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(Biosynthesis of chromophores in quinomycin antibiotics)

Involvement of Common Intermediate 3-Hydroxy-L-kynurenine in Chromophore Biosynthesis of Quinomycin Family Antibiotics†

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Abstract

Quinomycin antibiotics, represented by echinomycin, are an important class of antitumor antibiotics. We have recently succeeded in identification of biosynthetic gene clusters of echinomycin and SW-163D, and have achieved heterologous production of echinomycin in *Escherichia coli*. In addition, we have engineered echinomycin nonribosomal peptide synthetase (NRPS) to generate echinomycin derivatives. However, the biosynthetic pathways of intercalative chromophores quinoxaline-2-carboxylic acid (QXC) and 3-hydroxyquinaldic acid (HQA), which are important for biological activity, were not fully elucidated. Here, we report experiments involving incorporation of a putative advanced precursor, (2*S*, 3*R*)-[6'-²H]-3-hydroxy-L-kynurenine, and functional analysis of the enzymes Swb1 and Swb2 responsible for late-stage biosynthesis of HQA. Based on these experimental results, we propose biosynthetic pathways for both QXC and HQA *via* the common intermediate 3-hydroxy-L-kynurenine.

Keywords: *Streptomyces lasaliensis*; *Streptomyces* sp; echinomycin; SW-163D; biosynthesis

† Dedicated to the late Dr. C. Richard Hutchinson for his exceptional contributions to natural product biosynthesis, engineering, and drug discovery.

INTRODUCTION

Quinomycin class antibiotics, represented by echinomycin (**1**)¹ and SW-163D (**2**),²⁻⁴ are C₂-symmetric cyclic depsipeptides with a pair of intercalative chromophores that show potent antibacterial, anticancer, and antiviral activities (Figure 1). These antibiotics have potent DNA binding affinities at a level between nM to mM with different sequence selectivities. Reflecting these distinct affinities, quinomycin antibiotics show different inhibitory activities against various enzymes, such as DNA polymerase, RNA polymerase, reverse transcriptase, and topoisomerase.⁵ Although thiocoraline has promising antitumor activity, most members of this family of compounds are toxic to mammalian cells.⁵ To improve the profiles between antitumor activity and toxicity, we studied the biosynthetic pathway of **1** and synthesizing its derivatives by genetic engineering. Recently, we have achieved *de novo* production of **1** using *Escherichia coli* as a heterologous host.^{6,7} This facilitated synthesis of echinomycin derivatives by chemoenzymatic synthesis with EcmTE⁸ and by genetic engineering of nonribosomal peptide synthetase (NRPS).^{9,10}

Quinomycin class peptide antibiotics have characteristic chromophores, which are essential components for their biological activities by bisintercalation into DNA. Among more than 20 congeners of this class of antibiotics, only two major chromophores—quinoxaline-2-carboxylic acid (QXC, **6**) and 3-hydroxyquinaldic acid (HQA, **8**)—have been identified (Scheme 1).⁵ Although biosynthetic studies of these chromophores have been performed for **1**^{11,12} and triostin¹³ and several gene clusters have been reported,^{6,9,14,15} little experimental data were obtained. Here, we report chromophore biosynthesis from L-tryptophan by unique pathway switching *via* the common intermediate (2*S*, 3*R*)-3-hydroxy-L-kynurenine (**5**).

MATERIALS AND METHODS

Chemicals: (2*S*, 3*S*)-[6'-²H]-3-hydroxy-L-tryptophan,¹² 3-hydroxyquinaldic acid¹⁶ and (2*S*, 3*R*)-3-hydroxy-L-kynurenic acid¹⁷ were synthesized as reported previously. All reagents commercially supplied were used as received. Optical rotations were recorded on JASCO DIP-360 digital polarimeter. NMR spectra were obtained on JEOL ECP-300 and JEOL α -400 spectrometers for solution of CD₃OD. ¹H and ¹³C chemical shifts were referenced to the solvent signals: 3.30 and 49.0 ppm for CD₃OD. Mass spectra were recorded on JEOL JMS-T100CS (ESI). Flash column chromatography was performed

using Kanto Silica Gel 60N.

Bacterial Strains, Media, and Culture Conditions

Streptomyces lasaliensis ATCC 35851 and *Streptomyces* sp. SNA15896 was grown at 30 °C on R2YE medium containing 0.4% maltose, 1.0% malt extract (Difco) and 0.4% yeast extract (Difco). All *E. coli* strains BL21 (DE3) (Invitrogen) and Rosetta (Novagen) in this study were grown following standard protocols.

