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STRUCTURES OF PUWAINAPHYCINS A-E

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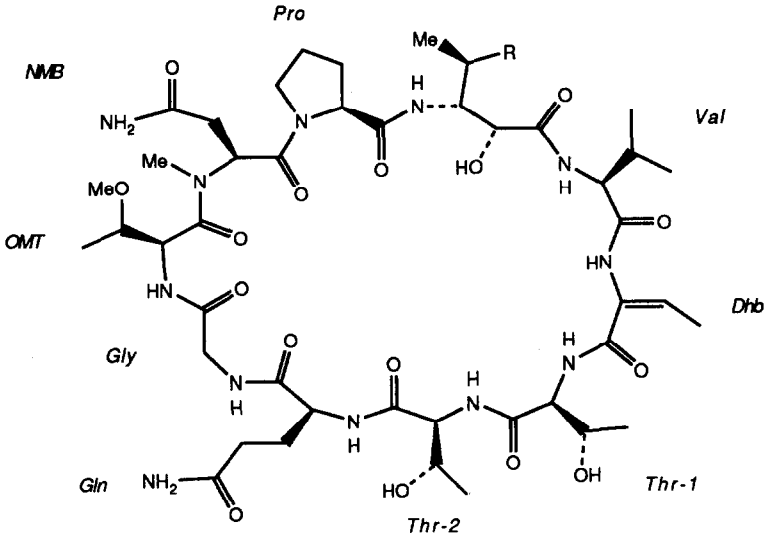
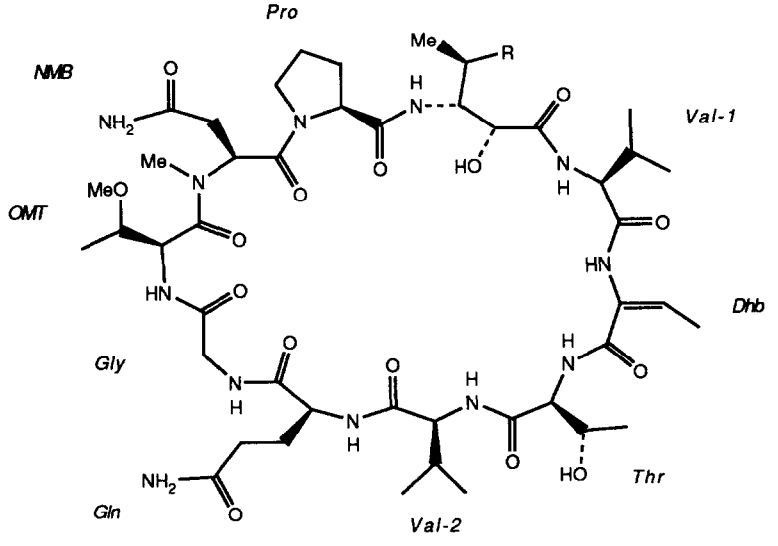
Abstract. Puwainaphycins A-E, one of which (C) is a potent cardioactive agent in isolated mouse atria, are cyclic decapeptides that have been isolated from a terrestrial blue-green alga *Anabaena* sp. BQ-16-1. The structures of puwainaphycins A-E, including most of the stereochemical features, have been elucidated by a combination of spectral and chemical methods.

The blue-green alga *Anabaena* sp. BQ-16-1 produces a chlorine-containing cyclic decapeptide, puwainaphycin C, which elicits a strong inotropic effect in isolated mouse atria (ED_{50} 0.2 ppm) without a concommitant chronotropic response. This cardioactive drug is accompanied by four structurally related, albeit markedly less active cyclic decapeptides, puwainaphycins A, B, D and E. In a previous paper we reported the gross structure determination of puwainaphycins C and D, where the 10 amino acid units of each peptide were identified by a detailed NMR analysis coupled with an acid hydrolysis study and sequenced using difference NOE spectroscopy plus two dimensional ^{13}C-^{13}C and carbon-detected ^{13}C-^{15}N correlation spectroscopy.[1] We describe here the gross structures of puwainaphycins A, B and E and the results of studies on the relative and absolute stereochemistry of this class of compounds.

