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Characterization of FabG and FabI of the *Streptomyces coelicolor* Dissociated Fatty Acid Synthase

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Streptomyces coelicolor produces fatty acids for both primary metabolism and for biosynthesis of the secondary metabolite undecylprodiginine. The first and last reductive steps during the chain elongation cycle of fatty acid biosynthesis are catalyzed by FabG and FabI. The *S. coelicolor* genome sequence has one *fabI* gene (SCO1814) and three likely *fabG* genes (SCO1815, SCO1345, and SCO1846). We report the expression, purification, and characterization of the corresponding gene products. Kinetic analyses revealed that all three FabGs and FabI are capable of utilizing both straight and branched-chain β-ketoacyl-NAC and enoyl-NAC substrates, respectively. Furthermore, only SCO1345 differentiates between ACPs from both biosynthetic pathways. The data presented provide the first experimental evidence that SCO1815, SCO1346, and SCO1814 have the catalytic capability to process intermediates in both fatty acid and undecylprodiginine biosynthesis.

Introduction

Streptomycetes, like many other bacteria, utilize a type II fatty acid synthase to catalyze the formation of fatty acids. They produce primarily branched-chain fatty acids with only a minor proportion of straight-chain fatty acids.[1] The first step in a type II fatty acid synthase (FAS) process is catalyzed by FabH (β-ketoacyl synthase III), which catalyzes a decarboxylative condensation of an acyl-CoA primer with a malonyl-acyl carrier protein (ACP; Scheme 1). For branched-chain fatty acids, the primer is typically either isobutyryl-CoA or methylbutyryl-CoA. For straight-chain fatty acids, acetyl-CoA and propionyl-CoA serve as the most common primers. The resulting 3-ketoacyl-ACP product is reduced by NADPH-dependent FabG to provide 3-hydroxyacyl-ACP, which is dehydrated by FabA to form enoyl-ACP. The NADH-dependent FabI completes the cycle by catalyzing a reduction to provide the corresponding acyl-ACP. In subsequent rounds of elongation, the condensation step with the acyl-ACP is catalyzed by FabF rather than FabH, which only catalyzes the chain initiating step. The malonyl-ACP utilized in each elongation step is generated from malonyl-CoA by the action of a malonyl CoA:ACP transacylase (encoded by *fabD*). In streptomycetes, *fabH, fabC* (encoding the ACP), *fabF*, and *fabD* are clustered together as an operon.[2]

Streptomycetes also produce a vast array of biologically active secondary metabolites widely used in human health, such as for antibacterial, immunosuppressant, anticancer, and antimalarial purposes,[3] and in a number of cases, these natural products use fatty acids as a building block. These fatty acids often differ in chain length or extent of branching from those made by the primary metabolic type II FAS (e.g., daptomycin, frenolicin, R1128, tunicamycin, and undecylprodiginine).[4–9] The biosynthesis of their alkane chain occurs by a type II FAS pathway, which must differ from the primary metabolic pathway in the type of acyl precursors and number of cycles used. One such example is undecylprodiginine, a tripyrrole red pigmented compound with a linear alkyl chain known to exhibit a wide range of biological activities, such as antibacterial, immunosuppressive, antimalarial, and anticancer activities.[10,11]

Studies have revealed that the control of the two processes providing fatty acids for primary and secondary metabolism lies partially in the initial condensing enzymes.[1,12,13] For undecylprodiginine biosynthesis in *Streptomyces coelicolor*, homologues of the condensing enzymes (FabH and FabF) and the ACP (FabC) are encoded by *redP*, *redR*, and *redQ*, respectively in the *red* gene cluster. RedP is proposed to initiate biosynthesis of undecylprodiginine's alkane chain by condensing an acyl-CoA with a malonyl-RedQ. The 3-keto group of the resulting 3-ketoacyl-RedQ is then reduced to provide butyryl-RedQ, presumably by the type II FAS enzymes FabG, FabA, and FabI. RedR would then catalyze the subsequent condensation steps with malonyl-RedQ, and type II FAS enzymes shared with primary metabolism would handle reduction of the 3-keto group during each elongation cycle. Recently, we have demonstrated that FabH and its homologue, RedP, which catalyze the initial condensation in fatty acid and undecylprodiginine biosynthesis, respectively, are highly selective for either straight- (RedP) or branched-chain (FabH) acyl-CoA substrates.[14] Specifically, RedP will only process acetyl-CoA, whereas FabH is more efficient with branched acyl-CoA substrates. Additionally, both enzymes have been shown to possess differing ACP specificities: RedP only processes RedQ, whereas FabH is more efficient with FabC. This combination of acyl-CoA and ACP specificity

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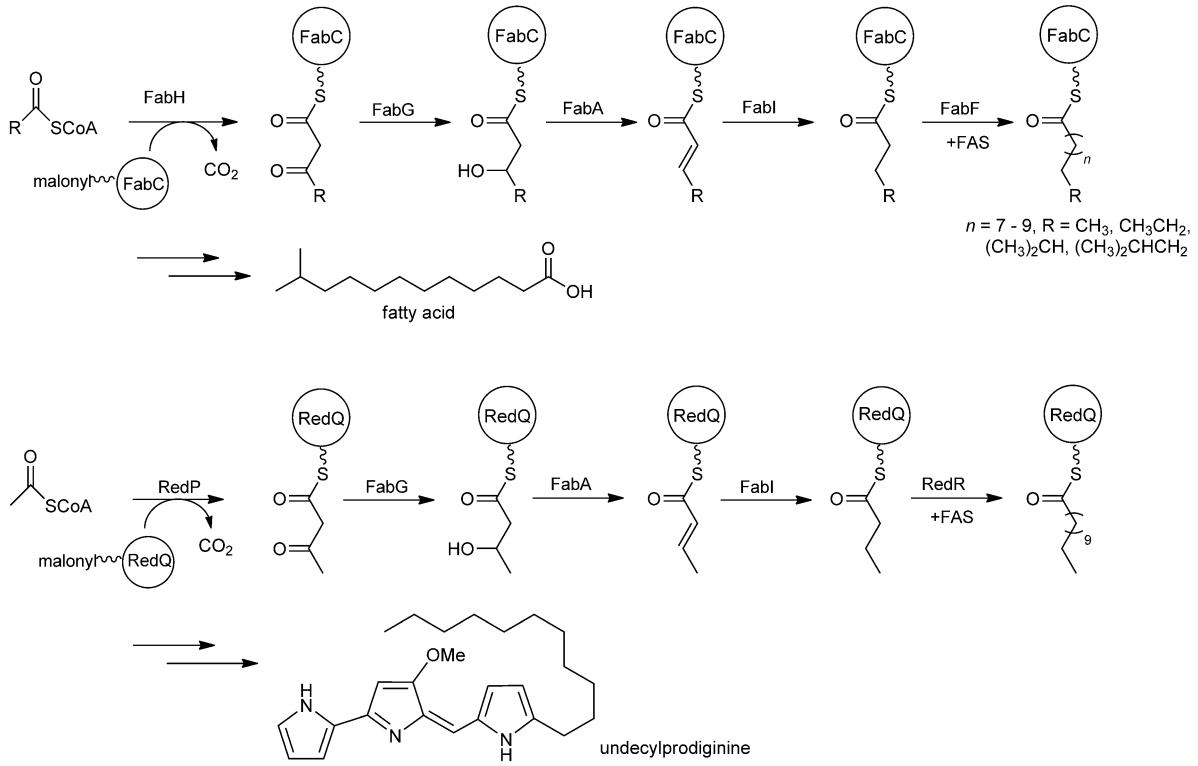


Scheme 1. Interface between fatty acid and undecylprodiginine biosynthesis in *S. coelicolor*.

provides for a separation of the two processes and control of the final product at the initiation step.

