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Stable Isotope Incorporation Evidence for the *De Novo* Biosynthesis of Terpenoic Acid Glycerides by Dorid Nudibranchs

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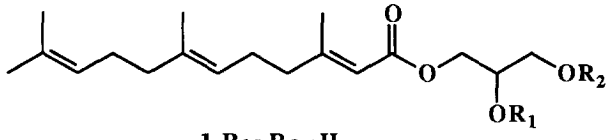
Abstract: Feeding experiments with [1,2-$^{13}C_2$]acetate have been used to demonstrate that the dorid nudibranchs *Archidoris odhneri* and *A. montereyensis* are capable of *de novo* biosynthesis of the terpenoic acid glycerides **1** and **4**. Feeding experiments using [2-^{13}C] mevalonolactone as a precursor have shown that *Sclerodoris tanya* is capable of *de novo* biosynthesis of the terpenoid fragment of tanyolide B (**7**).

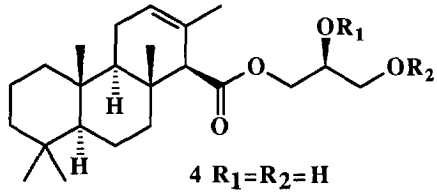
Terpenoic acid glycerides have been isolated from skin extracts of a number of dorid nudibranchs collected worldwide. The first examples reported were the farnesic acid glycerides **1** to **3** and the diterpenoic acid glycerides **4** to **6** isolated from the British Columbia dorids *Archidoris odhneri* and *A. montereyensis*.[1,2,3] Subsequently, terpenoic acid glycerides have been isolated from *Doris verrucosa* collected in the Bay of Naples,[4] from *Archidoris tuberculata* collected in Spain,[5] from *Archidoris pseudoargus* collected in the Eastern Atlantic,[6] from *Archidoris carvai* collected in southern Argentina,[7] from *Austrodoris kerguelensis* collected in the Antarctic,[8] and from *Sclerodoris tanya* collected in California.[9] It has been shown that terpenoic acid glycerides are toxic to freshwater fish[4,10] and antifeedant towards marine fish[2] in keeping with a putative role as defensive allomones. The true biosynthetic origin of the nudibranch terpenoic acid glycerides has been the subject of much speculation and some previous experimentation. Most dorid nudibranchs are known to sequester terpenoids unchanged from sponges, soft corals and other invertebrates that make up their diets and to store them in glands on their dorsums, presumably for defensive purposes.[11] The first evidence that nudibranchs were capable of *de novo* terpenoid biosynthesis came from the incorporation of ^{14}C labeled mevalonic acid into drimane sesquiterpenoids by the two Mediterranean dorids *Dendrodoris limbata* and *D. grandiflora*.[12] Subsequently, it was demonstrated that low levels of ^{14}C labeled mevalonic acid were incorporated into the terpenoid skeletons of the glycerides **1** and **4** by the B.C. nudibranchs *A. odhneri* and *A. montereyensis*.[2] Since the incorporation levels were low in *A. odhneri* and *A. montereyensis*, and other investigators were unable to observe incorporation of ^{14}C labeled mevalonic acid into terpenoic acid glycerides by *D. verrucosa*,[10] there has remained considerable uncertainty about the ability of dorids to make terpenoic acids *de novo*.

Biosynthetic studies utilizing NMR detection of stable isotopes provide unambiguous proof for the incorporation of labeled precursors. However, the successful use of stable isotope methodology requires levels

