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Analysis of YM-216391 Biosynthetic Gene Cluster and Improvement of the Cyclopeptide Production in a Heterologous Host

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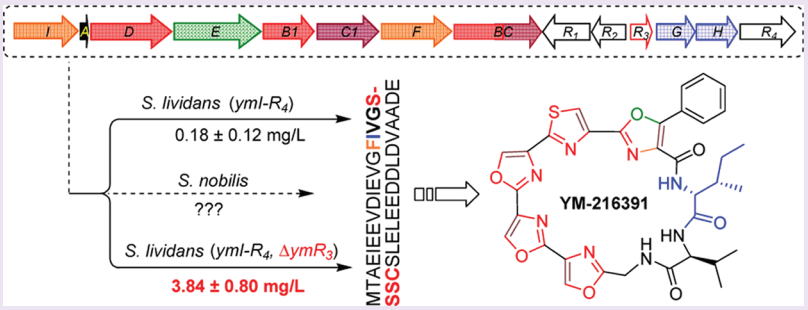
Supporting Information



ABSTRACT: YM-216391, an antitumor natural product, represents a new class of cyclic peptides containing a polyoxazole-thiazole moiety. Herein we describe its gene cluster encoding the biosynthetic paradigm featuring a ribosomally synthesizing precursor peptide followed by a series of novel posttranslational modifications, which include (i) cleavage of both N-terminal leader peptide and C-terminal extension peptide and cyclization in a head-to-tail fashion, (ii) conversion of an L-Ile to D-*allo*-Ile, and (iii) β-hydroxylation of Phe by a P450 monooxygenase followed by further heterocyclization and oxidation to form a phenyloxazole moiety. The cluster was heterologously expressed in *Streptomyces lividans* to bypass difficult genetic manipulation. Deletion of the *ymR*3 gene, encoding a putative transcriptional regulator, increased the YM-216391 yield about 20-fold higher than the original yields for the heterologous expression of wild-type cluster, which set the stage for further combinatorial biosynthesis.

The five-atom heterocycles, including thiazolines and oxazolines and subsequent oxidation products thiazoles and oxazoles, are a recurring motif in nature's medicinal chemistry toolbox of bioactive natural products.[1] Biosynthesis of the heterocycles involves heterocyclization and dehydration of X-Cys, X-Ser, or X-Thr dipeptide moieties to form thiazolines or oxazolines and then oxidation to thiazoles and oxazoles. This process includes two types of enzymatic strategies: nonribosomal peptide synthetase (NRPS) or posttranslational modification of a ribosomally synthesized prepeptide.[1,2] The conversion of X-Cys to thiazole moiety of epothilone and bleomycin was characterized as a NRPS based modification by the cyclization (Cy) domain and oxidation (Ox) domain.[3−5] Recently, the biosynthesis of heterocycle-containing natural products with ribosomal peptide frameworks has been actively pursued, including goadsporin,[6] cyanobactins,[7] streptolysin S,[8] thiopeptides,[9,10] and plantazolicins.[11,12] Generally speaking, this type of posttranslational modification involves several proteins/domains, including (i) a zinc-binding cyclodehydratase, (ii) an ATPase/GTPase likely docking protein, and (iii) a FMN dependent oxidase.[2]

As a novel family of heterocyclic natural products, the macrocyclic peptides containing highly constrained tandem or multiple heterocycles are always interesting to medicinal chemistry and biosynthetic studies.[1] Recently, an unusual polyoxazole-thiazole-based cyclopeptide, YM-216391 (Figure 1, **1**), was isolated from *Streptomyces nobilis*.[13,14] Structurally, this 24-membered macrocyclic system is characterized by a continuum of five azoles linked *via* a Gly-Val-D-*allo*-Ile tripeptide tether, shares homology with the marine anticancer natural products urukthapelstatin A (Figure 1, **2**)[15] and mechervharstatin (Figure 1, **3**),[16] and is similar to the potent telomerase inhibitor telomestatin (Figure 1, **4**).[17] The structure was assigned on the basis of NMR data, absolute configuration of the amino acid residues was determined by Marfey's analysis,[14] and the complete stereochemistry was further confirmed by total synthesis.[18,19] YM-216391 dose-dependently inhibited the growth of human cervical cancer HeLa S3 cells

