JANTHINOCINS A, B AND C, NOVEL PEPTIDE LACTONE ANTIBIOTICS PRODUCED BY *JANTHINOBACTERIUM LIVIDUM*

II. STRUCTURE ELUCIDATION

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The structures of janthinocins A, B and C, three novel macrocyclic peptide lactone antibiotics isolated from fermentations of *Janthinobacterium lividum*, were determined. The janthinocins are of particular interest because they contain three amino acid residues that have not previously been reported in natural products: Each contains *erythro*-β-hydroxy-D-leucine while janthinocins A and B also contain β-hydroxytryptophan and β-ketotryptophan, respectively.

The janthinocins were discovered as part of a screening program to detect antibiotics that interfere with cell-wall synthesis[1]. The isolation, physico-chemical characteristics and biological properties of janthinocins A, B and C (**1**, **2** and **3**, respectively) are reported in an accompanying paper[2]. In this paper we report on the structure elucidation of these peptide antibiotics.

L-Ile–D-*erythro*-βHL–L-Thr–L-Ser–D-*erythro*-βHL–ΔAbu–D-Ser–X–D-Orn–L-Phe

Janthinocin A (**1**) X = *threo*-β-Hydroxytryptophan.
Janthinocin B (**2**) X = β-Ketotryptophan.
Janthinocin C (**3**) X = Dehydrotryptophan.

ΔAbu is 2,3-dehydro-α-aminobutyric acid.
βHL is β-hydroxyleucine.

Structure Elucidation

The janthinocins were obtained as weakly basic, amorphous white solids and their MW's determined by FAB-MS. Janthinocins B and C give $(M+H)^+$ ions at *m/z* 1,191 and 1,175, indicating MW's of 1,190 and 1,174, respectively. Under identical conditions, the highest mass ion observed for janthinocin A was also at *m/z* 1,175. However, when the FAB mass spectrum was obtained with NaI added, the $(M+Na)^+$ peak at *m/z* 1,215 and $(M+I)^-$ at *m/z* 1,319 indicated that the true MW of janthinocin A is 1,192.

Several solvents were employed for NMR spectroscopic studies in order to shift signals in congested regions of the spectra and allow for further comparisons. The ^1H and ^{13}C NMR spectra of the janthinocins (Figs. 1~4) indicate that the three compounds are structurally related peptides, a fact also supported by the partial conversion of janthinocin A to janthinocin C under the acidic conditions used in the isolation. Conversion of janthinocin A to janthinocin C corresponds to a loss of 18 mass units, suggestive of dehydration. This supposition is consistent with the bathochromic shift observed in the UV spectrum (λ_{max} nm 276 and 287 in janthinocin A to 339 nm in janthinocin C) indicative of a dehydration that extends a conjugated system. The janthinocins all show an ester absorbance at 1742 cm^{-1} in their IR spectra.

Fig. 1. 400 MHz ^1H NMR spectrum of janthinocin A in CD_3CN-D_2O (1:4).

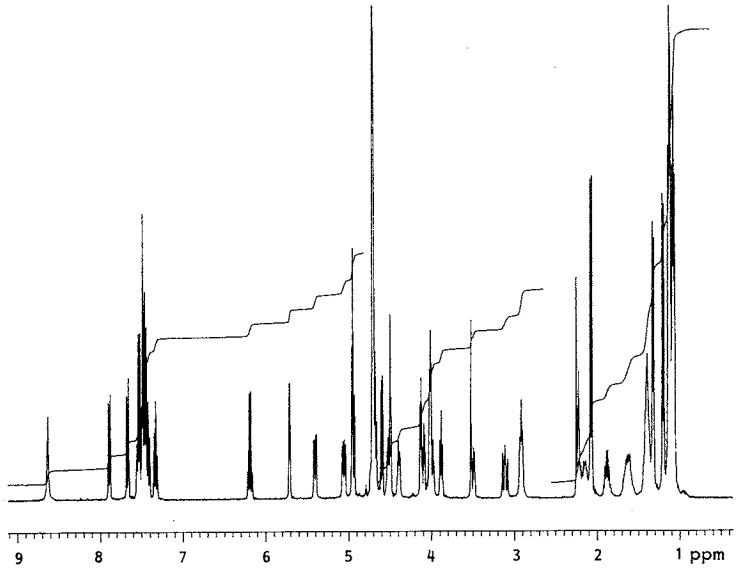


Fig. 2. 400 MHz ^1H NMR spectra of janthinocins B_2 and B_1.

(A) Janthinocin B_2 in CD_3CN-D_2O (1:1) (adjusted to pH 7.1 with 50 mM Na_2HPO_4 before exchanging with D_2O; ratio of B_2-B_1 is 4:1). (B) Janthinocin B_1 in CD_3CN-D_2O (1:4) (B_2-B_1 is 1:4).

