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# Modified peptides from a water bloom of the cyanobacterium *Nostoc* sp.

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**Abstract**—Six new metabilites, nostopeptin BN920 (**1**), nostoginin BN741 (**2**), nostoginin BN578 (**3**), banyascyclamide A (**4**) banyascyclamide B (**5**) and banyascyclamide C (**6**), were isolated from the hydrophilic extract of a *Nostoc* sp. The planar structure of compounds **1**–**6** was determined by homonuclear and inverse-heteronuclear 2D NMR techniques as well as high-resolution mass spectrometry. The absolute configuration of the asymmetric centers was studied using Marfey's method for HPLC. Compound **1** inhibits the serine-protease chymotrypsin while compound **2** inhibits amino-proteases. 

Heterocystous cyanobacteria species of the genus *Nostoc* are prolific producers of natural products that derive from polyketide-, terpenoid-, alkaloid- and non-ribosomal-peptide-biosynthesis. Several unusual groups of polyketides were isolated from *Nostoc* spp. including the only naturally occurring cyclophens, nostocyclopanes A–D from *Nostoc linckia*,[1] and nostocyclyne A from *Nostoc* sp.,[2] and the boric acid containing borophycin from *N. linckia*[3] and *Nostoc spongiaeforme* var. tenue.[4] The bacteriohopanoids isolated from *Nostoc* sp.,[5] and the four antibacterial diterpenoids isolated from *Nostoc commune*,[6,7] are the only terpenoides isolated from this group of cyanobacteria. Staurosporine derivatives from *Nostoc sphaericum*[8] and the violet pigment, nostocine A, from *N. spongiaeforme*,[9] are two examples of alkaloids produced by this genus of cyanobacteria. Non-ribosomal peptides are by far the dominant group of biologically active natural products that are produced by members of the genus *Nostoc*. Nostocyclamides A and M from *Nostoc* sp.,[10,11] and tenuecyclamides A–D from *N. spongiaeforme* var. tenue[4] are modified cyclic hexapeptides typically found in these cyanobacteria. A novel fernilatae linear modified peptide, muscoride A, was isolated from *Nostoc muscorum*.[12] Toxic microcystins[13] were isolated from a *Nostoc* sp. along with a novel cyclic peptide, nostophycin.[14] Cultures of *Nostoc minutum* produce two groups of elastase inhibitors—the cyclic peptides microviridins G and H[15] and the cyclic depsipeptides, nostopeptins A and B.[16] The cytotoxic depsipeptides, cryptophicins,[17] which are in clinical trails as anti-solid tumor drugs, are another excellent example of the diverse biosynthetic capabilities of this group of cyanobacteria.

*Keywords*: cyanobacteria; *Nostoc*; protease inhibitors; cyclichexapeptides.

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*Nostoc* sp.,[18] TAU strain IL-235, appears as clumps of green–yellow gelatinous material. Young colonies are initially attached to the rocky riverbed. When mature, they fill with air, detach and float as yellow clumps on the water surface. In the summer of 1999, we collected large quantities of the cyanobacterium clumps. The hydrophilic and lipophilic extracts of this bacterium yielded two UV-absorbing pigments, nostodione A and prenostodione.[19] In this paper we describe six additional metabolites isolated from the hydrophilic extract of a natural bloom of this cyanobacterium.

Nostopeptin BN920 (**1**) (Scheme 1) was isolated as an amorphous white solid. The molecular formula of **1**, $C_{46}H_{64}N_8O_{12}$, was deduced from high-resolution FAB MS measurements of its sodiated molecular cluster ion (*m/z* 943.4523). Examination of the $^1H$ and $^{13}C$ NMR spectra of compound **1** reveal that it is related in structure to the micropeptins previously isolated in our laboratory.[20] The $^1H$ NMR, in DMSO-$d_6$ revealed five doublet NH proton signals and two singlet-signals of the primary amide group between $\delta$ 6.71 and 8.36 ppm, pointing to seven amino acid residues (taking into account the NMe-aromatic amino acid and the Ahp residue that count as two amino acids). Analysis of the 1D ($^1H$, $^{13}C$ and DEPT) and 2D (COSY, TOCSY, ROESY, HMQC and HMBC) NMR data (Table 1) revealed the seven amino acid units and the acetic acid that constitute compound **1**, namely: valine, NMe-tyrosine, *N*,*N*-disubstituted phenylalanine, amino-hydroxy-piperidone (Ahp), leucine, threonine, glutamine and acetic acid. The ester linkage of **1** arises from the carbonyl of valine and the hydroxyl of threonine. All the proton and carbon signals of the latter residues, except the leucine carbonyl, were assigned from the COSY, TOCSY, HMQC and HMBC data. No HMBC correlation was observed between the carbonyl of leucine and the leucine side-chain protons. This

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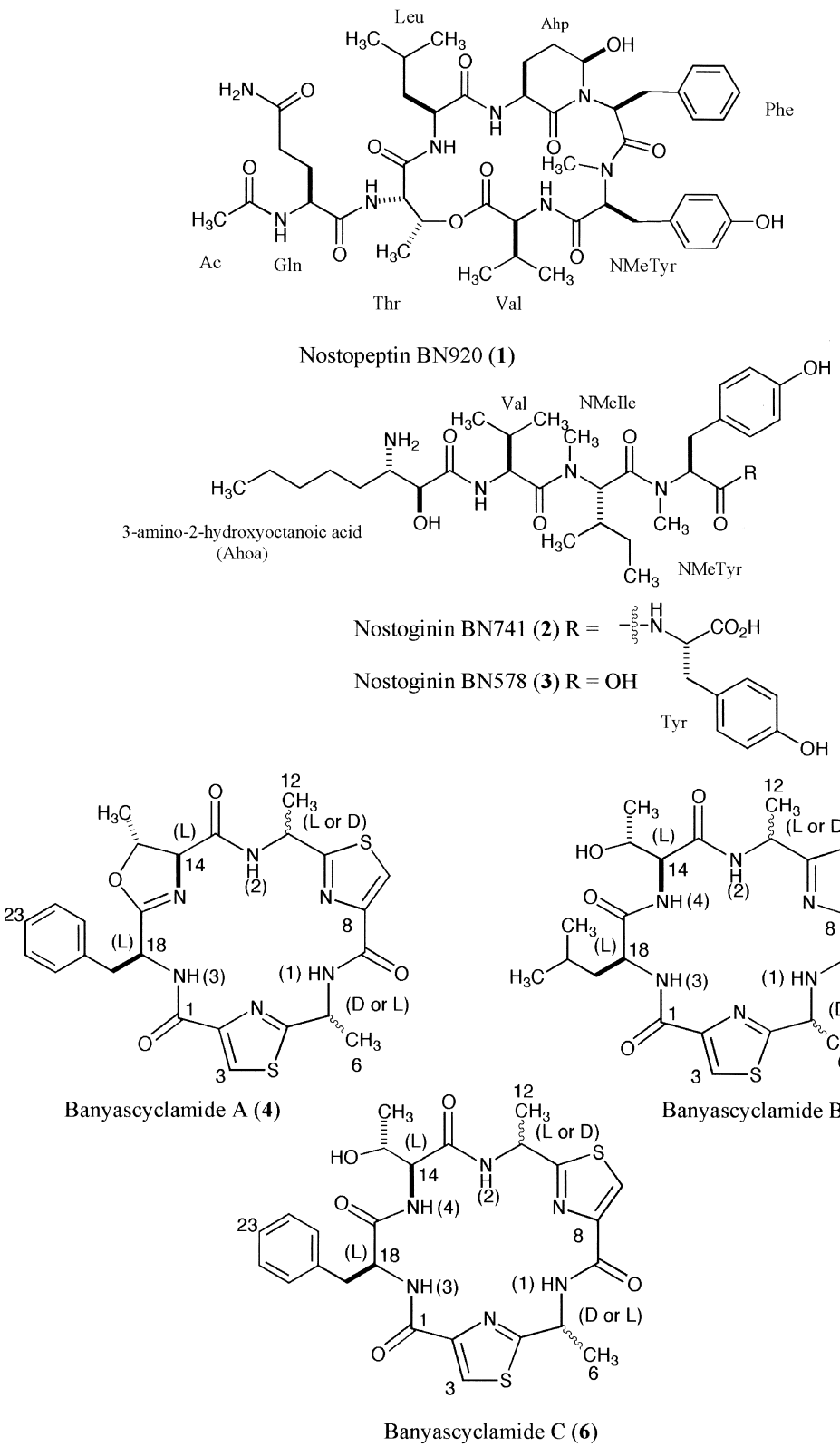


