Elucidation of the *Streptomyces coelicolor* pathway to 4-methoxy-2,2′-bipyrrole-5-carboxaldehyde, an intermediate in prodiginine biosynthesis†

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The biosynthetic pathway to 4-methoxy-2,2′-bipyrrole-5-carboxaldehyde (MBC), a key intermediate in the biosynthesis of prodiginine antibiotics in *Streptomyces coelicolor*, has been elucidated using a combination of gene replacements and feeding experiments with chemically synthesised MBC and a synthetic analogue of a pathway intermediate.

Prodiginines are a group of red tripyrrole antibiotics produced by several species and genera of actinobacteria as well as other eubacteria.[1] The potent biological actvities of members of this group of antibiotics has stimulated much recent interest in their synthesis, biosynthesis and mode of action.[1] Indeed GX-15-070, a synthetic analogue of prodigiosin, is currently in phase 1 and 2 oncology trials,[2] while PNU-156804, a synthetic analogue of undecylprodiginine **1**, has promising immunosuppressive activity.[3] *Streptomyces longispororuber* produces **1** and the cyclic derivative "ethyl-*meta*-cyclononylprodiginine" **2**.[4] Feeding experiments with ^{13}C-labelled precursors established that **1** and **2** are derived from one unit each of L-proline, L-serine and glycine, eight units of acetic acid, and the methyl group of L-methionine.[5] *Streptomyces coelicolor* A3(2) produces undecylprodiginine **1**[6] and the cyclic derivative streptorubin B **4** (C.C. and G.L.C., unpublished). The *red* gene cluster directing prodiginine biosynthesis in *S. coelicolor* was first identifed by Rudd and Hopwood.[7] Early investigations of the *red* cluster were reported by Hopwood and co-workers and an *S*-adenosyl methionine (SAM)-dependent *O*-methyl transferase involved in the formation of the methoxy group in **1** and **4** was identified and partially characterised.[8] The recently-completed genome sequence of *S. coelicolor* afforded the sequence of the entire *red* cluster (Fig. 1) and analysis of the proteins encoded by this gene cluster led Cerdeno *et al.* to propose a hypothetical pathway for the biosynthesis of **1** and **4**.[9] Subsequently reported genetic and biochemical experiments are consistent with several aspects of this proposed pathway.[10,11] 4-Methoxy-2,2′-bipyrrole-5-carboxaldehyde (MBC) **5** and 2-methyl-3-pentylpyrrole are known intermediates in prodigiosin biosynthesis in *Serratia* species.[1] A pathway for MBC biosynthesis in *Serratia* has recently been proposed on the basis of indirect evidence.[12] MBC and 2-undecylpyrrole **6** have been proposed as intermediates in the biosynthesis of **1**, **2** and **4** in *Streptomyces* species,[5,9] although direct evidence for this has been lacking until now.

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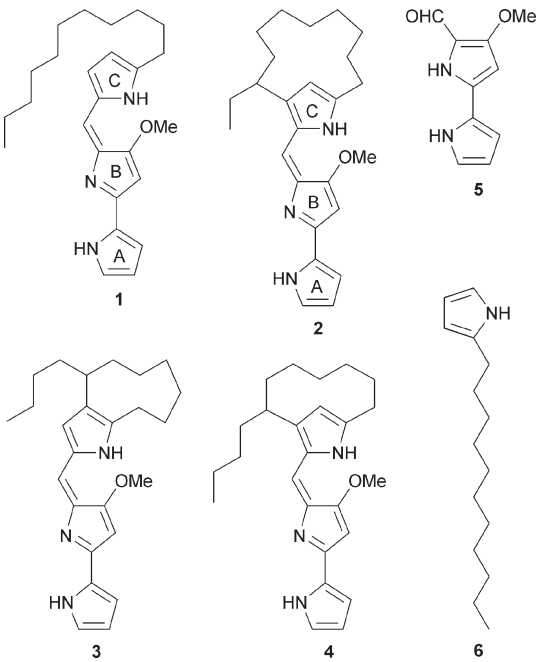


