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Citation	Chemical and pharmaceutical bulletin, 72(1), 80-85 https://doi.org/10.1248/cpb.c23-00776
Issue Date	2024-01-18
Doc URL	https://hdl.handle.net/2115/92020
Type	journal article
File Information	72_c23-00776.pdf



Regular Article

Five New Analogs of Streptogramin Antibiotic Viridogrisein Isolated from *Streptomyces niveoruber*

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Received November 11, 2023; accepted December 13, 2023

Five new viridogriseins B–F were isolated from *Streptomyces niveoruber*, along with viridogrisein and griseoviridin which belong to streptogramin family antibiotics. A combination of liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis and the advanced Marfey's method elucidated the structures of viridogriseins B–F, each featuring distinct constituent amino acids. Consistent with other streptogramin family antibiotics, these viridogrisein analogs exhibited potent antibacterial activity against *Staphylococcus aureus*. Furthermore, equimolar mixtures of each viridogrisein analog and griseoviridin inhibited the growth of *S. aureus* more potently than each analog treatment alone. Finally, an *in vitro* functional analysis of SgvY, encoded in the viridogrisein biosynthetic gene cluster, revealed that SgvY detoxifies viridogrisein against *S. aureus* by linearization. Considering that viridogrisein is not autotoxic to *S. niveoruber*, SgvY likely contributes to the self-resistance system against viridogrisein in *S. niveoruber*.

Key words viridogrisein, antibiotic, biosynthesis, depsipeptide, streptogramin, self-resistant system

Introduction

Natural product-based antibiotics have made valuable contributions to human health and well-being.¹⁾ However, a new challenge has arisen with the emergence of antimicrobial-resistant (AMR) bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and carbapenem-resistant *Enterobacteriales* (CRE).^{2,3)} To effectively combat bacterial infections, including those involving AMR strains, ongoing searches for novel antibacterial natural products are urgently required.

One of the last defense lines against serious bacterial infections is streptogramin family antibiotics.^{4,5)} The streptogramin family includes two subclasses, type A and type B. Some cyclic polyunsaturated macrolactones such as pristinamycin IIA^{6,7)} and mikamycin A⁸⁾ belong to type A, while depsipeptides such as pristinamycin IA⁷⁾ and mikamycin B⁹⁾ belong to type B. For their bactericidal activities, the type A and type B compounds both disrupt the elongation process of protein synthesis, by binding to a peptidyl transferase center and a peptide exit tunnel in the ribosome, respectively.^{10,11)} The combination of the two natural products, type A and type B, enhances the antibacterial activity by approx.100-fold relative to each individual natural product. Indeed, dalbapristin and quinupristin, semi-synthetic analogs of pristinamycin IIA and pristinamycin IA, respectively, are used in combination to combat vancomycin-resistant *Enterococci* (VRE) in clinical use.¹²⁾ Given their unique and potent activities even against Gram-positive bacteria resistant to front-line antibiotics, finding new streptogramins will facilitate the development of effective antibiotics.

Herein, we isolated griseoviridin, as well as viridogrisein and its five new analogs B–F, from *Streptomyces niveoruber* by antibacterial activity-guided screening. These natural products exhibited potent antibacterial activity against *Staphylococcus aureus*. Based on the griseoviridin biosynthetic gene cluster (BGC), SgvY encoded in the griseoviridin BGC apparently participates in the self-resistance system by linearizing griseoviridin in *S. niveoruber*.

Results and Discussion

To obtain novel antibacterial natural products, we screened about 100 bacterial culture extracts using an agar plate assay. In the course of screening, the culture extracts of *Streptomyces niveoruber* NBRC 15428 showed strong growth inhibition against *Kocuria rhizophila* NBRC 103217. Bioassay-guided fractionation of the crude extract from an 11 L culture of *S. niveoruber* yielded an antibacterial metabolite (**1**) with *m/z* 879.46, as a major constituent. A database search revealed that an *m/z* value of 879.46 is consistent with viridogrisein, previously isolated from *Streptomyces griseus*,¹³⁾ *Streptomyces* sp. CNS-575,¹⁴⁾ and *Streptomyces griseoviridis*.¹⁵⁾ Viridogrisein is a heptadepsipeptide with the amino acid sequence L-threonine (L-Thr)-D-leucine (D-Leu)-*cis*-4-hydroxy-D-proline (D-*cis*-Hyp)-*N*-methylated glycine (NMeGly)-*N*-methylated β -methyl-L-leucine (NMe β Me-L-Leu)-L-alanine (L-Ala)-*N*-methylated L-phenylglycine (NMe-L-Phg), blocked by a 3-hydroxy-2-pyridine carboxyl group (Hpc) at the *N*-terminus. The β -hydroxyl group of L-Thr is lactonized with the carboxylic group of NMe-L-Phg to form a depsipeptide. Although the ¹H-NMR spectrum of **1** was superimposable on that of viridogrisein from a previous report,¹⁴⁾ it showed complicated signals derived from three conformers in various NMR solvents. Since it was difficult to assign each signal to viridogrisein, an liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was conducted with the *seco*-**1** methylester, obtained by the methanolysis of **1**. From the major ion peak at *m/z* 911.50 chosen as the parent ion peak, the *b*-series of fragment ions were detected at *m/z* 449.45, 520.54, 661.52 and 732.50, revealing that the amino acid sequence of **1** was the same as that of viridogrisein (Supplementary Fig. S1). The configurations of the constituent amino acids in **1** were determined by applying the advanced Marfey's method^{16–18)} to the hydrolysate. The configurations of Thr, Ala, NMePhg, Leu, and Hyp in **1** were assigned as L-, L-, L-, D-, and D-*cis* amino acids, respectively. Although no standards of NMe β MeLeu were commercially available, the previous report assigned the configu-

