東北薬科大学
審査学位論文（博士）

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<th>氏名（本籍）</th>
<th>ボウインヤエト英悦（中国）</th>
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<td>海洋由来の放線菌と糸状菌からの生物活性物質の探索研究（Studies on the bioactive secondary metabolites produced by marine-derived actinomycetes and fungi）</td>
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論文審査委員

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<th>教授 山下幸和</th>
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<td>副査</td>
<td>教授 吉村祐一</td>
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<td>副査</td>
<td>教授 浪越通夫</td>
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海洋由来の放線菌と糸状菌からの
生物活性物質の探索研究

東北薬科大学大学院
薬学研究科
天然物化学教室
卜 英悦

2015年3月
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Chapter 1. Introduction-Past, Current Status, and Future Microbial Metabolites Research

Antibiotics, normally including antiviral, monoclonal, antibacterial, antifungal and antiparasitic drugs, have been utilized for the last 80 years to treat various infectious diseases, such as syphilis, tuberculosis, salmonella, and some forms of meningitis. Until the early 1940s, the importance of the antibiotics from microorganisms had been realized, some of them put into use in humans. In the long history, microorganisms have made a great contribution to people’s health. Concomitantly, widespread use of antibiotics, natural or synthetic, rises antibiotics resistance and leads to the emergence of antibiotic resistant bacteria, which reduce the efficacy of the drug or even not effective in humans any more. A report of World Health Organization states that antibiotic resistance is happening right now in any country and has been recognized as a major threat to public health. At this point, new types of antibiotics are required to treat infections caused by drug-resistant bacteria or new emerging pathogenic bacteria. Recently, marine-derived microorganisms, such as bacteria, fungi and actinomycetes, offer a great hope of the drug sighting and development due to the structural diversity and unique biological activities of their secondary metabolites, which have not been found among those isolated from the terrestrial sources yet. Many of those molecules possess antifungal, antibacterial, anti-inflammatory, antitumor, antiviral activities, the activity of kinase inhibitors and so on. The application of modern separation, genetic and bioassay screening techniques has accelerated novel microbial metabolites discovery.

1.1 History of Antibiotic Development

In the history, the development of pharmaceutical industry was oriented by the antibiotics produced by terrestrial microorganisms, such as soil-derived fungi and actinomycetes.

Penicillin antibiotics, among the most well-known antibiotics, were used as the first drugs to act effective against many previously serious pathogens caused by staphylococci and streptococci. In 1929, bacteriologist Alexander Fleming described the discovery of penicillin from *Penicillium notatum* and its activity against bacteria, and, later in 1940s, penicillin was developed into an antibiotic in humans by a group at Oxford (Bugni, T. S., 2004). During the World War II, penicillin played an important role in the control of pathogens in the battles and
greatly decreased the death of solders. Penicillins are usually very safe and still widely used today, though many types of penicillin-resistant bacteria emerged. They are sold in capsule, tablet, liquid and injectable forms.

Streptomycin, the first antibiotic active against tuberculosis, was discovered by Selman Abraham Waksman. In 1943, Selman Waksman and his team of students found that streptomycin from *Streptomyces griseus* was active against *Mycobacterium tuberculosis*. With the experimental equipment and financial support from Merck & Company, the results for the animals and clinical trials proved that streptomycin was effective chemotherapeutic treatment for tuberculosis.

What is the most significant is that Waksman believed that prices could be reduced if several companies could manufacture the drug. Waksman wanted to change the agreement giving Merck exclusive rights to the drug. Merck was generous to accept a non-exclusive license for the production of streptomycin.

In addition, including streptomycin and neomycin, Waksman's team discovered more than 15 antibiotics. Streptomycin and neomycin are extensively applied to treat various infectious diseases till now (Ginsberg, J., 2014).

Fleming and Waksman opened a new age of antibiotics studies and since 1940-1950s millions of strains from the soil microbiota have been extensively investigated all over the globe and numerous antibiotics have been discovered. Pharmaceutical industries have also focused on the soil microbes for more than 50 years (Abad, M. J., 2011).

Unfortunately, with the development and application of pharmaceutical drugs, drug-resistant bacteria have been continuously emerging. An outline of the history of antimicrobial agents, and emerging resistant organisms in response to antimicrobial agents are shown in Figure 1-1 (Saga, T.; Yamaguchi, K., 2009). Moreover, new antibiotics of the marketplace have fallen to less than half the previous level (Coates, A. R.; Hu, Y., 2006). However, bacteria, such as MRSA (meticillin-resistant *Staphylococcus aureus*), penicillin-resistant *S. pneumoniae* and *Pseudomonas aeruginosa*, have been rising to show high levels of resistance among pathogens (Coates, A.; Hu, Y., 2007).
1.2 Current Status of Microbial Metabolites Research

The importance of natural products in the discovery of pharmaceutical leads is provided by the fact that about half of the best-selling pharmaceuticals in 1991 were either natural products or their derivatives and in 1997, 42% were natural products or their derivatives and of these, 67% were antibiotics (Demain, A. L., 2009). Nowadays, researchers have been continuing searching for new types of metabolites from natural microorganism sources. Till 2005, among 22 500 biologically active compounds that have been obtained from microbes, 45% are produced by actinomycetes, 38% by fungi and 17% by unicellular bacteria (Berdy, J., 2005). Some of these compounds have been developed into antitumor drugs, enzyme inhibitors, immunosuppresants, hypocholesterolemic drugs, insecticides, antibacterial agents and so on (Demain, A. L., 2009).

On the other hand, after studied for several decades, metabolites from terrestrial microorganism sources appear repetitive. Researchers to some extent have become to preclude the study of terrestrial sources and turned to explore unique habitats, such as the marine environment (Bugni, T. S., 2004). Ocean covers 71% surface of the earth and inhabits about...
80% organism resources, which offers unlimited potential for biological and chemical diversity (Abad, M. J., 2011). Many natural products researchers continue to focus their effects on the studies of marine microorganisms. Novel and bioactive compounds from marine-derived microbes were discovered with a 10% increase in the number of compounds reported from the previous year, especially a 30% increase from 2010 to 2011 (Blunt, J. W., 2014). At the same time, modern genetic methods, high throughput screening techniques (HTS), separation technologies and recent advances in microbiological techniques have been developed and supported for the compounds discovery from microbes. In 1992, from marine-derived fungi, only 15 metabolites were discovered, and, till 2004, more than 272 new compounds were isolated (Bhadury, P., 2006). In addition, with the development of modern oceanographic science in recent years, humans have expanded to deep-sea regions by means of underwater detection and got a chance to collect marine deep-sea microorganisms. Researchers from major developed and developing countries are going on by using these advanced tools for drugs discovery from the sea. These years, the amount of new compounds discovered from marine organisms is rapidly going up and substantial numbers of those are derived from microorganisms (Figure 1-2.) (Blunt, J. W., 2013).

![Figure 1-2. Variation in number of publications of new marine natural products by country over the period 2006–2011.](image-url)
Some marine microbial metabolites possess strong bioactivities and currently are in clinical trials (Table 1-1) (Himaya, S. W. A., 2013) (Newman, D. J.; Cragg, G. M., 2014). Some of them will be introduced in this paper.

Table 1-1. Marine-derived compounds currently in clinical trials that have evidences of microbial origin.

<table>
<thead>
<tr>
<th>Clinical status</th>
<th>Trade mark</th>
<th>Compound</th>
<th>Biological activity</th>
<th>Microorganism</th>
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</thead>
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<tr>
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<td>Soblidotin</td>
<td>Peptide</td>
<td>Anticancer</td>
<td>Bacteria</td>
</tr>
<tr>
<td></td>
<td>Tectin</td>
<td>Tetrodotoxin</td>
<td>Antitumor</td>
<td>Bacteria</td>
</tr>
<tr>
<td>Phase II</td>
<td>Plinabulin</td>
<td>Diketopiperazine</td>
<td>Anticancer</td>
<td>Fungi</td>
</tr>
<tr>
<td></td>
<td>Isokahalide</td>
<td>Depsipeptide</td>
<td>Anticancer</td>
<td>Bacteria</td>
</tr>
<tr>
<td></td>
<td>Tasidotin</td>
<td>Peptide</td>
<td>Anticancer</td>
<td>Bacteria</td>
</tr>
<tr>
<td></td>
<td>Trabectedin / Yondelis</td>
<td>Alkaloid</td>
<td>Anticancer</td>
<td>Bacteria</td>
</tr>
<tr>
<td>Phase I</td>
<td>Bryostatin 1</td>
<td>Bryostatin 1</td>
<td>Anticancer</td>
<td>Bacteria</td>
</tr>
<tr>
<td></td>
<td>Marizomib</td>
<td>Beta-lactone-gamma-lactam</td>
<td>Anticancer</td>
<td>Bacteria</td>
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</table>

Tetrodotoxin, the well-known “marine toxin” acting as a selective blocker of Na+ channels, can be found in many marine organisms, such as pufferfish and sea stars. The origin of the toxin now has been considered as certain symbiotic marine microorganisms (Chau, R., 2011). The toxin was first isolated in 1909 by Dr. Yoshizumi Tahara (Clark, R. F., 1999). The toxin is now in Phase III trials as an agent (Tectin®) for cancer-related pain in Canada. (WEX Pharma INC.)

Plinabulin®, an experimental cancer drug, is in Phase II clinical development under Nereus Pharmaceuticals, INC. This agent is a simple modification of a marine fungal metabolite halimide (Hayashi, Y., 2013).

Tasidotin® is an orally active synthetic microtubule-targeted derivative of the marine depsipeptide dolastatin-15, is currently undergoing clinical evaluation for cancer treatment (Ray, A., 2007).

Salinosporamide A (Marizomib®), the compound discovered from a marine actinomycete,
Salinispora tropica, acts as a proteasome inhibitor for disrupting processes related to the growth and survival of cancer cells (Potts B.C., et al., 2011). The compound entered Phase I clinical trials.

Trabectedin® or Yondelis®, the first marine-derived anti-neoplastic drug for the treatment of advanced soft tissue sarcoma (D’Incalci M., 2014), currently is in Phases I to III. This agent was discovered from a mangrove tunicate, *Ecteinascidia turbinata*. Trabectedin was recognized to be produced by symbiotic bacteria (Javed, F., 2011). As only very little amount of the compound could be obtained from nature, PharmaMar produced this agent by the semi-synthesis from a precursor molecule, cyanosafracin B, from a marine-derived microorganism *Pseudomonas fluorescens* (Cuevas C, 2000).

Drugs discovery is still going on. Recently, numerous novel microbial metabolites have been continuously discovered. They act various bioactivities and show the potential to turn out into pharmaceutical products.

An alga-derived fungus *Pestalotia* sp. produced the potent antimicrobial metabolites, pestalone, which showed strong antimicrobial activity against vancomycin-resistant *Enterococcus faecium* (VREF) (MIC = 37 ng/mL) (Cueto, M., 2001). Two new lactones, pyrenocines D and E were isolated from the culture of a brown alga derived fungus, *Penicillium waksmanii*. Pyrenocine E showed moderate cytotoxicity against P388 leukemia cell line with an ED₅₀ of 1.30 μg/mL (Amagata, T., 1998). *Penicillium* strain CNC-350 produced two diketopiperazine dimmers, 11,11’-dideoxyverticillin A and 11’-deoxyverticillin A showing potent cytotoxicity against HCT-116 cell line with an IC₅₀ of 30 ng/mL (Son, B. W., 1999). Pseudoalteromonones A and B were obtained from Pseudoalteromonas sp., which was cytotoxic to MOLT-4 (human acute lymphoblastic leukaemia) cells (Chen, Y. H., 2012). Fradcarbazoles A-C were isolated from *Streptomces fradiae* from sediment collected from Jiaozhou Bay, Shandong, China. These compounds showed significant cytotoxicity against HTCLs and acted as the inhibitors of the kinase PKC-α (Fu, P., 2012). JBIR-109 to 111, analogues of trichostatin, were isolated from a sponge-derived *Streptomyces* strain collected from Kagoshima, Japan (Hosoya, T., 2012).

Overall, marine microbes are likely to become a rich source of novel effective drugs due to their immense diversity of genetic and biochemical properties. Pharmaceutical industries
now accept the ocean as an important aspect of medical research and are concentrating on natural products derived from marine microorganisms. PharmaMar (Spain & USA) has put the marine drugs research in a leading position (Thakur, N.L., 2005) (Bhatnagar, I.; Kim, S. K., 2010).

1.3 Future Trends of Microbial Metabolites Research

People are exploring the oceans with various high technologies. China, Japan, the United States, France and Russia have developed deep-water technology. For example, manned research submersibles JiaoLong (China) and Sinkai 6500 (JAMSTEC, Japan) can now dive to a depth of over 7,000 m and 6,500 m, which can be used to collect microorganisms from deep-sea. Very recently, several research groups have begun to seek novel microbial metabolites derived from deep-sea and rapidly there are some reports about their works. The deep-sea-derived *Streptomyces* sp. SCSIO 03032 was capable of producing new bisindole alkaloids, spiroindimicins A–D, which exhibited moderate cytotoxicities against several cancer cell lines (Zhang W., 2012). New spirotetronate antibiotics, Lobophorins H and I, were isolated from a South China Sea-Derived *Streptomyces* sp. 12A35 from the sediment collected at the depth of about 2000 metres. Lobophorin H showed a strong activity against *Bacillus subtilis* CMCC63501 with the MIC value of 3.13 μg/mL, while Lobophorin I possessed moderate activity with the MIC value of 6.25 μg/mL (Pan, H. Q., 2013). Besides the pharmaceutical leads, deep-sea derived microorganisms also offer other useful agents, like antifouling compounds. Some antifouling potentials of deep-sea-derived fungi from the South China Sea have been reported (Zhang, X. Y., 2014). Besides, new laboratory of deep-sea derived microorganisms has been set up very recently. Dr. McPhail’s research team of Oregon State University, USA started a program of elicitation of antibiotic natural products in multispecies cultures of deep-sea vent-derived microorganisms. The studies of deep-sea microorganisms have just started and the deep-sea microorganisms are potential to be a rich source for drugs discovery.

New culture techniques, such as fungal fermentation studies by adding histone deacetylases (HDAC) and histone acetyl transferases (HAT) inhibitors have been applied to metabolites discovery as reported by Oshima’s team, 2013. They found six new benzophenones cephalanones A–F from an endophytic fungus, *Graphiopsis Chlorocephala,*
only produced in the presence of an NAD\(^+\)-dependent HDAC inhibitor (Asai, T., 2013). Though the technique was applied to the terrestrial fungus, in the near future there could be several reports of marine-derived fungi.

The news reported in Oct. 2014 by Meera Senthilingam, for CNN had the headline “We're growing bacteria in space, and they could help us create new vaccines”. There is always a hope that space-derived new drugs can treat the refractoriness we are facing now, which the traditional types cannot do.

Overall, people are continuing inventing new technologies to yield microbial metabolites with unique biochemical and structural diversity or to find novel targets for vaccines and therapies, which offer a great hope of the human survival and the civilization development.