Puwainaphycins A and B were isolated as white amorphous solids from the cultured alga in yields of 0.1 and 0.15 %, respectively. The molecular formulas $C_{58}H_{98}N_{12}O_{17}$ and $C_{59}H_{100}N_{12}O_{16}$ were deduced for puwainaphycins A and B from detailed analyses of the ^{13}C and ^{1}H NMR spectra and high-resolution positive FAB mass spectral data [MH+ ions at *m/z* 1235.7169 (Δ -8.2 mmu), 1233.7423 (Δ -3.6 mmu)]. Extensive proton-proton decoupling studies, aided by homonuclear (^{1}H-^{1}H) phase-sensitive COSY, single and double RELAY,[2] TOCSY (HOHAHA[3]) and difference NOE experiments and a heteronuclear (^{1}H-^{13}C) CSCM or HMQC[4] experiment, led to 10 partial structures, eight of which were amino acid units inferred from acid hydrolysis (2 N HCl, 36-h reflux), viz. N-substituted glycyl, two threonyl, valyl, *O*-methylthreonyl (OMT), glutaminyl, *N*-methylasparaginyl (NMB), and prolyl units for puwainaphycin A and N-substituted glycyl, threonyl, two valyl, *O*-methylthreonyl, glutaminyl, *N*-methylasparaginyl, and prolyl units for puwainaphycin B. The remaining two partial structures, N-substituted 2-aminobut-2*E*-enyl (Dhb) and 3-amino-2-hydroxy-4-methyl-14-oxostearyl (Ahmos) residues, were consistent with 3-oxobutanoic acid and 3-amino-2-hydroxy-4-methyl-14-oxostearic acid which were also produced on acid hydrolysis. The signal patterns and chemical shifts for the carbons (Table 1) and hydrogens (Table 2) of the α-amino acid units in puwainaphycins A and B were essentially identical with those in puwainaphycins C and D, respectively. Moreover, the chemical shifts and coupling constants (Table 2) for the C-1 to C-5 portion of the β-amino acid unit (Ahmos) were comparable with those for the Champ unit in puwainaphycins C and D. The δ_C and δ_H-values for the terminal portion of the β-amino acid unit in puwainaphycins A and B, however, were clearly different from those in puwainaphycins C and D and an additional two methylene carbon signals were present which meant Ahmos was an octadecanoic acid. The most



A *Ahmos* R = $(CH_2)_9COCH_2CH_2CH_2CH_3$
C *Champ* R = $(CH_2)_9CHClCH_2CH_3$
E *Hamp* R = $(CH_2)_{11}CH_3$



B *Ahmos* R = $(CH_2)_9COCH_2CH_2CH_2CH_3$
D *Champ* R = $(CH_2)_9CHClCH_2CH_3$

Table 1. Carbon Chemical Shifts (ppm) of Puwainaphycins A-E in DMSO-d_6

Carbon Position		A	B	C	D	E	Carbon Position		A	B	C	D	E
Ahmos	1	169.6 s	169.6 s	169.5 s	169.6 s	169.8 s	*Thr-2*	1	174.6 s	171.0 s	174.4 s	171.8 s	174.6 s
or	2	69.6 d	69.6 d	69.6 d	69.3 d	69.9 d	or	2	56.9 d	61.1 d	56.8 d	61.4 d	57.1 d
Champ	3	56.1 d	56.1 d	56.0 d	56.7 d	56.2 d	*Val-2*	3	69.9 d	28.8 d	69.8 d	29.0 d	69.9 d
or	4	32.3 d	32.3 d	32.2 d	32.3 d	32.4 d		4	19.0 q	19.2 q	18.8 q	19.1 q	19.1 q
Hamp	Me on 4	16.0 q	16.0 q	15.9 q	16.0 q	16.1 q		4'		18.0 q		18.0 q	
	5	33.5 t	33.5 t	33.4 t	33.4 t	33.6 t							
	6	25.5 t	25.5 t	25.5 t	25.5 t	25.7 t	*Gln*	1	171.9 s	169.8 s	171.8 s	170.4 s	172.0 s
	7	29.8 t	29.8 t	29.7 t	29.7 t	29.8 t		2	53.2 d	53.5 d	53.1 d	53.6 d	53.3 d
	8	29.3 t	29.3 t	29.2 t	29.2 t	29.2 t		3	26.5 t	26.1 t	26.4 t	26.0 t	26.7 t
	9	29.0 t	29.0 t	29.0 t	29.0 t	29.2 t		4	31.8 t	31.8 t	31.7 t	31.8 t	32.0 t
	10	28.7 t	28.7 t	29.0 t	28.8 t	29.2 t		5	174.0 s	174.0 s	173.9 s	175.1 s	174.0 s
	11	25.6 t	25.6 t	28.5 t	28.6 t	29.2 t							
	12	29.1 t	29.1 t	25.9 t	26.0 t	29.4 t	*Gly*	1	168.9 s	169.0 s	168.8 s	169.6 s	169.0 s
	13	41.9 t	41.9 t	37.4 t	37.5 t	28.9 t		2	42.5 t	42.5 t	42.4 t	42.6 t	42.6 t
	14	210.4 s	210.4 s	66.3 d	66.7 d	31.5 t							
	15	41.6 t	41.6 t	30.9 t	31.0 t	22.3 t	*OMT*	1	169.8 s	171.9 s	169.7 s	172.4 s	170.0 s
	16	24.5 t	24.5 t	10.7 q	10.8 q	14.1 q		2	53.2 d	53.2 d	53.1 d	53.2 d	53.3 d
	17	21.8 t	21.8 t					3	74.9 d	74.9 d	74.8 d	74.9 d	75.0 d
	18	13.9 q	13.9 q					4	15.2 q	15.1 q	15.0 q	15.1 q	15.3 q
								OMe	55.9 q	55.8 q	55.7 q	56.0 q	56.0 q
Val	1	168.9 s	168.9 s	168.8 s	168.9 s	169.0 s							
or	2	55.8 d	56.9 d	55.7 d	57.1 d	56.0 d	*NMB*	1	167.5 s	167.5 s	167.5 s	167.9 s	167.7 s
Val-1	3	32.8 d	32.8 d	32.7 d	32.8 d	32.8 d		2	49.6 d	49.7 d	49.6 d	49.5 d	49.8 d
	4	19.0 q	19.2 q	18.9 q	19.1 q	19.1 q		3	34.0 t	34.0 t	33.9 t	33.8 t	34.1 t
	4'	18.5 q	18.4 q	18.4 q	18.3 q	18.6 q		4	171.6 s	171.6 s	171.5 s	172.1 s	171.7 s
								NMe	30.5 q	30.4 q	30.3 q	30.4 q	30.6 q
Dhb	1	164.1 s	164.1 s	164.0 s	164.6 s	164.2 s							
	2	132.4 s	132.4 s	132.3 s	131.9 s	132.3 s	*Pro*	1	171.1 s	171.1 s	171.0 s	171.9 s	171.2 s
	3	117.2 d	117.4 d	117.2 d	118.2 d	117.4 d		2	59.9 d	59.9 d	59.8 d	59.9 d	60.0 d
	4	12.4 q	12.4 q	12.2 q	12.4 q	12.4 q		3	30.2 t	30.2 t	30.0 t	30.2 t	30.2 t
								4	23.4 t	23.5 t	23.3 t	23.4 t	23.5 t
Thr	1	170.6 s	174.8 s	170.4 s	175.2 s	170.7 s		5	46.9 t	46.8 t	46.8 t	47.1 t	47.0 t
or	2	62.0 d	56.9 d	61.9 d	56.2 d	62.2 d							
Thr-1	3	65.4 d	70.0 d	65.3 d	70.3 d	65.5 d							
	4	20.8 q	19.0 q	20.6 q	19.1 q	20.8 q							

