Investigations of Sesquiterpenoid Biosynthesis by the Dorid Nudibranch *Acanthodoris nanaimoensis*

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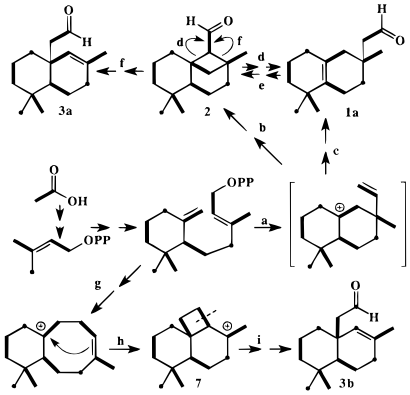
Marine invertebrates are an exceptionally rich source of terpenoids that have new carbon skeletons.[1] The structural diversity found in marine terpenoids has prompted investigations into their total synthesis,[2] their potential for drug development,[3] and their roles in chemical ecology.[4] Many proposals have been put forth attempting to rationalize the biogenetic origins of novel marine terpenoid carbon skeletons.[1] This active theoretical interest in the biogenesis of marine terpenoids has not been followed up with experimental tests of the hypothetical pathways. Only a small number of successful incorporations of isotopically labeled precursors into marine invertebrate terpenoid skeletons have been reported,[5] and these have all involved the use of either ^{14}C- or ^{3}H-labeled precursors. In most instances, the levels of incorporation were extremely low.

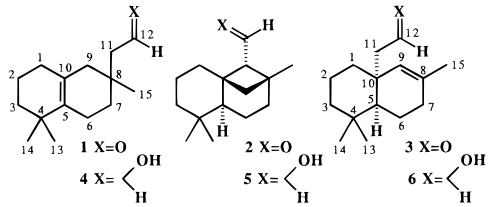
Skin extracts of dorid nudibranchs have yielded a wide variety of interesting terpenoids.[1,7−9] Several of these terpenoids appear to be effective feeding deterrents that play an important role in the survival of the shell-less molluscs.[6] The majority of terpenoids isolated from dorid nudibranchs are known to be sequestered from their diets.[7] Cimino et al. provided the first evidence for *de novo* terpenoid biosynthesis by nudibranchs when they showed that ^{14}C-labeled mevalonic acid was incorporated into sesquiterpenoids by *Dendrodoris limbata*.[8] Subsequently, our group reported the low-level incorporation of ^{14}C-labeled mevalonic acid into the terpenoic acid glyceride metabolites of the dorids *Archidoris montereyensis* and *Archidoris odhneri*.[9] Nudibranchs that sequester compounds from invertebrates in their diet frequently show significant variation in their terpenoid constituents from one collection site to another, reflecting a difference in the terpenoid content of their diet.[7] Those that make terpenoids via *de novo* biosynthesis would be expected to have similar terpenoid constituents at all sites. Thus, geographic invariance of terpenoid content, while not an absolute predictor of *de novo* biosynthesis, can be used to identify candidates for biosynthetic investigations.

Acanthodoris nanaimoensis is common on the outer coast of British Columbia. Skin extracts of *A. nanaimoensis* collected at many sites along the BC coast always contain nanaimoal (**1**), acanthodoral (**2**), and isoacanthodoral (**3**), which all have unprecedented sesquiterpenoid skeletons.[10] It has been proposed that the nanaimoane and isoacanthodorane skeletons might arise

Scheme 1



via opening of the cyclobutane ring in an acanthodorane precursor as shown in Scheme 1 (d and f).[10b] Alternatively, the nanaimoane skeleton could be formed via direct cyclization of a monocyclofarnesane precursor (Scheme 1, a, c),[10a] and it in turn could be a precursor to the acanthodorane skeleton (via e). Interest in the biogenesis of the three new sesquiterpenoid skeletons represented by **1**, **2**, and **3** prompted us to undertake isotope incorporation studies with *A. nanaimoensis*. The objectives were (i) to demonstrate that **1**−**3** were being made *de novo* by *A. nanaimoensis*, (ii) to increase the levels of precursor incorporation to a point where NMR detection of the stable isotope ^{13}C was feasible, and (iii) to compare the incorporation patterns with those predicted by the proposals in Scheme 1.