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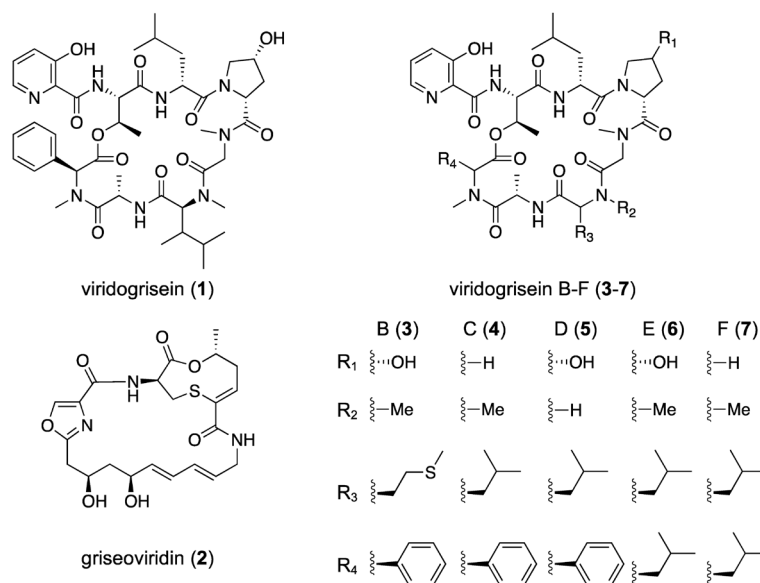


Fig. 1. Chemical Structures of Viridogrisein (1), Viridogriseins B–F (3–7), and Griseoviridin (2)

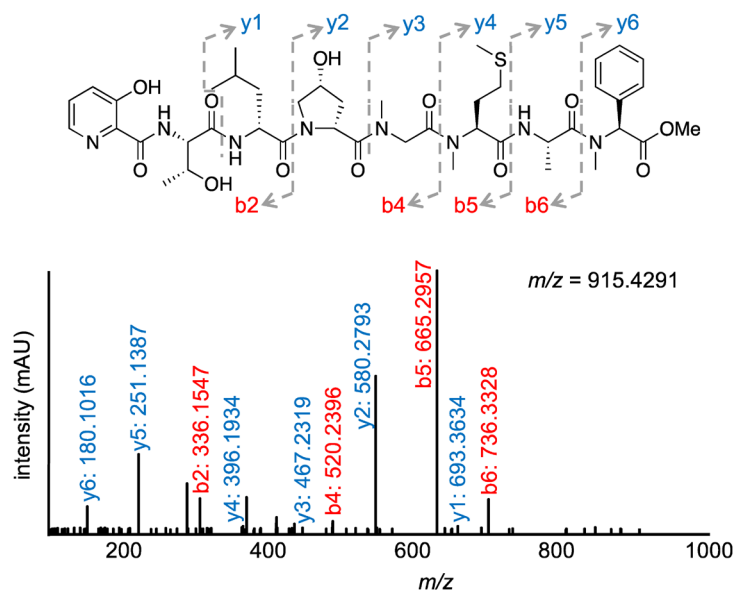


Fig. 2. ESI-TOF MS/MS Analysis of *seco*-Viridogrisein B Methyl ester

An ion peak of $[M + H]^+$ at m/z 915.4291 was chosen as a parent ion.

ration at the α position of $NMe\beta MeLeu$ in viridogrisein as the L-form, by the elution order of N^{α} -(5-fluoro-2,4-dinitrophenyl)-L-leucinamide (FDLA) derivatives.¹⁴ Consistently, the L-FDLA derivative of $NMe\beta MeLeu$ in **1** eluted earlier than the D-FDLA derivative, indicating that the configuration at the α position of $NMe\beta MeLeu$ is L. Although the configuration at the β position of $NMe\beta MeLeu$ has not been reported, and remains unknown, our NMR and LC-MS/MS analyses and application of the advanced Marfey's method indicated that **1** is identical to viridogrisein from *S. griseoviridis* (Fig. 1). *S. griseoviridis* is also known to produce a polyketide, griseoviridin (**2**), which works together with viridogrisein to exert synergistic antibacterial activity.¹⁹ In line with this, **2** was also isolated from *S. niveoruber* alongside **1** in this study (Fig. 1).