**1.4 Objective of This Research**

As discussed above, marine-derived microbial metabolites continue to be a potential source of new drugs discovery. The purpose of this research is to discover new compounds from marine-derived actinomycetes and fungi. Bioassay methods, including antibacterial, antifungal and anti-mycobacterial tests were used for the screening of culture broth of microbial strains. Bioassay-guided isolation from two fungi and one actinomycete strains extracts yielded 8 new compounds, and their bioassay activities are discussed.
Figure 1-3. Structures of selected marine-derived microbial agents currently in clinical trials
Figure 1-4. Structures of selected marine-derived microbial metabolites.
Figure 1-5. Structures of selected marine-derived microbial metabolites obtained by the application of new techniques.
Figure 1-5. Continued.
2. Isolation and Characterization of New Anti-Mycobacterial Nucleoside Antibiotics from a Marine-Derived *Streptomyces* sp. TPU1236A

2.1 Introduction

Tuberculosis (TB) is a high incidence of contagious disease in humans caused by various strains of mycobacteria, usually *Mycobacterium tuberculosis* (Kumar, V., 2007), which typically attacks the lungs (Konstantinos, A., 2010). It’s assumed that one-third of the world’s population have been infected with the *M. tuberculosis* complex, although not yet ill with active TB (WHO, 2010). The World Health Organization (WHO) estimates that active cases of tuberculosis afflict nearly 9 million people annually, and lead up to 1.4 million deaths per year (WHO, 2013). Tuberculosis chemotherapy involves a combination bacteriostatic antibiotics of isoniazid (INH) and rifampin (RFP) for at least 6 months, and pyrazinamide (PRZ) and ethambutol (EMB) or streptomycin (SPM) for the first 2 months of treatment (Askgaard, D.S., 1995). However, drugs for treating tuberculosis are far from ideal. Severe side effects associated with anti-TB drugs are common among patients hospitalized for pulmonary tuberculosis as reported (Gülbay, B. E., 2006). Besides, *M. tuberculosis* always acquires drug resistance mainly due to its lipid-rich cell wall acting as a permeability barrier from drugs and inadequate chemotherapy (Louw, G. E., 2009) (Michael, D., 1993), which mainly caused multi-drug-resistant tuberculosis (Dalton, T., 2012) (MDR-TB: resistance to at least isoniazid and rifampicin) and extensively-drug resistant TB (WHO, 2006) (M/XDR-TB: MDR resistance plus resistance to a fluoroquinolone and an amionoglycoside). The multi-drug-resistant type of tuberculosis further complicated the problem of TB control (Michael, D., 1993). More recently, the totally drug resistant TB (TDR-TB), which is resistant to all second-line drugs, have often been reported from India and the other Asian countries. Although a new drug, delmanid (Deltyba®), which inhibits the cell wall biosynthesis of mycobacteria, has been approved in 2014 for the treatment of MDR-TB in EU and Japan (Ryan, N.J., 2014) (Xavier, A.S., 2014), continuous efforts to discover new anti-TB agents with novel mechanisms of action and structural features are the emergent global demand. During our continuous research for anti-TB drugs produced by marine organisms, marine actinomycetes, as a new resource for natural products discovery, currently received considerable attention (Fenical, W., 2006). Increasing
evidences reveal that marine actinomycetes offer a great hope of the drug sighting and development due to the structural diversity and unique biological activities of their secondary metabolites, which have not been found among those isolated from the terrestrial sources yet (Zotchev, S. B., 2012).

As a part of our search for anti-TB compounds produced by marine actinomycetes collected from Iriomote Island, Okinawa, Japan, we evaluated the anti-mycobacterial activity against *Mycobacterium smegmatis* NBRC 3207 of EtOAc extracts of bacterial culture broth by the paper disc method. *M. smegmatis* has been the most widely studied due to its fast growing and non-pathogenicity compared to other mycobacterial species (Reyrat, J. M., 2001). The EtOAc extract of culture broth of a marine-derived *Streptomyces* sp. (strain TPU1236A) was found to exhibit the strongest antibacterial activity against *M. smegmatis* NBRC 3207. Activity-guided purification of the culture broth yielded five compounds, designated streptcytosines A–E (1–5) (Bu, Y. Y., 2014), along with six known compounds: de-amosaminyl-cytosamine (6), plicacetin (7), bamicetin (8), amicetin (9), collismycin A (10) and SF2738C (11) (Chen, R., 2013) (Haskell, T. H., 1958) (Hinman, J.W., 1953) (Gomi, S., 1994) (Figure 2-1 & 2-2).

![Figure 2-1. Structures of compounds 1–5.](image-url)
2.2 Results and Discussion

The strain TPU1236A was isolated from a seawater sample collected at Iriomote Island in Okinawa, Japan. The gene sequence used for the identification showed 100% identity with those of *Streptomyces badius* and *Streptomyces sindenensis*. The bacterium was cultured by shaking in a liquid medium for 7 days at 25 °C in the dark. The extracts of broth filtrates and mycelia were subjected to ODS silica gel column chromatography followed by preparative HPLC separation as described in the Experimental Section to afford compounds 1–11. Six known compounds were identified the structures by comparing the spectroscopic data for 6–11 with those of the reported values for de-amosaminyl-cytosamine (6), plicacetin (7), bamicetin (8), amicetin (9), collismycin A (10), and SF2738 C (11), respectively.

2.2.1 Screening Bioassay

Anti-mycobacterial screening was carried out using *M. smegmatis* NBRC 3207 by the paper disc method. Test microorganism was inoculated to a petri dish containing the 7H9 agar. A sterile antibiotic filter disc (diameter, 6 mm, Advantec, Tokyo, Japan) with the sample was placed in the dish and cultured for 2 days at 37 °C. The inhibition zone in diameter (mm) was measured and above 8 mm in diameter of the inhibition zone was considered as a positive result. The crude extract of the bacterium strain TPU1236A showed the strongest inhibitory
activity against *M. smegmatis* NBRC 3207 among 40 extracts of marine–derived microorganisms collected at Iriomote Island. (Figure 2-3)

Figure 2-3. Sampling sites of marine-derived actinomycetes (2012)
Table 2-1. Anti-mycobacterial screening (inhibition zone: nm) against *Mycobacterium smegmatis* NBRC 3207.

<table>
<thead>
<tr>
<th>Strain</th>
<th>EtOAc extract of broth (50 μg/disc)</th>
<th>Strain</th>
<th>EtOAc extract of broth (50 μg/disc)</th>
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<tbody>
<tr>
<td>TPU1201A</td>
<td>—</td>
<td>TPU1221A</td>
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<td>TPU1202A</td>
<td>—</td>
<td>TPU1222A</td>
<td>—</td>
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<td>TPU1203A</td>
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<td>TPU1220A</td>
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<td>TPU1240A</td>
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</table>

streptomycin sulfate $^b$ 30 methanol $^c$ —

$^a$: An inhibition zone was not detected. 
$^b$: Positive control (5 μg/disc). 
$^c$: Negative control (10 μL/disc).
2.2.2 Isolation of Compounds

The producing actinomycete, *Streptomyces* sp. (strain TPU1236A) was isolated from a seawater sample collected at Iriomote Island, Okinawa Prefecture, Japan. The bacterium was cultured on a liquid medium by shaking for 7 days at 25 °C in the dark. After cultivation, the colonies were harvested and extracted with methanol. The extracts were separated by combination of chromatographic procedures to afford streptcytosines A–E (1–5), de-amosaminyl-cytosamine (6), plicacetin (7), bamicetin (8), amicetin (9), collismycin A (10) and SF2738C (11). (Figure 2-4)

![Diagram](image)

*Streptomyces* sp.
TPU1236A

seed culture (30 mL, 27 °C, 150 r.p.m, 3 days)
main culture (10.0 L, 27 °C, 150 r.p.m, 21 days)

broth

filtered

filtrate
open column-HP20
washed with H$_2$O
eluted with MeOH

MeOH eluent

evaporated MeOH

extract (1.5 g)

open column-ODS

Fr.8

HPLC-ODS
60% MeOH, 0.05%
TFA

Fr.9

HPLC-ODS
45% MeOH,
0.05% TFA

Fr.10

HPLC-ODS
36% MeOH,
0.05% TFA

(3.2mg) (7.2mg)(8.1mg)(5.1mg)(12.0mg)(20.5mg)(12.3mg)(21.3mg)(11.2mg)(6.8 mg) (25.0mg)

Figure 2-4. Bioassay-guided isolation procedure for the whole broth extract.
2.2.3 Structure Determination of New Compounds

Streptcytosine A (1) was obtained as colorless oils. The molecular formula of C\textsubscript{30}H\textsubscript{42}N\textsubscript{6}O\textsubscript{9} was deduced from HRFABMS ([M+H]\textsuperscript{+} peak at m/z 631.3110, calcd for C\textsubscript{30}H\textsubscript{43}N\textsubscript{6}O\textsubscript{9}, Δ +1.9 mmu), \textsuperscript{1}H and \textsuperscript{13}C NMR spectral data analysis (Table 2-2). The \textsuperscript{1}H NMR spectrum (in CD\textsubscript{3}OD) displayed 37 proton signals. The other five active protons present in 1 were suggested by comparing with the amount of hydrogens in the molecular formula. The connectivity of proton and carbon atoms was established by the HMQC spectrum (Table 2-2). The presence of 30 carbons including five methyl, two \(sp^3\) methylene, one oxygenated \(sp^3\) methylene, one nitrogenated \(sp^3\) methylene, five oxygenated \(sp^3\) methine, one nitrogenated \(sp^3\) methine, two anomic \(sp^3\) methine, five \(sp^2\) methine, one nitrogenated \(sp^2\) methine, one \(sp^2\) quaternary, one nitrogenated \(sp^3\) quaternary, one nitrogenated \(sp^2\) quaternary, and three amide carbonyl carbons was decided by the analysis of the \textsuperscript{13}C NMR, DEPT and HMQC spectra.

Detailed 2D analysis revealed that 1 was composed of the C-2, C-4 to C-6 giving a cytosine (Zhang, G., 2012), the carbons from C-1’ to C-6’ and C-1” to C-6” containing a disaccharide, C-8 to C-14 forming a \(p\)-aminobenzoic acid (PABA) and 4-imidazolidinone by C-16, C-17, C-19, C-20 and C-21 (Figure 2-5). \textsuperscript{1}H-\textsuperscript{1}H COSY analysis gave the partial structures I to IV (Figure 2-5). The presence of a nucleotide cytosine in 1 was confirmed by the partial structure II and HMBC correlations (Figure 2-5) from H-6 to C-2 and from H-6 to C-4. A disaccharide unit in 1 was determined by two connected sugars. The partial structure III was cyclized to form a sugar called 3-hydroxyamicetose, which was deduced from the HMBC correlations (Figure 2-5) from H-1’ to C-5’ forming an oxygenated bond. Another oxygenated bond from H-1” to C-5” was also observed by the HMBC correlation (Figure 2-5) and, together with the partial structure IV, a pyranose ring structure was proved. Besides, two coupled nitrogenated \(sp^3\) methyls H-7” and 8” were observed and attached with C-4”’, which was suggested by the HMBC correlations (Figure 2-5) from H-7” and H-8” to C-4” to give the amino sugar called amosamine. 3-Hydroxyamicetose connected with amosamine by an \(α\)-(1→4)-glycoside bond, which was suggested by an HMBC correlation (Figure 2-5) form H-1” to C-4’. Cytosine and 3-hydroxyamicetose moieties were connected together by an observed HMBC correlation (Figure 2-5) from H-6 to the anomic C-1’. As plicacetin (7), the presence of PABA unit was deduced from two aromatic carbon signals at δ 130.8 and δ 120.6
(partial structure I) and an HMBC correlation (Figure 2-5) from H-14 to an amide carbonyl carbon at C-8, together with one nitrogenated $sp^2$ methine at C-12 and one $sp^2$ quaternary carbon at $\delta_C 131.6$. Unlike bamicetin (8) and amicetin (9) containing an $\alpha$-methyl-serine, a 4-imidazolidinone was present in 1, which was deduced from the HMBC correlations (Figure 2-5) from H-21 to C-16 and C-17, from H-19 to C-16 and C-17 and from H-20 to C-16, C-17 and C-19 (Figure 2-5). These data gave the planer structure of 1 as shown in Figure 2-5.
Table 2-2. $^{13}$C (100 MHz) and $^1$H (400 MHz) NMR data for streptycosine A (1) (CD$_3$OD).

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<tr>
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Figure 2-5. ^1_H-^1_H COSY and HMBC data for compound 1

Since streptcytosine A (1) was obtained together with plicacetin (7), bamicetin (8), and amicetin (9) from the strain TPU1236A, the absolute configuration of the sugar moiety will be the same, which was also confirmed by J values. The configuration of 1 at the C-17 position was also assigned to be the same as those of 8 and 9, since these compounds should be biosynthesized by the same pathway. Moreover, compounds 1 (+72), 7 (+96), 8 (+66), and 9 (+76) showed the same positive specific rotation. Therefore, streptcytosine A (1) may share the same configuration for whole asymmetric carbons as compounds 8 and 9, as depicted in Figures 2-1 and 2-2. Therefore, streptcytosine A (1) may share the same configuration for whole asymmetric carbons as compounds 8 and 9, as depicted in Figure 2-1 and 2-2.

Streptcytosine B (2) was isolated as pale yellow oils. The molecular formula of 2, C_{14}H_{19}N_{3}O_{4}S, was established by HRFABMS ([M+H]^+ peak at m/z 326.1180, caled for C_{14}H_{20} N_{3}O_{4}S, Δ +0.6 mmu), 1H and 13C NMR spectral data analysis (Table 2-3). The 13C NMR spectrum (in CD_{3}OD) showed the signals for two methyl, two sp^3 methylene, two oxygenated sp^3 methine, one anomeric sp^3 methine, two sp^2 methine, one nitrogenated sp^2 methine, one sulfureted sp^2 methine, one nitrogenated sp^2 quaternary, and two amide carbonyl carbons by the analysis of the DEPT and HMQC spectra.

1H and 13C data for 2 suggested the presence of 3-hydroxyamicetose and cytosine moieties in 2, the same as those in 1 by comparing their NMR spectra data (Table 2-2 and 2-3). Besides, the 1H-1H COSY analysis of 2 revealed the presence of the partial structure V (Figure 2-6) and attached with the carbonyl carbon at C-8 by the analysis of HMBC correlations from H-9 to C-8 and from H-10 to C-8. One sulfureted sp^3 methyl signal at δ 2.44 (H-11) was assumed to
bond to the partial structure V by HMBC correlations (Figure 2-6) from H-11 to C-10. The 
trans-configuration of the olefin bond at C-9 in partial structure V was determined by the 
coupling constant (14.5 Hz). Thus, the planer structure of 2 was determined. The relative 
configuration of 3-hydroxyamicetose moiety in 2 was considered as the same as 1 due to their 
similar 1H and 13C NMR signals (Table 2-2 and 2-3). Thus, the structure of 2 was determined.

Streptcytosine C (3) had the molecular formula of C15H21N3O4 as established from 
HRFABMS ([M+H]+ peak at m/z 308.1619, calcd for C15H22N3O4, A +0.9 mmu), and 1H and 
13C NMR spectral data analysis (Table 2-3). 1H NMR and 13C NMR data (Table 2-3) for 3 were 
similar to those for 2, except the presence of the partial structure VI (Figure 2-6) in the 
carboxylic acid moiety, showing the structure of 2-methyl-2-butoenoic acid, which was deduced 
from 1H-1H COSY and HMBC correlations (Figure 2-6) from H-12 to C-8, H-10 to C-8, H-10 
to C-12 and from H-11 to C-9. In VI, an NOE correlation was observed only between H-10 and 
H-11 but not between H-10 and H-12. 1H and 13C NMR data due to the 2-methyl-butoenoic acid 
moiety's in 3 were very similar to those of cytosaminomycin D (Shiomi, K., 1994), which 
indicated the trans-configuration for the double bond at C-9 (Figure 2-6). The configuration of 
the 3-hydroxyamicetose moiety in 3 was considered to be the same as 2 due to their similar 1H 
and 13C NMR signals (Table 2-3).

Streptcytosine D (4) was assigned to the same molecular formula as 3, C15H21N3O4, by 
HRFABMS ([M+H]+ peak at m/z 308.1601, calcd for C15H22N3O4, Δ-1.0 mmu), 1H and 13C 
NMR spectral data (Table 2-3). 1H and 13C NMR data (Table 2-3) for 4 and 3 were almost 
identical, except that a 3-methylcrotonic acid moiety existed in VII, which was identified by 
1H-1H COSY and HMBC correlations (Figure 2-6) from H-12 to C-10 and C-11 and from H-9 
to C-8, C-10 and C-11 (Figure 2-6). The configuration of 3-hydroxyamicetose moiety in 4 was 
considered to be the same as 3 due to their similar 1H and 13C NMR signals (Table 2-3).

