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Nostocyclin, A Novel 3-Amino-6-hydroxy-2-piperidone-containing Cyclic Depsipeptide from the Cyanobacterium *Nostoc* sp.

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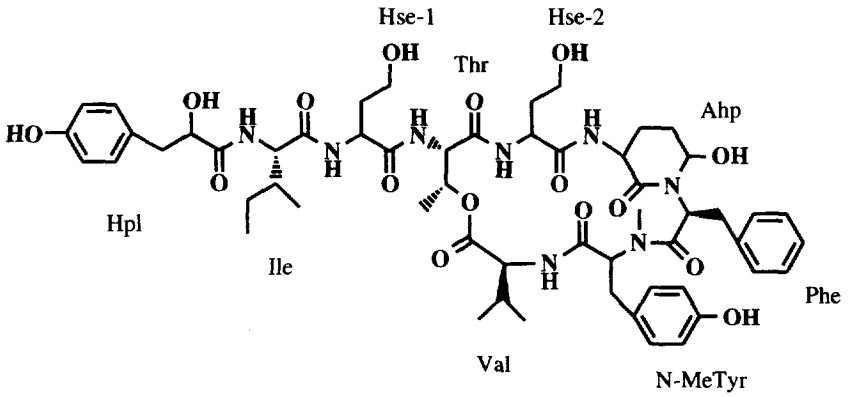
Abstract: Nostocyclin, a novel 3-amino-6-hydroxy-2-piperidone-containing cyclic peptide was isolated from a hepatotoxic strain of cyanobacterium *Nostoc* sp. Nostocyclin is non-toxic in acute *in vivo* bioassays, but inhibits protein phosphatase-1 activity at high concentration *in vitro*.

Populations of the cyanobacterium (blue-green alga) *Nostoc* have been traditionally used as a human food in several countries e.g. Bolivia, China, Mongolia and Russia[1]. However, some strains of *Nostoc* produce cyclic heptapeptides (microcyctins) which are potent hepatotoxins[2-4]. During investigations into toxins from newly-isolated *Nostoc* strains, we have found a novel cyclic depsipeptide and here present its isolation and structure elucidation **1**.



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Nostoc sp.(University of Dundee, strain DUN901) was isolated as a minority component from a bloom of the cyanobacterium *Nodularia spumigena*, collected from the brackish water Barrow Ski Club Lake, Lincolnshire, England. *Nostoc* sp. was grown axenically in batch culture in 8L volumes of Z8 minus nitrate medium[5], containing 25% (v/v) seawater, at 20-25°C. Cultures were sparged with air at about 7 L h^{-1} and light supplied by white fluorescent tubes giving an irradiance incident on the surface of the vessels of about 20μmol photon m^{-2} s^{-1}. Cells were harvested from stationary phase by centrifugation at 10,000 xg for 20 min, to give a pellet which was freeze-dried. Methanol extract from 10 g of freeze-dried cells was evaporated under reduced pressure. The remaining residue was suspended in 5% (v/v) acetic acid aqueous solution. The suspension was centrifuged at 2,000 rpm for 20 min and the supernatant retained. The inhibitor **1** was isolated by solid-phase

extraction using ODS cartridges(Sep-Pak ODS). The isolated inhibitor **1** was further purified by reverse-phase HPLC(Mightysil RP-18, 20 mm I.D. x 25 cm) with methanol(60%) containing 0.05 M phosphate buffer(pH 3.0) at 10 ml·min^{-1}. The yield of the inhibitor **1**, nostocyclin, was 35 mg. **1** was assayed for toxicity by intraperitoneal mouse bioassay[6], brine shrimp (*Artemia salina*)[7], and for the inhibition of protein phosphatase (PP1) using a rabbit skeletal muscle recombinant PP1 and colourimetric assay[8].

Nostocyclin (**1**) is a colorless amorphous solid: λmax(MeOH) 278 nm(ε: 2500). In the positive HRFABMS using glycerol as the matrix, the $[(M + H) - H_2O]^+$ ion was observed at m/z 1099.5414. In the case of negative FABMS, The $[M - H]^-$ ion was observed at m/z 1115.53. From the results, the molecular formula of **1** was established to be C_{56} H_{76} O_{16} N_8 (calcd for C_{56} H_{75} O_{15} N_8 : 1099.5476, Δ 6.2 mmu). The spectral data(Table 1) of ^{1}H - and ^{13}C-NMR of **1** suggest that nostocyclin is a depsipeptide. Moreover, *p*-hydroxyphenyllactic acid (Hpl) was contained in **1**, and was combined with the N-terminal. From amino acid analysis of the hydrolysate (6 N HCl, 110°C, 20 h), the detected amino acids were homoserine (Hse), threonine(Thr), phenylalanine (Phe), N-methyltyrosine(N-MeTyr), valine(Val) and isoleucine(Ile). All of the usual amino acid residues and N-MeTyr in **1** were shown to have the L-configuration by chiral GC analysis(Chirasil-L-Val, Alltech) of N-trifluoroacetyl isopropyl ester derivative of the hydrolysate[9]. The absolute configuration of Hpl in **1** was determined to be D-form by LC analysis[10].

