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Comparative Study of Toxic and Non-toxic Cyanobacterial Products: Novel Peptides from Toxic *Nodularia spumigena* AV1

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Abstract: Two types of novel peptides, cyclic peptides, nodulapeptins A (**1**) and B (**2**) and linear peptides, spumigins A~C (**3~6**), were isolated together with nodularin from toxic *Nodularia spumigena* AV1. Their structures were determined by 2D-NMR techniques, the advanced Marfey's method and MS/MS experiments.

It is well known that cyanobacteria produce several toxic compounds[1]. Microcystins and nodularin are known as lethal hepatotoxic peptides and the neurotoxins, anatoxin-a, anatoxin-a(s)[2] and saxitoxins, are also produced by some species of cyanobacteria[1,3]. We have found that peptides other than hepatotoxic peptides are produced together with microcystins by toxic cyanobacteria[3-7]. Aeruginopeptins were isolated not only from the cultured cells of toxic *Microcystis aeruginosa*[4] but also from bloom cells[5]. Anabaenopeptins[6] were isolated from *Anabaena flos-aquae* NRC 525-17 that simultaneously produces anatoxin-a(s) and microcystins. Anabaenopeptilides[7], whose structures are similar to those of aeruginopeptins, were also isolated together with anabaenopeptins[7] from toxic *Anabaena* species that co-produces microcystin. These results suggest that the production of these peptides is closely related to that of the toxic compounds. For the elucidation of the biosynthetic relationship between such peptides and hepatotoxic peptides, we compared the products from the toxic and non-toxic *Nodularia spumigena*, one of which is known to produce nodularin[8]. While we isolated two types of novel peptides, linear peptides and cyclic peptides such as anabaenopeptins, together with nodularin from toxic *N. spumigena*, two glycosidic compounds were isolated from non-toxic *N. spumigena* instead of these peptides. In this communication, we report the isolation and the structural determination of novel cyclic peptides, nodulapeptins, and linear peptides, spumigins, from toxic *N. spumigena* AV1[9]. The products from the non-toxic *N. spumigena* will be discussed in a subsequent paper.

Nodulapeptins A (**1**) and B (**2**) and spumigins A~C (**3~6**) were isolated together with nodularin from the 5% AcOH aq. extract of the cultured cells[9] and were purified by repeated silica gel and TOYOPEARL HW-40F chromatographies[10].

Nodulapeptins A (**1**) and B (**2**) were amorphous compounds: **1**; $[\alpha]_D^{26}$ -43.5° (*c* 0.030, MeOH), **2** ; $[\alpha]_D^{26}$ -44.7° (*c* 0.030, MeOH). The molecular formulae of **1** and **2** were established to be $C_{44}H_{63}N_7O_{14}S$ (*m/z* 930.4297 $[M+H]^+$ Δ +1.5 mmu) and $C_{44}H_{63}N_7O_{13}S$ (*m/z* 914.4438 $[M+H]^+$ Δ +0.5 mmu) based on the HRFABMS and NMR spectral data, indicating that the difference between **1** and **2** was that of an oxygen atom. Amino acid analysis of the 6 M HCl hydrolysates of **1** and **2** using the advanced Marfey's method[11] revealed the presence of L-methionine sulfone (Met(O_2)) and L-Met, respectively, in addition to the common constituent amino acids, L-Ser, *N*-methyl-L-homotyrosine (MeHty), L-homophenylalanine (Hph), D-Lys and L-Ile. Furthermore, the 2D-NMR analyses of **1** and **2** confirmed the presence of these amino acids. Ser detected from the hydrolysates of both **1** and **2** was derived from *O*-acetylserine (Ser(Ac)). The sequence of the constituent amino acids of **1** and **2** has been established with the help of the HMBC and NOESY spectra as shown in Fig. 1. Moreover, on the basis of their molecular formulae and FAB MS/MS experiments[12], Met detected from the hydrolysate of **2** was derived from methionine sulfoxide (Met(O))[13,14], because the difference of 16 mass units was observed in the resulting product ions containing the Met(O) or Met(O_2) moiety from $[M+H]^+$ of **1** and **2**. Therefore, nodulapeptins A (**1**) and B (**2**) have the cyclic pentapeptide moiety linked with Ile *via* the ureido bond and **2** has Met(O) in the place of Met(O_2) in **1** (Fig. 1).

