Biosynthesis of Alkylpyridines

Biogenesis of 3-Alkylpyridine Alkaloids in the Marine Mollusc *Haminoea Orbignyana***

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In comparison with our knowledge of terrestrial plant and microbial systems, little is known about the biogenesis of secondary metabolites in marine organisms, such as sponges, tunicates, algae and molluscs.[1] Although most of these compounds show more than one analogy with terrestrial metabolites, there are a few categories of products that seem to be structurally specific to marine species. One such group of compounds is formed by 3-alkylpyridine alkaloids (also named 3-alkylpiperidine alkaloids), a family of natural products that encompasses a very heterogeneous collection of molecules sharing an hypothetical common origin from a putative 3-alkylpyridine precursor (or a biochemical analogue).[2] Although several new members of this class of products were characterized over the last decade from Haplosclerida sponges[3] and Cephalaspidea molluscs,[4] no biosynthetic study has been reported in the literature to date. Herein, we describe the biosynthesis of haminol-2 (**1**) in the Mediterranean mollusc *Haminoea orbignyana*, the first in vivo evidence on the biogenesis of 3-alkylpyridines in marine organisms.

Mediterranean molluscs of the genus *Haminoea* (Opisthobranchia: Cephalaspidea) are chemically characterized by the presence of oxygenated 3-alkylpyridines, commonly named haminols, which when secreted in the mucus act as alarm pheromones inducing escape reaction in conspecifics.[5] The structure of **1**, which was first isolated along with its deacetyl derivative, **2**, from *H. orbignyana*,[5b] exemplifies well

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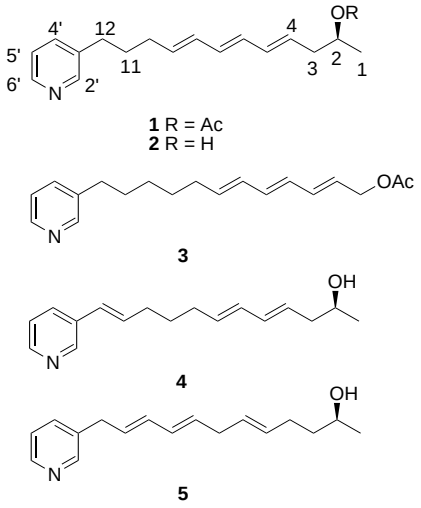
the peculiar characteristics of cephalaspidean alkylpyridines that, with the exception of **3**, has a polyunsaturated 12-membered chain with a hydroxy group at the *S*-configured C-2 center (e.g., **4** and **5**).[5]



To test the ability of *H. orbygniana* to produce de novo haminol-1 (**2**) and **1**, two preliminary experiments were performed by feeding either [2-^{14}C]-acetic acid (30 specimens, 0.3 μCi/specimen) or nicotinic acid-carboxy-^{14}C (18 specimens, 0.5 μCi/specimen). After the injection, the animals were starved three days in an aquarium before carrying out the extraction and purification of the secondary metabolites. Significant levels of radioactivity were recovered in the haminols (**1** and **2**) from both experiments, thus proving the de novo origin of the alarm pheromones in the cephalaspideans. Notably, incorporation of radioactive nicotinic acid into **2** and **1** provided the first evidence for the involvement of this molecule in the biogenesis of 3-alkylpyridines. To confirm these unexpected results and address the biosynthesis of **1** in *H. orbignyana*, other two groups of molluscs were injected twice over four days with either d_4-nicotinic acid ethyl ester or [1-^{13}C]-acetic acid. A third population of opisthobranchs was frozen and kept as control sample. Organic extracts of treated and control animals were prepared by soaking the frozen specimens in acetone, and **1** was purified from the resulting Et_2O-soluble fractions by radial TLC (8:2 *n*-hexane/ethyl acetate) on Chromatotron (Harrison Research). The analysis of the biosynthetic experiments was preceded by a complete assignment of the NMR data of **1** (^{1}H NMR: 400 MHz, $CDCl_3$, 19 °C; ^{13}C NMR: 100 MHz, $CDCl_3$, 19 °C; Table 1).

A feeding experiment with [D_4]nicotinic acid ethyl ester led to the labeling of the pheromones. In fact, after the injection of the deuterated precursor to 50 specimens of *H. orbignyana*, LC-MS (APCI; atmospheric pressure chemical ionization) analysis of the resulting product, **1**, showed a MS pseudomolecular ion at $m/z = 304$, which substantiated the retention of the four deuterium atoms (natural, $m/z = 300$ [$C_{19}H_{25}NO_2 + H^+$]; labeled, $m/z = 304$ [$C_{19}H_{21}D_4NO_2 + H^+$]). In SIM mode, integration of the HPLC peaks associated with natural and labeled **1** suggested that 5% of the whole content

Table 1: NMR data of haminol-2 (**1**) from natural and labeled samples.