Separate enzymes and dedicated ACPs thus appear to be key for separating the primary and secondary fatty acid biosynthetic processes. As only these genes are found in the secondary metabolite gene cluster, the remaining enzymes to complete each cycles are likely to be shared. To date, most work on this metabolic crosstalk has focused on FabD (malonyl-CoA:ACP transacylase), the most well-established and widespread example of a crosstalk between fatty acid biosynthesis (a primary metabolic process) and polyketide biosynthesis (a secondary metabolic process).[15] The gene for *fabD* is clustered with *fabH*, *fabF*, and *fabC*.[2] Studies have revealed that FabD has no significant ACP selectivity and plays an important role in both natural product and fatty acid biosynthetic processes.

The genes encoding 3-ketoacyl-ACP reductase (FabG), 3-hydroxyacyl-ACP dehydratase (FabA), and enoyl-ACP reductase (FabI), putatively shared between primary and secondary fatty acid synthesis, have not been characterized in Streptomyces (Scheme 1). The corresponding genes are not present within the streptomycetes's FAS gene cluster. Analysis of the *S. coelicolor* genome sequence revealed the presence of one *fabI* gene (SCO1814, encoding an enoyl-ACP reductase), and three potential *fabG* genes (SCO1815, SCO1345, and SCO1846, encoding β-ketoacyl-ACP reductase).[16] The putative FabI has not been previously characterized, and of the three possible candidates for *fabG*, only one (SCO1815, which lies immediately adjacent to *fabI*) has been partially characterized.[17] In this study, the structure and functional role of SCO1815 was determined. The ketoreductase activity of SCO1815 was monitored by employing the reverse reaction, that is, oxidation of 3-hydroxyacyl-ZhuG to 3-oxoacyl-ZhuG (ACP from R1128, an aromatic polyketide). The activity of this enzyme had not been previously measured with the cognate ACP (FabC or RedQ), and the specificity for the acyl group had not been determined. We posited that 1) FabI (encoded by SCO1814) and the putative FabG (encoded by SCO1815) would have enoyl-ACP reductase and 3-ketoacyl-ACP reductase activity, respectively, and 2) that neither would show selectivity for the ACP substrate (FabC or RedQ) or the branching in the acyl chain. This activity would permit them to process the acyl chains for both primary and secondary metabolism and would contrast the initiation enzyme selectivity. We also posited that SCO1815 would have 3-ketoacyl-ACP reductase activity, as it clusters with FabI similar to other organisms such as *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*.[16] Accordingly, the catalytic activity and substrate specificity of the FabG homologues (encoded by SCO1345 and SCO1346) might be dramatically different if their biological roles are unrelated to fatty acid biosynthesis.

Here we report the characterization of the three FabG and FabI homologues. Kinetic studies demonstrated that *S. coelicolor* FabI and FabG, encoded by SCO1814 and SCO1815, process both straight and branched acyl chains in their respective substrates. In addition, both enzymes can utilize either FabC or RedQ as the ACP components of the substrate. These findings are consistent with predictions. Surprisingly, 3-ketoacyl-ACP activity was also observed with the FabG homologues encoded by SCO1345 and SCO1346. However, differences in cofactor specificity, overall activity, and ACP selectivity were observed over that for the SCO1815 FabG.

Results and Discussion

β-Ketoacyl-ACP reductase (FabG)

Analysis of the *S. coelicolor* genome sequence revealed the presence of three likely *fabG* genes (SCO1815, SCO1345, and SCO1346, encoding β-ketoacyl-ACP reductase). To probe the physiological role of these three *S. coelicolor fabG* homologues, the genes were expressed in *Escherichia coli*, and the corresponding recombinant proteins were purified as a soluble N-terminal His_6-tag protein. *S. coelicolor* produces predominantly branched-chain fatty acids, whereas *E. coli* produces only straight chain fatty acids. In order to facilitate a comparison of *S. coelicolor* ketoacyl-ACP reductase acyl specificity with that of a straight-chain fatty acid producer, the *E. coli fabG* gene was also expressed and purified. Analysis of affinity purified proteins by SDS-PAGE showed a major band with an apparent molecular weight of approximately 28 kDa consistent with the expected molecular weight. On the basis of earlier studies which demonstrated that FabH (β-ketoacyl-ACP synthase III) from *E. coli* is specific for acetyl-CoA,[12] we hypothesized that *E. coli* FabG would be specific for straight-chain substrates. Despite the important role of FabG in FAS, analysis of specificity for straight- and branched-chain substrates has not been investigated. Thus, a range of straight- and branched-chain carboxylic acids were synthesized and activated as either a NAC or ACP derivatives and were tested with the three *S. coelicolor* FabG homologues and *E. coli* FabG.

β-Ketoacyl-NAC specificity

The activity of FabGs with a series of straight- and branched-chain β-ketoacyl-NAC substrates was determined by using a NADPH-based spectrophotometric assay. The apparent K_m values for all NAC substrates were greater than 2 mM (the high apparent K_m values can be attributed to using NAC thioesters in place of the natural ACP substrate), and limited substrate solubility prevented determination of V_{max} and k_{cat} values for these substrate mimics. Limited solubility also prevented assays with substrate mimics with chain lengths greater than C_{10}. Therefore, the catalytic efficiency (k_{cat}/K_m) was determined under substrate-limited conditions. Kinetic analysis revealed that all three FabG homologues were capable of utilizing both straight- and branched-chain substrates (Table 1). For the streptomycetes FabG homologues, this result is consistent with a FabG role in fatty acid and prodiginine biosynthesis, wherein it processes branched-chain products of FabH for primary metabolism, as well as straight-chain products of RedP for undecylprodiginine biosynthesis. Furthermore, all FabGs were also observed to process β-ketoacyl-NAC substrates with a range of alkyl chain lengths C_6–C_{10}, and catalytic efficiency generally increased as substrate chain length increased. This general trend was observed for all FabGs with branched-chain substrates. In the case of the straight-chain substrates, the greatest activity was observed with the C_8 substrates. The higher catalytic efficiency with longer chain substrates corresponds to the average chain length of the substrates that are processed in a fatty acid by FabG and potentially reflect greater occupancy of the appropriate acyl binding pocket in the overall catalytic process. FabG from *E. coli* showed an apparent efficiency several times higher than that of the *S. coelicolor* FabGs. This difference in catalytic activity could be innate or a result of assay conditions with substrate mimics. Finally, the data demonstrate, for the first time, that FabG from *E. coli* can utilize both straight- and branched-chain β-ketoacyl substrates with comparable efficiency. This observation is in contrast to the data with *E. coli* FabH, which has been shown to have a strong preference for straight-chain substrates such as acetyl and propionyl-CoA.[12] Although *E. coli* does not generate branched-chain fatty acids, production of small levels of them has been observed when the natural FabH is replaced with FabH which can process branched acyl-CoA starter units.[13,18] This production is presumably dependent upon the relaxed acyl group specificity of *E. coli* FabG.

