Enzymatic Synthesis of Molecular Skeletons of Complex Antitumor Antibiotics with Non-ribosomal Peptide Synthetases

Kenji Watanabe,¹ Hiroki Oguri,² and Hideaki Oikawa*²

¹University of Shizuoka, School of Pharmaceutical Sciences
52-1 Yada, Surugaku, Shizuoka 422-8526, Japan
²Division of Chemistry, Graduate School of Science, Hokkaido University
10-8 Kita-ku, Sapporo 060-0810, Japan

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Abstract: Nonribosomal peptide synthetase (NRPS) is a programmable modular machinery which produces a number of biologically active small–molecule peptides. Recent progress on understanding the catalytic mechanism of NRPS enabled us to engineer this complex catalytic system. We demonstrated both in vivo and in vitro enzymatic synthesis of complex natural antitumor peptides echinomycin and saframycin. An Escherichia coli–based expression system served as a flexible yet robust platform for producing complex natural products and their analogs by deletion, mutation and swapping of a specific subunit. The excised thioesterase domain of echinomycin exhibited remarkable substrate tolerance and created a cyclic peptide library. On the other hand, saframycin NRPS catalyzed highly unusual seven–step transformations to construct a pentacyclic tetrahydro–isoquinoline skeleton.

1. Introduction

For many years, organic synthesis has been the only method to provide a specific natural product except isolation of the corresponding compound from originally producing organisms. Thus, rare natural products possessing complex structures are good targets for total synthesis which contribute to providing target compounds and their derivatives, and to understanding their biological activities. In the last two decades, however, remarkable progress in the biosynthesis of natural products has been made by the biochemical analysis of biosynthetic enzymes and their genetic engineering.¹ Accumulative knowledge of detailed biosynthetic pathways of major families of natural products set a stage to synthesize natural products using biosynthetic enzymes in vitro and in vivo. In this account, we focus on the enzymatic synthesis of biologically active small molecule–polypeptides.

To create extraordinary diverse, natural, small molecule–polypeptides, nature adopted a programmable modular machinery, non-ribosomal polypeptide synthetase (NRPS).² NRPS consists of three essential domains in each module for constructing a polypeptide backbone as shown in Figure 1. At first, an adenylation (A) domain chooses a specific amino acid and loads it to a peptidyl carrier protein (PCP) domain or thiolation (T) domain. Then, a condensation (C) domain catalyzes condensation of two amino acid residues located at upstream and downstream modules. So, one module is required for a single amino acid extension. The feature that organization of modules determine the variety and number of a building unit is called as a colinearity rule. A matured peptide chain is cleaved off by a thioesterase (TE) or reductase (R) domain which is located at the terminal of the final module. Modification domains for tailoring an amino acid monomer or a peptide backbone such as methylation (M), epimerization (E) and heterocyclization provide options to create diversity of natural peptides.

Figure 1. Enzymatic reaction mechanism of nonribosomal peptide synthetase (NRPS).
According to the generally accepted mechanism (colinearity rule), NRPS produces polypeptides different in size and amino acid–sequence with various modifications. An important strategy that this enzyme adopts is that condensation substrates are always bound to the enzyme (T) and that the chain–elongated intermediates are never released from NRPS until the final cleavage. Therefore, this system guarantees reliable and safe delivery of the substrates. This allows rather broad substrate specificity of the C–domain. Each module is a self–consistent portable component. This suggests that replacing A–domain or a specific module may create new peptides in a programmable manner. Hence, the rational design of NRPS makes it possible to synthesize novel peptides just in the same way as organic synthesis. In this account, we describe an enzymatic synthesis of two important families of antitumor peptide antibiotics, echinomycin and saframycin.

2. Enzymatic Synthesis of Echinomycin and its Derivatives

Echinomycin (1) belongs to quinomycin bis–intercalator antibiotics (Figure 2). This class of antibiotics are dimeric cyclic peptides with a pair of chromophores and have potent DNA binding affinities at a level between nM to M with different sequence selectivities. Reflecting these distinct affinities, bis–intercalator antibiotics such as 1–4 show fairly different inhibitory activities against various enzymes such as DNA polymerase, RNA polymerase, reverse transcriptase and topoisomerase. Based on structure–activity relationships among this class of antibiotics, one can expect that minute structural difference may drastically change profiles between antitumor activity and toxicity. To examine this possibility, we became interested in synthesizing echinomycin derivatives.

