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Systematic isolation of microbial metabolites for natural products depository (NPDepo)*

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Abstract: A microbial fraction library has been constructed as a part of RIKEN Natural Products Depository (NPDepo) to discover and isolate novel metabolites with unique biological activity from microbial sources efficiently and rapidly. The fraction library was made by a systematic separation method based on basic chromatographic techniques. Each fraction in the library was analyzed by liquid chromatography/mass spectrometry (LC/MS) to reveal physicochemical properties of each metabolite within the fraction, and the results were applied to construct a database for rapid discovery of novel and structurally unique compounds. We developed a new type of metabolite database called MP (microbial products) plot to visualize each metabolite on a 2D area. The combination of the fraction library and the database led to the discovery and isolation of novel metabolites, verticilactam, which was a 16-membered macrolactam with an unprecedented β-keto-amide moiety, and spirotoamides A and B, which had a highly substituted 6,6-spiroaccetal moiety and a carboxamide moiety. Moreover, based on the utilization of the MP plot, the new capacity of a streptomycete to produce specific metabolites was discovered.

Keywords: biosynthesis; fraction library; mass spectrometry; microbial products; MP plot; natural products; spectral database; systematic isolation; UV–vis spectroscopy.

INTRODUCTION

Natural products have been an important screening source for therapeutic agents [1,2]. Secondary metabolites produced by microorganisms are a large part of such important natural products [3–5]. Microorganisms such as actinomycetes and fungi have been known to produce structurally diverse biologically active secondary metabolites [6,7]. Such metabolites are used not only in medicinal drugs but also bioprobes [8,9] as tools to investigate biological functions in chemical biology study [10,11]. We are constructing the RIKEN natural products depository (NPDepo), focusing on mainly microbial metabolites to discover the unknown potential of natural products [13].

A bioassay-guided separation method has been widely used to find and isolate such metabolites with the specific activity. However, this method might lose valuable active compounds for their small abundance or influences of co-existence materials, which decrease the potential activity of them, and inactive compounds for the specific assay system, which might have other useful activities. Also, we need to re-separate from the crude material by the guide of another assay system for the discovery of different active compounds.

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To overcome these problems, we systematically collected semi-purified samples of microbial fermentation broths, which are named "microbial metabolite fraction library" as a part of NPDepo [13]. We have already reported the advantage of the fraction library by the isolation of structurally new compounds, fraquinocins I and J [14], and 6-dimethylalylindole-3-carbaldehyde [15] from the fractions of *Streptomyces reveromycieticus* SN-593, which are known to be a producer of reveromycins [16,17]. Also, a marine natural products library based on a high-performance liquid chromatography/mass spectrometry (HPLC/MS) fractionation protocol has been reported with the advantage of rapid discovery of drug candidates [18].

The fraction library is constructed by a systematic separation method, using middle pressure liquid chromatography (MPLC) and HPLC based on the basic chromatographic techniques to maintain relatively high re-productivity, and contains all the eluted fractions of HPLC. The fractions, which are not completely purified and consist of unidentified minor components, might contain valuable compounds that have novel structures, unique activities, or key metabolites of a specific biosynthetic pathway. All the fractions have been analyzed by a photodiode array (PDA) detector attached LC/MS system. Therefore, it is possible to obtain more amounts of the same sample from the fermentation broth by the HPLC-guided purification. The utilization of the fraction library will enable us to make up the drawback of the bioassay-guided separation. The library is used for several screenings at once by the conventional bioassay and a newly developed chemical array method, which is a new platform for drug screening in order to save an amount of microbial products and an ultra-high-throughput screening method [19–21]. Based on the assay results, the fraction with a specific activity is easily picked up and used for identification of the active principal by the result of LC/MS analysis.

We are constructing the spectral database based on UV absorption and mass spectral data obtained from PDA-LC/MS analysis of the fraction library for identification of the compounds in the fractions without purification and avoiding the duplicate isolation. In addition to the usual spectral database, we are developing a unique database called MP (microbial products) plot, which is a distribution map for metabolites on a 2D area based on their physicochemical properties (retention time of HPLC and *m/z* value) of each metabolite obtained from the LC/MS analysis. The MP plot is useful and helpful for not only discovery of novel compounds but also search for specific metabolites group for each strain by comparison of distribution pattern between strains.

In this review, we describe the construction of a microbial fraction library, development of MP plot, and the recent progress of application of the fraction library on the discovery of novel metabolites and previously unknown capacity of an actinomycete to produce specific metabolites.

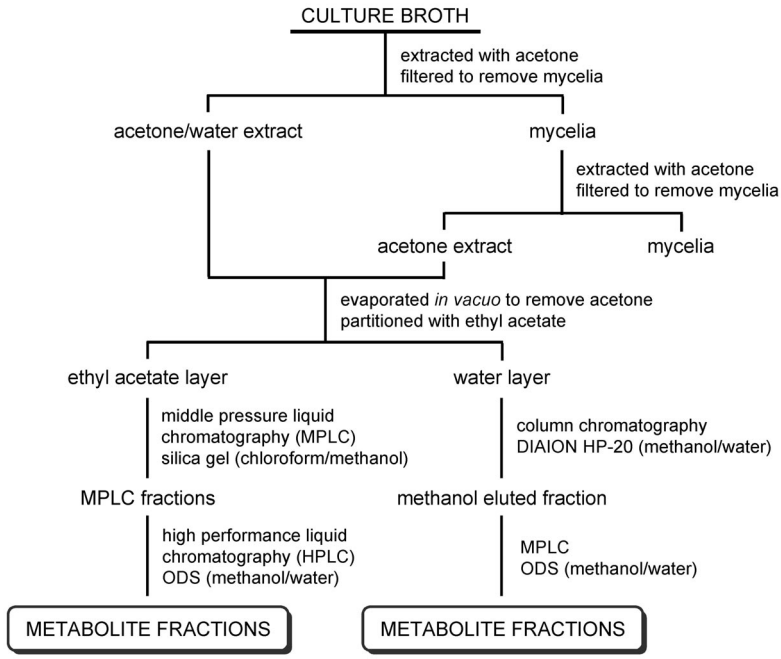
CONSTRUCTION OF A MICROBIAL METABOLITE FRACTION LIBRARY

A fraction library has been constructed for rapid discovery and isolation of novel metabolites with unique activity by a systematic separation method. The separation step is a key process and needs to be a simple and reliable method for high reproducibility, maintaining stable microbial culture and productivity. We developed a simple and systematic separation method for the construction of a fraction library based on basic chromatographic techniques.

