

Biosynthesis of the salinosporamide A polyketide synthase substrate chloroethylmalonyl-coenzyme A from S-adenosyl-L-methionine

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Polyketides are among the major classes of bioactive natural products used to treat microbial infections, cancer, and other diseases. Here we describe a pathway to chloroethylmalonyl-CoA as a polyketide synthase building block in the biosynthesis of salinosporamide A, a marine microbial metabolite whose chlorine atom is crucial for potent proteasome inhibition and anticancer activity. S-adenosyl-L-methionine (SAM) is converted to 5'-chloro-5'-deoxyadenosine (5'-CIDA) in a reaction catalyzed by a SAM-dependent chlorinase as previously reported. By using a combination of gene deletions, biochemical analyses, and chemical complementation experiments with putative intermediates, we now provide evidence that 5'-CIDA is converted to chloroethylmalonyl-CoA in a 7-step route via the penultimate intermediate 4-chlorocrotonyl-CoA. Because halogenation often increases the bioactivity of drugs, the availability of a halogenated polyketide building block may be useful in molecular engineering approaches toward polyketide scaffolds.

actinomycete | biological halogenation | marine natural product | proteasome inhibitor | *Salinispora tropica*

Polyketides are abundant microbial metabolites that possess a remarkable diversity in chemical structure and biological function. The enzymes that catalyze the assembly of these natural products, namely polyketide synthases (PKSs), belong to 3 protein families that similarly use small carboxylic acid building blocks as substrates. Polyketide biosynthetic pathways have evolved a myriad of ways to accommodate changes in the number and composition of their substrates, the manner in which they are assembled, and the further biochemical modification of the PKS product by tailoring enzymes to synthesize these often very complex organic molecules (1–4). The assembly line organization of modular type I PKSs in particular has facilitated their rational reengineering through combinatorial biosynthesis and mutasynthesis to yield new compound scaffolds that further extends their natural biosynthetic prowess (5).

Although PKSs exploit a wide assortment of priming carboxylic acid substrates to initiate the polyketide biosynthetic process (6), they are relegated to a small number of extending dicarboxylic acid units needed to elongate the growing polyketide chain via successive Claisen condensation reactions (7). The most common PKS extender units are malonyl-CoA, methylmalonyl-CoA, and, to a much lesser extent, ethylmalonyl-CoA that are selected by and attached to the PKS domain acyl carrier protein (ACP) by dedicated acyltransferases (ATs). These CoA-tethered PKS building blocks impart unreactive, aliphatic substituents (proton, methyl, and ethyl, respectively) to the polyketide backbone and contrast the second class of dedicated ACP-linked PKS extender units that instead harbor functionalized side chains. Methoxymalonyl-ACP, hydroxymalonyl-ACP, and aminomalonyl-ACP are relatively rare extender units that

supply methoxy, hydroxyl, and amino groups, respectively, to the polyketide molecule (8). The programmed introduction of these ACP-bound extender units results in the strategic placement of functional groups that confers important structural and biological properties to the polyketide.

We recently proposed that the PKS extender unit chloroethylmalonyl-CoA was involved in the biosynthesis of the anticancer agent salinosporamide A in the marine bacterium *Salinispora tropica* (9). This CoA-linked halogenated metabolite provides the reactive chloroethyl side chain germane to salinosporamide A's irreversible binding mechanism against the 20S proteasome (10). The biosynthesis of salinosporamide's chlorinated building block is initiated by the S-adenosyl-L-methionine (SAM)-dependent chlorinase (11), which catalyzes the conversion of SAM to 5'-chloro-5'-deoxyadenosine (5'-CIDA). Herein, we detail an 8-step biosynthetic pathway to chloroethylmalonyl-CoA and firmly establish this chlorinated metabolite as a PKS extender unit. Because halogen atoms not only favorably influence the bioactivity of drugs (12) but also offer chemically reactive handles for lead optimization by semisynthetic chemistry, this pathway to a halogenated PKS building block may facilitate the bioengineering of polyketide molecules for drug development.