During further investigations for antibacterial metabolites from the crude extract of *S. niveoruber*, five additional natu-

ral products were purified. These five compounds exhibited the same characteristic UV absorptions as that of the Hpc at the *N*-terminus of **1**, indicating that these compounds are viridogrisein analogs, viridogriseins B–F (**3–7**). Since their molecular weights did not match any known compounds in databases, these analogs appear to be new. Viridogrisein B (**3**) has the molecular formula $C_{42}H_{58}N_8O_{11}S$, established based on the high resolution electrospray ionization (HR-ESI)MS data (m/z 883.4030 $[M + H]^+$, Calcd. 883.4019). As the amount of **3** was too low to obtain adequate NMR data, we conducted an LC-MS/MS analysis with the *seco*-**3** methyl ester. From the major ion peak at m/z 915.43 chosen as the parent ion peak, the y -series of fragment ions were detected at m/z 693.36, 580.28, 467.23, 396.19, 251.14 and 180.10, revealing the amino acid sequence Thr-Leu (or isoleucine (Ile))-Hyp- $NMeGly$ - $NMeMet$ -Ala- $NMePhg$, blocked by Hpc at the *N*-terminus

Table 1. Antibacterial Activity of the Compounds Obtained in This Study against *Escherichia coli* (Top) and *Staphylococcus aureus* (bottom)

<i>Escherichia coli</i>		
	IC ₅₀ (μM)	IC ₅₀ (μM) + 2
1	>100	Not tested
<i>Staphylococcus aureus</i>		
	IC ₅₀ (μM)	IC ₅₀ (μM) + 2
1	0.28	0.47
2	>20	
3	4.25	0.90
4	2.31	0.79
5	3.97	0.81
6	>20	>2
7	>20	>2
8	>20	>2

Each IC₅₀ value was obtained in triplicate. The left row shows IC₅₀ for single-agent treatment of each compound. The right row shows IC₅₀ for equimolar mixture of each viridogrisein and griseoviridin.

(Fig. 2 and Supplementary Table S5). The *b*-series of fragment ions from the same parent ion peak was also consistent with the amino acid sequence deduced above. However, it was impossible to determine whether Leu or Ile was the second residue from the *N*-terminus, based on the MS/MS data alone. Therefore, by conducting the advanced Marfey's method for the hydrolysate of **3** with six possible second amino acids, D/L-Leu, D/L-Ile, and D/L-*allo*-Ile as standards, D-Leu was unambiguously detected. At the same time, the stereochemistries of the other constituent amino acids, Thr, Hyp, NMeMet, Ala, and NMePhg, were determined to be the L-, D-*cis*-, L-, L-, and L-forms, respectively (Fig. 1).

The chemical structures of viridogriseins C–F (**4**–**7**) were determined by LC-MS/MS analysis and the advanced Marfey's method, in the same way as **3** (Supplementary Fig. S2–S5). From the results, viridogriseins C–F were identified as new viridogrisein analogs (Fig. 1). Throughout **3**–**7**, the D-*cis*-Hyp, NMeβMe-L-Leu, and NMe-L-Phg of **1** were replaced by amino acid residues with relatively similar molecular sizes, such as D-Pro, L-Leu, NMe-L-Leu, and NMe-L-Met.

Having obtained these metabolites (**1**–**7**) by bioassay-guided screening against *K. rhizophila*, we next examined their antibacterial activities. Viridogrisein (**1**) did not suppress the growth of *Escherichia coli* even at a 100 μM concentration, whereas viridogrisein (**1**) and its analogs B (**3**), C (**4**), and D (**5**) exhibited potent antibacterial activity against *S. aureus* NBRC100910 with IC₅₀ values of 0.28, 4.25, 2.31, and 3.97 μM, respectively (Table 1). In addition, equimolar mixtures of each viridogrisein analog **3**, **4**, and **5** with **2** exhibited 2.9- to 4.9-fold more potent activity than the individual treatment with each viridogrisein analog (Table 1). However, no synergistic effect was observed with the equimolar mixture of **1** and **2**, likely due to the inherently potent activity of **1**. From these results, even the small changes between the chemical structures of these compounds **1**–**7** affected the potency of the synergistic bactericidal activity, indicating that careful tuning would be required to generate effective antibiotic cocktails of streptogramin family antibiotics.