**Scheme 1.**

is a known phenomenon in many micropeptins, which most probably arises from the dihedral angle between the carbonyl carbon and the side-chain protons of the amino acid at this position of the cyclic peptolide.[20] The assignment of the carbonyl at $\delta_C$ 170.3 ppm to leucine is based on the NOE correlation between the proton at position 2 of the leucine and Ahp-NH as well as the HMBC correlation of the Ahp-NH proton with this carbonyl. The amino acid sequence of nostopeptin BN920 (**1**) was determined from the HMBC correlations (Table 1) of the NH proton of an amino acid with the carbonyl of an adjacent amino acid (Val-NMe-Tyr, Ahp-Leu, Leu-Thr, Thr-Gln and Gln-Ac),

**Table 1**. NMR data of nostopeptin BN920 (**1**)

| Position | | $\delta_C$, mult.[a] | $\delta_H$, mult., $J$ (Hz) | LR H−C correlations[b] | NOE correlations[c] |
|---|---|---|---|---|---|
| Val | 1 | 172.3 s | | Val-2, Thr-3 | |
| | 2 | 55.9 d | 4.72 dd 9.6, 4.5 | Val-4,5,NH | Val-3,4,5,NH, Tyr-5,5′ |
| | 3 | 31.0 d | 2.07 m | Val-2,4,5 | Val-2 |
| | 4 | 17.4 q | 0.72 d 6.9 | Val-2,3,5 | Val-2 |
| | 5 | 19.4 q | 0.86 d 6.9 | Val-2,3,4 | Val-2 |
| | NH | | 7.40 d 9.6 | | Val-2, Tyr-2 |
| NMeTyr | 1 | 169.3 s | | Val-NH, Tyr-2 | |
| | 2 | 61.0 d | 4.90 dd 11.3, 2.3 | Tyr-NMe | Val-NH, Tyr-3,5,5′ |
| | 3 | 33.0 t | 2.71 dd 12.1, 11.3, 3.09 dd 12.1, 2.3 | Try-2,5,5′ | Tyr-Nme, Tyr-2,5,5′ |
| | 4 | 127.6 s | | Tyr-2,3,3′,5,5′,6,6′ | |
| | 5,5′ | 130.6 d | 6.99 d 8.4 | Tyr-3,3′,5′ | Tyr-2,3,6,6′, Val-2 |
| | 6,6′ | 115.5 d | 6.77 d 8.4 | Tyr-5,5′,6′,OH | Tyr-5,5′,OH |
| | 7 OH | 156.4 s | 9.34 s | Tyr-5,5′,6,6′,OH | Tyr-6,6′ |
| | NMe | 30.5 q | 2.74 s | | Tyr-2, Phe-2 |
| Phe | 1 | 170.5 s | | Tyr-2,NMe, Phe-2 | Phe-5,5′ |
| | 2 | 50.4 d | 4.73 dd 12.5, 5.0 | Phe-3′ | Phe-5,5′ |
| | 3 | 35.5 t | 1.78 dd 13.8, 5.0, 2.83 dd 13.8, 12.5 | Phe-2,5,5′ | Phe-5,5′, Phe-5,5′, Tyr-NMe, Ahp-5,5′,OH |
| | 4 | 136.9 s | | Phe-2,3′,6,6′ | |
| | 5,5′ | 129.5 d | 6.82 d 7.1 | Phe-3′,5′,7 | Phe-2,3,3′ |
| | 6,6′ | 127.9 d | 7.17 dd 7.1, 7.4 | | Phe-5,5′ |
| | 7 | 126.3 d | 7.13 t 7.4 | Phe-5,5′,6,6′ | |
| Ahp | 1 | 169.1 s | | Ahp-2,5 | |
| | 2 | 48.7 d | 3.62 ddd 15.8, 9.2, 6.7 | Ahp-NH | Ahp-3,NH, Phe-3,5,5′, Leu-6 |
| | 3 | 21.8 t | 2.41 dt 15.8, 12.1, 1.58 m | Ahp-2 | Ahp-2, Ahp-NH |
| | 4 | 29.4 t | 1.66 m, 1.54 m | | |
| | 5 | 73.9 d | 5.05 brs | Phe-2 | Ahp-3,4, Phe-3′,6,6′ |
| | OH | | 6.00 d 2.9 | | Phe-3′ |
| | NH | | 7.07 d 9.2 | | Leu-2 |
| Leu | 1 | 170.3 s | | Ahp-NH | |
| | 2 | 50.4 d | 4.21 ddd 11.1, 8.7, 3.0 | Leu-NH | Leu-3,4,5,6,NH |
| | 3 | 39.4 t | 1.69 m, 1.29 m | Leu-5,6 | Leu-2 |
| | 4 | 24.3 d | 1.44 m | Leu-5 | Leu-2 |
| | 5 | 23.4 q | 0.83 d 6.6 | Leu-6 | Leu-2 |
| | 6 | 21.0 q | 0.71 d 6.6 | Lys-5 | Leu-2, Ahp-2 |
| | NH | | 8.36 d 8.7 | | Thr-2,3 |
| Thr | 1 | 169.4 s | | Leu-NH, Thr-2,3 | |
| | 2 | 55.0 d | 4.55 brd 9.8 | Thr-4 | Leu-NH, Thr-4,NH |
| | 3 | 72.0 d | 5.38 brq 6.5 | Thr-2,4 | Leu-NH, Thr-4,NH |
| | 4 | 17.9 q | 1.17 d 6.5 | Thr-2,3 | Thr-2,3 |
| | NH | | 7.96 d 9.8 | | Gln-2 |
| Gln | 1 | 172.6 s | | Gln-2,Thr-2,NH | |
| | 2 | 52.2 d | 4.39 ddd 13.9, 7.6, 5.7 | Gln-4,4′,NH | Gln-3,4, Thr-NH, Ac-2 |
| | 3 | 28.1 t | 1.83 m, 1.65 m | Gln-2,4,4′,NH | Gln-2 |
| | 4 | 31.6 t | 2.11m (2H) | Gln-2,3,3′,$NH_2$ | Gln-2 |
| | 5 | 174.0 s | | Gln-4,4′, $NH_2$ | |
| | NH | | 8.25 d 7.6 | | Ac-2 |
| | $NH_2$ | | 6.71 s, 7.22 s | | $NH_2$-1, $NH_2$-2 |
| Ac | 1 | 169.4 s | | Ac-2, Gln-NH | |
| | 2 | 22.6 q | 1.84 s | | Gln-2,NH |

