A Mechanism of Benzoic Acid Biosynthesis in Plants and Bacteria that Mirrors Fatty Acid β-Oxidation

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The aromatic metabolite benzoic acid (**1**) is a biosynthetic building block of numerous benzoyl and benzyl groups that serve as important structural elements in a large number of natural products. In eukaryotes, for example, benzoate is a component of the zaragozic acids (**2**),[1] paclitaxel (taxol; **3**),[2] salicylic acid (**4**),[3] and cocaine (**5**)[4] (Figure 1). Although rarer in prokaryotic organisms, benzoyl-coenzyme A (benzoyl-CoA) is a starter unit for a few bacterial polyketides, such as enterocin (**6**) and the wailupemycins.[5]

Despite its simple structure and widespread occurrence, the biosynthesis of **1** and its thioester **1**-CoA are only partially understood. In the field of plant secondary metabolism, two major routes from L-phenylalanine (**7**) to benzoic acid have been reported: the β-oxidation-type pathway (Scheme 1, route a); and the so-called nonoxidative pathway, via benzaldehyde (**11**) (Scheme 1, route b).[3, 6, 7] Both routes possess common intermediates like cinnamoyl-CoA (**8**) and 3-hydroxy-3-phenylpropionyl-CoA (**9**-CoA) before branching to involve either oxidation and thiolation (route a) or retro-aldol cleavage followed by oxidation (route b). Until recently, the only known pathway from **7** to **1** in bacteria proceeded through two oxidative decarboxylation reactions involving the intermediates phenylpyruvate and phenylglyoxylate.[8]

We recently reported that the biosynthesis of **1** in the terrestrial plants *Nicotiana attenuata* and *Cucumis sativus*,[7] and the marine actinomycete "*Streptomyces maritimus*" proceeds by a similar pathway involving the intermediate 3-hydroxy-3-phenylpropionate.[9]

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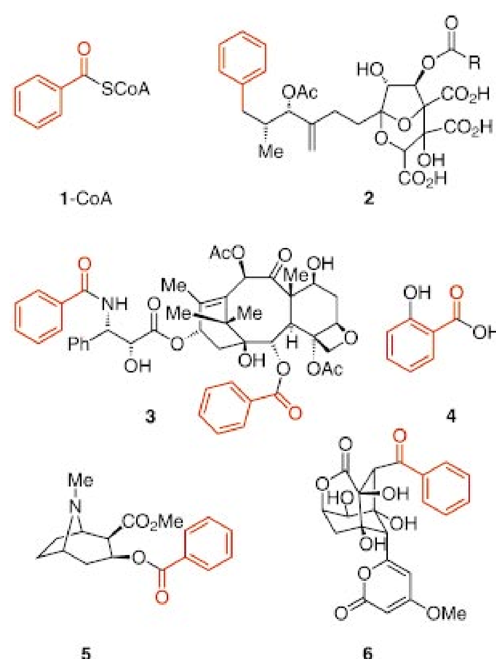
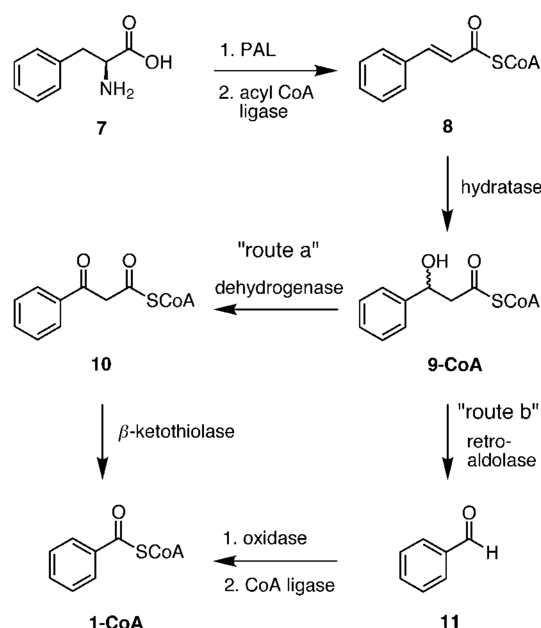


Figure 1. Structures of some important natural products containing benzoate-derived residues.



