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Origin of the C_3-unit in placidenes: further insights into taxa divergence of polypropionate biosynthesis in marine molluscs and fungi

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ABSTRACT

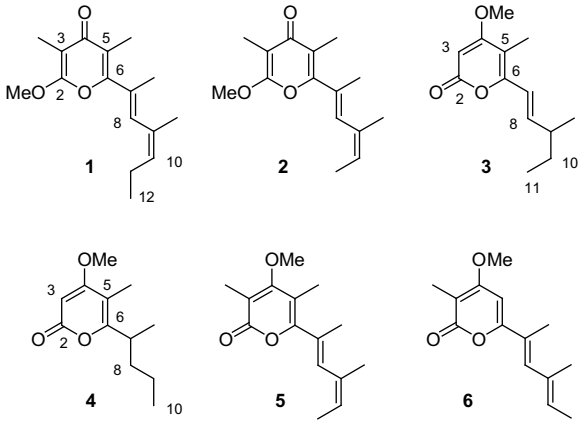
Structural diversity of polypropionates arises from the amazing combination of acetate and propionate units. Feeding experiments with single and doubly ^{13}C-labelled precursors prove that the Mediterranean slug *Placida dendritica* utilizes intact C_3-units for the biosynthesis of placidenes (e.g., **1**–**5**), prototypes of a family of pyrone-containing polypropionates largely represented in fungi and marine invertebrates. These results show that fungi and molluscs have elaborated two distinct polyketide pathways for the synthesis of similar or even identical compounds.

1. Introduction

Polypropionates form a large subgroup of bioactive natural products in which C_3-units partly or totally replace acetate in the carbon skeleton produced by polyketide synthases (PKSs). Their biosynthesis arises from two independent pathways involving either propionate elongation of the growing polyketide chain or *S*-adenosyl methionine (SAM)-dependent methylation of an acetate-derived PKS product. According to the literature, polypropionate assembly occurs by both acetate/SAM and propionate elongation in bacteria whereas only the acetate/SAM pathway has been reported in fungi.[1]

Polypropionates are also known from marine molluscs, where they are suggested to serve as chemical mediators for a plethora of ecological and physiological functions.[2] In particular, independent studies have highlighted the presence of an endogenous metabolic pathway that utilizes intact C_3-units to assemble the polypropionate carbon skeleton in marine gastropods.[3]

Here, feeding experiments with ^{13}C-labelled precursors have been used to determine the biosynthesis of placidenes, a family of regular and mixed polypropionates (of which the structures **1**–**5** show the main skeletons) of the marine mollusc *Placida dendritica*.[4] Placidenes prototype a large and diverse group of α- and γ-pyrone polypropionates world-wide found in fungi and gastropods. Our interest about the origin of these molecules has been raised by the recent report that the terrestrial fungus *Leptosphaeria maculans*[5] utilizes the acetate/SAM pathway to biosynthesize phomapyrone A (**6**), a 5-nor-9,10-*iso*-placidene F, which had been also described as 7-methyl-12-norcycercene B from the marine slug *Ercolania funerea*.[6]



2. Results and discussion

P. dendritica is a Mediterranean shell-less mollusc of the order Sacoglossa, which lives camouflaged upon the algae *Briopsis plumosa*. The molluscs (64 specimens) were collected in the Gulf of Naples in a period from January to March 2008 and transferred in an aquarium. After few days of acclimatization, 38 specimens were frozen and used as control. The remaining invertebrates were divided into three groups for the feeding experiments with sodium [1-^{13}C]-propionate, sodium [2-^{13}C]-acetate and sodium [1,2-$^{13}C_2$]-acetate. Each precursor

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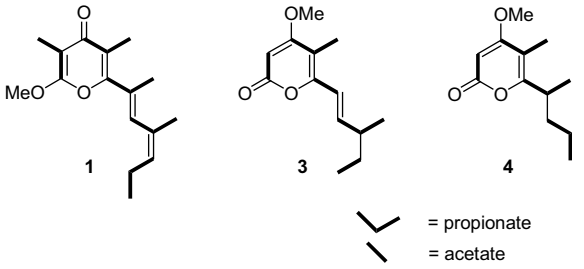
was dissolved at a final concentration of 100 μg/μl of distilled water and injected (50 μl/specimen) every other day for 1 week.

After purification (Experimental section), incorporation of ^{13}C-labelled material was tested by ^{13}C NMR in placidene A (**1**), placidene C (**3**) and placidene E (**4**), which have been chosen because they show carbon skeletons based on either regular propionate or mixed acetate/propionate pathways (Scheme 1).



