Escherichia coli allows efficient modular incorporation of newly isolated quinomycin biosynthetic enzyme into echinomycin biosynthetic pathway for rational design and synthesis of potent antibiotic unnatural natural product

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Abstract

Natural products display impressive activities against a wide range of targets, including viruses, microbes and tumors. However, their clinical use is hampered frequently by their scarcity and undesirable toxicity. Not only can engineering *Escherichia coli* for plasmid-based pharmacophore biosynthesis offer alternative means of simple and easily-scalable production of valuable yet hard-to-obtain compounds, but also carries a potential for providing a straightforward and efficient means of preparing natural product analogs. The quinomycin family of nonribosomal peptides, including echinomycin, trtiostin A and SW-163s, are important secondary metabolites imparting antibiotic antitumor activity via DNA bisintercalation. Previously we have shown the production of echinomycin and trtiostin A in *E. coli* using our convenient and modular plasmid system to introduce these heterologous biosynthetic pathways into *E. coli*. However, we have yet to develop a novel biosynthetic pathway capable of producing bioactive unnatural natural products in *E. coli*. Here we

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Supporting Information Available: Experimental procedures, two tables of DNA sequence information for the oligonucleotides, a figure for mass spectra of **3**, **4**, **5**, **6**, **7** isolated from labeled methionine-feeding experiment, and a table and two figures of spectrometric data on the *E. coli*-produced **14**.

report an identification of a new gene cluster responsible for the biosynthesis of SW-163s that involves previously unknown biosynthesis of (+)-(1*S*, 2*S*)-norcoronamic acid and generation of aliphatic side chains of various sizes via iterative methylation of an unactivated carbon center. Substituting an echinomycin biosynthetic gene with a gene from the newly identified SW-163 biosynthetic gene cluster, we were able to rationally re-engineer the plasmid-based echinomycin biosynthetic pathway for the production of a novel bioactive compound in *E. coli*.

Keywords

depsipeptide; hybrid molecule; nonribosomal peptide synthetase; engineered biosynthesis; *E. coli*

Introduction

With the growing global threat of multi-drug-resistant microbes and increasing economic and environmental concerns on synthetic production of natural products and their analogs, there is an increasing interest in developing alternate platforms for the discovery, production and development of pharmaceutically valuable compounds. To date, many biosynthetic gene clusters encoding polyketide synthases (PKSs), nonribosomal peptide (NRP) synthetases (NRPSs), mixed PKS—NRPSs and associated auxiliary enzymes have been found in various different organisms through recent genome and metagenome sequencing efforts[1]. However, many organisms are difficult to culture in large scale[2], and some are impractical to handle due to pathogenicity. Others produce natural products at very low level or maintain their gene clusters silent under conventional culture conditions[3]. One way to exploit the potential of these organisms and the genomic information for drug discovery and development is to establish a heterologous production system using a convenient host organism capable of expressing the exogenous genes and biosynthesizing the desired compounds. Several organisms, including certain *Streptomyces* strains and yeast, have been used as such hosts[4], but *Escherichia coli* carries certain advantages over others. First, a wealth of tools are available for incorporation, controlling expression, and modification of foreign genes in *E. coli*, such as plasmids with various useful properties, including plasmid compatibility and selectability. Additionally, methods of manipulating *E. coli* and its vectors are well-established and straightforward to perform. Furthermore, *E. coli* is forgiving in expressing exogenous genes and readily grows with a very short doubling time in a simple, inexpensive growth medium without requiring particularly difficult-to-obtain culture conditions. Although *E. coli* is not known for its ability to produce diverse secondary metabolites, the fact that *E. coli* is naturally capable of producing siderophore NRPs[5] and a PK—NRP hybrid molecule colibactin[6] suggests that its metabolic system is not entirely foreign to natural product biosynthesis. To introduce a heterologous biosynthetic pathway into *E. coli,* we chose a plasmid-based system. In this approach, plasmids are used to assemble and deliver genes necessary to reconstitute the target biosynthetic pathway into the host. Although this approach demands initial efforts of constructing plasmids bearing multiple genes of the target biosynthetic pathway, we have lowered the hurdle significantly by developing a cloning method that exploits iterative ligation of DNA fragments bearing compatible cohesive-ends. This led to the first successful production of a bioactive NRP natural product echinomycin **1** and triostin A **2** in *E. coli* (Figure 1)[7].