*Scheme 1. The two proposed biosynthetic routes to benzoyl-CoA (**1**-CoA), via cinnamoyl-CoA (**8**). PAL = phenylalanine ammonia-lyase.*

The plant study was conducted in the context of salicylic acid (**4**) biosynthesis—a process crucial for the onset of systemic acquired resistance in pathogen-infected plants.[3] The bacterial work, in contrast, focused on the biosynthesis of the rare benzoyl-CoA type II polyketide synthase starter unit in enterocin (**6**).[10] Both studies identified **9** as a key intermediate in benzoate biosynthesis and provided strong support in favor of a β-oxidation-like route (Scheme 1, route a). Here we report the absolute configuration of the hydroxy acid intermediate **9**, in

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both the eukaryotic and prokaryotic systems, and show that the stereochemistry is identical to that observed in fatty acid β-oxidation. In addition, sequence alignment of the enterocin cinnamoyl-CoA hydratase EncI with enoyl-CoA hydratases from fatty acid β-oxidation provides support for mechanistic identity in the hydration step.

To determine which enantiomer of **9** serves as an intermediate toward benzoic acid, we synthesized isotopically labeled forms of both optical isomers for use as metabolic probes. Two successful synthetic strategies were employed to produce (3*R*)- and (3*S*)-[ring-$^2H_5$]**9** with high enantiomeric excesses. An Evans aldol[11] condensation between [$^2H_5$]**11** and the lithium enolate of (4*R*)-3-acetyl-4-(1-methylethyl)-2-oxazolidinone, followed by separation of the diastereomers and hydrolysis of the carbamate group, gave (3*R*)- and (3*S*)-[$^2H_5$]**9** in $>95\%$ *ee*.[12, 13] Alternatively, enzymatic resolution of ($\pm$)-[$^2H_5$]**9** ethyl ester[7] with lipase PS-30 from *Pseudomonas* sp.[14] also gave access to the two enantiomeric hydroxy acids ($>95\%$ *ee*).

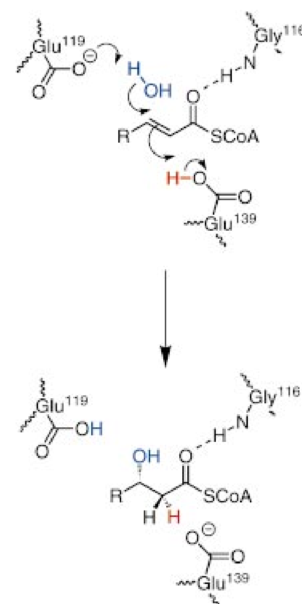
Cell-free extracts of *N. attenuata* converted only (*R*)-[$^2H_5$]**9** into [$^2H_5$]benzoic acid, a result comparable with that seen in cocaine biosynthesis (Table 1).[4] "*S. maritimus*" cultures, whether wild-type or one of two knock-out mutant strains, also preferentially incorporated (*R*)-[$^2H_5$]**9** into [$^2H_5$]enterocin. Since hydroxy acid **9** is downstream of the cinnamoyl-CoA hydration step, the mutant "*S. maritimus*" KI[24], which harbors the inactivated hydratase gene *encI*, produced wild-type levels of [$^2H_5$]enterocin. The thiolase *encJ* knock-out strain "*S. maritimus*" KJ produced very little polyketide, as expected. Incorporation of label by this mutant probably arises from complementation with the thiolase associated with fatty acid β-oxidation. The small, but nonzero, incorporation of (*S*)-[$^2H_5$]**9** into [$^2H_5$]**6** may be due to a lower stereoselectivity in the *Streptomyces* enzymes, but is more likely to result from partial dehydration or oxidation of (*S*)-**9** to the pathway intermediates **8** or **10**, respectively, by *Streptomyces* housekeeping enzymes, such as those involved in fatty acid β-oxidation. This observation was not made in the *N. attenuata* cell-free study, in which a much shorter incubation time was used (30 min vs. 1–5 days).