obvious difference was the presence of a ketone carbonyl carbon signal (210.4 ppm). In the ^{1}H NMR spectrum 4H signals appeared at 2.40 and 1.45 ppm, and there was a conspicuous 0.10 ppm upfield shift for the terminal methyl group. Its structure was further verified by characterization of Ahmos as the *N,O*-diacetate methyl ester. The same NOEs that were seen for puwainaphycins C and D[1] were seen for A and B, viz. between Pro H-2 and Ahmos NH, Ahmos H-2 and Val-1 NH, Ahmos OH and Val-1 NH, Val-1 H-2 and Dhb NH, Dhb NH and Dhb H-3, Dhb H_3-4 and Thr-1 NH, Thr-1 H-2 and Thr-2 NH (or Val-2 NH), Thr-1 H-3 and Thr-2 NH (or Val-2 NH), Thr-2 H-2 (or Val-2 H-2) and Gln NH, Gln NH and Gly NH, Gly H_2-2 and OMT NH, OMT H-2 and NMB *N*-Me, OMT H-2 and OMT *O*-Me, OMT H-3 and NMB *N*-Me, OMT H-3 and OMT *O*-Me, OMT H-3 and OMT H_3-4, NMB *N*-Me and NMB H_2-3, NMB H_2-3 and NMB NH_2, and NMB H-2 and Pro H_2-5, and this meant that the amino acid sequences in puwainaphycins A and B paralleled those in C and D, respectively. Furthermore HMBC[5] experiments rigorously supported the amino acid sequences depicted from the NOEs by showing cross peaks between (1) all of the NH protons and the carbonyl carbons of the adjacent amino acids two bonds away, viz. Ahmos NH and Pro C-1, Val-1 NH and Ahmos C-1, Dhb NH and Val-1 C-1, Thr NH and Dhb

