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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 non-proteinogenic amino acid cyclohexenylalanine (Scheme 1).⁷ The selection of the PKS extender unit is controlled by the acyltransferase domain AT₁ 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 analog salinosporamide D (**1**) would be similarly assembled from a propionate unit via the common PKS substrate (2S)-methylmalonyl-CoA (Scheme 1). We explored this assumption by administering [1-¹³C]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 ¹³C-enrichment (5%) at C-1, thereby confirming its assembly from propionate.

We next turned our attention to the propyl analog 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 [1-¹³C]propionate feeding experiment, and NMR analysis confirmed ¹³C-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% (Figure 1). These observations suggested that the C₅ 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-CoA⁹ 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-440¹⁰ 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 homolog *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 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 towards 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 \pm 0.7 min⁻¹; K_m 4.3 \pm 0.5 μ M) at comparable efficiency to 4-chlorocrotonyl-CoA (k_{cat} 23.1 \pm 2.6 min⁻¹; K_m 4.4 \pm 1.8 μ M), which is more efficient than that with crotonyl-CoA (k_{cat} 15.4 \pm 0.9 min⁻¹; K_m 20.7 \pm 4.2 μ M).⁶ Presumably, the CCR encoded by Strop_3612 has more substrate specificity towards 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 analogs were accepted as alternative *in vivo* substrates,¹³ elongated 2-alkenoates (C₆ to C₈) were not converted into new *sal* products as observed by HPLC-MS analysis. The capacity of a CCR homolog 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.

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 dentigerumycin¹⁵ 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

carboxylated¹⁶ and furthermore suggests that CCR protein engineering may readily afford unnatural malonyl-CoA precursors for the bioengineering of novel polyketide molecules.

Supplementary Material

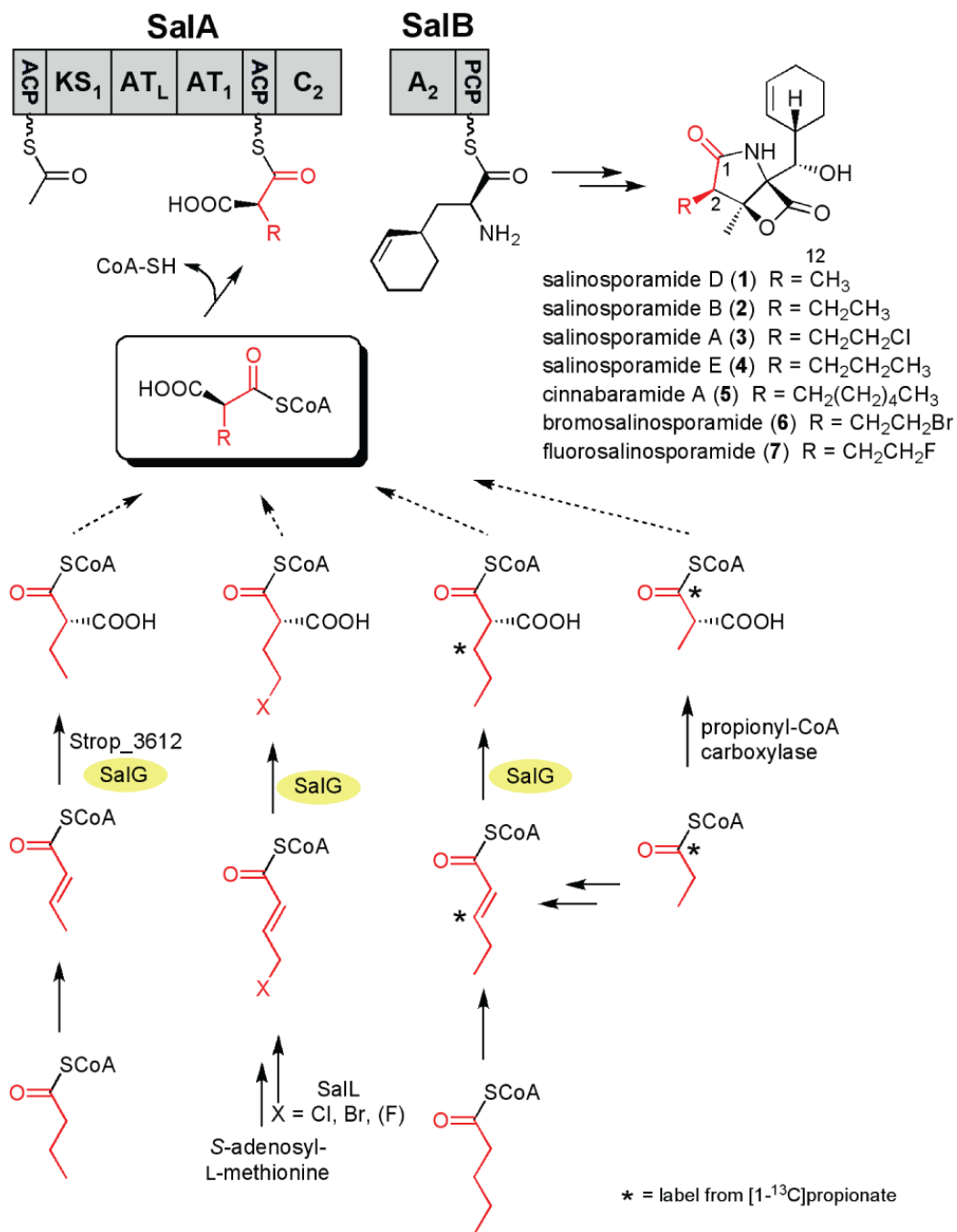
Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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**Scheme 1.**

