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NOTE

Hetiamacin B–D, new members of amicoumacin group antibiotics isolated from *Bacillus subtilis* PJS

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The amicoumacin group of antibiotics is a small family of isocoumarins with the common chromophore 3,4-dihydro-8-hydroxyisocoumarin, which shows specific UV absorbance at 246 and 314 nm in methanol.[1] In the previous report, PJS[2] (also named as Hetiamacin A[3]), a new member of the amicoumacin group, was discovered from the fermentation broth of an endophytic bacterium, *Bacillus subtilis* PJS. It contained a special hexahydropyrimidine ring in the side chain of its chemical structure, and exhibited outstanding activity against methicillin-resistant *Staphylococcus aureus* (MRSA). Subsequently, three new members of the amicoumacin group, designated as Hetiamacin B (**1**), C (**2**) and D (**3**) (Figure 1), were detected by ultra performance liquid chromatography–diode array detection–mass spectrometry (UPLC–DAD–MS) and bioassay, and purified by various chromatographies from the fermentation broth of strain PJS. In this paper, we describe the isolation, structural elucidation of **1**–**3** and antibacterial evaluation of **1**.

Culture and fermentation of the strain PJS were conducted as described previously.[2] The fermentation broth (20 l) was centrifuged and the supernatant was absorbed on a column containing 2 l of Diaion HP-20 (Mitsubishi Chemical Holdings Corp., Tokyo, Japan). The HP-20 column was then eluted successively with distilled water, 30, 50 and 80% acetone-distilled water (each 6 l) to yield four fractions (Fr. A–D), among which Fr. C showed the strongest activity against MRSA (American Type Culture Collection 33591). After being concentrated to dryness (2.2 g), the Fr. C was chromatographed on a LiChroprep RP-C_{18} column (1.0 × 50 cm, 40–63 μm, Merck Company, Darmstadt, Germany) and eluted successively with 30, 60 and 90% aqueous methanol (each 200 ml). Fractions were monitored by the UPLC–DAD–MS system (LC-20AD, SPD-M20A and LCMS-2020, Shimadzu Corp., Tokyo, Japan) with a Shim-Pack XR-ODS column (3.0 × 75 mm, 2.2 μm, Shimadzu Corp.). The fractions of 60% aqueous methanol containing amicoumacins were pooled and concentrated to give a yellow syrup (160.5 mg). After being dissolved in 1.0 ml of methanol, the sample was filtered through a syringe filter unit with 0.22-μm polytetrafluoroethylene membranes (Tianjin Jinteng Experiment Equipment Co. Ltd., Tianjin, China) and further purified by preparative HPLC (Agilent 1200, Agilent Technologies Inc., Santa Clara, CA, USA) on a Zorbax SB-C_{18} column (9.4 × 250 mm, 5 μm, Agilent Technologies Inc.) with methanol/water, 60:40 (v/v) at 2 ml min^{-1}. The peaks at R_t = 29, 26 and 33 min, with UV absorption maxima at 206, 246 and 314 nm, were collected and pooled to yield **1** (12.6 mg), **2** (2.8 mg) and **3** (1.5 mg), respectively.

Hetiamacin B (**1**) was obtained as a white powder, soluble in dimethyl sulfoxide (DMSO), CH_3OH, CH_2Cl_2 and $CHCl_3$. The molecular formula of **1** was established as $C_{23}H_{33}O_7N_3$ by positive high-resolution (HR)-ESI-MS (*m/z* found: 464.2389 $[M+H]^+$, calcd: 464.2391) with nine degrees of unsaturation. Analysis of the ^{13}C-NMR and DEPT spectra of **1** indicated the presence of 23 carbons, including 3 carbonyl carbons, 6 aromatic carbons and 14 aliphatic carbons including 6 carbons bonded to nitrogen or oxygen. The IR absorptions of **1** at 3290, 2957, 1662, 1528, 1462, 1231, 807 and 698 cm^{-1} indicated the presence of a benzoic acid moiety with a phenolic hydroxyl group and an amide group.[4,5] The UV absorption at λ_{max}^{MeOH}nm (ε): 203 (31 565), 246 (7014) and 314 (5139) was almost identical with that of isocoumarin compounds.[2–8] All data above revealed **1** had a chromophore similar to the 3,4-dihydro-8-hydroxyisocoumarin in its structure. By careful analysis of ^{1}H and ^{13}C-NMR, ^{1}H-^{1}H COSY, DEPT, ^{13}C-^{1}H COSY, HMBC and ROESY spectra in DMSO-d_6 and $CDCl_3$, chemical structure of **1** was elucidated. It consisted of two substructures, I and II, as shown in Figure 2. NMR data of **1** in DMSO-d_6 and $CDCl_3$ are listed in Table 1.

