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Oscillatorin, A Chymotrypsin Inhibitor from Toxic *Oscillatoria agardhii*

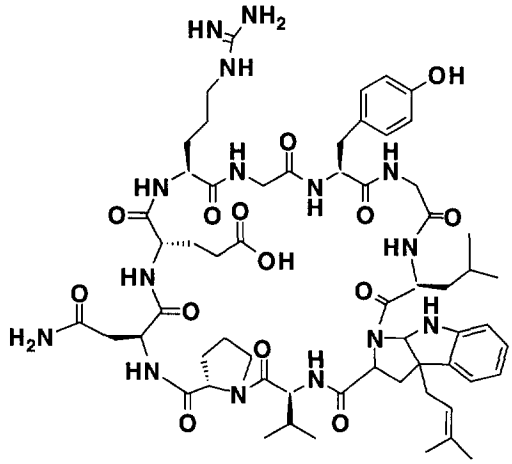
Tomoharu Sano and Kunimitsu Kaya

Environmental Chemistry Division, National Institute for Environmental Studies,
16-2, Onogawa, Tsukuba, Ibaraki 305, Japan

Abstract: Oscillatorin(**1**), a chymotrypsin inhibitor, was isolated from freshwater toxic cyanobacterium *Oscillatoria agardhii*. The structure was elucidated to be **1** by chemical degradation and 2D NMR analyses. Oscillatorin included an abnormal amino acid unit, (3a-*cis*)-1,2,3,3a,8,8a-hexahydro-3a-(3-methyl-2-butenyl)-pyrrolo[2,3-b]indol-2-carboxylic acid. Copyright © 1996 Elsevier Science Ltd

The toxic strain of freshwater cyanobacterium(blue-green algae) *Oscillatoria agardhii* forms waterblooms in freshwater lakes and drinking water reservoirs, and produces heptacyclic peptide hepatotoxins named microcystins[1].

During investigations of cyclic peptide toxins of the toxic strain of *O. agardhii*, we found a novel chymotrypsin-inhibiting cyclic decapeptide containing (3a-*cis*)-1,2,3,3a,8,8a-hexahydro-3a-(3-methyl-2-butenyl)-pyrrolo[2,3-b]indol-2-carboxylic acid (oscillatoric acid, Osc). We now describe the isolation and structure elucidation of oscillatorin(**1**).



1

O. agardhii (NIES-610 =CCAP 1459/22 =NIVA CYA 18) was cultured in 10L culture bottles with CT medium[2] [$Ca(NO_3)_2$•$4H_2O$ 15mg, KNO_3 10mg, β-Na_2 glycerophosphate 5mg, $MgSO_4$•$7H_2O$ 4mg, Vitamine B_{12} 0.01μg, Biotin 0.01μg, Thiamine HCl 1μg, $FeCl_3$•$6H_2O$ 0.06mg, $MnCl_2$•$4H_2O$ 0.01mg,