Scheme 1. Prediction of acetate and propionate units in placidene-A (**1**), -C (**3**) and -E (**4**).

As summarized in Table 1, every polyketide compound from animals fed with [1-^{13}C]-propionate revealed clear enrichment[7] with incorporation levels (Supplementary data), which were compatible with the results of previous biosynthetic studies in marine molluscs.[8] Placidene A (**1**) and placidene E (**4**) showed 30% increase of the ^{13}C NMR signals biosynthetically derived by C1 of propionate (Scheme 1). In placidene C (**3**), the expected enrichment of C4 (170.9 ppm) and C8 (145.3 ppm) was rather higher and reached almost 100% (Supplementary data).

According to these results, no incorporation was observed in the regular propionate skeleton of **1** after feeding studies with labelled acetates. On the contrary, use of these last precursors gave enrichment of the carbons of placidene C (**3**) and E (**4**) as predicted in the biogenesis of Scheme 1. Thus, origin from acetate of C2–C3, C6–C7 and C10–C11 was in agreement with enrichment of C3 (88.4 ppm), C7 (117.0 ppm) and C11 (11.7 ppm) of placidene C (**3**) after feeding 2-^{13}C acetate (Table 1). The incorporation of an intact acetate unit between C2 (164.8 ppm) and C3 (87.5 ppm) of placidene E (**4**) was unambiguously proven by the cross-peak between the two signals in ^{13}C–^{13}C-COSY spectrum (Fig. 1).

The above results demonstrate that the de novo origin of placidenes in the Mediterranean mollusc *P. dendritica* involves

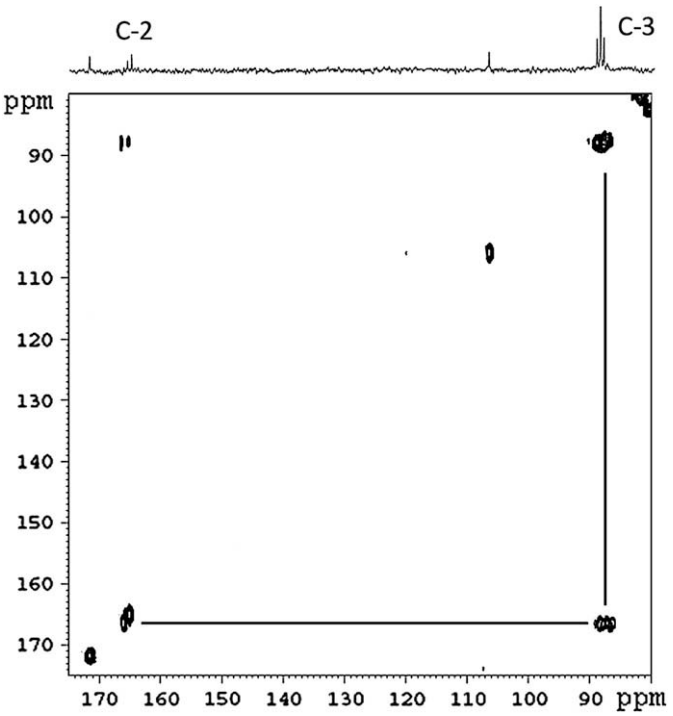


Figure 1. Down-field region (175–80 ppm) of ^{13}C–^{13}C-COSY spectrum ($CDCl_3$, 75 MHz) of placidene E (**3**) isolated from *P. dendritica* specimens after feeding experiments with [1,2-^{13}C]-acetate.

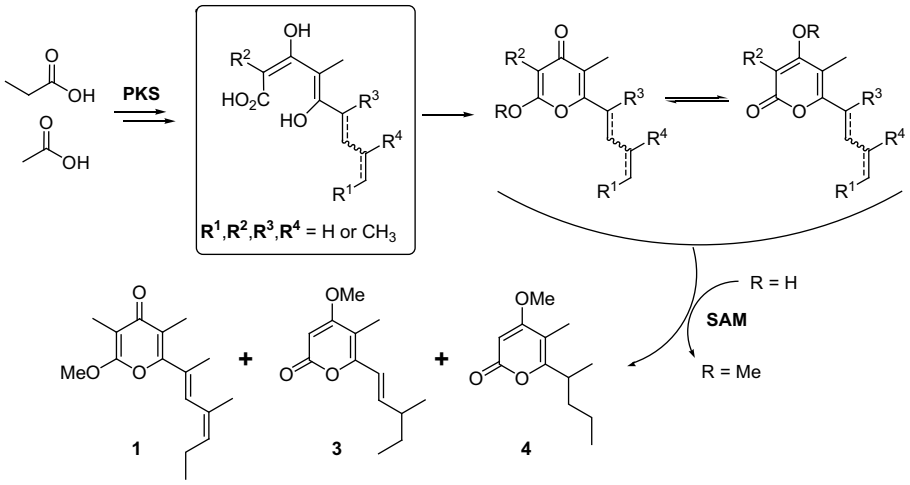
incorporation of intact C_3-units in the emerging chain. The labelling observed in compounds **1**, **3** and **4** excludes synthesis of the carbon skeleton through methylation of a pre-existent polyacetate chain, as otherwise demonstrated for the structurally related phomapyrone A (**6**) in the fungus *L. maculans*.[5] On these grounds, it is evident that synthesis of polypropionates in fungi and molluscs implies distinct metabolic pathways able to synthesize similar or even identical compounds.