Table 1. Kinetic data of *S. coelicolor* SCO1815, SCO1345, and SCO1346, and *E. coli* FabG

Substrate	k_{cat}/K_m [$mM^{-1}\,min^{-1}$]			
	SCO1815	SCO1345	SCO1346	*E. coli* FabG
Straight-chain				
3-ketohexanoic-NAC	38 ± 1.5	16 ± 1.2	160 ± 1.0	280 ± 17.0
3-ketooctanoic-NAC	93 ± 9.0	118 ± 6.0	205 ± 7.0	1912 ± 133.0
3-ketodecanoic-NAC	75 ± 3.0	46 ± 1.0	92 ± 5.0	1375 ± 32.0
Branched-chain				
5-methyl-3-ketohexanoic-NAC	11 ± 1.1	91 ± 8.0	15 ± 3.0	36 ± 4.0
6-methyl-3-ketoheptanoic-NAC	92 ± 5.0	123 ± 6.0	292 ± 5.0	703 ± 14.0
7-methyl-3-ketooctanoic-NAC	122 ± 2.0	385 ± 48.0	350 ± 47.0	1293 ± 20.0

The cofactor preference of all of the enzymes was also determined by using both NADPH and NADH. SCO1815, SCO1345, and *E. coli* FabG all demonstrated at least 10-fold higher catalytic activity with NADPH than NADH, consistent with previous studies of FabG proteins.[19–21] In contrast, SCO1346 was highly specific for NADH and, in this case, the catalytic activity was at least 100-fold higher than with NADPH (data not shown).

ACP specificity

As the acyl-NAC is a poor mimic of the natural substrate for FabG, activity of the latter was also determined with acyl-ACPs in an LC/MS assay. *S. coelicolor* SCO1815 was initially assayed by employing the reverse reaction, that is, oxidation of 3-hydroxyacyl-ZhuG to 3-oxoacyl-ZhuG (ZhuG is the ACP used in the biosynthesis of the polyketide R1128, a non-native pathway which has been introduced into *S. coelicolor*). This approach was taken to avoid problems associated with 3-ketoacyl-ACP preparation and stability.[17] Studies have shown that the acyl group specificity of an enzyme can change when using non-native ACPs.[22] Our approach was a) to generate the 3-ketoacyl-ACP enzymatically and then use it immediately to assay the reaction in the forward direction, and b) to test the *S. coelicolor* FabG homologues with the physiologically relevant

acyl-ACPs (RedQ and FabC). The phosphopantetheine ejection assay was carried out to monitor the reaction according to a previously described method.[23] Although e a detailed kinetic analysis was not possible because of limitations of acyl-ACP substrate production, a broad assessment of acyl and ACP specificity was possible for the all the FabG proteins.

Initially, FabH was incubated with butyryl-CoA and malonyl-FabC to generate the straight-chain β-ketoacyl-FabC, and the product was analyzed with LC/MS (Figure 1 A). A major *m/z* of 952 was observed, corresponding to the major mass-charge ratio for the condensed ACP bound product of FabH reaction (Figure 1 B). A MS^2 of *m/z* 952 produced a major *m/z* signal of 373, consistent with the mass of a phosphopantetheine thioester of the expected 3-keto condensed product of FabH (Figure 1 C). After addition of NADPH (or NADH) and all three FabGs to the β-ketoacyl-FabC product of FabH, the same analysis provided a major *m/z* of 375 (an increase in 2 atomic mass units), which is consistent with the expected mass of the β-hydroxyacyl-FabC product (Figure 1 D–F for SCO1815, SCO1345, and SCO1346, respectively). The level of reduction with SCO1345 could only be achieved under the same assay conditions by using tenfold higher levels of the enzyme. This level of difference in catalytic efficiency was not observed when the acyl-NAC substrate mimics were used. Assays were also carried out with branched-chain 3-ketoacyl-FabC. These were generated by using isobutyryl-CoA (in place of butyryl-CoA) and malonyl-FabC with FabH (Figure 1 G–I). These assays revealed conversion of the 3-ketoacyl-FabC with all three FabG homologues (Figure 1 J–L). The lower catalytic efficiency for SCO1345 was observed with both the straight- and branched-chain 3-ketoacyl-FabC substrates. The ability to process both straight and branched acyl-ACP substrates and the cofactor specificity of the FabG homologues are consistent with the observations on 3-ketoacyl- NAC substrate mimics.

Additionally, activity of each FabG was assayed with the 3-ketoacyl-ACP substrates by using both the FAS ACP (FabC) and

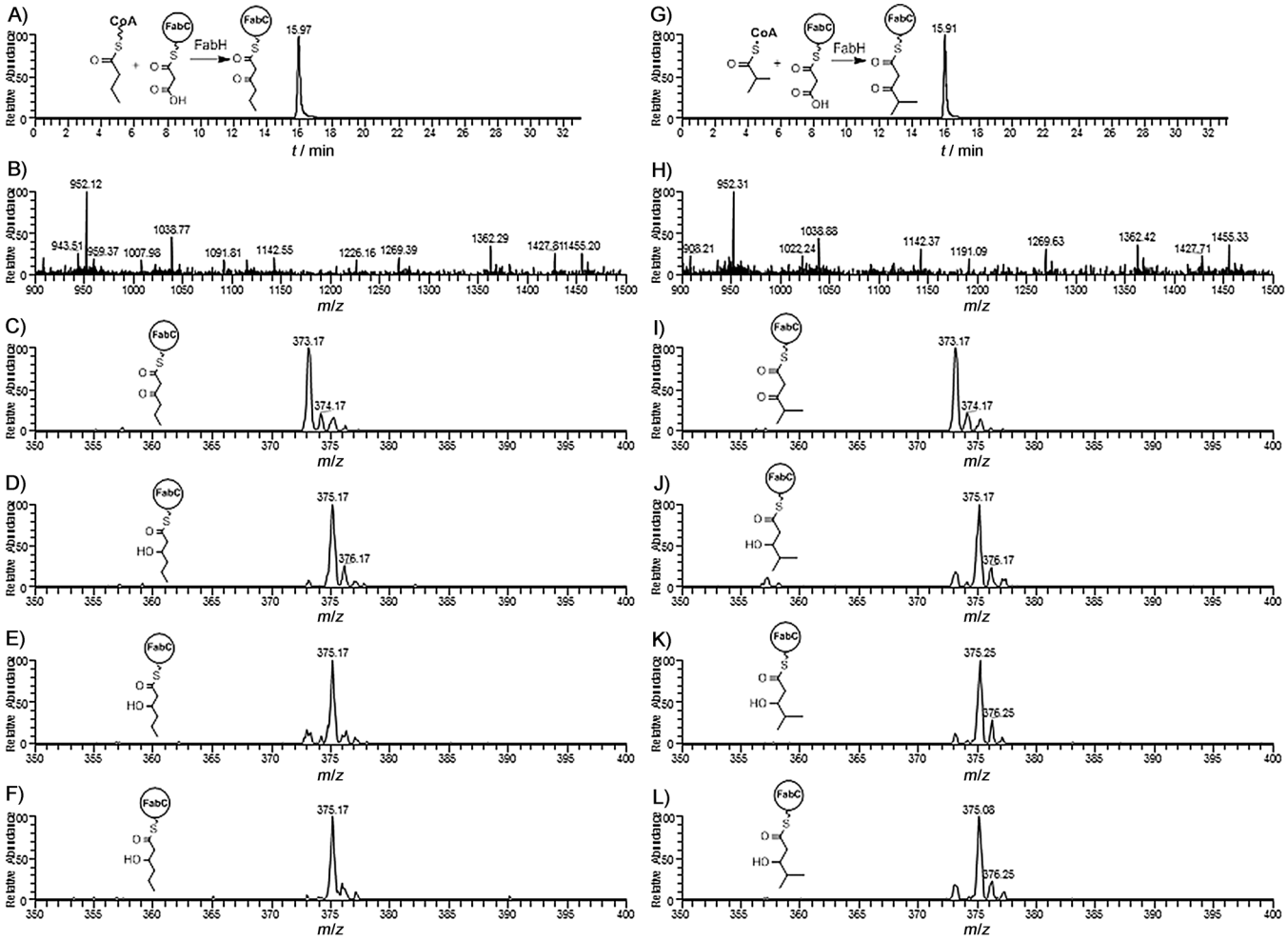


Figure 1. Coupled assay of FabH and FabG. Left: A) LC/MS extracted ion chromatogram of the FabH reaction with butyryl-CoA and malonyl-FabC. B) Total ion chromatogram; *m/z* of 952 corresponds to the major mass-charge ratio observed for ACP-bound 3-keto condensed product of FabH reaction. C) MS^2 spectra of 952 (373 corresponds to a phosphopantetheine thioester of expected 3-keto condensed product of FabH), and of the hydroxy product with D) SCO1815, E) SCO1345, and F) SCO1346. In comparison to SCO1815 and SCO1346, a tenfold higher concentration of SCO1345 was necessary to obtain the complete conversion of 3-ketoacyl substrate to a 3-hydroxyacyl product. Right: G) LC/MS–MS extracted ion chromatogram of the FabH reaction with isobutyryl-CoA and malonyl-FabC. H) Total ion chromatogram of the FabH reaction product. I) MS^2 spectra of 952. Phosphopantetheine fragment of hydroxy product of J) SCO1815, K) SCO1345, and L) SCO1346.