In parallel with the development of the *E. coli*-based system for heterologous production of secondary metabolites, we are also trying to identify new biosynthetic pathways that give rise to unique chemical structures found among natural products. Such knowledge is crucial in broadening the scope of combinatorial biosynthesis of various natural products and their analogs. Discovery of new enzymes that are capable of performing different transformations permits expansion of the toolbox with which we can devise novel biosynthetic pathways for production of interesting new compounds. Combined with the advantages of using *E. coli* as

a heterologous production host described earlier, *E. coli*-based plasmid-borne heterologous secondary metabolite biosynthetic system can not only facilitate the reconstitution of various biosynthetic gene clusters but also provide a flexible platform suitable for fast and simple rational re-designing of biosynthetic pathways through modular reassembling of the biosynthetic genes from various biosynthetic systems. Here, to put this concept to work, we have identified a new biosynthetic gene cluster responsible for the biosynthesis of the SW-163 series of NRPs involving several previously uncharacterized enzymatic transformations. Then we took this new knowledge and applied it to our previously established *E. coli*-based plasmid-borne NRP biosynthetic system to achieve a rationally engineered biosynthesis of unnatural bioactive NRP we named ecolimycin C, a hybrid compound of **2** and SW-163 C **3**.

Results

Isolation of SW-163 Biosynthetic Gene Cluster

Previously, we reported the total biosynthesis of two antitumor NRPs, **1** and its intermediate **2** in *E. coli*[7]. Originally identified and isolated from *Streptomyces* strains, both compounds were categorized as quinomycin antibiotics because of their quinoxaline or quinoline chromophores attached to the C_2-symmetric bicyclic depsipeptide core[8]. This family of antibiotics is well-characterized, and exhibits potent antibacterial, anticancer and antiviral activities[9]. While both compounds bis-intercalate into GC-rich DNA sequences, **1** and **2** exhibit distinct sequence preference. Now we have isolated an additional family of quinomycin antibiotics, SW-163C **3**, D **4**, E **5**, F **6** and G **7**[10,11] from *Streptomyces* sp. SNA15896 (Figure 1). All of them carry a pair of 3-hydroxyquinaldic acid (HQA, **8**) chromophores along with a non-proteinogenic amino acid, norcoronamic acid (NCA), whose biosynthetic mechanism has not been described to date. The absolute configuration of **4**, and hence the SW-163s, was determined by establishing the absolute configuration of the NCA residue to be (+)-(1*S*, 2*S*)-norcoronamic acid [(+)-NCA, **9**][11]. Also, like in **1**, compounds **4**, **5**, **6** and **7** all possess a central thioacetal cross-bridge formed by the chemical rearrangement of a disulfide bridge catalyzed by a post-NRPS modification enzyme, methyltransferase[7]. Most interestingly, however, the thioacetal bond is appended by a distinct alkyl side chain in these compounds, each varying by a single carbon unit.

Closely resembling the echinomycin biosynthetic gene cluster, the SW-163 biosynthetic gene cluster (Figure 2) was identified and isolated from the total DNA of *Streptomyces* sp. SNA15896 (Supporting Information Experimental Procedures). DNA sequence analysis of this 38 kilobase-long cluster revealed the presence of fifteen genes considered to be involved directly in the biosynthesis of SW-163s: seven genes (*swb*1, *swb*2, *swb*10, *swb*11, *swb*13, *swb*14 and *swb*18) likely forming the proposed SW-163 primer unit **8** (Figure 3a), three genes (*swb6, swb7, swb9*) assembling **9** (Figure 3b), and five genes (*swb8, swb12, swb16, swb17, swb20*) constructing and modifying the peptide backbone (Figure 3c). The cluster also contains a presumed resistance gene (*swb15*) and other genes, including *swb3*, *swb4*, *swb5* and *swb19*, presumably involved in the regulation of biosynthesis or export of the products.

Based on the results from our feeding experiments using isotope-labeled substrates[14] in conjunction with the previous BLAST[12] analyses of the genes from the SW-163, thiocoraline[13] and echinomycin[7] biosynthetic clusters, biosynthesis of **8** is thought to proceed similarly as previously proposed for the biosynthesis of quinoxaline-2-carboxylic acid (QXC **10**, see Figure 4b). That is, the biosynthesis proceeds via the initial β-hydroxylation of L-tryptophan to form (2*S*, 3*S*)-β-hydroxytryptophan **11**, followed by the formation of β-hydroxykynurenine **12**. Transamination of **12** by Swb1 can generate **13**, and oxidation and dehydration of **13** by Swb2 can afford **8** (Figure 3a).

Despite the structural similarity of **9** to coronamic acid, whose biosynthesis involves a cryptic chlorination step[15], no homologs of the coronamic acid biosynthetic genes were identified in the SW-163 biosynthetic gene cluster. Although the mechanism of biosynthesis of **9** remains unclear, based on previous reports of a radical SAM dehydrogenase and lysine 2, 3-aminomutase[16,17], we hypothesize that predicted radical SAM protein Swb7 and pyridoxal-5′-phosphate (PLP)-dependent aminotransferase Swb6 can work together closely to form **9** from L-Val via radical cyclopropanation (Figure 3b).

