3-Hydroxy-3-Methylglutaryl-CoA-Like Synthases Direct the Formation of Methyl and Ethyl Side Groups in the Biosynthesis of the Antibiotic Myxovirescin A

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Polyketides (PK), nonribosomal peptides (NRP) and their hybrids (PK/NRP) are classes of secondary metabolites whose members exhibit valuable activities with high potential for therapeutic applications. The program governing PK/NRP assembly is typically encoded within modular megasynthetases on which consecutive condensations of activated short-chain carboxylic acids (catalyzed by polyketide synthases; PKS) or amino acids (performed by nonribosomal peptide synthetases; NRPS) take place through a thiotemplated mechanism.[1]

Annotations of several PKS/NRPS gene clusters, including bacillaene, curacin and mupirocin,[2–8] indicated that a novel type of Claisen condensation mechanism is used in such natural product biosynthesis. Strikingly, all six biosynthetic gene clusters revealed (a) free-standing homologue(s) of 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS), generally surrounded by a set of genes encoding an acyl carrier protein (ACP), a mutant KS with a Cys-to-Ser active site substitution (KS^S) and two homologues of the enoyl-CoA hydratase (ECH) family.[4,7] HMG-CoA synthases are known to couple acetyl-CoA (Ac-CoA) onto the β-keto group of acetoacetyl-CoA to yield 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA).[9] It was therefore proposed that the above gene products condense Ac-CoA with structurally similar β-ketoacyl-S-ACP intermediates of polyketide origin to eventually result in the addition of the C2 acetate carbon in their respective structures.[2,4–6] This hypothetical scheme has been recently revised following in vitro reconstitution of the bacillaene enzyme homologues of *B. subtilis*[7,8] (cf. Scheme 1C). In comparison with other gene clusters,[4,7] the myxovirescin cluster contains two free-standing ACPs (TaB and TaE) and two HMG synthases (TaC and TaF; Scheme 1A). Curiously, the myxovirescin-labelling scheme indicates two atypical pendant groups branching from the basic polyketide backbone, the methylene group derived from the C2 portion of acetate attached to carbon C12, and the C2–C3 fragment derived from succinyl-CoA (Succ-CoA) at carbon C16[10] (Scheme 1B). Accordingly, the proposed biosynthetic scheme for myxovirescin assembly envisions two HMGS-like catalyzed reactions that would "intersect" the classical PK biochemistry by modifying

ACP-bound intermediates **2** and **5** (Scheme 1C). After each round of reactions, polyketide biosynthesis would be resumed and carried out by multimodular PKSs. The first one of these reactions is postulated to be carried out by the HMG-like synthase TaC by condensation of Ac-S-TaB with the C12 β-keto group of substrate **2** to yield **3** (Scheme 1C). In the second reaction, HMG-like synthase TaF should catalyze the condensation of propionyl-S-TaE (Pp-S-TaE) with the C16 β-keto group of intermediate **5** to yield **6**. In analogy to the KS^S PksG of *B. subtilis*,[7] KS^S TaK is thought to act twice during the biosynthesis by cleaving carboxyl groups from both malonyl-S-TaB (M-S-TaB) and methylmalonyl-S-TaE (Mm-S-TaE) in order to supply TaC and TaF, respectively, with their cognate substrates. The Mm-CoA is presumably a product of the Mm-CoA mutase-catalyzed conversion of Succ-CoA.[10] Similarly, based on in vitro data,[7,11] two enoyl-CoA hydratases TaX and TaY dehydrate and decarboxylate intermediates **3** and **6** to yield **4** and **7**, respectively (Scheme 1C).

