Title

Discovery of Novel Bioactive Compounds from a Rare Actinomycete *Amycolatopsis* sp.26-4

Keywords

Amycolatopsis, peptides, natural products, total synthesis, combined-culture

Abatract

Natural products play an indispensable role in the drug development process. It is estimated that ca. 35% of small-molecule drugs is either natural products or their synthetic derivatives.[1] Since the discovery of penicillin in 1928 and streptomycin in 1943, so many antibiotics have been successfully discovered from microbes, such as sulfonamide, bacitracin, chloramphenicol, tetracycline, vancomycin, etc. There is no doubt that antibiotic drugs are the most successful chemotherapy, since new antibiotics saved millions of lives from infectious diseases worldwide and increased life expectancy by up to two decades in recent history.[2] Therefore, compounds with novel skeletons and biological activities have always been the hotspot of natural products research.

In natural products research, microorganisms still remains as important source of new drug compounds. So far, among the bioactive compounds that have been isolated from microbes, 45 % are produced by actinomycetes, 38 % by fungi and 17 % by unicellular eubacteria.[3] Actinomycetes continue to play the most significant role in drug discovery and development.

Amycolatopsis, genus of a rare actinomycete, was misidetentified as *Streptomyces* and later as *Nocardia* in the past. In 1986, *Amycolatopsis* was recognized as a unique genus by Lechevalier.[4] *Amycolatopsis* is an important actinomycete in the industry of antibiotics, which produced many potent antibiotics on the market, such as balhimycin by *Amycolatopsis balhimycina*, thiazomycins by *Amycolatopsis fastidiosa*, rifamycins by *Amycolatopsis mediterranei*, azicemicins by *Amycolatopsis sulphurea*, and vancomycin by *Amycolatopsis orientalis*.[5] Among these antibiotics, rifamycin is one of the major drugs used for clinical

treatment of tuberculosis. Vancomycin is a famous antibiotics and recognized as the last line of defense against some resistant pathogenic bacteria. *Amycolatopsis* sp. exhibited the good ability of producing bioactive secondary metabolites.

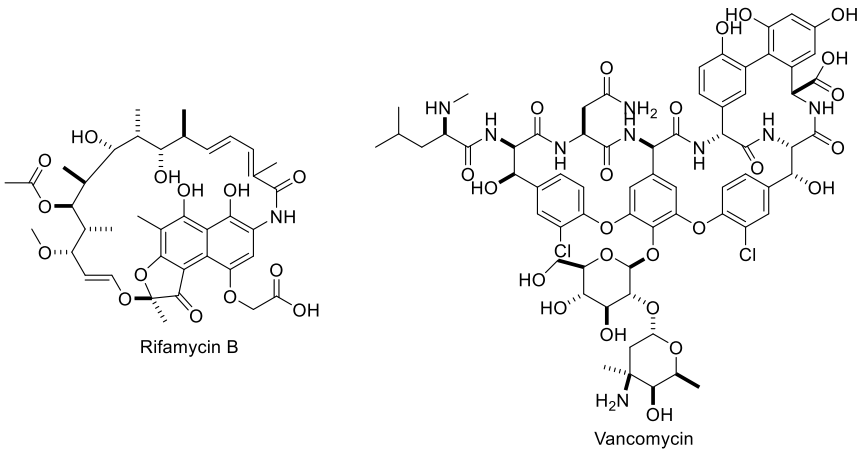


Figure 1 Structures of rifamycin B and vancomycin.

Sulfur, special element in natural products, always connected with aromatic ring as a thioether in the known compounds.[6] Thioethers in alphatic chain are rare, have never been discovered in peptides.[7] Sulfur often occurred as thiazoline or thiazole in peptides from microorganism and marine invertebrates, such as karamomycins, apratoxins, marthiapeptides, trunkamides, bistratamides, bisebromoamide, etc.[8] Most of them exhibited cytotoxicity against human cancer cells. It is worth noting that sulfur-containing heterocycle plays a critical role in several bioactive natural products and pharmaceutical agents.