(2*S*, 3*R*)-[6'-²H]-*N*-formyl-3-hydroxy-*L*-kynurenine (4a)

A stirred solution of (2*S*, 3*S*)-[6'-²H]-3-hydroxy-*L*-tryptophan (**3a**, 100 mg, 0.45 mmol) in MeOH (30 mL) was treated with ozone at -78 °C for 25 min. Excess dimethylsulfide (1 mL, 13.6 mmol) was added, and stirring was continued to room temperature. The volatile was removed in vacuo and the resultant residue was purified by HPLC (20% CH₃CN/0.1% HCOOH in H₂O, 0 – 10 min, 30%; 10 – 30 min, linear gradient 30 – 50%; 30 – 35 min, 50%; UV at 200 nm, 2 ml/min, 30 °C) with a column (Inertsil ODS-3, φ 3.0 x 250 mm, GL Science) to give **4a** (56.4 mg, 53%) along with an identified by-product (28 mg). **4a**: a colorless oil. ¹H-NMR (300 MHz, CD₃OD) δ 8.47 (1H, d, *J* = 8.3 Hz), 8.38 (1H, s), 7.84 (1H, s), 7.57 (1H, d, *J* = 8.3 Hz), 5.38 (1H, d, *J* = 3.4 Hz), 3.99 (1H, d, *J* = 3.4 Hz); ¹³C NMR (75 MHz, CD₃OD) δ 201.6, 170.3, 162.3, 139.0, 134.7, 132.0, 130.2, 125.7, 123.5, 73.2, 58.6; HRMS (ESI) calcd. for C₁₁H₁₂DN₂O₅ [M + H]⁺ 254.0886; found 254.0839; [α]_D²⁷ -14.0 (*c* 0.5, MeOH).

(2*S*, 3*R*)-[6'-²H]-3-hydroxy-*L*-kynurenine (5a)

To a stirred solution of amide **4a** (56 mg, 0.23 mmol) in H₂O (10 mL) was added trifluoroacetic acid (0.5 mL, 6.6 mmol) at room temperature. The mixture was stirred for 1 hr, and then lyophilized to give a residue which was purified by HPLC under the same conditions as **4a** to afford **5a** (50.5 mg, 95%). **5a**: an amorphous solid; ¹H-NMR (300 MHz, CD₃OD) δ 7.58 (1H, s), 7.25 (1H, d, *J* = 8.4 Hz), 6.73 (1H, d, *J* = 8.4 Hz), 5.43 (1H, d, *J* = 3.1 Hz), 4.06 (1H, d, *J* = 3.1 Hz); ¹³C-NMR (75 MHz, CDCl₃) δ 198.3, 170.6, 135.7, 130.3, 124.4, 122.3, 119.3, 118.3, 70.5, 57.6; HRMS (ESI) calcd. for C₁₀H₁₂DN₂O₄ [M + H]⁺ 226.0937; found 226.0902; [α]_D²⁷ +18.5 (*c* 0.08, H₂O).

Feeding Experiments with (2S, 3R)-[6'-²H]-3-hydroxy-L-kynurenine (5a)

Sterile R2YE medium (100 mL) was inoculated with spore suspension (H₂O, 5 ml) of *Streptomyces* sp. SNA15896 from a 7 day R2YE plate. The culture was incubated at 30°C and 130 rpm. On the third day after inoculation, the aqueous solution (5 mL) of 20 mg of isotopically labeled **5a** filtered through sterilized microfilter (0.22 µm) was added into the culture and incubation was continued for further 4 days. The cells were harvested by centrifugation at 5,000 x g. The resultant supernatants was extracted with EtOAc. Acetone extracts of the mycelia were concentrated and extracted with EtOAc. Both extracts from the supernatants and the mycelia were combined and concentrated in vacuo to yield the residue which was dissolved into MeOH (2 ml). The extract filtered with microfilter (0.22 µm) was directly analyzed by LC-ESI-MS (ESI positive mode) (ZORBAX XDB-C18, Agilent, φ 2.1 x 50 mm); 0.2 mL/min, 30 °C, solvent system, CH₃CN/0.1% TFA in H₂O, 0 – 5 min, 20%; 5 – 25 min, linear gradient 20 – 100%; 25 – 30 min, 100%.

Similar feeding experiment was employed to *S. lasaliensis* in the essentially same manner. Labeled precursor was fed on the second day after inoculation and incubation was continued for further 4 days.

Cloning, expression and purification of Swb1

The *swb1* gene was obtained by PCR amplification with SP-taq polymerase (LaboPass) using a genomic DNA from *Streptomyces* sp. SNA15896. Primers for *swb1* (*swb1*-Fw: 5'-AAAGAGAACCATATGGCAGCATCGGCGCCAG-3' and *swb1*-Rv: 5'-TTTGAGCTCGATGGCGACGCGCCGCTT-3') contained *Nde*I and *Xho*I sites (underlined, restriction endonuclease site) and the amplified gene was cloned into the corresponding sites of pET28b vector (Novagen) to yield pYHSwb1 for the production of Swb1. *E. coli*. BL21 (DE3) transformed with pYHSwb1 was incubated at 37 °C overnight in 10 mL LB medium supplemented with kanamycin (100 µg/mL). Each liter of fresh LB medium with the same antibiotics at the same concentration as described above was inoculated with 5 mL of the overnight culture and incubated at 37 °C until the optical density at 600 nm reached 0.6. Then, expression of *swb1* gene was induced with 100 µM IPTG at 15 °C. Incubation was continued for another 24 h, after which cells were harvested by centrifugation at 2,500 x g. All subsequent procedures were performed at 4 °C or on ice. Harvested cells were resuspended in disruption buffer [0.1

