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REVIEW

Beyond ethylmalonyl-CoA: The functional role of crotonyl-CoA carboxylase/reductase homologs in expanding polyketide diversityMicheal C. Wilson^a and Bradley S. Moore^{*ab}

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This review covers the emerging biosynthetic role of crotonyl-CoA carboxylase/reductase (CCR) homologs in extending the structural and functional diversity of polyketide natural products. CCRs catalyze the reductive carboxylation of α,β -unsaturated acyl-CoA substrates to produce a variety of substituted malonyl-CoA derivatives employed as polyketide synthase extender units. Here we discuss the history of CCRs in both primary and secondary metabolism, the mechanism by which they function, examples of new polyketide diversity from pathway specific CCRs, and the role of CCRs in facilitating the bioengineering novel polyketides.

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1 Introduction

Polyketides comprise a large class of pharmaceutically important natural products whose biological properties, including antimicrobial, immunosuppressant, and anticancer, are attributed to their specialized chemical structures derived from the condensation of simple carboxylic acid building blocks.^{1–3} Similar to the

way in which fatty acids (FAs) are constructed, polyketide assembly progresses through successive decarboxylative Claisen condensation reactions of activated alkylmalonate precursors and acyl thioesters catalyzed by multifunctional enzymes called polyketide synthases (PKSs).⁴ With each addition of either a coenzyme A (CoA)-linked or acyl carrier protein (ACP)-linked PKS extender unit, the polyketide chain grows by two carbon atoms (Fig. 1). Substitutions (*e.g.* alkyl, hydroxy, amino) at the C2 position of the activated malonate derivative impart structural complexity to the polyketide backbone, which can organize into a seemingly infinite number of combinations.

While polyketide starter units are incredibly varied in structure and size,³ extender units are much less chemically diverse.⁵ The most common PKS extender units include malonyl-CoA (**1**), methylmalonyl-CoA (**2**), and to a lesser extent ethylmalonyl-CoA

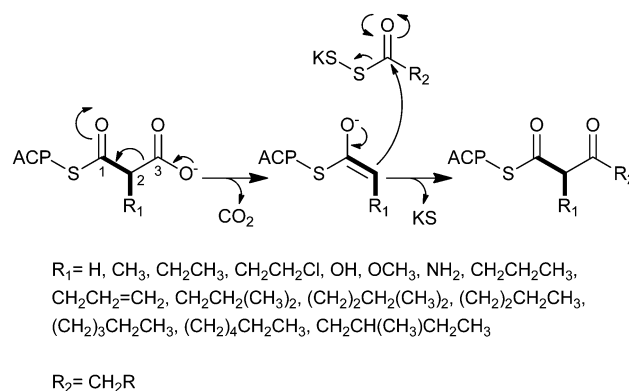
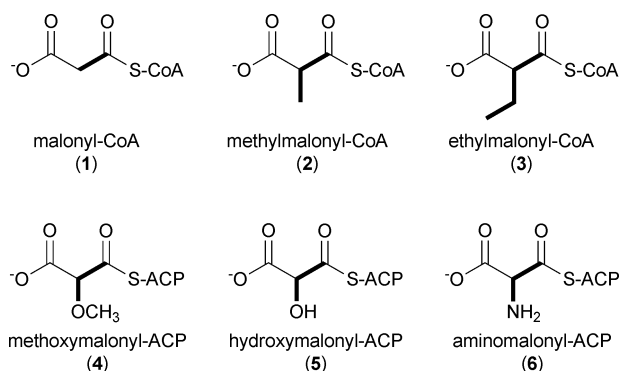


Fig. 1 The mechanism for decarboxylative Claisen condensation in type I PKS elongation.

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(3) that provide H, Me, and Et substituents, respectively, to the core polyketide molecule. A small subset of PKSs additionally utilize functionalized extender units linked directly to standalone ACPs, in particular methoxymalonyl-ACP (4), hydroxymalonyl-ACP (5), and aminomalonyl-ACP (6), that create programmed functional group diversity in the polyketide product.⁵



In 2009, a new paradigm for polyketide diversification emerged involving the biosynthesis of non-traditional CoA-linked PKS extender units by the reductive carboxylation of α,β -unsaturated acyl-CoA precursors by crotonyl-CoA carboxylase/reductase (CCR) homologs.^{6,7} This general mechanism for the biosynthesis of atypical PKS extender units (7–17) has more than doubled the list of defined CoA-linked precursors incorporated into polyketide structures (Fig. 2). Since these substrates are CoA-tethered, CCR homologs provide a unique opportunity to genetically engineer novel “unnatural” polyketides through extender unit redesign. Herein, we review recent developments in the characterization of these enzyme biocatalysts, discuss the functional roles they play in both primary and secondary metabolism, and consider their biotechnological applications.

2 Crotonyl-CoA carboxylases/reductases

CCRs and related enoyl-CoA carboxylase/reductases (ECRs) are members of the medium-chain reductase/dehydrogenase (MDR) protein superfamily that is conserved over all three domains of life.⁸ In addition to CCRs, the MDR superfamily includes NAD(P)-dependent alcohol dehydrogenase (ADH), quinone oxidoreductase (QOR), enoyl reductase (ER), and several other enzyme families that reduce either C=O or C=C bonds. Though known to catalyze the reduction of crotonyl-CoA (18) to butyryl-CoA (19) since the 1950s,^{9,10} it was not until 2007 that Alber and coworkers reported that a CCR homolog from the purple non-sulfur bacterium, *Rhodobacter sphaeroides*, also catalyzes the reductive carboxylation of 18 to (2S)-ethylmalonyl-CoA (3).¹¹ More intriguing was that the carboxylation was determined to be the physiologically favored reaction. This unprecedented reductive carboxylation has required a revision of the role CCR homologs in both primary and secondary metabolism.

2.1 CCRs in primary metabolism

In the mid-1950s, while the study of FA catabolism was in full swing, FA biosynthesis remained poorly understood. Early proposals suggested that FA biosynthesis occurred by condensation of acetyl-CoA precursors by a reversal of the β -oxidation pathway, however, biochemical evidence to support this hypothesis was lacking. While studying FA biosynthesis in rat liver, Langdon reported the NADPH-dependent enzymatic reduction of crotonyl-CoA (18) to butyryl-CoA (19), the starter unit for straight chain FAs.^{12,13} A dialyzed soluble fraction of homogenized rat liver reduced 18 to 19 in the presence of NADPH but was not able to reduce crotonate or substitute NADH as the electron donor. Later, Langdon *et al.* demonstrated that hex-2-enoyl-CoA could also oxidize NADPH at rates equivalent to 18.⁹ Thus, Langdon termed the enzyme TPN (NADPH) ethylene reductase. Around the same time, similar experiments were carried out with pig liver by Seubert *et al.* in



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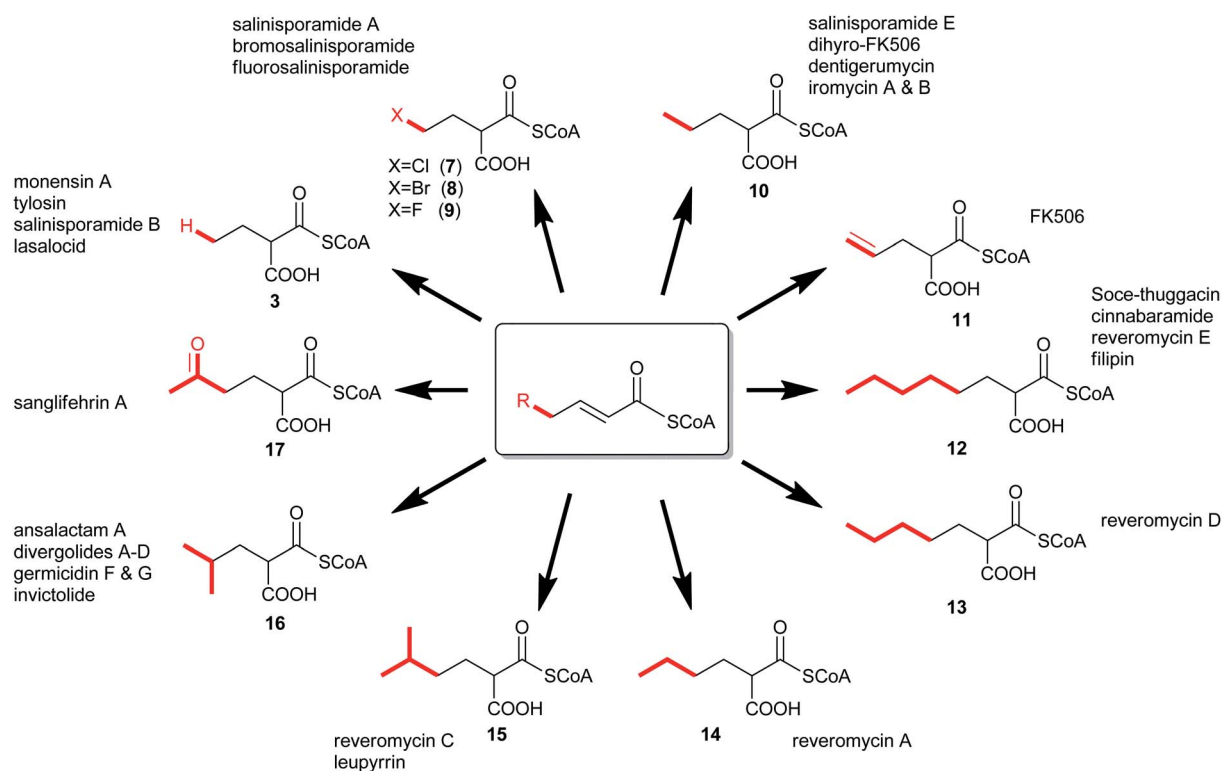


