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Isolation of 8′-Phosphate Ester Derivatives of Amicoumacins: Structure-activity Relationship of Hydroxy Amino Acid Moiety

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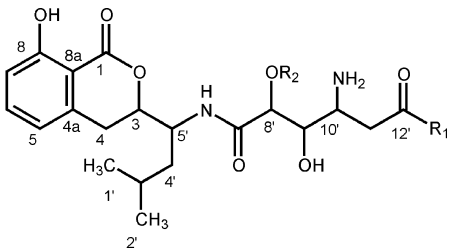
Abstract Two new compounds, 8′-phospho derivatives of amicoumacins A and B, were isolated from the culture broth of a strain of *Bacillus pumilus* together with amicoumacins A and B. Their structures were elucidated on the basis of spectroscopic methods and alkaline phosphatase treatments. Comparison of the antibacterial activities against methicillin-resistant *Staphylococcus aureus* (MRSA) of these compounds suggested that C-8′ hydroxyl and C-12′ amide group of amicoumacin A played a critical role for anti-MRSA activity.

Keywords amicoumacin, dihydroisocoumarin, anti-MRSA, phosphorylation, structure-activity relationship

Dihydroisocoumarin derivatives are mostly implicated as having physiological roles such as plant-microbe interactions [1～3]. The isolation of amicoumacins A (**1**) and B (**2**) [4～6] and closely related AI-77s [7, 8] from *Bacillus pumilus* evoked interest in medicinal research because of their characteristic biological activities including antibacterial, anti-inflammatory, antiulcer, gastroprotective, and anti-*Helicobacter pylori* activities [1～3, 7, 9, 10]. Their unique basic structures (Fig. 1) of the dihydroisocoumarin with a hydroxy amino acid side chain have attention from synthetic chemists, and there are a number of total syntheses and synthetic approaches, represented by Cappiello *et al.* [11] and references cited therein. The structurally related antibiotics with different hydroxy amino acid side chains, baciphelacin [12], Y-05460M-A [13], PM-94128 [14], xenocoumacins [15], and Sg17-1-4 [16], all with significant activities, represent a growing number of antibiotics in this class.

In our screening program for new antibiotics against methicillin-resistant *Staphylococcus aureus* (MRSA), **1** was isolated from the culture broth of strain MU313B which was newly isolated from a soil sample. The strain, which was identified as *Bacillus pumilus* by 16S rDNA analysis, also produced **2** [4～6] and two new amicoumacin derivatives (**3** and **4**). In this paper, we describe the structure determination of two new amicoumacin A derivatives and compare their parent compounds, **1** and **2**.



Compounds	R_1	R_2
1 (Amicoumacin A)	NH_2	H
2 (Amicoumacin B)	OH	H
3	OH	H_2PO_3
4	NH_2	H_2PO_3

Fig. 1 Structures of compounds **1** to **4**.

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Results and Discussion

After the initial identification of **1** as an anti-MRSA active substance, other peaks with similar UV spectra were detected in the culture broth, suggesting that these were derivatives of **1**.

Fig. 2 shows a typical time-course for production of compound **1** to **4** in the fermentation broth. Production of **1** began 24 hours after inoculation and increased until about 36 hours. The titre of **1** was then gradually decreased to 72 hours. **2** to **4** were only detected in significant amounts after 36 hours. These compounds were, thus isolated from 36-hour cultures.

The culture supernatant was treated with SepPak $VacC_{18}$, and the eluate with 70% aq MeOH was subjected to preparative HPLC to give pure **1** (Rt: 29~35 minutes, 6.0 mg). **2** to **4** were purified by the same procedure for **1**. **2** (4.0 mg) was obtained from the foregoing eluate by HPLC purification (Rt: 35~41 minutes). **3** (18 mg) and **4** (22 mg) were obtained from the 50% aq MeOH fraction of SepPak $VacC_{18}$ treatment followed by HPLC purification (Rt: 11~17 minutes for **3**; 17~23 minutes for **4**).

