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Nonenzymatic Oxidative Steps Accompanying Action of the Cytochrome P450 Enzymes StaP and RebP in the Biosynthesis of Staurosporine and Rebeccamycin

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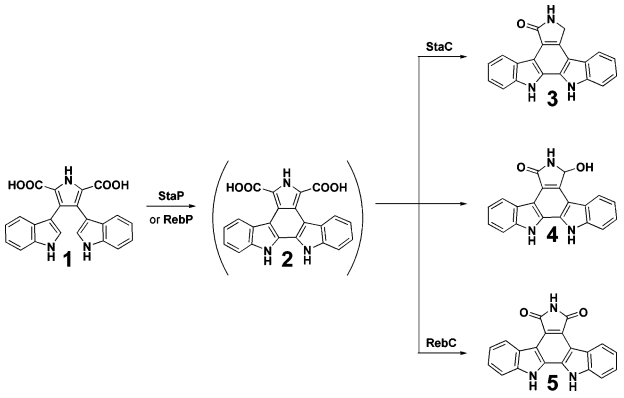
Biosynthesis of the antitumor indolocarbazoles rebeccamycin and staurosporine involves a complex series of oxidative coupling reactions, catalyzed by heme and flavin-based enzymes, starting from the common amino acid L-tryptophan.[1−3] Most intriguing among these is the four- to eight-electron oxidation of chromopyrrolic acid (CPA) **1** to the three aglycones, K252c **3**, 7-hydroxy-K252c **4**, and arcyriaflavin A **5**, which can be catalyzed by the single cytochrome P450 enzyme, StaP (or its homologue from the rebeccamycin pathway, RebP) (Scheme 1).[4] The presence of StaC enhances production of K252c **3** over other oxidation states of the aglycone, while RebC directs the preferential formation of arcyriaflavin A **5**. This activity is dependent on the presence of dioxygen.[4] Our previous work had suggested that aryl−aryl coupling preceded decarboxylation and oxidation of the pyrrole ring.[4] Hence, we postulated that the role of StaP/RebP may be simply to facilitate aryl−aryl coupling (a net two-electron oxidation), leading to the formation of 5,7-dicarboxyaglycone **2** (5,7-dicarboxy-12,13-dihydro-5*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole) (Scheme 2), with subsequent steps occurring spontaneously in solution.[4] In order to verify this assertion, here we explore a synthetic route toward the putative intermediate 5,7-dicarboxyaglycone **2** to assess its stability and its ability to convert to the aglycones **3**, **4**, and **5**. The presence of a spontaneous, nonenzymatic route from the aryl−aryl-coupled intermediate **2** to the aglycones would provide strong evidence that the singular role of StaP/RebP is to perform a two-electron aryl−aryl coupling reaction on CPA **1**. Although such aryl−aryl coupling catalysts are not uncommon in biosynthetic clusters, few have been studied in any detail.[5−8] We also probe the deuterium kinetic isotope effect of the StaP-mediated reaction to determine which of the aryl−aryl coupling or subsequent oxidative steps are rate-limiting.

The dimethyl ester of 5,7-dicarboxyaglycone was prepared by palladium-catalyzed aryl−aryl coupling[9] of the dimethyl ester of CPA (Supporting Information Scheme 1). This compound was easily isolable, and its identity was confirmed by NMR spectroscopic analysis. Upon deprotection in the presence of lithium hydroxide (in 1:1 methanol/water at room temperature), a suite of products were obtained, none of which corresponded to 5,7-dicarboxyaglycone **2**. The main two isolated products from this reaction mixture were 7-carboxy-K252c **7** and 7-carboxy-7-hydroxy-K252c **8**, as confirmed by NMR and mass spectroscopy (Scheme 2, Supporting Information), which were formed in a ratio of approximately 3:1. These data suggest that compounds **7** and **8** are the product of spontaneous reactivity of 5,7-dicarboxyaglycone **2** under mild aerobic conditions, and hence we suggest that they are representative of true intermediates formed in solution on reaction of StaP with CPA **1**.

We next sought to determine the reactivity of compounds **7** and **8**, either spontaneously or in the presence of StaP, StaC, or RebC,

Scheme 1. Overall Reaction Catalyzed by StaP/RebP, StaC, and RebC



to determine if they were in fact en route to the rebeccamycin and staurosporine aglycones, **3**, **4**, and **5**.

7-Carboxy-K252c **7** was highly unstable and converted spontaneously to two peaks by HPLC, the major peak corresponding to 7-hydroxy-K252c **4** and the minor one to arcyriaflavin A **5** (data not shown), which formed in a 2:1 ratio. This result was confirmed by mass spectroscopy: a clear peak at *m*/*z* 356, corresponding to the intact 7-carboxy-K252c **7**, was seen in the originally isolated sample; after 30 min in DMSO, this was replaced by the mass of 7-hydroxy-K252c **4** (*m*/*z* 328) (Scheme 2). Thus it seems that 7-carboxy-K252c **7** is directly en route to the two more oxidized aglycones (7-hydroxy-K252c **4** and arcyriaflavin A **5**), previously observed to form by StaP-mediated oxidation of CPA **1**. Interestingly, K252c **3** is not observed to form in the initial hydrolysis reaction nor in the reaction of 7-carboxy-K252c **7**, suggesting that spontaneous formation of this aglycone follows a divergent route.