the prodiginine ACP (RedQ; Figure 2). The expectation was that both ACP substrates would be used if FabG is involved in both processes. These assays were carried out in an analogous fashion to that used to look at straight- and branched-chain specificity. The phosphopantetheine fragment of the starting substrate, malonyl-FabC, exhibited an expected mass of 347 (Figure 2A). The β-ketoacyl-chain substrates were generated by using butyryl-CoA and malonyl-FabC with FabH and, after addition of FabG, the expected reduced product was observed (Figure 2C–F for SCO1815, SCO1345, and SCO1346 and *E. coli* FabG, respectively). Similarly, the β-ketoacyl-RedQ substrate was generated by the reaction of RedP with butyryl-CoA and malonyl-RedQ and was analyzed by LC/MS. The expected phosphopantetheine fragment of the starting substrate, malonyl-RedQ, was exhibited in the LC/MS assay (Figure 2G). Similar to the FabH product, a condensed product of RedP (β-ketoacyl-RedQ) was observed after incubation with malonyl-RedQ and butyryl-CoA (Figure 2H). Again, a reduced product was obtained after addition of FabG (Figure 2I, K and L, SCO1815, SCO1346 and *E. coli* FabG). In contrast, SCO1345 was not active with the RedQ substrates (Figure 2J). These data demonstrate that, of the three *S. coelicolor* FabGs enzymes, SCO1815 and SCO1346 have capability to process the ACPs from both fatty acid and undecylprodiginine biosynthesis. This is consistent with one or both of these FabGs processing 3-ketoacyl-FabC substrates for primary metabolism and 3-ketoacyl-RedQ substrates for secondary metabolism. The ability of *E. coli* FabG to process 3-ketoacyl-RedQ (Figure 2L) demonstrates that this enzyme can tolerate changes in both the acyl and ACP components of the 3-ketacyl-ACP substrate.

These analyses are consistent with the predictions for the activity and likely role of the FabG encoded by SCO1815. Notably, it uses both straight- and branched-chain 3-keotacyl-ACP substrates with either FabC or RedQ. These observations, in addition to the NADPH cofactor specificity and colocation of the gene with SCO1814 (and, detailed below, the enoyl-ACP reductase activity required for fatty acid biosynthesis), all support the hypothesis that this is the primary FabG used for both fatty acid and undecylprodiginine biosynthesis. Colocation of genes for FabG and FabI are also observed in other organisms,

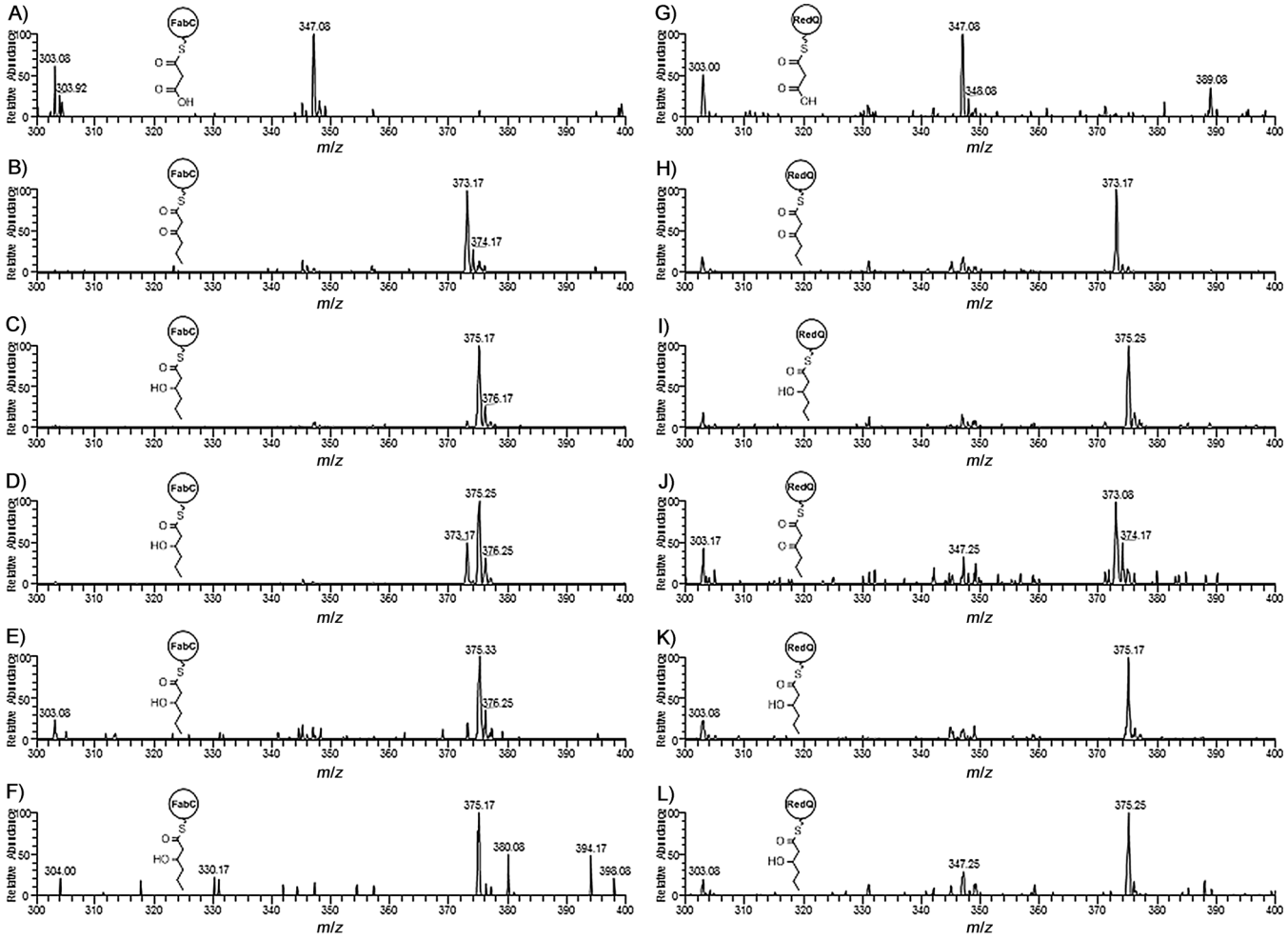
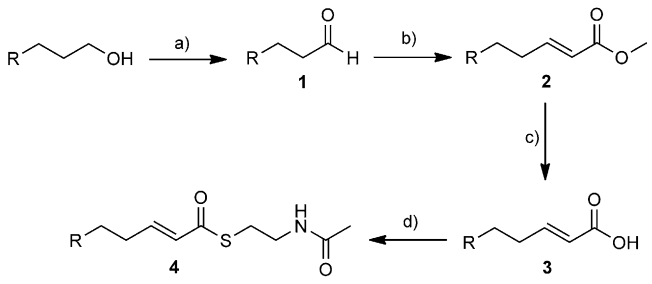


