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Biosynthesis

Intrinsic Ability of the β-Oxidation Pathway To Produce Bioactive Styrylpyrones

*Ying Huang, Sandra Hoefgen, Fabio Gherlone, and Vito Valiante**

Abstract: Naturally occurring α-pyrones with biological activities are mostly synthesised by polyketide synthases (PKSs) via iterative decarboxylative Claisen condensation steps. Remarkably, we found that some enzymes related to the fatty acid β-oxidation pathway in *Escherichia coli*, namely the CoA ligase FadD and the thiolases FadA and FadI, can synthesise styrylpyrones with phenylpropionic acids in vivo. The two thiolases directly utilise acetyl-CoA as an extender unit for carbon-chain elongation through a non-decarboxylative Claisen condensation, thus making the overall reaction more efficient in terms of carbon and energy consumption. Moreover, using a cell-free approach, different styrylpyrones were synthesised in vitro. Finally, targeted feeding experiments led to the detection of styrylpyrones in other species, demonstrating that the intrinsic ability of the β-oxidation pathway allows for the synthesis of such molecules in bacteria, revealing an important biological feature hitherto neglected.

Introduction

Pyrones are a class of oxygen-based heterocyclic compounds containing an unsaturated six-membered ring, with one oxygen atom and a ketone functional group, mainly found in two isomeric forms denoted as α-pyrone (2-pyrone) and γ-pyrone (4-pyrone). The carbonyl group of α-pyrones is in the α-position relative to the oxygen atom in the ring system (Figure 1A). α-pyrones are important fundamental moieties of various biologically active metabolites that are abundantly found in nature.[1] In particular, active 4-hydroxy-α-pyrones are the most commonly reported, with their C-3 and C-6 positions replaced by various substituents. Pseudopyronines A and B, probably the best examples, are 4-

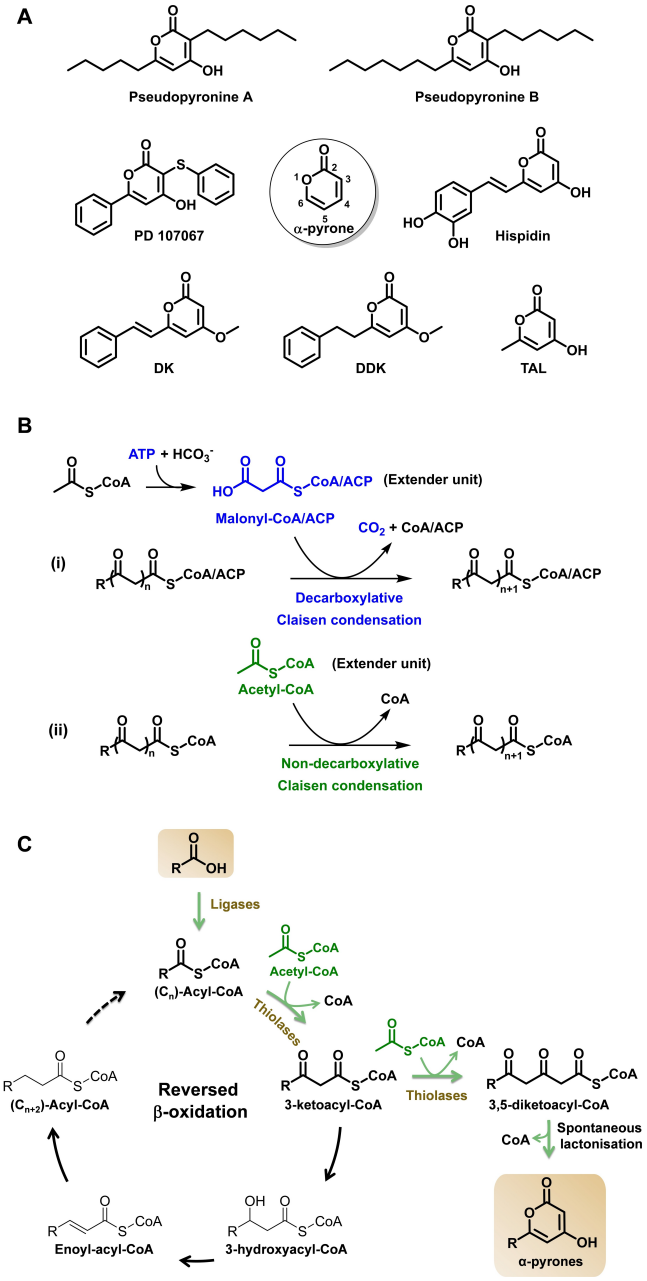

Figure 1. A) Structures of representative bioactive α-pyrones. B) Decarboxylative and non-decarboxylative Claisen condensation reactions in biological systems. C) Proposed biosynthesis of α-pyrones through degradative thiolases involved in reversal β-oxidation. The related pathways and enzymes are marked in green and dark gold, respectively.

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hydroxy-3,6-dialkyl-α-pyrones first isolated from *Pseudomonas fluorescens*,[2] having potent antibacterial,[3] algicidal[4] and antituberculosis[5] activities (Figure 1A). Other relevant α-pyrones are anti-HIV agents,[1b] such as the lead compound 4-hydroxy-6-phenyl-3-(phenylthio)-α-pyrone PD 107067 (Figure 1A),[6] and the well-known hispidin (Figure 1A), which serves as a key precursor of fungal luciferin[7] and exhibits anti-diabetic, antiviral, cytotoxic, cardioprotective and neuroprotective properties.[8] Lastly, it is worth mentioning the plant-derived psychoactive kavalactones with 4-methoxy groups like 5,6-dehydrokawain (DK) and dihydro-5,6-dehydrokawain (DDK) (Figure 1A), compounds with pharmaceutical potentials for treating anxiety, insomnia and pain.[9]

