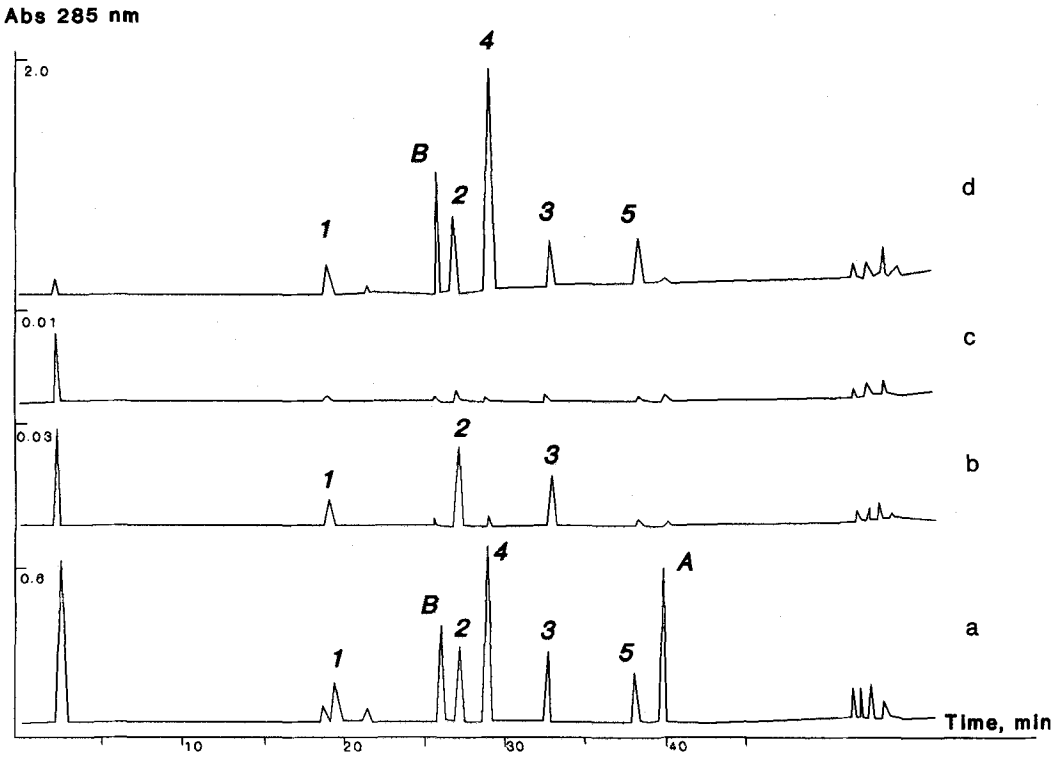

Figure 3. HPLC traces of the acetone extracts of *C. cristallina* cerata (*a*, 75 µg, 1/100 of the extract), mantle (*b*, 100 µg, 1/32 of the extract), digestive gland (*c*, 100 µg, 1/32 of the extract) and mucus (*d*, 200 µg, 1/5 of the extract). The tissues and mucus (~ 7 ml) were obtained from the same specimen. The digestive gland and cerata might be contaminated by traces of mantle tissue or mucus, respectively. This experiment was repeated three times. The amount of each metabolite varied, from specimen to specimen, from 0.02 to 0.3 mg, depending on the tissue and type of compound, but was always distributed as shown. (The various cyercenes are labeled on the diagram: *A*, *B*, 1–5, as in fig. 2.)

Table 1. Regenerative activity of cyercenes on *Hydra vulgaris* tentacles.

