A Bioactive Modified Peptide, Aeruginosamide, Isolated from the Cyanobacterium *Microcystis aeruginosa*

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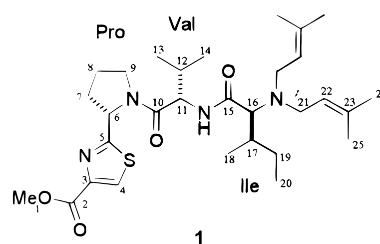
Introduction

In recent years, there has been increasing interest in isolating compounds with biological activity from cyanobacteria.[3] The cyanobacterium *Microcystis aeruginosa* is well-known for the production of the toxic cyclic peptide microcystins,[4] but it also produces several linear peptides that are potent protease inhibitors. These linear peptides, the aeruginosins[5] and microginins,[6] contain unusual amino acid residues unique to cyanobacteria. Recently, the X-ray crystal structures of the trypsin-aeruginosin 98B[7] and thrombin-aeruginosin 298A[8] complexes have been reported. In this paper, we report on the structure and biological activity of a novel peptide, aeruginosamide, isolated from a bloom of the cyanobacterium *M. aeruginosa*.

Results and Discussion

The sample of *M. aeruginosa* was collected from a bloom that occurred on Rutland Water, U.K., in September 1989. The sample was frozen, and the crude methanolic extract was purified using C18 reversed-phase chromatography. Analysis of this sample indicated that it contained microcystin-RR, -LR, -LY, -LW, and -LF along with a significant number of other less abundant microcystins.[9] During a routine isolation of the cyclic peptide microcystins, an additional compound was isolated. Initial evidence indicated that the compound was much less hydrophilic than the microcystins, as it showed a long retention time on reversed-phase silica. The compound was found to be soluble in $CDCl_3$, and the initial ^1H NMR spectrum indicated that it was not a



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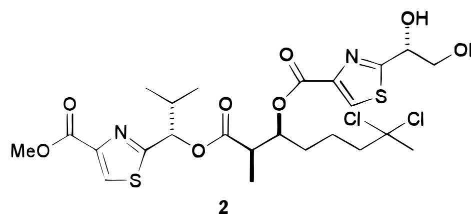

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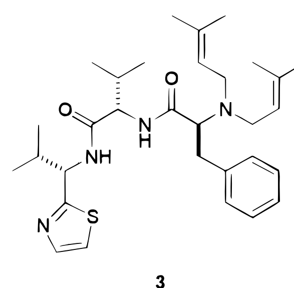

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microcystin. The ^1H NMR spectrum showed resonances that were consistent with a disubstituted thiazole ring (δ 8.03 s) and several amino acids, indicating the compound was a modified peptide. A combination of ^{13}C and DEPT-135 NMR showed that the compound contained seven quaternary carbons, eight methines, six methylenes, and nine methyl groups, giving an attached proton formula of $C_{30}H_{47}$. Low-resolution electrospray mass spectrometry gave an *m*/*z* 561, which taken with the APT-MF suggested a molecular formula of $C_{30}H_{48}N_4O_4S$, which was confirmed by high-resolution electrospray mass spectrometry (561.3516 Δ 4.1 mmu of calcd for $C_{30}H_{49}N_4O_4S$). Having established the peptidic nature of the compound, coupled with the fact that the ^1H NMR spectrum was well resolved (Figure 1), we decided to apply the selective one-dimensional TOCSY technique to rapidly elucidate the structure of the metabolite.[1,2] In the case of aeruginosamide, the first selective 1D TOCSY was performed on the NH at 6.8 ppm. As can be seen in Figure 1, this resulted in a spectrum that showed a doubled doublet at 4.64 ppm, a multiplet at 2.06 ppm, and two methyl doublets at 1.00 and 0.92 ppm. This immediately allowed us to assign this amino acid residue as valine, for which the reference chemical shifts are 4.18, 2.13, 0.97, and 0.94 ppm.[10] Similarly, the proline residue was assigned by selectively exciting the doubled doublet at 5.4 ppm. A selective 1D TOCSY experiment on the doublet at 2.9 ppm indicated the essential features of an isoleucine residue with the α proton heavily shielded compared to the expected chemical shift of 4.23 ppm. An additional non amino acid derived subunit was also found