To identify and isolate the echinomycin biosynthetic gene cluster, a cosmid library was constructed using S. lasaliensis total DNA. Screening with the PCR product from degenerate primers for NRPS afforded plasmids encoding the 36–kb echinomycin biosynthetic gene clusters. Homology of these genes enabled us to speculate their functions in the biosynthesis of quinoxaline–2–carboxylic acid (5, QXC) and peptide backbone construction. Based on previous findings that L–tryptophan has been identified as the precursor to QXC, and that nitrogen atoms at the 1– and 4–positions in QC have their origins in the indole and amino group of L–tryptophan, respectively, we proposed the QXC biosynthetic pathway as described in Scheme 1. According to similarity of Ecm13, Ecm1 and Ecm12 to those of the reported β–hydroxylation route enzymes, we expected three proteins to be involved in the β–hydroxylation of L–tryptophan. At first, a putative free–standing NRPS Ecm13 activates L–tryptophan and transfers it to the phosphopantetheiny1 arm of the T domain through thioester formation. Then, Ecm12 catalyzes the 3–hydroxylation of the tryptophan intermediate. The resulting covalently tethered product is hydrolyzed by a putative type II thioesterase (Ecm2) to liberate the free 3–hydroxytryptophan (6). Analogous to the metabolic pathway of tryptophan, oxidative cleavage of the indole 2,3–double bond by Ecm11 and subsequent dehydroxylation would afford 3–hydroxytryptophan (7). To provide a mechanistic rationale for the formation of the aryl–nitrogen bond between C5 and N4 in the quinoxaline ring, we hypothesized a novel oxidative rearrangement (7 to 8, via a spirocyclic intermediate) mediated by Ecm4. Finally, oxidation of the secondary alcohol of 8 by Ecm3 and subsequent decarboxylation–cyclization followed by spontaneous aromatization would furnish QXC.

Recently, we have cloned a biosynthetic gene cluster of another quinomycin analog SW–163D (4) which has a hydroxyquinoidal acid (9, HQA) moiety as a chromophore. This gene cluster also consists of four genes homologous to the genes for production of 3–hydroxykynurenine (7). After establishing the synthetic route of 3–hydroxytryptophan (6) and 3–hydroxykynurenine (7), we employed the feeding experiments of the isotopically labeled putative precursors 6a, 7a to establish their intermediacy in both echinomycin and SW–163 producing strains (Scheme 1). Successful incorporation of 6a and 7a into 1 and 4 strongly indicated that L–tryptophan is converted to 3–hydroxykynurenine (7) as a common precursor in the biosynthesis of two different chromophores (QXC and HQA). Enzymatic conversions of 7 to 9 via transamination product 10 with Swb1 and Swb2 provide further support to this hypothesis.

Based on the general mechanism of NRPS (colinearity rule) as shown above, amino acid specificity of the deduced...
adenylation (A) domain from the amino acid sequence and organization of ecm NRPS provide information on the reaction pathway of the peptide core formation (Scheme 2).\textsuperscript{5} Experimental results on a chromophore activating enzyme and an acyl carrier protein in the triostin biosynthesis suggest that QXC is activated and loaded onto NRPS Ecm6 by the catalysis of Ecm1 and FabC for fatty acid biosynthesis.\textsuperscript{11} The first module of Ecm6 catalyzes condensation with L-serine and the subsequent epimerization to give aryl capped D-serine intermediate, followed by condensation of L-alanine with the second module. The resultant dipeptide thioester was further condensed with N-methylated L-cysteine and L-valine with the third and fourth modules on the Ecm7 to afford the tetrapeptide intermediate. Furthermore, analysis of reported multimeric NRP systems, such as gramicidin S\textsuperscript{12} and enterobactin\textsuperscript{13} suggest that the echinomycin–synthesizing NRPS system is capped off with a thioesterase domain capable of peptide chain homodimerization and cyclorelease. Disulfide formation with the putative oxidoreductase Ecm17 would form triostin A (2a).

It has been proposed that triostin A (2a) is a direct precursor to echinomycin (1) (Scheme 3).\textsuperscript{14} DNA sequence data obtained for the gene cluster resolved a putative S-adenosyl-L-methionine (SAM)–dependent methyltransferase Ecm18, which has been thought to convert the disulfide bridge in 2a to the thioacetal bridge in 1 via a sulfoxonium
ylide intermediate. To verify this, we demonstrated in vitro that purified Ecm18 catalyzed the conversion of 2a to 1 in the presence of SAM. This is the first observation that a single methyltransferase is capable of transforming a disulfide bridge into a thioacetal moiety.

