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ORIGINAL ARTICLE

Naphthacemycins, novel circumventors of β-lactam resistance in MRSA, produced by *Streptomyces* sp. KB-3346-5. I. The taxonomy of the producing strain, and the fermentation, isolation and antibacterial activities

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Screening for circumventors of β-lactam resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) led us to find 17 novel antibiotics, naphthacemycins A_1–A_{11}, B_1–B_4 and C_1–C_2. The naphthacemycins were isolated from a cultured broth of *Streptomyces* sp. KB-3346-5 by repeated silica gel column chromatography and HPLC. Naphthacemycins enhanced imipenem activity 100–500 times against MRSA at 0.5 μg ml^{-1}, and naphthacemycins A_4–A_{11} themselves showed MIC_{50} values of 1–4 μg ml^{-1} against 22 MRSA strains.
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INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a causative microorganism of opportunistic infection that harms individuals in medical facilities who have compromised immune systems. Recently, it has also been gaining in significance as a cause of serious community-acquired infections among healthy people.[1] Although there are a few antibiotics used to combat MRSA (for example, vancomycin, teicoplanin, arbekacin, linezolid, daptomycin, tigecycline, ceftaroline, dalbavancin, oritavancin and tedizolid) microorganisms with resistance to some of them are increasingly being reported. In the course of screening for new antibiotics active against MRSA, we have recently found biverlactones, which are capable of circumventing arbekacin resistance in MRSA. They are thought to inhibit aminoglycoside-modifying enzymes.[2] We have also reported that cyslabdan enhances imipenem activity against MRSA.[3,4] Our continuous study to find microbial metabolites, like cyslabdan, that circumvent β-lactam resistance of MRSA, led us to discover further novel compounds, the naphthacemycins A_1–A_{11} (**1–11**), B_1–B_4 (**12–15**) and C_1–C_2 (**16–17**) (Figure 1), reported as KB-3346-5 substances in the patent by our group[5], isolated from a culture broth of *Streptomyces* sp. KB-3346-5. In this report, we describe the taxonomy of the producing strain, as well as the fermentation, isolation and antibacterial activities of these naphthacemycin compounds.

RESULTS AND DISCUSSION

Taxonomy of the producing organism

Strain KB-3346-5 was originally isolated from a soil sample collected in Okinawa Prefecture, Japan. The vegetative mycelia developed well on yeast extract–malt extract agar and nutrient agar, and the color was brown. The aerial mycelia were produced abundantly on yeast extract–malt extract agar, and the aerial mass color showed white to yellow. The mature spore chains were spiral and each had more than 20 spores per chain. The spores were cylindrical in shape, 0.5–0.6 × 0.7–0.8 mm in size and had a smooth surface (Figure 2). The isomer of diaminopimelic acid in whole-cell hydrolysates was LL-form. Major menaquinones were MK-9 (H_6) and MK-9 (H_8). Based on the taxonomic properties above, the microorganism was considered to belong to the genus *Streptomyces*[6] and was named *Streptomyces* sp. KB-3346-5. The strain was deposited at the

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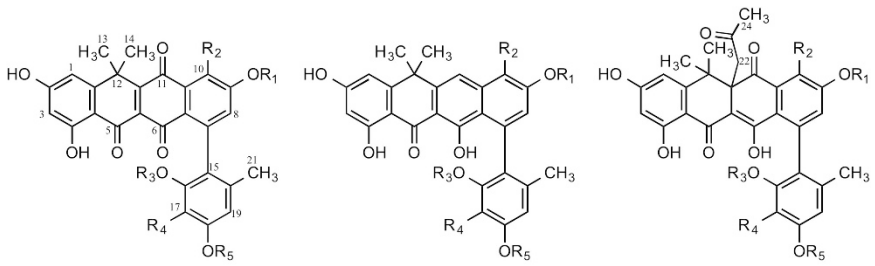