Compound	Concentration (µg/ml)	Average tentacle number ± SE	χ^2	P
Control	-	4.68 ± 0.10		
Extract (cerata)	50	7.00 ± 0.38	14.8	< 0.05
Cyercene-A	15	7.93 ± 0.55	49.5	< 0.001
Cyercene-B	40	5.60 ± 0.27	14.1	> 0.10
Cyercene-2	75	7.25 ± 0.59	12.6	> 0.10
Cyercene-3	75	8.00 ± 0.72	62.9	< 0.001
Cyercene-4	100	6.22 ± 0.28	11.6	> 0.10

The effect of cyercenes on *H. vulgaris* average tentacle number. Only the most abundant metabolites were assayed. At least 30 hydra were used for each test. The χ^2 values with relative P values for each test are also shown. The level of significance used was $p < 0.05$. The doses shown are the ones exerting the highest effect. At higher concentrations cyercenes were toxic to *H. vulgaris*.

As shown in table 1, cyercenes 2 and 3, which are, of the compounds tested, the only ones present in the mollusc mantle, exhibited an enhancement of *H. vulgaris* average tentacle number at 75 µg/ml, although the effect was not statistically significant for cyercene 2. Cyercenes B and 4, which are present both in the mollusc cerata and in the mucous secretion, did not exhibit any regenerating activity at dose up to 100 and 40 µg/ml respectively. In all cases, it was not possible to test higher doses of the compounds, owing to their toxicity to *H. vulgaris*. The highest effect was, however, shown by the compound which is only present in the cerata, cyercene A, which produced a 69% enhancement of average tentacle number at 15 µg/ml, thus corroborating the suggestion that it plays a biological role as a pivotal growth-inducing factor. However, although the activity exerted by cyercenes 2, 3 and, particularly, A accounts for most of the activity found in the crude acetone extract of *C. cristallina* cerata, and the tissue distribution of these metabolites supports their involvement in the regenerative processes, it is likely that other molecules, maybe present in hydrophilic extracts of the mollusc, act as the main factors which induce the rapid regeneration seen in *C. cristallina*.

The presence of cyercenes in the supposedly toxic mucous secretion of *C. cristallina* suggested their involvement in the previously reported chemical deterrence of

Cyerce species[2]. Compounds structurally similar to cyercenes and isolated from the Australian *C. nigricans* were not found to be active in a fish anti-feedant test[3]. However, since the putative toxic substances present in *C. cristallina* mucus would be readily dispersed in the marine environment in order to exert their deterrent action, we decided to test the effect of either the mucus ethereal extract or the purified cyercenes, dissolved in seawater, on the mosquito fish *Gambusia affinis*. In this commonly used ichthyotoxicity test[6, 7] six fish were used per test. As shown in table 2, all the compounds tested, as well as the mucus crude extract, were found to be toxic or very toxic to fish at a concentration of 10 µg/ml, thus confirming the toxicity of the mucus, and the involvement of cyercenes in chemical defense in *C. cristallina*. Cyercene 4 was the most active compound, being toxic at a concentration as low as 5 µg/ml.

Very interestingly, in both the ichthyotoxicity and the hydra regeneration assays, when two structurally similar compounds were used it was always the one containing one or two more methyl groups that exerted the highest activity (fig. 2, tables 1 and 2). Thus, in both assays, the 3-methyl-4-methoxy-α-pyrone cyercene 3 was more active than the lower homolog cyercene 2, and the γ-pyrone cyercene A was more active than the γ-pyrone cyercene B. The presence of one or two extra methyl groups in the molecule, by increasing the compound hydrophobicity, might facilitate its penetration through the cell membrane, thus enhancing its cellular effects. The two activities, however, are not necessarily related. Indeed, cyercene-4, which differs from cyercenes 2 and 3 in being a 4-methoxy-5-methyl-α-pyrone, is the most potent compound in the ichthyotoxicity test but has no regenerating activity on *Hydra vulgaris* (unfortunately, the available amount of the higher homolog of cyercene-4, cyercene-5, was not sufficient to assess its activity in the two assays). The complete absence of cyercenes, as well as of any other cyercene-like metabolite, from the acetone extract of the digestive gland, suggested that these compounds are not derived from dietary sources but are biosynthesized de novo by *C. cristallina*. This hypothesis was confirmed by in vivo biosynthesis experiments, using an isotope incorporation method which has already provided good results in experiments with other polypropionate metabolites[11]. Detachment of cerata was induced and the 'naked' molluscs incubated in seawater containing [^{14}C]-sodium propionate. After 5 days, the cerata, which had already reached an appreciable degree of regeneration (~ 50%, see fig. 1), were extracted as usual together with the mucus contained in them, and the extract was submitted either to reverse phase HPLC or to silica gel TLC (fig. 4a and 4b). Incorporation of almost 60% of the radioactivity contained in the extract was observed specifically in the fractions containing the seven cyercenes, the radioactivity profiles being perfectly superimposable on the UV profiles of the HPLC and TLC separations. In spite of the fact that some cyercenes are probably derived from the demethylation of others (fig. 5), the specific incorporation was very similar for six of the seven metabolites (table 3). In fact, the ^{14}C-propionic acid used was labeled on C-2, and demethylation would only involve the methyl groups corresponding to C-3 of the propionate precursor. The highest specific incorporation of label was found in cyercene A. This was not surprising, since the in vivo biosynthesis experiment was conducted during the early period of regeneration and, therefore, any compound uniquely contained in the cerata would be synthesized de novo in the presence of the labeled precursor.

