disulfide groups in the hormone structure due to the PDI activity.

After reduction of TPOR with glutathione the reduced enzyme was separated by an excess of thiol to avoid a turnover reaction of the enzyme. In fact the stoichiometric ratio between reduced-TPOR and insulin allows the enzyme to split only one of the three disulfide bonds in the hormone before being transformed into the oxidized (inactive) form.

The reaction of partially reduced insulin with iodo (1-^{14}C)acetamide shows that both the polypeptide chains of the hormone are alkylated by the radioactive reagent (fig. 3) with a ratio of approximately 2/1 for the A/B chains. The chymotrypsin digestion of the separated chains led to two radioactive fragments in the A-chain and two labeled peptides in the B-chain, as shown in figure 4. A summary of these results is reported in table 1.

Further evidence that the TPOR used in this work does not act sequentially on the disulfide bonds of insulin was obtained from the analysis of chymotryptic peptides A-1, A-2, B-1 and B-2, as shown in table 2. These results are in agreement with experimental C-14 ratios previously reported (A-1/A-2 = 3/1 and B-1/B-2 = 1/1) as well as with the reports of various authors[16-18] on the action of chymotrypsin on A and B chains of insulin.

It is not yet clear, from these results or those of various authors[8,10-12,19], whether different preparations of TPOR consist of a single enzyme, sometimes containing traces of proteases, or if there are distinct enzymes with TPOR and PDI activities. However, the results reported in this paper, obtained with a preparation of TPOR deprived of PDI activity, lead to the conclusion that this enzyme reduces aspecifically the disulfide bonds of insulin.

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Observations on the toxicity and metabolic relationships of polygodial, the chemical defense of the nudibranch *Dendrodoris limbata*[1]

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Summary. Polygodial (**1**), the defense metabolite stored in the skin of the nudibranch *Dendrodoris limbata,* is toxic for the mollusc itself when injected into the hepatopancreas. Biosynthetic experiments using labeled mevalonic acid were devised to investigate a possible metabolic relationship between **1** and the mixture of sesquiterpenoidic esters **2**, stored in the hepatopancreas. The results suggest that **1** and **2** are biosynthesized by independent pathways.

Key words. Polygodial; chemical defense; fish antifeedant; nudibranchs; *Dendrodoris limbata;* biosynthesis; marine natural products.

The recognized ability of many dorid nudibranchs to defend themselves by using secondary metabolites toxic or repellent to predators[2] raises questions about a) how the animal acquires its chemical defense; b) where the animal stores the noxious chemicals and c) how the animal manages to avoid suffering from the effects of its own chemical weapons.

Recently accumulated evidence showed that the majority of the defense metabolites are of dietary origin[3-5] (sponges) and suggested[2] that these metabolites are stored in skin glands from which they are delivered in response to a predator's attack. Dorid nudibranchs which extract defensive allomones from the diet have conceivably developed adaptations which allow the harmful sponge metabolites to pass through their digestive tracts.

However, the discovery of the ability of the dorid nudibranch *Dendrodoris limbata* to synthesize its chemical defensive weapon[6], polygodial (**1**), followed by other reports[7,8] in which the synthetic capacity of other nudibranchs is described, provides a unique opportunity to follow the metabolic fate of the defense metabolites by radiolabeling methods.

Polygodial (**1**) is contained only in the skin extracts of *D. limbata,* while from the digestive gland (hepatopancreas) of the same animal the biogenetically related and biologically inactive sesquiterpenoid esters (**2**) have been isolated. Since **1** and **2** are sesquiterpenes based on a common drimane skeleton it seems probable either that **1** is biosynthesized from the biologically inactive mixture **2** and then stored in skin glands or, vice versa, that the mixture **2** could represent the result of a detoxification process of **1** for its excretion since **1** could be toxic for the animal itself in view of its high chemical reactivity[9,10]. A third possibility is that **1** and **2** are biosynthesized in different sites by two independent pathways.

We report here results of experiments devised to test the above hypotheses as well as evidence that **1** is toxic for *D. limbata* when injected into the hepatopancreas.

Materials and methods. Two sets of experiments were performed in March and April 1984, in which labeled mevalonic acid was given to specimens of *D. limbata;* 9 and 14 animals were used respectively. 0.5 μCi of [2-^{14}C]-mevalonic acid-dibenzylethylenediamine salt (Amersham; 31 mCi/mmole) were injected into the

Table 1. Incorporation of label from [2-^{14}C]-mevalonic acid into the metabolites of *D. limbata* at various times (March 1984)[a]

Time	No. of animals	Polygodial (1)		Esters (2)		Euryfuran (3)
		mg	dpm/mg	mg	dpm/mg	dpm/mg
3 h	3	2[b]	965	27	127	185
19 h	3	12	11,300	35	2,300	3,700
28 h	3	9	16,500	33	5,500	10,800

[a] A total of 4.5 μCi (9.9×10^6 dpm) of [2-^{14}C]-mevalonic acid-dibenzylethylenediamine salt was injected into 9 animals. Specimens were taken at various times as indicated in the table. [b] The low amount of polygodial found in the specimens taken 3 h after injection of the labeled precursor could be due to the handling of the animals and consequent discharge of polygodial through the skin.

