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ORIGINAL ARTICLE

Maniwamycins: new quorum-sensing inhibitors against *Chromobacterium violaceum* CV026 were isolated from *Streptomyces* sp. TOHO-M025

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Quorum sensing is an important microbial signaling system that controls the expression of many virulence genes. Maniwamycins C–F, new compounds and quorum-sensing inhibitors, were isolated from the culture broth of *Streptomyces* sp. TOHO-M025 using a silica gel column and preparative HPLC. The structures of maniwamycins were elucidated by spectroscopic analyses, including NMR. The compounds each have an azoxy moiety. All maniwamycins inhibited violacein synthesis, which is controlled by quorum sensing, in *Chromobacterium violaceum* CV026.
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INTRODUCTION

Quorum sensing is an important signal transduction system among bacteria that is mediated by substances excreted from the bacterial cells into the environment.[1] The system regulates many virulence genes, including those that code for biofilm formation,[2] swarming motility,[3] antibiotic biosynthesis[4,5] and virulence factor production.[6,7] A new strategy to control infection is to discover drugs that inhibit microbial virulence without inhibiting growth, because this presents less selective pressure for the generation of resistance.[8,9]

Disruption of the quorum-sensing system in pathogenic *Burkholderia cepacia* and *Burkholderia pseudomallei* resulted in reduced pathogenicity in murine and hamster infections.[10,11] Erythromycin inhibits biofilm formation of *Pseudomonas aeruginosa* below the MIC.[12] LED209 is a non-toxic compound that does not inhibit pathogen growth; however, it markedly inhibits the virulence of several pathogens *in vitro* and *in vivo*.[13] Therefore, compounds that inhibit quorum sensing have great potential for the use in the treatment of bacterial infectious diseases.

On the basis of this new strategy of 'anti-infective drugs,' a screening system was established to search for quorum-sensing inhibitors. Piericidin E, a novel compound, was recently discovered from the culture broth of *Streptomyces* sp. TOHO-Y209.[14] During our screening program, an actinomycete strain, TOHO-M025, was found to produce compounds that inhibited quorum sensing in *Chromobacterium violaceum* CV026.[15] Activity-guided purification led to the discovery of novel compounds designated maniwamycins C–F (Figure 1). By structural elucidation, the fundamental skeleton of maniwamycins C–F was found to be similar to that of maniwamycin B, which has an azoxy moiety.[16]

In this study, the taxonomy of the producing strain, fermentation, isolation and structural elucidation of maniwamycins are described.

RESULTS

Taxonomy of the producing strain

Vegetative mycelia and aerial mycelia grew abundantly on a yeast extract–malt extract agar and other media, and did not show fragmentation into coccoid forms or bacillary elements. The vegetative mycelia were pale yellow in color, whereas the aerial mycelia were white. From scanning electron micrographs of the strain (Figure 2), the mature spore chains were observed to be spiral, and each had >20 spores per chain. The spores were cylindrical in shape, $0.8–1.0 \times 1.0–1.2$ μm in size and had smooth surfaces. Whirls, sclerotic granules, sporangia and flagellate spores were not observed. Melanoid and other soluble pigments were not produced.

The 1065 base sequences of the partial 16S ribosomal RNA were aligned. This sequence is available in GenBank, EMBL and DDBJ under the accession no. LC033903.

On the basis of the taxonomic properties and 16S ribosomal RNA sequence, strain TOHO-M025 was identified as belonging to the genus *Streptomyces*.

Fermentation and isolation

A seed culture of the strain TOHO-M025 was grown on yeast malt medium (0.4% yeast extract, 1.0% malt extract, 0.4% glucose, 2.0% agar, pH 7.0). The flasks were shaken on a rotary shaker at 27 °C for 3 days. The seed culture (3 ml) was transferred into flasks containing 300 ml of the yeast malt production medium. The fermentation was carried out at 27 °C for 10 days on a shaker, and the production of maniwamycins was observed at day 3 after inoculation, reaching a maximum at day 8 (Figure 3).

