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First Biosynthetic Evidence on the Phenyl-Containing Polyketides of the Marine Mollusc *Scaphander lignarius*

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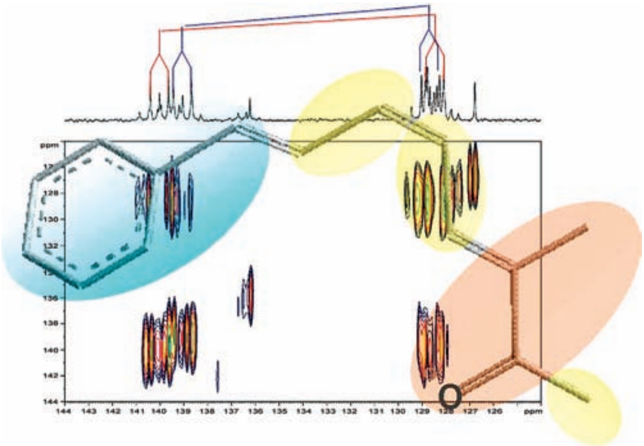
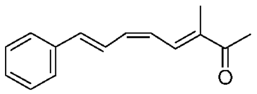
ABSTRACT



The biosynthesis of lignarenones 1 and 2, the major polyketides of the Mediterranean mollusc *Scaphander lignarius* is described. The process is primed by benzoic acid and requires acetate and propionate as extender units. The labeling pattern suggests PKS-like synthesis of an unusual *E*,Z,E-triene chain and origin of the benzoate unit from phenylalanine. ^{13}C-^{13}C NMR COSY has been used to establish the labeling positions due to incorporation of $^{13}C_2$-acetate.

Scaphander lignarius is a shelled cephalaspidean mollusc (Linné 1758) living at depths ranging from 50−200 m in the Mediterranean Sea.[1] Extracts of the animals display a mixture of aromatic compounds (here named lignarenones)[2] that have been suggested to function as intraspecific signals.[2b] Lignarenone A (**1**) and lignarenone B (**2**) are the predominant metabolites in these mixtures that also consist of a number of minor analogues differing in unsaturation or length of the alkyl chain.[3] In this work, we report the results of biosynthetic experiments designed to trace in vivo the origin of the alkyl backbone and phenyl group of this family of aromatic products.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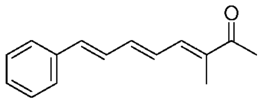
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S. lignarius was collected during the summer by dredging off Blanes (Mediterranean coast of Spain)[1] and immediately

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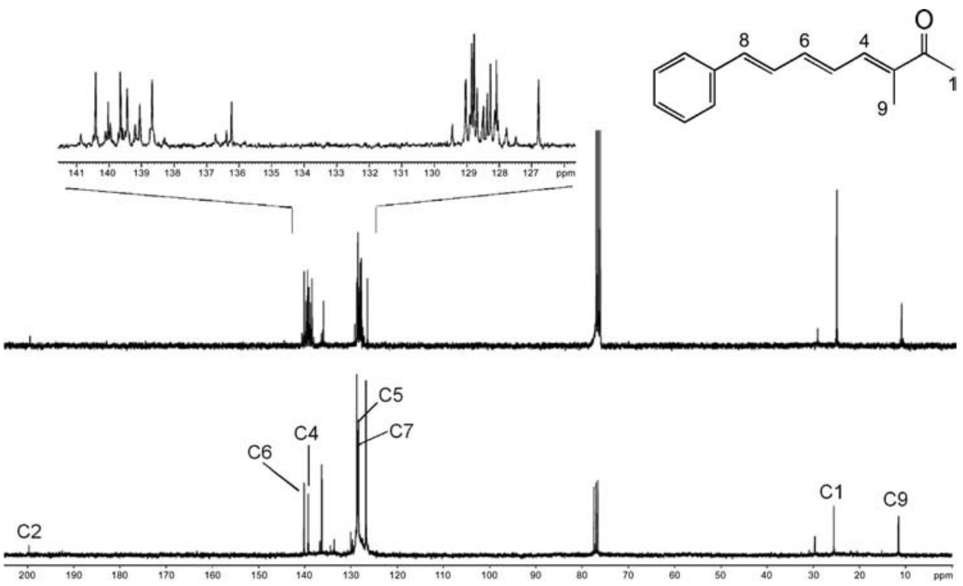
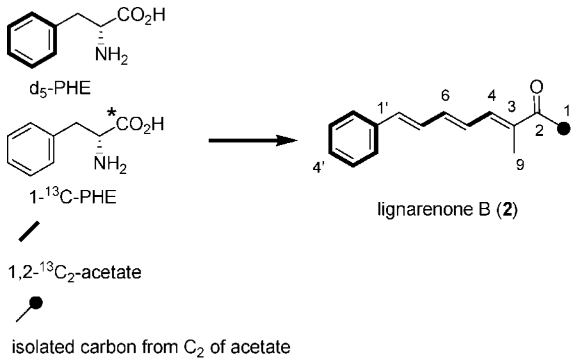