	$\delta(^1H)$	$\delta(^{13}C)$	% Apparent Enrichment[a]
1	1.20, s	19.5	−4.7
2	4.93, m	70.3	23.8
3	2.35, m; 2.31, m	39.2	−5.5
4	5.59, m	128.5	33.5
5	6.09, m	131.6	−3.3
6	6.09, m	130.7	33.6
7	6.09, m	133.3	0.0
8	6.09, m	131.1	25.3
9	5.67, m	133.8	−6.6
10	2.14, bq	32.1	23.1
11	1.73, m	30.6	−3.7
12	2.62, t	32.4	−2.1
2′	8.44, bs	149.9	6.0
3′	–	137.5	5.6
4′	7.49, bd	135.9	6.0
5′	7.20, dd	123.3	36.0
6′	8.44, bs	147.1	6.7
CO_{Ac}	–	170.6	116.5
Me_{Ac}	2.01, s	21.3	–

[a] From feeding experiments with [1-^{13}C]-acetic acid. The apparent enrichment was expressed as variation of the peak intensity in labeled and natural samples. Spectra were normalized to the signal at $\delta =$ 21.3 ppm (methyl group of the acetyl residue) and the values were calculated on the basis of the following formula: (labeled signal−natural signal)/natural signal.

of the pheromone derived from the exogenous precursor. The final evidence that nicotinic acid (**6**) was incorporated into the molecule of **1** came with ^{2}H NMR analysis (Figure 1). In fact, the ^{2}H NMR (62 MHz, CH_2Cl_2, 19 °C) spectrum (Figure 1, top) of labeled **1** showed three peaks at $\delta = 8.40$ (H-2′ and H-6′), 7.20 (H-5′), and 7.49 ppm (H-4′), which were in good agreement with the signals of the pyridine ring in the isotopically natural sample (Figure 1, bottom). The integration of these three resonances indicated a ratio of 2:1:1, thus confirming that there was complete retention of deuterium

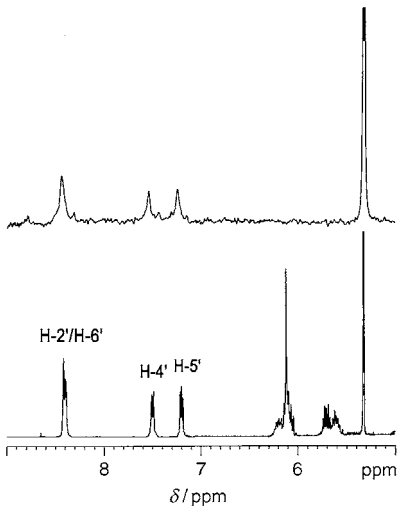


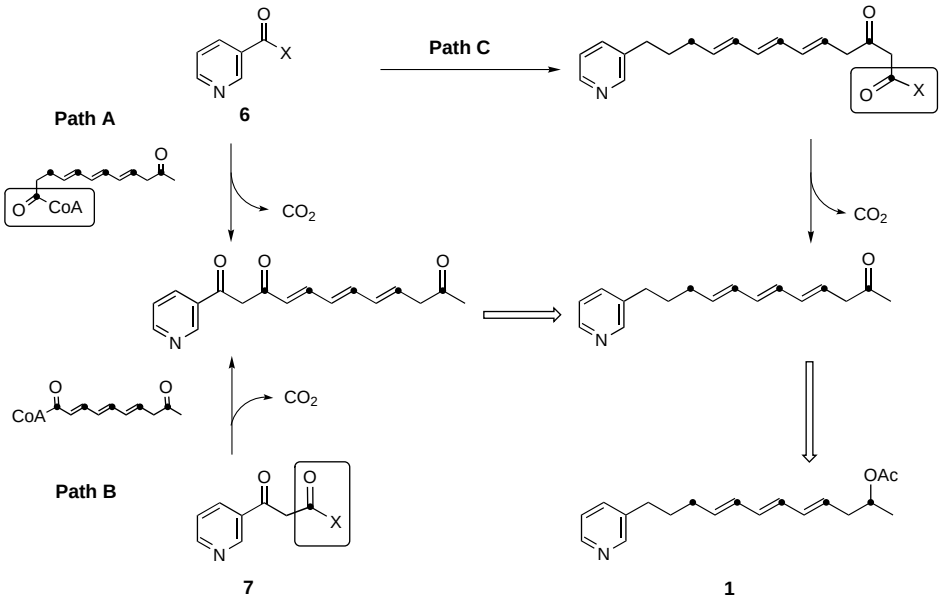
Figure 1. Incorporation of [D_4]nicotinic acid in haminol-2 (**1**): ^{2}H NMR (62 MHz, CH_2Cl_2, 19 °C) spectrum (top) and ^{1}H NMR (400 MHz, CD_2Cl_2, 19 °C) spectrum (bottom).

