Master's Thesis of Natural Science

Bio-synthetic Yield Improvement of Platensimycin through Co-culture of Two *Streptomyces* spp.: Identification of Inducing Factor produced by the Co-culture strain CMDD14E023

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이 논문을 이학석사 학위논문으로 제출함

2022년 2월

서울대학교 대학원

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강 은 모

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Abstract

Infectious diseases caused by bacteria have not been conquered yet since ancient times to the present. Since the development of penicillin which is the first antibiotic, the problem of infectious diseases originated from bacteria seemed to be solved. However, with the emergence of antibiotic-resistant bacteria, the mortality rate due to bacterial infection has been increasing repetitively. Therefore, antibiotics with new mechanisms of action that can act on resistant bacteria are regarded and natural antibiotics produced by microorganisms suggest the possibility of discovering new antibiotics. Among them, platensimycin is a broad-spectrum antibiotic against gram-positive pathogens and inhibits the fatty acid synthesis system. In this study, we increased the biosynthetic yield of platensimycin, a natural antibiotic produced by *Streptomyces platensis* and synthesized its derivatives. As a method to increase the biosynthetic yield, six media conditions and five extraction methods were conducted. As a result of media and extraction method study, the best condition that could synthesize and extract platensimycin was corn meal media and acidic ethyl acetate extraction. The liquid media extract of *S. platensis* grown for 14 days in media containing corn meal was separated from and purified by various chromatography techniques and chemical methods in order to obtain pure platensimycin. In addition, the yield was increased through a co-culture method using *Streptomyces champavatii*. It was confirmed that the yield was not increased with other strains. Furthermore, a compound made by *S. champavatii* increased the biosynthetic yield of platensimycin. The chemical structures of the extracted and purified compounds in this thesis were identified through NMR (Nuclear Magnetic Resonance), mass spectroscopy, and UV (Ultra Violet) spectroscopy. Through this study, the biosynthetic yield of platensimycin was increased by about four times and the causative substance (Inducer) was found and elucidated.

Keywords

natural products, antibiotics, platensimycin,

Streptomyces, biosynthetic yield improvement

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Chapter 1 Introduction

1.1 Antibiotics and resistant bacteria

The development of antibiotics, one of the greatest achievements of modern medicine, has enabled the complete cure of numerous infectious diseases. However, the emergence of antibiotic resistance bacteria has caused the failure of antibiotics treatment and fatal consequences of various infectious diseases within only 60 years since antibiotics were first introduced as clinical medicine.[1]

History of Antibiotics

Documents related to microbial infections, treatments and managements can be found in ancient Egypt, Greece, and China.[2] Modern antibiotics have begun the development of penicillin. The antibiotic penicillin which has saved millions of lives and marked as the first start of modern antibiotics was first discovered by Sir. Alexander Fleming.[2, 3] However, in 1948, penicillin-resistant bacteria appeared and were spread all around the world. Subsequently, antibiotics against strains with penicillin resistance were developed in 1950-60s.[4] Figures 1-1 and 1-2 show the simple history of antibiotic development and the occurrence of strains of various antibiotics.

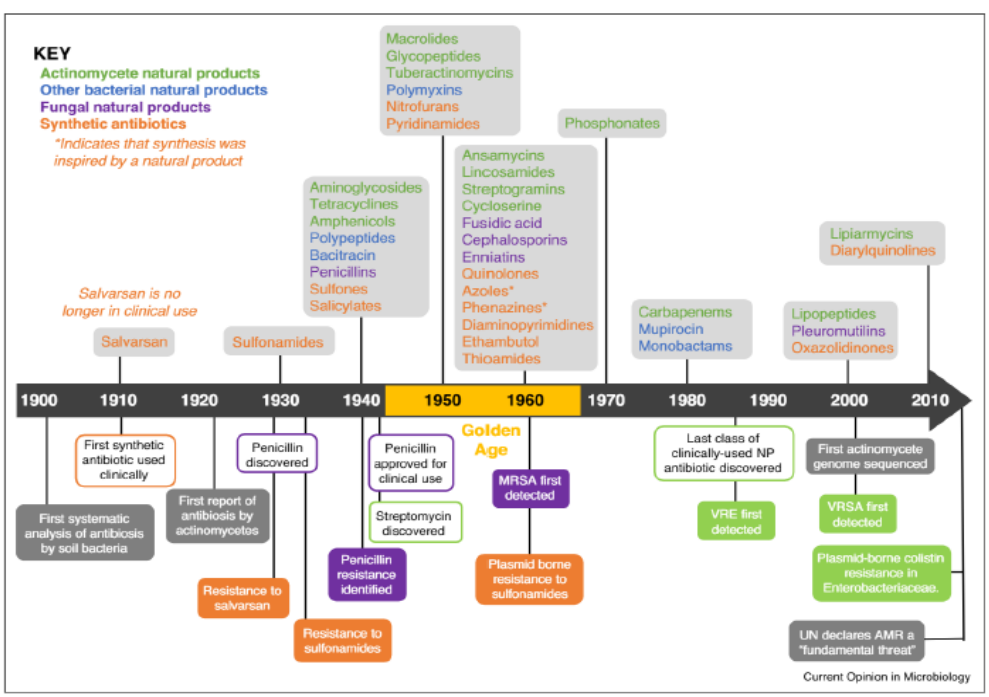


Figure 1-1. Timeline showing the decade new classed of antibiotic[1]

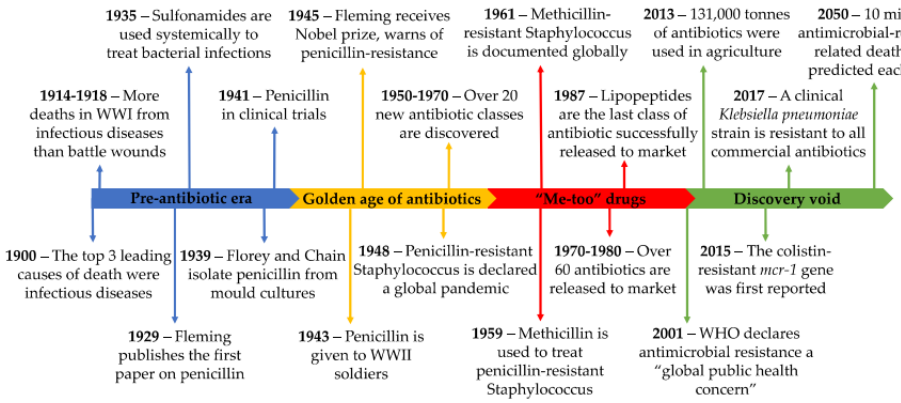


Figure 1-2. Events in the antibiotic and resistance timeline[4]

A resistance to therapeutic agents of bacterial infectious diseases has become a serious medical and social problem as they have the highest disease rates. Antibiotic resistance is a common problem in all developed and developing countries to the extent that the WHO (World Health Organization) defines it as a serious threat to global public health.[4] Figure 1-3 shows the comparison between antibiotic development and antibiotic-resistant.[5] As shown in figure 1-3, antibiotic resistance increases in a rapid pace, while the antibiotic development seem to be hampered and continues to decline. Therefore, it is necessary to develop not antibiotics that operate with existing mechanism of action but antibiotics with a new mechanisms of action.

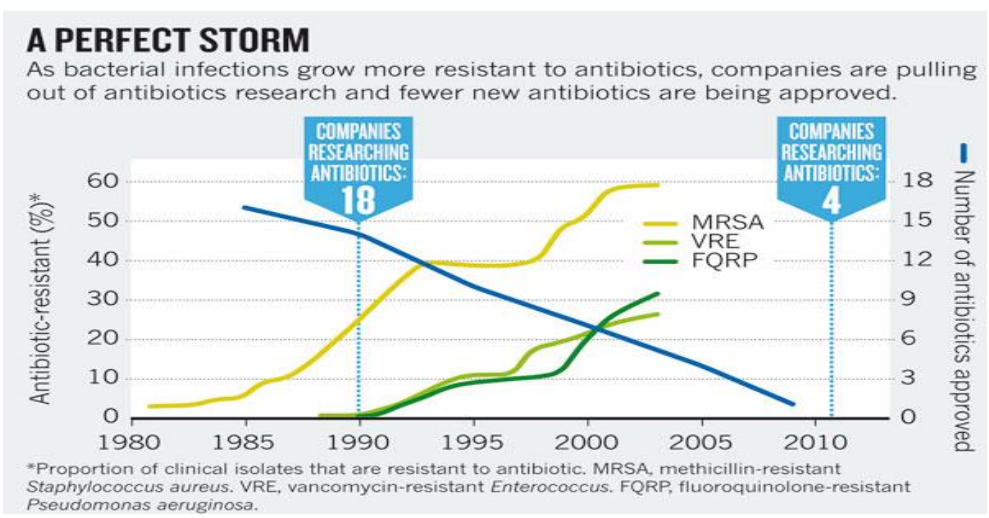


Figure 1-3. Comparison between antibiotic development and antibiotic-resistant[5]

Classes of antibiotics

There are several classes in antibiotics. Antibiotics from natural products (NPs) are classified based on the species of microorganisms. In the case of synthesized antibiotic drugs, they are often developed by obtaining an idea of moiety from existing NPs. In addition, most antibiotics are classified into individual antibiotic classes. Drugs with similar chemical and pharmacological properties can be grouped together. Their chemical structures are alike and drugs under the same class are effective to the identical or related bacteria. The groups are followings: Penicillins, Tetracyclines, Cephalosporins, Quinolones, Lincomycins, Macrolides, Sulfonamides, Glycopeptides, Aminoglycosides, Carbapenems and Lipopeptide. Each group acts on different types of bacteria and some antibiotics have side effects. The cephalosporin group is effective against MRSA (Methicillin-Resistant *Staphylococcus Aureus*) in the 5th generation, but it has a myelotoxicity. Also, the daptomycin of the lipopeptide group can kill resistant bacteria; however, it cannot be applied to pneumonia due to the lack of lung permeability. As there are various side effects, and many resistant bacteria that have not been conquered yet, the development of new antibiotics is necessary. Figure 1-4 and Table 1-1 below show the types and mechanisms of action of antibiotics that have been developed and used for treatment so far.

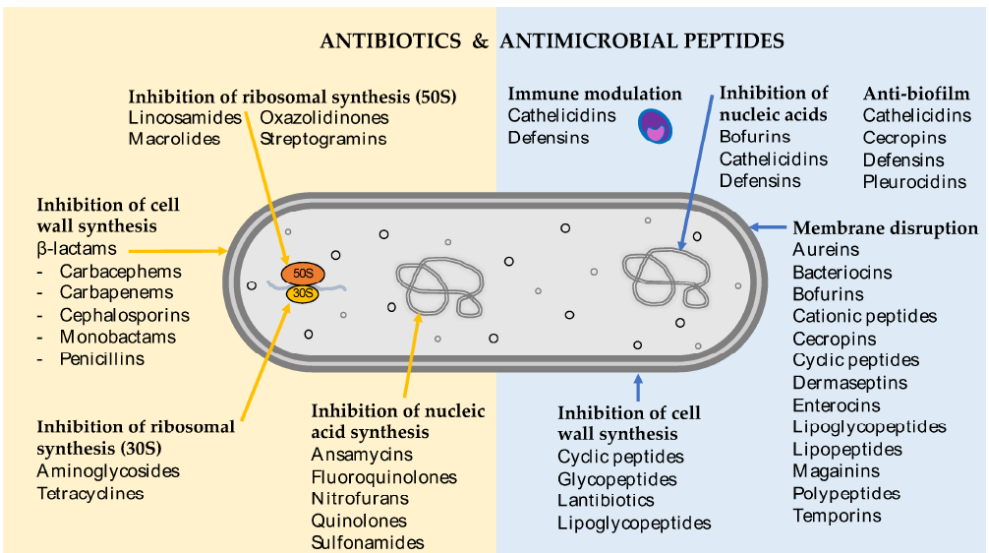


Figure 1-4. Antibiotics and their acting sites[4]

class	Introduced clinically	example	Molecular target
Antibiotics from actinomyces			
Aminoglycosides	1946	**Kanamycin A**	Protein synthesis : 30S ribosomal subunit
Tetracyclines	1948	**Tetracycline**	Protein synthesis : 30S ribosomal subunit
Amphenicols	1949	**Chloramphenicol**	Protein synthesis : 50S ribosomal subunit
Macrolides	1952	**Erythromycin**	Protein synthesis : 30S ribosomal subunit
Glycopeptides	1958	**Vancomycin**	Cell wall synthesis: D-ala-D-ala termini of lipid II
Carbapenems	1985	**Meropenem**	Cell wall synthesis: penicillin-binding proteins
Lipopeptides	2003	**Daptomycin**	Cell wall: cell membrane disruption
Lipiarmycins	2011	**Fidaxomicin**	Nucleic acid synthesis: RNA polymerase
Antibiotics from other bacteria Polypeptides			
Polypeptides	1941	**Gramicidin A**	Cell wall: forms ion channels that increase the permeability of the bacterial cell membrane
monobactams	1986	**Aztreonam**	Cell wall synthesis: penicillin-binding proteins
Antibiotics from fungi			
Penicillins	1943	**amoxicillin**	Cell wall synthesis: penicillin-binding proteins
Pleuromutilins	2007	**Retapamulin**	Protein synthesis: 50S ribosomal subunit
Synthetic antibiotics			
Sulfonamides	1936	**Mafenide**	Folate synthesis: inhibition of dihydropteroate synthetase
Pyridinamides	1952	**Isoniazid**	Cell wall: prodrug that inhibits the synthesis of mycolic acids
Oxazolidinones	2000	**Linezolid**	Protein synthesis: 50S ribosomal subunit
Diarylquinoline	2012	**Bedaquiline**	ATP synthesis: proton pump inhibition

Table 1-1. All classes of clinically used antibiotics and their source [1]

1.2 Natural products

Natural products (NPs)

Natural products are enormous structural and chemical diversity sources in therapeutics. They are optimized as drug-like molecules by evolution and remain the unique sources of drugs and drug candidates.[6] Making drugs by using NPs has different characteristics compared with making drugs by total synthesis. As mentioned above, NPs have various structures and chemical diversity, therefore, it generally has a large molecular weight, and its structure is complicated and has a large molecular rigidity.[7] That is why many natural product-based drugs have been released to date and are being clinically used. Among the approved drugs, the natural product section is "N", "NB" and "ND". These three sections account for only about 23.4% of all approved drugs, but when we include "S/NM" and "S*/NM" which mimic NPs, and "S*" of which pharmacophore came from NPs, it can be said that about 49% of them came from NPs. As such, it can be said that ideas and materials obtained from natural products play an important role in the development of new drugs.

