SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RAD: 13-179114- -6 **FECHA**: 2015-01-15 19:05:52
DEP: 2020 DIRECCION DE NUEVAS CREACIONES **EVE:** 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II **FOLIOS**: 23
ACT: 519 PRESENTACION

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
CARLOS REINALDO OLARTE GARCIA

Asunto: Radicación: 13-179114- -6
Trámite: 11
Evento: 378
Actuación: 519
Oficio: 241
Folios: 23

Patente de Invención	**X**			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	**X**	Capítulo I	**X**	Capítulo II	
No. Solicitud Internacional	PCT/EP2008/060689				
Fecha de Presentación Internacional	14/08/2008				

1. Documentos estudiados

Descripción:	N° 13-179114-00000-0000	29/Julio/2013
Reivindicaciones:	N° 13-179114-00000-0000	29/Julio/2013
Dibujos:	N° 13-179114-00000-0000	29/Julio/2013
Prioridad:	EP 07114507.2	17/Agosto/2007

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Presentación de solicitud Fraccionaria (Art 36 D 486)

Se acepta para examen la presente solicitud fraccionaria presentada e identificada como 2013-179114, la cual es divisional de la solicitud 10-017551, porque no implica ampliación de la materia contenida en la solicitud inicial.

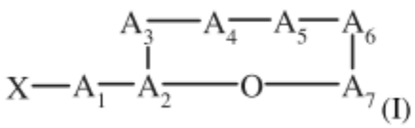
4. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del Título X de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. En el presente caso se estudiaran las reivindicaciones 1 a 14 del radicado N° 13-179114-00000-0000; sin embargo se le recuerda a la solicitante realizar el pago por las 4 reivindicaciones adicionales.

Título de la solicitud: “Depsipéptidos Cíclicos”.

El problema planteado en esta solicitud consiste en identificar depsipéptidos cíclicos producidos por *Chondromyces* que permitan modular la calicreina 7.

Para resolver este problema técnico la solicitud en estudio proporciona composiciones de compuestos de fórmula general (I) aislados de *Chondromyces.*



El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C07K 11/02; A61K 38/15.

5. De la solicitud de patente

Titulo

Se sugiere hacer el título más concreto teniendo en cuenta el objeto de la solicitud.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicaciones 1 a 14 se encuentran encabezadas como composición, sin embargo no se encuentra sustento en el capítulo descriptivo para el desarrollo de una composición, además, el único ingrediente que se encuentra definido es el activo. Se recuerda a la solicitante que una composición se caracteriza por sus ingredientes y las proporciones de los mismos.

Las reivindicaciones 1, 2, 3 no son concisas porque las definiciones de R incluyen géneros funcionales como heterociclilo, el cual aunque se pretende definir dando múltiples posibilidades en cuanto a números de átomos, posibles heteroátomos, número de ciclos, números de dobles enlaces, posibilidades de oxidación y posibles fusiones, esta forma de definir da lugar a un sinnúmero de posibilidades que no encuentran soporte en la descripción y que además restan claridad a la materia reclamada. Por lo tanto, se sugiere restringir la solicitud a una razonable generalización del tipo de compuestos, es decir, que se encuentran debidamente sustentados.

6. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: agosto 17 de 2007, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	NAMIKOSHI M. ET AL, Journal of Industrial Microbiology, Vol. 17, Págs. 373 - 384	"Bioactive Compounds by Cianobacteria"	1996
D2	ELERT E. ET AL, Journal of Natural Products, Vol. 68, Págs. 1324 - 1327	"Cyanopeptolin 954, a Chlorine-Containing Chymyotrypsin Inhibitor of *Microcystis aeruginosa* NIVA Cya 43"	08/Noviembre/2005

El documento D1[1] divulga compuestos producidos por cianobacterias, entre los cuales los péptidos cíclicos y depsipéptidos son los tipos de estructura más comunes (Resumen).

El documento D2[2] divulga un nuevo depsipéptido Cyanopeptolin 954 aislado de *Microcystis aeruginosa* (Resumen).

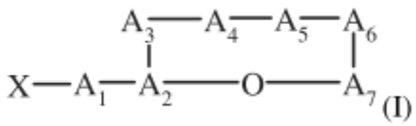
7. Examen de Patentabilidad (Art 14 D486)

Nivel inventivo (Art18 D486)

La reivindicación 1 consiste en *"Una composición farmacéutica que comprende un depsipéptido cíclico que tiene la formula estructural de la Fórmula (I):*

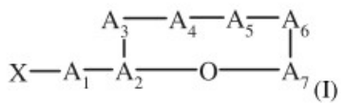
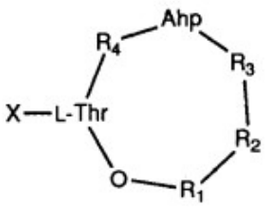
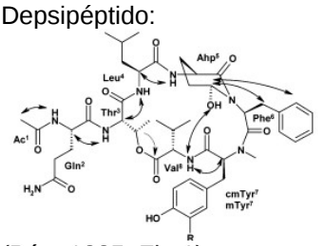
1 http://link.springer.com/article/10.1007%2FBF01574768

2 http://www.ncbi.nlm.nih.gov/pubmed/16180807



en donde se forma un enlace éster entre un grupo carboxilo de A_7 y un grupo hidroxilo de $A_{2...}$" (Radicado N° 13-179114-00000-0000).

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	**D1**	**D2**
Una composición que comprende:	Un depsipéptido	Un depsipéptido
Un depsipéptido cíclico de Fórmula (I)  en donde se forma un enlace éster entre un grupo carboxilo de A_7 y un grupo hidroxilo de A_2	Un depsipéptido de fórmula:  (Pág. 376, Fig. 7)	Depsipéptido:  (Pág. 1325, Fig 1)
X: $CH_3CH_2CH(CH_3)CO$ o $(CH_3)_2CHCO$	$CH_3(CH_2)_6CO$ $CH_3(CH_2)_4CO$ $CH_3(CH_2)_2CO$ (Pág. 377, Fig. 8)	CH_3CO
A_1: Glutamina, ácido glutamico	Gln, Glu	Gln
A_2: Treonina	Thr	Thr
A_3: Leucina	R_4:Leu	Leu
A_4: Ahp	Ahp	Ahp
A_5: Isoleucina	R_3: Ile	Phe
A_6: Tirosina, N-Me-Tirosina	R_2: Me Tyr	Tyr
A_7: Isoleucina o Valina	R_1: Ile, Val	Val
Vehículo farmacéuticamente aceptable	No se menciona	No se menciona

El documento D1 se considera el estado de la técnica cercano a la invención definida en la reivindicación 1 porque divulga compuestos producidos por cianobacterias, entre los cuales los péptidos cíclicos y depsipéptidos son los tipos de estructura más comunes (Resumen), esta anterioridad también enseña depsipéptidos que tienen actividad inhibitoria de las serina proteasas (Págs. 375 y

376).

Por su parte el documento D2 da a conocer el compuesto cyanopeptolin 954, el cual tiene actividad inhibitoria de quimiotripsina (Resumen; Pág. 1325, Fig. 1).

La diferencia entre la invención, definida en la reivindicación 1 y el depsipéptido divulgado en el documento D1 consiste en que el depsipéptido se encuentra en una composición farmacéutica junto con un vehículo, y en que el sustituyente X corresponde a $CH_3CH_2CH(CH_3)CO$ o $(CH_3)_2CHCO$, mientras que en el documento D1, este sustituyente puede ser $CH_3(CH_2)_6CO$, $CH_3(CH_2)_4CO$ o $CH_3(CH_2)_2CO$.

El efecto que logra el incluir $CH_3CH_2CH(CH_3)CO$ o $(CH_3)_2CHCO$ como sustituyente X es que los compuestos presentan actividad inhibitoria de Calicreina 7.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo proporcionar una composición que comprenda un depsipéptido con actividad inhibitoria de la calicreina 7”.

Sin embargo, el documento D1 muestra que compuestos como el Nostopeptin A, con una cadena alquílica en la posición equivalente a X (Pág. 377, Fig. 8) tienen actividad quimiotripsina y que otros depsipéptidos aislados de Cyanobacterium tienen actividad inhibitoria de serina proteasas (Pág. 375, Tabla 1). Así mismo, el documento D2 revela la actividad inhibitoria de quimiotripsina (Resumen) del compuesto Cyanopeptolin 954, el cual también tiene un metilo en la posición equivalente a X en el compuesto de la solicitud (Pág. 1325, Fig. 1).

Además, es de conocimiento de la persona normalmente versada en la materia que la calicreina 7 es una enzima quimiotríptica del estrato corneo, y que esta serina proteasa está asociada al cancer[3]. Nótese que en el documento D1 se realiza la determinación de la actividad inhibitoria de diferentes compuestos producidos por cianobacterias en diferentes serina proteasas, por lo tanto el hecho de determinar la actividad inhibitoria de calicreina 7 no constituye ningún salto técnico, y hace parte del quehacer rutinario de la persona normalmente versada en la materia. Así mismo es tarea rutinaria la obtención de una composición de un compuesto con actividad farmacológica[4].

En consecuencia, la persona del oficio normalmente versada en la materia, obtendría una composición de un depsipéptido alternativo al divulgado en el documento D1 a partir de las enseñanzas de esta anterioridad y del documento D2 para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

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3 SANTIN A. ET AL, “The Serine Protease Stratum Corneum Chymotryptic Enzyme (Kallikrein 7) is Highly Overexpressed in Squamous Cervical Cancer Cells”, Gynecologic Oncology, Vol. 94, 2004, Págs. 283 – 288.

4 GENNARO A., “Remington Farmacia”, Tomos 1 y 2, Edición 19, Editorial Médica Panamericana, 1998.

Las reivindicaciones dependientes 2 – 14 no mencionan alguna característica que haga inventiva a la composición de la reivindicación 1, por lo cual se considera que no tienen nivel inventivo.

Conclusión:
Las reivindicaciones 1 a 14 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

8. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	NAMIKOSHI M. ET AL, Journal of Industrial Microbiology, Vol. 17, Págs. 373 - 384	1 – 14	Nivel inventivo
D2	ELERT E. ET AL, Journal of Natural Products, Vol. 68, Págs. 1324 - 1327	1 – 14	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.

JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2015-01-20

Elaboró: Jeny Paola Villate Becerra
Revisó: Sandra Carolina Crespo

Journal of Industrial Microbiology (1996) 17, 373–384

Bioactive compounds produced by cyanobacteria

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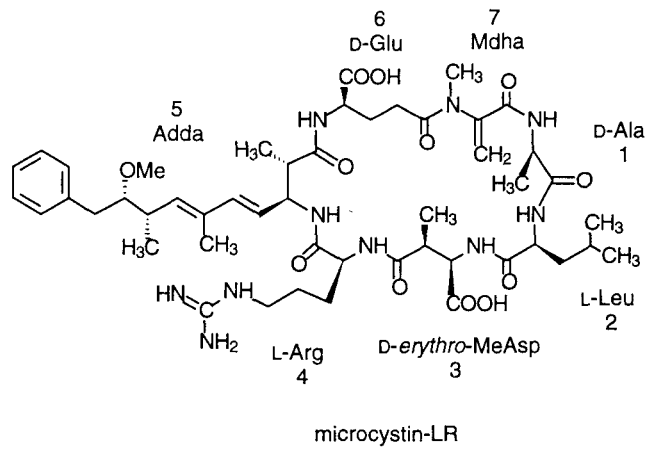
Cyanobacteria produce a large number of compounds with varying bioactivities. Prominent among these are toxins: hepatotoxins such as microcystins and nodularins and neurotoxins such as anatoxins and saxitoxins. Cytotoxicity to tumor cells has been demonstrated for other cyanobacterial products, including 9-deazaadenosine, dolastatin 13 and analogs. A number of compounds in cyanobacteria are inhibitors of proteases — micropeptins, cyanopeptolins, oscillapeptin, microviridin, aeruginosins — and other enzymes, while still other compounds have no recognized biological activities. In general cyclic peptides and depsipeptides are the most common structural types, but a wide variety of other types are also found: linear peptides, guanidines, phosphonates, purines and macrolides. The close similarity or identity in structures between cyanobacterial products and compounds isolated from sponges, tunicates and other marine invertebrates suggests the latter compounds may be derived from dietary or symbiotic blue-green algae.