Naturally occurring α-pyrones are synthesised by a variety of biosynthetic pathways. In most cases, however, they are generated via repeated decarboxylative Claisen condensation reactions catalysed by polyketide synthases (PKSs).[10] Nevertheless, PKSs are subject to inherent energy inefficiency and low carbon economy. During decarboxylative condensations, the biosynthesis of the commonly used extender unit malonyl-CoA from acetyl-CoA leads to additional ATP consumption, while decarboxylation during carbon-chain elongation causes the loss of carbon as a dioxide (Figure 1B, i). In contrast to PKSs, the recently reported thiolase-based polyketide biosynthesis can directly use acetyl-CoA as the extender unit to form α-pyrone triacetic acid lactone (TAL) (Figure 1A) through a non-decarboxylative Claisen condensation, enabling the product synthesis at maximum energy and carbon efficiency (Figure 1B, ii).[11] In fact, the ability of thiolases to catalyse the carbon-carbon bond formation has already been used for the synthesis of alcohols and carboxylic acids in combination with the reversal of the β-oxidation pathway.[12]

The β-oxidation pathway degrades 3-ketoacyl-CoA into acyl-CoA and acetyl-CoA, and when run in reverse, the involved degradative thiolases can synthesise 3-ketoacyl-CoA using acyl-CoA as the starting unit and acetyl-CoA as the extender unit. Thus, 3-ketoacyl-CoA with different functional groups other than acetoacetyl-CoA could also potentially be used as the starting unit for a second round of carbon-chain extension to obtain 3,5-diketoacyl-CoA, chemical intermediates that undergo spontaneous lactonization followed by the release of α-pyrones (Figure 1C).

To validate this hypothesis, we explored the substrate scopes of thiolases using various precursors provided by different ligases. Here, we show that the degradative thiolases FadA and FadI possess broad substrate specificity and can synthesise α-pyrones using a wide range of starting units. Then, after an in-depth characterization of the substrate promiscuity of different CoA ligases, we discovered that the long-chain fatty acid:CoA ligase FadD from *Escherichia coli* is able to esterify some phenylpropionic acid derivatives as well. With this, we were able to successfully produce bioactive styrylpyrones in *E. coli* simply by overexpressing FadD together with FadA or FadI, revealing an alternative pathway for the production of this class of bioactive compounds.

Results and Discussion

Potential α-Pyrone Biosynthetic Pathways in E. coli

We first treated *E. coli* BL21 (DE3) expressing a 4-coumarate:CoA ligase (4CL) from *Nicotiana tabacum*[13] with phenylpropionic acids and observed the production of 6-styryl- and 6-dihydrostyryl-α-pyrones (uniformly referred to as styrylpyrones) (Figure S1). This implies that some endogenous enzymes in *E. coli* can indeed catalyse the formation of α-pyrones using phenylpropionyl-CoAs. Notably however, feeding *E. coli* directly with 3-phenylpropionic acid also produced a trace amount of the corresponding styrylpyrone (Figure S1B), suggesting the presence of ligases that could potentially esterify the substrate and provide 3-phenylpropionyl-CoA.

Given the absence of type-III PKSs in *E. coli*, the biosynthesis of styrylpyrones upon feeding phenylpropionic acids could be explained through the involvement of thiolases, which could catalyse the condensation of acyl-CoA with acetyl-CoA to form 3-ketoacyl-CoA. Therefore, we hypothesised that four *E. coli* thiolase enzymes, namely AtoB, YqeF, FadA and FadI, could potentially catalyse the carbon-carbon extension. According to literatures, AtoB is an acetyl-CoA acetyltransferase (or biosynthetic thiolase) and exhibits high specificity for short-chain acyl-CoA;[14] YqeF is a predicted acyltransferase sharing high similarity to AtoB;[12a,15] FadA and FadI are both 3-ketoacyl-CoA thiolases (or degradative thiolases) with broad chain length specificity and play parallel roles in aerobic and anaerobic β-oxidation of fatty acids, respectively.[16]

Among them, AtoB has been reported to be employed in vitro for the production of TAL.[11] Nonetheless, we further investigated whether all the above mentioned thiolases could as well synthesise α-pyrones by the condensation of acetyl- and acetoacetyl-CoA. The results showed that the purified FadA, FadI, AtoB and YqeF (Figure S2) can all use acetyl- and acetoacetyl-CoA to synthesise TAL, with YqeF having a comparatively lower efficiency (Figure 2A). Additionally, the product of two rounds of condensations, the tetraacetic acid lactone, was also detected in the reactions containing each of the four thiolases (Figure S3). We then repeated the experiments by using acetyl-CoA as the only substrate, observing that FadA, FadI and AtoB underwent two rounds of condensations forming the TAL (Figure 2A). These results suggest that FadA, FadI and AtoB can utilise both acetoacetyl-CoA and acetyl-CoA as starting substrates. To further investigate this unexpected finding, we explored the substrate preference of these three thiolases for acetoacetyl-CoA and acetyl-CoA by labelling experiments. The ^{13}C-acetyl-CoA was synthesised from ^{13}C-malonate using purified malonyl-CoA synthetase (MatB) and malonyl-CoA decarboxylase (MatA) from *Rhizobium leguminosarum* (Figure 2B).[17] When both ^{13}C-acetyl-CoA and acetoacetyl-CoA were present, FadA, FadI and AtoB all mainly produced two-carbon-labelled TALs with only trace amounts of the fully-labelled TALs generated from ^{13}C-acetyl-CoA (Figure 2B). In addition, when only ^{13}C-acetyl-CoA was available, all

