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One Metabolite, Two Pathways: Convergence of Polypropionate Biosynthesis in Fungi and Marine Molluscs

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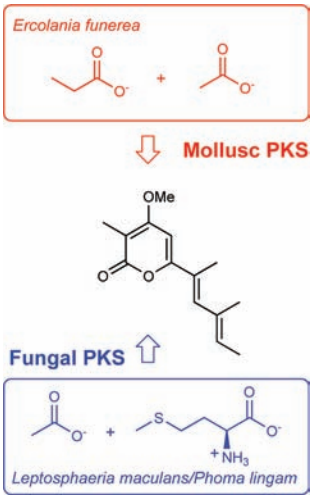
ABSTRACT



Structural similarity or even the identity of polyketide compounds does not necessarily imply unique biosynthesis. Feeding experiments with a ^{13}C labeled precursor establish that the C_3 units in 7-methyl-cyercene-1 (1) are derived from intact propionate in the marine mollusc *Ercolania funerea*. The same compound in the terrestrial fungus *Leptosphaeria maculans*/*Phoma lingam* is synthesized by an acetate/SAM pathway thus proving for the first time metabolic convergence of polyketide biosynthesis in eukaryotes. Traditional $^{1}H-^{13}C$ NMR correlation spectroscopy has been successfully applied to estimate ^{13}C incorporation in biosynthetic experiments.

Polypropionates are C_3-derived polyketide molecules which are found in bacteria, fungi, insects, and marine molluscs.[1] Despite the common structure, the biosynthesis of these polyketides changes in a phyla-specific manner. In actinomycetes and a few other bacteria, C_3-units are derived from propionate that is loaded as methylmalonyl CoA on modular type I PKS.[2] On the contrary, fungal polypropionates are always derived via SAM-mediated methylation of a linear acetate chain produced by iterative type I PKS.[3] In marine molluscs and insects, feeding experiments suggest a biochemical process similar to that operating in bacteria with incorporation of intact molecules of propionate.[1b,4,5] Interestingly, a few taxonomically restricted species of molluscs of the order Sacoglossa produce a family of 6-branched pyrones that are structurally

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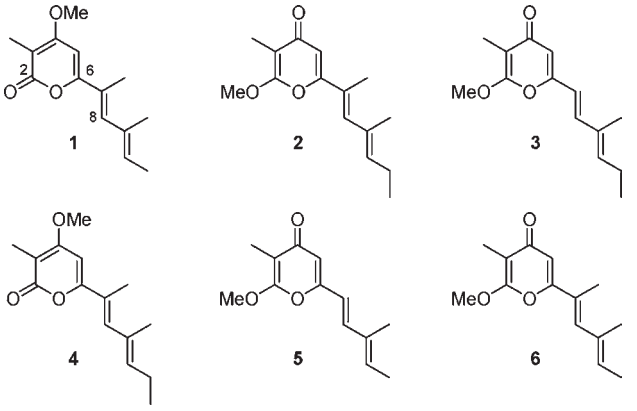
close or even identical to fungal compounds.[6] A significant example is provided by the branched-pyrone **1** that has been described as phomapyrone A from the terrestrial fungus *Leptosphaeria maculans*/*Phoma lingam*[7] and as 7-methyl cyercene-1 from the opisthobranch mollusc *Ercolania funerea*.[6c] Similar to the other fungal polypropionates, phomapyrone A is synthesized via a SAM-acetate pathway in *L. maculans*/*P. lingam*.[8] On the other hand, no information is available on 7-methyl-cyercene-1 although we have recently shown that other polyketides structurally related to **1** incorporate intact propionates in sister species of *E. funerea*.[4a] The main aim of this work is to determine the origin of the methyl branched units of **1** in the marine mollusc to verify the convergence of distinct pathways for the biosynthesis of polypropionate structures in molluscs and fungi.



Due to the mollusc size (0.5 cm average length) which prevented direct administration by injection, 157 specimens of *E. funerea* were maintained for 1 week in aerated seawater containing 1-^{13}C propionate (300 mg in 500 mL). A second group of animals (149 specimens) were kept as a controls and frozen immediately after collection. The two groups of molluscs were extracted according to our standard procedures.[9] Compound **1** is a minor component in the pool of polypropionates of *E. funerea* (**1**–**6**),[6c] and consequently the carbon spectrum of the purified product (0.5 mg) did not provide a signal-to-noise ratio suitable for quantitative evaluation of the labeling enrichment in monodimensional NMR spectroscopy. Selective labeling by ^{13}C enrichment of specific carbons is expected to enhance signal intensities in also bidimensional ^{1}H–^{13}C