Table 2. Incorporation of label from [2-^{14}C]-mevalonic acid into metabolites of *D. limbata* at various times (April 1984)[a]

Time	No. of animals	Polygodial (1)		Esters (2)		Euryfuran (3)
		mg	dpm/mg	mg	dpm/mg	dpm/mg
6 h	4	12	890	32	680	1,100
20 h	4	9	3,800	30	2,800	4,000
48 h	4	8	7,800	23	5,950	12,200
77 h	2	2[b]	14,500	12[b]	7,100	14,200

[a] Total of 7 μCi (15.4×10^6 dpm) of [2-^{14}C]-mevalonic acid-dibenzylethylenediamine salt was injected into 14 animals. Specimens were removed at various times as indicated in the table. [b] The animals were not fed during the incubation period so that, obviously, after a long time the amount of metabolites found is decreased.

hepatopancreas of each animal by means of a syringe. The animals were placed in aerated sea water (3 l) and specimens were withdrawn at selected times and killed by freezing. After dissection, the hepatopancreas and the mantles were separately extracted with acetone and the metabolites (**1** and **2**) isolated and purified to constant specific radioactivity as previously described[6]. Aliquots of the esters **2** from each experiment were converted[11] into euryfuran (**3**) in order to compare the specific radioactivity of polygodial with that due to the sesquiterpenoidic part of the esters **2**. Radioactivity was determined in a Tricarb liquid scintillation counter (Packard PRIAS, model PLD) equipped with an absolute radioactivity analyzer; the quenching was corrected by external standardization. The samples (0.5–2.0 mg) were dissolved in Insta-Fluor II (Packard) scintillation counting fluid.

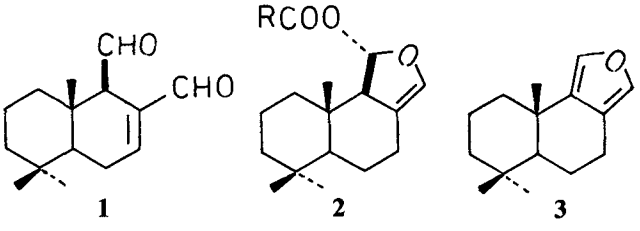
In the toxicity test, polygodial (**1**), dissolved in dimethylsulfoxide (2 mg per animal; 25 μl), was injected into the hepatopancreas of four specimens of *D. limbata*. The animals were placed in aerated sea water (3 l) and observed for 24 h. In a control experiment only dimethylsulfoxide (35 μl per animal) was injected into the hepatopancreas of three specimens and the animals were maintained in aerated sea water (3 l) for 24 h; during this period neither did death occur nor was suffering observed.

Results and discussion. Polygodial is toxic for *D. limbata* itself: when it was injected into four animals in an amount (2 mg) which is lower than the average value found in the skin of a single animal after extraction, suffering of the animals was evident and death occurred between 3 and 16 h. This finding implies that polygodial is probably synthesized and stored in specialized skin glands and, because of its toxicity, should be excreted through the skin or should be chemically modified (e.g. into **2**) to pass the excretory tract.

Two experiments were performed supplying labeled mevalonic acid in order to establish a precursor-product relationship between **1** and **2**. The results (tables 1 and 2) show that the radioactivity appears almost at the same time in both **1** and **2** and that there is a similar increase of the incorporated radioactivity over time in the two products. However, the specific radioactivity ratio between **1** and **2** is reversed passing from the March to the April experiment: in the first experiment (table 1) the radioactivity associated with the terpenoidic part of **2** (measured as **3**) is steadily lower than that associated with polygodial (**1**), while in the second experiment (table 2) this figure is reversed.

This finding, probably reflecting the difference in the metabolic rates due to the seasonal development of the animals, precludes that between **1** and **2** a precursor-product relationship exists.

It is expected in fact, as a general feature, that the specific



1 **2** **3**

radioactivity of a precursor will be higher than that found in its metabolic products or at least, when the radioactivity at different times is measured, that the label will appear first in the precursor and then in its metabolites: from this point of view the March experiments preclude that the esters **2** could be precursors of polygodial (**1**), while the April experiements exclude that **1** can be transformed into **2**. Therefore all these data, taken together, suggest that **1** and **2** are synthesized by independent pathways.

It can be suggested that the inverted ratio of the radioactivity found in the two products according to the date could reflect a difference in their metabolism, related to the developmental stage of the animal and its need to have a more or less significant amount of chemical defense.

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