

**Structural and Functional Analysis of Pantocin A: An Antibiotic from *Pantoea agglomerans* Discovered by Heterologous Expression of Cloned Genes\*\***

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The epiphytic bacterium *Erwinia amylovora* causes fire blight, a devastating disease of rosaceous plants such as apple and pear.<sup>[1]</sup> The closely related but nonpathogenic bacterium *Pantoea agglomerans* (formerly *E. herbicola*), which usually accompanies *E. amylovora* in the wild,<sup>[2]</sup> produces a family of antibiotics that inhibit *E. amylovora* in a poorly understood strain-dependent fashion.<sup>[3,4]</sup> The numerous members of this antibiotic family constitute a source of biocontrol agents for fire blight, a pool of new and potentially useful antibiotics against human bacterial pathogens, and a model of nature's ability to produce a "library" of biologically active molecules. The complexity of antibiotic production, especially the multiple antibiotics produced by a given strain and low production in liquid culture, has hampered chemical investigations. We have taken a genomic approach to deconvolute the multiple antibiotics and to address the production problems by a particular strain. Heterologous expression of a genomic DNA library from *P. agglomerans* in *Escherichia coli* provides access to the small-molecule antibiotics. Since both *P. agglomerans* and *E. coli* are members of the widely distributed *Enterobacteriaceae*, *E. coli* should be a suitable host for heterologous expression of *P. agglomerans* genes that encode antibiotics. Herein we report the isolation and structure determination of pantocin A, a new peptide-derived antibiotic, and its molecular target.

A genomic library of *P. agglomerans* strain Eh318 was constructed in *E. coli* strain DH5 $\alpha$ .<sup>[5]</sup> Two distinct antibiotic-

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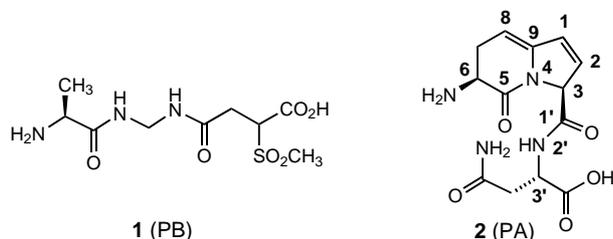
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producing cosmid clones, pCPP702 and pCPP719, were identified, and the two antibiotics they produced were trivially named pantocin A and B, respectively. One indication that the two antibiotics were different came from studies which showed that arginine suppresses the antibiotic activity of pantocin B while histidine suppresses the antibiotic activity of pantocin A. In previous work, pantocin B (PB, **1**) was structurally characterized, and its ability to potently inhibit *N*-



acetylornithine transaminase, which catalyzes a committed step in arginine biosynthesis, was established.<sup>[6–8]</sup> The characterization of pantocin A (PA, **2**) was plagued by inconsistent and low levels of production in liquid culture, together with its acid, base, and thermal lability. Significant reproducible production was eventually achieved by culturing *E. coli* XL2Blue harboring a 3.5 kb subclone of pCPP702 in the high copy number plasmid pUC19 (pUC449).

Isolation of **2** was guided by antibiotic activity against the *E. amylovora* strain Ea273 ( $IC_{50} = 200$  nM). Pantocin A (**2**) was purified from large-scale fermentation of *E. coli* XL2Blue-pUC449 by four chromatographic separations using anion-exchange, reverse-phase, and hydrophilic interactions. This compound is an optically active small molecule, and its structure was established by spectroscopic methods and synthesis of a hydrogenated derivative.