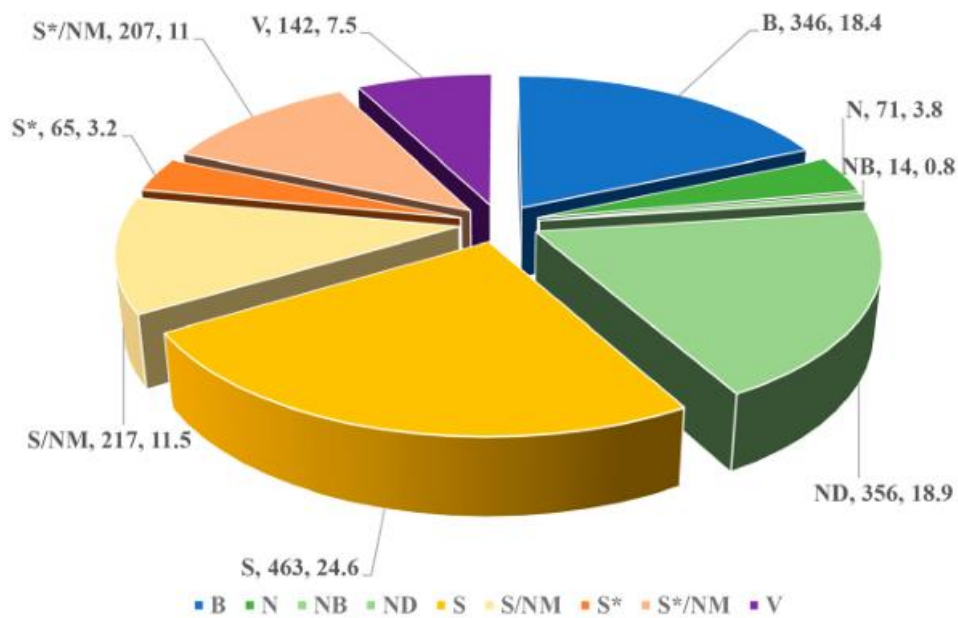


Figure 1-5. All new approved drugs during 01JAN81 to 30SEP19; n = 1881[8]

Figure 1-5 is a diagram showing the drug proportion that has been approved so far. The description of each category is as follows.;[8]

B: Biological

N: Natural product

NB: Botanical Natural product

ND: Derived from a natural product and usually a semisynthetic modification

S: Totally synthetic drug

S*: Total synthetic drug, but the pharmacophore is from NP

V: Vaccine

NM: Natural product mimic

Figure 1-6 shows the outline of natural product drug discovery. The purple boxes show the steps in the process of NPs drug discovery. The red boxes and green boxes represent the limitations and solutions respectively. The process starts with an extraction of natural products from organisms like bacteria, fungi, or even animals and plants. There are diverse extraction methods that can extract various compound classes of natural products. After identifying an extract with promising pharmacological activity, the bioactive fraction is continuously fractionated and purified until the pure bioactive compound is isolated.

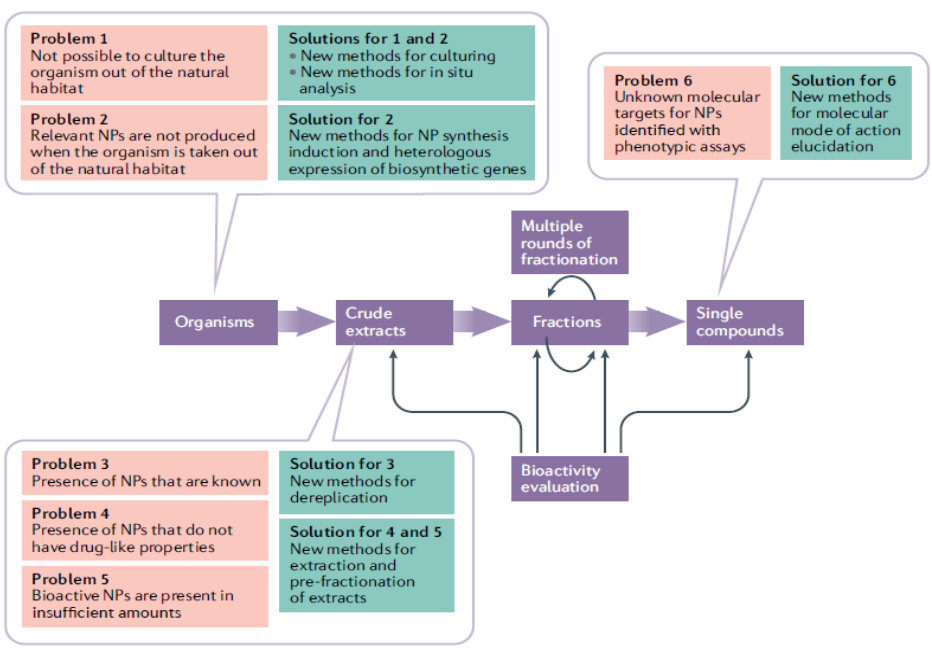


Figure 1-6. Outline of natural product drug discovery[7]

The problems and solutions represented in red and green boxes respectively are not perfectly addressed. The problems still exist despite of the development of sciences and the solutions also applied locally. Even though these challenges exist, the strength of the novel structure with bioactivity of natural products shows the possibility of the natural product.

Antibiotics based on NPs

Figure 1-7 is a diagram showing the developmental source of antibiotics. Excluding vaccines in the field of antibiotics, there are 130 drugs. Among these, the synthetic drug "S" section is only 7%. The remaining 93% are NPs with ideas or NPs. Unlike drugs with other diseases, the antibiotic field depends largely on natural products. Because bacteria have evolved to kill other microorganisms and are evolving further to kill antibiotic-resistant bacteria as well.[9] As a way to find a new antibiotic, it is expected that antibiotics with a novel structure can be found by the screening of existing NPs.

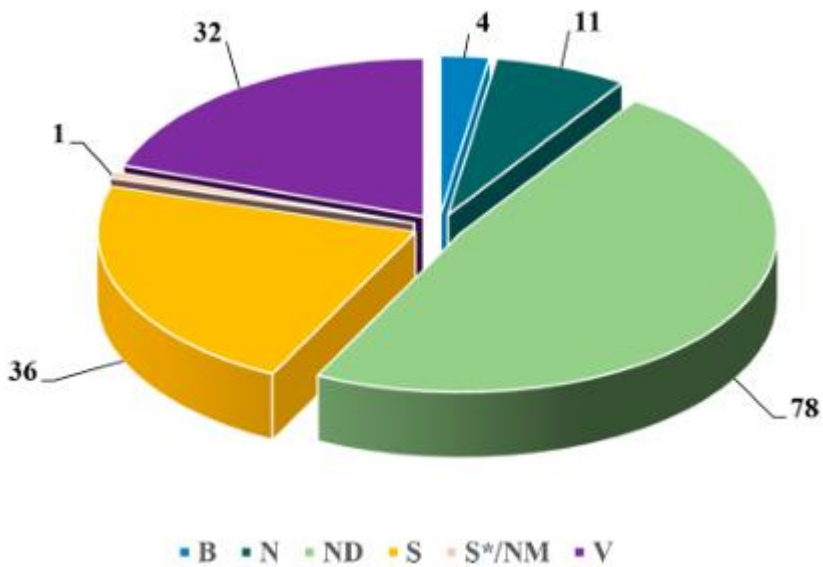


Figure 1-7. Antibacterial drugs by source; n = 162[8]

The small molecule antibiotics based on NPs continue to be found as shown in figure 1-8.[9] The **hymenosetin**, **1**, tetramate-containing NPs, is succumbed to total synthesis.[10] **Kibdelomycin**, **2**, exhibits broad-spectrum antibacterial activity by inhibiting DNA gyrase and topoisomerase IV.[11, 12] **Huminanamycin A**, **3**,

exhibit selectively active against the bacterial pathogen *Salmonella enterica* by inhibiting the synthesis of riboflavin.[13] **Penicyclones A-E**, **4**, with a highly functionalized cyclohexenone moiety show antibacterial polyketides and exhibit antimicrobial activity against the *S. aureus* with cytotoxicity.[14]

Polyenic amides, **5a-c**, **6, 7a-d** and **8**, exhibit antibacterial activity. **Inthomycin A, B** and **C**, **5a-c**, in which all isomer possessed the triene system, also exhibit antibacterial activity.[15] **Simoncyclinone D8**, **6**, containing aminococumarin, polyketide, and sugar subunits linked by a tetraene exhibit antibacterial activity against both gram-positive and negative bacteria by inhibiting DNA gyrase.[16] **Oxazolomycins**, **7a-d**, possessing complex polyene lactam-lactone substructures show antibacterial activity.[17] **Batumin**, **8**, inhibit bacterial cell motility which does not kill nor induce resistance.[18]

The unusual structures of antibiotics, **9** and **10**, have been reported. **Baulamycins A** and **B**, **9**, exhibit the antibacterial effect against both gram-positive and gram-negative.[19] **Artonin I**, **10**, inhibits multidrug resistance *S. aureus* and lower other antibiotic MIC values when it is used simultaneously.[20] These 'helper' systems provide the possibility of prolonging the life of existing clinically effective drugs without resistance.

The compounds **11** to **13** are total synthesis antibiotics. The first total synthetic compound **11**, **tetarimycin A**, has an antibacterial effect both on gram-positive and negative, and also active against MRSA (Methicillin-Resistant *S. aureus*) and VRE (Vancomycin-resistant *Enterococci*).[21] **Viridicatumtoxin**, **12**, possessed tetracyclin moiety shows inhibitory activity against MRSA and VRE.[22] **Hongoquercins**, **13a** and **b**, exhibit antibacterial activity against VRE and MRSA.[23]

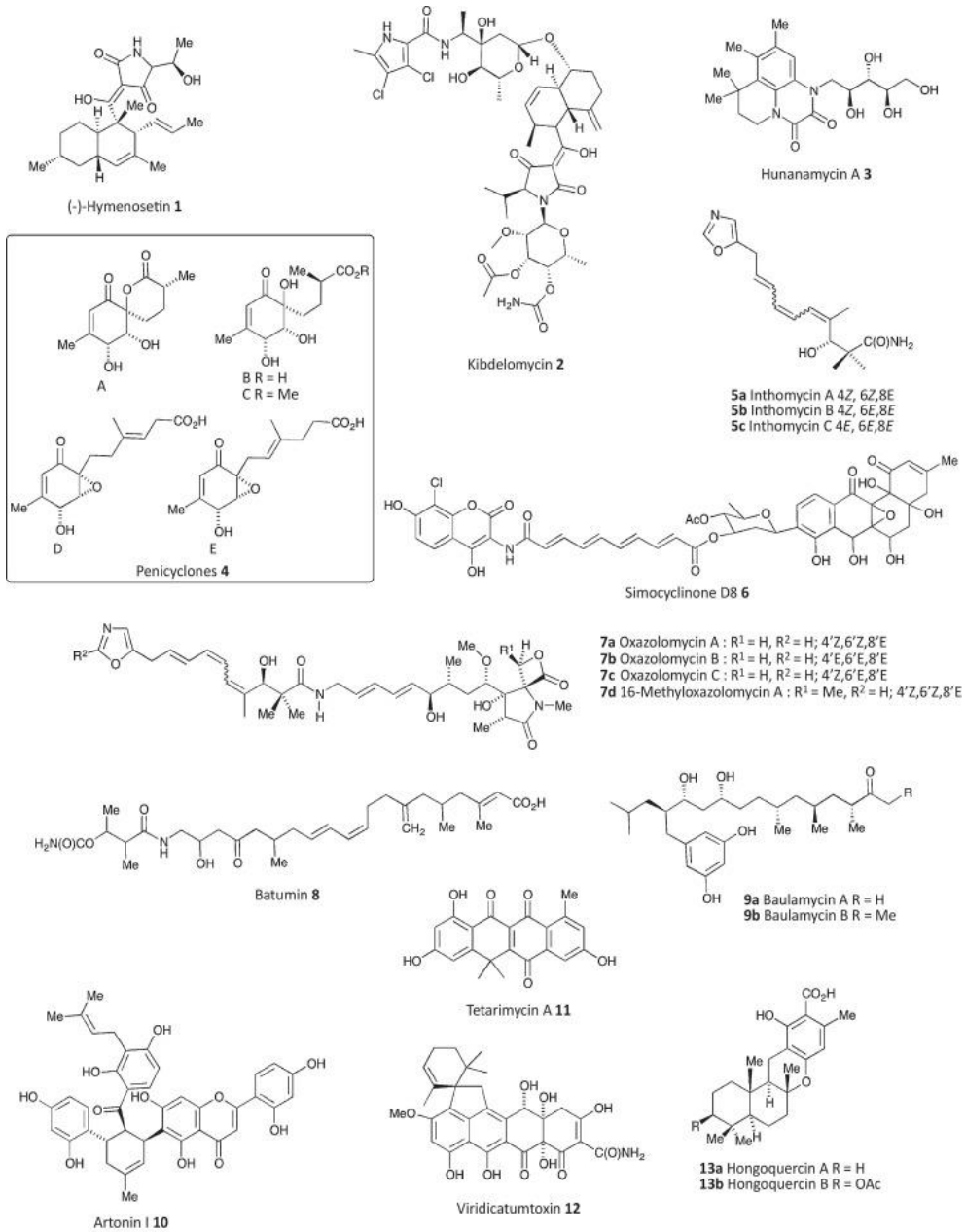
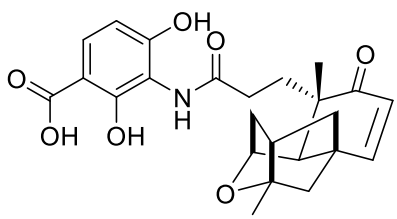


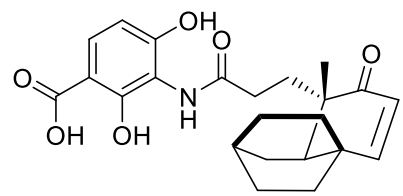
Figure 1-8. Antibacterial active NPs[9]

1.3 Platensimycin (PTM) and Platencin (PTN)

Platensimycin (PTM) is a natural antibiotic made by *Streptomyces platensis*. Platensimycin has a strong and broad spectrum gram-positive antibiotic effects. As mentioned in the previous introduction, we need antibiotics with new mechanisms of action. In this respect, platensimycin is an antibiotic that can satisfy our needs. Platensimycin shows antibiotic effects by inhibiting cellular lipid biosynthesis. Among them, PTM selectively inhibits β-ketoacyl-(acyl-carrier-protein (ACP)) synthase I/II (FabF/B).[24] The chemical structures of platensimycin (PTM) and platencin (PTN) are shown in figure 1-9. Platencin is also a natural antibiotic separated from *Streptomyces platensis*, showing similar medicinal effects as platensimycin but different location of action.