Even though PksG, the TaC homologue of *B. subtilis*, has been shown to catalyze the formation of HMG-S-ACP in vitro,[7] there has been no in vivo proof for the function of any HMGS in PKS/NRPS biosynthesis. Such evidence is especially needed given that, in most systems, HMGS homologues are predicted to form much more complex products than HMG-S-ACP.[7] For instance, TaC and TaF are predicted to act on carbons chains 15 and 19 atoms long while tethered to the ACPs of the giant 995-kDa PKS Ta-1, and to utilize atypical substrates, such as Pp-S-ACP, in addition to Ac-S-ACP.[6]

In an effort to provide such in vivo evidence, we have attempted to delete the HMGS homologues (*taC* and *taF*) and to analyze the engineered strains for the possible modifications these mutations would impose on the antibiotic structure. Consistent with the proposed working model, we envisioned three possible effects these deletions would have on myxovirescin biosynthesis. 1) The loss of TaC or TaF would not halt the myxovirescin biosynthetic machinery but only prevent condensation of the Ac (Pp) unit with the PKS/NRPS scaffold, therefore leading to the production of myxovirescin analogues carrying a ketone in the place of a methoxymethyl or ethyl group at the C12 and C16 positions, respectively (cf. Scheme 1B). In the case of loss of TaC, we assumed that this modification would also disrupt the C12–C14 diene system. 2) Antibiotic production would be blocked either by jamming of the reaction intermediates on the assembly line, or disruption of the megasynthetase complex. 3) Antibiotic production would be circumvented by complementation of function supplied by the other HMG-like synthase encoded in the gene cluster. Hence, in the case of loss of TaC, TaF would rescue antibiotic assembly by installing a Pp unit on both the C12 and C16 β-keto positions of intermediates **2** and **5** to produce myxovirescin analogues with two ethyl groups at respective positions. The ethyl group at C12 might be further modified into a hydroxyethyl or methoxyethyl group. Conversely, in the case of loss of TaF, TaC would attach two Ac groups onto intermediates **2** and **5**. After additional modification reactions on the C12 methyl carbon, a novel analogue of **1** carrying a methyl instead of an ethyl group at C16 would be formed.