Keywords: cyanobacteria; blue-green algae; toxins; enzyme inhibitors; peptides

Hepatotoxins from *Microcystis, Anabaena, Nostoc, Oscillatoria* and *Nodularia*

Toxic cyanobacterial (blue-green algal) waterblooms are found worldwide in eutrophic lakes, ponds, drinking water reservoirs and coastal waters, where they cause animal poisonings and pose risks to human health [8,9,11]. Several genera of cyanobacteria form these toxic waterblooms, with *Microcystis* being the most common [8,9,80]. Two types of toxins, hepatotoxins and neurotoxins, have been characterized from the toxic cyanobacteria, but hepatotoxicosis occurs more often than neurotoxicosis [8,9]; about 50% of *Microcystis* waterblooms show hepatotoxicity to mammals and other animals. The toxins responsible for the hepatotoxicity are the well known microcystins [10]. Microcystins are obtained not only from *Microcystis*, but also from *Anabaena, Nostoc* and *Oscillatoria* [74] which also form waterblooms.

The microcystins are cyclic heptapeptides like the representative microcystin-LR depicted in Figure 1 [72]. The unique structural feature in microcystins is the C_{20} amino acid, (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4*E*,6*E*-dienoic acid (Adda) [72], which plays an important role in their toxicity [21,54,55,59,74,82]. The suffix, LR, identifies the two variable L-amino acids at positions 2 and 4, ie, Leu and Arg [10]. More than 50 structural variations in microcystins have been found from the four genera of cyanobacteria [60,74]. The general structure of microcystins is cyclo(-D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha-), in which MeAsp is *erythro*-β-methylaspartic acid and Mdha indicates *N*-methyldehydroalanine [10,74]. X and Z are the commonly variable L-amino acids (positions 2 and 4), but structural modifications have been detected



microcystin-LR

Figure 1 Microcystin-LR.

in all seven amino acid units [60,74]. Some relationships between structural modifications and toxicity have been found for microcystins [21,54–57,59,60,74,82]. The structurally related toxins nodularins have been isolated from the brackish water-dwelling cyanobacterium *Nodularia spumigena* [59,72]. Nodularins are cyclic pentapeptides and have the Adda unit or its derivative (Figure 2).

Microcystins and nodularins show hepatotoxicity through inhibitory activity to protein phosphatases 1 and 2A [26,41,86] and are potent tumor promoters [15,61,62]. The cyclic structure of these toxins is essential for the toxicity, since linear compounds which have the same amino acid components as the cyclic peptides did not show apparent toxicity to mice [13,59,60].

Extensive efforts have been devoted to studies on cyanobacterial toxins resulting in understanding of the toxins and producing organisms. The results of studies on chemical structures, biosynthesis and structure-toxicity relationships of microcystins and nodularins have been summarized in our earlier review [74]. Microcystins are the most common causative agent of cyanobacterial waterbloom toxicosis,

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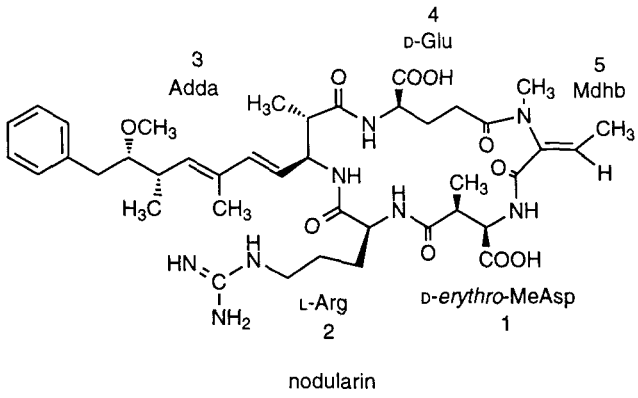


Figure 2 Nodularin isolated from the brackish water cyanobacterium *Nodularia spumigena*.

and show toxicity to mice at concentrations as low as 50 μg kg^{-1} (LD_{50}, intraperitoneal injection). The netpen liver disease of salmon observed on the Pacific Canadian coast is thought to be caused by microcystins [2]. A compound structurally related to nodularins, motuporin ([L-Val^2]nodularin), which has an L-Val unit instead of the L-Arg unit in nodularin, has been isolated from a marine sponge, *Theonella swinhoei* [14], but does not show cytotoxicity.

A structurally unrelated cyanobacterial hepatotoxin, cylindrospermopsin (Figure 3), has been isolated from *Cylindrospermopsis raciborskii* [63] and *Umezakia natans* [24] and has an acute (24 h) LD_{50} of 2.1 mg kg^{-1} (IP).

Neurotoxins from cyanobacteria

Neurotoxins (Figure 4) are the other group of cyanobacterial toxins (anatoxin-a from *Anabaena* and *Oscillatoria*, anatoxin-a(s) from *Anabaena flos-aquae*, homoanatoxin-a from *Oscillatoria rubescens*, saxitoxin and neosaxitoxin from *Aphanizomenon* and *Anabaena*) [8]. *Microcystis* species also produce anatoxin-a [67]. Saxitoxins are also produced by certain marine dinoflagellates [32,81].

During our studies on the metabolites of toxic cyanobacteria we isolated two nucleosides (Figure 5), 9-deazaadenosine and its 5′-α-D-glucopyranoside, from the freshwater cyanobacterium *Anabaena affinis* VS-1 [58]. These compounds showed cidal toxicity to a zooplankton, *Ceriodaphnia dubia* (LC_{50} = 0.1–0.5 μg ml^{-1}), and potent cytotoxicity to murine leukemia cells L1210 (IC_{50} = 0.002 and

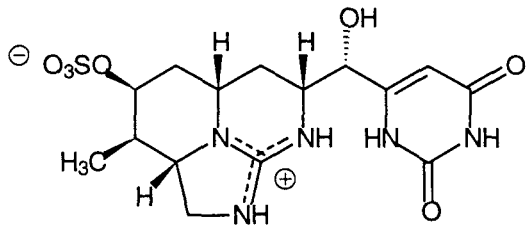


Figure 3 The structure of cylindrospermopsin.

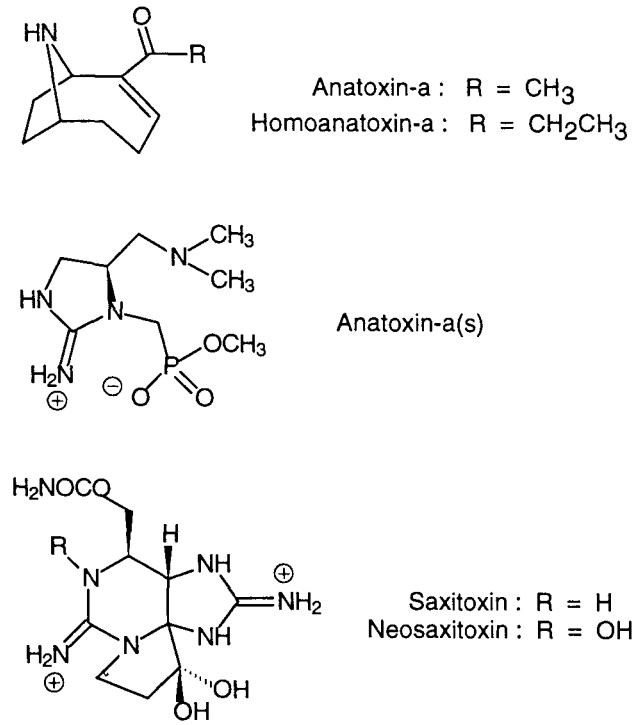


Figure 4 Anatoxin-a, homoanatoxin-a, anatoxin-a(s), saxitoxin and neosaxitoxin.

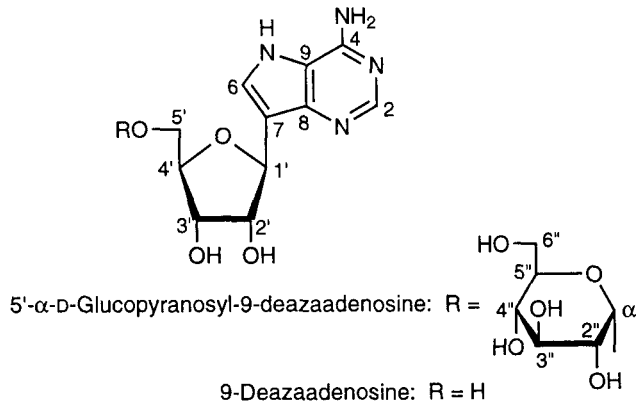


Figure 5 9-Deazaadenosine and its 5′-α-D-glucopyranoside isolated from the freshwater cyanobacterium *Anabaena affinis* strain VS-1.

0.02 μg ml^{-1}, respectively) [57,58]. The search for bioactive natural products, such as cytotoxins to tumor cells and antibacterial, antifungal and antiviral agents, from cyanobacteria has recently intensified [17,68]. The main effort for this purpose involved isolation of cyanobacteria strains from various environments and culturing of each strain to test for biological activities. Although toxins were the main subject of research on toxic cyanobacterial waterblooms, isolation of bioactive compounds other than biotoxins has recently been stressed.

Serine protease inhibitors from cyanobacteria

The production of bioactive components from *Microcystis* suggested a screening program for protease-inhibitory

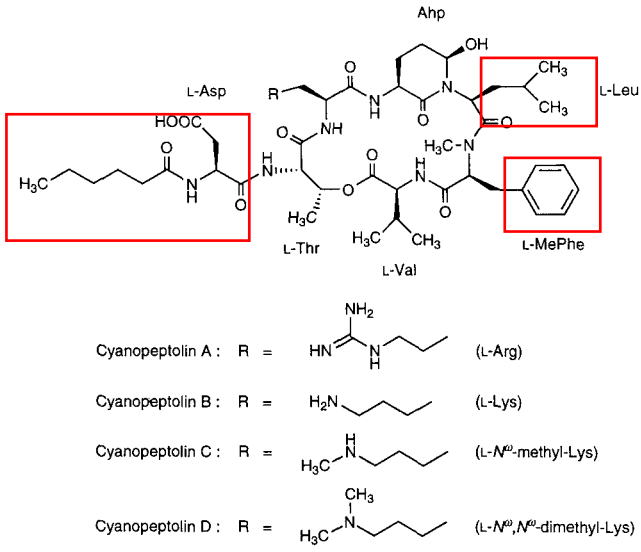


Figure 6 Cyanopeptolins A–D isolated from *Microcystis aeruginosa* PCC 7806.

activity of microalgal secondary metabolites [5]. The presence of non-toxic peptides in *Microcystis* in addition to microcystins was reported first by Weckesser and co-workers [4]. The structures of these peptides, termed cyanopeptolins A–D, isolated from microcystin-producing *Microcystis aeruginosa* PCC 7806, were assigned as shown in Figure 6 [42]. These compounds are cyclic depsipeptides (cyclic peptidolactones) and contain a unique amino acid unit, 3-amino-6-hydroxy-2-piperidone (Ahp). A number of closely related depsipeptides bearing the Ahp unit have been isolated since then from both toxic and non-toxic *Microcystis* species. The structures are summarized in Figure 7. Microcystilide A was isolated from *M. aeruginosa* NO-15-1840, which produced microcystins, and promoted cell differentiation of HL-60 cells at a concentration of 0.5 mg ml^{-1} [84]. Aeruginopeptins 95-A and B, and 228-A and B were isolated from *M. aeruginosa* strains TAC95 and M228, respectively [23]. Both strains produced microcystins, but no biological activity of these depsipeptides was noted. Five compounds belonging to this class were obtained as serine protease inhibitors. Micropeptins A and B, isolated from non-toxic *M. aeruginosa* NIES-100, showed inhibitory activities to plasmin and trypsin [65] and micropeptin 90 from non-toxic *M. aeruginosa* NIES-90 also inhibited plasmin and trypsin [28]. Cyanopeptolins S and SS were isolated together with microcystins from a waterbloom sample dominated by a *Microcystis* sp collected from a lake in Germany and showed inhibitory activity to trypsin, plasmin and thrombin [30,31]. The inhibitory activities to serine proteases by the depsipeptides isolated from *Microcystis* and other genera are summarized in Table 1 together with other serine protease inhibitors shown in Figures 8–10.