Elucidation of substructure I started from the three aromatic protons in the ^{1}H-NMR spectrum in DMSO-d_6, H-5 (δ_H 6.82, d, J = 7.2), H-6 (δ_H 7.48, dd, J = 8.4, 7.2) and H-7 (δ_H 6.84, d, J = 8.4), which displayed a 1, 2, 3-trisubstituted benzenoid ring in the ^{1}H-NMR and ^{1}H–^{1}H COSY spectra. Their corresponding carbons were assigned by ^{13}C–^{1}H COSY. The other three aromatic carbons were observed and assigned by tracing cross peaks from H-5 and H-7 to C-9 (δ_C 108.3), from H-6 to C-10 (δ_C 140.7) and C-8 (δ_C 160.8) in a HMBC spectrum run in DMSO-d_6. The chemical shift of C-8 in

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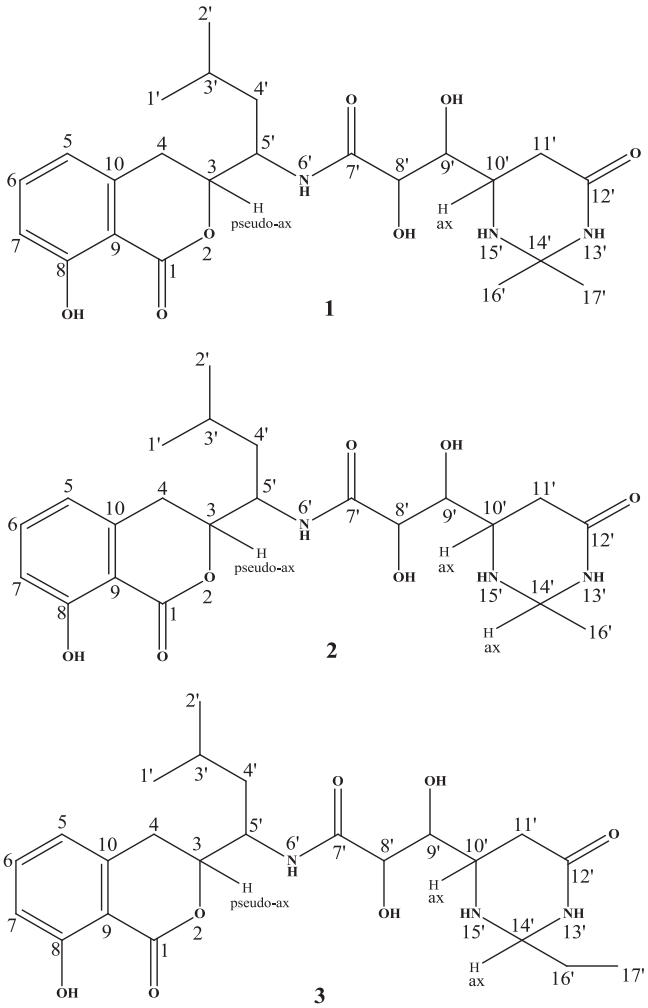

Figure 1 Chemical structures of Hetiamacin B (**1**), C (**2**) and D (**3**).