Figure 2. Coupled assay of FabH and FabG with butyryl-CoA and malonyl-FabC (or malonyl-RedQ). Left: Phosphopantetheine fragment of A) starting substrate malonyl-FabC, B) 3-keto product of FabH reaction with butyryl-CoA and malonyl-FabC, reduced product of C) SCO1815, D) SCO1345, and E) SCO1346, and F) *E. coli* FabG. Right: Phosphopantetheine fragment of G) malonyl-RedQ, H) 3-keto product of FabH reaction with butyryl-CoA and malonyl-RedQ, reduced product of I) SCO1815, K) SCO1346, and L) *E. coli* FabG; J) no product was observed with SCO1345.

notably mycobacterium.[16] Given all of this and the literature, which to date supports the role of a single FabG in a type II dissociated fatty acid biosynthetic process,[16] we were surprised to see 3-ketoacyl-NAC and 3-ketoacyl-ACP activity with FabG encoded by SCO1345 and SCO1346. The markedly lower activity of the FabG encoded by SCO1345 (compared to the other two FabG homologues) when 3-ketoacyl-ACP substrates were used indicates that these are not the physiological substrates, and that this reductase is involved in another cellular process. The same cannot be said for the FabG encoded by SCO1346, which differs from the SCO1815 encoded FabG by cofactor specificity. The physiological role of both of these FabG homologues thus remains elusive.

Enoyl-ACP reductase (FabI)

Analysis of the *S. coelicolor* genome sequence has revealed the presence of one putative *fabI* (SCO1814). In order to test the hypothesis that SCO1814 encodes the enoyl-ACP reductase used in fatty acid and undecylprodiginine biosynthesis, *fabI* (SCO1814) was amplified from *S. coelicolor* genomic DNA, expressed in *E. coli*, and purified. The purified protein had the expected molecular mass of approximately 30 kDa, as determined by SDS-PAGE. In addition to SCO1814, the previously characterized *E. coli fabI* gene[24,25] was also expressed and purified (also with an expected molecular mass of approximately 30 kDa). As *E. coli* generates only straight-chain fatty acids, we hypothesized that FabI from *E. coli* would have a strong preference for straight-chain substrates (as in the case of *E. coli* FabH). To date, no data has compared FabI activity with straight- and branched-chain substrates. Therefore, it would be useful to compare this FabI acyl specificity with that of the *S. coelicolor* FabI. In this study, a series of straight- and branched-chain substrates varying in chain length were synthesized (Scheme 2) and used in the spectrophotometric and LC/MS assay.



R = CH_3, C_3H_7, C_5H_{11}, $CH_2CH(CH_3)_2$, $(CH_2)_3CH(CH_3)_2$

Scheme 2. General scheme for synthesis of (*E*)-S-(2-acetamidoethyl) alk-2-enethioate (enoyl-NAC). a) oxalyl chloride, DMSO, CH_2Cl_2, −78 °C, 2.5 h; b) Methyl diethylphosphonoacetate, NaH, 1,2-dimethoxyethane, 0 °C to RT, 3 h; c) LiOH, THF/H_2O (1:1), reflux, 3 h; d) *N*-acetyl cysteamine, EDCI, DMAP, CH_2Cl_2, RT, 12 h.

Enoyl-NAC specificity

The activity of FabI with a series of straight- and branched-chain enoyl-NAC substrates (C_4–C_{10}) was determined in an NADH-dependent spectrophotometric assay. The apparent K_m value for all enoyl-NAC substrates was greater than 2 mM. Substrate solubility prevented the use of a longer substrate (greater than C_{10}) and a concentration well above the K_m value. Therefore, the apparent catalytic efficiency (k_{cat}/K_m) values were obtained under substrate-limited conditions, as in the case of FabG. The high K_m value likely reflects the use of NAC thioesters in place of ACPs. Nonetheless, these kinetic analyses demonstrated that *S. coelicolor* FabI has the capability of utilizing all of the straight- and branched-chain enoyl-NAC substrates tested (C_4–C_{10}; Table 2). No clear pattern was observed between catalytic efficiencies for straight and branched enoyl-NAC substrates of comparable chain length. These observations are consistent with the predicted activity of FabI and its role in both fatty acid and prodiginine biosynthesis (Scheme 1), wherein it processes both short- and long-chain products of FabH (or RedP) or FabF (or RedR), respectively. The catalytic efficiency of FabI with various straight-chain enoyl-NAC (chain length of C_4 to C_{10}) generally increased as substrate chain length increased, with a general trend of: oct-2-enoyl-NAC > dec-2-enoic-NAC > hex-2-enoic-NAC > but-2-enoic-NAC. A similar trend was also observed with the branched-chain substrates. The preference for processing longer straight-chain enoyl-substrates has previously been observed and is, again, likely the result of greater occupancy in the enoyl-group binding pocket of the enzyme.[26,27] Similar observations of processing both straight- and branched-chain enoyl-NAC substrates were seen with the *E. coli* FabI (Table 2). Thus, in both organisms that produce branched-chain fatty acids and those which produce only straight-chain fatty acids, the reductive enzymes FabI and FabG can process both straight- and branched-chain substrates. The apparent catalytic efficiency of *E. coli* FabI was several-fold higher than *S. coelicolor* FabI, an observation made previously when *E. coli* FabI was compared with FabI from other enzymes.[28] It has also been observed that enoyl-reductase (FabI) from other organisms prefers CoA over NAC thioesters, even though CoA is not the physiological substrate, and that NADH is the preferred cofactor.[28] Similar preferences were observed with the *S. coelicolor* FabI in this study and, in fact, no activity was observed with NADPH, even when enzyme concentration was increased 20-fold.

Table 2. Kinetic data of *S. coelicolor* and *E. coli* FabI.

Substrates	k_{cat}/K_m [mM^{-1} min^{-1}]	
	S. coelicolor FabI	*E. coli* FabI
Straight-chain		
but-2-enoic-NAC	10 ± 1.9	97 ± 6.0
hex-2-enoic-NAC	19 ± 1.0	231 ± 12.0
oct-2-enoic-NAC	49 ± 1.0	611 ± 15.0
dec-2-enoic-NAC	20 ± 1.2	157 ± 27.0
Branched-chain		
4-methylpent-2-enoic-NAC	17 ± 1.5	12 ± 2.0
5-methylhex-2-enoic-NAC	2 ± 0.1	0.37 ± 0.05
6-methylhept-2-enoic-NAC	29 ± 2.0	302 ± 6.0
7-methyloct-2-enoic-NAC	63 ± 4.0	361 ± 19.0
9-methyldec-2-enoic-NAC	9.0 ± 1.0	10 ± 1.0

ACP specificity

Similar to FabG, an LC/MS assay was carried out to determine the FabI specificity with the ACPs from both metabolic processes (Figure 3). In this study, an AcpP from *E. coli* fatty acid synthase was used because of technical difficulties in generating a sufficient amount of apo-FabC. Crotonoyl-AcpP was generated from apo-AcpP (the *E. coli* fatty acid synthase ACP) and crotonoyl-CoA by using Sfp (a phosphopantetheinyl transferase) and analyzed by LC/MS (Figure 3A). An *m/z* of 1115 was observed, corresponding to the mass-charge ratio observed for crotonoyl-AcpP (Figure 1B). An MS^2 of the parent (*m/z* 1115) provides a fragment of *m/z* 329, which is consistent with the expected mass of a phosphopantetheine thioester of the crotonoyl group (Figure 3C). The FabI catalyzed the reduction of crotonoyl-AcpP by NADH to the saturated product, butyryl-AcpP, with the predicted mass of 331 observed (Figure 3D). Similarly, crotonoyl-RedQ was generated to evaluate the role of FabI in secondary metabolism by the incubation of Sfp with apo-RedQ and crotonoyl-CoA (Figure 3E–G). Consistent with FabI's role in secondary metabolism, it was able to process crotonoyl-RedQ into the saturated product (Figure 3H).