The low levels of precursor incorporation into metabolites of marine invertebrates are usually attributed to a very slow rate of biosynthesis.[5] Nudibranch biology appeared to offer the possibility of overcoming this impediment.[7,9−11] When nudibranchs are collected in the field and transported live back to a laboratory for study, they usually shed most of the secondary metabolites that can be extracted by simply immersing them whole in solvent. The physical act of handling the nudibranchs perhaps simulates an attack by a predator, which causes them to release chemicals that might play a defensive role. Any manipulation that causes a nudibranch to shed a substantial portion of its secondary metabolites should in principle activate existing *de novo* biosynthetic pathways that are capable of replenishing the metabolites. With the biosynthetic pathway activated and the pool of unlabeled compounds depleted, conditions should be ideally suited to achieving high levels of incorporation of isotopically labeled precursors.

Specimens (95 animals) of *A. nanaimoensis* were collected via SCUBA in Barkley Sound, BC and transported back to UBC in refrigerated sea water. The nudibranchs were maintained at 12 °C in an aquarium filled with Barkley Sound sea water that was changed every 2 days. Individual specimens of *A. nanaimoensis* were given 100 μL injections of a 550 mM solution of [1,2-$^{13}C_2$]acetate every second day for 16 days. Two days after the last injection the specimens of *A. nanaimoensis*

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(2) (a) Liu, H.-J.; Ulibarri, G.; Nelson, L. A. K. *J. Chem. Soc., Chem. Commun.* **1990**, *20*, 1419−21. (b) Piers, E.; Wai, J. S. M. *Can. J. Chem.* **1994**, *72*, 146−57.
(3) Glaser, K. B.; de Carvalho, M. S.; Jacobs, R. S.; Kernan, M. R.; Faulkner, D. J. *Mol. Pharmacol.* **1989**, *36*, 782−8.
(4) *Ecological Roles of Marine Natural Products*; Paul, V. J., Ed.; Cornell University Press: Ithaca, NY, 1992.
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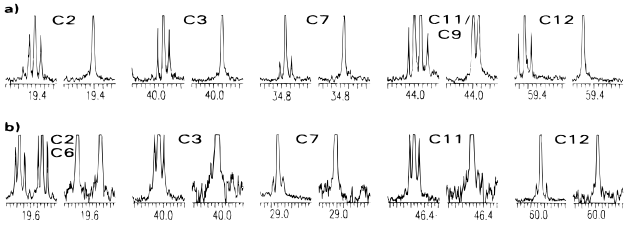

Figure 1. (a) ^{13}C NMR resonances ($CDCl_3$, 125 MHz) for isotopically labeled (left-hand resonances) and unlabeled (right-hand resonances) nanaimool (**4**). All truncated resonances have been normalized to the same peak height for the central singlet component. (b) Resonances for isoacanthodorol (**6**).

Table 1. ^{13}C NMR Data for Labeled Nanaimool (**4**) and Isoacanthodorol (**6**) Recorded in $CDCl_3$ at 125 MHz

	nanaimool (**4**)			isoacanthodorol (**6**)		
C	δ ^{13}C (ppm) (125 MHz)[a]	$J_{C,C}$ (Hz)	specific incorporation[b]	δ ^{13}C (ppm) (125 MHz)[a]	$J_{C,C}$ (Hz)	specific incorporation[b]
1	31.7	33.4	0.12	37.9	29.6	0.11
2	19.4	33.4	0.33	19.2	33.4	0.28
3	39.8	33.4	0.33	40.1	33.4	0.27
4	33.5	35.3	0.18	34.1	35.3	0.49
5	133.4	41.0	0.51	45.5	34.3	0.28
6	21.4	41.0	0.32	19.9	33.4	0.31
7	34.7	34.3	0.13	29.0	36.2	0.12
8	30.7	36.2	0.51	134.1	42.9	0.54
9	43.8	43.9	0.49	131.6	41.0	0.10
10	125.5	42.0	0.25	37.4	33.5	0.47
11	44.0	34.3	0.26	46.8	34.3	0.29
12	59.6	37.2	0.37	60.1	37.2	0.07
13	28.0	35.3	0.28	26.7	35.3	0.31
14	27.8	35.3	0.08	32.0	35.3	0.09
15	24.8	35.3	0.34	23.4	43.9	0.46

[a] Based on COSY, HMBC, and HMQC data. [b] See ref 12.

were carefully removed from the aquarium, and immediately immersed in methanol (250 mL). The EtOAc soluble portion of the methanol extract (650 mg) contained aldehydes **1**−**3**.