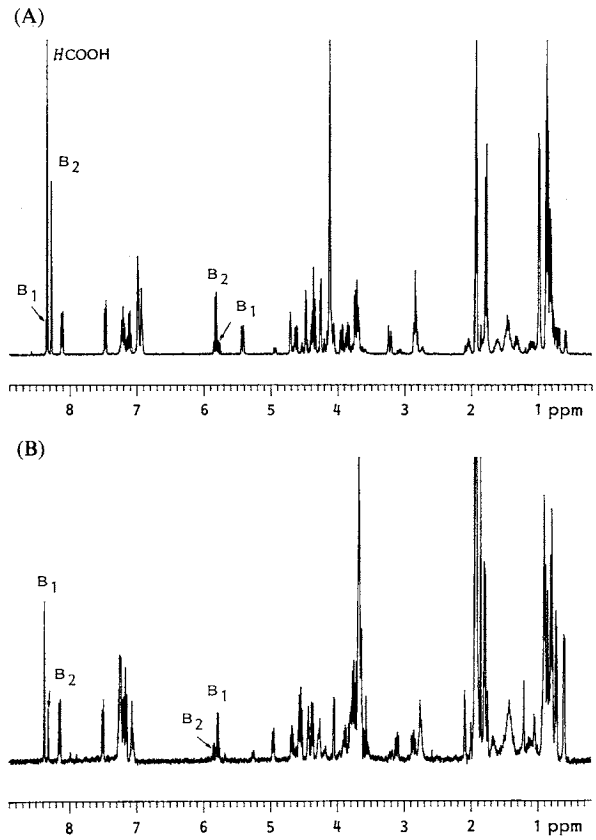


Fig. 3. 400 MHz ^{1}H NMR spectrum of janthinocin C in CD_3CN-D_2O (4:1).

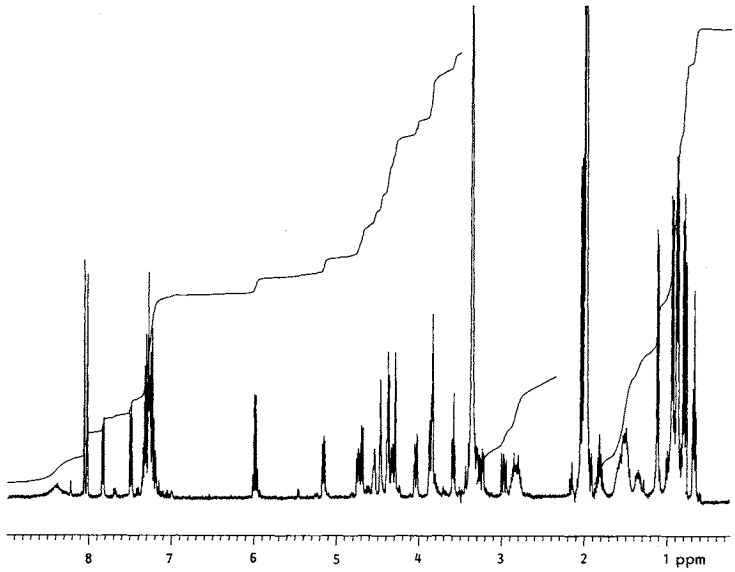


Fig. 4. 67.5 MHz ^{13}C NMR spectra of janthinocins A, B and C in CD_3CN-D_2O (4:1).
(A) Janthinocin A, (B) janthinocin B, (C) janthinocin C.

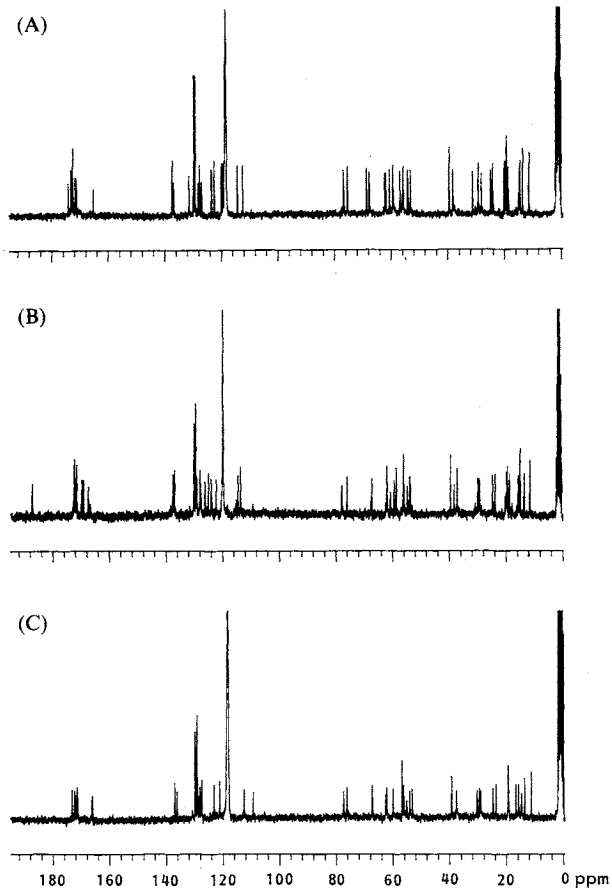
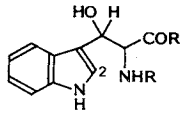
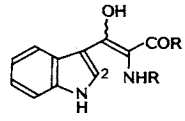
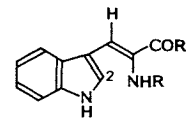


