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Can molluscs biosynthesize typical sponge metabolites? The case of the nudibranch *Doriopsilla areolata*

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Abstract—The first study on the biosynthesis of terpenoid metabolites in the porostome nudibranch *Doriopsilla areolata* is described herein. The de novo origin of two different groups of bi- and tri-cyclic sesquiterpenes, exhibiting opposite A/B ring junctions, has been clearly demonstrated by in vivo incorporation of labeled mevalonate. Surprisingly, metabolites of both series have been previously reported to co-occur in a sponge of genus *Dysidea*, that could be included in the diet of the nudibranch. © 2001 Published by Elsevier Science Ltd.

The discovery of terpenoids with opposite absolute stereochemistry in the same organism is quite unusual. However, a series of tricyclic sesquiterpenoids with opposite A/B ring junctions was first found in the corneous sponge *Dysidea*[1] and more recently in Pacific and Mediterranean specimens of the nudibranch *Doriopsilla areolata*.[2,3] The structural analogies between the two series of metabolites is striking. Both organisms contain (+)-euryfuran (**1**) and (−)-pallescensin-A (**2**) and structurally related metabolites. This surprising analogy prompted our interest in investigating the origin of the sesquiterpenes present in the nudibranch. *D. areolata* is a radula-less nudibranch of the family Dendrodoridae, characterized by species belonging to the two genera *Dendrodoris* and *Doriopsilla*, that are known to be eaters of corneous sponges. On this basis a dietary habit of *D. areolata* upon *Dysidea* sponges could be strongly suspected. But *Dendrodoris* species are also known to biosynthesize drimane sesquiterpenes de novo. The extent of such biosynthetic capacity is of particular interest because of questions as to how it has evolved.

Drimane sesquiterpenes are present in both genera.[4,5] First, there is a mixture of drimane esters (**3**). These have been found in the animals' internal organs, including the gonads,[2,6–9] suggesting that they somehow protect the developing eggs.[10] Secondly, there are structurally related compounds, such as polygodial (**4**) and its masked form, olepupuane (**5**). The fact that these are located in the skin, taken together with feeding experiments, makes it clear that these are defensive allomones.[7–10] Thirdly, a series of furanosesquiterpenes (**2**, **6** and **7**), exhibiting the *ent*-pallescensin skeleton, coexists with drimane metabolites in both Mediterranean and Atlantic *D. areolata*.[2,3] *ent*-Pallescensin-A (**2**) was isolated from the gonad, whereas the corresponding derivatives 15-acetoxy-*ent*-pallescensin-A (**6**) and 2,15-diacetoxy-*ent*-pallescensin-A (**7**) were found to be concentrated along the border of the mantle.[2,3] Drimane metabolites were absent in the mantle of the Mediterranean population,[2] while compounds **6** and **7** co-occurred with isodrimeninol (**8**) in the skin and with polygodial (**4**) in the mucus of the Atlantic population.[3] The biosynthesis de novo of drimane compounds, rigorously demonstrated for some *Dendrodoris* nudibranchs,[11–13] should also be active in *D. areolata*, whereas the origin of sesquiterpenes in *D. areolata* through bio-accumulation or by biosynthesis de novo is matter for discussion.

In this paper we report the study of the biosynthesis of the sesquiterpenes in Atlantic *D. areolata* by in vivo incorporation experiments with ^{14}C-labeled mevalonate.

D. areolata was collected in Outão, Setúbal, (Portugal) at a depth of 5–15 m, during April 2000.[3] Living animals (50 individuals, average size 2.5 cm) were transferred to the laboratory (ICMIB) and placed in aquaria. Nine specimens were each injected with 2.5 μCi DL-[2-^{14}C]-mevalonic acid dibenzylethylenediamine (DBED) salt dissolved in 100 μl of distilled water and kept in separated aerated seawater for