Through the structure elucidations of **3**–**7**, the six viridogrisein family compounds from *S. niveoruber* showed a wide

variety of chemical structures. To understand the chemical diversity of viridogrisein analogs in terms of their biosynthesis, we investigated the BGC of **1** in the *S. niveoruber* genome. In *S. griseoviridis* NRRL2427, the biosynthetic genes of **1** and **2**, composed of a large polyketide synthase (PKS)/non-ribosomal peptide synthetase (NRPS)-type BGC named *sgv* (NCBI accession number JX508597), are clustered and the expression is regulated by a γ-butyrolactone-mediated regulatory system.²⁰⁾ Consistent with *S. griseoviridis*, *S. niveoruber* encodes a comparable BGC, based on an antiSMASH prediction. In both BGCs, the backbone of viridogrisein is proposed to be biosynthesized by four NRPSs, SgvD1–D4, with several modification enzymes for providing the nonproteinogenic amino acids 3-Hpa, βMeLeu, and Phg (Fig. 3). Especially, given that the third, fifth, and seventh amino acids from the *N*-terminus of viridogriseins were variable, module 4 in SgvD3 and modules 6 and 8 in SgvD4 were likely responsible for the chemical diversity. Since competitive activity-based protein profiling (ABPP) has shown that some A domains could accept amino acids with molecular sizes comparable to the original substrates,²¹⁾ the structural variation of compounds **3**–**7** would be attributable to the promiscuity of the A domains in modules 4, 6, and 8.

While investigating the enzymes encoded in the BGC, we found SgvY, with an uncharacterized *in vitro* function. SgvY is a homolog of Vgb, encoded in a pathogenic *S. aureus* clinical isolate that tolerates virginiamycin, a viridogrisein derivative from *Streptomyces virginiae*.^{22,23)} Vgb detoxifies quinupristin, a synthetic virginiamycin analog, by cleaving the ester linkage between the carboxylic acid at the C-terminus and the hydroxyl group of a Thr to form dehydrobutyrine (Dhb).^{22,23)} Our *in vitro* analysis revealed that recombinant SgvY can convert viridogrisein into a linear peptide (**8**), replacing Thr with Dhb (Fig. 4 and Supplementary S8b, 8c). Notably, compound **8** did not inhibit the growth of *S. aureus*, even at a 20 μM concentration (Supplementary Fig. S7d). Since Ju and co-workers previously reported that the viridogrisein production level in the Δ*sgvY* mutant was consistent with that of wild-type, SgvY was not considered to be involved in the self-resistance systems against viridogrisein.²⁰⁾ Instead, the self-resistance systems reportedly arose from SgvT1, one of transporters encoded in *sgv*, by thwarting intracellular viridogrisein accumulation.²⁴⁾ However, our functional analysis of SgvY suggested that SgvY, at least in part or synergically, contributes to the self-resistance systems by converting viridogrisein to a harmless linear peptide form.

In this study, we discovered five antibacterial natural products, viridogriseins B–F (**3**–**7**), along with viridogrisein (**1**) and griseoviridin (**2**), from *S. niveoruber*. These viridogrisein analogs exerted potent bactericidal activities against some Gram-positive bacteria. Equimolar mixtures of griseoviridin and each viridogrisein analog exhibited more potent antibacterial activities against *S. aureus* than single-agent treatments with each viridogrisein analog. In addition, SgvY, encoded in the *sgv* BGC, detoxified **1** by linearizing the cyclic peptide in a unique manner, suggesting that SgvY is partially involved in the self-resistance system against viridogriseins in *S. niveoruber*.