Table 1. Characterization of the indole amino acid residue in janthinocins A, B and C.

Property	Janthinocin A	Janthinocin B	Janthinocin C
Structure			
Identity	β-Hydroxytryptophan	*E* and *Z* enols of β-ketotryptophan	Dehydrotryptophan
UV λ_{max} (H_2O)			
Measured	204, 276, 287	204, 242, 260, 312	220, 277, 339
Reported[a]	217, 278, 286	206, 246, 262, 310	220, 277, 290, 339
^1H NMR[b]	Hα δ 4.62, J=4.8 Hz[c]	B_2: 2-H, δ 8.36	Hβ δ 8.02[d]
	Hβ δ 5.48, J=4.8 Hz	B_1: 2-H, δ 8.33	2-H, δ 7.95
	2-H, δ 7.23		

[a] See refs 9 and 10.
[b] In CD_3CN-H_2O (4:1).
[c] Reported values [10] (CD_3OD) for *erythro*-isomer: δ 4.21, 5.22 J=7.3 Hz; for *threo*-isomer: δ 4.21, 5.58 J=4.6 Hz.
[d] Reported values[11] in DMSO-d_6 are Hβ δ 7.95 and 2-H δ 7.5.

Further, mild base hydrolysis of janthinocins A and B (0.05 M NH_4OH, 25°C, 17 hours) leads to the disappearance of this ester band and yields products (**4** and **5**, respectively) with $(M+H)^+$ ions 18 mass units higher in the FAB mass spectra, showing that the janthinocins are peptide lactones.

With this gross structural information in hand, the amino acid compositions of janthinocins A, B and C were determined by total acid hydrolysis and HPLC analysis of the phenyl-thiocarbamyl derivatives of the resulting amino acids[3]. Each of the hydrolysates contained serine, β-hydroxyleucine (βHL), threonine, isoleucine, phenylalanine and ornithine in a 2:2:1:1:1:1 ratio. No unidentified peaks were seen in the HPLC chromatograms. The amino acids were also analyzed by chiral capillary GC-MS[4] as the *N,O*-trifluoroacetyl isopropyl esters[5], and found to be L-isoleucine, D-serine, L-serine, *erythro*-β-hydroxy-D-leucine, L-threonine, L-phenylalanine and D-ornithine. This represents the first report of an *erythro*-β-hydroxy-D-leucine residue in naturally occurring peptides. In addition to these amino acids, the coupled signals at approximately δ 5.9 (1H, q, J=7.0 Hz) and 1.9 (3H, d, J=7.0 Hz) in the ^1H NMR spectra suggested that each of the antibiotics contains a 2,3-dehydro-α-aminobutyric acid (ΔAbu) residue. This amino acid has been reported in several natural products[6~8] and is destroyed during acid hydrolysis. The stereochemistry of the ΔAbu residue in the janthinocins was not determined. The nine amino acids common to the three antibiotics account for 990 daltons, suggesting that only one residue remained to be identified in each antibiotic.

The tenth residue, which proved to be unique in each of the janthinocins, was identified by interpretation of several pieces of data that are summarized in Table 1. Correlation of the HRFAB mass spectral results for janthinocin C ($(M+H)^+$ found 1,175.6115) with the partial molecular formula derived from the nine known constituent amino acids ($C_{46}H_{74}N_{10}O_{14}$) suggested a probable formula of $C_{57}H_{83}N_{12}O_{15}$ (Calcd 1,175.6101) for the $(M+H)^+$ pseudomolecular ion of janthinocin C. The tenth amino acid, then, would have a MW of 184+18 (H_2O) daltons and $C_{11}H_{10}N_2O_2$ as the molecular formula. In addition, this residue must account for the unassigned aromatic protons in the ^1H NMR spectrum and for the chromophore in the UV spectrum. These observations led to the conclusion that the tenth residue is dehydrotryptophan. Comparison of the UV spectral data reported for dehydrotryptophan in telomycin[9] with the data for

janthinocin C supports this identification. The stereochemistry of the dehydrotryptophan residue was not determined.