PA (**2**) has a molecular formula of  $C_{13}H_{16}N_4O_5$  as determined from HRFAB-MS studies. The  $^{13}C$  NMR (Table 1) and DEPT spectra indicate four carbonyl, four olefinic, three methine, and two methylene carbon atoms. Three vinylic, a highly deshielded methine, two  $C\alpha$  amino acid, four  $C\beta$  amino acid, and three exchangeable protons could be characterized from  $^1H$  and  $^1H$ - $^{13}C$  HMQC NMR spectra recorded in  $D_2O$  and  $[D_6]DMSO$ . A loss of  $m/z = 132$  in the tandem ESI-MS study suggested the presence of an asparagine residue, which was confirmed by 2D NMR studies. Two spin systems (H6/H7a,b/H8 and H1/H2/H3) identified by COSY experiments coupled with HMBC correlations (H6/C5, H8/C9, H1/C9, and H3/C1') established a continuous nine-carbon chain (C5 to C1') containing the non-asparagine carbon atoms in **2**. Two additional HMBC correlations (2'NH/C1' and H3'/C1') showed that the  $C_9$  and asparagine fragments must be connected by an amide bond.

$^{15}N$  NMR experiments on  $^{15}N$ -labeled **2**, which was made using  $^{15}N$  ammonium sulfate in minimal culture media, revealed the nitrogen connectivity. A highly downfield shifted  $^{15}N$  resonance ( $\delta = 145.1$  ppm) with correlations to H1, H2, H3, H6, and H8 required N4 to be connected to C3, C5, and C9. A  $^{15}N$  resonance at  $\delta = 36.5$  ppm, typical of an amine, and

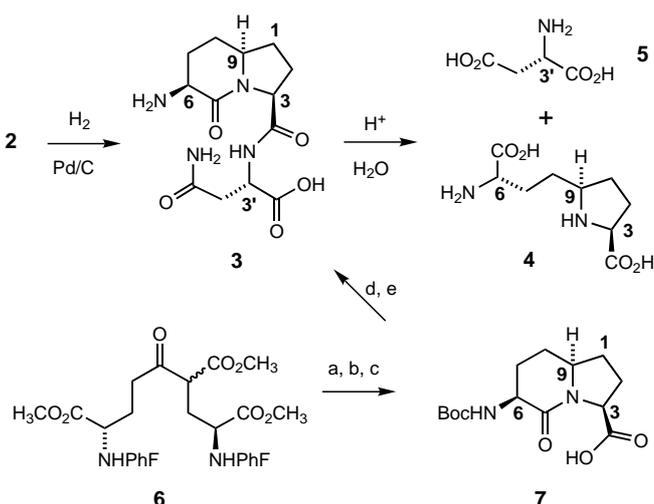
**Table 1:** NMR data for **2**.

Atom No.	$^{13}C$ [a]	$^1H$ [b]	$^{15}N$ [c]
1	126.3	6.35 (1 H, dd, 6.0, 2.0)	
2	131.5	6.15 (1 H, dd, 6.0, 1.7)	
3	66.4	5.21 (1 H, brs)	
4-N			145.1 (s)
5	167.9		
6	50.2	3.58 (1 H, dd, 13.6, 7.2)	
6-NH <sub>2</sub>			36.5 (s)
7	28.6	2.57 (1 H, m) 2.33 (1 H, m)	
8	95.5	5.12 (1 H, dd, 6.0, 2.4)	
9	143.3		
1'	166.2		
2'-NH		8.27 (1 H, d, 7.5)	126.0 (d, 93.0)
3'	49.8	4.29 (1 H, m)	
4'	37.9	2.50 (1 H, dd, 15.2, 7.0) 2.39 (1 H, dd, 15.2, 5.7)	
5'	171.6		
5'-NH <sub>2</sub>		6.84, 7.72 (2 H, brs)	113.6 (t, 90.0)
6'	172.6		

[a]  $^{13}C$  NMR (100 MHz, 25 °C,  $[D_6]DMSO$ ). [b]  $^1H$  NMR (500 MHz, 25 °C,  $[D_6]DMSO$ ) assignments based on  $^1H$ - $^{13}C$  HMQC. [c]  $^{15}N$  NMR (40.5 MHz, 25 °C,  $D_2O$ ). The  $^{15}N$  chemical shift was calibrated indirectly based on the gyromagnetic ratio of  $^{15}N$  and  $^1H$  according to reference [12].

its HMBC correlations with H6 and H7 required an amino group at C6, thus completing the planar structure of **2**.