M Tris-HCl (pH 7.2) and 20% (v/v) glycerol]. After addition of PLP (a final concentration 10 μ M), cells were disrupted by a French Press cell, and the lysate was clarified by centrifugation at 18,600 x *g*. The soluble fraction containing protein was applied to a Ni-NTA affinity column (GE Healthcare) previously equilibrated with binding buffer [0.1 M Tris-HCl (pH 7.2), 10 μ M PLP and 20% (v/v) glycerol] supplemented with 10 mM imidazole, at a flow rate of 1 mL/min. The column was washed with the binding buffer with 10 mM imidazole, then proteins were eluted with a gradient of 10 to 500 mM imidazole over 100 mL of the binding buffer. Fractions containing protein with target molecular weight were pooled. Buffer exchange into the reaction buffer (100 mM Tris-HCl, 20% (v/v) glycerol, pH 7.2) was carried out using a PD-10 column (GE Healthcare). Protein concentration was estimated using the Bio-Rad protein assay kit with bovine immunoglobulin G as a standard. Purified protein samples were analyzed by SDS-PAGE.

Cloning, expression and purification of Swb2

The *swb2* gene was obtained by PCR amplification with primers (swb2-Fw: 5'-AAAAGTGCGCATATGAGTGTGTCCGGCAAG -3' and swb2-Rv: 5'-TTTGAGCTCGCAGCAAGACGGCGG -3') contained *Nde*I and *Xho*I sites (underlined, restriction endonuclease site) and the amplified gene was cloned into the corresponding sites of pET28b vector (Novagen) to yield pYHSwb2 for the production of Swb2. The procedure from transformation to purification of Swb2 was same as that of Swb1 except use of *E. coli* (DE3) Rosetta cell in transformation and absence of PLP in the buffers during purification.

Enzymatic reactions with Swb1 and Swb2

Swb1 assay was performed in a total volume of 100 μ L in Tris-HCl buffer (100 mM, pH 7.2) with 14 μ M Swb1, 10 μ M PLP and 100 μ M α -ketoglutarate at 30 °C for 12 h. The substrate concentration was 100 μ M for 3-hydroxy-L-kynurenine (**5**) and L-kynurenine. Reactions were terminated by addition of MeOH (100 μ L) and the resultant mixture was vortexed and centrifugated at 13,000 x *g* for 2 min. After concentration of the supernatant using centrifugal evaporator, the residue was dissolved with water (100 μ L). The reaction products were analyzed by LC-ESI-MS (ESI positive mode) (Wakosil II 5C18, ϕ 2.1 x 50 mm); 0.2 mL/min, 30 °C, solvent system,

CH₃CN/0.1% TFA in H₂O, 0 – 5 min, 0%; 5 – 15 min, linear gradient 0 – 100%; 15 – 20 min, 100%. Swb2 assays were performed in a total volume of 100 μ L in Tris-HCl buffer (100 mM, pH 7.2) with 7 μ M Swb2 and 100 μ M NADPH at 30 °C for 12 h. The substrate concentration was 100 μ M for 3-hydroxykynurenic acid (**7**) and kynurenic acid. Work-up and analytical conditions were performed in the same manner as in the experiment with Swb1.

RESULTS

Bioinformatics analysis of quinomycin family antibiotics

In the biosynthetic study of echinomycin (**1**) and triostin, feeding experiments with isotope-labeled amino acids established that the QXC moiety was derived from L-tryptophan¹¹ and that the N1 and N4 of QXC were derived from the indole and amino groups of L-tryptophan, respectively.¹³ Essential genes for echinomycin biosynthesis have been identified by *de novo* production of **1** using *E. coli* as a heterologous host.⁶ Exclusion of genes for backbone biosynthesis, modification, regulation, and self-resistance leaves six genes suggested to be involved in the biosynthesis of QXC. Successful incorporation of (2*S*,3*S*)-[6'-²H]-3-hydroxy-L-tryptophan (**3a**) supported the intermediacy of **3**, indicating that β -hydroxylation of tryptophan involves covalent attachment to L-tryptophan to the ACP domain of the NRPS (Ecm13), hydroxylation with cytochrome P-450 monooxygenase (Ecm12), and release of the hydroxylated product by the thioesterase (Ecm2),¹² as in the case of tyrosine (novobiocin)¹⁸ and histidine (nikkomycin).¹⁹ Recently, four gene clusters of quinomycin-type antibiotics (Figure 1, **1**, **2**, triostin A and thiocoraline) have been identified,^{6,9,14,15} all of which share four homologous genes (identity %) [*ecm2/trsB/swb14/tioQ* (65% – 75%), *ecm11/trsC/swb10/tioF* (30% – 72%), *ecm12/trsB/swb13/tioI* (64% – 80%), and *ecm13/trsR/swb11/tioK* (58% – 76%)], which were proposed to be responsible for chromophore biosynthesis. While the clusters of **1** and triostin A consist of homologous genes *ecm3/trsP* (80%) and *ecm4/trsO* (72%) probably responsible for QXC biosynthesis,⁶ the clusters of **2** and thiocoraline have alternative unique genes *swb1/tioG* (57%) and *swb2/tioH* (58%) probably involved in HQA biosynthesis,⁹ as shown in Scheme 1. Among the four common genes for echinomycin chromophore biosynthesis, the putative function (L-tryptophan 2,3-dioxygenase) of the remaining gene *ecm11* suggested that 3-hydroxy-L-kynurenine (**5**) is a common intermediate in the