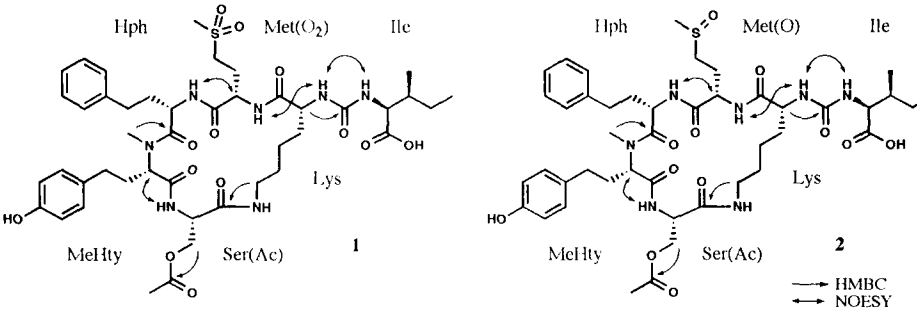

Fig. 1 Structures of nodulapeptins A (**1**) and B (**2**).

The physicochemical properties of spumigins A (**3**), B1 (**4**), B2 (**5**) and C (**6**) and their constituent amino acids determined by the advanced Marfey's method[11] are shown in Table 1. The sequence of the constituent segments of **4** was established with the help of the HMBC and NOESY spectra as shown in Fig. 2. However, it was difficult to determine the structures of other spumigins using the NMR technique alone due to limited amounts. In addition, their ^1H and ^{13}C-NMR signals appeared as a doublet, suggesting that this phenomenon is attributable to restricted rotation of the amide C-N bond between Pro and its adjacent amino acid, and **3** and **6** gave the more complicated ^1H-NMR spectra. Therefore, the sequence of the segments of **3**, **5** and **6** were carried out based on the analysis results of charge-remote fragmentation in product ion spectrum of **4** using FAB MS/MS[12] (Fig. 3, Table 2). The experiments and the results of the amino acid analyses indicated that spumigins B1 (**4**) and B2 (**5**) are the same linear peptides except for the absolute configuration of Arg. Spumigin A (**3**) has argininol (Argol) in the place of Arg and spumigin C (**6**) has Pro in the place of MePro in spumigin B. Additionally, spumigin C (**6**) is suggested to be an epimeric mixture due to the absolute configuration of Arg as shown in Fig. 2.

Table 1. Physicochemical properties and constituent segments of spumigins A (**3**), B1 (**4**), B2 (**5**) and C (**6**).

Peptides	$[\alpha]_D^{26}$ (MeOH)	Molecular formula (HRFAB MS data for $[M+H]^+$)	Segments
A (**3**)	−5.4° (*c* 0.025)	$C_{31}H_{44}N_6O_7$ (*m/z* 613.3342 Δ -0.7 mmu)	D-Hpla, D-Hty, L-MePro*, Argol**
B1 (**4**)	−9.9° (*c* 0.045)	$C_{31}H_{42}N_6O_8$ (*m/z* 627.3178 Δ +3.6 mmu)	D-Hpla, D-Hty, L-MePro*, L-Arg
B2 (**5**)	+2.8° (*c* 0.045)	$C_{31}H_{42}N_6O_8$ (*m/z* 627.3135 Δ -0.8 mmu)	D-Hpla, D-Hty, L-MePro*, D-Arg
C (**6**)	−2.5° (*c* 0.050)	$C_{30}H_{41}N_6O_8$ (*m/z* 613.3027 Δ +4.1 mmu)	D-Hpla, D-Hty, L-Pro, D,L-Arg

* This amino acid was identified as *cis* -4-methyl-L-proline by comparison with an authentic sample from the hydrolysate of leucinostatins (Ref. 19).