Table 2. Proton Chemical Shifts (ppm) for Puwainaphycins A-E in DMSO-*d6*

Proton Position		A	B	C	D	E	Proton Position		A	B	C	D	E
Ahmos	2	4.18 dd	4.17 dd	4.18 dd	4.18 dd	4.16 dd	*Gln*	2	4.06 ddd	4.12 ddd	4.06 ddd	4.10 ddd	4.10 ddd
or	C2-OH	5.56 d	5.56 d	5.56 d	5.56 d	5.54 d		C2-NH	7.37 d	7.52 d	7.37 d	7.52 d	7.38 d
Champ	3	3.95 ddd	3.95 ddd	3.96 ddd	3.96 ddd	3.96 ddd		3	2.02 m	2.02 m	2.05 m	2.04 m	2.04 m
or	C3-NH	6.85 d	6.85 d	6.86 d	6.84 d	6.86 d		3'	1.82 m	1.82 m	1.87 m	1.85 m	1.67 m
Hamp	4	1.68 m	1.68 m	1.68 m	1.68 m	1.72 m		4	2.17 m	2.20 m	2.17 m	2.20 m	1.99 m
	C4-Me	0.57 d	0.59 d	0.57 d	0.59 d	0.58 d		NH_2	7.30 s	7.39 s	7.30 s	7.37 s	7.28 s
	5	1.55 m	1.55 m	1.55 m	1.55 m	1.61 m		NH_2'	6.83 s	6.83 s	6.83 s	6.83 s	6.82 s
	5'	1.25 m	1.25 m	1.25 m	1.25 m	1.25 m							
	6-11	1.25 m	1.25 m	1.25 m	1.25 m	1.25 m	*Gly*	2	4.02 dd	4.00 dd	4.02 dd	3.99 dd	4.02 dd
	12	1.45 m	1.45 m	1.25 m	1.25 m	1.25 m		2'	3.24 dd	3.23 dd	3.24 dd	3.23 dd	3.22 dd
	13	2.40 t	2.40 t	1.66 m	1.66 m	1.25 m		C2-NH	7.97 dd	7.97 dd	7.97 dd	7.97 dd	7.96 dd
	14			4.00 p	4.00 p	1.25 m							
	15	2.40 t	2.40 t	1.66 dq	1.66 dq	1.25 m	*OMT*	2	4.73 dd	4.75 dd	4.73 dd	4.73 dd	4.72 dd
	16	1.45 m	1.45 m	0.97 t	0.98 t	0.82 t		C2-NH	6.82 d	6.82 d	6.82 d	6.82 d	6.81 d
	17	1.25 m	1.25 m					3	3.73 dq	3.73 dq	3.73 dq	3.73 dq	3.73 dq
	18	0.87 t	0.87 t					4	1.01 d	1.01 d	1.01 d	1.01 d	1.00 d
								OMe	3.18 s	3.18 s	3.18 s	3.18 s	3.17 s
Val	2	4.31 dd	4.31 dd	4.31 dd	4.32 dd	4.29 dd							
or	C2-NH	6.88 d	6.90 d	6.89 d	6.89 d	6.87 d	*NMB*	2	5.56 dd	5.56 dd	5.56 dd	5.56 dd	5.58 dd
Val-1	3	1.80 m	1.82 m	1.81 m	1.82 m	1.82 m		NMe	2.96 s	2.96 s	2.96 s	2.96 s	2.95 d
	4	0.90 d	0.89 d	0.90 d	0.89 d	0.89 d		3	3.04 dd	3.03 dd	3.04 dd	3.02 dd	3.03 dd
	4'	0.82 d	0.84 d	0.82 d	0.84 d	0.85 d		3'	1.99 dd	2.00 dd	1.99 dd	1.98 dd	1.99 dd
								NH_2	7.53 s	7.53 s	7.53 s	7.53 s	7.53 s
Dhb	C2-NH	9.10 s	9.10 s	9.10 s	9.10 s	9.08 s		NH_2'	6.03 s	6.03 s	6.03 s	6.03 s	5.98 s
	3	5.37 q	5.37 q	5.37 q	5.37 q	5.36 q							
	4	1.75 d	1.75 d	1.75 d	1.75 d	1.74 d	*Pro*	2	4.28 dd	4.29 dd	4.28 dd	4.28 dd	4.28 dd
								3	2.09 m	2.09 m	2.09 m	2.09 m	2.09 m
Thr	2	3.83 dd	4.87 dd	3.84 dd	4.86 dd	3.83 dd		3'	1.99 m	1.99 m	1.99 m	1.99 m	2.02 m
or	C2-NH	8.78 d	8.42 d	8.78 d	8.42 d	8.76 d		4	1.94 m	1.94 m	1.94 m	1.94 m	1.94 m
Thr-1	3	4.11 dq	4.60 dq	4.11 dq	4.60 dq	4.13 dq		4'	1.78 m	1.78 m	1.78 m	1.78 m	1.78 m
	C3-OH	5.21 s	4.08 s	5.20 s	4.08 s	5.21 s		5	3.17 m	3.17 m	3.17 m	3.17 m	3.18 m
	4	1.24 d	1.26 d	1.25 d	1.26 d	1.25 d		5'	4.24 m	4.24 m	4.24 m	4.24 m	4.24 m
Thr-2	2	4.90 dd	3.83 dd	4.90 dd	3.84 dd	4.90 dd							
or	C2-NH	8.38 d	8.62 d	8.36 d	8.61 d	8.36 d							
Val-2	3	4.60 dq	2.17 m	4.60 dq	2.17 m	4.59 dq							
	C3-OH	4.19 s		4.17 s		4.33 s							
	4	1.26 d	1.04 d	1.26 d	1.04 d	1.25 d							
	4'		1.02 d		1.02 d								