Systematic separation

The systematic separation method contains two-step separations consisting of silica gel and reversed-phase chromatography for hydrophobic materials and HP-20 resin and reversed-phase chromatography for hydrophilic materials. Since our goal for developing a library was to discover, identify, and isolate novel metabolites with unique activities, the method was developed in providing each fraction weight of at least 0.5 mg for various activity tests at the same concentration and reproductive assurance for re-isolation for future research.

Detail of the systematic isolation method is shown in Scheme 1. About 20 L of a metabolite rich culture broth was extracted twice with acetone to obtain aqueous acetone extract. It was evaporated to remove acetone under reduced pressure. The remaining water portion was then partitioned twice with ethyl acetate. The organic layer was evaporated to dryness to afford metabolite rich extract. It was subjected to MPLC on a silica gel column with a chloroform/methanol gradient system to obtain 8 fractions based on the UV chromatogram recorded at 254 nm. Each MPLC fraction was then separated by C_{18}-HPLC with a methanol/water gradient system into 48 fractions using time-constant slices, which were to become core components of the fraction library. The water layer was subjected to a DIAION HP-20 column chromatography with a methanol/water system to afford methanol-soluble material. It was separated by C_{18}-MPLC with a methanol/water gradient system into 48 fractions for the fraction library.



Scheme 1 Systematic separation method to prepare a metabolite fraction library.

We chose 22 microbial strains, which are known to produce structurally diverse compounds, consisting of 16 actinomycetes and 6 fungi as the fraction library, and prepared 6480 fractions from them. Approximately 90 % of the fractions (5869 fractions) contained fractionated materials of over 0.5 mg and could compare potential activities at the same concentration for several screenings. A part of each fraction has been stored as a dimethyl sulfoxide (DMSO) solution in 96 well plates at the concentration of 10 mg/mL for activity tests.

ACTIVITIES OF THE MICROBIAL METABOLITE FRACTION LIBRARY

The fraction library was evaluated by conventional bioassay against *Escherichia coli* with regard to antibacterial activity, *Magnaporthe oryzae* for antifungal activity, and human promyelocytic leukemia cell lines HL-60 for cytotoxic activity. The results are shown in Fig. 1 as a Venn diagram. The activities of the extracts before fractionation and MPLC fractions were also examined under the same conditions as with the fraction library (data not shown), and some fractions that were prepared from nonactive

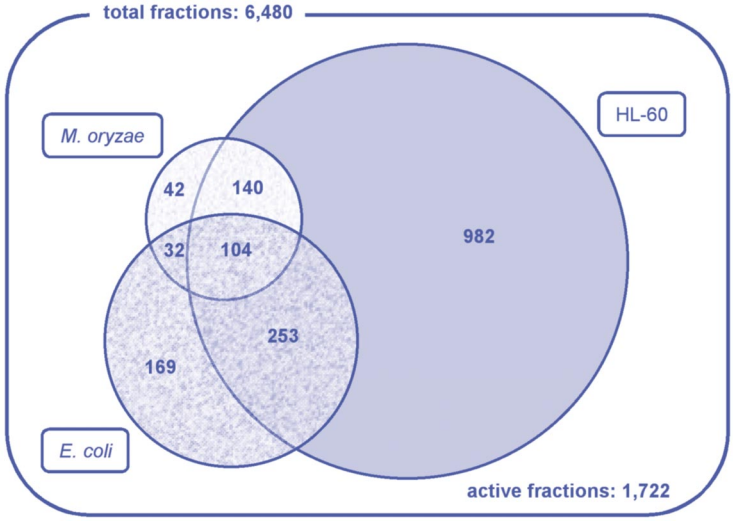


Fig. 1 The potential activities of the fraction library.

extracts showed robust activity. This result indicated an advantage of the fraction library, which revealed the activities of metabolites that resided in the extracts at low concentration or the effects of coexisting materials. Approximately 25 % of the fractions showed single or multiple activities.

LC/MS ANALYSIS OF THE FRACTION LIBRARY

To take advantage of the fraction library in discovering structurally unique compounds efficiently, we developed two types of databases, based on LC/MS analysis. Each fraction in the library was analyzed by PDA-LC/MS to determine the UV absorption and mass spectra of each metabolite in the fraction, which were used to construct the databases. One was a common spectral database, developed using ACD/Labs®. The other one is called MP plot, which displays each metabolite as a dot on a 2D map. The combination of these databases can be used to search for structurally novel and unique metabolites effectively and rapidly.

UV/MS spectral database

We used a commercial software program, ACD/Labs [22], as a spectral database to manage the spectral data from the PDA-LC/MS analysis. Thus, we could search easily for compounds with the target spectrum and known compounds using spectral data, such as maximum absorption value, *m/z* value, and similarities in spectral patterns.