Naphthacemycin A series　Naphthacemycin B series　Naphthacemycin C series

	R_1	R_2	R_3	R_4	R_5
Naphthacemycin A_1 (**1**)	H	H	CH_3	H	H
Naphthacemycin A_2 (**2**)	H	H	CH_3	Cl	H
Naphthacemycin A_3 (**3**)	CH_3	H	CH_3	H	H
Naphthacemycin A_4 (**4**)	CH_3	Cl	CH_3	H	H
Naphthacemycin A_5 (**5**)	H	H	CH_3	Cl	CH_3
Naphthacemycin A_6 (**6**)	CH_3	H	CH_3	Cl	H
Naphthacemycin A_7 (**7**)	CH_3	Cl	CH_3	Cl	H
Naphthacemycin A_8 (**8**)	CH_3	Cl	CH_3	H	CH_3
Naphthacemycin A_9 (**9**)	CH_3	H	CH_3	H	CH_3
Naphthacemycin A_{10} (**10**)	CH_3	Cl	CH_3	Cl	CH_3
Naphthacemycin A_{11} (**11**)	CH_3	H	CH_3	Cl	CH_3
Naphthacemycin B_1 (**12**)	H	H	H	H	H
Naphthacemycin B_2 (**13**)	H	H	CH_3	H	H
Naphthacemycin B_3 (**14**)	H	H	CH_3	Cl	H
Naphthacemycin B_4 (**15**)	H	Cl	CH_3	H	H
Naphthacemycin C_1 (**16**)	CH_3	Cl	CH_3	H	H
Naphthacemycin C_2 (**17**)	CH_3	H	CH_3	Cl	CH_3

Figure 1 Structures of naphthacemycins A_1–A_{11} (**1**–**11**), B_1–B_4 (**12**–**15**) and C_1–C_2 (**16**–**17**).

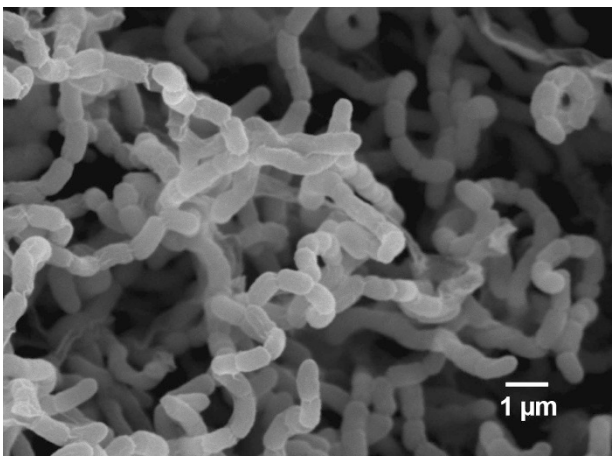

Figure 2 Scanning electron micrograph of the strain KB-3346-5 grown on yeast extract–malt extract agar at 27 °C for 2 weeks.

International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan, as FERM BP-10834.

Fermentation and isolation

A loop of cells of strain KB-3346-5 on the agar slant was inoculated into each of five test tubes containing 10 ml of a seed medium and shaken at 27 °C for 3 days. The seed broth (10 ml) was inoculated into each of five 500-ml Erlenmeyer flasks containing 100 ml of the seed medium and incubated on a rotary shaker at 27 °C for 3 days. The second seed broth (10 ml) was inoculated into each of fifty 500-ml Erlenmeyer flasks containing 100 ml of a production medium, and the fermentation was carried out on a rotary shaker at 28 °C for 8 days. The production of naphthacemycins began at 3–4 days and reached nearly maximum at 6 days (data not shown).

The cultured broth was extracted with acetone, and the extract was further extracted with ethyl acetate. The ethyl acetate extract was purified by silica gel column chromatography twice and HPLC to yield naphthacemycins A_1–A_{11} (**1–11**), B_1–B_4 (**12–15**) and C_1–C_2 (**16–17**). Their structures were elucidated by NMR experiments and X-ray analysis, as reported elsewhere.[7] Naphthacemycins displayed unique skeletons as natural products, consisting of a naphthacene ring monosubstituted with a phenyl residue at C-7 (Figure 1).

Biological activities

The circumvention of β-lactam resistance in MRSA was measured by enhancement of imipenem activity against MRSA. Naphthacemycins alone showed MIC values of 8–64 μg ml^{-1} against clinically isolated MRSA strain K24. In the presence of each 0.5 μg ml^{-1} of naphthacemycins, the MIC of imipenem was evaluated by the liquid dilution method. Without naphthacemycins, the MIC of imipenem was 32 μg ml^{-1}. As shown in Table 1, addition of naphthacemycins reduced the MIC of imipenem to between 0.06 and 0.25 μg ml^{-1}, yielding 128–512 times enhancement of imipenem activity. Among naphthacemycins, **3**, **6**, **7** and **13–15** showed 512 times enhancement.