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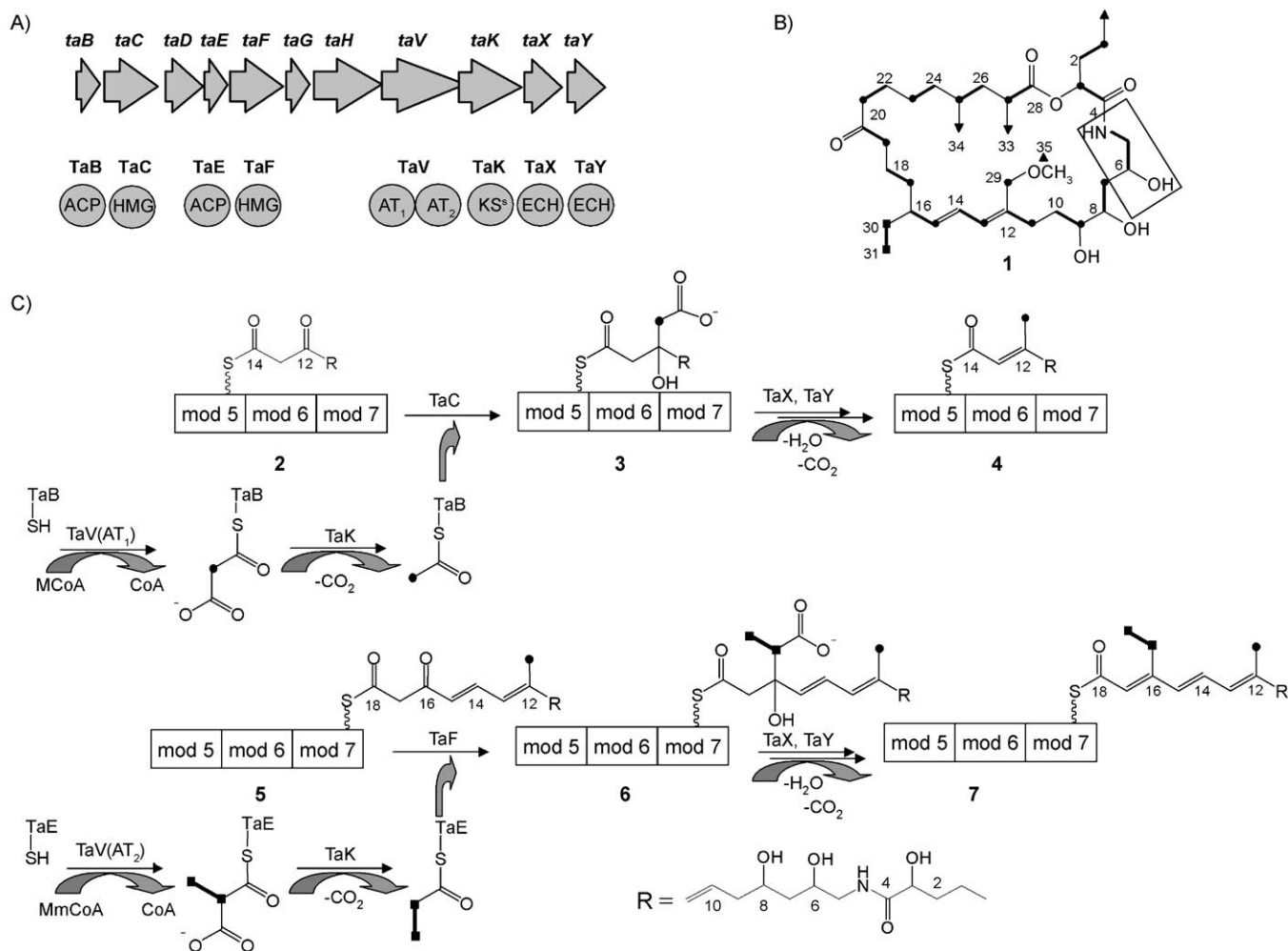
Scheme 1. A) 10.9-kb fragment of the myxovirescin A biosynthetic gene cluster encoding for monofunctional enzymes. TaB and TaE are putative ACPs, TaC and TaF homologues of HMG-CoA synthases, TaK is a mutant β-ketoacyl-ACP synthase (KS^S), and TaX and TaY are homologues of the crotonase family of enoyl-CoA hydratases. B) Structure of myxovirescin A (**1**) indicating the biosynthetic origin of its building units.[10] Boxed carbons originate from glycine, black circles indicate C2 of acetate, triangles indicate methyl groups derived from methionine, and connected squares show the ethyl group originating from carbons 2 and 3 of succinate. C) A working model of myxovirescin A assembly, based on refs. [6,7,11], depicts two rounds of modification reactions involving HMGS-like and ECH enzyme homologues taking place on the polyketide/nonribosomal peptide intermediates **2**, **3**, **5** and **6**. Two ATs encoded by *taV* load malonyl-CoA (M-CoA) and methylmalonyl-CoA (Mm-CoA) onto their cognate ACPs (TaB and TaE), which become substrates of the decarboxylase TaK. Acetyl-S-TaB and propionyl-S-TaE serve as second substrates for TaC and TaF HMGS-like synthases, respectively. The carbon-labelling pattern is described in (B). The C16=C17 double bond of intermediate **7** is presumably reduced by TaO PKS.

Following construction of two HMGS-like mutant strains, *M. xanthus* VS1011 (ΔtaC) and VS1012 (ΔtaF), the wild-type and mutant extracts were analyzed by HPLC-MS. Production of **1** was abolished in both VS1011 and VS1012 (data not shown). Masses of the predicted modified products could not be detected with a ΔtaC background. However, a unique, novel peak of $[M+H]^+ = 610$ with shorter retention time than **1** was apparent in the VS1012 extracts.

Evidence that the novel metabolite is a myxovirescin analogue was provided by tandem MS analysis. In addition, the UV$_{max}$ absorption at 239 nm of the novel myxovirescin ΔF suggested the same chromophore as in **1**. However, in comparison to the production of **1** by the wild-type strain, production of myxovirescin ΔF in VS1012 was reduced tenfold.