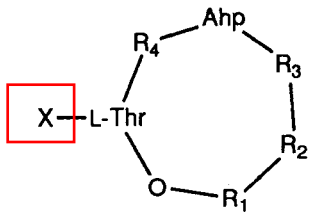
This class of depsipeptides has also been isolated from *Oscillatoria* spp and *Anabaena* spp. Oscillapeptin was obtained as a serine protease inhibitor from non-toxic *Oscillatoria agardhii* NIES-204 and inhibited elastase and chymotrypsin [78]. Oscillapeptin G was produced by *O. agardhii* NIES-610, together with microcystins, and showed inhibitory activity to tyrosinase at 1.0×10^{-4} M [77]. The isolation of anabaenopeptilides 90-A and B, and 202-A and B from *Anabaena circinalis* 90 and *Anabaena lemmermannii* 202 A2/41, respectively, was recently reported [16]. These two *Anabaena* spp were hepatotoxic, and microcystins have been characterized as the responsible toxins [79].

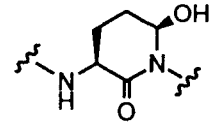
A closely related compound was isolated from a cyano-

Table 1 The inhibitory activity (IC_{50}) to serine proteases and tyrosinase by peptides isolated from cyanobacteria

	Trypsin	Plasmin	Thrombin	Elastase	Chymotrypsin	Tyrosinase	Ref
Micropeptin A	0.071[a]	0.026	n/i[b]	n/i	n/i		[65]
Micropeptin B	0.25	0.035	n/i	n/i	n/i		[65]
Micropeptin 90	2.0	0.1		n/i	n/i		[28]
Cyanopeptolin S	<0.2	<1	<5				[30]
Cyanopeptolin SS	<0.2	<1	<5				[31]
Oscillapeptin	n/i	n/i	n/i	0.3	2.2		[78]
Oscillapeptin G						1.0×10^{-4} M	[77]
A90720A	10 nM	30 nM	275 nM				[40]
Nostopeptin A	n/i	n/i	n/i	1.3	1.4		[44]
Microviridin A	n/i	n/i	n/i	n/i	n/i	3.3×10^{-4} M	[29]
Microviridin B	58	n/i	n/i	0.044	2.5		[66]
Microviridin C	32	n/i	n/i	0.084	4.9		[66]
Aeruginosin 298-A	1.0	n/i	0.3	n/i	n/i		[51]
Aeruginosin 98-A	0.6	6.0	7.0	n/i	n/i		[53]
Aeruginosin 98-B	0.6	7.0	10.0	n/i	n/i		[53]
Aeruginosin 98-C	3.9	5.0	3.3	n/i	n/i		[52]
Aeruginosin 102-A	0.2	0.3	0.04	n/i	n/i		[45]
Aeruginosin 102-B	1.1	0.8	0.1	n/i	n/i		[45]
Oscillamide Y					1.0×10^{-5} M		[76]

[a]The concentration is $\mu g\ ml^{-1}$, unless otherwise designated.
[b]Not inhibited (>10 or 100 $\mu g\ ml^{-1}$).





MePhe = *N*-methylphenylalanine
MeTyr = *N*-methyltyrosine
diMeTyr = *N,O*-dimethyltyrosine

Me(Cl)Tyr = *N*-methyl-3'-chlorotyrosine
MeLys = N^{ω}-methyllysine
diMeLys = N^{ω},N^{ω}-dimethyllysine
(H_4)Tyr = 1',2',3',4'-tetrahydrotyrosine
Hty = homotyrosine
Dhb = dehydrobutyrine

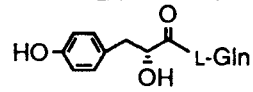
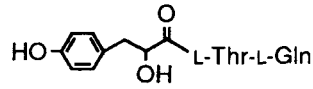
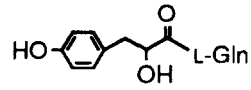
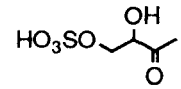
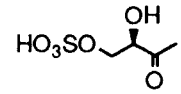
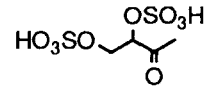
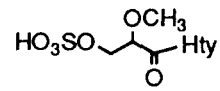
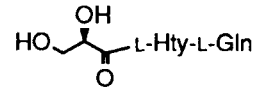
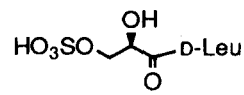
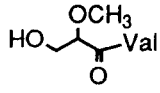
	R_1	R_2	R_3	R_4	X	Ref.
(from *Microcystis* spp.)						
Cyanopeptolin A	L-Val	L-MePhe	L-Leu	L-Arg	$CH_3(CH_2)_4CO$-L-Asp	[42]
Cyanopeptolin B	L-Val	L-MePhe	L-Leu	L-Lys	$CH_3(CH_2)_4CO$-L-Asp	[42]
Cyanopeptolin C	L-Val	L-MePhe	L-Leu	L-MeLys	$CH_3(CH_2)_4CO$-L-Asp	[42]
Cyanopeptolin D	L-Val	L-MePhe	L-Leu	L-diMeLys	$CH_3(CH_2)_4CO$-L-Asp	[42]
Microcystilide A	L-Ile	L-MeTyr	L-Leu	L-Tyr		[84]
Aeruginopeptin 95-A	L-Ile	L-MePhe	L-Thr	L-Tyr		[23]
Aeruginopeptin 95-B	L-Ile	L-MePhe	L-Thr	(H_4)Tyr		[23]
Aeruginopeptin 228-A	L-Ile	L-MePhe	L-Thr	L-Tyr		[23]
Aeruginopeptin 228-B	L-Ile	L-MePhe	L-Thr	(H_4)Tyr		[23]
Micropeptin A	L-Val	MeTyr	L-Leu	L-Lys	$CH_3(CH_2)_6CO$-L-Glu	[65]
Micropeptin B	L-Val	MeTyr	L-Leu	L-Lys	$CH_3(CH_2)_4CO$-L-Glu	[65]
Micropeptin 90	Val	MeTyr	L-Phe	Arg		[28]
Cyanopeptolin S	L-Ile	L-MePhe	L-Ile	L-Arg		[30]
Cyanopeptolin SS	L-Ile	L-MePhe	L-Ile	L-Arg		[31]
(from *Oscillatoria* spp.)						
Oscillapeptin	L-Ile	L-diMeTyr	L-Ile	Hty		[78]
Oscillapeptin G	L-Ile	L-MeTyr	L-Thr	L-Leu		[76]
(from *Anabaena* spp.)						
Anabaenopeptilide 90-A	L-Ile	L-diMeTyr	L-Thr	L-Hty	HCO-L-Gln	[16]
Anabaenopeptilide 90-B	L-Ile	L-Me(Cl)Tyr	L-Thr	L-Hty	HCO-L-Gln	[16]
Anabaenopeptilide 202-A	L-Ile	L-diMeTyr	L-Thr	L-Hty	HCO-L-Pro-L-Gln	[16]
Anabaenopeptilide 202-B	L-Ile	L-Me(Cl)Tyr	L-Thr	L-Hty	HCO-L-Pro-L-Gln	[16]
(from *Microchaete* sp.)						
A90720A	L-Val	L-MeTyr	L-Leu	L-Arg		[40]
(from *Dolabella auricularia*)						
Dolastatin 13	Val	MePhe	Phe	Dhb		[70]

Figure 7 19-Membered depsipeptides containing the 3-amino-6-hydroxy-2-piperidone (Ahp) unit.

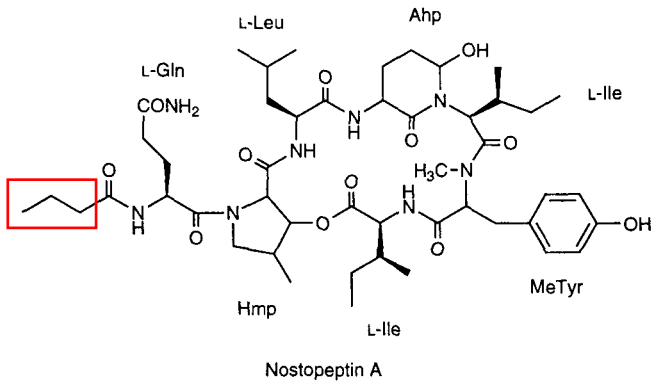


Figure 8 Nostopeptin A isolated from *Nostoc minutum* NIES-26.

bacterium other than the above species. *Microchaete loktakensis* produced A90720A, which showed inhibitory activity to trypsin, plasmin and thrombin similar to the other compounds.

A depsipeptide possessing the unique Ahp unit was first obtained from the Indian Ocean sea hare *Dolabella auricularia* and identified as a cytostatic agent [70]. This compound, dolastatin 13, has a very similar structure (Figure 7) to those of cyanobacterial metabolites, which would suggest that the primary producer of dolastatin 13 or its precursor may be a cyanobacterium on which the mollusc feeds.