during the biosynthesis and ruling out any change of the oxidation state of the pyridine. This was of particular interest since dihydropyridine intermediates are required for the decarboxylation of the nicotinic acid.[6] The presence of all deuterium atoms implied, therefore, the preservation of the carboxylic carbon of **6**, thus proving the incorporation of an intact molecule of the precursor. This result was also in full agreement with the results of the labeling of **1** recorded by feeding experiments with [1-^{14}C]-nicotinic acid.

Once the origin of both the C-12 center and the pyridine ring of **1** had been established, the question of the biosynthesis of the alkyl chain remained. A comparison of the ^{13}C NMR spectra of **1** from natural and treated molluscs demonstrated that C-2, C-4, C-6, C-8, and C-10 had been enriched after the injection of [1-^{13}C]-acetic acid(Table 1). The labeling pattern confirmed the acetogeninic origin of the alkyl chain and suggested that the hydroxy group at C-2 could be directly derived from the carbonyl moiety of an acetate unit. As expected, no incorporation was evident at C-12, whereas we recorded a significant increase of the signals for the acetyl group (C-1, $\delta = 170.6$ ppm) and C-5′ ($\delta =$ 123.3 ppm) of the pyridine moiety (Table 1). This latter finding is consistent with the involvement of acetate-derived glyceraldehyde 3-phosphate in the formation of nicotinic acid.[6] Although the experiments with ^{13}C-labeled acetates proved the incorporation of five acetate units into the aliphatic part of **1**, they did not clarify the mechanism leading to the assembly of the pheromone molecule. In fact, three different pathways could be proposed on the basis of the experimental data (Scheme 1). Paths A and B involve the condensation of a hexa- or pentaketide moieties with the nicotinic derivatives **6** and **7**, respectively. On the other hand, path C relies on incorporation of acetate via a polyketide biogenesis by using nicotinic acid as starter unit. In this view, the double bond position of **1** is not in agreement with the classical reduction of the growing polyketide intermediates, which suggests that shift of the double bonds might occur during the biosynthetic process. Every path described in Scheme 1, however, implies the loss of one carbon atom by decarboxylation of the acetate-derived chain.

In conclusion, this work proves the de novo biosynthesis of **1** in *H. orbignyana* and confirms the pioneering data reported by Fenical and co-workers with *Navanax inermis*.[7] The experiments prove the origin of the pyridine ring and C-12 from nicotinic acid, as well as the contribution of acetate to the formation of the alkyl chain. On the other hand, the way of assembling the molecule needs to be studied in more detail. Unfortunately, injection of ^{13}C-doubly labeled acetic acid, which should shed definitive light on these aspects, did not give clear evidence because of the limited number of molluscs (six specimens) that were available for the experiment. However, the polyketide route of Path C (Scheme 1) seems to us more likely, although nicotinic acid, or a metabolic equivalent, has been never reported as the starting unit in the biosynthesis of polyketides. This hypothesis is, however, not groundless if one considers the many examples of similar molecules that can be loaded by polyketide synthases of bacteria and fungi.[8] The short life-cycle of *H. orbignyana* (only 5–6 weeks) strongly limited our possibility of performing other feeding experiments (for example, with doubly labeled acetate) to shed light on these unresolved aspects.

Finally, as stated above, 3-alkylpyridines are generally envisaged as precursors of polycyclic sponge compounds including some of the most intriguing structures ever isolated from marine organisms, such as saraines[9] and manzamines.[10] Based on previous proposals, Andersen and co-workers have suggested a unified pathway for the biosynthesis of monomeric and oligomeric members of this family of natural products.[3] As already put forth by Baldwin and Whitehead,[11] one of the key points of this hypothesis is the origin of



Scheme 1. Possible pathways for the biosynthesis of **1**, based upon [1-^{13}C]-acetic acid (·) labeling studies. **X** is for -OH or a metabolically equivalent residue. Squares indicate the carbon atoms eventually lost by decarboxylation.

the 3-alkylpyridine motif from the condensation of ammonia, a C_{10} unit (a symmetrical dialdehyde) and a C_3 unit (an acrolein equivalent). Our data are not in agreement with this part of the proposal of Andersen and co-workers, although paths A and B closely resemble the biomimetic synthesis of keramaphidin B published by Baldwin.[11b] Yet, we cannot exclude that the biosynthesis of alkylpyridines in marine sponges may involve steps different from those occurring in *H. orbignyana.*

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