Results

Analysis of the *sal* Gene Cluster. Complete genome sequence analysis of *S. tropica* CNB-440 revealed 19 secondary metabolic gene clusters (13), including a 41-kb hybrid PKS-nonribosomal peptide synthetase (NRPS) gene set consistent with salinosporamide A biosynthesis (Fig. 1 and *SI Text*, Table S1). Before genome sequencing of CNB-440 was completed, we attempted to clone and sequence the *sal* cluster from *S. tropica* strain CNB-476 by using PCR-amplified PKS, NRPS, and crotonyl-CoA carboxylase/reductase (CCR) gene fragments as probes. Library screening led to the identification of a pOJ446 cosmid clone containing a 33-kb genomic insert 99% identical in DNA sequence to strain CNB-440 (Fig. 1).

Central to the *sal* cluster is the *salA* gene that codes for a

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. CP000667 (*S. tropica* CNB-440 complete genome sequence) and EF397502 (partial *sal* cluster from *S. tropica* CNB-476)].

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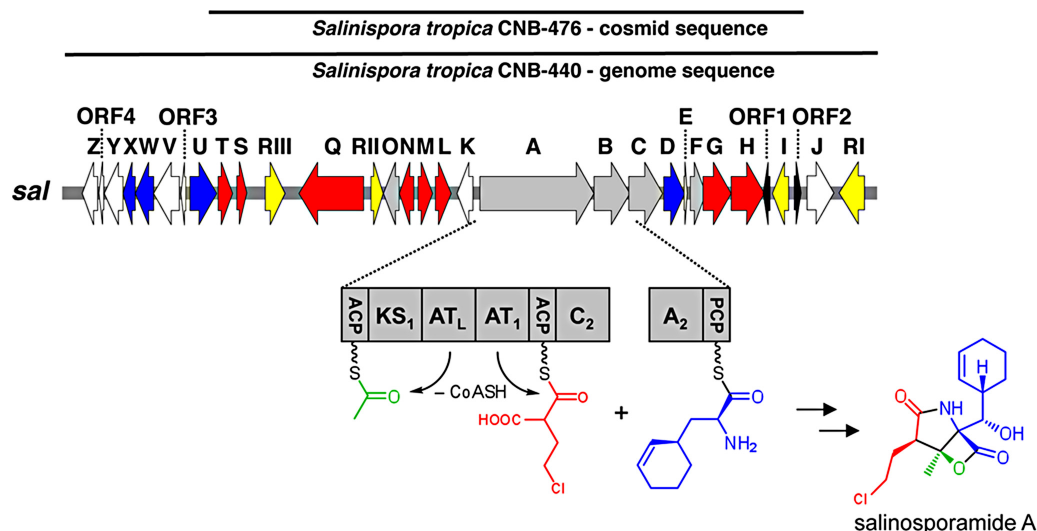


Fig. 1. Organization of the *sal*/biosynthetic gene cluster from *Salinispora tropica*. The *sal* DNA sequence in strains CNB-476 and CNB-440 is 99% identical. Genes putatively involved in the chloroethylmalonyl-CoA pathway (red), construction of the core γ -lactam- β -lactone ring system (gray), assembly of the nonproteinogenic amino acid L-3-cyclohex-2'-enylalanine (blue), regulation and resistance (yellow), unknown (white), and 2 partial transposases (black) are color-coded.

bimodular PKS of unusual domain organization harboring contiguous acyltransferase loading (AT_L) and extender (AT_1) (Fig. 1) domains rather than the standard AT_L -ACP $_L$ -KS $_1$ - AT_1 -ACP $_1$ assembly as observed in prototypical PKSs such as 6-deoxyerythronolide B synthase 1 (14). This noncanonical domain architecture is, however, found in several myxobacterial megasynthases such as those involved in stigmatellin, soraphen, and aurafuranone biosynthesis (15–17). Stable isotope feeding studies showed that salinosporamide A is biosynthesized from the building blocks acetate, the nonproteinogenic amino acid β -hydroxy-L-3-cyclohex-2'-enylalanine (CHA) and a sugar-derived chlorinated molecule that we hypothesized was a previously unknown PKS extender unit, namely chloroethylmalonyl-CoA (9).

