Role and substrate specificity of the *Streptomyces coelicolor* RedH enzyme in undecylprodiginine biosynthesis†

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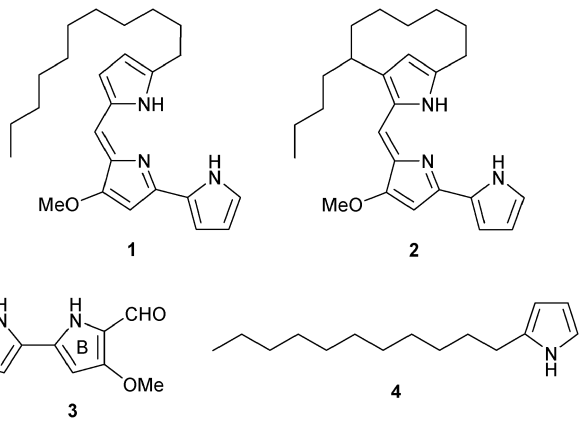
The function of RedH from *Streptomyces coelicolor* as an enzyme that catalyses the condensation of 4-methoxy-2,2′-bipyrrole-5-carboxaldehyde (MBC) and 2-undecylpyrrole to form the natural product undecylprodiginine has been experimentally proven, and the substrate specificity of RedH has been probed *in vivo* by examining its ability to condense chemically-synthesised MBC analogues with 2-undecylpyrrole to afford undecylprodiginine analogues.

Prodiginines are a group of red tripyrrole antibiotics, with potent immunosuppressant,[1] antimalarial[2] and anti-cancer activities,[3] which are produced by several actinobacteria and other eubacteria. The biological activities of prodiginines have stimulated much recent interest in their chemical synthesis, biosynthesis and mode of action.[4] Recently it has been discovered that some prodiginines bind to anti-apoptotic members of the BCL-2 protein family,[5] thus blocking the binding of pro-apoptotic BH3-only proteins, resulting in induction of apoptosis. These discoveries stimulated the development of obatoclax (GX150-70), a synthetic prodiginine analogue, which is currently in phase 1b and 2 clinical trials for treatment of a variety of leukaemia, lymphoma and solid tumour malignancies.[6] A thorough understanding of prodiginine biosynthesis may facilitate manipulation of the pathway to produce novel analogues with potent anti-cancer activity.

Streptomyces coelicolor A3(2) produces undecylprodiginine **1**[7] and the cyclic derivative streptorubin B **2**.[8] A cluster of 23 genes in *S. coelicolor* (the *red* cluster) directs the biosynthesis of **1** and **2** (Fig. 1).[9] 4-Methoxy-2,2′-bipyrrole-5-carboxaldehyde (MBC) **3** and 2-undecylpyrrole **4** are intermediates in the biosynthesis of **1** and **2**.[8,10] Most of the genes in the *red* cluster required for the biosynthesis of **1** and **2** have been experimentally identified[8,10] and the enzymology of early steps in MBC biosynthesis has been investigated.[11,12] The product of the *redH* gene has been proposed to catalyse the condensation of **3** and **4** to yield **1**,[13] but this hypothesis has not been investigated experimentally. Here we report the first experimental investigation of the role played by the RedH protein in the biosynthesis of **1** and the elucidation of the substrate specificity of this protein using a variety of chemically-synthesised analogues of MBC **3**.



To examine the function of *redH* in prodiginine biosynthesis, we replaced it on the chromosome of *S. coelicolor* M511 with a 'cassette' containing *oriT* and the *aac(3)IV* gene, which confers apramycin resistance, using PCR-targeting methodology to create *S. coelicolor* W33.[14] When grown on agar media, the W33 mutant produces no visible red pigment, indicating that *redH* is required for prodiginine biosynthesis. LC-MS/MS analysis of organic extracts of agar-grown mycelia of the W33 mutant revealed low levels of **1**. Control experiments suggested this was an artefact resulting from the extraction procedure. Complementation of the *redH:aac(3)IV* mutation by integration of a plasmid (pPKS1) containing *redH* under the control of the constitutive *ermE** promoter into the chromosome of *S. coelicolor* W33 restored wild type levels of prodiginine production.† Accumulation of **4** but not **3** could be detected in the W33 mutant by LC-MS/MS comparisons with chemically-synthesised[8,15] authentic standards.† However, the transient accumulation of **3** in *S. coelicolor* W33 could be inferred by the ability of this mutant to restore red pigment production in previously reported[10] MBC-deficient mutants of *S. coelicolor* by cross-feeding.† Unlike mutants of *S. coelicolor* blocked in the biosynthesis of **3** or **4**,[8,10] where feeding of chemically-synthesised MBC[15] and 2-undecylpyrrole,[8] respectively, restored production of **1** and **2**, feeding of synthetic **3** or **4** did not restore prodiginine production in the W33 mutant. To further investigate the role of RedH in the biosynthesis of **1**, we integrated pPKS1 (containing *redH* under the control of the constitutive *ermE** promoter) into the chromosome of *Streptomyces venezuelae* ATCC10712. LC-MS/MS analysis of