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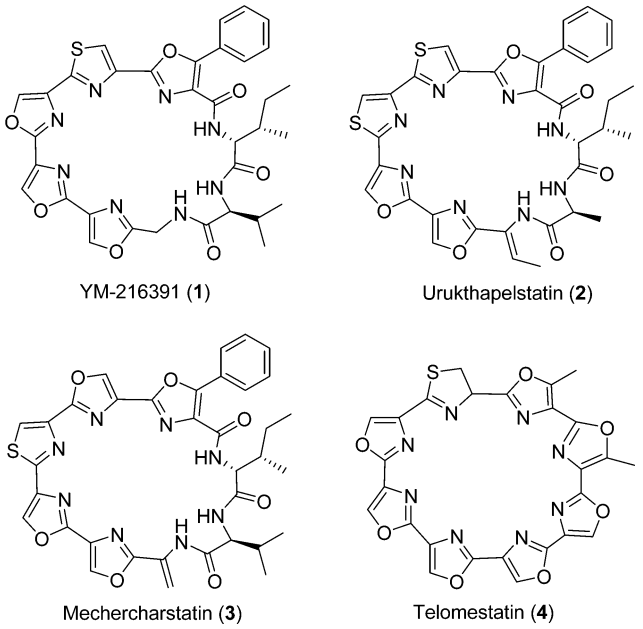

Figure 1. Structures of YM-216391 and related natural products.

with an IC_{50} value of 14 nM. It also showed potent cytotoxic activity against a human cancer cell line panel, and the mode of action remains to be investigated.[13] The unique structure, potent biological activity, and unprecedented mechanism have drawn considerable interest to further development of this class of natural products as potential anticancer drugs. However, until now little has been known about the biosynthesis of this family of sequential oxazoles containing macrocyclic peptides, so it still remains elusive to answer the question of whether this type of constrained tandem heterocycles arises from NRPSs or by the posttranslational modification of a ribosomally synthesized prepeptide.

To understand which biosynthetic process is used to construct the polyoxazole-thiazole-based framework of these macrocyclic peptides, we have undertaken the cloning of biosynthetic gene cluster of YM-216391 through genome scanning. The genomic DNA of producer *S. nobilis* was subjected to 454 sequencing, which yielded 9.29 Mb of consensus sequence. Bioinformatic analysis permitted the identification of a gene (assigned as *ymA*) encoding a 36-residue protein containing the F-I-V-G-S-S-S-C amino acid sequence at the middle region, which is identical to that predicted for the peptide core of YM-216319 (Figure 2). This finding suggested YM-216391 is most likely biosynthesized by a ribosomal process. To further prove that this gene cluster is necessary to YM-216391 production, a cosmid pTG1101 based on pJTU2554 vector[20] containing this DNA region was identified through library screening, followed by transformation of *S. lividans* 1326 by conjugation to obtain the heterologous expression strain *S. lividans* TG1101. The analysis of the metabolite accumulated by this mutant was performed by HPLC and LC−MS with *S. lividans* 1326 harboring pSET152 as control, which revealed that the production of YM-216391 absolutely depended on the cosmid pTG1101 (Figure 3I,II). The fraction of corresponding target was collected and further subjected to analysis by high-resolution MS, which yielded $(M + H)^+$ and $(M + NH_4)^+$ ions at m/z = 697.2174 and 714.2442 (Supplementary Figure S1), consistent with the molecular formula $C_{34}H_{32}N_8O_7S$ of YM-216391 [calculated 697.2193 for $(M + H)^+$ and 714.2458 for $(M + NH_4)^+$]. These results suggest that this gene cluster surrounding the *ymA* gene is necessary for the biosynthesis of YM-216391. Further sequence analysis of this region revealed 14 ORFs (Figure 2a and Table 1), from *ymI* to *ymR4*, which were proposed to constitute the YM-216391 gene cluster according to functional assignment of their deduced products.