Fig. 2 The expansion of known PKS extender units constructed by crotonyl-CoA carboxylase/reductase homologs and their associated natural products.

which 2-octenoyl-CoA was reduced to octanoyl-CoA in the presence of NADPH.¹⁰ In 1967, Fraser *et al.* purified an NADPH-dependent α,β -unsaturated ketone reductase from dog erythrocytes and human liver that could reduce **18** in addition to a variety of unsaturated ketones, including furfurylidene acetone and benzylidene acetone.¹⁴ The authors determined that the purified enzyme shared identical properties to the rat liver reductase reported by Langdon, which was also shown to reduce benzylidene acetone.¹⁴

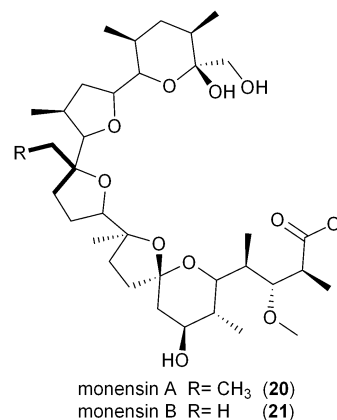
Over the next 30 years, NADPH-dependent enoyl reductase activity was reported in other mammals,¹⁵ protists,¹⁶ and bacteria¹⁷ before Reynolds and coworkers cloned and overexpressed the first *bona fide* CCR from *Streptomyces collinus*. *In vitro* characterization of the enzyme showed that the homodimeric *S. collinus* CCR catalyzed the final step in butyryl-CoA formation and was unable to accept any additional enoyl-CoA substrates or NADH as the electron donor.¹² Reynolds and colleagues later showed that CCRs in other actinomycetes were critical to the flux of precursors to both the FA and polyketide biosynthetic pools.^{13,18–21}

About a decade later, while investigating an alternate glyoxylate cycle for acetyl-CoA assimilation in *R. sphaeroides*, Alber and coworkers identified an NADPH-dependent enzyme that reductively carboxylates (*E*)-crotonyl-CoA (**18**) to (2*S*)-ethylmalonyl-CoA (**3**).^{11,22} This enzyme shared high sequence similarity (41/56% identity/similarity) to the CCR from *S. collinus*. In addition to the carboxylation reaction, the *R. sphaeroides* CCR could also reduce **18** to **19** in the absence of $\text{HCO}_3^-/\text{CO}_2$, although at 1/10th the rate of the carboxylation reaction. Since Alber's initial discovery, several additional CCR homologs have

been shown to also be carboxylating enzymes, thereby redefining CCRs as carboxylases with reductase activity being a remnant of their evolutionary history.²³ Ethylmalonyl-CoA (**3**) is further processed to glycolate to enter central metabolism or can be shunted into polyketide biosynthesis by providing butyrate units, as discussed below. The genes that make up this newly defined ethylmalonyl-CoA pathway are represented in about 8% of all sequenced bacteria, only 1% of which also have a complete glyoxylate pathway as defined by Kornberg and Krebs.^{23–25}

2.2 CCRs in secondary metabolism

The involvement of CCR in polyketide production was first reported by Reynolds and coworkers in association with the



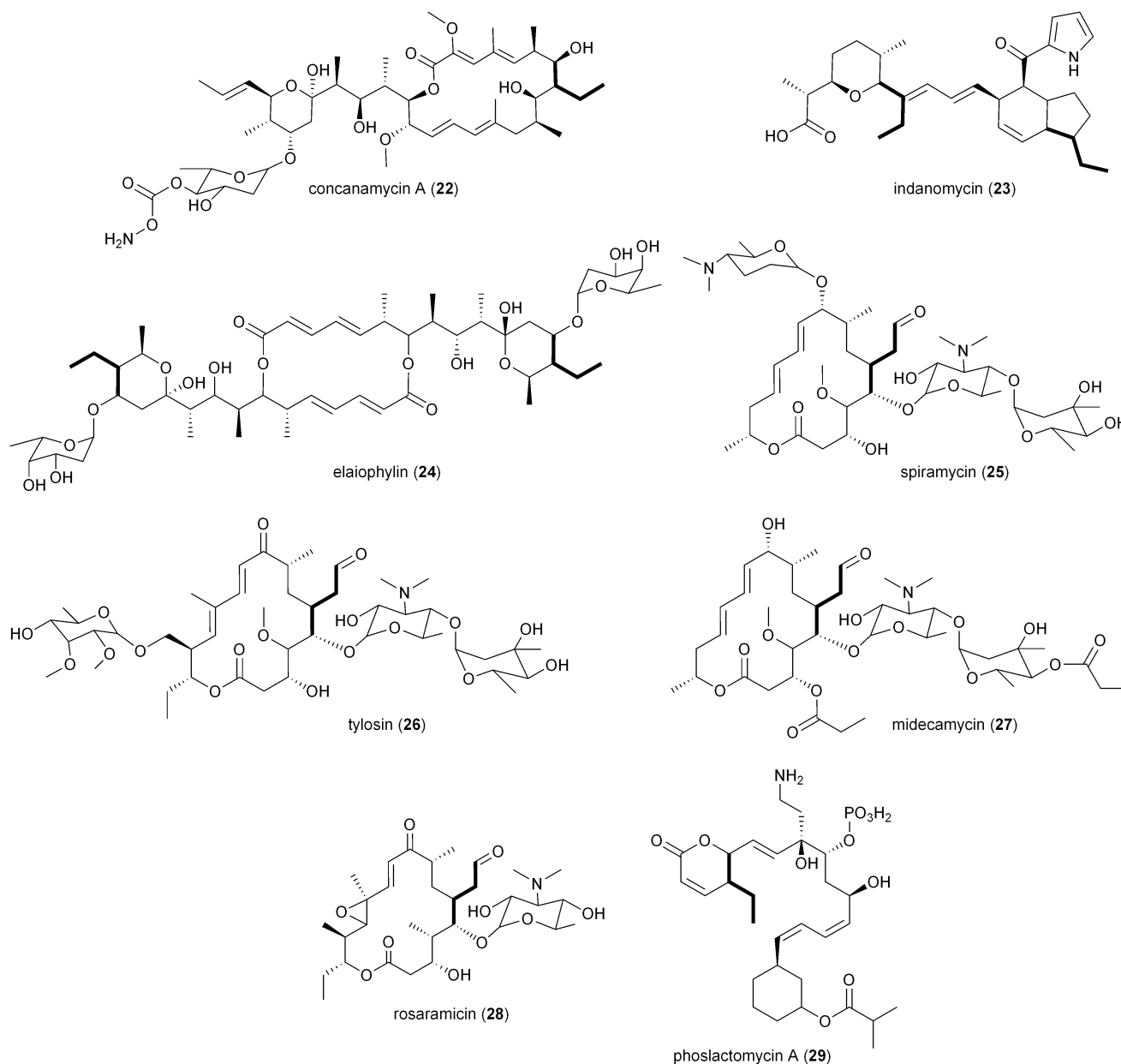
polyether antibiotic monensin **A** (**20**).²⁶ They carried out an exhaustive study in *Streptomyces cinnamomensis* on the role of CCR in determining the flux of propionate and butyrate precursors into monensins **A** (**20**) and **B** (**21**).²⁶ Disruption of the *S. cinnamomensis* *ccr* gene resulted in significant loss of **20** production while the addition of crotonic acid to wild-type cultures gave increased levels of **20**. In the case of *S. cinnamomensis*, the encoding *ccr* gene is located outside the monensin biosynthetic gene cluster and thus the primary ethylmalonyl-CoA pathway must also support polyketide biosynthesis.