Streptcytosine E (5) had the molecular formula of C15H23N3O4 determined from 
HRFABMS ([M+H]+ peak at m/z 310.1763, calcd for C15H24N3O4, Δ-0.4 mmu), 1H and 13C 
NMR data (Table 2-3). 1H and 13C NMR data (Table 2-3) for 5 were highly similar to those for 
4. The partial structure VIII, a propanoic acid moiety existing in carboxylic acid moiety was 
deduced to be different from 4 by 1H-1H COSY and HMBC correlations. The planar structure 
of 5 was confirmed by detailed 2D NMR data analysis (Figure 2-6).
Table 2-3. $^{13}$C (100 MHz) and $^1$H (400 MHz) NMR data for streptcytosines B—E (2–5) (CD$_3$OD).

<table>
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<th>$\delta_C$ (in ppm)</th>
<th>$\delta_H$, mult. (J in Hz)</th>
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<tr>
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2.2.4 Structure Determination of Known Compounds

Compound 6 was obtained as a colorless oil. The molecular weight and molecular formula, 225 and C_{10}H_{15}N_{3}O_{3}, were decided by the analysis of {\textsuperscript{1}H} and {\textsuperscript{13}C} NMR spectra and FABMS data. Compound 6 had very similar {\textsuperscript{1}H} NMR (CD_{3}OD, 400 MHz) spectrum as those of compounds 2–5. In the {\textsuperscript{13}C} NMR (CD_{3}OD, 100 MHz) spectrum of compound 6, similar chemical shifts at δ 161.9, 148.9, 146.4 and 95.3 of a cytosine moiety were observed. The planer structure of compound 6 was determined by HMBC, HMQC and {\textsuperscript{1}H–}{\textsuperscript{1}H} COSY correlations. The absolute configuration of compound 6 was considered to be the same as the known compound de-amosaminyl-cytosamine by comparing the chemical shifts with those of reported values (Chen, R., 2013). Therefore, the structure of compound 6 was determined.

Compound 7 was obtained as a yellow oil. The molecular weight and molecular formula, 517 and C_{25}H_{35}N_{5}O_{7}, were decided by the analysis of {\textsuperscript{1}H} and {\textsuperscript{13}C} NMR spectra and FABMS data. The {\textsuperscript{1}H} and {\textsuperscript{13}C} NMR spectra of compound 7 showed similar signals to those of compound 1. The planer structure of compound 7 was determined by HMBC, HMQC and {\textsuperscript{1}H–}{\textsuperscript{1}H} COSY correlations. Compound 7 had a positive specific rotation of +96 (c 0.1, MeOH) which was very close to the reported value of plicacetin. (Haskell, T. H., 1958) The {\textsuperscript{1}H} and {\textsuperscript{13}C} chemical shifts in CD_{3}OD of plicacetin were also reported by Chen, R., 2013. Thus, compound...
Compound 8 was considered as a known compound plicacetin.

Compound 8 was obtained as a pale yellow oil. The molecular weight and molecular formula, \(604\) and \(C_{28}H_{40}N_6O_9\), were decided by the analysis of \(^1H\) and \(^{13}C\) NMR spectra and FABMS data, which were the same as a *Streptomyces* derived antibiotic, bamicetin. The planer structure of compound 8 was determined by HMBC, HMQC and \(^1H-^1H\) COSY correlations. Compound 8 showed a positive specific rotation of \(+66\) (c 0.1, MeOH) and was confirmed as a known antibiotic bamicetin.

Compound 9 was obtained as a pale yellow oil. The molecular weight and molecular formula, \(618\) and \(C_{29}H_{42}N_6O_9\), were decided by the analysis of \(^1H\) and \(^{13}C\) NMR spectra and FABMS data. These data were identical with those of an anti-mycobacterial antibiotic, amicetin, which was isolated from *Streptomyces* sp. together with bamicetin (8) and plicacetin (7). The planer structure of compound 9 was determined by HMBC, HMQC and \(^1H-^1H\) COSY correlations. Compound 9 had a positive specific rotation of \(+76\) (c 0.1, MeOH) and was identified as amicetin.

Compound 10 was obtained as a colorless crystal. The molecular weight and molecular formula, \(275\) and \(C_{13}H_{14}N_3O_2S\), were decided by the analysis of \(^1H\) and \(^{13}C\) NMR spectra and FABMS data. The structure of compound 10 was established by HMBC, HMQC and \(^1H-^1H\) COSY correlations. The \(^1H\) NMR showed similar signals to that of collismycin A and the structure of compound 10 was confirmed.

Compound 11 was obtained as a pale yellow oil. The molecular weight and molecular formula, \(262\) and \(C_{13}H_{15}N_2O_2S\), were decided by the analysis of \(^1H\) and \(^{13}C\) NMR spectra and FABMS data. The \(^1H\) and \(^{13}C\) NMR chemical shifts of compound 11 were similar to those of compound 10. The structure of compound 10 was determined by HMBC, HMQC and \(^1H-^1H\) COSY correlations and was confirmed as SF2738C.

### 2.2.5 Biological Activity of Isolated Compounds

The antibacterial activities of compounds 1—11 against *M. smegmatis* NBRC 3207 were evaluated using the paper disc method (Ericsson, H., 1960), and MICs were determined by the liquid microdilution method using 96-well plastic plates (Table 2-4).

Compounds 1, 7–9, and 11 showed activity against *M. smegmatis* at 5 \(\mu\)g/disc. Amicetin (9) was reported to inhibit the growth of *M. tuberculosis* (Amsterdam, D., 1996) and showed strong activity against *M. smegmatis* in our experiment. The inhibition activity of compounds 1
and 7 (MIC = 32 μg/mL) was about a half of that of compounds 8 (MIC = 16 μg/mL). Therefore, the 2-methylserine moiety attached to the PABA unit will be important for the anti-mycobacterial activities of these compounds.

On the other hand, compounds 2–6 were not active against \textit{M. smegmatis} at 50 μg/disc. Consequently, the amino sugar (amosamine) will be essential for anti-mycobacterial activity.

Table 2-4. Anti-mycobacterial activities (inhibition zone: mm) of compounds 1–11 against \textit{Mycobacterium smegmatis} NBRC 3207.

<table>
<thead>
<tr>
<th>Compound</th>
<th>5 μg/disc</th>
<th>10 μg/disc</th>
<th>MIC (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9</td>
<td>12</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>—</td>
<td>—</td>
<td>n.d.</td>
</tr>
<tr>
<td>3</td>
<td>—</td>
<td>—</td>
<td>n.d.</td>
</tr>
<tr>
<td>4</td>
<td>—</td>
<td>—</td>
<td>n.d.</td>
</tr>
<tr>
<td>5</td>
<td>—</td>
<td>—</td>
<td>n.d.</td>
</tr>
<tr>
<td>6</td>
<td>—</td>
<td>—</td>
<td>n.d.</td>
</tr>
<tr>
<td>7</td>
<td>9</td>
<td>13</td>
<td>32</td>
</tr>
<tr>
<td>8</td>
<td>18</td>
<td>24</td>
<td>16</td>
</tr>
<tr>
<td>9</td>
<td>21</td>
<td>26</td>
<td>8</td>
</tr>
<tr>
<td>10</td>
<td>—</td>
<td>12</td>
<td>&gt;64</td>
</tr>
<tr>
<td>11</td>
<td>9</td>
<td>12</td>
<td>64</td>
</tr>
<tr>
<td>streptomycin sulfate</td>
<td>30</td>
<td>38</td>
<td>0.50</td>
</tr>
</tbody>
</table>

\textsuperscript{a}: An inhibition zone was not detected. \textsuperscript{b}: Not determined.

### 2.3 Experimental Section

#### 2.3.1 General Experimental Procedures

Optical rotations were measured with a JASCO P-2300 digital polarimeter (JASCO, Ltd., Japan). UV spectra were obtained on a Hitachi U-3310 UV-Visible spectrophotometer (Hitachi, Ltd., Japan) and IR spectra on a PerkinElmer Spectrum One Fourier transform infrared spectrometer (Waltham, MA, USA). NMR spectral data were obtained by a JEOL JNM-AL-400 NMR spectrometer (JEOL Ltd., Japan; 400 MHz for \textsuperscript{1}H and 100 MHz for \textsuperscript{13}C) in CDCl\textsubscript{3} (δ\textsubscript{H} 7.24, δ\textsubscript{C} 77.23), CD\textsubscript{3}OD (δ\textsubscript{H} 3.31, δ\textsubscript{C} 49.0) or DMSO-d\textsubscript{6} (δ\textsubscript{H} 2.50, δ\textsubscript{C} 39.51).
High-resolution FAB mass spectra were recorded on a JEOL JMS-MS 700 mass spectrometer (JEOL Ltd., Japan). Preparative HPLC was conducted using a Toyosoda CCPU instrument with a Tosoh UV-8010 detector.

2.3.2 Isolation, Identification and Fermentation of Producing Bacterium

*Streptomyces* sp. strain TPU1236A was isolated from a seawater sample collected at Iriomote Island, Okinawa Prefecture, Japan in September, 2012. Approximately 1 mL of the seawater was mixed with 25 mL of sterilized seawater with 1.0 % SDS, and 50 μL of the mixture was developed on an agar plate, containing glycerol 0.6%, arginine 0.1%, K₂HPO₄ 0.1%, MgSO₄ 0.05%, agar 1.5% and antibiotics (cycloheximide 100 μg/mL and rifampin 5 μg/mL) in natural seawater according to the method described by Fenical et al (Mincer, T. J., 2002). Identification of the strain TPU1236A was carried out by comparing partial sequence of the 16S rRNA gene. *S. badius* (732/732, 100%) and *S. sindenensis* (730/730, 100%) showed the highest similarity. Therefore, this producing strain TPU1236A was identified as *Streptomyces* sp. (Figure 2-7)

Figure 2-7. Picture of marine-derived actinomycete *Streptomyces* sp. TPU1236A grown on an agar plate.
The culture of strain TPU1236A was maintained on a Waksman agar slant (glucose 1%, peptone 0.5%, meat extract 0.5%, NaCl 0.3%, and agar 1.2% in deionized water) at 4 °C. The mycelia grown on the slant culture was inoculated in a 100-mL Erlenmeyer flask containing 40 mL of a seed medium (ASW-A medium: soluble starch 2%, glucose 1%, peptone 0.5%, yeast extract 0.5%, and CaCO₃ 0.3% in natural seawater, pH 7.0), and the flasks were shaken (150 rpm) at 25 °C for 3 days. The seed culture (2 mL) was transferred into 500-mL Erlenmeyer flasks containing each 200 mL of the same medium and incubated at 25 °C for 7 days at 150 rpm.

2.3.3 Bioassay

Anti-mycobacterial assay was carried out using *M. smegmatis* NBRC 3207 by the paper disc method and liquid microdilution method. The strain *M. smegmatis* NBRC 3207 was obtained from the Biological Resource Center (NBRC), NITE (Chiba, Japan) and maintained in 20% glycerol at −80 °C.

The test microorganism was cultured in Middlebrook 7H9 broth (BD, Franklin Lakes, NJ) containing 0.05% polysorbate 80 (BD), 0.5% glycerol, and 10% Middlebook OADC (BD) at 37 °C for 2 days and adjusted to 1.0 × 10⁶ CFU/mL. After incubation for 2 days at 37 °C, a petri dish was inoculated with the culture broth containing the bacterium. Then a sterile antibiotic filter disc (diameter, 6 mm, Advantec, Tokyo, Japan), treated with the sample solution in MeOH (2 μL), was placed in the dish. After evaporation of MeOH, the disc was placed in a plate and incubated for 2 days at 37 °C. The inhibition zone in diameter (mm) was measured. Streptomycin sulphate (5 and 10 μg) and MeOH were used as positive and negative controls, respectively.

MICs were measured by the liquid microdilution method (Koyama, N., 2010). After 85 μL of Middlebrook 7H9 broth containing 0.05% polysorbate 80, 0.5% glycerol, and 10% Middlebook OADC was added to each well of a 96-well microplate (Corning Inc., Corning, NY, USA), test compound dissolved in MeOH (5.0 μL) was added to each well at the final concentration of 0.125 to 64 μg/mL. Finally, the test microorganism (10 μL) was added at a concentration of 1.0 × 10⁶ CFU/mL. Microplates were incubated at 37 °C for 2 days. MIC was defined as the lowest concentration of test compound where the test microorganism did not grow.
2.3.4 Extraction and Isolation of Streptcytosines

The whole broth (10.0 L) was filtered and the mycelia were extracted with 1 L methanol and filtered. The broth filtrate was absorbed on a Diaion HP-20 column (500 mL), and the column was washed with water (2 L) and eluted with methanol (2 L). After evaporation of methanol in vacuum, the extracts from water phase and mycelia were combined to give about 1.5 g of solid material. The residue was fractioned by ODS column chromatography by stepwise elution with MeOH-H₂O to obtain 11 fractions. Streptcytosine A (1), eluted in the 70% MeOH fraction was purified by preparative reversed-phase HPLC (Senshu Scientific co., Ltd., PEGASIL ODS SP100 column, 250 mm × 10 mm, 5 μm; MeOH-H₂O, 6:4, 0.05% TFA; 2.0 mL/min; UV 210 nm). Streptcytosine A (1, 12.3 mg), plicacetin (7, 21.3 mg), SF2738C (11, 6.8 mg) eluted at 9.9, 15.0 and 12.2 min, respectively. Collismycin A (10, 11.2 mg) was obtained as a white crystal from 70% MeOH fraction. The 60% MeOH fraction was subjected to HPLC (ODS) and eluted with 45% aqueous MeOH, containing 0.05% TFA at a flow rate of 2 mL/min. Bamicetin (8, 20.5 mg), streptcytosines B (2, 3.2 mg), C (3, 7.2 mg) and D (4, 8.1 mg), de-amosaminyly-cytosamine (6, 12.0 mg) and streptcytosine E (5, 5.1 mg) eluted at 5.8, 16.8, 17.7, 19.3, 20.1 and 20.2 min, respectively. Amicetin (9, 25.0 mg), eluted in the 80% MeOH fraction, was isolated by similar HPLC separation procedures as the 60% MeOH fraction and eluted at 8.5 min.

**Streptcytosine A (1):** obtained as a colorless oil; [α]²³⁺D +72 (c 0.1, MeOH); UV λ_max (0.1 M HCl) nm (log ε): 269 (4.24), 276 (4.24), 317 (4.28); IR ν_max (KBr) cm⁻¹: 3500–3400, 2920, 1712, 1677, 1639, 1613, 1385, 1262, 1093, 802; HRFABMS (m/z) found: 631.3110, calcd: 631.3092 [M + H]⁺ for C₃₀H₄₃N₆O₉; ¹H and ¹³C NMR data in CD₃OD, see Table 2-2. ¹H NMR data in DMSO-d₆: δ 1.28 (3H, d, 5.9 Hz), 1.38 (3H, d, 6.0 Hz), 1.40 (3H, s), 1.47 (1H, d, 4.8 Hz), 1.54 (1H, d, 13.4 Hz), 1.75 (1H, dd, 21.0 Hz, 9.7 Hz), 1.99 (1H, d, 8.4 Hz), 2.31 (1H, m), 2.88 (6H, s), 3.11 (1H, t, 13.8 Hz), 3.30 (1H, td, 9.6 Hz, 3.6 Hz), 3.41 (1H, dd, 9.3 Hz, 3.2 Hz), 3.66 (2H, m), 3.83 (2H, m), 3.93 (1H, m), 4.93 (1H, d, 3.2 Hz), 5.23 (2H, q, 7.3 Hz), 5.74 (2H, 10.4 Hz), 7.35 (1H, d, 7.4 Hz), 7.82 (2H, d, 8.4 Hz), 8.12 (2H, d, 4.5 Hz), 8.17 (1H, d, 7.4 Hz), 8.94 (1H, brs).

**Streptcytosine B (2):** obtained as a pale yellow oil; [α]²³⁺D +24 (c 0.1, MeOH); UV λ_max (0.1 M HCl) nm (log ε): 249 (3.94), 338 (4.09); IR ν_max (KBr) cm⁻¹: 3500–3400, 2927, 1683, 1646,
1616, 1576, 1493, 1385, 1247, 1092; HRFABMS (m/z) found: 326.1180, calcd: 326.1175 [M + H]+ for C_{14}H_{20}N_{2}O_{4}S; 1H and 13C NMR data, see Table 2-3.