The circumvention activity was further evaluated by a larger panel of staphylococci for sensitivity testing of imipenem activity using 22 clinically isolated MRSA and five MSSA (methicillin-sensitive *S. aureus*) strains. Concentrations of naphthacemycins, of 0.2 μg ml^{-1} (for compounds **4–11**) and 1 μg ml^{-1} (for compounds **1–3** and **12–17**), did not inhibit the growth of tested bacteria. The ranges of

Table 1 Anti-MRSA activity of imipenem enhanced by naphthacemycins

Compound added	*MIC of naphthacemycin (μg ml^{-1})*	*MIC of imipenem (μg ml^{-1})*	*Ratio*
None		32	1
Naphthacemycin A_1 (**1**)	32	0.25	128
Naphthacemycin A_2 (**2**)	16	0.125	256
Naphthacemycin A_3 (**3**)	32	0.06	512
Naphthacemycin A_4 (**4**)	16	0.125	256
Naphthacemycin A_5 (**5**)	16	0.125	256
Naphthacemycin A_6 (**6**)	32	0.06	512
Naphthacemycin A_7 (**7**)	32	0.06	512
Naphthacemycin A_8 (**8**)	32	0.125	256
Naphthacemycin A_9 (**9**)	32	0.125	256
Naphthacemycin A_{10} (**10**)	32	0.125	256
Naphthacemycin A_{11} (**11**)	16	0.125	256
Naphthacemycin B_1 (**12**)	32	0.25	128
Naphthacemycin B_2 (**13**)	8	0.06	512
Naphthacemycin B_3 (**14**)	16	0.06	512
Naphthacemycin B_4 (**15**)	8	0.06	512
Naphthacemycin C_1 (**16**)	64	0.125	256
Naphthacemycin C_2 (**17**)	64	0.125	256

The concentration of each naphthacemycin was 0.5 μg ml^{-1}.

Table 2 MIC population of imipenem against MRSA and MSSA strains in the presence of naphthacemycins

		MRSA (22 strains)		*MSSA (5 strains)*
Compound added	*Dose (μg ml^{-1})*	*Range (μg ml^{-1})*	*MIC_{50} (μg ml^{-1})*	*Range (μg ml^{-1})*
None	–	4–64	32	0.015–0.03
Naphthacemycin A_1 (**1**)	1	0.5–64	32	0.015–0.03
Naphthacemycin A_2 (**2**)	1	0.125–64	1	0.015–0.03
Naphthacemycin A_3 (**3**)	1	0.125–64	8	0.015–0.03
Naphthacemycin A_4 (**4**)	0.2	0.25–64	32	0.015–0.03
Naphthacemycin A_5 (**5**)	0.2	0.25–64	8	0.015–0.03
Naphthacemycin A_6 (**6**)	0.2	0.25–64	4	0.015–0.03
Naphthacemycin A_7 (**7**)	0.2	0.25–64	2	0.015–0.03
Naphthacemycin A_8 (**8**)	0.2	0.5–64	16	0.015–0.03
Naphthacemycin A_9 (**9**)	0.2	0.5–64	16	0.015–0.03
Naphthacemycin A_{10} (**10**)	0.2	0.25–64	8	0.015–0.03
Naphthacemycin A_{11} (**11**)	0.2	1.0–64	16	0.015–0.03
Naphthacemycin B_1 (**12**)	1	0.125–64	32	0.015–0.03
Naphthacemycin B_2 (**13**)	1	0.125–64	2	0.015–0.03
Naphthacemycin B_3 (**14**)	1	0.125–64	2	0.015–0.03
Naphthacemycin B_4 (**15**)	1	0.125–64	8	0.015–0.03
Naphthacemycin C_1 (**16**)	1	0.5–64	16	0.015–0.03
Naphthacemycin C_2 (**17**)	1	0.5–64	32	0.015–0.03

MIC values and the values of MIC_{50} of the MRSA and MSSA strains are shown in Table 2. Most naphthacemycins reduced MIC_{50} values of imipenem and **2** reduced the value 32-fold. However, naphthacemycins had no effect on imipenem activity against MSSA strains.

