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Biosynthesis of Salinosporamides from \(\alpha,\beta\)-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 \(\beta\)-lactone-\(\gamma\)-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,\(^5\)\(^\dagger\),\(^6\) 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 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 \(\alpha,\beta\)-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 \([1\text{-}^{13}\text{C}]\) 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 \(^{13}\text{C}\)-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 \([1\text{-}^{13}\text{C}]\)propionate feeding experiment, and NMR analysis confirmed \(^{13}\text{C}\)-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% (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 (2S)-ethylmalonyl-CoA\(^9\) suggests the possibility of an analogous pathway to (2S)-propylmalonyl-CoA in *S. tropica* (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 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 \((k_{cat} 25.5 \pm 0.7 \text{ min}^{-1}; K_m 4.3 \pm 0.5 \mu M)\) at comparable efficiency to 4-chlorocrotonyl-CoA \((k_{cat} 23.1 \pm 2.6 \text{ min}^{-1}; K_m 4.4 \pm 1.8 \mu M)\), which is more efficient than that with crotonyl-CoA \((k_{cat} 15.4 \pm 0.9 \text{ min}^{-1}; K_m 20.7 \pm 4.2 \mu M)\). 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 2S-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. tropicasalL*-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 (2S)-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 pentylic building blocks in their polyketide products; to our knowledge only the macrolide immunosuppressant FKS06,\(^{14}\) 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\textsuperscript{16} and furthermore suggests that CCR protein engineering may readily afford unnatural malonyl-CoA precursors for the bioengineering of novel polyketide molecules.

Supplementary Material

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Acknowledgments

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(13). Administration of 4-bromocrotonic acid to the \textit{S. tropica salL} \textsuperscript{3} mutant also provided the chlorinated \textit{Figure 1, trace G} as a minor product. Its production presumably derives from the transchlorination of the substrate in the seawater-based A1 medium.
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) *S. tropicasalL* mutant, (C) *S. tropica* Strop_3612* mutant (D) *S. tropicasalG* mutant, (E) *S. tropicasalL* + 0.8 mM pentanoic acid, (F) *S. tropicasalL* + 0.8 mM *trans*-2-pentenoic acid, (G) *S. tropicasalL* + 0.12 mM 4-bromocrotonic acid, and (H) *S. tropicasalL* + 0.15 mM 4-fluorocrotonic acid. Salinosporamide analogs 1-4 and 6-7 are noted, while derivatives of 3 are marked with an asterisk.