The second product, 7-carboxy-7-hydroxy-K252c **8**, was found to be relatively stable under standard conditions at room temperature (Scheme 2). Furthermore, it was not a substrate for any combination of RebC, StaC, or StaP, as determined by HPLC analysis (data not shown). This more highly oxidized form of compound **7** therefore appears to be off-pathway with respect to aglycone formation in the rebeccamycin/staurosporine manifolds. That this apparently "dead-end" compound has not been isolated from StaP enzymatic assays suggests that it may not be formed in the StaP-catalyzed reaction.

These data suggest that aryl−aryl bond formation is indeed the only necessary enzyme-catalyzed step in the aglycone-forming reaction, with the remainder of the pathway occurring spontaneously. 7-Hydroxy-K252c **4** and arcyriaflavin A **5** form via the intermediacy of 7-carboxy-K252c **7**, whereas K252c **3** is likely formed via an alternative, less oxidized intermediate (Scheme 2). Therefore, we propose that K252c **3** derives from an intermediate

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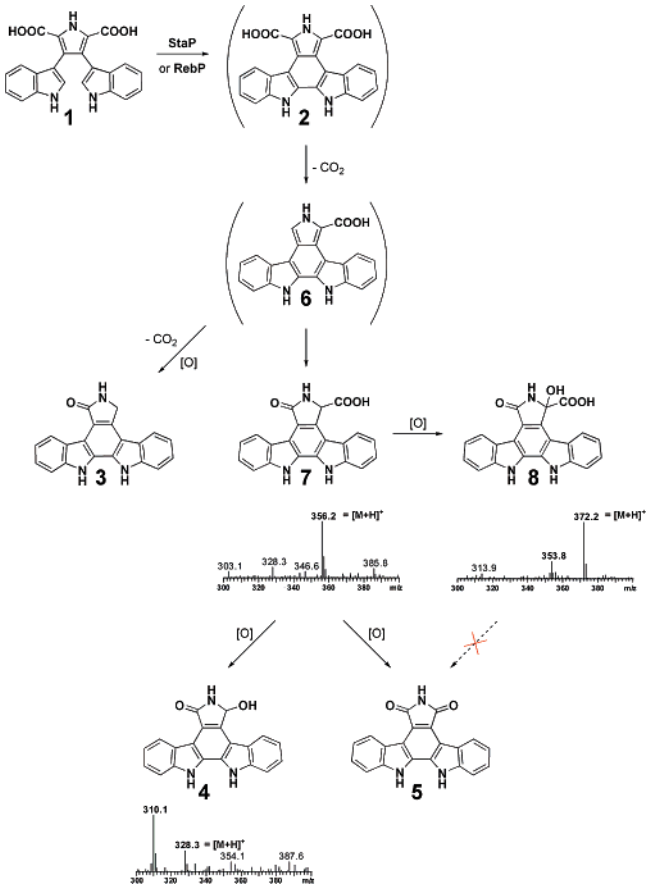
Scheme 2. Proposed StaP-Mediated and Spontaneous Steps in the Biosynthesis of Aglycones **3**, **4**, and **5**, Showing the Intermediacy of 7-Carboxy-K252c **7** and Divergence to Oxidized Off-Pathway Species, 7-Carboxy-7-hydroxy-K252c **8**



such as **6**, though this precursor has not been isolated. Nonetheless, it is apparent that 7-carboxy-7-hydroxy-K252c **8** is not an intermediate in the formation of aglycones **3**, **4**, or **5**. These data suggest a cascade of nonenzymatic decarboxylations and oxidations, totaling two to six electron oxidations, following the two-electron oxidative aryl−aryl coupling effected by the cytochrome P450 enzyme, StaP, or RebP (Scheme 2).

To further probe the manifold of the StaP-mediated reaction and its sequelae, we investigated if a deuterium kinetic isotope effect was manifested with fully ring-deuterated CPA. The StaP-mediated aryl−aryl coupling reaction likely occurs by P450-mediated hydrogen abstractions from the two indolyl C2 positions, with subsequent radical coupling giving intermediate **2**. If this is a rate-limiting step in the overall reaction, then the double C−D bond cleavage steps should lead to a significant kinetic isotope effect for deutero-CPA. Therefore, (indole-d_{10})-CPA was prepared enzymatically from (indole-d_5)-L-tryptophan using purified RebO and RebD according to a previously reported procedure (Supporting Information),[10] and its identity was confirmed by mass (m/z = 395, CPA mass + 10) and UV/visible spectroscopy and by HPLC coelution with an authentic protio-CPA standard **1**. Interestingly, the k_H/k_D rate ratio for the StaP-catalyzed reaction was not found to deviate substantially from unity when either K252c **3** or arcyriaflavin A **5** production was monitored (Table 1)—that is, there was no significant kinetic isotope effect. These results suggest that aryl−aryl coupling is not the rate-limiting step in the StaP-mediated formation of aglycones **3**, **4**, and **5**. Rather, subsequent decarboxylation and oxidation of the coupled scaffold, which we have determined to be spontaneous processes, are the rate-limiting stages of this transformation.