Thus, naphthacemycins circumvent imipenem resistance in MRSA, similar to cyslabdan, which we have reported previously,[4] but the enhancement ratio of cyslabdan, was much higher (128 times at population analysis) than with the naphthacemycins. It is interesting that naphthacemycins enhanced imipenem activity at the concentration lower than that of cyslabdan, although the enhancement ratio was lower than cyslabdan. Therefore, antibacterial activity of naphthacemycins alone against MRSA and MSSA strains were evaluated by a larger panel of staphylococci for sensitivity testing (Table 3). Naphthacemycins other than **1**, **12**, **16** and **17** showed equal antibacterial activity against both MRSA and MSSA strains, and the MIC_{50} of **11** was 1 μg ml^{-1} and that of **5–8** and **10** was 2 μg ml^{-1}, comparable to vancomycin.

The antibacterial activity of **8** and **9** (which were available in suitable quantities) against various *S. aureus* and a range of other bacteria was evaluated (Table 4). Compounds **8** and **9** showed antibacterial activity against Gram-positive bacteria. Their anti-MRSA activity was comparable to vancomycin, and they also inhibited the growth of

Table 3 MIC population of naphthacemycins against MRSA and MSSA strains

	MRSA (22 strains)		MSSA (5 strains)
Compound	Range (μg ml^{-1})	MIC_{50} (μg ml^{-1})	Range (μg ml^{-1})
Naphthacemycin A_1 (**1**)	>8	>8	>8
Naphthacemycin A_2 (**2**)	8	8	8
Naphthacemycin A_3 (**3**)	4–8	8	4
Naphthacemycin A_4 (**4**)	2–8	4	4
Naphthacemycin A_5 (**5**)	1–2	2	2
Naphthacemycin A_6 (**6**)	2–4	2	1–2
Naphthacemycin A_7 (**7**)	1–4	2	2
Naphthacemycin A_8 (**8**)	0.5–4	2	2
Naphthacemycin A_9 (**9**)	2–4	4	2–4
Naphthacemycin A_{10} (**10**)	1–2	2	1–2
Naphthacemycin A_{11} (**11**)	0.5–2	1	1
Naphthacemycin B_1 (**12**)	>8	>8	>8
Naphthacemycin B_2 (**13**)	8	8	8
Naphthacemycin B_3 (**14**)	4–8	8	4–8
Naphthacemycin B_4 (**15**)	4–8	8	4–8
Naphthacemycin C_1 (**16**)	>8	>8	>8
Naphthacemycin C_2 (**17**)	>8	>8	>8
Vancomycin	1–2	1	1

Table 4 Antibacterial activity of naphthacemycins A_8 (8) and A_9 (9) compared with vancomycin

	MIC (μg ml^{-1})		
Bacteria	**8**	**9**	Vancomycin
Staphylococcus aureus FDA209P (MSSA)	2	2	1
S. aureus Smith (MSSA)	1	1	2
S. aureus ISP447 (MSSA)	1	1	1
S. aureus ISP217 (MSSA)	1	1	1
S. aureus 70 (MRSA)	2	1	0.5
S. aureus KB 362 (MRSA)	1	1	1
S. aureus KB 363 (MRSA)	2	1	1
S. aureus Mu50 (MRSA)	2	2	8
S. aureus Linezolid resistant-6 (MRSA)	4	2	2
S. epidermidis	1	1	1
Kocuria rhizophila ATCC9341	0.5	1	1
Enterococcus faecalis ATCC21212	0.5	2	4
E. faecalis NCTC12201 (*VanA*)	0.5	2	>128
E. faecium NCTC12203 (*VanA*)	2	4	>128
Escherichia coli NIHJ JC-2	>128	>128	>128
Citrobactor freundii ATCC8090	>128	>128	>128
Klebsiella pneumoniae NCTN9632	>128	>128	>128
Proteus mirabilis IFO3849	>128	>128	>128
P. vulgaris OX-19	>128	>128	>128
Morganella morganii IID Kono	>128	>128	>128
Serratia marcescens IFO12648	>128	>128	>128
Enterobacter cloacae IFO13535	>128	>128	>128
E. aerogenes NCTC10006	>128	>128	>128
Pseudomonas aeruginosa 46001	>128	>128	>128
P. aeruginosa E-2	>128	>128	>128
Acinetobacter calcoaceticus IFO2552	>128	>128	>128

linezolid-resistant MRSA. Moreover, they showed good inhibition against vancomycin-resistant *Enterococcus faecalis* and *E. faecium*. Acute toxicity of **8** and **9** against mice was not observed at 100 mg kg^{-1} (s.c.).