The fact that *C. cristallina* employs a completely endogenous biosynthetic pathway, starting with propionic acid to produce cyercenes with different tissue distribution, supports our suggestion that members of this class of metabolites play a number of important biological roles. The evidence suggesting a primary function for these compounds as defense allomones is strong. Further investigations are required to isolate from *C. cristallina* chemical signals, other than cyercene-A, responsible for the impressive regeneration speed of the mollusc cerata, and to gain some understanding of the exact role played by cyercene A in this process. Future work will also be

Table 2. Ichthyotoxic activity of cyercenes on *Gambusia affinis*.

Compound	Concentration (µg/ml seawater)	Toxic	Very toxic
Extract (mucus)	10	–	Yes
Cyercene-A	10	–	Yes
Cyercene-B	10	Yes	No
Cyercene-2	10	Yes	No
Cyercene-3	10	–	Yes
Cyercene-4	10	–	Yes
Cyercene-4	5	Yes	No

The effect of cyercenes on the mosquito fish *Gambusia affinis*. Only the most abundant metabolites were assayed. The ranking of toxicity was assigned according to Coll et al.[8]. Cyercene 4 was active, albeit simply 'toxic', also at a 5 µM concentration.

Table 3. De novo biosynthesis of cyercenes: in vivo absorption experiments with ^{14}C-propionate.

Compound	Amount (µg)	Total incorporation (cpm)	Specific incorporation (cpm/mg)
Extract	4900	30000	6122
Cyercene-1	70	1026	14656
Cyercene-2	164	3747	22847
Cyercene-3	152	2880	18947
Cyercene-4	246	7080	28783
Cyercene-5	59	1080	18305
Cyercene-A	15	858	57200
Cyercene-B	36	663	18416

Total and specific incorporation of ^{14}C-propionate into cyercenes in vivo adsorption experiments with *C. cristallina*. The amount of each metabolite in this biosynthesis experiment was established by a comparison of the HPLC UV peaks at 285 nm (see fig. 4a) with those of known amounts of each cyercene. The values are for *C. cristallina* mucus plus partly (~ 50%) regenerated cerata. The amounts of cyercenes in completely regenerated cerata are normally different from those shown here (see fig. 3).