chromophore biosynthesis.

Feeding experiments of (2*S*,3*R*)-[6'-²H]-3-hydroxy-L-kynurenine (**5a**)

To confirm the intermediacy of **5**, feeding experiments with (2*S*,3*R*)-[6'-²H]-3-hydroxy-L-kynurenine (**5a**) were employed. The synthesis of **5a** began with [6'-²H]-3-hydroxy-L-tryptophan (**3a**).¹² Ozonolysis of **3a** afforded formamide **4a**, which was hydrolyzed with trifluoroacetic acid to give **5a** in 50% yield (2 steps). The resulting labeled putative precursor **5a** was administered to the echinomycin-producing strain *Streptomyces lasaliensis*. LC-MS analysis of echinomycin (**1**) thus obtained showed significant increases in 1 and 2 Da-shifted peak intensities at *m/z* 1102 [*d*₁-MH⁺] (41%) and 1103 [*d*₂-MH⁺] (25%) compared with the natural abundance peak at *m/z* 1101 [MH⁺] (Figure 2A). The data indicated that **5a** was incorporated into both QXC moieties of **1** in a highly efficient manner. Similarly, feeding of **5a** to *Streptomyces* sp. SNA15896 gave monodeuterated and dideuterated SW-163D, as evidenced by the observation of the peaks at *m/z* 1128 [*d*₁-MH⁺] (22%) and 1129 [*d*₂-MH⁺] (2%) were increased (Figure 2B). In this case, only one of the HQA moieties was incorporated into **2**, probably due to significant dilution of the labeled precursor prior to the dimerization step of the monomeric tetrapeptide precursor. These results from feeding experiments supported the suggestion that **5** is a common intermediate in the biosyntheses of both QXC and HQA.

Cloning, expression, and purification of Swb1 and Swb2

Based on the results of the bioinformatics analyses described above, we speculated that two gene products, Swb1 (aminotransferase) and Swb2 (oxidoreductase), were involved in the transformation of 3-hydroxy-L-kynurenine (**5**) to HQA. Thus, the last two steps are 1) transamination of **5** and concomitant imine formation giving 3-hydroxykynurenic acid (**7**); 2) the subsequent reduction and concomitant dehydration giving HQA (**8**), as shown in Scheme 1. To obtain direct support of this proposal, enzymatic conversion of **5** to **8** was examined.

Both *swb1* and *swb2* genes were amplified by PCR from genomic DNA. Amplicons were purified, digested with *Nde*I/*Xho*I, and then inserted into the pET28b vector with incorporation of an *N*-terminal His₆ tag. The resulting plasmids, pYHSwb1 and pYHSwb2, were used to transform *E. coli* BL21 (DE3) and Rosetta strains, respectively. The recombinant Swb1 and Swb2 were successfully expressed in *E. coli* strains at 15 °C as soluble proteins and purified by Ni-chelating affinity chromatography.

SDS-PAGE analysis indicated that the recombinant proteins obtained are major proteins (Figure 3).

Enzymatic activity of Swb1 and Swb2 and identification of the enzymatic reaction products

Enzyme assay of the putative aminotransferase Swb1 with **5a** was conducted in the presence of PLP and α -ketoglutarate. In LC-MS analysis of the reaction products, a novel peak was observed on selective ion monitoring (SIM) at m/z 207. Comparison of the enzymatic product with a synthetic standard **7** in LC-MS analysis, we concluded that the product was identical to **7** (Figure 4A). The second enzymatic reaction of the putative oxidoreductase Swb2 was carried out on **7** in the presence of NADPH. Again in LC-MS analysis, a new peak appeared at 12.7 min on monitoring m/z 190. This peak was identical to synthetic **8** (Figure 4B). Although Swb1 also converted from kynurenine (**9**) to kynurenic acid (**10**) (Figure 4C), Swb2 did not give any new peaks using **10** as a substrate. These results indicated that **5** is an intermediate in the biosynthesis of **8**.