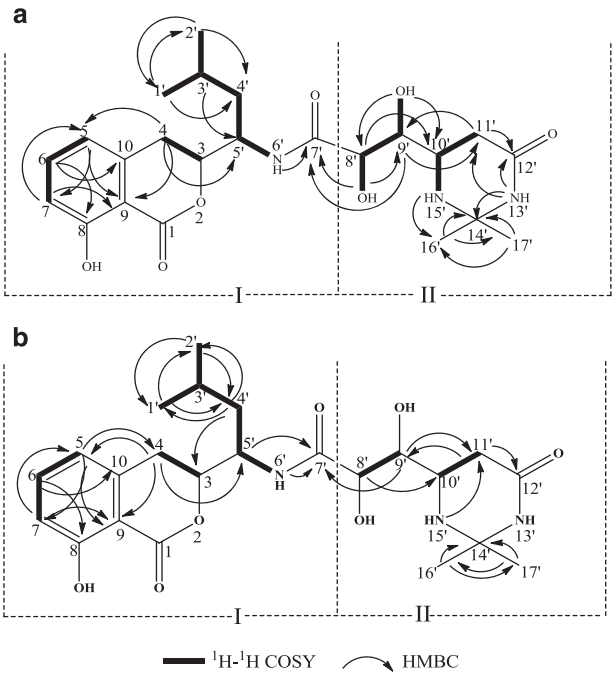

Figure 2 Key 2D NMR correlations of **1** in DMSO-d_6 (**a**) and $CDCl_3$ (**b**).

DMSO-d_6 suggested a hydroxyl group should be attached to C-8, which was supported by a proton (δ_H 10.78) as a broad peak from 8-OH in the downfield region of the ^{1}H-NMR spectrum in $CDCl_3$. The cross peaks between H-3 (δ_H 4.69) and H-4$_a$ (δ_H 2.85), H-4$_b$ (δ_H 3.03) in ^{1}H–^{1}H COSY together with HMBC correlations from H-4$_a$, H-4$_b$ to the aromatic carbons C-5 (δ_C 118.5) and C-9 in DMSO-d_6 suggested C-4 (δ_C 29.1) was connected with C-3 (δ_C 81.1) and C-10. The chemical shifts of H-3 and C-3 at low field indicated C-3 should be attached to the oxygen of the lactone ring to form the 3,4-dihydro-8-hydroxyisocoumarin skeleton. Identification of the isopentyl unit presented in substructure I started from the two methyl signals of 1′-CH_3 (δ_H 0.85) and 2′-CH_3 (δ_H 0.89), which were readily observed in the ^{1}H-NMR spectrum in DMSO-d_6. By tracing the cross peaks from H-1′, H-2′, H-4′$_a$ (δ_H 1.32) to H-3′ (δ_H 1.66) and from H-4′$_b$ (δ_H 1.66) to H-5′ (δ_H 4.20) in the ^{1}H-^{1}H COSY spectrum in DMSO-d_6, the isopentyl group was identified. The cross peaks between H-1′ and C-2′ (δ_C 23.3), between H-2′ and C-1′ (δ_C 21.5), between H-1′ and C-4′ (δ_C 39.1), between H-2′ and C-4′, and between H-3′ and C-5′ (δ_C 47.9) in the HMBC spectrum in DMSO-d_6 confirmed the presence of the isopentyl group. The HMBC correlations from H-4$_b$ to C-5′ in DMSO-d_6 and from H-4′$_a$, H-4′$_b$ to C-3 in $CDCl_3$ established the connectivity between C-3 and C-5′. Furthermore, a cross peak could be observed between H-5′ and 6′-XH proton (δ_H 7.64) in the ^{1}H–^{1}H COSY spectrum in DMSO-d_6, and this 6′-XH proton was considered as an NH proton on the basis of the relatively downfield chemical shifts of H-5′ and C-5′. The carbon C-7′ (δ_C 172.7, DMSO-d_6) in the downfield region revealed that C-7′ was a carbonyl, which was bound to 6′-NH to form an amide group by observation of HMBC correlations from the 6′-XH proton to C-7′ in DMSO-d_6, and from H-5′ to C-7′ in $CDCl_3$. All data above completed the construction of isocoumarin-type substructure I, which is the common structural moiety of all amicoumacins.