The high-resolution mass spectrum (HRMS) of myxovirescin ΔF (*m/z* 610.4287) suggested the molecular formula $C_{34}H_{60}NO_8^+$, and thus indicated the loss of one methylene group relative to **1**. In order to find out at which carbon position this modification occurred, myxovirescin ΔF was purified and subjected to NMR analysis (see the Supporting Information). Comparative analysis of ^{1}H NMR spectra of myxovirescins A and ΔF indicated changes specific to the aliphatic region (Figure 1A). The spectrum of myxovirescin ΔF lacked a triplet at $\delta_H = 0.86$ ppm assigned to the C31 methyl group of **1**. Instead, a new methyl group was visible as a doublet at $\delta_H = 1.00$ ppm ($J = 7$ Hz). ^{1}H,^{1}H COSY and HMBC analysis showed this new methyl group to be connected to the C16 methine group ($\delta_C = 38.3$ ppm, $\delta_H = 2.19$ ppm); this led to the assignment **8** (Figure 1B). Therefore, NMR analysis provided unambiguous evidence for the presence of a methyl, instead of an ethyl group, at C16 of **8**.
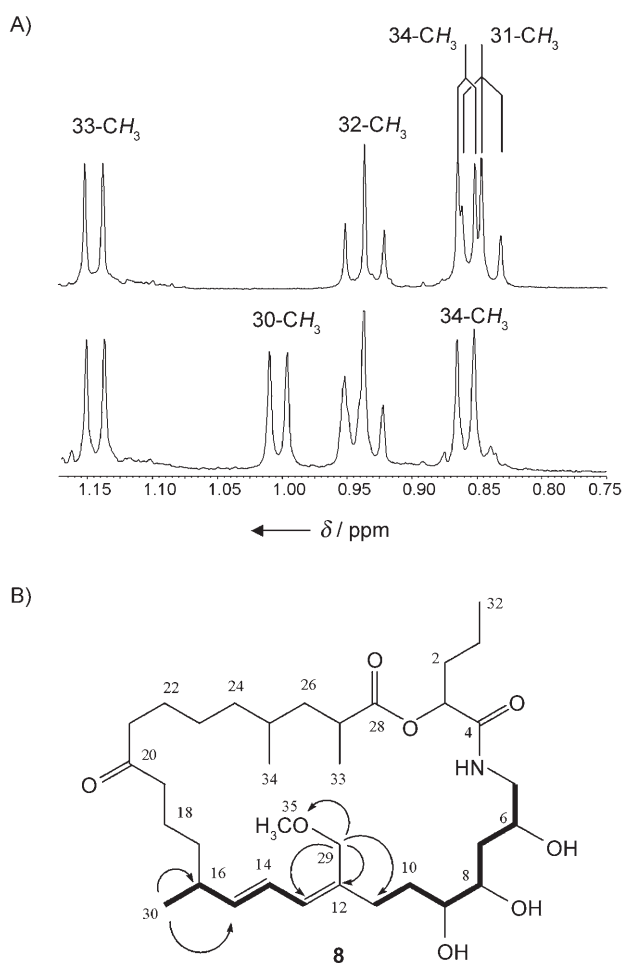
A)

B)

Figure 1. A) ^{1}H NMR spectral expansions of the aliphatic regions of myxovirescin A (upper) and the novel myxovirescin analogue ΔF (lower). B) The NMR-deduced structure of myxovirescin ΔF (**8**) reveals the presence of a methyl rather than ethyl group at C16. Bold lines indicate selected ^{1}H,^{1}H COSY and arrows show important HMBC correlations.

To evaluate whether another HMGS, besides TaC, could be directing production of **8**, the *M. xanthus* DK1622 genome was screened for genes encoding HMGS homologues. This search revealed only one candidate, MvaS (*E* value $= E^{-48}$), the HMGS of the mevalonate pathway.[12] Unlike in some other bacteria, disruption of *mvaS* in *M. xanthus* does not cause any growth defects, due to the efficient incorporation of leucine, as alternative precursor to HMG-CoA, and subsequently isoprenoids by a second pathway.[12, 13] Similarly, analysis of the Δ*taF* and the *mvaS*::*neo*, Δ*taF* double mutant (strain VS1044) extracts showed equal production of **8** (data not shown).