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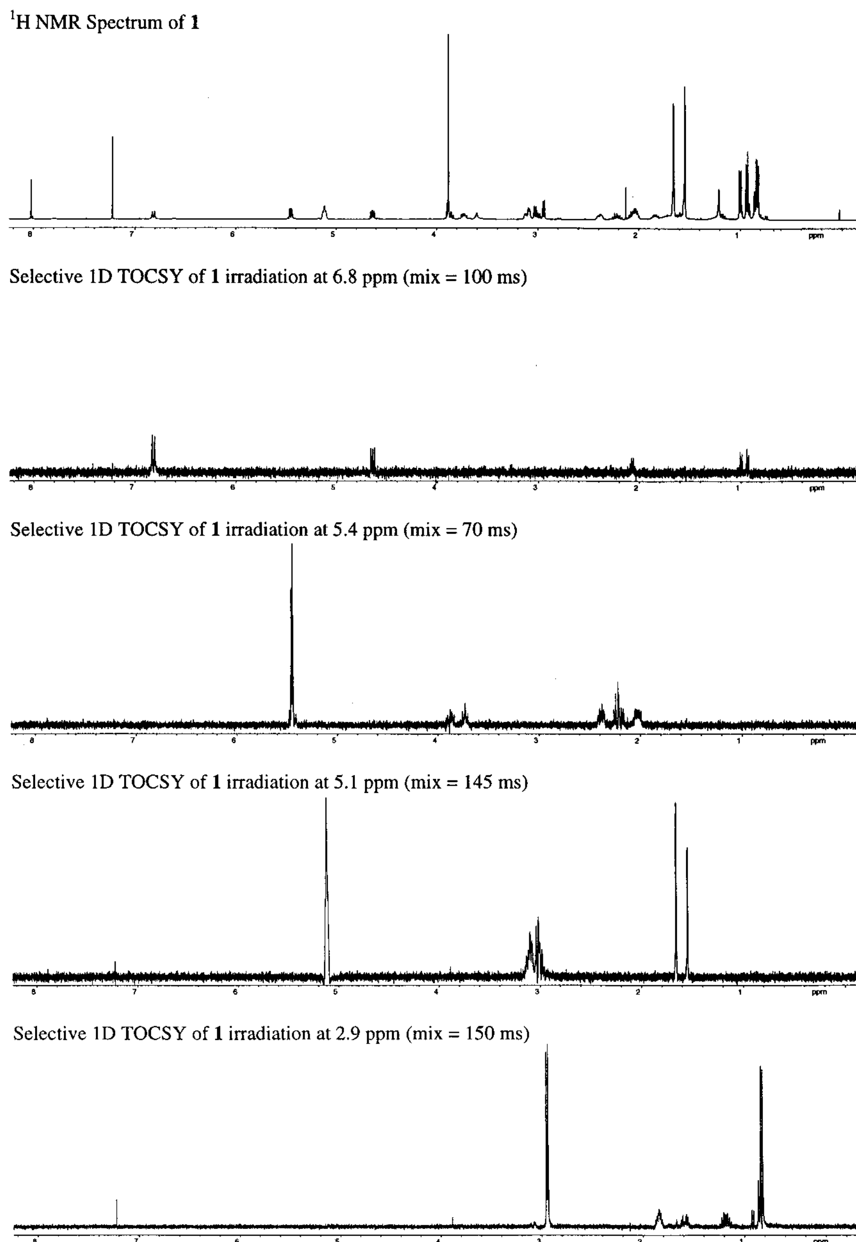

Figure 1. Selective 1D TOCSY spectra of **1**.

using this methodology by selectively exciting the triplet at 5.1 ppm, resulting in the appearance of an ABX system at 3.00 ppm and two methyl singlets at 1.66 and 1.55 ppm. The triplet at 5.1 ppm and the deshielded methyl singlets suggested a trisubstituted double bond. The CH_2 at 3.00 ppm was indicative of it being bonded to a nitrogen, which together with the integration led us to propose the diisoprenylamine in **1**. This was rapidly confirmed by matching chemical shifts for this unusual subunit to those for the same subunit found in virenamide A (**3**).[11] At this stage, all the ^{1}H resonances had been assigned except the methoxy singlet at 3.89 ppm. The next step of the procedure was to determine the sequence of the amino acids in aeruginosamide. This process was started by assigning all the protonated carbons using the gradient-filtered HSQC spectrum (Table 1).[12,13] Seven quaternary carbons remained to be assigned, and this was done using a gradient-filtered HMBC spectrum.[14] An HMBC correlation from δ_C 172.7 (C-15) to the α proton at δ_H 2.94 (H-16) completed the isoleucine residue. In addition, a correlation was found between C-15 and the NH, which in turn was correlated to the α proton of the valine residue at δ_H 4.64 (H-11), thus extending the chain. The valine carbonyl was found to be at δ_C 171.9 (C-10) due to its correlation to H-11. Correlations from C-16 to the isoprenyl CH_2 at δ_H 3.02, 3.10 confirmed that the diisoprenylamine unit was attached to C-16. Unfortunately we could find no HMBC correlations from the proline residue into the main chain of aeruginosamide, but the connection between the two