Carried out on an ARX-500 Bruker instrument.
[a] Multiplicity and assignment from HMQC experiment.
[b] Determined from HMBC experiment, $^nJ_{CH}$=8 Hz, recycle time 1 s.
[c] By ROESY experiment, mixing time 400 ms.

the NMe protons of NMe-Tyr with Phe-carbonyl and the Phe-H-2 with C-5 of the Ahp residue. The ester bond was assigned from an HMBC correlation between H-3 of threonine and the carbonyl of valine. The amino acid sequence could also be assembled from the ROESY data (Table 1). Fragmentations in the positive ion FAB MS of **1** farther support the suggested structure. The quasimolecular ion at *m/z* 943 [(M+Na)$^+$] produces two strong fragment ions at *m/z* 537 and 406, which are derived from the dissociation of the Thr-Val ester bond and the Leu-Ahp amide bond. The fragment ion at *m/z* 537 is composed of (NH-Ahp-Phe-NMeTyr-Val-CO+H)$^+$ while that at *m/z* 406 is composed of (Ac-Gln-Thr-Leu-CO−H+Na)$^+$. Acid hydrolysis of **1** and derivatization with Marfey's reagent,[21] followed by HPLC analysis, demonstrated the L-stereochemistry of the valine, NMe-tyrosine, phenylalanine, leucine, threonine and glutamine residues. Jones oxidation[22] of **1**, followed by a similar hydrolysis, derivatization and

**Table 2**. NMR data of nostoginin BN741 (**2**)

| Position | | $\delta_C$, mult.[a] | $\delta_H$, mult., $J$ (Hz) | LR H–C correlations[b] | NOE correlations[c] |
|---|---|---|---|---|---|
| Tyr | 1 | 172.9 s | | | |
| | 2 | 53.8 d | 4.35 ddd 5.1, 6.8, 7.9 | Tyr-3 | Tyr-3,5,NH, NMeTyr-2 |
| | 3 | 35.8 t | 2.81 dd 7.9, 14.3, 2.93; dd 5.1, 14.3 | Tyr-5,5′ | Tyr-2,3′,NH, Tyr-3,NH |
| | 4 | 127.6 s | | Tyr-3,3′,6,6′ | |
| | 5,5′ | 130.1 d | 6.95 d 8.3 | Tyr-3,3′ | Tyr-2, Val-3,4, Ahoa-3 |
| | 6,6′ | 115.1 d | 6.63 d 8.3 | Tyr-5,5′ | Val-2,3,4, Ahoa-3 |
| | 7 | 156.0 s | | Tyr-5,5′,6,6′,OH | |
| | OH | | 9.17 s | | |
| | NH | | 7.94 d 6.8 | | Tyr-2,3, NMeTyr-2,3 |
| NMeTyr | 1 | 170.1 s | | Tyr-NH | |
| | 2 | 55.8 d | 5.42 dd 5.1, 12.2 | NMeTyr-NMe | Tyr-2,NH, NMeIle-6 |
| | 3 | 33.4 t | 3.02 dd 5.1, 15.4, 2.73; dd 12.2, 15.4 | NMeTyr-5,5′ | Tyr-NH, NMeTyr-2,3′,5,5′, NMeTyr-3 |
| | 4 | 127.3 s | | NMeTyr-3,3′,6,6′ | |
| | 5,5′ | 129.5 d | 6.95 d 8.3 | NMeTyr-3,3′,5,5′ | NMeTyr-3,6,6′, NMeIle-NMe |
| | 6,6′ | 115.1 d | 6.63 d 8.3 | NMeTyr-5,5′ | NMeTyr-5,5′,OH, NMeIle-NMe |
| | 7 | 156.0 s | | NMeTyr-5,5′,6,6′,OH | |
| | OH | | 9.17 s | | NMeTyr-6,6′ |
| | NMe | 30.3 q | 2.62 s | | NMeTyr-3, NMeIle-2,3 |
| NMeIle | 1 | 169.6 s | | NMeIle-2, NMeTyr-NMe | |
| | 2 | 56.9 d | 4.90 d 10.6 | NMeIle-6,NMe | NMeIle-NMe, NMeTyr-NMe |
| | 3 | 32.6 d | 1.91 m | NMeIle-2,4,4′,6 | NMeIle-4,4′,5, NMeTyr-NMe |
| | 4 | 23.6 t | 0.70 m, 1.08 m | NMeIle-5,6 | NMeIle-3, NMeIle-2,3,5, Val-2 |
| | 5 | 11.2 q | 0.76 t 6.7 | NMeIle-4,6 | NMeIle-2,3,4, NMeTyr-5,5′,6,6′ |
| | 6 | 16.7 q | 0.67 d 6.3 | NMeIle-4′,5 | NMeIle-2,3,4′, NMeTyr-2 |
| | NMe | 29.2 q | 2.42 s | | NMeIle-2,3,4,4′, Val-2,3,4, NMeTyr-2,5,5′,6,6′ |
| Val | 1 | 170.9 s | | NMeIle-NMe | |
| | 2 | 53.2 d | 4.48 dd 4.5, 9.0 | Val-4,5 | Val-3,4,5, NMeIle-Nme |
| | 3 | 29.6 d | 1.59 m | Val-4,5 | Val-2,4,5,NH, NMeTyr-5,5 ′,6,6′ |
| | 4 | 20.1 q | 0.76 d 6.7 | Val-5 | Val-2,3,NH |
| | 5 | 15.9 q | 0.64 d 6.7 | Val-4 | Val-2,3, NMeTyr-Nme |
| | NH | | 7.70 d 9.0 | | Ahoa-2, Val-2,3,4, Ile-4′ |
| Ahoa | 1 | 170.3 s | | Ahoa-2, Val-NH | |
| | 2 | 70.9 d | 4.24 dd 3.0, 5.1 | Ahoa-2-OH,4 | Val-NH, Ahoa-2-OH,3,5 |
| | 2-OH | | 6.40 d 5.1 | | Ahoa-2 |
| | 3 | 53.2 d | 3.40 m | Ahoa-2,4 | Ahoa-2,7,8 |
| | 3-$NH_2$ | | 7.99 d 7.9 | | Ahoa-2,3,4 |
| | 4 | 27.1 t | 1.49 m, 1.33 m | | Ahoa-2,6,NH |
| | 5 | 24.6 t | 1.22 m, 1.35 m | | |
| | 6 | 31.2 t | 1.18 m | Ahoa-7,8 | Ahoa-2,3,4,NH |
| | 7 | 21.9 t | 1.23 m | Ahoa-8 | Ahoa-2,3,NH |
| | 8 | 14.0 q | 0.84 t 7.2 | Ahoa-6,7 | Ahoa-3 |