Figure 1. ^{13}C NMR spectra ($CDCl_3$, 75 MHz) of natural (bottom) and labeled (top) lignarenone B (**2**) after in vivo feeding experiments with [1,2-$^{13}C_2$]-acetate. The inset shows an enlarged view of the downfield region of the labeled product.

transferred to refrigerated aquaria. After a short period of acclimatization, three groups of 7 specimens were separately injected with 1-^{13}C phenylalanine, 1,2-$^{13}C_2$-acetate, and d_5-phenylalanine every second day for 1 week (Scheme 1). A fourth group of molluscs was frozen in liquid nitrogen and stored at −20 °C until analysis (control group). Because of the spontaneous and inevitable conversion of **1** into **2**, biosynthetic studies have been performed on a ^{13}C-enriched sample of the latter product. The labeling positions of the two compounds are identical, with occurrence of **2** consequent to isomerization of **1**.[2a]

Scheme 1. Biosynthetic Experiments Performed in Vivo with *S. lignarius* Resulting in Labeling of Lignarenone B (**2**)



Animals treated with d_5-phenylalanine (d_5-PHE) (5 mg/specimen in 150 μL distilled water) were extracted, and lignarenone B (**2**) was purified on silica gel column and fully characterized by 2D NMR experiments.[4] LC-ESI$^+$MS of this fraction showed a single chromatographic peak associated to two pseudomolecular [M+Na$^+$] ions at *m/z* 240.1 and 235.1 due to pentadeuterated and natural lignarenone B (**2**), respectively (Supporting Information). The deuterated isotopomer was largely predominant and indicated a significant incorporation of d_5-PHE in the mollusc product. Despite this, no labeling of ^{13}C carbon was obtained by feeding experiments with 1-^{13}C phenylalanine, thus suggesting that the aromatic amino acid undergoes chain-shortening. In fact, oxidative conversion of phenylalanine into benzoic acid prior to polyketide assembly explains the lack of labeling at C6 of lignarenone skeleton in experiments with 1-^{13}C phenylalanine and, at the same time, deuteration of the aromatic ring with d_5-phenylalanine.

Direct support to this hypothesis was achieved by experiments with ^{13}C-labeled acetate, which allowed us to address the biosynthesis of the alkyl chain of lignarenones and confirm that benzoate primes polyketide biosynthesis.

In fact, injection of sodium 1,2-$^{13}C_2$-acetate to *S. lignarius* produced a significant increase of the signal at 25.5 ppm (C1) and the appearance of a number of doublets in the downfield region of the ^{13}C NMR spectrum of **2** (Figure 1). Whereas the increase of C1 is attributable to breaking of the terminal C_2-unit by decarboxylation of the emerging polyketide chain (Scheme 2), the complexity arising from

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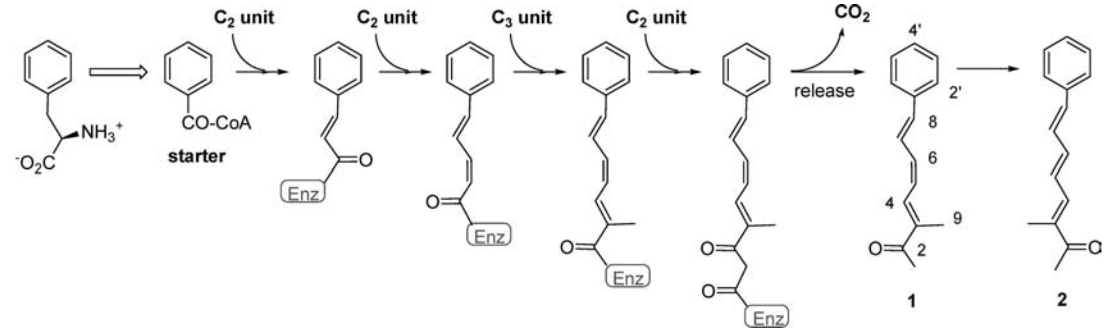
(3) Della Sala, G.; Cutignano, A.; Fontana, A.; Spinella, A.; Calabrese, G.; Domenech-Coll, A.; d'Ippolito, G.; Della Monica, C.; Cimino, G. *Tetrahedron* **2007**, *63*, 7256.