Experimental

Bacterial Strains *Streptomyces niveoruber* NBRC 15428,

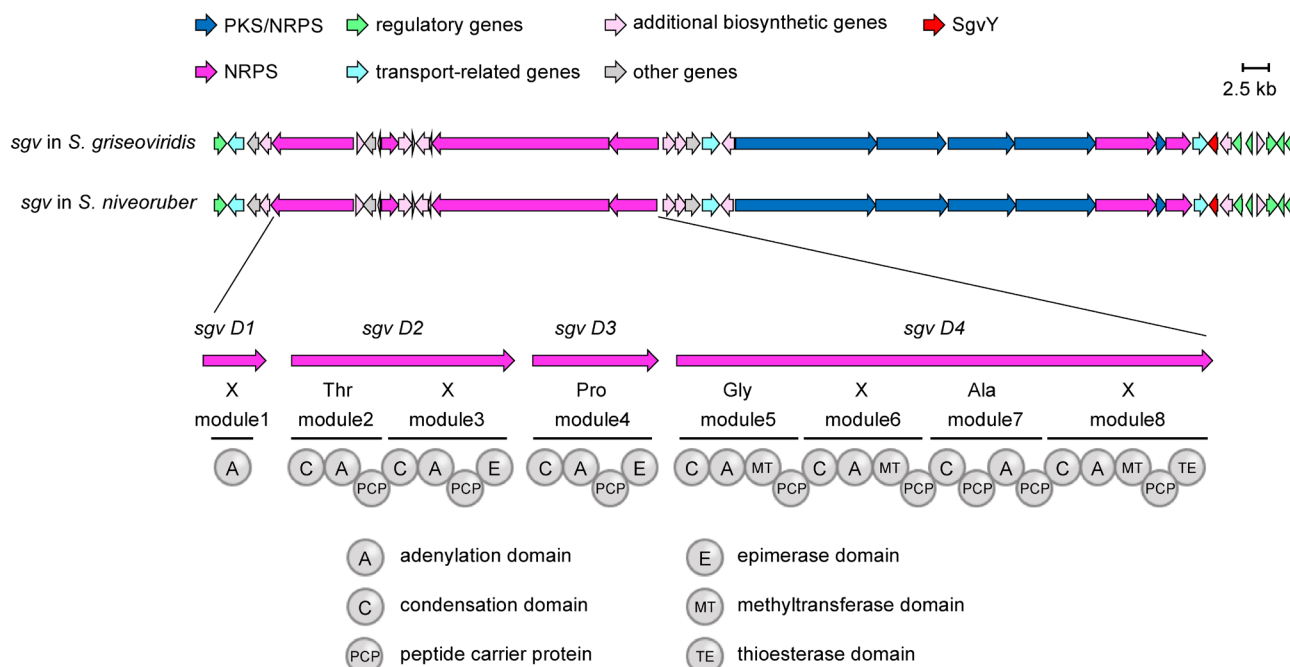


Fig. 3. Biosynthetic Gene Clusters of Griseoviridin and Viridigriseins in *S. griseoviridis* and *S. niveoruber*

All modules embedding several kinds of domains are shown in the enlarged view of *sgv D1–D4*. Amino acids on the modules indicate the substrate of the A domain predicted by antiSMASH. X represents an unpredicted substrate by antiSMASH. The accession number of the BGC in *S. griseoviridis* is JX508597.

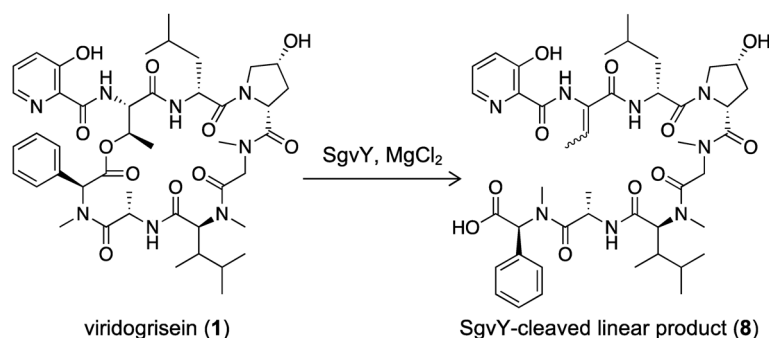


Fig. 4. Chemical Structure of a Linear Product (8) Enzymatically Converted from Viridigrisein (1) by SgvY

Kocuria rhizophila NBRC 103217, *Staphylococcus aureus* NBRC 100910 and screening strains (about 100 strains) were used throughout.

Materials and General Procedure All chemicals were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), Kanto chemicals (Tokyo, Japan) and Nacalai Tesque (Kyoto, Japan).

TSB was used to culture *S. niveoruber* and screening strains. Other media used: MH for evaluating antibacterial activity of the isolated compounds, 2xYT for cultivation of *E. coli* DH5 α and BL21(DE3). ¹H- and ¹³C-NMR spectra were collected at the Faculty of Pharmaceutical Sciences, Hokkaido University in a JEOL ECZ 500R (500MHz for ¹H-NMR). Chemical Shifts are denoted in δ (ppm) relative to residual solvent peaks as internal standards (CDCl₃: δ_H 7.26, δ_C 77.0, dimethyl sulfoxide (DMSO)-*d*₆: δ_H 2.50, δ_C 39.5). HRMS analyses were performed with Thermo Scientific LTQ Orbitrap XL (LTQ XL coupled with LTQ QR) spectrometer. Optical rotations were recorded on a JASCO P-1030 polarimeter. LC-MS experiments were performed with amaZon SL-NPC (Bruker Daltonics) or LCMS-2020 (Shimadzu) coupled with a

SHIMADZU HPLC system equipped with a LC-20AD intelligent pump. ESI-TOF MS/MS analyses were conducted by Thermo Scientific LTQ Orbitrap XL (LTQ XL coupled with LTQ QR) spectrometer or amaZon SL-NPC (Bruker Daltonics) coupled with a SHIMADZU HPLC system equipped with a LC-20AD intelligent pump. Optical density at 600nm (OD600) was measured using a UV-VIS spectrophotometer UV-1900 (Shimadzu).

Culture Extracts of Screening Bacteria About 100 distinct bacterial strains were streaked out individually onto TSB plates and grown at 30°C for a couple of days. The resulting colonies from each agar plate were inoculated to 5mL of TSB in a test tube. After growth at 30°C/320rpm for a couple of days, 50 μ L of the saturated culture was inoculated to a test tube, carrying 5mL of TSB medium. After growth at 30°C/320rpm for 3 d, the culture was separated to a cell pellet and a supernatant by centrifugation. The supernatant was applied directly to a Sep-Pak C18 Plus Short Cartridge (Waters, U.S.A.). After application of the supernatant, the column was washed with 1mL each of 0, 50 and 100% MeOH in water. The 50 and 100% MeOH fractions were combined and dried

in vacuo. On the other hand, the cell pellet was resuspended with 3 mL of acetone. The unsuspended debris was removed by centrifugation and the acetone fraction was collected and dried *in vacuo*. The 50 and 100% MeOH fractions from the supernatant and the acetone extract from the cell pellet were combined and dried *in vacuo* to prepare for the agar-plate assay.

