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Regio and Stereodivergent Antibiotic Oxidative Carbocyclizations Catalyzed by Rieske Oxygenase-Like Enzymes

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Abstract

Oxidative cyclizations, exemplified by the biosynthetic assembly of the penicillin nucleus from a tripeptide precursor, are arguably the most synthetically-powerful implementation of C-H activation reactions in Nature. Here we show that Rieske oxygenase-like enzymes mediate regio and stereodivergent oxidative cyclizations to form 10- and 12-membered carbocyclic rings in the key steps of the biosynthesis of the antibiotics streptorubin B and metacycloprodigiosin, respectively. These reactions represent the first examples of oxidative carbocyclizations catalyzed by non-heme iron-dependent oxidases and define a novel type of catalytic activity for Rieske enzymes. A better understanding of how these enzymes achieve such remarkable regio and stereocontrol in the functionalization of unactivated hydrocarbon chains will greatly facilitate the development of selective manmade C-H activation catalysts.

Oxidative cyclization reactions are key steps in the biosynthesis of several important bioactive natural products, including clinically-used compounds such as penicillins (e.g. isopenicillin N **2**), clavulanic acid (derived from (*3S*, *5S*)-dihydroclavaminic acid **4**), fosfomycin **6** and vancomycin (Fig. 1)[1–5]. Such reactions are typically catalyzed by enzymes utilizing non-heme iron cofactors and molecular oxygen as a co-substrate[3, 6–10]. However, examples of heme or flavin and oxygen-dependent oxidative cyclases are also known[5, 11–13]. In the non-heme enzymes, iron-bound molecular oxygen is generally assumed to undergo reductive cleavage of the O=O bond to generate an Fe(IV)=O intermediate that carries out regio- and stereo-specific cleavage of a C-H bond to generate a carbon-centered radical (or a

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Additional Information

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metal-bound equivalent) as the key intermediate in oxidative cyclization[14–17]. Despite intense recent interest in C-H activation chemistry, similar non-enzymatic reactions have hitherto not been developed as part of the repertoire of the synthetic chemist. Greater understanding of how oxidative cyclization reactions are catalyzed in Nature may significantly aid the development of man-made catalysts.

In 1974 Wasserman and coworkers proposed that the *ansa*-bridged 12-membered carbocyclic ring of metacycloprodigiosin **7** is biosynthesized in *Streptomyces longispororuber* by an oxidative cyclization reaction of the co-metabolite undecylprodigiosin **8** (Figure 2A)[18]. Metacycloprodigiosin **7** and undecylprodigiosin **8** belong to a large family of structurally-related metabolites called the prodiginines, which also includes streptorubin B **9**, prodigiosin R1 **10** and roseophilin **11** (Figure 2A)[19–23]. The prodiginines have attracted considerable recent interest as a result of their potent biological activities. The most significant among these appears to be their ability to disrupt protein-protein interactions between B-cell lymphoma 2 (Bcl-2) family anti-apoptotic proteins and Bcl-2 homology 3 (BH3) domain-containing pro-apoptotic proteins which has led to the development of obatoclax, a synthetic analogue of streptorubin B **9**, metacycloprodigiosin **7** and prodigiosin R1 **10** that is currently in a range of Phase 1 and 2 oncology trials[24].

