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Biosynthesis of Salinosporamides from α,β-Unsaturated Fatty Acids: Implications for Extending Polyketide Synthase Diversity

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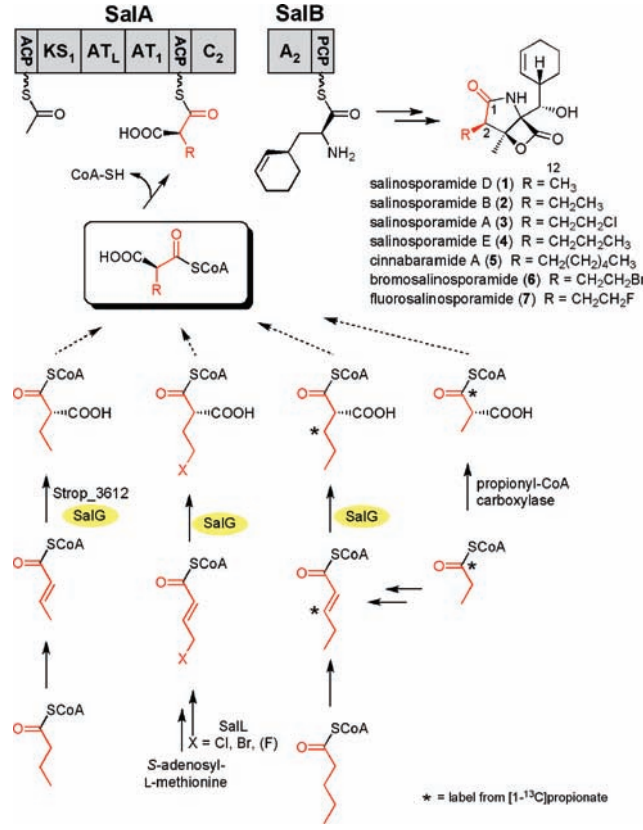
The salinosporamides comprise a natural product family of potent anticancer agents produced by the marine bacterium *Salinispora tropica*. This group of densely functionalized β-lactone-γ-lactam proteasome inhibitors is largely distinguished through structural differences at C-2 bearing methyl, ethyl, chloroethyl, and propyl substituents per salinosporamides D (**1**), B (**2**), A (**3**), and E (**4**), respectively (Scheme 1).[1−3] The recent discovery of the related metabolite cinnabaramide A (**5**) from a terrestrial streptomycete,[4] which instead harbors a C-2 hexyl chain, further extends the natural salinosporamide structural family. We recently reported that salinosporamides A and B are biosynthetic products derived from an unusual hybrid polyketide synthase−nonribosomal peptide synthetase (PKS−NRPS) pathway initiated by the chain elongation of acetyl-S−ACP by chloroethylmalonyl-CoA or ethylmalonyl-CoA,[5,6] respectively, followed by the nonproteinogenic amino acid cyclohexenylalanine (Scheme 1).[7] The selection of the PKS extender unit is controlled by the acyltransferase domain AT_1 from the hexadomained SalA synthase. Herein we report that salinosporamides D and E are respectively accessed from methylmalonyl-CoA and propylmalonyl-CoA, the latter of which is a newly described PKS extender unit that belongs to a growing family of PKS substrates derived from α,β-unsaturated fatty acids.

Based on the biosynthetic assembly of salinosporamide B from a butyrate building block via ethylmalonyl-CoA,[5,6] we reasoned that the methyl analogue salinosporamide D (**1**) would be similarly assembled from a propionate unit via the common PKS substrate (2*S*)-methylmalonyl-CoA (Scheme 1). We explored this assumption by administering [1-^{13}C]propionate to the *S. tropica salL*-deficient mutant, which is specifically unable to synthesize the chlorinated salinosporamide A as the major product of the *sal* pathway due to inactivated 5′-chloro-5′-deoxyadenosine synthase SalL.[8] Isolation and characterization of the resultant salinosporamide D by NMR revealed the specific ^{13}C-enrichment (5%) at C-1, thereby confirming its assembly from propionate.

We next turned our attention to the propyl analogue salinosporamide E (**4**), which would presumably derive from a pentanoate building block. If so, this would imply the prospect of a new PKS building block, namely propylmalonyl-CoA. Salinosporamide E was similarly isolated from the [1-^{13}C]propionate feeding experiment, and NMR analysis confirmed ^{13}C-enrichment (40%) at C-12, thereby suggesting an origin from pentanoate derived from propionate and acetate precursors (Scheme 1). While the administration of pentanoic acid to the *S. tropica salL*-deficient mutant resulted in a significant increase (∼500%) in salinosporamide E titers, unsaturated *trans*-2-pentenoic acid had a much greater effect in enhancing its production at ∼1500%

Scheme 1. Biosynthesis of salinosporamide A (**3**), Its Analogues, and Their Substituted Malonyl-CoA PKS Building Blocks (Boxed)[a]



[a] Abbreviations: ACP, acyl carrier protein; KS, ketosynthase; AT, acyltransferase; C, condensing domain; A, adenylation domain; PCP, peptidyl carrier protein.

(Figure 1). These observations suggested that the C_5 substrate is limiting and that the α,β-unsaturated carboxylic acid is a more advanced biosynthetic intermediate.