Strain TOHO-M025 was cultivated in the yeast malt medium (10 l) at 27 °C for 10 days. The cultured broth was extracted with 10 l of ethyl acetate. Ethyl acetate extract (966 mg) was applied to a silica gel

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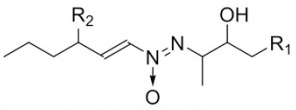

	R_1	R_2
maniwamycin C	H	OH
maniwamycin D	H	H
maniwamycin E	H	H
maniwamycin F	$CONH_2$	H

Figure 1 Structures of maniwamycins.

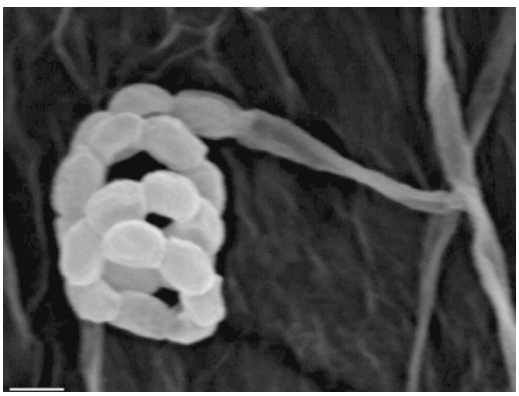

Figure 2 Scanning electron micrograph of *Streptomyces* sp. TOHO-M025. Bar represents 1 μm.

column (30 g of silica gel (0.063–0.210 mm), Wako Co., Osaka, Japan), and eluted with hexane–ethyl acetate (50:50), ethyl acetate, chloroform–methanol (100:5) and methanol. Active fractions eluted with hexane–ethyl acetate (50:50) and chloroform–methanol (100:5) were concentrated to yield crude materials I (297 mg) and II (86 mg), respectively. Crude material I was separated by centrifugation into two oil layers, and then the upper, yellow oil (160 mg) was purified by HPLC (column, Shim-pack PREP-ODS (Shimadzu Co., Kyoto, Japan), 20 × 250 mm; mobile phase, 65% methanol; flow rate, 8.0 ml min^{-1}; detection, UV at 240 nm). Under these conditions, maniwamycins C (18.4 mg), D (5.6 mg) and E (29.6 mg) were eluted at their peaks, with retention times of 11.0, 25.5 and 27.0 min, respectively. Crude material II was purified by HPLC under the same conditions to yield maniwamycin F (8.4 mg), with a retention time of 14.0 min.

Structural elucidation

Physico-chemical properties of maniwamycins are summarized in Table 1. The molecular formula of maniwamycin E was determined to be $C_{10}H_{20}N_2O_2$ on the basis of high-resolution FAB-MS (HR FAB-MS) measurement. A characteristic band at 1462 cm^{-1} in the IR spectrum suggested the presence of an azoxy group. The ^{13}C NMR spectrum showed 10 resolved peaks, which were classified into three methyl carbons, three methylene carbons, two sp^3 methine carbons and two sp^2 methine carbons based on the analysis of the DEPT spectra (Table 2). The ^1H NMR spectrum (in $CDCl_3$) displayed 20 proton signals, and the ^{15}N NMR spectrum displayed two signals (Table 3). The connection between the proton and carbon atoms was established by the HMQC spectrum (Table 2). Analyses of the ^1H–^1H COSY revealed the presence of two partial structures as shown in Figure 4. Furthermore, ^1H–^{15}N long-range couplings of ^2J and ^3J observed in the ^1H–^{15}N HMBC spectrum (Figure 5) revealed a connection between fragments I and II as follows: the cross-peaks

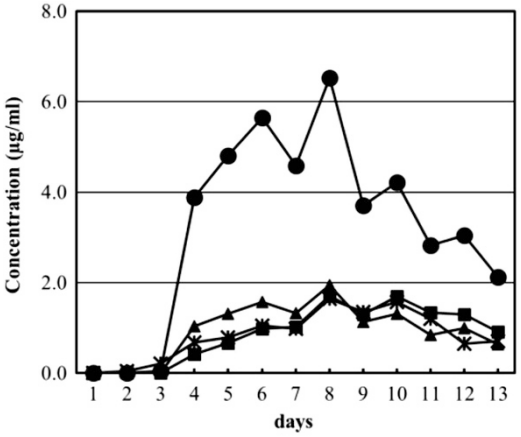

Figure 3 A time course of maniwamycin production by *Streptomyces* sp. TOHO-M025. Square, maniwamycin C; triangle, maniwamycin D; circular, maniwamycin E; asterisk, maniwamycin F.