Coupling constants for puwainaphycin A in Hz. *Ahmos*: 2,OH = 5.5; 2,3 = 5.5; 3,NH = 11.3; 4,Me = 6.6; 17,18 = 7.2. *Val*: 2,NH = 9.9; 2,3 = 7.0; 3,4 = 7.0; 3,4' = 7.0. *Dhb*: 3,4 = 7.3. *Thr-1*: 2,NH = 3.8; 2,3 = 4.0; 3,4 = 6.7; 3,OH = 6.4. *Thr-2*: 2,NH = 9.4; 2,3 = 2.4; 3,4 = 7.3; 3,OH = <1. *Gln*: 2,NH = 7.9. *Gly*: 2,NH = 2',NH = 6.3; 2,2' = -16.0. *OMT*: 2,NH = 9.0; 2,3 = 2.6; 3,4 = 6.2. *NMB*: 2,3 = 11.2; 2,3' = 2.8; 3,3' = -15.7.

Coupling constants for puwainaphycin B are essentially the same as those for A, with the following exceptions. *Val-2* (in lieu of Thr-2): 2,NH = 3.8; 2,3 = 3.9; 3,4 = 7.6; 3,4' = 7.6. *Gly*: 2,NH = 8.2; 2',NH = 7.0; 2,2' = -16.8.

Coupling constants for puwainaphycin C in Hz. *Champ*: 2,OH = 5.4; 2,3 = 5.5; 3,NH = 10.2; 4,Me = 6.5; 17,18 = 7.7. *Val*: 2,NH = 8.2; 2,3 = 6.8; 3,4 = 6.7; 3,4' = 6.7. *Dhb*: 3,4 = 7.2. *Thr-1*: 2,NH = 3.7; 2,3 = 3.7; 3,4 = 6.2; 3,OH = 4.4. *Thr-2*: 2,NH = 9.3; 2,3 = 2.5; 3,4 = 7.3; 3,OH = <1. *Gln*: 2,NH = 7.5; 2,3 = 10.2; 2,3' = <1; 3,3' = -14.0; 3,4 = 3,4' = 6.9. *Gly*: 2,NH = 2',NH = 6.1; 2,2' = -15.8. *OMT*: 2,NH = 9.1; 2,3 = 2.8; 3,4 = 6.1. *NMB*: 2,3 = 11.5; 2,3' = 4.9; 3,3' = -15.9. *Pro*: 2,3 = 9.7.

Coupling constants for puwainaphycin D are essentially the same as those for C, with the following exceptions. *Val-2* (in lieu of Thr-2): 2,NH = 3.5; 2,3 = 3.5; 3,4 = 6.3; 3,4' = 6.3. *Gly*: 2,NH = 8.1; 2',NH = 6.7; 2,2' = -16.9.

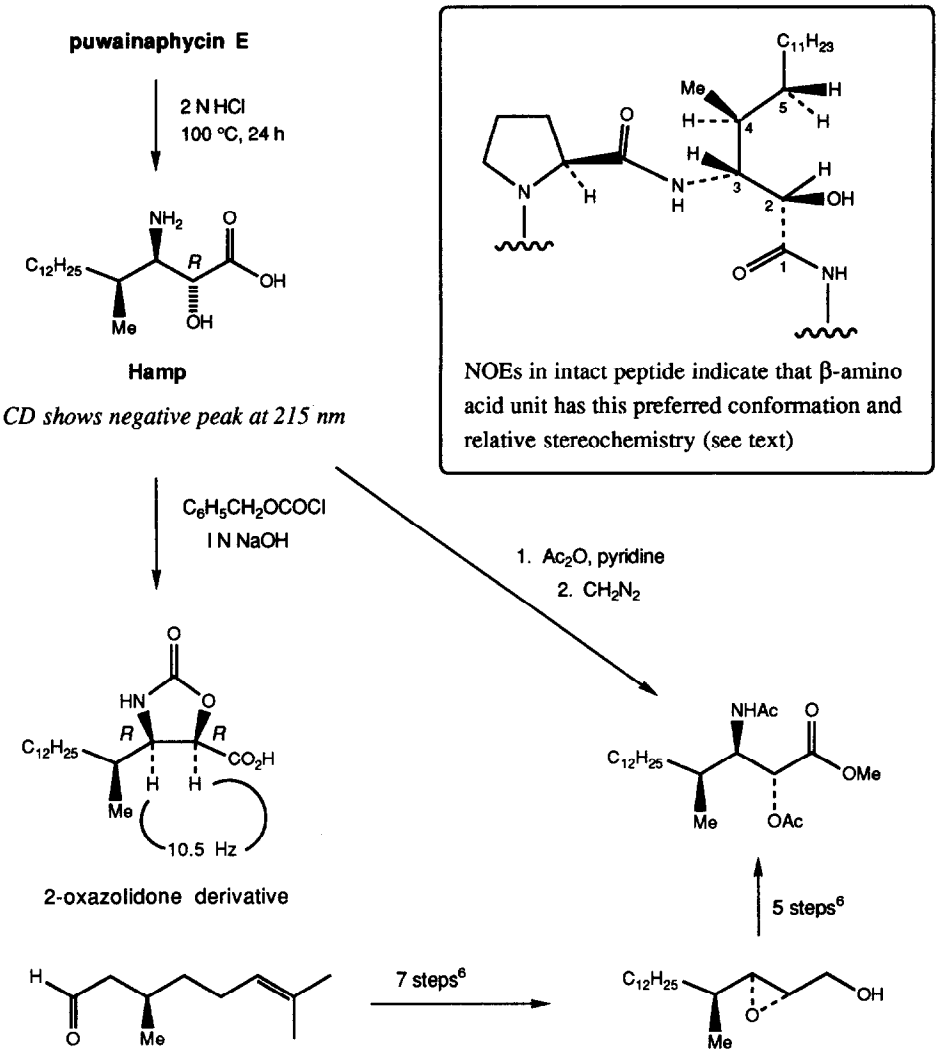
Coupling constants for puwainaphycin E are essentially the same as those for C.