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† Electronic supplementary information (ESI) available: Experimental procedures for the construction and genetic complementation of the *S. coelicolor* W33 mutant, the construction of *S. venezuelae*/pPKS1, the synthesis of **7a–d**, **13** and **14a–d** and the feeding experiments; LC-MS data showing accumulation of **4** and **14a–d**; cross-feeding data showing accumulation of **3**; spectroscopic data for **3**, **5b**, **6**, **7a–d**, **11–13** and **14a–d**. See DOI: 10.1039/b801677a

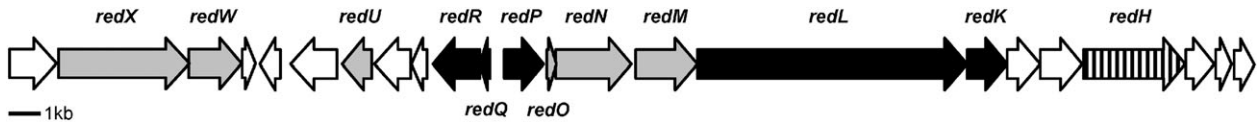


Fig. 1 Organisation of the *red* cluster that directs biosynthesis of **1** and **2** in *S. coelicolor*. Genes known to participate in assembly of MBC **3** are shaded black; genes known to participate in the assembly of 2-undecylpyrrole **4** are shaded grey. The *redH* gene investigated in this study is highlighted with stripes.

wild type *S. venezuelae* showed that it does not produce prodiginines on agar media and analysis of the complete genome sequence of this organism indicates that it does not contain a similar gene cluster to the *red* cluster, nor does it contain a gene with significant similarity to *redH* (M. J. Bibb, personal communication). Feeding of MBC **3** and 2-undecylpyrrole **4** to wild type *S. venezuelae* grown on agar media did not result in visible red-pigment production. However LC-MS/MS analysis of acidified organic extracts of the mycelia from this experiment revealed trace quantities of **1**, likely resulting from the extraction procedure. On the other hand feeding of MBC **3** and 2-undecylpyrrole **4** to *S. venezuelae*/pPKS1 ($redH^+$) grown on agar media resulted in visible red-pigment production and LC-MS/MS analysis of acidified organic extracts of the mycelia from this experiment revealed substantial quantities of **1** (Fig. 2). Together these data provide strong evidence that RedH catalyses condensation of the known intermediates MBC **3** and 2-undecylpyrrole **4** to form **1** in the *S. coelicolor* prodiginine biosynthetic pathway.

RedH is predicted to encode a protein with three functional domains.[9] These domains show sequence similarity to the ATP-binding domains and the phosphotransfer domains, respectively, of phosphoenol pyruvate synthase (PEPS) and pyruvate-phosphate dikinase (PPDK).[9] The third functional domain of RedH does not show sequence similarity to any proteins or protein domains of known function.[9] By analogy with the known catalytic mechanism of PEPS and PPDK, we hypothesised that RedH catalyses the ATP-dependent phosphorylation of the aldehyde oxygen atom of MBC **3**, thus activating the carbonyl carbon atom towards nucleophilic attack by C-5 of 2-undecylpyrrole **4** and assisting subsequent loss of the aldehyde oxygen atom as phosphate. We thus thought it would be interesting to probe the substrate specificity of RedH by examining the ability of our previously constructed *S. coelicolor* W39 (*redM::aac(3)IV*) mutant (which is blocked in the biosynthesis of **3**) to catalyse condensation of chemically-synthesised MBC analogues with the 2-undecylpyrrole that accumulates in this mutant.

