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Enzymatic Assembly of the Bis-Indole Core of Rebeccamycin

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Rebeccamycin **1** (Figure 1), a natural product produced by the bacterium *Lechevalieria aerocolonigenes* ATCC 39243, is a member of the family of indolocarbazole antibiotics.[1] Rebeccamycin and the structurally related compound, staurosporin **2**, exhibit broad spectrum antitumor activity through their ability to inhibit topoisomerase I and II or protein kinase C, respectively.[2−5] The indolocarbazole framework is derived from two molecules of tryptophan.[6,7] Disruption of biosynthetic genes in *L. aerocolonigenes* and the structural characterization of putative pathway intermediates has provided insight into the order and mechanism of assembly of rebeccamycin.[8] However, only partial information is available about the enzymes involved in indolocarbazole biosynthesis. To date, in vitro work with purified enzymes has been limited to the first steps of rebeccamycin biosynthesis, namely, halogenation of tryptophan **4a** by RebH and the subsequent oxidative deamination of 7-chlorotryptophan **4b** by RebO, leading to 7-chloroindolepyruvic acid **6b** (Scheme 1).[9,10] Here we describe the characterization of RebD, the first report of an enzyme involved in formation of the bis-indole system.

The *rebD* gene encodes a protein of 1013 amino acid residues. RebD shows 54% sequence identity to the homologous enzyme from the staurosporin pathway (StaD) and 34% sequence identity to VioB that is involved in violacein **3** biosynthesis.[11,12] However, no other sequence in the available databases exhibits significant homology to RebD over the entire polypeptide. A putative heme binding motif ([FY]-[LIVMK]-X-X-H-P-[GA]-G) of the cytochrome b_5 family is located in the N-terminal region of the enzyme.[13] Typically, *b*-type cytochromes are coordinated noncovalently via the conserved histidine residue found within the sequence motif. A putative flavin-binding motif (G-X-G-X-X-G-X-X-X-[G/A] and GG doublet) was located in the central region of RebD.[14] The function of RebD is proposed to control formation of the central pyrrole ring through oxidative coupling of two tryptophan-derived substrates.[8,15,16] The identified sequence motifs match well with the chemistry required to perform this transformation as cytochromes are commonly involved in performing C−C coupling reactions. The flavin-binding domain would be a good candidate to relay electron equivalents between the heme and the ultimate oxidizing agent. His-tagged RebD was purified from recombinant *E. coli* cells and analyzed for the presence of heme and flavin cofactors. The presence of a *b*-type cytochrome cofactor could be substantiated through the absorption spectrum characteristics of RebD and also through heme-staining using a colorimetric assay (see Supporting Information). The latter analysis also demonstrated that the cytochrome cofactor is indeed noncovalently bound to the enzyme. However, neither fluorometric analysis, UV wavelength scanning, nor HPLC analysis revealed any evidence for the presence of a flavin cofactor co-purifying with heterologously expressed RebD.

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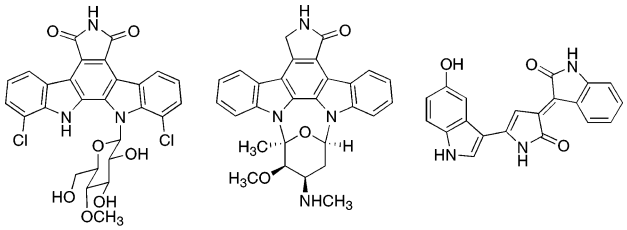
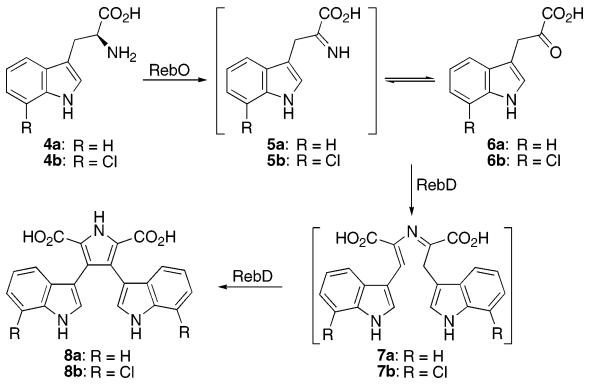


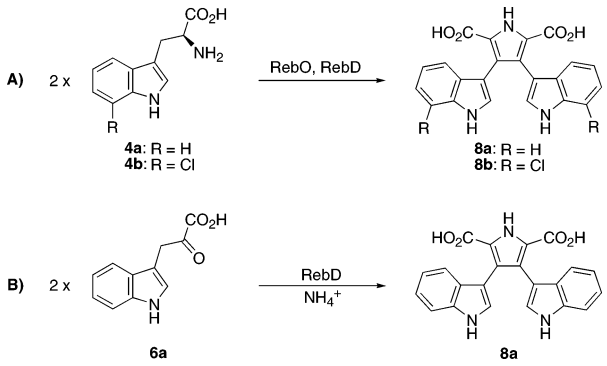
1 **2** **3**

Figure 1. Structures of rebeccamycin **1**, staurosporin **2**, and violacein **3**.