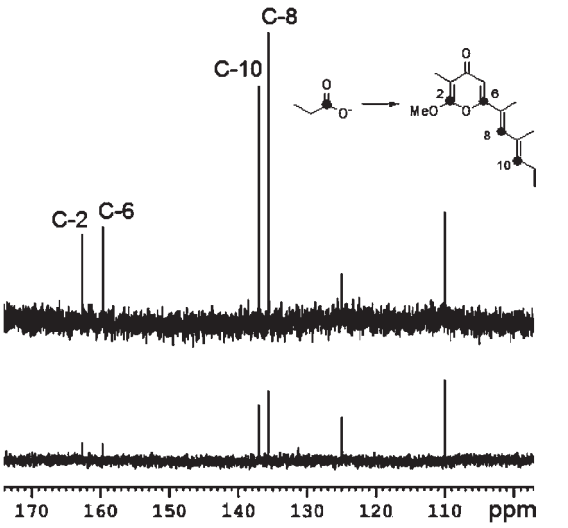


Figure 1. Downfield region of ^{13}C NMR spectrum of natural (below) and enriched (above) sample of 7-methyl-cyercene B (**2**). Labeled carbons are highlighted by black dot.

Table 1. Carbon Enrichments in Compound **2** after Feeding with 1-^{13}C Propionate; Variation Is Calculated As $(I_{lab} - I_{nat})/I_{nat}$ %[a]

		^{13}C	HSQC	HMBC	
C	ppm	Integral Variation %	Integral Variation %	Integral Variation %	Correlation[b]
2	162.4	99.4	–	209.0	OMe
				230.1	Me-3
3	100.8	n.d.	–	ref.	Me-3
				−6.2	H-5
4	181.6	n.d.	–	9.0	Me-3
5	109.8	−3.8	−20.9	n.d.	
6	159.5	275.7	–	188.5	H-5
				142.1	Me-7
7	124.8	–	–	−9.9	Me-7
				−36.1	H-5
8[c]	135.4	148.1	221.0	132.1	Me-7
				96.6	H-10
9	131.1	n.d.	–	−4.9	H-11
				6.8	Me-9
10[c]	136.8	122.9	171.7	197.7	H-12
				195.3	H-11
				129.2	Me-9
11	21.7	−27.1	−32.3	−12.6	H-12
12	13.8	−33.6	−29.5	−2.4	H-11
Me-3	6.7	−16.9	−14.4	n.d.	
Me-7	14.1	−30.0	−17.6	−54.2	H-8
Me-9	16.6	−18.2	1.3	−28.8	H-10
OMe	55.7	ref	ref	n.d.	

[a] NMR spectra have been recorded in $CDCl_3$ at 600 and 400 MHz. [b] Proton assignments are reported in ref 11. [c] HSQC and HMBC correlations suggested revision of C-8 and C-10 assignment. The values of these carbons are now reversed. n.d. = not detectable; ref = reference signal.

(7) Pedras, M. S. P.; Morales, V. M.; Taylor, J. L. *Phytochemistry* **1994**, *36*, 1315–1318.

(8) Pedras, M. S. C.; Chumala, P. B. *Phytochemistry* **2005**, *66*, 81–87.

(9) Frozen specimens were sonicated in acetone (3 × 20 mL), and the aqueous residue after evaporation of the organic solvent was diluted with distilled water and partitioned with diethyl ether (3 × 20 mL). The ether extract was then fractionated on a silica gel column by using a petroleum ether/diethyl ether stepwise gradient. Final purification of propionates **1**–**6** was achieved by HPLC on an ODS1 column (Phenomenex, MeOH/H_2O 70:30, flow 0.6 mL/min, λ_{max} 270 nm).

(10) (a) Terashima, N.; Akiyama, T.; Ralph, S.; Evtuguin, D.; Neto, C. P.; Parkas, J.; Paulsson, M.; Westermark, U.; Ralph, J. *Holzforschung* **2009**, *63*, 379–384. (b) Tsvetanova, B.; Kiemle, D. J.; Price, N. P. J. *J. Biol. Chem.* **2002**, *277*, 35289–35296. (c) Wang, Y. G.; Morinaca, B. I.; Reyes, J. C. P.; Wolff, J. J.; Romo, D.; Molinski, T. F. *J. Nat. Prod.* **2010**, *73*, 428–434.