MP plot

Although ACD/Labs is a powerful spectral database, it is unable to compare or distinguish metabolite groups between strains, which is the first step in the discovery of novel metabolites. Thus, we developed the MP plot, as shown in Fig. 2A, to complement the typical spectral database. It is a 2D plot, comprising an *X*-axis for retention time and a *Y*-axis for *m/z* values, based on LC/MS analysis; each metabolite appears as a dot on the 2D map.

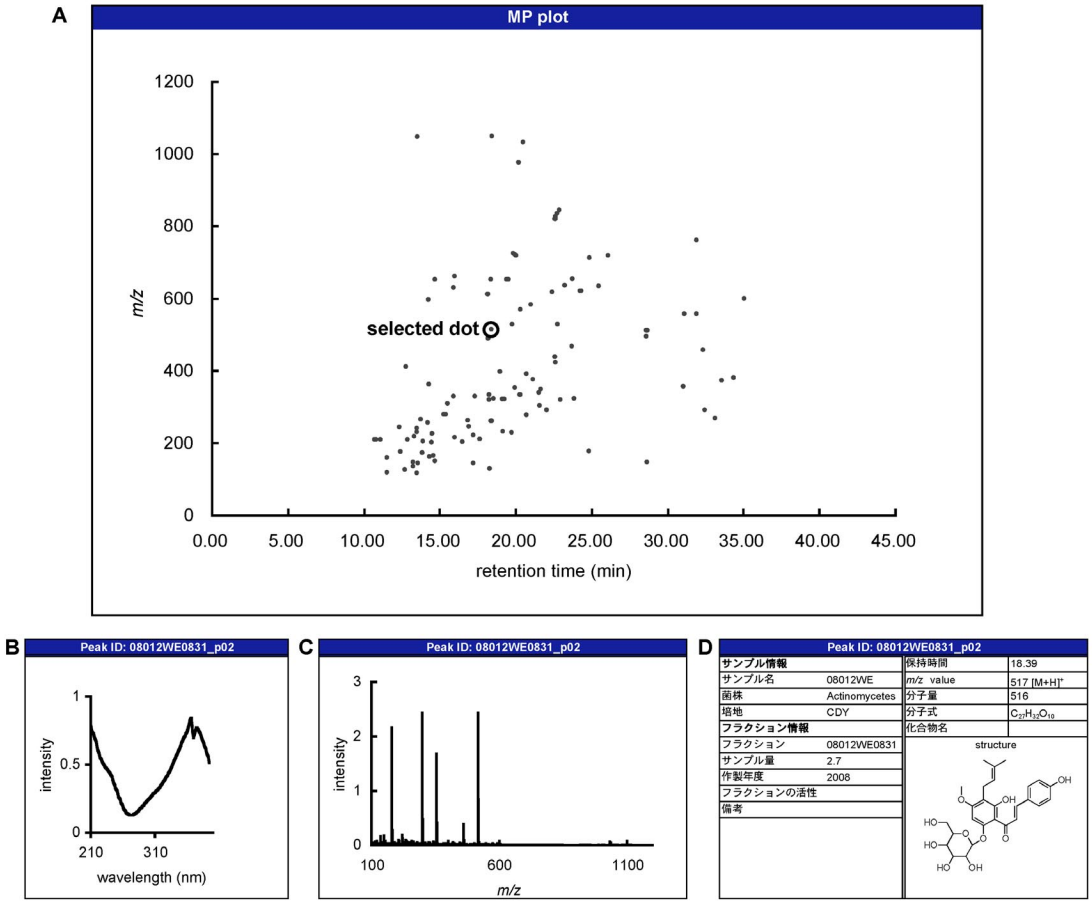


Fig. 2 2D MP plot and information windows for the selected dot. **A:** MP plot, **B:** UV absorption spectrum for the selected dot, **C:** mass spectrum for the selected dot, **D:** sample information table for the selected dot.

MP plot is a map of metabolite distribution and a collection of strain fingerprints, based on the physicochemical properties of each metabolite. We can distinguish metabolite groups for each strain easily by comparing the distribution of dots between strains.

The plot was developed using HTML and can be displayed in a common web browser, such as Internet Explorer®, Firefox®, and Safari®, without special software. Each dot links to the UV absorption spectrum (Fig. 2B), mass spectrum (Fig. 2C), and sample information table (Fig. 2D), which appear in a new window. One can select several dots simultaneously and compare their spectral data. Also, one can select a single or multiple strains and compare metabolite distributions in a single window. These features can be used to distinguish characteristic metabolites in each strain from frequently appearing compounds and discover novel metabolites.

SEARCH FOR STRUCTUALLY UNIQUE COMPOUNDS AND DISTINCT METABOLITE GROUPS BASED ON THE DATABASE AND MP PLOT

A search of the spectral database and MP plot was performed to discover structurally unique compounds, and three new metabolites, verticilactam (**1**) [23], and spirotoamides A (**2**) and B (**3**) were discovered and isolated (Fig. 3). They were isolated and purified easily in a single HPLC step from the

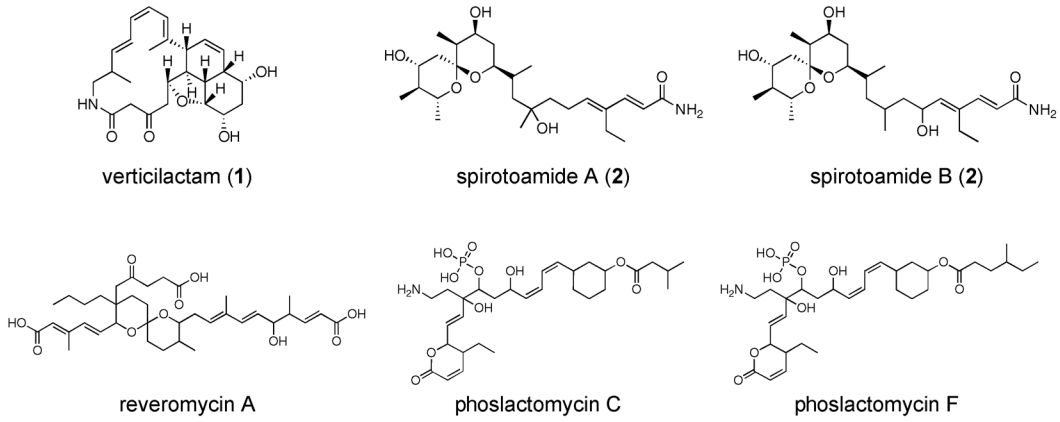


Fig. 3 Structures of verticilactam (**1**), spirotoamides A (**2**) and B (**3**), and identified compounds.

