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Identification and Stereochemical Assignment of the β-Hydroxytryptophan Intermediate in the Echinomycin Biosynthetic Pathway

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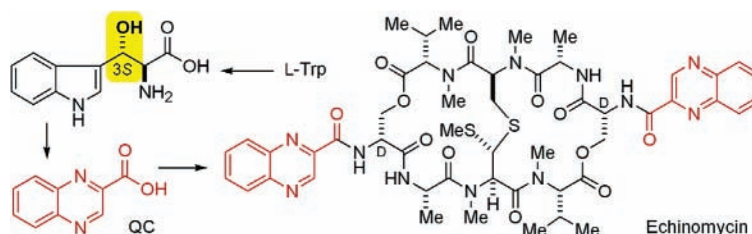
ABSTRACT



Little is known about how quinoxaline-2-carboxylic acid (QC) is synthesized in nature. On the basis of analysis of echinomycin biosynthetic gene clusters as well as feeding experiments with labeled precursors, we have proposed a biosynthetic pathway to QC and identified the (2*S*,3*S*)-β-hydroxytryptophan as a key intermediate.

Quinoxaline antibiotics are chromodepsipeptides produced by various species of streptomycetes that are widely distributed in nature.[1] Echinomycin (**1**, quinomycin A) and triostin A (**2**) are probably the best known members of the group exhibiting potent antibacterial, anticancer, and antiviral activities.[2] This class of antibiotics contains two quinoxaline-2-carboxyl moieties linked to a cross-bridged cyclic octapeptide dilactone core (Figure 1).[3] Echinomycin (**1**) is reported to be a strong inhibitor of transcription[4] as well as DNA synthesis,[5] which has been attributed to its ability to bind to double-stranded DNA sandwiching two base pairs within its U-shaped conformation.[6]

Apart from the biological investigation of quinoxaline antibiotics, the biosynthetic studies have been limited to feeding experiments with labeled amino acid precursors[7] and directed biosynthesis of the heteroaromatic carboxylic acids.[8] To gain a better understanding of the biosynthesis, we recently cloned the entire gene cluster (*ecm*) encoding the megaenzyme complexes responsible for the formation of **1**. On the basis of sequencing and homology analysis of the *ecm* cluster as well as preliminary biochemical experimenta-

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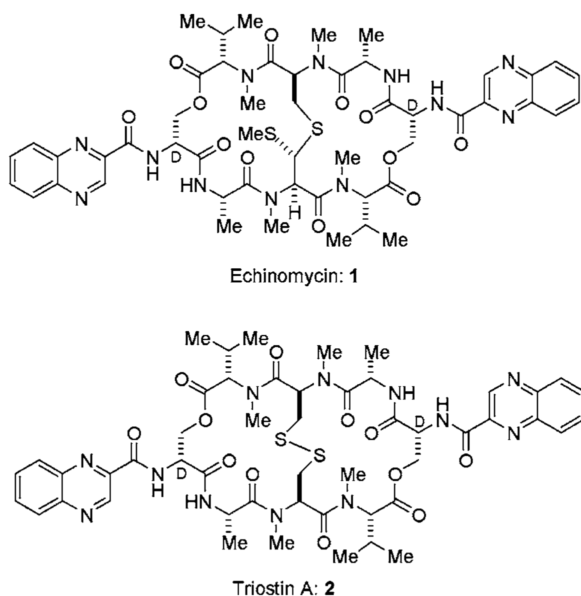

Figure 1. Structures of quinoxaline antibiotics.

tion, we predicted that the biosynthesis of quinoxaline-2-carboxylic acid (**12**, QC) involves eight genes (*ecm2−4, 8, 11−14*), whereas five genes (*ecm1, 6, 7, 17, 18*) encoding a modular nonribosomal peptide synthetase (NRPS)[9] are responsible for the peptide core synthesis and modifications (Figure 2). The NRPS assembly line lacks a carrier protein (ArCP) loading QC; instead, a fatty acid synthase acyl carrier protein (FabC) mediates the loading as well as the condensation of QC with serine, as previously reported for related biosynthesis of **2** by *Streptomyces triostinicus* and *Streptomyces echinatus.*[10] Having identified the gene cluster responsible for biosynthesis of echinomycin, we were able to introduce the 14 open reading frames (ORFs) from the cluster (*ecm1−4, 6−8, 11−14, 16−18*), *S. lasaliensis fabC* and *Bacillus subtilis* phosphopantetheine transferase gene *sfp* into *Escherichia coli* and successfully engineer complete biosynthesis of **1**.[11]