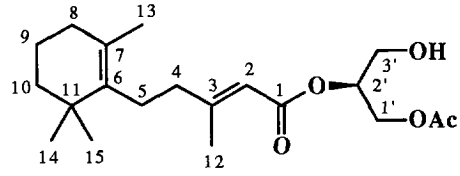
of incorporation that are sufficiently high to be detected by NMR. To date, the observed levels of incorporation of either radiolabeled acetate or mevalonic acid into terpenoid skeletons as a result of feeding studies conducted with marine invertebrates has generally been very low, which has discouraged the attempted use of stable isotopes.[13] Recently, it has been shown that it is possible to get sufficiently high levels of incorporation of [1,2-$^{13}C_2$]acetate into nudibranch terpenoids to provide unambiguous NMR proof for *de novo* biosynthesis and furthermore to test biogenetic hypotheses.[14] Although the previous experiments using ^{14}C labeled mevalonic acid had provided evidence for *de novo* terpenoid biosynthesis by *A. odhneri* and *A. montereyensis*, the uncertainty surrounding this result made it desirable to obtain unambiguous confirmation of *de novo* biosynthesis via stable isotope incorporation methodology. While this work was in progress at UBC, it was learned that the La Jolla group was also investigating the use of stable isotopes to demonstrate *de novo* biosynthesis of the terpenoic acid glyceride tanyolide B (**7**) by the California dorid *S. tanya*. Presented below is positive stable isotope incorporation evidence for the *de novo* biosynthesis of terpenoic acid glycerides by all three dorids, *A. odhneri*, *A. montereyensis*, and *S. tanya*.



1 $R_1=R_2=H$
2 $R_1=H$, $R_2=Ac$
3 $R_1=Ac$, $R_2=H$
8 $R_1=R_2=Ac$



4 $R_1=R_2=H$
5 $R_1=H$, $R_2=Ac$
6 $R_1=Ac$, $R_2=H$



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The experiments at Vancouver followed the protocol recently developed by the UBC group to demonstrate the *de novo* biosynthesis of the sesquiterpenoids nanaimoal and isoacanthodoral by the dorid *Acanthodoris nanaimoensis*.[14] This protocol is based on the observation that when nudibranchs are collected via SCUBA in the field and transported back to a laboratory for study, they usually shed most if not all of the secondary metabolites that can be extracted by simply immersing them whole in solvent. It is possible that the physical act of handling them simulates an attack by a predator, which causes them to release chemicals that might play a defensive role. From the perspective of a biosynthetic study, any manipulation that causes a nudibranch to shed some portion of its complement of unlabeled compounds should in principle activate any existing *de novo* biosynthetic pathway that can lead to replacement of the putative defensive compounds. With

the biosynthetic pathway activated, and the pool of unlabeled compounds depleted, conditions would appear to be ideally suited to realizing the desired goal of achieving high levels of incorporation of isotopically labeled precursors. Doubly labeled acetate was choosen as a precursor in the present experiments in order to generate doublet resonances for enriched carbons in labeled samples in order to provide visually convincing incorporation data that did not depend on measuring what were anticipated to be small peak height differences of singlet resonances that would result from low level incorporation of singly labeled precursors.

Specimens of *A. odhneri* and *A. montereyensis* were collected by hand using SCUBA in Barkley Sound B.C. and transported to Vancouver in refrigerated seawater. The nudibranchs were maintained at 12 °C in Barkley Sound seawater that was changed every second day for the duration of the experiments. Individual specimens of *A. odhneri* and *A. montereyensis* were given 100 μL injections of a 550 mM solution of [1,2-$^{13}C_2$]acetate every second day for 16 days. The handling of the animals required for each injection caused the nudibranchs to shed mucus and terpenoids, resulting in a partial turnover of the pool of terpenoic acid glycerides. On the eighteenth day, two days after the last injection, the nudibranchs were immediately immersed whole in methanol.

The methanol extracts from the injected specimens (20 animals) of *A. odhneri* were fractionated to give 130 mg of the major farnesic acid glyceride (**1**). Acetylation of glyceride **1** with acetic anhydride and pyridine at room temperature for 12 h gave the diacetate **8** in essentially quantitative yield. The methyl and carbonyl carbons of the acetate units in **8** provided an internal control of unlabeled carbons. Shown in Figure 1 are the truncated ^{13}C NMR resonances, all normalized to the same peak height for the central singlet component, for the terpenoid, glycerol and acetate carbons in the proton noise decoupled ^{13}C NMR spectrum of the labeled sample of **8**. The resonances assigned to the terpenoid carbons 1, 2, 3, 5, 6, 7, 9, 10, 11, 13, 14 and 15 as well as the resonances assigned to the glycerol carbons 1', 2' and 3' in the spectrum of labeled **8** all appear as central singlets due to natural abundance ^{13}C flanked by clear doublets resulting from specific incorporation of intact acetate units. As expected, the acetate methyls show only a singlet resonance resulting from natural abundance ^{13}C. The ^{13}C NMR spectrum of a control sample of completely unlabeled diacetate **8** acquired at the same concentration on the same spectrometer and for the same number of scans did not show any evidence for the doublet components (Figure 1). The intensity of the doublet components in the labeled spectrum of **8** indicates an average specific incorporation of intact acetate units of 0.09%.[15] Analysis of the coupling constants observed for the doublet components in the terpenoid carbon resonances (Table 1) revealed the pattern of intact acetate incorporation shown in **1a** (Scheme 1) that was completely consistent with the expected biosynthesis from mevalonic acid.

