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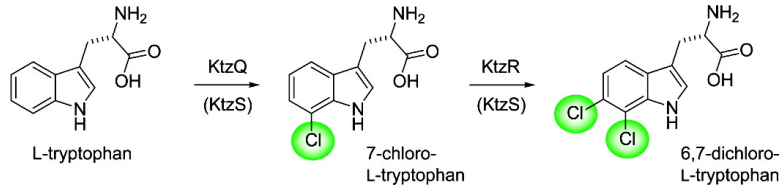

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Tandem Action of the O_2- and $FADH_2$-Dependent Halogenases KtzQ and KtzR Produce 6,7-Dichlorotryptophan for Kutzneride Assembly

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Kutznerides are antifungal nonribosomal hexadepsipeptides produced by the soil actinomycete *Kutzneria* sp. 744.[1] The macrolactone scaffold is assembled from six unusual building blocks. The lactone oxygen is derived from *tert*-butylglycolate while the five amino acid monomers are all nonproteinogenic. In some kutznerides, for example, kutzneride 2 (**1**) (Figure 1), there are two chlorinated residues, a tricyclic dihalogenated (2*S*,3a*R*,8a*S*)-6,7-dichloro-3a-hydroxy-hexahydropyrrolo[2,3-*b*]indole-2-carboxylic acid (diClPIC) and a γ-chloro-piperazate. In addition, we previously suggested that the unusual 2-(1-methylcyclopropyl)-D-glycine (MecPG) residue might arise by cryptic chlorination of an isoleucyl side chain, setting up subsequent closure to the β,γ-cyclopropane.[2,3] With the expectation that several halogenation events are operative in kutneride biosynthesis, the cognizant gene cluster in kutzneria was identified by degenerate primer-based PCR amplication through the use of $FADH_2$-dependent and nonheme Fe^{II}-dependent halogenase probes.[2] Bioinformatic analysis indicated a putative flavin reductase KtzS, two flavin-dependent halogenases KtzQ and KtzR and one mononuclear iron halogenase KtzD, the first example of both flavin and iron halogenase genes encoded together within the same gene cluster.

In parallel studies,[4] we have observed that the nonheme iron halogenase KtzD chlorinates isoleucine bound in thioester linkage to carrier protein KtzC, thus suggesting that KtzQ and KtzR could be responsible for the halogenation in the diClPIC and/or γ-chloro-piperazic acid residues. Comparison to other known flavin halogenases revealed KtzQ shares 60% identity with ThaL, a tryptophan 6-halogenase from *Steptomyces albogriseolus*,[5] whereas KtzR shares 54% identity with PyrH, a tryptophan 5-halogenase from *Streptomyces rugosporus*,[6] suggesting KtzQ and KtzR halogenate tryptophan prior to pyrroloindole formation. In this study, we validate that purified KtzQ and KtzR are tandem acting halogenases that catalyze the regiospecific dichlorination of L-tryptophan to produce 6,7-dichloro-L-tryptophan.

To evaluate the role of the two putative O_2- and $FADH_2$-dependent 50 kDa halogenases, the *ktzQ* and *ktzR* genes were amplified from genomic DNA isolated from *Kutzneria* sp. 744 and subcloned for translation as N-terminally His_6-tagged enzymes. Soluble protein (1.8 mg/L culture) was obtained for the KtzQ construct when heterologously expressed in and purified from *E.coli* BL21 (DE3), whereas heterologous expression in *Pseudeomonas putida* KT2440 was required to provide the KtzR construct in soluble form (1.1 mg/L culture). Both proteins were obtained in over 90% purity after Ni-NTA affinity and gel exclusion chromatographies (Supporting Information, Figure S1). The purified enzymes did not contain any cofactor with absorbance in the visible range of the spectrum. To provide the $FADH_2$ anticipated as substrate for the two halogenases, KtzS was expressed in *E. coli* BL21 (DE3), purified as the C-His_6-tagged construct, and shown to be an NADH-utilizing FAD reductase. The previously characterized *E. coli* NAD(P)H dependent flavin reductase SsuE was also prepared.[7] Assays for halogenation were conducted in the presence of soluble chloride ions with FAD, NADH, and excess KtzS or SsuE to generate the diffusible $FADH_2$ required for halogenase catalysis.

