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Terpene Biosynthesis in the Nudibranch *Doriopsilla areolata*

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Received July 2, 2002

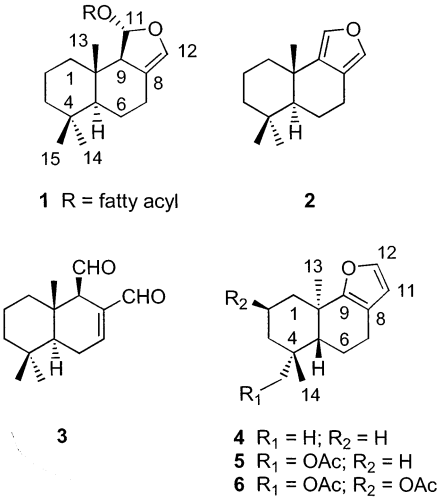
Biogenesis of the enantiomeric sesquiterpenes **1** and **5** of the marine nudibranch *Doriopsilla areolata* was investigated by feeding of [1-^{13}C]glucose, [1,2-$^{13}C_2$]glucose, and [1,2-$^{13}C_2$]acetate. Evidence is presented that supports de novo origin of both compounds via mevalonic acid.

Introduction

In the course of evolution nudibranchs, a group of shell-less sea slugs, have developed a complex strategy of protection[1] that includes the use of a variety of secondary metabolites which either deter feeding of or kill harmful organisms.[2] By analogy with insect—plant interactions, many of these compounds have structures consistent with a dietary origin, including a large number of sponge and algal metabolites.[2] A few other metabolites, however, have more uncertain sources, and for them biosynthesis in the molluscs has been postulated.[2] This last ability is regarded as an evolutionary step beyond the simple accumulation of dietary metabolites, since the production of noxious substances generally benefits the producer organism that becomes independent of specific food availability.[3]

The European nudibranch *Doriopsilla areolata*, Bergh 1980, is characterized by an array of defensive sesquiterpenoids showing two enantiomeric skeletons related to drimane (**1**—**3**) and *ent*-pallescensin A (**4**—**6**).[4] The co-occurrence of enantiomeric structures in *D. areolata* is unique within opisthobranch molluscs, but molecules based on drimane and the *ent*-pallescensin skeleton have been reported from sponges of the genus *Dysidea*.[5] The co-occurrence of similar compounds in different phyla is rather unusual, and the case of *D. areolata* is even more singular since the nudibranch and the sponge may represent a prey—predator pair.

In this paper we describe the results of isotope incorporation in *D. areolata*. Three distinct groups of molluscs



were treated every second day for 2 weeks with [1-^{13}C]-glucose, [1,2-$^{13}C_2$]glucose, or [1,2-$^{13}C_2$]acetate. The objectives of the biosynthetic studies were (i) to clarify the biochemical pathways leading to **1**—**6**, (ii) to investigate the biochemical relationships between drimane compounds and pallescensin derivatives, and (iii) to suggest a sound explanation for the co-occurrence of similar compounds, such as **2** and **4**, in taxonomically distinct organisms. Furthermore, the use of [1-^{13}C]-labeled glucose allowed confirmation that the carbon skeletons of **1**—**6** arise by the classical mevalonic pathway, turning down other biogenetic routes.[6]

Results and Discussion

D. areolata (39 specimens) was collected by hand by scuba diving at depths of 10—15 m along the northwest coasts of Spain in May 2001. In agreement with previous studies,[4] nudibranch extracts showed a tissue-dependent distribution of the secondary metabolites: euryfuran (**2**) and (—)-*ent*-pallescensin A (**4**) were found in mantle and viscera, drimane esters (**1**) were found specifically in the

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10.1021/jo026131v CCC: $25.00
Published on Web 02/19/2003