Although early precursor feeding experiments established that QC originates from L-tryptophan,[7a] little is known about how QC is synthesized in nature. The putative functions of each of the eight genes (*ecm2−4, 8, 11−14*) responsible for QC biosynthesis have led to the proposal of a biosynthetic transformation of **3** into **12** as illustrated in Figure 2.[12] First, Ecm13 exhibits homology with free-standing adenylation−peptidyl carrier protein (A-PCP) paired domains of NRPS subunits, which is surmised to activate **3** and transfer it to the HS-pant-PCP domain through thioester formation (**3** → **4**). Second, Ecm12 is responsible for the β-hydroxylation of the tryptophan intermediate (**4** → **5**). The resulting covalently tethered **5** is then hydrolyzed by a putative type II thioesterase (Ecm2)[9b,13] to liberate the free β-hydroxytryptophan **6**. Analogous to the metabolic pathway of tryptophan, oxidative cleavage of the indole 2,3-double bond by Ecm11 (**6** → **7**) and subsequent deformylation by Ecm14 would afford 3-hydroxykynurenine **8**. To provide a mechanistic rationale for the formation of the aryl−nitrogen bond between C5 and N4 in the quinoxaline ring, we currently hypothesized a novel oxidative rearrangement (**8** → **9**, via a spirocyclic intermediate) mediated by Ecm4. Finally, oxidation of the secondary alcohol of **9** by Ecm3 and subsequent cyclization (**10** → **11**) followed by spontaneous aromatization would furnish QC (**12**). Notably, our proposal is consistent with a previous report that nitrogen atoms at the 1- and 4-positions in QC have their origins in the indole and amino group of L-tryptophan, respectively.[7b]

As an initial step to verify the biosynthetic proposal, we directed our attention to the free β-hydroxytryptophan intermediate **6**. We assumed that **6** would have water-soluble and cell-penetrable properties and be an effective small-molecule probe for the precursor-directed biosynthetic studies. To date, detailed biosynthetic investigations of the β-hydroxylation of amino acids through an aminoacyl thioester intermediate have been limited to tyrosine[14] and histidine,[15] and little is known about the β-hydroxylation of tryptophan in the context of NRPS.[9,16−17] In this paper, a pair of diastereomers of the ^{2}H-labeled β-hydroxytryptophans have been synthesized. Precursor-directed biosynthetic studies on *Streptomyces lasaliensis* have identified the native biosynthetic intermediate and also established its relative and absolute configuration for the first time.

Synthesis of the β-hydroxyl tryptophans, (2*S*,3*R*)-**16** and (2*S*,3*S*)-**17**, commenced with the incorporation of a ^{2}H label at the indole C5 position via lithiation of a 5-bromoindole derivative (**18** → **19**) as shown in Scheme 1.[18,19] Stepwise installation of the α,β-unsaturated ester followed by protec-