The naphthacemycin A series has a unique skeleton of 7-phenylnaphthacene-5,6,11(12*H*)-trione. Most structurally related compounds, tetarimycin A (**18**, Figure 3)[8] and fasamycins (**19**, **20**)[9], were recently reported by Brady and co-workers as antibacterial agents. The antibacterial activity mechanism was identified as inhibition of FabF, one of the enzymes involved in type II fatty acid biosynthesis.[10] Therefore, biological properties of naphthacemycins might be related to FabF inhibition, though we have not tested whether they could show inhibitory activity against FabF and type II fatty acid biosynthesis or not yet. Tetracycline (**21**) produced by *Streptomyces* spp. also has a naphthacene skeleton, but it is much more saturated and has a kink between rings A and B, which is believed to be involved in its binding to ribosomes.[11] Chelocardin (**22**), produced by *Nocardia sulphurea*, has a much more planar skeleton.[12] Its bactericidal activity is not caused by protein synthesis inhibition but is believed to be due to membrane disruption.[13] Tetracenomycin C (**23**), produced by *S. glaucescens*, is an another type of naphthacene antibiotic.[14,15] It binds to DNA and mainly inhibits the growth of actinobacteria. EA-371α (**24**), produced by *Streptomyces* sp., was isolated as an inhibitor of the MexAB-OprM efflux pump, which is involved in intrinsic antibiotic resistance of *Pseudomonas aeruginosa*.[16] Though its skeleton is 8-oxobenzo[*a*]naphthacene, it has a 1,3-dihydroxy-10,10-dimethylanthrone unit, similar to the naphthacemycins. Its desulfonated analog, benastatin A (**25**), is active against Gram-positive bacteria.[17] Bischloroanthrabenzoxocinone (**26**), produced by *Streptomyces* sp., also has the same unit.[18,19] It also inhibits bacterial type II fatty acid biosynthesis and shows antibacterial activity against Gram-positive bacteria. The currently unknown mode of action of naphthacemycins may be similar to that of one of the above compounds.

METHODS

Taxonomy of the producing organism

The International *Streptomyces* Project media recommended by Shirling and Gottlieb[20] as well as media recommended by Waksman[21] were used to investigate the cultural characteristics. Cultures were observed after incubation for 2 weeks at 28 °C. The morphological properties were observed using a scanning electron microscope JSM-5600 (JEOL, Akishima, Japan). Isomers of diaminopimelic acid in whole-cell hydrolysates were elucidated by TLC[22] and menaquinones were analyzed by HPLC.[23]

Media

The agar slant medium for the stock culture of the strain KB-3346-5 consisted of starch (Kanto Chemical Co., Tokyo, Japan) 1.0%, NZ amine (Wako Pure Chemical Industries, Osaka, Japan) 0.3%, yeast extract (Oriental Yeast Co., Tokyo, Japan) 0.1%, meat extract (Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan) 0.1%, $CaCO_3$ 0.3% and agar (Shimizu Shokuhin Kaisha, Shizuoka, Japan) 1.5%, adjusted to pH 7.0 before sterilization. The seed medium consisted of starch 2.4%, glucose 0.1%, peptone (Kyokuto Pharmaceutical Industrial Co.) 0.3%, yeast extract 0.5%, meat extract 0.3% and $CaCO_3$ 0.4%, adjusted to pH 7.0 before sterilization. The production medium consisted of glucose 0.5%, corn steep powder (Marcor Development Co., Carlstadt, NJ, USA) 0.5%, oatmeal (Nippon Food Manufacturer, Sapporo, Japan) 1.0%, Pharmamedia (Traders Protein, Lubbock, TX, USA) 1.0%, K_2HPO_4 0.5%, $MgSO_4{\cdot}7H_2O$ 0.5%, $FeSO_4{\cdot}7H_2O$ 0.0001%, $MnCl_2{\cdot}4H_2O$ 0.0001%, $ZnSO_4{\cdot}7H_2O$ 0.0001%, $CuSO_4{\cdot}5H_2O$ 0.0001% and $CoCl_2{\cdot}6H_2O$ 0.0001%, adjusted to pH 7.0 before sterilization.