DISCUSSION

We performed a feeding experiment with [6'-²H]-3-hydroxy-L-kynurenine (**5a**) to strains producing echinomycin and SW-163D. Efficient incorporation of **5a** confirmed its intermediacy in the biosynthesis of both chromophores, QXC **6** and HQA **8**. Intermediacy of **3** and **5** was further supported by the results of bioinformatics studies indicating that common gene homologs (*ecm2/trsB/swb14/tioQ*, *ecm11/trsC/swb10/tioF*, *ecm12/trsB/swb13/tioI*, and *ecm13/trsR/swb11/tioK*) for β -hydroxylation of L-tryptophan and oxidative cleavage of indole were found in the gene clusters of four quinomycin family members. *In vitro* functional analysis of enzymes Swb1 and Swb2 established conversion of **5** to HQA **8**.

In a previous study of the biosynthesis of thiocoraline, Salas et al. proposed that kynurenine (**9**) derived from L-tryptophan with TioF (L-tryptophan-2,3-dioxygenase) and TioL (aryl formamidase) is an intermediate that is further transformed with TioH (oxidoreductase) and TioI (cytochrome P-450 monooxygenase) to HQA **8**.¹⁵ Tsodikova et al. reported functional analysis of TioF, which converted tryptophan to **9**, supporting the intermediacy of **9**.²⁰ On the other hand, we unambiguously established enzymatic conversion of **5** to HQA **8** with Swb1 (TioG

homolog) and Swb2 (TioH homolog). In addition, our data in the feeding experiment and bioinformatics analysis strongly suggest that cytochrome P-450 monooxygenase homologs Ecm12/TrsB/Swb13/TioI are involved in hydroxylation of L-tryptophan but not that of quinaldic acid. These data did not fit Salas' proposed pathway. We speculated that both 2,3-dioxygenase homologs Swb10 and TioF can catalyze oxidative cleavage of not only tryptophan but also **3**, indicating functional analysis of TioF is not conclusive evidence on the intermediacy of **9**.

Analysis of chromophore biosynthesis can yield insight into one of the strategies by which microorganisms create novel pathways for secondary metabolites. Natural peptides often incorporate hydroxylated amino acids.^{18,19,21,22} In chromophore biosynthesis, the producing strains utilize similar β -hydroxylation system consisting of cytochrome P-450, NRPS, and discrete thioesterase for conversion of L-tryptophan to **3**.^{18,19} Chromophore biosynthetic clusters for **1** and **2** consist of genes encoding enzymes for tryptophan metabolism, such as kynurenic acid²³ and nicotinic acid,^{24,25} which are presumably different from the constitutive enzymes (Scheme 2). After conversion of **3** to **5** with tryptophan-2,3-dioxygenase homolog, this common intermediate is transformed into either **7** with aminotransferase homolog or rearranged product with monooxygenase homolog (Scheme 1). Final transformations to chromophores **6** and **8** are catalyzed by specific redox enzymes such as Ecm3 and Swb2. Therefore, the strategy for creating novel pathways can be achieved by 1) incorporation of modification enzymes transforming a common precursor to an unusual precursor; 2) duplication of existing genes and mutation to change function; 3) recruiting additional enzyme to optimize target structure.

Although arylformamidase is an essential enzyme for kynurenine biosynthesis, the corresponding gene is not found in any of the four quinomycin family gene clusters. Initially, *tioL* and *ecm14* (*trsF*) were proposed to catalyze this reaction^{6,15} but low sequence homology and absence of the *N*-formamidase gene in the SW-163 gene cluster suggested lack of this gene in all quinomycin gene clusters. In echinomycin biosynthesis, the gene for the aryl carrier protein for loading of QXC chromophore is not present on the gene cluster but FabC involved in the fatty acid biosynthesis is acted as an alternative acyl carrier protein.²⁶ Therefore, arylformamidase in the biosynthesis of nicotinic acid pathway may be used as an alternative.

We have already established the *de novo* synthesis of echinomycin by

introducing three plasmids carrying NRPS genes, QXC biosynthetic genes and other auxiliary genes into *E. coli*. The findings presented in this paper will lead to switching of the chromophore from QXC to HQA by exchanging two genes, *i.e.*, *ecm3-ecm4* to *swb1-swb2*. This will add options for diversification of quinomycin skeleton with engineering of biosynthetic pathways.^{27,28}