Platensimycin (PTM)
Molecular Weight: 441.48
Chemical Formula: $C_{24}H_{27}NO_7$



Platencin (PTN)
Molecular Weight: 413.47
Chemical Formula: $C_{23}H_{27}NO_6$

Figure 1-9. Chemical structure of PTM and PTN

The chemical structures of PTM and PTN are different yet similar. First of all, the structure can be divided into three groups; it is a group with a common 3-amino-2,4-dihydroxybenzoic acid (ADHBA) is a group with different aliphatic ketolide cages, and an amide bond group that connects the two. The ADHBA group, it is a part that strongly binds to FabF. The Aliphatic ketolide cage group causes PTM and PTN to act on different proteins in Fatty acid synthase II. The exact positions of proteins and cycles inhibited by PTM and PTN are shown in Figure 1-10.

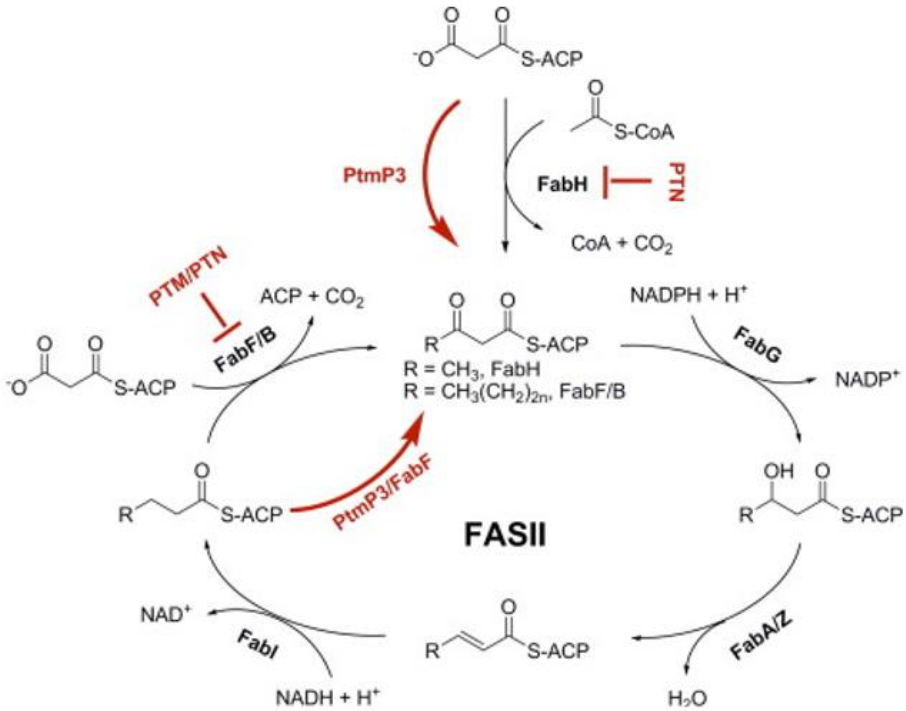


Figure 1-10. Mode of action of PTM and PTN[25]

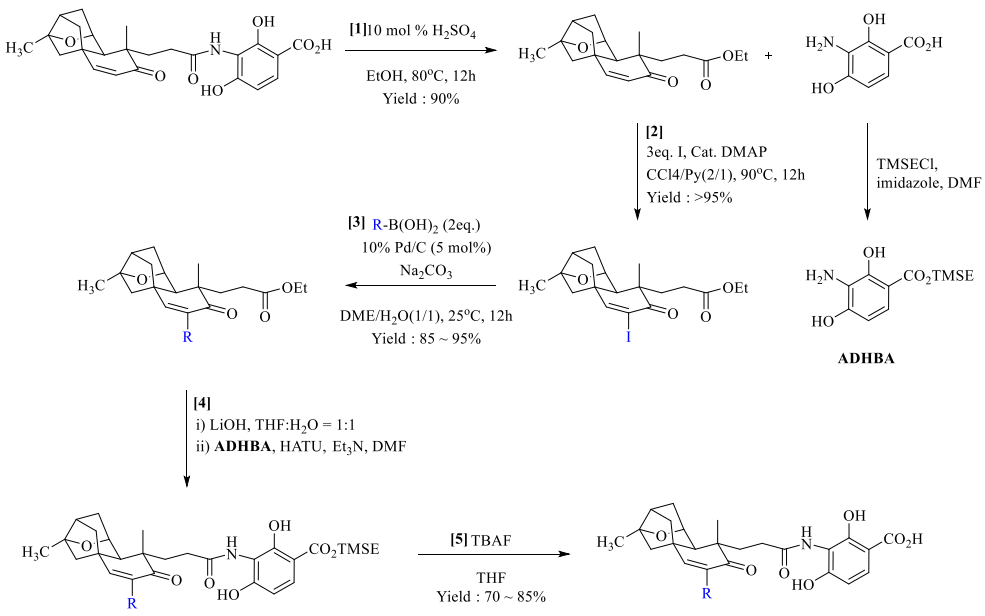
PTM and PTN act in the bacterial type II fatty-acid synthesis (FASII) as shown in Figure 1-10. FASII is known as a good target for antibiotic research, and FabF and FabH are essential steps in the process of fatty acid biosynthesis. (FabH is the initiation condensation enzyme of fatty acid biosynthesis, and FabF/B is the elongation condensation enzyme)[26-28] The actual *in vitro* result is shown in Table 1-2, and it does not have cross-resistance to major antibiotic-resistant bacteria around the world. In addition, mammalian cell toxicity was significantly low (>1,000 μg/ml), and the antifungal activity was also low (>64 μg/ml), indicating that it selectively acts on the bacterial fatty acid synthase.[24] MIC results of all other platensimycin derivatives against *S. aureus* two clinical MSSA and four MRSA strains using the agar dilution method show at least two or more times less active than platensimycin and linezolid (positive control).[29]

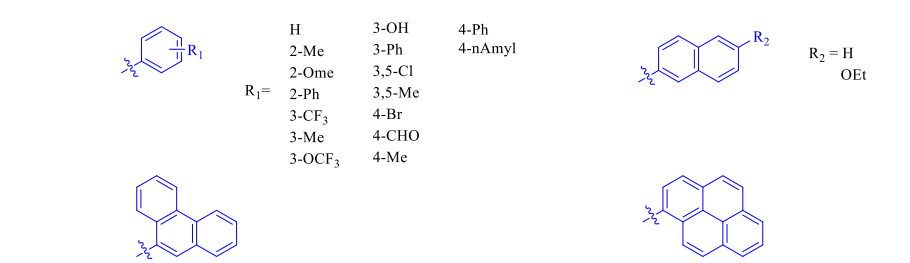
Organism and genotype	platensimycin	linezolid
Antibacterial activity (MIC, μg/ml)		
S. aureus (MSSA)	0.5	4
S. aureus + serum	2	4
S. aureus (MRSA)	0.5	2
S. aureus (MRSA, macrolide^{R})	0.5	2
S. aureus (MRSA, linezolide^{R})	1	32
S. aureus (VISA, vancomycin^{I})	0.5	2
Enterococcus faecalis (macrolide^{R})	1	1
Enterococcus faecalis (VRE)	0.1	2
S. pneumonia	1	1
E. coli (wild-type)	>64	>64
E. coli (tolC)	16	32
Toxicity		
HeLa MTT (IC_{50})	>1,000	>100
Candida albicans (MIC)	>64	>64

Table 1-2. MIC and toxicity of Platensimycin and linezolid[24]

Semi-synthesis of platensimycin derivatives

The semi-synthesis of platensimycin derivatives are shown in figure 1-11.[29] In accordance with the paper referenced, structure activity relationship (SAR) was carried out based on Suzuki-Miyaura cross-coupling reactions, and the reaction is as follows. The reaction is largely divided into five steps. Referring to the reaction [1] in Figure 1-11, hydrolysis was performed on the ADHBA part and the ketolide part of platensimycin through the acid catalyzed hydrolysis reaction. The [2] reaction is a reaction in which a proton of α position is substituted with iodine. The reaction [3] is a Suzuki-Miyaura cross-coupling reaction. The [4] reaction consists of two processes. The i) reaction is the process of detecting the Et group, which is the protecting group of the carboxylic acid in the reaction. The ii) reaction is the most widely used reaction to produce amide bond, which connects the amine of ADHBA and carboxylic acid using HATU. The [5] reaction is the process of removing the TMSE group that protects the carboxylic acid possessed in ADHBA group.





*Py: Pyridine; DMAP: 4-(dimethylamino)pyridine; TMSE: trimethylsulylethyl; DMF: N,N-dimethylformamide; DME: dimethooxyethane; HATU: 2-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate; Et_3N:triethylamine; TBAF: tetrabutylammonium fluoride

Figure 1-11. Semi-synthesis of platensimycin derivatives[29-31]

1.4 genus *Streptomyces*

Among various sources of natural products, microorganisms contribute a lot to natural diversity in terms of chemical diversity of compounds and are an enormous source of physiologically and biologically unique and active compounds that play leading roles in the pharmaceutical industry and medical treatment.[9] *Streptomyces* is the largest genus of actinobacteria, and genus *Streptomyces* is a rich source for antifungals, antibiotics and chemotherapeutic drugs. During the golden age (1940-50), when antibiotic development was active, 70-80% of antibiotics came from actinobacteria. As a result, *Streptomyces* is being studied further for the discovery of new therapeutic agents to overcome the emergence of antibiotic resistance bacteria.[32]

In this thesis, two bacteria from the genus *Streptomyces* are used: *Streptomyces platensis* and *Streptomyces champavatii*. The first strain we used, *S. platensis* is a strain that produces the antibiotic platensimycin, and *S. champavatii* used for co-culture is a strain that produces substances related to increasing biosynthesis of platensimycin. *Streptomyces platensis* produce **platensimycin(PTM)** and **platencin(PTN)**, **migrastatin**, **isomigrastatin**, **terramycine** and **dorrigocin A** and **B**.[33, 34] (figure 1-12) In this thesis, *Streptomyces platensis* MA7331 was used to obtain platensimycin.

We used *Streptomyces champavatii* NRRL b-5682 for co-culture. *Streptomyces champavatii* is a strain separated from the sediment in Bohai Bay, China. The code name used in this thesis is 14E023, and the species name is NRRL B-5682. *S. champavatii* (14E023, NRRL B-5682) produces lots of compounds such as **ferrioxamine**, **enterocine**, **surugamide**, and **kitamycin**.[35, 36] Figure 1-13 shows that previously discovered chemical structures of compounds produced by *S. champavatii*. Compound **a, deferoxamine** as a group of ferrioxamine, is a bacterial siderophore involved in iron metabolism of bacteria. Compound **b**, **surugamide**, which is acyclic octapeptide with four d-amino acid residues shows **cathepsin B** inhibitory activity. Compound **c**, **enterocine**, shows antibacterial activity. Compound d, **kitamycin**, is an antimycin-type antibiotic. In addition, it produces a number of derivatives. In the course of searching for secondary metabolite from *Streptomyces champavatii*, we obtained compound that showed a biosynthetic yield

improvement of platensimycin. The isolation and structural elucidation of inducer, m/z 268.2, was described in this thesis.

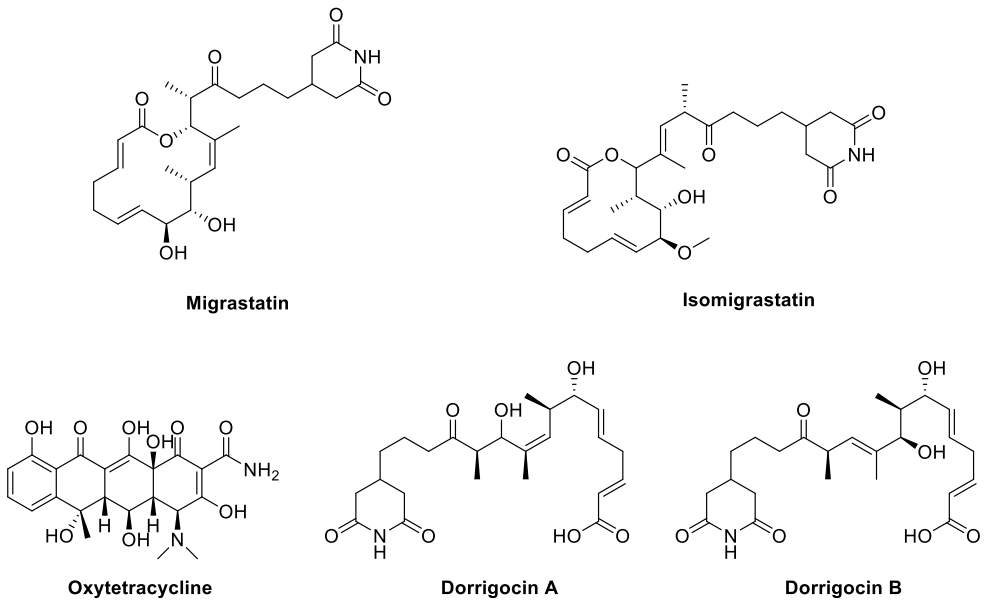


Figure 1-12. Natural products from *Streptomyces platensis*

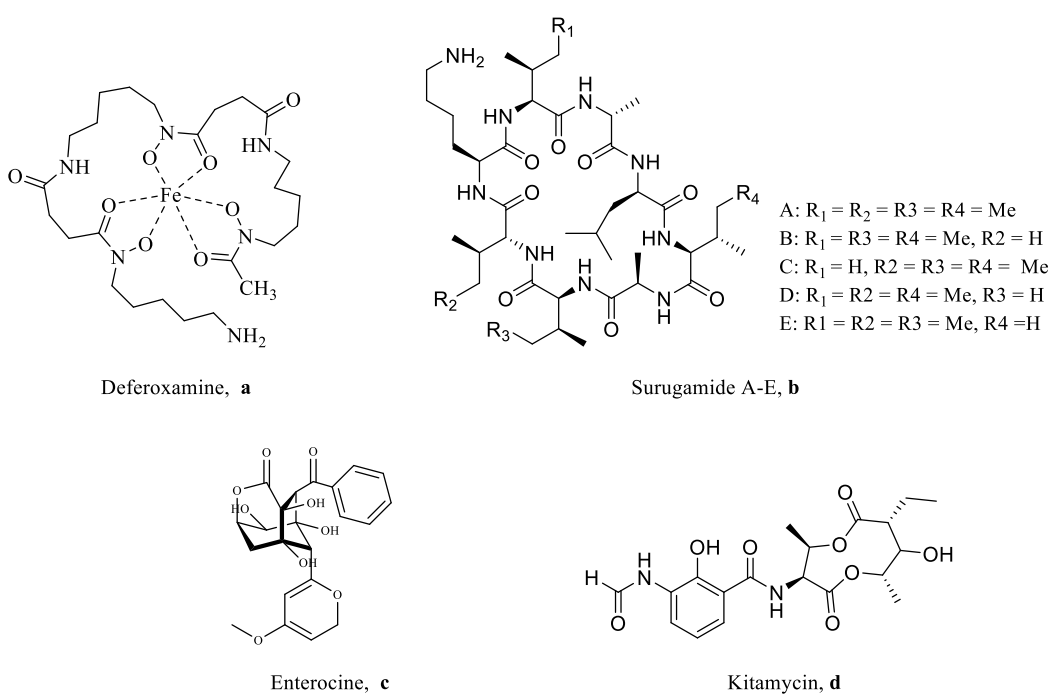


Figure 1-13. Natural products from *Streptomyces champavatii*

1.5 Co-culture

We tried to increase the yield of platensimycin through co-culture. Co-culture was conducted for two reasons. The first reason is a competitive reason based on natural selection. Co-culture of different bacteria describing the natural state of bacteria colonies can produce more compounds or even new compounds due to the nutrition or space competition.[37] The second reason is a siderophore. (figure 1-14) Siderophore, secondary metabolites of bacteria or plant, plays a key role to help absorb iron and used for intracellular coenzyme and various enzymes from the environment around the bacteria. Certain bacteria make siderophores with high iron affinity to absorb iron from the surrounding environment, and the ferric iron is taken into the organism. And the presence of this siderophore helps the growth of bacteria, thereby increasing material production.[38] For the above reasons, co-culture was conducted, and the strain *S. champavatii* was selected. *S. champavatii* creates a siderophore such as perrioxamine, the same genus as *Streptomyces*.