Since dehydration of janthinocin A leads to janthinocin C and their amino acid analyses are identical, we propose that the tenth amino acid residue in janthinocin A is β-hydroxytryptophan, an amino acid hitherto unreported in nature. This assignment is supported by comparison of the UV spectral data reported for β-hydroxytryptophan[10] with those for janthinocin A. Further, the β-hydroxytryptophan residue in janthinocin A appears to be *threo*, rather than *erythro*, based on a comparison of ^{1}H NMR data for the peptide with published data for both amino acid isomers[9]. The ^{1}H and ^{13}C NMR changes that accompany the conversion of janthinocin A to janthinocin C are also consistent with the proposed structures. To elaborate, the signals found at δ 4.62 (1H, d, J=4.8 Hz) and 5.48 (1H, d, J=4.8 Hz) in CD_3CN-D_2O (4:1) were found to be coupled in the COSY spectrum of janthinocin A and were assigned to the α and β protons, respectively, of the β-hydroxytryptophan residue. These signals are absent in janthinocin C but one of the new singlets at *ca.* 8 ppm could be assigned to the olefinic β proton. By integration, the C_2 proton of the indole ring of janthinocin A is found at δ 7.23 in janthinocin A and this signal is absent after dehydration to janthinocin C. The second proton singlet at *ca.* 8 ppm of janthinocin C is thus presumed to be due to the deshielded C_2 indole proton. These assignments for the janthinocin C dehydrotryptophan residue are in close agreement with chemical shifts reported for the corresponding protons in telomycin[11]. In addition, the ^{13}C NMR spectrum of janthinocin C shows two signals at δ 166 (consistent with the presence of both ΔAbu and a second α,β-unsaturated amide) while only one is detected for janthinocin A.

Since janthinocin B is two mass units lower in MW than janthinocin A while containing the same non-indolic amino acids, the unknown residue in janthinocin B is β-ketotryptophan. This assignment is supported by comparison of the UV spectral data for janthinocin B with those reported for *N*-acetyl-β-keto-D,L-tryptophanamide[12]. This also represents the first report of a β-ketotryptophan residue in a natural product.

The relationship among janthinocins A, B and C was further demonstrated by conversion of janthinocin B to janthinocin A (and the three other diastereomeric peptides that arise from reduction of the β-ketotryptophan residue to β-hydroxytryptophan) by reduction with sodium borohydride. Acid treatment of these reduced peptides produced janthinocin C.

The absolute configuration of the β-hydroxytryptophan residue in janthinocin A was not determined due to the lability of this amino acid. However, tryptophan side chain oxidases that preferentially oxidize L-tryptophan, in a mixture of D- and L-tryptophan, to both *threo*- and *erythro*-β-hydroxytryptophan have been isolated from a *Pseudomonas* sp.[13]. This same enzyme also catalyzes the preferential conversion of the L-*threo*-isomer to β-ketotryptophan.

The question of configuration of the β-ketotryptophan residue in janthinocin B is irrelevant since it exists in solution as a mixture of the *E* and *Z* enols and not in the keto form to any measureable extent. The two enol forms can be separated chromatographically but slowly interconvert to give a pH and solvent dependent equilibrium mixture[2]. From the ^{1}H NMR spectra in Fig. 2, it can be seen that solvent conditions favoring a 4:1 excess of either enol, termed B_1 and B_2, are known. Although we had hoped to verify that janthinocin B contains the two enols of the β-ketotryptophan residue by observing NH resonance multiplicities, all of the nonexchanging NMR solvents tried gave unsuitably broad signals. However, evidence that the β-ketotryptophan is found as a mixture of enols and not in the keto form is supported

by the IR, UV, and NMR spectra. First, no ketone carbonyl absorption is seen in the IR spectrum of janthinocin B (the IR absorbance for an enol, $1640 \sim 1580\,cm^{-1}$, would be obscured by the amide absorbance). Secondly, the UV spectra of both isomers in janthinocin B are superimposable, a result not consistent with one isomer being the keto and the other the enol tautomer. Thirdly, the β carbons of both isomers are found at δ 187.0, a chemical shift consistent with the proposed structure. We were unable to assign the α carbon resonances by inspection since the presence of the two isomers complicates the ^{13}C spectrum. Finally, the C_2 proton of the indole ring is found as a sharp singlet at *ca.* δ 8.4 in both janthinocins B_1 and B_2, similar to the chemical shift found for that proton in the dehydrotryptophan residue of janthinocin C (δ 7.95) and telomycin (δ 7.48; DMSO-d_6 - D_2O) and substantially downfield from the corresponding proton in tryptophan itself (δ 7.3). No singlet assignable as the α proton for β-ketotryptophan is apparent in either ^1H NMR spectrum.