Table 1. *Isotopic enrichment of **1** and **6** in cell-free extracts of* N. attenuata *and cultures of "*S. maritimus*", respectively, following incubation with (3*R*)- or (3*S*)-[$^2H_5$]**9**.*

Species	Isotopic enrichment [%]			*R*:*S*
	($\pm$)-[$^2H_5$]**9**	(3*R*)-[$^2H_5$]**9**	(3*S*)-[$^2H_5$]**9**	
Nicotiana attenuata	n.a.	3.7	0[a]	$>40:1$
"*Streptomyces maritimus*"	25.7	45.7	9.2	5:1
"*S. maritimus*" KI mutant	n.a.	50.4	10.2	5:1
"*S. maritimus*" KJ mutant	n.a.	5.5	0.9	6:1

[a] Below limit of detection (approx. 0.1%). – n.a. = not analyzed.

Since "route a" in Scheme 1 is analogous to fatty acid β-oxidation, we compared the absolute configuration of **9** with those of its fatty acid counterparts. β-Oxidation always proceeds via (3*S*)-3-hydroxyacyl-CoA intermediates, and examination of Schemes 2 and 3 reveals that the configuration of (3*R*)-**9** and (3*S*)-3-hydroxyacyl-CoA at C3 is the same (the change in nomenclature



Scheme 2. *Proposed roles of Glu[119] and Glu[139] in enoyl-CoA hydration,[15] illustrating the analogy between fatty acid and cinnamic acid hydratases. R = Ph, alkyl.*

is due to higher priority of the phenyl group in **9**). In *Escherichia coli*, the enoyl-CoA hydratase involved in fatty acid β-oxidation catalyzes the *syn* addition of water across the C=C double bond. Moreover, the essential catalytic residues (Gly[116], Glu[119], and Glu[139]; Scheme 2) are highly conserved in fatty acid enoyl-CoA hydratases, suggesting a common mechanism in many species.[15] Sequence alignment of the cinnamoyl-CoA hydratase EncI from "*S. maritimus*"[10] with enoyl-CoA hydratases from various fatty acid oxidation pathways shows considerable similarity (Figure 2).[19–22] Crucially, the highly conserved residues Gly[116], Glu[119], and Glu[139] are also present in the "*S. maritimus*" protein. It is noteworthy that the coumaroyl-CoA hydratase/lyase from *Pseudomonas fluorescens* does not possess an equivalent to Glu[119].[22] This enzyme converts coumaroyl-CoA to 4-hydroxy-

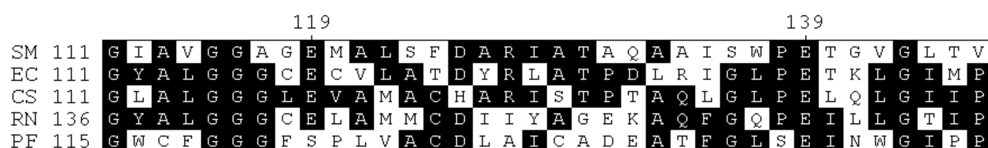
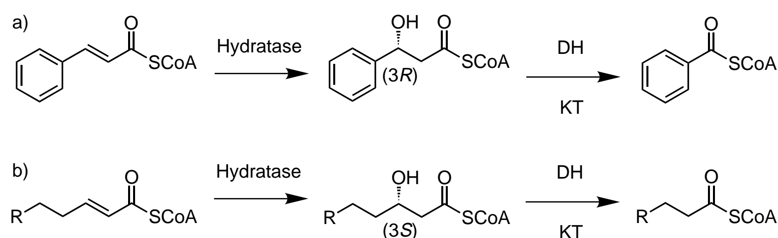


Figure 2. *Amino acid sequence alignment of cinnamoyl-CoA hydratase from "*S. maritimus*" (SM);[10] fatty acid enoyl-CoA hydratases from* E. coli *(EC),[19]* Cucumis sativus *(CS)[20] and* Rattus norvegicus *(RN);[21] coumaroyl-CoA hydratase/lyase from* Pseudomonas fluorescens *(PF).[22]*

benzaldehyde by a retro-aldol reaction and thus has some analogy to "route b" in Scheme 1.[22, 23]