We recently reported that the *red* gene cluster of *Streptomyces coelicolor* A3(2) (Figure 2B) directs the biosynthesis of streptorubin B **9** and identified 4-methoxy-2, 2'-bipyrrole-5-carbaldehyde (MBC) **12** and 2-undecylpyrrole **13** as key precursors of streptorubin B **9** and its co-metabolite undecylprodigiosin **8**[25, 26]. The RedH enzyme has been shown to catalyze condensation of MBC **12** and 2-undecylpyrrole **13** to form undecylprodigiosin **8** (Figure 2C), and streptorubin B **9** was proposed to derive from undecylprodigiosin **8** via an oxidative cyclization reaction catalyzed by RedG (Figure 2A)[27, 28]. Sequence comparisons suggest that RedG is a Rieske oxygenase-like enzyme. The universally conserved CXH and CXXH sequence motifs within the amino-terminal domain of Rieske oxygenases contain two Cys and two His residues that ligate the iron atoms within the Fe_2S_2 Rieske cluster that is characteristic of this family of enzymes[29]. Within the carboxy-terminal domain of Rieske oxygenases, the conserved $DXHX_4H$ motif contains two His residues that ligate the non-heme iron center and an Asp residue that is proposed to mediate electron transfer from the Rieske cluster to the non-heme iron atom via a hydrogen bond network between an oxygen atom of the Asp side chain and one His residue each from the Rieske cluster and non-heme iron binding sites[29]. All three universally conserved sequence motifs are found within the RedG protein, with only one change from Asp to Glu in the $DXHX_4H$ motif that is unlikely to be of functional significance (Figure 2D).

Rieske non-heme iron-dependent oxygenases typically catalyze a range of oxidation reactions such as *cis*-dihydroxylation of aromatic compounds (e.g. of naphthalene **14** to form **15**) and N-oxidation of aromatic amines to the corresponding nitro compounds (e.g. of **16** to afford pyrrolnitrin **17**) (Figure 2E)[30, 31]. They invariably require a reductase partner protein to provide electrons to the non-heme iron center via the Rieske center and in some cases a ferredoxin is also required to mediate this process[32]. There are no known Rieske oxygenases that catalyze oxidative cyclization reactions. Here we report that RedG catalyzes an oxidative cyclization reaction to form the 10-membered carbocycle of streptorubin B **9**

and that the RedG ortholog McpG of *Streptomyces longispororuber* catalyzes an analogous reaction to form the 12-membered carbocycle of metacycloprodigiosin **7**.

Results

To investigate the role of RedG in streptorubin B biosynthesis, we deleted the *redG* gene from the chromosome of *S. coelicolor* using polymerase chain reaction (PCR)-targeting-based mutagenesis technology[33]. Liquid chromatography-mass spectrometry (LC-MS) analysis of mycelial extracts of the *redG* mutant showed that it still produces undecylprodigiosin **8**, but no longer produces streptorubin B **9** (Figure 3A). In *trans* expression of *redG* in this mutant under the control of the constitutive *ermE** promoter restored production of streptorubin B **9** (Figure 3A). These experiments unequivocally implicated RedG in the biosynthesis of streptorubin B **9** and ruled out its involvement in undecylprodigiosin **8** biosynthesis.

We next sought to establish whether RedG is the only enzyme encoded by the *red* cluster required for streptorubin B **9** biosynthesis, in addition to those known to be required for undecylprodigiosin **8** biosynthesis. We recently showed that feeding synthetic MBC **12** and 2-undecylpyrrole **13** to *Streptomyces venezuelae* (which does not produce prodiginines or contain a prodiginine biosynthetic gene cluster) that had been genetically engineered to constitutively express *redH* results in undecylprodigiosin **8** production[27]. Thus we genetically engineered *Streptomyces venezuelae* to constitutively co-express *redG* and *redH*, and carried out an analogous feeding experiment with MBC **12** and 2-undecylpyrrole **13**. LC-MS analysis of mycelial extracts showed that streptorubin B **9** is produced in addition to undecylprodigiosin **8** by this strain (Figure 3B). This firmly established that RedG is the only enzyme required in addition to RedH for the assembly of streptorubin B **9** from MBC **12**and 2-undecylpyrrole **13**, clearly demonstrating that RedG effects regiospecific C-H activation in the oxidative cyclization reaction that forms the strained 10- membered carbocycle of streptorubin B **9**.