Isolation

A mixture of the cultured broth (5 l) and the same volume of acetone was shaken for 30 min. Acetone in the solution was removed by evaporation and

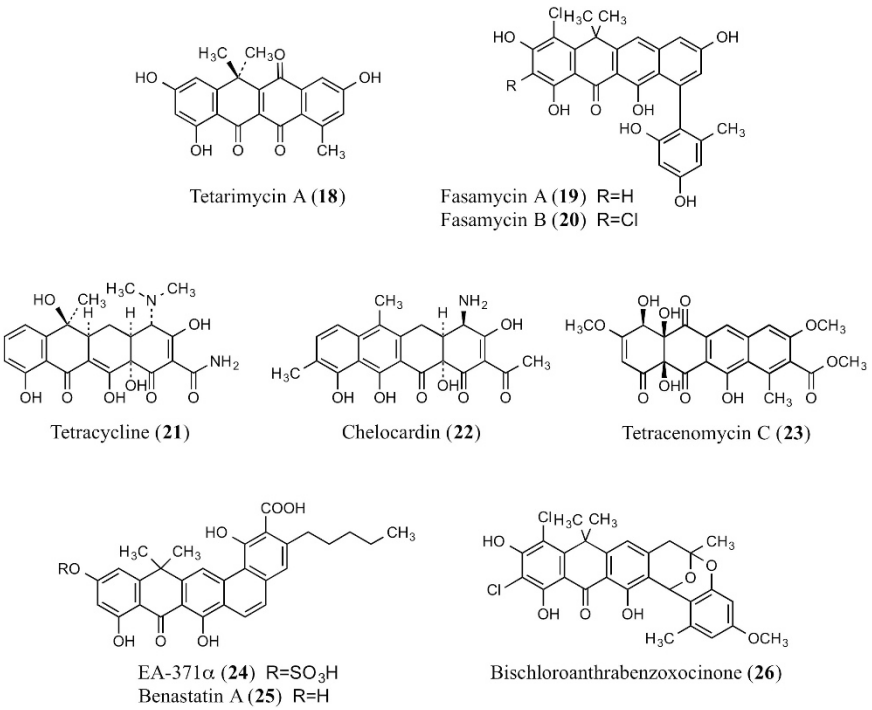

Figure 3 Structures of naphthacemycin-related antibiotics.

the remaining water solution was partitioned with 10 l of ethyl acetate. The ethyl acetate extract (6.73 g) was applied to a silica gel column (220 g of silica gel 60 (0.063–0.200 mm), Merck KGaA, Darmstadt, Germany), washed with $CHCl_3$ and eluted with $CHCl_3$–MeOH (100:1, 100:2 and 100:10). Active fractions eluted with $CHCl_3$–MeOH (100:1) and $CHCl_3$–MeOH (100:10) were concentrated to yield crude materials I (4.37 g) and II (0.83 g), respectively.

A total of 1.0 g of the crude material I was used for the further purification. It was applied to a silica gel column (30 g of silica gel 60 (0.040–0.063 mm), Merck KGaA) and eluted with $CHCl_3$–MeOH (100:0, 100:1 and 100:2). Naphthacemycins were eluted at 100:0 to 100:2 ratio solutions to yield crude materials III (472 mg) and IV (69.2 mg). The crude material III was separated by ODS HPLC (column, Pegasil ODS, φ20 × 250 mm, Senshu Scientific Co., Tokyo, Japan; mobile phase, 60% CH_3CN; flow rate, 8 ml min^{-1}). Crude material V (295 mg) and naphthacemycins A_{10} (**10**, 14.7 mg), A_{11} (**11**, 55.7 mg) and C_2 (**17**, 9.2 mg) were eluted at 43, 49, 51 and 55 min, respectively. The crude material V was further purified by ODS HPLC (column, Pegasil ODS, φ20 × 250 mm; mobile phase, 85% CH_3CN; flow rate, 8 ml min^{-1}) to yield naphthacemycins A_8 (**8**, 106 mg) and A_9 (**9**, 175 mg) at the eluate of 23 and 27 min, respectively. Crude material IV was purified by Pegasil ODS HPLC using 55% CH_3CN (flow rate, 8 ml min^{-1}) to yield naphthacemycins A_5 (**5**, 26.4 mg), A_6 (**6**, 11.5 mg) and A_7 (**7**, 14.3 mg) at the retention times of 25, 36 and 38 min, respectively.