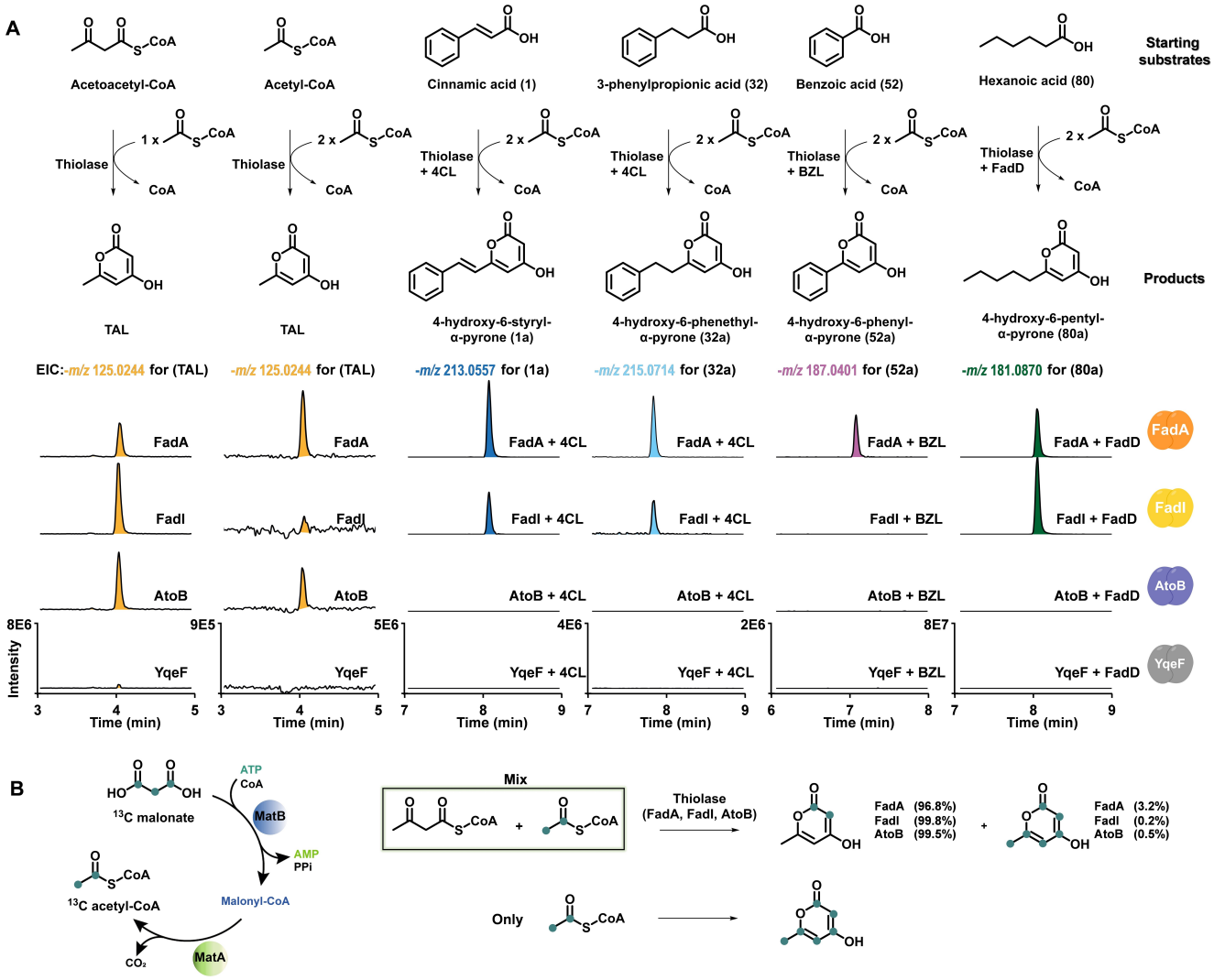


Figure 2. In vitro characterization of the functions of *E. coli* thiolases FadA, FadI, AtoB and YqeF. A) Proposed pathways for the synthesis of different α-pyrones by thiolases from various starting substrates activated by ligases 4CL, BZL and FadD, and LC-HRMS extracted ion chromatograms (EIC) of products from corresponding in vitro enzymatic reactions. The EICs of tetraacetic acid and diketide intermediates are shown in Figure S3. B) Exploring the substrate preference of thiolases FadA, FadI and AtoB by using ^{13}C-acetyl CoA, which was generated from ^{13}C-malonate using malonyl-CoA synthetase (MatB) and malonyl-CoA decarboxylase (MatA). The percentages show the relative ratios of the produced TAL having either two or fully labelled carbons.

three thiolases produced fully-labelled TALs, but at lower yields (about 20 times less when compared to acetoacetyl-CoA).

The in vitro assays performed with the isolated thiolases revealed their biosynthetic potential, which is in contrast with their assigned function. In particular, FadI and FadA are generally involved in the β-oxidation pathway as degradative thiolases. Moreover, the assay confirmed the highest preference for acetoacetyl-CoA as starting units, which make the overall reactions performed by the thiolases thermodynamically more favourable if compared to the necessity of producing the initial acetoacetyl-CoA from two molecules of acetyl-CoA.[18] Additionally, this experiment revealed that FadA, FadI and AtoB may utilise alternative acyl-CoAs as initial substrates.

Inspired by the previous experiment, we repeated the in vitro tests by using cinnamic acid (**1**) and 3-phenylpropionic acid (**32**). The corresponding CoA-esters were obtained by using the purified 4CL from *N. tabacum*. As shown, FadA and FadI produced 4-hydroxy-6-styryl-α-pyrone (**1a**) and 4-hydroxy-6-phenethyl-α-pyrone (**32a**) through two rounds of iterative non-decarboxylative Claisen condensation steps, while AtoB and YqeF could not use these phenylpropionyl-CoA precursors as substrates (Figure 2A). Afterwards, we also tested benzoic acid (**52**), which was esterified via a benzoate:CoA ligase (BZL) from *Rhodopseudomonas palustris*. Among the four thiolases, only FadA synthesised 4-hydroxy-6-phenyl-α-pyrone (**52a**) (Figure 2A), while AtoB and YqeF used the substrate and one unit of acetyl-CoA forming a diketide intermediate (Figure S3). Next, we employed the *E. coli* long-chain fatty acid:CoA ligase FadD

to produce hexanoyl-CoA (**80**), which was successfully used by FadA and FadI to yield 4-hydroxy-6-pentyl-α-pyrone (**80a**) (Figure 2A), while AtoB and YqeF catalysed again only one round of non-decarboxylative condensation to form the corresponding diketide intermediate (Figure S3).