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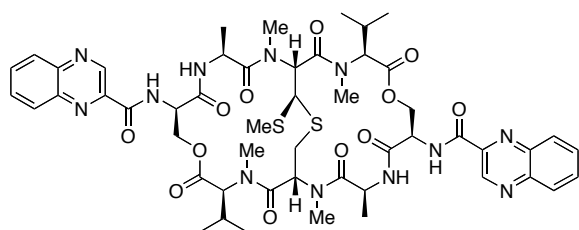
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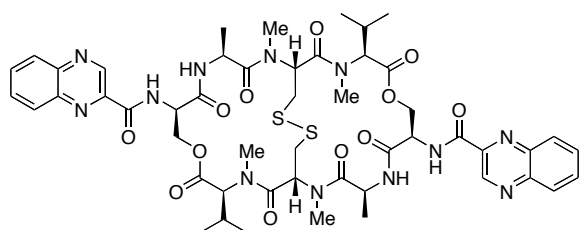
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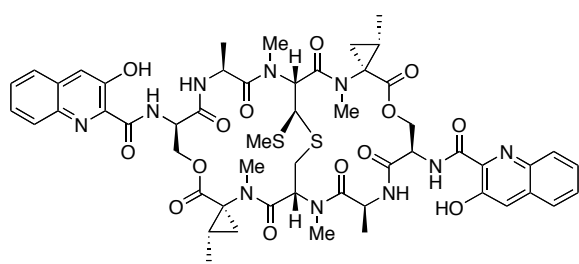
Legends for Figures and Schemes



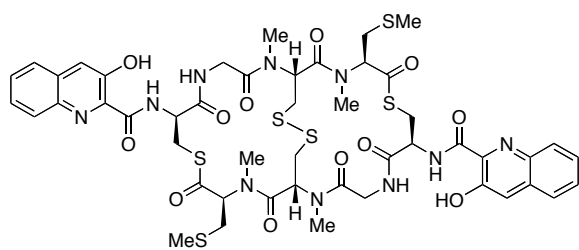
echinomycin (1)



triostin A



SW-163D (2)



thiocoraline

Figure 1. Quinomycin family antibiotics.

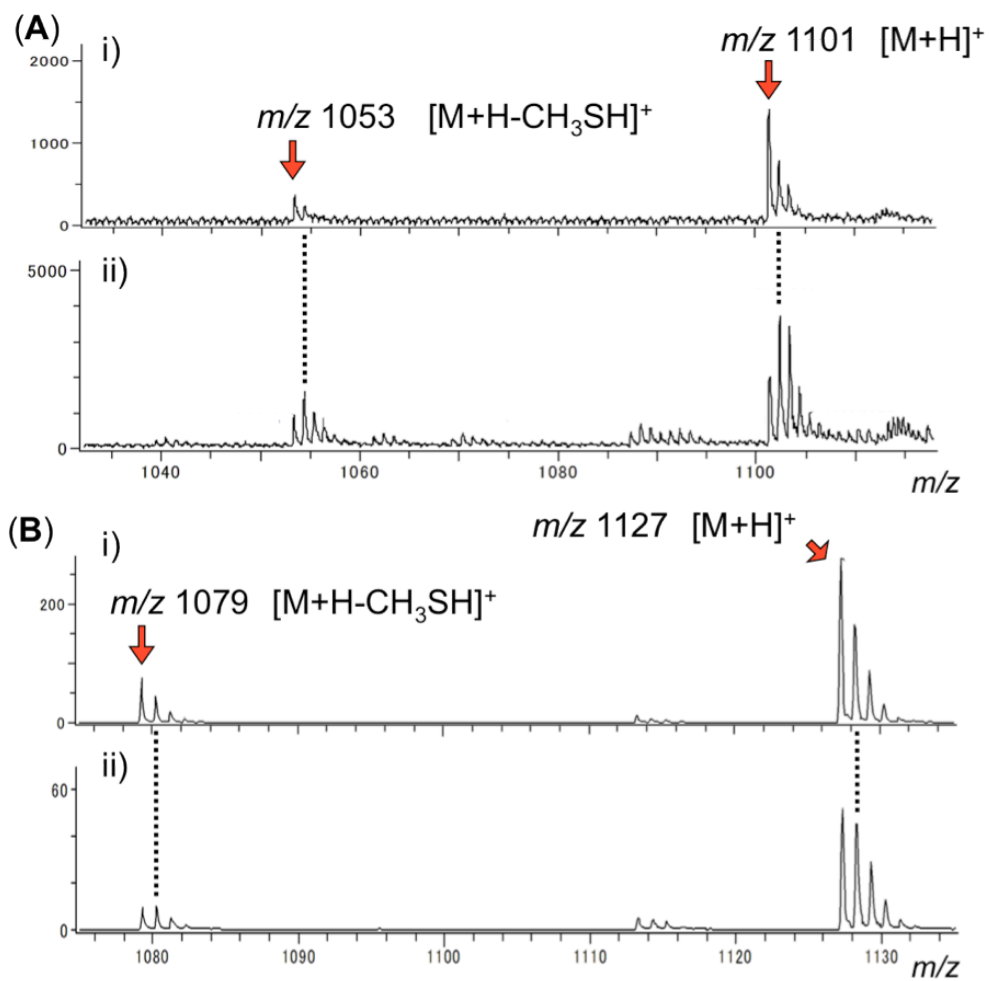


Figure 2. ESI-MS spectra of echinomycin (**1**) and SW-163D (**2**) from feeding experiments with (2*S*, 3*R*)-[6-²H]-3-hydroxy-L-kynurenine (**5a**). (A) i) non-labeled **1**; ii) **1** obtained from feeding experiment. (B) i) non-labeled **2**; ii) **2** obtained from feeding experiment. Assays and HPLC conditions are available in Experimental Section.