from H-2 (δ 3.92) to N-1, from H-3 (δ 4.14) to N-1 and N-2, from H_3-4 (δ 1.12) to N-1, from H-1′ (δ 6.89) to N-2 (δ 329) and from H-2′ (δ 6.96) to N-2 suggested connections between partial structures I and II via azoxy linkages. IR absorption at 3355 cm^{-1} suggested the presence of a hydroxy residue as a connection at the C-3 position in the structure. In addition, the J coupling constant (14 Hz) between H-1′ and H-2′ indicated a 1*E*-configuration. The structure of maniwamycin E that was elucidated is shown in Figure 1.

The molecular formula of maniwamycin D was established as $C_{10}H_{20}N_2O_2$ by HR FAB-MS. The ^1H, ^{13}C and ^{15}N NMR spectra of maniwamycin D were quite similar to those of maniwamycin E, except at C-2 and C-3 (Tables 2 and 3). The planar structure of maniwamycin D was established by COSY, HMQC and ^1H–^{15}N HMBC; therefore, the planar structure of maniwamycin D was found to be the same as that of maniwamycin E. Moreover, the ^1H and ^{13}C NMR spectra of maniwamycins D and E, using the solvent dimethyl sulfoxide-*d6*, were compared with the previously reported spectra of maniwamycin B[17] (Table 4). However, the optical rotation of maniwamycin E was different from that of maniwamycin D (Table 1). Thus, the structure of maniwamycin D was determined to be the same as *epi*-maniwamycin E.

The molecular formula of maniwamycin C was established as $C_{10}H_{20}N_2O_3$ by HR FAB-MS, with one hydroxy residue believed to bind to maniwamycin E. The ^1H, ^{13}C and ^{15}N NMR spectra of maniwamycin D were quite similar to those of maniwamycin E, except at position C-3′ (Tables 2 and 3). The C-3′ position was a methine carbon, and a chemical shift moved it downfield. Therefore, the structure of maniwamycin C that was elucidated was the same as 3′-hydroxy-maniwamycin E.

The molecular formula of maniwamycin F was established as $C_{11}H_{21}N_3O_3$ by HR FAB-MS. The ^1H, ^{13}C and ^{15}N NMR spectra of maniwamycin F were quite similar to those of maniwamycin E, except at position C-1 (Tables 2 and 3). The cross-peak from H_2–N (δ 5.59, 5.95) to N (δ 105) was detected in the ^1H–^{15}N HMQC. The cross-peaks from H_2–N to C-1 (δ 38.6) and C–R_2 (δ 174.5), from H-2 (δ 4.13) to C-1 and C–R_2, and from H_2-1 (δ 2.40) to C-2 (δ 70.8) and C–R_2 were detected in the ^1H–^{13}C HMBC. The signal at 1663 cm^{-1} was detected in the IR absorption. These data indicated the presence of an amide residue at the C-1 position in the structure. Therefore, the structure of maniwamycin F that was elucidated was the same as 1-amide-maniwamycin E.