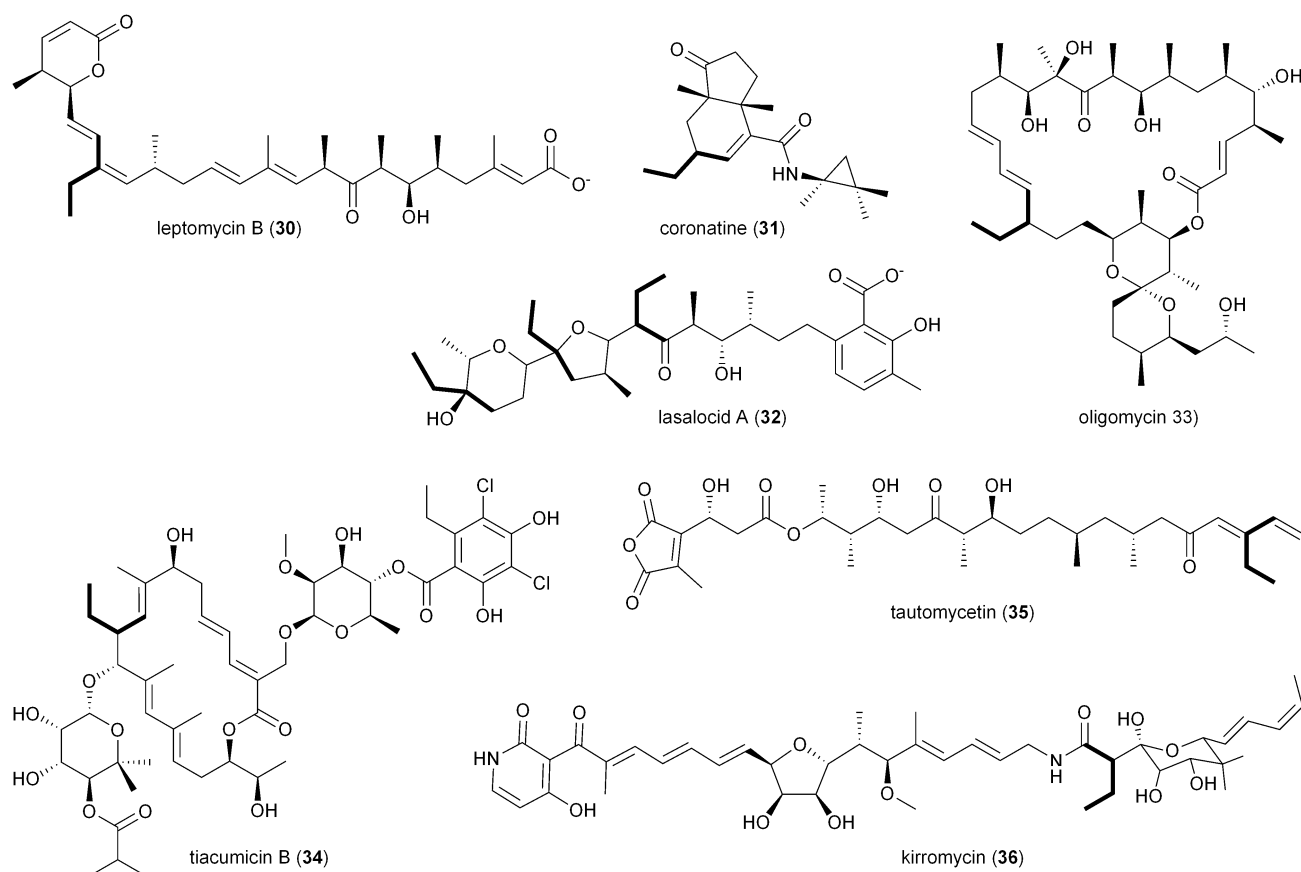
While there is more than one pathway to (2*S*)-ethylmalonyl-CoA (**3**),⁵ the CCR-dependent pathway appears to be the dominant source of this precursor for polyketide formation. Sequence analysis of PKS gene clusters associated with butyryl-containing polyketides supports that, unlike monensin,²⁰ most polyketide gene clusters retain a secondary copy of the CCR

encoding gene to likely synchronize **3** synthesis from acetoacetyl-CoA with polyketide assembly. These include the biosynthetic gene clusters for concanamycin (**22**),²⁷ indanomycin (**23**),²⁸ elaiophyllin (**24**),²⁹ spiramycin (**25**),³⁰ tylosin (**26**),³¹ midecamycin (**27**),³² rosaramicin (**28**),³³ phoslactomycin **A** (**29**),³⁴ leptomycin **B** (**30**),³⁵ crononatine (**31**),³⁶ lasalocid **A** (**32**),³⁷ oligomycin (**33**),³⁸ tiacumicin **B** (**34**),³⁹ tautomycetin (**35**),⁴⁰ kirromycin (**36**),⁴¹ FK520 (**52**),⁴² sanglifehrin **A** (**64**), and divergolides **A–D** (**66–69**).⁴³ Thus, although the monensin system was the first established pathway to require CCR biochemistry, it and the *S. coelicolor* A3(2) type III PKS germicidin synthase⁴⁴ are in the minority to draw upon **3** from primary metabolism.

2.3 Mechanism of CCRs

The regiospecificity of the reduction reaction of CCR was first established by Robinson *et al.* in 1962 using tritium labeled





NADPH³ incubated with **18** and enzyme preparations from rat brain and adipose tissue.⁴⁵ When the isolated butyrate was degraded and analyzed, it was determined that the majority (*ca.* 94%) of the H³ was found on the β -carbon. The mechanism and stereochemistry of CCR's reductive carboxylation and reduction reactions were ultimately established by Alber and coworkers with recombinant *R. sphaeroides* CCR.⁴⁶ The reductive carboxylation of (*E*)-crotonyl-CoA (**18**) to (2*S*)-ethylmalonyl-CoA (**3**) and the hydrogenation of **18** to butyryl-CoA (**19**) utilize NADPH as the reductant (Fig. 3a). For the carboxylation reaction it was determined that CO₂ is the preferred reactive species instead of HCO₃⁻.⁴⁶ As mentioned above, reduction of **18** to **19** occurs in the absence of HCO₃⁻/CO₂, but only at a fraction of the rate of the preferred reductive carboxylation reaction. The proposed mechanism for the reductive carboxylation of α,β -unsaturated acyl-CoA substrates begins with transfer of the hydride from NADPH onto the β -carbon to give a thioester enolate, followed by electrophilic attack of the CO₂ at the α -carbon. The pro-(4*R*) hydrogen of NADPH is transferred in both CCR reactions to the *re* face of the C3 position of crotonyl-CoA (Fig. 3b–c). The stereospecificity of the reduction reaction alone had previously been determined to be pro-(4*S*) specific for the CCR of *S. collinus*,⁴⁷ however, a re-evaluation of the data suggested that the stereochemistry of the labeled NADPH was misassigned.^{46,48} Depending on whether the carboxylation or reduction reaction proceeds, either CO₂ or a solvent proton are incorporated in an *anti* fashion at C2 of crotonyl-CoA, respectively (Fig. 3a).

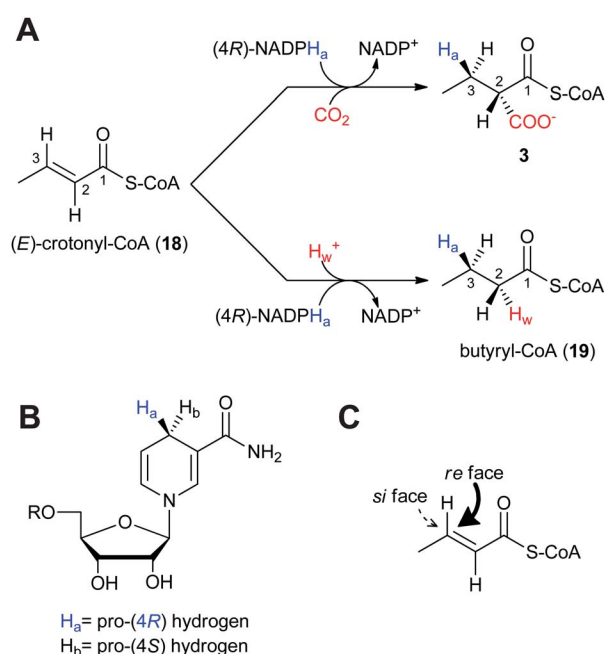


Fig. 3 The stereochemistry and regiospecificity of CCR enzymatic reactions. A) Reductive carboxylation of (*E*)-crotonyl-CoA (**18**) to (2*S*)-ethylmalonyl-CoA (**3**) and reduction of **18** to butyryl-CoA (**8**). B) The pro-(4*R*) hydrogen (H_a) of NADPH is transferred to the C) *re* face of (*E*)-crotonyl-CoA (**18**).

3 Polyketide diversification by CCR homologs

Sequence analysis of PKS biosynthetic gene clusters from predominantly actinomycete bacteria has recently identified a growing trend of co-clustered CCR and PKS genes involving polyketides that assimilate building blocks other than butyrate. This co-occurrence suggested that CCR homologs may use dedicated substrate derivatives in the synthesis of novel C2-modified malonyl-CoA molecules for polyketide assembly. With the first established example in 2009 of a CCR homolog involved in chloroethylmalonyl-CoA (7) biosynthesis,⁷ several other cases of alkylmalonyl-CoA production quickly followed over the next two years to establish CCR-based biochemistry as a major metabolic driver in PKS extender unit diversification. Phylogenetic analysis of PKS-associated CCRs shows that CCRs specific for the production of ethylmalonyl-CoA (3) largely clade

separately from those responsible for production of more esoteric extender units (Fig. 4). However, unlike PKS acyl-transferase (AT) or ketoacylsynthase (KS) domains, *in silico* prediction of the substrate specificity of CCR homologs is not readily achieved by phylogenetic analysis.^{49–51} This is due in part to the rare nature of some CCR substrates as well as the fact that a number of pathway specific CCR homologs can accept a variety of enoyl-CoA thioesters, as exemplified by the salinosporamide,^{6,52–55} FK506,⁵⁶ and reveromycin^{57,58} series of metabolites. In this section, we highlight the recently established roles of CCR homologs in polyketide assembly.

