Investigations of Terpenoid Biosynthesis by the Dorid Nudibranch *Cadlina luteomarginata*

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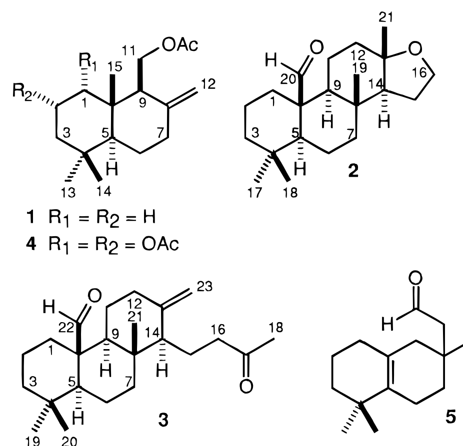
Stable isotope incorporation studies with [1,2-$^{13}C_2$]acetate, [1,2-$^{13}C_2$,$^{18}O_1$]acetate, and [2-^{13}C]mevalonolactone have been used to investigate the biosynthesis of the terpenoids albicanyl acetate (**1**), cadlinaldehyde (**2**), and luteone (**3**) by the dorid nudibranch *Cadlina luteomarginata*. The results have shown that all three terpenoids are synthesized *de novo* by *C. luteomarginata* and the incorporation patterns are consistent with the biogenetic proposal that the new cadlinalane and luteane carbon skeletons are formed by degradation of a sesterterpenoid precursor. This represents the first demonstration of sesterterpenoid biosynthesis by a marine mollusc. Quantitative analysis has shown that only a small turnover of metabolites takes place during the feeding experiments but that the newly formed molecules have extremely high levels of incorporation of labeled precursors.

Introduction

A remarkable collection of terpenoid metabolites has been isolated from the skin extracts and egg masses of the northeastern Pacific dorid nudibranch *Cadlina luteomarginata*.[1−8] To date, a total of 37 compounds representing 21 different regular, rearranged, and degraded monoterpenoid, sesquiterpenoid, diterpenoid, and sesterterpenoid carbon skeletons have been reported.[8] Three of the carbon skeletons were first encountered in *C. luteomarginata* terpenoids.[1,4,8] The majority of compounds isolated from *C. luteomarginata* skin extracts have also been isolated from sponges that comprise the diet of the nudibranchs. A smaller group of the compounds, that have not yet been found in dietary sponges, have chemical structures that are closely related to known sponge terpenoids and they are presumed to have a dietary origin as well. All of the *C. luteomarginata* skin extract terpenoids with known or putative sponge origins show significant collection-site-dependent variation in their occurrence, reflecting the differences in sponge diet at each locale.

Three terpenoids, albicanyl acetate (**1**), cadlinaldehyde (**2**), and luteone (**3**), have been found in skin extracts of specimens of *C. luteomarginata* collected at all sites along the British Columbia (BC) coast and, therefore, within this range don't show the geographic variability that is characteristic of compounds sequestered from dietary sponges.[8] It has been proposed that such a lack of geographic variability in occurrence of molluscan metabolites is a good predictor of *de novo* biosynthesis.[9] A number of additional pieces of circumstantial evidence support the possibility of *de novo* biosynthesis of terpenoids **1**, **2**, and **3** by *C. luteomarginata*. The 21-carbon cadlinalane and 23-carbon luteane skeletons found in cadlinaldehyde (**2**) and luteone (**3**), respectively, have not been encountered in known sponge terpenoids. Thus, there is no "structural similarity" argument for a sponge origin of **2** and **3**. Drimane sesquiterpenoids are known from marine sponges making it impossible to immediately rule out a sponge source for **1**.[10] However, 1α,2α-diacetoxyalbicanyl acetate (**4**) has recently been found in an egg mass of *C. luteomarginata*.[8] Compound **4** has never been found in skin extracts or whole body extracts of the nudibranch and, therefore, it seems likely that it is being made in whole or in part by *C. luteomarginata*. Albicanyl acetate (**1**) is a reasonably potent fish antifeedant which suggests that the diacetoxy derivative **4** might play a protective role in the egg masses.[3] From an evolutionary perspective, it makes sense for the nudibranch to produce **1** and **4** *de novo* in order to have direct control over the critical issue of protecting its eggs.



Prompted by the circumstantial evidence presented above for *de novo* biosynthesis of **1**, **2**, **3**, and **4**, we have

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Table 1. Yields of Terpenoids 1, 2, 3 Obtained per 20 Specimens of *C. luteomarginata*

collection site	collection date	experiment	yield of metabolite (mg) per 20 specimens		
			1	**2**	**3**
Jervis Inlet	May 2, 1996	injection of [1,2-$^{13}C_2$]sodium acetate	12.2	3.2	1.8
Barkley Sound	May 22, 1996	injection of [2-^{13}C]mevalonolactone	7.8	3.8	7.1
Barkley Sound	July 14, 1996	control	9.0	1.8	4.1
Barkley Sound	July 14, 1996	injection of [1,2-$^{13}C_2$]sodium acetate	78.4	2.3	4.1
Barkley Sound	September 11, 1996	control	4.1	$<$1.0	1.6
Barkley Sound	September 11, 1996	injection of [1,2-$^{13}C_2{}^{18}O_1$]sodium acetate	34.7	$<$1.0	2.4

undertaken an investigation of terpenoid biosynthesis by *C. luteomarginata*. The objectives of the investigation were: (i) to demonstrate that albicanyl acetate (**1**), cadlinaldehyde (**2**), and luteone (**3**) are made *de novo* by *C. luteomarginata* as indicated by the geographic invariance of their occurrence in skin extracts, and (ii) to verify that the new cadlinalane and luteane carbon skeletons found in cadlinaldehyde (**2**) and luteone (**3**), respectively, arise from degradation of a sesterterpenoid precursor as proposed in Scheme 2. Dorid nudibranchs are known to be capable of *de novo* sesquiterpenoid[11−14] and diterpenoid[12,14] biosynthesis, but to date there has been no demonstration of sesterterpenoid biosynthesis by any marine mollusc.