YmA, a 36-residue prepeptide, shows no sequence homology in the data bank. The first 13-residue with MTAEIEEVDIEVG amino sequence of the prepeptide is proposed to serve as a leader sequence for processing, which is rich in acidic amino acids Asp (D) and Glu (E) but without a double Gly motif.[21] However, the prepeptide also contains an 18-residue C-terminal extension with SLELEEDDLDVAADE amino sequence likely for recognition, which is richer in acidic amino acids Asp and Glu. So this prepeptide is a highly acidic protein with a calculated pI of 3.17 and contains 46% of acidic amino acids in the N-terminal leader sequence and C-terminal extension that need to be removed during the maturation of final product (Figure 2b).

Biosynthesis of the polyoxazole-thiazole-based framework is one of the most remarkable processes of this family of natural products. Two genes, *ymD* and *ymBC*, encoding a docking protein and a cyclodehydratase-oxidase didomain enzyme, were assigned responsibility for conversion of the G-S-S-S-C into Gly-oxazole-oxazole-oxazole-thiazole moiety (Figure 2b). YmD, containing a conserved Pro-rich C terminus (PDPHPFPHPLP), is a YcaO-like protein found in many bacteriocin biosynthetic pathways as a docking protein with ATPase/GTPase activity.[8,22] YmBC is a 492-residue protein; it contains a C-terminal domain with NADPH and FMN binding motif and shows sequence homology to the oxidase domain of epoB, the NRPS of the epothilone biosynthesis pathway.[4,5] The N-terminal half of YmBC does not include any obvious motif and shows no sequence homology in the data bank. Further analysis for this domain allowed the identification of two groups of CxxC motifs (CLGC and CRWC), which are invariant elements comprising a zinc-tetrathiolate for the cyclodehydratase.[8,23] Previously, biochemical characterization of microcin B17 biosynthesis showed that the multiple heterocycles are generated by a zinc-binding cyclodehydratase McbB, a docking protein McbD with ATPase activity, and a FMN dependent oxidase McbC.[22−25] Recently, the biosynthetic studies of goadsporin,[6] cyanobactins,[7] and thiopeptides[9,10] revealed that the cyclodehydratase and docking protein are fused into one protein, while in a widely distributed toxin biosynthetic pathway, either three separated enzymes or two enzymes (oxidase and fused cyclodehydratase-docking protein) are present.[8] However, in the biosynthesis of YM-216391, the zinc-binding cyclodehydratase and the FMN-dependent oxidase are fused together as a two-functional enzyme and the docking protein as a monofunctional discrete enzyme.

The analysis of YM-216391 gene cluster indicated more tailoring steps in the biosynthetic pathway. A cytochrome P450 monooxygenase, encoded by *ymE*, containing a typical heme binding motif FGGGRRSCPG, may be a candidate enzyme for β-hydroxylation of Phe. The P450 oxygenases for β-hydroxylation usually bind the amino acid substrate either free in solution or covalently linked to the peptide carrier protein (PCP) domain of NRPS as aminoacyl-*S*-PCP.[26,27] In the biosynthetic pathway of YM-216391, the β-hydroxylation of Phe most likely occurred in the prepeptide stage after the formation of polyoxazole-thiazole moiety (Figure 2b). In