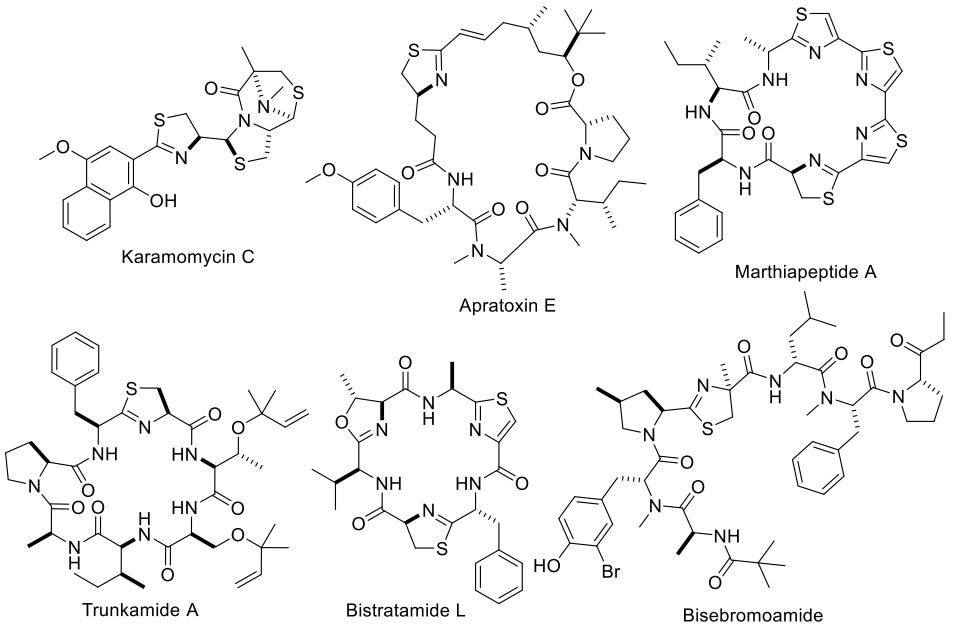


Figure 2 Structures of thiazole/thiazoline containing peptides.

Because of the unique structure and bioactivity, total synthesis of sulfur-containing peptides has been considering as an attractive research topic. Several compounds have been successfully synthesized in recent years.[9] Chemical synthesis of thiazole ring was easily accessible by various approaches and Hantzsch reaction discovered in 1889 remains one of the most reliable routes.[10] However, synthesis of thiazoline is not so easy due to the epimerization of chiral carbon. The condensation of L-cysteine and aryl nitriles or *α*,*α*-difluoroalkyl amines is an alternative way to obtain optically active thiazoline in good yields.[11] Dehydration reaction between thioamide and alcohol is commonly used in thiazoline containing peptide synthesis.

Since the rise of antibiotic resistance, finding novel natural antibiotics to fight infection has become an urgent need.[12] In this research, I performed a systematic research for the secondry metabelites of *Amycolatopsis* sp. 26-4 derived from Iriomote island near Okinawa, Japan. Based on previous screenings for structurally unique and biologically interesting natural products, I isolated and identified a series of novel sulfur-containing heterocyclic lipopeptides named thioamycolamides from the culture broth of *Amycolatopsis* sp. 26-4. (**Chapter 1-1**). A bioinspired concise total synthesis of thioamycolamide A has been accomplished (**Chapter 1-2**). Two novel decapeptides, Amycolapeptin A and Amycolapeptin B, which were not detected in monoculture of *Amycolatopsis* sp. 26-4, have been isolated from the combined-culture broth of *Amycolatopsis* sp. 26-4 and *Tsukamurella pulmonsis* TP-B0596 (**Chapter 2**).

Chapter 1. Studies on Thioamycolamides Produced by *Amycolatopsis* sp. 26-4

Chapter 1-1. Isolation, Structure Determination, and Cytotoxicity of Sulfur-Containing Cyclic Lipopeptides Thioamycolamides

In natural products research, chemical screening without information of bioactivity is also an important method to discover novel metabolites.[13] From research for structurally unique and biologically interesting natural products, I investigated novel natural products from a library of rare actinomycetes derived from Iriomote island near Okinawa, Japan. Based on my preliminary screenings using physicochemical properties including both HRMS and UV spectroscopy, I discovered a series of compounds with molecular weight from 420 to 480 *m/z* from the culture broth of *Amycolatopsis* sp. 26-4. The chemical formular of these compounds were not recorded in the natural product mass databass (AntiBase), suggested that moleculars haven't been discovered before. Coumponds were subsequently isolated and identified as novel sulfur-containing heterocyclic lipopeptides named thioamycolamides A-E. Among these five metabolites with unprecedented carbon skeletons, thioamycolamide A (**1**) was produced as the major product by this Gram-positive bacterium (Figure 3).

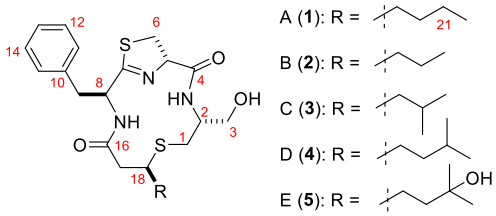


Figure 3 Structures of thioamycolamides A-E (**1-5**).

