Kasumigamide, an Antialgal Peptide from the Cyanobacterium *Microcystis aeruginosa*

Keishi Ishida and Masahiro Murakami*

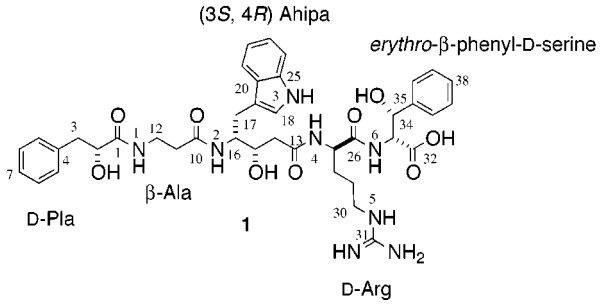
Laboratory of Marine Biochemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

amura@hongo.ecc.u-tokyo.ac.jp

Received December 15, 1999

Kasumigamide (**1**), a novel antialgal tetrapeptide containing an *N*-terminal α-hydroxy acid, was isolated from the freshwater cyanobacterium *Microcystis aeruginosa* (NIES-87). Its structure was elucidated by two-dimensional $^1H-^1H$ and $^1H-^{13}C$ NMR correlation experiments and confirmed by mass spectral and amino acid analyses. The absolute stereochemistry of **1** was determined by chemical studies. This peptide showed an antialgal activity against the green alga *Chlamydomonas neglecta* (NIES-439).

The freshwater cyanobacteria have been well-known to be a rich source of compounds having unique activities and structures. Among these compounds, cyanobacterin[1] and fischerellin A[2] have been reported to exhibit allelopathic activities against microalgae. Cyanobacterin has been isolated from the culture filtrate of *Scytonema hofmanni* (UTEX 1581) and inhibits the growth of various algae. Fischerellin A from *Fischerella muscicola* exhibits a MIC of 14 nM against the cyanobacterium *Synechcoccus* PCC6911. Cyanobacterin and fischerellin A might play a role in chemical defense. To discover novel allelochemical compounds, we carried out the anti-green algal screening of the freshwater cyanobacteria. We found that the freshwater cyanobacterium *Microcystis aeruginosa* (NIES-87) produced an antialgal compound against the green alga *Chlamydomonas neglecta* (NIES-439). This compound was named kasumigamide (**1**), a new linear tetrapeptide containing an *N*-terminal α-hydroxy acid. In this paper we report the isolation and structural elucidation of **1**.



Microcystis aeruginosa (NIES-87)[4] was obtained from the NIES collection and cultured in our laboratory to yield 18.2 g (dry weight) from 100 L of culture. The 80% methanol extract of freeze-dried alga was partitioned between water and diethyl ether. The aqueous layer was further extracted with *n*-butanol, and the *n*-butanol layer was fractionated by ODS flash column chromatography (20−100% MeOH elution) followed by reversed-phase HPLC, using 0.05% TFA in aqueous MeCN to yield kasumigamide (**1**, 10.0 mg).[5]

The molecular formula of **1** was established to be $C_{40}H_{50}N_8O_9$ by the HRFABMS and NMR spectral data (Table 1). Its peptidic nature was suggested by the ^1H and ^{13}C NMR spectra, and the amino acid analysis of the hydrolysate which gave *β*-Ala and Arg. The extensive NMR analyses including $^1H-^1H$ COSY, HMQC, and HMBC spectra (see Supporting Information) disclosed the presence of these two usual amino acids and other three units containing hydroxy groups (*δ* 5.04, 5.55, and 5.76). These three units were determined as follows. First, in the $^1H-^1H$ COSY spectrum, the H-2 oxy-methine proton (*δ* 4.01) was correlated to H-3 methylene protons (*δ* 2.65, 2.95) and hydroxyl proton (*δ* 5.55), and the correlations between H-2 and H-3/C-1 (*δ* 173.2), H-2 and H-3/C-4 (*δ* 138.6) and H-3/C-5, 9 (*δ* 129.4) in the HMBC spectrum were observed, indicating the presence of the phenyllactic acid (Pla) unit. Second, the connectivity from 6-NH (*δ* 7.99) to 34-OH (*δ* 5.76) was determined by the $^1H-^1H$ COSY, and the HMBC correlations (H-33 and H-34/C-32, and H-34/C-35, C-36 and C-40) allowed the presence of the *β*-phenylserine unit. Finally, eight ^{13}C signals at *δ* 136.1 (C-25), 127.6 (C-20), 122.9 (C-18), 120.7 (C-23), 118.3 (C-21), 118.0 (C-22), 111.4 (C-19), and 111.2 (C-24) and a singlet proton (*δ* 10.71, 3-NH) indicated the presence of an indolyl group. Interpretation of $^1H-^1H$ COSY and HMBC spectra supported the presence of the indolyl group. In the $^1H-^1H$ COSY spectrum, the connectivities from H-14 (*δ* 2.28, 2.35) to H-15 (*δ* 3.84) and from 2-NH (*δ* 7.75) to H-17 (*δ* 2.72, 3.06) were determined, and the HMBC correlation (H-17/C-15 (*δ* 70.1))

* Author to whom correspondence should be addressed. Phone: +81-3-5841-5298. Fax: +81-3-5841-8166.