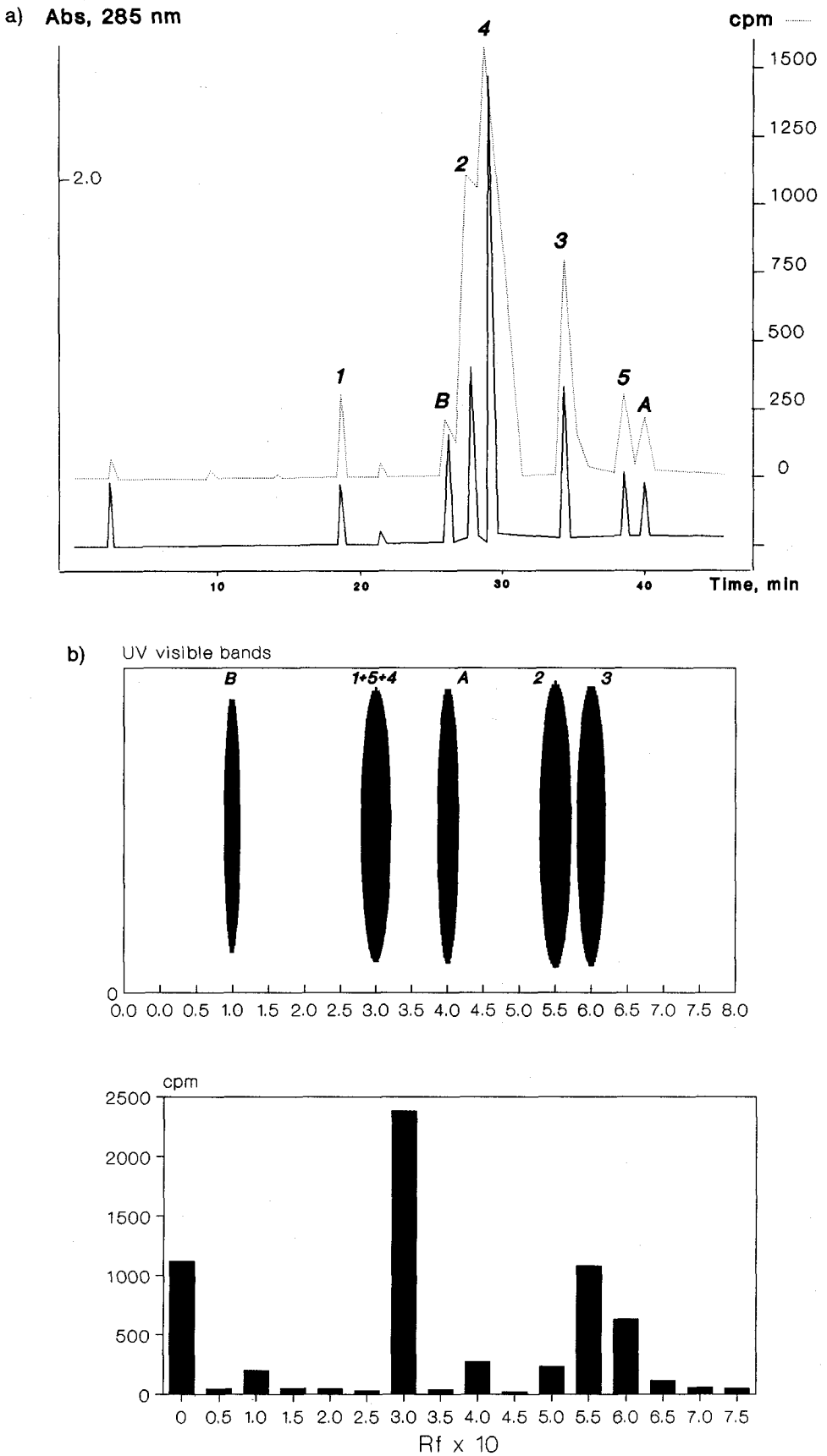

Figure 4. HPLC (*a*) and preparative TLC (*b*) radioactivity and UV profiles of aliquots (1/3) of the extract from the mucus plus partly regenerated cerata of the *C. cristallina* specimens from in vivo adsorption experiments with ^{14}C-propionate.