Scheme 1



Scheme 2



The in vitro activity of RebD was first investigated in a coupled assay together with RebO (Scheme 2A). RebO is purified with its flavin cofactor tightly bound when expressed in *E. coli* and does not require the addition of any other cofactors for turnover.[10] A typical reaction mixture contained 0.5−2 mM substrate, 0.5 μM RebO, 1 μM RebD, and 100 units of catalase. The reaction was allowed to proceed for 6−15 h, quenched through addition of aqueous HCl, and the reaction products were extracted with ethyl acetate. The products were analyzed by HPLC equipped with a photodiode array detector. When **4b** was incubated in the presence of RebO and RebD, a new compound was detected that exhibited absorption maxima at 223 and 270 nm. Purified product was

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subjected to electrospray mass spectrometry and showed a molecular ion peak at *m*/*z* 452.0 ($M-H^+$) consistent with chlorochromopyrrolic acid **8b** along with the expected isotope pattern. MS/MS analysis provided two fragment ions corresponding to loss of one or two molecules of CO_2. Similar MS and UV−vis spectrum results were previously reported for purified **8b**.[8] Notably, the reaction did not require the addition of any cofactors, such as flavin or nicotinamide. We have already demonstrated that RebO possesses a certain degree of substrate flexibility in that it also converts **4a** to **6a** (Scheme 1).[10] Therefore, we set out to investigate if the same flexibility was also observed for RebD in an analogous experiment to the one described above. Indeed, when tryptophan was incubated with RebO and RebD, a new compound was formed with the same absorption maxima as **8b** and a molecular ion peak of *m*/*z* 384.1 ($M-H^+$) consistent with chromopyrrolic acid **8a**. Furthermore, the compound was shown to be identical to a synthetic standard of **8a** (see Supporting Information). In contrast, no conversion of **4a** or **4b** by RebD was observed in the absence of RebO (data not shown), demonstrating that the amino acid cannot function as the sole substrate for RebD.

Next, we investigated the substrate specificity of RebD more directly using the pyruvic acid derivative **6a** (Scheme 2B). In the absence of an exogenously added nitrogen source, no new products were observed. However, when NH_4^+ was included in the reaction, **8a** was readily formed. Exchanging the nitrogen source to $^{15}NH_4^+$ resulted in the expected shift of the molecular ion peak from *m*/*z* 384.1 to 385.0 consistent with incorporation of ^{15}N. Thus, RebD on its own is fully competent to perform the condensation and the oxidative coupling leading to the central pyrrole moiety of **8**. Furthermore, no exogenous cofactors or enzymes are required to recycle the cytochrome *b* of RebD as would be usually the case for heme−iron enzymes. This experiment also demonstrates that RebD can incorporate NH_4^+ into the pyrrole ring of **8**. These results are consistent with observations made from precursor incorporation experiments that strongly indicated that the α-amino group from tryptophan did not significantly contribute to the phthalimide nitrogen of **1**.[6]

It is noteworthy that the results on the biosynthesis of rebeccamycin, obtained both from in vivo and also from the in vitro work described here, postulate fundamentally different mechanisms of bis-indole formation for the biosynthesis of rebeccamycin **1** and violacein **3** even though RebD and VioB are related enzymes. First, the central ring in **3** is formed with concomitant rearrangement of the carbon skeleton, but this is not the case in **1** or **2**.[6,17] Second, the nitrogen atom in the central ring in **3** is exclusively derived from the right-side tryptophan and not from an exogenous source as can be the case for rebeccamycin **1**.[6,18] However, our results obtained from the coupled enzymatic conversion with RebO and RebD do indicate that the nitrogen can be indirectly derived from tryptophan via the imine **5**.[19]

On the basis of the results described above, we propose the following mechanism for the pyrrole formation leading to chromopyrrolic acid **8**. The most likely substrate for RebD is either the indolepyruvic acid **6** or its imine derivative **5**. This is supported by the ability of RebD to catalyze the coupling of **6** to give **8** in the presence of NH_4^+ (Scheme 2B). Hence, the RebD-catalyzed pyrrole formation is a two-electron oxidation mediated through the *b*-type cytochrome cofactor with molecular oxygen as the final electron acceptor. As the reduction of O_2 to two molecules of H_2O requires four electrons, RebD might be able to perform two oxidative couplings from one molecule of O_2. However, experiments on oxygen consumption will have to be conducted in order to investigate this further. Furthermore, as no products were observed when RebD was reacted with **6** in the absence of NH_4^+, the condensation of **5** and **6** to give imine **7** is likely to precede the oxidative coupling as shown in Scheme 1.

To summarize, we have shown that RebD is responsible for all catalytic steps forming the central pyrrole ring of **8** from **6** (or **5**) without requiring any additional cofactors. This constitutes the first step of bis-indole formation in the biosynthesis of rebeccamycin **1** and most likely also in the biosynthesis of staurosporin **2**. Furthermore, it is probably at this stage that the rebeccamycin pathway diverges from the biosynthesis of violacein **3**, making RebD and its homologues (StaD and VioB) key enzymes in the biosynthetic pathways leading to indolocarbazole natural products.

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Supporting Information Available: Experimental procedures, analytical data, and complete ref 4. This material is available free of charge via the Internet at http://pubs.acs.org.

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