(1) Mason, C. P.; Edwards, K. R.; Carlson, R. E.; Pignatello, J.; Gleason, F. K.; Wood, J. M. *Science* **1982**, *215*, 400−402.

(2) (a) Hagmann, L.; Jüttner, F. *Tetrahedron Lett.* **1996**, *37*, 6539−6542. (b) Strivastava, A.; Jüttner, F.; Strasser, R. J. *Biochim. Biophys. Acta* **1998**, *1364*, 326−336.

(3) Namikoshi, M.; Rinehart, K. L. *J. Ind. Microbiol.* **1996**, *17*, 373−384.

(4) Watanabe, M. M.; Hiroki, M. *NIES−Collection List of Strains*, 5th ed., *Microalgae and Protozoa Microbial Culture Collection*; National Institute of Environmental Studies: Tsukuba, Japan, 1997; p79−80.

(5) Kasumigamide: $[\alpha]^{22}_D$ −11.2° (*c* 0.5, MeOH); UV (MeOH) λ_{max} 282 (*ϵ* 3840), 291 (*ϵ* 3340) nm; HRFABMS (neg) *m/z* 785.3654 ($C_{40}H_{49}N_8O_9$, Δ +3.2 mmu). Kasumigamide (115.7 mg) was also obtained from freeze-dried alga (116 g) of *M. aeruginosa* TAC-91 in our laboratory.

10.1021/jo991918f CCC: $19.00 © 2000 American Chemical Society
Published on Web 08/31/2000

Table 1. ^{1}H and ^{13}C NMR Data for Kasumigamide in DMSO-d_6

	^{13}C	^{1}H (mult J (Hz))	HMBC (C no.)
		Phenyllactic Acid	
1	173.2		
2	72.1	4.01 (dd, 8.5, 3.9)	1, 3
3a	40.3	2.65 (dd, 13.8, 8.5)	1, 2, 4, 5, 9
3b		2.95 (dd, 13.8, 3.9)	1, 2, 4, 5, 9
4	138.6		
5,9	129.4	7.18 (d, 8.1, 2H)	3, 5, 7, 9
6,8	127.9	7.23 (dd, 8.1, 7.7, 2H)	4, 5, 6, 8, 9
7	125.9	7.17 (t, 7.7)	5, 9
2-OH		5.55 (br)	
		β-Ala	
10	170.3		
11a	35.3	2.10 (ddd, 14.5, 7.5, 7.1)	10, 12
11b		2.21 (ddd, 14.5, 7.5, 7.1)	10, 12
12	35.0	3.17 (m, 2H)	1, 10, 11
N-1		7.75 (t, 5.0)	1, 10, 12
		4-Amino-3-hydroxy-5-indolylpentanoic Acid	
13	171.2		
14a	40.0	2.28 (dd, 14.4, 3.7)	13, 15
14b		2.35 (dd, 14.4, 9.1)	
15	70.1	3.84 (ddd, 9.1, 6.4, 3.7)	
16	54.0	3.90 (dddd, 9.2, 9.0, 6.4, 3.5)	
17a	25.0	2.72 (dd, 15.0, 9.2)	15, 18, 19, 20
17b		3.06 (dd, 15.0, 3.5)	15, 18, 19, 20
18	122.9	7.06 (s)	17, 19, 20, 25
19	111.4		
20	127.6		
21	118.3	7.53 (d, 7.7)	19, 23, 25
22	118.0	6.95 (dd, 7.7, 7.3)	20, 24
23	120.7	7.03 (dd, 7.7, 7.3)	21, 25
24	111.2	7.31 (d, 7.7)	20, 22
25	136.1		
N-2		7.75 (d, 9.0)	10, 16
N-3		10.70 (s)	18, 19, 25
15-OH		5.04 (br)	
		Arginine	
26	171.4		
27	51.8	4.31 (ddd, 8.5, 7.8, 5.6)	26, 28, 29
28a	29.2	1.40 (m)	29
28b		1.56 (m)	29
29	24.8	1.36 (m, 2H)	
30	40.3	3.01 (m, 2H)	28, 29, 31
31	156.6		
N-4		7.94 (d, 8.5)	13, 26, 27
N-5		7.45 (t, 5.4)	
		β-Phenylserine	
32	171.4		
33	58.4	4.47 (dd, 8.8, 7.3)	26, 32, 34, 35
34	72.7	4.86 (d, 7.3)	32, 33, 35, 36, 40
35	141.8		
36,40	126.6	7.37 (d, 7.7, 2H)	34, 36, 38, 40
37,39	127.7	7.29 (t, 7.7, 2H)	35, 37, 39
38	127.2	7.22 (t, 7.7)	36, 40
N-6		7.99 (d, 8.8)	26
34-OH		5.76 (br)	

