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STRUCTURE–ANTIBACTERIAL RELATIONSHIP OF NIGERICIN DERIVATIVES

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*Two new polyether antibiotics **3**, **5** together with three known ones **1**, **2**, **4** were isolated from* Streptomyces hygroscopicus *XM201. Based on the unambiguous NMR data assignments, their structures were determined to be 30-acetyl nigericin (**1**), 1-*O*-methyl-30-acetyl nigericin (**2**), 1,29-*O*-dimethyl-30-acetyl nigericin (**3**), nigericin (**4**), and 29-*O*-methyl abierixin (**5**), respectively. The antibacterial activities of the nigericin derivatives **1–4** were studied. Compounds **1** and **4** showed strong activities against* Staphylococcus aureus *ATCC25923 and* Bacillus cereus *1126 with MIC of 0.25 μg/mL and 0.125 μg/mL, respectively. No inhibitory activities were observed against* Escherichia coli *CMCC44103 at a concentration of 25 μg/mL. Only **1** and **4** showed distinguished effects on the protoplast regeneration clones of* B. cereus *1126 and* E. coli *CMCC44103 at a concentration of 1 μg/mL.*

Key words: polyether antibiotics, nigericin, abierixin, *Streptomyces hygroscopicus* XM201.

Polyether antibiotics belong to natural products, which are mainly produced by the *Streptomyces* genus [1]. Because they possess the ability to transport monovalent cations through biological membranes with lipophilic complexes [2], several of them are used as anticoccidial feed additives. Nigericin was classified as an ionophore because of a similar ability to transport alkali ions across lipid barriers [3]. Epinigericin and abierixin were isolated from *Streptomyces albus* NRRLB-1865 [2, 4]. In this article, two new derivatives of nigericin and abierixin with three known ones were isolated and identified from the fermentation extracts of S*treptomyces hygroscopicus* XM201, and the antibacterial activities of the four derivatives of nigericin **1–4** are discussed.

Compound **3**. The molecular formula was determined to be $C_{44}H_{74}O_{12}$ by ESIMS based on a quasi-molecular ion peak at *m/z* 817.8 $[M+Na]^+$ and NMR spectra. According to some empirical characteristic signals [5], **3** has a very similar structure to nigericin (**4**) except for three methyls (MeOOC-1, MeO-29, CH_3COO-30). HMQC and HMBC showed that the proton at δ 3.74 has a direct correlation with the carbon at δ 51.7(MeOOC-1), and the proton at δ 2.09 has a direct correlation with δ 21.9 (CH_3COO-30) and long-range correlation with the carbon at δ 171.1. In the data comparison with those of nigericin, the carboxyl at C-1 was esterified, the hydroxyl at C-29 was replaced by an oxygen-substituted methyl, and the hydroxyl at C-30 was replaced by acetyl in compound **3**. The compound was determined to be 1,29-*O*-dimethyl-30-acetyl nigericin. The detailed comparison is shown in Table 1.

Compound **5** was obtained as a colorless powder. Its molecular formula was determined to be $C_{41}H_{70}O_{11}$ on the basis of a quasi-molecular ion peak at *m/z* 761.6 and NMR spectra. In a comparison of the spectra of **5** with those of abierixin (**6**), a methoxyl at C-29 was observed in **5**. The HMQC spectra showed that the proton at δ 3.46 (MeO-29) has a direct correlation with the carbon at δ 48.0, which indicated that the hydroxyl at C-29 in abierixin [3] was replaced by an oxygen-substituted methyl in compound **5**. The compound was determined to be 29-*O*-methyl abierixin. A detailed comparison of ^{13}C spectra with abierixin is shown in Table 2.

