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Zwittermicin A, an Antifungal and Plant Protection Agent from *Bacillus cereus*

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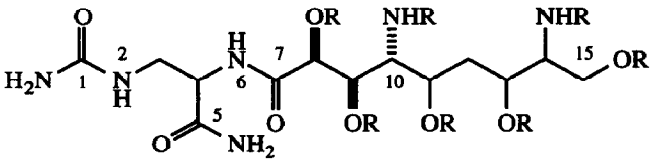
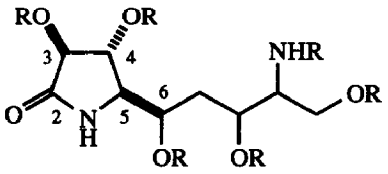
Summary: Zwittermicin A (**1**), a new linear aminopolyol, was isolated from *Bacillus cereus* UW85 and its structure was determined by spectroscopic methods.

As part of a program to discover novel agriculturally useful chemicals, we investigated *Bacillus cereus* UW85 because this bacterium suppressed diseases of alfalfa[1], tobacco[2], cucumber[3] and peanuts[4] caused by a wide variety of pathogenic fungi. Bioassay-guided fractionation of UW85 cultures led to the isolation of two antibiotics that inhibit the growth of the plant pathogen *Phytophthora medicaginis* at low concentrations and reduced the infection of alfalfa.[5] In this communication, we report the isolation and structural elucidation of the more potent antibiotic, zwittermicin A, as **1**.



1 R = H
2 R = Ac



3 R = H
4 R = Ac

B. cereus UW85 was grown in half-strength trypticase soy broth (15 g/L).[5] One liter of a three-day-old fully sporulated culture of UW85 was centrifuged to remove spores, and the supernatant, adjusted to pH 7.0 with HCl, was loaded on a CM-Sephadex cation exchange column (ammonium form). The column was washed with 5 mM NH_4HPO_4 (pH 7.0) and eluted with 0.1 M NH_4OH (pH 10.3). Active fractions were combined and evaporated under reduced pressure at 45 °C. Further purification by paper electrophoresis at pH

9.2 with 2000 volts for 1 h and at pH 1.7 with 3000 volts for 15 m gave zwittermicin A in an average yield of 2-4 mg/L of culture.

Zwittermicin A (**1**)[6] is an amorphous ninhydrin-positive powder with a molecular formula of $C_{13}H_{28}N_6O_8$ (HRFABMS), and since the ^{13}C NMR spectrum (Table 1) shows three carbonyl signals (δ 177.9, 177.1, and 164.6), it must be acyclic. The ^1H NMR spectral data of **1** (D_2O), in conjunction with ^1H-^1H COSY, identify two spin systems: from C-3 to C-4 and from C-8 to C-15. The ^{13}C NMR data, assigned by $^1H\{^{13}C\}$-HMQC and HMBC experiments (Table 1) indicate that C-8, C-9, C-11, C-13 and C-15 are attached to oxygens while C-3, C-4, C-10 and C-14 are attached to nitrogens.

The ^1H NMR spectrum (DMSO-d_6) of acetylation product **2**[7] contains two mutually coupled and D_2O-exchangeable protons at ∂ 7.19 and 7.05—typical for a primary amide. The amide is placed at C-5 due to a strong nOe between one of the amide NH_2 protons (δ 7.19) and H-4 (δ 3.98) in the 2-D ROESY of **2** and a two-bond ^1H-^{13}C correlation from H-4 (δ 4.45) to C-5 (δ 177.1) in the HMBC (D_2O, J = 6 Hz) of **1**. The ^{13}C NMR signal at δ 164.6 is attributed to a urea carbon. Since both signals of the C-3 methylene are coupled to the proton at N-2 (δ 6.23) in the COSY of **2** and show three-bond correlations to C-1 in the HMBC of **1**, N-2 is connected to C-3 and is one of the urea nitrogens. The broad signal at δ 5.67 (2H, D_2O-exchangable) of **2**, which correlates to the N-2 proton in ROESY but exhibits no correlations to any protons in COSY, is assigned to the urea NH_2, thereby defining the left part of the molecule from C-1 to C-5.