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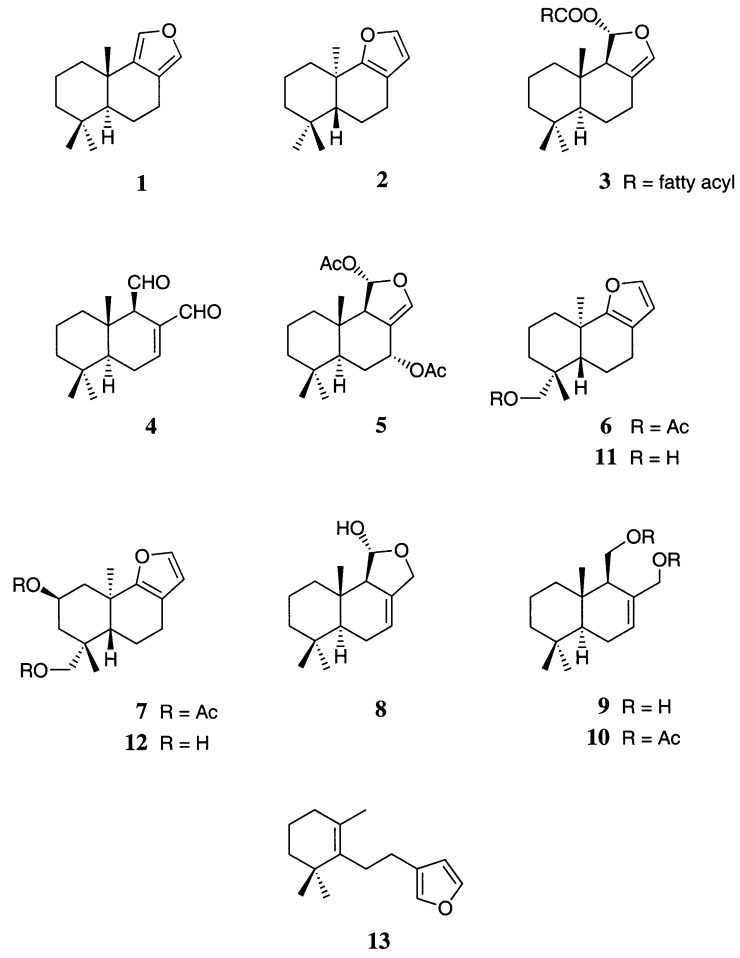

1 **2** **3** R = fatty acyl

4 **5** **6** R = Ac
11 R = H

7 R = Ac
12 R = H **8** **9** R = H
10 R = Ac

13

7 days. Then the molluscs were killed by freezing at −20°C. The remaining 41 individuals were also frozen and chemically analyzed.[3]

Labeled specimens were carefully dissected so as to separate mantle tissues from internal organs, and then separately extracted with acetone. The ether soluble fractions of both acetone extracts were analyzed by TLC chromatography. In agreement with previous results,[3] the internal part extract (256 mg, 9,100 cpm/mg) contained, in addition to sterols and fatty acids, euryfuran (**1**), *ent*-pallescensin-A (**2**) and the drimane ester mixture (**3**), identified by comparison with standard samples, whereas the skin extract (107 mg, 22,700 cpm/mg) was characterized by the presence of 15-acetoxy-*ent*-pallescensin-A (**6**) and 2,15-diacetoxy-*ent*-pallescensin-A (**7**). Polygodial (**4**), a component of the mucous secretion,[3] was also found in this skin extract, probably because the mucus secreted by the animals while they were being manipulated was not separated from the mantle tissue. Isodrimeninol (**8**), a masked form of **4**, previously isolated in very low levels from the mantle of *D. areolata*,[3] was not detected.

Following the chromatographic procedure already described,[3] both ether extracts were fractionated by Si-gel columns, using a light petroleum ether/diethyl ether gradient: quite pure **4**, **6** and **7** were isolated from the skin extract, whereas the esters **3**, along with an unresolved mixture of compounds **1** and **2**, were obtained from the extract of internal parts. All fractions recovered from both chromatographic columns were counted. Significant radioactivity was found only associated with sterols and with fractions containing sesquiterpenoid compounds: **1**+**2** (3.5 mg, 33,700 cpm/mg), **3** (81.0 mg, 17,500 cpm/mg) and sterol fraction (18 mg, 2,700 cpm/mg) from the digestive gland; **4** (3.2 mg, 34,200 cpm/mg), **6** (3.3 mg, 65,700 cpm/mg), **7** (3.6 mg, 47,600 cpm/mg) and sterol fraction (28.5 mg, 39,000 cpm/mg) from the skin. All these labeled fractions, except that containing compounds **1** and **2**, were submitted to further purification steps, as reported in Section 1, and the radioactivity was measured after each purification step. Fig. 1 shows the specific radioactivity (expressed in cpm/mg) for all compounds isolated after the last purification step. In agreement with the previous results on *Dendrodoris*,[11,13] drimane metabolites (the mixture **3** from internal organs and **4** from the mantle) showed a good incorporation of labeled mevalonate, as illustrated in Fig. 1. In particular, the specific incorporation in the drimane skeleton of **3** was demonstrated by converting **3** into euryfuran (**1**), which was labeled (15,000 cpm/mg) after TLC purification, whereas no radioactivity was found associated with the fatty acid mixture obtained by methanolysis of **3**. A good level of incorporation was observed in polygodial (**4**), as it was secured by transforming **4** into diol **9**, which was subsequently acetylated and re-purified giving labeled **10** (32,600 cpm/mg). However, higher incorporation was obtained in the two derivatives of *ent*-pallescensin-A, compounds **6** and **7**. The significant