Ornithine was shown to be attached through the α- and not the δ-amino group by observing the effect of pD on the chemical shift of the δ methylene protons in janthinocin A. Under neutral conditions these protons are found at δ 2.72; addition of NaOD to the sample shifts the resonance to δ 2.18.

Direct sequencing of the janthinocins by MS-MS was unsuccessful because no significant fragment ions were observed. This problem was also encountered with lysobactin[14], another macrocyclic peptide lactone antibiotic discovered from the same screen. Likewise, early attempts to sequence the janthinocins by FAB mass spectral analysis of partial acid hydrolysates were complicated by the cyclic nature of the peptides. To simplify the sequence information, the acid labile dehydroamino acid residues in janthinocin C were stabilized by reduction to α-aminobutyric acid and tryptophan residues, and the resulting lactone, **6**, was hydrolyzed with base to give an acyclic peptide, **7**. Mild acid hydrolysis of **7** and FAB mass spectroscopic analysis of the resultant mixture, Table 2, led to the partial sequence Ile-βHL-Thr-Ser-βHL-Abu-Ser-(Orn, Trp, Phe).

To determine the sequence of the tryptophan, ornithine and phenylalanine residues, the *C*-terminus of the acyclic peptide was investigated by several methods. We thought that the position of these residues could be facilely determined by degradation from the carboxy terminus with carboxypeptidase Y[15]. Peptide **7** was incubated with this enzyme at 37°C for 24 hours and samples were taken at various times and subjected to amino acid analysis. Partial liberation of phenylalanine was seen after 24 hours, but neither ornithine nor tryptophan was observed, a fact later explained by the presence of D-ornithine as the penultimate residue. Phenylalanine was confirmed as the carboxy terminal residue by reduction of the lactone function in janthinocin A with sodium borohydride, followed by total acid hydrolysis and amino acid analysis of the resulting hydrolysate. Seven of the eight acid-stable constituent amino acids were seen and only phenylalanine was absent. Similar treatment of the acyclic peptide (**4**) from basic hydrolysis of janthinocin A gave an amino acid analysis identical with the parent compound. This established phenylalanine as the carboxyl portion of the lactone and therefore, as the *C*-terminal residue.

Table 2. Peptides produced by mild acid hydrolysis of **7**.

Peptide	$(M+H)^+$ (*m/z*)
Ile-βHL	261
Ile-βHL-Thr	362
Ile-βHL-Thr-Ser	449
Ile-βHL-Thr-Ser-βHL (peptide **8**)	578
Ile-βHL-Thr-Ser-βHL (-H_2O)	560
Ile-βHL-Thr-Ser-βHL-Abu	663
Ile-βHL-Thr-Ser-βHL-Abu-Ser	750
Ile-βHL-Thr-Ser-βHL-Abu-Ser-(Orn, Trp)	1,050 (weak)

The relative positions of the ornithine and tryptophan residues were established by interpretation of the FAB mass spectrum of the peptides

Table 3. Amino acid composition of peptides from mild acid hydrolysis of tetrahydrojanthinocin A (**6**)[a].

Ile	βHL	Thr	Ser	βHL	Abu[b]	Ser	Trp	Orn	Phe	$(M+H)^+$ (m/z)
+	+								+	408
+	+	+							+	509
+	+							+	+	522
+	+	+	+						+	596
+	+	+						+	+	623
+	+	+	+					+	+	710
+	+	+	+	+					+	725
+	+					+	+	+	+	795
	+	+	+	+	+			+	+	839
+	+	+				+	+	+	+	895
	+			+	+	+	+	+	+	895
+	+	+	+			+	+	+	+	983
	+		+	+	+	+	+	+	+	983
+	+		+	+	+	+	+	+	+	1,009 (weak)

[a] Composition deduced from low resolution MS measurements: + indicates that the residue is present.
[b] α-Amino butyric acid.

produced from mild acid hydrolysis of tetrahydrojanthinocin A (**6**). In addition to the first six ions listed in Table 2, numerous positive ions were seen in the tetrahydrojanthinocin A hydrolysate that were not seen in the mild acid hydrolysate of **7**. The sequences assigned for these ions, Table 3, identify β-hydroxyleucine as the hydroxyl component of the lactone and ornithine rather than β-hydroxytryptophan as the penultimate residue and lead to the structure shown for peptide **7**. This sequence was confirmed by Edman degradation of peptide **7** and FAB mass spectral analysis of the resultant intermediates. Chromic acid oxidation of janthinocin A (**1**) and the mild base hydrolysis product (**4**) of **1**, followed by total acid hydrolysis and a comparison of amino acid content, confirmed that the hydroxyl group of βHL is involved in the lactone bond[6]. It is interesting to note that the hydroxyl donor to the lactone bond could not be readily identified by inspection of the FAB mass spectrum from the mild acid hydrolysate of janthinocin A itself since most of the significant ions seen in this spectrum corresponded to peptides containing Ile, βHL, Thr and Phe. Only a moderately strong ion at $(M+H)^+$ m/z 408 and a weak ion at m/z 522 (Table 3) suggested that βHL was the hydroxyl donor of the lactone.