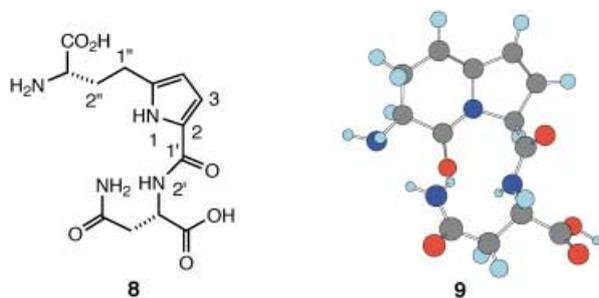
The stereocenters at C3', C3, and C6 were defined by the hydrogenation of **2**, NOESY experiments, and synthesis. Hydrogenation of **2** gave **3** as a single diastereomer, and NOESY correlations between H3/H9 and H6/H9 require H3 and H6 to be on the same face (Scheme 1). Hydrolysis of **3** gave **4** and L-aspartic acid (**5**), which set the absolute configuration at C3' as *S*. Both **3** and its 3(*R*),6(*R*),9(*R*) diastereomer were prepared from **6**, which was synthesized from



**Scheme 1.** a) NaOH/dioxane;  $CH_3I/K_2CO_3/DMSO$ ; b)  $H_2/Pd-C$ ; c)  $(Boc)_2O/TEA/DMF$ ; NaOH/ $CH_3OH-H_2O$ ; d) H-Asn-O $\dagger$ Bu-HCl, EDC/NMM/DMF; e) TFA/ $CH_2Cl_2$ . Boc = *tert*-butoxycarbonyl, TEA = triethylamine, EDC = 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide, NMM = *N*-methylmorpholine, TFA = trifluoroacetic acid.

L- or D-Glu(OMe)-OMe following a literature procedure<sup>[9]</sup> (Scheme 1). HPLC analysis of synthetic **3** and **3** derived from **2** indicated that the absolute configuration of **3** was 3(*S*),6(*S*). Thus, C3, C6, and C3' all have the absolute configuration corresponding to an L-amino acid, and the absolute stereostructure of **2** is as shown.

Perhaps the most striking feature of **2**, which to the best of our knowledge has no precedents in reported natural or synthetic compounds, is its very existence given the apparent ease with which it could aromatize to a pyrrole. Removal of a proton from C3, which is  $\alpha$  to the carbonyl group at C1', followed by reprotonation at C8 would generate a pyrrole, and this process likely underlies the lability of **2**. A minor inactive compound was also recovered on isolating **2**, and this was characterized as **8**. Compound **8** arises from the aromatization and hydrolysis of the bicyclic amide bond. No

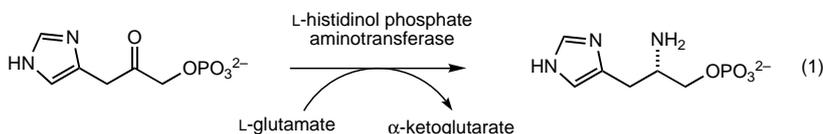


material representing aromatization unaccompanied by hydrolysis was isolated—an observation consistent with the known rapid hydrolysis of *N*-acylpyrroles.<sup>[10]</sup> While **2** is sensitive to acid, base, and elevated temperatures, it survives for days at room temperature in aqueous solution under neutral pH conditions. Two structural features of **2** undoubtedly contribute to its modest persistence: the C3–H bond is shown in energy-minimized structures of **2** not to be aligned with the  $\pi$  bond of the C1' carbonyl group, and this lack of conjugation creates a kinetic barrier to proton removal (see structure **9**). The out of conjugation conformation is at least partially enforced by hydrogen bonds from asparagine-derived NH groups to the C5 carbonyl oxygen atom. The electron-withdrawing effect of the C5 carbonyl group also diminishes the thermodynamic benefit of aromatization, and this electron-withdrawing effect is enhanced by the same hydrogen bonds.