The C-7 to N-6 amide bond is established by several pieces of spectroscopic evidence: (a) in the 2-D ROESY of **2**, the N-6 proton (δ 8.20) couples with H-4 (δ 3.98), shows strong nOes to H-4 and H-8 (d 5.17), and weaker nOes to H-3 (δ 3.14) and H-9 (δ 5.34); (b) H-8 and H-9 both correlate to C-7 (δ 177.9) in the HMBC of **1**. The total number of hydroxyl and amino groups in zwittermicin A (**1**) is established by the seven acetyl signals in ^1H NMR spectrum of **2**. The positions of the amino groups in **1** are confirmed by the presence of two amide protons at δ 8.06 and 8.03 that are coupled to H-10 (δ 4.38) and H-14 (δ 4.10) in **2**. Additional support for the hydroxyl positions comes from the significant downfield acetylation shifts of the methine protons H-8, H-9, H-11 and H-13, as well as the methylene protons H_2-15. Thus the planar structure of zwittermicin A is formulated as **1**.

Zwittermicin A (**1**) is stable under both neutral and acidic (1 N HCl, rt, 10 h) conditions but is hydrolyzed to **3** under basic conditions (1 M NaOH, rt, 10 h). The hydrolysis product **3**[8] is purified by paper electrophoresis and identified on the basis of spectroscopic data and derivatization. A molecular formula of $C_9H_{18}N_2O_6$ (FABMS) requires two degrees of unsaturation. Since there is only one carbonyl signal in the

^{13}C NMR, **3** must be cyclic. The proton connectivity from H-3 to H_2-10 (note change in numbering) is revealed by a COSY analysis, and the locations of oxygens and nitrogens are determined by ^{13}C NMR, assigned on the basis of HMBC, and by comparison with the partial structure of **1** from C-8 to C-15. Treatment of **3** with acetic anhydride in pyridine furnished **4**[9] which displays 6 acetyl signals in its ^1H NMR spectrum. The proton at N-1 in **4** exhibits a much smaller acetylation shift (0.27 ppm) than the corresponding proton (NH-10) in **2** (0.95 ppm), indicating that on hydrolysis under basic condition, the nitrogen on C-10 attacks carbonyl C-7 forming a γ-lactam. The relative configurations at C-3, C-4 and C-5 in **3** are established by the strong nOe between H-3 and H-5 in NOEDS. An nOe between H-4 and H_2-7 is also observed, but irradiation at H-3 or H-5 does not give an nOe for H-4. The stereochemistry of **1** at the corresponding centers is deduced from **3**.

Zwittermicin A (**1**), a novel aminopolyol, differs both chemically and biologically from the peptidal antibiotics previously isolated from *Bacillus* spp.[10] The isolation and identification of this unique antibiotic is an important step towards understanding the mechanism of plant protection by UW85 at the molecular level.

Table 1. NMR spectral data of zwittermicin A (1) and acetylation product 2.

Atom	Chemical Shift		
	^{13}C (100 MHz)[a]	^1H (400 MHz, mult, *J* in Hz)	
	1 (D_2O)		**2** (DMSO-d_6)
1	164.6 (s)		
NH_2-1			5.67 (2H, br)[b]
2			6.23 (br)[b]
3	43.5 (t)	3.62 (dd, 14.5, 3.5)	3.30 (m)
		3.49 (dd, 14.5, 7)	3.14 (dd), 11, 5.2)
4	57.3 (d)	4.45 (dd, 7, 3.5)	3.98 (m)
5	177.1 (s)		
NH_2-5			7.19 and 7.05 (br)[b]
NH-6			8.20 (d, 6.4)[b]
7	177.9		
8	74.7 (d)	4.55 (d, 2)	5.17 (d, 2)
9	72.1 (d)	4.35 (dd, 4.5, 2)	5.34 (dd, 11.2, 2)
10	60.6 (d)	3.56 (dd, 6, 4.5)	4.38 (ddd, 11.2, 10, 2.4)
NH-10			8.06 (d, 10)[b]
11	68.4 (d)	4.28 (m)	4.52 (ddd, 12, 2, 2)
12	37.3 (t)	1.80 (m) and 1.76 (m)	1.85 (m) and 1.75 (m)
13	68.8 (d)	4.19 (br d, 10)	4.88 (ddd, 10.4, 6.8, 2.4)
14	59.7 (d)	3.43 (ddd, 8.5, 4, 4)	4.10 (m)
NH-14			8.03 (d, 9.2)[b]
15	60.1 (t)	3.94 (dd, 12.5, 4)	4.00 (m)
		3.78 (dd, 12.5, 8.5)	3.90 (dd, 11.2, 7.2)
Ac			2.11, 2.04, 1.96, 1.91, 1.84, 1.83, 1.74 (s, 3H)

a ------ Assigned by HMBC experiment.
b ------ Exchangeable with D_2O.