Biosynthesis of salinosporamide A (3), its analogs and their substituted malonyl-CoA PKS building blocks (boxed). Abbreviations: ACP, acyl carrier protein; KS, ketosynthase; AT, acyltransferase; C, condensing domain; A adenylation domain; PCP, peptidyl carrier protein.

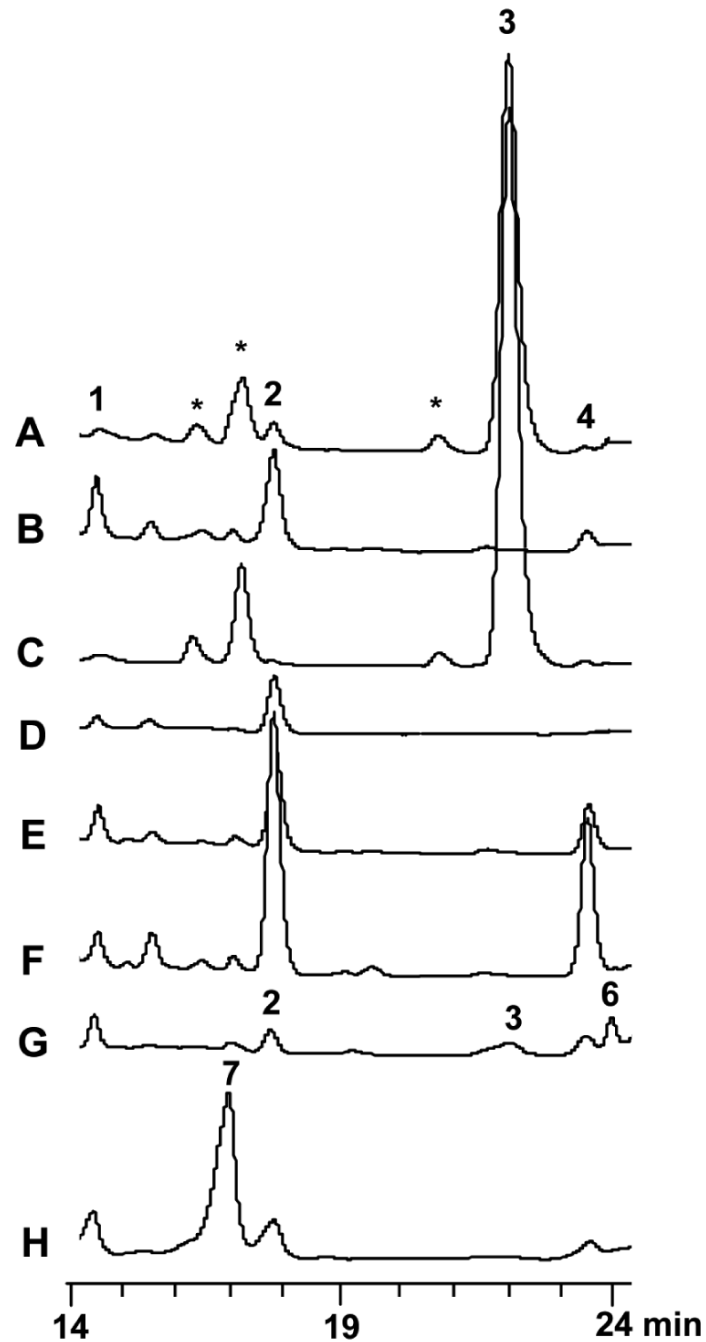


Figure 1.

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