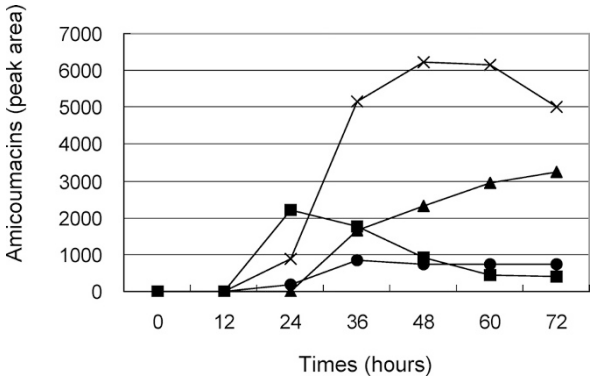

Fig. 2 A typical time-course of amicoumacins production.

Production of amicoumacins was estimated from the peak intensity of HPLC analyses with detection by wavelength at 315 nm. The injection volume of culture broth was 100 μl. —■; **1**, —●; **2**, —▲; **3**, —×; **4**.

The physico-chemical properties of **1** to **4** are summarized in Table 1. The molecular formula of **1** was revealed to be $C_{20}H_{29}N_3O_7$ by HR-ESI-MS analysis. Based on the MS and NMR data as well as its characteristic UV absorbance at 315 nm, **1** was elucidated as amicoumacin A which was previously isolated from *Bacillus pumilus* [4~6]. **2** was identified as amicoumacin B [4~6] in a similar manner, and its structure was also confirmed with the direct comparison with an authentic sample.

The physico-chemical properties and NMR spectral data of **4** and **3** were very similar to those of amicoumacin A and B [6], respectively (Tables 1 and 2). The molecular formula of **4** was revealed to be $C_{20}H_{30}N_3O_{10}P$ by HR-ESI-MS analysis. Comparison of NMR and MS data between **1** and **4** indicated that **4** was a mono phosphate ester. The presence of a phosphate moiety was suggested by the ^{31}P-NMR signals: 0.09 ppm in **4**. The position of phosphate moiety was assigned to C-8′ based on its down field shift effect on NMR spectra: ^{1}H, 4.16 ppm in **1** to 4.68 ppm in **4**; ^{13}C, 73.6 ppm in **1** to 76.1 ppm in **4**. Thus, **4** was determined to be the 8′-phospho derivative of amicoumacin A. Similarly, **3** was determined the 8′-phospho derivatives of amicoumacin B by comparison with **2**.

To confirm the structures of **4** and **3**, these compounds were treated with alkaline phosphatase at pH 8.8. As a result, compounds **4** or **3** were converted to **1** or **2**,

Table 1 Physico-chemical properties of **1** to **4**

	1 (Amicoumacin A)	**2** (Amicoumacin B)	**3**	**4**
Molecular weight	423	424	504	503
Molecular formula	$C_{20}H_{29}N_3O_7$	$C_{20}H_{28}N_2O_8$	$C_{20}H_{29}N_2O_{11}P$	$C_{20}H_{30}N_3O_{10}P$
HR-ESI-MS (*m/z*)				
Pos $[M+H]^+$				
Calcd	424.2084	425.1924	505.1587	504.1747
Found	424.2067	425.1940	505.1582	504.1745
Neg $[M-H]^-$				
Calcd	422.1927	423.1767	503.1431	502.1591
Found	422.1941	423.1776	503.1432	502.1572
UV λ_{max} (50% MeOH+0.1% NH_4OAc)	248, 315	248, 315	248, 315	248, 315
$[\alpha]_D^{23}$ *c* 0.01 (MeOH)	−81.3	−46.5	−55.4	−101.8