These depsipeptides, listed in Figure 7, incorporated an L-Thr unit in a lactone linkage as another common structural feature, in addition to the Ahp unit. All compounds have an *N*-methylated amino acid unit as R_2. The R_4 amino

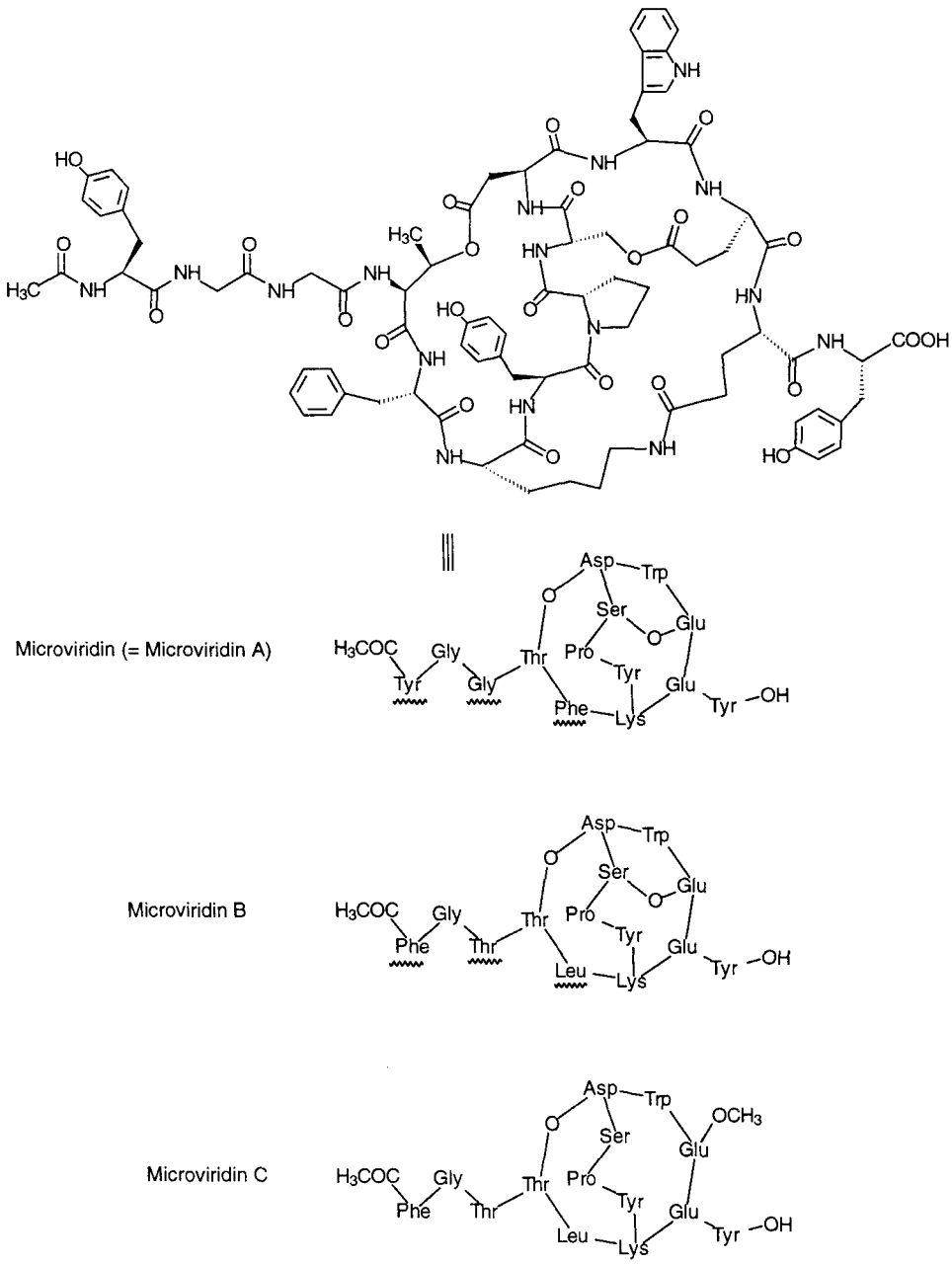
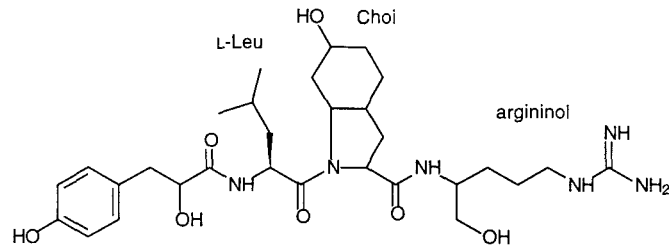
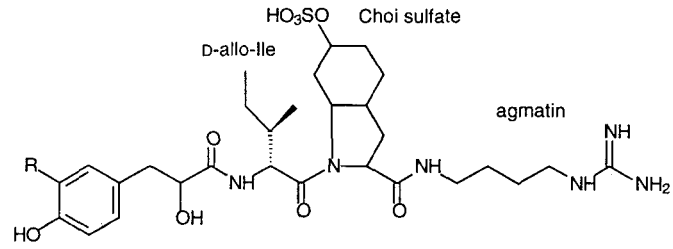


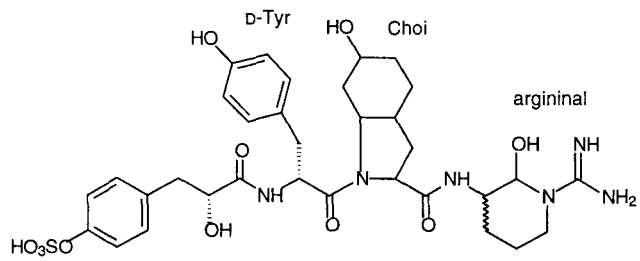
Figure 9 Tricyclic depsipeptides, microviridins A–C. (All amino acid components are the L-form.)



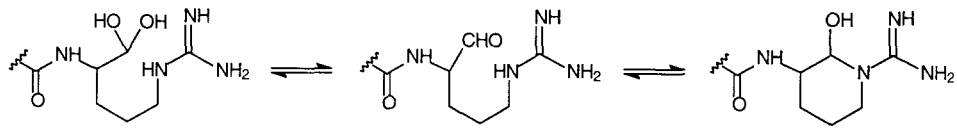
Aeruginosin 298-A



Aeruginosin 98-A R = Cl
Aeruginosin 98-B R = H
Aeruginosin 98-C R = Br



Aeruginosin 102-A : L-argininal
Aeruginosin 102-B : D-argininal



Tautomerism of argininal

Figure 10 Aeruginosins 298-A, 98-A, 98-B, 98-C, 102-A and 102-B isolated from *Microcystis* spp.

acid unit shows wide variety, while R_1 is either Val or Ile. The side chains attached to the amino group of L-Thr unit can be divided into four types, ie, the *N*-terminus is acylated by four types of carboxylic acids. One type is a hexanoyl or octanoyl acidic amino acid (Asp or Glu) residue. A second type has a *p*-hydroxyphenyllactyl unit attached to an amino acid or dipeptide unit. The third type of acid is a modified (sulfated, methylated) glyceric acid attached to the L-Thr unit directly or through an amino acid residue. The stereochemistry of glyceric acid in three compounds was determined to be D-. The fourth type is a formyl group attached to an amino acid or dipeptide.

The unusual amino acid (H_4)Tyr, 1′,2′,3′4′-tetrahydrotyrosine, was detected in aeruginopeptins 95-B and 228-B isolated from *Microcystis* spp. This amino acid was first found in microcystin-(H_4)YR obtained from a waterbloom of *Microcystis* spp [55,60]. Homotyrosine (Hty), found in the compounds from *Anabaena* spp and *Oscillatoria* spp, is also an amino acid component in microcystins isolated from *Anabaena* spp [22,56]. Dehydrobutyrine (Dhb), an amino acid component of dolastatin 13, is also found in nodularins as its *N*-methyl derivative (Figure 2).

The partial structures of the compounds in Figure 7 at the Thr unit, with a variable side chain attached to its amino group and its hydroxyl in a lactone bond in the ring, resemble those of didemnins isolated from the colonial tunicate *Tridemnum solidum* [73]. Didemnins are thought to be derived at least in part from symbiotic cyanobacterial metabolite(s).

A similar depsipeptide has been isolated from *Nostoc*

minutum NIES-26 as a serine protease inhibitor [44]. The compound, nostopeptin A, has the Ahp unit and a rare amino acid component, 3-hydroxy-4-methylproline (Hmp). The Hmp unit forms the lactone bond in the ring system, like the Thr unit in the above compounds. The 19-membered depsipeptides possessing the Ahp unit appear to be common metabolites in the freshwater cyanobacteria.

The second type of depsipeptides has the unique structural feature of a tricyclic ring system (Figure 9). The first example was isolated from the toxic *Microcystis viridis* NIES-102, which produced microcystins, and was named microviridin after the producing organism [29]. Although all amino acid components were L-, the structure assigned was quite unusual. Microviridin was reported to inhibit tyrosinase activity at a concentration of 3.3×10^{-14} M (Table 1). Two compounds closely related to microviridin have been reported recently from toxic *M. aeruginosa* NIES-298 [66] and named microviridins B and C. Another compound isolated from the same organism together with microviridins B and C was identified as microviridin and it has been suggested to rename microviridin as microviridin A. The structural differences between microviridins A and B are rather small: three amino acid components of Tyr, Gly and Phe in microviridin A are replaced by Phe, Thr and Leu, respectively, in microviridin B as indicated by underlining in Figure 9. The ester bond between the Ser hydroxyl group and the γ-carboxyl of the Glu unit found in microviridins A and B is missing in microviridin C, and the Glu γ-carboxyl is present as its methyl ester.

Microviridins B and C inhibited the activities of elastase, chymotrypsin and tyrosinase (Table 1), while microviridin A did not inhibit these serine proteases at 100 μg ml^{-1} [66]. The mechanism of action and structure-activity relationship of these compounds to serine proteases are interesting research targets.

The other structural type of serine protease inhibitors, aeruginosins, have been isolated from toxic and non-toxic *Microcystis* spp (Figure 10). They are linear peptides and have the unusual amino acid unit, 2-carboxy-6-hydroxyoctahydroindole (Choi), as the common amino acid component. Aeruginosin 298-A was isolated from the toxic strain of *M. aeruginosa* NIES-298 and inhibited thrombin and trypsin (Table 1) [51]. Microviridins A, B and C were also obtained from this strain (NIES-298), as noted above, together with microcystins. The non-toxic strain of *M. aeruginosa* NIES-98 produced aeruginosins 98-A, B and C as inhibitors of trypsin, thrombin and plasmin (Table 1) [52,53]. Aeruginosins 102-A and B were found in toxic *M. viridis* NIES-102 and inhibited thrombin, trypsin and plasmin (Table 1) [45]. This strain produced microcystins and microviridin A [29].

These compounds have *p*-hydroxyphenyllactic acid or its derivative at the *N*-terminus as the common structural unit. This acid unit is also found in five depsipeptides listed in Figure 7 as the masking group at the *N*-terminus. Aeruginosin 298-A has an L-Leu unit, while the other compounds have a D-amino acid unit at the same position.

The guanidine-containing units at the *C*-terminus are divided into three types, all derived from arginine. The argininal unit in aeruginosins 102-A and B is the aldehyde variant of Arg. These two compounds each showed three inseparable peaks on reversed-phase HPLC, which might be explained by the equilibria shown in Figure 10 [45]. The argininol unit in aeruginosin 298-A is a further reduced form of Arg containing a hydroxymethyl group. The third unit type, found in aeruginosins 98-A, B and C, is the decarboxyl variant of Arg, agmatin.

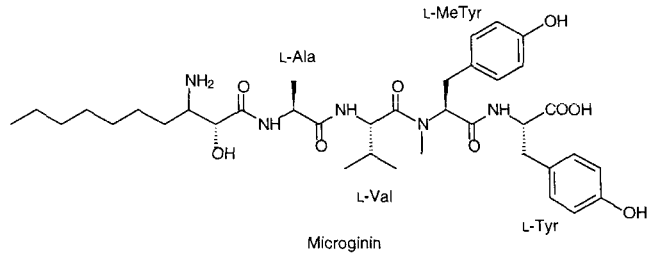
Cyanobacterial peptides with other bioactivities

Figure 11 shows another linear peptide isolated from the non-toxic strain of *M. aeruginosa* NIES-100. This strain also produced micropeptins A and B (see above). The linear peptide named microginin consisted of three usual and one *N*-methylated L-amino acids and a new β-amino acid at the *N*-terminus [64]. Microginin inhibited angiotensin-converting enzyme (LC_{50} = 7.0 μg ml^{-1}) but did not inbibit serine proteases.

Aeruginoguanidines 98-A, B and C (Figure 12) were obtained from *M. aeruginosa* NIES-98 during the isolation of serine protease inhibitors aeruginosins 98-A, B and C [52]. Aeruginoguanidines have unusual structures containing two *N*-methylated arginines with the *C*-terminus marked by an unusual highly sulfated aromatic amine for all three compounds, and two guanidine groups derivatized with prenyl and geranyl groups in aeruginoguanidine 98-A. The structures of aeruginoguanidines 98-B and C differ from that of 98-A in the isoprenoid units attached to the guanidino groups. These compounds were reported to show weak cytotoxicity to P388.