With the structure of **2** established, we addressed how such a polar molecule gains entry into *E. amylovora* cells and the nature of its molecular target.<sup>[11]</sup> Addition of the synthetic tripeptide Ala-Gly-Gly to the test medium, in amounts exceeding three times that of **2**, suppresses the antibiotic activity of **2**. Thus, **2** likely gains entry through the tripeptide transporter, and the added tripeptide monopolizes the transporter thereby limiting cellular entry of **2**.<sup>[6,11]</sup>

Since addition of histidine suppresses the activity of **2**, the cellular target was sought on the histidine biosynthetic pathway.<sup>[11]</sup> The common strategy of feeding intermediates

along a biosynthetic pathway has limited applicability in histidine biosynthesis since many of the intermediates are ionized phosphate esters that have poor cellular access. Using the well-characterized histidine biosynthetic enzymes of *E. coli*, the step catalyzed by L-histidinol phosphate aminotransferase, which converts imidazole acetol phosphate to L-histidinol phosphate [Eq. (1)], was identified as the target of **2**.<sup>[11]</sup>



L-histidinol phosphate aminotransferase is a pyridoxal-5'-phosphate (PLP) dependent enzyme that couples the reductive amination of imidazole acetol phosphate with the conversion of L-glutamate into  $\alpha$ -ketoglutarate.

Pantocin A and B share a close functional relationship that is not apparent from their structures. Both gain access to their cells via the tripeptide transporter and inhibit transaminase-catalyzed amino acid biosynthetic steps that introduce nitrogen functionality.<sup>[6,11]</sup> Whereas **1** inhibits arginine biosynthesis, **2** inhibits histidine biosynthesis. This functional similarity is unsurprising since they have the common goal of suppressing the growth of bacterial competitors in a nitrogen-poor environment—an environment where import by tripeptide transporters and amino acid biosynthesis are essential.

The structural and functional analysis of **2** (PA) reported here used techniques of small-molecule chemistry and biochemical analysis. The cosmid library/heterologous expression approach provides ready access to the DNA conferring production of **2** on *E. coli* XL2Blue-pUC449. Analysis of this DNA provides insights into the biosynthesis of **2** and identifies strains producing antibiotics related to **2** as discussed in the following communication.

## Experimental Section

**Cultivation and isolation:** *E. coli* XL2Blue-pUC449 was grown in a shaker maintained at 30 °C for 24 h in minimal media supplemented with 100  $\mu\text{g mL}^{-1}$  ampicillin.<sup>[5]</sup> The culture supernatant was applied to a Dowex 1X8-200 ( $\text{HCO}_3^-$ ) anion-exchange column and eluted with  $\text{CO}_2$ -saturated  $\text{H}_2\text{O}$ . The active fractions were lyophilized, then applied to a C18 solid-phase extraction column and eluted with  $\text{H}_2\text{O}/\text{CH}_3\text{CN}$  (100/0 to 90/10). Further fractionation by HPLC, first with a reverse-phase column (Vydac, 218TP510) using  $\text{H}_2\text{O}/0.1\text{M NH}_4\text{OAc}/\text{CH}_3\text{CN}$  (94/5/1), then with a polyhydroxyethyl A column (Poly LC, Inc.) using  $\text{H}_2\text{O}/0.1\text{M NH}_4\text{OAc}/\text{CH}_3\text{CN}$  (20/5/75) gave compounds **2** and **8** in yields of approximately 1  $\text{mg L}^{-1}$  and 0.2  $\text{mg L}^{-1}$ , respectively.