related fraction, suggesting that some fractions were well separated and primarily contained a single metabolite. Also, we compared the MP plots between actinomycetes to find distinct metabolite groups and identified strain-specific metabolites.

Verticilactam (1), a 16-membered macrolactam with an unprecedented β-keto-amide moiety, isolated from the fraction library of *Streptomyces spiroverticillatus*

In the fraction library of *S. spiroverticillatus* JC-8444, one fraction contained a peak with a λ_{max} value of 270 (sh) and 280 nm and an *m/z* value of 428 $[M + H]^+$, as shown in Fig. 4. This strain produces tautomycin [24,25], which is a member of a class of polyketides and has a 6,6-spiroacetal core structure and a unique 2,3-dialkylmaleic anhydride moiety [26]. The UV and mass spectra of the peak, however, were not identical to those of tautomycin, and a search of the spectral database revealed that the compound had not been isolated.

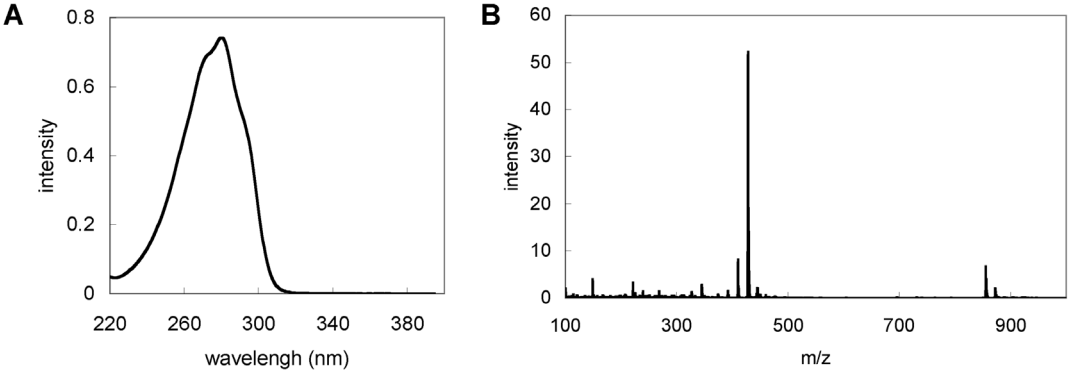


Fig. 4 UV absorption (**A**) and mass (**B**) spectra of **1**.

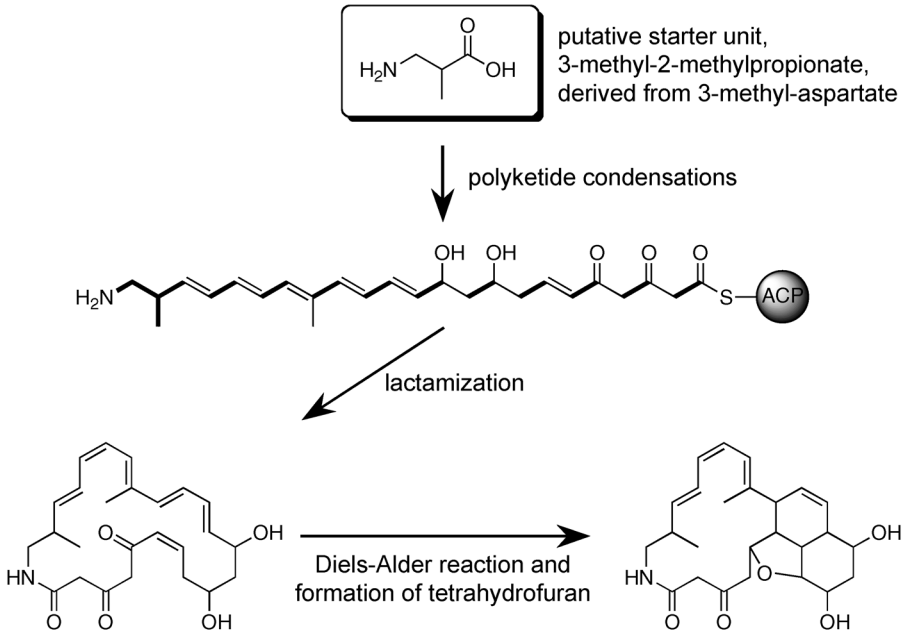
Thus, we decided to isolate the compound. The fraction was purified by C_{18}-HPLC by isocratic elution with acetonitrile/water, yielding compound **1** as a colorless amorphous solid.

The molecular formula of compound **1** was determined to be $C_{25}H_{33}NO_5$ by high-resolution mass spectrometry (HRMS). The planar structure was determined by analysis of the 1D and 2D NMR spec-

tra. The geometries of the double bonds were deduced, based on coupling constants in the ^{1}H NMR spectrum and ^{13}C NMR chemical shift values of the related carbons. The relative stereochemistry around the cyclohexane was assigned, based on the nuclear Overhauser effect correlated spectroscopy (NOESY) data and typical ^{1}H NMR chemical shift values on the cyclohexane. The cyclohexane and cyclohexene were determined to be in chair and pseudochair forms, respectively.