Substructure II was identified starting from a methine proton H-8′, which was readily observed as a triplet signal at δ_H 3.90 in the ^{1}H-NMR spectrum in DMSO-d_6. By tracing the cross peaks in the ^{1}H–^{1}H COSY spectrum in DMSO-d_6 between H-8′ and H-9′ (δ_H 3.64), between H-10′ (δ_H 3.19) and H-11′$_a$ (δ_H 2.02), H-11′$_b$ (δ_H 2.03), together with HMBC correlations from H-8′ to C-10′ (δ_C 48.2), from H-9′ to C-11′ (δ_C 31.7) and from H-11′$_a$, H-11′$_b$ to C-9′ (δ_C 73.5), structural moiety as –CH(8′)–CH(9′)–CH(10′)–CH_2(11′)– was established. The chemical shifts of H-8′, C-8′ (δ_C 72.4) and H-9′, C-9′ in DMSO-d_6 indicated C-8′ and C-9′ should be attached to an oxygen or nitrogen atom. This finding was confirmed by ^{1}H–^{1}H COSY correlations between H-8′ and 8′-OH proton (δ_H 5.59), between H-9′ and 9′-OH proton (δ_H 4.97), as well as HMBC correlations from 8′-OH proton to C-9′, from 9′-OH proton to C-8′ and C-10′ in DMSO-d_6. A carbon signal downfield at δ_C 169.1 in the ^{13}C-NMR spectrum in DMSO-d_6 suggested the presence of 12′-C=O, which was connected to C-11′ by observation of cross peaks between H-11′$_a$, H-11′$_b$ and C-12′ in the HMBC spectrum in DMSO-d_6. A singlet proton signal from 13′-NH proton (δ_H 7.74) was readily observed in the downfield region of the ^{1}H-NMR spectrum in DMSO-d_6. HMBC correlations from 13′-NH proton to C-11′, C-12′ and C-14′ (δ_C 66.8) suggested 13′-NH was attached to both C-12′ and C-14′. Two singlet methyl proton signals, 16′-CH_3 (δ_H 1.18) and 17′-CH_3 (δ_H 1.24), showing HMBC correlations to the quaternary carbon C-14′, established the isopropylidene unit CH_3(16′)-C(14′)-CH_3(17′). Herein, 32 out of the 33 protons in **1** had been assigned, and only one doublet proton signal at δ_H 1.92 in the ^{1}H-NMR spectrum in DMSO-d_6 remained to be