Ile-βHL-Thr-Ser-βHL-Abu-Ser-Trp-Orn-Phe (**7**)

Ile-βHL-Thr-Ser-βHL (**8**)

The relative positions of the D- and L-serine residues were elucidated by hydrolysis of pentapeptide fragment **8**, isolated from the mild acid hydrolysate of janthinocin A. The serine residue in this peptide was shown to possess the L configuration by GC-MS.

Thus, the amino acid composition and sequence information lead to structure **1** for janthinocin A, **2** for janthinocin B and **3** for janthinocin C. While these antibiotics show good activity against Gram-positive bacteria *in vitro* and *in vivo*, hydrolysis of the lactone functionality in each of the three peptides and reduction of janthinocin C to tetrahydrojanthinocin C cause a major loss of the activity.

Experimental

NMR spectra were obtained with Jeol GX-400 and Jeol GX-270 spectrometers; chemical shifts (δ)

are reported in ppm downfield from internal tetramethylsilane. FAB mass spectra (8 keV Xe) were obtained from a thioglycerol or a dithiothreitol-dithioerythritol matrix with a VG-ZAB-2F mass spectrometer (Vacuum Generators, Ltd.). HR analyses were performed at 1:8,000 resolution by peak matching using polyethylene glycol as an internal standard. CI-MS analyses were performed using a Finningan TSQ-4600 mass spectrometer equipped with a model 9610 gas chromatograph (Finnigan MAT Corp.). A CH_4-NO_2 mixture was used for negative CI. UV spectra were recorded on a Shimadzu UV-260 instrument and IR spectra were measured in KBr with a Mattson Sirius 100 FT-IR spectrometer.

HPLC was performed with a Varian 5000 HPLC using a 4.1 × 150 mm, 10 μm Hamilton PRP-1 column eluted isocratically at 1 ml/minute with 1% NaH_2PO_4 in acetonitrile-water (34:66). The effluent was monitored with an LKB 2140 photodiode array detector. Under these conditions, the Rt's for janthinocins A, B_1, B_2 and C were 7.39, 2.17, 4.43, and 2.68 minutes, respectively.

Total acid hydrolysates were prepared by heating the peptide in 6 M HCl in a sealed, evacuated ampule at 110°C for 48 hours (incomplete hydrolysis was noted at 24 hours).

Amino acid analyses were performed according to a published procedure[2], modified by performing the HPLC analyses of the PTC amino acids on a Perkin-Elmer CR C_{18} cartridge column (4 × 30 mm, 3 μm particle size), eluting with a gradient of acetonitrile-0.5 M sodium acetate buffer, pH 6.5.

Janthinocins A, B and C

The janthinocins were isolated as described in a companion paper[2]. Typically, 60 liters of fermentation broth gave 130 mg of janthinocin A and 100 mg of janthinocin B. The amount of janthinocin C in the fermentations is variable (5 to 20 mg) and may be an artifact of the isolation procedure since janthinocin A is converted to janthinocin C under acidic conditions. HRFAB-MS $(M+H)^+$ *m/z*: Janthinocin A, 1,193.6201 ($C_{57}H_{85}N_{12}O_{16}$ Calcd 1,193.6207); janthinocin B, 1,191.6028 ($C_{57}H_{83}N_{12}O_{16}$ Calcd 1,191.6050); janthinocin C, 1,175.6115 ($C_{57}H_{83}N_{12}O_{15}$ Calcd 1,175.6101). Full assignments for janthinocin NMR data will be the subject of a separate publication (N. H. ANDERSEN, J. BEALE and C. CHEN, manuscript in preparation).

Amino Acid Chirality Determinations

Amino acid standards and peptide total acid hydrolysates were derivatized for analysis by conversion to the *N,O*-trifluoroacetyl isopropyl esters according to a published procedure[4]. The amino acid derivatives were separated by GC using a 25 m × 0.31 mm Chirasil-VAL III fused silica capillary column (Alltech Associates, Inc.) run isothermally at 70°C for 3 minutes, then heated to 200°C at 4°C/minute.