The above data are consistent with either 2-undecylpyrrole **13** or undecylprodigiosin **8** being the substrate of RedG. Insight into the timing of the oxidative cyclization reaction in streptorubin B **9** biosynthesis came from analysis of the metabolites that accumulate in a *redI* mutant of *S. coelicolor*. The *redI* gene encodes a protein with sequence similarity to S-adenosylmethionine (SAM)-dependent methyl transferases[28]. LC-MS analyses of mycelial extracts of the *redI* mutant showed that it produces neither undecylprodigiosin **8** nor streptorubin B **9**. Instead they indicate that the mutant produces desmethylundecylprodigiosin **18** (Figures 2A and 3C), a supposition that was confirmed by chemical conversion of the accumulated metabolite to undecylprodigiosin **8** using trimethylsilyldiazomethane. The two peaks observed for desmethylundecylprodigiosin **18** in the LC-MS analysis reflect the fact that this compound exists as two isomers or tautomers that interconvert more slowly than the timescale of the analysis. This was confirmed by separately collecting the compound under each peak and reanalyzing it. In both cases the original mixture of the two compounds was observed upon reanalysis. No desmethylstreptorubin B could be detected in the mutant, indicating that oxidative carbocyclization occurs after RedH-mediated condensation of MBC **12** and 2-

undecylpyrrole **13** and that the MBC-derived portion of undecylprodigiosin **8** is required for the reaction to occur. To directly probe whether undecylprodigiosin **8** can be converted to streptorubin B **9** by RedG, we incubated chemically-synthesized undecylprodigiosin **8**[27] with *S. venezuelae* expressing the *redG* and *redH* genes or just the *redG* gene. In both cases LC-MS/MS analyses of mycelia extracts indicated that streptorubin B **9** was formed (Figure 3D and supporting information), although a larger amount of streptorubin B **9** relative to undecylprodigiosin **8** was formed in the strain expressing *redG* and *redH*, suggesting that RedG and RedH may form a complex *in vivo*. We also investigated whether RedG can catalyze the oxidative carbocyclization of 2-undecylpyrrole **13** by feeding it to *S. venezuelae* expressing the *redG* and *redH* genes. None of the carbocyclic derivative of 2-undecylpyrrole could be detected in this experiment by comparison with a chemically-synthesized authentic standard[34] (see supporting information). If oxidative carbocyclization can occur before the condensation of MBC **12** with 2-undecylpyrrole **13**, we would expect to observe accumulation of the carbocyclic derivative of 2-undecylpyrrole in mutants of *S. coelicolor* blocked in MBC biosynthesis[26]. Again, by comparison with the authentic standard we could find no evidence for this (see supporting information).

Our attention now turned to formation of the 12-membered carbocycle of metacycloprodigiosin **7** which we surmised would proceed via an analogous oxidative cyclization reaction catalyzed by a RedG ortholog of *Streptomyces longispororuber*. Using degenerate PCR primers designed to anneal with conserved regions within the *redH* and *redG* genes, we amplified a 637 base pair fragment of *S. longispororuber* genomic deoxyribonucleic acid (DNA). Sequencing of the amplimer revealed two partial coding sequences (CDSs) with a high degree of similarity to regions of the *redH* and *redG* genes. We constructed a genomic fosmid library of *S. longispororuber* and screened it by PCR for clones containing the identified CDSs. One clone was selected for further analysis and the entire sequences of the identified CDSs together with a third partial coding sequence were obtained by walking out from the sequence obtained from the amplimer (Figure 2B) (Genbank accession no. BankIt1432020 Streptomyces JF288762). The complete CDSs, which were named *mcpH* and *mcpG* encoded proteins with 78% and 75% similarity, respectively, to RedH and RedG. The partial CDS encoded a protein (129 amino acids) with 79% similarity to the 102 carboxy-terminal amino acids of RedI. To examine the role of McpG in carbocycle formation during metacycloprodigiosin **7** biosynthesis, we introduced *mcpG* under the control of the *ermE** promoter into the *redG* mutant of *S. coelicolor*. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of mycelia extracts of the resulting strain indicated that it produces undecylprodigiosin **8** and a carbocyclic derivative (Figure 3E), which was purified by semi-preparative reverse-phase high pressure liquid chromatography (HPLC) and shown by ^{1}H nuclear magnetic resonance (NMR) and circular dichroism (CD) spectroscopic analyses to be identical to an authentic sample of metacycloprodigiosin **7** isolated from *S. longispororuber* (Figures 3F and 3G). There was no evidence for formation of any streptorubin B **9** in this experiment. These results unequivocally show that McpG mediates an analogous oxidative carbocyclization reaction in metacycloprodigiosin **7** biosynthesis to the oxidative carbocyclization reaction catalyzed by RedG in streptorubin B **9** biosynthesis. Intriguingly, however, the reactions mediated by RedG and McpG involve regiospecific C-H activation at different positions along the