3.1 Chloroethylmalonyl-CoA and bromoethylmalonyl-CoA

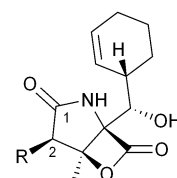
The salinosporamide family of potent proteasome inhibitors (37–43) from the obligate marine bacteria *Salinispora tropica*⁵⁹



Fig. 4 Phylogenetic analysis of CCR homologs from characterized biosynthetic gene clusters. Their evolutionary history was inferred using the UPGMA method from 1000 replicates. The analysis involved 47 protein sequences. Evolutionary analyses were conducted in MEGA5.⁹⁴

and *Salinispora pacifica*⁵⁴ structurally differ at C2 of the γ -lactam ring, possessing H, Me, Et, Pr, EtCl, and EtBr substituents. Stable isotope tracer experiments by Moore and coworkers revealed the varied biogeneses of these biochemical precursors that were ultimately linked to a hybrid PKS–nonribosomal peptide synthetase enzymatic assembly line.⁵² The *S. tropica* salinosporamide biosynthetic gene cluster contains the CCR homolog *salG* that originally was hypothesized as supplying the butyrate unit for salinosporamide B assembly.⁷ Genetic inactivation of the *salG* gene, however, did not lead to significant loss in salinosporamide B production; rather, disruption of the primary metabolic CCR encoding gene *strop_3612* resulted in its large reduction in yield. Deletion of *salG*, on the other hand, eliminated the production of the chlorinated analog salinosporamide A in which the C2 chloroethyl side chain was shown to derive from the ribose unit of *S*-adenosylmethionine (SAM).^{7,60} Further encoded within the *sal* biosynthetic gene cluster are seven additional genes that constitute the newly established chloroethylmalonyl-CoA pathway unique to *S. tropica*.⁷ Extensive *in vivo* studies utilizing gene inactivation and chemical complementation, along with *in vitro* biochemical and structural characterization of the gene products from the salinosporamide pathway defined the chloroethylmalonyl-CoA (7) pathway, as shown in Fig. 5a.^{7,61} The biosynthesis of 7 begins with the halogenation of SAM by the chlorinase SalL to yield 5'-chloro-5'-deoxyadenosine (5'-CIDA, 45). Six enzyme-catalyzed reactions convert 5'-CIDA to 4-chlorocrotonyl-CoA (46), which is subjected to reductive carboxylation by the CCR homolog SalG

to yield 7. Biochemical analysis of recombinant SalG revealed its substrate preference for 4-chlorocrotonyl-CoA (46) over crotonyl-CoA (18) and its preferred reductive carboxylation turnover.⁷ Based on SalG's high similarity with primary CCRs, it is presumed that this class of enzymes yields the same 2*S*-stereochemical outcome. In addition to this natural pathway to (2*S*)-chloroethylmalonyl-CoA (7), SalL can also accommodate bromide ion as the nucleophilic halogen to initiate the SAM-dependent pathway to give bromoethylmalonyl-CoA (8) for PKS incorporation into bromosalinosporamide (38).^{6,60} The haloethylmalonyl-CoA biosynthetic pathway is unique to *S. tropica* and represents an orthogonal mode of polyketide halogenation that typically involves post-PKS modifying halogenating enzymes.^{60,62}



salinosporamide A	R = CH ₂ CH ₂ Cl	(37)
bromosalinosporamide	R = CH ₂ CH ₂ Br	(38)
fluorosalinoparamide	R = CH ₂ CH ₂ F	(39)
salinosporamide B	R = CH ₂ CH ₃	(40)
salinosporamide D	R = CH ₃	(41)
salinosporamide E	R = CH ₂ CH ₂ CH ₃	(42)
salinosporamide K	R = H	(43)
cinnabaramide A	R = CH ₂ (CH ₂) ₄ CH ₃	(44)

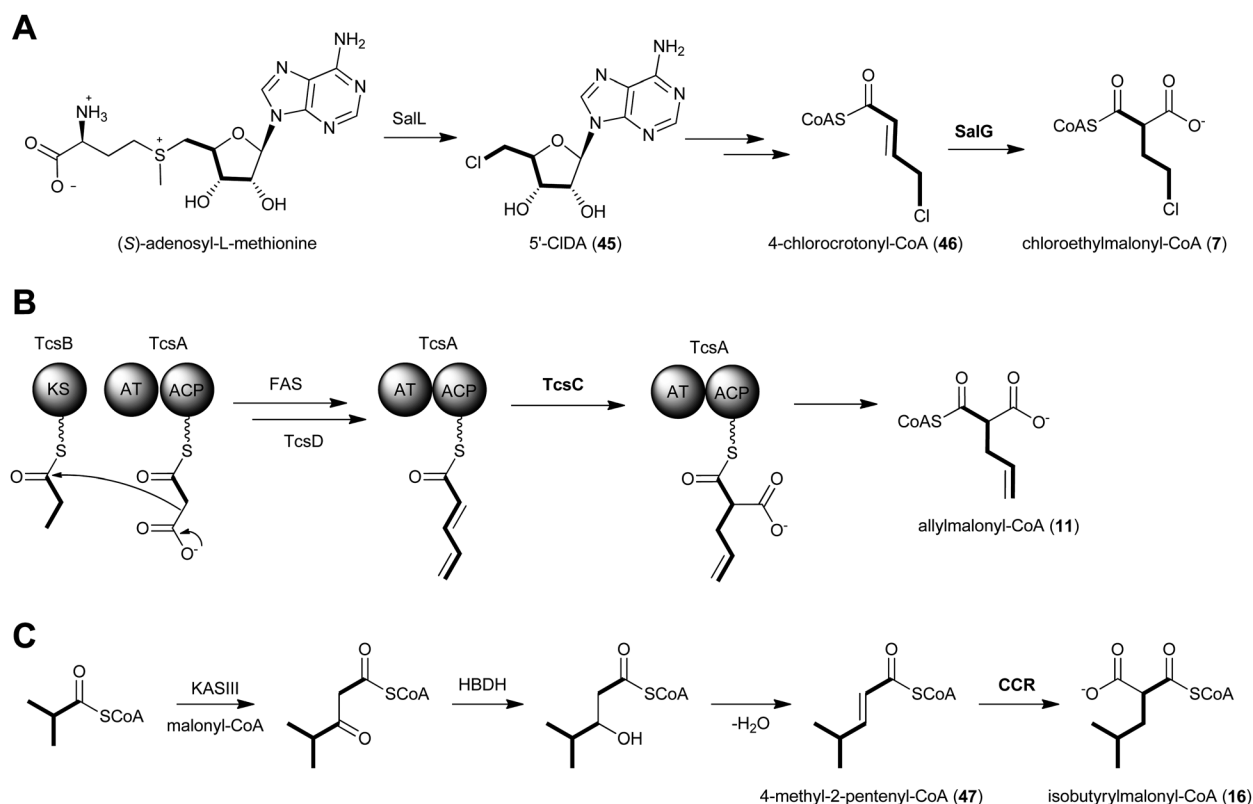
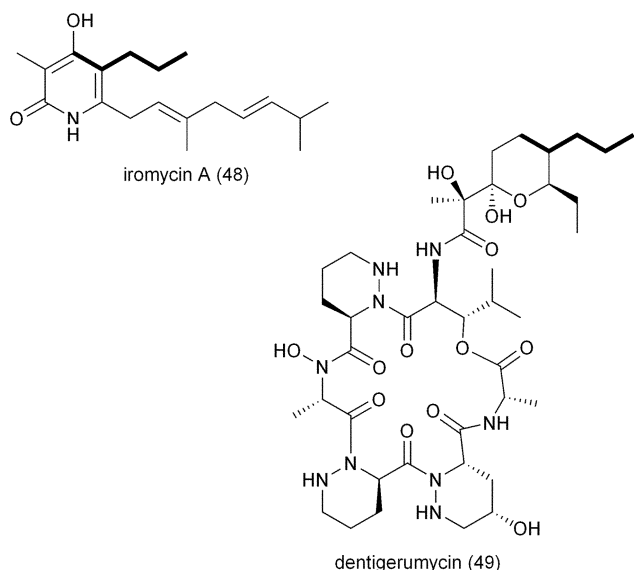


Fig. 5 Proposed biosynthetic pathways for the dedicated PKS extender units A) chloroethylmalonyl-CoA (7), B) allylmalonyl-CoA (11) and C) isobutyrylmalonyl-CoA (16).

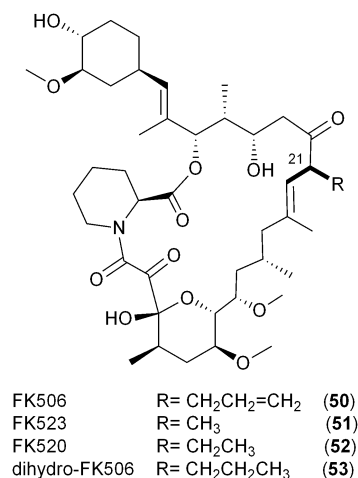
3.2 Propylmalonyl-CoA

The genetic inactivation of the *S. tropica* CCR *salG* gene also led to a second loss in salinosporamide production, that of salinosporamide E (**42**), which contains a C2 propyl group.⁶ This observation suggested that SalG had yet another biological activity as a *trans*-2-pentenyl-CoA reductase/carboxylase to give (2*S*)-propylmalonyl-CoA (**10**) and that the associated SalA PKS acyltransferase (AT₁) has broad substrate tolerance. Stable isotope experiments supported its biogenesis from acetate and propionate precursors, and biochemical interrogation of the SalG recombinant protein confirmed its enzymatic activity as having similar kinetics to that with **7**. While SalG has broadened substrate tolerance over the co-occurring and related primary CCR Strop_3612, attempts to accommodate longer chain 2-alkenyl-CoA substrates were unsuccessful,⁶ yet opened the door to the possibility of related CCRs in supplying alternative PKS extender units by this strategy. While it is rare for PKSs to incorporate pentyl building blocks into their polyketide products, a few other examples include the nitric oxide synthase inhibitor iromycin A (**48**),⁶³ the acyl depsipeptide dentigerumycin (**49**),⁶⁴ and the macrolide immunosuppressant dihydro-FK506 (**53**).⁶⁵