Overall, we concluded that the thiolases FadA, FadI, AtoB and YqeF generally use up to two units of acetyl-CoA for two rounds of iterative non-decarboxylative Claisen condensation reactions. Because of the strict specificity of AtoB and YqeF for short-chain acyl-CoA, they utilise relatively short precursors such as acetyl-CoA, acetoacetyl-CoA, benzoyl-CoA and hexanoyl-CoA. YqeF uses these precursors to undergo only one round of non-decarboxylative Claisen condensation, whereas AtoB catalyses both one and two rounds of condensation for acetyl-CoA and acetoacetyl-CoA, and only one round for benzoyl-CoA and hexanoyl-CoA (Figure S3 and S4).

The above results have shown that there is a connection between the substrates used and the number of performed iterative condensations. The mechanism that determines the elongation steps during polyketide formation is still unsolved, but based on the results obtained here, we can hypothesise that bulkier starting units may reduce the space in the catalytic site by prematurely interrupting the iterative elongation, similarly to what has been previously suggested for type-III PKSs.[19] From another point of view, triketide intermediate is highly reactive, which may be the reason why the third and more rounds of condensation did not occur. Upon the occurrence of two rounds of the Claisen condensation, the enzyme forms a 3–5-diketoacyl-CoA intermediate, which then spontaneously off-loads through lactonisation, leading to the α-pyrones. Therefore, FadI and FadA, especially the latter one, have the broadest substrate specificity but can only catalyse up to two rounds of condensations to form α-pyrones.

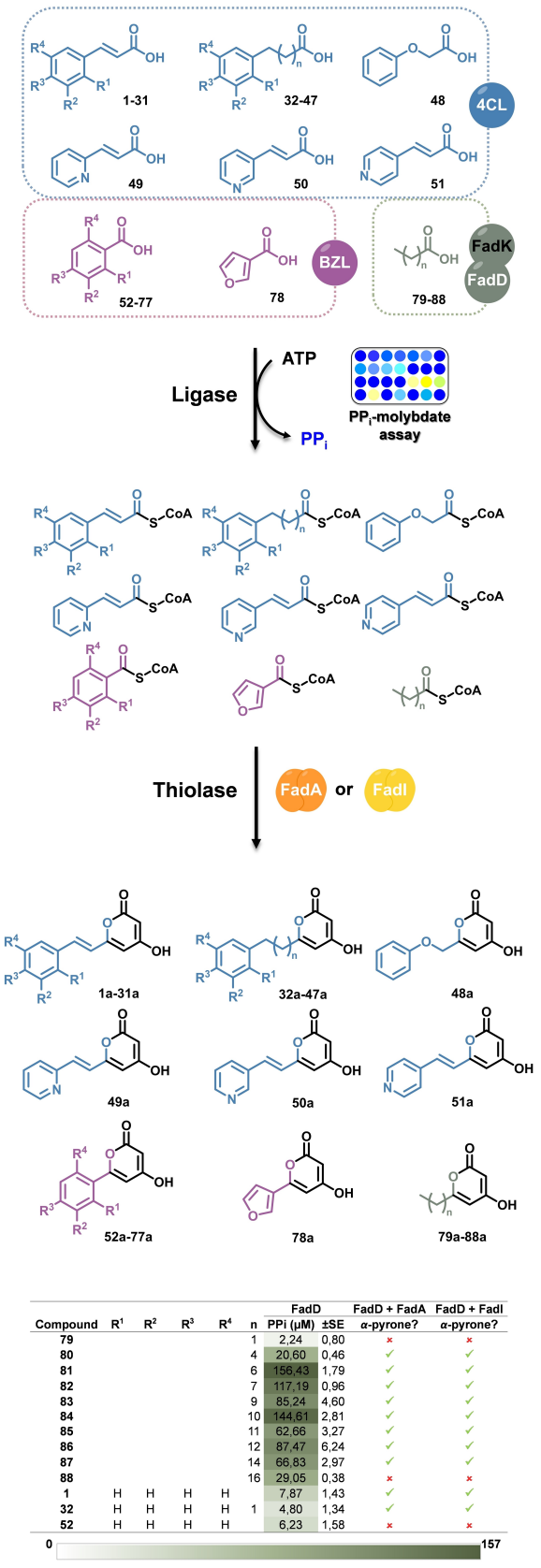
In-Depth Characterization of Substrate Specificity of CoA Ligases and Thiolases

To provide a variety of CoA precursors for the studied thiolases, so as to explore their substrate specificity and simultaneously synthesise a vast array of α-pyrones, we investigated the substrate promiscuity of the used ligases 4CL, BZL and FadD. For a more comprehensive array, we also added the *E. coli* medium-chain fatty acid:CoA ligase FadK in our experiments.[20] The high-throughput colorimetric assay used here was based on the detection of pyrophosphate (PP_i), as reported before.[21] The PP_i was released upon ATP consumption during CoA-esterification, forming stable phosphomolybdate complexes. Thus, the PP_i values are related to the ease of formation of CoA esters catalysed by the ligases. We tested 88 different substrates in three main classes, which are phenylpropionic acid derivatives, benzoic acid derivatives and fatty acids (Figure 3). For the substrate specificity of 4CL, we tested phenylpropionic acid derivatives (**1**–**44**), phenylacetic acid (**45**), phenylbutyric acid (**46**), phenylvaleric acid (**47**), phenoxyacetic acid (**48**) and pyridylacrylic acids (**49**–**51**). As for BZL, we tested benzoic acid derivatives (**52**–**77**) as well as 3-furoic acid (**78**). Moreover, because of the intrinsic ability of *E. coli* of producing **32a**, we also tested whether the fatty acid:CoA ligases FadD and FadK could be used to esterify **1**, **32** and **52**. Lastly, we included the substrate specificity of FadD and FadK for different lengths of fatty acids (**79**–**88**).