These assays clearly demonstrate that SCO1814 (FabI) has both enoyl-NAC and enoyl-ACP reductase activity. In both cases, FabI utilizes various chain lengths of straight- and branched-chain substrates. Furthermore, FabI does not discriminate between ACPs from primary metabolism and secondary metabolism. A gene encoding the enoyl-ACP reductase (FabI) is not present in the *red* biosynthetic gene cluster, and thus, all evidence support a role for SCO1814 in both fatty acid and undecylprodiginine biosynthetic processes in *S. coelicolor* (Scheme 1). These results provide an explanation for a previous observation, wherein it was shown that FabI inhibitors decrease the biosynthetic yield of undecylprodiginine, containing a fatty acid chain generated by primary metabolism. The biosynthetic yield was not decreased for secondary metabolites not containing fatty acid-derived moieties.[29]

Conclusions

In summary, undecylprodiginine biosynthesis involves numerous intriguing enzymes and a fascinating interface with fatty acid biosynthesis. We cloned all three 3-ketoacyl-ACP reductases homologues (SCO1815, SCO1345, and SCO1346), along with

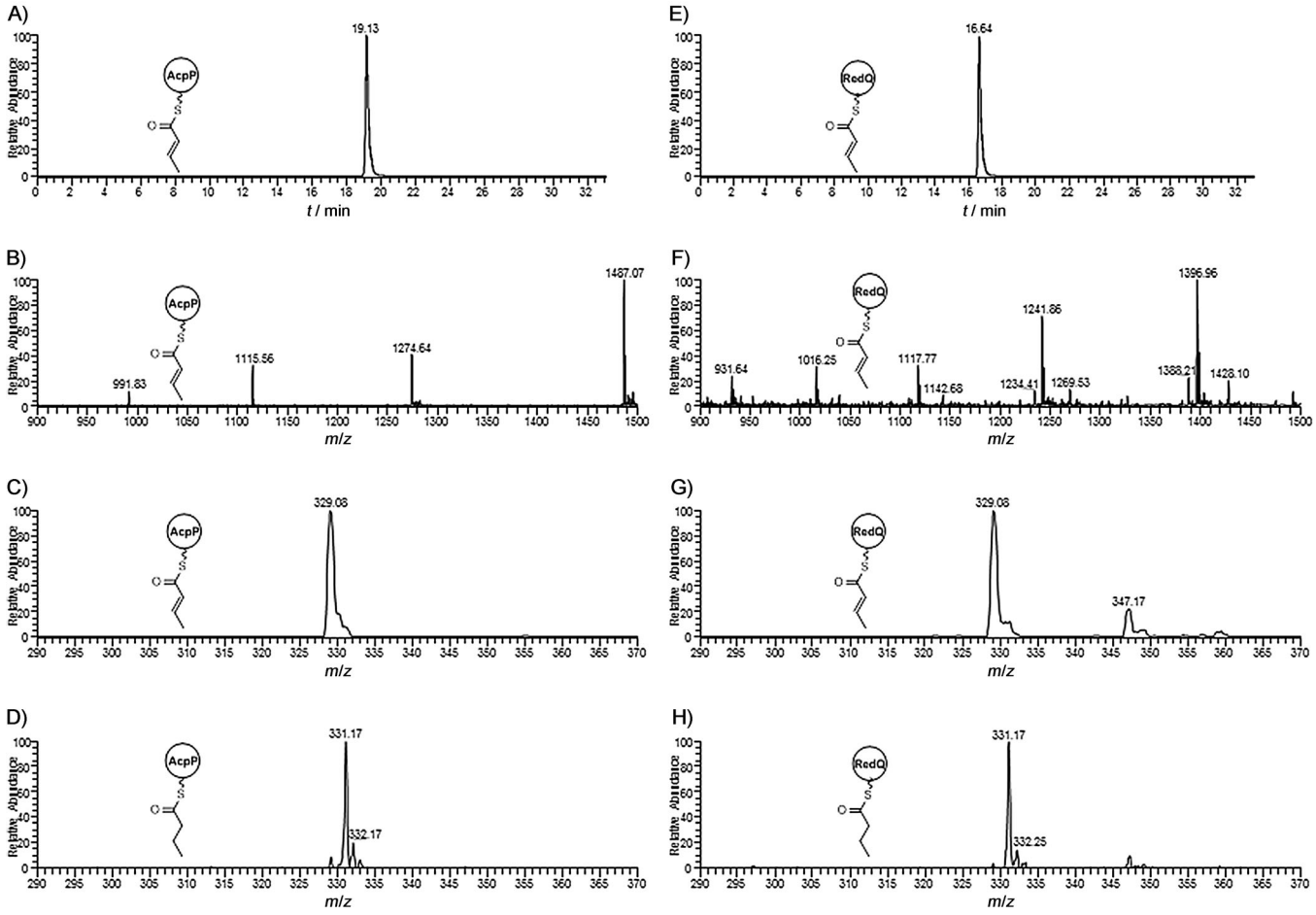


Figure 3. LC/MS analysis of FabI reaction with crotonoyl-AcpP (or crotonoyl-RedQ) and NADH. Left: A) Mass spectrum of the starting substrate, crotonoyl-AcpP. B) Total ion chromatogram, *m/z*. C) MS^2 spectra of 1115 (329 corresponds to a phosphopantetheine thioester of a crotonoyl group). D) Saturated product of FabI with crotonoyl-AcpP and NADH. Right: E) Mass spectrum of crotonoyl-RedQ. F) *m/z* of crotonoyl-RedQ. G) MS^2 spectra of 1016. H) Saturated product of InhA.

the SCO1814 enoyl-ACP reductase homologue from *S. coelicolor*, and purified all proteins. We carried out the first kinetic analyses of these streptomycetes enzymes and demonstrated that all three FabGs homologues have 3-ketoacyl-ACP reductase activity, with the FabI homologue having enoyl-ACP reductase activity. The activities of the SCO1815-encoded FabG and the SCO1814 FabI provide compelling support for a role of these enzymes in providing both fatty acids for primary metabolism and dodecanoic acid for undecylprodiginine biosynthesis (Scheme 1). Thus, the data now provide experimental evidence to support the role of FabG and FabI, in addition to FabD, in both processes and suggests they might exert no control over the products. In contrast, the condensing enzymes (FabH and RedP) are the key controlling factors.[14]

Experimental Section

Materials: β-NADH and β-NADPH were from Research Products International Corp. All other chemicals, including unlabeled CoA derivatives, were from Sigma. Cosmid 3F7 and 4A7, containing *S. coelicolor* genomic DNA, were kindly provided by the John Innes Institute. FabC, RedQ, and *E. coli* ACP were sourced as described previously.[1,30,31] Preparation of malonyl-ACPs and crotonoyl-ACPs was carried out as described previously.[14]

***S. coelicolor* and *E. coli fabG*:** The genes encoding SCO1815, SCO1345, and SCO1346 (*fabG*) were amplified from the appropriate *S. coelicolor* cosmids by PCR by using the primers 5′-CATATG AGCCGC TCGGTT CTC-3′ and 5′-GGATCC TCAGTG ACCCAT TCCCAG TCC-3′ (SCO1815); 5′-CATATG ACTGAA CTGCCC GAGCCC TCC-3′ and 5′-GGATCC TCAGGC CACGCC GGCGTT C-3′ (SCO1345); and 5′-CATATG TCCACC ACTGAG CAGCG-3′ and 5′-GGATCC CTAGTC GAGCGG TCCGCC-3′ (SCO1346). The *E. coli fabG* was amplified from *E. coli* genomic DNA by PCR by using 5′-CATATG AATTTT GAAGGA AAAATC GCACTG-3′ and 5′-GGATCC TCAGAC CATGTA CATCCC GCCG-3′ (restriction sites underlined). The amplified inserts were digested with NdeI and BamHI and cloned into the pET15b vector to provide the expression plasmid for SCO1815 (pRS1), SCO1345 (pRS2), and SCO1346 (pRS3), as well as *E. coli fabG* (pRSE1).