It is interesting to note that the resonances for the terpenoid carbons 4 and 8 in the ^{13}C NMR spectrum of the labeled diacetate **8** (Figure 1) also appear as singlets flanked by doublet components. In the case of these two resonances, the doublet components are less intense than those observed for the other terpenoid carbons. Despite their relatively low intensity, a comparison with the control spectrum of unlabeled **8** (Figure 1) indicates that the doublet components flanking the resonances for carbons 4 and 8 in the spectrum of labeled **8** must also be the result of incorporation of ^{13}C labeled acetate units. C-8 could be coupled to either or both of C-7 and C-9, and C-4 could be coupled to either or both of C-3 and C-5. Coupling constant data for the C-4 and C-8 resonances did not clearly indicate which neighboring carbons were responsible for the coupling.

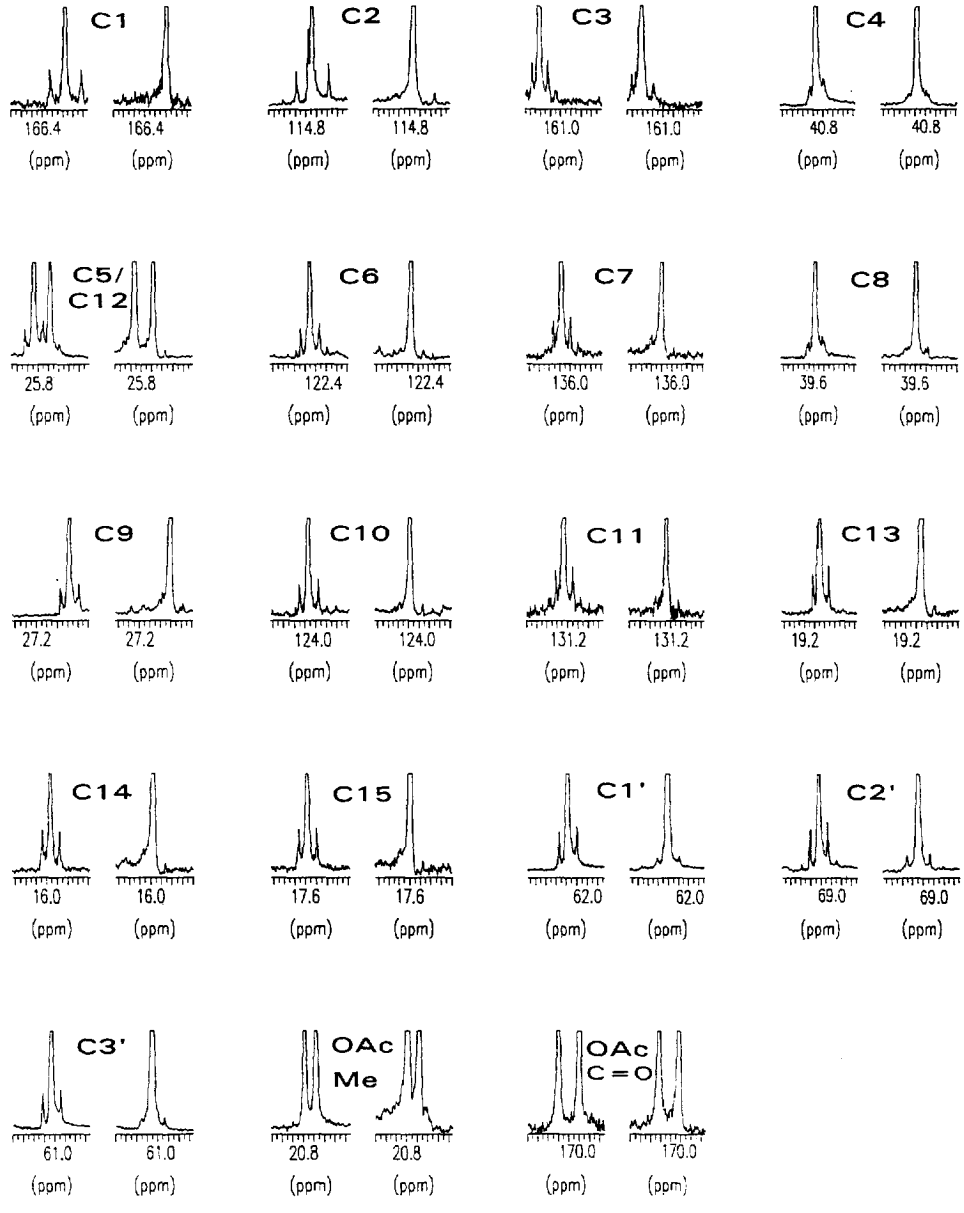
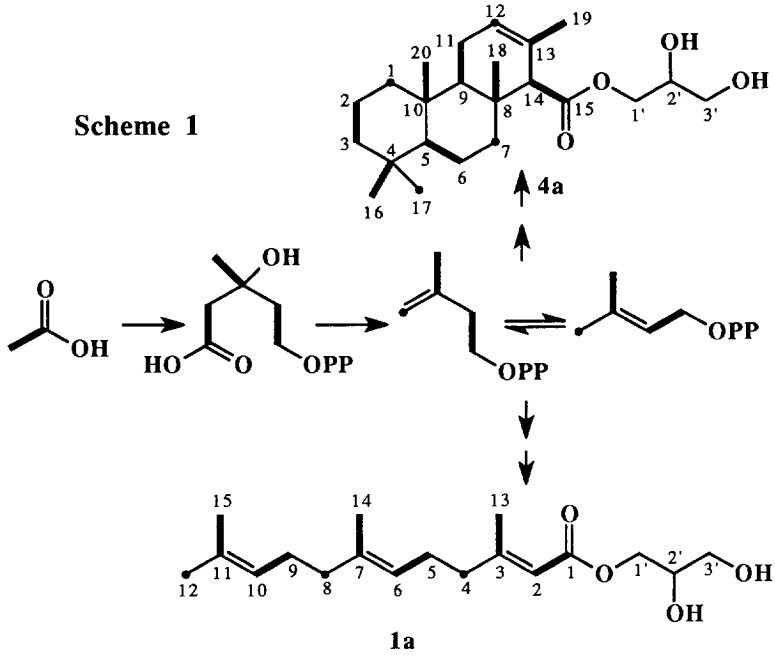


Figure 1. ^{13}C NMR resonances (125 MHz, $CDCl_3$) for labelled (left) and unlabelled (right) samples of the farnesic acid glyceride diacetate **8**. The central singlets have all been plotted at the same peak height for comparison purposes and then truncated for presentation.

Scheme 1



The most likely explanation for the coupling observed in the C-4 and C-8 carbons of labeled **8** is that it originates from incorporation of more than one ^{13}C labeled acetate unit into a single farnesic acid molecule.[14,15] The nudibranchs were starved during the 16 day injection period and this could well have led to a highly labeled acetate pool that in turn could have resulted in a reasonable probability of more than one labeled acetate unit being incorporated into individual mevalonic acid molecules and/or more than one labeled mevalonic acid unit being incorporated into a single farnesic acid residue. As a result, the observed C-4 coupling would arise from molecules of farnesic acid glyceride diacetate **8** having a singly labeled carbon at C-4 and an intact acetate unit at either C-3/C-13 or C-5/C-6. Similar clusters of three adjacent ^{13}C labeled carbon atoms in single molecules of **8** could lead to the coupling observed in C-8. Although the arguments presented above account for all of the features of the weak flanking doublets observed in the ^{13}C NMR spectrum of **8**, alternate origins for these doublets involving different incorporation patterns of intact acetate units into isopentenyl pyrophosphate and dimethylallyl pyrophosphate cannot be completely ruled out with the current data.

