Stable isotope incorporation evidence for a polyacetate origin of the acyl residues in triophamine, a diacylguanidine metabolite obtained from the dorid nudibranch *Triopha catalinae*

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Biosynthetic feeding experiments with sodium [1,2-$^{13}C_2$]-acetate have shown that the acyl residues of triophamine 1 are formed *de novo* in *Triopha catalinae* from five intact acetate units.

Skin extracts obtained from dorid nudibranchs have proven to be a very rich source of novel secondary metabolites.[1] Most of the metabolites reported from dorid skin extracts are known to be sequestered unchanged from the sponges, soft corals, bryozoans and tunicates that make up their diets.[2] A small number of dorids have been shown to be capable of *de novo* biosynthesis of their skin metabolites.[3] Extracts of nudibranchs that sequester secondary metabolites from their diets frequently show a significant variation in metabolite content at different collecting sites, reflecting the changing secondary metabolite content of their dietary organisms.[2,4] Nudibranchs that make secondary metabolites *via de novo* biosynthesis give extracts of constant metabolite composition over their entire geographic range.[3c,d]

The dorid nudibranch *Triopha catalinae*, commonly called the 'clown nudibranch' because of the bright orange tubercles covering its whitish-gray body, is found in rocky subtidal habitats all along the western coast of North America.[5] Skin extracts of *T. catalinae* contain the unique diacylguanidine metabolite triophamine **1** at all collecting sites from Alaska to southern California.[6] *T. catalinae*'s diet is made up exclusively of bryozoans. Careful examination of extracts made from all of the obvious species of bryozoans collected from the same habitats as *T. catalinae* has never resulted in the isolation of triophamine **1**. The geographic invariance of triophamine **1** occurrence in *T. catalinae* extracts,[2,3] the apparent lack of a dietary origin, and its novel diacyl guanidine structure combined to make triophamine **1** an ideal candidate for biosynthetic investigations.

Examination of the structure of triophamine **1** suggested a polyketide biogenetic origin for the ten carbon acyl residues. Of particular interest was the origin of the two ethyl branches on the linear C-1 (C-1′) to C-6 (C-6′) or C-1 (C-1′) to C-8 (C-8′) chains. Ethyl branches are only rarely encountered in natural products formed by polyketide biosynthesis. Two examples can be found in the structures of the microbial metabolites lasalocid A and monensin. Biosynthetic experiments have shown that the ethyl branches in lasalocid A and monensin arise from butyrate presumably *via* ethyl malonate.[7] Several investigations of polyketide biosynthesis by marine pulmonate molluscs have been previously reported.[8] These have all focused on metabolites such as denticulatin A that have a polypropionate biogenetic origin[8b] and in all instances radiolabelled precursors were used to detect incorporation. The demonstration that molluscs are capable of polypropionate biosynthesis raised the possibility that the ethyl branches in the acyl residues in triophamine came from propionate plus some one carbon unit.

Recently, we have shown that it is possible to use stable isotope methodology to investigate the biosynthesis of terpenoid metabolites by dorid nudibranchs.[3c,3d] Thus, our initial biosynthetic investigation of triophamine had two objectives: (*i*) to demonstrate that **1** was being made *via de novo* biosynthesis by *T. catalinae* as indicated by the geographic invariance of its occurrence, and (*ii*) to extend the use of stable isotope methodology to the study of polyketide biosynthesis in marine molluscs. The precursor chosen for the initial feeding experiment was sodium [1,2-$^{13}C_2$]acetate.

Specimens of *T. catalinae* (30 animals) were collected by hand using SCUBA at depths of 5 to 10 m in surge channels off Sanford and Fleming Islands, Barkley Sound, BC, Canada and carefully transferred to a running seawater tank at the Bamfield Marine Station. Four hours after collection each animal was injected with 100 μl of a freshly prepared 550 mM solution of sodium [1,2-$^{13}C_2$]acetate in distilled water. Injections were made through the dorsum directly into the large digestive gland. Each animal was given a second identical injection of doubly labelled acetate 24 h after the first and then the animals were left unmolested and without food in the running seawater tank for 9 days. The experiment was terminated by gently removing the animals from the running seawater and immediately immersing them in methanol (250 ml).

Triophamine **1** was isolated from the *T. catalinae* methanol extract as previously described[6] and the ^{13}C NMR assignments (125 MHz, $CDCl_3$) listed in Table 1 were confirmed by analysis of COSY, HMQC and HMBC data. Shown in Fig. 1 are the ^{13}C NMR resonances for a labelled sample and an unlabelled sample of triophamine **1**. The central singlets in all of the resonances have been normalized to the same peak height and the resonances have been truncated in order to highlight the flanking doublets in the labelled spectrum. It was obvious from examination of the ^{13}C NMR data that intact units of doubly labelled acetate had been incorporated into triophamine **1** in the feeding experiment with an average specific incorporation of 0.24%‡ Analysis of the coupling constants for the flanking doublets (Table 1) clearly showed the presence of intact acetate units at C-9/C-10 (C-9′/C-10′), C-7/C-8 (C-7′/C-8′) and C-5/C-6 (C-5′/C-6′). The non-protonated carbons C-1 (C-1′) at δ 185.6 and C-4 (C-4′) at δ 139.0 gave resonances that were too weak