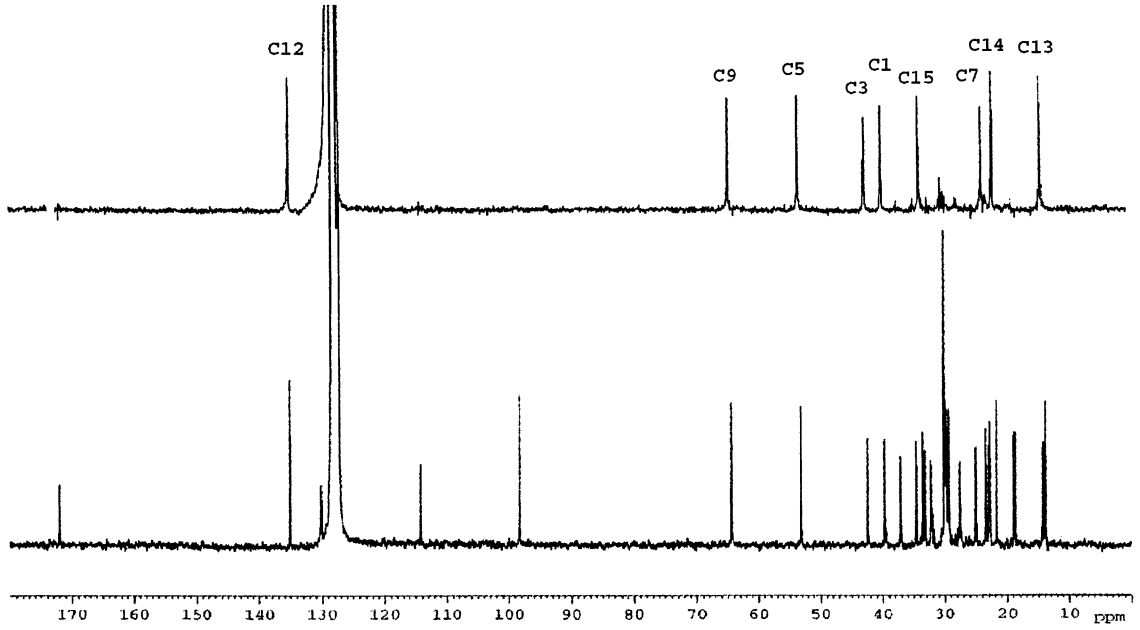
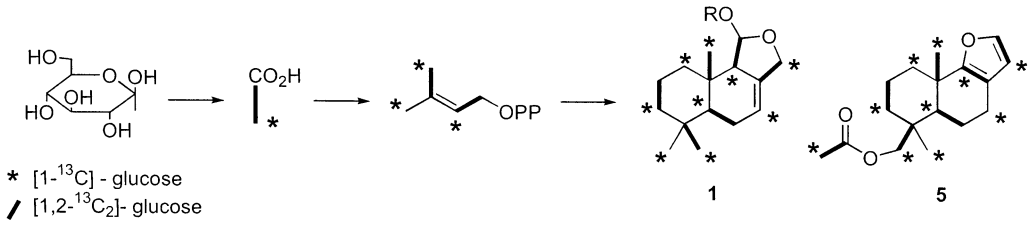


FIGURE 1. ^{13}C NMR (bottom) and ^{13}C NMR difference (top) spectra of drimane esters (**1**). The difference spectrum was obtained by subtracting the spectrum of the natural sample from that of the ^{13}C-enriched one.

SCHEME 1



hermaphrodite gland, and acetoxy-*ent*-pallescensins A (**5** and **6**) and polygodial (**3**) were detected only in the skin of the molluscs. However, the ecological role, tissue distribution, and biochemical relationships of *Doriopsilla* sesquiterpenoids have already been outlined in previous studies.[4]

Injection of [1-^{13}C]glucose in *D. areolata* led to an enrichment pattern of **1** and **5** in total agreement with an origin by the acetate/mevalonate pathway (Scheme 1).[6] ^{13}C signal enhancements were very clear by comparing the peak height of corresponding signals (normalized to C4 for **1** and C8 for **5**) in the spectra of isotopically labeled and natural abundance compounds (Table 1). The spectrum of labeled **1** showed enrichment of C1, C3, C5, C7, C9, C12, C13, C14, and C15 in agreement with the predicted labeling pattern (Figure 1). A similar enrichment model was also observed in the spectra of 15-acetoxy-*ent*-pallescensin A (**5**) (Figure 2). Interestingly, the acyl residues linked to the terpenoid part recorded different labeling patterns. While the acetate unit at C15 of **5** was as efficiently labeled as the terpene carbons, no incorporation was observed in the acetate-derived chains of the fatty acids of **1**. These results implied that exogenous precursor was used for the synthesis of both terpenes and the acetyl residue, whereas the acyl substituents of **1** were likely derived from a preexisting reserve of fatty acids.