Scheme 3. Proposed mechanism for the thioacetal formation with the methyltransferase Ecm18.

3. \textit{de novo} Biosynthesis of Quinomycin Antibiotics in \textit{E. coli}

The methods for synthesizing complex natural products such as echinomycin (I) by simply expressing biosynthetic gene clusters that are responsible for the production of these compounds have been extensively studied.\textsuperscript{15,16} For this purpose, the wild-type biosynthetic genes have been successfully expressed and represented \textit{in vivo} and \textit{in vitro} enzymatic productions by using several expression hosts. Among various heterologous hosts, \textit{E. coli} for manipulation of biosynthetic genes and metabolic engineering will provide us with the following advantages: the availability of well-established molecular biological techniques, a fast life cycle, abundance of expression plasmids with a variety of replication origins, and a comprehensive map of its genome.\textsuperscript{17} Thus, we chose \textit{E. coli} as a heterologous host to synthesize echinomycin (I) using biosynthetic enzymes.

In general, \textit{E. coli} does not retain multiple plasmids. This indicates that introduction of more than ten genes into a heterologous host requires an efficient method to construct expression plasmid carrying multiple biosynthetic genes. In addition, our approach requires multiple monocistronic gene cassettes. This approach simplifies plasmid construction and re-construction because it eliminates the need to clone every gene into the construct in the same transcriptional direction. For this purpose, we created a “promoter–gene–terminator” cassette that can ligate into a vector carrying another “promoter–gene–terminator” cassette(s).\textsuperscript{5} To insure the stable retention of three plasmids in \textit{E. coli}, we decided to use the orthogonal origins of replication and three antibiotic resistance genes. Also, we believe that our approach has improved our chances and ability to incorporate an even greater number of heterologous genes into \textit{E. coli} in a simple and reliable fashion.

Before construction of artificial gene clusters, we examined the feasibility for expression of each of the fifteen \textit{S. lasaliensis} genes in \textit{E. coli} by individual expression plasmids. Once SDS–PAGE analysis of all biosynthetic enzymes expressed was confirmed, we then assembled the fifteen genes and the gene for phosphopantetheinylation of NRPS into three separate vectors allowing each gene to carry its own T7 promoter and ribosome-binding site. The resultant three plasmids were introduced into \textit{E. coli}. Subjecting \textit{E. coli} strain BL21 (DE3) carrying our three plasmids to an 8-day–long fed–batch fermentation in M9 minimal medium, we were able to purify echinomycin (I) from the culture extract through a series of chromatographic steps to obtain a final yield of 0.3 mg of 1 per liter of culture.\textsuperscript{5} Furthermore, to demonstrate the ease and effectiveness of modifying the \textit{E. coli}–based heterologous biosynthetic system, we chose a version of the echinomycin biosynthetic pathway into a triostin A biosynthetic pathway by simply modifying our plasmids and removing \textit{ecm18}. The further modified strain produced the expected compound, triostin A (2a) at a yield of 0.6 mg per liter of culture.\textsuperscript{3} Adding QXC significantly improved the yield of 2a more than 20 times (13 mg/L).\textsuperscript{18}

In cells, organisms can produce complex natural products using abundant substrates such as amino acids, sugars and acyl CoAs. An important feature of our approach using an artificial gene cluster is that this methodology is applicable to synthesizing any type of natural products (Figure 3). Our results confirmed that replacement of the polycistronic cassette with a multi–monocistronic cassette can consistently exhibit high–level expression of every gene in our constructs and ameliorate plasmid construction. We believe these improvements have made \textit{de novo} biosynthesis of complex natural products possible.