The *redM*, *redW* and *redO* genes of *S. coelicolor* are proposed to be involved in the biosynthesis of **5** (Fig. 1).[9,10] RedM catalyses reaction of the carboxyl group of L-proline with ATP to form an aminoacyl adenylate, which is transferred to the phosphopantetheine thiol of the peptidyl carrier protein (PCP) RedO; L-prolyl-RedO undergoes subsequent conversion to L-pyrrolyl-RedO, catalysed by the FAD-dependent oxidase RedW (Scheme 1).[10] These observations together with the previously reported incorporation of L-proline into the A- and B-rings of **1** and **2** in *S. longispororuber*,[5] are consistent with the proposed involvement of *redM*, *redW* and *redO* in the biosynthesis of **5**. To further examine this hypothesis, we replaced *redM* and *redW* on the chromosome of *S. coelicolor* M511 with a "cassette" containing *oriT* and the *aac(3)IV* apramycin resistance gene using a recently developed PCR-targeting method (Fig. 1).[13] Neither **1** nor **4** could be detected in organic extracts of the *redM::aac(3)IV* or the *redW::aac(3)IV* mutants of *S. coelicolor* M511 when grown on solid or in liquid supplemented minimal medium. LC-MS/MS analysis of organic extracts of the mycelia of these mutants and comparison with synthetic standards of **5**[2] and **6**[4] showed that the mutants both accumulate **6**, but not **5**. Feeding of synthetic **5** to these mutants restored production of **1** and **4**, confirming that **5** is an intermediate in their biosynthesis and that *redM* and *redW* are required for assembly of **5**.

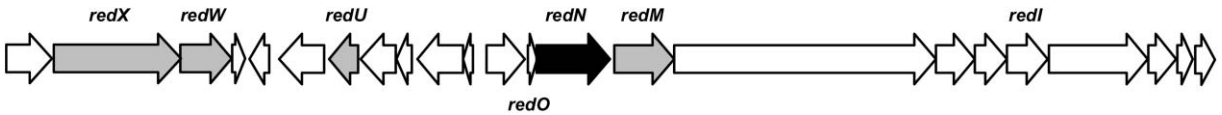


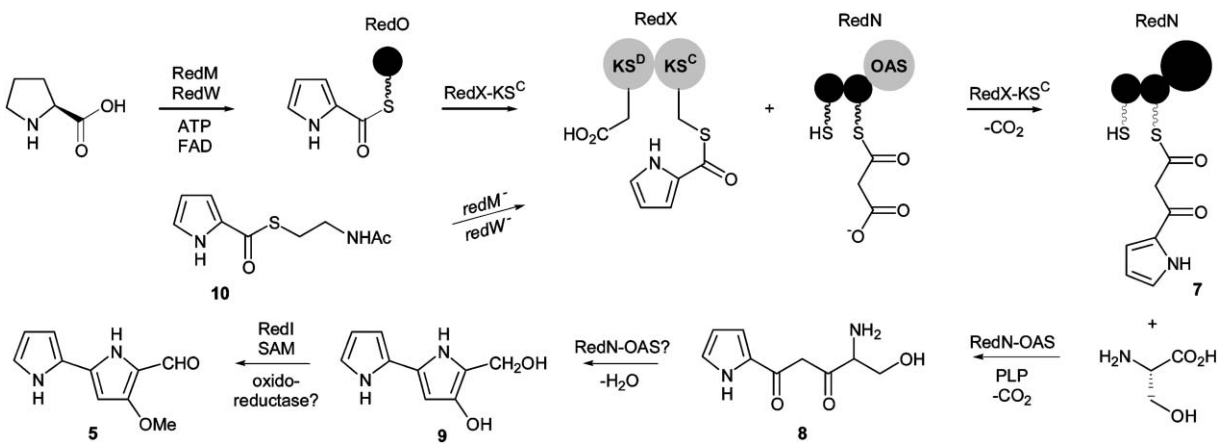
Fig. 1 Organisation of the *red* cluster that directs biosynthesis of **1** and **4** in *S. coelicolor*. Genes proposed to participate in assembly of MBC **5** are labelled.