To gain more insight into the labeling patterns, two feeding experiments with [1,2-$^{13}C_2$]glucose and [1,2-$^{13}C_2$]-acetate were next carried out.[8] Figure 3 shows ^{13}C NMR resonances of drimane esters (**1**) obtained by injection of

TABLE 1. ^{13}C NMR Data of 1 and 5 Isolated from *D. areolata* Fed with [1-^{13}C]Glucose, [1,2-^{13}C]Glucose, and [1,2-^{13}C]Acetate

	compound **1**			compound **5**		
no.	δ	increase[a]	flanking signals[b] (Hz)	δ	increase[a]	flanking signals[b] (Hz)
1	39.7	3.05		35.8	2.11	
2	18.9	0.96	33.2	18.5	1.08	32.3
3	42.4	2.77	33.2	36.7	1.76	32.3
4	33.2	1.00	35.3	36.9	1.09	nd
5	53.2	2.72	34.4	52.8	1.92	34.4
6	22.9	1.06	34.4	20.1	1.12	34.4
7	23.5	2.81	–	23.3	1.88	–
8	114.3	0.82	80.1	114.0	1.00	br m
9	64.5	2.66	41.8	159.3	1.97	br m
10	37.2	0.88	36.5	36.9	1.09	nd
11	98.3	1.05	41.8	110.3	1.86	70.7
12	135.2	2.70	80.1	140.5	1.13	70.7
13	14.0	2.87	36.5	21.7	1.87	34.4
14	21.8	2.90	35.3	27.6	1.80	–
15	33.6	3.06	–	66.8	2.08	38.4
CO	171.1	0.73	–	170.2	–	61.1
$(CH_2)_n$	30.3	1.01	–	–	–	–
CH3	14.3	1.09	–	20.4	2.33	61.1

[a] From feeding experiments with [1-^{13}C]glucose. The results obtained by the comparison of peak heights were further supported by a similar analysis based on the manual integration of peak areas. Spectra were normalized to C4 for **1** and C8 for **5**. [b] From feeding experiments with [1,2-$^{13}C_2$]glucose and [1,2-$^{13}C_2$]acetate. nd = not detectable due to signal overlapping; br m = broad multiplet.

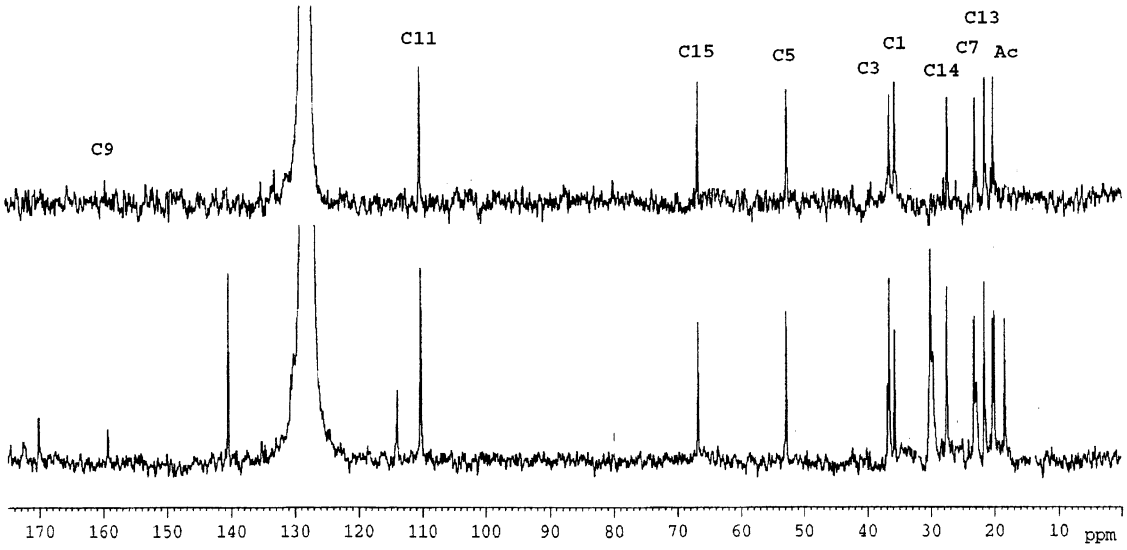


FIGURE 2. ^{13}C NMR (bottom) and ^{13}C NMR difference (top) spectra of 15-acetoxy-*ent*-pallescensin (**5**). The difference spectrum was obtained by subtracting the spectrum of the natural sample from that of the ^{13}C-enriched one.