**Cloning of *E. coli*-pUC449:** Pantocin A producing cosmid clone pCPP702 was subcloned to generate pCPP1051, from which a XbaI-HindIII fragment was further subcloned into pBluescript to yield plasmid pCPP717.<sup>[5]</sup> The cloned insert was sequenced (GenBank accession No. U81376). A 3.5 kb DNA fragment was then amplified by a polymerase chain reaction from pCPP717 using primer (5'-CCGACTCTAGAGTAGGTATGAC-3') and primer (5'-ATACTCTGCAGAGTTGGTGCTCCA-3'), and ligated into pUC19 at the XbaI and PstI sites. Recombinants were selected

from transformed *E. coli* XL2 Blue cells on Luria–Bertani plates supplemented with ampicillin at 100  $\mu\text{g mL}^{-1}$ , to yield clone *E. coli* XL2 Blue-pUC449.

**2**:  $[\alpha]_{\text{D}}^{25} = -291.1^\circ$  ( $c = 1.8$ ,  $\text{H}_2\text{O}$ ); IR (KBr):  $\tilde{\nu}_{\text{max}} = 3700\text{--}2400$  (br), 1676, 1578, 1402  $\text{cm}^{-1}$ ; UV ( $\text{H}_2\text{O}$ ):  $\lambda_{\text{max}}$  ( $\epsilon$ ) = 200 (12089), 272 nm (5447); HRFAB-MS:  $m/z$ : 331.1018  $[M+\text{Na}]^+$ , calcd  $m/z$ : 331.1018 for  $\text{C}_{13}\text{H}_{16}\text{N}_4\text{O}_5\text{Na}$ ; ESI-MS/MS:  $m/z$ : 309  $[M+\text{H}]^+$ , 177, 159, 151, 149.

**8**:  $^1\text{H NMR}$  (500 MHz,  $\text{D}_2\text{O}$ , 25  $^\circ\text{C}$ ):  $\delta = 6.81$  (1H, d,  $J = 3.7$  Hz, H3), 6.12 (1H, d,  $J = 3.7$  Hz, H4), 4.66 (1H, dd,  $J = 9.0$ , 4.5 Hz, H3' (Asn  $\alpha\text{H}$ )), 3.75 (1H, t,  $J = 6.5$  Hz, H3''), 2.85 (1H, dd,  $J = 15.0$ , 4.5 Hz, H4'a), 2.80–2.69 (3H, m, H1'' and H4'b), 2.25–2.10 ppm (2H, m, H2'');  $^{13}\text{C NMR}$  (100 MHz, 5%  $\text{CD}_3\text{OD}$  in  $\text{D}_2\text{O}$ , 25  $^\circ\text{C}$ ):  $\delta = 178.6$  (C6', COOH), 177.0 (C5', CONH<sub>2</sub>), 175.4 (C4'') 163.4 (C1'), 137.4 (C5), 125.0 (C2), 113.5 (C3), 108.6 (C4), 55.3 (C3''), 52.9 (C3'), 39.1 (C4'), 31.5 (C2''), 23.8 ppm (C1''); ESI-MS/MS/MS:  $m/z$ : 327  $[M+\text{H}]^+$ , 310, 195, 177, 149, 132, 106; UV ( $\text{H}_2\text{O}$ ):  $\lambda_{\text{max}}$  ( $\epsilon$ ) = 200 (6438), 220 (2994, sh), 275 nm (10887); IR (thin film):  $\tilde{\nu}_{\text{max}} = 3700\text{--}2400$  (br), 1668, 1604, 1533, 1404  $\text{cm}^{-1}$ .