Isotope-labelling data for **1** (Scheme 1B) demonstrate that both C30 and C31 (the ethyl group at C16) originate from Mm-CoA. Therefore, the appearance of a methyl group at C16 of **8** suggests that, in the absence of TaF, the megasynthetase fails to incorporate Mm-CoA. The absence of the ethyl moiety in **8** is also supported by the observation that addition of vitamin B_{12} to VS1012 fermentations does not stimulate the production of **8**; this is in contrast to the observed two- to three-fold increase it causes in the production of **1** by the wild-type

strain DK1622 (data not shown). This argues for a role of vitamin B_{12} in the production of Mm-CoA, which takes place through a vitamin B_{12}-dependent Mm-CoA mutase-catalyzed conversion of Succ-CoA into Mm-CoA. Indeed, evidence that TaF is directly involved in the formation of the ethyl group at C16 is also supported by the complete loss of production of **1** and appearance of **8** in the Δ*taF* background.

Furthermore, the tenfold reduction in the production of **8** in comparison to **1** indicates severe impairment of the metabolic flux through the megasynthetase upon removal of TaF, with only 2 mg of **8** being produced from 45 L of VS1012 culture (Supporting Information). In contrast, elimination of myxovirescin accessory enzymes, such as the methyltransferase TaQ,[6] and one of the oxygenating enzymes (V.S. and R.M., unpublished data) results in production of the respective desmethyl and deoxy analogues of **1** at 80–100% of the wild-type yield. In addition, loss of TaC abolishes myxovirescin production. Taken together, these results support the part of our biosynthetic model that depicts TaC and TaF acting directly on the "assembly line" rather than after assembly of the antibiotic scaffold.[6] Moreover, these insights back the in vitro studies performed with the HMGS homologue PksG of *B. subtilis*.[7]

Therefore, having excluded the involvement of the primary metabolic HMGS MvaS in the addition of the C30 methyl group of **8**, our results argue for a model in which, in the absence of TaF, TaC rescues myxovirescin assembly by installing two Ac building blocks, one at the C12 and the other one at the C16 β-keto positions of intermediates **2** and **5**, respectively. This results in a novel antibiotic endowed with two methyl groups, one of which is further oxygenated and methylated (**8**; Figure 1B). It appears that this rather discrete complementation by TaC in the Δ*taF* background and the failure of TaF to complement TaC in the Δ*taC* background highlight the differences between the TaC and TaF HMG-like synthases, probably not only in the choice of their cognate substrates, but also in the specificity of their docking interactions with the other enzymes comprising the megasynthase complex. Alternatively, incorporation of the larger ethyl group early in biosynthesis might result in the rejection of the modified intermediate at later biosynthetic steps.

In summary, this work provides one of the first in vivo studies (related work on the mupirocin system was reported at a recent conference; T. J. Simpson, personal communication) directed toward the elucidation of a novel pathway leading to PKS/NRPS natural product assembly, which takes place by a Claisen condensation mechanism previously thought to be specific only to HMG-CoA synthases of the primary metabolism. In contrast to the *S*-adenosylmethionine (SAM)-dependent methyltransferases (MT), which use an electrophilic substrate (SAM) to methylate the activated C2 carbons of the β-ketoacyl-*S*-ACP intermediate,[9] HMGSs use nucleophilic substrates to methylate the C3 carbon of the same type of substrate.[7] Due to enzymatic differences between HMGSs and SAM-dependent MTs, introduction of the C30 methyl group onto the C16 carbon by SAM MT appears mechanistically unfeasible. HMGSs, in combination with the set of enzymes mentioned above, can form methyl or ethyl groups, which may be further processed

into methylmethoxy,[6] *exo*-methylene,[14] cyclopropyl[4] or vinyl chloride groups.[5] Knowledge gained from these and further studies might benefit future biocombinatorial efforts directed towards rational redesigning of known or the discovery of novel molecules with therapeutic potential.

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