Table 1 Physicochemical properties of maniwamycins

	Maniwamycin C	*Maniwamycin D*	*Maniwamycin E*	*Maniwamycin F*
Appearance	Brown oil	Brown oil	Brown oil	Brown oil
Molecular formula	$C_{10}H_{20}N_2O_3$	$C_{10}H_{20}N_2O_2$	$C_{10}H_{20}N_2O_2$	$C_{11}H_{21}N_3O_3$
MW	216	200	200	243
Pos. FAB-MS (*m/z*)	217 $[M+H]^+$	201$[M+H]^+$	201$[M+H]^+$	244$[M+H]^+$
HR Pos. FAB-MS (m/z)				
Obsd.	217.1533	201.1604	201.1602	244.1662
Calcd.	217.1552 ($C_{10}H_{21}N_2O_3$)	201.1603 ($C_{10}H_{21}N_2O_2$)	201.1603 ($C_{10}H_{21}N_2O_2$)	244.1661 ($C_{11}H_{22}N_3O_3$)
$[\alpha]_D^{25}$	−11.9 (*c* 0.18, CH_3OH)	−38.6 (*c* 0.03, CH_3OH)	−100.2 (*c* 0.1, CH_3OH)	−32.8 (*c* 0.85, CH_3OH)
UV λ_{max}^{MeOH} nm (log ε)	231 (4.02)	234 (4.02)	234 (4.02)	234 (4.09)
IR ν_{max}^{KBr} cm^{-1}	3349, 2923, 1631, 1589, 1465, 1376	3390, 2829, 1650, 1473, 1028	3355, 2924, 2855, 1669, 1462, 1028	3392, 1663, 1475, 1434, 1320
Solubility				
Soluble	CH_3OH, EtOAc	CH_3OH, EtOAc	CH_3OH, EtOAc	CH_3OH, EtOAc
Insoluble	H_2O, *n*-hexane	H_2O, *n*-hexane	H_2O, *n*-hexane	H_2O, *n*-hexane

Table 2 ^1H and ^{13}C NMR data of maniwamycins in $CDCl_3$

	Maniwamycin C		*Maniwamycin D*		*Maniwamycin E*		*Maniwamycin F*		*Epi-maniwamycin B*[23]
No.	δ_C *(M)*	δ_H *(Int, M, J = Hz)*	δ_C *(M)*	δ_H *(Int, M, J = Hz)*	δ_C *(M)*	δ_H *(Int, M, J = Hz)*	δ_C *(M)*	δ_H *(Int, M, J = Hz)*	δ_H *(Int, M, J = Hz)*
1	18.8 (q)	1.21 (3H, d, *J*=7)	20.1 (q)	1.20 (3H, d, *J*=7)	18.7 (q)	1.19 (3H, d, *J*=7)	38.6 (t)	2.40 (2H, d, *J*=3)	1.22
2	69.7 (d)	3.94 (1H, dq, *J*=4, 7)	70.6 (d)	3.87 (1H, dq, *J*=7, 7)	69.6 (d)	3.92 (1H, dq, *J*=4, 7)	70.8 (d)	4.13 (1H, m)	3.91–4.02
3	60.9 (d)	4.16 (1H, dq, *J*=4, 7)	61.5 (d)	4.02 (1H, dq, *J*=7, 7)	60.6 (d)	4.14 (1H, dq, *J*=4, 7)	59.5 (d)	4.20 (1H, dq, *J*=6, 7)	4.18
4	10.7 (q)	1.14 (3H, d, *J*=7)	12.8 (q)	1.12 (3H, d, *J*=7)	10.7 (q)	1.12 (3H, d, *J*=7)	11.9 (q)	1.18 (3H, d, *J*=7)	1.16
1′	138.0 (d)	7.11 (1H, d, *J*=13)	137.9 (d)	6.91 (1H, d, *J*=13)	137.8 (d)	6.89 (1H, d, *J*=14)	137.8 (d)	6.89 (1H, d, *J*=14)	7.01
2′	136.2 (d)	6.98 (1H, dd, *J*=5, 13)	134.6 (d)	6.98 (1H, dt, *J*=7, 13)	134.6 (d)	6.96 (1H, dt, *J*=7, 14)	134.9 (d)	6.96 (1H, dt, *J*=7, 14)	6.91
3′	68.9 (d)	4.41 (1H, dt, *J*=5, 6)	28.1 (t)	2.20 (2H, dt, *J*=7, 7)	28.1 (t)	2.18 (2H, dt, *J*=7, 7)	28.1 (t)	2.19 (2H, dt, *J*=7, 7)	2.22
4′	39.1 (t)	1.61 (2H, m)	30.3 (t)	1.45 (2H, m)	30.3 (t)	1.44 (2H, m)	30.3 (t)	1.45 (2H, m)	1.24–1.52
5′	18.4 (t)	1.44 (2H, m)	22.2 (t)	1.35 (2H, m)	22.1 (t)	1.34 (2H, m)	22.2 (t)	1.35 (2H, m)	1.24–1.52
6′	13.8 (q)	0.94 (3H, t, *J*=7)	13.7 (q)	0.91 (3H, t, *J*=7)	13.7 (q)	0.89 (3H, t, *J*=7)	13.7 (q)	0.90 (3H, t, *J*=7)	0.92
R_1	—		—		—		174.5 (s)		
NH_2	—		—		—			5.59, 5.95 (brd)	