Table 1 NMR spectroscopic data for compounds 1, 2 and 3

	*Hetiamacin B (**1**)*				*Hetiamacin C (**2**)*		*Hetiamacin D (**3**)*	
	DMSO-d_6		*$CDCl_3$*		*$CDCl_3$*		*$CDCl_3$*	
Position	δ_H[a] *(mult, J Hz)*	δ_C[b]	δ_H[c] *(mult, J Hz)*	δ_C[d]	δ_H[a] *(mult, J Hz)*	δ_C[b]	δ_H[a] *(mult, J Hz)*	δ_C[b]
1	—	169.1	—	169.5	—	169.5	—	169.5
3	4.69 (d, 12.6)	81.1	4.61 (d, 12.5)	81.1	4.61 (d, 12.6)	81.1	4.62 (d, 12.6)	81.0
4_a	2.85 (dd,16.2, 3.0)	29.1	2.81 (d, 15.5)	30.3	2.82 (d, 15.6)	30.3	2.83 (d, 15.6)	30.3
4_b	3.03 (dd, 16.2, 12.6)		3.06 (dd, 15.5, 13.5)		3.07 (dd, 15.6, 13.2)		3.07 (dd, 15.6, 14.4)	
5	6.82 (d, 7.2)	118.5	6.69 (d, 7.0)	118.3	6.70 (d, 7.2)	118.3	6.70 (d, 6.6)	118.3
6	7.48 (dd, 8.4, 7.2)	136.3	7.41 (dd, 8.5, 7.0)	136.5	7.41 (dd, 8.4, 7.2)	136.5	7.42 (dd, 7.8, 6.6)	136.5
7	6.84 (d, 8.4)	115.2	6.87 (d, 8.5)	116.2	6.87 (d, 8.4)	116.2	6.88 (d, 7.8)	116.3
8	—	160.8	—	162.1	—	162.1	—	162.2
8-OH		—	10.78 (brs)		10.74 (brs)	—	10.79 (brs)	—
9	—	108.3	—	108.1	—	108.0	—	108.0
10	—	140.7	—	139.3	—	139.4	—	139.3
1′	0.85 (d, 6.6)	21.5	0.95 (d, 7.0)	21.8	0.94 (d, 6.6)	21.8	0.95 (d, 6.6)	21.8
2′	0.89 (d, 6.6)	23.3	0.96 (d, 7.0)	23.1	0.96 (d, 7.2)	23.1	0.97 (d, 7.2)	23.1
3′	1.66 (m)	24.0	1.63 (m)	24.8	1.63 (m)	24.8	1.63 (m)	24.8
$4'_a$	1.32 (m)	39.1	1.49 (m)	40.6	1.47 (m)	40.5	1.49 (m)	40.6
$4'_b$	1.66 (m)		1.84 (m)		1.84 (m)		1.85 (m)	
5′	4.20 (m)	47.9	4.34 (m)	48.8	4.35 (m)	48.8	4.35 (m)	48.7
6′-NH	7.64 (d, 9.6)	—	7.27 (d, 11.0)		7.29 (d, 9.6)	—	7.24 (overlapped)	—
7′	—	172.7	—	174.7	—	174.5	—	174.4
8′	3.90 (t, 6.0)	72.4	4.01 (d, 9.0)	72.4	4.02 (d, 9.0)	72.1	4.06 (d, 7.8)	73.1
8′-OH	5.59 (d, 6.0)	—	Unidentified		6.06 (brs)	—	6.15 (brs)	—
9′	3.64 (dd, 11.4, 5.4)	73.5	3.60 (dd, 8.5, 6.5)	73.3	3.64 (m)	73.4	3.53 (m)	73.2
9′-OH	4.97 (d, 5.4)	—	4.86 (brs)		4.84 (brs)	—	4.87 (brs)	—
10′	3.19 (m)	48.2	3.44 (m)	51.0	3.27 (m)	55.1	3.24 (m)	56.4
$11'_a$	2.02 (m)	31.7	2.21 (dd, 17.0, 11.5)	32.5	2.28 (dd, 16.2, 11.4)	32.4	2.27 (dd, 16.2, 12.6)	34.1
$11'_b$	2.03 (m)		2.58 (d, 15.5)		2.57 (d, 16.2)		2.73 (d, 15.0)	
12′	—	169.1	—	170.2	—	171.1	—	170.5
13′-NH	7.74 (s)	—	Unidentified	—	6.41 (brs)		Unidentified	
14′	—	66.8	—	68.2	4.44 (brs)	63.9	4.26 (brs)	68.8
15′-NH	1.92 (d, 12.6)	—	6.31 (s)	—	Unidentified		Unidentified	
16′	1.18 (s)	28.4	1.41 (s)	28.6	1.33 (d, 5.4)	22.5	1.63 (overlapped)	29.4
17′	1.24 (s)	30.6	1.42 (s)	31.4			1.00 (overlapped)	8.64

[a]^{1}H-NMR spectral data were recorded at 600 MHz.
[b]^{13}C-NMR spectral data were recorded at 150 MHz.
[c]^{1}H-NMR spectral data were recorded at 500 MHz.
[d]^{13}C-NMR spectral data were recorded at 125 MHz.

identified. By calculation of element composition and degrees of unsaturation of **1**, it was speculated that a tetrahydro-4-pyrimidinone ring must exist in substructure II and was composed of 10′-CH, 11′-CH_2, 12′-C=O, 13′-NH, 14′-C and 15′-NH, which was the last unassigned proton signal at δ_H 1.92. Cross peaks in the HMBC spectrum from 15′-NH proton to C-16′ (δ_C 28.4) in DMSO-d_6, and to C-11′ in $CDCl_3$, further supported this assignment. Thus, substructure II was elucidated.