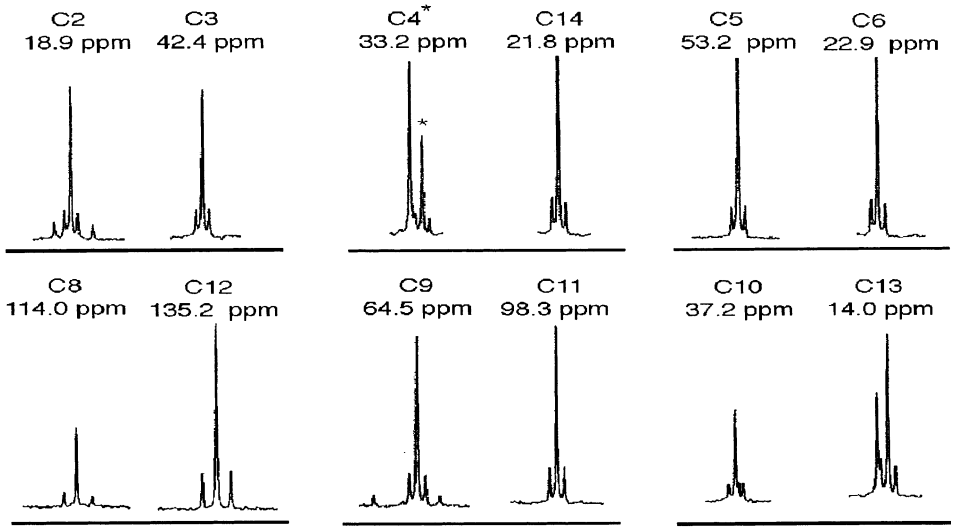


FIGURE 3. Selected ^{13}C NMR signals of **1** isolated from the feeding experiment with [1,2-$^{13}C_2$]glucose.

doubly labeled glucose. Since intact acetate units preserve their $^{13}C-^{13}C$ coupling, the ^{13}C NMR spectra manifest incorporation through the presence of doublets flanking the natural isotopomer signals. The presence of two or more intact units in a single molecule is not statistically favored, and in general, a simple analysis of the coupling constants allows one to detect the couples of biogenetically related carbons. On this basis, the ^{13}C NMR spectrum of **1** revealed incorporation of intact acetate into C2−C3, C4−C14, C5−C6, C8−C12, C9−C11, and C10−C13 (Figure 3). The coupling constant analysis (Table 1) was in total agreement with the biogenesis of the drimane skeleton predicted by cyclization of farnesyl diphosphate (Scheme 2).

Similar results were recorded by feeding [1,2-$^{13}C_2$]-acetate, although the incorporation was significantly higher (1.65%) than that with glucose (0.35%). Feeding of doubly labeled acetate, furthermore, caused the appearance of unexpected doublets flanking C1, C7, and C15 (Supporting Information). The origin of these spurious signals in biosynthetic experiments with marine molluscs has already been interpreted by Andersen and co-workers as the result of the incorporation of more than one acetate unit into a single mevalonic acid molecule.[8] The ^{13}C NMR spectrum of **1** obtained from experiments with [1,2-$^{13}C_2$]acetate also showed an apparent lowering of the spectral resolution. The phenomenon is quite common in nonspecific ^{13}C enrichment, as can occur in biosynthetic experiments, and it is due to long-range couplings ($J \approx 7-10$ Hz) that appear within and between multilabeled isoprene units.[9] As a consequence, the