C-1, Thr-2 NH (or Val-2 NH) and Thr-1 C-1, Gln NH and Thr-2 C-1 (or Val-2 C-1), Gly NH and Gln C-1, OMT NH and Gly C-1 and (2) all of the α-carbon protons and the carbonyl carbons of the adjacent amino acids three bonds away, viz. Val-1 H-2 and Ahmos C-1, Thr-1 H-2 and Dhb C-1, Thr-2 H-2 (or Val-2 H-2) and Thr-1 C-1, Gln H-2 and Thr-2 C-1 (or Val-2 C-1), Gly H_2-2 and Gln C-1, OMT H-2 and Gly C-1, NMB H-2 and OMT C-1, and Pro H-2 and NMB C-1. An HMBC correlation was also observed between the NMB N-methyl protons and the OMT carbonyl carbon.

Puwainaphycin E was also isolated as a white amorphous solid from the cultured alga, but its yield was much lower (0.01%). The molecular formula for E was concluded to be $C_{56}H_{96}N_{12}O_{16}$ based on a detailed analysis of ^{13}C and ^{1}H NMR spectra and the positive FAB mass spectrum (MH^+ ion at *m/z* 1193; shifts to *m/z* 1215 for MNa^+). Compared with the other puwainaphycins, the most noticeable difference was the absence of both keto and chloro groups. The chemical shifts for the carbons (Table 1) and hydrogens (Table 2) of the α-amino acid units in E were virtually the same as those in A and C. For the β-amino acid unit, however, the NMR data for the C-1 to C-5 portion were identical with the NMR data for the Ahmos unit in A and B and the Champ unit in C and D, but the δ_C and δ_H-values for the terminal portion of the β-amino acid unit in E were different. The ^{1}H and ^{13}C signals for the chlorine-bearing methine were missing and the chemical shifts were consistent with a normal alkyl chain. NMR analysis indicated that the β-amino acid unit was a 2-hydroxy-3-amino-4-methylpalmitic acid (Hamp) unit. Its structure was verified by acid hydrolysis of A and B to the free fatty acid and direct comparison with Hamp from the acid hydrolyzate of calophycin, a closely-related antifungal cyclic decapeptide from the terrestrial blue-green alga *Calothrix fusca* EU-10-1.[6] Finally the amino acid sequence in E was verified from NOE and HMBC experiments.

As expected puwainaphycins A and B showed the same α-amino acid profiles as puwainaphycins C and D, respectively, and the α-amino acid pattern for puwainaphycin E was similar to those of puwainaphycins A and C. Marfey analysis[7] of the acid hydrolyzates of puwainaphycins A-E indicated that *all* of the α-amino acids were L. The β-amino acid from puwainaphycin E was found to be (2*R*,3*R*,4*S*)-2-hydroxy-3-amino-4-methylpalmitic acid, since the corresponding diacetate methyl ester was identical in all respects, including optical properties, with the Hamp derivative from calophycin, but more importantly with a synthetic sample prepared from (*R*)-(+)-citronellal.[6]

The absolute stereochemistry at C-2, C-3 and C-4 in the β-amino acid units of puwainaphycins A-D was determined in the following way: (1) The CD spectra of Ahmos, Champ and Hamp all exhibited a strong negative Cotton effect near 215 nm, strongly suggesting that the absolute configuration at C-2 was *R* for all of the puwainaphycins.[8] (2) The coupling constants between H-2 and H-3 (J = 10.5 Hz) in the corresponding 2-oxazolidone derivatives of these β-amino acids indicated that these two protons were cis to each other in the derivatives and this meant that the absolute configuration at C-3 was *R* in all of the puwainaphycins.[9] (3) The same NOEs that were observed between Hamp protons in calophycin were seen between Ahmos and Champ protons for the puwainaphycins, viz. NOEs were observed between H-2 and H-3, H-2 and H-5, H-2 and H-5', H-3 and the OH on C-2, H-4 and the NH, and H-3 and the methyl group on C-4,[6] strongly suggesting that the absolute configuration at C-4 was *S* for all of the puwainaphycins.