**Streptcytosine C (3):** obtained as a pale yellow oil; [α]_{23}^{20}D +36 (c 0.1, MeOH); UV λ_{max} (0.1 M HCl) nm (log ε): 257 (3.95), 311 (4.18); IR ν_{max} (KBr) cm⁻¹: 3500–3400, 2928, 1683, 1646, 1613, 1561, 1489, 1385, 1262, 1092; HRFABMS (m/z) found: 308.1619, calcd: 308.1610 [M + H]+ for C_{15}H_{22}N_{3}O_{4}; 1H and 13C NMR data, see Table 2-3.

**Streptcytosine (4):** obtained as a pale yellow oil; [α]_{23}^{20}D +68 (c 0.1, MeOH); UV λ_{max} (0.1 M HCl) nm (log ε): 217 (3.96), 263 (4.16), 309 (4.32); IR ν_{max} (KBr) cm⁻¹: 3500–3400, 2937, 1735, 1674, 1634, 1561, 1492, 1396, 1272, 1095; HRFABMS (m/z) found: 308.1601, calcd: 308.1610 [M + H]+ for C_{15}H_{22}N_{3}O_{4}; 1H and 13C NMR data, see Table 2-3.

**Streptcytosine (5):** obtained as a pale yellow oil; [α]_{23}^{20}D +62 (c 0.1, MeOH); UV λ_{max} (0.1 M HCl) nm (log ε): 237 (3.61), 310 (4.04); IR ν_{max} (KBr) cm⁻¹: 3500–3400, 2963, 1720, 1677, 1624, 1571, 1494, 1383, 1276, 1095; HRFABMS (m/z) found: 310.1763, calcd: 310.1767 [M + H]+ for C_{15}H_{24}N_{4}O_{4}; 1H and 13C NMR data, see Table 2-3.

**De-amosaminyl-cytosamine (6):** obtained as a colorless oil; [α]_{23}^{20}D +34 (c 0.1, MeOH); UV (0.1 M HCl) λ_{max} nm (log ε) 277 (4.11); IR ν_{max} (KBr) cm⁻¹ 3500–3400, 1723, 1683, 1204, 1095; 1H NMR (CD_{3}OD, 400 MHz) δ 8.01 d (7.8 Hz), 6.11 d (7.8 Hz), 5.65 dd (10.5 Hz, 2.1 Hz), 3.49 m, 3.24 m, 2.15 m, 2.03 m, 1.77 m, 1.64 m, 1.31 d (6.1 Hz). 13C NMR (CD_{3}OD, 100 MHz) δ 161.9, 148.9, 146.4, 95.3, 84.1, 80.7, 71.6, 32.3, 31.0, 18.5; FABMS m/z 226 [M + H]+.

**Plicacetin (7):** obtained as a yellow oil; [α]_{23}^{20}D +96 (c 0.1, MeOH); UV λ_{max} (0.1 M HCl) nm (log ε) 255 (4.08), 315 (4.17); IR ν_{max} (KBr) cm⁻¹ 3500–3400, 1679, 1643, 1605, 1567, 1487, 1314, 1257, 1202, 1053; 1H NMR (CD_{3}OD, 400 MHz) δ 8.2 (d, 7.2 Hz), 7.81 (d, 8.7 Hz), 7.43 (d, 6.4 Hz), 6.75 (d, 8.7 Hz), 5.76 (d, 8.4 Hz), 5.00 (d, 3.7 Hz), 4.08 m, 3.98 (br t 10.4 Hz), 3.75 m, 3.57 (dd, 9.1 Hz, 3.8 Hz), 3.43 (td, 9.4 Hz, 4.6 Hz), 3.12 (t, 10.3 Hz), 3.01 s, 2.38 m, 2.15 (br d, 10.1 Hz), 1.68 (br d, 9.2 Hz), 1.67 m, 1.46 (d, 6.1 Hz), 1.37 (d, 6.1 Hz). FABMS m/z 518, [M + H]+.

**Bamicetin (8):** obtained as a pale yellow oil; [α]_{23}^{20}D +66 (c 0.1, MeOH); UV λ_{max} (0.1 M HCl) nm (log ε) 251 (4.21), 257 (4.21), 321 (4.42); IR ν_{max} (KBr) cm⁻¹ 3500–3400, 2934, 1683, 1644, 1605, 1487, 1404, 1254, 1204, 1136; 1H NMR (CD_{3}OD, 400 MHz) δ 8.21 (d, 7.5 Hz), 8.00 (d,
8.0 Hz), 7.83 (d, 8.0 Hz), 7.56 (d, 7.5 Hz), 5.78 (d, 8.6 Hz), 5.03 (d, 3.5 Hz), 4.12 (d, 12.0 Hz), 4.07, m, 3.88 (br t, 10.0 Hz), 3.84 (d, 10.0 Hz), 3.76 m, 3.53 (dd, 9.1 Hz, 3.8 Hz), 3.44 (td, 9.6 Hz, 3.9 Hz), 2.88 (t, 9.9 Hz), 2.79 s, 2.41 m, 2.17 (br d 10.2 Hz), 1.70 (br d, 10.2 Hz), 1.68 m, 1.65 s, 1.38 (d, 6.0 Hz); $^{13}$C NMR (CD$_3$OD, 100 MHz) δ 170.2, 168.4, 164.7, 156.6, 146.7, 143.7, 130.3, 131.0, 121.3, 98.7, 96.7, 84.6, 78.2, 76.2, 73.6, 68.7, 65.7, 65.3, 64.7, 63.4, 42.3, 30.8, 27.8, 19.0, 18.7, 18.0; FABMS m/z 605, [M + H]$^+$.  

**Amicetin (9):** obtained as a pale yellow oil; [α]$_D^{23}$ +76 (c 0.1, MeOH); UV $\lambda_{\text{max}}$ (0.1 M HCl) nm (log ε) 257 (4.05), 323 (4.31); IR $\nu_{\text{max}}$ (KBr) cm$^{-1}$ 3500–3400, 2934, 1679, 1602, 1484, 1405, 1306, 1254, 1202, 1136, 1056; $^1$H NMR (CD$_3$OD, 400 MHz) δ 8.19 (d, 7.4 Hz), 7.99 (d, 8.8 Hz), 7.82 (d, 8.8 Hz), 7.59 (d, 7.4 Hz), 5.78 (d, 7.9 Hz), 5.02 (d, 3.8 Hz), 4.12 (d, 12.0 Hz), 4.09 m, 3.98 (br. t, 9.8 Hz), 3.85 (d, 12.0 Hz), 3.76 m, 3.57 (dd, 9.4 Hz, 4.3 Hz), 3.44 (td, 9.6 Hz, 4.0 Hz), 3.12 (t, 10.2 Hz), 3.02 s, 2.40 m, 2.18 (br d 9.1 Hz), 1.71 (br d, 9.1 Hz), 1.69 m, 1.66 s, 1.47 (d, 6.1 Hz), 1.38 (d, 6.0 Hz); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 170.5, 168.6, 164.9, 157.3, 146.8, 144.0, 130.5, 130.4, 121.5, 99.0, 96.8, 84.8, 78.4, 76.6, 74.0, 72.1, 68.1, 65.6, 64.2, 63.7, 42.6, 31.1, 28.1, 19.3, 19.1, 19.0; FABMS m/z 619, [M + H]$^+$.  

**Collismycin A (10):** obtained as a needle crystal; $^1$H NMR (DMSO-d$_6$, 400 MHz) δ 11.75 s, 8.73 (br d, 1.3 Hz), 8.70 (d, 4.3 Hz), 8.38 (d, 7.9 Hz), 8.00 br s, 7.96 (t, 7.9 Hz), 7.48 (dd, 7.9 Hz, 6.8 Hz), 4.05 s, 2.34 (br d, 1.3 Hz). $^{13}$C NMR (DMSO-d$_6$, 100 MHz) δ 167.1, 156.2, 154.7, 152.9, 149.5, 147.1, 137.6, 124.9, 121.3, 121.0, 103.1, 56.4, 17.8. FABMS $m/z$ 276, [M + H]$^+$. C$_{13}$H$_{14}$N$_3$O$_2$. $^1$H NMR data in CDCl$_3$: δ 2.36 (3H, s), 4.10 (3H, s), 7.32 (1H, dd, 7.7 Hz, 4.4 Hz), 7.84 (1H, td, 7.8 Hz, 1.5 Hz), 8.04 (1H, s), 8.53 (1H, d, 8.0 Hz), 8.65 (1H, d, 4.0 Hz), 9.08 (1H, s), 10.06 (1H, brs).  

**SF2738C (11):** obtained as a pale yellow oil; $^1$H NMR (CD$_3$OD, 400 MHz) δ 8.87 d (4.7), 8.62 d (7.8), 8.21 td (7.8, 1.4), 8.20 s, 7.74 dd (7.2, 5.2), 5.10 s, 4.36 s, 2.47. FABMS $m/z$ 263, [M + H]$^+$. C$_{13}$H$_{15}$N$_2$O$_2$S. $^1$H NMR data in CDCl$_3$: 2.33 (3H, s), 4.12 (3H, s), 4.97 (2H, s), 7.50 (1H, dd, 7.1 Hz, 4.8 Hz), 7.88 (1H, s), 7.99 (1H, m), 8.40 (1H, d, 7.7 Hz), 8.84 (1H, d, 4.4 Hz).
2.4 Supporting Spectral Data for Compounds 1–11

Figure 2-S1. UV spectrum of streptcytosine A (1).

Figure 2-S2. IR spectrum of streptcytosine A (1) (KBr).
Figure 2-S3. Positive FABMS spectrum of streptcytosine A (1).

Figure 2-S4. Negative FABMS spectrum of streptcytosine A (1).
Figure 2-S5. $^1$H NMR spectrum of streptcytosine A (1) in CD$_3$OD.

Figure 2-S6. $^1$H NMR spectrum of streptcytosine A (1) in DMSO-$d_6$. 

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Figure 2-S7. $^{13}$C NMR and DEPT spectra of streptcytosine A (1) in CD$_3$OD.

Figure 2-S8. $^1$H-$^1$H COSY spectrum of streptcytosine A (1) in CD$_3$OD.
Figure 2-S9. HMQC spectrum of streptcytosine A (1) in CD$_3$OD.

Figure 2-S10. HMBC spectrum of streptcytosine A (1) in CD$_3$OD.
Figure 2-S11. UV spectrum of streptcytosine B (2).

Figure 2-S12. IR spectrum of streptcytosine B (2) (KBr).
Figure 2-S13. Positive FABMS spectrum of streptcytosine B (2).

Figure 2-S14. Negative FABMS spectrum of streptcytosine B (2).
Figure 2-S15. $^1$H NMR spectrum of streptcytosine B (2) in CD$_3$OD.

Figure 2-S16. $^{13}$C NMR and DEPT spectra of streptcytosine B (2) in CD$_3$OD.
Figure 2-S17. $^1$H-$^1$H COSY spectrum of streptycytosine B (2) in CD$_3$OD.

Figure 2-S18. HMQC spectrum of streptycytosine B (2) in CD$_3$OD.
Figure 2-S19. HMBC spectrum of streptcytosine B (2) in CD$_3$OD.

Figure 2-S20. UV spectrum of streptcytosine C (3).
Figure 2-S21. IR spectrum of streptcytosine C (3) (KBr).

Figure 2-S22. Positive FABMS spectrum of streptcytosine C (3).
Figure 2-S-23. Negative FABMS spectrum of streptcytosine C (3).

Figure 2-S24. $^1$H NMR spectrum of streptcytosine C (3) in CD$_3$OD.
Figure 2-S25. $^{13}$C NMR and DEPT spectra of streptcytosine C (3) in CD$_3$OD.

Figure 2-S26. $^1$H-$^1$H COSY spectrum of streptcytosine C (3) in CD$_3$OD.
Figure 2-S27. HMQC spectrum of streptcytosine C (3) in CD$_3$OD.

Figure 2-S28. HMBC spectrum of streptcytosine C (3) in CD$_3$OD.
Figure 2-S29. UV spectrum of streptycytosine D (4).

Figure 2-S30. IR spectrum of streptycytosine D (4) (KBr).
Figure 2-S31. Positive FABMS spectrum of streptocytosine D (4).

Figure 2-S32. Negative FABMS spectrum of streptocytosine D (4).
Figure 2-S33. $^1$H NMR spectrum of streptcytosine D (4) in CD$_3$OD.

Figure 2-S34. $^{13}$C NMR and DEPT spectra of streptcytosine D (4) in CD$_3$OD.
Figure 2-S35. $^1$H-$^1$H COSY spectrum of streptcytosine D (4) in CD$_3$OD.

Figure 2-S36. HMQC spectrum of streptcytosine D (4) in CD$_3$OD.
Figure 2-S37. HMBC spectrum of streptcytosine D (4) in CD$_3$OD.

Figure 2-S38. UV spectrum of streptcytosine E (5).
Figure 2-S39. IR spectrum of streptycytosine E (5) (KBr).

Figure 2-S40. Positive FABMS spectrum of streptycytosine E (5).
Figure 2-S41. Negative FABMS spectrum of streptycytosine E (5).

Figure 2-S42. $^1$H NMR spectrum of streptycytosine E (5) in CD$_3$OD.
Figure 2-S43. $^{13}$C NMR and DEPT spectra of streptcytosine E (5) in CD$_3$OD.

Figure 2-S44. $^1$H-$^1$H COSY spectrum of streptcytosine E (5) in CD$_3$OD.
Figure 2-S45. HMQC spectrum of streptcytosine E (5) in CD$_3$OD.

Figure 2-S46. HMBC spectrum of streptcytosine E (5) in CD$_3$OD.
Figure 2-S47. UV spectrum of de-amosaminyl-cytosamine (6).

Figure 2-S48. IR spectrum of de-amosaminyl-cytosamine (6) (KBr).
Figure 2-S49. Positive FABMS spectrum of de-amosaminyl-cytosamine (6).

Figure 2-S50. $^1$H NMR spectrum of de-amosaminyl-cytosamine (6) in CD$_3$OD.
Figure 2-S51. $^{13}$C NMR and DEPT spectra of de-amosaminyl-cytosamine (6) in CD$_3$OD.

Figure 2-S52. $^1$H-$^1$H COSY spectrum of de-amosaminyl-cytosamine (6) in CD$_3$OD.
Figure 2-S53. HMQC spectrum of de-amosaminyl-cytosamine (6) in CD$_3$OD.

Figure 2-S54. HMBC spectrum of de-amosaminyl-cytosamine (6) in CD$_3$OD.
Figure 2-S55. UV spectrum of plicacetin (7).

Figure 2-S56. IR spectrum of plicacetin (7) (KBr).
Figure 2-S57. Negative FABMS spectrum of plicacetin (7).

Figure 2-S58. $^1$H NMR spectrum of plicacetin (7) in CD$_3$OD.
Figure 2-S59. $^1$H-$^1$H COSY spectrum of plicacetin (7) in CD$_3$OD.

Figure 2-S60. HMQC spectrum of plicacetin (7) in CD$_3$OD.
Figure 2-S61. HMBC spectrum of plicacetin (7) in CD$_3$OD.

Figure 2-S62. UV spectrum of bamicetin (8).
Figure 2-S63. IR spectrum of bamicetin (8) (KBr).

Figure 2-S64. Positive FABMS spectrum of bamicetin (8).
Figure 2-S65. \( ^1\)H NMR spectrum of bamicetin (8) in CD\(_3\)OD.

Figure 2-S66. \( ^{13}\)C NMR and DEPT spectra of bamicetin (8) in CD\(_3\)OD.
Figure 2-S67. $^1$H-$^1$H COSY spectrum of bamicetin (8) in CD$_3$OD.

Figure 2-S68. HMQC spectrum of bamicetin (8) in CD$_3$OD.
Figure 2-S69. HMBC spectrum of bamicetin (8) in CD$_3$OD.

Figure 2-S70. UV spectrum of amicetin (9).
Figure 2-S71. IR spectrum of amicetin (9) (KBr).