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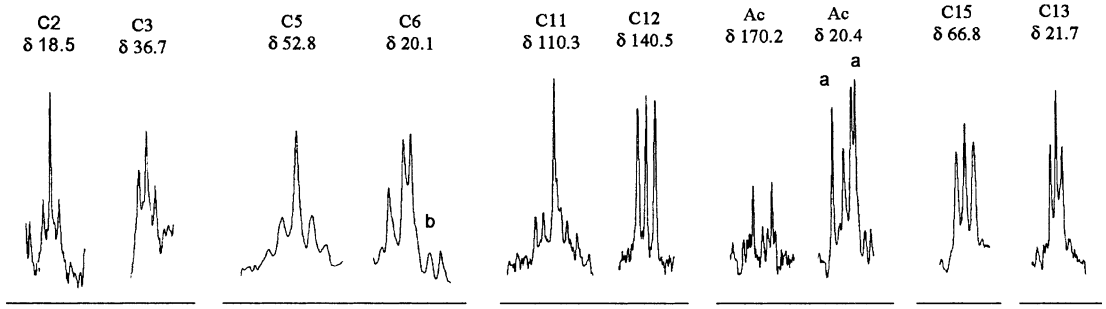
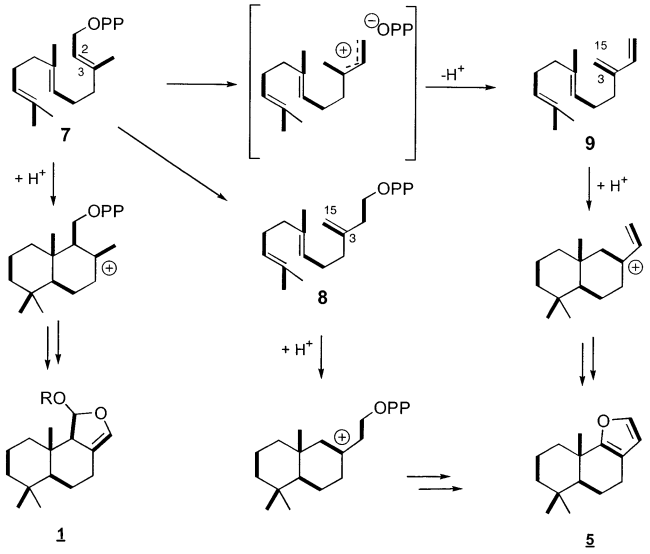


FIGURE 4. Selected ^{13}C NMR signals of **5** isolated from the feeding experiment with [1,2-$^{13}C_2$]acetate. The peaks marked with an "a" are doublets partially overlapped to other signals, and the peaks marked with an asterisk are coupled signals.

SCHEME 2. Biogenetic Proposal for the Formation of 1 and 5 from Farnesyl Diphosphate (7)



flanking signals are affected by strong line-broadening that prevents quantitative measurement of the $^{13}C-^{13}C$ correlations. Once more, fatty acid chains were not labeled with either precursor.

Spectra of 15-acetoxy-*ent*-pallescensin A (**5**) obtained from feeding experiments with [1,2-$^{13}C_2$]acetate or [1,2-$^{13}C_2$]glucose showed severe signal overlapping (Figure 4), thus making it impossible to use coupling constants to establish the complete set of data. However, clear correlations were obtained for C2−C3, C5−C6, and C11−C12 and for the acetyl group.

The presence of an intact acetate at C11−C12 was of particular interest since it suggests the transformation of the *trans*,*trans*-farnesol skeleton, as indicated in the biogenetic hypothesis described in Scheme 2. A number of other resonances showed relatively weak doublets or more complex multiplets that, however, supported the incorporation of doubly labeled acetate (Table 1). Signal overlapping of C4 and C10 did not allow us to determine clearly the couplings of the methyl appendages (C13, C14, C15), but the intense doublets centered at 21.7 ppm (J_{C-C} = 34.4 Hz, C13) and 66.8 ppm (J_{C-C} = 38.4 Hz, C15) did not leave any doubt about the incorporation of intact C_2 units at C4−C15 and C10−C13 (Figure 4).