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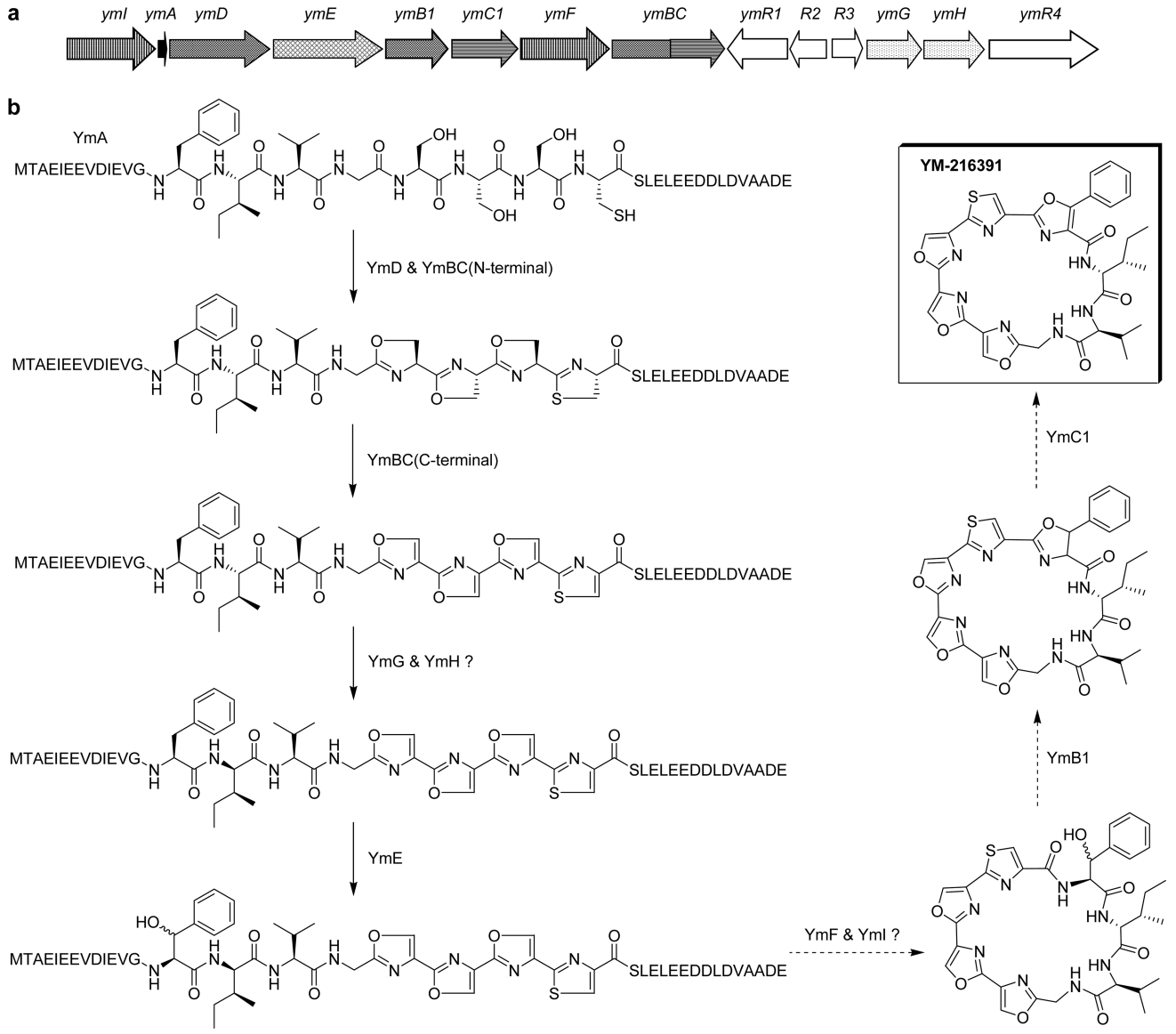

Figure 2. Biosynthetic pathway of YM-216391. (a) Organization of gene cluster. (b) Proposed model for the biosynthesis of YM-216391.