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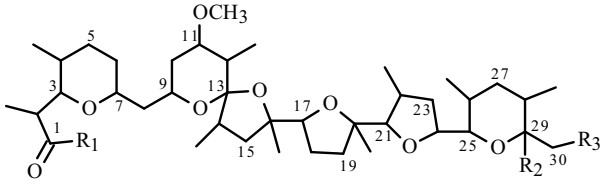
TABLE 1. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) Spectra of Compounds **1–4** in $CDCl_3$ (mult, J/Hz)

C atom	**1**		**2**		**3**		**4**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	176.3 s	–	176.5 s	–	176.4 s	–	177.5 s	–
MeOOC-1	–	–	51.6 q	3.72 (s)	51.7 q	3.74 (s)	–	–
2	44.1 d	2.32 (m)	42.9 d	2.22 (m)	43.1 d	2.65 (m)	44.2 d	2.22 (m)
2a	12.9 q	1.05 (d, J = 11.8)	12.9 q	1.24 (m)	12.7 q	1.05 (m)	13.06 q	1.24 (m)
3	73.2 d	3.77 (d, J = 10.0)	73.7 d	3.63 (m)	73.6 d	3.77 (m)	73.0 d	3.63 (m)
4	27.6 d	1.82 (m)	27.6 d	1.49 (m)	27.7 d	1.81 (m)	27.5 d	1.49 (m)
4a	10.9 q	0.93 (d, J = 7.0)	10.9 q	1.05 (d, J = 6.9)	10.9 d	0.97 (d, J = 8.40)	10.8 q	1.05 (m)
5	25.8 t	1.66 (m)	25.7 t	1.42 (m)	25.0 t	1.66 (m)	25.7 t	1.42 (m)
		1.96 (m)		1.49 (m)		1.96 (m)		1.49 (m)
6	23.5 t	2.06 (m)	21.8 t	1.90 (m)	21.5 t	2.06 (m)	23.1 t	1.74 (m)
7	68.9 d	4.08 (br.d, J = 10.8)	69.5 d	1.74 (m)	69.6 d	4.10 (m)	69.0 d	3.84 (m)
8	35.6 t	2.50 (m)	36.5 t	2.50 (m)	36.6 t	2.50 (m)	35.2 t	2.50 (m)
		0.97 (m)		1.13 (m)		0.97 (m)		1.13 (m)
9	60.3 d	4.18 (br.t, J = 7.5)	60.6 d	3.84 (m)	60.4 d	4.19 (m)	60.3 d	4.03 (m)
10	31.8 t	2.31 (m)	32.7 t	4.03 (m)	36.4 t	2.31 (m)	31.7 t	1.90 (m)
11	78.1 d	3.37 (m)	78.7 d	3.27 (m)	78.8 d	3.40 (m)	78.0 d	3.27 (m)
MeO-11	57.5 q	3.36 (s)	57.8 q	3.34 (s)	58.2 q	3.31 (s)	57.4 q	3.34 (s)
12	37.2 d	1.78 (m)	36.9 d	1.49 (m)	36.5 d	1.81 (m)	37.1 d	1.49 (m)
12a	13.1 q	1.02 (d, J = 7.5)	13.0 q	1.12 (m)	12.93 q	1.03 (m)	13.13 q	1.12 (m)
13	108.2 s	–	107.8 s	–	107.3 s	–	108.2 s	–
14	39.0 d	2.07 (m)	39.4 d	1.74 (m)	39.5 d	2.07 (m)	39.0 d	1.74 (m)
14a	13.2 q	0.87 (d)	13.2 q	0.88 (d, J = 10.0)	12.99 q	0.90 (d, J = 8.54)	13.3 q	0.88 (m)
15	42.6 t	1.78 (m)	40.0 t	1.49 (m)	43.2 t	1.78 (m)	43.2 t	1.49 (m)