Table 1. Rates of Aglycone Production by StaP Using Protio-CPA (k_H) and (Indole-d_{10})-CPA (k_D) as Substrates

aglycone produced	k_H (min^{-1})	k_D (min^{-1})	k_H/k_D
K252c **3**	0.046 ± 0.001	0.048 ± 0.005	0.96 ± 0.11
arcyriaflavin A **5**	0.031 ± 0.002	0.033 ± 0.004	0.94 ± 0.14

The indolocarbazole scaffolds of the rebeccamycin and staurosporine natural products, and perhaps also the rearranged scaffolds of the violaceins[11,12] and chromoviridins,[13] arise by a set of enzyme-mediated oxidations using flavin and heme cofactors, admixed with autoxidative processes that reflect the instability of electron-rich pyrrole frameworks in aerobic environments. The results described in this report substantially simplify the role of StaP (and its homologue RebP) in mediating formation of a suite of aglycones by four- to eight-electron oxidation of CPA **1**. That is, StaP/RebP is simply responsible for the two-electron intramolecular aryl−aryl coupling of CPA **1** to give the six-ring intermediate **2**, with all subsequent steps occurring nonenzymatically in solution.

In the two enzyme StaP/StaC or StaP/RebC systems,[4] the partner flavoenzymes StaC and RebC likely act to intercept intermediate **2** or subsequent intermediates such as **6** or **7** formed en route to the aglycones (Scheme 2). Recent crystallographic work has identified density attributable to a tautomer of **7** in the active site of RebC.[14] Our current study on the nonenzymatic steps following StaP-mediated aryl−aryl bond formation corroborates the proposed role of RebC (and by extension StaC) in intercepting and redirecting intermediates en route to the aglycones, **3**, **4**, and **5**.

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Supporting Information Available: Experimental procedures and complete ref 6. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Sánchez, C.; Butovich, I. A.; Braña, A. F.; Rohr, J.; Méndez, C.; Salas, J. A. *Chem. Biol.* **2002**, *9*, 519−531.
(2) Onaka, H.; Taniguchi, S.-I.; Igarashi, Y.; Furumai, T. *Biosci., Biotechnol., Biochem.* **2003**, *67*, 127−138.
(3) Onaka, H.; Taniguchi, S.-I.; Igarashi, Y.; Furumai, T. *J. Antibiot.* **2002**, *55*, 1063−1071.
(4) Howard-Jones, A. R.; Walsh, C. T. *J. Am. Chem. Soc.* **2006**, *128*, 12289−12298.
(5) Funa, N.; Funabashi, M.; Ohnishi, Y.; Horinouchi, S. *J. Bacteriol.* **2005**, *187*, 8149−8155.
(6) Zhao, B.; et al. *J. Biol. Chem.* **2005**, *280*, 11599−11607.
(7) Zerbe, K.; Woithe, K.; Li, D. B.; Vitali, F.; Bigler, L.; Robinson, J. A. *Angew. Chem., Int. Ed.* **2004**, *43*, 6709−6713.
(8) Woithe, K.; Geib, N.; Zerbe, K.; Li, D. B.; Heck, M.; Fournier-Rousset, S.; Meyer, O.; Vitali, F.; Matoba, N.; Abou-Hadeed, K.; Robinson, J. A. *J. Am. Chem. Soc.* **2007**, *129*, 6887−6895.
(9) Harris, W.; Hill, C. H.; Keech, E.; Malsher, P. *Tetrahedron Lett.* **1993**, *34*, 8361−8364.
(10) Howard-Jones, A. R.; Walsh, C. T. *Biochemistry* **2005**, *44*, 15652−15663.
(11) Demoss, R. D.; Evans, N. R. *J. Bacteriol.* **1960**, *79*, 729−733.
(12) Hoshino, T.; Kondo, T.; Uchiyama, T.; Ogasawara, N. *Agric. Biol. Chem.* **1987**, *51*, 965−968.
(13) Momen, A. Z.; Mizuoka, T.; Hoshino, T. *J. Chem. Soc., Perkin Trans. 1* **1998**, 3087−3093.
(14) Ryan, K. S.; Howard-Jones, A. R.; Hamill, M. J.; Elliot, S. J.; Walsh, C. T.; Drennan, C. L. *Proc. Natl. Acad. Sci. U.S.A.*, in press.

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