and the ROESY correlation (H-15/H-17) connected C-15 with C-16. These results and the HMBC correlations between H-14/C-13 (δ 171.2) and H-17/C-18 and C-20 revealed the structure of the 4-amino-3-hydroxy-5-indolylpentanoic acid (Ahipa) unit.

The sequence of these five units was determined by the HMBC correlations between β-Ala H-12 and 1-NH/Pla C-1, Ahipa H-16 and 2-NH/β-Ala C-10, Arg 4-NH/Ahipa C-13, and β-phenylserine H-33 and 6-NH/Arg C-26).

The stereochemistry of Arg was determined as D by the HPLC analysis of the derivatives of the acid hydrolysate of **1** with L-Marfey's reagent.[6] The stereochemistry of Pla was determined to be D by the HPLC analysis of menthyl ester derivative of the acid hydrolysate of **1**.[7]

To determine the stereochemistry of β-phenylserine, **1** was hydrolyzed with 6 N HCl, and β-phenylserine was purified by reversed-phase HPLC. The HPLC analysis indicated that β-phenylserine in **1** was either *erythro*-β-phenyl-D- or -L-serine.[8] The negative optical rotation value[8] of β-phenylserine in **1** revealed that this hydroxy amino acid is *erythro*-β-phenyl-D-serine. Furthermore, the HPLC analysis of Marfey derivatives of four stereoisomers also supported that β-phenylserine in **1** is *erythro*-β-phenyl-D-serine.[9] To determine the stereochemistry of Ahipa, four stereoisomers were synthesized from Boc Trp (For) as previously described by Maibaum et al.[10] Boc Trp (For) was activated by *N*,*N*′-carbonyldiimidazole followed by the treatment with magnesium enolate of hydrogen

(6) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591−596.

(7) Shin, H. J.; Matsuda, H.; Murakami, M.; Yamaguchi, K. *J. Org. Chem.* **1997**, *62*, 1810−1813.

(8) *erythro*-β-Phenyl-D,L-serine was prepared from *threo*-β-phenyl-D,L-serine. (a) Bolhofer, W. A. *J. Am. Chem. Soc.* **1952**, *74*, 5459−5461. (b) Fones, W. S. *J. Biol. Chem.* **1953**, *204*, 323−328. For experimental details of the synthetic procedures, see Supporting Information. β-Phenylserine was analyzed by reversed-phase HPLC (Cosmosil 5C18-MS, 4.6 × 250 mm; 2% MeCN containing 0.05% TFA; UV detection 210 nm; flow rate 1.0 mL/min). Retention times (min) in standards: *erythro*-β-phenyl-D,L-serine (8.0) and *threo*-β-phenyl-D,L-serine (9.8). Retention time (min) in β-phenylserine of **1**: (8.0). Natural β-phenylserine: $[\alpha]^{25}_D$ − 79.7° (*c* 0.12, 6 N HCl) [lit.[8b] $[\alpha]^{25}_D$ (2% solution in 6 N HCl) *threo*-β-phenyl-L-serine, − 48.6°; *threo*-β-phenyl-D-serine, + 48.0°; *erythro*-β-phenyl-L-serine, + 81.3°; *erythro*-β-phenyl-D-serine, − 82.0°].

(9) For experimental details of the synthetic procedures, see Supporting Information. β-Phenylserine was derivatized with L-Marfey's reagent and analyzed by reversed-phase HPLC (Cosmosil 5C18-MS, 4.6 × 250 mm; 35% MeCN containing 0.1% TFA; UV detection 340 nm; flow rate 1.0 mL/min). Retention times in the derivatives of the standards (min): *erythro*-β-phenyl-L-serine (14.8), *threo*-β-phenyl-L-serine (15.6), *erythro*-β-phenyl-D-serine (19.2), *threo*-β-phenyl-D-serine (24.8). Retention time in the derivatives of β-phenylserine of **1** (min): (19.2).