Based on these results, the structure of verticilactam (**1**) was determined, as shown in Fig. 3. Verticilactam (**1**) is also a polyketide, but its structure is unrelated to that of tautomycin. Moreover, **1** had an unusual β-keto-amide moiety in the 16-membered macrolactam skeleton. The compound having a β-keto-amide in a macrolactam was the first reported as a natural product and also had not been reported as a synthetic compound.

The biosynthesis pathway of **1** was proposed, as shown in Scheme 2, in which verticilactam (**1**) is biosynthesized by type I polyketide synthase (PKS). Based on its structural similarity to the 20-membered macrolactam vicenistatin [27], a 3-methylaspartate might be utilized as a starter unit, followed by polyketide condensation, which produces a putative post-PKS product, a 24-membered lactam. It might be modified by potential Diels–Alder cycloaddition; tetrahydrofuran formation via C-9 oxidation by P450 follows, producing verticilactam.



Scheme 2 Proposed biosynthesis pathway of **1**.

The MP plot of the *S. spiroverticillatus* fraction library is shown in Fig. 5A, in which dot 'e' in Fig. 5B at 17.9 min of retention time and *m/z* value of 428 $[M + H]^{+}$ is verticilactam (**1**) (Fig. 5E). Nearby, there are 2 dots (dots c and d in Fig. 5B) with the same *m/z* value. In addition, their UV spectra are identical to that of **1**, as shown in Figs. 5C, D, and E. These observations suggest that the compounds at dots c and d are structural isomers of **1**, with slightly polar properties, as deduced from the shorter retention times on the plot.

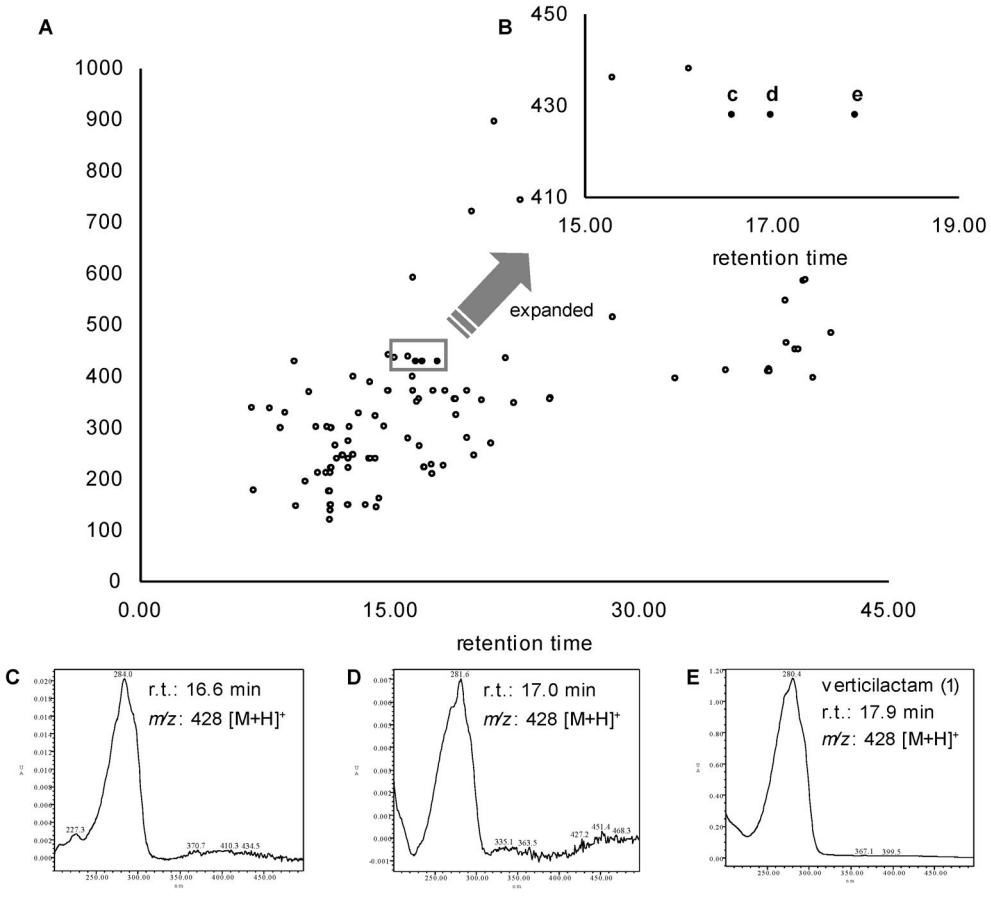


Fig. 5 MP plot for *S. spiroverticilatus* fraction library. **A:** MP plot, **B:** expansion of MP plot, **C:** spectral data of dot c, **D:** spectral data of dot d, **E:** spectral data of dot e (verticilactam).

Spirotoamides A (2) and B (3), spiroacetal polyketides isolated from the fraction library of *Streptomyces griseochromogenes*

In the fraction library of *S. griseochromogenes* JC82-1223, which produces the polyketide compound tautomycetin [28,29], a fraction contained a peak that had a λ_{max} value of 260 nm and an *m/z* value of 468 $[M + H]^+$, as shown in Fig. 6. The next fraction contained a peak with identical UV and mass spectra. However, these peaks had disparate retention times by LC/MS analysis. A search of the spectral database revealed that these compounds were not identical to tautomycetin and had not been isolated. Thus, these fractions were purified by C_{18}-HPLC, yielding compounds **2** and **3** as pale yellow amorphous solids. The structures of **2** and **3** were determined in an extensive analysis of spectroscopic data, including NMR and mass spectral data (Fig. 3).