Finally, substructures I and II were linked through 7′-C=O and 8′-CH on the basis of HMBC correlations from 8′-OH proton and H-9′ to C-7′ in DMSO-d_6. Therefore, the planar structure of **1** was completed.

Hetiamacin C (**2**) was obtained as a white powder with UV absorption maxima at 203 (ε 20 264), 246 (4503) and 314 (3493), which suggested that this compound also had 3,4-dihydro-8-hydroxyisocoumarin skeleton in its structure. The molecular formula of compound **2** was determined as $C_{22}H_{31}O_7N_3$ by HR-ESI-MS (*m/z* found: 450.2252 $[M+H]^+$, calcd: 450.2248). The MW of **2** was 14 Da smaller than that of **1**, suggesting that the structures of **1** and **2** likely differed by the absence of a methyl group. This finding was fully supported by comparison of the NMR spectral data of **1** and **2** in $CDCl_3$ (Table 1). The ^{1}H-NMR spectrum of **2** was closely similar to that of **1**, but only three methyl groups presented in highfield of the ^{1}H-NMR spectrum of **2** rather than four in that of **1**. The three methyl groups of **2** were assigned as 1′-CH_3 (δ_H 0.94), 2′-CH_3 (δ_H 0.96) attached to C-3′ (δ_C 24.8), and 16′-CH_3 (δ_H 1.33) attached to C-14′ (δ_C 63.9) by observation of ^{1}H–^{1}H COSY correlations between H-1′, H-2′ and H-3′, and between H-16′ and H-14′, respectively. The doublet signal of H-16′ in the ^{1}H-NMR spectrum along with the tertiary carbon signal of C-14′ in the DEPT spectrum revealed that C-14′ carried only one methyl group (16′-CH_3). Thus, the structure of **2** was determined as in Figure 3.

Hetiamacin D (**3**) had a molecular formula of $C_{23}H_{33}O_7N_3$ determined by HR-ESI-MS (*m/z* found: 464.2388 $[M+H]^+$, calcd: 464.2391). The similar UV absorption maxima at 203 (ε 22 353), 246 (4756) and 314 (3090) suggested **3** was also a member of

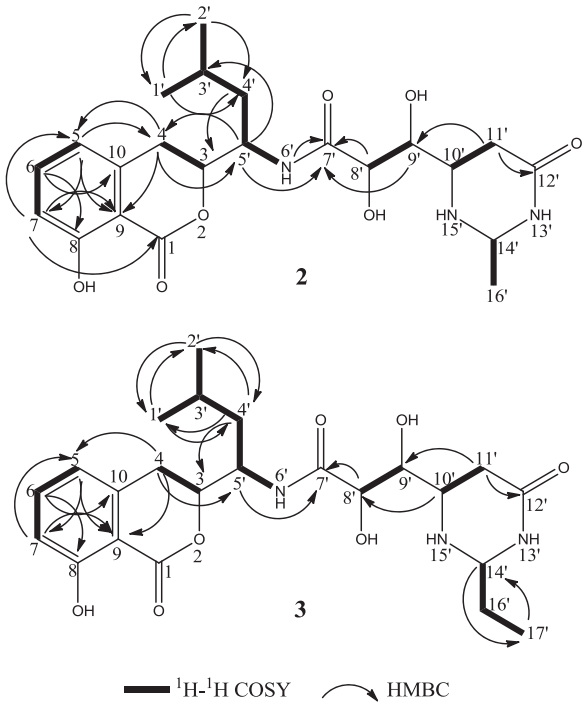

Figure 3 Key 2D NMR correlations of **2** and **3** in $CDCl_3$.