The 19-membered cyclic peptides shown in Figure 13 are not metabolites of *Microcystis* spp but were produced by toxic strains of *Anabaena* spp and an *Oscillatoria* sp. *Anabaena flos-aquae* NRC 525-17 gave anabaenopeptins A and B together with microcystins and anatoxin-a(s) [25]. These peptides showed weak relaxation activity to norepinephrine-induced constriction of rat aortic preparations. *A. circinalis* 90 produced anabaenopeptins A, B and C, and anabaenopeptins B and D were isolated from *A. lemmermannii* 202 A2/41 [16]. Both strains contained microcystins. Oscillamide Y was obtained as a serine protease inhibitor from the microcystin-producing strain of *O. agardhii* NIES-601 [76], which also produced oscillapeptin G. Oscillamide Y inhibited the activity of chymotrypsin (Table 1), which suggests that the other members of the class might also have this activity.

Anabaenopeptins have been detected from six *Anabaena* and two *Oscillatoria* strains of fresh-water cyanobacteria and a brackish-water cyanobacterium *Nodularia spumi-*



Microginin

Figure 11 Microginin isolated from *Microcystis aeruginosa*.

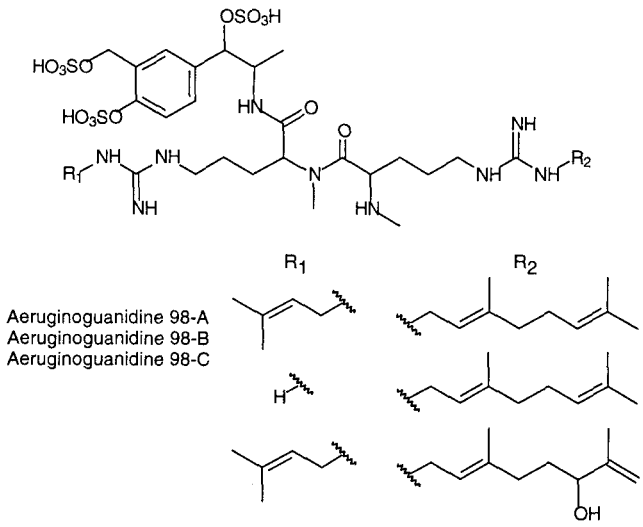
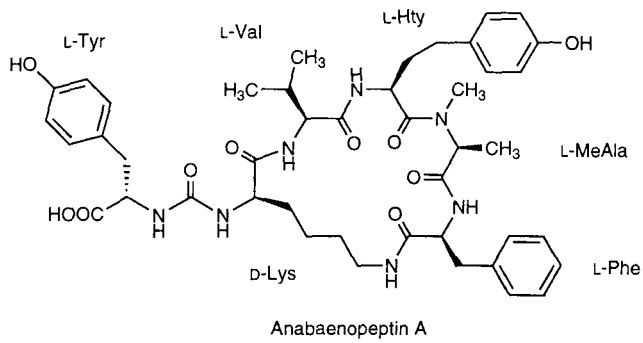
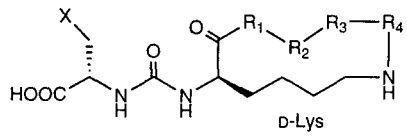


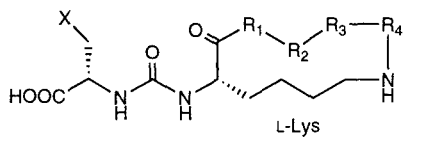
Figure 12 Aeruginoguanidines 98-A, 98-B and 98-C isolated from *Microcystis aeruginosa*.



Anabaenopeptin A



	R_1	R_2	R_3	R_4	X
Anabaenopeptin A	L-Val	L-Hty	L-MeAla	L-Phe	(L-Tyr)
Anabaenopeptin B	L-Val	L-Hty	L-MeAla	L-Phe	(L-Arg)
Anabaenopeptin C	L-Val	L-Hty	L-MeAla	L-Phe	(L-Lys)
Anabaenopeptin D	L-Val	L-Hty	L-MeAla	L-Phe	(L-Phe)
Oscillamide Y	L-Ile	Hty	MeAla	L-Phe	(L-Tyr)



	R_1	R_2	R_3	R_4	X
Konbamide	L-Ala	L-Leu	L-MeLeu	BhTrp	(L-Leu)
Keramamide A	L-Leu	L-Leu	MeCht	L-Phe	(L-Phe)

BhTrp = 2-bromo-5-hydroxytryptophan
MeCht = 6-chloro-5-hydroxy-*N*-methyltryptophan

Figure 13 19-Membered cyclic peptides isolated from *Anabaena* spp, *Oscillatoria* sp and marine sponges.

gena, together with hepatotoxins microcystins or nodularins (Table 2) [16].

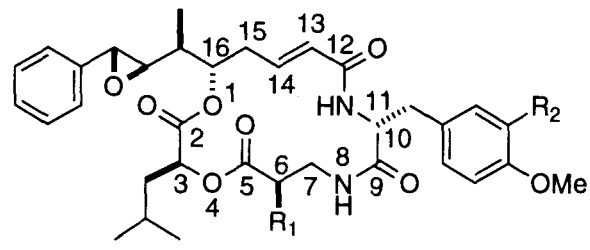
The unique structural features of these compounds are the ureido group and the ω-amido linkage of the D-Lys unit with the *C*-terminal amino acid unit (L-Phe). Interestingly, the stereochemistry of this Lys unit (D-) is different from the other amino acid units. The amino acid units R_2, R_3 and R_4 are the same in the five peptides and R_3 is *N*-methylated. The terminal amino acid (X) forming the ureido unit shows wide variety among these peptides, however.

Peptides structurally related to these compounds have been isolated from two species of marine sponges belonging to the *Theonella* genus. Konbamide [36] and keramamide A [37] have cyclic structures possessing the ureido unit and the ω-amide linkage similar to the above compounds (Figure 13). The position of the *N*-methylated amino acid unit is also the same as in the cyanobacterial metabolites. The difference is observed in the Lys unit: the compounds from marine sponges have an L-Lys unit instead of the D-form of the cyanobacterial metabolites. Konbamide and keramamide A each have one unique tryptophan derivative (BhTrp and MeCht) as shown in Figure 13.

The cyanobacterial genus *Microcystis* is a rich source of cyclic and linear peptides possessing a variety of biological activities, including the inhibition of certain enzyme activities. Structurally related peptides are also obtained from *Anabaena, Nostoc* and *Oscillatoria* spp. These peptides could be common secondary metabolites of the cyanobacteria which form toxic waterblooms, but since both toxic and non-toxic strains produce similar peptides, a relationship between these peptides and biotoxin production is still unclear.

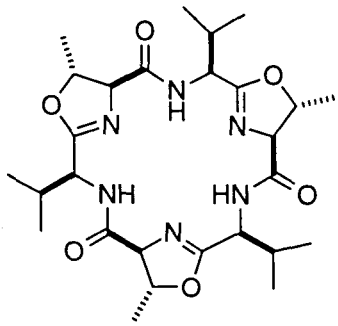
Relationship between cyanobacterial and invertebrate products

During studies on cyanobacterial secondary metabolites, structural relationships between the cyanobacterial secondary metabolites and compounds isolated from marine invertebrates have been observed. The structure of dolastatin 13, from the sea hare, resembles the structures of the cyanobacterial 19-membered depsipeptides in Figure 7, which are also similar to structures of didemnins from a colonial tunicate, as noted above. The 19-membered cyclic peptides shown in Figure 13 have structures related to those of compounds from marine sponges. The structural near-identity between nodularins from the cyanobacterium and



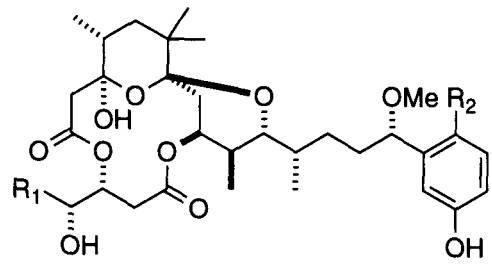
cryptophycin-1: $R_1 = CH_3$, $R_2 = Cl$
arenastatin A (cryptophycin-24): $R_1 = H$, $R_2 = H$

Figure 14 Cryptophycin A from a cyanobacterium and arenastatin from a sponge.



cycloxazoline (westiellamide)

Figure 15 Cycloxazoline, from a tunicate, the same as westiellamide, from a cyanobacterium.

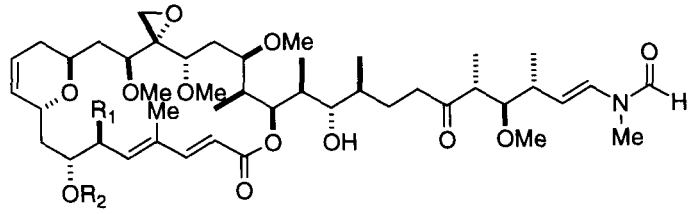


aplysiatoxin: $R_1 = CH_3$, $R_2 = Br$
oscillatoxin A: $R_1 = R_2 = H$

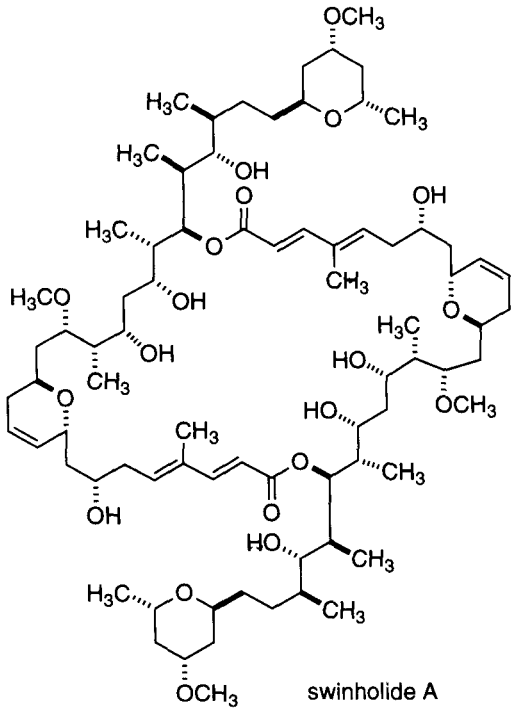
Figure 17 Aplysiatoxin, from a sea hare, and oscillatoxin A, from a cyanobacterium.

motuporin from a sponge was also noted above. Examples of these relationships are increasing as more structures of cyanobacterial secondary metabolites are assigned.