Table 2. Carbon Enrichments in Compound **1** after Feeding with 1-^{13}C Propionate; Variation Is Calculated As $(I_{lab} - I_{nat})/I_{nat}$ %[a]

		HSQC	HMBC	
		Integral Variation	Integral Variation	Correlation[b]
C	ppm	%	%	
2	164.9	–	221.6	Me-3
3	101.9	–	−7.0	Me-3
			30.6	H-5
4	165.8	–	ref	H-5
5	92.2	−19.5	n.d.	n.d.
6	160.8	–	347.9	Me-7
			430.4	H-5
			200.0	H-8
7	123.7	–	17.0	H-5
			8.8	H-8
			13.8	Me-7
8	136.7	143.0	521.0	H-10
			519.3	Me-9
			350.1	Me-7
9	132.9	–	26.2	H-11
10	129.0	−1.6	19.8	H-11
11	14.0	−8.7	−8.9	H-10
Me-3	8.5	−12.9	n.d.	
Me-7	13.9	−26.6	26.1	H-8
Me-9	16.4	−27.4	14.3	H-10
OMe	56.0	ref	n.d.	

[a] NMR spectra have been recorded in $CDCl_3$ at 600 MHz. Assignments were in accordance with ref 7. [b] Proton assignments are reported in ref 11. n.d. = not detectable; ref = reference signal.

NMR spectra. HSQC sequences have been already used to estimate isotopic enrichment in metabolic studies[10] even if there is no application of HMBC experiments to detect labeling in biosynthetic studies. Considering the net increase of sensitivity due to indirect detection of the less sensitive nucleus (^{13}C) through the spin coupled proton, this approach should be appropriate to overcome the drawbacks of monodimensional ^{13}C NMR spectroscopy to estimate ^{13}C incorporation in the metabolite **1**. The correctness of our hypothesis was first tested on samples of natural glucose enriched with linearly increasing concentration of the 1-^{13}C isotopomer (data not shown) and then validated on another propionate of *E. funerea*, namely 7-methyl-cyercene-B (**2**). This last metabolite, in contrast to **1**, was sufficiently abundant to give monodimensional ^{13}C NMR spectra proving enrichment at C-2, C-6, C-8, and C-10 (Figure 1). Integration of cross peaks in the HSQC and HMBC spectra of natural and enriched **2** gave an identical labeling pattern. As for the 1D-^{13}C spectrum, significant increases were observable only for signals of C-2, C-6, C-8, and C-10, whereas the other carbons showed variation that was close to zero or slightly negative due to random baseline or signal distorsions.

The experiments on labeled 7-methyl-cyercene-B (**2**) unambigously confirmed the correspondence of mono- and bidimensional NMR spectra in evaluating the relative enrichment of specific carbon signals in biosynthetic studies (Table 1).

A quantitative interpretation of cross-peaks in routine HMBC is in principle not generally possible due to the evolution under ^{1}H−^{1}H homonuclear couplings during the pulse sequence.

Nevertheless, the above data prove that a relative quantitation, resulting from a comparison between spectra of natural and enriched samples recorded under the same experimental conditions, is still possible with HMBC sequences that can be therefore used to evaluate the position of ^{13}C labeling. Furthermore, since each carbon shows correlations with more protons, ^{13}C-enrichments can be usually assessed by more cross-peaks thus increasing the consistency of the assignment (Table 1).

With these results in hand, we proceeded with analysis of HSQC and HMBC of **1** (Table 2).

Among the signals expected to be enriched, only C-8 gave a cross-peak in the HSQC which proved the incorporation in this position. This result was confirmed by the HMBC experiments that also indicated selective labeling at the quaternary C-2 and C-6. These carbons correlated with more than one proton, and on the average each resulting cross-peak showed a nearly 4-fold area increase in comparison with the natural sample (Table 2). This is clearly evident in the three-dimensional plot of HMBC spectra reported for both samples in Figure 2. The considerable signal enhancement was largely stronger than possible fluctuation due to baseline distortion, thus proving unambiguously incorporation of 1-^{13}C propionate in specific positions of the polyketide skeleton.

As described in Figure 3, the resulting biosynthesis suggests the contribution of an enzyme able to utilize acetate and propionate for chain extension. The feature of loading different building blocks is typical of Type I polyketide synthases of prokaryotes,[2] whereas it is in strict contrast with the molecular architecture of the iterative processes described in fungi. Consequently, the study excludes the origin of 7-methyl-cyercene-1 (**1**) in symbiotic fungi, as well as proves the existence of two biochemical processes committed to the synthesis of the same compound in organisms of two different phyla of life. Involvement of bacteria in the synthesis of polyketides **1**−**6** cannot be excluded on the basis of the labeling results even if it is to note that modular polyketide synthases with catalytic activities similar to those of bacteria have been also described in the genome of marine invertebrates.[12]

(11) ^{1}H NMR data ($CDCl_3$, 600 MHz). 7-methyl-cyercene-1 (**1**), δ, m, *J* (Hz): 6.15 (H-5, s); 7.0 (H-8, brd); 5.64 (H-10, q, 6.8); 1.76 (H_3-11, d, 6.8); 1.94 (Me-3, s); 2.03 (Me-7, brs); 1.83 (Me-9, brs); 3.91 (OMe, s). 7-methyl-cyercene-B (**2**): 6.27 (H-5, s); 6.71 (H-8, brs); 5.53 (H-10, t, 7.4); 2.17 (H_2-11, q, 7.4); 1.03 (H_3-12, t, 7.4); 1.86 (Me-3, s); 2.02 (Me-7, brs); 1.83 (Me-9, brs); 4.04 (OMe, s).