4. Synthesis of Echinomycin Derivatives by Inactivation of Specific Domains and Replacing NRPS Protein

By employing the advantages of our \textit{E. coli}–based plasmid–borne biosynthetic gene cluster, unprecedented \textit{in vivo} synthesis of unnatural echinomycin derivatives was examined. The first target was des–\textit{N}–tetramethyl triostin A (2b) or TANDEM, which bis–intercalates into DNA and exhibits a distinct preference for AT sequences in contrast to echinomycin displaying a different DNA preference for GC–rich sequences.\textsuperscript{4}

In order to produce 2b in \textit{E. coli}, we hypothesize the need to 1) inactivate the two methylation (M) domains of the 340–kDa bimodular NRPS Ecm7, responsible for the \textit{N}–methylation of the cysteine and the valine residues in the core peptide backbone, and 2) omitting Ecm18, responsible for forming the thioacetal–bridge.\textsuperscript{5} To this end, we had to redesign the echinomycin biosynthetic pathway into a TANDEM biosynthetic pathway. It was discovered that three motifs in the NRPS M domain are highly conserved. The first of the three conserved motifs, GXGXG, is analogous to a glycine–rich \textit{S}–adenosyl–l–methionine–binding motif commonly found in other types of methyltransferases.\textsuperscript{19} Therefore, we constructed a hexaplet glycine mutant in the two
active sites of Ecm7 termed Ecm7* which carries GXGXG to SXSSXS mutations in each of the two modules (Scheme 4(a)). We verified its production in E. coli, strain BL21 (DE3), by SDS–PAGE analysis.20 The engineered E. coli was able to produce our expected molecule 2b to suggest that the mutagenic enzyme Ecm7* was not entirely removed of its activity and inactivation of its M domain was an isolated change to allow for production of the des–N–methylated compound.

The second example is ecolimycin (11) which is a hybrid peptide between echinomycin and SW–163. This approach is relatively more demanding to direct biosynthesis of NRP analogs. However, we anticipate that the efficiency of our heterologous system will enable to reconstitute the hybrid biosynthetic pathway using two NRP producing gene clusters and to produce our target compound. As in the case of isolation of echinomycin biosynthetic genes, the SW–163 biosynthetic gene cluster was identified and isolated from the genome of Streptomyces sp. SNA15896. DNA sequence analysis of a 38 kilobase–long cluster revealed the presence of genes responsible for biosynthesizing the proposed primer unit for SW163s, 3–hydroxyquinaldic acid, assembling (+)–(1S,2S)–norcoronamic acid (Scheme 4(b), 12) as a proposed extender unit, and forging the peptide backbone. Since differences in the chemical structures of triostin A (2a) and SW–163C (Figure 2, 3) are trivial, the potential for engineering the backbone was obvious requiring the recruitment of nonproteinogenic amino acid, as a substrate for the peptide–scaffold of SW–163 which is commissioned by bimodular NRPS.5 To produce compound 11 using E. coli, expression of swb17 containing an adenylation domain able to accept 12 as an extender unit, a single plasmid was used to incorporate genes encoding two megasynthetase NRPSs, Ecm6 and Swb17 (Scheme 4(b)). BL21 (DE3), a strain of E. coli, was transformed with our three plasmids and subjected to shake flask cultivation for a duration of seven days in M9–minimal medium supplemented with 12, an extender unit which was obtained via chemical synthesis. Before synthesizing 12 for supplying the substrate during biosynthesis of 4, the absolute configuration of SW–163D (Figure 2, 4) was determined together with that of 12 as a constituent of its peptide backbone.21 Then, the synthetic method of the unusual amino acid, (+)–(1S,2S)–norcoronamic acid (12) was developed for culture supplementation.21 Subjecting culture extract isolated from the fermentation broth of the genetically engineered E. coli to a series of chromatographic steps, 11 was isolated at a final yield of 0.6 mg of compound per liter of culture.8

Our successful productions of two unnatural natural peptides suggest that the unnatural straight–chain peptides were accepted as substrates for elongation by succeeding NRPS modules, and eventually homodimerized and cycloreleased by the TE domain. In addition, a putative disulfide bond–forming enzyme Ecm17 has relatively relaxed substrate specificity against the cyclic peptide intermediates and converts to the corresponding products. In the experiment shown above, we can demonstrate the capacity for module–module interaction in our system between two NRPSs of differing origin. In conclusion, the E. coli plasmid–based system allows fast and simple transfer of heterologous biosynthetic pathways of interest from its original producing hosts, such as streptomycetes, into an easy–to–handle heterologous host. Along with the described implications, the system also facilitates the desired engineering of compatible pathways for production of unnatural natural products.