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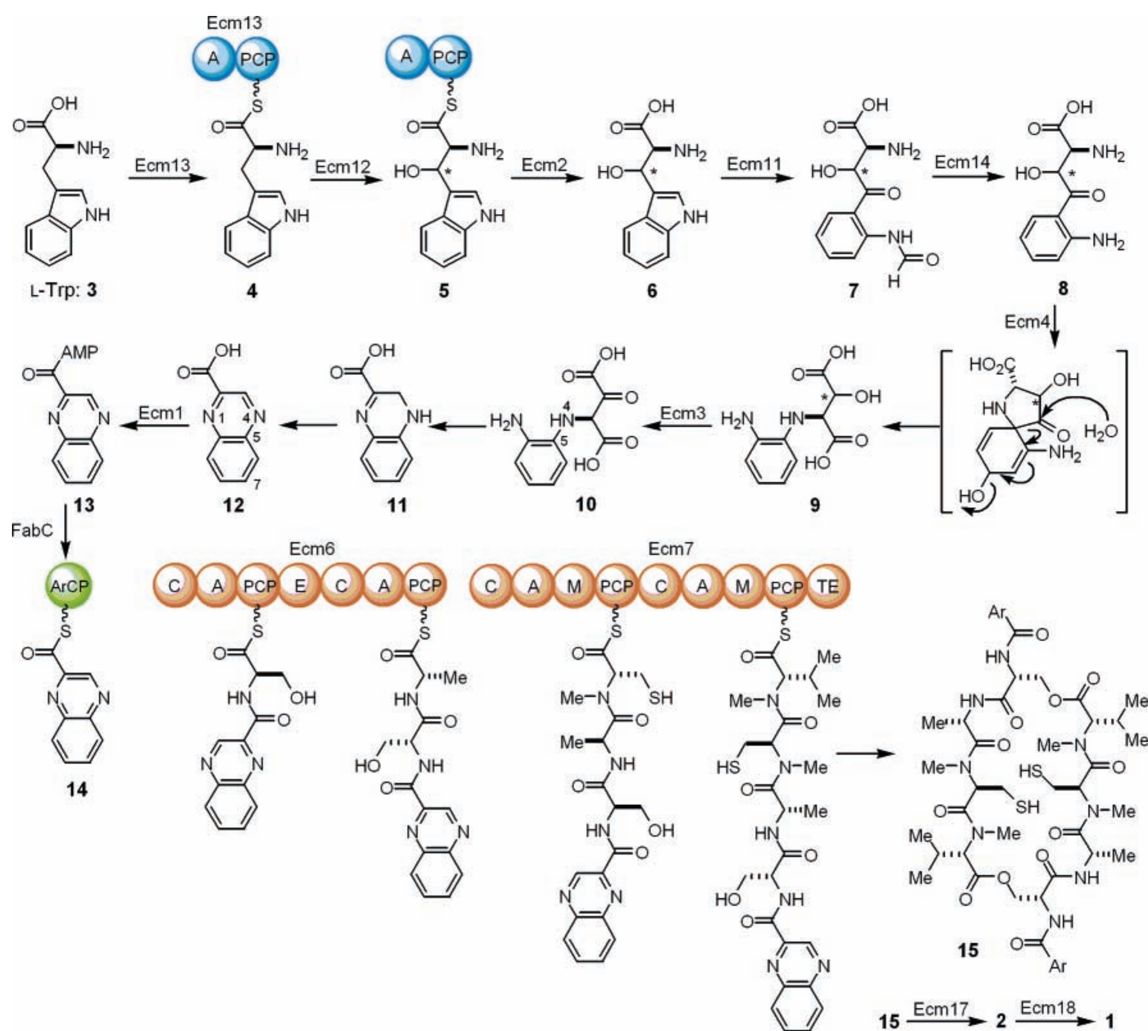

Figure 2. Proposed pathway for quinoxaline-2-carboxylic acid (QC) biosynthesis and model for the NRPS assembly line of echinomycin. Deduced functions (sequence homologues) of genes involved in echinomycin biosynthesis are as follows: *ecm1*, QC activation (peptide arylation enzyme *entE*); *ecm2*, QC biosynthesis (type II thioesterase *grsT*); *ecm3*, QC biosynthesis (isopropylmalate dehydrogenase *leuB*); *ecm4*, QC biosynthesis (FAD-dependent oxidoreductase *ubiH*); *ecm6*, NRPS module 1-2 (nonribosomal peptide synthetase *teiC*); *ecm7*, NRPS module 3-4 (nonribosomal peptide synthetase *acmC*); *ecm8*, unknown (MbtH-like protein *mbtH*); *ecm11*, QC biosynthesis (tryptophan 2,3-dioxygenase *tdo2*); *ecm12*, QC biosynthesis (cytochrome P450 oxidase *cypX*); *ecm13*, QC biosynthesis (mannopeptimycin peptide synthetase *mppB*); *ecm14*, QC biosynthesis (erythromycin A esterase *ereB*).