Results and Discussion

Stable isotope labeled precursor feeding experiments with *C. luteomarginata* followed the general protocol used by our group in recent investigations of terpenoid[13,14] and polyketide[15] biosynthesis in dorid nudibranchs. Three terpenoid precursors, [1,2-$^{13}C_2$]acetate, [1,2-$^{13}C_2$,$^{18}O_1$]-acetate, and [2-^{13}C]mevalonolactone, were used in the current study. Specimens of *C. luteomarginata* were collected via SCUBA in Barkley Sound and Jervis Inlet, BC, and transported back to UBC in refrigerated seawater. The nudibranchs were maintained at 12 °C in an aquarium filled with Barkley Sound seawater that was changed every 2 days. In the first experiment, individual specimens of *C. luteomarginata* were given 100−200 μL injections of a 550 mM solution of [1,2-$^{13}C_2$]acetate every second day for 15 days (seven injections total). The physical act of handling the nudibranchs for injections caused them to partially shed the terpenoids in their dorsums. Two days after the last injection the *C. luteomarginata* specimens were carefully removed from the aquarium seawater and the intact animals were immediately immersed in methanol. Fractionation of the methanol extract as previously described[8] gave pure samples of albicanyl acetate (**1**), cadlinaldehyde (**2**), and luteone (**3**). Table 1 gives a comparison of the yields of **1**, **2**, and **3** obtained from the [1,2-$^{13}C_2$]acetate feeding experiment with the yields from control animals. It is interesting to note that in the July feeding experiment with Barkley Sound animals, the injection of labeled acetate led to a nearly 10-fold increase in the isolated yield of albicanyl acetate (**1**) (9 mg/20 animals for the control vs 78 mg/20 animals for the feeding experiment).

The ^{13}C NMR spectra obtained for **1**, **2**, and **3** isolated from animals injected with [1,2-$^{13}C_2$]acetate showed clear

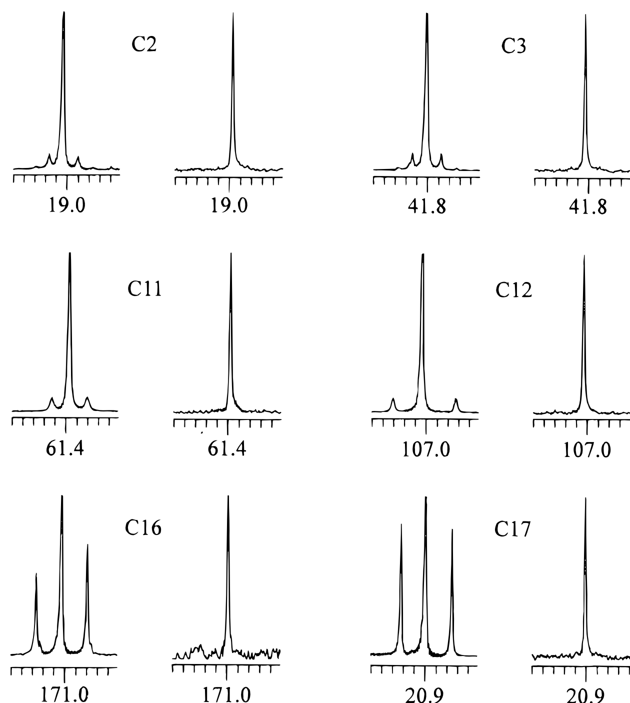

Figure 1. Selected ^{13}C NMR resonances from the spectrum of albicanyl acetate (**1**) ($CDCl_3$, 125 MHz) isolated from *C. luteomarginata*. For each carbon, the left-hand resonance is from specimens fed [1,2-$^{13}C_2$]sodium acetate and the right-hand resonance is from an unlabeled control sample. All horizontal expansions in this and other figures span a 1.00 ppm range. The resonances have been normalized to a common central peak height and then truncated for ease of visual comparison.

evidence for the *de novo* biosynthesis of all three terpenoids by *C. luteomarginata*. Figure 1 shows selected resonances from the spectrum of albicanyl acetate (**1**) obtained from the feeding experiment along with the corresponding resonances from an unlabeled control sample. The weak doublets flanking the strong natural abundance central singlet in the C-2 (δ 19.0), C-3 (41.8), C-11 (61.4), C-12 (107.0), C-16 (171.0), and C-17 (20.9) resonances result from incorporation of intact [1,2-$^{13}C_2$]acetate units into **1**. Analysis of the coupling constants for all of the resonances in the spectrum (Table 2) confirms that the pattern of acetate incorporation is identical to that predicted by the biogenetic pathway shown in Scheme 1. The data also show that there is a significant difference in the efficiency of acetate incorporation into the terpenoid skeleton (average specific incorporation 0.19%) and into the acetyl group (specific incorporation 0.82%). In several replicates of this feeding experiment it was found that in all cases the level of incorporation into the acetyl group was high, whereas in some experiments it was impossible to find conclusive evidence for incorporation into the terpenoid skeleton. Interestingly, the levels of incorporation into the terpe-