The crude material II was applied to a silica gel column (25 g of silica gel 60 (0.040–0.063 mm), Merck KGaA) and eluted with $CHCl_3$–MeOH (100:2 and 100:10). Naphthacemycins eluted at 100:2 and 100:10 ratios were collected to yield crude materials VI (170 mg) and VII (251 mg), respectively. The crude material VI was separated by Pegasil ODS HPLC using 75% MeOH (flow rate, 8 ml min^{-1}), and naphthacemycins A_2 (**2**, 13.9 mg), B_3 (**14**, 10.0 mg) and B_4 (**15**, 30.5 mg) and crude materials VIII (20.2 mg) and IX (61.2 mg) were eluted at 17, 19, 27, 22 and 36 min, respectively. The crude material VIII was applied to Pegasil ODS HPLC using 55% CH_3CN (flow rate, 8 ml min^{-1}), and the eluted peak of 18 min was collected to yield naphthacemycin C_1 (**16**, 14.2 mg). The crude material IX was further purified by Pegasil ODS HPLC using 55% CH_3CN (flow rate, 8 ml min^{-1}) to yield naphthacemycins A_3 (**3**, 21.5 mg) and A_4 (**4**, 32.8 mg) at the eluate of 21 and 24 min, respectively. The crude material VII was separated by Pegasil ODS HPLC using 50% CH_3CN (flow rate, 8 ml min^{-1}), and crude material X (29.9 mg) and naphthacemycin B_2 (**13**, 89.6 mg) were eluted at 12 and 19 min, respectively. The crude material X was further purified by Pegasil ODS HPLC using 65% MeOH (flow rate, 7 ml min^{-1}) to yield naphthacemycins B_1 (**10**, 10.0 mg) and A_1 (**1**, 14.2 mg) at the eluate of 36 and 44 min, respectively.

Assay of antibacterial activity

Measurement of anti-MRSA activity of imipenem enhanced by naphthacemycins were carried out by the liquid microdilution method[24] using MRSA strain K24, as reported previously.[4]

A larger panel for sensitivity testing of clinically isolated MRSA (22 strains) and drug susceptible (MSSA) organisms (5 strains) were carried out by the agar dilution method[25] in the presence of imipenem and/or naphthacemycins. An overnight culture of MRSA and MSSA strains was diluted with fresh medium to the appropriate bacterial density and spread onto a plate of Difco Mueller Hinton Agar (Becton, Dickinson and Company, Sparks, MD, USA) containing serial twofold dilutions of imipenem or naphthacemycins. To measure imipenem activity enhancement, 0.2 or 1 μg ml^{-1} of naphthacemycins was added with imipenem. To measure naphthacemycins activity, naphthacemycins alone were added. The plates were incubated at 37 °C for 20 h. MIC_{50} was defined as the concentration at which 50% of the strains could not grow.

Antibacterial activity of naphthacemycins against a variety of bacteria were measured by the agar dilution method.[25]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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1 Diep, B. A. & Otto, M. The role of virulence determinants in community-associated MRSA pathogenesis. *Trends Microbiol.* **16**, 361–369 (2008).
2 Iwatsuki, M. *et al*. Biverlactones A-D, new circumventors of arbekacin resistance in MRSA, produced by *Penicillium* sp. FKI-4429. *Tetrahedron* **67**, 6644–6648 (2011).