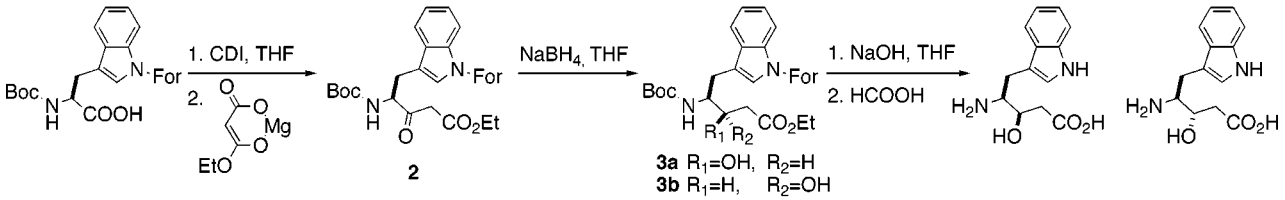
(10) Maibaum, J.; Rich, D. H. *J. Org. Chem.* **1988**, *53*, 869−873. For experimental details of the synthetic procedures, see Supporting Information.

(11) Otani, I.; Kusumi, T.; Kashman, Y.; Kakisawa, H. *J. Am. Chem. Soc.* **1991**, *113*, 4092−4096.

(12) Ahipa from **1** (100 μg) was derivatized with L-FDAA[6] and analyzed by reversed-phase HPLC (Cosmosil 5C18-MS, 4.6 × 250 mm, 20−60% (1%/min) MeCN containing 0.05% TFA, UV detection 340 nm, flow rate 1.0 mL/min). Retention times (min) of standards: (3*R*,4*S*)-Ahipa (28.8), (3*S*,4*S*)-Ahipa (29.4), (3*S*,4*R*)-Ahipa (30.6), (3*R*,4*R*)-Ahipa (32.0), derivative from **1** (30.6).