The recent functional revision of crotonyl-CoA reductase (CCR) as a carboxylase that catalyzes the reductive carboxylation of crotonyl-CoA to (2*S*)-ethylmalonyl-CoA[9] suggests the possibility of an analogous pathway to (2*S*)-propylmalonyl-CoA in *S. tropica* from 2-pentenyl-CoA (Scheme 1). Inspection of the complete genome sequence of *S. tropica* CNB-440[10] revealed two CCR-encoding genes. We previously inactivated each by PCR-targeted mutagenesis and showed that Strop_3612 encodes a primary CCR involved in salinosporamide B biosynthesis while the homologue *salG* codes for a novel chlorocrotonyl-CoA reductase/carboxylase associated with salinosporamide A biosynthesis.[6] Upon further analysis of the *S. tropica* CCR mutants, we observed that production

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of salinosporamide E was exclusively lost in the *salG* knockout mutant whereas it was maintained in the Strop_3612 mutant (Figure 1). Hence, this in vivo experiment suggests that SalG has relaxed substrate specificity toward 2-alkenyl-CoAs and is able to also reductively carboxylate 2-pentenyl-CoA in addition to 4-chlorocrotonyl-CoA as previously reported.[6] In vitro analysis of recombinant octahistidyl-tagged SalG confirmed that it is able to reductively carboxylate 2-pentenyl-CoA (k_{cat} 25.5 ± 0.7 min^{-1}; K_m 4.3 ± 0.5 μM) at comparable efficiency to 4-chlorocrotonyl-CoA (k_{cat} 23.1 ± 2.6 min^{-1}; K_m 4.4 ± 1.8 μM), which is more efficient than that with crotonyl-CoA (k_{cat} 15.4 ± 0.9 min^{-1}; K_m 20.7 ± 4.2 μM).[6] Presumably, the CCR encoded by Strop_3612 is more specific toward crotonyl-CoA, although attempts to verify this hypothesis were unsuccessful due to issues with soluble expression of the protein. However, we did observe in parallel experiments that the CCR originally isolated from *Streptomyces collinus*[11] uses crotonyl-CoA and not 2-pentenyl-CoA as a substrate for reductive carboxylation. Thus this CCR and SalG have markedly different substrate specificities yet likely have the same 2*S*-stereochemical outcome as in other homologous CCRs and medium-chain dehydrogenases/reductases.[9]

Given the relaxed in vitro and in vivo substrate specificity of SalG, we explored other substrates including 4-bromo- and 4-fluorocrotonate, which are anticipated substrates of natural bromosalinosporamide (**6**)[3] and engineered fluorosalinosporamide (**7**),[12] respectively (Scheme 1). Administration of the 4-halocrotonic acids to the *S. tropica salL*-deficient mutant resulted in the production of **6** and **7** as anticipated (Figure 1), thereby establishing bromoethylmalonyl-CoA and fluoroethylmalonyl-CoA as additional PKS extender units unique to the salinosporamide biosynthetic pathway. While the 4-halo analogues were accepted as alternative in vivo substrates,[13] elongated 2-alkenoates (C_6 to C_8) were not converted into new *sal* products as observed by HPLC-MS analysis. The capacity of a CCR homologue to preferentially accommodate a longer chain 2-alkenyl-CoA, however, is strongly suggestive in the biosynthesis of cinnabaramide A (**5**),[4] which based on the salinosporamide biosynthetic model would incorporate (2*S*)-hexylmalonyl-CoA derived from the reductive carboxylation of 2-octenyl-CoA.

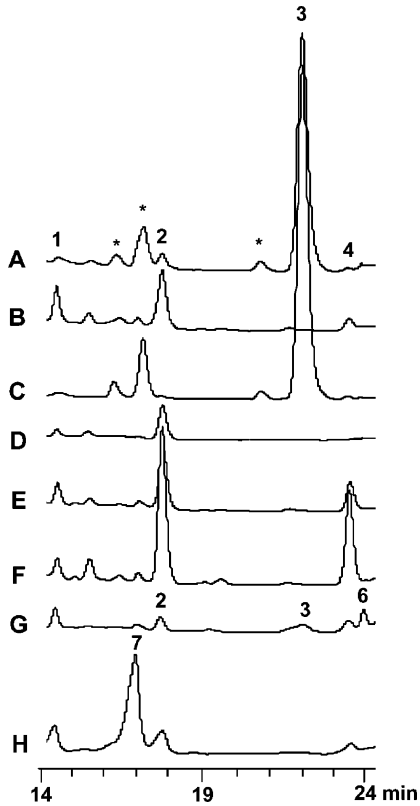

Figure 1. HPLC analysis at 210 nm of organic fractions of (A) wild-type *S. tropica*, (B) *S. tropica salL*$^-$ mutant, (C) *S. tropica* Strop_3612$^-$ mutant, (D) *S. tropica salG*$^-$ mutant, (E) *S. tropica salL*$^-$ + 0.8 mM pentanoic acid, (F) *S. tropica salL*$^-$ + 0.8 mM *trans*-2-pentenoic acid, (G) *S. tropica salL*$^-$ + 0.12 mM 4-bromocrotonoic acid, and (H) *S. tropica salL*$^-$ + 0.15 mM 4-fluorocrotonoic acid. Salinosporamide analogues **1**−**4** and **6**−**7** are noted, while derivatives of **3** are marked with an asterisk.

In conclusion, we discovered the new PKS extender unit propylmalonyl-CoA, in the context of salinosporamide E biosynthesis. It is rare for PKSs to incorporate pentyl building blocks in their polyketide products; to our knowledge only the macrolide immunosuppressant FK506,[14] which carries an allyl side chain, and the acyl depsipeptide dentigerumycin[15] may similarly incorporate propylmalonyl-CoA units. This discovery exemplifies a new strategy in PKS extender unit biochemistry in which α,β-unsaturated acyl-CoA thioesters are reductively carboxylated[16] and furthermore suggests that CCR protein engineering may readily afford unnatural malonyl-CoA precursors for the bioengineering of novel polyketide molecules.

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Supporting Information Available: Experimental procedures, NMR data, and SalG kinetic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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