Scheme 4. (a) In vivo enzymatic synthesis of TANDEM (2b) by E. coli carrying the mutated NRPS Ecm7* whose methylation domains were inactivated. (b) In vivo enzymatic synthesis of a hybrid antibiotic ecolimycin C (11) by E. coli carrying the swapped NRPS Swb17 in the presence of the synthetic extender unit 12.
5. Chemoenzymatic Synthesis of Echinomycin Derivatives using a Thioesterase (TE) Domain Excised from the Echinomycin NRPS

It has been speculated that wild-type TE domain, an integral NRPS domain that is located at the C terminus, was responsible for catalyzing the intramolecular cyclization of macrocyclic peptides at the final step. Using several excised TEs from the gramicidin,12 enterobactin,13 surfactin22 and tyrocidine23 NRPS biosynthetic assembly modules has helped to elucidate the activity involved during this process and corroborate their proposed function. These enzymes were analyzed with substrates that were esterified with N–acetylcysteamine (SNAC) to mimic their natural substrates and ultimately establish their substrate–tolerance. Interestingly, from previous studies, TEs of NRPS are known to accept a variety of analog peptides for use as substrates and catalyze a number of cyclorelease reactions. Echinomycin TE,24 a domain located in the Ecm7 module of the echinomycin NRPS, catalyzes the homodimerization and its cyclorelease during biosynthesis of echinomycin. Difficulty to handle N–desmethyltetrapeptidyl thioester due to its inherent reactivity of the free thiol in byproducts formation by thiol/thioester exchange prompted us to use N–desmethylocta-peptidyl substrates. To synthesize various echinomycin analogs, the substrate tolerance of the enzyme for cyclization was examined with substrates which were chemically synthesized to include varying amino acid residues and chromophores relative to its model substrate, 13 (Figure 4). Grati-fyingly, all substrates we synthesized were converted into cyclization products although some substrates reduced their catalytic efficiencies. Substrates without a disulfide bridge caused significant reduction of reaction rates, indicating that a disulfide bridge played an important role in facilitating peptide preorganization into product–like conformations. This remarkable substrate tolerance may allow us to create further diversification of echinomycin analogs.

One of the major drawbacks of Ecm TE catalyzed reactions was hydrolysis of cyclization products. However, circumvention of this problem came from the intrinsic property of bis–intercalator cyclic peptides. In vitro studies with the addition of DNA containing AT–rich sequences improved the yield of target cyclic product with a complete reversal of the cyclization:hydrolysis ratio, from 1:2 to 18:1. Since the improvements were correlated with the DNA unwinding ability of the products, it may be attributed to DNA acting as a scaffold to capture the cyclic product via bis–intercalation and shielding it from potential hydrolytic degradation. This favorable outcome will provide more avenues to producing vast TE culminating cyclic peptides. Based on observed improvement with DNA, a screening system exploiting this observation will facilitate attempts to identify a candidate molecule from a cyclic peptide library by its ability to bis–intercalate to specific DNA sequences. We can benefit from the inherent diversity of this class of compounds and exploit our increasing appreciation of their mechanism of assembly to logically generate natural product analogs with improved bioactivity.

6. Enzymatic Synthesis of Core Skeleton of Antitumor Antibiotics Saframycin

Saframycin A (14) is a representative member of the tetrahydroisoquinoline antibiotics (Figure 5).25 The members of this family have been isolated from various soil bacteria and marine vertebrates such as sponges and ascidians,26 and revealed potent antitumor activity which is originated from iminium ions via aminoacetal moiety and its equivalents. Among the saframycin analogues, ecteinascidin 743 (15, ET–743) revealed a remarkably potent antitumor activity not only with common tumor cell lines but also resistant cell lines. Recently, ET–743 15 was approved as an anticancer drug in the European Union for patients with soft tissue sarcoma.27 Because of a limited supply from natural sources, ET–743 15 is currently produced by the semisynthetic process.28 However, this synthesis required more than 25–step chemical conversions. As an alternative strategy for generating important antibiotics, biosynthetic engineering is strongly desired in view of an efficient and an environmentally benign production. Therefore, to realize this methodology, understanding of the detailed biosynthetic mechanism is in high
demand for the success of this alternative route.

A series of biosynthetic studies on saframycin (14) established its biosynthetic precursors as L-alanine, glycine, and 2 moles of modified amino acid (18) derived from L-tyrosine. To date, three biosynthetic gene clusters of tetrahydroisoquinoline antibiotics 14, safracin B (Figure 5, 16) and saframycin Mx1 (17) have been identified and their bioinformatic analysis indicated that a saframycin backbone is constructed by NRPS. Recently, Wen and coworkers proposed that the saframycin NRPS (SfmA, SfmB, SfmC) unusually assembles the core skeleton via a putative tetrapeptidyl intermediate using SfmC in an iterative manner. However, detailed biosynthetic mechanism of this unusual transformation has not been uncovered. To elucidate it and develop novel saframycin derivatives with enzymatic synthesis, we started to investigate enzymatic reactions with NRPS SfmC.