Co-culture of *Streptomyces platensis* MA7331 with a *Streptomyces Champavatii* NRRL B-5682 was carried out to stimulate the production of platensimycin in this study, whereas single cultivation was conducted as a control group. Since a corn meal was used as media, the transparency of the media after sterilization was low, making it difficult to measure optical density (O.D.) through UV/VIS absorbance. So the co-culture experimental method was designed over time. As an experimental group in which *S. platensis* MA7331 was incubated on the corn meal media a day and two days earlier, an experimental group in which *S. champavatii* was incubated a day and two days earlier. And a control group which raised with only one strain and incubated at the same time was prepared. After that, the time extended to five days.

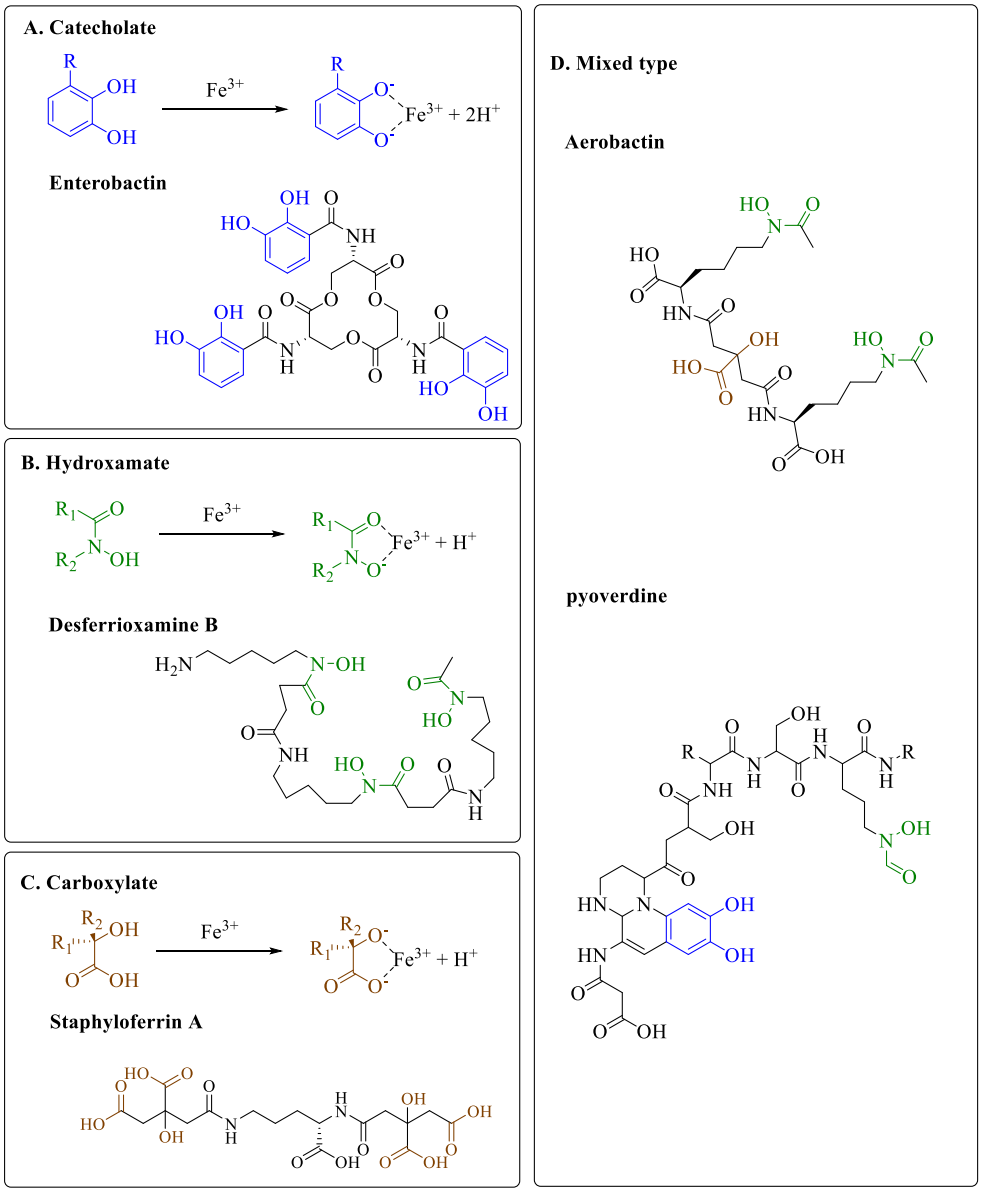


Figure 1-14. The types of siderophore and examples

Three common and most frequently found types of siderophore are shown in figure 1-14. There are Catecholate, Hydroxamate and Carboxylate, and the three types can be mixed and entered in one compound as shown in D. mixed type in figure 1-14. Deferoxamine, a type of siderophore found in *S. champavatii*, is a hydroxamate type siderophore.

Chapter 2 Yield improvement of biosynthesis of platensimycin

2.1 Media, extraction and time study

The amount of PTM generated would differ depending on the media conditions. The experiment was conducted with six different media referring to various papers. In addition, five extraction methods were used to experiment with which extraction conditions can be the best for extracting PTM. All six media were extracted using five extraction conditions. Table 2-1 and 2-2 show the ingredients of six different media and five different extraction conditions respectively.

	1	2	3	4	5	6
Corn meal		40				
Dextrin	40			40		
Malt extract						15
Dextrose						6
Lactose	40	40	25	60		
Yeast extract	5					6
MOPS			5	20	5	20
Glucose			30			
Ammonium Sulfate			0.5	2		
L-Aspartic Acid			1			
Starch					70	
Soybean Flour					15	
$CaCO_3$					5	
$MnCl_2$					0.015	
$(NH_4)_6Mo_7O_{24}$					0.03	

Table 2-1. Ingredient table of six different media[39-44]

Extraction Methods		
1	DCM (Dichloromathane)	Adjust to pH 3
2		pH 7
3	EA (Ethyl acetate)	Adjust to pH 3
4		pH 7
5	XAD-7, pH 7	

Table 2-2. Five different extraction methods

The corn meal media (media #2) among six media is the best condition to make *S. Platensis* (MA7331) to bio-synthesis of platensimycin. The following figure is data from detecting platensimycin with a molecular weight of 441 by using the ion extraction method of LC-MS after extraction of six media. Figure 2-1 shows the result of detected PTM of all six media. The red box shows the platensimycin. It was confirmed that corn meal and malt media (media #2 and media #6) produced more PTM than other four media. All of these media were extracted by five different extraction methods and compared.

The previous researcher's extraction method was performed using EA in acid condition. A problem was discovered at that time, but the most difficult thing to extract was that the sticky ingredients of the cornmeal mixed with EA, blocking the entrance of the funnel. To address this problem, the media with bacteria proceeded with centrifuge, divided into pellets and supernatant and then extracted in acid condition. Among the five extraction methods shown in table 2-2, it was verified that extraction with ethyl acetate adjusted to pH 3 (method #3) was the best way to extract PTM. When extraction was performed under neutral conditions in both DCM and EA, a smaller amount of platensimycin was detected under acid conditions as shown in figure 2-2. The extraction conducted with XAD-7 resin rarely extracted PTM. As a result, the final media and extraction method were corn meal and acidic EA extraction.

The time study was conducted to determine the time required for the growth of platensimycin. After the incubation, 100 mL of sample was extracted every day from five to ten days, and it was confirmed that saturated 8 days after

inoculation. Based on the results of this experiment, we decided to grow for a total of 12 days, including seed culture.

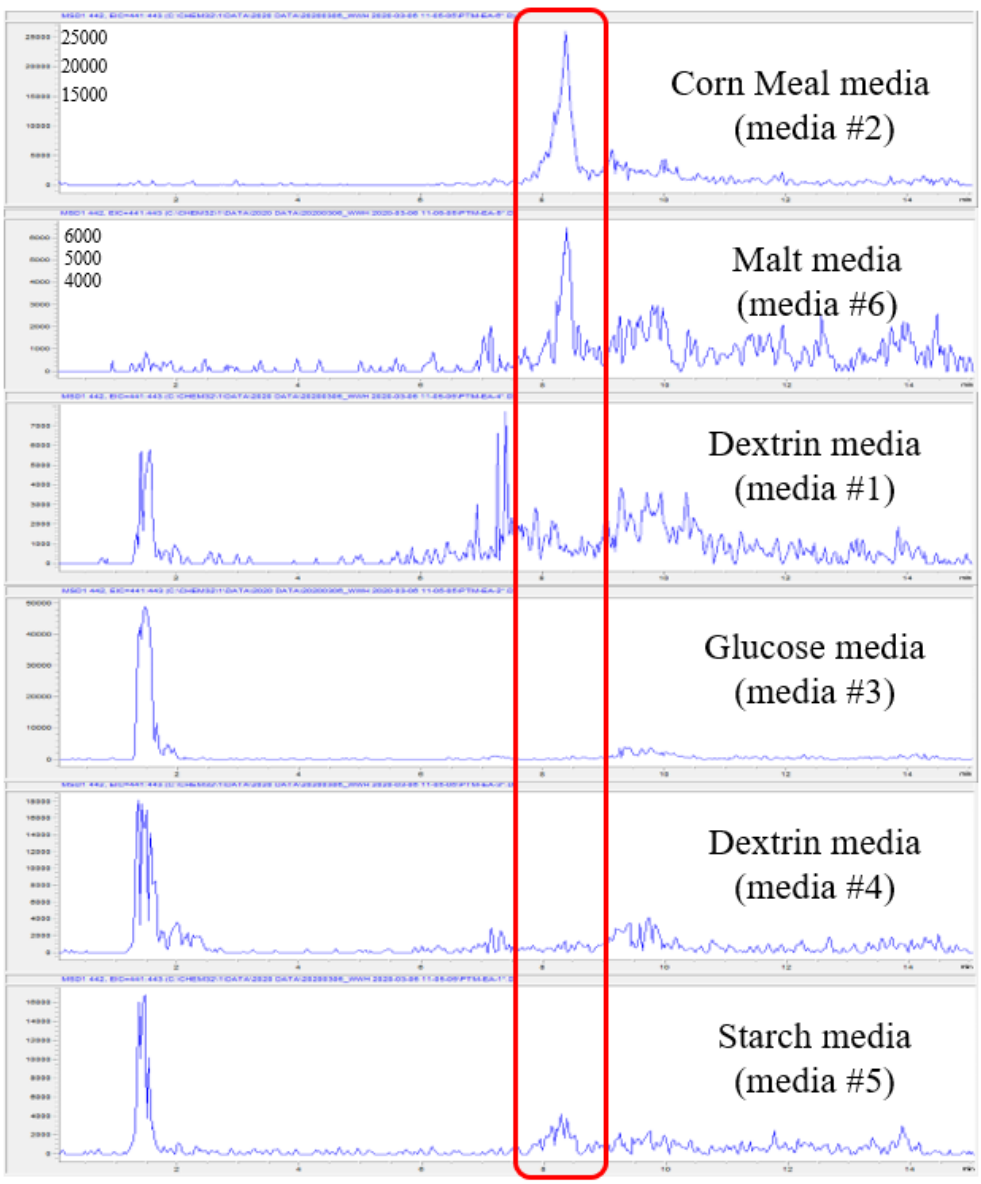


Figure 2-1. The relative amount of platensimycin

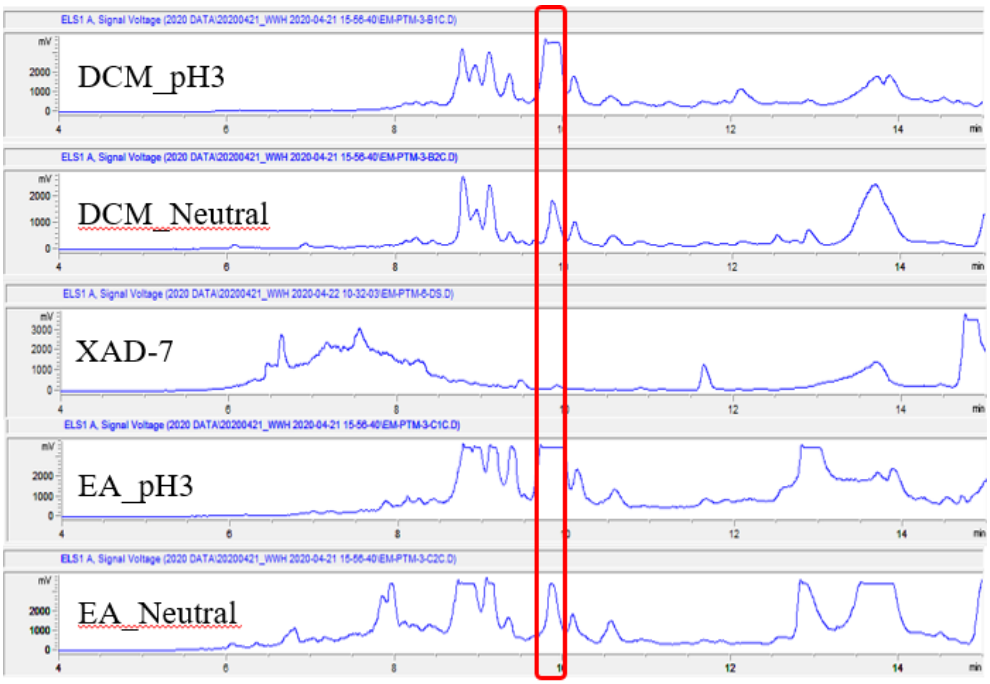


Figure 2-2. Five different extraction methods

Detailed culture condition was shown in Materials and Methods chapter.