We propose that the hexa-domained SalA is involved in the selection, attachment and condensation of acetyl-CoA and chloroethylmalonyl-CoA, to generate a β -keto thioester intermediate. The loading module is of the ACP $_1$ / AT_L -type that recognizes monocarboxylic acid starter units such as acetyl-CoA rather than the KSQ-type that accepts dicarboxylic acids like malonyl-CoA (6). The possibility of chloroethylmalonyl-CoA as an extender unit prompted us to examine the phylogeny of AT_1 in more detail (SI Text and Fig. S1), because AT domain divergence is a critical factor in the evolution of polyketide structural diversity (18). Malonyl-CoA- and methylmalonyl-CoA-specific AT domains share a common ancestor, diverging at some point of evolution to form 2 distinct groups, whereas the relatively rare ethylmalonyl-CoA and methoxymalonyl-ACP AT s appear to have evolved more than once because the known sequences reside in either clade (18). Our analysis with representative AT domains reconstructs that scenario and places SalA_ AT_1 in the methylmalonyl-CoA group, more closely related to some myxobacterial AT s although forming its own subclade (SI Text and Fig. S1). In addition, the distinct signature motifs (14) apparent from sequence alignments are also in agreement with AT_1 accepting an unreported extender unit (SI Text and Fig. S1B).

Moreover, the detection of salinosporamide analogs with different side chains at C2 (ethyl and methyl, Fig. 2), points to the promiscuity of AT_1 in accepting not only chloroethylmalonyl-CoA (salinosporamide A), but also other substituted malonyl extender units such as ethylmalonyl-CoA (salinosporamide B) and methylmalonyl-CoA (salinosporamide D). To probe the

central role of *salA*, we disrupted it via a single-cross-over homologous recombination event. Inactivation of *salA* abolished the biosynthesis of all salinosporamides, thereby confirming that this family of β -lactones is indeed derived from a PKS pathway (Fig. 2).

The Chloroethylmalonyl-CoA Pathway. Based on the gene organization of the *sal* cluster, we propose a route to chloroethylmalonyl-CoA as illustrated in Fig. 3A. Biosynthesis of ethylmalonyl-CoA as a precursor of salinosporamide B is shown for comparison (Fig. 3B) and is not encoded in the *sal* locus but constitutes rather a primary metabolic pathway for acetate assimilation and a source of building blocks for secondary metabolite production (19, 20).

We recently reported that chlorine incorporation into salinosporamide A is catalyzed by the SAM-dependent chlorinase SalL in an orthogonal manner to other known enzymatic chlorination reactions, but analogous to fluorinase FIA of *Streptomyces cattleya*, a fluoroacetate and 4-fluorothreonine producer (11, 21). Earlier studies using ^{13}C -labeled glucose showed an incorporation pattern in the chlorobutyrate moiety of salinosporamide A consistent with the ribose unit of SAM being the ultimate precursor (9). Interestingly, the *sal* cluster also harbors the FIB homolog SalT. FIB is the second enzyme in the pathway to fluorometabolites in *S. cattleya*, catalyzing the phosphorolytic cleavage of 5'-FDA to produce

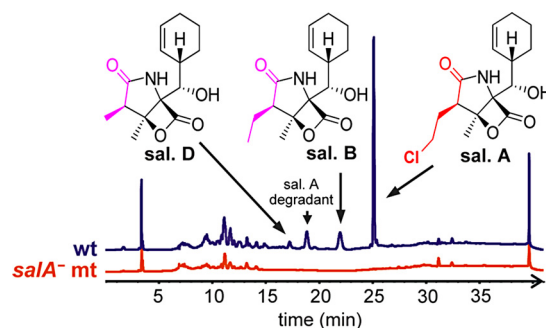


Fig. 2. Inactivation of the PKS gene *salA* completely abolishes salinosporamide (sal.) production. HPLC chromatograms of culture extracts with detection at 210 nm. Mt, mutant; wt, wild-type.