		1.60 (m)		1.42 (m)		1.60 (m)		1.42 (m)
16	81.5 s	–	82.3 s	–	83.6 s	–	81.5 s	–
16a	27.9 q	1.40 (s)	26.4 q	2.18 (s)	23.9 q	1.25 (s)	28.0 q	2.18 (s)
17	82.5 d	3.52 (dd, J = 5.0, 10.0)	82.3 d	3.36 (m)	80.0 d	3.40 (m)	82.4 d	3.36 (m)
18	26.2 t	1.82 (m)	26.7 t	1.49 (m)	27.6 t	1.81 (m)	26.1 t	1.49 (m)
		1.49 (m)		1.42 (m)		1.49 (m)		1.42 (m)
19	30.7 t	2.17 (m)	32.0 t	1.75 (m)	33.0 t	2.20 (m)	31.0 t	1.75 (m)
		1.37 (m)		1.35 (m)		1.37 (m)		1.35 (m)
20	83.7 s	–	83.8 s	–	83.7 s	–	83.5 s	–
20a	22.8 q	1.10 (s)	23.1 q	2.11 (s)	23.0 q	1.15 (s)	22.7 q	2.11 (s)
21	85.6 d	3.97 (d, J = 3.5)	85.7 d	3.76 (m)	86.1 d	3.87 (m)	85.8 q	3.76 (m)
22	35.2 d	2.24 (m)	35.0 d	1.80 (m)	35.7 d	2.20 (m)	35.1 d	1.80 (m)
22a	16.0 q	0.85 (d, J = 8.5)	16.0 q	0.87 (m)	15.8 q	0.87 (d, J = 7.12)	15.6 q	0.87 (m)
23	32.2 t	2.24 (m)	34.7 t	1.80 (m)	36.4 t	2.20 (m)	32.3 t	1.80 (m)
		1.37 (m)		1.35 (m)		1.35 (m)		1.35 (m)
24	77.2 d	4.35 (m)	76.7 d	4.20 (m)	77.3 d	4.25 (m)	76.7 d	4.20 (m)
25	75.1 d	3.94 (d, J = 10.6)	76.6 d	3.74 (m)	76.8 d	4.07 (m)	74.4 d	3.74 (m)
26	32.4 d	1.37 (m)	32.4 d	1.35 (m)	32.6 d	1.37 (m)	32.5 d	1.35 (m)
26a	17.4 q	0.84 (d, J = 7.2)	17.4 q	0.86 (d, J = 6.7)	17.5 q	0.84 (d, J = 8.34)	17.3 q	0.86 (m)
27	36.8 t	1.46 (m)	37.1 t	1.42 (m)	39.6 t	1.46 (m)	37.2 t	1.35 (m)
28	35.7 d	1.43 (m)	35.5 d	1.35 (m)	35.8 d	1.40 (m)	35.7 d	1.42 (m)
28a	16.2 q	1.66 (m)	16.2 q	1.42 (m)	16.0 q	1.66 (m)	16.3 q	0.92 (m)
29	95.9 s	0.90 (d)	96.0 s	0.92 (d, J = 6.6)	98.1 s	0.93 (d, J = 9.13)	97.0 s	–
MeO-29		–	–	–	48.3 q	3.27 (s)	–	–
30	68.2 t	4.30 (m)	68.0 t	4.25 (m)	64.9 t	3.27 (s)	68.3 t	4.25 (m)
		3.90 (d, J = 10.6)		3.73 (m)		4.25 (m)		3.73 (m)
AcO-30 ($\underline{CH_3}COO$)	21.0 q	2.09 (s)	21.0 q	3.34 (s)	21.0 q	2.09 (s)	–	–
($CH_3\underline{COO}$)	171.1 s	–	171.1 s	–	171.1 s	–	–	–