Preparation of **3** from **2**: A solution of **2** (2.4 mg) in deionized  $\text{H}_2\text{O}$  (300  $\mu\text{L}$ ) was treated with Pd/C (10 wt %, 0.66 mg) and stirred under hydrogen at room temperature for 8 h. The reaction mixture was filtered over celite, washed with deionized  $\text{H}_2\text{O}$  (2 mL), and the combined aqueous fractions were dried to give 1.7 mg of **3**.  $^1\text{H NMR}$  (500 MHz,  $\text{D}_2\text{O}$ , 25  $^\circ\text{C}$ ):  $\delta = 4.46$  (1H, d,  $J = 9.0$  Hz, H3), 4.40 (1H, dd,  $J = 8.0$ , 5.0 Hz, H3'), 3.93 (1H, t,  $J = 8.5$  Hz, H6), 3.74 (1H, m, H9), 2.76 (1H, dd,  $J = 15.0$ , 5.0 Hz, H4'a), 2.65 (1H, dd,  $J = 15.0$ , 8.0 Hz, H4'b), 2.35 (1H, m, H7a), 2.25–2.10 (4H, m, H2a, 1a, 8a, 2b), 1.91 (1H, m, H7b), 1.67 (1H, m, H8b), 1.64 ppm (1H, m, H1b);  $^{13}\text{C NMR}$  (100 MHz,  $\text{D}_2\text{O}$  + 5%  $\text{CD}_3\text{OD}$ , 25  $^\circ\text{C}$ ):  $\delta = 177.8$  (C6'), 176.6 (C5'), 173.6 (C1'), 169.5 (C5), 61.0 (C3), 59.2 (C9), 53.2 (C3'), 49.6 (C6), 38.6 (C4'), 32.3 (C1), 30.2 (C2), 26.2 (C8), 25.6 (C7);  $[\alpha]_{\text{D}}^{25} = -57.8^\circ$  ( $c = 0.16$ ,  $\text{H}_2\text{O}$ ); UV ( $\text{H}_2\text{O}$ ):  $\lambda_{\text{max}}$  ( $\epsilon$ ) = 200 nm (2200); IR (thin film):  $\tilde{\nu}_{\text{max}} = 3700\text{--}2400$  (br), 2954 (sh), 1668, 1529 (sh), 1448, 1392, 1342  $\text{cm}^{-1}$ ; ESI-MS/MS:  $m/z$ : 313  $[M+\text{H}]^+$ , 181, 153, 125.

Chiral TLC: Chiral TLC analyses were performed on CHIRAL-PLATE (Macherey-Nagel) using solvent A ( $\text{CH}_3\text{OH}/\text{H}_2\text{O}/\text{CH}_3\text{CN}$ , 5/5/20) or B (acetone/ $\text{H}_2\text{O}/\text{CH}_3\text{OH}$ , 10/2/2). **3** (0.15 mg, 0.5  $\mu\text{mol}$ ) was heated in 6 M HCl (300  $\mu\text{L}$ ) at 85  $^\circ\text{C}$  for 19 h to give **4** and **5**. Authentic D- and L- Asn were also treated in the same manner. Compound **5** migrated the same distance as treated L-Asn ( $R_f = 0.47$  in A, 0.66 in B), which was easily distinguishable from treated D-Asn ( $R_f = 0.40$  in A, 0.58 in B).

**3**: As shown in Scheme 1, N-(PhF) L- or D-dimethyl glutamate was used in a Claisen condensation, reductive amination, and lactam-cyclization sequence to give enantiopure (3S,6S,9S)-**7** or (3R,6R,9R)-**7** following the procedure of Lombart and Lubell.<sup>[9]</sup> Coupling with *tert*-butyl-L-asparagine ester followed by removal of protecting groups then gave (3S,6S,9S)-**3** or (3R,6R,9R)-**3** in 7% overall yield.