Carried out on an ARX-500 Bruker instrument.
[a] Multiplicity and assignment from HMQC experiment.
[b] Determined from HMBC experiment, $^nJ_{CH}$=8 Hz, recycle time 1 s.
[c] By ROESY experiment, mixing time 500 ms.

HPLC analysis, demonstrated an L-stereochemistry (*S*) for the Glu derived from the Ahp residue (the oxidation and subsequent hydrolysis liberated glutamic acid from Ahp). The stereochemistry of C-6 of the Ahp was determined as *R* on the basis of the *J*-values of H-6, <2.5, which point to an equatorial orientation of this proton and the chemical shift of the axial H-4, $\delta_H$ 2.40 brq, which is down-field shifted by the axial hydroxyl at position 6.

Nostoginin BN741 (**2**) was isolated as an amorphous white solid. High-resolution FAB MS measurements furnished, for nostoginin BN741 (**2**), a molecular formula of $C_{39}H_{59}N_5O_9$. Nostoginin BN741 (**2**), is a linear peptide consisting of a β-amino acid residue, 3-amino-2-hydroxy octanoic acid (Ahoa), two *N*-methylated amino acids, NMe-Ile and NMe-Tyr, and two normal amino acid residues (Val, Tyr). Nostoginin BN741 (**2**) is closely related in structure to microginin SD755, recently isolated in our group from *Microcystis aeruginosa*.[20] Both compounds share the same amino-acid sequence except for the β-amino-acid, Ahoa in nostoginin BN741 (**2**) versus NMe-Ahoa in microginin SD755. The structures of the acid residues were determined by analysis of the 1D ($^1$H, $^{13}$C and DEPT) and 2D (COSY, TOCSY, ROESY, HMQC and HMBC) NMR data (Table 2). The assignment of the carbonyl carbons of Ile and Ahoa is based on HMBC correlations of the proton at position 2 of the acid residue with the carbonyl carbon of the residue. In the cases of Tyr, NMeTyr and Val, such correlations were not observed and the assignment of the carbonyl carbons were made after

**Table 3**. NMR data of nostoginin BN578 (**3**)

| Position | | $\delta_C$, mult.[a] | $\delta_H$, mult., $J$ (Hz) | LR H–C correlations[b] | NOE correlations[c] |
|---|---|---|---|---|---|
| NMeTyr | 1 | 172.1 s | | NMeTyr-2 | |
| | 2 | 57.6 d | 5.18 dd 3.8, 11.3 | NMeTyr-3,3′,NMe | NMeIle-3,5,NMe |
| | 3 | 32.9 t | 3.11 dd 3.8, 15.4, 2.90; dd 11.3, 15.4 | | NMeTyr-2,3′,5,5′, NMeTyr-3 |
| | 4 | 127.5 s | | NmeTyr-3,3′,6,6′ | |
| | 5,5′ | 129.5 d | 6.93 d 8.3 | NMeTyr-3,3′,5,5′ | NMeTyr-2,3,6,6′, NMe, NMeIle-5 |
| | 6,6′ | 115.1 d | 6.62 d 8.3 | NMeTyr-5,5′,6,6′,OH | NMeTyr-5,5′,OH, NMeIle-5 |
| | 7 | 156.1 s | | NMeTyr-5,5′,6,6′,OH | |
| | OH | | 9.20 brs | | NMeTyr-6,6′ |
| | NMe | 29.9 q | 2.72 s | | NMeTyr-2,5 |
| NMeIle | 1 | 169.6 s | | NMeIle-2, NMeTyr-NMe | |
| | 2 | 56.5 d | 4.95 d 10.7 | NMeIle-5,6,Nme | NMeIle-6,NMe, NMeTyr-NMe |
| | 3 | 32.5 d | 1.95 m | NMeIle-2,6 | NMeIle-NMe |
| | 4 | 23.6 t | 0.75 m, 1.15 m | NMeIle-5 | NMeIle-Nme |
| | 5 | 11.1 q | 0.75 t 6.7 | NMeIle-4 | NMeTyr-5,5′,6,6′ |
| | 6 | 16.8 q | 0.69 d 6.7 | NMeIle-4′ | NMeIle-2,3,4′, NMeTyr-2 |
| | NMe | 29.6 q | 2.55 s | | NMeIle-3,4,6, Val-2,3 |
| Val | 1 | 171.0 s | | NMeIle-NMe, Val-2 | |
| | 2 | 53.2 d | 4.49 dd 5.2, 8.7 | Val-4,5 | Val-3,4,5, NMeIle-Nme |
| | 3 | 29.9 d | 1.65 m | Val-2,4,5 | Val-2, NMeIle-NMe |
| | 4 | 20.0 q | 0.76 d 6.7 | Val-5 | Val-2 |
| | 5 | 15.8 q | 0.74 d 6.7 | Val-4 | Val-2 |
| | NH | | 7.71 d 8.7 | | Ahoa-2, Val-2,3,5 |
| Ahoa | 1 | 170.4 s | | Ahoa-2, Val-NH | |
| | 2 | 70.9 d | 4.24 dd 3.0, 5.1 | Ahoa-2-OH,4 | Val-NH, Ahoa-2-OH,3,$NH_2$ |
| | 2-OH | | 6.40 d 5.1 | | Ahoa-2 |
| | 3 | 53.3 d | 3.40 m | | Ahoa-4,5 |
| | 3-$NH_2$ | | 7.99 d 7.9 | | Ahoa-2,4 |
| | 4 | 28.9 t | 1.49 m, 1.33 m | | Ahoa-3,$NH_2$ |
| | 5 | 24.6 t | 1.22 m, 1.35 m | Ahoa-6,7,8 | |
| | 6 | 31.2 t | 1.18 m | Ahoa-5,8 | Ahoa-2,3,4 |
| | 7 | 21.9 t | 1.23 m | Ahoa-5 | Ahoa-2,3 |
| | 8 | 14.0 q | 0.83 t 7.2 | Ahoa-6,7 | Ahoa-3 |

Carried out on an Avance 400 Bruker instrument.
[a] Multiplicity and assignment from HMQC experiment.
[b] Determined from HMBC experiment, $^nJ_{CH}$=8 Hz, recycle time 1 s.
[c] By ROESY experiment, mixing time 500 ms.

