Pantocin B, an Antibiotic from *Erwinia herbicola* Discovered by Heterologous Expression of Cloned Genes

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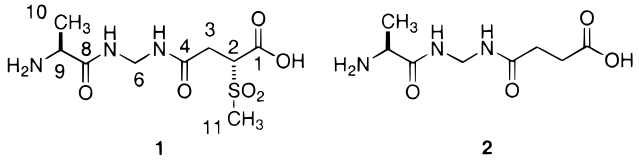
Fire blight devastates apples, pears, and other rosaceous plants with such severe symptoms that at one time lightning was suspected as the cause. The real pathogenic agent is a bacterium, *Erwinia amylovora*, that colonizes the external surfaces of the plants' flowers, buds, and leaves. A closely related bacterium, *E. herbicola* (syn. *Pantoea agglomerans*), also colonizes the same plant surfaces[1−3] and produces antibiotics that effectively control *E. amylovora*.[4,5] *E. herbicola* has been studied as a biological control agent for *E. amylovora* in orchards where trees are currently treated with copper compounds or streptomycin.[2,6−10] Establishing the molecular nature of the *E. herbicola* antibiotics has been hampered by a complex biosynthetic pattern in which a single strain of *E. herbicola* produces multiple antibiotics, and different strains produce different multiple antibiotic complexes. Most of the antibiotics seem to share a common feature: antibiotic activity can be suppressed by the addition of an amino acid to the test medium.[11−13] The complexity of the *E. herbicola* antibiotics is not known since the only characterization available is the pattern of amino acids that suppress antibiotic activity. As a first step, we elected to decipher the molecular structure and genetics of one antibiotic using an approach based on cloning and heterologously expressing individual biosynthetic pathways. Secondary metabolite biosynthetic and resistance genes are often found clustered together on bacterial chromosomes, facilitating the cloning and heterologous expression of bacterial natural products from single continuous fragments of genomic DNA.[14] A cosmid library of *E. herbicola* strain 318 (Eh318) DNA was constructed in *Escherichia coli*, and two independent antibiotic producing clones were identified.[15] The two antibiotics, which have been named pantocin A and B, have their antibiotic activity suppressed by histidine and arginine, respectively. In this paper we report the isolation, structure determination, and mechanism of action of pantocin B (**1**).

Table 1. ^{1}H and ^{13}C NMR data for Pantocin B (**1**)

position	^{13}C[a]	^{1}H[b,c]
1	172.4	
2	70.4	4.30 (dd, 8.0, 6.5)
3	34.8	2.93 (m)
4	174.9	
5	NH	
6	47.2	4.62 (s and q,[d] 13.5)
7	NH	
8	173.8	
9	51.8	4.03 (q, 7.0)
10	19.2	1.50 (d, 7.0)
11	42.2	3.20 (s)

[a] ^{13}C spectrum was recorded at 100 MHz in D_2O (external reference with TSP in D_2O). [b] ^{1}H spectrum was recorded at 500 MHz in D_2O (referenced to HDO at 4.82 ppm). [c] Assignments are based on ^{1}H−^{13}C HMQC. [d] AB quartet.

Large-scale fermentations of the pantocin B (**1**) producing subclone pCPP719 of Eh318 cosmid pCPP704 produce two related small molecules. Cation- and anion-exchange chromatography followed by HPLC gave pantocin B as the minor component (1 mg/L of culture) along with a major component **2** (3 mg/L).[16]



In minimal media, pantocin B has picomolar activity against *E. amylovora* (IC_{50} = 750 pM); compound **2** has no detectable antibiotic activity. Inhibition studies were performed in minimal media because the antibiosis of pantocin B is suppressed by arginine, which is invariably present in other more complex microbial culture broths. Pantocin B is a water soluble, optically active ($[\alpha]^{25}_{D}$ +31.5°, *c* 0.32, H_2O) small molecule; a molecular formula of $C_9H_{17}N_3O_6S$ was established with high-resolution FAB MS (*m*/*z* $[M + H]^+$ 296.0910 obs., 296.0916 calcd for $C_9H_{18}N_3O_6S$).

Analysis of one- and two-dimensional ^{1}H and ^{13}C NMR spectra led to the planar structure corresponding to **1** (Table 1). Both two-carbon spin systems—C2−C3 and C9−C10—were easily identified. An HMBC correlation from the C8 carbonyl to the C10 methyl protons and the downfield shift of the C9 proton suggested an alanine fragment, which was confirmed by acid hydrolysis. A methylenediamine was readily apparent from the coupling of both amide NH protons to the C6 methylene protons, and an HMBC correlation from C8 to the C6 methylene protons links the methylenediamine to alanine. Additional HMBC correlations from C4 to the C6 methylene protons as well as the