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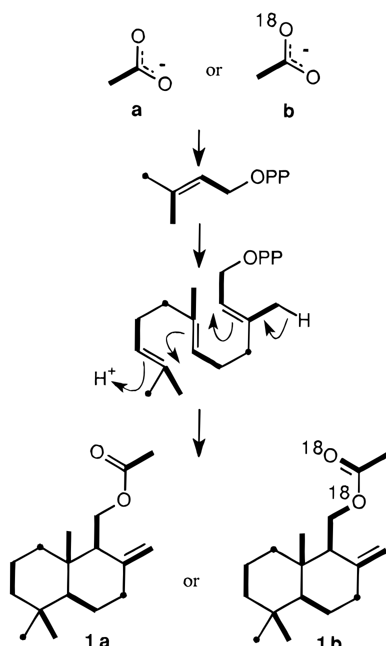
Table 2. ^{13}C NMR Data for Albicanyl Acetate (1) Obtained from the [1,2-$^{13}C_2$]Sodium Acetate Feeding Experiment

carbon no.	^{13}C δ (ppm)	$J_{(C-C)}$ (Hz)
1	38.9	
2	19.0	33.4
3	41.8	33.4
4	33.3	38.2
5	55.0	33.4
6	23.8	33.8
7	37.5	
8	146.6	72.5
9	54.6	40.1
10	38.8	38.1
11	61.4	42.0
12	107.0	72.5
13	33.5	
14	21.6	36.2
15	15.0	37.2
16	171.0	60.1
17	20.9	59.1

Scheme 1. Proposed Biogenesis of Albicanyl Acetate (1) from [1,2-$^{13}C_2$]Acetate (a) and [1,2-$^{13}C_2$,$^{18}O_1$]Acetate (b)



noid skeleton appeared to be seasonal and the highest levels were found in animals collected during egg laying season.

Figures 2 and 3 show selected resonances from the ^{13}C NMR spectra of cadlinaldehyde (**2**) and luteone (**3**) obtained from the [1,2-$^{13}C_2$]acetate feeding experiment. The resonances in each Figure have again been normalized to a constant central peak height and then truncated. Three resonances at δ 16.6 (Me-19), 20.8 (Me-18 and Me-21), and 206.4 (C-20) in the ^{13}C NMR spectrum of cadlinaldehyde (**2**) (Figure 2) showed relatively intense doublets flanking a central natural abundance singlet indicating incorporation of intact acetate units into the molecule. A number of other resonances showed relatively weak doublets (i.e., δ 31.9 (C-17) and 64.9 (C-16)) or more complex multiplets (i.e., δ 22.9 (C-15) and 41.7 (C-3)) flanking the central singlet that were also indications of incorporation of ^{13}C-labeled acetate units. The low signal-to-noise ratio in the remaining peaks in the spectrum (Supporting Information) made it impossible

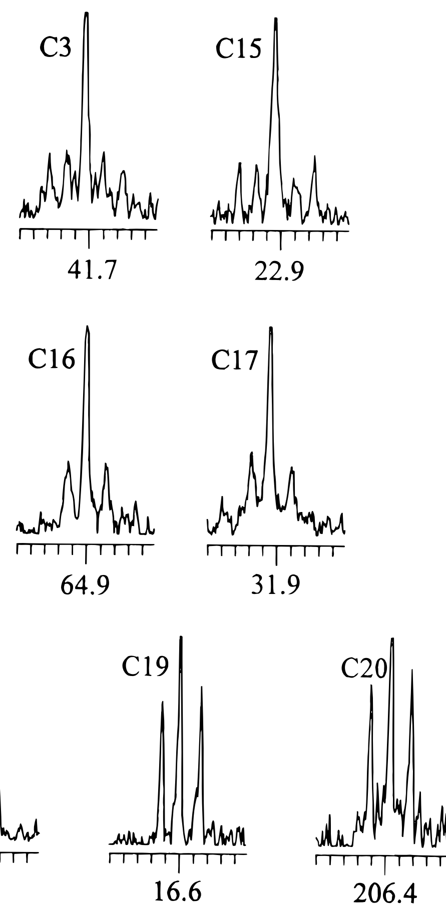


Figure 2. Selected ^{13}C NMR resonances from the spectrum of cadinaldehyde (**2**) ($CDCl_3$, 125 MHz) isolated from *C. luteomarginata* fed [1,2-$^{13}C_2$]sodium acetate.

to detect conclusive evidence for incorporation of ^{13}C-labeled acetate into the carbons assigned to these resonances.

Scheme 2 shows that the methyl appendages C-18, C-19, C-20, and C-21 should all be part of intact acetate units if cadlinaldehyde (**2**) is formed via standard terpenoid biosynthesis. Indeed, the clear flanking doublets in the C-19 (δ 16.6) and C-20 (δ 206.4) resonances are consistent with incorporation of intact doubly labeled acetate units at C-8/C-19 and C-10/C-20 in **2**. The C-18 and C-21 methyl carbon resonances in the spectrum of **2** coincidentally have identical chemical shifts of δ 20.8 making it impossible to be absolutely certain that the resonances for both methyls have relatively intense flanking doublets. However, the flanking doublet in the δ 20.8 resonance has essentially the same intensity relative to the central singlet as do the flanking doublets in the C-19 and C-20 resonances, which is consistent with both the C-18 and C-21 methyls being derived from intact acetate units as expected.

The biogenetic proposal in Scheme 2 predicts that C-16 and C-17 in **2** are both derived from C-2 of cleaved acetate units. Thus, if during the incorporation of acetate into cadlinaldehyde (**2**) there was high dilution of labeled precursor the resonances for C-16 and C-17 should appear only as enriched singlets. However, inspection of the resonances for C-16 and C-17 in Figure 2 shows that there are weak doublets flanking the central singlets. The most likely explanation for the flanking doublets observed in the C-16 and C-17 resonances is that they originate from incorporation of more than one ^{13}C-