The molecular formula of compound **2** was determined to be $C_{26}H_{45}NO_6$ by HRMS. The IR spectrum indicated the presence of hydroxy groups and an amide group. The planar structure was elucidated by analysis of the 1D and 2D NMR spectra. The presence of a 6,6-spiroacetal moiety was established, based on the typical ^{13}C NMR chemical shift value for the acetal carbon [30–35] and correlations in the rotating frame Overhauser effect spectroscopy (ROESY) spectrum. The geometries of both double bonds were confirmed, based on the coupling constant in the ^1H NMR spectrum and the ^{13}C NMR chemical shift value. The relative stereochemistry of the 6,6-spiroacetal structure was exam-

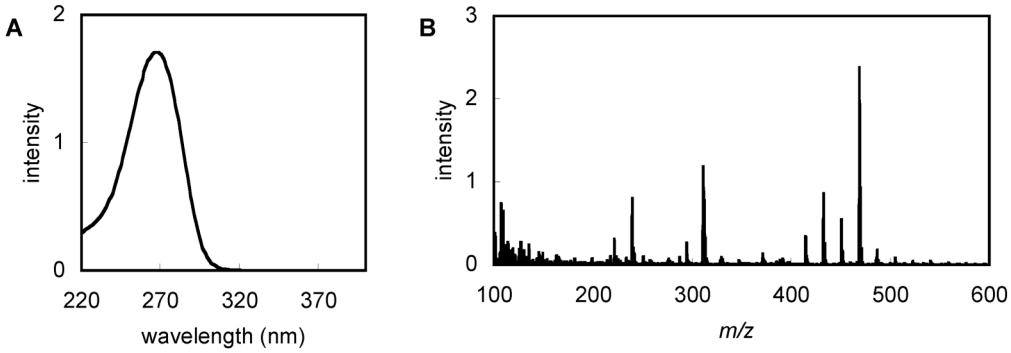
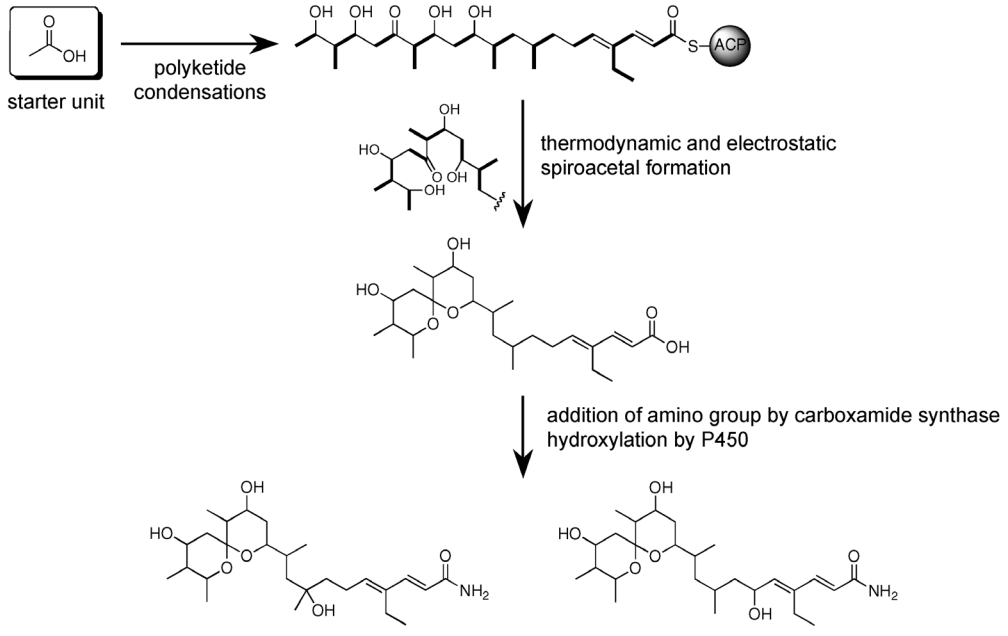


Fig. 6 UV absorption (**A**) and mass (**B**) spectra of **2**.

ined using the ROESY correlations and coupling constants in the ^{1}H NMR spectrum. Both tetrahydropyrans assumed chair forms, and the configuration at C-15 was determined to be *S**.

Compound **3** had the same molecular formula ($C_{26}H_{45}NO_6$) as **2** by HRMS. The ^{1}H and ^{13}C NMR spectra were similar to those of **2**, indicating that **3** was a structural isomer of **2**, with the difference of a substitution of the hydroxy group at C-9. The compound was determined to be spirotoamide B, as shown in Fig. 3, by analysis of the NMR data, and the stereochemistry was confirmed to be the same as that of **2**.

The proposed biosynthetic pathways of spirotoamides A (**2**) and B (**3**) are shown in Scheme 3. The polyketide backbone is expected to be synthesized by type I PKS. The putative dihydroxy ketone might be involved in forming the spiroacetal core structure, based on the biosynthesis of tautomycin [36] and avermectin [37]. Recently, the biosynthesis of reveromycin A, which also has a 6,6-spiroacetal core structure (Fig. 3), was reported, and revJ was identified as the enzyme that catalyzed the formation of the spiroacetal core from the dihydroxy ketone precursor [38].



Scheme 3 Proposed biosynthesis pathway of **2** and **3**.

Based on these findings, we checked for the existence of a revJ homolog in *S. griseochromogenes* by Southern hybridization using a *revJ* probe and confirmed that this strain did not have a revJ homolog, suggesting that the spiroacetal core was synthesized thermodynamically from a dihydroxy ketone, as in tautomycetin and avermectin biosynthesis. The transfer of an amino group by a carboxamide synthase and hydroxylation by P450 might be involved in the formation of compounds **2** and **3**.