Base Hydrolysis of Janthinocins A and B (**1** and **2**) to Give Acyclic Peptides **4** and **5**

The hydrolysis of **1** (33 mg) and **2** (23 mg), in 2 ml of 0.05 M NH_4OH, to produce peptides **4** and **5** was monitored by HPLC. The lactones of **1** and **2** were completely cleaved after 17 hours at 25°C or after 5 hours at 37°C. Rt's for the acyclic products **4** and **5** were 1.49 and 1.63 minutes, respectively. The reaction mixtures were taken to dryness *in vacuo* and purification effected by chromatography on MCI gel CHP20P resin, eluting with CH_3CN-H_2O-HCOOH (250:750:2) to give 26 mg of **4**: FAB-MS *m/z* 1,211 $(M+H)^+$; IR (KBr) cm^{-1} 3316, 3066, 2967, 1657, 1595, 1524, 1384; and 21 mg of **5**: FAB-MS *m/z* 1,209 $(M+H)^+$; IR (KBr) cm^{-1} 3328, 3068, 2967, 2935, 1655, 1593, 1537, 1385.

Conversion of Janthinocin A (**1**) to Janthinocin C (**3**)

The conversion of **1** to **3** in dilute HCl at 25°C was monitored by HPLC and was complete after 24 hours in 0.01 M HCl or after 4 hour in 0.1 M HCl.

Conversion of Janthinocin B (**2**) to Janthinocin A (**1**) and Janthinocin C (**3**)

To 12 mg of **2** in 3 ml of CH_3CN-H_2O (2:1) was added 1 ml of 4 M Na_2HPO_4 adjusted to pH 6.8 with H_3PO_4 and *ca.* 5 mg of $NaBH_4$ (final pH *ca.* 9). The reaction mixture was stirred at room temperature for 2 hours and monitored by HPLC. At 90 minutes, no janthinocin B remained by inspection of the UV spectra of the eluted components. Four new products with UV spectra indistinguishable from that of janthinocin A were formed with Rt (area) of 1.28 (22%), 1.94 and 2.03 (47%) and 6.86 minutes (24%), as well as a peak (5%) corresponding to janthinocin A itself in both Rt and UV spectrum. The reaction

mixture was adjusted to pH 6.0 with 1 M HCl and the products partially resolved by chromatography of a portion of the reaction mixture on MCI gel CHP20P resin, eluting with CH_3CN-H_2O-HCOOH (110:390:1). The first material to elute had an $(M+H)^+$ ion at *m/z* 1,197, consistent with reduction of both the ketone and lactone of janthinocin B. The next material to emerge was a mixture of two components, Rt of 1.94 and 2.03 minutes, with an $(M+H)^+$ ion at *m/z* 1,193, from reduction of the β-ketotryptophan residue to two of the four possible diastereoisomers of β-hydroxytryptophan. The ^1H NMR spectrum shows β protons for the two isomers at δ 5.45, $J=8.4$ Hz and 5.36, $J=7.0$ Hz. The next fraction contained not only those two isomers, but also a third isomer, $(M+H)^+$ 1,193, with the β proton at δ 5.50, $J=4.0$ Hz. The small amount of janthinocin A produced (the fourth possible β-hydroxytryptophan diastereoisomer) was not eluted from the MCI gel CHP20P column under these conditions. HPLC and MS analysis of a solution of the crude reaction mixture in 0.1 M HCl showed complete conversion to janthinocin C after 2 hour at 25°C.

Preparation of Tetrahydrojanthinocin C (**6**) and Decapeptide **7**

A solution of 50 mg of janthinocin C in 10 ml of 2-propanol-water (8:2) was stirred with 10% Pd-C under hydrogen for 16 hours at 25°C. A ^1H NMR spectrum showed incomplete reduction of the dehydrotryptophan residue. Hydrogenation was therefore continued with fresh 10% Pd-C for an additional 48 hours after which the ^1H NMR spectrum showed complete reduction of the dehydroamino acid residues. Removal of the solvent and the catalyst gave 48 mg of tetrahydrojanthinocin C (**6**): FAB-MS *m/z* 1,179 $(M+H)^+$; IR (KBr) cm^{-1} 1744 (ester), 1658, 1531, 1458, 1384, 1097; UV $\lambda_{max}^{H_2O}$ nm 205, 218 (sh), 275, 282, 289.

A solution of 30 mg of **6** in 5 ml of 0.03 M NH_4OH was kept at 37°C for 4 hours. The IR spectrum of the product still showed lactone absorbance at 1742 cm^{-1} so hydrolysis was continued in 0.1 M NH_4OH at 37°C for 2 hours. The solution was taken to dryness *in vacuo* to give decapeptide **7**: FAB-MS *m/z* 1,197 $(M+H)^+$, 1,195 $(M-H)^-$; IR (KBr) cm^{-1} 1763 (v weak), 1657, 1537, 1458, 1385.