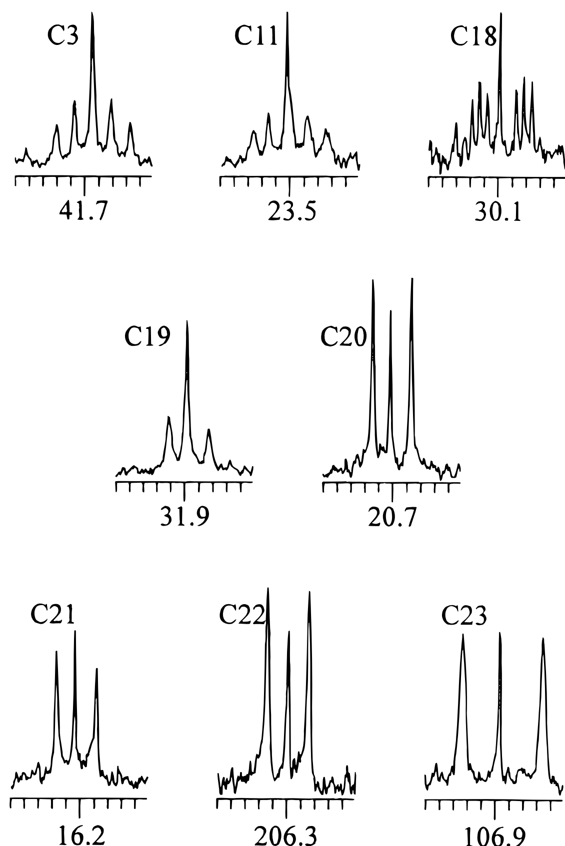
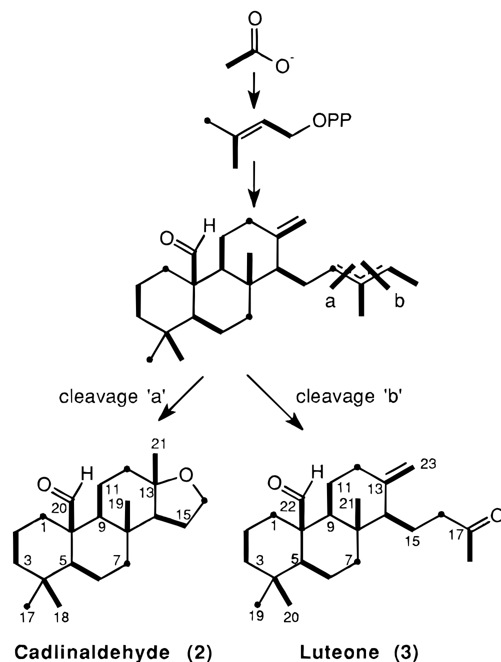


Figure 3. Selected ^{13}C NMR resonances from the spectrum of luteone (**3**) ($CDCl_3$, 125 MHz) isolated from *C. luteomarginata* fed [1,2-$^{13}C_2$]sodium acetate.

Scheme 2. Proposed Biogenesis of Cadlinaldehyde (2) and Luteone (3) from [1,2-$^{13}C_2$]Acetate



labeled acetate unit into a single cadlinaldehyde (**2**) molecule. *C. luteomarginata* specimens were starved during the 16 day injection period and this could have led to a highly labeled acetate pool and a reasonable probability of more than one labeled acetate unit being incorporated into individual mevalonic acid molecules

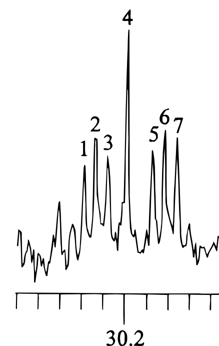


Figure 4. Expanded ^{13}C NMR resonance for C-18 from the spectrum of luteone (**3**) ($CDCl_3$, 125 MHz) isolated from *C. luteomarginata* fed [1,2-$^{13}C_2$]sodium acetate.

and/or more than one labeled mevalonic acid unit being incorporated into individual molecules of **2**. As a result, the C-16 doublet shown in Figure 2 would arise from molecules of **2** having a singly enriched carbon at C-16 and a labeled intact acetate unit at C-14/C-15. Similarly, the C-17 weak doublet would arise from having a singly labeled carbon at C-17 and a labeled intact acetate unit at C-4/C-18.

Figure 3 shows selected resonances from the ^{13}C NMR spectrum of luteone (**3**) isolated from the [1,2-$^{13}C_2$]acetate feeding experiment. Once again there are a series of resonances which have relatively intense doublets flanking the central singlet (δ 16.2 (C-21), 20.7 (C-20), 206.3 (C-22), and 106.9 (C-23)), one that shows a relatively weak doublet flanking the central singlet (δ 31.9 (C-19)), a group that has complex multiplets flanking the central singlet (i.e., δ 30.1 (C-18), 41.7 (C-3), and 23.5 (C-11)), and several in which the signal-to-noise ratio is so low that it is impossible to detect conclusive evidence for incorporation of labeled acetate (Supporting Information). The biogenetic proposal presented in Scheme 2 requires that the C-20, C-21, C-22, and C-23 methyl appendages in **3** should all be formed from intact acetate units. This is supported by the observation of relatively intense flanking doublets in the resonances assigned to these carbons in the labeled sample of luteone (**3**). The average apparent specific incorporation at these four sites is 3.6% in luteone (**3**), which is roughly four times the apparent specific incorporation observed for the methyl appendage carbons (average in **2** = 0.86%) in cadlinaldehyde (**2**) in the same feeding experiment.

Recently it has been pointed out that one of the difficulties encountered in carrying out biosynthetic feeding experiments with marine invertebrates is that "the rate of metabolite turnover may be low and, therefore, utilization of precursors must be assessed against a high background level of unlabeled material".[16] Using the [1,2-$^{13}C_2$]acetate incorporation data for luteone (**3**) it is possible to get a quantitative estimate of both (i) the percentage of molecules that have been formed during the feeding experiment, and (ii) the extent to which these molecules have incorporated the labeled precursor. The basis for this calculation is the fortuitous observation of a well-resolved multiplet structure in the C-18 resonance (Figure 4).