determination of the peptide sequence, mainly from NOE correlations. HMBC correlation of the NMeTyr-NMe with the carbonyl at $\delta_C$ 170.9 ppm and NOE correlation of the same NMe with Val-H-2 established the latter carbonyl to be Val-C-1. HMBC correlation of Tyr-NH with the carbonyl at $\delta_C$ 170.1 ppm and the NOE of the latter proton and Tyr-2 with NMeTyr-2 established this carbonyl as NMeTyr-C-1. The remaining carbonyl at $\delta_C$ 172.9 ppm was assigned to C-1 of Tyr. The amino acid sequence of nostoginin BN741 (**2**) was assembled by HMBC correlations (Table 2) of the NH protons with the vicinal carbonyl carbons (Val-Ahoa, the NMe protons of NMeTyr with the carbonyl of NMeIle, and from NOEs between Tyr-NH and H-2 with H-2 of NMeTyr, and NMeTyr-NMe with Val-H-2. Fragmentations in the positive ion FAB MS of **2** farther support its suggested structure. The protonated molecular ion of **2**, at *m/z* 742, consequently losses Tyr, NMeTyr and NMeIle from the carboxylic-end of the peptide to produce the fragment ions at *m/z* 561 (9%), 384 (100%) and 256 (20%), respectively.

Acid hydrolysis of **2** and derivatization with Marfey's reagent,[21] followed by HPLC analysis, demonstrated the L-stereochemistry of the valine, tyrosine, NMe-isoleucine and NMe-tyrosine. The assignment of the (2*S*,3*S*) stereochemistry to the stereogenic centers of Ahoa, in **2**, is based on the comparison of the NMR data of the protons and carbons at these centers (2-CH–OH: $\delta_C$ 70.9 d, $\delta_H$ 4.24 dd, $J$=5.1, 3.0 Hz; 3-CH–$NH_2$: $\delta_C$ 53.2 d, $\delta_H$ 3.40 m) with those of (2*S*,3*R*)-3-amino-2-hdroxydecanoic acid (Ahda) (2-CH–OH: $\delta_C$ 69.4 d, $\delta_H$ 4.05 brs; 3-CH–$NH_2$: $\delta_C$ 53.0 d, $\delta_H$ 3.22 m) in microginin[23] and (2*S*,3*S*)-Ahda (2-CH–OH: $\delta_C$ 70.6 d, $\delta_H$ 4.20 dd, $J$=5.6, 3.0 Hz; 3-CH–$NH_2$: $\delta_C$ 53.0 d, $\delta_H$ 3.37 m) in microginin 299-C.[24]

Nostoginin BN578 (**3**) was isolated as an amorphous white solid. Its proton and carbon NMR spectra resembled those of **2**. Comparison of the NMR spectra of both compounds (**2** and **3**) clearly pointed to the conclusion that **3** lacks one of the two tyrosine-moieties that appear in **2**. The

**Table 4**. NMR data of banyascyclamide A (**4**), B (**5**) and C (**6**)

| Position | Banyascyclamide A (**3**)[a] | | Banyascyclamide B (**4**)[a] | | Banyascyclamide C (**5**)[b] | |
|---|---|---|---|---|---|---|
| $\delta_C$, mult.[c] | $\delta_H$, mult., *J* (Hz) | $\delta_C$, mult.[a] | $\delta_H$, mult., *J* (Hz) | $\delta_C$, mult.[a] | $\delta_H$, mult., *J* (Hz) | $\delta_C$, mult.[a] |
| 1 | 159.3 s | | 159.6 s | | 159.5 s | |
| 2 | 148.5 s | | 148.8 s | | 148.0 s | |
| 3 | 123.8 d | 8.17 s | 125.3 d | 8.28 s | 125.3 d | 8.20 s |
| 4 | 172.1 s | | 171.5 s | | 171.5 s | |
| 5 | 48.0 d | 5.48 dq 6.3, 6.7 | 46.6 d | 5.63 dq 8.2, 6.8 | 46.6 d | 5.62 dq 8.4, 6.8 |
| 6 | 24.9 q | 1.75 d 6.7 | 24.5 q | 1.59 d 6.8 | 24.2 q | 1.60 d 6.8 |
| $N_1H$ | | 8.59 d 6.3 | | 8.69 d 8.2 | | 8.68 d 8.4 |
| 7 | 159.6 s | | 159.3 s | | 159.5 s | |
| 8 | 149.2 s | | 148.1 s | | 148.5 s | |
| 9 | 123.3 d | 8.07 s | 124.9 d | 8.30 s | 124.9 d | 8.29 s |
| 10 | 170.6 s | | 171.4 s | | 171.5 s | |
| 11 | 46.8 d | 5.23 dq 7.5, 6.7 | 46.8 d | 5.55 dq 8.4, 6.8 | 46.8 d | 5.56 dq 10.0, 6.8 |
| 12 | 24.9 q | 1.40 d 6.7 | 24.2 q | 1.49 d 6.8 | 24.2 q | 1.56 d 6.8 |
| $N_2H$ | | 7.58 d 7.5 | | 8.19 d 8.4 | | 8.28 d 10.0 |
| 13 | 169.3 s | | 170.2 s | | 170.2 s | |
| 14 | 73.9 d | 4.04 dd 9.3, 2.2 | 58.9 d | 4.32 dd 10.1, 1.9 | 59.0 d | 4.26 dd 10.0, 2.1 |
| 15 | 81.9 d | 4.85 dq 9.3, 6.2 | 67.9 d | 4.17 dq 1.9, 6.3 | 67.9 d | 4.13 dq 2.1, 6.4 |
| 16 | 21.7 q | 1.63 d 6.2 | 20.9 q | 1.03 d 6.3 | 20.8 q | 0.86 d 6.4 |
| $N_4H$ | | | | 8.28 d 10.1 | | 8.32 d 10.0 |
| 17 | 167.6 s | | 170.9 s | | 170.2 s | |
| 18 | 48.2 d | 5.21 dddd 2.2, 4.0, 4.5, 8.4 | 53.5 d | 4.75 ddd 15.4, 10.3, 5.6 | 55.9 d | 4.99 ddd 10.4, 8.4, 7.4 |
| 19 | 37.4 t | 3.22 dd 13.8, 4.0, 3.25 dd 13.8, 4.5 | 42.9 t | 1.63 m, 1.79 m | 39.1 t | 3.14 dd 14.5, 8.4, 3.23 dd 14.5, 7.4 |
| 20 | 135.4 s | | 24.2 d | 1.59 m | 137.2 s | |
| 21,21′ | 127.7 d | 7.14 d 7.1 | 22.8 q[d] | 0.96 d 6.1 | 129.1 d | 7.29 d 7.1 |
| 22,22′ | 128.3 d | 7.22 t 7.1 | 22.2 q[e] | 0.96 d 6.1 | 128.5 d | 7.24 t 7.1 |
| 23 | 127.2 d | 7.18 t 7.1 | | | 126.7 d | 7.19 t 7.1 |
| $N_3H$ | | 8.09 d 8.4 | | 8.79 d 10.3 | | 8.85 d 10.4 |