Agar-Plate Assay *K. rhizophila* was streaked out onto a MH agar plate and grown at 30 °C overnight. The resulting colonies were inoculated to 5 mL of MH in a 15 mL falcon tube and grown at 30 °C/210 rpm for 1 d. The culture extracts were dissolved in MeOH to be 10 mg/mL. Paper discs were infused with 30 μ L of each sample, allowed to dry, and placed on a soft MH agar (1% agar) plate containing 1% saturated culture of *K. rhizophila*. The agar plate was incubated at 30 °C for 16 h and then inspected visually.

Purification and Structure Elucidation of Viridogriseins *S. niveoruber* was streaked out onto a TSB agar plate and grown at 30 °C for 2 d. The resulting colonies were inoculated to 10 mL of TSB in a test tube. After growth at 30 °C/320 rpm for 2 d, 1 mL of the saturated culture was inoculated to 110 $\times$ 500 mL baffled flask carrying 100 mL of TSB medium. After growth at 30 °C/140 rpm for 72 h, the cells spun down to separate the supernatant and the pellet. Then, the supernatant was extracted with ethyl acetate and concentrated *in vacuo* to obtain a crude residue. The residue was dissolved in MeOH and subjected to a Sephadex LH-20 gel filtration column. After monitoring fractions exhibit antibacterial activity, the fractions were combined and concentrated *in vacuo*. The residue was subjected onto Cosmosil 140C₁₈-OPN (Nacalai Tesque) column and eluted with 0, 20, 40, 60, 80, and 100% MeOH in water. The 80 and 100% MeOH fractions were combined and dried *in vacuo*. The material was subjected onto HPLC on COSMOSIL 5C₁₈-MS-II column 10.0 ID $\times$ 250 mm (nacalai tesque) with H₂O/MeCN (1/1) as mobile phase to yield viridogrisein (4.2 mg, t_R : 34.4 min). In the same condition, viridogrisein B–D (3–5) were obtained (t_R : 23.0, 19.9 and 41.5 min, respectively). Viridogrisein E (6) and F (7) were eluted at 24.9 and 28.6 min, respectively under the condition (COSMOSIL 5C₁₈-MS-II column 10.0 ID $\times$ 250 mm (nacalai tesque) with H₂O/MeCN (42/58) as mobile phase).

The molecular formula of viridogrisein was established as C₄₄H₆₂N₈O₁₁ based on the HR-ESI-MS data (m/z 879.4620 [M + H]⁺, Calcd. 879.4611). The planer structure was determined by analysis of ¹H-NMR, correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond connectivity (HMBC) spectra. Absolute configurations were determined using the advanced Marfey's method with an L-FDLA reagent. Viridogrisein (approx. 0.1 mg) was hydrolyzed with 6 M HCl (0.1 mL) at 100 °C for 2 h. The reaction mixture was dried *in vacuo*. The hydrolysate dissolved in H₂O (50 μ L) was treated with 1 M NaHCO₃ (20 μ L) and D- or L-FDLA (1% (w/v) in acetone, 100 μ L). The mixture was stirred at 60 °C for 1 h. The reaction mixture was quenched with 1 M HCl (20 μ L), evaporated, and then dissolved in MeOH. LC-MS analysis of the samples was performed on a COSMOSIL 5C₁₈ MS-II 3.0 ID $\times$ 150 mm (nacalai tesque) with a gradient elution of H₂O/MeCN (9/1) to H₂O/MeCN (4/6) over 45 min (both water and MeCN containing 0.05% trifluoroacetic acid). LC-MS analysis of a hydroxy proline conjugated with L-FDLA was performed with H₂O/MeCN

(77/23) (both water and MeCN containing 0.1% formic acid). Further LC-MS analysis of a leucine conjugated with L-FDLA was performed with H₂O/MeCN (60/40) (both water and MeCN containing 0.1% formic acid). The synthetic standard for an N-methylated-D/L-phenylglycine were synthesized according to the previous report.²⁵ The result of the advanced Marfey's analysis is shown in Supplementary Table S5 and Figure S6.