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6. Zwittermicin A (**1**). Colorless powder, readily soluble in H_2O but only slightly soluble in CH_3OH or pyridine; $[\alpha]_D$ = +8.9° (c 0.9, H_2O); FABMS *m/z* 419 (MNa^+, 45%), 397.2055 (MH^+, 51%, $C_{13}H_{29}N_6O_8$ requires 397.2047), ^1H and ^{13}C NMR spectral data in D_2O (see Table I).

7. Compound **2** was made by the following procedure. To a mixture of zwittermicin A (**1**, 2 mg) in H_2O (0.2 mL) and pyridine (0.2 mL), acetic anhydride (0.3 mL) was added. The resulting solution was stirred at rt for 1 h, followed by evaporation under reduced pressure to dryness. The residue was redissolved in pyridine (0.2 mL) and acetic anhydride (0.2 mL) and the solution was stirred at rt for 12 h. After the excess reagents were evaporated, the residue was purified by reverse phase HPLC on a Whatman C18 column (CH_3OH-H_2O, 3:1) to obtain **2** (1.7 mg) as a colorless powder. FABMS *m/z* 691 (MH^+, 80%); ^1H NMR spectral data in DMSO-d_6 (see Table I); ^1H NMR (1:3 CD_3OD/D_2O) δ 3.40 (d, *J* = 7 Hz, H-3), 3.39 (d, *J* = 8.5 Hz, H-3'), 4.26 (m, H-4), 5.27 (d, *J* = 2.5 Hz, H-8), 5.37 (dd, *J* = 13, 2.5 Hz, H-9), 4.50 (dd, *J* = 13, 3.5 Hz, H-10), 4.70 (m, H-11), 1.80 (m, 2H, H_2-12), 4.94 (m, H-13), 4.25 (m, H-14), 4.11 (d, 2H, H_2-15), 2.16, 2.11, 2.03, 2.01, 1.97, 1.94, 1.92 (s, 3H, Ac).

8. Compound **3**. Colorless powder, $[a]_D$ = +4.0° (c 0.2, H_2O); ^{13}C NMR (D_2O, assigned by HMBC experiment) δ 177.3 (C-2), 77.8 (C-3), 75.5 (C-4), 59.5 (C-5), 67.4 (C-6), 36.7 (C-7), 67.6 (C-8), 58.6 (C-9), 62.8 (C-10); ^1H NMR (D_2O) δ 4.34 (d, *J* = 8 Hz, H-3), 4.16 (t, *J* = 8 Hz, H-4), 3.48 (dd, *J* = 7, 3 Hz, H-5), 4.05 (dt, *J* = 9.5, 3 Hz, H-6), 1.70 (m, 2H, H_2-7), 4.17 (m, H-8), 3.39 (dt, *J* = 9, 3.5 Hz, H-9), 3.92 (dd, *J* = 11.5, 4 Hz, H-10), 3.75 (dd, *J* = 11.5, 9 Hz, H-10'); FABMS *m/z* 251.1236 (MH^+, 75%, $C_9H_{19}N_2O_6$ requires 251.1243).

9. Compound **4**. Colorless powder; ^1H NMR (1:3 CD_3OD/D_2O) δ 5.31 (d, *J* = 5.5 Hz, H-3), 5.42 (dd, *J* = 6, 5 Hz, H-4), 3.75 (dd, *J* = 5, 3 Hz, H-5), 5.12 (m, H-6), 1.80 (m, H_2-7), 5.02 (br dd, *J* = 10.5, 10.5 Hz, H-8), 4.26 (m, H-9), 4.09 (d, *J* = 4.5 Hz, H-10), 4.08 (d, *J* = 7 Hz, H-10'), 2.11, 2.10, 2.05, 1.96 (s, 3H, Ac), 1.95 (s, 6H, Ac); FABMS *m/z* 503 (MH^+, 50%).

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