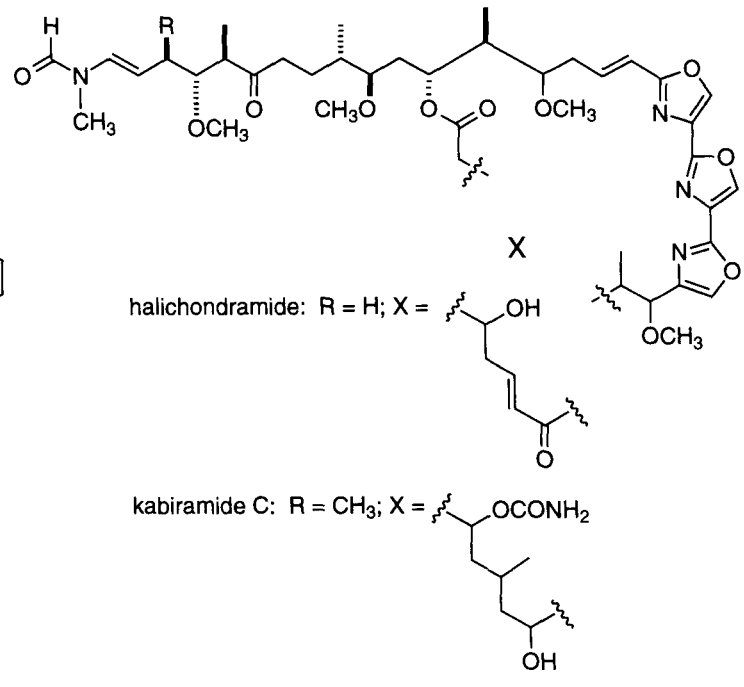
Several additional examples follow. Cryptophycins [18,83], isolated from terrestrial cyanobacteria (*Nostoc* spp) as antitumor agents, have structures very similar to that of arenastatin A [39], from the marine sponge *Dysidea arenaria* (Figure 14). A cytotoxic cyclic peptide cycloxazoline [20] from the colonial tunicate *Lissoclinum bistratum* is the same compound as westiellamide [71] obtained from the terrestrial cyanobacterium *Westiellopsis prolifica* (Figure 15). The terrestrial cyanobacteria *Scytonema* spp and *Tolypothrix* spp produce cytotoxic scytophycins and tolytoxins [6,27] whose structures are related to those of marine sponge metabolites swinholide A [7,35,38] from *Theonella*



tolytoxin: $R_1 = OH$, $R_2 = Me$
scytophycin B: $R_1 = R_2 = H$



swinholide A



halichondramide: R = H; X =

kabiramide C: R = CH_3; X =

Figure 16 Tolytoxin and scytophycin B, from cyanobacteria, swinholide A and halichondramide, from sponges, and kabiramide from nudibranch egg masses.

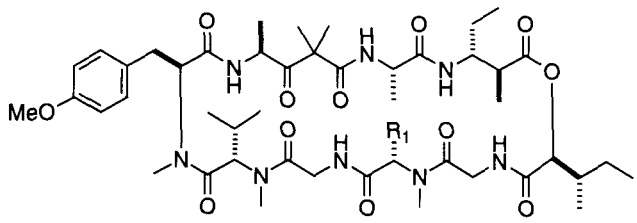


Figure 18 Majusculamide C, from a cyanobacterium, and dolastatin 11, from a sea hare.

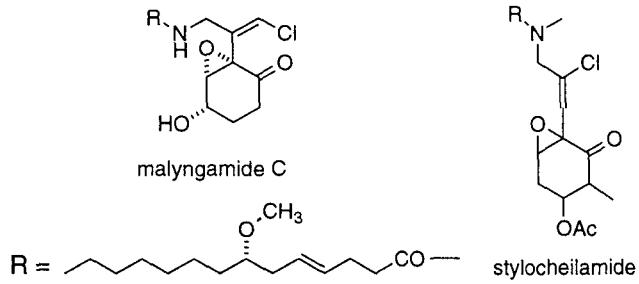


Figure 19 Malyngamide C, from a cyanobacterium, and stylocheilamide, from a sea hare.

Table 2 Detection of anabaenopeptins in hepatotoxic cyanobacteria [16]

Cyanobacteria	Hepatotoxins	Anabaenopeptins
Anabaena flos-aquae NRC 525-17	microcystins	A, B
Anabaena flos-aquae CYA 83/1	microcystins	B, D
Anabaena flos-aquae 202 A1	microcystins	B, D
Anabaena lemmermannii 66	microcystins	B, D
Anabaena lemmermannii 202 A2/41	microcystins	B, C
Anabaena circinalis 90	microcystins	A, B, C
Oscillatoria agardhii 97	microcystins	B, C
Oscillatoria agardhii CYA 128	microcystins	A, C
Nodularia spumigena BY1	nodularins	B

swinhoei and halichondramide [34] from a *Halichondria* sp, and to kabiramide C [46] from nudibranch eggmasses (Figure 16). Aplysiatoxins (Figure 17) were isolated from sea hares [33] and also from the marine cyanobacteria *Lyngbya* spp and *Oscillatoria* spp [49]. The marine cyanobacterial metabolites majusculamide C [12] and malyngamide C [1] from *L. majuscula* have structures similar to those of compounds from sea hares, dolastatin 11 [3,69] from *Dolabella auricularia* and stylocheilamide [75] from *Stylocheilus longicauda*, respectively (Figures 18 and 19). Majusculamide C was recently isolated from a sponge but was postulated to originate in a *Lyngbya majuscula* contaminant which provided the peptide through filter feeding by the sponge [85].

The sea hares apparently obtain these compounds or their precursors from their diets since certain marine cyanobacteria are favorite foods for these molluscs. The structural relationships between metabolites of terrestrial cyanobacteria and marine invertebrates, such as sponges and tunicates, are interesting. The primary producers of these compounds from sponges and tunicates may be symbiotic or associated cyanobacteria. In any case, it is quite interesting that marine and terrestrial cyanobacteria produce similar and sometimes the same secondary metabolites. Thus, it can be suggested that certain secondary metabolites isolated from marine invertebrates can be derived from certain microalgae.

General similarities in structural features are also observed between compounds from marine and terrestrial sources. Halogenated and sulfated compounds are characteristically found in marine organisms and have also been obtained from terrestrial cyanobacteria. Some examples were noted above in secondary metabolites from freshwater cyanobacteria. A number of sulfated metabolites have been obtained as bioactive metabolites (Figures 7, 10 and 12) and halogenated metabolites such as anabaenopeptilides 90-A and 202-B (Figure 7) and aeruginosins 98-A and C (Figure 10) are also isolated from freshwater cyanobacteria. Halogenated compounds are generally much more abundant in cyanobacteria than in other terrestrial organisms. Other examples of halogenated metabolites of terrestrial cyanobacteria are cyanobacterin from *Scytonema hofmanni* [43], hapalindole A from *Hapalosiphon fontinalis* [50], nostocyclophane D from *Nostoc linckia* [47] and puwainaphycins C and D from an *Anabaena* sp [19,48].

Both marine and terrestrial cyanobacteria produce structurally related secondary metabolites and it is of interest to speculate why the cyanobacteria produce such metabolites. One can imagine that ancient cyanobacteria evolved in the seas and lived together with certain marine invertebrates, providing metabolites produced by photosynthesis, and that some of the cyanobacteria left the seas and adapted to freshwater habitats and other terrestrial environments. The search for bioactive secondary metabolites from cyanobacteria is providing increasing numbers of useful and structurally characteristic compounds. Future research should yield more evidence for the biological relationships between marine invertebrates and cyanobacteria in marine and terrestrial habitats.

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Cyanopeptolin 954, a Chlorine-Containing Chymotrypsin Inhibitor of *Microcystis aeruginosa* NIVA Cya 43

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A new depsipeptide, cyanopeptolin 954 (**1**), was isolated from the freshwater cyanobacterium *Microcystis aeruginosa* NIVA Cya 43. The structure of the compound was elucidated by chemical and spectroscopic analyses, including 2D NMR and GC-MS of the hydrolysate. The major structural differences compared to previously characterized heptadepsipeptides of *Microcystis* are the replacement of the basic amino acid in position 4 by L-leucine, the presence of L-phenylalanine in position 6, and the uncommon residue 3′-chloro-*N*-Me-L-tyrosine in position 7. Cyanopeptolin 954 inhibited chymotrypsin with an IC_{50} value of 45 nM. Nostopeptin BN920, formerly isolated from the cyanobacterium *Nostoc*,[1] was isolated from the same strain of *Microcystis*, and a cis amide bond between Phe (6) and N-Me-Tyr (7) was shown. Nostopeptin BN920 inhibited chymotrypsin with an IC_{50} value of 31 nM.

The vast majority of the toxic cyanobacterial waterblooms worldwide are formed by cyanobacteria of the genus *Microcystis*. In most cases toxicity in *Microcystis* is due to the synthesis of hepatotoxins of the microcystin family. However, the extensive search for new protease inhibitors in recent years has revealed that only about 50% of the *Microcystis* water blooms show hepatotoxicity to mammals and other animals, but almost all contain protease inhibitors.[2,3] It can be hypothesized that under natural conditions these inhibitors may act as important defense molecules against potential grazers of *Microcystis*. Several groups of inhibitors of serine proteases were isolated from toxic and nontoxic *Microcystis* species. The most abundant are depsipeptides that contain the modified amino acid 3-amino-6-hydroxy-2-piperidone (Ahp). Considering Ahp as a derivative of glutamate, hexa-, hepta-, and octadepsipeptides have been found, all of which contain a six-amino-acid-membered ring with a lactone structure between the C-terminus and the hydroxy group of threonine. In all Ahp-containing depsipeptides in *Microcystis* either isoleucine or valine is the C-terminal amino acid, and the amino group of threonine or the N-terminal amino acid of the side chain is linked to a short-chain carboxylic acid. The amino acid adjacent to the C-terminus is an N-methylated aromatic amino acid (Phe, Tyr, ring-chlorinated Tyr[4], kynurenine[5]) or an N-methylated heteroaromatic amino acid (Trp[5−7]). Depsipeptides of *Microcystis* that show these basic structures haven been labeled cyanopeptolins,[8] micropeptins,[9] aeruginopeptins,[6] and microcystilides.[10] Following the reasoning of Bister at al.[11] that the cyanopeptolins were the first variants to be reported with a complete stereochemistry, we use this trivial name for the new compound cyanopeptolin 954.

Results and Discussion

Bioassay-guided fractionation of the extract of *Microcystis* NIVA Cya 43 by solid-phase extraction and subsequent separation on a reversed-phase HPLC resulted in two compounds displaying inhibitory activity against chymotrypsin. Upon analysis with LC-ESIMS, the MS/MS fragmentation pattern of one compound indicated the presence of one chlorine and suggested it to be an as yet unknown cyanopeptolin. The new compound, cyanopeptolin 954, exhibited a quasi molecular ion $[M + Na]^+$ at *m/z* 977.5. The second compound exhibited a quasi molecular ion $[M + Na]^+$ at *m/z* 943.5, which suggested it to be identical to the depsipeptide nostopeptin BN920 previously reported of the cyanobacterial genus *Nostoc*.[1] The peptide nature of cyanopeptolin 954 and nostopeptin BN920 was suggested by amino acid analysis of the hydrolysate that indicated the presence of Val, Leu, Glu, Thr, and Phe in both compounds and the presence of chloro-N-Me Tyr in cyanopeptolin 954 and of N-Me Tyr in nostopeptin BN920.

High-resolution electrospray mass spectrometry (ESI^+-MS) of the sodium adduct of cyanopeptolin 954 led to a base peak of *m/z* 977.4141, yielding the molecular formula $C_{46}H_{63}N_8O_{12}ClNa$ with a deviation of 0.5 ppm from the calculated exact mass. Therefore, uncharged cyanopeptolin 954 has the molecular formula $C_{46}H_{63}N_8O_{12}Cl$. The isotopic distribution of the ions indicated the presence of chlorine. The following N-acylated amino acids could be established from ^1H and ^{13}C 1D and homo- and heteronuclear 2D NMR spectra of cyanopeptolin 954 (Table 1): Glu, Leu, Val, 3-amino-6-hydroxy-2-piperidone (Ahp), O-acylated Thr, N-alkylated Phe, and N-methylated 3′-chloro-Tyr. NMR data of Ahp in cyanopeptolin 954 were consistent with previously published data of Ahp in other depsipeptides (see Supporting Information). The structure of N-methylated 3′-chloro-Tyr was deduced from the 2D NMR spectrum and confirmed by comparison of the NMR data with those published from scyptolins A and B[12] and micropeptins 478-A and -B.[13] An additional singlet in the ^1H spectrum was identified as an N-acetyl group. ROESY correlations (Table 1) from Phe (6) to Ahp (5) confirm that the nitrogen of Phe (6) is part of the Ahp moiety with both amino acids forming the typical hemiaminal structure.