Figure 2-S72. Positive FABMS spectrum of amicetin (9).
Figure 2-S73. Negative FABMS spectrum of amicetin (9).

Figure 2-S74. $^1$H NMR spectrum of amicetin (9) in CD$_3$OD.
Figure 2-S75. $^{13}$C NMR and DEPT spectra of amicetin (9) in CD$_3$OD.

Figure 2-S76. $^1$H-$^1$H COSY spectrum of amicetin (9) in CD$_3$OD.
Figure 2-S77. HMQC spectrum of amicetin (9) in CD$_3$OD.

Figure 2-S78. HMBC spectrum of amicetin (9) in CD$_3$OD.
Figure 2-S79. EIMS spectrum of collismycin A (10).

Figure 2-S80. $^1$H NMR spectrum of collismycin A (10) in DMSO-$d_6$. 
Figure 2-S81. $^1$H NMR spectrum of collismycin A (10) in CDCl$_3$.

Figure 2-S82. $^{13}$C NMR and DEPT spectra of collismycin A (10) in DMSO-$d_6$. 
Figure 2-S83. $^1$H-$^1$H COSY spectrum of collismycin A (10) in DMSO-$d_6$.

Figure 2-S84. HMQC spectrum of collismycin A (10) in DMSO-$d_6$. 
Figure 2-S85. HMBC spectrum of collisycin A (10) in DMSO-$d_6$. 

Figure 2-S86. Positive FABMS spectrum of SF2738C (11).
Figure 2-S87. $^1$H NMR spectrum of SF2738C (11) in CD$_3$OD.

Figure 2-S88. $^1$H NMR spectrum of SF2738C (11) in CDCl$_3$. 
3. Isolation and Characterization of New Pyrone-Type Polyketides from a Marine-Derived *Penicillium* sp. TPU1271

3.1 Introduction

Marine sources provided numbers of biologically active compounds, including anti-tumor, anti-cancer, anti-microtubule, anti-proliferative, cytotoxic, photo protective, as well as antibiotic and antifouling properties (Bhatnaga, I., 2010). Marine microorganisms, especially marine-derived fungi, have become an important source of pharmacologically active metabolites (Bugni, T. S., 2004). Compounds from marine-derived fungi have been recognized as one of the richest sources of structurally novel and biologically active metabolites (Jensen, P. R.; Fenical, W., 2000).

In the course of our continuous search for new compounds produced by marine-derived fungi, a marine-derived *Penicillium* sp. (strain TPU1271) was found to produce various types of secondary metabolites, including pyrone-type and acetylenic-type polyketides, diketopiperazines, quinoline alkaloids, benzodiazepines, cyclic dipeptides and sesquiterpenoids. Further isolation from the culture broth of *Penicillium* sp. TPU1271 obtained two new pyrone-type polyketides, compound (12a) and 9-epi-compound (13a) (Figure 3-1), along with nine known compounds, verrucosidin (14) (Wilson, B. J., 1981) (Burka, L. T. 1983) (Hodge, R. P., 1988), fructigenine A (15) (Arai, K., 1989) (Kozlovsky, A. G., 2001), verrucofortine (16) (Hodge, R. P., 1988) (Arai, K., 1989) , cyclopenol (17) (Birkinshaw, J. H.,1963), cyclopenin (18) (Bracken, A., 1954), penipratynolene (19) (Nakahara, S., 2004) (Jian, Y. J., 2010), cyclo-(L-Trp-L-Phe) (20) (Kimura, Y., 1996) (Chu, D., 2011), aspterric acid (21) (Shimada, A., 2002) and viridicatol (22) (Birkinshaw, J. H.,1963) (Yurchenko, A. N., 2010). In this study, the isolation, structural determination and antimicrobial activity of compound (12a), 9-epi-compound (13a) and their acetate derivatives (12b) and (13b) are described.
**Figure 3-1.** Structures of new compounds 12a, 13a and their acetates 12b, 13b

**Figure 3-2.** Structures of known compounds 14–22.
3.2 Results and Discussion

The *Penicillium* sp. strain TPU1271 was isolated from the organic matter attached with cultivating oysters collected at the Oshika Peninsula, Miyagi Prefecture, Japan (Figure 3-3). The crude extracts of the whole broth were absorbed on ODS silica gel and subjected to the column chromatography. The elutes were further separated by preparative HPLC to yield two new polyketides, compound (12a) and 9-epi-compound (13a), and nine known compounds. The structures of known compounds 14−22 were determined on the basis of their spectroscopic data and comparison with the reported values for verrucosidin (14) (Wilson, B. J., 1981) (Burka, L. T. 1983) (Hodge, R. P., 1988), fructigenine A (15) (Arai, K., 1989) (Kozlovsky, A. G., 2001), verrucofortine (16) (Hodge, R. P., 1988) (Arai, K., 1989), cycloopenol (17) (Birkinshaw, J. H., 1963), cycloopenin (18) (Bracken, A., 1954), penipratynolene (19) (Nakahara, S., 2004) (Jian, Y. J., 2010), cyclo-(L-Trp-L-Phe) (20) (Kimura, Y., 1996) (Chu, D., 2011), aspterric acid (21) (Shimada, A., 2002) and viridicatol (22) (Birkinshaw, J. H., 1963) (Yurchenko, A. N., 2010).

![Figure 3-3. Sampling side of marine-derived fungi (2012)](image)
3.2.1 Isolation of Compounds

The producing fungus was cultured on a liquid medium for 21 days. The culture was added a half volume of cetone and filtered. After the evaporation of acetone, the filtrate was separated with an ODS column followed by repeated HPLC (ODS) to obtain compounds 12a, 13a and 14 – 22 (Figure 3-4).

Figure 3-4. Isolation procedure for the whole broth extract of strain TPU1271.
3.2.2 Structure Determination of New Compounds

Compound 12a was obtained as colorless oils. The molecular weight and formula, 434 and \(C_{24}H_{34}O_7\), were deduced from HRFABMS and NMR data. The \(^1\text{H}\) NMR spectrum of 12a showed nine \(sp^3\) methyl, two olefin \(sp^2\) methane and three oxygenated \(sp^3\) methine signals and suggested the presence of two activated hydrogens. The \(^1\text{H}\) and \(^{13}\text{C}\) NMR signals were assigned by the analysis of \(^1\text{H}-^1\text{H}\) COSY, HMQC and HMBC data (Table 3-1). \(^1\text{H}-^1\text{H}\) COSY data revealed the partial structures I and II and their connectivity in compound 12a was established by the analysis of HMBC correlations.(Figure 3-5)

Compound 12a was a geometric isomer of verrucosidinol (Yu, K., 2010) at C-7 to 9 and they had similar \(^1\text{H}\) NMR and \(^{13}\text{C}\) NMR data (Table 3-1). The chemical shifts and coupling constants of the \(\alpha\)-pyrone and furan units were very similar to those of verrucosidinol (Yu, K., 2010). The differences between the structures of compound 12a and verrucosidinol were the types of heptadiene units (Figure 3-5). The heptadiene unit of compound 12a was revealed to connect with the furan unit by the analysis of HMBC correlations from H-11 to C-12 and C-13 and from H-23 to C-11 (Figure 3-5). The connectivity of the heptadiene unit to \(\alpha\)-pyrone unit was confirmed by HMBC correlations from H-20 to C-5 and from H-7 to C-5. Therefore, the gross structure of compound 12a was established (Figure 3-5).

![Verrucosidinol](image)

Verrucosidinol (Yu, K., 2010)

Compound 13a had the same molecular formula and weight as 12a, which was deduced from the analysis of HRFABMS and NMR data. The \(^1\text{H}\) and \(^{13}\text{C}\) NMR signals were assigned by the analysis of \(^1\text{H}-^1\text{H}\) COSY, HMQC and HMBC data (Table 3-1). The \(^1\text{H}\) and \(^{13}\text{C}\) NMR signals were very close to those of compound 12a (Table 3-1). Compound 13a had the same \(^1\text{H}-^1\text{H}\) COSY, HMQC and HMBC data as 12a and the skeletal structure of compound 13a was established as the same as 12a (Figure 3-5).
Table 3-1. $^{13}$C (100 MHz) and $^1$H (400 MHz) NMR data (CD$_3$OD) for compounds 12a, 13a.

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Figure 3-5. $^1$H-$^1$H COSY and key HMBC correlations of compounds 12a.

The relative configurations of compounds 12a and 13a were determined by the analysis of NOESY correlations of their acetate derivatives 12b and 13b (Figure 3-6). From the NOESY correlations of 12b and 13b, a 9S*-configuration in compound 12a and a 9R*-configuration in compound 13a were determined, respectively (Figure 3-6).

Figure 3-6. NOESY correlations of compounds 12b and 13b.
3.2.3 Structure Determination of Known Compounds

Compound 14 was obtained as a pale yellow oil. The molecular weight and formula, 416 and C_{24}H_{32}O_{6}, were deduced from EIMS and NMR data. The \(^1\)H NMR spectrum of 14 showed nine \(sp^3\) methyl, two olefin \(sp^2\) methane and three oxygenated \(sp^3\) methine signals, which could be confirmed that compound 14 had no activated hydrogens. The planer structure of compound 14 was determined by HMBC, HMQC and \(^1\)H-\(^1\)H COSY correlations. The absolute configuration of compound 14 was considered as the same as the known compound verrucosidin by comparing the chemical shifts with those reported values. Therefore, the structure of compound 14 was determined.

Compound 15 was obtained as a yellow oil. The molecular weight and molecular formula, 443 and C_{27}H_{29}N_{3}O_{3}, were decided by the analysis of \(^1\)H and \(^{13}\)C NMR spectra and EIMS data. The \(^1\)H NMR in CDCl\(_3\) showed 29 signals. From \(^1\)H NMR spectrum, germinal methyl (\(\delta\) 0.88 and 1.03 ppm), terminal olefin (\(\delta\) 5.02, d, \(J = 17.5\) Hz; \(\delta\) 5.04, d, \(J = 10.8\) Hz and \(\delta\) 5.67, dd, \(J = 17.3\) Hz, 11.0 Hz) could be confirmed. The structure of compound 15 was determined by HMBC, HMQC and \(^1\)H-\(^1\)H COSY correlations. Further comparison of \(^1\)H and \(^{13}\)C NMR data for 15 with the reported values for fructigenin A confirmed the structure of 15.

Compound 16 was obtained as a yellow oil. The molecular weight and molecular formula, 409 and C_{24}H_{31}N_{3}O_{3}, were decided by the analysis of \(^1\)H and \(^{13}\)C NMR spectra and EIMS data. The \(^1\)H NMR spectrum of compound 16 was similar to that of fructigenine A. The difference between 15 and 16 was detected at the side chain, that is, compound 16 had an isopropyl group instead of the phenyl group in 15. The \(^1\)H NMR spectrum of compound 16 was very close to that of verrucofortrine as reported. Thus, compound 16 was identified as a known compound.

Compound 17 was obtained as a pale yellow powder. The molecular weight and molecular formula, 310 and C_{17}H_{14}N_{2}O_{4}, were decided by the analysis of \(^1\)H and \(^{13}\)C NMR spectra and EIMS data. Compound 18 was obtained as a pale yellow oil. Compound 18 had the molecular weight and molecular formula of 294 and C_{17}H_{14}N_{2}O_{3}. From \(^1\)H NMR spectrum of compound 18, a benzene ring was confirmed in the field of \(\delta\) 7.0-7.6 Hz. The molecular weight of compound 17 was 16 (O) more than that of compound 18. A hydroxyl group was considered to be attached to the benzene ring, and compound 17 and 18 were confirmed as cyclopenol, cyclopenin, respectively.
Compound 19 was obtained as a colorless oil. The molecular weight and molecular formula of 220 and C₁₂H₁₂O₄ were decided from 1D NMR and EIMS data. The signals due to a methoxy proton signal (δ 3.81) and one 1,4-di-substituted phenyl group were observed in the ¹H NMR spectrum. The structure of 19 was established by HMBC correlations and was confirmed as penipratynolene.

Compound 20 was obtained as a white powder. The molecular weight and molecular formula of 333 and C₂₀H₁₉N₃O₂ were decided from 1D NMR and EIMS data. The ¹H NMR spectrum of compound 20 showed that 20 was a diketopiperazine. The structure of compound 20 was decided by HMBC correlations and confirmed as cyclo-(L-Trp-L-Phe).

Compound 21 was obtained as a brown powder. EIMS showed a peak at m/z 266, and the molecular formula of C₁₅H₂₂O₄ was decided by the analysis of 1D NMR spectrum. The structure of compound 21 was decided by ¹H-¹H COSY, HMQC and HMBC correlations and was confirmed as aspterric acid (21).

Compound 22 was obtained as a pale orange oil. The molecular weight of 253 was determined by EIMS. The molecular formula (C₁₅H₁₁O₃N) of compound 22 was decided from ¹H and ¹³C NMR data. All the signals of ¹H and ¹³C NMR were in the lower-field region. The structure of compound 22 was decided by ¹H-¹H COSY, HMQC and HMBC correlations. Compound 22 was identified as viridicatol.

### 3.2.4 Biological Activity

The antibacterial activity against *M. smegmatis* NBRC 3207 of compounds 12a, 12b, 13a, 13b and 14–22 were evaluated by the paper disc method (Ericsson, H., 1960). Only verrucosidin (14) showed modest activity (11 mm at 40 μg/disc). Verrucosidin (14), acting as a potent neurotoxin to the mammal animals, was first isolated from the fungus *Penicillium verrucosum* var. *cyclopium* (Wilson, B. J., 1981), and reported to inhibit dose-dependently the expression of GRP78 promoter with the IC₅₀ value of 25 nM (Choo, S. J., 2005). In this study, verrucosidin (14) was first reported to show anti-mycobacterial activity against *M. smegmatis*.

Although new compounds 12a, 13a and their acetates 12b, 13b showed no activity against *M. smegmatis*, these compounds have close structural relationship to the polyene α-pyrene mycotoxins, such as verrucosidin (14) and citreoviridin (Sakabe, N., 1964), most of which showed potent inhibitory activity against mitochondrial ATPase and oxidative phosphorylation
3.3 Experimental Section

3.3.1 General Experimental Procedures

NMR spectral data were obtained by a JEOL JNM-AL-400 NMR spectrometer (JEOL Ltd., Japan; 400 MHz for $^1$H and 100 MHz for $^{13}$C) in CD$_3$OD ($\delta_H$ 3.31, $\delta_C$ 49.15), CDCl$_3$ ($\delta_H$ 7.24, $\delta_C$ 77.23), acetone-$d_6$ ($\delta_H$ 2.05, $\delta_C$ 29.92, 206.68) and DMSO-$d_6$ ($\delta_H$ 2.50, $\delta_C$ 39.51). Mass spectra were recorded with a JEOL JMS-MS 700 mass spectrometer (EI or FAB mode with $m$-nitrobenzyl alcohol or glycerol as the matrix). UV spectra were obtained on a Hitachi U-3310 UV-Visible spectrophotometer (Hitachi, Ltd., Japan) and IR spectra on a PerkinElmer Spectrum One Fourier transform infrared spectrometer (Waltham, MA, USA). Optical rotations were measured with a JASCO P-2300 digital polarimeter (JASCO, Ltd., Japan). Preparative HPLC was conducted using a Toyosoda CCPU instrument equipped with a Tosoh UV-8010 detector.

3.3.2 Isolation, Identification, and Fermentation of Producing Bacterium

The fungus strain, TPU1271, was isolated from the organic matter attached with cultivating oysters collected at a depth of 10 meters under the seawater at the Oshika Peninsula, Miyagi Prefecture, Japan in June, 2012. The organic matter was diluted 25 times with sterile seawater and 200 μL of the resulted liquid was plated to an agar medium (PDA, 90% seawater). The isolated fungus was identified as *Penicillium* sp. relied on morphological and gross physiological characters.