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(13) Fontana, A. In *Biogenetic proposals and biosynthetic studies on secondary metabolites of marine molluscs*; Cimino, G., Gavagnin, M., Eds.; Progress in Molecular and Subcellular Biology Series; Springer: Heidelberg, Germany, 2006; Vol. 43, pp 303−332.

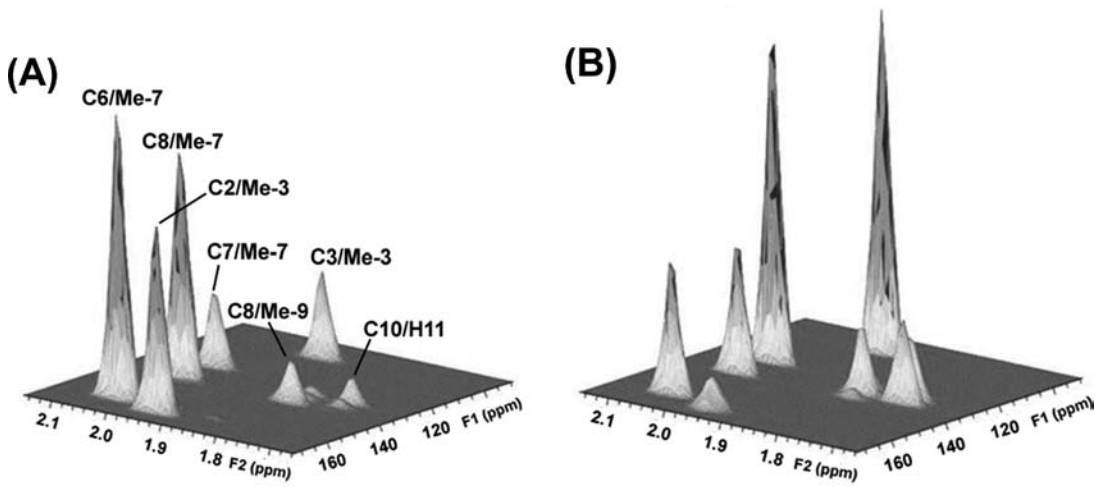

Figure 2. Three dimensional plots of a selected HMBC region of labeled (A) and natural (B) pyrone propionate **1**.

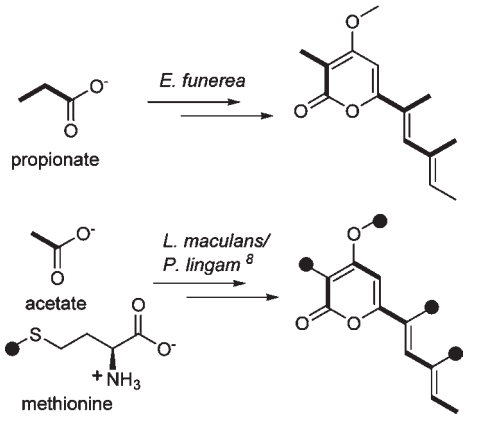

Figure 3. Origin of C_3 unit in 7-methyl-cyercene 1/phomapyrone A (**1**) based upon feeding experiments.

In conclusion, we used routine HSQC and HMBC spectra to establish the incorporation of intact ^{13}C-labeled propionate in the polyketides **1** and **2** of the marine sacoglossan mollusc *E. funerea*. *De novo* biosynthesis of secondary metabolites is a common trait within sacoglossans and other marine opisthobranchs.[13] Even if no direct studies have hitherto addressed the origin of these molecules by symbiotic or associated microorganisms, it is commonly accepted that these molluscs have elaborated their own biochemical pathways that are committed to the synthesis of natural products serving for the survival of the population or individual. The biosynthetic assembly of **1** and **2** is in line with our knowledge of polypropiogenesis in opisthobranchs and confirms for the first time in a direct manner that the same polypropionate **1** is synthesized by two different pathways in fungi and in these marine invertebrates. To the best of our knowledge, there are very few examples of compounds synthesized by two different pathways.[14] All of them concern divergence between eukaryotes and prokaryotes. Biosynthesis of 7-methyl-cyercene-1/phomapyrone A (**1**) would be the first case of biosynthetic convergence between two eukaryotes.

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Supporting Information Available. General experimental procedures, NMR spectra (Figures S1−S11) and NMR data (Tables T1−T5) of natural and labeled **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.