7. Enzymatic Reaction Catalyzed by SfmC for Construction of a Pentacyclic Tetrahydroisoquinoline Skeleton

Inspection of three tetrahydroisoquinoline biosynthetic NRPSs allowed us to realize the first module which might activate a fatty acyl chain and load it onto the downstream module. To examine this possibility, we synthesized modified tyrosine 18 and several dipeptidyl-S-CoA esters (19a–19d) with various fatty acyl chains. Each synthetic peptidyl-S-CoA was incubated with SfmC in the presence of ATP, Mg2⁺ and NADPH or NADH at 30 °C. HPLC–MS analysis of the reaction mixture indicated that three new products were generated in the reaction with the N-myristoyl-L-Ala–Gly–S-CoA (19a) and NADPH (Scheme 5). Based on spectral analysis including 2D–NMR data, the structure of the products was determined as a pentacyclic tetrahydroisoquinoline 20a, a bicyclic tetrahydroisoquinoline product 22 and a simple reduction product 21. Treatment of

Scheme 5. In vivo enzymatic synthesis of the saframycin core skeleton with saframycin NRPS SfmC.

Scheme 6. Proposed mechanism for the formation of the pentacyclic tetrahydroisoquinoline scaffold with the saframycin NRPS SfmC involving highly unusual iterative reductions and Pictet–Spengler reactions.
the reaction mixture with potassium cyanide was employed to give a cyanoadduct 23, providing further evidence that this compound has a characteristic carbinolamine functional group in the saframycin family. The N-palmitoyl–L-Ala–Gly–S-CoA 19b also provided the pentacyclic compound 20b (Scheme 5). On the other hand, the reaction with the acetylated peptidyl–S-CoA 19c and the free amine peptidyl–S-CoA 19d, however, did not provide any detectable enzymatic products. These facts obviously indicated that the long-chain fatty acid connected to the linear peptidyl chain is a cryptic functional group during the saframycins biosynthesis and a critical element for the SfmC reaction.

On the basis of these results of the SfmC reaction, we proposed the plausible assembly mechanism of the pentacyclic tetrahydroisoquinoline skeleton from N-myristoyl–L-Ala–Gly–S-CoA 19a as shown in Scheme 6. The reaction sequence is as follows: 1) the R domain of SfmC reduces the thioester linkage of the peptidyl–S-CoA 19a in a NADPH-dependent manner and provides the fatty acyl dipeptidyl aldehyde 21; 2) the A domain activates the first molecule of modified tyrosine 18 as an aminoaetyl–AMP and loads onto the thiol group of the phosphopantetheinyl arm attached with T4; 3) the first Pictet–Spengler type reaction between the aldehyde 21 and the amino group of the modified tyrosine 18 gives the bicyclic tetrahydroisoquinoline–S–T4; 4) this intermediate is reduced again by the R domain to release the bicyclic aldehyde 22 from the SfmC; 5) the second molecule of modified tyrosine is reloaded onto the T4; 6) the second Pictet–Spengler reaction mediated by the C domain between the bicyclic aldehyde 22 and the modified tyrosine–S–T4 affords a tetracyclic tetrahydroisoquinoline product; 7) reduction of the thioester linkage of the tetrcyclic–S–T4 intermediate by the R domain to furnish the pentacyclic tetrahydroisoquinoline skeleton 20a, termed as presaframycin, via the spontaneous intramolecular cyclization of the aldehyde 24. The observation of the bicyclic compound 22 strongly indicated that the pentacyclic tetrahydroisoquinoline skeleton is assembled in a stepwise manner as shown in Scheme 6. Based on the observation that the R domain accepts three structurally different substrates, we speculate that SfmC accepts various synthetic analogs. Currently, we are synthesizing saframycin derivatives by chemoenzymatic process which can lead to a synthetic intermediate of ET–743 15.