If the component lines in the C-18 resonance (Figure 4) are numbered from 1 to 7 starting with the highest frequency component, then it is possible to assign the

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individual peaks to the various forms of labeled luteone (**3**) present in the sample obtained from the feeding experiment. The central component (line #4) in the multiplet can be assigned to a natural abundance ^{13}C singlet arising from (i) unlabeled luteone molecules that were present in *C. luteomarginata* at the outset of the feeding experiment, and (ii) from luteone molecules that have incorporated [1,2-$^{13}C_2$]acetate during the feeding experiment but not at positions C-16, C-17, or C-18. Component lines #2 and #6 can be assigned to a doublet (J = 41 Hz) arising from molecules that have incorporated an intact [1,2-$^{13}C_2$]acetate unit at C-17 and C-18 but have a ^{12}C atom at C-16. The remaining component lines (#s 1,3,5,7) can be assigned to a doublet of doublets (J = 14, 41 Hz) arising from molecules that have incorporated an intact [1,2-$^{13}C_2$]acetate unit at C-17/C-18 and a single ^{13}C label from C-2 of a [1,2-$^{13}C_2$]acetate unit at C-16. This latter assignment is in good agreement with the observation that geminal ^{13}C-^{13}C couplings are typically ≈15 Hz when the central carbon is a ketone as is the case in the C-16/C-18 coupling observed in the triply labeled luteone (**3**) molecules.[17]

In order to calculate (i) the percentage of molecules that have been formed during the feeding experiment and, (ii) the extent to which these molecules have incorporated the labeled precursor, it has been assumed that the nudibranchs have a sizable pool of unlabeled metabolites at the outset of the experiment and only a small pool of new molecules is made in the presence of the isotopically labeled precursor. Therefore, the isolated products at the end of the experiment are really made up of two components: (1) molecules which were present at the outset and are completely unlabeled and, (2) labeled molecules that have been made during the experiment. Consequently, the "apparent specific incorporation" of ^{13}C label at any particular site is a weighted average of the incorporation in the original molecules (zero) and the specific incorporation in the labeled molecules. This can be expressed as follows if it is assumed that there is uniform incorporation of acetate at all positions in luteone (**3**):

$$\mathrm{sa} = \mathrm{si}\cdot x + 0\cdot(1 - x) \qquad (1)$$

where sa is the apparent specific incorporation observed in the whole sample, si is the specific incorporation in the newly formed metabolites that were made during the labeling experiment, and x is the fraction of molecules made during the labeling experiment.

In the [1,2-$^{13}C_2$]acetate feeding experiment the total intensity of the doublet and the doublet of doublet components present in the C-18 resonance of luteone (**3**) can be used to directly calculate sa at C-18 as follows:

$$\mathrm{sa} = \%\ \text{enrichment above natural abundance} = 1.1\%\ X\,(\text{combined integrated peak area of enriched satellites: lines 1,2,3,5,6,7})/(\text{peak area of the natural abundance singlet: line 4})$$

Using the same arguments presented above, the apparent specific incorporation of an intact [1,2-$^{13}C_2$]acetate unit at C-17/C-18 as well as a single acetate label at C-16 in a single molecule of **3** can be calculated as follows:

$$\mathrm{sa}_2 = \mathrm{si}\cdot\mathrm{si}\cdot x + 0\cdot(1 - x) \qquad (2)$$

where sa_2 is the apparent specific incorporation for the doublet of doublets, si·si represents the probability of having two adjacent ^{13}C labeled carbons arising from different labeled acetate units in a single molecule of **3** formed during the labeling experiment, x represents the fraction of labeled molecules, and $1 - x$ represents the fraction of unlabeled molecules present at the outset.

The calculation of sa_2 would also use the equation

$$\mathrm{sa}_2 = \%\ \text{enrichment above natural abundance} = 1.1\%\ X\,(\text{combined integrated peak area of doublet of doublet components: lines 1,3,5,7})/(\text{peak area of the natural abundance singlet: line 4})$$

The measured relative intensities of the component lines in the C-18 multiplet were as follows: line 1: 0.38; line 2: 0.68; line 3: 0.48; line 4: 1.0; line 5: 0.52; line 6: 0.65; line 7: 0.58. Using the values for sa and sa_2 obtained from the component line relative intensity measurements it is possible to solve eq 1 and 2 for si and x. The calculated values are: si = 0.60 and x = 0.06. Therefore, the specific incorporation in the labeled luteone (**3**) molecules is actually 60%, but only 6% of the molecules in the entire pool are labeled. These numbers are completely consistent with the argument that the acetate pool in the nudibranchs during the injection experiment is highly labeled and, therefore, molecules made from this pool should be highly labeled. The si and x values are also consistent with our expectation that only a relatively small percentage of the metabolite pool is being synthesized in these experiments.

The [1,2-$^{13}C_2$]acetate feeding experiment provided conclusive evidence for the *de novo* biosynthesis of albicanyl acetate (**1**), cadlinaldehyde (**2**), and luteone (**3**) by *C. luteomarginata*. However, the complexity of the multiplet patterns in the ^{13}C NMR spectra of labeled **2** and **3** made it impossible to use coupling constant information to establish the complete pattern of acetate incorporation in either molecule. Scheme 2 proposes a degraded sesterterpenoid biogenetic origin for both cadlinaldehyde (**2**) and luteone (**3**). In order to test this proposal, a second feeding experiment was conducted utilizing [2-^{13}C]mevalonolactone as a precursor. Once again, individual specimens of *C. luteomarginata* were given 100−200 μL injections of a 550 mM solution of [2-^{13}C]mevalonolactone every second day for 15 days (seven injections total). Two days after the last injection the specimens of *C. luteomarginata* were carefully removed from the aquarium seawater and immersed whole in methanol. Fractionation of the methanol skin extract gave pure samples of albicanyl acetate (**1**), cadlinaldehyde (**2**), and luteone (**3**).

The ^{13}C NMR spectrum of albicanyl acetate (**1**) obtained from the [2-^{13}C]mevalonolactone feeding experiment failed to show any evidence for incorporation of labeled precursor. Figure 5 shows the ^{13}C NMR spectrum of cadlinaldehyde (**2**) isolated from the [2-^{13}C]mevalonolactone feeding experiment. Five resonances in the spectrum, assigned to C-1 (δ 34.3), C-7 (40.4), C-12 (39.2), C-16 (64.9), and C-17 (31.9), show clear enrichment above natural abundance (Table 3). This labeling pattern is in complete agreement with the biogenetic proposal that **2** is a degraded sesterterpenoid as shown in Scheme 3.