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Table 1. ^{1}H and ^{13}C Assignments for Aeruginosamide (1) in $CDCl_3$ at 400 MHz

atom no.	$\delta^{13}C$ (mult)	$\delta^{1}H$ (mult, J Hz)	$^{1}H-^{1}H$ COSY correlations	HMBC $^{13}C \rightarrow {}^{1}H$ correlations
1	52.5 q	3.89 s		
2	162.0 s			1, 4
3	146.8 s			4
4	127.6 d	8.03 s		
5	173.6 s			4, 6, 7
6	59.1 d	5.45 dd, 2.9, 8.3	7	7, 9
7	31.9 t	2.37 m	6, 8	6, 9
		2.23 dq, 12.4, 8.0		
8	24.7 t	2.04 m	7, 9	6, 7, 9
9	47.8 t	3.87 m	8	6, 8
		3.73 ddd, 4.9, 6.7, 9.9		
10	171.9 s			11
11	55.4 d	4.64 dd, 6.1, 8.8	NH, 12	NH, 13, 14
12	31.1 d	2.06 m	11, 13, 14	11, 13, 14
13	20.1 q	1.00 d, 6.7	12	11, 12, 14
14	18.2 q	0.92 d, 6.7	12	11, 13
15	172.7 s			NH, 16
16	68.7 d	2.94 d, 7.3	17	18, 21
17	33.6 d	1.84 m	16, 18	16, 18, 19, 20
18	16.0 q	0.83 d, 6.7	17, 19	16
19	27.0 t	1.59 m	18, 20	16, 18, 20
		1.18 m		
20	11.6 q	0.85 t, 7.3	19	19
21	48.0 t	3.10 dd, 5.6, 14.6	24, 25	16
		3.02 dd, 7.6, 14.6		
22	122.5 d	5.12 t, 6.1	24, 25	21, 24, 25
23	134.6 s			21, 24, 25
24	26.1 q	1.66 bs	21, 22	22, 25
25	18.2 q	1.55 bs	21, 22	21, 24
NH		6.80 d, 9.0	11	

was made by the observation of a T-ROESY[15,16] correlation between the proline δ protons at δ_H 3.73/3.87 (H-9) and the valine α proton at δ_H 4.64 (H-11). At this stage, the structure was complete from C-6 to C-25, and the δ_H 8.03 s suggested a disubstituted thiazole moiety, with the methoxy singlet at δ_H 3.89 indicating perhaps a methoxy ester functionality. Using the HMBC correlations from C-2 to H-1, H-4; C-3 to H-4; and C-5 to H-4, H-6, and H-7, a thiazole with a methoxy ester group at C-3 and the proline residue at C-5 was proposed, and this was confirmed by chemical shift comparison to a similar unit found in dolabellin (**2**).[17] The sequence of subunits was confirmed by the electrospray cone voltage-induced dissociation mass spectrum. The loss of one and two isoprenyl units is observed at *m/z* 493 and 425, and the $(isoprene)_2N$ fragment is observed at *m/z* 154. A fragmentation between the NH and C-15 is deduced from fragments at *m/z* 312 and 252. A final fragmentation was witnessed between C-10 and the proline N at *m/z* 212 and 349. The unique proline-thiazole-methoxy ester connectivity has, to our knowledge, not been reported before, making aeruginosamide a novel structural type. It can be seen that the above methodology represents an advance on previous methods that are used to elucidate the structures of modified peptides. The absolute stereochemistry of the amino acid residues was determined by an acid digestion of aeruginosamide followed by chiral TLC.[18] Using this method, the stereochemistry was determined to be that for the naturally occurring amino acids L-Pro, L-Ile, and L-Val.

The two unusual features of aeruginosamide, the diisoprenylamine and the carboxylated thiazole moiety, have not been reported in cyanobacterial natural products before but have been reported in species where cyanobacterial symbiosis is suspected. Dolabellin was isolated from the sea hare *Dolabella auricularia*, which has long been suspected to harbor cyanobacterial symbionts, and recently, analogues of *Dolabella* metabolites, the dolastatins, have been isolated from cyanobacterial cultures.[19,20] The virenamides were isolated from *Diplosoma virens*, a colonial ascidian that contains symbiotic prokaryotic algae in its cloacal cavity. It is therefore possible that the virenamides and dolabellin are in fact produced by symbiotic cyanobacteria and not by the host organism itself. Aeruginosamide showed mild cytotoxicity to A2780 human ovarian tumor cells and K562 human leukaemia cells with ID_{50}'s of 2.9 and 5.2 μM, respectively.