The presence of doublets flanking the singlet resonances for all three glycerol carbons 1', 2' and 3' indicates that intact acetate units have been incorporated at both positions C-1'/C-2' and at C-2'/C-3' in the glycerol residue. This incorporation pattern would result from esterification at both primary carbons of an achiral glycerol intermediate containing one intact acetate unit. Thus, the specific incorporation of acetate into the glycerol residue is the sum of the incorporations at the C-1'/C-2' and C-2'/C-3' positions.

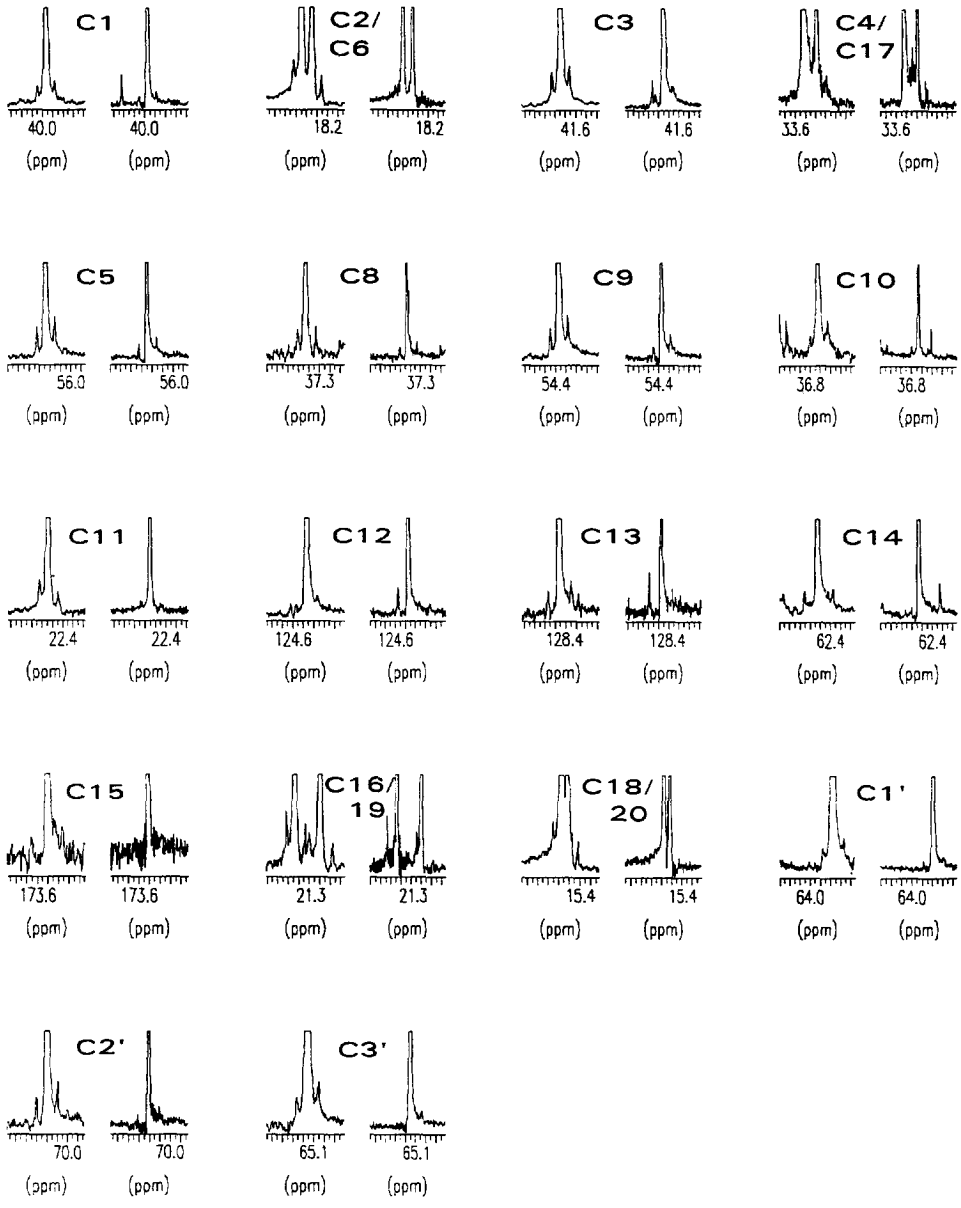


Figure 2. ^{13}C NMR resonances (125 MHz, $CDCl_3$) for labelled (left) and unlabelled (right) samples of the diterpenoic acid glyceride **4**. The central singlets have all been plotted at the same peak height for comparison purposes and then truncated for presentation.

The methanol extracts from the injected specimens of *A. montereyensis* (20 animals) were fractionated as previously described[3] to give a crystalline sample (38 mg) of the major diterpenoic acid glyceride **4**. Examination of the proton noise decoupled ^{13}C NMR spectrum for labeled **4** (Figure 2) revealed that intact acetate units had been incorporated into both the terpenoid and glycerol fragments although at lower levels than were observed for **1**. Analysis of the $^{13}C/^{13}C$ coupling constants in the spectrum of labeled **4** (Table 1) demonstrated that the incorporation pattern of intact acetate units in the terpenoid fragment was as shown in **4a** in Scheme 1.