Table 1 ^{1}H and ^{13}C NMR Data for Nostocyclin in Dimethylformamide-d_7

position		^{1}H *J* (Hz)			^{13}C	position		^{1}H *J* (Hz)			^{13}C
Hpl	1				173.5	Ahp	1				170.0
	2	4.19	(m)		73.7		2	3.79	(m)		50.0
	3	3.03	(dd	13.73, 3.51)	40.6		3	2.59	(m)		22.4
		2.73	(m)					1.67	(m)		
	4				129.4		4	1.84	(m)		30.6
	5,9	7.09	(d	8.55)	131.2			1.67	(m)		
	6,8	6.74	(d	8.54)	115.4		5	5.26	(s)		75.1
	7				157.0		NH	7.27	(d	8.58)	
Ile	1				171.9	Phe	1				171.6
	2	4.42	(dd	8.95, 6.51)	57.0		2	4.99	(dd	11.60, 4.27)	51.5
	3	1.79	(m)		38.7		3	2.98	(m)		35.5
	4	1.45	(m)		25.1			2.73	(m)		
		1.04	(m)				4				137.9
	5	0.84	(t	7.32)	11.5		5,9	6.96	(d	7.02)	130.3
	6	0.87	(d	6.71)	15.7		6,8	7.20	(t	7.32)	128.4
	NH	7.58	(d	9.15)			7	7.15	(d	7.32)	126.8
Hse-1	1				173.1	N-Me-Tyr	1				170.5
	2	4.69	(dd	13.47, 7.73)	51.5		2	5.13	(dd	11.29, 8.54)	62.0
	3	2.03	(m)		30.6		3	3.25	(m)		33.8
		1.85	(m)					2.78	(m)		
	4	3.62	(m)		58.9		4				128.7
	NH	8.22	(d	7.32)			5,9	7.11	(d	8.54)	131.2
Thr	1				170.2		6,8	6.70	(d	8.55)	116.2
	2	4.81	(d	9.46)	49.5		7				157.4
	3	5.60	(q	6.40)	72.8		Me	2.84	(s)		30.4
	4	1.29	(d	6.71)	18.3	Val	1				173.2
	NH	8.04	(d	11.90)			2	4.63	(dd	9.15, 5.19)	57.4
Hse-2	1				171.1		3	2.13	(m)		31.6
	2	4.45	(m)		50.8		4	0.91	(d	7.02)	19.6
	3	2.21	(m)		33.8		5	0.81	(d	7.01)	17.9
		1.64	(m)				NH	7.65	(d	9.16)	
	4	3.50	(m)		59.1						
	NH	8.38	(d	8.24)							

Extensive NMR analysis by ^{1}H-^{1}H COSY and HMBC spectra revealed the spin systems of six amino acids but not Phe. The presence of an N,N-disubstituted derivative of Phe was suggested due to the absence of the amide proton. On the Thr unit, the chemical shift of H-3(δ, 5.60 ppm) suggested that the hydroxyl group of Thr was acylated. The structure of 3-amino-6-hydroxy-2-piperidone(Ahp) was deduced by COSY and HMBC spectra. In the COSY spectra, the relation of the connectivities from NH(δ, 7.27 ppm) to 6-OH(δ, 6.20 ppm) of Ahp was determined. On the Ahp unit, the chemical shift of H-6(δ, 5.26 ppm) and C-6(δ, 75.1 ppm) suggested that the C-6 was substituted with O and N. Furthermore C-2 and C-6 of Ahp correlated with α-H of the Phe derivative in the HMBC spectrum. Consequently, Ahp was deduced to be a part of a hemiaminol structure formed from glutamate γ-semialdehyde and Phe.

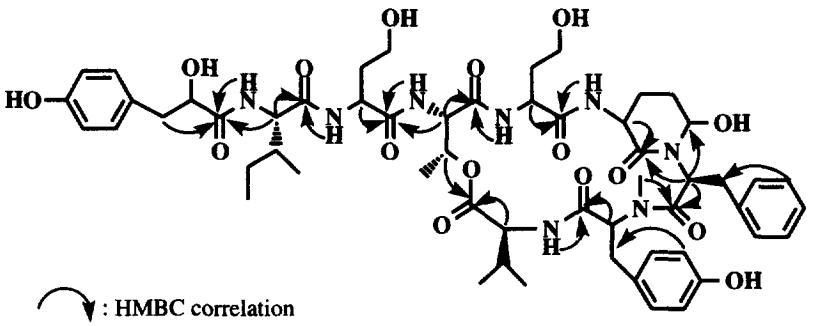

Fig.1. HMBC correlations of nostocyclin

The sequence of **1** was mostly deduced by HMBC correlations from N-H to C=O. The HMBC correlation from β-H of Thr to C=O of Val confirmed the ester formation between Thr and Val. The methyl proton of N-MeTyr correlated with C=O of Phe in the HMBC spectrum. Furthermore, Phe and Ahp were connected as a hemiaminol mentioned above. From the results, the structure of nostocyclin was established as **1**.

Ahp-containing cyclic depsipeptides have been isolated from cyanobactria[10-15]. Nostocyclin is an Ahp-containing cyclic depsipeptide but it also contains *p*-hydroxyphenyllactic acid and two molecules of homoserine.

Nostocyclin was not toxic by intraperitoneal mouse bioassay at up to 2500 μg·kg body wt^{-1} or by brine shrimp bioassay at up to 5 μM. In the *in vitro* PP1 inhibition assay, inhibition by nostocyclin was found, although with a relatively high IC_{50} of 64 μM (1280-fold higher than microcystin-LR in this colourimetric assay). Other naturally-occurring PP1 inhibitors vary widely in IC_{50} values(e.g. 600-fold range) and inhibit other protein phosphatases to varying extent[16,17]. The biological function(s) and significance of nostocyclin are unknown: further screening is in progress.

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