Extraction method: acidic EA extract (centrifuged)

Incubation condition;

i) 25 mL seed: 28 ℃ for three days

ii) 100 mL fermentation: 28 ℃ for ten days

LC-MS preparation;

i) Sample concentration & Injection volume: 1 mg/mL & 30 μL

ii) Methods: CMDD STD

run time 15 min. pump A: H_2O, pump B: ACN (Acetonitrile)

0 to 2 min.: isocratic to A95/B5

2 to 12.5 min.: gradient A95/B5 to A0/B100

12.5 to 15min.: isocratic to A0/B100

2.2 Co-culture

The co-culture method was used to improve the yield of PTM. Co-culture strain was a *Streptomyces champavatii*. Co-culture media was a corn meal. After raising the seed for 4 days, it increased for 10 additional days to a total of 14 days as same as before. The co-culture plan proceeded under the conditions shown in the table 2-3. We prepared a total of 7 samples. The sample #1 and #2 are control groups, raising only MA7331 and 14E023 respectively. The sample #3 and #4 were inoculated with MA7331 first, #3 was inoculated with 14E023 a day later, and #4 was inoculated with 14E023 two days later. The sample #5 and #6 were inoculated with 14E023 first, #5 was inoculated with MA7331 a day later, and #6 was inoculated with MA7331 two days later. The sample #7 was inoculated at the same time.

Sample number	First Inoculation Strain	Second Inoculation Strain	Condition
1	MA7331	-	MA7331 only
2	14E023	-	14E023 only
3	MA7331	14E023	Day 1, 14E023 inoculation
4	MA7331	14E023	Day 2, 14E023 inoculation
5	14E023	MA7331	Day 1, MA7331 inoculation
6	14E023	MA7331	Day 2, MA7331 inoculation
7	MA7331 & 14E023		Simultaneous

Table 2-3. Co-Culture plans and sample condition

The following figure 2-3 was the LC-MS results of six samples. In order to help quantitative analysis with an internal standard, the peak in the 11 minutes (blue box) was added in the same concentration and amount as the seven samples. By using these internal standards, the amount of PTM was determined only by looking at UV absorption. As a result, it was found that adding 14E023 first and then MA7331 could increase the biosynthetic yield. The duplication experiment was conducted and confirmed that the result was duplicated. In addition, we

conducted a time study to determine the perfect time for inoculation. The compound which was used in the internal standard (blue box) was **djalonensone** (3,7-dihydroxy-9-methoxy-1-methyl-6H-benzo[c]chromen-6-one, $C_{15}H_{12}O_5$, MW: 272.26).

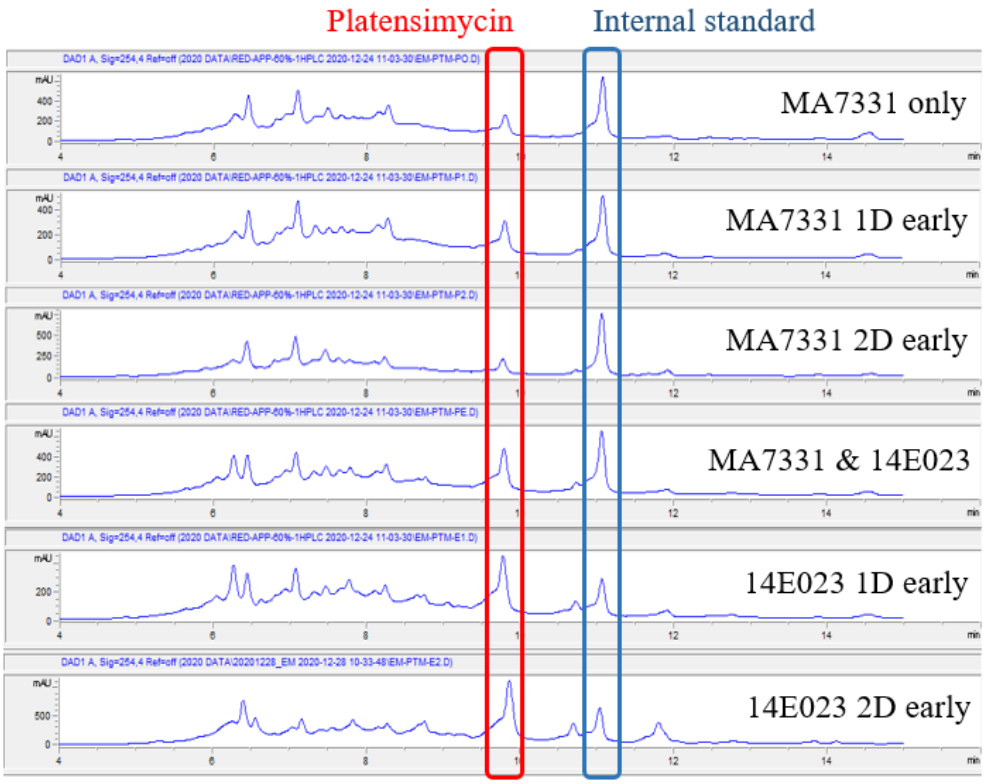


Figure 2-3. Co-cultured with MA7331 & 14E023

LC-MS preparation;

i) Sample concentration & Injection volume: 1 mg/mL & 30 μL

ii) Methods: CMDD STD

The time study for co-culture was also conducted. Previous experimental results revealed that 14E023 should be inoculated on the media before MA7331. It was confirmed that more PTM was generated when 14E023 was inoculated one and two days earlier. But it was necessary to check how it was generated three or more days earlier. Therefore, time study was conducted. The results of time study are shown in Figure 2-4. Since there was little difference between the three and four days earlier, the experiment of putting it in earlier than 5 days was not

conducted.

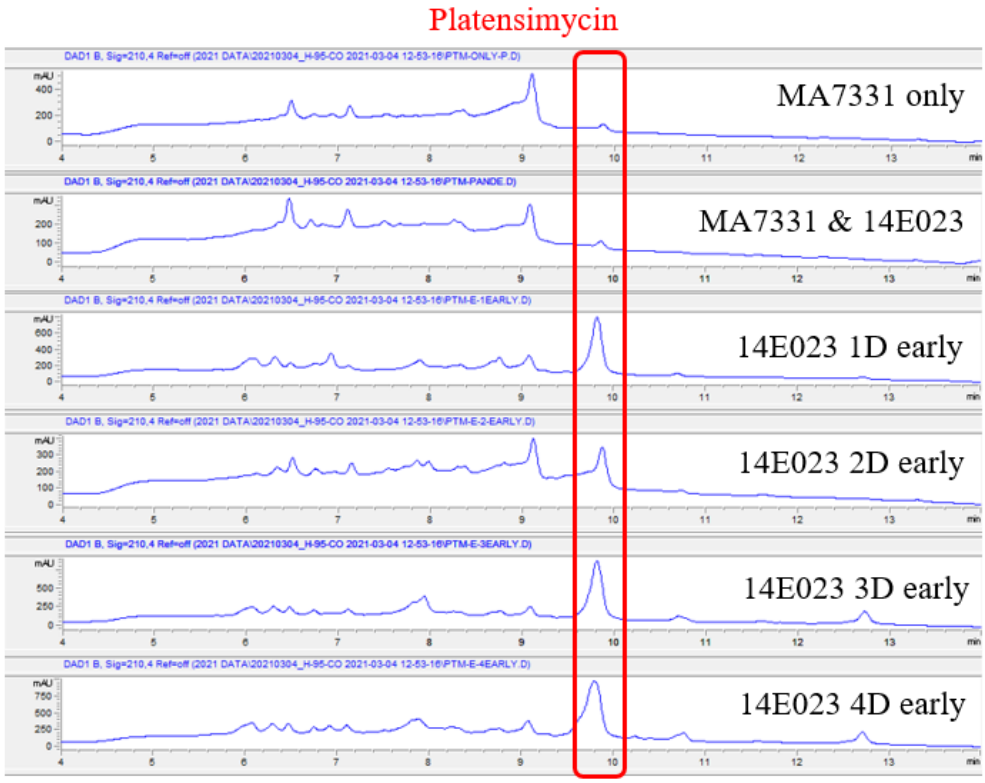


Figure 2-4. Time study for co-culture

LC-MS preparation:

i) Sample concentration & Injection volume: 1 mg/mL & 30 μL

ii) Methods: CMDD STD

Through the co-culture experiment, it was confirmed that more PTM was generated when 14E023 was added first. It was necessary to consider what would make them synthesize more platensimycin. The first hypothesis is that due to the nature of *S. platensis* which produces antibiotics that kill other bacteria, more PTM would be made when other bacteria exist. The second hypothesis is that the products from 14E023 make MA7331 produce more PTM. To verify the first hypothesis, the *Streptomyces cavourensis* (NRRL 2740) was co-cultured. Figure 2-5 is the result of co-culture of *S. platensis* with 14E023 and *S. cavournensis* respectively. Although the experiment was conducted with only one strain, it could be determined that the presence or absence of other bacteria did not affect the production of PTM.

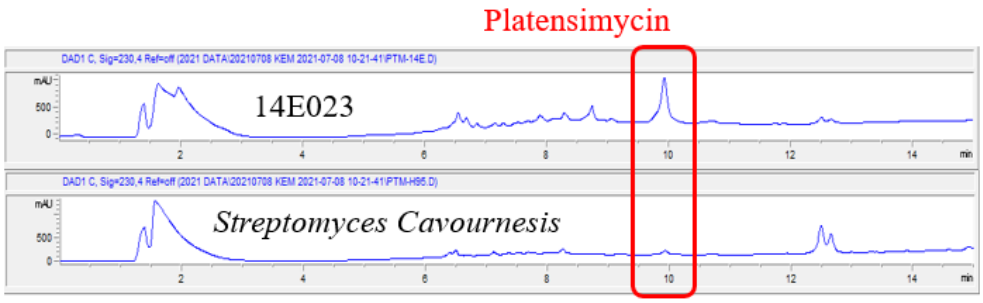


Figure 2-5. The influence of the existence of other bacteria

LC-MS preparation:

i) Sample concentration & Injection volume: 1 mg/mL & 30 μL

ii) Methods: CMDD STD

2.3 Defining causative substances

Since the presence or absence of other bacteria had no effect on PTM production, an experiment was designed to confirm that compounds or something else produced by 14E023 increase PTM production. The material produced by 14E023 was extracted and fractionated, then MA7331 was raised in media with extracted fractions. The 7 fractions were obtained and weighted. Each fraction was injected into 1 L of corn meal media to make the corn meal media + fraction 1 to 7.

The 2 L of corn meal without any fraction was prepared as a control group. One of two was inoculated only MA7331 and the other was inoculated 14E023 first, and MA7331 later. All 9 corn meal media were extracted and analyzed. Figure 2-6 shows the effect of injected fraction. In fraction 5 and 6, more PTM was synthesized than MA7331 only and even MA7331 and 14E023. The other fractions make less PTM than MA7331 only.

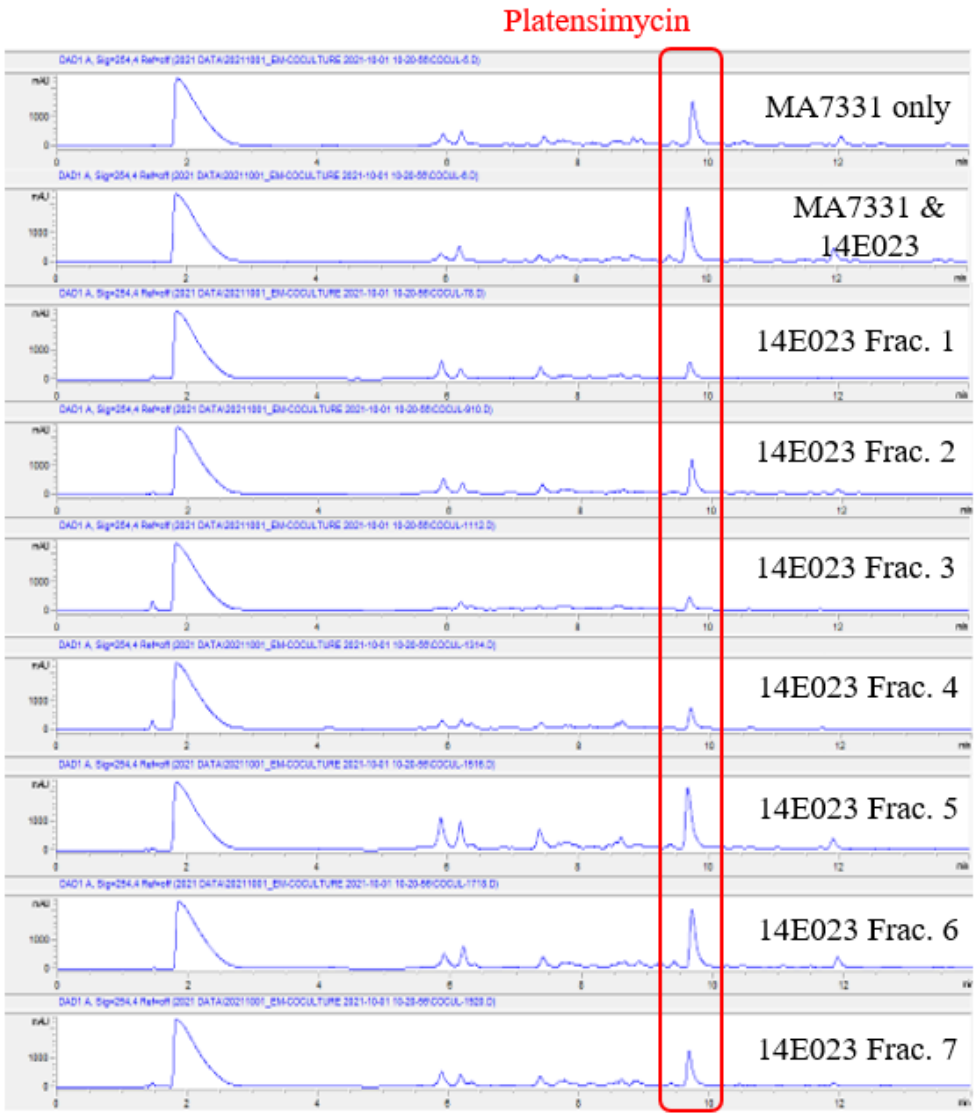


Figure 2-6. The effect of injected fraction

LC-MS preparation;

i) Sample concentration & Injection volume: 5 mg/mL & 30 μL

ii) Methods: CMDD STD

To clarify a causative material (inducer), fraction 5 was separated into six peaks by HPLC technique. All six peaks were injected into each media, and *S. platensis* was raised in the media for 14 days. And the results are shown in Figure 2-7. The red box shows the UV absorption of platensimycin. The LC-MS figure marked MA7331 only was grown in corn meal media without other peaks used as

control. It was confirmed that the largest amount of platensimycin was formed in peak 1. Comparing the amount of the extracted media with the amount of the extracted platensimycin, peak 1 and peak 6 make MA7331 synthesize more platensimycin. The absorbance of peak 1, however, is high enough to consider that a larger amount of platensimycin was generated.