The MP plot for the fraction library of *S. griseochromogenes* is shown in Fig. 7, in which four dots appear side by side with the same *m/z* values (dots c~f in Fig. 7B). Dots e and d are spirotoamides A and B. The remaining two dots are unknown, but they have identical UV absorption spectra as those of **2** and **3**, suggesting that they are also spirotoamides and structural isomers of **2** and **3**.

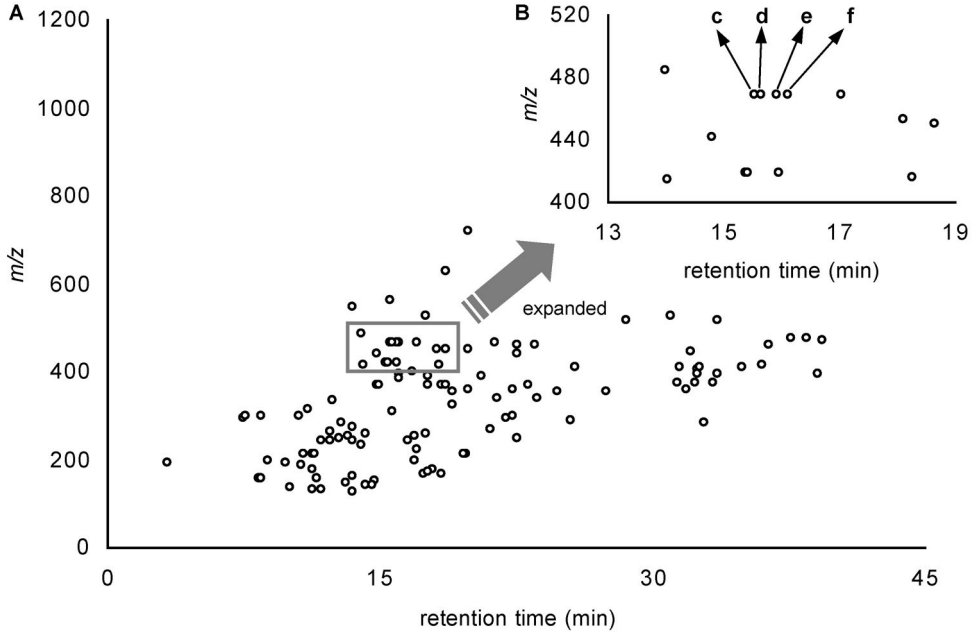


Fig. 7 MP plot for *S. griseochromogenes* fraction library. **A:** MP plot, **B:** expansion of MP plot, **dot c:** unknown spirotoamide derivative, **dot d:** spirotoamide B, **dot e:** spirotoamide A, **dot f:** unknown spirotoamide derivative.

Search for a distinctive metabolite group through a comparison of MP plots

Structurally related compounds have essentially similar physicochemical properties, implying that their dots are expected to appear in proximity and cluster on the MP plot, like spirotoamides on the plot in Fig. 7. Thus, we compared MP plots for 9 actinomycetes (Fig. 8). Some plots had an enclosed area, which indicated dots showing distinct distribution.

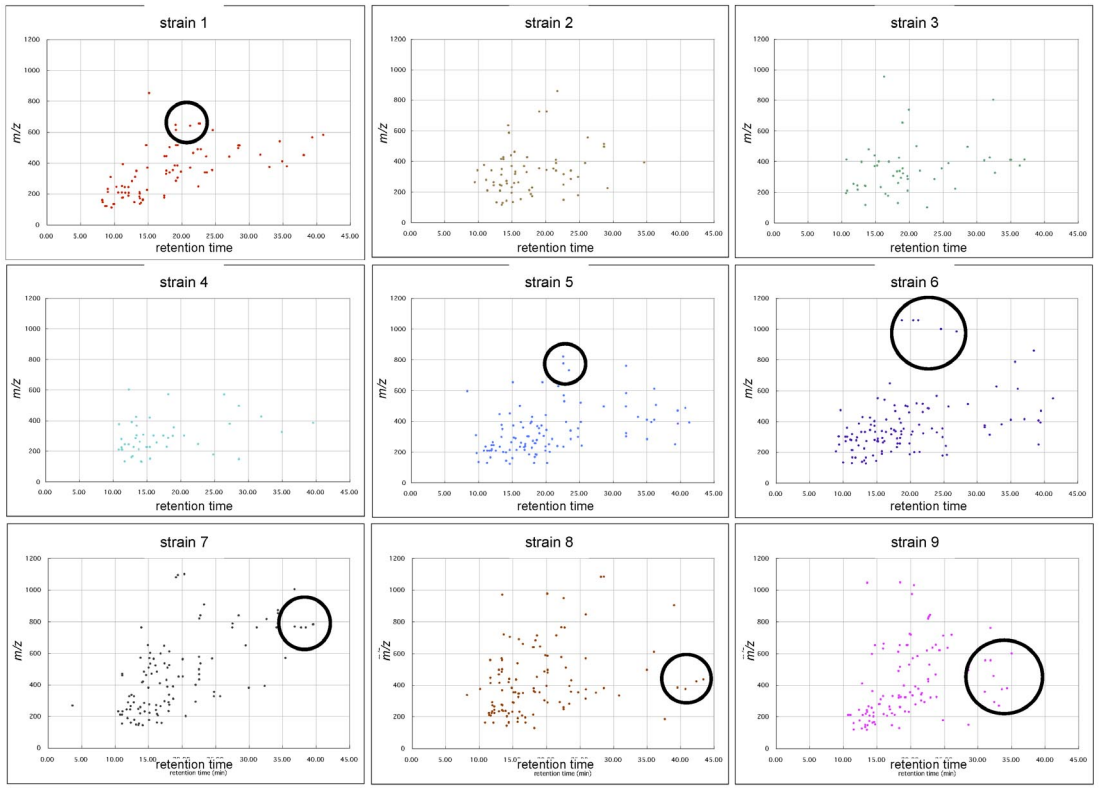


Fig. 8 Comparison of MP plot. Enclosed area showed a characteristic distribution for each strain.