Assembling of the SW-163 peptide scaffold from L-Ser, L-Ala, L-Cys and **9** appears to be accomplished by two bimodular NRPSs, Swb16 and Swb17. The terminal TE domain in Swb17 likely homodimerizes and cycloreleases the peptide chain (Figure 3c)[18]. Following cyclization of the linear peptide, an oxidoreductase Swb20 presumably catalyzes an oxidation reaction to generate the disulfide bond in **3**. Subsequently, the disulfide bridge in **3** can be converted to a thioacetal bridge to form **4** (Figure 3d) by a predicted methyltransferase Swb8, homolog of the SAM-dependent methyltransferase Ecm18 that catalyzes the equivalent step in the echinomycin biosynthetic pathway[7].

Lastly but most notably, we propose that Swb9, a predicted radical SAM protein, performs the further iterative methylation of the thioacetal bridge. With the finding of Swb9, we are able to come up with a more plausible mechanism for the iterative methylation of the thioacetal bridge (Figure 3e) than our previously proposed mechanism[7]. Using the well-established method[19] for isotopically enriching the SAM-derived methyl groups with a labeled L-methionine, we were able to observe that, in addition to the four *N*-methyl groups of the cyclic depsipeptide core, every proton in the *S*-alkyl chains of **4**, **5**, **6** and **7** became deuterated upon feeding of [*methyl*-D_3]-L-methionine to the SW-163-producing host (Supporting Information Figure 1). These results were consistent with the *S*-alkyl side chains formed by step-wise transfer of a methyl group derived from SAM by a methyltransferase. As proposed for the methylation during the fosfomycin biosynthesis[20], a radical SAM protein can perform a methyl transfer reaction on a non-activated carbon at the cost of one molecule of SAM and methylcobalamine (MeCbl). We hypothesize that this unique radical SAM methyltransferase can iteratively methylate **4** to generate ethyl- **5**, *i*-propyl- **6**, and *sec*-butyl **7** derivatives (Figure 3e). Further studies to support this idea are currently underway.

E. coli Biosynthesis of Novel NRP with Antibiotic Activity

To further challenge the versatility of our system, we aimed to biosynthesize *in vivo* in *E. coli* a new, bioactive compound. For this purpose, as an initial attempt we chose to replace L-Val in the depsipeptide scaffold of **2** with **9** to yield a hybrid molecule **14** we termed ecolimycin C. The crystal structure of the complex between **2** and its DNA substrate showed L-Val minimally participating in the DNA recognition[21]. Also, the fact that the SW-163 antibiotics carry this moiety indicated that the new compound would most likely retain antibiotic activity.

To convert the triostin A biosynthetic pathway into a biosynthetic pathway for **14**, Ecm7 was replaced with Swb17, a bimodular NRPS showing high production level in *E. coli* (Supporting Information Figure 2a) that contains the adenylation domain capable of accepting **9** as an extender unit and the thioesterase domain capable of homodimerizing and cycloreleasing the peptide chain containing **9**. To minimize plasmid instability, gene assemblies were kept to a minimal length while maintaining as many genes within a contiguous metabolic pathway on a single plasmid as possible; the genes for the biosynthesis of **10** were put together on one plasmid (pKW532), two megasynthetases Ecm6 and Swb17 on another (pKW755, Supporting Information Figure 3a), and the rest of the genes on the third plasmid (pKW539, Supporting Information Figure 3b). Also, to prevent potential premature termination and mRNA degradation[22] while transcribing an extremely long polycistronic gene assembly, all genes were assembled in a multi-monocistronic fashion.

Because **14** may possess antibiotic activity, the growth of *E. coli* can be hampered by its accumulation in the cytosol. Thus, a self-resistance mechanism was included to circumvent this potential problem. Previously, the echinomycin resistance-conferring gene *ecm16*, a close homolog of the daunorubicin resistance-conferring gene *drrC* that provides non-destructive resistance against daunorubicin in *S. lividans* and *E. coli*[23], was used successfully for our *E. coli in vivo* production of **1** and **2**[7]. Considering this previous report, along with the observation that the expression level of *swb15* in *E. coli* (Supporting Information Figure 2b) was comparable to that of *ecm16*, we reasoned that either Ecm16 or Swb15, UvrA-like protein with high (90%) sequence homology to Ecm16 (Figure 2b), can provide *E. coli* resistance against **14**. To ascertain the effectiveness of the two genes on the culture viability and titer of **14**, two separate constructs were prepared to include either *ecm16* (pKW539) or *swb15* (pKW756) in the expression system (Supporting Information Figure 3b). However, since there was almost no difference in the titer between the two systems, Ecm16 was used throughout the studies.