***S. coelicolor* and *E. coli fabI*:** The SCO1814 gene (encoding FabI) was amplified from the appropriate *S. coelicolor* cosmid by using the forward primer 5′-CATATG AGCGGA ATTCTC GAGGGC AAG-3′ (introducing a NdeI restriction site) and a reverse primer 5′-GGATCC TCAGGC GCCGAT GGCGTG C-3′ (introducing a BamHI restriction site). The *E. coli fabI* was amplified from *E. coli* genomic DNA by using the forward primer 5′-CATATG GGTTTT CTTTCC GGTAAG-3′ (introducing a NdeI restriction site) and reverse primer 5′-GGATCC TTATTT CAGTTC GAGTTC G-3′ (introducing a BamHI restriction site). The resulting PCR products were digested with NdeI and BamHI and ligated into the corresponding sites in pET15b to provide the *S. coelicolor fabI* expression plasmid, pRS4, and the *E. coli fabI* expression plasmid, pRSE2.

Protein expression and purification: All plasmids were transform into *E. coli* BL21(DE3) cells. The resulting transformants were grown at 37 °C in LB medium containing ampicillin (100 μg mL^{-1}) until $A_{600}=0.6$ was reached, induced with isopropyl-β-D-thiogalactopyranoside (0.1 mM), and incubated for 3 h at 37 °C. Cells were harvested by centrifugation for 10 min at 12000 *g* and 4 °C and stored at −80 °C.

The resulting *E. coli* cell pellets were suspended in lysis buffer A (50 mM sodium phosphate buffer pH 7.2, 300 mM NaCl, 5 mM 2-mercaptoethanol, 10% glycerol, 0.05% (*v*/*v*) Tween-20) with 10 mM imidazole and lysozyme (1 mg mL^{-1}). The resulting cell suspension was incubated on ice for 30 min, then the cell lysate was cleared by centrifugation at 16000 g for 20 min. The crude cell extract was loaded onto a Ni-NTA resin column. The N-terminal polyhistidine-tagged protein was eluted by using buffer A with imidazole (300 mM). Fractions containing pure protein were pooled, exchanged with 50 mM sodium phosphate buffer pH 7.2, and stored in 20% glycerol at −80 °C.

General procedures for synthesis of (*E*)-S-(2-acetamidoethyl) alk-2-enethioate (enoyl-NAC thioester) substrates for FabI assays: Straight- and branched-chain enoyl-NAC compounds, ranging from C_4 to C_{10} in alkyl chain length, were synthesized according to general Scheme 2. Reactions were monitored by TLC, and all compounds were characterized by ^{1}H NMR spectroscopy.

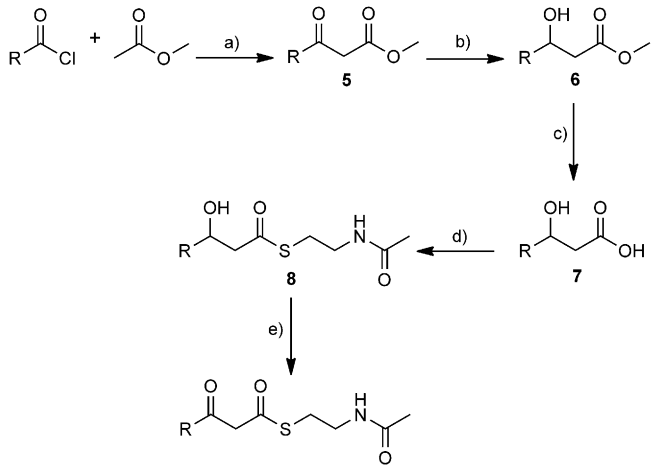
Synthesis of alkanal 1: Dimethyl sulfoxide (6.2 mL, 88 mmol) was added dropwise to a stirred solution of oxalyl chloride (3.80 mL, 44 mmol) in anhydrous CH_2Cl_2 (100 mL) at −78 °C under argon. After 30 min, a solution of the starting alcohol (3.0 g, 29 mmol) was added dropwise, and the reaction mixture was stirred at −78 °C for 1.5 h. Triethyl amine (20.45 mL, 147 mmol) was then added dropwise, and the reaction mixture was allowed to warm to room temperature. Saturated aqueous NH_4Cl (30 mL) was added, and the organic layer was separated. The resulting aqueous phase was extracted with CH_2Cl_2 (3×40 mL), and the combined organic layers were dried over anhydrous sodium sulfate (Na_2SO_4) and concentrated in vacuo to give aldehyde **1**.

Synthesis of (*E*)-methyl alk-2-enoate (2): Methyl diethylphosphonoacetate (5.86 g, 27 mmol) was added dropwise to a stirred solution of the sodium hydride (0.66 g, 27 mmol) in 1.2-dimethoxyethane (15 mL) at 0 °C under argon atmosphere and stirred for 30 min. After dropwise addition of the aldehyde (**1**; 2.0 g, 23 mmol), the reaction mixture was allowed to warm to room temperature and stirred for 3 h. The reaction mixture was quenched with cold water and extracted with EtOAc (3×15 mL). The combined organic layers were washed with brine (20 mL), dried over Na_2SO_4, and concentrated in vacuo. The crude product was purified by column chromatography (EtOAc/hexane, 7:93) to yield the desired ester **2**.

Synthesis of (*E*)-alk-2-enoic acid (3): LiOH (1.0 g, 42.2 mmol) was added to a stirred solution of ester **2** (2.0 g, 14 mmol) in tetrahydrofuran/H_2O (1:1, 40 mL), and the mixture was heated at reflux. After 2.5 h, the reaction mixture was cooled to 0 °C and acidified with 1 N HCl. The layers were separated, and the aqueous phase was extracted with EtOAc (3×30 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated in vacuo to give the corresponding carboxylic acid (**3**).

Synthesis of (*E*)-S-(2-acetamidoethyl) alk-2-enethioate (4): *N*-Acetyl cysteamine (0.94 mL, 8.98 mmol) was added to a stirred solution of carboxylic acid **3** (1.0 g, 7.81 mmol) in anhydrous CH_2Cl_2 (20 mL) at 0 °C under nitrogen atmosphere, followed by 4-(*N*,*N*-dimethylamino)pyridine (DMAP; 0.24 mg, 1.95 mmol), and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDCI; 1.5 g, 7.81 mmol). The reaction mixture was allowed to warm to room temperature and stirred overnight. The reaction was quenched with saturated aqueous NH_4Cl (15 mL) and extracted with CH_2Cl_2 (3×20 mL). The combined organic layers were dried over Na_2SO_4, concentrated in vacuo, and the residue was purified by column chromatography (hexane/EtOAc, 40:60) to yield desired product **4**.