undecyl chain of undecylprodigiosin **8** (Figure 2A). Equally intriguing is the fact that the predominant stereoisomer of streptorubin B **9** and metacycloprodigiosin **7** isolated from *S. coelicolor* and *S. longispororuber*, respectively, have recently been shown to have opposite absolute configurations (Figure 2A)[34, 35], as suggested by the opposite Cotton effects observed in the CD spectra of these compounds. Thus the oxidative cyclization reactions catalyzed by RedG and McpG are not only regiodivergent, but also stereodivergent.

Discussion

Here we have identified RedG and McpG as two members of a family of Rieske-oxygenase-like enzymes that mediate remarkable regio- and stereodivergent oxidative carbocyclization reactions in the biosynthesis of streptorubin B **9** and metacycloprodigiosin **7**, respectively. Such reactions are unprecedented in other biosynthetic pathways and represent a completely new type of catalytic activity for the Rieske non-heme iron-dependent oxygenase-like enzymes.

Very recently, a gene cluster believed to direct the biosynthesis of roseophilin **11** and prodigiosin R1 **10** in *Streptomyces griseoviridis* was identified, cloned and sequenced[36]. Four genes within this cluster encode RedG homologues. It is tempting to speculate that they are involved in an oxidative carbocyclization reaction to form prodigiosin R1 **10** and two oxidative carbocyclization reactions in roseophilin **11** biosynthesis, as well as the oxidative conversion of the central ring in a presumed bipyrrolylpyromethene precursor of roseophilin to the corresponding furan (Figure 2A).

One intriguing question raised by our results is whether RedG and McpG utilize an Fe(IV)=O intermediate for hydrogen abstraction like other non-heme iron-dependent enzymes that catalyze oxidative cyclizations, or whether they use Fe(III)-OOH/(HO)Fe(V)=O intermediates, which have recently been proposed to be the oxidants in Rieske oxygenases such as naphthalene dioxygenase[37, 38]. The Fe(III)-OOH intermediate in Rieske oxygenases is generated by reduction of a Fe(II)-O_2 complex with an electron supplied by the Fe_2S_2 center. Electrons from reduced nicotinamide adenine dinucleotide (NADH), or its phosphorylated derivative, are supplied to the Fe_2S_2 center by an external reductase (a ferredoxin is often also involved)[32]. No such reductases are encoded by genes within the *red* gene cluster of *S. coelicolor*, but several candidates are encoded by genes located elsewhere on the chromosome. To address the questions of the involvement of molecular oxygen and a specific reductase, as well as the nature of the reactive intermediate in the RedG and McpG-catalyzed reactions these enzymes will have to be reconstituted *in vitro*.

Future studies will focus on exploiting these novel enzymes in the chemoenzymatic synthesis of streptorubin B **9** and metacycloprodigiosin **7** analogues that are not easily accessible by conventional synthetic methods. Elucidating the unique catalytic mechanisms of these enzymes will be another important goal.

C-H activation and oxidative cyclization reactions have attracted considerable recent interest as powerful new tools for organic synthesis[39, 40]. The findings reported here should

stimulate attempts to introduce oxidative carbocyclizations via selective C-H activation into the modern synthetic repertoire.

