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Dorid Nudibranch Elaborates Its Own Chemical Defense

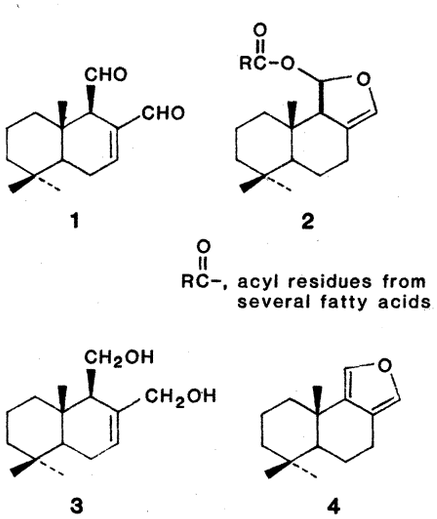
Abstract. *A biosynthetic experiment with mevalonic acid labeled with carbon-14 showed that the nudibranch* Dendrodoris limbata *elaborates polygodial, a sesquiterpenoid dialdehyde stored in the mantle, which constitutes its chemical defense against predators. Previously described nudibranchs drew defensive chemicals from their preys.*

Among the different defensive mechanisms (*1*) employed by nudibranchs to escape predators, chemical defense has received attention only recently. Several of these shell-less mollusks secrete a mucus that decreases the animal's likelihood of being eaten (*2*), since the mucus may contain toxic (*3*) or unpleasant compounds (*4, 5*). Most of the nudibranchs studied from a chemical point of view belong to the suborder Doridacea; these invertebrates feed mainly on sponges (*6*), which are said to be repellent to most other animals.

The metabolites found in dorid nudibranchs have been traced in some cases to a particular sponge (*3, 4, 7–10*), suggesting that these mollusks concentrate metabolites from their sponge diet in skin glands and employ these metabolites as a defensive secretion (*10*).

When such a predator-prey relation was not determined, it was suggested that the metabolites found in the nudibranchs arose by predation on unidentified sponge species (*7, 11*).

We reported earlier (*12*) the presence of polygodial (structure **1**) in the skin extracts of *Dendrodoris limbata* (phylum Mollusca, class Gastropoda, subclass Opisthobranchia, order Nudibranchia, suborder Doridacea). This compound showed antifeedant activity against marine and freshwater fish. A mixture of sesquiterpenoid esters (structure **2**), which were inactive in the feeding inhibition bioassay (*12*), was isolated (*13*) from the digestive gland of the same nudibranch.



We were unable to find either compound **1** or **2** in several sponges collected in the area in which *D. limbata* was located, and we therefore investigated the possibility that these metabolites are synthesized in the nudibranch. Since both compounds **1** and **2** have a sesquiterpenoid skeleton, mevalonic acid was selected as the most suitable precursor for the biosynthetic experiments in vivo. Seven specimens of *D. limbata* were placed in aerated seawater (3 liters), and 2 μCi of [2-^{14}C]mevalonic acid–dibenzylethylenediamine salt (Amersham; 31 mCi/mmole) in distilled water solution (0.01 ml) was injected into the hepatopancreas of each animal by means of a syringe.

After 24 hours, the animals were killed by freezing and were carefully dissected. The hepatopancreas and the mantles were separately extracted with acetone. The solvent was removed at reduced pressure from the acetone extracts, and the residues were partitioned between diethyl ether and water. The resulting ether layers were evaporated and then subjected to chromatography on two distinct silica gel columns with petrol and increasing amounts of diethyl ether (*12, 13*).

Polygodial (compound **1**; 15 mg) was recovered from the mantle extract. This compound was pure, as judged by thin-layer chromatography; to ascertain whether the radioactivity found (Table 1) was due to polygodial itself or to accompanying impurities, compound **1** was further purified by preparative silica gel thin-layer chromatography (Merck; C_6H_6–diethyl ether, 8:2) to yield 9 mg of material that was subsequently reduced with $NaBH_4$ in methanol to the dialcohol (structure **3**) and purified again by preparative thin-layer chromatography (petrol–diethyl ether, 3:7), with 5 mg recovered.

The radioactive counts in the samples were determined (*14*) after each purification step (Table 1); the specific radioactivity in disintegrations per minute per milligram remained practically constant. The results indicate good incorporation (1.6 percent) of mevalonic acid into polygodial. The esters (**2**) obtained from the column chromatography were contaminated by colored material; they were therefore purified by preparative thin-layer chromatography (petrol–diethyl ether, 95:5), and the radioactive counts were determined.

To establish whether the radioactivity found was associated, as expected, with the terpenoid part of the molecule, we converted mixture **2**, by heating in the presence of a catalytic amount of silica gel (*13*), into the furan (structure **4**) and into the corresponding mixture of free fatty acids. The furan was purified by preparative thin-layer chromatography (petrol). The mixture of the fatty acids was purified with the same system, but with a different eluent (petrol–diethyl ether, 1:1). The esters, **2**, were also biosynthesized de novo by the nudibranch. The labeled mevalonic acid was specifically incorporated into the terpenoid part of the molecule, the radioactivity, after conversion, being almost completely associated with the furan (*15*).