(13) (3*R*,4*S*)-4-Amino-3-hydroxy-5-indolylpentanoic acid: $[\alpha]^{23}_D$ −26.7° (*c* 0.2, 50% MeOH); HRFABMS *m*/*z* 249.1238 $[M + H]^+$ ($C_{13}H_{17}N_2O_3$, Δ − 0.1 mmu); ^{1}H NMR (DMSO-d_6, 600 MHz) δ 2.37 (dd, 15.4, 6.0, 1H, $COCH_2$), 2.41 (dd, 15.4, 5.1, 1H, $COCH_2$), 2.74 (dd, 14.5, 8.1, 1H, CH_2Ar), 3.02 (dd, 14.5, 5.1, 1H, CH_2Ar), 3.15 (br, 1H, CH), 3.72 (m, 1H, C*H*OH), 6.98 (t, 8.1, 1H, ind.), 7.06 (t, 8.1, 1H, ind.), 7.20 (s, 1H, ind.), 7.32 (d, 8.1, 1H, ind.), 7.54 (d, 8.1, 1H, ind.), 10.88 (s, 1H, NH). (3*S*,4*S*)-4-Amino-3-hydroxy-5-indolylpentanoic acid: $[\alpha]^{23}_D$ −14.4° (*c* 0.1, 50% MeOH); HRFABMS *m*/*z* 249.1249 $[M + H]^+$ ($C_{13}H_{17}N_2O_3$, Δ + 1.0 mmu); ^{1}H NMR (DMSO-d_6, 600 MHz) δ 2.26 (dd, 15.8, 4.7, 1H, $COCH_2$), 2.39 (dd, 15.8, 5.6, 1H, $COCH_2$), 2.77 (dd, 14.5, 6.8, 1H, CH_2-Ar), 2.96 (dd, 14.5, 7.3, 1H, CH_2Ar), 3.09 (m, 1H, CH), 3.73 (br, 1H, C*H*OH), 6.98 (t, 8.1, 1H, ind.), 7.06 (t, 8.1, 1H, ind.), 7.18 (s, 1H, ind.), 7.32 (d, 8.1, 1H, ind.), 7.54 (d, 8.1, 1H, ind.), 10.87 (s, 1H, NH). (3*S*,4*R*)-4-Amino-3-hydroxy-5-indolylpentanoic acid: $[\alpha]^{23}_D$ + 28.4° (*c* 0.1, 50% MeOH); HRFABMS *m*/*z* 249.1232 $[M + H]^+$ ($C_{13}H_{17}N_2O_3$, Δ − 0.7 mmu); ^{1}H NMR (DMSO-d_6, 600 MHz) δ 2.37 (dd, 15.4, 6.0, 1H, $COCH_2$), 2.40 (dd, 15.4, 5.0, 1H, $COCH_2$), 2.73 (dd, 14.5, 8.6, 1H, CH_2Ar), 3.02 (dd, 14.5, 5.1, 1H, CH_2Ar), 3.14 (m, 1H, CH), 3.70 (m, 1H, C*H*OH), 6.98 (t, 8.1, 1H, ind.), 7.06 (t, 8.1, 1H, ind.), 7.19 (s, 1H, ind.), 7.32 (d, 8.1, 1H, ind.), 7.54 (d, 8.1, 1H, ind.), 10.88 (s, 1H, NH). (3*R*,4*R*)-4-Amino-3-hydroxy-5-indolylpentanoic acid: $[\alpha]^{23}_D$ + 18.3° (*c* 0.1, 50% MeOH); HRFABMS *m*/*z* 249.1245 $[M + H]^+$ ($C_{13}H_{17}N_2O_3$, Δ + 0.6 mmu), ^{1}H NMR (DMSO-d_6, 600 MHz) δ 2.25 (dd, 15.8, 4.7, 1H, $COCH_2$), 2.38 (dd, 15.8, 5.6, 1H, $COCH_2$), 2.77 (dd, 14.1, 6.8, 1H, CH_2Ar), 2.95 (dd, 14.1, 7.3, 1H, CH_2Ar), 3.08 (m, 1H, CH), 3.73 (br, 1H, C*H*OH), 6.98 (t, 8.1, 1H, ind.), 7.06 (t, 8.1, 1H, ind.), 7.18 (s, 1H, ind.), 7.34 (d, 8.1, 1H, ind.), 7.54 (d, 8.1, 1H, ind.), 10.88 (s, 1H, NH). Ahipa from **1**: $[\alpha]^{23}_D$ + 26.3° (*c* 0.1, 50% MeOH); HRFABMS *m*/*z* 249.1249 $[M + H]^+$ (Δ + 1.0 mmu); ^{1}H NMR (DMSO-d_6, 600 MHz) δ 2.37 (dd, 15.4, 6.0, 1H, $COCH_2$), 2.42 (dd, 15.4, 5.1, 1H, $COCH_2$), 2.74 (dd, 14.5, 8.1, 1H, CH_2Ar), 3.02 (dd, 14.5, 5.1, 1H, CH_2Ar), 3.15 (br, 1H, CH), 3.71 (m, 1H, C*H*OH), 6.97 (t, 8.1, 1H, ind.), 7.06 (t, 8.1, 1H, ind.), 7.20 (s, 1H, ind.), 7.34 (d, 8.1, 1H, ind.), 7.54 (d, 8.1, 1H, ind.), 10.88 (s, 1H, NH).

Scheme 1



ethyl malonate to yield β-keto ester **2**, which was purified by column chromatography on silica gel (Scheme 1). Boc-(3*R*,4*S*)- and -(3*S*,4*S*)-Ahipa (For) ethyl esters (**3a** and **3b**) were obtained after $NaBH_4$ reduction of β-keto ester, followed by chromatography (Scheme 1), and the stereochemistry on C-3 was determined by the NMR analysis of MTPA ester derivatives.[11] Two additional diastereomers Boc-(3*S*,4*R*)- and -(3*R*,4*R*)-Ahipa (For) ethyl ester were also obtained from Boc D-Trp (For). The absolute stereochemistry of Ahipa was determined to be 3*S*,4*R* by the HPLC analysis of Marfey derivatives[12] and chemical properties[13] of deprotected stereoisomers (Scheme 1).

Kasumigamide showed antialgal activity against the green alga *C. neglecta* (NIES-439) at a concentration of 2 μg/mL (minimum effective dose). From the observation under a light microscope, *C. neglecta* precipitated and the movement of flagella stopped by the treatment of kasumigamide. Regarding cyanobacterin,[1] it has been shown by electron microscopy that the cell membrane and thylakoids of microalgae are the major targets for algicidal activity. Fischerellin A[2] has been reported to inhibit photosystem II of plants including microalgae. As the structure of kasumigamide is not related with these compounds, the elucidation of the mechanism of its antialgal activity is intriguing.

Acknowledgment. We thank Prof. K. Yamaguchi, Tokyo University of Agriculture and Technology, for reading this manuscript and valuable discussion. This work was partly supported by a Grant-in-Aids for "Research for the Future" Program from the Japan Society for the Promotion of Science. K. Ishida is financially supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

Supporting Information Available: All experimental procedures, ^1H, ^{13}C, and 2D NMR data. This material is available free of charge via the Internet at http://pubs.acs.org.

JO991918F