3 Fukumoto, A. *et al.* Cyslabdan, a new potentiator of imipenem activity against methicillin-resistant *Staphylococcus aureus*, produced by *Streptomyces* sp. K04-0144. I. Taxonomy, fermentation, isolation and structural elucidation. *J. Antibiot.* **61**, 1–6 (2008).
4 Fukumoto, A. *et al.* Cyslabdan, a new potentiator of imipenem activity against methicillin-resistant *Staphylococcus aureus*, produced by *Streptomyces* sp. K04-0144. II. Biological activities. *J. Antibiot.* **61**, 7–10 (2008).
5 Omura, S. *et al.* (Kitasato Institute, Japan; Kyowa Hakko Kirin Co., Ltd., Japan). KB-3346-5 substances, their fermentative manufacture, and antibacterial agents containing them. *Jpn. Kokai Tokkyo Koho*, JP2009046404A (2009).
6 Williams, S. T., Goodfellow, M. & Alderson, G. in: *Bergey's Manual of Systematic Bacteriology*, Vol. 4 (eds Wiliams, S. T. *et al.*) 2452–2492 (Williams & Wilkins, Baltimore, MD, USA, 1989).
7 Fukumoto, A. *et al.* Naphthacemycins, novel circumventors of β-lactam resistance in MRSA, produced by *Streptomyces* sp. KB-3346-5. II. structure elucidation. *J. Antibiot.* (doi:10.1038/ja.2017.29).
8 Kallifidas, D., Kang, H.-S. & Brady, S. F. Tetarimycin A, an MRSA-active antibiotic identified through induced expression of environmental DNA gene clusters. *J. Am. Chem. Soc.* **134**, 19552–19555 (2012).
9 Feng, Z., Kallifidas, D. & Brady, S. F. Functional analysis of environmental DNA-derived type II polyketide synthases reveals structurally diverse secondary metabolites. *Proc. Natl Acad. USA* **108**, 12629–12634 (2011).
10 Feng, Z., Chakraborty, D., Dewell, S. B., Reddy, B. V. B. & Brady, S. F. Environmental DNA-encoded antibiotics fasamycins A and B inhibit FabF in type II fatty acid biosynthesis. *J. Am. Chem. Soc.* **134**, 2981–2987 (2012).
11 Thaker, M., Spanogiannopoulos, P. & Wright, G. D. The tetracycline resistome. *Cell Mol. Life Sci.* **67**, 419–431 (2010).
12 Oliver, T. J., Prokop, J. F., Bower, R. R. & Otto, R. H. Chelocardin, a new broad-spectrum antibiotic. I. Discovery and biological properties. *Antimicrob. Agents Chemother.* **1962**, 583–591 (1963).
13 Chopra, I. Tetracycline analogs whose primary target is not the bacterial ribosome. *Antimicrob. Agents Chemother.* **38**, 637–640 (1994).
14 Weber, W., Zähner, H., Siebers, J., Schröder, K. & Zeeck, A. Stoffwechselprodukte von Mikroorganismen. 175. Mitteilung. Tetracenomycin C. *Arch. Microbiol.* **121**, 111–116 (1979).
15 Weber, W., Zähner, H., Siebers, J., Schröder, K. & Zeeck, A. in: *Actinomycetes. Zbl. Bakt. Suppl. 11* (eds Schaal, K. P. & Pulverer, G.) 465–468 (Gustav Fischer Verlag, Stuttgart, 1981).
16 Lee, M. D. *et al.* Microbial fermentation-derived inhibitors of efflux-pump-mediated drug resistance. *Farmaco* **56**, 81–85 (2001).
17 Aoyagi, T. *et al.* Benastatins A and B, new inhibitors of glutathione *S*-transferase, produced by *Streptomyces* sp. M1384-DF12. I. Taxonomy, production, isolation, physico-chemical properties and biological activities. *J. Antibiot.* **45**, 1385–1390 (1992).
18 Kodali, S. *et al.* Determination of selectivity and efficacy of fatty acid synthesis inhibitors. *J. Biol. Chem.* **280**, 1669–1677 (2005).
19 Herath, K. B. *et al.* Anthrabenzoxocinones from *Streptomyces* sp. as liver X receptor ligands and antibacterial agents. *J. Nat. Prod.* **68**, 1437–1440 (2005).
20 Shiring, E. B. & Gottlieb, D. Methods for characterization of *Streptomyces* species. *Int. J. Syst. Bacteriol.* **16**, 313–340 (1966).
21 Waksman S. A. (ed.) In *The Actinomycetes* Vol.2, (Williams & Wilkins, Baltimore, 1961).
22 Hasegawa, T., Takizawa, M. & Tanida, S. A rapid analysis for chemical grouping of aerobic actinomycetes. *J. Gen. Appl. Microbiol.* **29**, 319–322 (1982).
23 Collins, M. D., Pirouz, T., Goodfellow, M. & Minnikin, D. E. Distribution of menaquinones in actinomycetes and corynebacteria. *J. Gen. Microbiol.* **100**, 221–230 (1977).
24 Japanese Society of Chemotherapy. Report of the committee for Japanese standards for antimicrobial susceptibility testing for bacteria. *Chemotherapy* **38**, 102–105 (1990).
25 Nagayama, A. *et al.* Final report from the Committee on Antimicrobial Susceptibility Testing, Japanese Society of Chemotherapy, on the agar dilution method (2007). *J. Infect. Chemother.* **14**, 383–392 (2008).