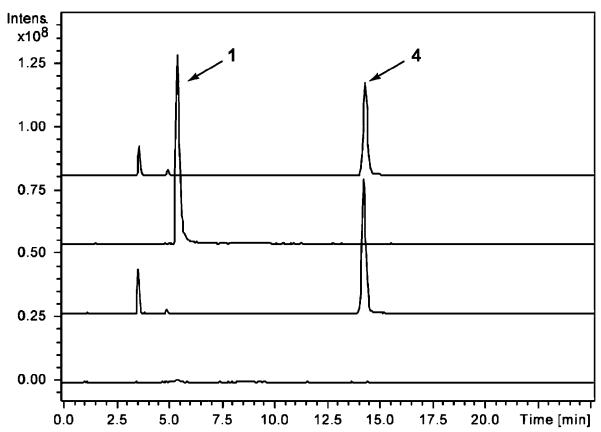
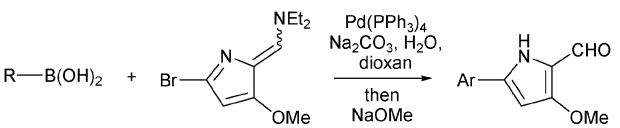


Fig. 2 LC-MS analysis of the production of **1** by wild type *S. venezuelae* and *S. venezuelae*/pPKS1 fed with synthetic **3** and **4**. Extracted ion chromatograms (EICs) at $m/z = 222$ (top trace) and $m/z = 394$ (second from top trace) for extracts from *S. venezuelae*/pPKS1. EICs at $m/z = 222$ (second from bottom trace) and $m/z = 394$ (bottom trace) for extracts from wild type *S. venezuelae*.

Analogues **7a–d** of MBC, with other aromatic rings in place of the monosubstituted pyrrole ring, were synthesised *via* Suzuki coupling of the appropriate aryl boronic acid **5a–d** with vinyl bromide **6** (Scheme 1). Similar conditions to those recently reported for the synthesis of MBC *via* Suzuki coupling of *N*-BOC-pyrrole-2-boronic acid with **6** were used.[15]

Roseophilin **8**, a natural product from *Streptomyces griseoviridis*,[16] contains an identical conjugated structure to the prodiginines, except that the B-ring is a furan instead of a pyrrole (Scheme 2). Reasoning that roseophilin and the prodiginines could be biosynthesised *via* analogous pathways, we also synthesised the analogue **13** of MBC with the trisubstituted pyrrole replaced by a furan, to examine whether this compound could be utilised by the *S. coelicolor* W39 (*redM::aac(3)IV*) mutant to biosynthesise a roseophilin–undecylprodiginine hybrid structure **9** (Scheme 2). 4-Methoxy-2(5*H*)-furanone **10** was converted to enamine **11** by reaction with excess dimethylformamide dimethyl acetal, distilling methanol from the reaction as it was formed. Compound **11** was reacted with phosphorus oxybromide to form the corresponding bromo-enamine which was converted to the bromo-aldehyde **12** by hydrolysis. This compound was unstable and decomposed to a black tar on storage. Thus it was used immediately in a Suzuki reaction with *N*-BOC-pyrrole-2-boronic acid which afforded the *N*-BOC derivative of MBC analogue **13**. The BOC group was removed from this compound by base-promoted hydrolysis (Scheme 2).

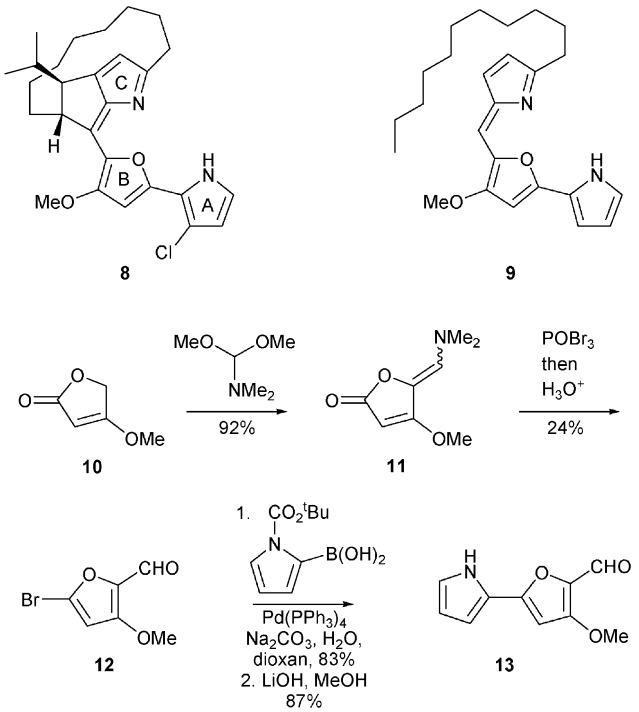
Feeding of MBC analogues **7a–d** to the *S. coelicolor* W39 (*redM::aac(3)IV*) mutant resulted in production of the corresponding undecylprodiginine analogues **14a–d** (Scheme 3).† Rather than relying solely on UV–Vis spectroscopic and low