Experimental Section

General Methods. Liquid chromatography was performed using cartridge flash and high-pressure liquid chromatography. Solvents were HPLC grade and used as supplied. Mass spectra were acquired in the electrospray ionization mode. Selective 1D TOCSYs were acquired by creating a shaped pulse at the desired resonance frequency and incorporating this into the 1D TOCSY pulse sequence. An array of spectra were acquired at different mixing times (10−180 ms in 10 ms increments), using four transients per increment. The optimum mixing time for each substructure is given in Figure 1.

Isolation. The sample of *M. aeruginosa* was collected from a bloom that occurred on Rutland Water, Leicestershire, U.K., in September 1989. The material was identified as *M. aeruginosa* after microscopic examination by Linda Lawton, and concentrated cells were stored at −20 °C until required. Cells were thawed (4 L of concentrated scum, approximately 128 g) and then extracted with MeOH for 1 h prior to centrifugation at 1500*g* for 30 min. The pellet was reextracted a further two times, and then the pooled extracts were diluted with water to 20% MeOH. The diluted extract was mixed, allowed to stand for 30 min prior to filtration (GF/C), and then passed through a flash chromatography column (C18 cartridge, 40 mm × 15 cm). The column was eluted in a stepwise fashion from 0 to 100% MeOH in 10% increments, each of 1 L. All fractions were analyzed by HPLC[9] with a high-resolution diode array detector with resolution set at 1.2 nm and spectra observed between 200 and 300 nm. Samples were separated on a C18 column (25 × 0.46 cm) using water and acetonitrile, both containing 0.05% (v/v) trifluoroacetic acid (TFA), as eluents. Separation was achieved using a linear gradient starting at 30% (v/v) acetonitrile increasing to 35% over 10 min followed by an increase to 70% over the next 30 min. Analysis revealed a significant nonmicrocystin peak in the 90% MeOH fraction; however, its retention time of 21.7 min suggested that the compound was more hydrophilic under acidic conditions. The 90% MeOH flash eluate was dark green in appearance, suggesting the copurification of pigment. To facilitate the removal of much of this pigment, 5 L of acidified water (0.05% TFA) was added to the extract, which was reapplied to the flash chromatography cartridge and eluted as before. Acidification caused the aeruginosamide to be eluted in an earlier fraction (70% MeOH), thus removing most of the pigment from the sample and yielding 60 mg of **1** as a pale green oil.

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Aeruginosamide (1): pale green oil; $[\alpha]_D$ −71.4° (*c* 0.01, $CHCl_3$); IR ν (cm^{-1}) 2938, 2897, 1740, 1640, 1525, 1461, 1240; HRESIMS 561.3516 Δ 4.1 mmu of calcd for $C_{30}H_{49}N_4O_4S$; LRESIMS cone voltage induced dissociation *m*/*z* 561 (90, $[M + H]^+$), 493 (50), 425 (5), 349 (15), 312 (27), 281 (90, $[M + 2H]^{2+}$), 253 (30), 222 (32), 212 (100), 185 (17), 154 (38). For NMR data, see Table 1.

Determination of Stereochemistry. Aeruginosamide was subjected to acid hydrolysis in 6 N HCl at 110 °C for 72 h. The acid digest was subjected to chiral TLC using chiral plates (ODS impregnated with a proline derivative and Cu^{2+})[18] and visualized using ninhydrin spray reagent. Two different solvent systems were utilized. For proline, the solvent system used was MeOH/H_2O/MeCN 1:1:4. The R_f's for standard D-Pro and L-Pro were 0.50 and 0.63, respectively. The acid digest of aeruginosamide showed a TLC spot at R_f 0.64, indicating it to contain L-Pro. For isoleucine and valine, the solvent used system was MeOH/H_2O/MeCN 5:5:3. Standard R_f's were D-Ile (0.60), L-Ile (0.81), D-Val (0.58), L-Val (0.69). The aeruginosamide digest in the same solvent system showed spots at R_f's of 0.80 and 0.68, indicating it to contain L-Ile and L-Val.

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Additions and Corrections

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Thierry Billard, Nicolas Roques, and Bernard Langlois*. Synthetic Uses of Thio- and Selenoesters of Trifluoromethylated Acids. 1. Preparation of Trifluoromethyl Sulfides and Selenides.

Page 3813. The following Supporting Information paragraph should be added.

Supporting Information Available: NMR spectra for compounds **1a**−**j**, **5a**,**b**,**d**−**i**, and **10**. This material is available free of charge via the Internet at http://pubs.acs.org.

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