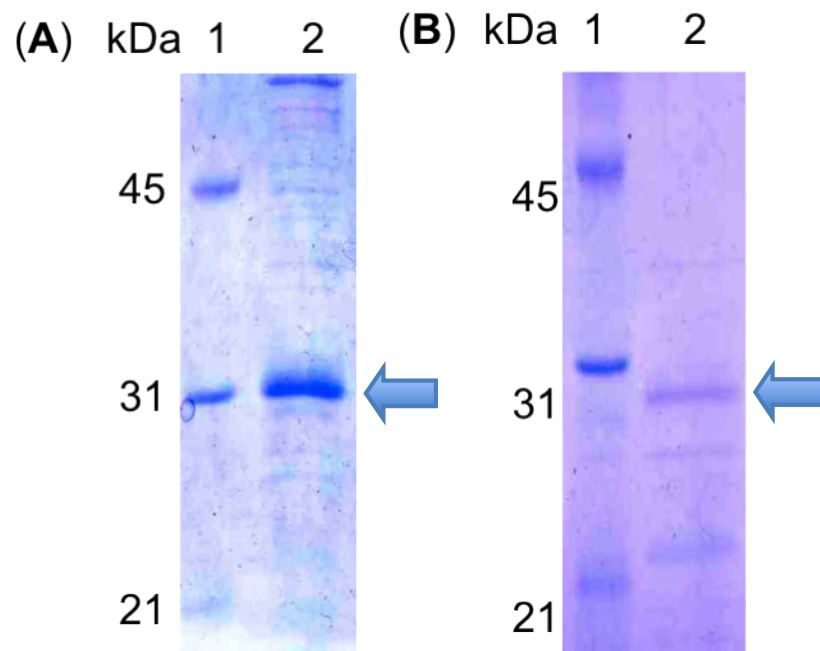


Figure 3. SDS-PAGE analysis showing partially purified Swb1(A) and Swb2 (B): lane 1, size markers; lane 2, partially purified Swb1 or Swb2.