Methods

Materials and methods are described in the supporting information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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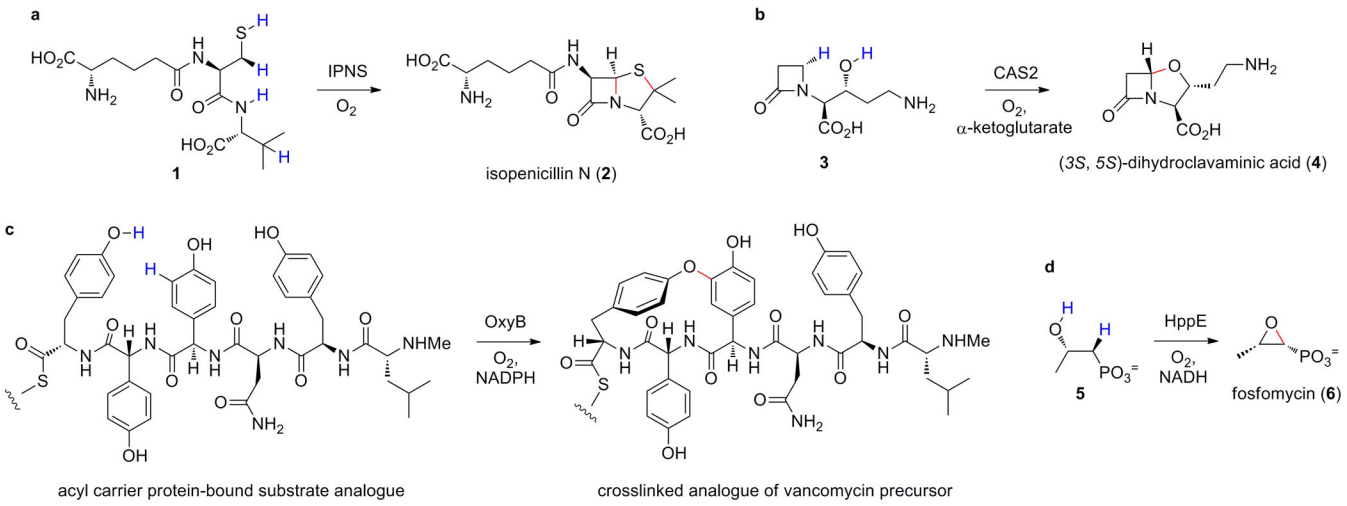

Figure 1.

Key oxidative cyclization reactions in the biosynthesis of clinically-used natural products. Hydrogen atoms removed in the reactions are highlighted in blue and new bonds formed are highlighted in red. (**A**) Isopenicillin N synthase (IPNS) is a non-heme iron-dependent enzyme that catalyzes two oxidative cyclization reactions within the tripeptide **1** to form the bicyclic nucleus of isopenicillin N **2** at the expense of a molecule of oxygen. (**B**) Clavaminate synthase 2 (CAS2) is a non-heme iron-dependent enzyme that catalyzes the oxidative cyclization of the monocyclic β-lactam **3** to form (*3S*, *5S*)-dihydroclavaminic acid **4**, an intermediate in the biosynthesis of the bicyclic β-lactamase inhibitor clavulanic acid, utilizing a molecule of oxygen together with two electrons from α-ketoglutarate. (**C**) HppE is a non-heme iron dependent enzyme that catalyzes the oxidative cyclization of the β-hydroxyphosphonate **5** using molecular oxygen and two electrons from NADH to form the key biologically-active epoxide group in the antibiotic fosfomycin **6**. (**D**) OxyB is a cytochrome P450 that has been shown to catalyze the oxidative cyclization of an acyl carrier protein-bound synthetic analogue of a nonribosomally biosynthesized peptide thioester to form an analogue of a key cross-linked vancomycin precursor at the expense of a molecule of oxygen and two electrons from NADPH.