The PP_i values indicated that most of the tested substrates were successfully esterified by the corresponding ligases. Thus, except for those substrates with very low catalytic values, all the esterified substrates were used to verify the substrate specificity of FadA and FadI by detecting the formation of the corresponding α-pyrones (Figure S5–S8). The results showed that the majority of substrates esterified by the 4CL were used by both FadA and FadI, with molecules ranging from C6–C2 to C6–C5, even including 3-(2-pyridyl)acrylic acid (**49**) (Figure 3). This led to the production of various interesting compounds, including bisnoryangonin (**16a**) and hispidin (**23a**).[22] We also observed that only a few benzoyl-CoA precursors were utilised by FadA and none could be used by FadI (Figure 3). Concerning FadK, this enzyme has been reported to be maximally active in esterifying C_6 and C_8 fatty acids;[20] yet we found that FadK has an overall much lower affinity for all the tested fatty acids if compared to FadD. Among the esterified fatty acids, only the C_8, C_9, and C_{11} fatty acyl-CoA were used by FadA and FadI, producing trace amounts of the corresponding α-pyrones. Notably however, our results showed that FadD has an even broader substrate scope than it has been reported before.[23] Indeed, we could demonstrate that FadD is able to esterify not only even carbon number fatty acids (C_6, C_8, C_{12}, C_{14} and C_{16}), but also odd carbon number fatty acids (C_9, C_{11} and C_{13}) and even **1** and **32**, which when coupled with FadA and FadI yielded the α-pyrones **1a** and **32a**, respectively (Figure 3 and Figure S7). This implies that *E. coli* contains a complete set of available enzymes to synthesise styrylpyrones upon the feeding with the right precursor.

In Vivo Functional Characterization of FadA and FadI

Since the in vitro analyses showed the possibility of synthesising styrylpyrones using the bacterial native ligases and thiolases, we overexpressed in *E. coli* BL21 the ligases (FadD and FadK) and thiolases (FadA and FadI) in different combinations, and heterologously expressed 4CL and BZL to serve as controls. For this purpose, we employed the single plasmid multigene expression system previously reported.[21] When **1** and **32** were fed separately to *E. coli* expressing the empty plasmid, only a small amount of **32a** was observed (Figure 4A, i). The individual expression of FadA and FadK resulted in no changes (Figure 4A, ii and v), while the single expression of FadI or FadD led to the detection of **1a** and the increase of **32a** (Figure 4A, iii and iv). Co-expression of FadD with FadA or FadI resulted in significantly higher yields of both **1a** and **32a** (Figure 4A, vi and vii), similar to the co-expression of FadA or FadI with the 4CL (Figure 4A, xi and xii). This suggests that in vivo, the catalytic efficiency of FadD in the esterification of



Compound	R¹	R²	R³	R⁴	n	4CL PPi (µM)	±SE	4CL + FadA α-pyrone?	4CL + FadI α-pyrone?
1	H	H	H	H		31,46	4,12	✓	✓
2	OH	H	H	H		27,20	2,24	✓	✓
3	OCH_3	H	H	H		2,38	0,57	NT	NT
4	NH_2	H	H	H		21,10	0,53	✓	✓
5	NO_2	H	H	H		0,00	2,90	NT	NT
6	F	H	H	H		36,04	1,74	✓	✓
7	Cl	H	H	H		2,85	1,35	NT	NT
8	Br	H	H	H		0,00	1,17	NT	NT
9	H	OH	H	H		37,28	5,57	✓	✓
10	H	OCH_3	H	H		38,20	1,38	✓	✓
11	H	NH_2	H	H		51,07	1,51	✓	×
12	H	NO_2	H	H		10,31	1,22	✓	✓
13	H	F	H	H		26,17	1,05	✓	✓
14	H	Cl	H	H		30,33	0,73	✓	✓
15	H	Br	H	H		39,49	4,30	✓	✓
16	H	H	OH	H		60,12	4,46	✓	✓
17	H	H	OCH_3	H		23,43	2,12	✓	✓
18	H	H	NH_2	H		93,39	1,90	✓	✓
19	H	H	NO_2	H		0,86	1,85	NT	NT
20	H	H	F	H		24,74	1,01	✓	✓
21	H	H	Cl	H		23,31	2,10	✓	✓
22	H	H	CF_3	H		9,93	1,53	✓	✓
23	H	OH	OH	H		44,97	2,88	×	✓
24	H	OCH_3	OH	H		53,20	5,34	✓	✓
25	H	OCH_3	OCH_3	H		1,32	1,69	NT	NT
26	OH	H	OH	H		34,96	1,63	×	×
27	H	OH	H	OH		25,84	4,72	✓	✓
28	OCH_2O		H	H		12,54	1,36	✓	✓
29	H	OCH_2O		H		28,82	2,13	✓	✓
30	H	OH	OH	OH		40,62	1,80	×	×
31	H	OCH_3	OH	OCH_3		4,35	1,69	NT	NT
32	H	H	H	H	1	18,52	1,69	✓	✓
33	NO_2	H	H	H	1	10,29	1,97	✓	✓
34	Cl	H	H	H	1	10,36	2,28	✓	✓
35	H	NO_2	H	H	1	8,57	1,66	✓	✓
36	H	Cl	H	H	1	19,47	3,78	✓	✓
37	H	H	OH	H	1	64,64	7,11	✓	✓
38	H	H	OCH_3	H	1	7,87	2,00	✓	✓
39	H	H	NO_2	H	1	9,11	1,72	✓	✓
40	H	H	Cl	H	1	8,57	3,35	✓	✓
41	H	H	CF_3	H	1	9,19	1,85	✓	✓
42	H	OCH_3	OCH_3	H	1	8,13	1,84	×	×
43	H	OCH_2O		H	1	8,81	2,84	✓	✓
44	H	OCH_3	OH	OCH_3	1	7,96	1,52	×	×
45	H	H	H	H	0	5,83	0,06	✓	✓
46	H	H	H	H	2	6,31	0,37	✓	✓
47	H	H	H	H	3	9,69	1,86	✓	✓
48						8,01	1,43	✓	✓
49						25,44	1,67	✓	✓
50						4,22	1,21	NT	NT
51						3,02	0,59	NT	NT