3.3 Allylmalonyl-CoA

Like the salinosporamides that owe their structural diversity and hence biological properties to PKS extender unit-derived chemistry, the FK506 family of immunosuppressant polyketides (**50–53**) also differs in substitution pattern at C21 of the macrolide as a result of broad PKS extender unit tolerance. In addition to the recently established allylmalonyl-CoA (**11**) unit that uniquely distinguishes FK506 (tacrolimus, **50**),^{56,66} derivatives can be substituted with methylmalonyl-CoA (FK523, **51**), ethylmalonyl-CoA (FK520, ascomycin, **52**) or propylmalonyl-CoA (dihydro-FK506, **53**).⁵⁶



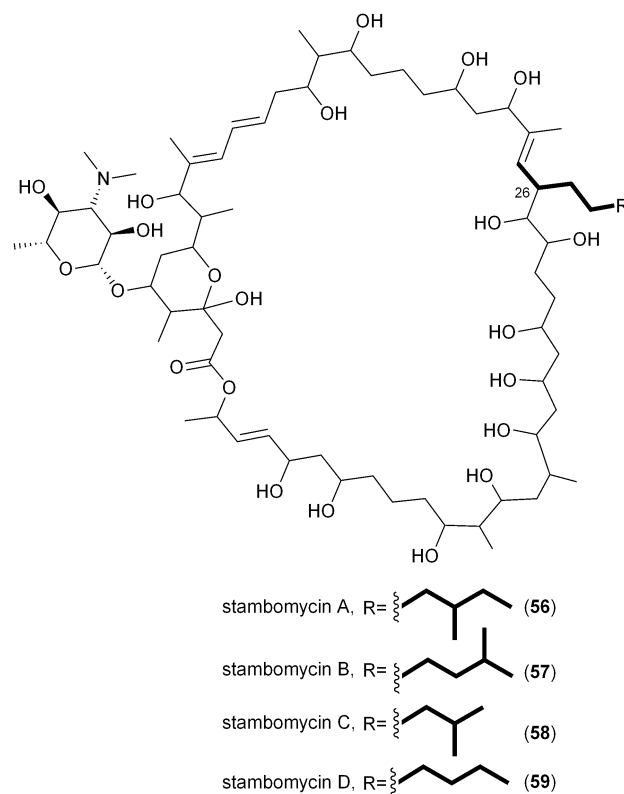
A partial biosynthetic gene cluster for FK506 was originally cloned and sequenced from *Streptomyces* sp. MA6548 but only contained the core PKS genes and two genes determined to be involved in post-PKS modification.⁶⁵ More than a decade later, two groups independently discovered the molecular and biochemical basis for the introduction of the allyl group in FK506 from the novel PKS extender unit allylmalonyl-CoA (**11**).^{56,66} Initially reported in 2010, Petkovic and coworkers identified 15 open reading frames located upstream of the FK506 PKS core biosynthetic genes in *S. tsukubaensis* NRRL 18488 that were not present in the previously sequenced FK520 biosynthetic gene cluster from *S. hygroscopicus* var. *ascomyceticus*.^{42,66} In addition to the CCR homolog, AllR, the so-called *all* subcluster encodes a diketide synthase system comprised of an AT-ACP didomain protein (AllA) and an unusual ketosynthase (KS) didomain (AllK). Deletion of the KS-containing gene *allK* established its essential role in FK506 biosynthesis and supported a dedicated precursor pathway to **11**. Further support for this pathway was achieved by chemical complementation with allylmalonyl-SNAC that restored FK506 production.⁶⁶ The allylmalonyl-CoA (**11**) biosynthetic pathway was independently established the following year by Yoon and coworkers in a comprehensive chemical, biochemical, and genetic study of three FK506 biosynthetic gene clusters.⁵⁶ In addition to identifying the four genes supporting allylmalonyl-CoA biosynthesis encoded in the *tcs* subcluster (synonymous to the *all* genes reported by Petkovic), their enzymatic functions were established through the *in vitro* reconstitution of the pathway (Fig. 5b). The TcsA/B (aka AllA/K) diketide synthase putatively gives rise to the β-ketopentyl-ACP_{TcsA} product that interacts with the endogenous fatty acid synthase system to give *trans*-2-pentenyl-ACP_{TcsA}. Reductive carboxylation by the CCR homolog TcsC (aka AllR) is next proposed with this ACP-bound substrate, which is a departure from previously characterized CCRs that rather function on CoA thioester-based molecules. The TcsC- and TcsD-catalyzed NADPH-dependent carboxylation and FAD-dependent dehydrogenation reactions, respectively, were monitored by ESI-MS and support the preference for ACP-based substrates in which carboxylation precedes the introduction of the double bond in the construction of allylmalonyl-ACP_{TcsA}. The authors suggest thioester migration to allylmalonyl-CoA (**11**) before acyltransfer by the allylmalonyl-specific AT₄ of the FK506 PKS.

While the origin of the five-carbon PKS extender unit derived from **11** and propylmalonyl-CoA (**10**) in the FK506 and dihydro-FK506 macrolides, respectively, originates from a dedicated diketide synthase pathway,^{56,66} an alternative pathway to **10** that may involve the β -oxidation of odd-chain fatty acids likely supports salinosporamide E (**42**) biosynthesis since the *alltcs* subcluster is absent in the *S. tropica* genome.⁶⁷ It thus remains to be seen which metabolic pathway predominates in the other pentyl-containing polyketide systems.

3.4 Hexylmalonyl-CoA and other straight chain extender units

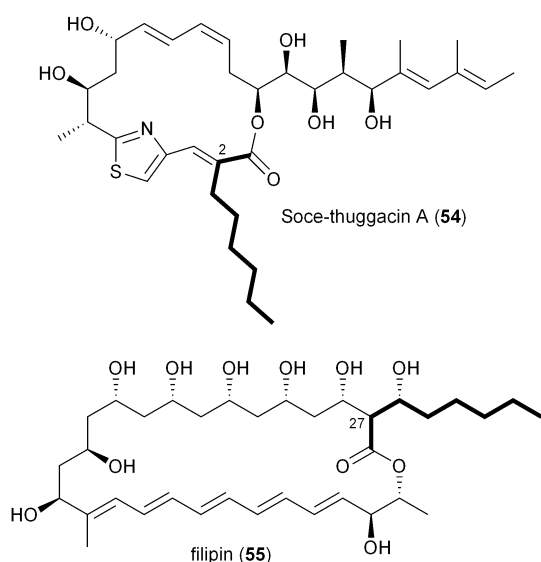
A number of polyketides, in particular cinnabaramide A (**44**),^{55,68} Soce-thuggacin A (**54**),⁶⁹ filipin (**55**),^{70,71} stambomycin D (**59**),⁷² and reveromycin E (**63**),^{73,74} contain hexyl side chains suggestive of the PKS extender unit hexylmalonyl-CoA (**12**). Its biosynthesis was first proposed in 2010 by Müller and coworkers through comparative analysis of the biosynthetic gene clusters for the thuggacin macrolide antibiotics from two myxobacteria, *Sorangium cellulosum* So ce895 and *Chondromyces crocatus* Cm c5.⁷⁵ The most notable difference between the products of these highly analogous pathways involves the final PKS extension in which Cmc-thuggacin A from *C. crocatus* incorporates a standard propionate unit derived from methylmalonyl-CoA, while Soce-thuggacin A (**54**) from *S. cellulosum* integrates an unusual octanoyl unit⁶⁹ presumably derived from **12**. The *S. cellulosum* thuggacin biosynthetic gene cluster uniquely contained the CCR homologous gene *tgaD* that could putatively be involved in **12** formation *via* the reductive carboxylation of 2-octenoyl-CoA.⁷⁵ As predicted, inactivation of *tgaD* yielded a **54**-negative mutant supporting the central role of TgaD in its construction. Unlike the previous examples involving the salinosporamide and FK506 systems in which the primary metabolites methyl- and ethylmalonyl-CoA could chemically complement the pathway specific CCR mutant to give natural product analogs, no such recovery was observed in the thuggacin system suggesting that the gatekeeper AT₁₁ domain is specific toward the longer chain hexylmalonate, which is consistent with the absence of natural C2 variants in *S. cellulosum*. However, due to TgaD's relatively low

similarity to characterized CCR homologs, a more complete biochemical characterization is needed to confirm its anticipated function and to determine whether it operates with CoA- or ACP-linked substrates.