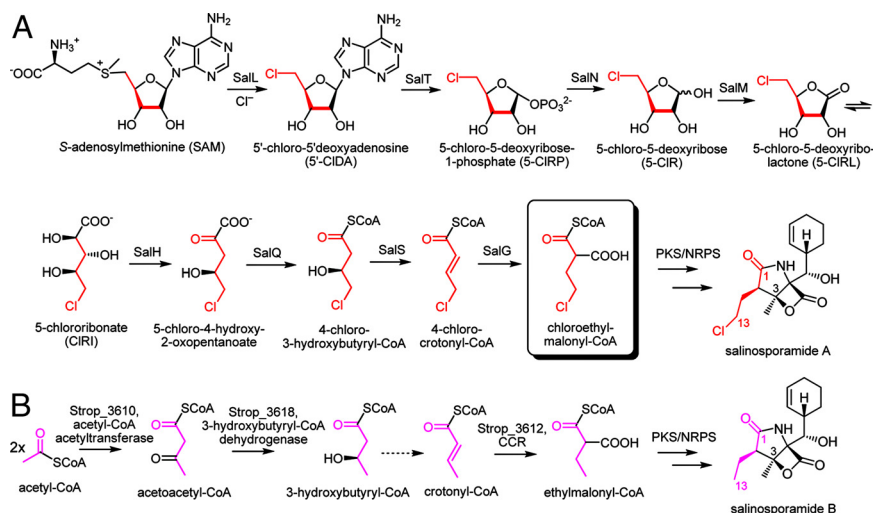


Fig. 3. Comparison of chloroethylmalonyl-CoA and ethylmalonyl-CoA biosynthetic pathways. (A) Proposed pathway to chloroethylmalonyl-CoA as a PKS extender unit in salinosporamide A biosynthesis. (B) The corresponding ethylmalonyl-CoA moiety in salinosporamide B is derived from acetate (9). Crotonyl-CoA carboxylase/reductase (CCR) is a key enzyme in the ethylmalonyl-CoA pathway (19).

5-fluoro-5-deoxy-D-ribose-1-phosphate (5-FRP) (22). Although SalT likely catalyzes the analogous conversion of 5'-CIDA to 5-CIRP (Fig. 3A), the 2 pathways appear to diverge at this point as no other structural homologous genes are shared between the *sal* and *fl* clusters (22). For fluorometabolites it has been speculated that an isomerase catalyzes the ring opening of 5-FRP to 5-fluoro-5-deoxy-D-ribulose-1-phosphate, in analogy to the known metabolism of 5'-methylthioadenosine (23). Indeed, the in vitro reconstitution of 4-fluorothreonine biosynthesis has been recently achieved by using besides FIA and FIB, 3 enzymes not coded in the *fl* cluster, that is, an isomerase, a surrogate aldolase and a pyridoxal (PLP)-dependent transaldolase (24).

For chloroethylmalonyl-CoA, we propose instead that the phosphatase homolog SalN and the dehydrogenase/reductase SalM catalyze the dephosphorylation of 5-CIRP to 5-chloro-5-deoxy-D-ribose (5-CIR) followed by oxidation to 5-chlororibonate (5-CIRI) possibly via the intermediate 5-chloro-5-deoxy-D-ribono-1,4-lactone (5-CIRL) (Fig. 3A). SalH, a dihydroxyacid dehydratase homolog, then putatively converts 5-CIRI to 5-chloro-4-hydroxy-2-oxopentanoate, which is then subjected to SalQ-catalyzed oxidative decarboxylation to 4-chloro-3-hydroxybutyryl-CoA. This product importantly results in a 4-carbon chlorometabolite consistent with the C1/C2/C12/C13 salinosporamide A fragment (9). SalQ shares >50% sequence identity with α -oxoacid ferredoxin ox-

doreductases known to catalyze the oxidative decarboxylation of α -ketoacids with reduction of ferredoxin (Fd) to the corresponding CoA derivative, the prototype of which is pyruvate ferredoxin oxoreductase (25).