To determine chemical structures of viridogriseins B–F (3–7), each molecular formula of viridogriseins B–F (3–7) was established based on the HR-ESI-MS data (Supplementary Table S2). Then, MS/MS analysis was conducted with *seco*-viridogrisein B–F methylester. To obtain *seco*-viridogrisein methylesters, 0.1 mg of viridogriseins B–F in each microtube were dissolved in 300 μ L of 7 M NH₃ in MeOH. The solutions were incubated at 100 °C for 1 d in microwave (initiator, Biotage). After incubation, the samples were dried *in vacuo* and resuspended in MeOH, which was subjected to MS/MS analysis. All absolute configurations of viridogriseins B–F (3–7) were determined using the advanced Marfey's method as described above. The standard for an N-methylated-L-methionine and an N-methylated-D-methionine were synthesized according to the previous report.²⁶ The results of the advanced Marfey's analysis are shown in Supplementary Table S5 and Fig. S6.

To yield griseoviridin, the fractions of the gel filtration containing griseoviridin, as judged by ESI-MS, were combined, concentrated *in vacuo*. Then the material was subjected onto HPLC on COSMOSIL Cholesterol column 10.0 ID $\times$ 250 mm (Nacalai Tesque) with H₂O/MeCN (4/1) as mobile phase to yield griseoviridin (2.6 mg, t_R : 27.8 min). The molecular formula of griseoviridin was established as C₂₂H₂₈N₃O₇ based on the HR-ESI-MS data (m/z 478.4980 [M + H]⁺, Calcd. 478.4975). The planer structure was determined by analysis of the NMR spectra.

Evaluation of Antibacterial Activity The antibacterial effect of the isolated compounds was assessed using *Staphylococcus aureus* NBRC 100910 and *Escherichia coli* DH5 α . *S. aureus* was streaked out onto an MH agar plate and grown at 30 °C overnight. The resulting colonies were inoculated to 5 mL of MH medium in a 15 mL falcon tube. After growth at 30 °C/210 rpm for 1 d, the saturated culture was inoculated to 100 μ L/well of MH medium in a 96-well plate to an initial OD₆₀₀ of 0.05. Cells were exposed to purified compounds dissolved in DMSO (2.5% (v/v)) at 30 °C for 16 h. After incubation, the turbidity was measured at OD₆₀₀ by using a microplate reader (infinite M200, TECAN). Antibacterial activity against *E. coli* was conducted in the same way as *S. aureus*.

Expression and Purification of the Recombinant SgvY To construct an expression plasmid for recombinant SgvY with a His tag fused at its C-terminus, the *sgvY* gene was amplified with 0.5 μ M specific primers (*sgvY*_pET28a_Fw and *sgvY*_pET28a_Rv, Supplementary Table S1), using DNA polymerase 1 U KOD One[®] PCR Master mix -Blue- (TOYOBO) and the *S. niveoruber* genome as a template. Then, the PCR product was inserted into the pUC19 vector (TaKaRa) and transformed into *E. coli* DH5 α chemically competent cells. The plasmid pUC19-*sgvY*-His was extracted and the sequence was confirmed. The plasmid was double digested with NdeI/HindIII and ligated with the pET-28a(+) expression vector (Novagen), and transformed into *E. coli* BL21(DE3) chemical-

ly competent cells. A single colony of *E. coli* BL21(DE3) harboring the plasmid pET-28a(+)-sgvY-His was picked and inoculated into 5 mL of 2xYT medium with 50 µg/mL kanamycin, and the culture was incubated overnight at 37°C/230 rpm. The 2 mL of the overnight culture was inoculated into 200 mL of 2xYT medium and cultured at 37°C/140 rpm for 3 h. To induce protein expression, isopropyl-1-thio-β-D-galactopyranoside (IPTG) at a final concentration of 1 mM was added to the cultures, and then the cells were cultivated at 16°C for 15 h. The cells were collected by centrifugation at 4000 rpm for 15 min at 4°C and resuspended in 20 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl (buffer A). The cells were sonicated, and the lysate was centrifuged at 15000 rpm for 10 min at 4°C. The supernatant was loaded onto a Ni-NTA agarose column (Wako) equilibrated with buffer A. After washing the resin with buffer A and buffer A containing 50 mM imidazole, SgvY-His was eluted with buffer A containing 500 mM imidazole. The elution fraction was desalted using a Vivaspin 6 10000 MWCO centrifugal filter. Protein concentration was measured by Bio-Rad protein assay kit. Protein purification was checked by SDS-PAGE (Supplementary Fig. S8a).

Functional Analysis of the Recombinant SgvY One hundred microliters of reaction mixture containing 50 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.5), 1 mM MgCl₂, 15 µM SgvY and 200 µM viridogrisein (**1**) dissolved in DMSO was incubated at 30°C for 15 h. Reaction was quenched by 500 µL of MeOH and the centrifuged at 15000 rpm for 10 min at 4°C. The supernatant was analyzed by LC-MS. LC-MS analysis of the samples was performed on a COSMOSIL 5C₁₈ MS-II 2.0 ID × 100 mm (nacalai tesque) with a gradient elution of H₂O/MeCN (9/1) to H₂O/MeCN (4/6) over 22 min (both water and MeCN containing 0.1% formic acid).