BL21 (DE3) was transformed with three plasmids pKW532, pKW755 and pKW539, and subjected to shake-flask cultivation for seven days in M9 minimal medium supplemented with chemically synthesized **9** (Figure 4a). After subjecting the *E. coli* culture extract to a series of chromatographic steps to obtain purity necessary for collecting well-resolved 2D NMR spectra, the final yield of **14** was 0.6 mg per liter of culture. The expected mass of $[M+H]^+$ ion at m/z = 1083.3849 was obtained on the purified **14** by FAB—HR—MS (Figure 4c and Supporting Information Table 3). The compound was characterized further by ^{1}H, ^{13}C and 2D NMR (Figure 4d, 4e and Supporting Information Table 3, Figures 4 and 5) to reveal a characteristic resonance at δ 9.50 (s, 1 H, QXC H-3′). Ultimately, total correlated spectroscopy (TOCSY) (Supporting Information Figure 5b) confirmed unambiguously that our engineered bacterium was capable of producing **14**. Furthermore, the antibiotic activity of this new hybrid NRP compound was established to be as effective if not more potent than **3** as determined by halo formation assay against *Bacillus subtilis* (Figure 5).

Discussion

The *E. coli*-based biosynthetic system clearly exhibits its ease of manipulation when one attempts to introduce planned modifications into the target biosynthetic pathway. Taking full advantage of these features and challenging our plasmid-borne NRP biosynthetic system further, we decided to combine two NRP biosynthetic pathways from two different sources to biosynthesize a novel hybrid NRP which we named ecolimycin C **14**. To prepare this hybrid molecule, we applied part of the biosynthetic gene cluster for the quinomycin antibiotics SW163C—G that we have newly discovered to our plasmid-borne triostin A biosynthetic pathway. Apart from the very interesting thioacetal bridge alkyl side chain formation that appears to be accomplished by unprecedented iterative methylation of non-activated carbon centers by a radical SAM methyltransferase Swb9 (Figure 3e), the SW-163 biosynthetic pathway involves the production of non-proteinogenic amino acid **9** (Figure 3b) and its incorporation into the homodimeric cyclic depsipeptide core by the NRPS Swb17 (Figure 3c). By replacing Ecm7 in the triostin A biosynthetic pathway with Swb17, we envisioned the production of **14**, which carries the chromophores of **2** while having a sterically more constrained, α-branched amino acid residue of **3** in its backbone (Figure 3b). The HQA-to-QXC and L-Val-to-(+)-NCA switch seemed modest enough to preserve the antibiotic activity of the parent compounds **2** and **3**, yet different enough to see possible alterations in the substrate DNA affinity and/or sequence selectivity[24].

Although it was necessary to feed *E. coli* chemically synthesized **9** to accomplish the biosynthesis of **14**, the observed titer of **14** was significantly higher than that of **1** or **2** without requiring feeding of **10**. This unexpected high yield of an unnatural NRP could be due to higher expression level of *swb17* (Supporting Information Figure 2a). Also, lower metabolic turnover