Table 1: Specific incorporation data for the [1,2-$^{13}C_2$]acetate feeding experiments with *Archidoris odhneri* and *A. montereyensis*.

	Farnesic acid glyceride diacetate (**8**)			Diterpenoic acid glyceride (**4**)		
C#	δ ^{13}C(ppm) (125 MHz)[a]	$J_{C,C}$(Hz)	% specific incorporation[c]	δ ^{13}C(ppm) (125 MHz)[a]	$J_{C,C}$(Hz)	% specific incorporation[c]
1	165.9	76.3	0.15	39.9	(34.3)[b]	(0.03)[b]
2	114.7	76.3	0.10	18.7	33.6	0.06
3	161.4	40.0	0.08	41.9	33.6	0.08
4	40.9	(33.4)[b]	(0.03)[b]	33.0	35.1	0.05
5	25.9	43.9	0.07	56.5	34.3	0.07
6	122.7	43.9	0.07	18.5	35.1	0.10
7	136.1	42.0	0.08	41.9		
8	39.6	(33.4/ 41.0)[b]	(0.04)	37.4	35.9	0.12
9	26.6	43.9	0.08	54.3	34.3	0.06
10	124.1	44.8	0.07	36.6	35.9	0.08
11	131.2	42.0	0.09	22.7	35.1	0.06
12	25.4	(44.8)[b]	(0.007)[b]	124.3	(41.2)[b]	
13	18.9	39.1	0.10	128.5	44.2	0.09
14	15.9	42.0	0.08	62.6	57.2	0.05
15	17.6	42.0	0.09	173.4	59.5	(0.07)[b]
16				21.6	35.9	0.06
17				33.2		
18				15.6	35.9	0.08
19				21.2	44.2	0.05
20				15.7	35.1	0.05
1'	62.4	42.9	0.07	63.5	40.4	0.04
2'	69.2	42.9	0.09	70.4	41.2	0.07
3'	61.3	43.9	0.07	65.1	41.2	0.05

a) ^{13}C NMR assignments are based on COSY, HMQC and HMBC data.
b) These doublets are very weak making it difficult to obtain accurate coupling constant or specific incorporation measurements.
c) see footnote 16.