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(1) Eriskine, H. M.; Lopatekci, L. E. *Can. J. Microbiol.* **1975**, *21*, 35−41.
(2) Wrather, J. A.; Kuc, J.; Williams, E. B. *Phytopathology* **1973**, *63*, 1075−1076
(3) Billings, E.; Baker, L. A. E. *J. Appl. Bacteriol.* **1963**, *26*, 58−65.
(4) Vanneste, J. L.; Yu, J.; Beer, S. V. *J. Bacteriol.* **1992**, *174*, 2785−2796.
(5) Wodzinski, R. S.; Umholtz, T. E.; Rundle, J. R.; Beer, S. V. *J. Appl. Bacteriol.* **1994**, *76*, 22−29.
(6) Beer, S. V.; Rundle, J. R.; Norelli, J. L. *Acta Hortic.* **1984**, *151*, 195−201.
(7) Vanneste, J. L.; Yu, J. *Acta Hortic.* **1990**, *273*, 409−410.
(8) McIntyre, J. L.; Kuc, J.; Williams, E. B. *Phytophathology* **1973**, *63*, 872−877.
(9) Riggle, J. H.; Klos, E. J. *Can. J. Bot.* **1972**, *50*, 1077−1083.
(10) Wison, M.; Epton, H. A. S.; Sigee, D. C. *Plant Pathol.* **1990**, *39*, 301−308.
(11) Wodzinski, R. S.; Paulin, J.-P. *J. Appl. Bacteriol.* **1994**, *76*, 603−607.
(12) El-Goorani, M. A.; Beer, S. V. *Phytopathology* **1991**, *81*, 121.
(13) Ishimaru, C. A.; Klos, E. J.; Brubaker, R. *Phytopathology* **1988**, *78*, 746−750.
(14) Martin, J. F. *J. Ind. Microbiol.* **1992**, *9*, 73−90.
(15) Wright, S. A. I. The Genetics of antibiotic production and the role of antibiotics in biological control of *Erwinia Amylovora* by *Erwinia Herbicola*. Ph.D. Thesis, Cornell University, August 1997.

(16) Pantocin B was obtained from 10 L cultures of pCPP719 grown at 21 °C for 48 h in minimal media supplemented with 50 μg/mL ampicillin. The cells were removed by centrifugation, and the antibacterial active cell-free culture broth was applied to a Dowex 50Wx4-200 (H^+) cation-exchange column. The active fractions eluted from the cation-exchange column with 0.5 M NH_4OH were then applied to an AG1x8-400 (HCO_3^-) anion-exchange column and eluted with CO_2 saturated H_2O. Compounds **1** and **2**, typically in a 1:3 ratio, were then purified by HPLC using a reversed phase cyano column, first with 94:1:5 H_2O/0.1 M NH_4OH/CH_3CN and then with 0.1% CH_3CO_2H in H_2O as the mobile phase.

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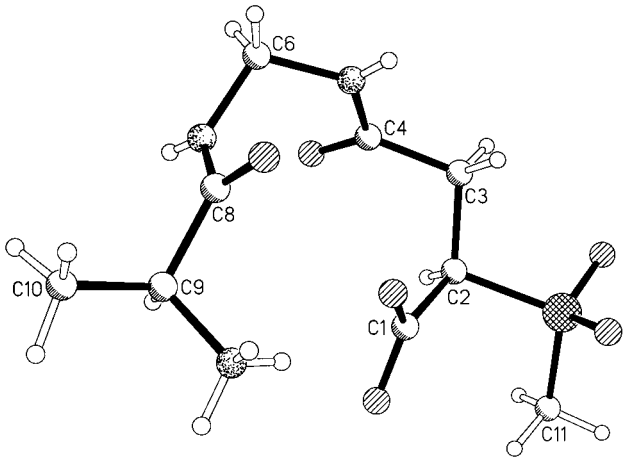
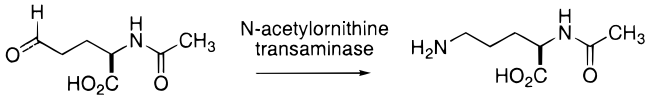


Figure 1. A perspective drawing of the final X-ray model of pantocin B (**1**). The absolute configuration shown was deduced from the X-ray experiment and from identification of L-alanine as a hydrolysis product. Partly shaded spheres indicate carbons; diagonal lines, oxygens; dots, nitrogens; and crosshatching, sulfur.

protons at C2 and C3 allow most of the structure to be established. The available NMR data do not allow an unambiguous placement of the methyl sulfone at C2 or C3. The ^{1}H NMR spectra in D_2O shows exchange of the C2 proton (75%, 6 days, 20 °C). In addition, the NMR spectra indicate that pantocin B, as isolated, is a roughly 3:1 diastereomeric mixture at C2. The major compound (**2**) lacks the methyl sulfone fragment.

The relative and absolute stereochemistry of pantocin B (**1**) was determined with a single-crystal X-ray diffraction analysis (Figure 1).[17] The absolute stereochemistry deduced in the X-ray analysis was additionally verified by a chiral mobile phase HPLC analysis of the alanine released upon acid hydrolysis. Comigration of the hydrolysis product with L- but not D-alanine in the presence of *N*,*N*-dipropyl L-alanine and cupric acetate agrees with the X-ray determined absolute configuration.[18] In the solid state, pantocin B adopts a horseshoe shape in which the N- and C-termini are close to each other (Figure 1) and linked by a strong hydrogen bond (2.88 Å). With this hydrogen bond, the overall conformation of pantocin B is relatively rigid with only limited flexibility about some bonds.