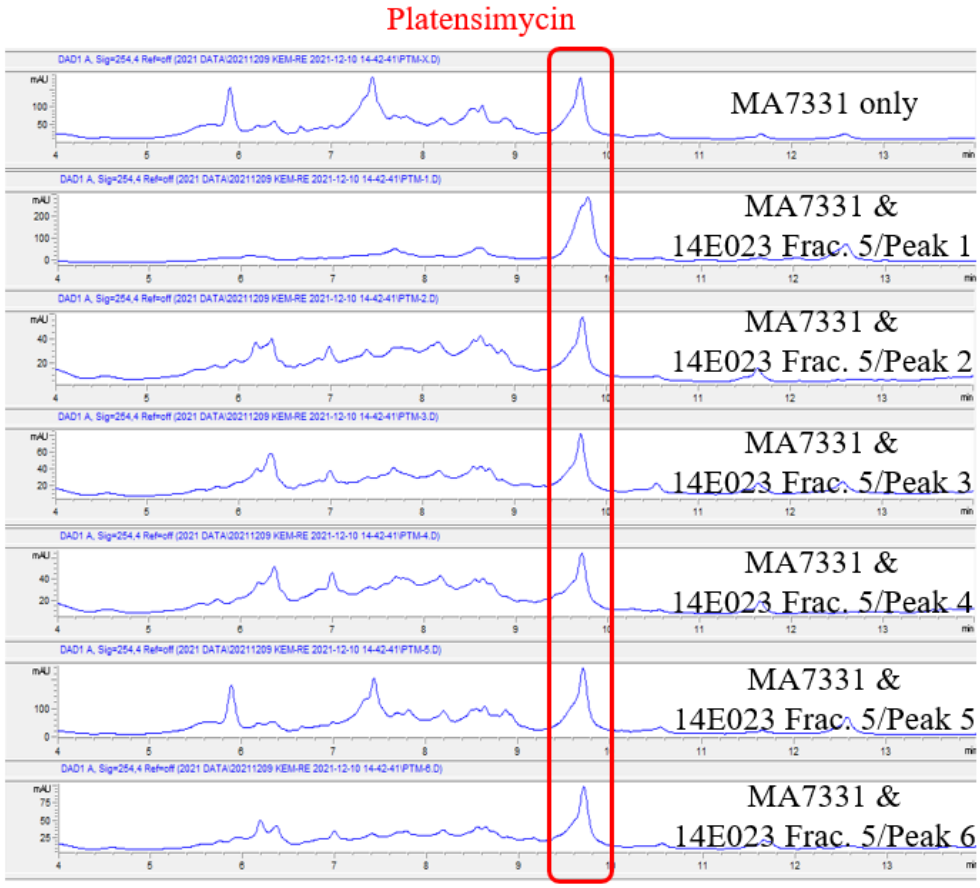


Figure 2-7. The effect of injected peaks from fraction 5

The substance that occupies the most in peak 1 was separated and purified, and it was confirmed that the substance was adenosine. Adenosine, isolated as colorless amorphous powder, showed UV absorption at λ_{max} 210 nm and 254 nm. Its molecular formula $C_{10}H_{13}N_5O_4$ was established by combined analysis of NMR and ESIMS with ion peak at *m/z* 268.2 $[M+H]^+$.

Chapter 3 Structural elucidation of inducer

To synthesize platensimycin derivatives, platensimycin should be purified up to synthetic grade. After purification of PTM, several organic chemical reactions were conducted.

3.1 Purification of platensimycin

The crude extract from corn meal media was fractionated through Sephadex LH-20, which is a semi-purification. The purification of platensimycin containing fractions confirmed by ESI-MS were conducted further through open column chromatography and HPLC technique. Because the silica open column method using acetone/hexane/acetic acid = 30/70/1 does not work well, HPLC technique was used to afford platensimycin.

Platensimycin, isolated as amorphous powder, showed UV absorption at λ_{max} 230 nm and 300nm. Its molecular formula $C_{24}H_{27}NO_7$ was established by combined analysis of NMR and ESI-MS with ion peak at *m/z* 442.2 $[M+H]^+$. ^1H NMR (700 MHz, DMSO-d_6) δ 11.72 (bs, 1H), δ 10.10 (s, 1H), δ 8.98 (s, 1H), δ 7.54 (d, j = 8.82, 1H), δ 6.42 (d, j = 8.82, 1H), δ 6.67 (d, j = 10, 1H), δ 5.58 (d, j = 10, 1H), δ 4.38 (bs, 1H), δ 2.35 (m), δ 2.34 (m), δ 2.27 (bs, 1H), δ 2.06 (m), δ 2.03 (m), δ 1.98 (m) δ 1.94 (bs, 1H), δ 1.90 (m), δ 1.78 (dd, j = 11, 6.4 2H), δ 1.70 (bs, 1H), δ 1.66 (m), δ 1.36 (s, 1H), δ 1.15 (s, 1H)

3.2 Purification and elucidation of adenosine conformers

Adenosine, isolated as a colorless powder, showed UV absorption at λ_{max} 210 nm and 254 nm. Its molecular formula $C_{10}H_{13}N_5O_4$ was established by combined analysis of NMR and ESI-MS with ion peak at *m/z* 268.2 $[M+H]^+$. ^1H NMR (700 MHz, DMSO-d_6) δ_H 8.34 (s, 1H), 8.13 (s, 1H), 7.35-7.29 (s, 3H), 7.30 5.96, 5.87 (d, j = 3.5, 4.1, 1H), 5.48 (bs, 1H), 5.18 (bs, 1H), 5.16 (bs, 1H), 4.59-4.56 (m, 1H), 4.30, 4.26, 4.14 (m, 1H) 4.03, 3.96 (m, 1H), 3.66 - 3.51 (m, 1H). ^{13}C NMR (175 MHz, DMSO-d_6) δ_C 156.22 (C-6), 152.47 (C-2), 149.11 (C-4), 140.01 (C-8), 119.39 (C-5), 87.99 (C-1´), 85.98 (C-4´), 73.57 (C-2´), 70.71 (C-3´), 62.58 (C-5´).

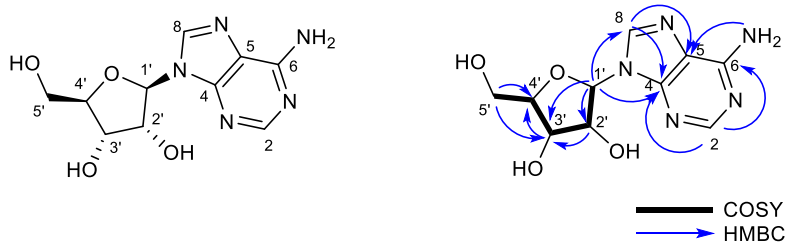


Figure 3-1. Structure of adenosine with COSY and HMBC correlations

Table 3-1. Spectral properties of adenosine (DMSO-d_6)

No.	δ_C, type	δ_H, m (J in Hz)	COSY	HMBC
2	152.47, CH	8.13, s	-	4, 6
4	149.11, C	-	-	
5	119.39, C	-	-	
6	156.22, C	-	-	
8	140.01, CH	8.34-8.32, s	-	4, 5, 1´
1´	87.99, CH	5.96, d (3.5)	2’	4, 8, 2´, 3´
		5.87, d (4.1)		
2´	73.57, CH	4.59-4.56, m	1’,3’	1´, 3´, 4´
3´	70.71, CH	4.30, 4.26, 4.14, m	2’,4’	1´, 2´, 4´, 5´
4´	85.98, CH	4.03,3.96, m	3’,5’	2´, 3´, 5´
5´	62.58, CH_2	3.66-3.51, m	4’	3´, 4´
2´-OH	-	5.16, bs	-	-
3´-OH	-	5.18, bs	-	-
5´-OH	-	5.48, bs	-	-
6-NH_3	-	7.35-7.29, s	-	5

700 MHz for ^1H and all 2D NMR experiments, 175 MHz for ^{13}C

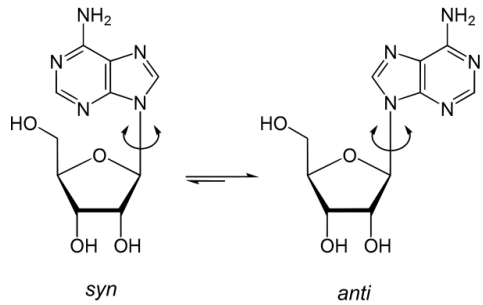


Figure 3-2. Conformational isomers of adenosine

Conformational isomers (conformers) are a form of stereoisomers that are interconverted by rotations of single bonds. The forms corresponding to the local minima of the potential energy surface are specifically called conformational isomers or conformers.[45] The conformer corresponding to the local maximum of the energy surface is the transition state between the local-minima conformational isomers. When the energy barrier is low, free rotation occurs, and the compound exists as a mixture in which a number of conformers are rapidly equilibrated. If the energy barrier is high enough, rotation can be restricted, and molecules can exist for relatively long periods as stable rotational isomers. This is enough to be confirmed by NMR, and the isolated adenosine in this thesis corresponds to this case. As shown in Figure 3-2, in the syn and anti-conformer, the lone pair electron of nitrogen in purine and the aromatic ring affect the chemical shift of carbon and hydrogen in the 5-membered ring, and vice versa. Therefore, each conformer has a slightly different NMR chemical shift.

Table 3-2. NMR data for adenosine (DMSO-d_6)

	Conformer 1		Conformer 2		Conformer 3	
No.	**δ_C, type**	**δ_H, m (J in Hz)**	**δ_C, type**	**δ_H, m (J in Hz)**	**δ_C, type**	**δ_H, m (J in Hz)**
2	152.469, CH	8.13, s	152.426, CH	8.13, s	152.426, CH	8.13, s
4	149.115, C	-	149.115, C	-	149.047, CH	-
5	119.391, C	-	119.235, C	-	119.190, C	-
6	156.218, C	-	156.171, C		156.152, C	-
8	140.013, CH	8.3486[a], s	139.987, CH	8.3413[a], s	139.930, CH	8.3177[a], s
1´	87.987, CH	5.96, d (3.5)	90.970, CH	5.87, m	90.852, CH	5.87, m
2´	73.571, CH	4.59, m	80.628, CH	4.58, m	81.145	4.56, m
3´	70.707, CH	4.30, m	77.333, CH	4.26, m	76.763	4.14, m
4´	85.979, CH	4.03, m	87.658, CH	3.96, m	85.979	3.96, m
5´	62.578, CH_2	3.66, m	61.736, CH	3.55, m	62.440	3.54, m
2´-OH	-	5.16, bs	-	5.16, bs		5.16, bs
3´-OH	-	5.18, bs	-	5.18, bs		5.18, bs
5´-OH	-	5.48, bs	-	5.48, bs		5.48, bs
6-NH_3	-	7.3508, s	-	7.3091,s		7.2920, s

700 MHz for ^{1}H and all 2D NMR experiments, 175 MHz for ^{13}C

[a] The assignment may be interchanged

Chapter 4 Materials and Methods

4.1 Instruments and Data collection

The reagent including all commercial chemicals used in this study were purchased from Aldrich (U.S.A.), Sigma (U.S.A.), Merck (Germany), Combi-Blocks (U.S.A.), Bepharm (U.S.A.), TCI (Japan) and ThermoFisher scientific (Korea). All glassware was oven-dried before being used. Thin-Layer Chromatography (TLC) was performed on pre-coated silica gel glass plate (silica gel 60 F254, 0.25 nm, Merck and 60 CN $F_{254}S$) and silica gel 60 (silica gel 230-400mesh ASTM) was used for flash column chromatography.

High-performance liquid chromatography was performed with a Waters HPLC (Waters Corporation, Massachusetts, U.S.A.) equipped with a HPLC WATERS 1525 binary HPLC pump and a WATERS 2489 UV/visible detector using Luna 5 μm C18 (250 × 10 mm 5 micron) at flow rate 2.0 mL/min. ESI low resolution mass data was acquired by Agilent Technologies 6120 quadrupole mass system coupled with an Agilent Technologies 1260 series HPLC with a reversed-phase Phenomenex luna 5 μm C18 (100 × 4.6 mm, 5 micron). Preparative High-performance liquid chromatography was performed with a JAI HPLC (Japan Analytic Industry, Japan) equipped with a HPLC pump P-9104A and a JAI UV detector 3702 using JAIGEL-ODS-BP-L, SP-120-15 (500 × 20 mm 15 micron) at flow rate 6 mL/min.

All NMR spectra were recorded on Bruker Ascend TM 700 spectrometer using methoanol-d_4, DMSO-d_6 and $CDCl_3$ as solvents. Chemical shifts were reported with reference to the respective solvent peaks [δ_H 3.31 and δ_C 49.0 for methanol-d_4, δ_H 2.05 and δ_C 39.52 for DMSO-d_6, δ_H 7.26 and δ_C 77.16 for $CDCl_3$]. Multiplicity of peaks is expressed as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and bs (broad single).

First grade solvents from Dae-Jung Chemicals & Metals Co. Ltd, Korea were used for fractionation of extracts and for chemical reactions. HPLC grade solvents from J. T. Baker and Dae-Jung Chemicals & Metals Co. Ltd were used for HPLC. NMR solvents were purchased from EUROSO-TOP.

4.2 General procedure

All reactions were performed under an inert nitrogen atmosphere and stirred at about 1000 rpm. The progress of the reaction was predicted by TLC. The TLC plates were detected by 254 nm wavelength UV light or Ninhydrin staining solution. The reaction mixture which could be regarded as the end of the reaction was diluted with dichloromethane or ethyl acetate and extracted several times with distilled water until there is no organic compound. The organic layer was separated and an aqueous layer was extracted with a small amount of organic solvent twice. The collected organic layer was dried off through anhydrous sodium sulfate (Na_2SO_4). After the drying agent was filtered out, the solvent was evaporated. The resulting material was purified by silica column chromatography or HPLC technique. The collected compound with solvent was dried by rotary evaporator and remaining solvent was removed by high-vacuum pump. The resulting compound was identified by NMR spectrum and kept sealed. Chemical shifts are reported with parts per million (δ).