The experiments at La Jolla used [2-^{13}C] mevalonolactone as a precursor in a feeding experiment to determine whether *S. tanya* was capable of *de novo* biosynthesis of the tanyolides. Ten specimens were injected with 25 µl (5 mg) of [2-^{13}C] mevalonolactone in 1:1 EtOH/seawater and maintained in aquaria. Five animals were extracted with acetone after 24 hours, and the remaining 5 were extracted after 6 days. Tanyolide B (**7**) was purified from both treatments and ^{13}C spectra were recorded and compared with the spectrum of an unlabeled sample. Peak heights were normalized to C2', which should not have been affected by the labelling

experiment. The difference between the normalized height of each peak in a labeled sample and the corresponding normalized peak height from a control sample was calculated, to identify peaks showing ^{13}C enrichment (see Experimental and Table 2). Biogenetic theory predicts that C-4, C-8 and C-14/15 should all derive from the C-2 carbon of mevalonate. After 24 hours, the only centers showing significant enrichment were C-4 and C-8. In the 6 day treatment, C-4, C-8 and C-14/15 all showed greater enrichment than any other peak. When analyzed statistically with a one-tailed Mann Whitney U test,[17] this result was significant ($p<0.005$). The specific incorporation of [2-^{13}C] mevalonolactone was calculated to be 0.15%. Some enrichment was seen for the methyl group of the acetate, suggesting that some of the mevalonate had been degraded to [2-^{13}C] acetate and used to acetylate the glycerol portion of the molecule.

In summary, the experiments described above provide the first unambiguous proof for the *de novo* biosynthesis of terpenoic acid glycerides by dorid nudibranchs and they confirm the results of previous radioisotope investigations of the biosynthesis of **1** and **4**.[2] These experiments also provide a further demonstration[14] that stable isotope methodology can be used to investigate the biosynthesis of terpenoid metabolites by marine invertebrates.

Table 2: Peak height data for labeled and unlabeled tanyolide B (**7**) from *S. tanya* fed [2-^{13}C] mevalonolactone.

Carbon number	Unlabeled tanyolide B (**7**) peak height (mm)	Normalized to C-2'	Six day labeled tanyolide B (**7**) peak height (mm)	Normalized to C-2'	Difference
1	29	0.397	32.5	0.369	-0.03
2	72	0.986	90	1.023	0.04
3	36	0.493	47.5	0.540	0.05
4	**73**	**1.0**	**98.5**	**1.126**	**0.13**
5	75	1.027	91	1.040	0.01
6	30	0.411	41.5	0.472	0.06
7	34	0.466	42	0.477	0.01
8	**72**	**0.986**	**101**	**1.1154**	**0.17**
9	70	0.959	86	0.983	0.02
10	77	1.055	90	1.023	-0.03
11	41.5	0.569	51	0.583	0.01
12	54	0.740	68	0.777	0.04
13	53	0.726	62	0.709	-0.02
14/15	**150**	**2.055**	**196**	**2.240**	**0.19**
$COCH_3$	18.5	0.253	26.5	0.301	0.05
$COCH_3$	49	0.671	67	0.766	0.09
baseline noise	3	0.027	4	0.034	0.01

Experimental

[1,2-$^{13}C_2$]Acetate Incorporation Studies with *Archidoris odhneri* and *A. montereyenesis*: Forty specimens each of *A. odhneri* and *A. montereyensis* were collected in Barkley Sound, B.C. at depths of -20 to -30 m for the former and 0 to -10 m, for the latter. Twenty animals of each species were immediately immersed in 500 mL MeOH at the surface and used as control samples. The remaining animals were transported back to Vancouver in 12 °C Barkley Sound seawater, and each species was stored in separate

insulated containers in a cold room equilibrated to an air temperature of 12 °C. Sixteen hours after arrival in the laboratory, the seawater in each container was changed with fresh Barkley Sound sea water that had also been pre-equilibrated to 12 °C. Each animal was then injected through the dorsum on the ventral side between the rhinopores and gills with 100 μL of a freshly prepared 550 mM solution of [1,2-$^{13}C_2$]sodium acetate in doubly distilled water. Seven subsequent injections were performed at 48 h intervals, with a seawater change accompanying each injection. Forty-eight hours after the final injection, the remaining 20 animals of each species were sacrificed by immediate immersion in 500 mL MeOH.