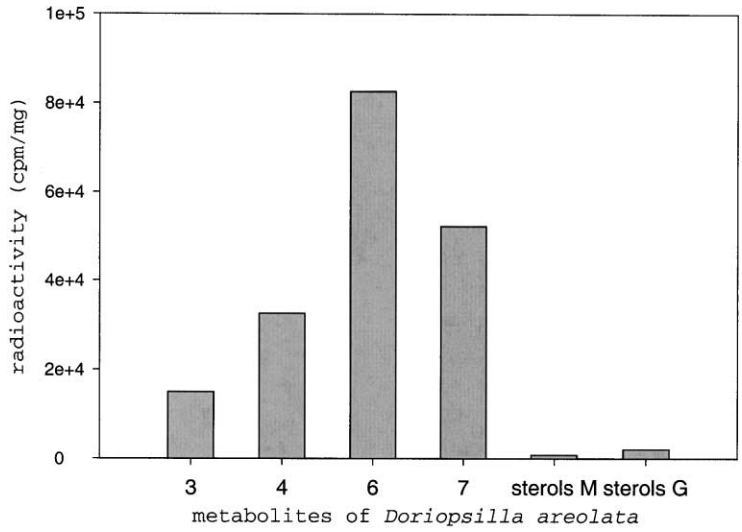

Figure 1. Incorporation of labeled mevalonate in sesquiterpenes and sterols (M=mantle, G=gland) in *D. areolata*.

level of radioactivity associated to the carbon skeleton of *ent*-pallescensin-A was confirmed by converting **6** and **7** into the corresponding alcohols **11** and **12**, which were purified and then submitted to acetylation to give starting compounds **6** (82,700 cpm/mg) and **7** (52,300 cpm/mg). The incorporation of labeled mevalonate into *ent*-pallescensin-A (**2**) was not precisely evaluated due to the difficulty of separating the mixture of the two non-polar compounds **1** and **2**, eluted together from the column of the ether extract of internal organs. Sterols from both internal and external parts were found to be clearly labeled, but the specific incorporation was very low compared to that observed for sesquiterpenes.

These results prove that the sesquiterpenes of *D. areolata* are biosynthesized de novo. They also clearly demonstrate that, by a mevalonate pathway, the animal is able to synthesize de novo two different series of sesquiterpenes, exhibiting opposite carbon skeletons. Such co-occurrence of compounds with opposite skeletons is very unusual, but as mentioned above, both **1** and **2** have been reported from a sponge of the genus *Dysidea*, that could be included in the diet of the mollusc. According to the authors' suggestion, a single 'cyclizing enzyme' could lead to both skeletons starting from the same monocyclic precursor **13**.[1] However, the mollusc is also able to biotransform **2** into two other metabolites, the acetyl derivatives **6** and **7**. Such biotransformations could be suggested as the starting point for the 'retrosynthetic' evolution of de novo synthesis in nudibranchs.[14] Once the enzyme that effects the final biotransformation has arisen, supposedly through gene duplication, earlier steps in the biosynthetic pathway can be added by further gene duplication. Likewise the furanosesquiterpenoids include other metabolites located in the skin. Polygodial (**4**) is thought to be produced there, supposedly through enzymatic action, from the less toxic olepupuane (**5**) when the nudibranch comes under attack. A retrosynthetic evolution of the series of enzymes necessary for the biosynthesis de novo is a possibility for these metabolites as well.

Enzyme specificity is not absolute, and it is likely that biotransformation of one kind of metabolite has been the starting point for biotransformation of something similar. It would be interesting to find out whether the group of metabolites shares a family of enzymes with a common ancestry. The two series of terpenoids are structurally distinct and record different labeling levels (Fig. 1), suggesting the presence of two independent pathways starting from mevalonate. Although that is a distinct possibility, they may arise from a common intermediate derived from mevalonate (e.g. **13**), as already suggested for the sponge *Dysidea*.[1] If so, then the number of evolutionary innovations needed to account for the animals' biosynthetic capacity is less than one might suppose.