4.3 Culture of MA7331 and 14E023

MA7331

The stock solution containing MA7331 (*Streptomyces Platensis*, P-502884, F-155,515) was inoculated in seed media and incubated at 28 ℃ to the laboratory for four days. Seed media contained the following ingredients; in g/L: soluble starch 20.0; dextrose 10.0; $CaCO_3$ 1.0; NZ amine type E 5.0; Difco beef extract 3.0; Difco bacto peptone 5.0; Difco yeast extract 5.0. After four days, 5 mL of MA 7331 raised in seed media were inoculated into 2.5 L ULTRA YIELDTM flasks, each containing 1 L of corn meal media. Corn meal media contained the following ingredients; in g/L: corn meal 40.0; lactose 40.0. The fermentation was carried out in a shaking incubator (220 rpm) at 28 ℃ for ten days.

14E023

The stock solution containing 14E023 (*Streptomyces Champavatii*, NRRL B-5682) which had been separated from the sediment layer of Bohai bay in China was inoculated in seed media and incubated at 28 ℃ to the laboratory for three days. Seed media and culture media were same as follow; SYP Media in g/L: starch 10; yeast extract 4; peptone 2. After three days, 5 mL of 14E023 raised in seed media were inoculated into 2.5 L ULTRA YIELDTM flasks, each containing 1 L of SYP media. The fermentation was carried out in a shaking incubator (220 rpm) at 28 ℃ for four days.

4.4 Extraction and Isolation

Platensimycin

The separation method using a separate funnel had to be changed to a new method because of the characteristics of the corn meal media. When the media was mixed with ethyl acetate (EtOAC, EA) or DCM (Dichloromethane), it formed a micelle and became sticky, which finally could not pass though the outlet of the funnel. After nine days, the broth was centrifuged at 2400 rpm for 30 minutes. The supernatant and pellet were collected separately. The supernatant was adjusted to pH 3 by adding HCl and partitioned with ethyl acetate. Also the collected pellet was treated with 1:1 ratio of EA and distilled water followed by pH adjustment, and centrifuged at 2400 rpm for 30 minutes. The EA from 2nd centrifuged media was collected. The collected EA from 1st and 2nd partition was extracted with distilled water once again to get rid of HCl, then evaporated.

The crude extract was fractionated through sephadex LH-20 column chromatography using acetone 100% to afford 10 fractions. After that fraction 4 to 6 were subjected to further purification by HPLC technique. Fraction 4 to 6 were subjected to reversed-phase HPLC (Phenomenex Luna C18, 5 μm, 100 Å, 250 × 100 mm, 2.0 mL/min, UV = 230 nm, 300 nm), eluting with 40% CH_3CN in H_2O with 0.05% trifluoroacetic acid to afford platensimycin.

Adenosine from fraction 5

The 14E023 in 16 L of SYP media was extracted by ethyl acetate using a separate funnel. The EtOAc layer was evaporated in vacuo, and weighted (687.1 mg). The crude extract was fractionated by sephadex LH-20 column chromatography using methanol (MeOH): dichloromethane (DCM) = 1: 1 to afford 7 fractions. After that fraction 5 was subjected to further purification by HPLC technique. Fraction 5 was subjected to reversed-phase HPLC (Phenomenex Luna C18, 5 μm, 100 Å, 250 × 100 mm, 2.0 mL/min, UV = 210 nm, 255 nm), eluting with 35% CH_3CN in H_2O to afford peak 1. Peak 1 was subjected to reversed-phase HPLC (Phenomenex Luna C18, 5 μm, 100 Å, 250 × 100 mm, 2.0 mL/min, UV = 210 nm, 255 nm), eluting with 35% CH_3CN in H_2O to afford adenosine.