Abbreviation: M, multiplicity.
^1H NMR: 500 MHz in $CDCl_3$ (ref. 7.24 p.p.m., *J* value in Hz), ^{13}C NMR: 125 MHz in $CDCl_3$ (ref. 77.0 p.p.m.)

Table 3 ^{15}N NMR data of maniwamycins

	Maniwamycin C	*Maniwamycin D*	*Maniwamycin E*	*Maniwamycin F*
1 (–N)	359 p.p.m.	350 p.p.m.	353 p.p.m.	354 p.p.m.
2 (–NO)	328 p.p.m.	328 p.p.m.	329 p.p.m.	328 p.p.m.
$–NH_2$	—	—	—	105 p.p.m.

^{15}N NMR: 50 MHz in $CDCl_3$ (ref. 317 p.p.m., using pyridine as an external standard)

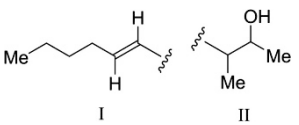

Figure 4 Partial structures of maniwamycin E.

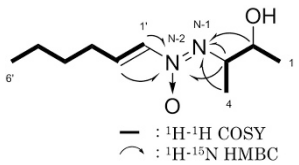

Figure 5 ^1H–^1H COSY and ^1H–^{15}N HMBC experiments with maniwamycin E.

Effect of maniwamycins on *C. violaceum* CV026 and other bacteria

We investigated the quorum-sensing inhibitory effects of maniwamycins on *C. violaceum* CV026 (Figure 6). The maniwamycins inhibited purple pigment (violacein) synthesis, controlled by quorum sensing, and this inhibitory effect was found to be dose dependent in the range of 0.01–1 mg ml^{-1}. Maniwamycins D and E showed greater activity than maniwamycins C and F. The IC_{50} of maniwamycin E was 0.12 mg ml^{-1}. The antimicrobial activities, the MICs, of the maniwamycins against *C. violaceum* CV026, *Staphylococcus aureus*

Table 4 ^{1}H and ^{13}C NMR data of maniwamycin D and E in DMSO-*d6*

	Maniwamycin D		*Maniwamycin E*		*Maniwamycin B*[17]	
No.	δ_C *(M)*	δ_H *(M, J = Hz)*	δ_C *(M)*	δ_H *(M, J = Hz)*	δ_C *(M)*	δ_H *(M, J = Hz)*
1	19.3 (q)	1.04 (d, *J*=6.5)	20.3 (q)	1.03 (d, *J*=6.0)	19.2 (q)	1.06 (d, *J*=6.5)
2	67.4 (d)	3.74 (m)	68.2 (d)	3.68 (m)	67.5 (d)	3.69 ~ 3.80 (m)
3	60.4 (d)	3.98 (quintet, *J*=6.5)	60.9 (d)	3.89 (quintet, *J*=6.5)	60.5 (d)	3.99 (quintet *J*=6.5)
4	11.6 (q)	0.98 (d, *J*=6.5)	12.4 (q)	1.03 (d, *J*=6.5)	11.6 (q)	0.99 (d, *J*=6.5)
1′	138.0 (d)	7.06 (d *J*=13.5)	138.1 (d)	7.07 (dt *J*=13.5)	138.1 (d)	7.08 (d, *J*=14.0)
2′	133.8 (d)	6.85 (dt *J*=13.5, 7.5)	134.0 (d)	6.85 (dt *J*=13.5, 7.5)	133.7 (d)	6.87 (dt *J*=14.0, 7.5)
3′	27.1 (d)	2.19 (dt, *J*=7.5, 8.5)	27.2 (d)	2.19 (dt, *J*=7.5, 7.5)	27.2 (d)	2.20 (q, *J*=7.5)
4′	29.8 (t)	1.41 (m)	29.9 (t)	1.40 (m)	29.9 (t)	1.24 ~ 1.47 (m)
5′	21.6 (t)	1.30 (m)	21.7 (t)	1.29 (m)	21.7 (t)	1.24 ~1.47 (m)
6′	13.6 (q)	0.88 (t, *J*=7.0)	13.6 (q)	0.87 (t, *J*=7.0)	13.6 (q)	0.89 (t, *J*=7.0)
OH		4.61 (d, *J*=5)		4.70 (d, *J*=5.5)		