The *redN* gene has previously been shown to be required for biosynthesis of **1** and **4**.[9] To determine whether *redX* is also required for the biosynthesis of **1** and **4**, we constructed a *redX::aac(3)IV* mutant of *S. coelicolor* M511 (Fig. 1). LC-MS/MS analysis of organic extracts of mycelia of this mutant showed that the production of both metabolites was abrogated. RedN and RedX have been proposed to participate in the biosynthesis of **6**.[9] However, feeding of synthetic **6** to the previously reported *ΔredN* mutant[9] of *S. coelicolor* M511 did not restore production of **1** and **4**. Furthermore, LC-MS/MS analysis of organic extracts of mycelia of the *ΔredN* and *redX:aac(3)IV* mutants showed that they accumulate **6** and not **5**. Feeding of synthetic **5** to either of these mutants restored production of **1** and **4** showing that RedN and RedX participate in the biosynthesis of **5** and not **6** as previously proposed.[9]

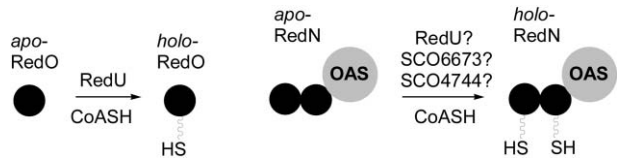
Together with the previous sequence analysis of proteins encoded by the *red* cluster,[9] these results lead us to propose a revised pathway for the biosynthesis of **5** in *S. coelicolor* (Scheme 1). Thus, transfer of the pyrrole-2-carboxyl group attached to RedO onto the active site cysteine residue of the C-terminal KS^C domain in RedX could be followed by decarboxylation of a malonyl group attached to one of the two acyl carrier protein (ACP) domains of RedN and concomitant condensation with pyrrole-2-carboxyl-RedX to give the corresponding β-keto-β-pyrrolyl-propanoyl thioester **7**. Condensation of L-serine with pyridoxal phosphate (PLP) bound to the C-terminal α-oxoamine synthase (OAS) domain of RedN would yield the corresponding seryl imine, which could undergo a similar series of reactions to those proposed to be catalysed by 8-amino-7-oxo-nonanoate synthase in biotin biosynthesis to give **8**.[14] Cyclisation and dehydration of **8** would give 4-hydroxy-2,2′-bipyrrole-4-methanol (HBM) **9**. Methylation of the 4-hydroxy group in this intermediate could be catalysed by the previously identified methyl transferase encoded by *redI*,[8,9] although it is at present unclear at which stage(s) this occurs in the biosynthetic pathways to **1** and **4**. Any one of three potential dehydrogenases/oxidases encoded by genes in the *red* cluster could catalyse the oxidation of the hydroxyl group in HBM to the corresponding aldehyde; further experiments will be required to elucidate which enzyme catalyses this transformation.

The pathway proposed above for the biosynthesis of **9** in *S. coelicolor* raises several intriguing questions. How is the pyrrole-2-carboxyl group formed on RedO transferred to the active site cysteine residue in the KS^C domain of RedX? What is the role of the dual ACP domains in RedN? How are the ACP domains in RedN loaded with malonyl thioester extender units? Does RedN catalyse the cyclisation and dehydration of the α,γ-dioxoamine intermediate **8** to afford **9**? What is the role of the KS^D domain in RedX? Which *S. coelicolor* phosphopantetheinyl transferase (PPTase) catalyses post-translational phosphopantetheinylation of the ACP RedO and the ACP domains in RedN?

We used the *N*-acetylcysteamine (NAC) thioester derivative of pyrrole-2-carboxylic acid **10**[10] (Scheme 1) to investigate the mechanism for transfer of the pyrrole-2-carboxyl group from RedO to the KS^C domain of RedX. NAC thioester analogues effectively mimic acylated ACP and PCP intermediates in biosynthetic pathways and serve as substrates for polyketide synthase KS domains and nonribosomal peptide synthetase condensation domains[15] that process acyl ACP or PCP intermediates. Feeding of **10** to the *redM::aac(3)IV* and *redW::aac(3)IV* mutants of *S. coelicolor* M511 restored production of **1** and **4**, whereas feeding of **10** to the *redX::aac(3)IV* and *ΔredN* mutants did not. In contrast, feeding of pyrrole-2-carboxylic acid (PCA) did not restore production of **1** and **4** in



Scheme 1 Proposed pathway for biosynthesis of MBC **5** illustrating roles of RedM, RedW and RedO and proposed roles of RedX and RedN. ACPs and ACP domains are represented by black spheres. KS^C = ketosynthase domain containing an active site Cys residue, KS^D = ketosynthase domain containing Asp in place of the active site Cys residue, OAS = PLP-dependent α-oxoamine synthase domain.