The amino acid sequence of cyanopeptolin 954 could be deduced from sequential ROESY correlations (NOE) like α-$N_{i,\ i+1}$, β-$N_{i,i+1}$, δ-$\beta_{i,i+1}$, or $\alpha-\alpha_{i,i+1}$. The $\alpha-\alpha$ NOE from Phe (6) to *N*-methyl-3′-chloro-Tyr (7) indicated a cis amide

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Table 1. NMR Data of Cyanopeptolin 954 (**1**) in DMSO-d_6

amino acid	position	δ_C	δ_H	selected ROESY correlations	selected HMBC correlations
Ac[1]	1	169.6			Gln-NH, Ac-2
	2	22.8	1.85	Gln-NH	
Gln[2]	1	172.9			Thr-NH, Gln-2
	2	52.4	4.40	Thr-NH	
	3	31.9	2.08/2.11		
	4	174.2			Gln-3
	NH		8.05		
	NH_2		7.21/6.72		
Thr[3]	1	169.6			Leu-NH, Thr-2
	2	55.1	4.55	Leu-NH	
	3	72.2	5.39	Leu-NH, Ahp-NH	
	4	18.1	1.17		
	NH		7.97		
Leu[4]	1	170.6			Ahp-NH
	2	50.6	4.22	Ahp-NH	
	3	39.1	1.68/1.30		
	4	24.5	1.45		
	5	23.7/21.2	0.84/0.73		
	NH		8.35	Ahp-NH	
Ahp[5]	1	169.3			Ahp-2
	2	48.9	3.64		
	3	22.0	2.40/1.55		
	4	29.7	1.70/1.58		
	5	74.1	5.07	Phe-3, Phe-2′/6′	
	NH		7.07		
	OH		6.03	Val-NH	
Phe[6]	1	170.7			cmTyr-NCH_3
	2	50.7	4.72	cmTyr-2	
	3	35.8	2.89/1.83		
	1′	136.9			
	2′/6′	129.7	6.80		
	3′/5′	128.2	7.17		
	4′	126.6	7.13		
cmTyr[7]	1	169.6			Val-NH, cmTyr-2
	2	61.0	4.90	Val-NH	
	3	32.9	3.11/2.75		
	1′	129.5			
	2′	130.9	7.16		
	3′	120.0			
	4′	152.3			
	5′	117.0	6.99		
	6′	129.6	6.96		
	NCH_3	30.7	2.77	Val-NH	
	OH		10.1		
Val[8]	1	172.3			Thr-3
	2	56.1	4.74		
	3	31.2	2.08		
	4	19.7/17.6	0.87/0.73		
	NH		7.42		

bond between these two amino acids, as reported for cyanopeptolins A−D,[8] scyptolins A and B,[12] hofmannolin,[14] and A90720A.[15] In the cyanopeptolins with cis amide bonds published before, the two amino acids that form the cis amide bond are N-Me-Phe and either Leu (cyanopeptolins A−D[8]) or Thr (scyptolins A and B[12]), or the cis amide bonds are formed by N-Me-Tyr and either Leu (A90720A[15]) or 4′-O-Me-Tyr in hofmannolin[14] instead of *N*-methyl-(3′-chloro)-Tyr and Phe in the new compound. In cyanopeptolin 954 all other amide bonds were trans, and the sequential NOEs are listed in Table 1. The lactone ring closure between Thr-3 and Val-8 was verified by HMBC correlation from the β-proton of Thr-3 to the quaternary carbonyl C atom of Val-8. A common feature of many cyanopeptolins is the moiety located N-terminally from Thr-2. This moiety was composed of Gln and acetic acid. The acetylation of the α-NH_2 group of Gln was confirmed by the strong sequential

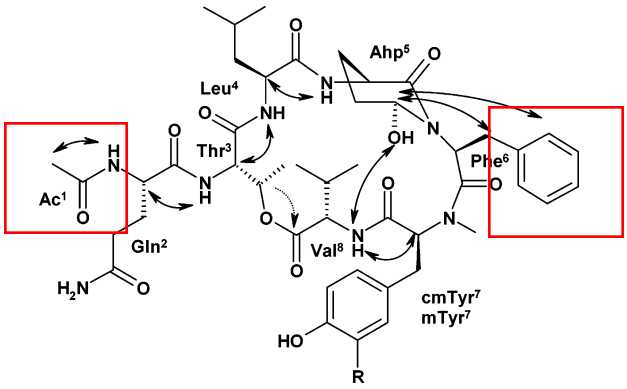

Figure 1. Chemical structure of cyanopeptolin 954 (**1**, R = Cl) and nostopeptin BN920 (**2**, R = H). Double-headed arrows designate important ROESY correlations, and the dotted arrow indicates an HMBC correlation. **mTyr**, *N*-methyl-Tyr; **cmTyr**, 3′-chloro-*N*-methyl-Tyr.

NOE of the Gln-NH to the acetyl group (Table 1). Thus the gross structure was determined as **1**.

The absolute configuration of cyanopeptolin 954 was determined by spectral and chemical methods. The stereochemistry of usual amino acids (Thr, Leu, Phe, and Val) was determined by chiral GC-MS amino acid analysis of the hydrolysate of **1**, which allowed us to assign the L-configurations to all these amino acids. Upon acid hydrolysis, Gln was converted to Glu and determined to be the L-enantiomer, indicating the L-configuration of Gln. Oxidation of cyanopeptolin 954 with CrO_3[17] followed by acid-catalyzed hydrolysis led to the formation of L-Glu as the major degradation product of Ahp, indicating the l-configuration of the α-position of Ahp. The absolute stereochemistry of (3*S*,6*R*)-L-Ahp was deduced from NOEs and corresponded to the configuration observed in other cyanopeptolines[18] (see Supporting Information). The stereochemistry of chloro-N-Me-Tyr was investigated by HPLC analysis with Marfey's reagent[19] and was determined to be the L-enantiomer. The inner ring current effect of the aromatic ring from N-Me-Phe or N-Me-Tyr induced a highfield shift to the β-, γ-, and δ-protons of either Leu or Thr in those cyanopeptolins.[8,12,14] This effect was also observed in cyanopeptolin 954, where H-β' of Phe-6 had a normal shift of 2.89 ppm but H-β'' was shifted upfield to 1.83 ppm and the aromatic protons H 2′/6′ of Phe-6 were shifted upfield to 6.80 ppm. A strong characteristic NOE between Ahp-δ-OH (5) and Val-NH (8) was detected in cyanopeptolin 954 and nostopeptin BN920, as well as in cyanopeptolins A−D,[8] cyanopeptolin S,[16] scyptolins A and B,[12] and hofmannolin.[14] This confirms the assumption of identical configurations of the amino acids in this part of the molecule. Therefore the amino acid *N*-methyl-(3′-chloro)-Tyr is in the L-configuration.

The molecular formula of nostopeptin BN920 was deduced from the base peak of ESI^+MS (*m/z* 943.4537) yielding the molecular formula $C_{46}H_{64}N_8O_{12}Na$ with a deviation of 0.2 ppm from the calculated exact mass. Therefore, uncharged nostopeptin BN920 has the molecular formula $C_{46}H_{64}N_8O_{12}$. With the exception of N-methylated 3′-chloro-Tyr the same amino acids as in **1** were found in the ^1H and ^{13}C 2D NMR spectra of nostopeptin BN920 (data not shown). Instead of N-methylated 3′-chloro-Tyr, N-methylated Tyr was detected. The amino acid sequence of nostopeptin BN920 was deduced from sequential NOEs and suggested this peptide to be identical to nostopeptin BN920.[1] However, this peptide has not been reported in *Microcystis* before, and sequential NOEs unequivocally revealed a cis amide bond between Phe (6) and N-Me-Tyr

(7) (Table 1). In Ploutno and Carmeli[1] the amide bond between Phe (6) and N-Me-Tyr (7) is not defined in the text; however, the structure shows the trans-conformer. Our results suggest that the peptide isolated from *Microcystis* is a different conformer of nostopeptin BN920 and hence is named nostopeptin BN920 according to Ploutno and Carmeli.[1] In this study all other amide bonds in nostopeptin BN920 were determined to be trans. The stereochemistry of the amino acids and of Ahp was found to be identical to those in **1**. The stereochemistry of N-Me-l-Tyr was determined by HPLC analysis with Marfey's reagent.[19] Similar to **1** the lactone ring closure between Thr-3 and Val-8 was verified by HMBC correlation, and the structure was determined as **2**.

Cyanopeptolin 954 (**1**) and nostopeptin BN920 (**2**) inhibited bovine chymotrypsin with IC_{50}'s of 4.1 and 3.0 ng mL^{-1} (44.5 and 31.2 nM), respectively. Both compounds did not inhibit bovine trypsin at 15.9 and 10.6 μg mL^{-1} (17.3 and 11.4 μM), respectively. Thus cyanopeptolin 954 and nostopeptin BN920 are among the strongest Ahp-containing chymotrypsin inhibitors. The strongest chymotrypsin inhibitor so far described is micropeptin T-20 with an IC_{50} of 2.5 nM,[4] while the majority of cyanopeptolins exhibit IC_{50} values of 2−4 μM. Among the heptadepsipeptides of *Microcystis* inhibition of trypsin or chymotrypsin is mutually exclusive. Compounds that display inhibitory activity against trypsin but not against chymotrypsin (cyanopeptolin A,[20] micropeptins A and B,[9] micropeptin SF995,[21] micropeptins SD944 and SD999,[5] micropeptins EI964 and EI992[22]) are characterized by a basic amino acid in position 4, while heptadepsipeptides that inhibit chymotrypsin but not trypsin (micropeptin 88A,[23] micropeptins SD979 and SD1002,[5] cyanopeptolin 963A,[11] cyanopeptolins 954 and 920, this study) are characterized by a neutral (Leu) or aromatic (Phe, Tyr) amino acid in position 4. This corresponds to the known specificity of chymotrypsin (preferred cleavage of peptide bonds at Phe and Tyr) and trypsin (cleavage at Lys or Arg). Trypsins and chymotrypsins have recently been shown to be the major digestive proteases in the most important natural grazer of *Microcystis*,[24] and it remains to be tested if the natural grazing pressure drives the evolution of new variants of depsipeptides in natural ecosystems.

Experimental Section

General Experimental Procedures. Optical rotations were determined on a Perkin-Elmer 241 polarimeter using a quartz cell of 10 cm length and 1 mL volume. UV spectra were recorded on a Cary 50 photometer (Varian). NMR spectra were recorded on a Bruker DMX500 (Bruker-Daltonics, Fällanden, Switzerland) spectrometer using a triple inverse probe for the 1D-^{1}H and 2D spectra. The sample concentration was 0.5−1 mM in DMSO-d_6. ^{1}H and ^{13}C shifts are referred to DMSO-d_6 = 2.49 and 39.9 ppm, respectively The following NMR experiments were carried out: 1D-^{1}H, ^{1}H−^{1}H-COSY, ^{1}H−^{1}H-ROESY, ^{1}H−^{1}H-TOCSY, ^{1}H−^{13}C-COSY (HSQC). A Bruker DRX500 with TXI-CryoProbe was used for the ^{1}H−^{13}C-long-range COSY (HMBC) and a DRX400 with ^{13}C CryoProbe for the ^{13}C spectra. ESIMS spectra were recorded in positive and negative ion mode, using a 9.4 T Apex-III FT-MS (Bruker Daltonics, Fällanden, Switzerland). HPLC-ESI-MS-derived mass spectra were obtained on a LC-MS (LCQ Duo mass spectrometer, Finnigan Thermoquest) equipped with an electrospray source (ESI). The composition of the derivatized hydrolysate was determined using GC-MS (Finnigan GCQ). HPLC separations were performed on a Shimadzu 10AVP system equipped with a diode array detector and an autosampler.