Isolation of Farnesic Acid Glyceride (1) from *A. odhneri:* The control and injected specimens of *A. odhneri* were dealt with in exactly the same fashion as outlined below. After 24 h, the original 500 mL MeOH extract was decanted and filtered. Four more 500 mL extracts were made from the animals in the following order: MeOH, 2 X 1:1 MeOH: CH_2Cl_2, MeOH. All extracts were decanted, filtered, combined, and reduced *in vacuo* to yield an aqueous suspension that was diluted up to 500 mL with distilled water and extracted with four 500 mL portions of EtOAc. The combined EtOAc extracts were dried over $MgSO_4$, filtered, and reduced *in vacuo* to yield an orange oil. The orange oil was separated via silica gel flash chromatography (eluent: 1:1 hexanes/ EtOAc) to yield a fraction enriched in farnesic acid glyceride (**1**). This fraction was repeatedly subjected to reversed phase HPLC (eluent: 4:1 MeOH/ H_2O) to yield a pure sample of **1**: (yield: control 71 mg; injected specimens 130 mg).

One mL of pyridine and 2 mL of acetic anhydride was added to each sample of **1** and this reaction mixture was allowed to stir for 16 h at room temperature. Purification of the labeled and unlabeled diacetates via silica gel flash chromatography (eluent: 85/15 hexane: EtOAc) yielded pure samples of labeled and unlabeled diacetate **8** in essentially quantitative yield.

Isolation of the Diterpenoic Acid Glyceride 4 from *A. montereyensis:* Extracts were made of each group of *A. montereyensis* specimens in exactly the same manner as for *A. odhneri,* as outlined above. The oil that resulted from the EtOAc/ H_2O partitioning of the combined crude extracts was subjected to silica gel flash chromatography (eluent: 1:1 hexanes/EtOAc) to yield a fraction that was highly enriched in the diterpenoic acid glyceride (**4**). This fraction was recrystallized from hexane (HPLC grade) to yield pure (**4**) as white needles. (yield: control 52.6 mg; injected specimens 38.0 mg).

[2-^{13}C] Mevalonolactone Incorporation with *Sclerodoris tanya*: Ten specimens were injected with 25 μl (5 mg) of [2-^{13}C] mevalonolactone (Isotech Inc.) dissolved in 1:1 EtOH/seawater into the body cavity of the animal. Five animals died after 24 hours and were extracted together in acetone. The remaining five animals survived for 6 days, at which point they were sacrificed and immersed in acetone. Pure tanyolides were obtained with the same partitioning and chromatography scheme as before.[9] ^{13}C spectra were recorded for labeled and unlabeled samples of **7** of equivalent mass using identical acquisition parameters. Peak heights were measured as millimeters above the baseline, and normalized to the height of C-2'. Significant positive differences in normalized height indicate incorporation. After 24 h, only C-4 (+0.07) and C-8 (+0.08) showed incorporation. After 6 days, significant incorporation was seen for C-4 (+0.13), C-8 (+0.17) and C-14/15 (+0.19); all other changes were < 0.06. The labeled peaks were ranked according to normalized height increase and analyzed nonparametrically using a one-tailed Mann Whitney U test.[17] This test tells you the probablity of randomly obtaining a particular ranking and it is used when the numerical data are not in themselves reliable

due to sampling errors, a situation that arises in the measurement of peak heights in ^{13}C NMR spectroscopy. The results for the 6 day trial were highly significant ($p < 0.005$).

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16. Numbers listed for specific incorporations = % enrichments above natural abundance = 1.1% X (combined integrated peak area of enriched satellites minus the combined theoretical peak area for these same satellites resulting from natural abundance coupling)/(peak height of the natural abundance singlet plus the combined theoretical peak area for all satellites resulting from natural abundance coupling). The probability of observing natural abundance coupling between a pair of adjacent carbons is 0.011 X 0.011 = 0.000121. The intensity of this signal would be split between the two doublet components resulting in a predicted abundance of 0.0000605 for each component. Thus the doublet components should each be (0.0000605/(0.011 - n X 0.000121)) X 100 = 0.55% of the intensity of the unenriched central singlet for each coupling interaction. (n is the number of next neighbor carbons and it must have values between 1 and 4. Therefore, 0.011 - n X 0.000121 is always $\approx$ 0.011.).

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