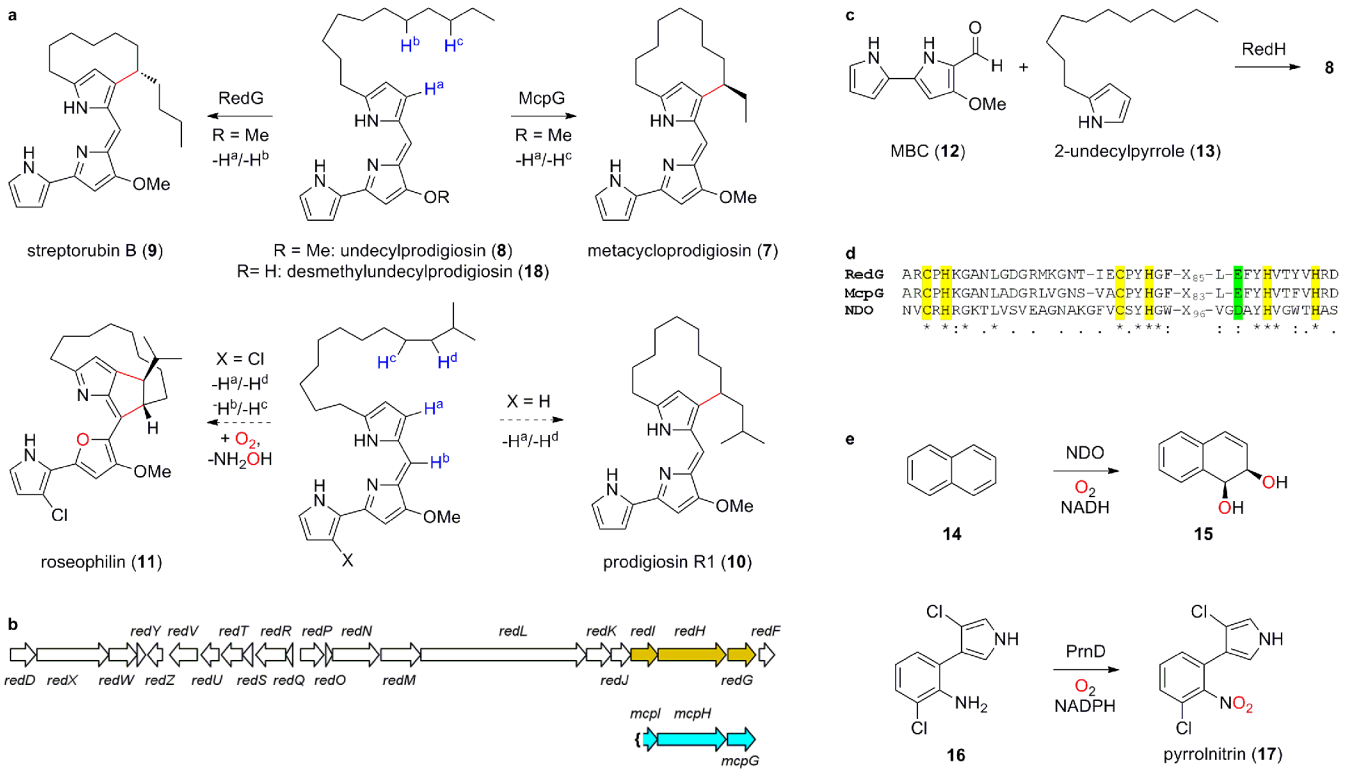

Figure 2.