addition, the conversion of L-Ile to D-*allo*-Ile was also proposed in this stage, possibly catalyzed by YmG and YmH, although the order of these two modification steps remains to be determined experimentally (Figure 2b). The YmG enzyme is similar to aminomutase but without radical SAM binding motif CxxxCxxC. It contains Cys47 and Cys123, possibly functioning as the catalytic acid/base residues for deprotonation/protonation of the cofactor-independent amino racemases,[28] although they do not show the obvious sequence homology. Bibb and co-workers identified the biosynthetic gene cluster of L-*allo*-Ile containing cypemycin and proposed CypI as a candidate isomerase.[29] However, any gene products including YmG found in the YM-216391 gene cluster show no homology with CypI. YmH does not share the obvious homology to the known enzymes and is proposed to be functionally related to YmG because the two genes are translationally coupled for their overlapping stop and start codons. Although D-*allo*-Ile residues also were identified in the cyclic peptides aerucyclamides from cyanobacterial,[30] the epimerase responsible for the conversion of L-Ile into D-*allo*-Ile still remains to be explored.

Another important step of ribosomal peptides biosynthesis is excision of the N-terminal leader peptide and C-terminal extension and concomitant cyclization. YmF, though it does not share the obvious homology to the known proteases,[31,32] is supposed functionally similar to cleave *β*-hydroxylPhe-D-*allo*-Ile-Val-Gly-oxazole-oxazole-oxazole-thiazole out of the full-length precursor at both N- and C-termini, followed by N−C terminal cyclization (Figure 2b). The high percent of acidic amino acids (46% of D/E) in both N-terminal leader sequence and C-terminal extension requires an alkaline protease to bind, recognize, and then catalyze the amide bond hydrolysis reaction. YmF is the only candidate enzyme with calculated pI of 8.68 except for another alkaline protein, YmBC (with calculated pI of 8.99), which was already assigned as cyclodehydratase-oxidase for the formation of polyoxazole-thiazole moiety. YmI, a hypothetical protein belonging to the cupin family, cannot be assigned function on the basis of only the sequence analysis. This formation process of cyclic peptide may be similar to the split intein circular ligation model[33] but requiring YmF and/or YmI assistance, while the enzymatic mechanism needs to be further investigated.

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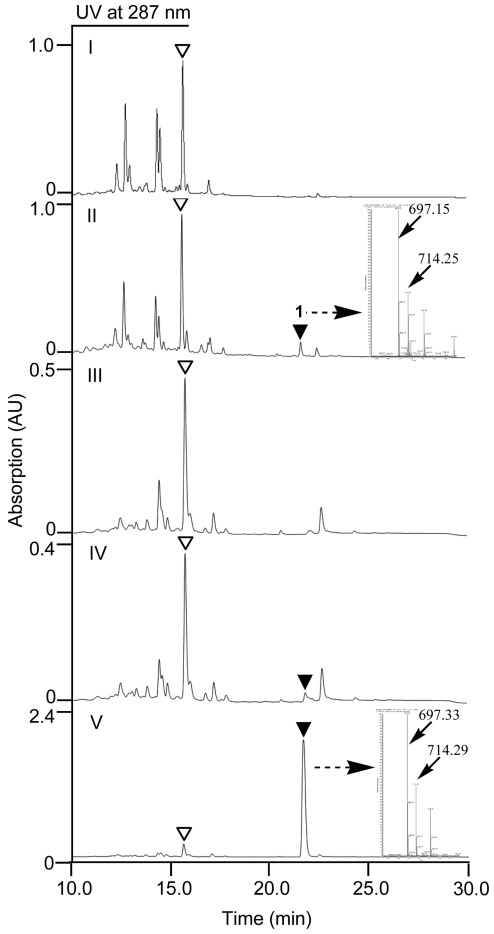