Scheme 2 Phosphopantetheinylation of RedO requires RedU, whereas the PPTase(s) involved in modification of the RedN ACP domains are uncertain.

any of the mutants. These results show that PCA is not an intermediate in the biosynthesis of **5** and implicate direct transacylation from RedO to the RedX KS^C domain as the likely transfer mechanism. They also suggest that **10** is an effective mimic of pyrrole-2-carboxyl-RedO *in vivo*.

Post-translational phosphopantetheinylation of ACPs and PCPs with coenzyme A catalysed by PPTases is required to provide the thiol to which acyl intermediates in biosynthetic pathways are thioesterified.[16] A total of six ACP and PCP domains or discrete proteins involved in both the MBC and 2-undecylpyrrole branches of the pathway to **1** and **4** are encoded by genes in the *red* cluster. The *redU* gene within the *red* cluster is one of three genes in the *S. coelicolor* genome sequence encoding a protein with significant sequence similarity to known PPTases.[9] The other putative PPTases are encoded by SCO4744 and SCO6673. The former shows similarity to AcpS-type PPTases and has been shown to catalyse phosphopantetheinylation of a relatively broad range of ACPs *in vitro*.[17] The latter has not been experimentally investigated, but shows significant similarity to SePptII of *Saccharopolyspora erythrea*, which has recently been shown to catalyse efficient phosphopantetheinylation of 6-deoxyerythronolide synthase ACP domains.[18] To investigate the role of RedU in the biosynthesis of **1**, **4** and **5**, we constructed a *redU::aac(3)IV* mutant of *S. coelicolor* M511 (Fig. 1). LC-MS analysis of organic extracts of mycelia of this mutant showed that production of **1** and **4** was abrogated and **6** accumulated. Feeding of synthetic MBC to the mutant restored production of **1** and **4** demonstrating that RedU is only required for phosphopantetheinylation of ACPs involved in MBC biosynthesis. To examine whether RedU is required for phosphopantetheinylation of both the RedN ACP domains and the RedO ACP we fed pyrrole-2-carboxylic acid NAC thioester **10** to the mutant. This restored production of **1** and **4** indicating that RedU is only required for phosphopantetheinylation of the RedO ACP in *S. coelicolor* (Scheme 2). Further experiments will be required to determine whether RedU is a RedO-specific PPTase or whether it can also catalyse phosphopantetheinylation of the other five carrier proteins involved in the biosynthesis of **1** and **4**. Regardless of the outcome of these experiments, it is clear that SCO6673 and/or SCO4744 must be capable of efficiently phosphopantetheinylating the RedQ ACP and the four ACP domains in RedN and RedL.

The answers to other questions raised by our proposed pathway to **9** will require further experiments. However, it is tempting to speculate that the KS^D domain of RedX may serve as an acyl transferase capable of catalysing acylation of the RedN ACP thiol groups with malonyl CoA, possibly *via* a malonate–aspartate mixed anhydride covalent intermediate. The dual ACP domains of RedN may be required as a "holding station" for sequestering the β-keto-β-pyrrolyl-propanoyl thioester intermediate prior to undergoing the decarboxylative condensation with serine and subsequent cyclisation and dehydration reactions, which may proceed with a low overall catalytic rate.

In conclusion, we have provided direct evidence for the involvement of RedM, RedW, RedX, RedN and RedU in the assembly of MBC **5**, which we have shown is an intermediate in *S. coelicolor* prodiginine biosynthesis. Combined with previous experiments defining the biochemical functions of RedM, RedW, RedO and RedI,[8,10] and sequence analyses of the proteins encoded by the *red* cluster,[9] these data lead us to propose a revised pathway for MBC biosynthesis in *S. coelicolor* involving a hybrid NRPS-PKS system containing an unusual α-oxoamine synthase domain.

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