(3S,6S,9S)-**3**:  $[\alpha]_{\text{D}}^{25} = -50.7^\circ$  ( $c = 1.01$ ,  $\text{H}_2\text{O}$ );  $^1\text{H NMR}$  (500 MHz,  $\text{D}_2\text{O}$ , 25  $^\circ\text{C}$ ):  $\delta = 4.61$  (1H, dd,  $J = 6.0$ , 7.0 Hz), 4.51 (1H, d,  $J = 8.8$  Hz), 4.06 (1H, t,  $J = 8.5$  Hz), 3.78 (1H, m), 2.87–2.77 (2H, m), 2.42 (1H, m), 2.31–2.18 (3H, m), 2.13 (1H, m), 1.97 (1H, m), 1.73–1.63 ppm (2H, m). (The small differences in  $\delta$  values between (3S,6S,9S)-**3** and **3** derived from **2** are the result of differences in pH values and concentration. Mixing two samples resulted in overlapping peaks).  $^{13}\text{C NMR}$  (100 MHz,  $\text{D}_2\text{O}$  + 10%  $\text{CD}_3\text{OD}$ , 25  $^\circ\text{C}$ ):  $\delta = 175.9$ , 175.6, 174.0, 168.0, 60.8, 59.1, 51.1, 49.4, 37.5, 32.3, 30.2, 26.3, 25.0 ppm; IR (thin film):  $\tilde{\nu}_{\text{max}} = 3700\text{--}2400$  (br), 2956 (sh), 1670, 1530 (sh), 1448, 1390, 1340  $\text{cm}^{-1}$ ; ESI-MS/MS:  $m/z$ : 313  $[M+\text{H}]^+$ , 181, 153, 125.

(3R,6R,9R)-**3**:  $[\alpha]_{\text{D}}^{25} = +45.7^\circ$  ( $c = 0.98$ ,  $\text{H}_2\text{O}$ );  $^1\text{H NMR}$  (500 MHz,  $\text{D}_2\text{O}$ , 25  $^\circ\text{C}$ ):  $\delta = 4.62$  (1H, dd,  $J = 8.0$ , 5.0 Hz), 4.53 (1H, d,  $J = 8.8$  Hz), 4.06 (1H, t,  $J = 8.5$  Hz), 3.78 (1H, m), 2.84 (1H, dd,  $J = 15.5$ , 5.0 Hz), 2.71 (1H, dd,  $J = 15.5$ , 8.0 Hz), 2.42 (1H, m), 2.30–2.17 (3H, m), 2.05 (1H, m), 1.96 (1H, m), 1.75–1.64 ppm (2H, m);  $^{13}\text{C NMR}$  (100 MHz,  $\text{D}_2\text{O}$  + 10%  $\text{CD}_3\text{OD}$ , 25  $^\circ\text{C}$ ):  $\delta = 175.9$ , 175.3, 173.9, 168.1, 60.8, 59.1, 50.9, 49.5, 37.6, 32.3, 30.6, 26.3, 25.0 ppm; IR

(KBr):  $\tilde{\nu}_{\text{max}} = 3700\text{--}2400$  (br), 2955 (sh), 1671, 1528, 1447  $\text{cm}^{-1}$ ; ESI-MS/MS:  $m/z$ : 313  $[M+\text{H}]^+$ , 181, 153, 125.

HPLC analysis was performed on an Aqua C18 column (250  $\times$  4.6 mm, 5  $\mu\text{m}$ , 125  $\text{\AA}$ , Phenomenex) with a mobile phase of  $\text{H}_2\text{O}$  (0.1% TFA)/ $\text{CH}_3\text{CN}$  (0.1% TFA) with a gradient of 100/0 (0–5 min); 100/0 to 85/15 (5–20 min) at 1  $\text{mL min}^{-1}$ . (3R,6R,9R)-**3** and (3S,6S,9S)-**3** showed base-line separation with retention times of 15.6 and 15.9 min, respectively. **3** derived from **2** co-migrated with (3S,6S,9S)-**3** but not (3R,6R,9R)-**3**.

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**Keywords:** antibiotics · gene expression · natural products · structure elucidation

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