Roles of Rieske non-heme iron-dependent oxygenases and oxygenase-like enzymes, together with associated enzymes, in the biosynthesis of natural products and the degradation of organic compounds. (**A**) Oxidative carbocyclization reactions proposed to be mediated by Rieske oxygenase-like enzymes in metacycloprodigiosin **7**, streptorubin B **9**, prodigiosin R1 **10** and roseophilin **11** biosynthesis. The hydrogen atoms removed in the reactions are highlighted in blue and the new bonds formed are highlighted in red. A Rieske oxygenase is also proposed to effect the replacement of a nitrogen atom with an oxygen atom (highlighted in red) in roseophilin **11** biosynthesis. (**B**) Organization of the *red* gene cluster that directs streptorubin B **9** biosynthesis in *S. coelicolor*. The *redG*, *redH* and *redI* genes discussed in this study are highlighted in orange. The *mcpG*, *mcpH* and *mcpI* genes, which are involved in the biosynthesis of metacycloprodigiosin **7** and are homologues of *redG*, *redH* and *redI*, are found in the same relative order on the chromosome of *S. longispororuber* (highlighted in blue). (**C**) Role of RedH in undecylprodigiosin **8** biosynthesis. (**D**) Sequence alignment of the Rieske oxygenase-like enzymes RedG and McpG that mediate oxidative carbocyclization reactions in streptorubin B **9** and metacycloprodigiosin **7** biosynthesis, respectively, with the structurally-characterized Rieske oxygenase naphthalene dioxygenase (NDO). Conserved residues within RedG and McpG that ligate the [2Fe-2S] cluster and Fe(II) atom in NDO are highlighted in yellow. An Asp residue of NDO (mutated to Glu in RedG and McpG) proposed to mediate electron transfer between the [2Fe-2S] cluster and the Fe(II) atom is highlighted in green. (**E**) Typical reactions catalyzed by Rieske oxygenases. Naphthalene dioxygenase (NDO) catalyzes the dihydroxylation of naphthalene **14** to form **15** using molecular oxygen and two electrons

derived from NADH. PrnD catalyzes the oxidation of an amino group in **16** to afford the nitro group in pyrrolnitrin **17** utilizing molecular oxygen and electrons from NADPH. Oxygen atoms derived from molecular oxygen that are introduced into the products are highlighted in red.