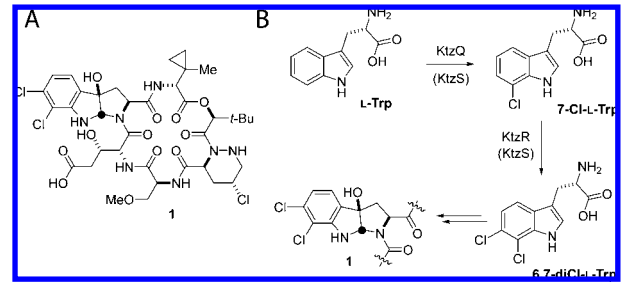

Figure 1. (A) Kutzneride 2, **1**; (B) formation of 6,7-dichloro-L-tryptophan (6,7-diCl-L-trp) during kutzneride biosynthesis.

KtzQ showed no activity for chlorination of piperazic acid or γ,δ-dehydropiperazic acid but was active when incubated with L-Trp (Figure 2a) and yielded a product that comigrated with authentic 7-chloro-L-tryptophan (7-Cl-L-Trp) by HPLC analysis. Mass analysis of the reaction product by ESI gave a pair of $[M - H]^-$ ions at m/z = 237.0 and 239.0 in a ~3:1 ratio, consistent with a monochlorinated Trp compound (Figure S2). Comparison of the ^1H NMR data of the product and authentic 7-Cl-L-Trp further confirmed the site of chlorination at position 7 of the indole ring (Figure S3). When authentic 6-chloro-L-tryptophan (6-Cl-L-Trp) was tested as substrate for KtzQ, a new peak appeared that coeluted with a chemically synthesized standard of 6,7-dichloro-L-tryptophan (6,7-diCl-L-Trp) (Figure 2b). ESI-MS analysis afforded three $[M - H]^-$ ions at m/z = 270.9, 272.9, and 274.9 in a ~10:6:1 ratio, consistent with a dichlorinated Trp compound (Figure S4).

To gain further insight into the substrate specificity of KtzQ, kinetic studies were undertaken with L-Trp and 6-Cl-L-Trp. At pH 7.0 under the coupled enzyme assay conditions (KtzS and KtzQ) the k_{cat} for L-Trp to 7-Cl-L-Trp is 0.19 min^{-1} while for 6-Cl-L-Trp to 6,7-diCl-Trp the k_{cat} is 0.07 min^{-1}. The K_m for both Trp and 6-Cl-Trp as substrates is $\leq 2\ \mu M$, at the sensitivity limit of the assay. From these results we conclude KtzQ is a regiospecific tryptophan-7-halogenase which has a preference for halogenation at the 7 position of unmodified L-Trp but will also take the 6-chloro-substrate.

KtzR in turn also chlorinated L-Trp and the halogenated product comigrated with authentic 6-Cl-L-Trp (Figure 2c). The identity of the monochlorinated reaction product was further confirmed by ESI-MS, yielding a pair of $[M - H]^-$ ions at m/z = 237.0 and 239.0 in a ~3:1 ratio (Figure S5). When presented with 7-Cl-L-Trp as a halogenation substrate, KtzR was again a regioselective 6-chlorination catalyst, yielding 6,7-diCl-L-Trp as product (Figure 2d). The

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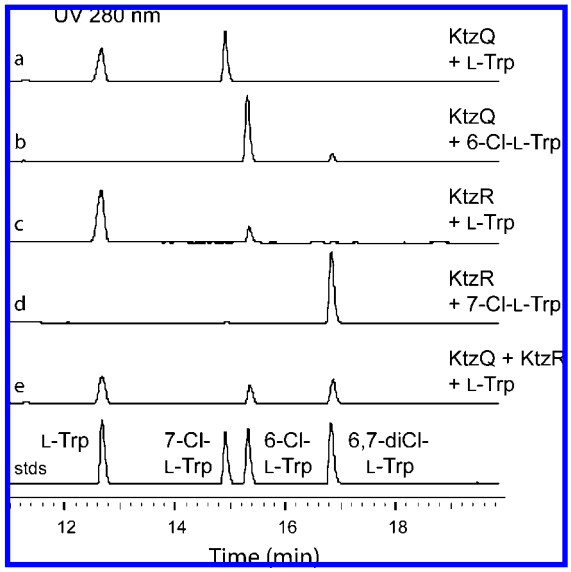

Figure 2. HPLC analysis of the KtzQ and KtzR catalyzed halogenations: (a) KtzQ incubated with L-Trp; (b) KtzQ incubated with 6-Cl-L-Trp; (c) KtzR incubated with L-Trp; (d) KtzR incubated with 7-Cl- L-Trp; (e) KtzQ and KtzR incubated with L-Trp.