(17) Kalinowski, H. O.; Berger, S.; Braun, S. *Carbon-13 NMR Spectroscopy*; John Wiley & Sons: New York, 1988; p 559.

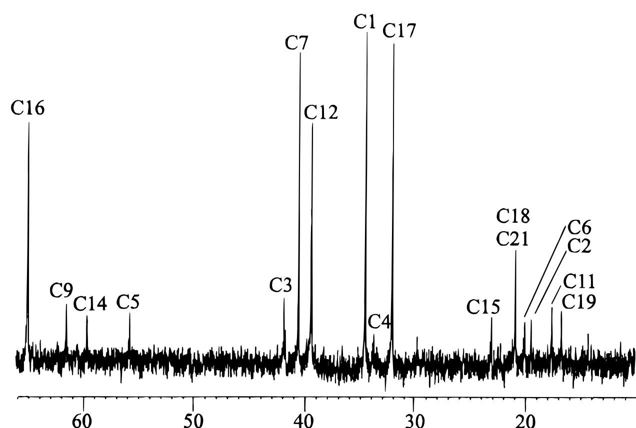


Figure 5. Upfield region of the ^{13}C NMR spectrum of cadlinaldehyde (**2**) ($CDCl_3$, 125 MHz) isolated from *C. luteomarginata* fed [2-^{13}C]mevalonolactone.

Table 3. Specific Incorporation of [2-^{13}C]Mevalonolactone into Cadlinaldehyde (2) and Luteone (3)

	cadlinaldehyde (**2**)		luteone (**3**)	
carbon no.	^{13}C δ (ppm)	specific incorptn (%)	^{13}C δ (ppm)	specific incorptn (%)
1	34.3	6.57	34.4	13.9
2	19.3		19.4	
3	41.7		41.7	
4	33.6		33.6	
5	55.6		55.1	
6	19.9		18.4	
7	40.4	6.30	39.5	14.7
8	36.5		39.8	
9	61.4		60.2	
10	53.4		53.8	
11	17.4		23.5	
12	39.2	6.54	37.7	14.5
13	79.5		147.1	
14	59.5		55.2	
15	22.9		17.6	
16	64.9	5.11	42.6	18.4
17	31.9	5.83	209.2	
18	20.8		30.1	
19	16.6		31.9	13.2
20	206.4		20.7	
21	20.8		16.2	
22	--		206.3	
23	--		106.9	

Of particular significance is the enrichment of the C-16 resonance, which clearly demonstrates that this carbon originates from C-2 of mevalonic acid, ruling out any alternate type of homologation of a diterpenoid to give the 21-carbon cadlinalane skeleton. The ^{13}C NMR spectrum of luteone (**3**) (Supporting Information) obtained from the [2-^{13}C]mevalonolactone feeding experiment also showed clear enrichment above natural abundance at C-1 (δ 34.4), C-7 (39.5), C-12 (37.7), C-16 (42.6), and C-19 (31.9) (Table 3), once again in complete agreement with the proposed biogenesis of the luteane skeleton from a sesterterpenoid precursor as shown in Scheme 3.

A central issue in a concurrent investigation of the biosynthesis of nanaimoal (**5**), acanthodoral, and isoacanthodoral by the dorid nudibranch *Acanthdoris nanaimoensis* was the origin of the oxygen atom present in the aldehyde functionality.[13] One possibility was that the alcohol oxygen atom in farnesyl pyrophosphate was retained in the aldehyde functionalities of these three sesquiterpenoids. In order to test this possibility, it seemed reasonable to feed [1,2-$^{13}C_2$,$^{18}O_1$]acetate and then look for the ^{18}O isotope-induced shift in the doublets

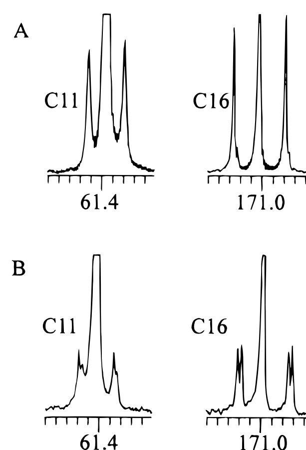
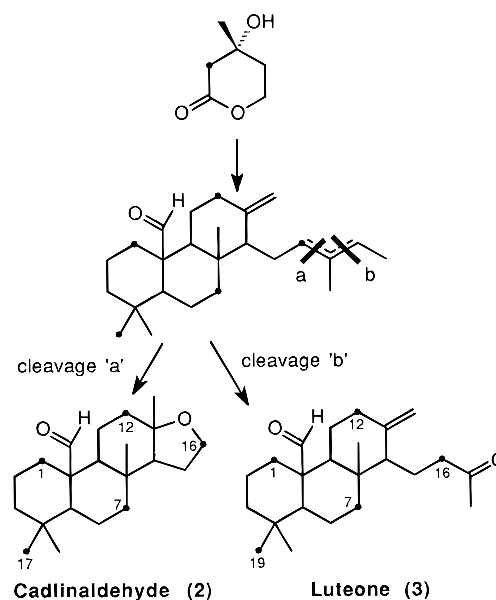


Figure 6. ^{13}C NMR resonances for C-11 and C-16 of albicanyl acetate (**1**) ($CDCl_3$, 125 MHz) from *C. luteomarginata* fed: (A) [1,2-$^{13}C_2$]sodium acetate, and (B) [1,2-$^{13}C_2$-$^{18}O_1$]sodium acetate.