Table 1 Specific incorporation data for triophamine **1**

Carbon no.	δ(ppm)	*J*/Hz	Specific incorporation‡
1, 1′	185.6 (br)	—	—
2, 2′	50.2	51.9	0.16
3, 3′	39.3	42.7	0.20
4, 4′	139.0	—	—
5, 5′	120.5	43.5	0.28
6, 6′	13.1	42.7	0.34
7, 7′	22.6	33.6	0.15
8, 8′	12.7	32.8	0.24
9, 9′	25.4	34.3	0.24
10, 10′	12.1	35.1	0.29
11	157.4	—	—

and noisy to allow detection of flanking doublets. However, the resonances for C-2 (C-2′) and C-3 (C-3′) clearly showed flanking doublets indicating that they had been labelled by intact acetate units. The intact acetate generating the coupling observed in the C-2 (C-2′) resonance could be located at either C-1/C-2 (C-1′/C-2′) or C-2/C-3 (C-2′/C-3′). It was apparent from the magnitudes of the coupling constants observed for the C-2 (C-2′) (*J* 51.9 Hz) and C-3 (C-3′) (*J* 42.3 Hz) flanking doublets that C-2 (C-2′) and C-3 (C-3′) were not coupled to each other and, therefore, the intact acetate units had to be located at C-1/C-2 (C-1′/C-2′) and C-3/C-4 (C-3′/C-4′) giving the overall labelling pattern for triophamine **1** shown in Scheme 1.

The sodium [1,2-$^{13}C_2$]acetate feeding experiment has demonstrated that triophamine **1** is biosynthesized *de novo* by *T. catalinae*. Analysis of the ^{13}C NMR data for the labelled compound **1** has shown that the ten carbon acyl residues are formed from five intact acetate units. The current study represents the first demonstration that it is feasible to use stable isotopes to investigate polyketide biosynthesis in a marine invertebrate and it is the first demonstration that dorid nudibranchs are capable of polyketide biosynthesis. Further experiments designed to identify advanced intermediates involved in the formation of the ten carbon acyl residues from acetate are in progress.

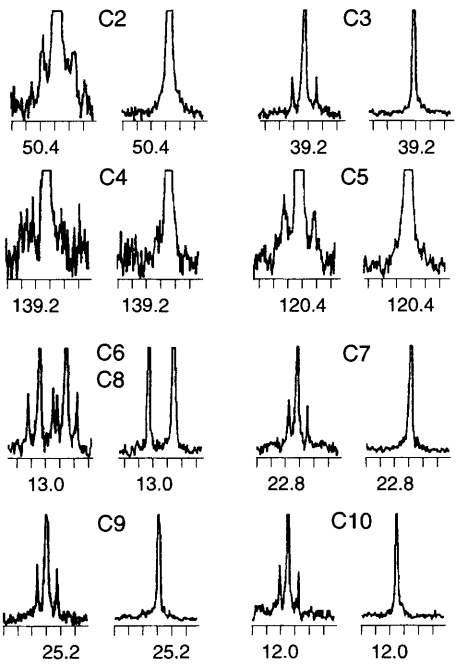
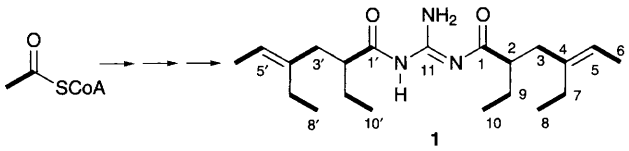


Fig. 1 Proton noise decoupled ^{13}C NMR resonances for labelled (left hand resonances) and unlabelled (right hand resonances) samples of triophamine **1**. All resonances have been normalized to the same peak height and then truncated.



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Scheme 1

Financial support was provided by the Natural Sciences and Engineering Research Council of Canada. The authors thank Mike LeBlanc and the staff of the Bamfield Marine Station for assistance.

Footnotes

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‡ Numbers listed for specific incorporations = % 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)]. The probability of observing natural abundance coupling between a pair of adjacent carbons is 0.011 × 0.011 = 0.000 121. The intensity of this signal would be split between the two doublet components resulting in a predicted abundance of 0.000 060 5 for each component. Thus the doublet components should each be [0.000 060 5/(0.011 − *n* 0.000 121] × 100 ~ 0.55% of the intensity of the unenriched central singlet for each coupling interaction (*n* is the number of next neighbour carbons and it must have a value between 1 and 4; therefore, 0.011 − *n* 0.000 121 is always ~ 0.011). In practice the corrections for natural abundance coupling that have been included in the above calculation for specific incorporation are so small that they have a negligible effect on the resulting values.

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Received, 11th July 1996; Com. 6/04871D