The single colony of strain TPU1271 grown on the PDA was inoculated into a 100-mL Erlenmeyer flask containing 40 mL of the seed medium (2% glucose, 0.5% polypeptone, 0.05% MgSO$_4$·7H$_2$O, 0.2% yeast extract, 0.1% KH$_2$PO$_4$, 0.1% agar and seawater, pH 6.0). The flask was shaken for 3 days at 25 °C, and 2 mL of the culture broth was transferred into 500-mL Erlenmeyer flasks containing each 200 mL of the production medium (3% sucrose, 3% soluble starch, 1% malt extract, 0.3% Ebios (Asahi Food & Healthcare Co. Ltd., Tokyo, Japan), 0.5% KH$_2$PO$_4$ and 0.05% MgSO$_4$·7H$_2$O and natural seawater, pH 6.0). Fifteen 500-mL Erlenmeyer-flasks were cultured and maintained static at room temperature for 3 weeks.
3.3.3 Bioassay

Compounds 12a, 12b, 13a, 13b and 14–22 were tested for antimicrobial activity against Mycobacterium smegmatis NBRC 3207 (48 hours at 37°C). A sterile antibiotic filter disc (6 mm in diameter), treated with the test sample was placed in the dish. After incubation, the inhibition zone in diameter (mm) was measured.

3.3.4 Extraction and Isolation of New Polyketides

The whole broth (3 L) was added an equal amount of acetone and filtered. The mixture was evaporated under reduced pressure to remove acetone, and the aqueous solution was subjected to ODS column chromatography. The column was eluted with a stepped gradient of H₂O-MeOH mixtures (100:0 to 0:100, v/v) to obtain 10 fractions. Compound (12a, 5.5 mg), compound (13a, 4.1 mg), cyclopenin (18, 11.2 mg), penipratynolene (19, 2.4 mg), cyclo-(L-Trp-L-Phe) (20, 2.1 mg) and viridicatol (22, 5.5 mg) were obtained by separation of the 50% MeOH fraction with preparative reversed-phase HPLC (Senshu Scientific Co., Ltd., Pegasil ODS SP100 column, 250 × 10 mm, 5 μm; MeOH-H₂O=50:50; 2.0 mL/min; UV 210 nm). Fructigenine A (15, 19.0 mg), verrucofortine (16, 2.6 mg) and aspterric acid (21, 80.5 mg)
were isolated from 70% MeOH fraction with the same HPLC systems (MeOH-H$_2$O=70:30; 2.0 mL/min; UV 210 nm). Verrucosidin (14, 17.5 mg) were purified with HPLC separation by eluting with the solution of MeOH-H$_2$O=80:20. The 30% MeOH fraction was chromatographed on a Sephadex LH-20 column with MeOH-H$_2$O=30:70 to yield cyclopenol (17, 90.0 mg).

**Compound (12a):** [α]$^20_D$ +110 (c 0.1, MeOH); UV $\lambda_{max}$ (MeOH) nm (log ε): 306 (3.40), 394 (1.25); IR $\nu_{max}$ (KBr) cm$^{-1}$: 2978, 2929, 2857, 1688, 1558, 1451, 1377, 1095, 1043, 1012; HRFABMS ($m/z$) found: 435.2389, calcd: 435.2383 [M + H]$^+$ for C$_{24}$H$_{35}$O$_7$; $^1$H and $^{13}$C NMR data in CD$_3$OD, see Table 3-1;

**12a-acetate (12b):** [α]$^21_D$ +122 (c 0.1, MeOH); UV $\lambda_{max}$ (MeOH) nm (log ε): 300 (3.97), 368 (0.82), 387 (1.25), 394 (1.69); IR $\nu_{max}$ (KBr) cm$^{-1}$: 2973, 2929, 1739, 1714, 1690, 1561, 1454, 1372, 1355, 1237, 1089, 1043, 1029; HRFABMS ($m/z$) found: 477.2492, calcd: 477.2488 [M + H]$^+$ for C$_{26}$H$_{37}$O$_8$; $^1$H NMR data in CD$_3$OD: δ 5.84 (1H, t, 0.9 Hz, H-$\gamma$-7), 5.32 (1H, s, H-$\gamma$-9), 5.65 (1H, t, 0.9 Hz, H-$\gamma$-11), 3.54 (1H, s, H-$\gamma$-13), 4.02 (1H, q, 6.8 Hz, H-$\gamma$-15), 1.11 (3H, d, 6.8 Hz, H-16), 2.018 (3H, s, H-17), 3.85 (3H, s, H-18), 2.08 (3H, s, H-19), 1.61 (3H, s, H-20), 1.39 (3H, br d, 1.0 Hz, H-21), 1.76 (3H, br d, 0.7 Hz, H-22), 1.30 (3H, s, H-23), 1.41 (3H, s, H-24), 2.05 (3H, s, H-26).

**Compound (13a):** [α]$^20_D$ +96 (c 0.1, MeOH); UV $\lambda_{max}$ (MeOH) nm (log ε): 304 (3.63), 393 (1.43); IR $\nu_{max}$ (KBr) cm$^{-1}$: 2978, 2934, 2863, 1683, 1558, 1451, 1380, 1092, 1048, 1026; HRFABMS ($m/z$) found: 435.2391, calcd: 435.2383 [M + H]$^+$ for C$_{24}$H$_{35}$O$_7$; $^1$H and $^{13}$C NMR data in CD$_3$OD, see Table 3-1;

**13a-acetate (13b):** [α]$^{21}_D$ +118 (c 0.1, MeOH); UV $\lambda_{max}$ (MeOH) nm (log ε): 297 (3.85), 386 (1.80), 397 (1.29); IR $\nu_{max}$ (KBr) cm$^{-1}$: 2978, 2934, 1742, 1714, 1690, 1561, 1451, 1372, 1353, 1235, 1089, 1078, 1040, 1026; HRFABMS ($m/z$) found: 477.2498, calcd: 477.2488 [M + H]$^+$ for C$_{26}$H$_{37}$O$_8$; $^1$H NMR data in CD$_3$OD: δ 5.82 (1H, t, 0.9 Hz, H-7), 5.30 (1H, s, H-9), 5.67 (1H, t, 0.9 Hz, H-11), 3.55 (1H, s, H-13), 4.02 (1H, q, 6.8 Hz, H-15), 1.11 (3H, d, 6.8 Hz, H-16), 2.020 (3H, s, H-17), 3.85 (3H, s, H-18), 2.08 (3H, s, H-19), 1.62 (3H, s, H-20), 1.38 (3H, br d, 1.0 Hz, H-21), 1.76 (3H, br d, 0.7 Hz, H-22), 1.30 (3H, s, H-23), 1.42 (3H, s, H-24), 2.024 (3H, s, H-26).

**Verrucosidin (14):** obtained as a pale yellow oil. [α]$^{22}_D$ +70 (c 0.1, MeOH); EIMS ($m/z$) 416;
\(^1\)H NMR data in CDCl\(_3\): \(\delta\) 1.16 (3H, d, 6.8 Hz), 1.39 (3H, s), 1.40 (3H, s), 1.44 (3H, s), 1.87 (3H, brs), 1.93 (3H, brs), 2.01 (3H, s), 2.02 (3H, s), 3.40 (1H, s), 3.46 (1H, s), 3.80 (3H, s), 4.10 (1H, q, 6.8 Hz), 5.44 (1H, s), 5.83 (1H, s).

**Fructigenine A (15):** obtained as a yellow oil; EIMS \(m/z\) at 443; Molecular formula: C\(_{27}\)H\(_{29}\)N\(_3\)O\(_3\); \(^1\)H NMR data in CDCl\(_3\): \(\delta\) 0.88 (3H, s), 1.03 (3H, s), 2.14 (1H, t, 12.0 Hz), 2.46 (1H, dd, 5.6 Hz), 2.57 (3H, s), 2.74 (1H, dd, 14.4 Hz, 9.4 Hz), 3.44 (1H, dd, 14.3 Hz, 3.5 Hz), 3.70 (1H, dd, 10.3 Hz, 5.0 Hz), 4.15 (1H, dd, 10.1 Hz, 2.3 Hz), 5.02 (1H, d, 17.5 Hz), 5.04 (1H, d, 10.8 Hz), 5.67 (1H, dd, 17.3 Hz, 11.0 Hz), 5.74 (1H, brs, exchangeable with D\(_2\)O), 5.95 (1H, brs), 7.02-7.27 (8H, m), 7.93 (1H, brs).

**Verrucofortine (16):** obtained as a yellow oil; EIMS \(m/z\) at 409; Molecular formula: C\(_{24}\)H\(_{31}\)N\(_3\)O\(_3\); \(^1\)H NMR data in DMSO-\(d_6\): \(\delta\) 0.83 (3H, d, 6.8), 0.85 (3H, d, 6.8), 0.88 (3H, s), 1.06 (3H, s), 1.72 (1H, m), 1.76 (1H, m), 1.84 (1H, m), 2.26 (1H, t, 12 Hz), 2.55 (3H, s), 3.18 (2H, d, 4.8 Hz), 3.80 (1H, dd, 11.3 Hz, 6.0 Hz), 4.03 (1H, m), 5.06 (1H, d, 10.2 Hz), 5.10 (1H, d, 16.1 Hz), 5.87 (1H, dd, 11.0 Hz, 10.2 Hz), 5.97 (1H, brs), 7.17 (1H, t, 7.3), 7.30 (1H, t, 7.6 Hz), 7.47 (1H, d, 7.6), 7.84 (1H, d, 8.0 Hz), 8.11 (1H, brs); \(^1\)H NMR data in CDCl\(_3\): \(\delta\) 0.88 (3H, 6, 6, 6), 0.97 (3Hx2, d, 6, 2, 1, 13 (3H, s), 1.55 (1H, m), 1.73 (1H, m), 1.99 (1H, m), 2.38 (1H, dd, 11.7 Hz, 8.5 Hz), 2.60 (1H, d, 6.3 Hz), 2.61 (3H, s), 3.85 (1H, m), 3.94 (1H, m), 5.09 (1H, d, 17.9 Hz), 5.10 (1H, d, 10.6 Hz), 5.77 (1H, m), 5.98 (1H, brs), 7.13 (1H, t, 7.0 Hz), 7.27 (1H, m), 7.30 (1H, d, 7.3 Hz), 7.98 (1H, brs).

**Cyclopenol (17):** obtained as a yellow powder; EIMS \(m/z\) at 310; Molecular formula: C\(_{17}\)H\(_{14}\)N\(_2\)O\(_4\); \(^1\)H NMR data in CD\(_3\)OD: \(\delta\) 3.19 (3H, s), 4.08 (1H, s), 6.10 (1H, d, 7.3), 6.15 (1H, t, 2.2), 6.71 (1H, br d, 8.0 Hz), 7.01 (1H, t, 8.0), 7.15-7.18 (1Hx3, m), 7.55 (1H, m). \(^1\)H NMR data in acetone-\(d_6\): \(\delta\) 3.13 (3H, s), 4.12 (1H, s), 6.1-6.3 (2H, m), 6.7-7.6 (6H, m), 8.42 (1H, brs), 9.66 (1H, brs).

**Cyclopenin (18):** obtained as a pale yellow oil; EIMS \(m/z\) at 294; Molecular formula: C\(_{17}\)H\(_{14}\)N\(_2\)O\(_3\); \(^1\)H NMR data in CD\(_3\)OD: \(\delta\) 3.16 (3H, s), 4.15 (1H, s), 6.66 (1Hx2, d, 7.6 Hz), 7.02 (1H, dd, 8.0 Hz, 0.8 Hz), 7.13 (1Hx2, m), 7.19 (1Hx2, br d, 7.8), 7.27 (1H, t, 7.4), 7.53 (1H, td, 5.9 Hz, 1.5 Hz). \(^1\)H NMR data in CDCl\(_3\): \(\delta\) 3.22 (3H, s), 3.98 (1H, s), 6.6-6.7 (2H, m), 7.0-7.6 (7H, m), 8.82 (1H, brs).

**Penipratynolene (19):** obtained as a colorless oil; EIMS \(m/z\) at 220; Molecular formula:
C_{12}H_{12}O_4; \textsuperscript{1}H NMR data in DMSO-\textit{d}_6: \delta 3.42 (1H, d, 2.2 Hz), 3.81 (3H, s), 4.08 (2H, m), 4.59 (1H, ddd, 8.3, 6.6 Hz, 2.2 Hz), 5.87 (1H, d, 6.1 Hz), 7.07 (2H, d, 9.1), 7.91 (2H, d, 9.1). \textsuperscript{1}H NMR data in acetone-\textit{d}_6: \delta 2.98 (1H, d, 2.0 Hz), 3.84 (3H, s), 4.19 (2H, m), 4.75 (1H, m), 4.91 (1H, brs), 7.07 (2H, d, 8.9 Hz), 7.97 (2H, d, 8.9 Hz).

Cyclo-(L-Trp-L-Phe) (20): obtained as a white powder; EIMS (m/z) at 333; Molecular formula: C_{20}H_{19}N_3O_2; \textsuperscript{1}H NMR data in DMSO-\textit{d}_6: \delta 1.85 (1H, dd, 13.6 Hz, 6.9 Hz), 2.45 (1H, dd, 13.2 Hz, 4.6 Hz), 2.52 (1H, dd, 14.3 Hz, 5.9 Hz), 2.80 (1H, dd, 14.5 Hz, 4.3 Hz), 3.85 (1H, m), 3.97 (1H, m), 6.71 (2H, m), 6.96 (1H, d, 2.4 Hz), 7.00 (1H, m), 7.07 (1H, t, 7.0 Hz), 7.17 (1H, m), 7.32 (1H, d, 7.5 Hz), 7.49 (1H, d, 7.9 Hz), 7.71 (1H, d, 2.0 Hz), 7.92 (1H, d, 1.8 Hz), 10.90 (1H, s).

Aspterric acid (21): obtained as a brown powder; EIMS (m/z) at 266; Molecular formula: C_{15}H_{22}O_4; \textsuperscript{1}H NMR data in DMSO-\textit{d}_6: 1.53 (1H, m), 1.61 (1H, m), 1.72 (3H, s), 1.77 (1H, m), 1.80 (1H, m), 1.85 (3H, s), 2.13 (1H, brs), 2.16 (1H, brs), 2.28 (1H, m), 2.42 (1H, dd, 14.7 Hz, 8.7 Hz), 2.53 (1H, br d, 11.9 Hz), 3.48 (1H, d, 1.1 Hz), 3.82 (1H, d, 8.1 Hz), 4.43 (1H, d, 8.4 Hz); \textsuperscript{1}H NMR data in CDCl\textsubscript{3}: \delta 1.52 (1H, m), 1.59 (3H, s), 1.70 (3H, s), 1.73 (1H, m), 1.74 (1H, m), 2.03 (1H, m), 2.14 (1H, m), 2.18 (1H, m), 2.27 (1H, m), 2.3-2.4 (3H, m), 2.41 (1H, m), 3.50 (1H, d, 8.4 Hz), 3.92 (1H, d, 8.4 Hz), 4.28 (1H, d, 8.5 Hz).

Viridicatol (22): obtained as a pale orange oil; EIMS (m/z) at 253; Molecular formula: C_{15}H_{11}O_3N; \textsuperscript{1}H NMR data in DMSO-\textit{d}_6: \delta 6.71 (1H, m), 6.72 (1H, m), 6.82 (1H, m), 7.07 (1H, m), 7.10 (1H, m), 7.29 (1H, t, 7.4), 7.31 (1H, m), 7.32 (1H, m), 9.14 (1H, s), 9.54 (1H, s), 12.21 (1H, s).
3.4 Supporting Spectral Data for Compounds 12a, 12b, 13a, 13b and 14–22

Figure 3-S1. UV spectrum of compound (12a).

Figure 3-S2. IR spectrum of compound (12a).
Figure 3-S3. Positive FABMS spectrum of compound (12a).

Figure 3-S4. $^1$H NMR spectrum of compound (12a) in CD$_3$OD.
Figure 3-S5. $^{13}$C NMR and DEPT spectra of compound (12a) in CD$_3$OD.

Figure 3-S6. $^1$H-$^1$H COSY spectrum of compound (12a) in CD$_3$OD.
Figure 3-S7. HMQC spectrum of compound (12a) in CD$_3$OD.

Figure 3-S8. HMBC spectrum of compound (12a) in CD$_3$OD.
Figure 3-S9. UV spectrum of 12a-acetate (12b).