Domain analysis of the putative biosynthetic enzyme SalS suggests that it belongs to the hotdog fold superfamily that includes FabZ, a β -hydroxyacyl-ACP dehydratase involved in bacterial fatty acid biosynthesis (26). SalS's closest characterized homolog (47% amino acid identity) is Rv0130 from *Mycobacterium tuberculosis*, a 16-kDa protein shown to catalyze the reversible hydration of crotonyl-CoA to hydroxybutyryl-CoA in vitro (27). However, it has been shown that these enzymes function as dehydratases in vivo when coupled to a reductase (28). Hence, we propose that SalS catalyzes the reversible dehydration of 4-chloro-3-hydroxybutyryl-CoA to 4-chlorocrotonyl-CoA.

The final reaction toward chloroethylmalonyl-CoA is putatively catalyzed by SalG, which shows sequence identity (>60%) to crotonyl-CoA carboxylase/reductases (CCR). The biological function of CCR was recently revised to catalyze the last step of ethylmalonyl-CoA biosynthesis (19) (Fig. 3B), and similarly we hypothesize that SalG catalyzes the reductive carboxylation of 4-chlorocrotonyl-CoA to chloroethylmalonyl-CoA.

Gene Inactivation and Chemical Complementation. To functionally identify the chloroethylmalonyl-CoA pathway enzymes, we inacti-

Table 1. Salinosporamide production by *S. tropica* mutants compared to the wild-type

Strain	Protein function	Sal. A, % [†]	Sal. B, % [†]	Strop homolog, sequence identity
wild-type	—	100 ± 10	100 ± 13	—
<i>salA</i> [−]	PKS	n.d.	n.d.	—
<i>salL</i> [−]	Chlorinase (11)	n.d.	90 ± 20	Strop_1405, 35%
<i>salT</i> [−]	Purine nucleoside phosphorylase	50 ± 8	91 ± 10	Strop_0986, 69%
<i>salN</i> [−]	Phosphatase	16 ± 3	92 ± 9	—
<i>salM</i> [−]	Dehydrogenase/reductase	2.2 ± 0.2	120 ± 20	Strop_2799, 35%
<i>salH</i> [−]	Dihydroxyacid dehydratase	3.8 ± 0.7	70 ± 15	Strop_1231, 36%
<i>salQ</i> [−]	α-ketoacid decarboxylase	25 ± 6	98 ± 16	Strop_1050 [‡]
<i>salS</i> [−]	AcyI dehydratase	39 ± 14	95 ± 30	—
<i>salG</i> [−]	Cl-CCR	n.d.	94 ± 30	Strop_3612, 53%
<i>Strop_3612</i> [−]	CCR	112 ± 8	52 ± 6	<i>salG</i> , 53%

Sal., salinosporamide; n.d., not detected (<0.2%).

[†]Values are average of at least two independent experiments \pm deviation.

[‡]Strop_1050 represents a partial duplication of *sa/Q* (894 of 3,450 nt).

technology kit for PCR-targeting, A. Lapidus from the Joint Genome Institute (Walnut Creek, CA) for *sal* fosmids, and W. Fenical and P. R. Jensen (Scripps Institution of Oceanography, La Jolla, CA) for *S. tropica* strains. A.S.E. is a Tularik Postdoctoral Fellow of the Life Sciences Research Foundation, and L.L.B. was a

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