As described in Scheme 2, the extraordinary chemical variety of polyketides described in *P. dendritica* and taxonomically-related organisms is therefore the result of a peculiar capacity of the putative PKS to accept and combine acetate or propionate units at

Table 1
^{13}C NMR data of placidene-A (**1**), -C (**3**) and -E (**4**) after feeding experiments with [1-^{13}C]-propionate and [2-^{13}C]-acetate

C	**1**		**3**			**4**	
		1-^{13}C propionate		1-^{13}C propionate	2-^{13}C acetate		1-^{13}C propionate
	ppm	Increase (%)[a]	ppm	Increase (%)[a]	Increase (%)[a]	ppm	Increase (%)[a]
2	161.9	*81.8*	163.9	—	—	164.8	—
3	99.3	—	88.4	—	*30.7*	87.5	—
4	181.4	*26.3*	170.9	*123.2*	—	171.0	*60.5*
5	117.9	—	106.3	—	—	106.2	—
6	158.3	*73.5*	154.3	—	—	164.1	*49.7*
7	127.9	—	117.0	—	*43.0*	34.5	—
8	135.1	*20.3*	145.3	*116.4*	—	36.6	*43.6*
9	130.2	—	39.2	—	—	20.7	—
10	132.3	*48.4*	29.3	—	—	14.0	—
11	22.6	—	11.7	—	*15.5*		
12	13.9	—					
CH_3-3	6.9	—					
CH_3-5	11.9	—	8.7	—	—	9.0	—
CH_3-7	16.0	—				18.3	—
CH_3-9	23.1	—	19.7	—	—		
OCH_3	55.2	Int. ref.	56.0	Int. ref.	Int. ref.	56.0	Int. ref.

[a] Carbon enrichment was calculated as follows: Increase=(labelled signal area−natural signal area)/natural signal area. Carbon signal of –OCH_3 was used to normalize spectra of natural and labelled **1**, **3** and **4**. Int. ref.=internal reference.



Scheme 2. Proposed biosynthesis of placidene-A (**1**), -C (**3**) and -E (**4**) according to a PKS-like assembly using both propionate and acetate building blocks. Formation and methylation of the pyrone ring is in agreement with Ref. 9.

different stages of the biosynthetic assembly (Scheme 2). The biogenetic proposal also takes into account that α- and γ-pyrone rings are formed by spontaneous cyclization followed by methylation, after release of the polyketide chain from the putative thiotemplate.[9]

Direct use of intact C_3-units, as methylmalonyl-CoA, by PKSs has been so far proved only in bacteria,[1,10] thus suggesting that polyketide biosynthesis of placidenes, as well as of other polypropionates produced de novo in marine gastropods,[3] may be ultimately traced back to prokaryotes living associated with or in symbiosis with the invertebrates. In reality, recent studies of Calestani and co-workers on the presence and function of the polyketide synthase gene SpPks in the sea urchin genome[11] open to new possibilities for the origin of placidenes and other related compounds in marine molluscs. In fact, following the arguments of these authors about the hitherto unknown evolution of PKS genes in animals,[11b] it should not be excluded a priori that biosynthesis of placidenes, and more generally of polypropionates, involves functional PKSs present in the genome of *P. dendritica* and other marine gastropods.

In conclusion, as Shen outlined a few years ago,[12] the presence of many different PKSs in nature is not an issue, whereas it is less clear what are the factors governing the selection of these variants. Here, we add another facet concerning two different putative PKS pathways committed to similar or identical molecules in two different taxa. The above biosynthetic results are sufficient to exclude the contribution of fungi in the synthesis of placidenes and other related metabolites from marine molluscs, as one could imagine from the studies on phomapyrones,[5] but the absence of molecular data leaves open the question about the bacterial origin of these molecules. Experiments are in due course to trace the molecular basis of PKS activity in polyketide-producing molluscs.