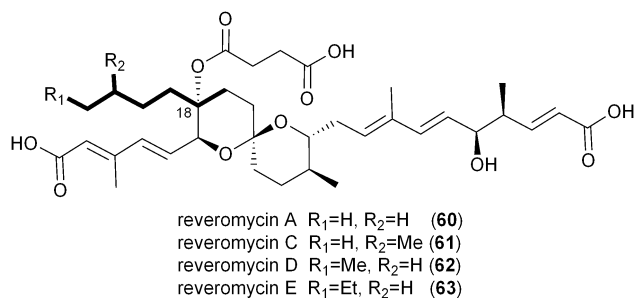


A second example of hexylmalonyl-CoA (**12**) as a PKS extender unit involves cinnabaramide A (**44**) from the terrestrial *Streptomyces* species strain JS360 that belongs to the salinosporamide family of proteasome inhibitors.⁶⁸ The cinnabaramides are distinguished from the salinosporamides by the putative incorporation of **12** by AT₂ of the cinnabaramide PKS, CinA. As proposed in Soce-thuggacin A (**54**) biosynthesis, **12** is similarly derived from the reductive carboxylation of 2-octenoyl-CoA by a pathway specific CCR, which in this case is encoded by *cinF*. Interestingly, the sequence similarity between CinF and TgaG is very low considering that they are predicted to catalyze the same reaction and may rather reflect their different taxonomic origins. As with the thuggacins, gene inactivation of *cinF* generated a mutant incapable of producing the cinnabaramides, prompting Müller and coworkers to conclude that CinF is responsible for **12** production in *Streptomyces* sp. JS360.⁵⁵

While the Soce-thuggacins and cinnabaramides strictly maintain hexyl side chains in their polyketide structures, the reveromycin family (**60–63**) of osteoclast-targeting polyketides contains four natural C18 variants suggestive of a mixture of alkylmalonyl-CoA precursors (**12–15**).^{73,76,77} Though the current biosynthetic literature by Osada and coworkers is focused on reveromycin's novel spiroketal formation,⁵⁸ an equally fascinating feature involves the incorporation of four rare or unique PKS extender units in a single position on their structures. The reveromycins putatively incorporate butyryl- (**14**), pentyl- (**13**), isopentyl- (**15**), and hexylmalonyl-CoA (**12**) precursors as the



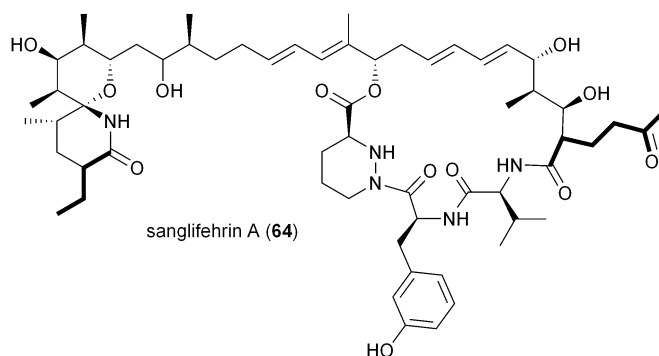
fourth extender unit of their polyketide backbone. As expected based on the pathways described thus far, the reveromycin biosynthetic gene cluster contains a CCR homolog (*revT*) with potentially promiscuous substrate specificity.



The first *in vitro* characterization of a 2-octenoyl-CoA carboxylase/reductase was reported in 2011 by Kwon and coworkers with the CCR homolog PteB from *Streptomyces avermitilis*.⁷⁰ The *pteB* gene is located within the biosynthetic gene cluster of filipin (**55**),³⁸ an antifungal polyene macrolide originally isolated in 1955 that incorporates an octanoate unit during the final PKS extension.^{70,71} Recombinant PteB had dual activity, reducing 2-octenoyl-CoA to octanoyl-CoA in the absence of bicarbonate, and in its presence, reductively carboxylating it to **12**. PteB exhibited broad substrate tolerance toward crotonyl-, 2-hexenoyl-, 2-octenoyl-, and 2-decenoyl-CoAs, consistent with structure analogs, such as chainin,⁷⁸ strevertene,⁷⁹ and takanawaene,⁸⁰ from other actinomycetes that differ in the nature of the final extender unit. Though gene clusters for the **55** analogs have not been reported, their conserved structures suggest a common biosynthetic pathway with modified substrate specificity of a CCR and associated gatekeeper AT domain to account for differing side-chains at C27.

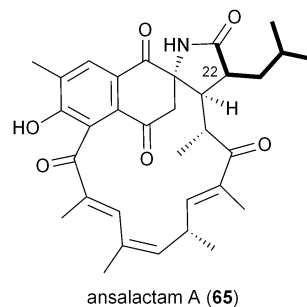
In a departure from the CCR-based biosynthesis of **12**, *Streptomyces ambifaciens* appears to generate its dedicated alkylmalonyl-CoA substrates for stambomycin assembly by an alternate route.⁷² DNA sequence analysis of the 150 kb stambomycin biosynthetic gene cluster failed to identify a pathway specific CCR homolog; instead Laureti *et al.* observed the gene *samR0483* that encodes a biotin-dependent carboxylase related to propionyl-CoA carboxylase that may function to directly carboxylate saturated octanoyl-CoA and derivatives to ultimately provide stambomycin D (**59**) and other C26 structure analogs.

The biosynthesis of the potent cyclophilin inhibitor sanglifehrin A (**64**) was recently established through the sequence analysis of its biosynthetic machinery in *Streptomyces flaveolus* to reveal an intriguing possibility of the oxidized PKS extender unit 2-oxobutylmalonyl-CoA (**17**).⁸¹ Liu and colleagues suggest its biosynthesis from the dedicated iterative type I PKS SfaK to make a putative unsaturated triacetate intermediate for reductive carboxylation to 2-oxobutylmalonyl-CoA; however, the authors have not identified a specific CCR for this reaction as found in the allylmalonyl-CoA (**11**) pathway that also involves a separate PKS for its construction. While the sanglifehrin biosynthesis gene cluster does indeed harbor a CCR gene, *sfaR*, it rather functions as a *bone fide* CCR responsible for **3** biosynthesis linked to the formation of the proposed PKS primer (2*R*)-ethylmalonamyl-CoA.



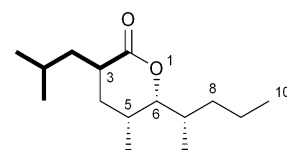
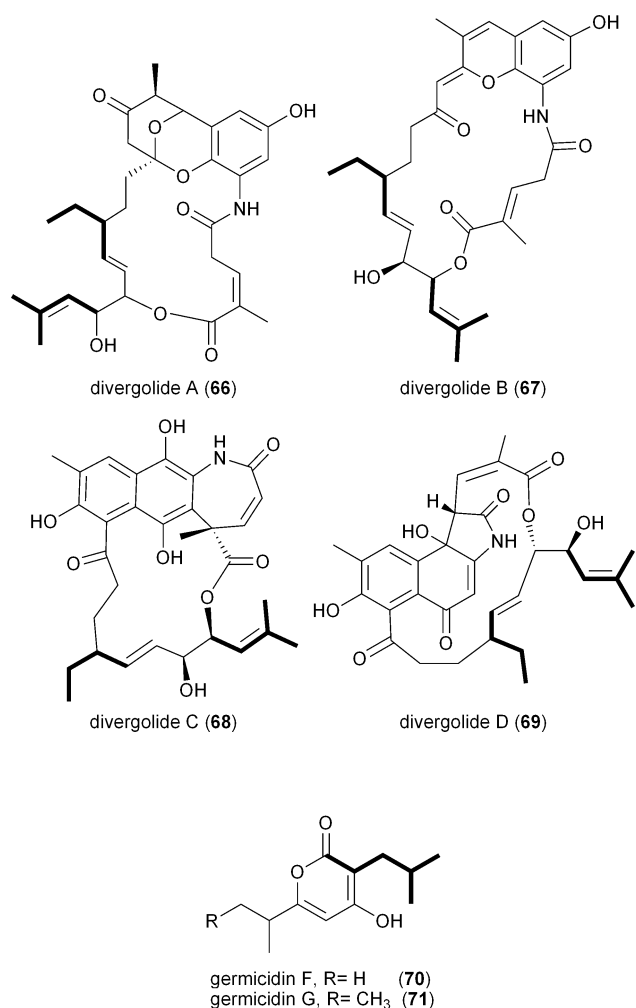
3.5 Isobutyrylmalonyl-CoA and other branched chain extender units

A series of polyketides independently isolated and characterized in early 2011 by the Moore and Hertweck groups suggested the involvement of the novel branched extender unit isobutyrylmalonyl-CoA (**16**) and its synthesis *via* a CCR mechanism. The discovery of the ansa macrolide natural product ansalactam A (**65**) from marine-derived *Streptomyces* sp. CNH-189 suggested an unusual branched side chain at C22 that was proposed to originate from the reductive carboxylation of 4-methyl-2-pentenyl-CoA (**47**).⁸² Moore and coworkers explored its biogenesis with stable isotopes and ruled out a leucine origin of this moiety that rather is derived from the condensation of isobutyrate and acetate to 4-methyl-2-pentenoate.



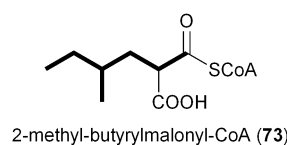
Hertweck and coworkers similarly isolated a novel group of ansa macrolide antibiotics, divergolides A–D (**66–69**), from the mangrove endophyte *Streptomyces* sp. HKI0576 that also contain the unsaturated version of the isobutyryl polyketide side chain.⁸³ The polyketide backbone that contains this branched substituent is disrupted with an oxygen atom that could originate *via* a Baeyer–Villiger-type oxidation reaction. Precursor experiments with D₇-isobutyrate not only labeled **66–69** as anticipated but also identified two new germicidin derivatives, F (**70**) and G (**71**), originating from this branched building block.⁴³ Shotgun sequence analysis provided the divergolide biosynthetic gene cluster and illuminated the molecular basis for isobutyrylmalonyl-CoA (**16**) biosynthesis (Fig. 5c). Two copies of CCR-encoding genes were identified in the type I PKS gene cluster, one (*DivD*) suggested for ethylmalonyl-CoA (**3**) biosynthesis and the second (*DivR*) for isobutyrylmalonyl-CoA (**16**) assembly. The *divR* gene resides in a three-gene cassette that also includes the FabH-like KS *divS* and the 3-hydroxybutyryl-CoA dehydrogenase *divT* that together encodes **16** synthesis

(Fig. 5c) as supported by its heterologous expression with the germicidin type III PKS in *Streptomyces albus* to give **70** and **71**.