Table 2 ^{1}H-, ^{13}C- and ^{31}P-NMR chemical shifts of **3** and **4**

Position	**3**			**4**		
	δ_C[a]	δ_H[a] (*J* in Hz)	δ_p[b]	δ_C[a]	δ_H[a] (*J* in Hz)	δ_p[b]
1	171.1			171.1		
3	82.4	4.63 (m)		82.4	4.64 (ddd, *J*=12.8, 3.2, 2.7)	
4	30.5	2.90 (m)		30.5	2.90 (dd, *J*=16.5, 2.7)	
		3.14 (dd, *J*=16.5, 12.4)			3.13 (dd, *J*=16.5, 12.8)	
4a	141.8			141.7		
5	119.6	6.78 (d, *J*=7.8)		119.6	6.77 (d, *J*=7.3)	
6	137.4	7.42 (dd, *J*=8.2, 7.8)		137.4	7.42 (dd, *J*=8.2, 7.3)	
7	116.6	6.81 (d, *J*=8.2)		116.6	6.81 (d, *J*=8.2)	
8	163.2			163.2		
8a	109.5			109.5		
1′	21.9	0.92 (d, *J*=6.4)		21.9	0.91 (d, *J*=6.4)	
2′	23.9	0.96 (d, *J*=6.4)		23.9	0.96 (d, *J*=6.4)	
3′	25.5	1.77 (m)		25.6	1.74 (m)	
4′	40.3	1.44 (m)		40.3	1.43 (ddd, *J*=13.7, 9.6, 3.7)	
		1.79 (m)			1.82 (m)	
5′	50.6	4.36 (ddd, *J*=11.0, 3.7, 3.2)		50.6	4.35 (ddd, *J*=11.0, 3.7, 3.2)	
7′	172.5			172.6		
8′	76.3	4.66 (m)		76.1	4.68 (m)	
9′	72.6	4.14 (m)		72.4	4.17 (m)	
10′	52.5	3.70 (m)		52.6	3.74 (ddd, *J*=10.5, 3.7, 3.2)	
11′	34.5	2.66 (dd, *J*=16.9, 10.1)		33.4	2.67 (dd, *J*=16.9, 10.5)	
		2.90 (m)			2.96 (dd, *J*=16.9, 3.2)	
12′	175.8			175.4		
			−0.31			0.09

[a] Chemical shifts of ^{1}H- and ^{13}C-NMR are shown with reference to CD_3OD as 3.30 ppm and 49.0 ppm, respectively.

[b] The δ values referenced to 0.3% phosphoric acid in MeOH as external standard at 0 ppm.

respectively (data not shown). These results showed that **4** or **3** were 8′-phospho derivatives of amicoumacin A or B, respectively. Treatment of **4** with alkaline phosphatase also afforded **2** and **3** together with **1**. **4** may be converted to **3** under these conditions as **1** is similarly converted to **2**.

To clarify the structure-activity relationship of hydroxy amino acid moiety of amicoumacins, antibacterial activity of **1** to **4** against *Staphylococcus aureus* ATCC 43300 was measured. The MICs of these compounds were shown in Table 3. The activity of **1** was almost equivalent to that of vancomycin. On the other hand, **2** to **4** showed no activity at the concentrations tested. These results suggested that C-8′ hydroxyl and C-12′ amide group of **1** play a critical role for anti-MRSA activity.

The amicoumacins are a family of structurally diverse products that possess a broad range of pharmacological properties such as antibacterial, anti-inflammatory, antiulcer, gastroprotective, and anti-*Helicobacter pyroli* activities. Their varying activities may be related to the hydroxy amino acid moiety, because these compounds have the common dihydroisocoumarin structure. This is the first report of the isolation of C-8′ modified amicoumacin derivatives and their evaluation for antibacterial activity, though some studies of structure-activity relationships have been reported in these compounds. In this study, we showed

Table 3 MICs of **1** to **4** against *Staphylococcus aureus* ATCC 43300

Compounds	MIC (μg/ml)
1	4
2	256
3	256
4	>256
Vancomycin	2.5

that C-8′ hydroxyl and C-12′ amide group of **1** play a critical role for anti-MRSA activity. The importance of the C-12′ amide group for anti-MRSA activity agrees with the structural requirements for other biological activities [6]. This study has shown that the antibacterial activity of amicoumacin A is a dramatically decreased by phosphorylation at the C-8′ hydroxyl group.

Time course experiments showed that **4** was produced as concentrations of **1** declined. Phosphorylative inactivation of antibiotics is known [17～20], and considered as one of the resistance mechanisms to antibiotics. Two alternative hydroxyl groups, C-8′ and C-9′, are available for *O*-phosphorylation in the amicoumacin structures. Our analysis revealed the exclusive phosphorylation of the C-8′ hydroxyl group. Phosphorylation of amicoumacins is, thus an interesting finding possibly in relation to self-resistance and export of amicoumacins. Interestingly, amicoumacin production is distributed not only in *B. pumilus* but also in the other *Bacillus* species [21], and its biological significance is of great interest in the *Bacillus* genus.