Abbreviations: DMSO, dimethyl sulfoxide; M, multiplicity.
^{1}H NMR: 500 MHz in DMSO-*d6* (ref. 2.49 p.p.m., *J* value in Hz), ^{13}C NMR: 125 MHz in DMSO-*d6* (ref. 39.0 p.p.m.)

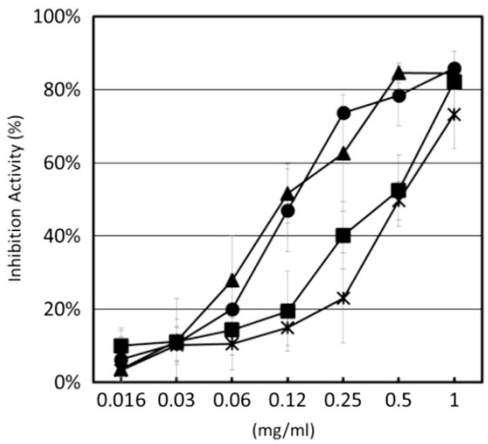

Figure 6 Effect of maniwamycins on violacein production in *Chromobacterium violaceum* CV026. Square, maniwamycin C; triangle, maniwamycin D; circular, maniwamycin E; asterisk, maniwamycin F.

ATCC25923, *Pseudomonas aeruginosa* ATCC27853, *Escherichia coli* ATCC25922, *Candida albicans* JCM1542 and *Aspergillus flavus* JCM1738 were $>1\ mg\ ml^{-1}$.

DISCUSSION

In this study, maniwamycins C–F were isolated from streptomycete metabolites, and each was found to contain an azoxy moiety. Maniwamycins A and B were first isolated from a *Streptomyces* sp. and described as having anti-fungal activity and lacked anti-bacterial activity.[17] There are a few natural compounds that contain an α,β-unsaturated azoxy moiety.[18] Among these are elaiomycin,[19] LL-BG872α,[20] jietacin[21] and azoxyalkene.[22]

The planar geometric structures of maniwamycins C–F were elucidated, but the absolute stereochemical configurations of these compounds remain undetermined. The NMR spectra of maniwamycins D and E were very similar to those of maniwamycin B and *epi*-maniwamycin B (Tables 2 and 4). Therefore, we conclude that the planar structures of these compounds were the same.

On the other hand, the optical rotations of maniwamycins D and E were − 38.6 and − 100.2, respectively (Table 1). The optical rotation of maniwamycin B has a (*2S,3S*) configuration of +108, whereas the optical rotation of synthetic 2-*epi*-maniwamycin B has a (*2R,3S*) configuration of +35.4.[17,23] Therefore, we estimated that the configurations of maniwamycins D and E were (*2S,3R*) and (*2R,3R*), respectively.