Scheme 1. Determination of the absolute stereochemistry of puwainaphycin E. Similar experiments were carried out to determine the absolute stereochemistries of puwainaphycins A-D. The CD spectra of Ahmos, the β-amino acid from acid hydrolysis of puwainaphycins A and B, and a 1:1 mixture of C-14 epimers of 3-amino-2,14-dihydroxy-4-methylpalmitic acid (Dhamp), the β-amino acid product from acid hydrolysis of puwainaphycins C and D, also showed negative peaks near 215 nm. The ^{1}H NMR spectra of the 2-oxazolidone derivatives of Ahmos and Dhamp also showed 10.5 Hz coupling constants between the ring methine protons. Ahmos and Dhamp were not compared with synthetic samples.

Experimental Section

Spectral Analysis. NMR spectra were determined on 11.75 and 7.05 tesla instruments operating at 500 and 300 MHz for ^1H and 125 and 75 MHz for ^{13}C, respectively. Proton chemical shifts are referenced in dimethyl sulfoxide-d_6, acetone-d_6 and $CDCl_3$ to the residual DMSO-d_5 (2.49 ppm), acetone-d_5 (2.04 ppm) and $CHCl_3$ (7.26 ppm) signals; carbon chemical shifts are referenced in dimethyl sulfoxide-d_6 to the solvent (39.5 ppm).

Isolation. Puwainaphycins A-E were isolated from cultured *Anabaena* sp. (UH isolate BQ-16-1) as previously described[1] in respective yields of 0.1, 0.15, 0.3, 0.2 and 0.01 % (based on the dry weight of the alga).

Puwainaphycin A: amorphous white solid, $[\alpha]_D$ -37° (1:1 MeCN/H_2O, c 0.4); UV (MeCN) λ_{max} nm (ε) 204 (21,300), 243 (4,800); high resolution FABMS, *m/z* 1235.7169 ($C_{58}H_{98}N_{12}O_{17}$, Δ-8.2 mmu).

Puwainaphycin B: amorphous white solid, $[\alpha]_D$ -36° (1:1 MeCN/H_2O, c 0.4); UV (MeCN) λ_{max} nm (ε) 201 (20,600), 243 (3,100); high resolution FABMS, *m/z* 1233.7423 ($C_{59}H_{100}N_{12}O_{16}$, Δ-3.6 mmu).

Puwainaphycin E: amorphous white solid; positive ion FABMS, *m/z* 1194 (MH^+).

Acid Hydrolysis. A 24-36 h reflux of 50 mg of puwainaphycin A or B in 6 mL of 2 N HCl gave a hydrolyzate that was separated into two fractions by reverse-phase chromatography on C-18 (Analytichem BondElut). The fraction eluted with water was subjected to HPLC with 0.1% trifluoroacetic acid (TFA) as previously described[1] to give 2-oxobutanoic acid, glycine, threonine, glutamic acid, valine, *O*-methylthreonine, *N*-methylaspartic acid, and proline.

The C-18 BondElut fraction eluted with MeOH was treated first with Ac_2O in pyridine followed by CH_2N_2 in CH_2Cl_2. Gradient HPLC of the resulting Ahmos-related *N,O*-diacetyl methyl esters on silica with 10-50% EtOH in 1:1 hexane/CH_2Cl_2 gave 3-acetamido-2-acetoxy-4-methyl-14-oxostearate, $[\alpha]_D$ +17° (1:1 MeCN/H_2O, c 1.6); EIMS *m/z* (rel. intensity) 441 (2), 410 (2), 399 (3), 398 (2), 384 (8), 342 (43), 311 (33), 310 (100), 300 (19), 282 (22), 268 (66), 240 (6), 202 (11), 160 (31), 142 (41); high resolution EIMS, *m/z* 441.3091 ($C_{24}H_{43}NO_6$, Δ-0.1 mmu), 342.2284 ($C_{18}H_{32}NO_5$, Δ-0.4 mmu), 300.2159 ($C_{16}H_{30}NO_4$, Δ1.5 mmu), 282.2081 ($C_{16}H_{28}NO_3$, Δ-1.2 mmu), 240.1973 ($C_{14}H_{26}NO_2$, Δ-0.3 mmu). ^1H NMR (DMSO-d_6) δ 7.73 (d, J = 9.7 Hz, NH), 4.78 (d, J = 7.9 Hz, H-2), 4.29 (ddd, J = 9.7, 7.9 and 4.0 Hz, H-3), 3.65 (s, OMe), 2.40 (t, J = 7.3 Hz, H_2-13,15), 2.12 (s, OAc), 1.85 (s, OAc), 1.75 (m, H-4), 1.40 (m, H_2-12,16), 1.25 (br s, H_2-5,6,7,8,9,10,11,17), 0.86 (d, J= 6.9 Hz, Me on C-4), 0.86 (t, J = 7.2 Hz, H_3-18). ^{13}C NMR (DMSO-d_6) δ 210.4 (s, C14), 169.74 (s), 169.71 (s), 168.8 (s), 72.3 (d, C-2), 52.0 (s, OMe), 50.7 (d, C-3), 41.7 (t, C-13), 41.4 (t, C-15), 33.3 (t), 32.3 (t), 28.9 (t), 28.7 (t, 3 carbons), 28.5 (t), 26.2 (t), 25.3 (t), 23.2 (t), 22.3 (t), 21.7 (t), 20.3 (t), 14.2 (q, Me on C-4), 13.7 (q, C-18).