5a: R = phenyl
5b: R = N-BOC-2-indolyl
5c: R = 2-furyl
5d: R = 2-thienyl

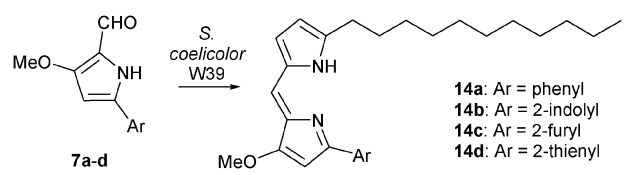
7a: Ar = phenyl, 70%
7b: Ar = 2-indolyl, 78%
7c: Ar = 2-furyl, 60%
7d: Ar = 2-thienyl, 56%

Scheme 1 Synthesis of MBC analogues with other aromatic rings in place of the monosubstituted pyrrole.



Scheme 2 Structures of roseophilin **8** and the undecylprodiginine–roseophilin hybrid **9**, and synthesis of MBC analogue **13**.

resolution MS data to characterise these undecylprodiginine analogues, which do not unambiguously confirm their structures, authentic standards of **14a–d** were prepared by acid-catalysed condensation of **7a–d** with 2-undecylpyrrole[17] and shown to be identical by LC-MS/MS analyses to the undecylprodiginine analogues produced in the feeding experiments. In critically important control experiments designed to discriminate between enzymatic and non-enzymatic condensation reactions, **7a–d** were fed to the *S. coelicolor* W33 (*redH::aac(3)IV*) mutant. LC-MS/MS analyses indicated that **14a–d** were produced in all of these experiments, probably as a result of the isolation procedure.† While the quantity of **14a** produced relative to the accumulated 2-undecylpyrrole **4** was modestly greater when **7a** was fed to the W33 (*redH::aac(3)IV*) mutant than when **7a** was fed to the W39 (*redM::aac(3)IV*) mutant, the quantities of **14b–d** produced by the W39 mutant were very much higher than the quantities produced by the W33 mutant (relative to accumulated **4**), indicating unequivocally that **7b–d** are substrates of RedH.† In contrast, feeding of MBC analogue **13** to the *S. coelicolor* W39 mutant did not result in production of roseophilin–undecylprodiginine hybrid **9**. This may be because **13** is not a substrate of RedH or because it cannot enter *S. coelicolor* cells.



Scheme 3 Structures of undecylprodiginine analogues produced by feeding the corresponding synthetic MBC analogues to the *S. coelicolor* W39 mutant.

In conclusion, we have demonstrated that the *S. coelicolor* RedH enzyme catalyses condensation of MBC **3** and 2-undecylpyrrole **4** in the biosynthesis of undecylprodigine **1**. We have also shown that RedH can catalyse condensation of a variety of synthetic MBC analogues **7a–d** with 2-undecylpyrrole **4** to form several undecylprodiginine analogues **14a–d**, albeit with varying efficiences.[18] The results of these experiments indicate that the nitrogen atom in the A-ring of MBC is not required for catalysis, but that a heteroatom capable of donating a lone-pair to the aromatic ring is important. They also indicate that RedH does not impose a strong steric constraint on the A-ring of MBC. While we suspect that the nitrogen atom in the B-ring of MBC may play an important role in the catalytic mechanism of RedH, the data obtained here do not allow us to conclusively confirm or deny this hypothesis. Finally these experiments demonstrate the feasibility of preparing prodiginine analogues *via* a mutasynthesis approach. It will be particularly interesting to see whether this mutasynthesis approach can be extended to the preparation of analogues of streptorubin B **2**, which unlike analogues of undecylprodiginine **1** and prodigiosin are not readily accessible by total synthesis strategies.

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