of the building block **9** may have helped boost the yield further. Nevertheless, this result indicates that, in addition to Ecm7, another NRPS Swb17 can also accept, elongate, homodimerize and cyclorelease unnatural peptide chain. Furthermore, Ecm17 exhibited relaxed substrate specificity by accepting the unnatural intermediate to **14**. Most importantly, however, our result demonstrates that two NRPSs from differing origin were able to establish productive *in vivo* intermodular interactions and produce **14** in *E. coli*. The fact that **14** was obtained at a comparable or better yield than the parent compounds in *E. coli* by a simple substitution of an enzyme in the corresponding heterologous biosynthetic pathway without any extensive modifications of the pathway nor its constituents is especially encouraging for the future prospect of our system. In addition, the efficiency and versatility of the *E. coli* platform can be improved further by incorporating various technologies currently being investigated, including the development of efficient fed-batch fermentation protocols[25], use of quantitative metabolic flux analyses for identifying the possible routes to improving the system[26], application of directed evolution for improving the protein-protein interactions among foreign enzymes and their domains[27], and manipulation of the transcriptional machineries for tailoring the global cellular phenotype for improved heterologous secondary metabolite production[28]. Taken together, we believe that our results have laid down the foundation for the future development and refinement of *E. coli* as a flexible, robust platform for fast and simple large-scale production of pharmaceutically useful molecules and their analogs in an environmentally friendly fashion from simple carbon and nitrogen sources.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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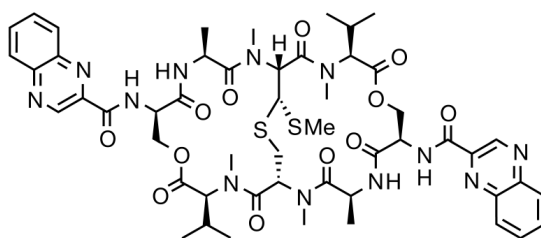
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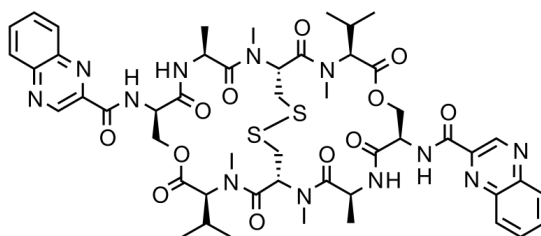
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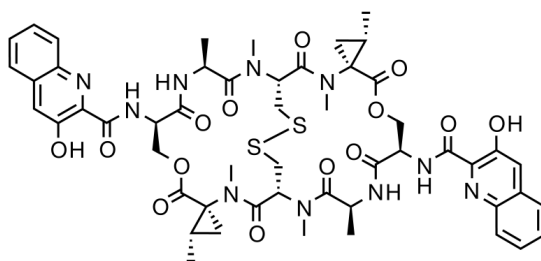
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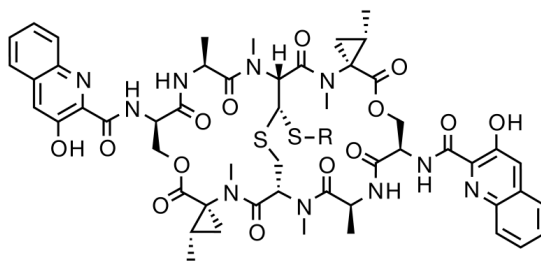
SW-163D **4** R=Me
SW-163E **5** R=Et
SW-163F **6** R=*i*-Pr
SW-163G **7** R=*sec*-Bu

