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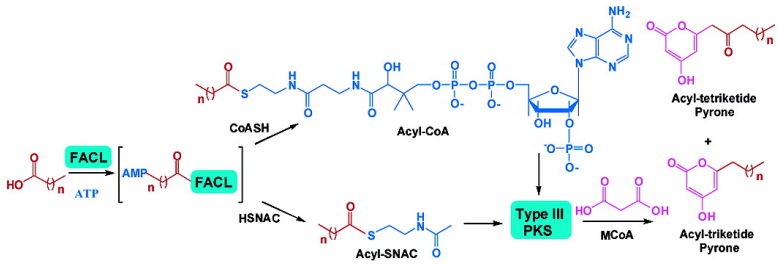

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Promiscuous Fatty Acyl CoA Ligases Produce Acyl-CoA and Acyl-SNAC Precursors for Polyketide Biosynthesis

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The study of bioactive natural products has undergone rapid advancement with the cloning and sequencing of large numbers of gene clusters.[1] Along with the development of expression systems, the genetic reconstitution necessitates that the heterologous hosts possess substrate pools that could be coordinately supplied for biosynthesis.[2] Polyketide synthases (PKS) utilize acyl-coenzyme A (CoA) precursors and synthesize polyketides by repetitive decarboxylative condensations.[2] Acyl-CoAs in the cell are synthesized through multiple metabolic routes. The biotin-dependent acyl-CoA carboxylases are the major source of dicarboxylate extender units.[3] Another route involves the activation of carboxylic acids by acyl-activating enzymes (AAE), primarily through the formation of a thioester bond. The potential of these proteins to produce varied CoA precursors and their utilization in polyketide biosynthesis have not been investigated. Incidentally, such protein domains are recognized in biosynthetic clusters of several PKSs and non-ribosomal peptide synthetases (NRPSs).[4] Here, we report remarkable substrate tolerance of fatty acyl-CoA ligases (FACLs) to produce diverse acyl-CoAs as well as *N*-acetylcysteamine (NAC) analogues from their corresponding acids. In combination with promiscuous starter unit specificity of multifunctional types I and -III PKSs, these precursors generate diverse metabolites, demonstrating new prospects to expand polyketide diversity.

The *Mycobacterium tuberculosis* genome encodes for 34 AAE proteins (annotated as FadD).[5] We recently delineated these proteins into two distinct classes, namely fatty acyl-AMP ligases (FAAL) and fatty acyl-CoA ligases (FACL).[6] We now investigate which CoA starter substrates are generated by FACLs that could support polyketide biosynthesis. Toward this goal several FadD proteins were cloned and expressed in *Escherichia coli*. We chose FadD6, 15, and 19 proteins as representative FACLs for this study, as these were hyperexpressed in a soluble form. Biochemical characterization of FadD6, FadD19, and FadD15 showed good catalytic efficiency for the formation of lauroyl-CoA (k_{cat}/K_M = 2667, 1925, and 2246 s^{-1} M^{-1}). FACL proteins are known to contain overlapping substrate specificities[7] and preliminary studies suggested FadD19 to be specific for medium-chain fatty acids, while FadD6 and 15 preferred long-chain fatty acids. The tolerance of FadD proteins was further investigated by using methylated, hydroxylated, and unsaturated fatty acids. 2-Methylhexadecanoic acid and 3-methylundecanoic acid were gifts from Paul P Van Veldhoven.[8] The formation of fatty acyl-CoA thioesters were monitored on reversed phase HPLC (Figure 1) and were confirmed by tandem nanospray mass spectrometry (MS). To our surprise, modifications at α-, *β*-, *ω*-, and (*ω*−*ν*)- positions of various medium- and long-chain acids did not block their conversion to CoA esters. The competition experiments carried out by using ^{14}C-lauric acid also confirmed broad substrate flexibility (Figure S4). In fact FadD6 showed quantitative conversions with many of the fatty acids. This robust synthesis of acyl-CoAs prompted to investigate whether these CoAs could be channelled for producing polyketides in a cell-free system.