(4) Lignarenone B (**2**): yellow oil; ^{1}H NMR ($CDCl_3$), UV (CH_3OH), IR, and EIMS data are in agreement with the literature;[2a] ^{13}C NMR ($CDCl_3$) 199.6 (C-2), 140.1 (C-6), 139.2 (C-4), 136.7 (C-3), 136.3 (C-1′ and C-8), 128.7 (C-3′ and C-5′), 128.6 (C-5), 128.4 (C-7), 128.3 (C-4′), 126.8 (C-2′ and C6′), 25.5 (C-1), 11.6 (C-9); ESI$^+$ MS *m/z* 235.1 (for $C_{15}H_{16}ONa^+$).

(5) (a) Cutignano, A.; Tramice, A.; De Caro, S.; Villani, G.; Cimino, G.; Fontana, A. *Angew. Chem., Int. Ed.* **2003**, *42*, 2633. (b) Cutignano, A.; Cimino, G.; Giordano, A.; d'Ippolito, G.; Fontana, A. *Tetrahedron Lett.* **2004**, *45*, 2627.

Scheme 2. Proposed Biosynthesis of Lignarenones in *S. lignarius*[a]



[a] The tag "Enz" indicates the putative enzyme-bound intermediates of the emerging polyketide chain.

the carbon-carbon couplings required further experiments for the assignment of the downfield signals in the NMR spectrum of the doubly labeled product. Fortunately, ^{13}C homonuclear correlation spectroscopy (^{13}C-^{13}C COSY) enabled the analysis of this part of the spectrum of **2** (Figure 2) and established clearly the correlation between signals at 139.2 (C4) and 128.6 (C5) ($J = 56.5$ Hz), as well as between those at 140.1 (C6) and 128.4 (C7) ($J = 57.6$ Hz).

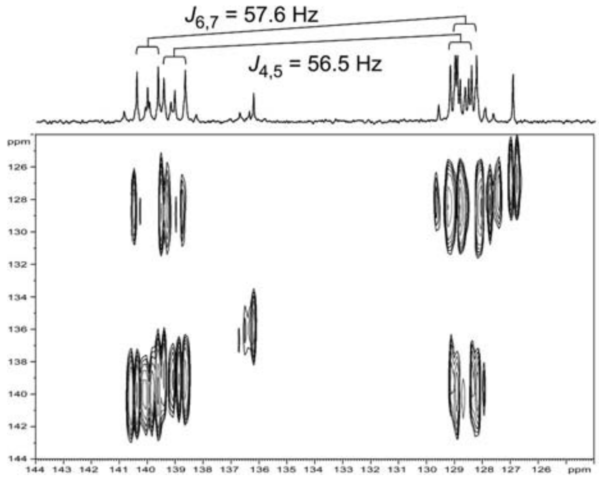


Figure 2. $^{13}C-^{13}C$ COSY NMR experiment ($CDCl_3$, 75 MHz) of ^{13}C-labeled lignarenone B (**2**) after feeding experiments with [1,2-$^{13}C_2$]-acetate.

The pathway resulting from the above experiments is summarized in the biosynthetic proposal of Scheme 2. The data agree with a polyketide assembly that requires benzoic acid as starter unit and incorporation of intact C_2-units at C4/C5 and C6/C7 of the alkyl chain. Enrichment of C1 is in agreement with decarboxylation of the emerging molecule **1** that undergoes spontaneous isomerization to give **2**. The absence of labeling of carbons C2, C3, and C9 is strongly suggestive of the origin of this part of the molecule from propionate, presumably as methyl malonyl-CoA.

In conclusion, along with synthesis of haminols from nicotinic acid,[5] biosynthesis of lignarenones represents the second example of an aromatic unit priming polyketide elongation in marine invertebrates.[6] It is worth noting that the suggested polyketide pathway implies an unusual synthesis of a *E*,Z,*E*-triene chain and origin of benzoate from phenylalanine. In plants, the latter process is under control of the ubiquitous enzyme phenylalanine-ammonia-lyase (PAL) and proceeds through oxidation of a cinnamoyl intermediate to benzoyl-CoA.[7] PAL-dependent processes have also been reported in bacteria[8] and yeast,[9] whereas in animals, synthesis of benzoic acid from phenylalanine occurs only for catabolic deammination/oxidation of the aromatic aminoacid.[10] This might suggest origin or contribution of symbiotic microorganisms in the biosynthesis of lignarenones. It is however worth pointing out that no direct piece of evidence in support or opposition to symbiotic origin has been so far reported for metabolites de novo produced by opisthobranchs, a varied group of animals showing tissue and body specialization far higher than that of other marine invertebrates (e.g., sponges and cnidarians).[11] Feeding experiments to confirm the origin of the C_3-unit from methylmalonate and the biochemical details for the conver-

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sion of phenylalanine into benzoic acid are programmed in due course as soon as the animals are available again.

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Supporting Information Available: Experimental procedures, compound characterizations, and NMR and MS spectra of the biosynthetic precursors and target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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