Figure 1.
Chemical structures of quinomycin antibiotics.
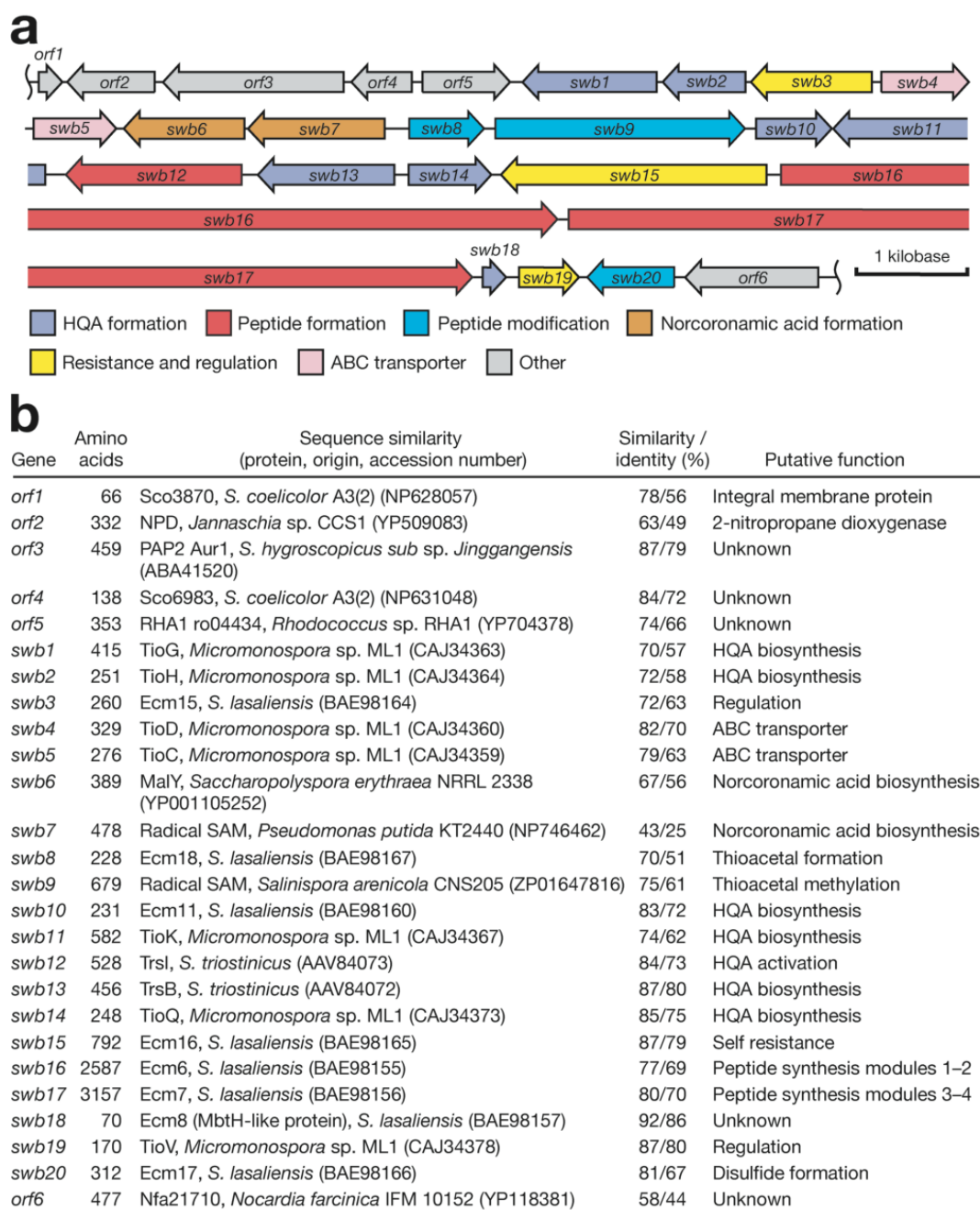
Figure 2.
Biosynthetic cluster of SW-163 from *Streptomyces* sp. SNA15896. (a) Organization of the SW-163 biosynthetic gene cluster. (b) Deduced functions of the ORFs identified within the SW-163 biosynthetic gene cluster based on the percentage sequence similarity/identity to known proteins determined using BLAST.
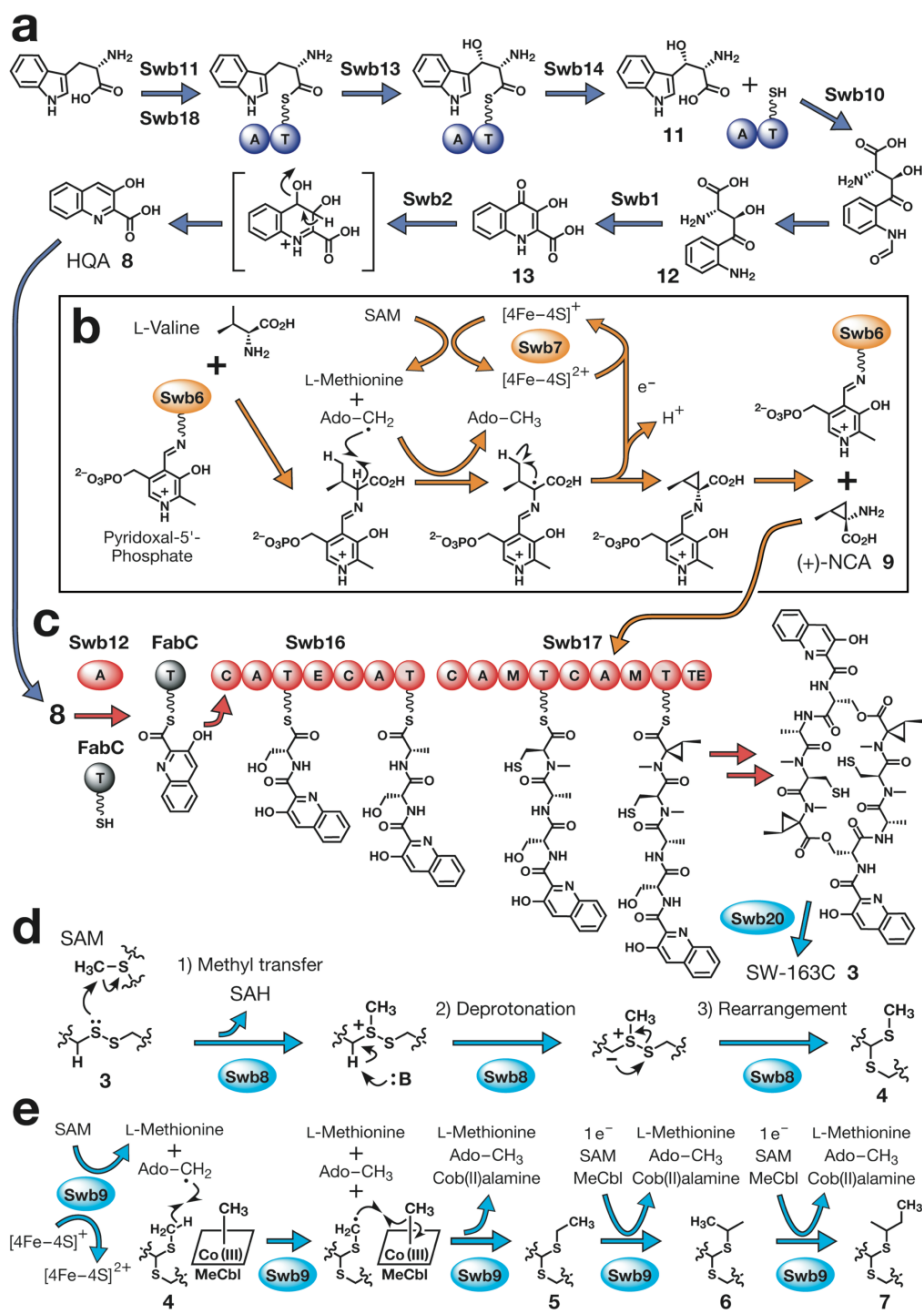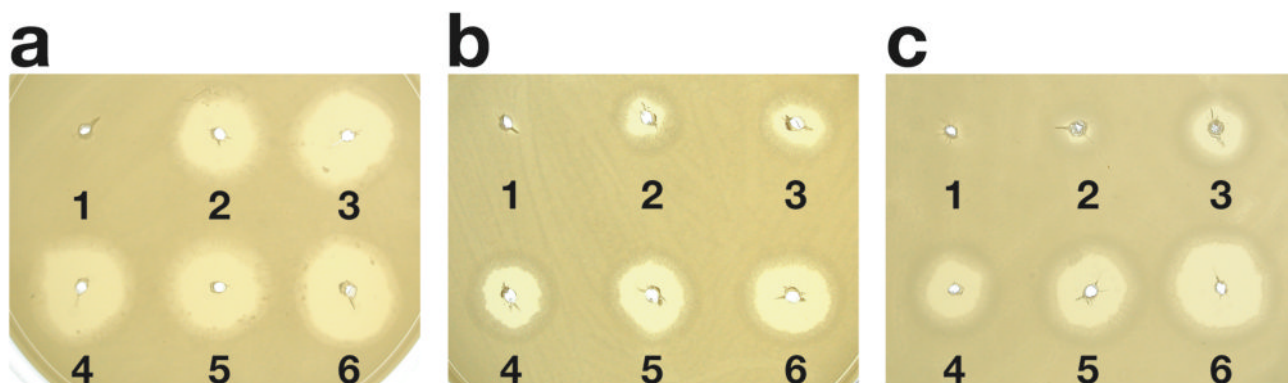
Figure 3.
Proposed mechanism for the SW-163 biosynthesis. (a) Proposed pathway for the biosynthesis of HQA **8**. (b) Proposed mechanism for the formation of (+)-(1*S*, 2*S*)-norcoronamic acid [**9** (+)-NCA]. (c) Proposed pathway for the biosynthesis of the octadepsipeptide core. (d) Proposed mechanism for the formation of a thioacetal bridge. (e) Proposed mechanism for the formation of a series of alkyl substituents differing by one carbon unit. NRPS domain name abbreviations are: A, adenylation; C, condensation; E, epimerization; M, *N*-methyltransferase ; M*, inactive methylation; T, thiolation; and TE, thioesterase. Other abbreviations are: Ado-CH_3, 5′-deoxyadenosine; AMP adenosine 5′-monophosphate ; HQA, 3-hydroxyquinaldic acid;