Argol (argininol) was not detected from the hydrolysate of **3 by the advanced Marfey's method.

Hpla: 4-hydroxyphenyllactic acid, Hty: homotyrosine, MePro: 4-methylproline

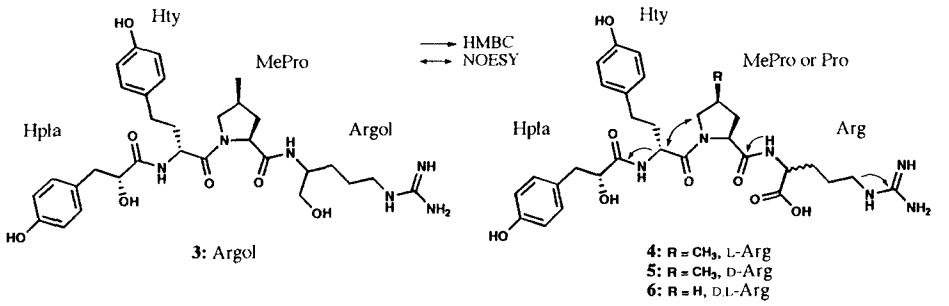

Fig. 2 Structures of spumigins A (**3**), B1 (**4**), B2 (**5**) and C (**6**).

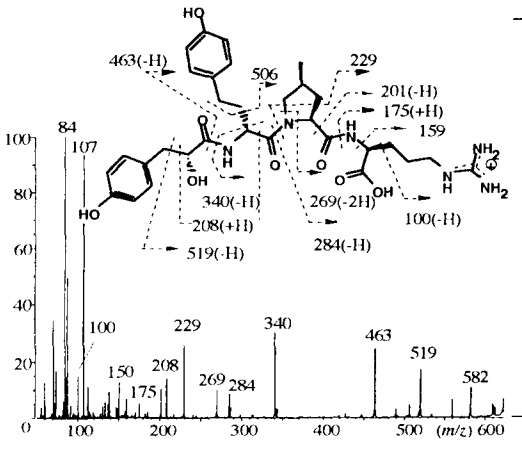

Fig. 3 Product ion spectrum for $[M+H]^+$ at *m/z* 627 of spumigin B1 (**4**) under positive FABMS conditions.

Table 2. Product ion spectral data (*m/z*) for $[M+H]^+$ of spumigins.

4, 5	Assignment of product ion	**6**	**3**
627	M+H	613 (-14)	613 (-14)
582	M+H –COOH	568 (-14)	–
519	M –(CH_2-Ph-OH)	505 (-14)	505 (-14)
506	M+H –R(Hty)	492 (-14)	492 (-14)
463	M+2H –Hpla	449 (-14)	449 (-14)
340	NH-CH-CO-MePro-Arg-OH	326 (-14)	326 (-14)
284	M –(Hpla-Hty)	270 (-14)	270 (-14)
269	M-H –(Hpla-Hty-N(MePro))	255 (-14)	255 (-14)
229	(N-CH-CO-Arg-OH)+H	229	215 (-14)
208	(HO-CH-CO-NH-CH-R(Hty))+H	208	208
201	(CO-Arg-OH)	201	187 (-14)
175	(H-Arg-OH)+H	175	–
159	(R(Arg)-CH-COOH)+H	159	144 Argol –NH
150	Hty*	150	150
107	(CH_2-Ph-OH)	107	107
100	R(Arg)	100	100 R(Argol)
84	MePro*	70 Pro*	84

* immonium ion

The structures of the nodulapeptins are similar to those of the anabaenopeptins[6,7] which were isolated from the toxic *Anabaena* species that co-produces microcystins. Recently, Murakami *et al.* reported the isolation and the structural determination of aeruginosins[15-17] from *Microcystis aeruginosa*, which is well known to produce microcystins, and the established structures of spumigins[18] are similar to these compounds. Furthermore, spumigins containing Arg are suggested as a precursor of aeruginosins containing Argol, argininal and agmatin.

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