Experimental

General

HPLC analysis was performed with a TOSOH 8020 system fitted with a diode array detector. Preparative HPLC was performed using a Waters 600E system fitted with diode array detector. ^{1}H-NMR (400 MHz), ^{13}C-NMR (100 MHz) and ^{31}P-NMR (162 MHz) spectra were recorded on a JEOL JNM-ECX400 spectrometer. Liquid chromatography time-of-flight mass spectrometry (LC TOF-MS) data were obtained under electron spray ionization (ESI) on a Waters LCT Premier^{TM}. Optical rotation was measured on a digital polarimeter P-1020 (JASCO).

Microorganism and Taxonomy

The producing strain MU313B was isolated from a soil sample collected in Tsukuba City, Ibaraki prefecture, Japan. A stock culture of the strain was maintained on yeast extract - malt extract agar medium consisting of yeast extract 0.4%, malt extract 1.0%, glucose 0.4%, and agar 1.8% (pH 7.3) at 30°C. A segment of the 16S rDNA was amplified by colony-PCR using the primer sets of 9F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1541R (5′-AAGGAGGTGATCCAGCC-3′) [22] in a final volume of 100 μl for 30 cycles of amplification using a step program (30 seconds at 95°C, 30 seconds at 55°C, and 1 minute at 72°C). Gel-purified PCR products were subjected to DNA sequencing service (the Bio Matrix Inc., Japan). The producing organism, the strain MU313B, was identified as *Bacillus pumilus* based on the complete identity of the 16S rDNA sequence with that (accession no. DQ870735) in the GenBank database.

Culture Conditions

A maintenance culture of the strain MU313B was inoculated into 500-ml Erlenmeyer flask containing 50 ml of culture medium composed of glucose 2.0%, $NaNO_3$ 0.2%, K_2HPO_4 0.1%, KCl 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%, peptone 0.5%, yeast extract 0.1%, and soybean meal 2.0% (pH 7.0, before sterilization), and cultured at 30°C for 36 hours on a rotary shaker (200 rpm).

HPLC Analysis

1 to **4** in the fermentation broth and conversion experiments were analyzed under the following conditions: column; SunFire (4.6 i.d.×150 mm, Waters), column temperature 40°C, gradient elution, solvent A (MeOH) and solvent B (0.2% ammonium acetate in water), gradient rate; 0～10 minutes (50% A), 10～13 minutes (50→100% A, linear), 13～17 minutes (100% A), 17～20 minutes (100→50% A, linear), flow rate; 1.0 ml/minute, detection; wavelength between at 230～400 nm using photo diode array.

Isolation of 1 to 4

For isolation of **1** to **4**, the culture broth (4.9 liter) was centrifuged at 1,600 *g* for 20 minutes to obtain the supernatant which was treated with SepPak $VacC_{18}$ cartridge (Waters). After washing with 50% aq MeOH, the active substance was eluted from the cartridge with 70% aq MeOH. These material were lyophilized and, were redissolved in 50% aq MeOH containing 0.1% ammonium acetate to subject a reversed-phase HPLC under following condition: column; SunFire (19 i.d.×150 mm, Waters), column temperature 40°C, gradient elution, solvent A (MeOH) and solvent B (0.2% ammonium acetate in water), gradient rate; 0～35 minutes (45% A), 35～38 minutes (45→100% A, linear), 38～47 minutes (100% A), flow rate; 7.0 ml/minute, detection; wavelength between at 210～400 nm using photo diode array. These fractions were then desalted using same column with 50～60% aq MeOH containing 0.5% AcOH as a mobile phase at the flow rate of 7.0 ml/minute.

Dephosphorylation of 3 and 4

A solution (200 μl) of bovine alkaline phosphatase (0.5 u, sigma P7640) and 0.3 mM of **3** or **4** in 50 mM Tris-HCl (pH 8.8) was incubated at 37°C for 5 hours. The reaction was stopped by adding to same volume of MeOH and then centrifuged to obtain a sample solution. The solution was subjected to a reversed-phase HPLC.

Antimicrobial Activity

The MICs (μg/ml) for *Staphylococcus aureus* ATCC 43300 (MRSA) were determined by microdilution technique according to NCCLS guidelines [23]. The MICs by a serial two-fold dilution (0.6~256 μg/ml) in Mueller-Hinton broth II (Becton, Dickinson and Company) were defined as the lowest concentration of amicoumacins which allowed no visible growth of the test organism in 96-well plate.

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