Table 2 Antibacterial activity of 1

Test organisms	*MIC (μg ml^{-1})*
S. aureus ATCC 29213 (MSSA)	1
S. aureus 09-6 (MSSA)[a]	1
S. aureus ATCC 33591 (MRSA)	1
S. aureus 2630 (MRSA)[a]	1
S. aureus Mu50 (VISA)	4
S. epidermidis ATCC 12228 (MSSE)	1
S. epidermidis 12-6 (MSSE)[a]	1
S. epidermidis 12-8 (MRSE)[a]	2
S. haemolyticus 2818 (MRSH)[a]	2
Enterococcus faecalis ATCC 29212 (VSE)	64
E. faecalis ATCC 51299 (VRE)	64
E. faecium ATCC 700221 (VRE)	64
Escherichia coli ATCC 25922	64
Klebsiella pneumonia ATCC 700603	>64
Pseudomonas aeruginosa ATCC 27853	>64
Acinetobacter calcoacetious ATCC 19606	>64
Enterobacter cloacae ATCC 43560	>64
Proteus mirabilis 12-6[a]	>64
Citrobacter freundii ATCC 43864	64

Abbreviations: ATCC, American Type Culture Collection; MRSA, methicillin-resistant *S. aureus*; MRSE, methicillin-resistant *S. epidermidis*; MSSA, methicillin-sensitive *S. aureus*; MSSE, methicillin-sensitive *S. epidermidis*; VISA, vancomycin-intermediate *S. aureus*; MRSH, methicillin-resistant *S. haemolyticus*; VSE, vancomycin-sensitive enterococci; VRE, vancomycin-resistant enterococci.
[a]The strain was isolated from the clinic.

amicoumacin group antibiotics. Detailed analysis of the ^{1}H-NMR and ^{13}C-NMR spectra of **3** in $CDCl_3$ (Table 1) suggested **3** had the same scaffold as **1** and **2**, except that C-14′ (δ_C 68.8) was linked with an ethyl group assigned as –CH_2(16′)–CH_3(17′). This assignment was supported by the cross peaks between 14′-CH (δ_H 4.26) and 16′-CH_2 (δ_H 1.63), and between 16′-CH_2 and 17′-CH_3 (δ_H 1.00) in the ^{1}H–^{1}H COSY spectrum, and further confirmed by HMBC correlations from H-17′ to C-14′, and from H-14′ to C-17′ (δ_C 8.64). Therefore, the structure of **3** was determined as in Figure 3.

The relative configurations of compounds **1, 2** and **3** were elucidated by analysis of ^{1}H–^{1}H COSY coupling constants and cross peaks observation in the ROESY spectra. In the ^{1}H-NMR spectrum in DMSO-d_6 of compound **1**, a large coupling constant (12.6 Hz) between H-3 and H-4$_b$ demonstrated that H-3 was in a pseudo-axial orientation. In the ^{1}H-NMR spectrum in $CDCl_3$ of compound **1**, a large coupling constant (11.5 Hz) between H-10′ and H-11′$_a$ indicated H-10′ had a axial orientation. For **2** and **3**, the relative configurations of H-3 and H-10′ were the same as **1** by comparison of similar ^{1}H–^{1}H COSY coupling constants with those of **1**. The H-14′ of **2** and **3** was established to be both in the axial orientation by the cross peak observed between H-10′ and H-14′ in the ROESY spectrum in $CDCl_3$. Thus, the relative stereochemistry of compounds **1–3** were determined as in Figure 1.

In vitro antibacterial activity of **1** was evaluated by the broth microdilution method according to Clinical and Laboratory Standards Institute guidelines.[9] The MIC values against bacteria of **1** are listed in Table 2. Compound **1** showed strong inhibitory activities against *S. aureus*, *S. epidermidis* and *S. haemolyticus* including drug-resistant isolates, MRSA, methicillin-resistant *S. epidermidis*, methicillin-resistant *S. haemolyticus* and vancomycin-intermediate *S. aureus*, with MIC values of 1–4 μg ml^{-1}. Compound **1** exhibited weak or no activity against tested Gram-negative bacteria, with MIC values ⩾64 μg ml^{-1}. The antibacterial activities of compounds **2** and **3** were not be evaluated because of their inadequate amounts.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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