1. Experimental

1.1. General procedures

Radioactivity was counted by a Packard 1600 TR liquid scintillation analyzer, using Wallac Optiphase scintillation counting fluid (Packard). DL-[2-^{14}C] mevalonic acid DBED salt (58 mCi/mmol) was purchased from NEN® Life Science. Si-gel chromatography was performed by using precoated Merck F_{254} plates and Merck Kieselgel 60 powder.

1.2. Biological material

D. areolata (average size 2.5 cm) was collected in Outão, Setúbal, Portugal (38°29.6′N 8°55.7′W) between 5 and 15 m depth during April 2000 and identified by G. Calado. Fifty individuals were transferred living to Naples and placed in aquaria. Nine specimens were used for incorporation experiments, while the remaining individuals were frozen and then extracted and analyzed as already reported.[3]

1.3. Incorporation experiments

Each of nine *D. areolata* specimens was injected with 2.5 μCi DL-[2-^{14}C]-mevalonic acid dibenzylethylenediamine (DBED) salt dissolved in 100 μl of distilled

water. The animals were kept in separated aerated seawater for 7 days, then the mollusks were killed by freezing at −20°C.

1.4. Isolation and purification of labeled metabolites

Extraction of frozen labeled *D. areolata* individuals was carried out according to previous reported procedures for unlabeled material.[3] The ether soluble portion (256 mg, 9,100 cpm/mg) of the acetone extract of internal organs was fractionated by Si-gel column, using light petroleum ether/diethyl ether gradient, giving, in order of increasing polarity, an unresolved mixture of **1** and **2** (3.5 mg, 33,700 cpm/mg), esters **3** (81.0 mg, 17,500 cpm/mg) and sterol fraction (18.0 mg, 2,700 cpm/mg). The ether soluble portion (107 mg, 22,700 cpm/mg) of the acetone extract of the skin was fractionated by Si-gel column, using light petroleum ether/diethyl ether gradient, giving, in order of increasing polarity, fractions containing crude compounds **6** (3.3 mg, 65,700 cpm/mg), **7** (3.6 mg, 47,600 cpm/mg), and **4** (3.2 mg, 34,200) and sterol fraction (28.5 mg, 39,000 cpm/mg). Labeled fractions obtained by the first fractionation step of both mantle and internal organs extracts were further purified as follows. Where not indicated, Si-gel column chromatographic separations were achieved by using light petroleum ether/diethyl ether gradient as eluent. Labeled molecules were identified by TLC comparison with unlabeled standard compounds.

An aliquot (10.8 mg) of the fraction containing the crude mixture **3** (81.0 mg, 17,500 cpm/mg) was dissolved in light petroleum ether and heated with Si-gel. The product obtained was eluted by light petroleum ether and purified by Si-gel column to give pure **1** (1.0 mg, 15,000 cpm/mg). Another aliquot (11.9 mg) of the fraction containing crude **3** was submitted to methanolysis (anh. MeOH, anh. Na_2CO_3, 6 h). The reaction product was fractionated to give a mixture of unlabeled fatty acid methyl esters (7.0 mg).

Crude polygodial (**4**) (3.2 mg, 34,200 cpm/mg) was reduced by $NaBH_4$/MeOH. After usual work-up, the reduction product was chromatographed to give diol **9** (1.1 mg, 33,000 cpm/mg) which was acetylated by Ac_2O/Py and re-purified to afford the acetyl derivative **10** (1.3 mg, 32,600 cpm/mg).

Both crude *ent*-pallescensin-A derivatives, **6** (3.3 mg, 65,700 cpm/mg) and **7** (3.6 mg, 47,600 mg), were submitted to methanolysis (anh. MeOH, anh. Na_2CO_3). Crude reaction products were fractionated by column obtaining **11** (1.6 mg, 80,500 cpm/mg) and **12** (2.4 mg, 51,700 cpm/mg), respectively. Compounds **11** and **12** were acetylated by Ac_2O/Py and subsequently purified to afford pure **6** (1.4 mg, 82,700 cpm/mg) and **7** (2.1 mg, 52,300 cpm/mg), respectively.

Aliquots of sterol-containing fractions from both extracts were first purified by Si-gel column chromatography by eluting with chloroform, to obtain 4 mg (9,900 cpm/mg) from the skin and 15 mg (1,700 cpm/mg) from the digestive gland of quite pure sterol mixtures. Both fractions were acetylated by Ac_2O/Py and subsequently purified by Si-gel column to afford pure acetyl sterols, 1.5 mg (800 cpm/mg) from the skin and 12.3 mg (2,100 cpm/mg) from internal organs.

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