Figure 3. HPLC analysis of YM-216391 production by heterologous expression. (I) *Streptomyces lividans* 1326 containing plasmid pSET152 as control; (II) *S. lividans* TG1101 [*S. lividans* 1326 harboring cosmid pTG1101 (wild-type cluster)]; (III) mutant *S. lividans* TG1102 [*S. lividans* 1326 harboring cosmid pTG1102 (Δ*ymR1*)]; (IV) mutant *S. lividans* TG1103 [*S. lividans* 1326 harboring cosmid pTG1103 (Δ*ymR2*)]; (V) mutant *S. lividans* TG1104 [*S. lividans* 1326 harboring cosmid pTG1104 (Δ*ymR3*)]. (▼) YM-216391 (**1**); (▽) an unknown metabolite whose production is independent of YM-216391 biosynthesis.

The final step in YM-216391 maturation is the heterocyclization of thiazole-β-hydroxylPhe and further oxidation to form a thiazole-phenyloxazole moiety. Two genes, *ymB1* and *ymC1*, encoding a cyclodehydratase and an oxidase, are likely responsible for formation of this special five-atom heterocycle (Figure 2b). YmB1 does not keep the conserved CxxC motifs, which usually exist in the cyclodehydratase as zinc-binding sites using prepeptide as substrate, but contains two nearby DxxE sequences (DLGE and DNPE), a known metal-binding motif. This type of special cyclodehydratases has been mentioned as an exception in the analysis of toxin biosynthetic cluster.[8] YmC1 shows weak homology to the FAD-binding monooxygenase, which just fit the request of oxidizing phenyloxazoline into phenyloxazole. Compared to the classical three-component system McbBCD[25] and recently characterized heterocyclization in cyanobactin biosynthesis,[34] the special two-component cyclodehydratase-oxidase system proposed here requires neither a leader peptide nor a C-terminal extension for recognition, which provides an opportunity to explore a potentially different enzymatic system to do the similar chemical process.

The difficult genetic manipulations in recalcitrant endogenous producer and poor, unstable production of YM-216391 hinder the further investigation in a native producing strain (*S. nobilis* JCM 4274), and heterologous expression has to be used to bypass these obstacles. Although we successfully transferred the gene cluster into the model host *S. lividans* 1326 and achieved approximately 176 ± 115 μg/L yield of compound, more efforts are still required to improve the titer of YM-216391 for further combinatory biosynthesis. Recently, several successful cases about improvement of secondary metabolite production by manipulating pathway-specific regulators[35] inspire us to characterize the putative regulatory genes in YM-216391 gene cluster. Two genes, *ymR1* and *ymR2*, encode putative regulatory proteins belonging to AraC family and Mar family transcription regulator, respectively. We then replaced *ymR1* and *ymR2* with flippase recognition target (FRT) scar using λ-Red-mediated recombination and got the resulting cosmid pTG1102 (Δ*ymR1*) and pTG1103 (Δ*ymR2*), respectively (the identification of genotype in Supplementary Figure S2). Conjugative transfer and expression of pTG1102 in *S. lividans* failed to produce YM-216391, while the recombinant strain *S. lividans* TG1103 containing pTG1103 still yielded the product with an obviously decreased level (Figure 3III,IV). However, when we delete *ymR3*, encoding a protein showing high similarity to a hypothetical protein with unknown function and moderate sequence homologous to the 4-oxalocrotonate tautomerase, the resulting heterologous expression strain *S. lividans* TG1104 [harboring cosmid pTG1104 (Δ*ymR3*)] produced YM-216391 up to a yield of 3.84 ± 0.80 mg/L (Figure 3V). This titer is 20-fold greater than the original yields for the heterologous expression of wild-type cluster, making the production and isolation of large amounts of this compound vastly more efficient. Indeed, we easily isolated enough compound from heterologous expression strain *S. lividans* TG1104 and further confirmed the structure by ^{1}H NMR and ^{13}C NMR (Supplementary Figures S3, S4 and Supplementary Table S1). This result suggested that *ymR3* should encode a negative regulator, and this finding underscores once again the effectiveness of the heterologous expression and manipulating pathway regulation to overcome genetic system and production improvement. The last protein, YmR4, is a transmembrane efflux protein, may contribute to resistance.