0 — 94

Compound	R¹	R²	R³	R⁴	BZL PPi (µM)	±SE	BZL + FadA α-pyrone?	BZL + FadI α-pyrone?
52	H	H	H	H	30,74	0,19	✓	×
53	CH_3	H	H	H	14,25	1,89	×	×
54	H	CH_3	H	H	11,93	1,58	✓	×
55	H	H	CH_3	H	13,19	1,62	✓	×
56	OH	H	H	H	10,76	1,40	×	×
57	H	OH	H	H	21,63	1,34	✓	×
58	H	H	OH	H	13,72	1,58	✓	×
59	OCH_3	H	H	H	10,31	0,13	×	×
60	H	OCH_3	H	H	11,20	2,63	×	×
61	H	H	OCH_3	H	10,58	2,48	×	×
62	NH_2	H	H	H	9,78	1,64	×	×
63	H	NH_2	H	H	9,25	1,26	×	×
64	H	H	NH_2	H	4,60	0,99	×	×
65	NO_2	H	H	H	5,63	1,91	×	×
66	H	NO_2	H	H	3,69	0,63	×	×
67	H	H	NO_2	H	3,33	0,17	×	×
68	F	H	H	H	62,29	3,84	✓	×
69	H	F	H	H	59,83	0,53	✓	×
70	H	H	F	H	41,04	2,56	✓	×
71	Cl	H	H	H	13,40	2,35	×	×
72	H	Cl	H	H	11,91	2,26	✓	×
73	H	H	Cl	H	5,50	0,73	✓	×
74	OH	OH	H	H	4,25	0,79	×	×
75	OH	H	H	CH_3	5,89	2,32	×	×
76	OH	H	OH	CH_3	6,75	2,19	×	×
77	H	OCH_2O		H	5,52	2,38	×	×
78					24,49	2,03	×	×

0 — 63

Compound	R¹	R²	R³	R⁴	n	FadD PPi (µM)	±SE	FadD + FadA α-pyrone?	FadD + FadI α-pyrone?
79					1	2,24	0,80	×	×
80					4	20,60	0,46	✓	✓
81					6	156,43	1,79	✓	✓
82					7	117,19	0,96	✓	✓
83					9	85,24	4,60	✓	✓
84					10	144,61	2,81	✓	✓
85					11	62,66	3,27	✓	✓
86					12	87,47	6,24	✓	✓
87					14	66,83	2,97	✓	✓
88					16	29,05	0,38	×	×
1	H	H	H	H		7,87	1,43	✓	✓
32	H	H	H	H	1	4,80	1,34	✓	✓
52	H	H	H	H		6,23	1,58	×	×

0 — 157

Compound	R¹	R²	R³	R⁴	n	FadK PPi (µM)	±SE	FadK + FadA α-pyrone?	FadK + FadI α-pyrone?
79					1	7,55	1,12	×	×
80					4	6,20	0,65	×	×
81					6	1,74	0,61	✓	✓
82					7	15,65	2,06	✓	×
83					9	23,37	0,45	✓	✓
84					10	6,22	1,50	×	×
85					11	17,66	1,46	×	×
86					12	13,10	0,88	×	×
87					14	12,19	1,77	×	×
88					16	12,22	2,05	×	×
1	H	H	H	H		2,87	0,60	×	×
32	H	H	H	H	1	8,92	0,37	×	×
52	H	H	H	H		9,51	0,84	×	×

Figure 3. Substrate promiscuity of ligases (4CL, BZL, FadD and FadK) and thiolases (FadA and FadI). For determining the ligase specificity in vitro, a molybdate-based activity assay was performed using substrates **1**–**88**. Based on the PP_i values, the substrates (**1**, **2**, **4**, **6**, **9**–**18**, **20**–**24**, **26**–**30**, **32**–**49**, **52**–**88**) that were shown to be taken by the corresponding ligase were used to further verify the substrate promiscuity of thiolases. And this was achieved by producing α-pyrone derivatives in vitro with the purified ligases and thiolases. The production of α-pyrones was detected via LC-HRMS (generated is indicated by a green tick, otherwise by a red cross; NT means not tested).