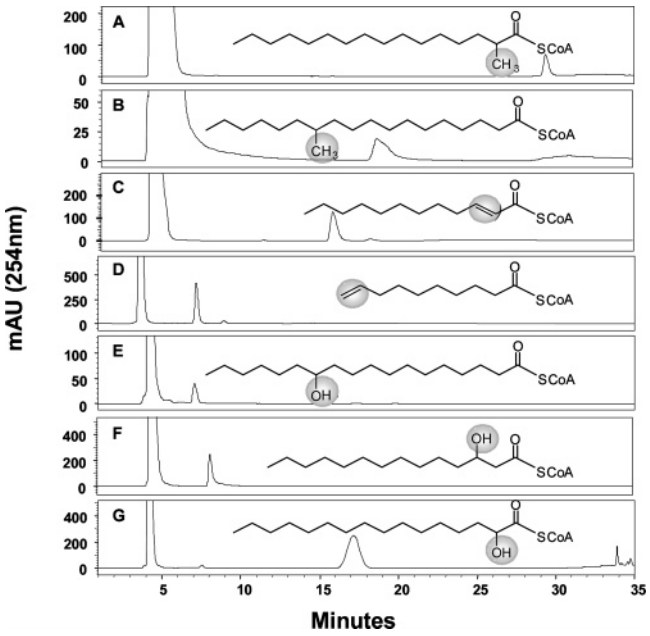

Figure 1. Reverse phase HPLC chromatograms for the enzymatic synthesis of diverse acyl-CoAs by FadD6 protein (SI).

The enzymatic coupling to produce polyketide products was examined by using PKS18 and mycocerosic acid synthase (MAS) proteins from *M. tuberculosis*. The PKS18 protein synthesizes triketide and tetraketide pyrones by condensing two or three malonyl-CoA units with starter fatty acyl-CoA substrates.[9] Our crystallographic investigations had revealed a unique substrate binding tunnel from the active site to the surface of the protein that had suggested the feasibility of using a wider range of starter substrates.[9] Using the FadD−Pks18 coupled system, a series of pyrone products were synthesized and analyzed using LC−MS/MS (Table 1). The MAS is a multifunctional type-I iterative polyketide synthase, which synthesizes mycocerosic acid by iterative condensation of fatty acyl-CoAs with methylmalonyl-CoA.[10] FadD6 and Mas protein were co-incubated with various fatty acids, and the synthesis of mycocerosic acids was monitored by radio-TLC (Figure S6). FadD19 and FadD15 proteins also supported polyketide biosynthesis, demonstrating that FACL proteins in general could provide pools of CoA thioesters.

The remarkably relaxed substrate specificity displayed by FadD proteins prompted us to explore their tolerance toward shorter sulfhydryl substrates such as NAC, the acceptor terminal portion of CoASH. Acyl-SNAC substrates have been successfully used to exogenously provide precursors in modular PKSs and NRPSs.[11] When NAC was used for CoASH in the FadD19 enzymatic assays with ^{14}C-lauric acid, a new product was observed on the radio-TLC (R_f = 0.85). Further analysis on HPLC with in-line radioactive detector showed a new peak at 36.5 min, which produced a

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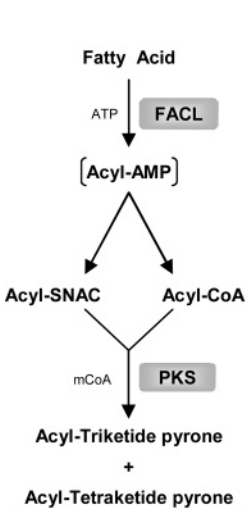
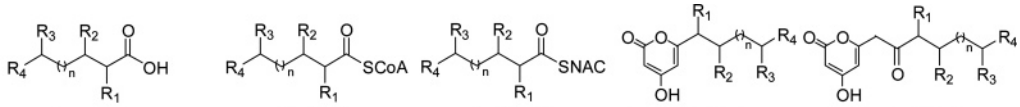
Table 1. MS Characterization of Intermediates and Final Products Generated during FadD−Pks18 Coupled Polyketide Synthesis[a]