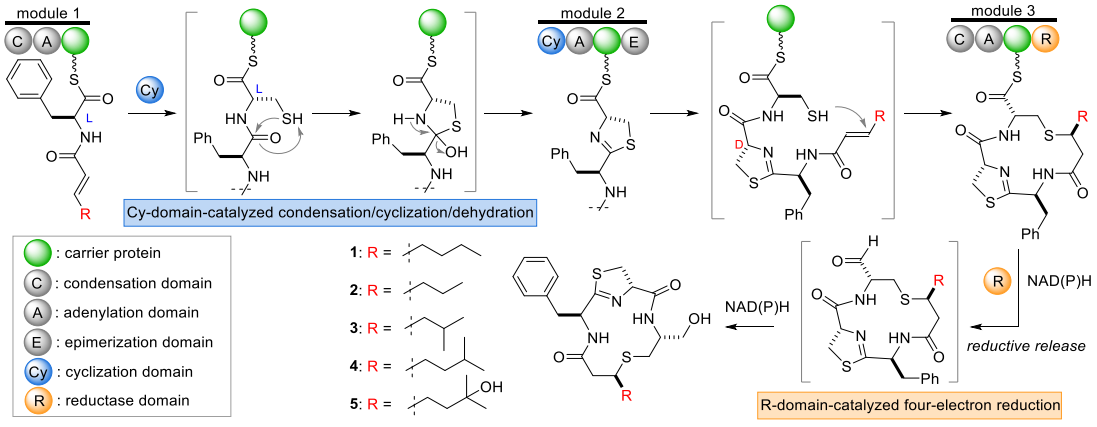
The planar structural elucidation was accomplished by HRMS and 1D/2D NMR spectroscopic data analyses. The absolute configurations were determined by Marfey's method, CD spectroscopy, and synthesis of partial structures. The side chain at lipid moiety of each analogue varied in terms of the number of carbon atoms and the branching pattern of the methyl groups. The carbon numbers found in the side chains of thioamycolamides A–E were three, four and five, and the terminal structure of the side chains was normal- (without branched methyl) or iso-type. These structural moieties were confirmed by HRMS and NMR analyses.

The cytotoxicities of all isolated compounds were evaluated in two human cancer cell lines, HT1080 and HeLa S3. Thioamycolamides A and D with a four carbon-chain length at side chain

exhibited moderate cytotoxicity with IC_{50} values of 6.53–21.22 μM, whereas thioamycolamide E with a 2,2-dimethyl-2-butanol side chain had an IC_{50} value of greater than 100 μM, indicating the importance of the aliphatic property for their biological activities. Moreover, thioamycolamides B and C, both of which have a three carbon-chain length at side chain, showed weak (IC_{50}, 78.57, 67.88 μM, respectively) or no cytotoxicity even at 100 μM, respectively. These structure-activity relationships (SARs) suggest that both hydrophobicity and the length of the side chain at side chain are crucial for exhibiting the cytotoxicity presumably based on the difference of membrane permeability.

Chapter 1-2. Bioinspired Concise Total Synthesis of Thioamycolamide A

As described in chapter 1-1, rare sulfur-containing cyclic lipopeptide thioamycolamide A (**1**, Figure 1) along with the minor analogues thioamycolamides B-E (**2**-**5**) were isolated. The peptide **1** shows moderate inhibitory activities against several human cancer cell lines, and chemical structure of **1** was established by the combination of spectroscopic analyses and chemical synthesis of their partial structure. Its cyclic skeletal structure bears a D-configured thiazoline, a thioether bridge, a fatty acid-side chain, and a reduced C-terminus. Although its biosynthetic gene cluster has not been identified, these structural features suggest that the cytotoxin **1** was assembled by a nonribosomal peptide synthetase (NRPS) as illustrated in Scheme 1.



Scheme 1 Putative biosynthetic pathway of **1**-**5**.

To get more amount and different derivatives of thioamycolamides for further biological activity research, a concise total synthesis of thioamycolamide A has been accomplished based on its putative biosynthesis. The peptide chain was elongated by an isomerization-suppressed

procedure, and the thioether linkage was constructed by thio-Michael addition as a key reaction in the final step.

Furthermore, the structural identity between natural and synthetic thioamicolamide A was confirmed by the LC-MS experiments with highly sensitive Marfey's method.[14] In the LC-MS experiments, stereochemical purity of chemically constructed thioamicolamide A was surely verified, which corroborates the efficiency of our bioinspired synthesis and strongly supports the proposed thio-Michael addition pathway for its thioether biosynthesis.