Hydrolysis was carried out on 10 mg of puwainaphycin E in a similar manner. The crude β-amino acid in the C-18 BondElut fraction eluted with MeOH was treated with Ac_2O in pyridine followed by CH_2N_2 in CH_2Cl_2 to give methyl 3-acetamido-2-acetoxy--4-methylpalmitate which was purified by HPLC on silica (Alltech Econosil, 10μ) with 1:1 hexane/THF, mp 44 °C, $[\alpha]_D$ +17° ($CHCl_3$, c 0.8), CD (*p*-dioxane) $[\theta]_{211}$ -3300; EIMS *m/z* 399 (M^+); ^1H NMR ($CDCl_3$) δ 5.51 (d, J = 10.5 Hz, NH), 5.02 (d, J = 5.7 Hz, H-2), 4.47 (ddd, J = 10.5, 5.7 and 4.8 Hz, H-3), 3.74 (s, OMe), 2.14 (s, OAc), 2.02 (s, OAc), 1.72 (m, H-4), 1.49 (m, H_2), 1.32 (m, H_2), 1.25 (m, H_{18}), 0.90 (d, J= 6.6 Hz, Me on C-4), 0.88 (t, J = 6.7 Hz, H_3-16).

Determination of Absolute Configurations of Amino Acids. To 0.5 mg of each α-amino acid in 100 μL of water was added successively 200 μL of a 1% solution of 1 fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, Marfey's reagent) in acetone and 40 mL of 1.0 M sodium bicarbonate solution. The mixture was heated at 40 °C for 1 h and cooled. Then 20 μL of 2 M HCl was added and the mixture was degassed. An aliquot was analyzed on a 10 cm x 4.6 mm reversed-phase C-18 column (Pierce Spheri-5) using a linear gradient of 10 to 40% acetonitrile in 0.05 M triethylamine phosphate at pH 3.0 (flow rate 2.0 mL/min, 25 °C, UV detection at 340 nm) over a period of 45 min. Marfey derivatives of authentic L- and D-amino acids had the following respective t_R values: 14.1 and 17.5 min for L- and D-glutamate, 14.4 and 12.3 min for L- and D-*N*-methylaspartate, 11.9 and 17.9 min for L- and D-threonine, 11.6 min for L-*allo*-threonine, 18.2 min for L-*O*-methylthreonine, 24.3 and 36.3 min for L- and D-valine, and 17.7 and 23.7 min for L- and D-proline. The retention times for the Marfey derivatives of amino acids from hydrolysis of puwainaphycins A, B, C and D were 14.1-14.3 for L-glutamate, 14.6-14.7 for L-*N*-methylaspartate, 11.9-12.1 for L-threonine, 17.8-18.3 for L-*O*-methylthreonine, 24.1-24.5 for L-valine, and 17.8-18.2 for L-proline.

The crude β-amino acid (1.5 mg) in 1 mL of 1 M NaOH solution cooled to 5 °C was treated with 250 mL of benzyl chloroformate. After stirring for an hour, the mixture was acidified with 1 N HCl and extracted several times with ethyl acetate. The combined extract was dried and evaporated to give a residue that was purified on a silica BondElut column with 30% EtOAc in hexane. The 2-oxazolidone carboxylic acid derivatives showed the following common ^{1}H NMR signals in acetone-d_6: δ 6.38 (d, J = 9.9 Hz, NH), 4.95 (d, J = 10.5 Hz, H-2), 4.23 (ddd, J = 10.5, 9.9, 4.8 Hz, H-3), 1.82 (m, H-4), and 0.90 (d, J = 6.6 Hz, Me on C-4).

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