8. Concluding Remarks

In this account, we demonstrate in vitro and in vivo enzymatic synthesis of complex natural peptides. An E. coli–based expression system which we developed provides a powerful method for rational genetic engineering of NRPS by deletion, mutation and swapping of modules and subunits. This process is successfully applied to the enzymatic synthesis of echinomycin and its derivatives. Our studies described in this account clearly demonstrated that multimodular enzyme NRPS have relatively broad substrate specificity and tolerant, structurally different intermediates to process them down-stream as shown in this account and related literatures. This suggests that combinatorial biosynthesis using portability of domains and modules in NRPS is a promising approach to synthesize a number of peptide antibiotic derivatives. In addition, in vitro synthesis of the pentacyclic tetrahydroisoquinoline core of saframycin with a single NRPS clearly demonstrated novel functions of both C and R domains in NRPS. As shown in the studies of echinomycin and saframycin NRPS, chemoenzymatic and enzymatic reactions of artificial substrates further produce diversity of various peptide products.

A rate limiting step of enzymatic synthesis of natural products is the identification of biosynthetic genes. Nevertheless, innovative DNA sequencing technology enables us rapid access of target genes. Recent success of in vitro synthesis of complex polyketide enterocin with twelve enzymes added further support to the usefulness of this powerful technology since this synthesis can be achieved by simply mixing substrates and co–factors with the corresponding enzymes. Pioneering works on these enzymatic syntheses of natural products both in vitro and in vivo have revealed a promising future for this technology. In the near future, this approach will be an alternative method in preparing complex natural products and their analogs with potent biological activities that are available only at low yields or produced by difficult–to–culture organisms.

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References


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Kenji Watanabe: Associate Professor, School of Pharmaceutical Sciences, University of Shizuoka

Kenji Watanabe received his B.A. in 1996 and Ph.D. in 2000 in biological chemistry from Hokkaido University in the Department of Agriculture. His postdoctoral fellowships included a year at the School of Pharmacy, University of Wisconsin–Madison and a two-year appointment at Stanford University in the Department of Chemistry and Chemical Engineering. In 2003, he returned to Hokkaido University as an assistant professor in the Department of Agriculture. In 2004, he joined the Department of Pharmaceutical Sciences at the School of Pharmacy, University of Southern California as a senior research associate and was promoted to research assistant professor, in 2006. He returned to Hokkaido University as a designated assistant professor in the Division of Chemistry, Graduate School of Science, and he joined the Research Core for Interdisciplinary Sciences, Okayama University in 2008. In 2009, he moved to Shizuoka to join the School of Pharmaceutical Sciences, University of Shizuoka as an associate professor. e-mail: watanabe.kenji@gmail.com

Hiroki Oguri is an Associate Professor of the Graduate School of Science, Hokkaido University. He was born in 1970 in Tokyo. He received B.Sc (1993) and Ph.D. (1998) degrees from Tohoku University, and was then appointed as an Assistant Professor in the research group of Prof. Masahiro Hirama (1998–2003). In 2003–2004, he worked with Prof. Stuart L. Schreiber at Harvard University as a visiting scientist; then, in 2004, he joined Hokkaido University in the research group of Prof. Hideaki Oikawa as an Associate Professor. Since 2007, he has also been serving as an investigator at Creative Research Initiative “Sousei” (CRIS), Hokkaido University. He was honored with the Inoue Research Award for Young Scientists (2000), the Young Scientist’s Research Award in Natural Product Chemistry (2002), and the Chemical Society of Japan Award for Young Chemists (2005). His current research interests include design, synthesis and diversification of natural product analogs and modulation of cellular functions using synthetic molecules.

Hideaki Oikawa is a Professor at the Division of Chemistry and the Graduate School of Science, Hokkaido University. He received his Ph.D. (1984) in organic chemistry from Hokkaido University. He worked as a postdoctoral fellow with Prof. D. E. Cane at Brown University from 1984 and with Dr. K. Isono at RIKEN in Japan from 1985. He joined the Department of Agriculture, Hokkaido University, as an Assistant Professor in 1986 and was promoted to Associate Professor in 1999. He moved to the Division of Chemistry and Graduate School of Science at Hokkaido University as a Professor in 2003. His present research focuses on the engineering of biosynthesis of natural products, combinatorial biochemistry, and the reaction mechanism on C–C bond-forming enzymes. He is a recipient of the Award for Encouragement of Young Scientists from the Japan Society of Bioscience, Biotechnology, and Agrochemistry (1993) and the Chemical Society of Japan Award for Creative Work (2004).