General procedures for the synthesis of S-(2-acetamidoethyl) 3-oxoalkanethioate (3-ketoacyl-NAC thioester) substrates for FabG assays: Straight and branched 3-keto acyl-NAC compounds, with alkyl chain lengths ranging from C_4 to C_{10}, were synthesized according to general Scheme 3. Reactions were monitored by TLC, and all compounds were characterized by ^{1}H NMR spectroscopy.



R = nC_3H_7, nC_5H_{11}, nC_7H_{15}, $CH_2CH(CH_3)_2$, $(CH_2)_2CH(CH_3)_2$ $(CH_2)_3CH(CH_3)_2$

Scheme 3. General scheme for synthesis of *S*-(2-acetamidoethyl) 3-oxoalkanethioate (3-ketoacyl-NAC). a) Lithium diisopropylamide, THF, −78 °C to RT, 3 h; b) $NaBH_4$, CH_3OH, 0 °C, 15 min; c) LiOH, THF/H_2O (1:1), reflux, 3 h; d) *N*-acetyl cysteamine, EDCI, DMAP, CH_2Cl_2, RT, 12 h; e) pyridinium chlorochromate, CH_2Cl_2, RT, 1 h.

Synthesis of methyl 3-oxoalkanoate (5): Methyl acetate (7.50 mL, 93.8 mmol) was added to a stirred solution of lithium diisopropylamide (LDA; 39.0 mL of 1.8 M solution in THF, 70.3 mmol) in THF (100 mL) at −78 °C. After stirring for 15 min, a solution of the starting acyl chloride in THF was added dropwise, and the reaction mixture was stirred at 78 °C for 1 h before being warmed to room temperature and stirred for 3 h. The reaction mixture was quenched with 1 N HCl and extracted with EtOAc (3×100 mL). The combined organic extracts were washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The crude product was purified by column chromatography (hexane/EtOAc, 95:5) to afford the desired ester (**5**).

Synthesis of methyl 3-hydroxyalkanoate (6): $NaBH_4$ (1.90 g, 50 mmol) was added slowly to a stirred solution of **5** (3.0 g, 50 mmol) in anhydrous methanol (20 mL) at 0 °C, and the reaction mixture was stirred at 0 °C for 20 min. The resulting solution was quenched with 1 N HCl and extracted with EtOAc (3×20 mL). The combined organic layers were dried over Na_2SO_4, concentrated in vacuo, and purified with column chromatography to give the corresponding methyl 3-hydroxyalkanoate (**6**).

Synthesis of S-(2-acetamidoethyl) 3-hydroxyalkanoate (8): Hydrolysis of **6** to provide **7**, and subsequent conversion to **8**, was analogous to the steps for formation of formation of enoyl-NAC (described above).

Synthesis of S-(2-acetamidoethyl) 3-oxoalkanethioate (9): A suspension of pyridinium chlorochromate (1.44 g, 6.72 mmol) in anhydrous CH_2Cl_2 (50 mL) was treated with **8** (1.0 g, 4.48 mmol). The mixture was stirred at room temperature for 2 h. Et_2O (100 mL) was added to precipitate the chromium salts. After filtration through a silica gel pad, the solvent was evaporated to yield the crude keto compound, which was purified by column chromatography (hexane/EtOAc 50:50) to afford desired compound **9**.

FabG and FabI assays: Spectrophotometric assays were used to assess the substrate specificity of FabG and FabI. FabG activity was determined by monitoring the conversion of the 3-ketoacyl-NAC substrate to 3-hydroxyacyl-NAC. The conversion of the enoyl-NAC substrate to acyl-NAC was monitored to determine the activity of FabI. Assays were performed in sodium phosphate buffer (50 mM, pH 7.2), NADH (or NADPH 0.4 mM),substrate (1.0 mM, suspended in DMSO to give a final concentration of 1%), and protein (0.05 μg) in a final volume of 100 μL. The reaction mixture was incubated at room temperature for 10 min, and enzymatic activity was measured by monitoring the decrease in absorbance at 340 nm due to the consumption of NADH (or NADPH). Steady-state kinetic measurements were obtained by determining FabG or FabI activity at various concentrations (0.125–16.0 mM) of either 3-ketoacyl-NAC or enoyl-NAC and a constant concentration (400 μM) of either NADPH or NADH, respectively. All reactions were performed at least in triplicate, and nonlinear regression analysis with Grafit version 4.012 (Erithacus Software, Horley, United Kingdom) was used to determine k_{cat} and K_m values.

FabG and FabI activities were also determined with 3-ketoacyl-ACP and enoyl-ACP, respectively, in an LC-MS-based assay under similar reaction conditions as described above. Briefly, FabI was incubated with NADH and either crotonoyl-AcpP (the *E. coli* fatty acid synthase ACP) or crotonoyl-RedQ in a final volume of 20 μL for 10 min. Coupled assays were carried out to obtain the FabG activity. First FabH (or RedP) was incubated with butyryl-CoA (or isobutyryl-CoA) and with malonyl-FabC (or malonyl-RedQ) in a similar way as described before[14] (50 mM sodium phosphate buffer (pH 7.2); total volume of 20 μL). The reaction mixture was incubated at 25 °C for 10 min (leading to generation of the 3-ketoacyl ACP) and then treated with FabG and either NADPH or NADH for 10 min. All reactions were quenched with 10% formic acid (20 μL), and products were analyzed by LC-ESI-MS, performed with a high-resolution mass spectrometer externally mass-calibrated prior to analysis to obtain a mass accuracy within ±5 ppm. Chromatography was performed with an Accela HPLC system (Thermo Fisher Scientific) by using a Discovery (3 μm, 15 cm×2.1 mm, Supelco) reversed-phase column at a flow rate of 200 μL min^{-1}. Solvent A was water with 0.05% formic, and solvent B was acetonitrile with 0.5% formic acid. The solvent gradient initiated with solvent A (99%) for 5 min and then went to 99% of solvent B over 25 min. A final 2 min at 99% Solvent B was used before re-equilibration with solvent A. The HPLC eluent was directed to a ThermoElectron LTQ-Orbitrap XL Discovery instrument (San Jose, CA, USA), equipped with an ESI ion max source. The ionization interface was operated in the positive mode with the following settings: source voltage: 4 kV; sheath and aux gas flow rates: 50 and 10 units respectively; tube lens voltage: 100 V; capillary voltage: 14 V; and capillary temperature: 300 °C. LTQ with collision-induced dissociation (CID) values of 30 was used for LC-ESI-MS/MS experiments.

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Keywords: beta-ketoacyl-acyl carrier protein reductase · enoyl-ACP reductase FabG · FabI · InhA · *Streptomyces coelicolor*

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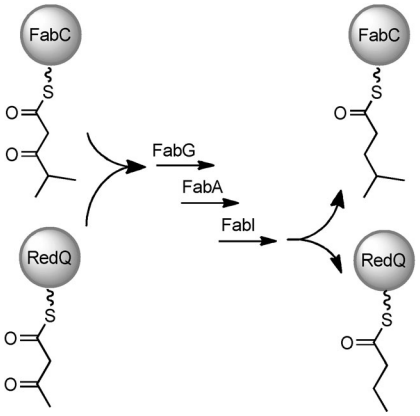
Functional crosstalk: β-Ketoacyl-acyl carrier protein (ACP; FabG) and enoyl-ACP reductase (FabI) from *Streptomyces coelicolor* were identified and characterized. Kinetic analysis demonstrated that these enzymes process straight and branched-chain substrates along with the ACPs from both fatty acid and undecylprodiginine biosynthetic pathways. This relaxed substrate specificity allow these enzymes to be involved in both processes.



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Characterization of FabG and FabI of the *Streptomyces coelicolor* Dissociated Fatty Acid Synthase