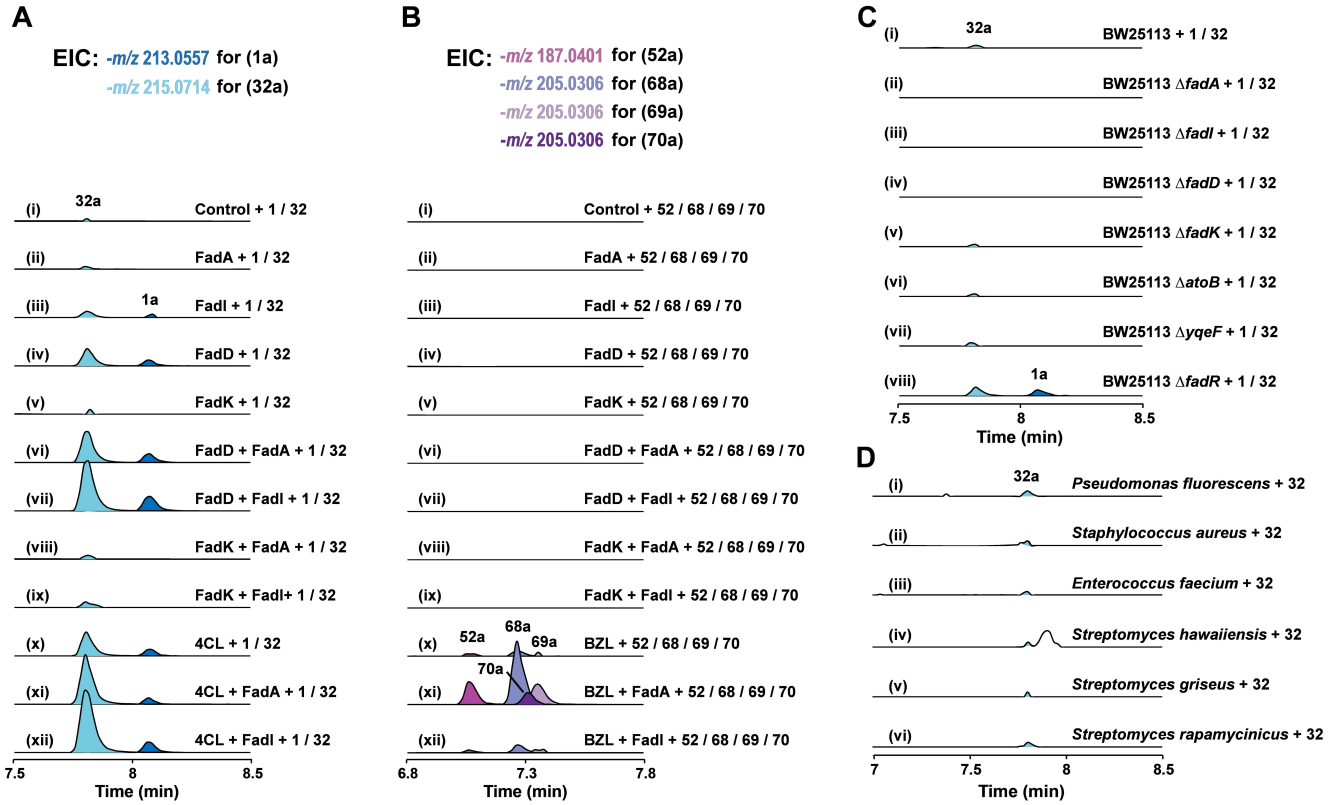


Figure 4. Production of α-pyrones in *E. coli* and other bacteria. LC-HRMS EIC showing the production of α-pyrone derivatives (**1a**, **32a**, **52a**, **68a**, **69a** and **70a**) from *E. coli* strains containing different enzymes expressed from pMGE-T7 vector fed with A) phenylpropionic acid derivatives **1** or **32**; B) benzoic acid derivatives **52**, **68**, **69** or **70**. C) Production of 6-dihydrostyryl-α-pyrone **32a** in *E. coli* K-12 BW25113 single-gene knockout mutants. D) Different bacterial species produced **32a** after direct feeding **32**.

phenylpropionic acid derivatives is somehow similar to that of the plant-derived 4CL. Subsequently, we fed other phenylpropionic acid derivatives (**16**, **17**, **22**, **29**, **37**, **38**, **41** and **43**) to *E. coli* co-expressing FadD in combination with FadA or FadI, resulting in the production of corresponding bioactive 6-styryl and 6-dihydrostyryl-α-pyrones (Figure S9).[24] As for benzoic acid derivatives, in vitro assays showed higher yields of α-pyrones **68a**, **69a** and **70a** produced with fluoro-substituted benzoic acids **68**, **69** and **70** as starting substrates. Therefore, **52**, **68**, **69** and **70** were fed individually to *E. coli.* The results showed that only the *E. coli* expressing the BZL was able to use benzoic acid derivatives to produce the corresponding α-pyrones (Figure 4B). Hence, both in vitro and in vivo results indicated that FadD is not able to catalyse the formation of the CoA-esters from benzoic acid derivatives. Meanwhile, production of **52a**, **68a**, **69a** and **70a** in *E. coli* containing BZL again confirmed that FadA can utilise benzoyl-CoA derivatives as starting units.

Given the observed production of **32a** in *E. coli*, we further validated the functions of *fadD*, *fadK*, *fadA*, *fadI*, *atoB* and *yqeF* using the *E. coli* K-12 BW25113 single-gene knockout mutants. The deletion of *fadK*, *atoB* and *yqeF* had no effect on the production of **32a** (Figure 4C, v–vii), while the deletion of *fadA*, *fadI* and *fadD* was crucial, confirming that the ligase FadD and thiolases FadA and FadI are the key enzymes involved in the α-pyrones biosynthesis in vivo (Figure 4C, ii–iv). Consequently, the deletion of *fadR*, negative regulator of the *fad* operon, resulted in the detection of **1a** and the increase of **32a** (Figure 4C, viii).

Knowing the ubiquity of the β-oxidation pathway and its related enzymes, we speculate that other bacteria may synthesise styrylpyrones. Therefore, we tested available Gram-positive and Gram-negative bacteria kept in our laboratory and treated them directly with **32**. As shown, they all produced trace amounts of **32a** under different culture conditions (Figure 4D). Additionally, we detected the TAL production in many of these species without precursor feeding. Moreover, in *Enterococcus faecium*, although no TAL was detected, we observed the production of fatty acid-derived pyrones (**80a** and **81a**) (Figure S10). These findings could substantiate the natural competence of bacteria in producing α-pyrones.