Chapter 2. Studies on Combined-Culture of *Amycolatopsis* sp. 26-4 and *Tsukamurella pulmonsis* TP-B0596

In 2011, Onaka reported mycolic acid-containing bacteria *Tsukamurella pulmonis* TP-B0596 can influence the biosynthesis of cryptic natural products in *Streptomyces lividans* TK23. The subsequent research showed *Tsukamurella pulmonis* TP-B0596 is an activator strain which could change secondary metabolism in ~90% of *Streptomyces* species in combined-culture compared with single culture.[15] This new coculture method was called "combined-culture". Although their biological roles in the complex microbial communities are still largely unknown, "combined-culture" fermentation method has been recently proved as a simple and powerful tool to search potential metabolites. Several novel bioactive compounds produced by the combined-culture were discovered such as arcyriaflavin E, chojalactones, dracolactams, catenulobactins, niizalactams, etc.[16]

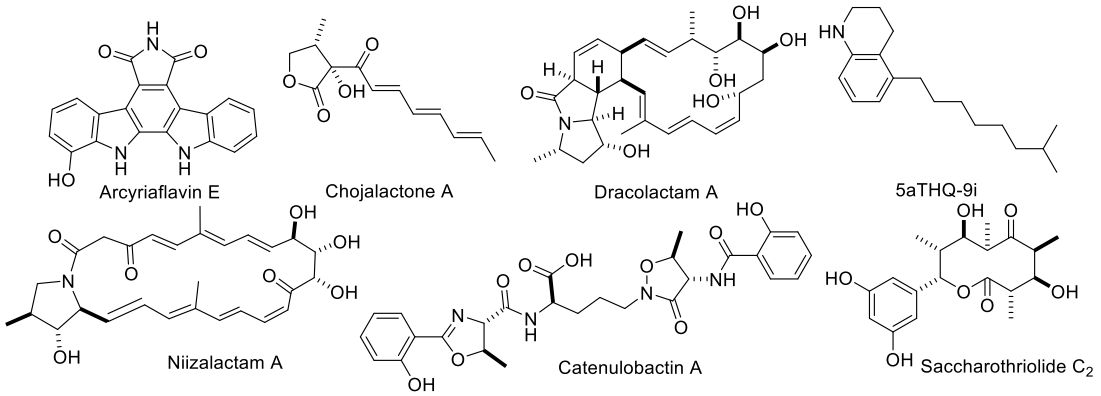


Figure 4 Structures of novel compounds produced by the combined-culture.

By applying combined-culture method, our laboratory priviously discovered several novel 5-alkyl-1,2,3,4-tetrahydroquinolines (5aTHQs) from the combined-culture broth of *Streptomyces nigrescens* and *Tsukamurella pulmonis* TP-B0596 and saccharothriolide C_2 from *Saccharothrix* sp. A1506 and *Tsukamurella pulmonis* TP-B0596.[17]

Amycolatopsis sp. 26-4 exhibited the ability of producing structurally unique secondary metabolites in the thioamicolamides research. In order to activate silent cryptic biosynthetic gene clusters and obtain more novel skeleton metabolites, combined-culture method was applied to strain *Amycolatopsis* sp. 26-4 with *Tsukamurella pulmonis* TP-B0596. Two novel nonapeptides, amycolapeptins A and B, which were not produced in a single culture of *Amycolatopsis* sp. 26-4, isolated from the combined-culture broth, with 22-membered depsipeptide, *β*-hydroxytyrosine,

and highly modified tryptophan moieties. The chemical structures including the absolute configuration were elucidated by spectroscopic analysis, chemical synthesis of fragments, a highly sensitive Marfey's method, and CD spectroscopy.

Conclusions

In this thesis, I summarized the complete research of novel cyclic sulfur-containing lipopeptides, thioamycolamides, including isolation, structure elucidation, absolute configuration determination, total synthesis, biological evaluation and plausible biosynthetic pathway. Moreover, two novel nonapeptides, amycolapeptins A and B, which were not produced by a single culture of *Amycolatopsis* sp. 26-4, were successfully isolated from the combined-culture broth. The structures of amycolapeptins were also confirmed.

The differences in the activities of thioamycolamides A-E suggests that further SARs study in the C18 side chain would be helpful for possible anticancer development of this class of compounds. Although the biological roles of strains *Amycolatopsis* sp. 26-4 and *Tsukamurella pulmonis* TP-B0596 in the microbial communities are unknown, further studies on combined-culture may provide answers to these interesting questions. The biosynthetic mechanisms of thioamycolamides and amycolapeptins in the producing organism are also of considerable interest, because of their highly modified skeletons. I believe that my results in my thesis would be helpful for drug development, microorganism biosynthesis, and microbial communication research in the near future.

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