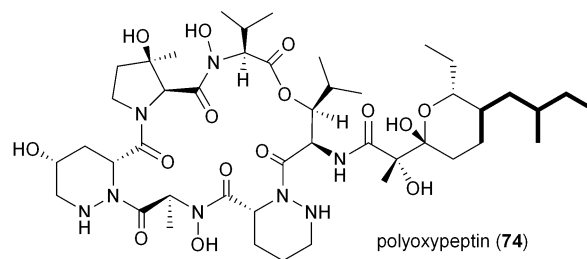


3-demethyl-3-isobuteryl-3,6,7-tri-epi-invictolide (**72**)

Inspection of publicly available genomic information suggests that the isobutyrylmalonyl-CoA (**16**) gene cassette resides in a number of orphan biosynthetic systems, suggesting a greater frequency of this newly described PKS extender unit in actinomycetes (Fig. 6). Related branched-chain extender units, such as the proposed 2-methyl-butrylmalonyl-CoA (**73**) and isopentylmalonyl-CoA (**15**), may be synthesized in a similar FabH-based fashion to give polyoxypeptin A (**74**)⁸⁵ and reveromycin C (**61**),⁵⁸ respectively. In the case of **61**, a FabH-like KS, *revR*, and CoA-ligase, *revS*, are located directly upstream of the reveromycin CCR, *revT*. Similar to **65–72**, this three gene cassette is proposed to function in the biosynthesis of the unusual extender units of the reveromycins (**12–15**).⁵⁸



2-methyl-butrylmalonyl-CoA (**73**)



polyoxypeptin (**74**)

Recently two new analogs related to the queen recognition pheromone invictolide were described by Sato and coworkers from a marine-derived actinomycete most closely related to *Nocardiopsis tangguensis*.⁸⁴ The tetrapropionate δ -lactone 3,6,7-tri-epi-invictolide differs from the analog **72** in the fourth and final ketide residue that hypothetically derives instead from **16**.

4 Engineering new polyketides utilizing pathway specific CCR homologs

In the majority of cases involving CCR-based PKS extender units, the associated acyltransferase (AT) domain displays relaxed specificity toward its selection of malonate substrates.

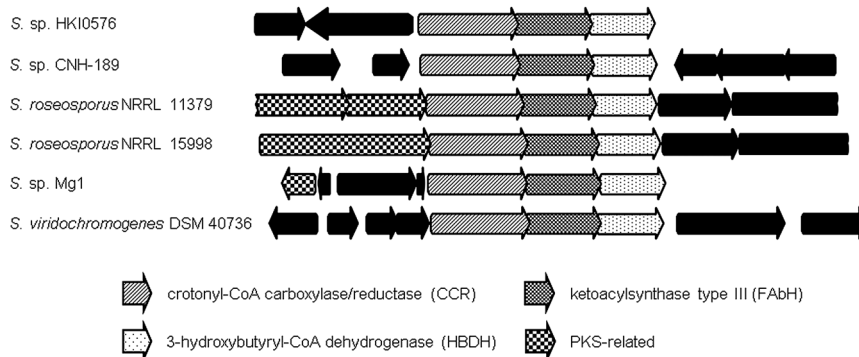


Fig. 6 Orthologous gene neighborhoods to the isobutyrylmalonyl-CoA (**16**) pathway of the divergolide (**66–69**) producer, *Streptomyces* sp. HKI0576, putatively involved in the biosynthesis of branched PKS extender units.

This observation is evident from the number of derivatives of the salinosporamide (37–43), FK506 (50–53), monensin (20–21), reveromycin (60–63), germicidin (71–72), and thuggacin (54) natural product series. Previous phylogenetic analyses of AT domains suggest that ATs with the same substrate specificity

share a common evolutionary origin with malonyl- and methylmalonyl-specific ATs clustering into distinct clades (Fig. 7).^{86,87} The less common ethylmalonyl-specific AT, however, does not form a distinct clade but rather resides in subclades within the methylmalonyl group, which is suggestive

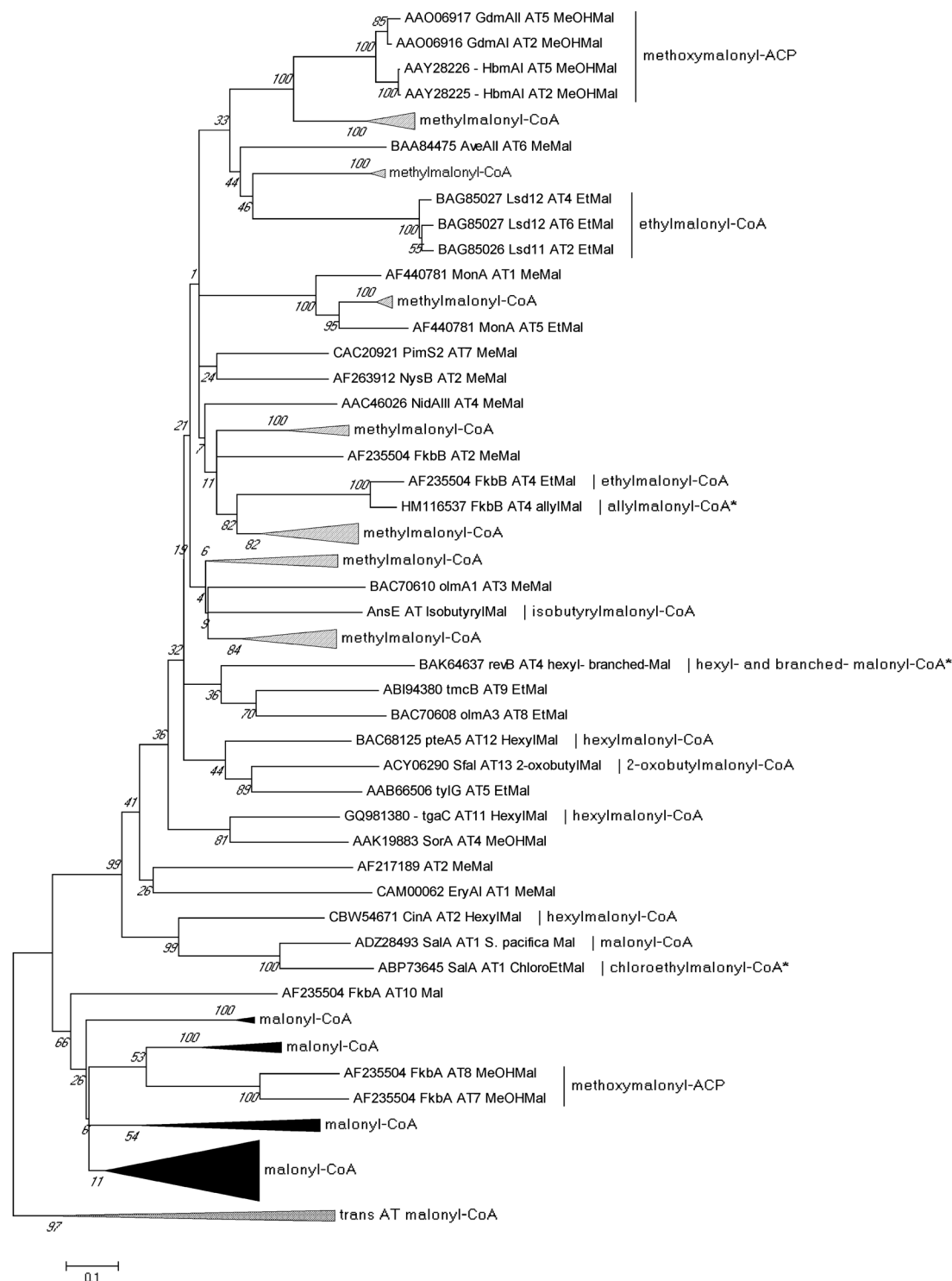


Fig. 7 Phylogenetic analysis of 130 acyltransferase (AT) domains from characterized and putative PKS proteins. The evolutionary history was inferred using the Neighbor-Joining method, and the bootstrap consensus tree was generated from 1000 replicates. Evolutionary analyses were conducted in MEGA5.⁹⁴ Asterisks (*) indicate that AT domains accept multiple extender units.

that this group may have evolved more than once. Examination of the haloethylmalonyl-preferred SalA AT₁⁷ and the allylmalonyl-preferred FkbB AT₄⁸⁸ associated with the CCR homologs SalG and TcsC, respectively, similarly placed them in the methylmalonyl clade. A more thorough phylogenetic analysis of the AT domains associated with the CCR-derived PKS substrates highlighted in this review article is shown in Fig. 7. Similar to AT domains that accept ethylmalonyl-CoA (3), our analysis shows that AT domains specific for less common CCR-derived extender units also reside in subclades within the methylmalonyl group. Moreover, AT domains specific for the same atypical extender unit may have also evolved separately. This is perhaps best exemplified by the AT domains specific for hexylmalonyl-CoA in the cinnabaramide, thuggacin, and filipin pathways (Fig. 7).