Culture of *Microcystis*. *M. aeruginosa* NIVA Cya 43 (Culture Collection of Algae, Norwegian Institute for Water Research) was grown in 10 L glass bottles in mineral medium[25] with aeration (filtered, 0.3 L min^{-1}, without additional CO_2). Cultures were illuminated continuously with fluorescent tubes at an intensity of 60 μmol $m^{-2}\cdot s^{-1}$ at 20 °C for 20−30 days. Then cells were harvested by centrifugation (4.000*g*), lyophilized, and kept in a freezer at −20 °C until extraction.

Extraction and Isolation. Portions of the lyophilized cyanobacterial biomass (1 g) were extracted with MeOH (100 mL) for 12 h in the dark, and the extract was separated by centrifugation from the residue. The supernatant was diluted with ultrapure water to a 10% methanol solution and was allowed to pass under vacuum through an equilibrated reversed-phase ODS cartridge (10 g sorbent; Varian, Darmstadt, Germany). Material retained on the cartridge was eluted in steps with 50 mL each of 20, 40, 60, and 80% methanol in water and finally with 100% methanol. The fraction eluting with 60% methanol from the cartridge was evaporated in a vacuum rotary evaporator at 40 °C. The residue was dissolved in 50% methanol (1 mL) and fractionated by reversed-phase HPLC (Nucleosil 150-5 C18, 250 × 4 mm, Macherey-Nagel, Düren, Germany; DAD at 220 nm, 1 mL min^{-1} flow rate with 33% solvent B; solvent A: H_2O and 0.05% TFA; solvent B: 90:10 acetonitrile/water and 0.05% TFA). The peaks eluting at 8.8 and 12.7 min were collected in about 50 HPLC runs. Subsequently the collected volume was diluted with the same volume of H_2O and applied to an ODS cartridge (Varian, equilibrated with 20% MeOH). The cartridge was washed with 5% MeOH to neutral pH; the inhibitors were eluted with 50 mL of MeOH, evaporated to dryness, and dissolved in MeOH (1 mL) to obtain **1** in 0.32% and **2** in 0.16% yield based on the dry weight of the cyanobacterium.

Analysis of the Hydrolysate. The amino acids of the peptides were determined with GC-MS after hydrolysis of 100 μg of each highly purified cyanopeptolin in 6 M HCl at 110 °C for 48 h. The derivatization of the dried hydrolysate and of equivalent amounts of amino acid standards was performed with MTBSTFA in tetrahydrofurane and TFA (20:25:0.05) according to Blom et al.[26] Analyses were conducted on a capillary column (30 m DB-1301, 0.32 mm i.d., 0.25 μm film thickness under the following separation conditions: 1 min at 120 °C, 120 to 250 °C at a rate of 10 °C min^{-1}. The retention times (min) of amino acid standards were as follows: Val (9.84), Leu (10.35), Glu (16.22), Thr (13.58), Phe (14.42), N-MeTyr (17.12), 3′-chloro-N-MeTyr (19.55).

To determine the configuration of the amino acids, the hydrolysate was dried and subsequently acylated with trifluoroacetic acid anhydride (Fluka, Buchs, Switzerland) at 80 °C for 1 h. Analyses were conducted on a Chirasil-Val column (Permabond-L-Chirasil-Val; 25 m × 0.25 mm; Macherey-Nagel, Düren, Germany) under the following separation conditions: 2 min at 80 °C, 80 to 180 °C at the rate of 8 °C min^{-1}, 10 min at 180 °C. The retention times (min) of enantiomeric amino acids on the Chirasil Val column were as follows: D-Val (6.54), L-Val (6.64), D-Thr (6.86), l-Thr (6.98), D-Leu (8.71), L-Leu (8.92), D-Glu (13.64), L-Glu (13.72), D-Phe (14.81), L-Phe (14.87). The determination of the absolute configuration of each amino acid was confirmed by spiking the derivatized hydrolysates with the derivatized authentic amino acids.

Oxidation of Cyanopeptolin 954 and Nostopeptin BN920 by CrO_3. CrO_3 oxidation was performed according to Itou et al.[17] Compound **1** or **2** (30 μg) was dissolved in 500 μL of a solution of CrO_3 in ethanol (1 mg mL^{-1}) and stirred at room temperature for 2 h. Then 500 μL of ultrapure water was added and the sample stored at 4 °C for 12 h. Subsequently the sample was filled up to 10 mL with 90/10 water/ethanol and subjected to an ODS cartridge (Varian, equilibrated with 10% MeOH; v/v). The cartridge was flushed with 10% MeOH and the sample eluted with 100% MeOH. The eluate was evaporated to dryness (40 °C) and hydrolyzed as described above. Glu that was obtained after hydrolysis allowed the determination of the configuration of the α-position of Ahp.

Synthesis of N-Me-L-Tyr. *N*-methyl-L-tyrosine was prepared according to a slightly modified protocol.[27] *N*-Benzyl-L-tyrosine: To a stirred solution of 9.06 g (50 mmol) of L-tyrosine in 30 mL of a 4 M aqueous sodium hydroxide solution was

added 5 mL (50 mmol) of freshly distilled benzaldehyde as a single portion. After 30 min of stirring, the mixture became homogeneous and 570 mg (150 mmol) of sodium borohydride was added carefully in small portions, keeping the temperature below 15 °C. After stirring for another 30 min the same procedure was repeated with the above given amounts of benzaldehyde and sodium borohydride. After stirring for an additional 2 h, the reaction mixture was extracted two times with 30 mL of diethyl ether each and neutralized with 1 M aqueous hydrochloric acid to give a precipitate. The crude *N*-benzyl-L-tyrosine was filtered off, washed with water, dried under vacuum (yield 88%), and used for the methylation without further purification. *N*-Benzyl-*N*-methyl-L-tyrosine: 5.15 g (20 mmol) of finely powdered *N*-benzyl-L-tyrosine was treated with a mixture of 2.3 mL (60 mmol) of formic acid and 2 mL (24 mmol) of aqueous formaldehyde solution (38−40%) at 100 °C. After 3 h the reaction was finished as monitored by TLC. Evaporation to dryness and recrystallization of the residue from water/ethanol gave the desired compound as a colorless solid (yield: 78%). *N*-Methyl-L-tyrosine: A solution of 4 g (14.7 mmol) of *N*-benzyl-*N*-methyl-L-tyrosine was dissolved in 60 mL of a mixture consisting of 58 mL of glacial acetic acid and 2 mL of concentrated hydrochloric acid. The mixture was hydrogenated with 200 mg of palladium on charcoal (5 mol % Pd). After hydrogenation the catalyst was filtered off and the solution was evaporated to dryness. Recrystallization from water/ethanol gave the desired compound in 71% yield, $[\alpha]^{20}_D$ +30.4 [*c* 1, 1:1 6 M HCl/HOAc (v:v)].

Synthesis of 3′-Chloro-N-Me-Tyr. N-Me-D-Tyr was obtained from Bachem (Bubendorf, Switzerland). A mixture of 60 mg of N-Me-D-Tyr or N-Me-L-Tyr and 1 mL of freshly distilled sulfuryl chloride was warmed at 80 °C (3 min) according to Ishida et al.[13] until gas no longer evolved. Then 2 mL of sulfuryl chloride was added and warmed at 80 °C (1 h). Thereafter, excess sulfuryl chloride was removed by evaporation and freeze-drying.

HPLC Analysis of N-Me-Tyr and chloro-N-Me-Tyr. The enantiomers of N-Me-Tyr and chloro-N-Me-Tyr were determined by HPLC using Marfey's method.[19] To the acid hydrolysate (6 M HCl at 110 °C for 24 h) of 100 μg of **1** or **2** was added 50 μL of 1-fluoro-2,4-dinitrophenyl-5-L-alanineamide (L-FDAA$NaHCO_3$, Novabiochem, Läufelfingen, Switzerland) in acetone (10 mg mL^{-1}). The mixtures were heated at 80 °C for 3 min. After cooling, 120 μL of 1 M HCl and 300 μL of 50% acetonitrile were added. The derivatives were analyzed on a C-18 Supelco-Sil ODS reversed-phase column (4.6 × 250 mm; Supelco; Bellefonte, PA); the mobile phase was acetonitrile/H_2O/trifluoroacetic acid (23/77/0.05; v/v/v). The retention times of standards (min) were as follows: N-Me D-Tyr L-FDAA (29.7 min), N-Me L-Tyr L-FDAA (30.2 min), chloro-N-Me D-Tyr L-FDAA (49.8 min), and chloro-N-Me L-Tyr L-FDAA (51.9 min). The retention times of N-Me Tyr L-FDAA in the acid hydrolysate of nostopeptin BN920 was 30.3 min, and that of chloro-N-Me Tyr L-FDAA in the acid hydrolysate of cyanopeptolin 954 was 51.8 min.

Molar Absorption Coefficient. The molar absorption coefficient was determined by quantitative analysis of L-Leu after acidic hydrolysis of **1** and **2**. L-U-$^{13}C_6$ Leu (Euriso-top, Saarbrücken, Germany) was added as an internal standard before hydrolysis. After derivatization with MTBSTFA and GC-EIMS, the integrals of the fragment ions *m/z* 200, 274, 302, and 360 were used for quantitative analysis of Leu and *m/z* 205, 279, 308, and 366 for the ^{13}C-labeled Leu. These values were correlated to the UV absorption of **1** and **2** measured before hydrolysis.

Inhibition of Proteases. The inhibition of trypsin and chymotrypsin was measured according to Von Elert et al.[24] Solutions of *N*-α-benzoyl-DL-arginine 4-nitroanilide hydrochloride (Sigma, 187 μM) and *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine 4-nitroanilide (Sigma, 125 μM) in potassium phosphate buffer (0.1 M, pH 7.5) were used as substrates for both trypsin (T-4665, Sigma) and α-chymotrypsin (C-4129, Sigma) from bovine pancreas. The absorption change was measured for 5 min at 20 °C at 390 nm in 1 mL with a Cary 50 photometer.

Cyanopeptolin 954 (1): amorphous powder; UV (MeOH) λ_{max} 282 (ϵ 2000) nm; ^1H and ^{13}C NMR, see Table 1; ESIMS (positive mode) *m/z* 976.9 (100%), 497.2 (80%), 936.9 (20−25%), 940.8 (20−25%), 993.9, 489.3; ESIMS (negative mode) *m/z* 250.1 (100%), 953.0 (20%), 988.9 (15%); HRESIMS (positive) *m/z* 977.4141 ($C_{46}H_{63}N_8O_{12}ClNa$, relative mass error $\Delta_m = 0.5$ ppm).

Nostopeptin BN920 (2): amorphous powder; UV (MeOH) λ_{max} 279 (ϵ 1600) nm; ESIMS (positive mode) *m/z* 942.9 (100%), 480.4 (50%), 903.0 (10−15%), 940.8, 958.9, 472.2, ESIMS (negative mode) *m/z* 250.1 (100%), 919.1 (20%), 955.0 (15%); HRESIMS (positive) *m/z* 943.4537 ($C_{46}H_{64}N_8O_{12}Na$, relative mass error $\Delta_m = 0.5$ ppm).

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Supporting Information Available: NMR data of 3-amino-6-hydroxy-2-piperidone (Ahp) in previously published depsipeptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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