Table 1. ^{1}H and ^{13}C NMR spectra data of Oscillatorin in DMF-*d7* at 500MHz

position		^{1}H	*J*(Hz)	^{13}C	position		^{1}H	*J*(Hz)	^{13}C
Val	1			169.7	Tyr	1			173.2
	2	4.27	(dd, 8.9, 9.5)	55.8		2	4.77	(m)	54.8
	3	1.4	(m)	32.8		3	2.88	(dd, 4.9, 13.2)	39.7
	4	0.78	(d, 6.4)	19.0			2.75	(dd, 8.0, 13.2)	
	4'	0.24	(d, 6.4)	19.2		4			128.5
	NH	7.28	(d, 9.5)			5,9	7.00	(d, 8.2)	131.2
Pro	1			173.0		6,8	6.71	(d, 8.2)	115.6
	2	4.38	(dd, 3.5, 5.2)	59.8		7			157.0
	3	2.0	(m)	30.2		NH	7.38	(d, 9.2)	
	4	1.82	(m)	24.7	Gly 1	1			168.1
	5	3.51	(m)	48.3		2	4.45	(dd, 7.2, 17)	42.1
Asn	1			174.0			3.64	(dd, 4.9, 17)	
	2	4.59	(m)	49.1		NH	8.56	(dd, 4.9, 7.2)	
	3	3.57	(dd, 4.5, 17)	36.8	Leu	1			172.6
		2.95	(dd, 4.1, 17)			2	4.37	(m)	49.4
	4			175.2		3	1.4	(m)	43.5
	NH	8.05	(d, 8.9)			4	1.57	(m)	25.0
Glu	1			172.8		5	0.92	(d, 6.4)	21.5
	2	4.02	(m)	57.2		5'	0.86	(d, 6.7)	23.8
	3	2.07	(m)	27.0		NH	7.65	(d, 8.6)	
	4	2.35	(m)	34.5	Osc	1			
	5			179.5		2	4.48	(dd, 2, 7)	62.4
	NH	10.99	(bs)			3	2.89	(dd, 14, 7)	43.0
Arg	1			172.8			2.41	(dd, 14, 2)	
	2	4.35	(m)	53.4		3a			55.6
	3	2.15	(m)	28.9		3b			134.0
		1.96	(m)			4	7.13	(d, 7.6)	123.7
	4	1.7	(m)	26.6		5	6.65	(t, 7.6)	119.3
	5	3.25	(m)	41.5		6	6.99	(t, 7.6)	128.9
	7			158.7		7	6.61	(d, 7.6)	109.9
	2-NH	7.90	(d, 9.1)			7a			149.8
Gly 2	1			169.2		8	6.92	(d, 3.4)	
	2	4.07	(dd, 7.5, 17.0)	43.5		8a	5.62	(d, 3.4)	82.6
		3.33	(dd, 4.3, 17.0)			9	2.49	(dd, 7.9, 14.4)	38.4
	NH	8.26	(dd, 4.3, 7.5)				2.42	(dd, 6.1, 14.4)	
						10	5.05	(dd, 6.1, 7.9)	120.1
						11			135.1
						12	1.56	(s)	25.9
						13	1.64	(s)	18.0
						14			171.1

$ZnSO_4 \cdot 7H_2O$ 7μg, $CoCl_2 \cdot 6H_2O$ 1.2μg, $Na_2MoO_4 \cdot 2H_2O$ 0.75μg, $Na_2EDTA \cdot 2H_2O$ 0.3mg, TAPS(*N*-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid) 40mg, distilled water 100mL, pH 8.2] . The cells were grown isothermally at 20°C(light intensity, below 250 μmol photon m^{-2} s^{-1}; aeration rate, 1.5 L min^{-1}).

The chloroform-methanol-water(1:3:1, v/v) extract from freeze-dried cells(99.1 g) was suspended with 5% acetic acid aqueous solution. The suspension was filtered. The filtrate inhibited chymotrypsin potently[3]. The inhibitor in the filtrate was fractionated by solid-phase extraction using ODS cartridge(Sep-Pak ODS). The inhibitor fraction was further purified by reverse-phase HPLC(Purosphare ODS, 20 mm I.D. × 25 cm, flow rate, 6.0 ml/min) with methanol(75%) containing 0.05 M phosphate (pH 3.0). The yield of the inhibitor **1**, oscillatorin, was 8.5 mg.

Oscillatorin inhibited chymotrypsin activity at 1.3×10^{-5}M. The inhibitor **1** is a colorless amorphous solid: λmax(H_2O) 283nm(logε 3.4). The $[M+H]^+$ ion of the positive HRFABMS using glycerol as a matrix was observed at m/z 1240.6456. From the results, the molecular formula of **1** was established to be $C_{60}H_{85}O_{14}N_{15}$