Reduction of the mixture of aldehydes with $NaBH_4$ followed by reversed phase HPLC purification (eluent: 4:1 $MeOH/H_2O$) gave pure samples of **4** (10 mg, 0.1 mg/animal) and **6** (7.0 mg, 0.07 mg/animal) and only a trace of **5**. Shown in Figure 1a are the truncated ^{13}C NMR resonances for a representative group of carbon atoms in both a labeled sample and an unlabeled control sample of **4**. All of the the resonances shown in Figure 1a, and indeed all of the resonances in the ^{13}C NMR spectrum of labeled **4** (supporting information), clearly show flanking doublets resulting from the incorporation of ^{13}C-labeled acetate units. An analysis of the intensity of the flanking doublets (Table 1) indicates that there is one set of relatively intense doublets flanking the resonances assigned to C-2, C-3, C-5, C-6, C-8, C-9, C-10, C-11, C-12, C-13, and C-15 (average specific incorporation 0.35%)[12] and another set of relatively weak doublets flanking the resonances for C-1, C-7, and C-14 (average specific incorporation 0.11%). A complete analysis of the $^{13}C/^{13}C$ coupling constants (Table 1) for the intense doublets revealed the pattern of intact acetate unit incorporation for nanaimoal indicated in **1a**, which is consistent with biogenesis from mevalonic acid following either pathway a, c or a, b, d in Scheme 1.

Pathways a, c or a, b, d predict that the ^{13}C label incorporated at C-1, C-7, and C-14 in labeled **4** should not be part of intact acetate units. Therefore, if only one labeled acetate unit was incorporated per labeled molecule of **4**, the resonances for carbon atoms C-1, C-7, and C-14 should appear as enriched singlets. The most likely explanation for the weak flanking doublets observed in the C-1, C-7, and C-14 resonances of labeled **4** is that they originate from incorporation of more than one ^{13}C-labeled acetate unit into a single nanaimoal (**1**) molecule.[13] *A. nanaimoensis* 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 and/or more than one labeled mevalonic acid unit being incorporated into individual molecules of **1**. As a result, the C-7 doublet would arise from molecules of **4** having a singly enriched carbon at C-7 and an intact acetate unit at either C-5/C-6 or C-8/C-15. Similar clusters of three adjacent ^{13}C-labeled carbon atoms in single molecules of **4** could lead to the couplings observed in C-1 and C-14.

The ^{13}C NMR spectrum of labeled **6** also showed evidence for significant levels of labeled acetate incorporation (Figure 1b and supporting information). Once again there was one set of relatively intense doublets and another set of relatively weak doublets resulting from incorporation of isolated intact acetate units and incorporation of more than one adjacent labeled acetate unit per molecule, respectively. An analysis of the coupling constants observed for the complete set of intense doublets (Table 1) revealed the pattern of acetate incorporation shown in **3b** in Scheme 1. Of particular significance was the obvious difference in intensity of the doublets flanking the C-11 and C-12 resonances, which provided a clear demonstration that C-11 and C-12 do not arise from an intact acetate unit. This result was unexpected, and it ruled out the proposed pathway f to the isoacanthodorane skeleton shown in Scheme 1. An alternate pathway g, h, i to the rearranged isoacanthdorane skeleton, which is consistent with the observed acetate incorporation pattern, is presented in Scheme 1.

The present work represents the first successful use of stable isotope detection by NMR to study the biosynthesis of terpenoid carbon skeletons by a marine invertebrate. Incorporation of ^{13}C-labeled acetate into nanaimoal (**1**) and isoacanthodoral (**3**) has demonstrated that *A. nanaimoensis* is capable of *de novo* terpenoid biosynthesis, and a detailed analysis of the incorporation patterns has uncovered an unanticipated rearrangement in the biogenetic pathway to the isoacanthodorane skeleton.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of isotopically labeled **4** and **6** (7 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(12) Numbers listed for specific incorporations = percent enrichments above natural abundance = 1.1% × (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).

(13) For a precedent, see: Needham, J.; Hu, T.; McLachlan, J.; Walter, J.; Wright, J. *J. Chem. Soc., Chem. Commun.* **1995**, 1623−4.