In summary, we have successfully identified a biosynthetic gene cluster encoding the ribosomally polyoxazole-thiazole-based macrocyclic peptide by genome sequencing and confirmed its function by heterologous expression. The YM-216391 gene cluster exhibits several unusual features of the ribosomal based posttranslational modification, most remarkably the putative epimerases catalyzing the conversion of an L-Ile to D-*allo*-Ile and a P450 monooxygenase responsible for β-hydroxylation of Phe in the precursor peptide stage. In addition, the precursor peptide needs to be cleaved both N-terminal and C-terminal prepeptide and ligated in a head-to-tail fashion. In addition to converting S-S-S-C into polyoxazole-thiazole moiety, this biosynthetic system provides the first example of heterocyclization and oxidation of β-hydroxyl-Phe to form a phenyloxazole moiety. Deletion of a hypothetical regulatory gene *ymR3* from the cluster in the heterologous host allowed us to obtain strains with production of YM-216391 increased 20-fold. The work described here thus provides a foundation to investigate the biosynthetic mechanisms of the

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Table 1. Deduced Functions of ORFs in YM-216391 Biosynthetic Gene Cluster[a]

Gene	aa*	Protein homologue	Similarity / Identity (%)	Proposed function
ymI	340	Hypothetical protein (CAE81053); *Bdellovibrio bacteriovorus* HD100	50/34	Unknown
ymA	36	No	No	Precursor peptide: MTAEI-EEVDIEVG**FIVGSSSC**SL-ELEEDDLDVAADE
ymD	476	TpdO (ACS83760) ; *Nonomuraea* sp. WU8817	38/29	Docking protein
ymE	500	Cytochrome P450 family protein (EDM74971); *Plesiocystis pacifica* SIR-1	50/34	P-450 hydroxylase
ymB1	261	OsJ_21831 (EAZ37497); *Burkholderia rhizoxina*	41/34	Cyclodehydratase
ymC1	318	Nitrite reductase large subunit (CAM04176); *Saccharopolyspora erythraea* NRRL 2338	41/27	FAD-binding monooxygenase
ymF	381	Predicted protein (ACO63355); *Micromonas* sp. RCC299	37/51	Hypothetical protease
ymBC	492	Nitroreductase (ABQ92956); *Roseiflexus* sp. RS-1	55/41	Cyclodehydratase-oxidoreductase
ymR1	239	AraC family regulator (EFB65384); *Streptomyces* sp. ACTE	70/55	Transcriptional regulator
ymR2	166	Mar family regulator (ACZ86477); *Streptosporangium roseum* DSM 43021	86/70	Transcriptional regulator
ymR3	132	Hypothetical protein SBI_03728 (ADI06849); *Streptomyces bingchenggensis* BCW-1	80/60	Transcriptional regulator
ymG	210	Arginine aminomutase (AAP03121); *Streptomyces griseochromogenes*	63/55	Hypothetical epimerase
ymH	244	Hypothetical protein Amir_1171 (ACU35125); *Actinosynnema mirum* DSM 43827	58/42	Unknown
ymR4	520	Transmembrane efflux protein (EDY62713); *Streptomyces pristinaespiralis* ATCC 25486	92/88	Transporter

[a]aa*: amino acid.

novel posttranslational modification of a ribosomally synthesized prepeptide and also sets the stage for engineering the pathway for novel analogues to develop useful anticancer drugs.

■ ASSOCIATED CONTENT

Supporting Information

Materials and methods, the HRMS and NMR data of YM-216391, and genotype analysis of gene replacement mutants. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

The sequence reported in this paper has been deposited into GenBank under accession no. JN411915.

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Notes

The authors declare no competing financial interest.

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