Scheme 3. Proposed Biogenesis of Cadlinaldehyde (2) and Luteone (3) from [2-^{13}C]Mevalonolactone



arising from intact incorporation of acetate units. Albicanyl acetate biosynthesis by *C. luteomarginata* appeared to offer an ideal model to test the feasibility of this approach since the farnesyl pyrophosphate alcohol oxygen atom was expected to be retained at C-11 in this molecule (Scheme 1). Thus, *C. luteomarginata* was fed [1,2-$^{13}C_2$,$^{18}O_1$]acetate following the protocol described above for the [1,2-$^{13}C_2$]acetate feeding experiment. The [1,2-$^{13}C_2$,$^{18}O_1$]acetate was prepared by reacting [1,2-$^{13}C_2$]acetyl chloride with $H_2{}^{18}O$ (>99% ^{18}O) to give [1,2-$^{13}C_2$,$^{18}O_1$]acetate that was $\approx$50% labeled with ^{18}O.

Figure 6 shows the acetate carbonyl and C-11 resonances in the ^{13}C NMR spectrum of albicanyl acetate (**1**) obtained from the [1,2-$^{13}C_2$,$^{18}O_1$]acetate feeding experiment and the same resonances in a sample of **1** obtained from a [1,2-$^{13}C_2$]acetate feeding experiment. The carbonyl resonance at δ 171.0 in the sample of **1** obtained from the [1,2-$^{13}C_2$,$^{18}O_1$]acetate feeding experiment shows two sets of essentially equal intensity doublets flanking the central natural abundance singlet. Comparison with the carbonyl resonance in the spectrum of **1** obtained from the [1,2-$^{13}C_2$]acetate feeding experiment showed that the downfield doublet arises from molecules (**1a**) that have

^{16}O in the acetate carbonyl functionality and, therefore, the upfield doublet arises from molecules (**1b**) which have the acetate carbonyl oxygen labeled with ^{18}O. The observed isotope shift in the δ 171.0 resonance was 0.03 ppm which is in the reported range of 0.030–0.55 ppm for ^{18}O-labeled carbonyl carbons.[18] It is interesting to note that the nearly 1:1 ratio of the two doublets demonstrates that there has been virtually no exchange of the acetate oxygen atoms during the course of the feeding experiment. Examination of the C-11 resonance (δ 61.4) in the sample of **1** obtained from the [1,2-$^{13}C_2$,$^{18}O_1$]acetate feeding experiment also reveals the presence of two doublets flanking the central natural abundance singlet, confirming that the farnesyl pyrophosphate alcohol oxygen atom is retained in the biosynthesis. Once again, the observed isotope shift of 0.025 ppm in the C-11 resonance was within the reported range of 0.01–0.035 ppm for C-O carbons.[18] In the case of C-11, the upfield flanking doublet resulting from molecules (**1b**) that have the alkoxy oxygen labeled with ^{18}O is less intense than the downfield doublet arising from molecules (**1a**) with ^{16}O at the alkoxy oxygen. This difference in intensity indicates that there is some limited exchange of the oxygen atoms during the processing of acetate in the terpenoid pathway.

Conclusions

The results presented above have clearly demonstrated that albicanyl acetate (**1**), cadlinaldehyde (**2**), and luteone (**3**) are biosynthesized *de novo* by *C. luteomarginata* as predicted by the geographic invariance of their occurrence in BC specimens of the nudibranch. In all of the feeding experiments that were attempted, there was efficient incorporation of either labeled acetate or mevalonolactone into **2** and **3**, but a number of feeding experiments failed to show incorporation of labeled precursors into the terpenoid portion of albicanyl acetate (**1**). The highest levels of incorporation of labeled acetate into the drimane portion of **1** were obtained with animals that were collected during egg-laying season suggesting some reproductive cycle control on the biosynthesis of this compound. This observation is consistent with the recent discovery of 1α,2α-diacetoxyalbicanyl acetate (**4**), a diacetoxy derivative of **1**, in egg masses of *C. luteomarginata*, where it is presumed to be playing a defensive role.

The mevalonolactone incorporation results support a sesterterpenoid origin for the new degraded cadlinalane and luteane terpenoid carbon skeletons as proposed in Scheme 3. This represents the first demonstration of *de novo* sesterterpenoid biosynthesis in marine molluscs, which have previously been shown to be capable of sesquiterpenoid and diterpenoid biosynthesis.[11–14] There is only one previous report of the successful incorporation of a stable isotope-labeled mevalonic acid equivalent into a marine invertebrate terpenoid during a feeding study.[14] In that example, the levels of incorporation were quite low and the labeling pattern could only be discerned by statistical analysis of ^{13}C NMR peak heights in the labeled sample. The levels of incorporation of [2-^{13}C]mevalonolactone into cadlinaldehyde (**2**) and luteone (**3**) in the current feeding experiment with *C. luteomarginata* were unexpectedly high, allowing for routine visual confirmation of the labeling pattern. In cadlinaldehyde (**2**), the average apparent specific incorporation of ^{13}C at the five labeled carbons was 6.1%, and in luteone (**3**) it was 14.9%. Quantitative analysis of the [1,2-$^{13}C_2$]acetate incorporation into luteone (**3**) showed that only ≈6% of the isolated molecules were made during the feeding experiment but that this small pool of luteone was exceptionally highly labeled (i.e., specific incorporation of ≈60% at C-18). This result suggests that it is probably not the effectiveness of incorporation of precursors into biosynthetic products that is the limiting factor in the use of stable isotopes to investigate the biosynthesis of marine invertebrate terpenoids but rather it is the large pool of unlabeled compound against which incorporation must be assessed.