Carried out on an ARX-500 Bruker instrument.
[a] In $CDCl_3$.
[b] In DMSO-$d_6$.
[c] Multiplicity and assignment from HMQC experiment.
[d] Position 21.
[e] Position 22.

protonated-molecular ion cluster of the positive FAB mass-spectrum at *m/z* 579 and the high-resolution measurements of this cluster ion agreed with this finding and established the molecular formula of **3** as $C_{30}H_{51}N_4O_7$. The acid residues were determined by analysis of the 1D ($^1H$, $^{13}C$ and DEPT) and 2D (COSY, TOCSY, ROESY, HMQC and HMBC) NMR data (Table 3). The amino acid sequence of nostoginin BN578 (**3**) was assembled from HMBC correlations (Table 3) of the NH proton with the vicinal carbonyl carbon (Val-NMe and Ahoa), the NMe protons of NMeIle with the carbonyl of Val and the NMe protons of NMeTyr with the carbonyl of NMeIle. The ROESY correlations (Table 3) supported this assignment. Similar to compound **2**, the FAB MS of **3** shows a base peak at *m/z* 384, which is derived from the fragmentation of NMeTyr from the carboxylic-end of the peptide. Acid hydrolysis of **3** and derivatization with Marfey's reagent,[21] followed by HPLC analysis, demonstrated the L-stereochemistry of the valine, NMe-isoleucine and NMe-tyrosine. The assignment of the (2*S*,3*S*) stereochemistry to the stereogenic centers of Ahoa, in **3** is based on the same arguments as those discussed for **2**.

Banyascyclamides A–C (**4**–**6**) are modified-cyclic-hexapeptides, which are closely related in structure. They are composed of two thiazole–alanine units, threonine and phenylalanine (in **4** and **6**) or leucine (in **5**). Banyascyclamide A (**4**) was isolated as a white amorphous solid. The molecular formula of **4**, $C_{25}H_{26}N_6O_4S_2$ (HREIMS), indicated 16 double-bond equivalents. The hexapeptide nature of **4** was deduced from the six nitrogen atoms in the formula and six $sp^2$ carbon signals in the amide region of the $^{13}C$ NMR spectrum. The presence of only three amide protons, two singlet aromatic protons and a downfield shifted methyl carbinol unit in the $^1H$ NMR spectrum suggested three cyclically modified amino acids. Three standard amino acid residues—two alanyl-moieties and a phenyl-alanyl-moiety—and three modified amino acids—two thiazole and one methyloxazoline—were established on the basis of analyses of $^1H$, $^{13}C$ (Table 4), COSY, HMQC and HMBC spectra. The two thiazole–alanyl units could be established on the basis of the long range H–C correlations between the aromatic protons (H-3 with H-9) and the neighbouring carbons (H-3 with C-1, C-2 and C-4, H-9 with C-7, C-8 and C-10), of H-5 with C-4 and H-11 with C-10 and on the basis of the COSY correlations of H-5 with $N_1H$ and $CH_3$-6, and H-11 with $N_2H$ and $CH_3$-12. An H–H COSY experiment established the vicinities of H-14 to H-15 and H-15 to $CH_3$-16, H-18 to H-19 and H-19′, H-21 and H-21′ to H-22 and H-22′, of H-22 and H-22′ to H-23, as well as, the homoallylic 2.2 Hz coupling of H-14 with H-18. The chemical shifts and multiplicity of protons 14 to 16 pointed to a cyclically modified threonine moiety while those of protons 18 to 23 pointed to a modified phenylalanine moiety.[25] Long-range H–C correlations between C-13 and H-14 and H-15 established the modified-threonine moiety.

Long-range H–C correlations between C-17 and H-19, H-19′ and $N_3H$ and between C-20 and H-19, H-19′, H-22 and H-22′ established the phenylalanine moiety. HMBC correlation between H-14 and C-17, as well as the homoallylic coupling between H-14 and H-18, confirmed the assembly of the methyloxazoline moiety. The three units could be sequenced to the total gross structure by the HMBC correlations of the NH signals of one unit to the amide carbonyl of the neighbouring unit; $N_1H$ with C-7, $N_2H$ with C-13 and $N_3H$ with C-1.

Banyascyclamide B (**5**), had a molecular formula of $C_{22}H_{30}N_6O_5S_2$ based on HREIMS data (*m/z* 522.1719). The electron impact mass spectrum showed some important fragment ions at $[M-18]^+$ (loss of water from the threonine moiety) and $[M-44]^+$ (base peak, loss of formaldehyde from the side-chain of threonine and $[M-74]^+$ (loss of water from threonine and isobutene from the side-chain of leucine), indicative of threonine and leucine residues. Four standard amino acid residues—two alanines, leucine and threonine—and the two thiazoles were established from the analysis of the $^1H$, $^{13}C$, COSY, HMQC and HMBC spectra (Table 4). The two thiazole–alanine units were established on the basis of the long-range H–C correlation of the aromatic protons (H-3 and H-9) with the neighboring carbons, the protons of the alanyl-methyls (6- and 12-$H_3$) with the thioimide carbons (C-4 and C-10, respectively) and from the COSY correlation within the alanyl-spin system, as described for **4**. The threonine moiety was established from the COSY correlations of H-14 with H-15 and $N_4H$, H-15 with Me-16 and the HMBC correlation of H-14 with C-13. The leucine moiety was determined from the COSY correlation of H-18 with H-19 and 19′, and H-20 with H-19, 19′, Me-21 and Me-22. HMBC correlation between $N_2H$ and C-13 established the fragment C-8 to $N_4H$, which was further extended by the correlation of $N_4H$ with the carbonyl at $\delta_C$ 170.9 ppm. The latter carbonyl did not show any other HMBC correlation but was assigned as the carbonyl of the leucine unit by comparison of its chemical shift with the carbonyl carbons of **4** and tenuecyclamides A–D.[4] This argument extends the established fragment to include C-8 to $N_3H$. The two other carbonyl carbons in the spectrum that did not show correlations with their side-chains ($\delta_C$ 159.3 and 159.6 ppm) are obviously conjugated with the thiazole moieties (by comparison with **4**). HMBC correlations of $N_3H$ with the carbonyl that resonates at $\delta_C$ 159.6 ppm and $N_1H$ with the carbonyl that resonates at $\delta_C$ 159.3 ppm allows only the assignment of the former as C-1 and the later as C-7. This concludes the assignment of the planar structure of **5**.