product identity was also confirmed by ESI-MS ($[M - H]^-$ m/z = 270.9, 272.9, and 274.9) (Figure S6). Comparison of ^{1}H NMR data of the product and authentic 6,7-diCl-L-Trp confirmed the site of chlorination at position 6 of the indole ring (Figure S7). Remarkably, the catalytic efficiency for halogenation of position 6 of 7-Cl-L-Trp was better than for L-Trp itself. The k_{cat} ratio for 7-Cl-L-Trp/L-Trp as substrate (1.4 min^{-1}/0.08 min^{-1}) favors 7-Cl-L-Trp by ~18-fold, and the K_m value for 7-Cl-L-Trp at 114 μM is ~7-fold lower than for L-Trp at 808 μM, thus the presence of a chloride at the 7-position of the indole ring results in a ~120-fold increase in KtzR efficiency relative to umodified L-Trp. KtzR, like KtzQ, was not active for introduction of chloride to piperazic acid or γ,δ-dehydropiperazic acid.

Tandem incubations of L-Trp with KtzQ and KtzR along with the flavin reductase KtzS or SsuE and NADH and FAD yielded the 6,7-diCl-L-Trp product (Figure 2e). From these results and the individual enzyme kinetics, we conclude that the tandemly organized *ktzQ* and *ktzR* genes encode two $FADH_2$-dependent halogenases that work sequentially on free L-Trp in the order of KtzQ followed by KtzR to form 6,7-diCl-L-Trp (Figure 1B). It is then likely that 6,7-diCl-L-Trp is the monomer incorporated into the growing kutzneride assembly line by the KtzH adenylation domain. The final conversion to the diClPIC moiety in mature kutzneride is postulated to be achieved by epoxidation of the indole 2,3-double bond by the hemeprotein KtzM with intramolecular capture of the epoxide by the amide nitrogen.[2]

KtzQ is a regiospecific tryptophan-7-halogenase analogous to the enzymes RebH[8] and PrnA[9] that act in the first steps of rebeccamycin and pyrrolnitrin biosynthesis, respectively. The regioselectivity for introduction of chlorine at the 6-position of the indole ring of L-Trp and 7-Cl-L-Trp renders KtzR a homologue of ThaL[6] in the thienodolin pathway. ThaL has a turnover of 2.8 min^{-1} while the Trp-7-halogenases PrnA and RebH perform at 0.1 to 1.4 chlorinations per minute; these values are within range of those measured for KtzQ and KtzR. The k_{cat} values may be faster in vivo if enzyme complexes exist to channel the $FADH_2$ and resultant FAD(C4a)-OOH toward productive chlorination (via generation of HOCl, which is proposed to affect N-chlorination of an active-site lysine side chain to form lysine chloramine as the proximal halogenating species)[9c] versus competing autoxidation to FAD.

Whereas KtzQ is analogous to all of the previously characterized tryptophan halogenases in its prefence for unmodified L-Trp, KtzR is novel in that it has a ~120 fold prefence for 7-Cl-L-Trp over L-Trp. The substrate binding site in KtzQ is well conserved with PrnA, RebH, ThaL, and PyrH and all of the amino acids responsible for binding tryptophan are present. In contrast, two aromatic residues which stack against the indole ring of tryptophan in the PrnA and RebH structures, H101 and W455 in PrnA and H109 and W466 in RebH, are replaced with glutamine and leucine residues respectively in KtzR (Table S1). Structural analysis is warranted to determine if and then how these changes in the active site residues allow KtzR to differentiate 7-Cl-L-Trp from L-Trp.

In summary, our initial characterization of the three enzymes KtzQRS make it likely that the regioselective double halogenation in kutzneride biosynthesis occurs on free L-Trp by sequential action of the KtzQR pair. Kinetic characterization shows KtzQ acts first to chlorinate at the 7-position of L-Trp and then KtzR installs the second chlorine at the 6-position of 7-Cl-L-Trp to generate 6,7-diCl-L-Trp. Thus, while KtzD, Q, and R are now characterized halogenases, the chlorination of the piperazate moiety has not yet been accounted for and may require further genomic analysis of the *Kutzneria* producer.

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Supporting Information Available: Figures S1−S7, Table S1, detailed experimental procedures and spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

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