Figure 3-S10. IR spectrum of 12a-acetate (12b).
Figure 3-S11. MS spectrum of 12a-acetate (12b).

Figure 3-S12. $^1$H NMR spectrum of 12a-acetate (12b).
Figure 3-S13. UV spectrum of compound (13a).

Figure 3-S14. IR spectrum of compound (13a).
Figure 3-S15. Positive FABMS spectrum of compound (13a).

Figure 3-S16. $^1$H NMR spectrum of compound (13a) in CD$_3$OD.
Figure 3-S17. $^{13}$C NMR and DEPT spectra of compound (13a) in CD$_3$OD.

Figure 3-S18. $^1$H-$^1$H COSY spectrum of compound (13a) in CD$_3$OD.
Figure 3-S19. HMQC spectrum of compound (13a) in CD$_3$OD.

Figure 3-S20. HMBC spectrum of compound (13a) in CD$_3$OD.
Figure 3-S21. UV spectrum of 13a-acetate (13b).

Figure 3-S22. IR spectrum of 13a-acetate (13b).
Figure 3-S23. MS spectrum of 13a-acetate (13b).

Figure 3-S24. $^1$H NMR spectrum of 13a-acetate (13b).
**Figure 3-S25.** EIMS spectrum of verrucosidin (14).

**Figure 3-S26.** $^1$H NMR spectrum of verrucosidin (14) in CDCl$_3$. 
Figure 3-S27. $^{13}$C NMR and DEPT spectra of verrucosidin (14) in CDCl$_3$.

Figure 3-S28. $^1$H-$^1$H COSY spectrum of verrucosidin (14) in CDCl$_3$. 
Figure 3-S29. HMQC spectrum of verrucosidin (14) in CDCl₃.

Figure 3-S30. HMBC spectrum of verrucosidin (14) in CDCl₃.
Figure 3-S31. EIMS spectrum of fructigenine A (15).

Figure 3-S32. 1H NMR spectrum of fructigenine A (15) in CDCl₃.
Figure 3-S33. $^{13}$C NMR and DEPT spectra of fructigenine A (15) in CDCl$_3$.

Figure 3-S34. $^1$H-$^1$H COSY spectrum of fructigenine A (15) in CDCl$_3$. 
Figure 3-S35. HMQC spectrum of fructigenine A (15) in CDCl₃.

Figure 3-S36. HMBC spectrum of fructigenine A (15) in CDCl₃.
Figure 3-S37. EIMS spectrum of verrucofortine (16).

Figure 3-S38. $^1$H NMR spectrum of verrucofortine (16) in DMSO-$d_6$. 
Figure 3-S39. $^1$H NMR spectrum of verrucofortine (16) in CDCl$_3$.

Figure 3-S40. EIMS spectrum of cyclopenol (17).
Figure 3-S41. $^1$H NMR spectrum of cyclopenol (17) in CD$_3$OD.

Figure 3-S42. $^1$H NMR spectrum of cyclopenol (17) in acetone-$d_6$. 
Figure 3-S43. $^{13}$C NMR and DEPT spectra of cyclopenol (17) in CD$_3$OD.

Figure 3-S44. $^1$H-$^1$H COSY spectrum of cyclopenol (17) in CD$_3$OD.
Figure 3-S45. HMQC spectrum of cyclopenol (17) in CD$_3$OD.

Figure 3-S46. HMBC spectrum of cyclopenol (17) in CD$_3$OD.
Figure 3-S47. EIMS spectrum of cyclopenin (18).

Figure 3-S48. $^1$H NMR spectrum of cyclopenin (18) in CD$_3$OD.
Figure 3-S49. $^1$H NMR spectrum of cyclopenin (18) in CDCl$_3$.

Figure 3-S50. $^{13}$C NMR and DEPT spectra of cyclopenin (18) in CD$_3$OD.
Figure 3-S51. $^1$H-$^1$H COSY spectrum of cyclopenin (18) in CD$_3$OD.

Figure 3-S52. HMQC spectrum of cyclopenin (18) in CD$_3$OD.
Figure 3-S53. HMBC spectrum of cyclopenin (18) in CD$_3$OD.

Figure 3-S54. EIMS spectrum of penipratynolene (19).
Figure 3-S55. $^1$H NMR spectrum of penipratynolene (19) in DMSO-$d_6$. 

Figure 3-S56. $^1$H NMR spectrum of penipratynolene (19) in acetone-$d_6$. 
Figure 3-S57. $^{13}$C NMR and DEPT spectra of penipratynolene (19) in DMSO-$d_6$.

Figure 3-S58. $^1$H-$^1$H COSY spectrum of penipratynolene (19) in DMSO-$d_6$. 
Figure 3-S59. HMQC spectrum of penipratynolene (19) in DMSO-\textit{d}_6.

Figure 3-S60. HMBC spectrum of penipratynolene (19) in DMSO-\textit{d}_6.
Figure 3-S61. EIMS spectrum of cyclo-(L-Trp-L-Phe) (20).

Figure 3-S62. $^1$H NMR spectrum of cyclo-(L-Trp-L-Phe) (20) in DMSO-$d_6$. 
Figure 3-S63. $^{13}$C NMR and DEPT spectra of cyclo-(L-Trp-L-Phe) (20) in DMSO-$d_6$.

Figure 3-S64. $^1$H-$^1$H COSY spectrum of cyclo-(L-Trp-L-Phe) (20) in DMSO-$d_6$. 
Figure 3-S65. HMQC spectrum of cyclo-(L-Trp-L-Phe) (20) in DMSO-$d_6$.

Figure 3-S66. HMBC spectrum of cyclo-(L-Trp-L-Phe) (20) in DMSO-$d_6$. 
Figure 3-S67. EIMS spectrum of aspterric acid (21).

Figure 3-S68. $^1$H NMR spectrum of aspterric acid (21) in CD$_3$OD.
Figure 3-S69. $^1$H NMR spectrum of aspterric acid (21) in CDCl$_3$.

Figure 3-S70. $^{13}$C NMR and DEPT spectra of aspterric acid (21) in CD$_3$OD.
Figure 3-S71. $^1$H-$^1$H COSY spectrum of aspterric acid (21) in CD$_3$OD.

Figure 3-S72. HMQC spectrum of aspterric acid (21) in CD$_3$OD.
Figure 3-S73. HMBC spectrum of aspterric acid (21) in CD$_3$OD.

Figure 3-S74. EIMS spectrum of viridicatol (22) in DMSO-$d_6$. 
Figure 3-S75. $^1$H NMR spectrum of viridicatol (22) in DMSO-$d_6$.

Figure 3-S76. $^{13}$C NMR spectrum of viridicatol (22) in DMSO-$d_6$. 
Figure 3-S77. $^1$H-$^1$H COSY spectrum of viridicatol (22) in DMSO-$d_6$.

Figure 3-S78. HMOC spectrum of viridicatol (22) in DMSO-$d_6$. 
Figure 3-S79. HMBC spectrum of viridicatol (22) in DMSO-$d_6$. 
4. Isolation and Characterization of Antifungal Compounds from a Marine-Derived *Penicillium copticola* TPU1270

4.1 Introduction

Microorganisms have made a phenomenal contribution to the health of people (Sanchez, S., 2009). Marine-derived fungi have proven to be a rich source for the discovery of new natural products (Bhatnagar, I., 2010). During our continuous work for new compounds discovery, a strain of *Penicillium copticola* TPU1270, isolated from a marine bubble sample collected at Iriomote Island, Okinawa Prefecture, Japan showed antifungal activity against *Mucor hiemalis* (inhibition zone of 20 mm at 200 μg/disc). Bioassay-guided isolation from the culture broth of strain TPU1270 yielded a new natural open-chain hemisuccinimide, named penicillimide (23), together with five known eremophilane sesquiterpenes: sporogen-AO 1 (24) (Tirilly Y., 1983) (Tanaka S., 1984), 3-acetyl-13-deoxyphomenone (25) (Schneider G., 1997) (Huang Y. F., 2008), 6-dehydropetasol (26) (Sugawara, F., 1993), 7-hydroxypetasol (27) (Sugawara, F., 1993), and petasol (28) (Sugama K., 1983). The isolation and antifungal activities of compounds 23–28 are described.

![Chemical structures of compounds](image)

Figure 4-1. Structures of compounds 23–28 isolated from *Penicillium copticola* TPU1270.
4.2 Results and Discussion

A new natural open-chain hemisuccinimide, named penicillimide (23) and five known compounds 24–28. Penicillimide (23) was first isolated from a culture broth of fungus were obtained from the culture broth of strain TPU1270.


4.2.1 Screening Bioassay

Antifungal screening was carried out using Mucor hiemalis IAM 6088 by the paper disc method. The test microorganism was inoculated to a petri dish containing the PDA (Potato dextrose agar). A sterile antibiotic filter disc (diameter, 6 mm, Advantec, Tokyo, Japan) with the sample was placed in the dish and cultured for 2 days at 25°C. The inhibition zone in diameter (mm) was measured.

The crude extract of the fungual strain TPU1270 showed the inhibitory activity against M. genevensis (inhibition zone: 20 mm at 200 μg/disc) among 10 crude extracts of marine–derived fungi, while other strains showed no activity.

4.2.2 Isolation of Compounds

The whole broth after a 21-d static culture was added a half volume of acetone and filtered. After the evaporation of acetone, the filtrate was separated with an ODS column followed by repeated HPLC (ODS) to afford compounds 23–28. (Figure 4-2)
**Penicillium copticola** (strain TPU1270)

- Seed culture (30 mL, 27°C, 150 r.p.m., 3 days)
- Main culture (3.0 L, RT, static, 21 days)

Broth

- Added acetone and filtered
- Filtrate
- Evaporate acetone
- Absorbed on an ODS column
- Eluted with a stepwise gradient of H₂O/MeOH

<table>
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<th>Fr.3 (30%)</th>
<th>Fr.4 (40%)</th>
<th>Fr.5 (50%)</th>
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Figure 4-2. Isolation procedure for the whole broth extract.

### 4.2.3 Structure Determination of New Natural Compound

Penicillimide (23) was obtained as a pale yellow solid with the molecular formula C₁₃H₁₄NO₅Cl determined by HRESIMS (m/z 299.0555 [M⁺]). The ¹H and ¹³C NMR spectra revealed 12 proton and 13 carbon signals (Table 4-1). The ¹H and ¹³C NMR signals were assigned by the analysis of ¹H-¹H COSY, HMQC and HMBC correlations (Table 4-1). In the ¹³C NMR spectrum of 23, one oxygenated methyl, three sp³ methylene, three sp² methine, two sp² quaternary, one oxygenated sp² quaternary and three carbonyl carbons were assigned by the analysis of 1D and 2D NMR spectra. ¹H-¹H COSY correlations were observed between H-3 (δ 2.61) / H-4 (δ 2.84) and H-13 (δ 6.86) / H-14 (δ 7.03) and gave the partial structures I and II. These partial structures were connected by the HMBC correlations (Figure 4-3).

The differences in the molecular formula and weight between compound 23 and coniothyriomycin were two protons and 2 Da. NMR data due to the 2-(3-chloro-4-hydroxyphenyl)acetylamide moiety in 23 were very similar to those of the same
moiety in coniothyriomycin, which was previously identified as an antifungal metabolite from the fungus *Coniothyrium* sp. (Krohn, K., 1992). A difference in the structures of compound 23 and coniothyriomycin was detected at the dicarboxylic acid units. Compound 23 had the succinic acid unit while coniothyriomycin had the fumaric acid unit (Δ2'-succinic acid unit). Thus, the structure of compound 23 was assigned as shown in Figure 4-3 and named penicillimide (Note 4-1).

Table 4-1. $^{13}$C (100 MHz) and $^1$H (400 MHz) NMR data for compound 23 (acetone-$d_6$ and CD$_3$OD).

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</table>

Figure 4-3. $^1$H-$^1$H COSY and HMBC correlations for compound 23
4.2.4 Structure Determination of Known Compounds

Compound 24 was obtained as a colorless oil. The molecular weight and formula (248 and C_{13}H_{20}O_{3}) of 24 was determined by ^1H and ^13C NMR and EIMS data. The structure of compound 24 was determined by the HMBC, HMQC and ^1H-^1H COSY correlations and compound 24 was confirmed as sporogen-AO 1.

Compound 25 was obtained as a pale yellow oil. The molecular weight and formula (290 and C_{17}H_{22}O_{4}) of 25 was determined by ^1H and ^13C NMR and EIMS data. The additional acetyl group was confirmed by ^13C NMR spectrum and molecular formula. Thus, compound 25 was confirmed as 3-acetyl-13-deoxyphomenone.

Compound 26 was obtained as a colorless oil. The molecular weight and formula (232 and C_{15}H_{20}O_{2}) of 26 was decided by ^1H and ^13C NMR and EIMS data. One singlet signal at δ 6.86 of H-6 in ^1H NMR, together with the comparison with the ^1H NMR of petasol, compound 26 was determined as 6-dehydropetasol.

Compound 27 was obtained as a colorless oil. The molecular weight and formula (250 and C_{15}H_{22}O_{3}) of 27 was determined by ^1H and ^13C NMR and EIMS data. The ^1H NMR spectrum showed similar signals as that of sporogen-AO 1. The difference in the ^1H NMR spectra between 26 and 27 was that two doublets at δ 2.32 and 1.75 were observed in the ^1H NMR spectrum of compound 27, and one singlet at δ 3.31 in the spectrum of 26. Thus, compound 27 was identified as 7-hydroxypetasol.

Compound 28 was obtained as a colorless oil. The molecular weight and formula (234 and C_{15}H_{22}O_{2}) of 28 was determined by ^1H and ^13C NMR and EIMS spectra. The lower-field region of ^1H NMR spectrum was similar to that of 7-hydroxypetasol while the higher-field region showed some differences. The comparison of the molecular formula of 28 and 27 revealed the difference one oxygen atom. Based on these information, compound 28 was identified as Petasol.

4.2.5 Biological Activity

The antifungal activity against M. hiemalis, A. fumigates and the anti-mycobacterial activity against Mycobacterium smegmatis NBRC 3207 of compounds 23—28 were evaluated by the paper disc method (Ericsson, H., 1960) and the results of antifungal activity were shown in Table 4-2.
Table 4-2. Antifungal activity (inhibition zone, mm) against *Mucor hiemalis* and *Aspergillus fumigatus*.

<table>
<thead>
<tr>
<th>Compound</th>
<th><em>M. hiemalis</em></th>
<th><em>A. fumigatus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>40 μg/disc</td>
<td>80 μg/disc</td>
</tr>
<tr>
<td></td>
<td>40 μg/disc</td>
<td>80 μg/disc</td>
</tr>
<tr>
<td>23</td>
<td>—&lt;sup&gt;a&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>24</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>25</td>
<td>—</td>
<td>9</td>
</tr>
<tr>
<td>26</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>27</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>28</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Amphotericin B (10 μg/disc)</td>
<td>12</td>
<td>13</td>
</tr>
</tbody>
</table>

<sup>a</sup> An inhibition zone was not detected.

Compounds 24, 25, 26 and 28 showed antifungal activity against both *Mucor hiemalis* and *Aspergillus fumigatus*, but no activity against *Mycobacterium smegmatis* NBRC 3207 of compounds 23—28 was observed.

Butenoate derivatives such as coniothyriomycin (Krohn, K. 1992, 2003) and 3-phenylcarbamoyl-arylic acid methyl ester (Bobkova, T.S. 1977), were reported to possess the remarkable antifungal and herbicidal activities. Compound 23, the derivative of coniothyriomycin, was detected successfully from two days’ culture broth by EIMS measurement, while coniothyriomycin was not found. Thus, compound 23 was considered as originally produced by the fungus strain, TPU1270. Unfortunately, the results showed compound 23 had no antifungal activity against *M. hiemalis* and *A. fumigatus* at 80 μg/disc. Therefore, the fumaric acid partial structure will be essential for the antifungal activity of butenoate derivatives.