In Vivo Synthesis of Kavalactones DK and DDK

With the acquired knowledge, we decided to exploit the *E. coli* thiolases and ligase to produce plant-derived kavalactones.[9a,b] Naturally occurring kavalactones, DK and DDK (Figure 1A), are structurally the 6-styryl- and 6-dihydrostyryl-α-pyrones (**1a** and **32a**) with *O*-methylation at

C-4 position.[10c] For their synthesis, the kava *O*-methyltransferase 1 (KOMT1) was used to methylate the C-4 hydroxyl group (Figure 5A). Subsequent feeding of *E. coli* co-expressing FadD, FadA/FadI and KOMT1 with **1** and **32** resulted in the production of DK and DDK, respectively (Figure 5B). For DK synthesis, the FadI-based pathway produced ≈0.1 mg L^{-1}, 20 times higher than the FadA-based pathway. However, both pathways had similar yields for DDK (≈1.0 mg L^{-1}), which was 10-fold higher than DK (Figure 5C). Here, we showed the possibility of synthesising kavalactones using FadD, FadA/FadI and KOMT1, so that the production of other psychoactive kavalactones using different phenylpropionic acid substrates is achievable. Moreover, this approach, in combination with further optimization, has the potential to improve the productivity of target product.

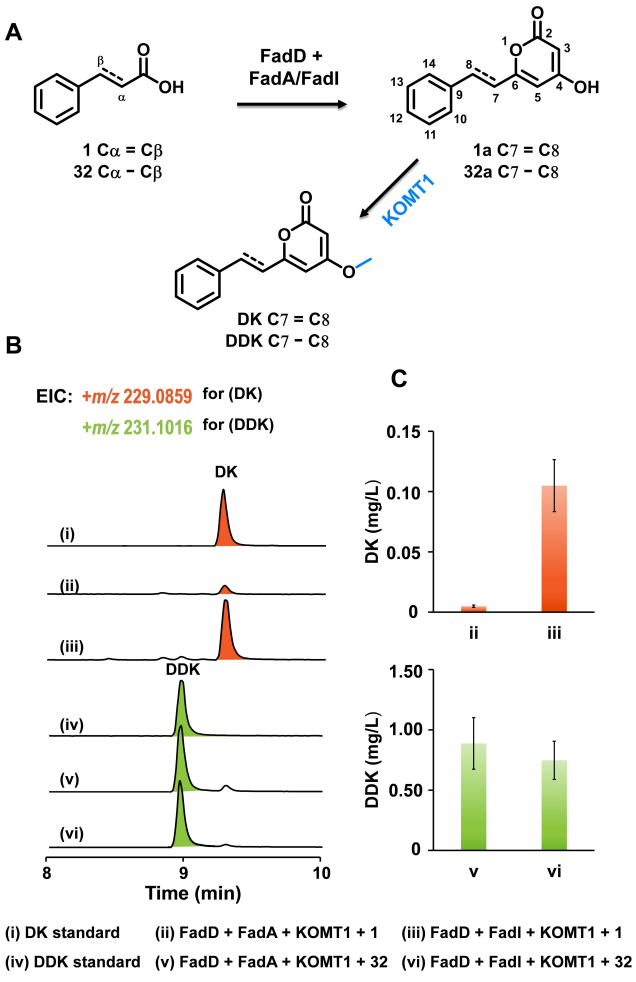


Figure 5. Formation of kavalactones DK and DDK through non-decarboxylative Claisen condensation in *E. coli*. A) The proposed pathway for the synthesis of DK and DDK using FadD, FadA (or FadI) and KOMT1. KOMT1 methylates the hydroxy group at C-4 position (shown in blue). B) LC-HRMS EIC of DK and DDK produced by *E. coli* expressing FadD, FadA/FadI and KOMT1 fed with **1** and **32**, respectively. C) DK and DDK titers in vivo. The error bars represent the standard deviations calculated from three independent biological replicates.

Conclusion

The reversal of the β-oxidation cycle has been long investigated for the opportunity of using its biosynthetic potential to produce valuable chemicals and fuels.[12a] So far, the main focus was to exploit the thiolases to produce linear short-/middle-chain fatty acids through a non-decarboxylative Claisen condensation.[25] Moreover, we have recently learned that thiolases can also be employed to produce alkylresorcinol derivatives and α-pyrones, revealing novel biochemical concepts.[11] Here, we moved one step forward, demonstrating the synthesis of styrylpyrones using the β-oxidation-related thiolases FadA and FadI. The fact that these enzymes could use phenylpropionyl-CoA derivatives was unexpected and revealed novel biosynthetic routes. So far, the biosynthesis of styrylpyrones has been mainly connected to the plant-derived type III-PKSs.[26] In other organisms, such as fungi, the production of bioactive styrylpyrone like hispidin was instead connected to NRPS-PKS coding genes.[7]

One mandatory aspect in producing styrylpyrones from thiolases is the availability of the esterified precursors. Interestingly, FadD, the *E. coli* long-chain fatty acid:CoA ligase, was shown here to be capable of esterifying a wide range of phenylpropionic acid derivatives. Given the presence of the fatty acids β-oxidation pathway in all living organisms, the biosynthetic capacity of producing styrylpyrones should be present in all bacteria. Indeed, feeding experiments on a few Gram-positive and Gram-negative bacteria confirmed the presence and production of the α-pyrone **32a** in wild-type species. From an ecological point of view, if we assume that phenylpropionic acid derivatives, as products of the degradation of lignin and other aromatic molecules by plants,[27] are widely present in nature, then we can imagine that all bacteria have the potential to acquire and transform these precursors and produce styrylpyrones. Lastly, we have shown here that the studied thiolases can be employed to produce relevant compounds, such as the plant-derived kavalactones DK and DDK, in *E. coli*, already revealing the biotechnological potential of exploiting more favourable non-decarboxylative Claisen condensation steps to produce bioactive styrylpyrones.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the Supporting Information of this article.

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