In conclusion, feeding experiments with ^{13}C-labeled glucose or acetate provided conclusive evidence of de novo biosynthesis of **1** and **5** via the acetate/mevalonate pathway in *D. areolata*. Acetoxypallescensins (**5** and **6**) are supposed to play a defensive role against potential predators,[3] whereas the drimane esters (**1**) may have an antibacterial function in the egg masses.[10] It makes sense for the nudibranchs, therefore, to produce **1** and **5** to protect themselves and their own species. Since previous studies proved that both **1** and **5** are assembled in the viscera of the molluscs, it is reasonable to suggest that a single pool of *trans*,*trans*-farnesyl diphosphate (**7**) might be involved in the biosynthesis of both compounds. Following this line of reasoning, the co-occurrence of the enantiomeric skeletons could be due to the conversion of **7** to **8** or other related compounds, e.g., β-farnesene (**9**). The proposal (Scheme 2) implies two different ways of cyclization, the first of which relies on a preliminary cleavage of the diphosphate group, leaving behind a reactive allylic carbocation from which β-farnesene (**9**) is generated by loss of H^+.[11] The second mechanism involves an acidic catalysis that initiates the process without the cleavage of the diphosphate group. Next, the diphosphate might be involved in the formation of the oxygenated five-membered rings of **1** and **5**. It is worth noting that the biogenetic proposal of Scheme 2 is based on the present knowledge of the enzyme-catalyzed cyclization of sesquiterpenes in terrestrial organisms (mainly plants, bacteria, and fungi).[12] In fact, mechanistic and genetic aspects of the terpene biosynthesis in marine molluscs have been never investigated so far.[13]

Finally, our experiments rule out acquisition of the sesquiterpenoids or their potential skeleton precursors from dietary sources, but they do not exclude production by a symbiont, although this possibility seems unlikely. This finding leaves unresolved the questions about the co-occurrence of identical chemical patterns in taxonomically distinct organisms, such as *Dysidea* and *Doriopsilla*.

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Experimental Section

Feeding Experiment. *D. areolata* (39 specimens) was collected by hand by scuba diving at depths of 10−15 m along the northwest coasts of Spain in May 2001. Part of the molluscs (15 specimens) were treated as controls and not subjected to any experiment. These animals were extracted with acetone and worked up as already reported.[4a,b] The remaining animals were divided into three groups (eight specimens per group) and kept alive in aquaria (800 mL of sterilized marine water) for 14 days. On days 1, 6, and 10, the animals were injected into the hepatopancreas with [1,2-$^{13}C_2$]-glucose (6 mg/specimen in 100 μL of distilled water), [1,2-$^{13}C_2$]-acetate (6 mg/specimen in 100 μL of distilled water), or [1-^{13}C]glucose (6 mg/specimen in 100 μL of distilled water). On day 15 all the animals were frozen at −80° C, and each group of molluscs was processed separately following the same procedure.

Purification of the Metabolites. The inner organs and mantle of the frozen molluscs were separated by anatomical dissection. Each section was separately immersed in acetone (three times) and the release of secondary metabolites promoted by using an ultrasound treatment over 2 min. The acetone fractions were combined, reduced in vacuo, and partitioned between Et_2O and water (three times). The ether layers were dried over anhydrous sodium sulfate and reduced in vacuo to yield (on average per eight specimens) 65 mg of yellow oil from the mantles and 55 mg of oil from the glands. The gland extract was fractionated on silica gel columns using an increasing polarity gradient of eluents (Et_2O in petroleum ether) to give pure **1** (approximately 0.8 mg/specimen). In the same manner, the purification of the mantle extracts yielded pure **5** (approximately 0.4 mg/specimen). ^{13}C NMR spectra (32 kB) of natural and isotopically labeled samples were recorded at 75 MHz in C_6D_6 with decoupling during acquisition and an interval delay of 5s (ns = 15000; NMR sample concentration, $\mathbf{1}_{nat}$, 0.04 M; $\mathbf{1}_{lab}$, 0.04 M; $\mathbf{5}_{nat}$, 0.03 M $\mathbf{5}_{lab}$, 0.02 M).

Acknowledgment. This research was partially supported by a Pharmamar grant. Dr G. Villani is gratefully acknowledged for the biological assistance.

Supporting Information Available: ^{13}C NMR spectra with doubly labeled precursors of **1** and **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

JO026131V