The NMR and MS spectra of banyascyclamide C (**6**) showed great similarities to those of **4**. In the proton NMR spectrum of **6** (when compared with the spectrum of **4**) an additional doublet amide proton is apparent and some changes in the chemical shifts of the signals of the threonine moiety are recognized. The EIMS showed a molecular ion-radical at *m/z* 556, which is consistent with the molecular formula $C_{25}H_{28}N_6O_5S_2$ (HREIMS), and fragment ions derived from loss of water and formaldehyde from the threonie moiety (*m/z* 538 and 512, respectively) and benzyl radical from the phenylalanine moiety. The findings described above, suggest that the methyloxazoline-phenyl-alanyl moiety in **4** is substituted by threonine–phenyl-alanine in **6**. The structure of **6** was confirmed in the same manner as the two former compounds, **4** and **5**. The sub-structures C-2 to C-7 and C-8 to C-1 were assembled from COSY and HMBC correlations. The latter two fragments could be connected in only one way (C-1 to C-2 and C-7 to C-8) considering the peptide origin of this compound.

To determine the absolute configuration of the amino acids in banyascyclamides A–C (**4**–**6**), the three compounds were hydrolysed (6 M HCl, 120°C) derivatized with Marfey's reagent and then analysed using Marfey's method for HPLC.[21] The hydrolytic conditions generated L-threonine and L-phenylalanine from banyascyclamides A (**4**) and banyascyclamides C (**6**), and L-threonine and L-leucine from banyascyclamides B (**5**) along with the same 1:1 mixture of the *R*- and *S*-thiazole-alanyl moieties. These thiazole-alanyl moieties were identified by comparison with authentic compounds isolated under the same conditions from tenuecyclamide A.[4] Therefore, the three compounds contain one D-alanine unit and one L-alanine unit; but the stereochemistry of positions 5 and 1, in **4**–**6** could not be determined.

The inhibitory activity of Nostopeptin BN920 (**1**) was determined for two enzymes, the serine proteases trypsin and chymotrypsin. Nostopeptin BN920 (**1**) inhibits cymotrypsin with $IC_{50}$ of 0.11 μM, but not trypsin at 45.0 μg/mL. Nostoginin BN741 (**5**) inhibits Bovine Amino Peptidase N (APN) with $IC_{90}$ of 1.3 μM, but does not inhibit Bovine Neutral Endo Peptidase (NEP) and Angiotensin Converting Enzyme (ACE) at a concentration of 48.0 μg/mL.

## 1. Experimental

### 1.1. Instrumentation

High resolution MS were recorded on a Fisons VG AutoSpecQ M 250 instrument. UV spectra were recorded on a Kontron 931 plus spectrophotometer. NMR spectra were recorded on a Bruker ARX-500 spectrometer at 500.136 MHz for $^1H$ and 125.76 MHz for $^{13}C$ and a Bruker Avance 400 spectrometer at 400.13 MHz for $^1H$ and 100.62 MHz for $^{13}C$. $^1H$, $^{13}C$, DEPT, gCOSY, gTOCSY, gROESY, gHMQC and gHMBC spectra were recorded using standard Bruker pulse sequences. HPLC separations were performed on an ISCO HPLC system (model 2350 pump and model 2360 gradient programmer) equipped with an Applied Biosystem Inc. diode-array detector.

### 1.2. Biological material

*Nostoc* sp., TAU strain IL-235, was collected, in July 1999, from the spring pool of the Banyas stream, one of the tributaries that flow into the Jordan River, in Israel.

### 1.3. Isolation procedure

The naturally-collected, freeze-dried cells (318 g) were extracted with MeOH: $H_2O$ (7:3) (×3) and then with MeOH:

chloroform (1:1) (×3). The combined hydrophilic extract was concentrated under reduced pressure to afford 8.66 g of crude extract. The crude extract was separated on an ODS (YMC-GEL, 120A, 4.4×6.4 cm) flash column with increasing amounts of MeOH in water. Fractions 5–7 (2:3, 1:1 and 3:2 MeOH/$H_2O$) were found to be active in protease inhibition assays (chymotrypsin and APN) and thus, separated on a Sephadex LH-20 gel-filtration column with 1:1 $CHCl_3$/MeOH. Fraction 3 (50 mg), from the Sephadex LH-20 column, inhibited chymotrypsin and was subjected to a reversed-phase HPLC (YMC ODS-A 10 μm, 250 mm× 20.0 mm, DAD at 210 nm, flow rate 5.0 mL/min) in 55:45 MeOH/water to obtain pure compound **1**. Compound **1** (10.0 mg), 0.11% yield based on the weight of the crude extract, was eluted from the column with a retention time of 70.0 min. Fraction 5 (22 mg), from the Sephadex LH-20 column, inhibited APN and was subjected to a reversed-phase HPLC (YMC ODS-A 10 μm, 250 mm×20.0 mm, DAD at 210 nm, flow rate 5.0 mL/min) in 7:3 MeOH/water to obtain semi-pure **2** (10.4 mg). Semi-pure **2**, was chromatographed on the same column with 6:4 MeOH/ water to obtain pure **2**. Compound **2** (3.0 mg), 0.034% yield based on the weight of the crude extract, was eluted from the column with a retention time of 23.1 min. Fractions 6–8, from the Sephadex LH-20 column, were not active in the protease inhibition assays but rather exhibited proton NMR spectra indicative of thiazole moieties. The combined fraction (6–8, 73 mg) was subjected to a reversed-phase HPLC (YMC ODS-A 10 μm, 250 mm×20.0 mm, DAD at 210 nm, flow rate 5.0 mL/min) in 55:45 MeOH/water to obtain a fraction (6, 9 mg) that exhibited NMR spectra similar to those of compound **2** and two fractions (7 and 8) that exhibited the thiazole-characteristic NMR spectrum. Fraction 6 was purified on a semi-preparative HPLC column (YMC ODS-A 10 μm, 250 mm×10.0 mm, DAD at 210 nm, flow rate 2.0 mL/min) in 35:65 acetonitrile/water to obtain pure **3** (4.9 mg, 0.056% yield based on the weight of the crude extract) with two broad peaks having retention times of 8 and 10 min. Fraction 7 (22 mg), was subjected to a reversed-phase HPLC (YMC ODS-A 5 μm, 250 mm×10 mm, DAD at 210 nm, 1:1 acetonitrile/water, flow rate 3.0 mL/min). Compound **4** (7.1 mg), 0.082% yield based on the dry weight of the crude extract, was eluted from the column with a retention time of 18.2 min. Fraction 8 (27 mg) was subjected to a reversed-phase HPLC (YMC ODS-A 5 μm, 250 mm×10 mm, DAD at 210 nm, 35:65 acetonitrile/water, flow rate 2.0 mL/min). Compound **5** (1.4 mg), 0.016% yield based on the dry weight of the crude extract, was eluted from the column with a retention time of 20.7 min and compound **6** (1.0 mg), 0.012% yield based on the dry weight of the crude extract, was eluted from the column with a retention time of 23.1 min.