These results show that the nudi-

Table 1. Radioactivity found in the metabolites of *Dendrodoris limbata* and in their derivatives after each purification step. A total of 14 μCi (30.8×10^6 dpm) of [2-^{14}C] mevalonic acid–dibenzylethylenediamine salt was injected into seven specimens.

Compound	Amount (mg)	Radioactivity (dpm/mg)
1 (first purification)	15	32,400
1 (second purification)	9	28,000
3 (from reduction of **1**)	5	32,500
2 (second purification)	53	6,500
4 (from thermolysis of **2**)	10	13,100
Fatty acids (from thermolysis of **2**)	15	330

branch *D. limbata* biosynthesizes polygodial (**1**), which constitutes its chemical defense and is stored in the mantle. Thus the ability of a nudibranch to elaborate its chemical defense has been demonstrated. *Dendrodoris limbata* also synthesizes the mixture of sesquiterpenoid esters (**2**) found in the digestive glands. We believe that these esters should be regarded as products of further metabolism of polygodial as a result of a detoxication process. Polygodial is a reactive molecule that readily interacts with NH_2 groups (*16*) and therefore could be toxic for *D. limbata* itself if stored for a long time. When the animal is molested, the compound is secreted through the skin. The esters (**2**), which are found into the hepatopancreas only, could represent the normal excretory metabolites of polygodial.

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need an effective, nontoxic drug that will kill the parasite and some form of therapy to suppress the major autoimmune mechanisms that produce the lesions of Chagas' disease.

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Rheumatoid Factors and Chagas' Disease

Clarkson and Mellow (*1*) report that they found antibodies with rheumatoid factor properties in the serum of lactating rats. They point out that such immunoglobulin "accounts for the unusual resistance of previously uninfected lactating rats and their suckling pups to infection with *T. lewisi*," and that "a similar rheumatoid factor . . . which is induced late in the usual course of infection with *T. lewisi* in nonlactating rats, amplifies an earlier IgG response and terminates the infection." They then draw a parallel between *Trypanosoma lewisi* and *T. cruzi* infections and suggest that "it might be possible to treat Chagas' disease by temporarily inducing the production of rheumatoid factor." Although the results of their experiments are interesting, I do not agree with certain aspects of their conclusions and I offer the following comments:

In early work with my colleagues (*2*) it was shown that humans infected with *T. cruzi* (Chagas' disease) produce rheumatoid factor-like antibodies. Such factors are highly reactive with heterologous γ-globulin (*3*) and are demonstrable with the Waaler-Rose reaction (*4*); they are composed of immunoglobulin M (IgM) and are less responsive to human γ-globulin (*3*) prepared for the Singer and Plotz test (*5*). High titers of these rheumatoid factors were found in the serum of 95 percent of patients (infants, children, and adults) from the time of clinical onset (acute phase) of the disease to about 1 year (*3*). However, these factors were also found, intermittently, in 25 percent of patients with chronic Chagas' disease (*3*). Such factors were found in patients with or without evident clinical lesions. These findings suggest that there is no correlation between the presence of rheumatoid factor and the course of infection in Chagas' disease, and that IgM therefore plays a marginal role in modulating *T. cruzi* infections.

Although it is possible that the rheumatoid factors produced during Chagas' disease are a natural protective response of the host to *T. cruzi*, it is also possible that these factors, because of their reactivity with γ-globulin, are autoantibodies. There is evidence that several tissue lesions in Chagas' disease result from humoral and cellular autoimmune reactions (*3*, *6*).

It seems unlikely, therefore, that the rheumatoid factors will be useful in the treatment of Chagas' disease. For adequate treatment of this disease we still

Cabral (*1*) raises doubt about the possible extension of our findings (*2*) with *Trypanosoma lewisi* in rats to *T. cruzi* in humans. Although we considered this possibility only as a hypothesis to guide future research, there is other evidence that implicates a positive role for rheumatoid factors (RF's) in *Trypanosome* infections as well as in autoimmune disease.

Cabral notes that RF appears in the serum of most patients with Chagas' disease, generally before the development of a complement fixation titer (CFT), and states that RF is not correlated with protection. However, a negative CFT does not imply absence of parasite-specific immunoglobulin G (IgG). Also rheumatoid factors have a broad range of potential epitope specificities; an immunoglobulin M (IgM) that is specific for one epitope on a particular IgG subclass may be protective, whereas another RF, although generally reactive with bound or aggregated IgG, may not be protective. An analogy can be made to the overall immunoglobulin response to *T. cruzi* and many other infectious agents. With rare exceptions, all infected animals show an increase in serum immunoglobulins whether they resist or succumb to the disease. If one did not know that the specificity of these immu-