The MP plot for strain 1, which was plotted for the fraction library of *Actinomadura azurea*, had the enclosed area, as shown in Fig. 9A. This strain produces the polyether compounds cationomycins [39,40]. In the circle, there were four dots (Fig. 9B) with identical UV absorption spectra and approximate *m/z* values, as shown in Figs. 9C~F, indicating that they are analogs. However, the UV spectra were not identical to those of cationomycins. Moreover, the mass spectra implied that they had a phosphate group on the structure by typical fragmenting patterns for phosphate ester compounds.

Based on these observations, we searched a database by UV and mass spectra, and two dots (c and e) were identified as phoslactomycins C and F [41,42], respectively (Fig. 3). However, the two remaining dots (d and f) were not identified as phoslactomycins, suggesting that they are novel phoslactomycin derivatives. This is the first report that *A. azurea* produces phoslactomycins.

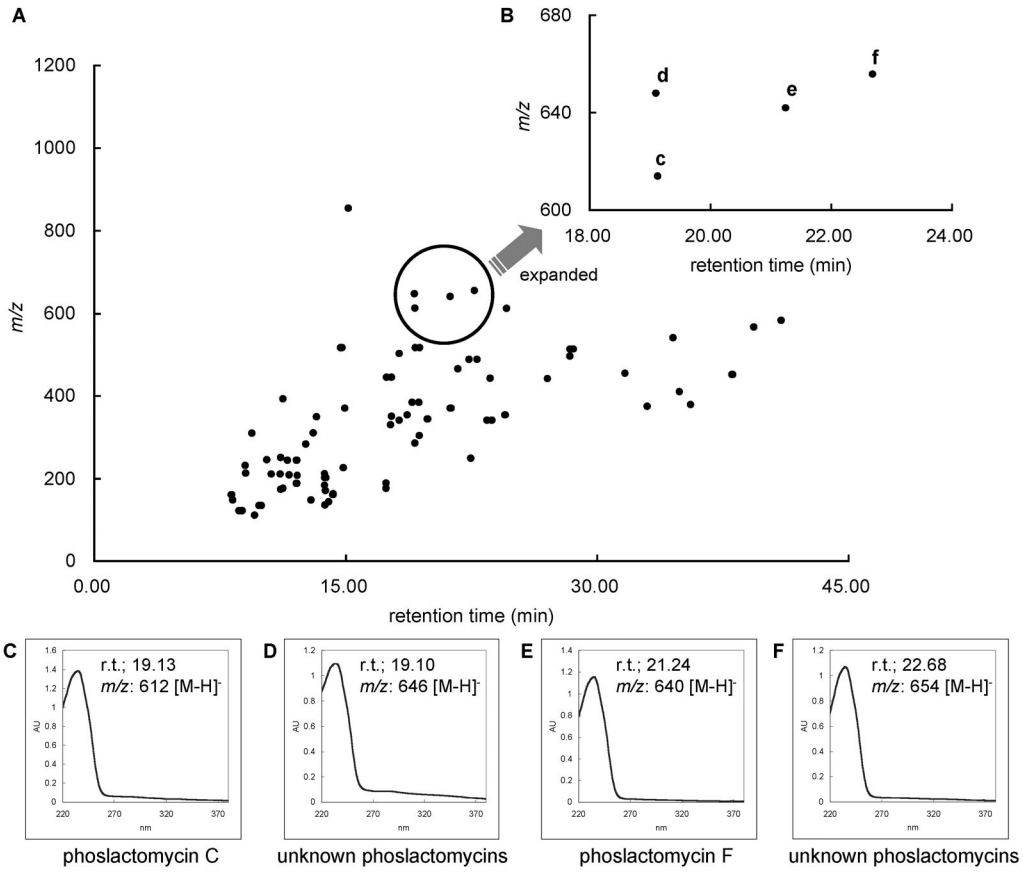


Fig. 9 MP plot for strain 1 in Fig. 8. **A:** MP plot for strain 1, **B:** expansion of MP plot, **C:** spectral data of dot c (phoslactomycin C), **D:** spectral data of dot d, **E:** spectral data of dot e (phoslactomycin F), **F:** spectral data of dot f.

CONCLUSIONS

We constructed a microbial metabolite fraction library, comprising 6480 fractions from 22 microbial strains, by systematic separation, which was developed as a simple and highly reproducible method that is based on routine chromatography. Each fraction in the library was analyzed by PDA-LC/MS to construct a spectral database and MP plot, which visualized and compared metabolites on a 2D area by physicochemical property.

Three new metabolites, verticilactam and spirotoamides A and B, were discovered using a combination of a database search and the MP plot and isolated from the fraction library in a single HPLC purification step. Verticilactam has an unusual β-keto-amide moiety on a 16-membered macrolactam skeleton, which is the first example having the β-keto-amide moiety. In contrast, the spirotoamides have a highly substituted 6,6-spiroacetal core structure and a carboxamide moiety. Moreover, we revealed that *A. azurea* had the capacity to produce phoslactomycins, based on our comparison of metabolite distribution on the MP plots and spectral database searches.

These results suggest that our methodology in constructing a fraction library with the MP plot and spectral database is a promising means of discovering and isolating novel compounds and revealing the potential of microorganisms that synthesize valuable secondary metabolites. Moreover, the fraction library can be used to generate new drug candidates as a part of RIKEN NPDepo.

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