Acknowledgments We thank K. Matsuda (Hokkaido University) for providing bacterial strains to investigate antibacterial natural products.

Funding This work was partly supported by Hokkaido University, Global Facility Center (GFC), Pharma Science Open Unit (PSOU), funded by MEXT under “Support Program for Implementation of New Equipment Sharing System,” the Takeda Science Foundation, SUNTORY Foundation for life sciences, Japan Science and Technology Agency (JST, ACT-X grant no. JPMJAX22BO), Japan Agency for Medical Research and Development (AMED Grant nos. JP23ama121039, JP23ak0101163) and Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan (JSPS KAKENHI Grant Nos., 22H05128, 21H02635, 20K16023).

Author Contributions A.Y. and T.W. designed the experiments. T.H. isolated the secondary metabolites. A.Y., T.H. and T.W. performed structure determination of the secondary

metabolites. T.H. performed antibacterial assay and *in vitro* functional analysis of SgvY. A.Y., T.H. and T.W. analyzed the data. A.Y. and T.W. wrote the paper.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials This article contains supplementary materials.

References

- 1) Hutchings M. I., Truman A. W., Wilkinson B., *Curr. Opin. Microbiol.*, **51**, 72–80 (2019).
- 2) Brown E. D., Wright G. D., *Nature* (London), **529**, 336–343 (2016).
- 3) Chellat M. F., Raguz L., Riedl R., *Angew. Chem. Int. Ed.*, **55**, 6600–6626 (2016).
- 4) Cocito C., *Microbiol. Rev.*, **43**, 145–192 (1979).
- 5) Mast Y., Wohlleben W., *Int. J. Med. Microbiol.*, **304**, 44–50 (2014).
- 6) Kingston D. G. I., Kolpak M. X., *J. Am. Chem. Soc.*, **102**, 5964–5966 (1980).
- 7) Laforest H., Fourgeaud M., Richet H., Lagrange P. H., *Antimicrob. Agents Chemother.*, **32**, 1094–1096 (1988).
- 8) Arai M., Karasawa K., Nakamura S., Yonehara H., Umezawa H., *J. Antibiot.*, **11**, 14–20 (1958).
- 9) Watanabe K., Yonehara H., Umezawa H., Sumiki Y., *J. Antibiot.*, **13**, 291–292 (1960).
- 10) Li Q., Pellegrino J., Lee D. J., *et al.*, *Nature* (London), **586**, 145–150 (2020).
- 11) Noeske J., Huang J., Olivier N. B., Giacobbe R. A., Zambrowski M., Cate J. H. D., *Antimicrob. Agents Chemother.*, **58**, 5269–5279 (2014).
- 12) Allington D. R., Rivey M. R., *Clin. Ther.*, **23**, 24–44 (2001).
- 13) Sheehan J. C., Zachau H. G., Lawson W. B., *J. Am. Chem. Soc.*, **80**, 3349–3355 (1958).
- 14) Haste N. M., Perera V. R., Maloney K. N., Tran D. N., Jensen P., Fenical W., Nizet V., Hensler M. E., *J. Antibiot.*, **63**, 219–224 (2010).
- 15) Bartz R. Q., Ehrlich J. D., Smith M. R., United States Patent Office, patented 27 February, 1962.
- 16) Fujii K., Ikai Y., Oka H., Suzuki M., Harada K., *Anal. Chem.*, **69**, 5146–5151 (1997).
- 17) Fujii K., Ikai Y., Mayumi T., Oka H., Suzuki M., Harada K., *Anal. Chem.*, **69**, 3346–3352 (1997).
- 18) Marfey P., *Carlsberg Res. Commun.*, **49**, 591–594 (1984).
- 19) Hosoda K., Koyama N., Kanamoto A., Tomoda H., *Molecules*, **24**, 1495 (2019).
- 20) Xie Y., Wang B., Liu J., Zhou J., Ma J., Huang H., Ju J., *ChemBioChem*, **13**, 2745–2757 (2012).
- 21) Kasai S., Konno S., Ishikawa F., Kakeya H., *Chem. Commun.*, **51**, 15764–15767 (2015).
- 22) Mukhtar T. A., Koteva K. P., Hughes D. W., Wright G. D., *Biochemistry*, **40**, 8877–8886 (2001).
- 23) Korczynska M., Mukhtar T. A., Wright G. D., Berghuis A. M., *Proc. Natl. Acad. Sci. U.S.A.*, **104**, 10388–10393 (2007).
- 24) Xie Y., Ma J., Qin X., Li Q., Ju J., *Microb. Cell Fact.*, **16**, 177 (2017).
- 25) Jakob A. K. M. H., Sundermann T. R., Klein C. D., *Bioorg. Med. Chem. Lett.*, **29**, 1913–1917 (2019).
- 26) Aurelio L., Box J. S., Brownlee R. T. C., Hughes A. B., Sleebs M. M., *J. Org. Chem.*, **68**, 2652–2667 (2003).