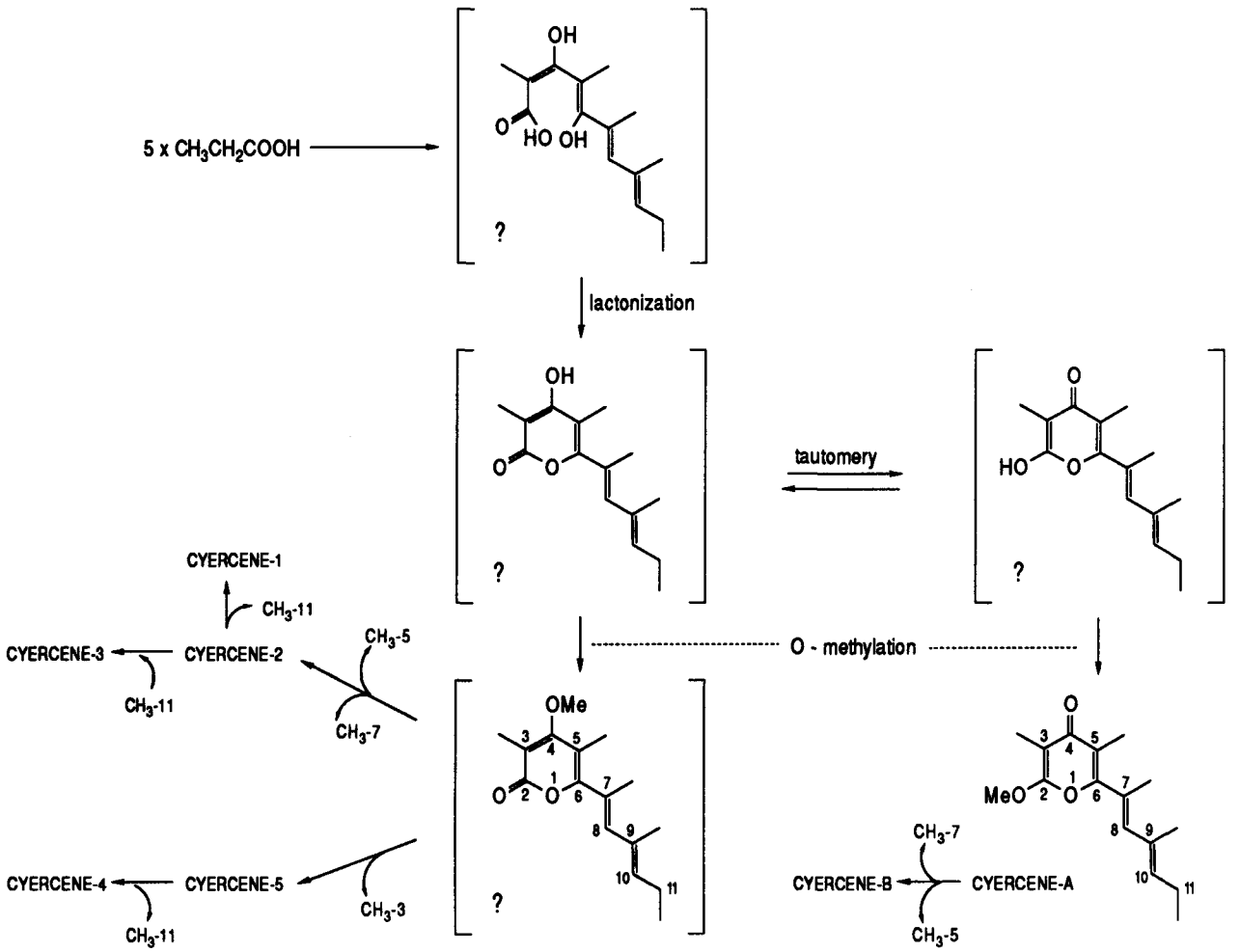

Figure 5. Proposed pathway for the de novo biosynthesis of cyercenes from propionic acid. This scheme, although plausible, needs further experimental evidence; the structures between square brackets, and accompanied by question marks, are likely, albeit hypothetical, intermediaries.

aimed at attempting to isolate some of the possible cyercene precursors accompanied by question marks in figure 5. However, the present study has confirmed the importance of marine gastropod molluscs as a source of fascinating biological observations, as well as of simple models for the study of some of the key issues of cell biology such as cell growth and differentiation.

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