C. luteomarginata is only the second marine mollusc that is known to be capable of both sequestering skin extract terpenoids from dietary sources and producing them via *de novo* biosynthesis. The other example is the Mediterranean dorid nudibranch *Dendrodoris grandiflora* which also makes drimane sesquiterpenoids that it deploys on its dorsum and in its egg masses much like *C. luteomarginata*.[19] One unresolved feature of *C. luteomarginata* terpenoid chemistry is the observation that albicanyl acetate (**1**), cadlinaldehyde (**2**), and luteone (**3**) are found in all BC specimens of the nudibranch but they are apparently not present at all in southern California specimens. This suggests that there are chemically distinct sub-populations of *C. luteomarginata* even though classical taxonomic analysis based on morphology indicates that there is only a single species all along the western coast of North America.[20]

Experimental Section

Specimens of *Cadlina luteomarginata* were collected by hand using SCUBA at depths of 10–50 ft in both Agamemnon Channel, Jervis Inlet, BC and Barkley Sound, BC. Between May and September 1996 six collections were made of between 20 and 44 specimens each time. Weight of individual animals varied between 0.6 g and 6.0 g (wet). Two collections were treated as control experiments and, therefore, not subjected to injections. These animals were immediately immersed in methanol and worked up according to the procedure outlined below.

Collections targeted for injection experiments were treated as follows. Animals were kept alive in aquaria at 12 °C for 17 days following collection. On days 3, 5, 7, 9, 11, 13, and 15 each animal was subjected to injection of [1,2-$^{13}C_2$]acetate, [1,2-$^{13}C_2$,$^{18}O_1$]acetate, or [2-^{13}C]mevalonolactone. The precursor was dissolved in distilled water to a concentration of 0.55 mol·L^{-1}. An injection of 100–200 μL (depending on size of animal) was administered by 26G1/2 needle and syringe to the left dorsum (presumably into the hepatopancreas). On day 17 all specimens were immersed in methanol.

Each collection of animals was processed separately following the same protocol. Following immersion, the animals were extracted exhaustively over 5 days with methanol (two times) and methanol/methylene chloride (1:1) (three times). The extracts were combined, reduced *in vacuo*, and partitioned between water and ethyl acetate. The ethyl acetate extracts were dried over anhydrous magnesium sulfate, combined, and reduced *in vacuo* to yield a fragrant yellow oil (~500 mg per 20 animals). This oil was fractionated on a silica gel flash column using a gradient eluent system (9:1 hexanes/ethyl acetate to ethyl acetate). Fractions were further purified by C_{18} reversed-phase high-performance liquid chromatography (9:1 methanol/water) to yield pure albicanyl acetate (**1**), cadlinaldehyde (**2**), and luteone (**3**). Yields of **1**, **2**, and **3**

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obtained from the various feeding experiments are given in Table 1. ^{13}C NMR spectra of isotopically labeled samples were recorded at 125 MHz in $CDCl_3$ with proton noise decoupling.

Albicanyl acetate (1). obtained from the [1,2-$^{13}C_2$]acetate feeding experiment; colorless oil; $[\alpha]^{25}_D$ ($CHCl_3$) = +24°; IR ($CHCl_3$) ν_{max}: 2920, 2860, 1740, 1460, 1440, 1400, 1380, 1240, 1040, 900 cm^{-1}; ^1H NMR ($CDCl_3$): 0.71 (s, Me15), 0.77 (s, Me14), 0.84 (s, Me13), 1.97 (s, Me17), 2.36 (ddd, J = 13, 4, 2, H7′), 4.14 (dd, J = 11, 9, H12), 4.29 (dd, J = 11, 4, H12′), 4.47 (d, J = 1, H11), 4.81 (d, J = 1, H11′); ^{13}C NMR (see Table 2); HREIMS m/z: 264.2088 ($C_{17}H_{28}O_2$ requires 264.2082). ^1H and ^{13}C NMR spectra of the sample of **1** obtained from the [1,2-$^{13}C_2$,$^{18}O_1$]acetate feeding experiment are available in the Supporting Information.

Cadlinaldehyde (2). obtained from the [1,2-$^{13}C_2$]acetate feeding experiment; white amorphous solid subsequently recrystallized from methanol to yield white needles; $[\alpha]^{25}_D$ ($CHCl_3$) = +25°; UV ($CHCl_3$) λ_{max}: 246 (log ϵ = 2.66) nm, 250 sh (log ϵ = 2.59); IR (film) ν_{max}: 2926, 2750, 1705, 1457, 1377 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.70 (s, Me19), 0.77 (s, Me18), 0.92 (s, Me17), 0.98 (s, Me21), 3.80 (dd, J = 8, 17, H16), 3.88 (dt, J = 9, 3, H16′), 10.07 (s, H20) ; ^{13}C NMR (see Table 3); HREIMS m/z: 318.2489 ($C_{21}H_{38}O_2$ requires 318.2589). ^1H and ^{13}C NMR spectra of the sample of **2** obtained from the [2-^{13}C]mevalonolactone feeding experiment are available in the Supporting Information.

Luteone (3). obtained from the [1,2-$^{13}C_2$]acetate feeding experiment; white crystalline solid; $[\alpha]^{25}_D$ ($CHCl_3$) = +12°; UV ($CHCl_3$) λ_{max}: 244 (log ϵ = 2.42) nm; IR (film) ν_{max}: 3078, 2931, 2849, 2750, 1708, 1646, 1457, 1363, 1161 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.57 (s, Me21), 0.74 (s, Me20), 0.91 (s, Me19), 2.09 (s, Me18), 2.30 (m, H16), 2.57 (ddd, J = 5, 8, 18, H16′), 4.40 (bs, H23), 4.79 (d, J = 1, H23′), 10.07 (bs, H22) ; ^{13}C NMR (see Table 3); HREIMS m/z: 344.2717 ($C_{23}H_{36}O_2$ requires 344.2715). ^1H and ^{13}C NMR spectra of the sample of **3** obtained from the [2-^{13}C]mevalonolactone feeding experiment are available in the Supporting Information.

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Supporting Information Available: 1D ^1H and ^{13}C NMR spectra for all samples of isotopically labeled **1**, **2**, and **3**. Expansions of all ^{13}C NMR resonances for **1**, **2**, and **3** obtained from the [1,2-$^{13}C_2$]acetate feeding experiment (19 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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