(Calcd for M+H:1240.6478, Δ-2.2 mmu). The spectral data(Table 1) of ^{1}H- and ^{13}C-NMR of **1** suggested the compound is a peptide. The detected amino acids by amino acid analysis of the hydrolysate(6N HCl, 110°C, 20 h) were valine(Val), proline(Pro), aspartic acid(Asp), glutamic acid(Glu), arginine(Arg), glycine(Gly), tyrosine(Tyr) and leucine(Leu). All amino acids were identified to be L-configuration by chiral GC analysis(Chirasil-L-Val capillary column, 0.25 mm I.D. × 25 m) of *N*-trifluoroacetyl isopropyl ester derivative of the hydrolysate[4]. The extensive NMR analyses of ^{1}H-^{1}H COSY and HMBC spectra revealed an abnormal amino acid residue which was (3a-*cis*)-1,2,3,3a,8,8a-hexahydro-3a-(3-methyl-2-butenyl)-pyrrolo[2,3-b]indol-2-carboxylic acid (oscillatoric acid, Osc)(Fig 1.). The ROESY spectra of **1** suggested that the configuration of H-8a and 3-methyl-2-butenyl moiety was *cis* in close analogy with physostigmine. This moiety may be derived from intramolecular cyclization of tryptophane and addition with isoprene unit. Studies on the absolute configuration of Osc are in progress. In order to confirm the position of free carboxyl group and amide in **1**, the peptide was reacted with diphenylphosphonic azide(DPPA)[5] and [bis(trifluoroacetoxy)-iodo]benzene[6], but the reactions did not proceed in the least. Therefore Asn and Glu were determined by the pH dependency of ^{1}H-NMR chemical shift of ω−1 position. The proton signal of Glu H-4 was shifted to 2.5 ppm at acidic condition(0.1M DCl) from 2.3ppm at neutral or basic condition. At the same condition, the proton signals of Asn H-3 were not shifted.

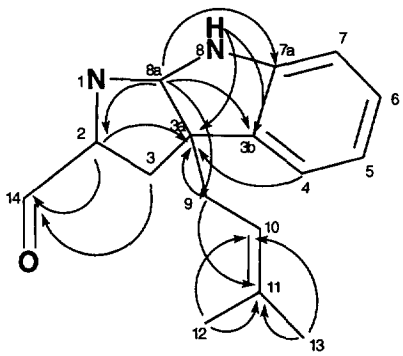


Fig. 1. HMBC correlations of oscillatoric acid residue in **1**

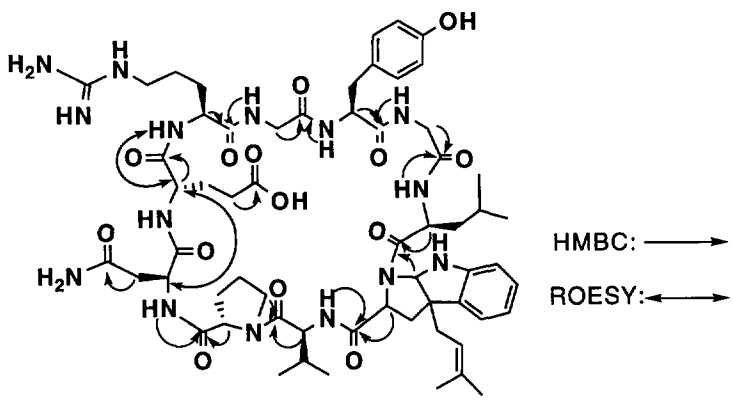
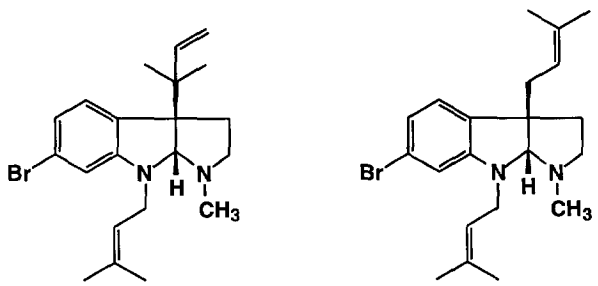


Fig. 2. HMBC and ROESY correlations of oscillatorin

The sequence of **1** was mostly deduced by HMBC correlations from N-H to C=O (Fig. 2.). The sequence of -Arg-Glu-Asn- in **1** was elucidated by ROESY correlations from α-H to N-H and α-H (Fig. 2.).

Flustramine A and B (hexahydro-3a-(3-methyl-2-butenyl)-pyrrolo[2,3-b]indole compounds) were isolated from marine bryozoa, *Flustra foliacea*[7]. Their fundamental skeleton resembled closely that of oscillatoric acid. These facts suggested that these alkaloids may be produced by the symbiotic cyanobacteria in marine bryozoa.



Flustramine A Flustramine B

Fig. 3. Structures of flustramine A and B

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