The position of the oxygen atom in the azoxy group of each maniwamycin was determined to be on the side of the olefin group by the proton chemical shift to the azoxy group and the UV absorption maximum.[24] In addition, the ^{15}N chemical shifts of two nitrogen atoms included in the azoxy group indicated this position.[25]

Moreover, the *syn–anti* configurations of the azoxy moieties in all isolated maniwamycins in this paper were determined to be the *Z*-form. In a previous report, ^{15}N chemical shifts of two nitrogen atoms with an *anti*-configuration were higher field than those in a *syn*-configuration.[25] All reported compounds of actinomycete origin have *Z*-form azoxy moieties.[18]

Maniwamycin B has anti-*C. albicans* IFM40001 activity at a concentration of $50\ \mu g\ ml^{-1}$,[15] but the compounds we isolated in this study had no effect on *Candida*. Moreover, maniwamycins C–F showed no anti-bacterial activity, as mentioned above. However, maniwamycins C–F were effective against the quorum sensing of *C. violaceum* CV026. Maniwamycins D and E showed stronger inhibition of quorum-sensing activity than maniwamycins C and F.

Several compounds have been reported to inhibit quorum sensing,[26] including patulin from *Penicillium*,[27] halogenated furanones from the marine alga *Delisea pulchra*,[28] azithromycin[29] and chloramphenicol.[30] However, to date, there are no reports on the quorum-sensing inhibitory activities of maniwamycin derivatives.

In conclusion, four new compounds, designated as maniwamycins C–F, were isolated from the culture broth of *Streptomyces* sp. TOHO-M025. They inhibited quorum sensing of *C. violaceum* CV026 without antimicrobial activity.

MATERIALS AND METHODS

General experimental procedures

Bacterial strain, TOHO-M025, was isolated from a soil sample collected in Iwaki, Fukushima, Japan, and was used for the production of maniwamycins C–F. *C. violaceum* CV026,[15] used to evaluate quorum-sensing inhibition activity, was kindly provided by Dr T. Ikeda (Utsunomiya University, Japan).

UV spectra were recorded on a spectrophotometer (GeneQuant1300, GE Healthcare Life Sciences, Little Chalfont, UK). IR spectra were recorded on a Fourier transform IR spectrometer (FT/IR-4100, JASCO, Tokyo, Japan). Optical rotations were measured with a digital polarimeter (P-2200, JASCO).

FAB-MS and HR FAB-MS spectra were recorded using a mass spectrometer (JMS-700 V, JEOL, Tokyo, Japan). The various NMR spectra were also determined with a spectrometer (JNM-ECA500, JEOL). 16S ribosomal RNA fragments of TOHO-M025 was amplified by PCR using the general bacterial 16S ribosomal RNA primers, 10F (5′-AGTTTGATCCTGGCTC-3′) and 1100R (5′-CAGGAAGGGTTGCGCT-3′). The DNA sequences of the amplified fragments were determined by cycle sequencing with the chain termination technique and dye-labeled dideoxynucleotides using a Genetic Analyzer 3500 (Applied Biosystems, Foster City, CA, USA).

Analysis of maniwamycin production

To determine the amount of maniwamycins in a culture broth, whole-culture broths (300 µl) were removed from flasks and extracted with ethyl acetate. The samples, dissolved in MeOH, were analyzed using a HPLC system under the following conditions: column, ODS-Tm80 (4.6 × 150 mm, TOSOH Co., Tokyo, Japan); flow rate, 0.8 ml min^{-1}; mobile phase, 40% CH_3CN/0.06% trifluoroacetic acid. Maniwamycins C, D, E and F were detected at 3.9, 13.6, 14.4 and 4.6 min, respectively.

Quorum-sensing inhibitory assay

The compounds were serially diluted with methanol. These dilutions were added to wells in microtiter plates and air dried over a clean bench until the methanol was completely evaporated. To each well, 200 µl of Luria-Bertani soft agar, *C. violaceum* CV026 and *N*-hexanoyl-L-homoserinelactone (Santa Cruz Biotech. Co., Santa Cruz, CA, USA) were added, and then the plates were incubated at 27 °C for 24 h. Each plate was incubated at 45 °C until it was dry, and then 100 µl of dimethyl sulfoxide was added and the plates were shaken for 2 h to extract violacein. The OD of the dimethyl sulfoxide extract was measured at 570 nm (OD_{570}).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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