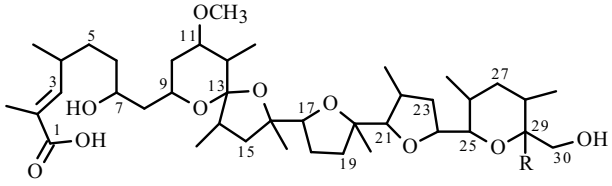
TABLE 2. The ^{13}C NMR (125 MHz) Spectra of **5** (C_5D_5N) and Abierixin

C atom	**5**	Abierixin	C atom	**5**	Abierixin
	δ_C (in C_5D_5N)	δ_C (in $CDCl_3$)		δ_C (in C_5D_5N)	δ_C (in $CDCl_3$)
1	170.7 s	172.07	18	44.1 t	
2	128.0 s	125.9	19	34.5 t	
2a	13.1 q	15.0 q	20	84.7 s	84.1 s
3	147.7 d	149.7	20a	23.3 q	19.9 q
4	33.6 d		21	86.6 d	86.5 d
4a	20.5 q	12.2 q	22	35.9 d	35.5 d
5	33.5 t				35.2 t
6	28.1 t				35.0 t
7	70.0 d	71.4 d			34.8 d
8	38.0 t	36.8 t			34.4 t
9	64.0 d	65.5 d			34.4 d
10	36.4 t	32.9 t			33.3 d
11	78.6 d	78.3 d	22a	16.1 q	15.9 q
MeO-11	57.5 q	58.0 q	23	34.1 t	32.3 t
12	37.2 d	36.9 d	24	78.3 d	76.3 d
12a	13.3 q	12.9 q	25	78.1 d	77.3 d
13	108.3 s	108.3 s	26	33.7 d	
14	39.9 d	39.6 d	26a	18.0 q	17.4 q
14a	13.5 q	13.2 q	27	34.7 t	
15	40.2 t	40.3 t	28	35.3 d	
		42.3 t	28a	16.5 q	
16	84.3 s	84.1 s	29	100.5 s	97.3 s
16a	24.6 q	27.3 q	MeO-29	48.1 q	
17	81.6 d	81.8 d	30	64.4 t	67.7 t



1 - 4

1: $R_1 = R_2 = OH$, $R_3 = CH_3COO$
2: $R_1 = OCH_3$, $R_2 = OH$, $R_3 = CH_3COO$
3: $R_1 = OCH_3$, $R_2 = OCH_3$, $R_3 = CH_3COO$
4: $R_1 = R_2 = R_3 = OH$



5, 6

5: R = OCH_3
6: R = OH

Two new polyether antibiotics **3**, **5** together with three known ones **1**, **2**, **4** were isolated from *Streptomyces hygroscopicus* XM201 and elucidated as 30-acetyl nigericin (**1**), 1-*O*-methyl-30-acetyl nigericin (**2**), 1,29-*O*-dimethyl-30-acetyl nigericin (**3**), nigericin (**4**), and 29-*O*-methyl abierixin (**5**), respectively. The antibacterial activities of the nigericin derivatives **1**–**4** were tested.

Compound **1** showed the same antibiotic activities as **4**, the MIC of **1** against *B. cereus* 1126 and *S. aureus* ATCC25923 were 0.25 μg/mL and 0.125 μg/mL, respectively, and no inhibitory activities were observed against *E. coli* CMCC44103 at the concentration of 25 μg/mL. Compounds **2** and **3** showed less antibiotic activities against bacteria, and no inhibitory activities against *B. cereus* 1126, *S. aureus* ATCC25923, and *E. coli* CMCC44103 at the concentration of 25 μg/mL. In the regeneration experiments, **1** and **4** (< 20 μg/mL) showed distinguished effects on the regeneration protoplast of *B. cereus* 1126 and *E. coli* CMCC 44103 at the concentration of 1 μg/mL, whereas **2** and **3** (>10^4 μg/mL) showed no effects.

Ionophores are well-documented polyether antibiotics, many of which have been isolated and the antibiotic activities tested. Nigericin was sensitive to *B. cereus*, and abierixin was not [4], but almost all of the polyethers showed weak inhibitory activities against G^- bacterium because the cell wall of G^- bacterium does not permit the penetration of hydrophobic molecules with molecular weights of 600 and above [6]. The regeneration effects of **1** and **4** on the regeneration protoplast of *E. coli* CMCC44103 also suggested that the cell wall of G^- bacterium is a barrier to interaction.