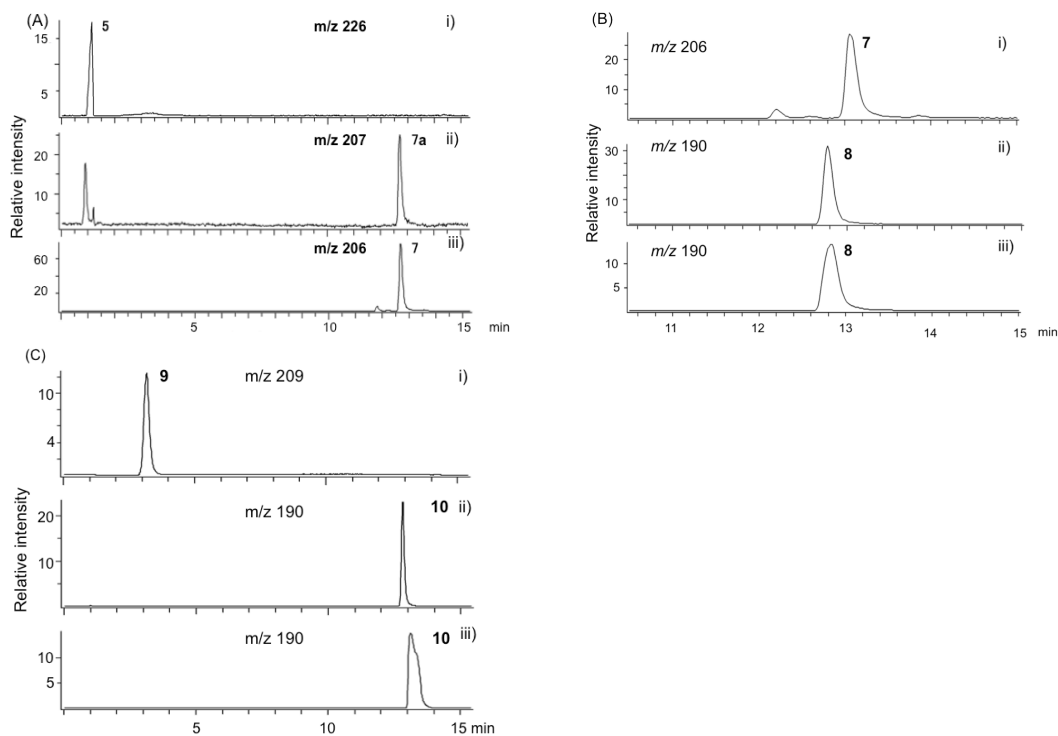
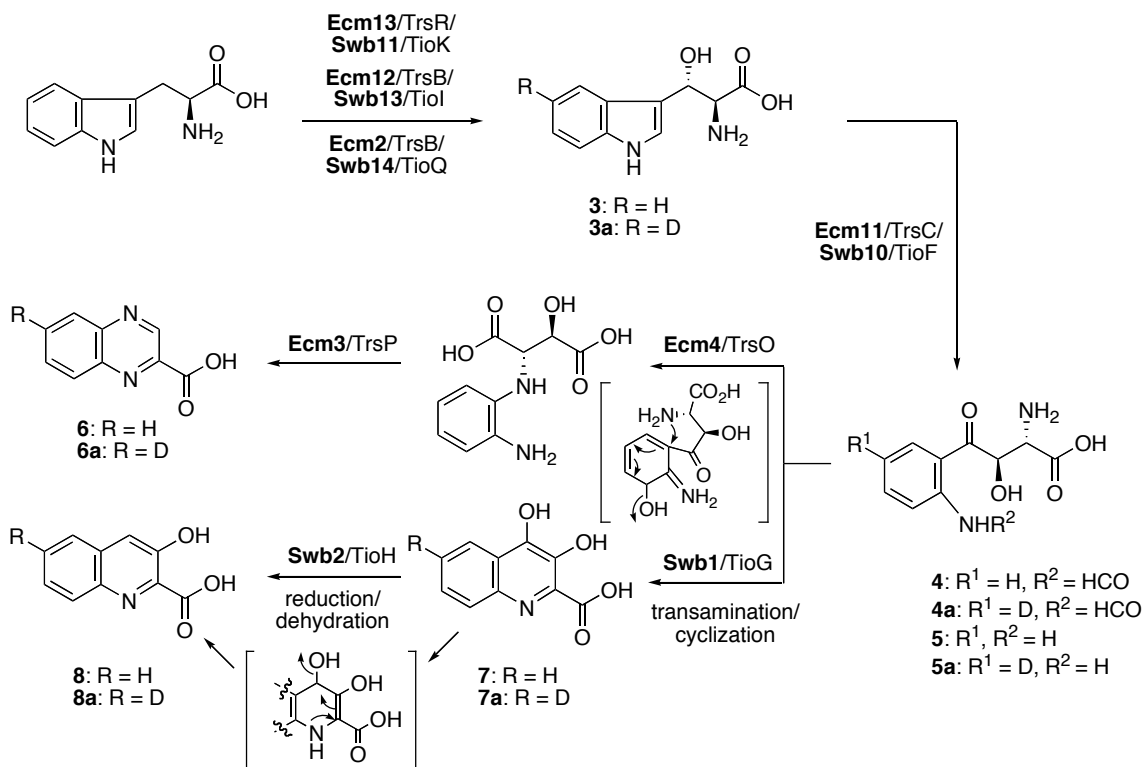


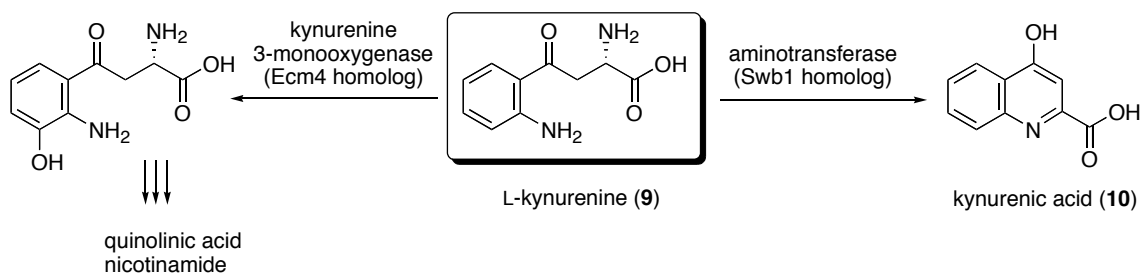
Figure 4. LC-MS analysis of enzymatic reaction products with Swb1 and Swb2 (ESI positive mode). (A). i) the substrate **5**; ii) reaction products with Swb1; iii) authentic sample of the product **7**. (B). i) the substrate **7**; ii) reaction products with Swb2; iii) authentic sample of the product **8**. (C). i) the substrate **9**; ii) reaction products with Swb1; iii) authentic sample of the product **10**. Assays and HPLC conditions are available in Experimental Section.

Scheme 1. Proposed biosynthetic pathway of chromophores of echinomycin (**1**) and SW-163D (**2**).



Scheme 2. Gene homologs found in primary and secondary metabolites.

A) Tryptophan metabolism



B) Chromophore biosynthesis