tion of the indole nitrogen yielded a common intermediate **20**. In efforts to synthesize **16**, we utilized the Sharpless catalytic asymmetric aminohydroxylation. Treatment of **20** with CbzNClNa in the presence of $K_2OsO_2(OH)_2$ and $(DHQD)_2AQN$ afforded C3-hydroxytryptophan derivative **21** in 45% yield and 78% ee,[20] which was subjected to a Pd-mediated hydrogenolysis to give **22**. Hydrolysis of **22** under basic conditions to liberate **16** was not a trivial step due to both the retro-aldol reaction and the epimerization. After considerable experimentation, conversion of **22** to **16** was finally realized by enzymatic hydrolysis using Amano protease P to produce **16** in excellent yield (96% for two steps).[21] Having a carefully controlled synthetic procedure for the fragile molecule, we then turned our attention to the synthesis of the other diastereomer **17**.[22] The Sharpless asymmetric dihydroxylation of **20** produced a diol product in 81% yield and >99% ee, which was then subjected to regioselective mono-*p*-nitrobenzenesulfonylation to afford **23** in 64% yield.[23] Nucleophilic substitution of **23** with NaN_3 and subsequent exposure with Pd/C under a H_2 atmosphere yielded **25**. Finally, enzymatic hydrolysis of **25** proceeded smoothly to produce **17** in good yield.

With a pair of the diastereomeric β-hydroxytryptophan derivatives, **16** and **17**, in hand, feeding experiments using *S. lasaliensis* were then investigated. The crude extracts from the culture broth were purified by silica gel chromatography

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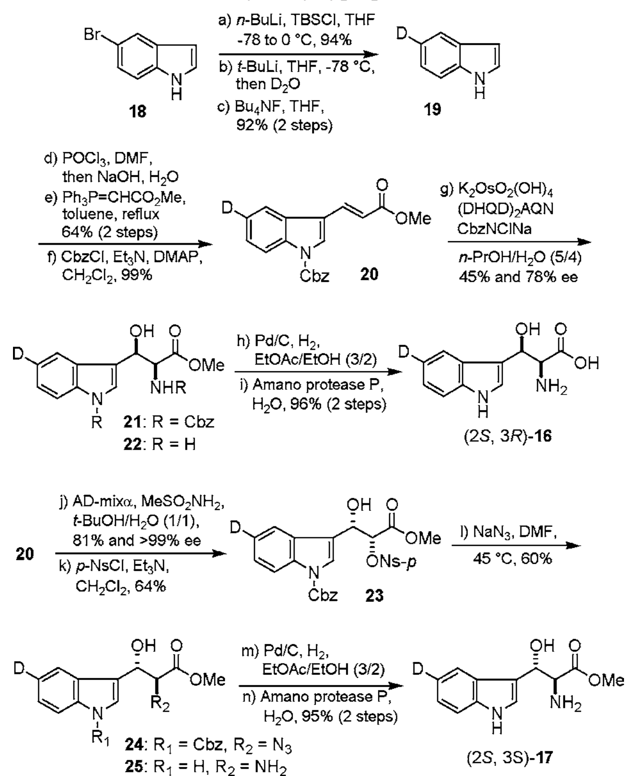
Scheme 1. Synthesis of a Diastereomeric Pair of 3-Hydroxytryptophans