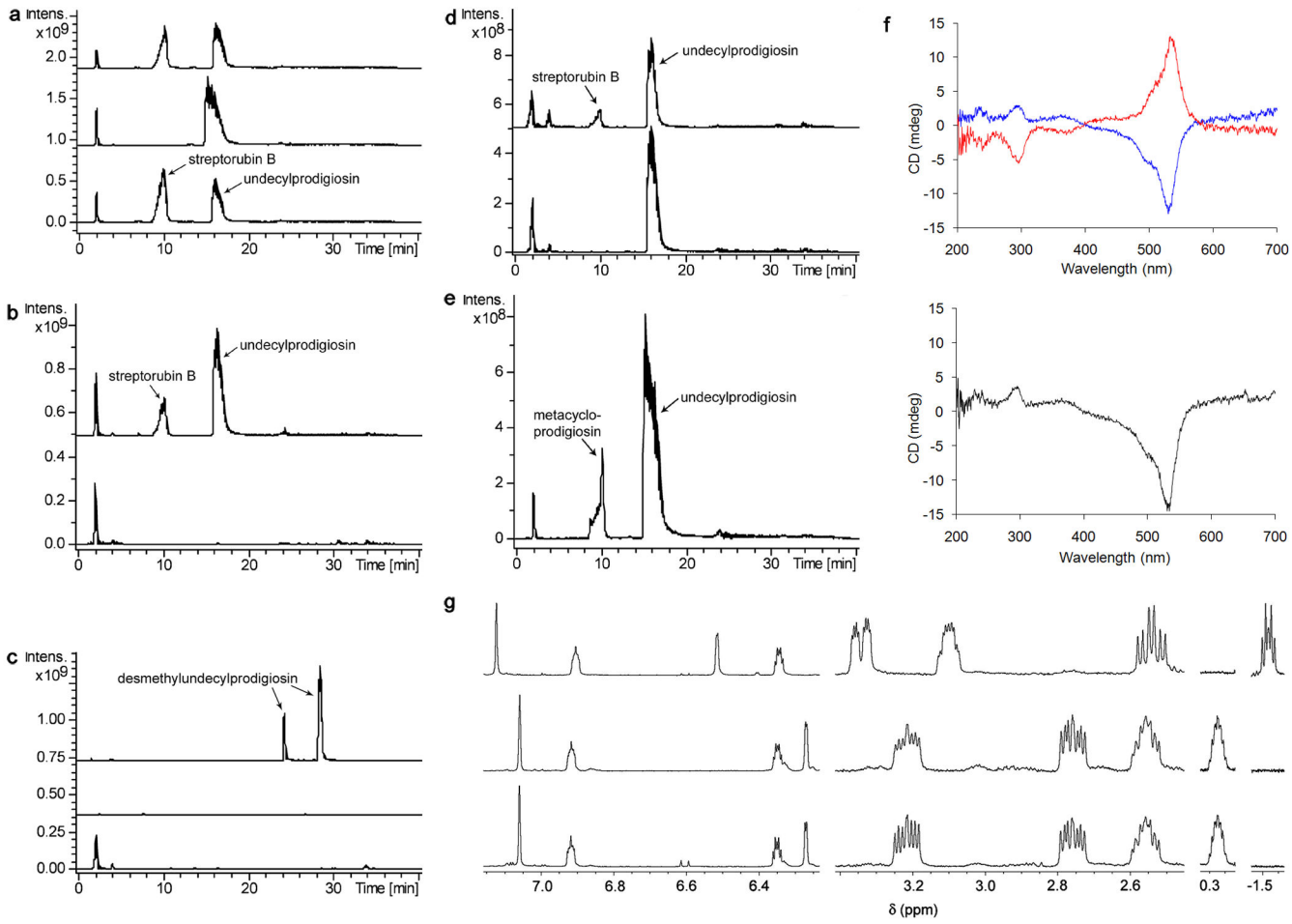

Figure 3.

Data that elucidate the role of RedG and McpG in streptorubin B **9** and metacycloprodigiosin **7** biosynthesis, respectively. (**A**) Extracted ion chromatograms (EICs) for *m/z* range 392–394 from LC-MS analyses in positive ion mode of extracts of *S. coelicolor* M511 (top trace), a Δ*redG* mutant of *S. coelicolor* M511 (middle trace) and the Δ*redG* mutant expressing *redG* in *trans* (bottom trace). (**B**) EICs for *m/z* range 392–394 from LC-MS analyses of extracts of *S. venezuelae* fed with MBC **12** and 2-undecylpyrrole **13** (bottom trace) and *S. venezuelae* expressing *redH* and *redG* fed with MBC **12** and 2-undecylpyrrole **13** (top trace). (**C**) EIC for *m/z* 380 (top trace), *m/z* 378 (middle trace) and *m/z* range 392–394 (bottom trace) from LC-MS analyses of extracts of a *redI::oriT-apr* mutant of *S. coelicolor*. (**D**) EICs for *m/z* range 392–394 from LC-MS analyses of extracts of *S. venezuelae* (bottom trace) and *S. venezuelae* expressing *redH* and *redG* (top trace), both fed with synthetic undecylprodigiosin **8**. (**E**) EICs for *m/z* range 392–394 from LCMS analyses of extracts of *S. coelicolor* Δ*redG* mutant expressing *mcpG* in *trans*. (**F**) Top: CD spectra of streptorubin B **9** (red) and metacycloprodigiosin **7** (blue). Bottom: CD spectrum of the cyclic derivative of undecylprodigiosin isolated from the *S. coelicolor* Δ*redG* mutant expressing *mcpG* in *trans* (black). (**G**) Diagnostic regions of the ^{1}H NMR spectra of streptorubin B **9** (top trace) metacycloprodigiosin **7** (middle trace) and the cyclic derivative

of undecylprodigiosin isolated from the *S. coelicolor* Δ*redG* mutant expressing *mcpG* in *trans* (bottom trace).