Mild Acid Hydrolysis of Tetrahydrojanthinocin (**6**) and Decapeptide **7**

Solutions of 2 mg of **6** and 2 mg of **7** each in 1 ml of 1 M HCl were heated in evacuated ampules at 110°C for 2 hours and then taken to dryness *in vacuo*. The residues were analyzed by FAB-MS.

Carboxypeptidase Y Digest of Decapeptide **7**

In order to reactivate the lyophilized enzyme[15], 5 mg of carboxypeptidase Y (Sigma Chemical Company) was dissolved in 0.5 ml of H_2O and stored at 5°C overnight. To a solution of 5 mg of **7** in 0.5 ml of 0.1 M pyridine-acetate buffer, pH 5.5, was added 0.1 ml of the carboxypeptidase Y solution. The mixture was kept at 37°C for 24 hours. Aliquots were taken at 1, 2, 4, 8 and 24 hours, immersed in boiling water for 5 minutes and then stored at 0°C for later amino acid analysis.

Sodium Borohydride Reduction of Janthinocin A (**1**), Peptide **4** and Tetrahydrojanthinocin C (**6**)

Each peptide, 0.5 mg, was dissolved in 1.0 ml of methanol-water (1:1) and a small amount of sodium borohydride was added. After 3 days at 0°C, a drop of glacial acetic acid was added and the reaction mixtures were taken to dryness *in vacuo*. The residues were hydrolyzed and subjected to amino acid analysis. Amino acid analysis indicated that the amino acids Ser, Ile, βHL, Thr, α-aminobutyric acid, tryptophan, Orn and Phe were present in the following ratios: Reduced **1**: 2:1:2:1:0:0:1:0; reduced **4**: 2:1:2:1:0:0:1:1; reduced **6**: 2:1:2:1:1:~0.5:1:0.

Mild Acid Hydrolysis of Janthinocin A (**1**) and Isolation of Pentapeptide **8**

A solution of 16 mg of **1** in 3 ml of 1 M HCl was heated under N_2 at 110°C for 4.5 hours. The hydrolysate was extracted with ether and the aqueous layer taken to dryness *in vacuo*: FAB-MS *m/z* 839, 725, 710, 623, 596, 578, 560, 542, 522, 509, 408, 362 in the positive mode; 915, 838, 723, 708, 621, 576, 558, 505, 360 in the negative mode. The hydrolysate was chromatographed on a 1.1×24 cm column of Dowex 50W-X2 resin, pyridinium form, eluting with 90 ml of water, followed by a linear gradient of 5% acetic acid to 1 M pyridine-acetic acid pH 5.1 (300 ml). Pentapeptide **8**, 1.4 mg, eluted between 85 and 115 ml of the gradient: FAB-MS *m/z* 578 $(M+H)^+$, 576 $(M-H)^-$.

Edman Sequencing of 7

Edman sequencing of 7 (8 mg) was performed according to a published procedure[14], with minor modifications. For each of the first four steps of the degradation, the diphenylthiocarbamyl (diPTC) peptide was prepared, triturated with toluene, cleaved with TFA and then triturated with ether to remove the soluble *N*-terminal PTC-amino acid from the residual PTC-ornithine-containing peptide. The PTC-amino acid was then converted to the PTH-amino acid with aqueous acid. During steps five through seven of the Edman sequencing, the diPTC peptides proved to be toluene soluble. In these cases, toluene extraction was omitted and the crude reaction mixtures were evaporated under a stream of nitrogen, cleaved with TFA and triturated with ether as described above. PTH-amino acids through step 7 were detected by CI-MS and the residual PTC-peptides through step 6 were detected by FAB-MS analysis.

Oxidation of Janthinocin A (1) and Acyclic Peptide 4

To 1.8 mg of **1** and 14. mg of **4** was added 150 μl of a solution containing 100 μg of chromic acid in 100 μl of pyridine and 3 ml of AcOH. The reaction mixtures were kept at room temperature for 20 hours. The oxidized peptides were partially purified by sorbing on 1 ml of MCI gel CHP20P resin, washing with water and eluting the peptides with CH_3CN-H_2O-HCOOH (70:30:1). Eluates were taken to dryness *in vacuo* and the residues were hydrolyzed and subjected to amino acid analysis. **1**: Ser (0.09), Thr (0.00), βHL (0.38), Ile (0.81), Phe (1.00) and Orn (0.53). **4**: Ser (0.13), Thr (0.03), βHL (0.05), Ile (0.60), Phe (1.00) and Orn (0.68).

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