Nigericin has been proved to possess the ability to carry cations through membranes [7]. The antibacterial results of nigericin (**4**) and its closed derivates **1**–**3** suggested that the carboxyl at C-1 is necessary for transport of cations through biological membranes, but the hydroxyl at C-30 is not necessary.

EXPERIMENTAL

General Experimental Procedures. Mass spectra were measured on a VG Auto Spec-3000 spectrometer. NMR spectra were measured on Bruker DRX-500 spectrometers with TMS as internal standard, and ^1H and ^{13}C NMR spectra were measured in $CDCl_3$ or C_5D_5N at room temperature. The reversed-phase (RP) C_{18} silica gel for the column chromatography was obtained from Merck and the Sephadex LH-20 from Amersham Biosciences.

Fermentation and Extraction. The strain was cultured at 28°C on yeast extract/malt extract medium (g/L): yeast extract 3.0, peptone 5.0, malt extract 3.0, glucose 10.0, addition $MgCl_2$ (2.5 mol/L) diluted up to 18 L. After two weeks of cultivation the cultures were extracted three times by 80% ethyl acetate, 15% methanol, and 5% formic acid. The organic solutions were collected by filtration and removed under vacuum at 40°C to yield the crude extract (6.0 g).

Isolation of the Compounds. The extracts (6.0 g) were dissolved with methanol and run on MPLC over a reversed-phase (RP-18) silica gel (170 g) column, eluting with water containing increasing amounts of methanol to produce four fractions (fraction A – D). Fraction D (3 g) was subjected to column chromatography over Sephadex LH-20 (140 g) and eluted with methanol. The combined fraction (2 g) was further subjected to MPLC over reversed-phase silica gel (RP-18, 120 g) column, eluting with acetone-water (6:4; 7:3, v/v) to produce two fractions (fraction Da and Db). Fractions Da (0.2 g) and Db (1.5 g) were further purified by normal silica gel column, eluting with petroleum ether - acetone (50:1; 30:1, v/v) to obtain compounds **1** (20 mg), **2** (30 mg), **3** (9 mg), **4** (100 mg), and **5** (10 mg).

Bioassay Procedures. The antimicrobial activity of compounds **1**–**4** was tested against *S. aureus* ATCC25923, *E. coli* CMCC44103, and *B. cereus* 1126 in a 96-well microtiter plate using a modified method [8]. All of the compounds are tenfold diluted to 1 mg/mL in stock solution, then twice diluted from 0.1×10^{-3} μg/mL to 500 μg/mL with medium containing yeast extract 10 g, peptone 5 g, and NaCl 10 g in 1 liter tap water (or medium containing yeast extract 10 g, peptone 20 g, and glucose 20 g in 1 liter tap water), and dispensed into microtiter plates; each of compounds was tested three times. Bacteria and fungi were adjusted to 10^5 CFU/mL and 10^4 CFU/mL, respectively, for each microtiter plate. The microtiter plates were incubated at 37°C and 28°C for 24–48 hours, respectively. The results of inhibition were determined by direct observation. The lowest concentration of the compounds where no growth of microorganisms was observed was regarded as the minimum inhibitory concentrations (MIC).

To get more information on the mechanisms of ion transportation through biological membranes by polyether compounds, protoplast of bacterium was used. *E. coli* CMCC44103 and *B. cereus* 1126 were converted to protoplast with a modified method [9]. At the regeneration stage, 1 μg/mL of each compound was added to the regeneration medium containing yeast extract 10g, peptone 5g, NaCl 10g, sucrose 171 g, and $MgCl_2\cdot6H_2O$ 4.06 g, pH 7.2 in 1 liter tap water, with monensin (1 μg/mL) and blank as control. The plates were incubated at 37°C for 18–24 hours.

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