In conclusion, (3*R*)-3-hydroxy-3-phenylpropionyl-CoA is an intermediate in the biosynthesis of benzoic acid in *N. attenuata* and "*S. maritimus*". The configuration of this intermediate is identical to that of its analogues in fatty acid β-oxidation, and there is considerable amino acid sequence similarity between the "*S. maritimus*" cinnamoyl-CoA hydratase EncI and fatty acid enoyl-CoA hydratases. In particular, the EncI amino acid residues Gly^{116}, Glu^{119}, and Glu^{139}, which are essential residues for catalytic activity in homologous fatty-acid-type hydratases, suggest that the two enzyme systems operate by a common mechanism. These observations shed new light on benzoate biosynthesis and provide the strongest evidence yet for the existence of a β-oxidation-like pathway leading to **1** (Scheme 3). The similarity of the plant and bacterial pathways suggests that "*S. maritimus*" could serve as a useful model organism for studying the molecular basis of this important metabolic pathway.



Scheme 3. *Similarities between the principal transformations in the a) benzoate and b) fatty acid pathways. DH = dehydrogenase, KT = ketothiolase.*

Experimental Section

Synthesis of (3*R*)- and (3*S*)-[$^2H_5$]-3-hydroxy-3-phenylpropionic acid [(3*R*)- and (3*S*)-[$^2H_5$]9]

i) Enzymatic resolution: (±)-[$^2H_5$]-3-hydroxy-3-phenylpropionic acid ethyl ester[7] was subjected to enzymatic hydrolysis using lipase PS-30,[14] giving (3*S*)-[$^2H_5$]-3-hydroxy-3-phenylpropionic acid (yield = 50.4%). The recovered ester was saponified (KOH) to give (3*R*)-[$^2H_5$]-3-hydroxy-3-phenylpropionic acid (yield = 86.0%). ii) Evans method: [$^2H_5$]benzaldehyde ([$^2H_5$]**11**) (0.5 mmol) was alkylated by using a mixture of lithium diisopropylamide (0.6 mmol) and (4*R*)-2-acetyl-4-(1-methylethyl)-2-oxazolidinone (0.6 mmol) in THF at −78 °C. Standard work-up and separation of the diastereoisomers on silica yielded (3*R*,4′*R*)-adduct (56%), $[\alpha]_D^{25} = -40.4$ ($c = 1.08$, CH_2Cl_2), and (3*S*,4′*R*)-adduct (27%), $[\alpha]_D^{25} = -114.2$ ($c = 1.08$, CH_2Cl_2).[12, 13] Subsequent hydrolysis of the carboximides with lithium hydroperoxide[16] and recrystallization from *tert*-butyl methyl ether provided the 3-hydroxy acids in 89–92% yield. Both synthetic approaches gave chiral 3-hydroxy-3-phenylpropionates with spectral data identical to those of the racemate,[7] and *ee* values > 95% (determined by GC of Mosher's esters). (3*R*)-[$^2H_5$]-3-hydroxy-3-phenylpropionic acid: $[\alpha]_D^{25} = +18.8$ ($c = 1.03$, EtOH); (3*S*)-[$^2H_5$]-3-hydroxy-3-phenylpropionic acid: $[\alpha]_D^{25} = -19.1$ ($c = 1.06$, EtOH).

Feeding experiments: i) Cell-free extracts of *N. attenuata* were prepared as described by Guo and Wagner.[17] An aliquot of this extract (1 mL) was added to 3 mL of buffer[18] containing ATP, CoA, and either (*R*)-[$^2H_5$]- or (*S*)-[$^2H_5$]**9** (10 μM). The product mixture was extracted, derivatized, and analyzed by GC–MS, as described previously.[7] ii) Fermentation of "*S. maritimus*", extraction, and analyses of the extracts were carried out as described previously.[9, 10] (±)-, (3*R*)-, and (3*S*)-[$^2H_5$]**9** were added as sterile solutions (40 μmol in 100 μL DMSO per 100 mL A1 culture) at the time of inoculation with "*S. maritimus*" preculture, and the cultures were harvested 5 days later. The mutants "*S. maritimus*" KI and KJ were conversely grown on A1 plates containing apramycin at 37 °C for 1 day.

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