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Appendix

Figure S1. ^{1}H NMR spectrum of platensimycin in DMSO-d_6

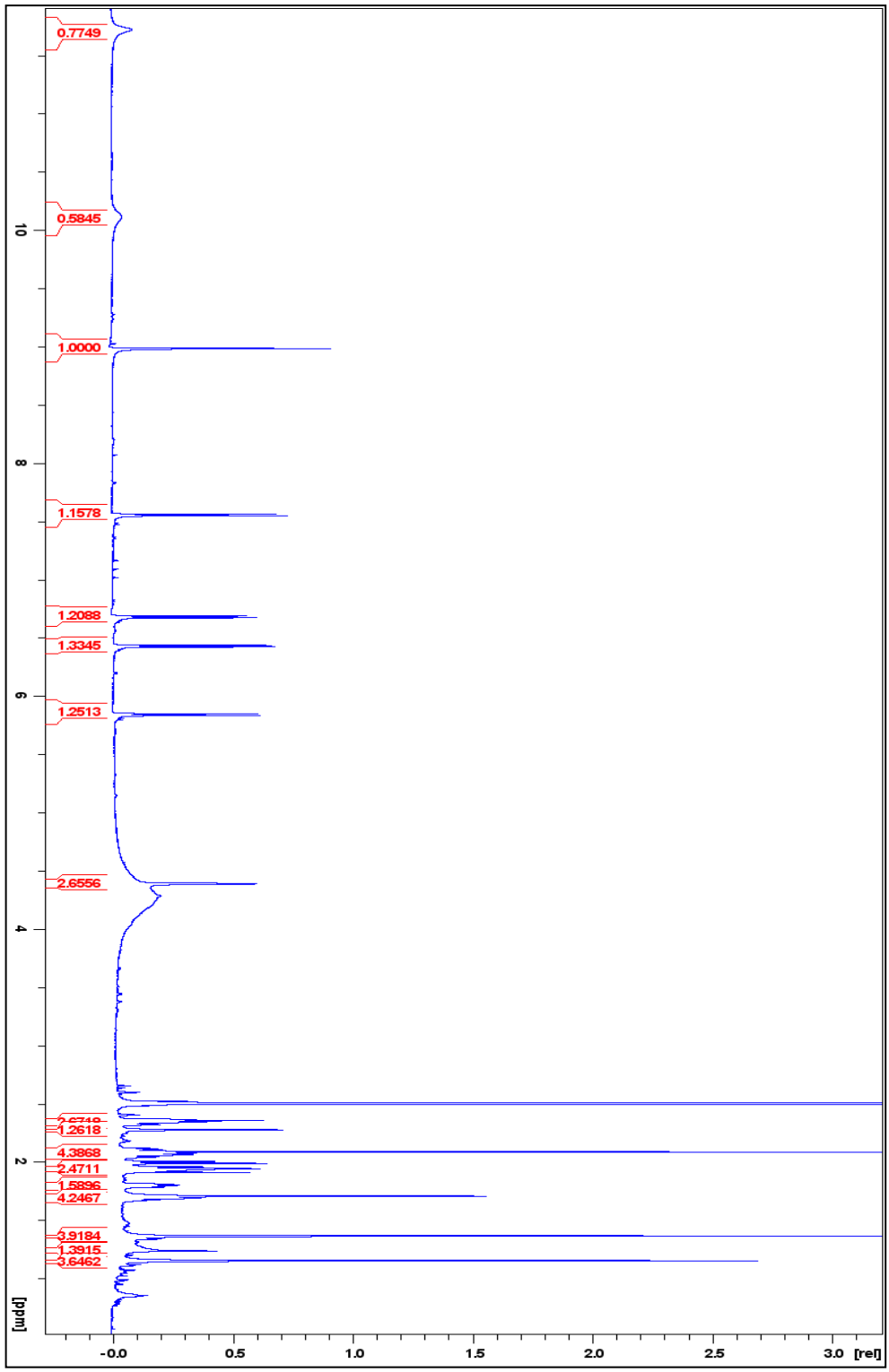


Figure S2. ^{1}H NMR spectrum of adenosine in DMSO-d_6

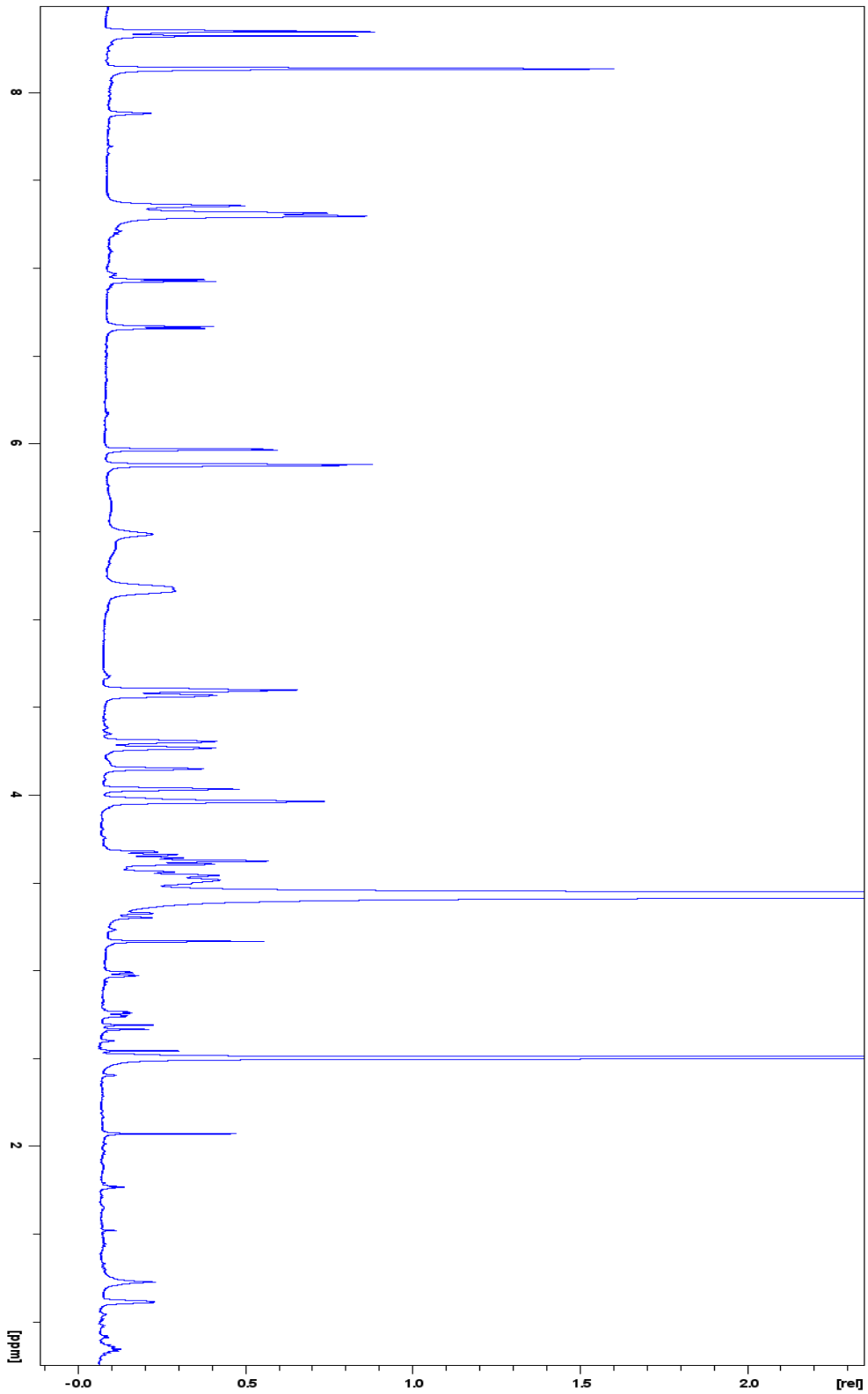


Figure S3. ^{13}C NMR spectrum of adenosine in DMSO-d_6

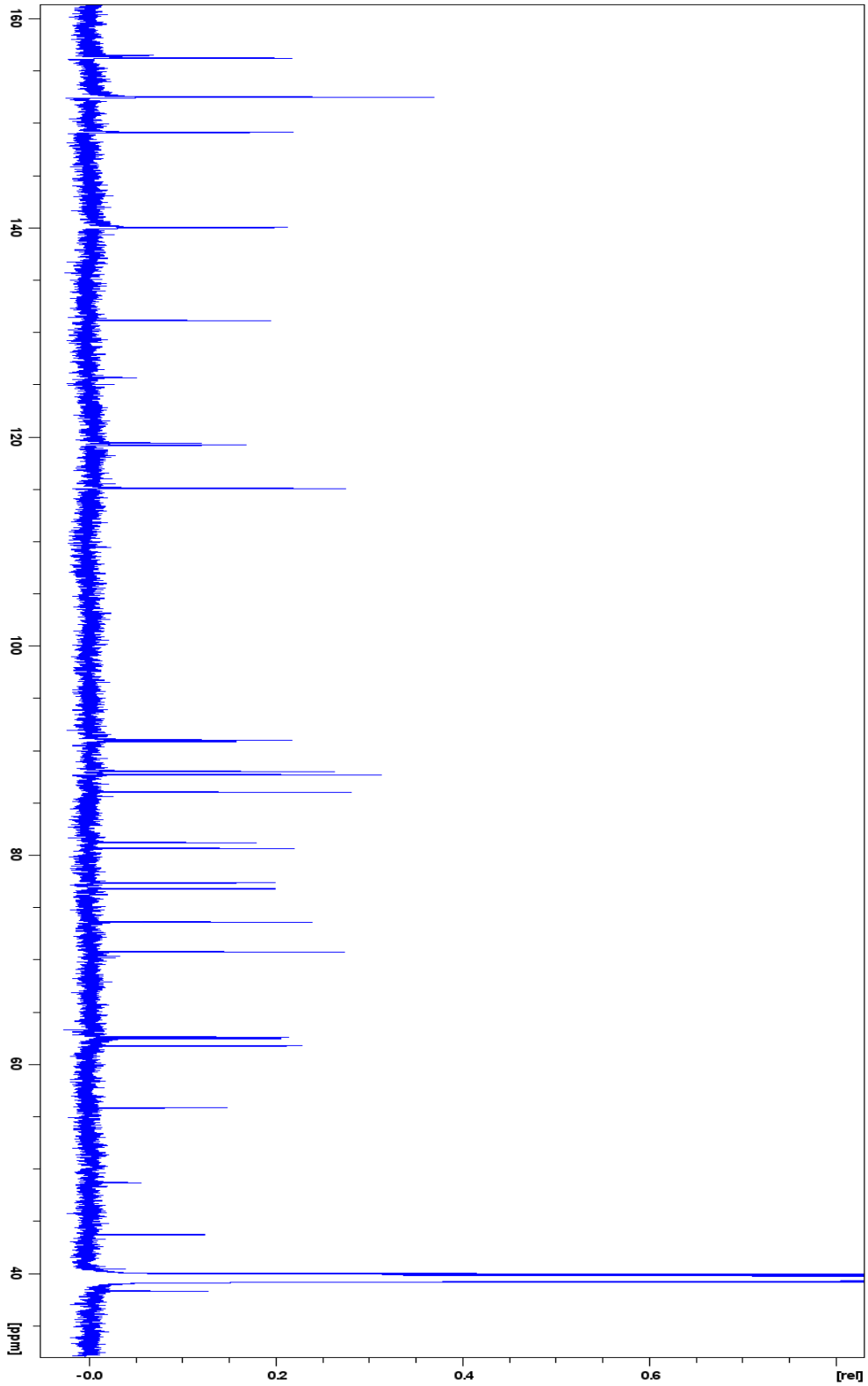


Figure S4. COSY NMR spectrum of adenosine in DMSO-d_6

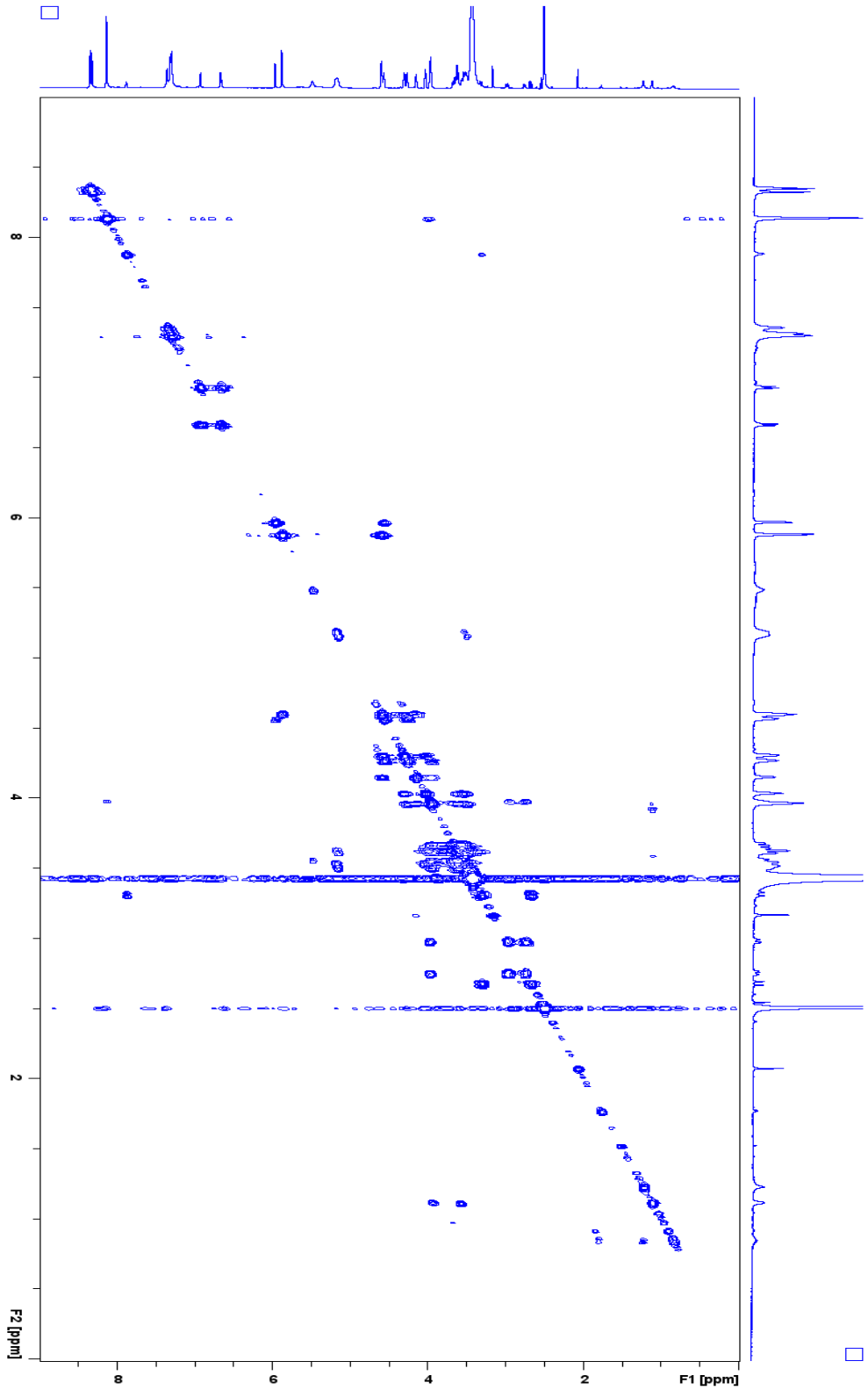


Figure S5. HSQC NMR spectrum of adenosine in DMSO-d_6

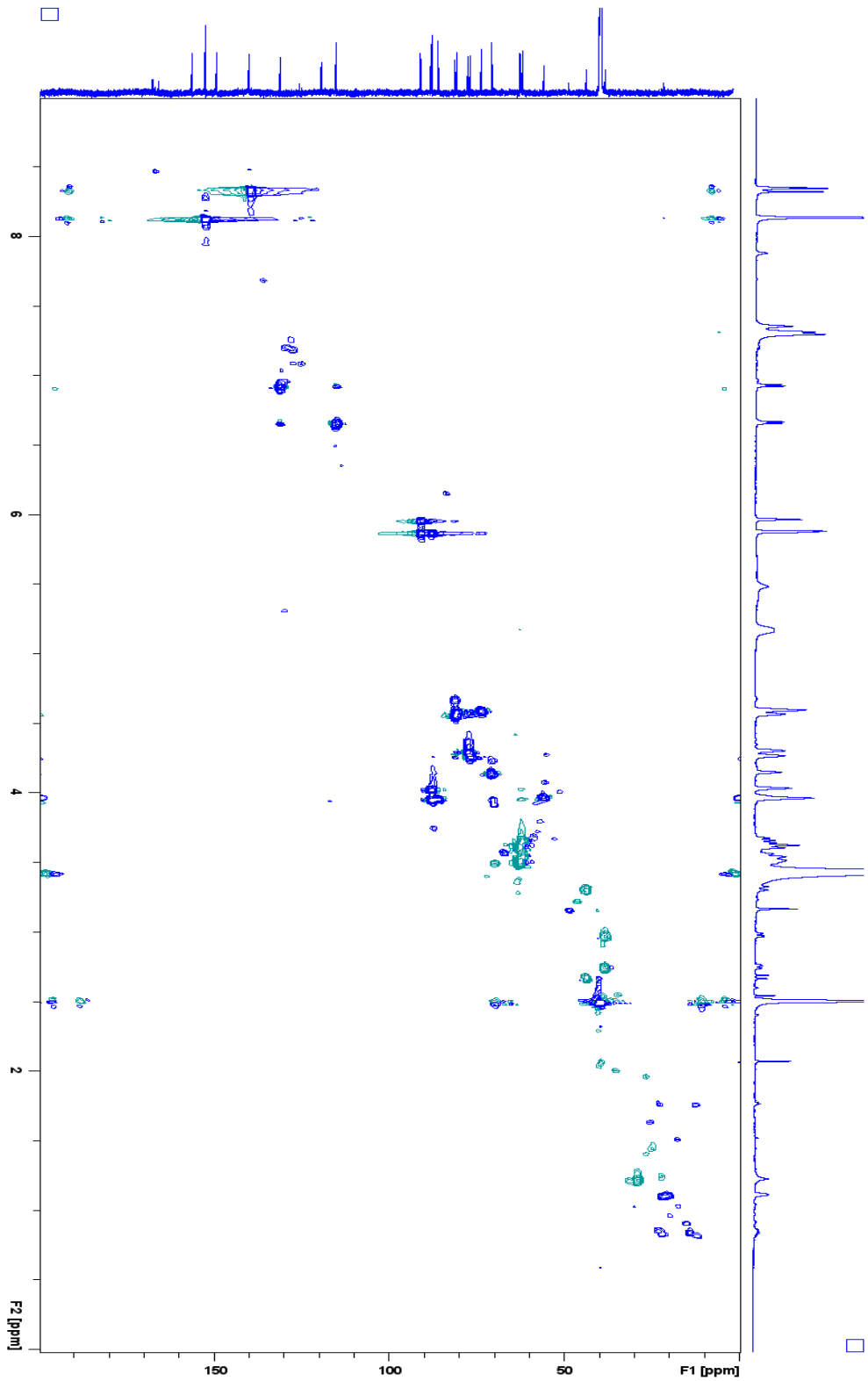
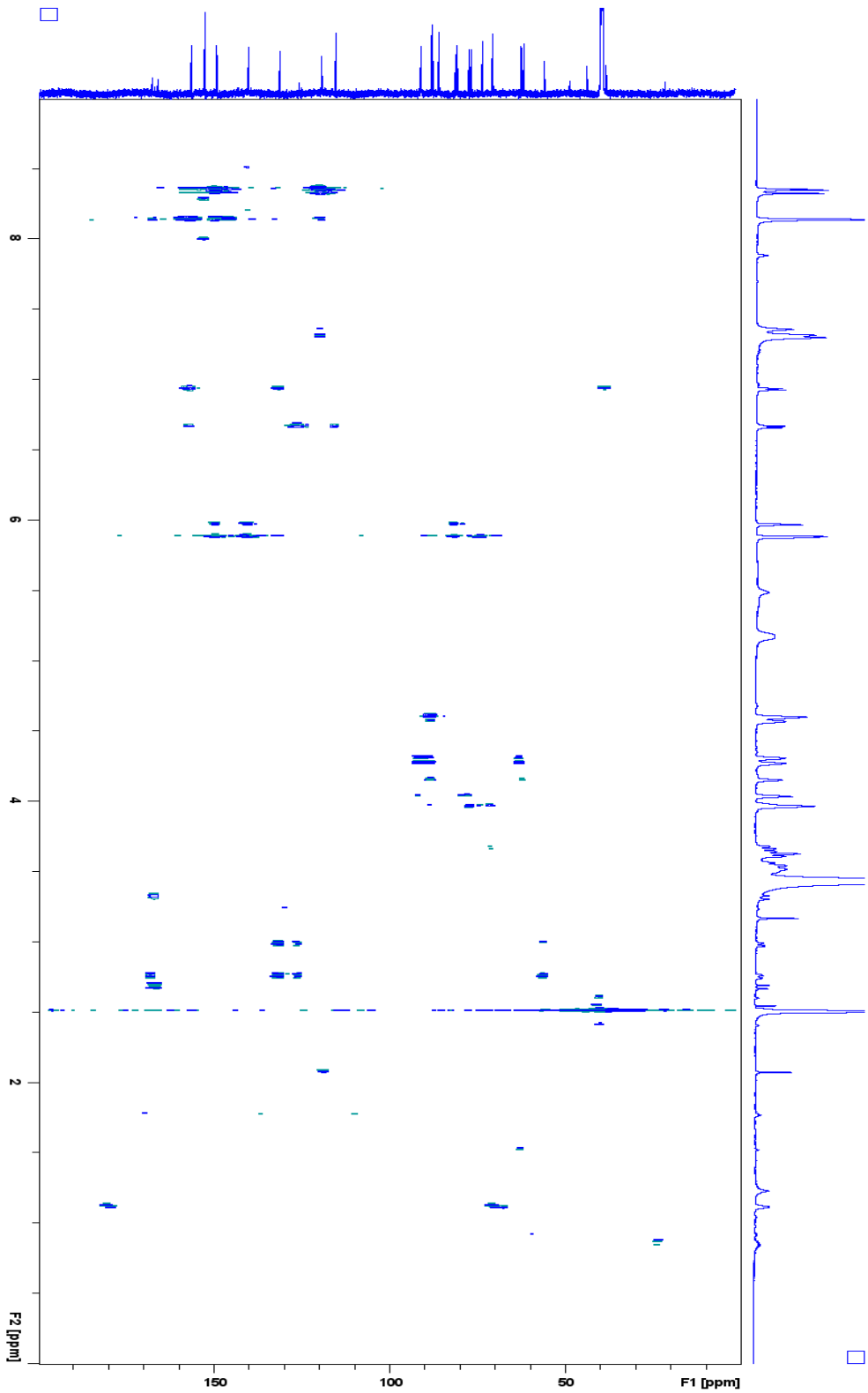


Figure S6. HMBC NMR spectrum of adenosine in DMSO-d_6



한글 초록

세균에 의한 감염병은 고대에서부터 현재까지 정복되지 않은 질병이다. 최초의 항생제인 페니실린이 개발된 이후 세균에 의한 감염병 문제가 해결되는 듯 보였으나, 항생제 내성균들의 등장으로 세균 감염에 의한 사망률이 다시 증가하고 있다. 따라서 내성균에게도 작용할 수 있는 새로운 작용 기작을 가진 항생제가 필요하고, 미생물로부터 생합성되어 나오는 천연 항생물질들은 신규 항생 물질의 발견 가능성을 제시하고 있다. 그 중 플라텐시마이신은 그람양성균의 강한 광범위항생제로 지방산 합성 시스템을 저해하는 독특한 방식으로 항생 효과를 보인다. 본 연구에서는 미생물 *Streptomyces platensis*가 생합성하는 천연 항생물질 플라텐시마이신의 생합성 수율을 증대할 방법을 연구하였다. 생합성 수율을 증대하는 방법으로 여섯 가지 배지 조건과 공동 배양을 이용하였으며, 추출양 최적화를 위한 다섯 가지 추출 방법을 시도하였다. 수득률이 가장 높고, 추출되는 양이 가장 많은 조건은 다음과 같다. 옥수수 가루를 이용한 배지에서 혼합배양 균주(*Streptomyces* sp. CMDD14E023)를 3일 먼저 키운 뒤, *Streptomyces platensis*를 접종하고 추가적으로 11일간 더 배양한다. 그 이후 산성 조건의 아세트산 에틸을 이용하여 추출한다. 14일간 키워진 *S. platensis*의 액체 배지 추출물은 다양한 크로마토그래피 기법과 화학적 방법으로 분리 및 정제하여 순수한 플라텐시마이신을 얻었다. 또한 *Streptomyces champavatii* 균주를 이용한 혼합배양 방식을 통해 수율을 증대시켰으며, *S. champavatii* 가 아닌 다른 균주로 진행한 실험에서는 수율이 증대되지 않음을 확인하였다. 또한 *S. champavatii*가 만들어내는 물질 중 생합성 수율을 증가시키는 화합물을 발견하였다. 추출 및 정제된 물질들의 화학적 구조는 NMR, 질량분석과 UV 분광법을 통해 규명하였다. 본 연구를 통해 플라텐시마이신의 생합성 수득량이 약 4배 증가하였으며, 수득량 증대에 기여하는 화합물을 확인하였다.