to afford **1**. Analysis of the resulting **1** by ESI-MS as well as ^{1}H and ^{2}H NMR revealed that only **17** was integrated into **1** incorporating a deuterium label, whereas no integration of **16** was observed on the basis of the amount of ^{2}H-labeled **1** (Figures 3 and 4). We observed the integration of **17** into **1** through mass spectrometric analysis (Figure 3c), in which the major peak for **1** was shifted to 1124.35 corresponding to the expected **1** (calcd for $C_{51}H_{63}DN_{12}O_{12}S_2Na$ $[M + Na]^+$ 1124.41) having a single deuterium label. As shown in Figure 4, the ^{2}H NMR spectrum of a CH_2Cl_2 solution of the labeled **1** showed a broad peak at 7.8 ppm corresponding to deuterium at H7 of the quinoxaline ring. Thus, these experiments have not only identified the native biosynthetic intermediate but also established its relative and absolute configuration, **17**, as depicted in Figure 3. The strict stereochemical differentiation of the β-hydroxytryptophan intermediates lends support to the notion that the enzymatic hydroxylation of amino acids during the covalent tethering as aminoacyl-*S*-PCP intermediates is a general strategy in peptide antibiotic biosynthesis.[9b,16a] In addition, upon the administration of **17**, the incorporation efficiency exceeded 55% as determined from the ^{2}H NMR spectra with reference to natural abundance solvent peaks of CH_2Cl_2. This indicates that the substrate **17** is capable of penetrating the cell membrane and also of progressing to the site of biosynthesis in a highly efficient manner. Overall, the results reported here substantiate our current biosynthetic proposal. Further investigation to verify the downstream biosynthetic pathway to QC is currently underway in our laboratories.

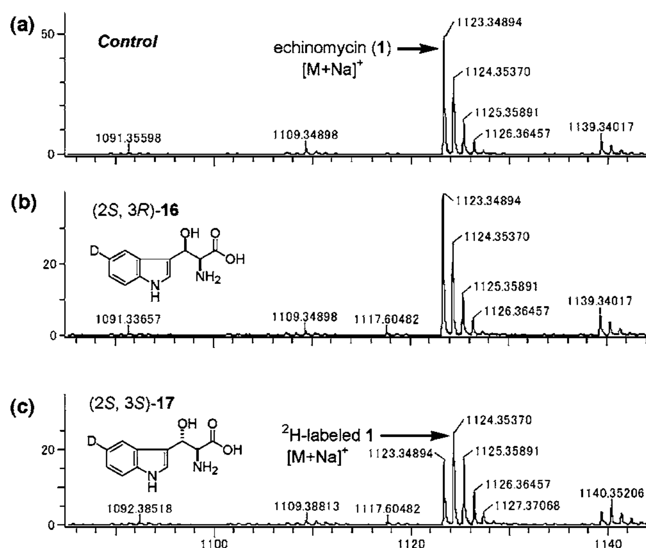


Figure 3. Comparative mass spectrometric analysis of echinomycins isolated from culture broth extracts administered with or without deuterium-labeled β-hydroxytryptophans. (a) Echinomycin standard. (b,c) Echinomycins obtained from the cultures of *S. lasaliensis* in the presence of **16** and **17**, respectively.

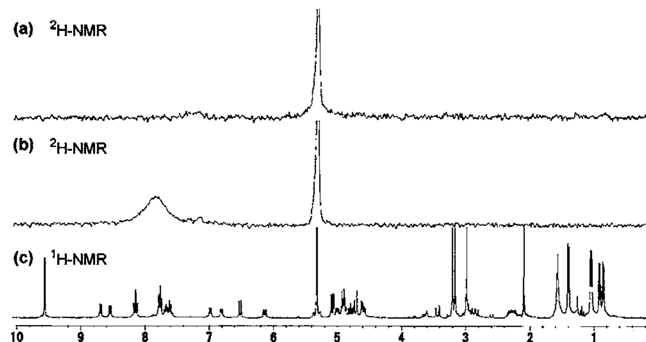


Figure 4. NMR analysis of echinomycins. (a,b) ^{2}H NMR spectra in CH_2Cl_2 of echinomycins obtained from the cultures of *S. lasaliensis* in the presence of **16** and **17**, respectively. (c) ^{1}H NMR spectrum in CD_2Cl_2 of the echinomycin standard.

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Supporting Information Available: Experimental procedures and spectroscopic data for **16**, **17**, **20**, **21**, **23**, and **24**. This material is available free of charge via the Internet at http://pubs.acs.org.

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