MeCbl, methylcobalamine; (+)-NCA, (+)-(1*S*, 2*S*)-norcoronamic acid; SAM, *S*-adenosyl-L-methionine; and SAH, *S*-adenosyl-L-homocysteine.
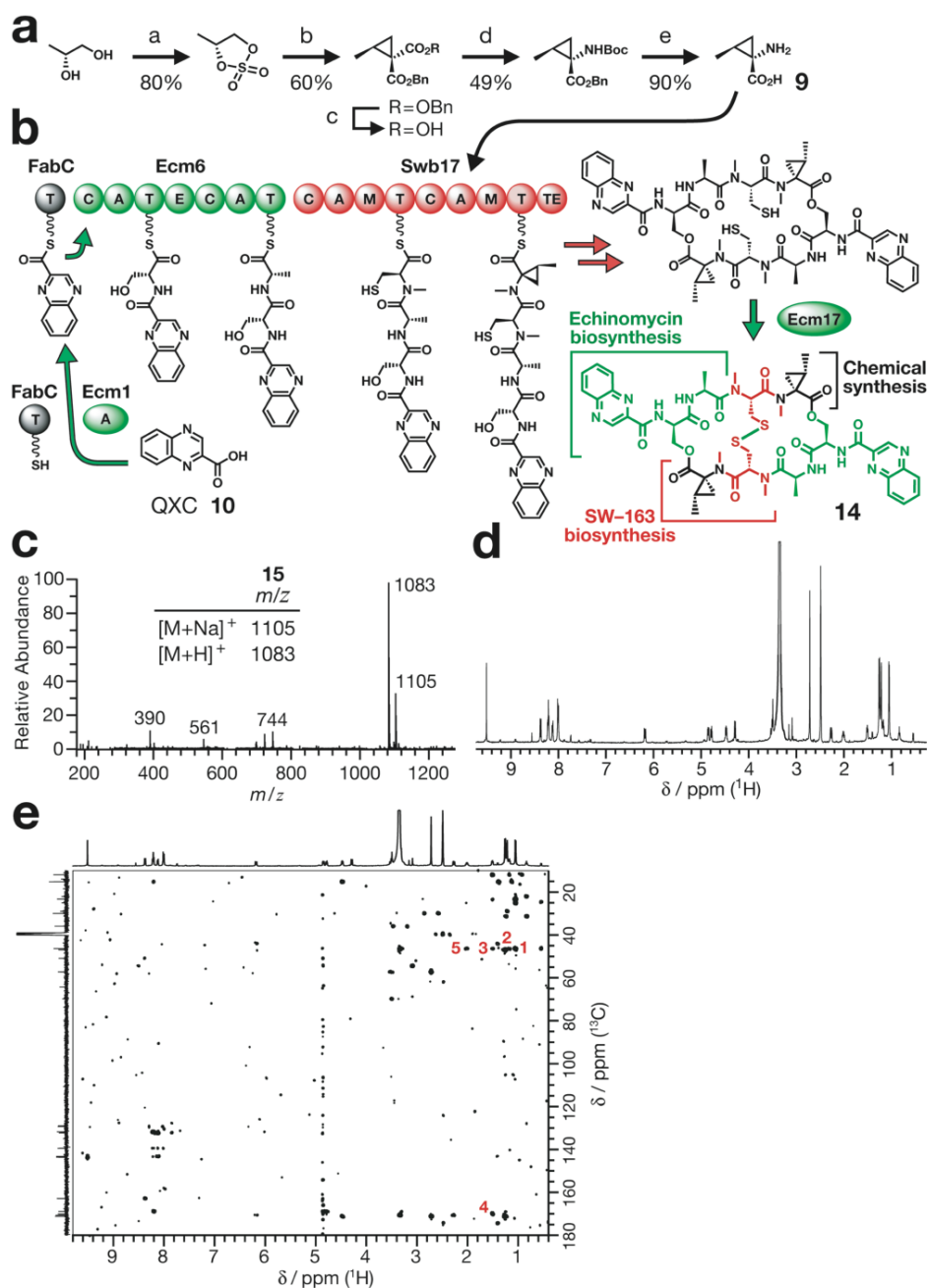
Figure 4.
Biosynthesis of a novel antibiotic ecolimycin C **14** in *E. coli*. (a) Chemical synthesis of the extender unit **9** fed to *E. coli* for the biosynthesis of **14**. a, i) $SOCl_2$, $ClCH_2CH_2Cl$, ii) cat. $RuCl_3$, $NaIO_4$, CH_3CN—H_2O; b, dibenzyl malonate, NaH, 1,2-dimethoxyethane; c, 1 M NaOH, MeOH; d, diphenylphosphoryl azide, Et_3N, *t*-BuOH; e, i) H_2, 10% Pd/C, EtOAc, ii) 6 M HCl, MeOH. Product yield of each chemical reaction step is reported in percentages. (b) Schematics of the pathway for the *E. coli* biosynthesis of **14**. Abbreviation: QXC, quinoxaline-2-carboxylic acid. (c) LC—MS data, (d) ^{1}H NMR spectrum and (e) HMBC spectrum of **14**. Important correlations (in red) are 1: δ 1.05 (NCA—C*H₃*) and 46.4 (NCA—*C*α); 2: δ 1.18 (NCA—C*H₂*) and 46.4 (NCA—*C*α); 3: δ 1.51 (NCA—C*H₂*) and 46.4 (NCA—

*C*α); 4: δ 1.51 (NCA—C*H*$_2$) and 170.1 (NCA—*C*O); 5: δ 2.01 (NCA—C*H*) and 46.4 (NCA—*C*α).

Figure 5.
Halo-formation assay for assessing the antibiotic activity of **2**, **3** and **14**. A tryptic soy agar plate plated with *B. subtilis* was spotted with (a) **2**, (b) **3** and (c) **14**. Each well contained the corresponding compound at an incremental amount of 2: 1.0 ng; 3: 2.0 ng; 4: 4.0 ng; 5: 8.0 ng and 6: 16 ng. Well 1 is a negative control.