Given the apparent relaxed natural specificity of ATs associated with CCR-derived substrates, several investigators exploited this enzymatic promiscuity to selectively bioengineer novel structure analogs *via* mutasynthesis and genetic engineering methods (Fig. 8). Moore and coworkers first expanded

salinosporamide biosynthesis to give the unnatural derivative fluorosalinosporamide (39), a very potent “reversible” proteasome inhibitor, through mutasynthesis with the *salL* chlorinase knockout mutant supplemented with 5'-fluoro-5'-deoxyadenosine.⁸⁹ By complementing the mutant with simple 4-substituted crotonates, they later could rescue the natural biosynthesis of salinosporamides A (37), B (40), E (42), and bromosalinosporamide (38) as well as create the unnatural 39.⁶ Fluorosalinosporamide (39) was also genetically engineered by replacing the *salL* chlorinase gene with the *flA* fluorinase gene from *Streptomyces cattleya*, representing the first engineered biosynthetic experiment to generate a fluoroorganic molecule from inorganic fluoride.⁹⁰

Relaxed substrate specificity was also observed in the FK506 and cinnabaramide series. The genetic disruption of the *tcsB* KS gene prevented *de novo* allylmalonyl-CoA biosynthesis and allowed for the mutasynthesis of FK506 analogs derived from a series of carboxylic acids.⁵⁶ Specifically, *trans*-2-hexenoic acid (76), 4-fluorocrotonic acid (75), and 4-methylpentanoic acid (77) provided 36,37-dihydro-37-methyl-FK506 (78),

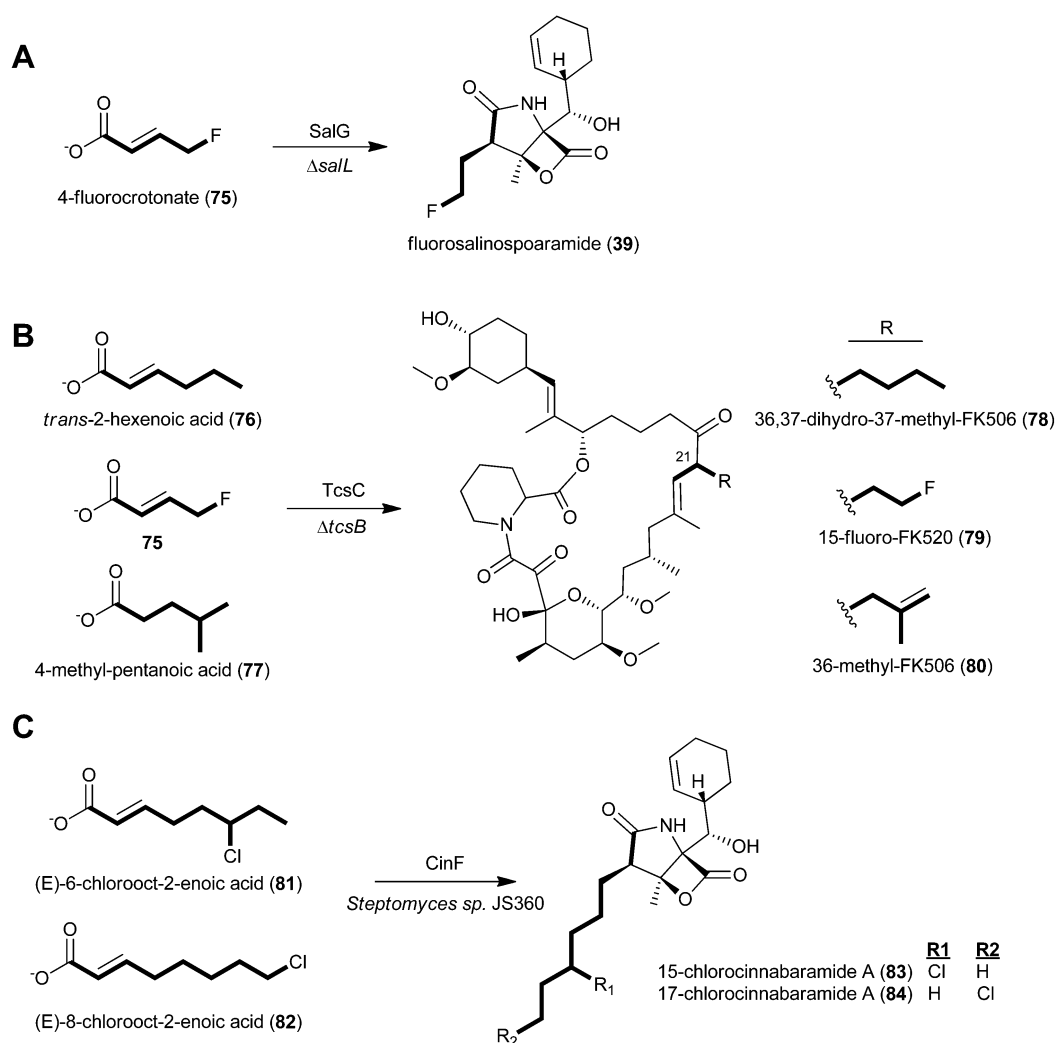


Fig. 8 Engineering of natural product analogs through chemical complementation of A) the *S. tropica* chlorinase (*salL*) deficient mutant to generate fluorosalinosporamide (39), B) the *Streptomyces* sp. KCTC 11604BP *tcsB* mutant to generate three unnatural FK506 analogs (78–80), and C) the wild-type cinnabaramide producer, *Streptomyces* sp. JS360, to generate chlorinated cinnabaramide analogs (83–84).

15-fluoro-FK520 (**79**), and 36-methyl-FK506 (**80**), respectively. The latter molecule, **80**, is noteworthy in two regards: first, the unnatural precursor was presumably modified by the TcsD dehydrogenase to introduce the double bond, as in FK506 (**50**), and second, **80** showed improved neurite outgrowth activity. With the discovery of fluoroethylmalonyl-CoA (**9**)⁹⁰ and isobutyrylmalonyl-CoA (**16**)⁸³ biosynthetic pathways, the genetic engineering of **79** and **80**, respectively, is now feasible. In the case of cinnabaramide diversification, Müller and coworkers took a precursor-directed biosynthesis approach with the wild-type strain since the background synthesis of the proposed natural substrate 2-octenoyl-CoA furnished from fatty acid metabolism could not be eliminated.⁵⁵ Administration of (*E*)-6-chlorooct-2-enoic acid (**81**), (*E*)-8-chlorooct-2-enoic acid (**22**), and their corresponding *N*-acetylcysteamine thioesters resulted in new cinnabaramide derivatives with 15- and 17-chloro groups (**83–84**) that on average exhibited superior bioactivity over the parent cinnabaramide.

5 Outlook

The 2007 functional revision of *R. sphaeroides* CCR as a reductive carboxylase¹¹ followed by the 2009 discovery of the *S. tropica* CCR homolog SalG as having alternate substrate specificity^{6,7} exposed a new strategy in PKS extender unit diversification from α,β -unsaturated acyl thioesters. Several CCR-mediated pathways to linear and branched-chain (halo)alkylmalonyl-CoA molecules have since been uncovered, making these related pathways, although rare, the most functionally accommodating in providing diverse PKS substrates⁵ (Fig. 2). To date, all characterized pathways originate from actinomycetes⁹¹ and myxobacteria,⁹² arguably two of the most metabolically rich groups of prokaryotes. Additional pathways are undoubtedly awaiting discovery not just from these bacterial groups, but also from others based on genomic scanning of publicly available sequences. For instance, CCRs are widespread amongst the proteobacteria and are also present in the genomes of firmicutes and spirochaetes. Even more intriguing is that the genome of the firmicute *Ruminococcus* sp. 18P13 contains a CCR (CBL16354) that is located directly upstream of a FabH-like ketoacylsynthase and adjacent to genes predicted to be involved in polyketide and non-ribosomal peptide assembly. To the best of our knowledge, no secondary metabolite has been associated with these biosynthetic genes.

The polyketide engineering potential has only been scratched with the successful analoging in the salinosporamide,⁶ cinnabaramide,⁵⁵ and FK506⁵⁶ series in which the broad natural substrate tolerance of native CCRs and associated gatekeeper ATs accommodated modest changes in precursor structure. Whether more ambitious modifications in substrate or in product design are biochemically feasible with rationally engineered biosynthetic systems is to be seen. Early AT engineering in the all-methylmalonate erythromycin PKS allowed for the successful swap of an ethylmalonate unit,⁹³ thereby illuminating the concept of extender unit remodeling. To address the renewed engineering potential with alkylmalonyl-CoAs will require a firmer understanding of the structural and mechanistic basis of substrate selectivity and binding in CCR homologs and in alkylmalonyl-specific ATs. Based on the variety of substrates

accommodated by CCR and AT homologs, structure-based enzyme engineering is anticipated to provide active site changes that correlate to substrate selectivity. Its application toward designer polyketide molecules is significant since the longer acyl chains in CCR-derived PKS extender units provide greater structural design flexibility as seen in the reactive chemical handles of the haloethylmalonyl and allylmalonyl series that engender key biological features to their polyketide products. Precursor supply of the α,β -unsaturated acyl thioester substrates will also have to be addressed as the pathways so far elucidated show that these molecules come from diverse metabolic origins involving dedicated, as well as general, pathways. Hence the outlook for the further diversification of the ethylmalonyl-CoA pathway in polyketide biosynthesis and bioengineering is promising with new insight likely right around the corner.

6 Acknowledgements

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