**1.3.1. Nostopeptin BN920 (1).** $[\alpha]_D^{25}=-30.0$ (*c* 0.1, MeOH); UV $\lambda_{max}$ (MeOH) 218 nm (ε 19600), 278 nm (ε 5400). For NMR data Table 1. Positive FABMS (DTT/DTE) *m/z* (relative intensity) 959 ($MK^+$, 55%), 943 ($MNa^+$, 100%), 537 (50%), 406 (90%), 393 (73%), 262 (76%). Negative FABMS (DTT/DTE) *m/z* 919 $[M-H]^-$; HRFABMS *m/z* 943.4523 ($MNa^+$, calcd for $C_{46}H_{64}N_8NaO_{12}$, 943.4541). HPLC analysis of the derivatized hydrolysates of **1** established L-Val, L-Leu, L-Phe, L-Thr, L-Gln and L-NMe-Tyr. HPLC analysis of the FDAA derivatives, of oxidized-**1** hydrolysates, established an additional L-Glu and thus confirmed the (2*S*) configuration of the Ahp units in this compound.

**1.3.2. Nostoginin BN741 (2).** $[\alpha]_D^{25}=-56.0$ (*c* 0.2, MeOH); UV $\lambda_{max}$ (MeOH) 244 nm (ε 13400), 279 nm (ε 3100). For NMR data Table 2. Positive FABMS (TEA) *m/z* (relative intensity) 780 ($MK^+$, 10%), 764 ($MNa^+$, 19%), 742 ($MH^+$, 8%), 561 (9%), 384 (100%), 288 (10%), 256 (20%), 229 (24%). Negative FABMS (TEA) *m/z* 740 $[M-H]^-$; HRFABMS *m/z* 764.4177 ($MNa^+$, calcd for $C_{39}H_{59}N_5NaO_9$, 764.4210). HPLC analysis of the derivatized hydrolysates of **2** established L-Val, L-NMeIle, L-Tyr and L-NMe-Tyr.

**1.3.3. Nostoginin BN578 (3).** $[\alpha]_D^{25}=-40.4$ (*c* 0.5, MeOH); UV $\lambda_{max}$ (MeOH) 235 nm (ε 11400), 277 nm (ε 2900). For NMR data Table 3. Positive FABMS (TEA) *m/z* (relative intensity) 579 ($MH^+$, 8%), 384 (100%); HRFABMS *m/z* 579.3758 ($MH^+$, calcd for $C_{30}H_{51}N_4O_7$, 579.3757). HPLC analysis of the derivatized hydrolysates of **3** established L-Val, L-NMeIle and L-NMe-Tyr.

**1.3.4. Banyascyclamide A (4).** $[\alpha]_D^{25}=-19.3$ (*c* 0.12, MeOH); UV $\lambda_{max}$ (MeOH) 218 nm (ε 17300), 238 nm (ε 17900). For NMR data Table 4. EIMS *m/z* (relative intensity) 538 ($M^+$, 100%), 447 (69%), 162 (35%), 138 (42%); HREIMS *m/z* 538.1460 ($M^+$, calcd for $C_{25}H_{26}N_6O_4S_2$, 538.1456). HPLC analysis of the derivatized hydrolysates of **4** established L-Alanyl-Thiazolic acid, D-Alanyl-Thiazolic acid, L-Phe and L-Thr.

**1.3.5. Banyascyclamide B (5).** For NMR data Table 4. EIMS *m/z* (relative intensity) 522 ($M^+$, 7%), 504 (42%), 478 (100%), 461 (21%), 448 (50%), 378 (55%); HREIMS *m/z* 522.1719 ($M^+$, calcd for $C_{22}H_{30}N_6O_5S_2$, 522.1719). HPLC analysis of the derivatized hydrolysates of **5** established L-alanyl-thiazolic acid, D-alanyl-thiazolic acid, L-Leu and L-Thr.

**1.3.6. Banyascyclamide C (6).** $[\alpha]_D^{25}=50.1$ (*c* 0.1, MeOH); UV $\lambda_{max}$ (MeOH) 222 nm (ε 15100), 238 nm (ε 16300). For NMR data Table 4. EIMS *m/z* (relative intensity) 556 ($M^+$, 14%), 538 (61%), 512 (100%), 465 (28%), 447 (43%), 412 (21%), 326 (27%), 310 (32%), 181 (30%), 155 (55%), 138 (80%); HREIMS *m/z* 556.1563 ($MH^+$, calcd for $C_{25}H_{28}N_6O_5S_2$, 556.1562). HPLC analysis of the derivatized hydrolysates of **4** established L-alanyl-thiazolic acid, D-alanyl-thiazolic acid, L-Phe and L-Thr.

### 1.4. Determination of the absolute configuration of the amino acids

0.5 mg portions of compounds **1**–**6** were dissolved in 6 M HCl (1 mL). The reaction mixture was then placed in a sealed glass bomb at 110°C for 20 h. In another experiment, 0.25 mg portion of compound **1** was first oxidized with Jones reagent (1 drop) in acetone (1 mL) at 0°C for 10 min. Following the usual work-up, the residue was dissolved in 6 M HCl (1 mL) and placed in a sealed glass bomb at 108°C for 18 h. After removal of the HCl, by repeated evaporation in vacuo, the hydrolysate was resuspended in water (40 μL)

and derivatized with Marfey's reagent (1-fluoro-2,4-dinitrophenyl)-5-L-alanine amide (FDAA). The *N*-[(1,5-dinitrophenyl)-4-L-alanine amide]-2-amino acid (AA) derivatives, from hydrolysates, were compared with similary derivatized standard AA by HPLC analysis: Knauer GmbH Eurospher 100 C18, 10μ, 4.6 mm×300 mm, flow rate: 1 mL/min, UV detection at 340 nm, linear gradient elution from 9:1 50 mM triethylammonium phosphate (TEAP) buffer (pH 3)/acetonitrile to 1:1 TEAP/acetonitrile within 60 min. The determination of the absolute configuration of each amino acid was confirmed by spiking the derivatized hydrolysates with the derivatized authentic amino acids.

### 1.5. Protease inhibition assays

Trypsin and chymotrypsin were purchased from Sigma Chemical Co. Trypsin was dissolved in 50 mM Tris–HCl/100 mM NaCl/1 mM $CaCl_2$ to prepare a 1 mg/mL solution. Chymotrypsin was dissolved in 50 mM Tris–HCl/100 mM NaCl/1 mM $CaCl_2$/1 mM HCl to prepare a 1 mg/mL solution. A 2 mM solution of *N*-benzoyl-D, L-arginine-*p*-nitroanilide (for trypsin) and Suc-Gly-Gly-*p*-nitroanilide (for chymotrypsin) in the appropriate buffer solution was used as a substrate solution. The test sample was dissolved in ethanol and diluted with the same buffer solution as that used for the enzyme and substrate. A 100 μL buffer solution, 10 μL enzyme solution and 10 μL of test solution were added to each microtiter plate well and preincubated at 37°C for 5 min. Then, 100 μL of substrate solution was added to begin the reaction. The absorbance of the well was immediately measured at 405 nm. The developed color was measured after incubation at 37°C for 30 min. The procedure for the inhibition assays of the amino proteases APN, NEP and ACE was published elsewhere.[26]

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