Since the antibiotic activity of pantocin B (**1**) can be suppressed by adding arginine to the test medium; its target was sought along the arginine biosynthetic pathway. Arginine biosynthetic intermediates were tested for their ability to suppress pantocin B antibiosis, and *N*-acetylornithine (**4**) was the last such intermediate.



3 **4**

Earlier intermediates in the pathway did not suppress antibiosis, suggesting *N*-acetylornithine transaminase as pantocin B's target. Inhibition studies using crude *E. amylovora* cell lysate showed pantocin B to be an inhibitor of *N*-acetylornithine transaminase—competitive with *N*-acetylornithine ($K_i \approx 250\ \mu$M) and uncompetitive with glutamine. The in vitro assay measures the consumption of *N*-acetylornithine (**4**), and the modest K_i relative to the potent in vivo cytotoxicity reflects the high levels of *N*-acetylornithine, which compete with pantocin B for the same binding site, present in the enzyme assay system.[20,21]

The structure of pantocin B has some interesting biosynthetic features. The N-terminus undoubtedly derives from alanine, and the methylenediamine piece, which has been noted in one other microbial antibiotic,[22] could come from glycine via a Hofmann-type rearrangement. The origin of the succinic acid fragment is obscure. The smallest clone coding pantocin B biosynthesis is 19−20 kb, and a fuller understanding of the biosynthetic pathway will undoubtedly be revealed by the ongoing DNA sequencing efforts.

The antibiotics pantocin A and B are largely responsible for the ability of Eh318 to control *E. amylovora*. A knockout mutant, made by marker-exchange, of Eh318 deficient in pantocin B biosynthesis, Eh439, is still effective in controlling *E. amylovora*, and a marker-exchange mutant of Eh318 deficient in pantocin A biosynthesis, Eh421, is also effective. However, the marker-exchange mutant deficient in both pantocin A and B biosynthesis, Eh440, is markedly less effective than Eh318 in controlling *E. amylovora*.

The cloning and heterologous expression of known natural product biosynthetic pathways is now a standard laboratory technique. The current study illustrates the power of extending this approach to the isolation and characterization of previously unknown natural products.

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Supporting Information Available: NMR spectral data for both antibiotics and archival X-ray data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(17) Crystals of pantocin B (**1**) are monoclinic, space group $P2_1$ with a = 11.776(4) Å, b = 5.806(2) Å, c = 12.296(4) Å, and β = 116.28(1)°. A total of 913 unique reflections ($2\theta \leq 42°$, Cu Kα) were collected with variable speed $\omega/2\theta$ scans. The structure was solved by direct methods and refined to a conventional R-factor of 4.32% for the 756 reflections with $|F_o| \geq 4\sigma(F_o)$. The absolute structure parameter (Flack) was 0.0(3) indicating that the refined structure has the correct absolute configuration. Crystallographic data for pantocin B have been deposited with the Cambridge Crystallographic Data Center. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, U.K. (fax: +44-(0)1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).

(18) Pantocin B was hydrolzyed to release the alanine by heating 1 mg in 200 μL of 6 N HCl for 20 min at 150 °C. A control tripeptide of L-Ala-Gly-Glu in addition to L- and D-alanine were also heated in the same manner. L- and D-alanine were then separated by analytical chiral mobile phase HPLC (C-18 column 250 × 4.6 mm) using 8 mM *N*,*N*-di-*n*-propyl-L-alanine and 4 mM cupric acetate as additives to the 100% H_2O mobile phase run at 0.5 mL/minute.[19] Both the alanine from pantocin B and the L-alanine from the control tripeptide comigrate with L-alanine (9.0 min) and not D-alanine (8.2 min) under these conditions.

(19) Weinstein, S.; Engel, M. H.; Hare, P. E. *Anal. Biochem.* **1982**, *121*, 370−377.

(20) The in vitro enzyme assay for *N*-acetylornithine transaminase converts *N*-acetyl-L-ornithine to *N*-acetyl-L-glutamic semialdehyde, the reverse of arginine biosynthesis. *N*-Acetyl-L-glutamic semialdehyde is detected by reacting pyrryoline-5-carboxylic, the cyclized form of the semialdehyde, with *o*-aminobenzaldehyde to yield a yellow dihydroquinazolinium compound, which is detected spectrophotometrically.

(21) Vogel, H. J.; Jones, E. E. *Methods Enzymol.* **1970**, *17A*, 260−264.

(22) Ikeda, Y.; Kondo, S.; Ikeda, D.; Yokose, K.; Furutani, H.; Ikeda, T.; Hamada, M.; Ishizuka, M.; Takeuchi, T.; Umezawa, H. *J. Antibiot.* **1986**, *39*, 476−478.