3. Experimental section

3.1. General

NMR spectra were recorded on a Bruker Avance DRX 600 equipped with a cryoprobe operating at 600 MHz for proton or on a Bruker Avance DPX 300 operating at 300 MHz for proton. Spectra were referenced to $CDCl_3$ (δ_H 7.26, δ_C 77.0 ppm) as internal standard.

3.2. Feeding experiments

Specimens of *P. dendritica* were collected during January–March 2008 in the Gulf of Naples. The animals were transferred into an aquarium at 15 °C to allow acclimatization and after 7–10 days they were divided into groups for feeding experiments with the labelled precursors (5 mg/50 ml of distilled water) by injection. Thirty-eight specimens not subjected to any treatment were used as control of the biosynthetic experiments. At the end of each experiment, the animals were frozen and kept at −20 °C until analysis.

3.3. Extraction procedures and metabolite purification

The frozen animals were extracted with acetone by sonication. Acetone extracts were concentrated under reduced pressure and the water residue was partitioned with diethyl ether (3×50 mL). The combined ether extracts of the mantle were dried under vacuum and subjected to column chromatography on silica gel using a gradient elution of diethyl ether in light petroleum ether from 10% to 50% as previously reported.[4b] According to the reported procedures, placidenes eluting with 20% and 30% diethyl ether were further purified by HPLC on ODS-1 column (Phenomenex, 4.6×250 mm, 100A, μm) by using a MeOH/H_2O isocratic elution (70:30), flow 0.7 ml/min and monitoring the absorbance at 240 nm. NMR and MS data of compounds **1**–**5** are in full agreement with those reported in the literature.[4]

3.4. Experiment with [1-^{13}C]-propionate

Thirty-two specimens were injected with Na [1-^{13}C]-propionate (5 mg/50 μl distilled water) three times every other day. A second experiment was carried out with another 17 animals according to the same procedure. Forty-three animals from the two experiments that survived after the three injections were kept for 3 days in aquarium and then frozen. For metabolite analysis, the ether extracts obtained as previously indicated (31.7 mg, yellow-green oil) was purified on silica gel column and HPLC affording **1** (1.9 mg, pale yellow oil), **3** (1.6 mg, pale yellow oil) and **4** (1.3 mg, pale yellow oil).

3.5. Experiment with [2-^{13}C]-acetate

Twenty-five specimens were treated with Na [2-^{13}C]-acetate (5 mg/50 μl distilled water) three times every other day. Twenty-three animals that completed the experiment were frozen until analysis. Metabolite purification from the ether extract (15.2 mg, brown-yellow oil) afforded **1** (1.4 mg, pale yellow oil), **3** (1.5 mg, pale yellow oil) and **4** (0.2 mg, pale yellow oil).

3.6. Experiment with [1,2-^{13}C]-acetate

Twenty-two specimens were fed with the labelled precursor (5 mg/50 μl distilled water) three times every other day. A second (six specimens) and a third (five specimens) group of animals was subjected to the same treatment. Combined ethereal extracts (44.3 mg, brown-yellow oil) of frozen animals were fractionated as indicated and gave **1** (0.2 mg, colourless oil), **3** (0.2 mg, colourless oil) and **4** (2.1 mg, pale yellow oil).

3.7. Control group

Thirty-eight specimens were immediately frozen after collection and treated as control group for biosynthetic experiments. Extraction of the animals and purification of polypropionates from the ether extract (23 mg) were carried out with the usual procedures and afforded **1** (1.3 mg), **3** (1.4 mg) and **4** (1.0 mg). NMR and MS data of these compounds are in full agreement with those previously reported in the literature.[4]

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Supplementary data

This material contains NMR spectra of labelled placidenes. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2009.07.078.

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7. The ^{13}C NMR signals were integrated by using the Integration tool of the Bruker XWIN-NMR master programme (Version 3.5). To have normalization of the signal areas in natural and labelled samples, ^{13}C signal of OCH_3 (not labelled according with both acetate and propionate) was used as internal standard in **1**, **3** and **4**. An arbitrary value of 100 was assigned to these signals during the integration procedure. Signal increase was then determined according to the following calculation: Increase S#1=(Area S#1$_L$−Area S#1$_N$)/(Area S#1$_N$) S#1=^{13}C NMR signal of interest S#1$_L$=^{13}C NMR signal of interest in labelled sample after normalization. S#1$_N$=^{13}C NMR signal of interest in natural sample after normalization.
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