Compounds 24, 26 and 28 showed the inhibition zone against *M. hiemalis* and *A. fumigatus* at 40 μg/disc. Compound 25 was active against *M. hiemalis* and *A. fumigatus* at 80 μg/disc and compound 27 showed no activity. Related sesquiterpenoids were isolated from various fungi, such as *Penicillium citrinum* (Yurchenko, A.N. 2013), *Petasies fragrans* (Sugama,
K. 1983), Penicillium sp. SS080624SCf1 (Motohashi, K., 2009), Drechslera gigantea (Bunkers, G., 1990), Penicillium sp. BL27-2 (Huang, Y. F., 2008), Hansfordia sp. (Schneider, G., 1997), Aspergillus oryzae (Tanaka, S., 1984), Sarcographa tricosa (Le, D. H., 2013) and Apiognomonia sp. f24023 (Kawahara, T., 2013). The cytotoxic, antimicrobial and phytotoxic activities were described in these reports. The results for similar antifungal activity in this study suggested that the hydroxyl group attached to C-7 will lead to the loss of the antifungal activity of these sesquiterpenoids.

4.3 Experimental Section

4.3.1 General Experimental Procedures

NMR spectral data were obtained by a JEOL JNM-AL-400 NMR spectrometer (JEOL Ltd., Japan; 400 MHz for $^1$H and 100 MHz for $^{13}$C) in CDCl$_3$ ($\delta_H$ 7.24, $\delta_C$ 77.23) or CD$_3$OD ($\delta_H$ 3.31, $\delta_C$ 49.15). Mass spectra were recorded with a JEOL JMS-MS 700 mass spectrometer (EI or FAB mode with m-nitrobenzyl alcohol or glycerol as the matrix). UV spectra were obtained on a Hitachi U-3310 UV-Visible spectrophotometer (Hitachi, Ltd., Japan) and IR spectra on a PerkinElmer Spectrum One Fourier transform infrared spectrometer (Waltham, MA, USA). Optical rotations were measured with a JASCO P-2300 digital polarimeter (JASCO, Ltd., Japan). Preparative HPLC was conducted using a Toyosoda CCPU instrument equipped with a Tosoh UV-8010 detector.

4.3.2 Isolation, Identification, and Fermentation of Producing Bacterium

The fungus strain TPU1270 was isolated from a marine bubble sample at the beach of Iriomote Island in Okinawa, Japan in July, 2012. Approximately 1 mL of the seawater was macerated in 25 mL sterilized seawater with 1.0 % SDS and 50 μL of the suspension was plated to a agar plate (glycerol 0.6%, arginine 0.1%, K$_2$HPO$_4$ 0.1%, MgSO$_4$ 0.05%, agar 1.5% and the antibiotics (cycloheximide 100 μg/mL, rifampin 5 μg/mL) in natural seawater). The fungus strain TPU1270 was identified as Penicillium copticola by a comparison of 228 bp ITS1 rDNA sequences (100% match).
Figure 4-4. Pure cultures of *Penicillium copticola* TPU1270 in dish

The culture of strain TPU1270 was maintained on a PDA (Difco Laboratories, Detroit, MI, USA) plate. The mycelia grown on the agar plate was inoculated in a 100-mL Erlenmeyer flask containing 50 mL of PD broth as the seed medium. The flasks were shaken on a shaker (150 r.p.m) at 27 °C for 3 days. Aliquots (2 mL) of the seed culture were inoculated to 500-mL Erlenmeyer flasks containing each 200 mL of the main medium (3% sucrose, 3% soluble starch, 1% malt extract, 0.3% Ebios (Asahi Food & Healthcare Co. Ltd., Tokyo, Japan), 0.5% KH$_2$PO$_4$ and 0.05% MgSO$_4$·7H$_2$O; adjusted to pH 6.0 before sterilization). The production culture was carried out at room temperature for 21 days.

4.3.3 Bioassay

Compounds 23–28 were tested for antimicrobial activity against *Mycobacterium smegmatis* NBRC 3207 (48 hours at 37°C), *Asperillus fumigatus* IAM 13869 (48 hours at 25°C) and *Mucor hiemalis* IAM 6088 (48 hours at 25°C). A sterile antibiotic filter disk (6 mm in diameter), treated with the test sample was placed in the dish. After incubation, the inhibition zone in diameter (mm) was measured.
4.3.4 Extraction and Isolation of Compounds

The whole broth (3 L) was added the same volume of acetone and filtered. After evaporation under reduced pressure to remove acetone, the aqueous solution was subjected to ODS column chromatography by the stepwise gradient elution with H₂O-MeOH mixtures (100:0 to 0:100, v/v) to afford 10 fractions. About 30 mg of penicillimide (23) was obtained as a crystal from 40% MeOH fraction. Sporogen-AO 1 (24, 15.0 mg) was obtained by separation of the 50% MeOH fraction with preparative reversed-phase HPLC (Senshu Scientific co., Ltd., Pegasil ODS SP100 column, 250 × 10 mm, 5 μm; MeOH-H₂O=55:45; 2.0 ml/min; UV 210 nm). 3-Acetyl-13-deoxyphomenone (25, 4.0 mg) was isolated from the 70% MeOH fraction by preparative HPLC (ODS, MeOH-H₂O= 75:25), and 6-dehydropetasol (26, 15.1 mg) and petasol (28, 12.0 mg) were obtained from the 60% MeOH fraction by preparative ODS HPLC (MeOH-H₂O = 66:34). Preparative HPLC (ODS, MeOH-H₂O=33:64) of the 30% MeOH fraction yielded 7-hydroxypetasol (27, 5.5 mg).

Figure 4-5. Chromatography for the analysis of penicillimide (23) with HPLC systems (ODS Senshu Scientific co., Ltd., Pegasil ODS SP100 column, 250 × 10 mm, 5 μm; MeOH-H₂O=44:56; 2.0 ml/min; UV 210 nm)
Penicillimide (23): pale yellow crystals. UV (MeOH) $\lambda_{\text{max}}$ nm (log $\epsilon$) 202 (3.69), 280 (2.80). IR (KBr) $\nu_{\text{max}}$ 3419, 1739, 1734, 1635, 1501, 1424, 1364, 1287, 1059, 1048 cm$^{-1}$. HREIMS ($\text{m/z}$) 299.0555 ($\text{[M]}^+$; calcd for C$_{13}$H$_{14}$NO$_5$Cl, 299.0561).

IR (KBr) $\nu_{\text{max}}$ 3419, 1739, 1734, 1635, 1501, 1424, 1364, 1287, 1059, 1048 cm$^{-1}$. HREIMS ($\text{m/z}$) 299.0555 ($\text{[M]}^+$; calcd for C$_{13}$H$_{14}$NO$_5$Cl, 299.0561).

Sporogen-AO 1 (24): obtained as a colorless oil; EIMS ($\text{m/z}$): 248; Molecular formula: C$_{15}$H$_{20}$O$_3$; $[\alpha]_{D}^{23} +14$ (c 0.1, MeOH); $^1$H NMR data in CDCl$_3$: $\delta$ 1.20 (3H, s), 1.24 (3H, d, 6.8 Hz), 1.42 (1H, m), 1.79 (1H, m), 1.85 (3H, brs), 2.13 (1H, m), 2.32 (1H, ddd, 14.2 Hz, 3.2 Hz, 2.6 Hz), 2.63 (1H, tdd, 14.1 Hz, 4.6 Hz, 1.8 Hz), 3.31 (1H, s), 3.60 (1H, ddd, 10.8 Hz, 4.5 Hz), 5.08 (1H, m), 5.09 (1H, brs), 5.74 (1H, d, 2.0 Hz).

3-Acetyl-13-deoxyphomenone (25): obtained as a pale yellow oil; EIMS ($\text{m/z}$): 290; Molecular formula: C$_{17}$H$_{22}$O$_4$; $[\alpha]_{D}^{22} +126$ (c 0.1, MeOH); $^1$H NMR data in CDCl$_3$: $\delta$ 1.10 (3H, d, 6.8 Hz), 1.24 (3H, s), 1.43 (1H, m), 1.71 (1H, t, 8.7 Hz), 1.85 (3H, s), 2.04 (1H, m), 2.15 (1H, m), 2.33 (1H, ddd, 14.8 Hz, 4.2 Hz, 2.7 Hz), 2.54 (1H, ddd, 15.1 Hz, 13.9 Hz, 4.6 Hz, 2.2 Hz), 3.30 (1H, s), 4.84 (1H, ddd, 10.7 Hz, 9.4 Hz, 4.8 Hz), 5.09 (1H, s), 5.75 (1H, d, 1.9 Hz).

6-Dehydropetasol (26): obtained as a colorless oil; EIMS ($\text{m/z}$): 232; Molecular formula: C$_{15}$H$_{20}$O$_2$; $[\alpha]_{D}^{22} +70$ (c 0.1, MeOH); $[\alpha]_{D}^{22} +300$ (c 0.1, MeOH); $^1$H NMR data in CDCl$_3$: $\delta$ 1.13 (3H, s), 1.21 (3H, d, 6.7 Hz), 1.36 (1H, m), 1.41 (1H, m), 1.80 (1H, brs), 1.95 (3H, s), 2.22 (1H, m), 2.35 (1H, ddd, 14.3 Hz, 4.2 Hz, 2.3 Hz), 2.49 (1H, ddd, 14.1 Hz, 13.9 Hz, 5.6 Hz, 1.1 Hz), 3.64 (1H, m), 5.06 (1H, brs), 5.16 (1H, brs), 6.05 (1H, d, 1.0 Hz), 6.86 (1H, s). Exact Mass: 232.1463

7-hydroxypetasol (27): obtained as a colorless oil; EIMS ($\text{m/z}$): 250; Molecular formula: C$_{15}$H$_{22}$O$_3$; $[\alpha]_{D}^{23} -47$ (c 0.1, MeOH); $^1$H NMR data in CDCl$_3$: $\delta$ 1.11 (3H, d, 6.7 Hz), 1.14 (3H, s), 1.38 (1H, m), 1.64 (1H, m), 1.75 (1H, d, 14.8 Hz), 1.77 (3H, s), 2.18 (1H, m), 2.31 (1H, m), 2.32 (1H, d, 14.8 Hz), 2.53 (1H, ddd, 13.9 Hz, 9.6 Hz, 4.3 Hz), 3.61 (1H, ddd, 10.5 Hz, 10.3 Hz, 5.4 Hz), 4.83 (1H, s), 4.94 (1H, s), 5.92 (1H, brs).
**Petasol (28):** obtained as a colorless oil; EIMS (m/z): 234; Molecular formula: C_{15}H_{22}O_{2}; [α]^{23}_D +124 (c 0.1, MeOH); $^1$H NMR data in CDCl$_3$: δ 1.04 (3H, 6.7 Hz), 1.14 (3H, s), 1.30 (1H, m), 1.41 (1H, m), 1.70 (3H, brs), 1.84 (1H, t, 13.6 Hz), 1.99 (1H, dd, 12.9 Hz, 4.5 Hz), 2.13 (1H, m), 2.31 (1H, ddd, 14.9 Hz, 4.8 Hz, 2.6 Hz), 2.41 (1H, tdd, 14.9 Hz, 5.0 Hz, 2.0 Hz), 3.07 (1H, dd, 14.2 Hz, 4.6 Hz), 3.59 (1H, td, 10.6 Hz, 4.3 Hz), 4.79 (1H, s), 4.95 (1H, s), 5.74 (1H, d, 1.4 Hz).
4.4 Supporting Spectral Data for Compounds 23—28

Figure 4-S1. UV spectrum of penicillimide (23).

Figure 4-S2. IR spectrum of penicillimide (23) (KBr).
Figure 4-S3. EIMS spectrum of penicillimide (23).

Figure 4-S4. $^1$H NMR spectrum of penicillimide (23) in CD$_3$OD.
Figure 4-S5. $^1$H NMR spectrum of penicillimide (23) in acetone-$d_6$.

Figure 4-S6. $^{13}$C NMR spectrum of penicillimide (23) in CD$_3$OD.
Figure 4-S7. $^{13}$C NMR spectrum of penicillimide (23) in acetone-$d_6$.

Figure 4-S8. $^1$H-$^1$H COSY spectrum of penicillimide (23) in CD$_3$OD.
Figure 4-S9. HMQC spectrum of penicillimide (23) in CD$_3$OD.

Figure 4-S10. HMBC spectrum of penicillimide (23) in CD$_3$OD.
Figure 4-S11. EIMS spectrum of sporogen-AO 1 (24).

Figure 4-S12. $^1$H NMR spectrum of sporogen-AO 1 (24) in CD$_3$OD.
Figure 4-S13. $^1$H NMR spectrum of sporogen-AO 1 (24) in CDCl$_3$.

Figure 4-S14. $^{13}$C NMR spectrum of sporogen-AO 1 (24) in CD$_3$OD.
Figure 4-S15. $^1$H-$^1$H COSY spectrum of sporogen-AO 1 (24) in CD$_3$OD.

Figure 4-S16. HMQC spectrum of sporogen-AO 1 (24) in CD$_3$OD.
Figure 4-S17. HMBC spectrum of sporogen-AO 1 (24) in CD$_3$OD.

Figure 4-S18. UV spectrum of 3-acetyl-13-deoxyphomenone (25).
Figure 4-S19. IR spectrum of 3-acetyl-13-deoxyphomenone (25).

Figure 4-S20. EIMS spectrum of 3-acetyl-13-deoxyphomenone (25).
Figure 4-S21. $^1$H NMR spectrum of 3-acetyl-13-deoxyphomenone (25) in CD$_3$OD.

Figure 4-S22. $^1$H NMR spectrum of 3-acetyl-13-deoxyphomenone (25) in CDCl$_3$. 
Figure 4-S23. $^{13}$C NMR spectrum of 3-acetyl-13-deoxyphomenone (25) in CD$_3$OD.

Figure 4-S24. $^1$H-$^1$H COSY spectrum of 3-acetyl-13-deoxyphomenone (25) in CD$_3$OD.
Figure 4-S25. HMQC spectrum of 3-acetyl-13-deoxyphomenone (25) in CD$_3$OD.

Figure 4-S26. HMBC spectrum of 3-acetyl-13-deoxyphomenone (25) in CD$_3$OD.
Figure 4-S27. EIMS spectrum of 6-dehydropetasol (26).

Figure 4-S28. $^1$H NMR spectrum of 6-dehydropetasol (26) in CDCl$_3$. 
Figure 4-S29. $^{13}$C NMR spectrum of 6-dehydropetasol (26) in CDCl$_3$.

Figure 4-S30. $^1$H-$^1$H COSY spectrum of 6-dehydropetasol (26) in CDCl$_3$.
Figure 4-S31. HMQC spectrum of 6-dehydropetasol (26) in CDCl₃.

Figure 4-S32. HMBC spectrum of 6-dehydropetasol (26) in CDCl₃.
Figure 4-S33. Positive FABMS spectrum of 7-hydroxypetasol (27).

Figure 4-S34. Negative FABMS spectrum of 7-hydroxypetasol (27).
Figure 4-S35. $^1$H NMR spectrum of 7-hydroxypetasol (27) in CD$_3$OD.

Figure 4-S36. $^1$H NMR spectrum of 7-hydroxypetasol (27) in CDCl$_3$. 
Figure 4-S37. $^{13}$C NMR spectrum of 7-hydroxyptasol (27) in CD$_3$OD.

Figure 4-S38. EIMS spectrum of ptasol (28).
Figure 4-S39. $^1$H NMR spectrum of petasol (28) in CDCl$_3$.

Figure 4-S40. $^{13}$C NMR spectrum of petasol (28) in CDCl$_3$. 
Figure 4-S41. $^1$H-$^1$H COSY spectrum of petasol (28) in CDCl$_3$.

Figure 4-S42. HMQC spectrum of petasol (28) in CDCl$_3$. 
Figure 4-S43. HMBC spectrum of petasol (28) in CDCl₃.
References and Notes


**Note 4-1:** Penicillimide (23) might be named as dihydroconiothyrionmycin, but a suffix “mycin” will not be suitable since compound 23 was a fungal metabolite.


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Publications and Presentations

Publications


Presentations:


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To those who love me and those I love, I dedicate this thesis.