	[M-H]$^{-2}$ *m/z*	[M+H]$^{+}$*m/z*	[M-H]$^{-}$ *m/z*	[M-H]$^{-}$*m/z*
1: n=11, R_1=OH, R_2=H, R_3=H, R_4=H	1020.28	374.19	ND	379.31
2: n=9, R_1=H, R_2=OH, R_3=H, R_4=H	991.74	346.18	309.22	ND
3: n=8, R_1=H, R_2=H, R_3=OH, R_4=C_6H_{13}	1047.66	402.21	365.27	407.28
4: n=10, R_1=H, R_2=H, R_3=H, R_4=OH	1006.34	360.24	323.28	365.29
5: n=11, R_1=CH_3, R_2=H, R_3=H, R_4=H	1018.34	372.24	363.27	405.31
6: n=6, R_1=H, R_2=CH_3, R_3=H, R_4=H	948.18	302.22	265.18	307.21
7: n=8, R_1=H, R_2=H, R_3=CH_3, R_4=C_6H_{13}	1046.38	400.32	363.30	405.31
8: n=8, R_1=OH, R_2=H, R_3=CH_3, R_4=H	976.11	330.25	293.22	335.23
9: n=4, R_1=H, R_2=H, R_3=H, R_4=$C_{10}H_{22}$	1029.56	384.29	359.26	417.27
10: n=5, R_1=H, R_2=H, R_3=H, R_4=CH_2	918.26	272.16	235.13	277.14
11: n=7, R_1=H, R2=H, R3=H , R_4=H	948.08	302.21	265.18	307.21

[a] ND, not detected.

molecular ion peak at $(M + H)^+$ at *m/z* 302.21. The fragmentation pattern for this molecular ion yielded a fragment of *m/z* 120.04 corresponding to NAC ion $(C_4H_{14}O_1N_1S_1)^+$, confirming the enzymatic synthesis of lauroyl-SNAC. A simple model for acyl-SNAC formation would involve transfer of acyl moiety from the adenylates to sulfhydryl group of HSNAC analogous to CoASH. Interestingly, the FAAL proteins showed inefficient acyl-SNAC biosynthesis. Steady-state kinetic studies with FadD6 protein displayed 6-fold higher apparent K_M for HSNAC than for CoASH. In addition, HSNAC showed competitive inhibition with CoASH with a K_i of 91 μM. These studies suggest that both CoASH and HSNAC bind at an overlapping site on the enzyme and that acyl-SNAC synthesis proceeds in a mechanistically comparable manner to acyl-CoA formation. Preparative scale-up showed good acyl-SNAC conversions and 1 μmole of FadD6 protein synthesized more than 200 μmoles of lauroyl-SNAC product.

Since utilization of acyl-SNAC precursors by iterative PKSs have not been explored, other than a very recent study with chalcone synthase,[12] we investigated whether FadD−PKS18 coupled system would generate pyrone products with HSNAC. Indeed, in situ generated acyl-SNACs could be successfully incorporated to produce acyl-pyrones. The efficiency of catalysis suggested that PKS18 might possess similar affinity for acyl-CoA and acyl-SNAC substrates. Steady-state kinetic studies showed that K_M of lauroyl-SNAC was comparable to that of lauroyl-CoA. The broad substrate specificity to incorporate a variety of acyl-NAC-thioesters highlights the relaxed substrate specificity of type-III PKS enzymes.

In summary, the AAE proteins provide an attractive route to expand the precursors available for polyketide biosynthesis. The substrate specificity of FACL proteins shows remarkable tolerance to activate variety of modified fatty acids to their corresponding CoA and SNAC analogues. The incorporation of acyl-SNAC primers by PKS18 protein suggests that these synthetic analogues could be used to exogenously supplement starter units. Together, the promiscuous activity of FACL and PKSs provides new opportunities to expand the repertoire of natural products.

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Supporting Information Available: Materials and methods, enzyme kinetics, and MS characterization of products; complete ref 7. This material is available free of charge via the Internet at http://pubs.acs.org.

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