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Isolation and Structures of Nostopeptolides A1, A2 and A3 from the Cyanobacterium *Nostoc* sp. GSV224

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Abstract—The isolation and total structure determinations of nostopeptolides A1 (**1**), A2 (**2**) and A3 (**3**) are described. These cyclic depsipeptides, which are devoid of cytotoxic, antifungal and inhibition of protease activities, are characteristic constituents of the cryptophycin-producing cyanobacterium *Nostoc* sp. GSV224. Structure elucidation by NMR analysis was made more challenging by the existence of each nostopeptolide in three conformations. One-dimensional TOCSY experiments proved to be very useful in isolating and identifying the nine amino acid residues and the butyryl group in each compound. The absolute stereochemistries of **1**–**3** were determined by comparing the amino acids in the acid hydrolyzates directly with authentic standards.

Introduction

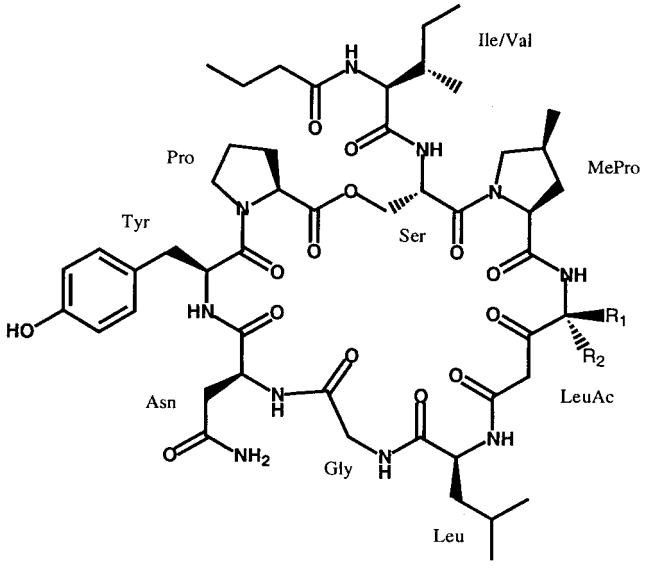
Nostoc sp. GSV224, isolated from a terrestrial sample collected in India,[1] and *Nostoc* sp. ATCC53789, isolated from a lichen collected in Scotland at Arron Island,[2] are to date the only known cryptophycin-producing cyanobacteria.[2–6] Interestingly each cyanobacterium elaborates essentially the same spectrum of >25 cryptophycins as determined by HPLC analysis.[7] Nevertheless, these two cyanobacteria are significantly different phylogenetically, as their 16S rDNAs differ by 2.8% in homology (GenBank Accession AF062637 for GSV224 and AF062638 for ATCC53789). Moreover, the other secondary metabolites that are found in GSV224 appear to be quite different from the ones found in ATCC53789. For example, GSV224 produces a class of peptolides (nostopeptolides) which are totally absent in the ATCC53789 species. The genes that encode for the biosynthesis of the nostopeptolides in GSV224 (GenBank Accession AF204805)[8] are not found in ATCC53789. Conversely the ATCC53789 species produces an unusual class of cyclic peptides (nostocyclopeptides)[9] which are not present in the GSV224 species. In this paper we report the isolation and structure determination of three of the nostopeptolides found in GSV224, viz. nostopeptolide A1 (**1**), A2 (**2**) and A3 (**3**). The biological significance of the nostopeptolides is presently unknown.[10]

Lyophilized GSV224 was extracted with 4:1 acetonitrile/dichloromethane and the concentrated extract was fractionated by reversed-phase flash chromatography using various mixtures of water in acetonitrile. Nostopeptolides A1, A2 and A3 were eluted in the 1:1 H_2O/CH_3CN fraction. Reversed-phase HPLC led to isolation of the three new peptolides (**1**–**3**) in yields of 0.032, 0.0016 and 0.002%, respectively.

Results and Discussion

Gross structure determination

Mass spectrometry established a molecular weight of



1	$R = CH_3$	$R_1 = CH_2CH(CH_3)_2$; $R_2 = H$
2	$R = H$	$R_1 = CH_2CH(CH_3)_2$; $R_2 = H$
3	$R = CH_3$	$R_1 = H$; $R_2 = CH_2CH(CH_3)_2$

Keywords: nostopeptolides; depsipeptides; cyanobacterium.
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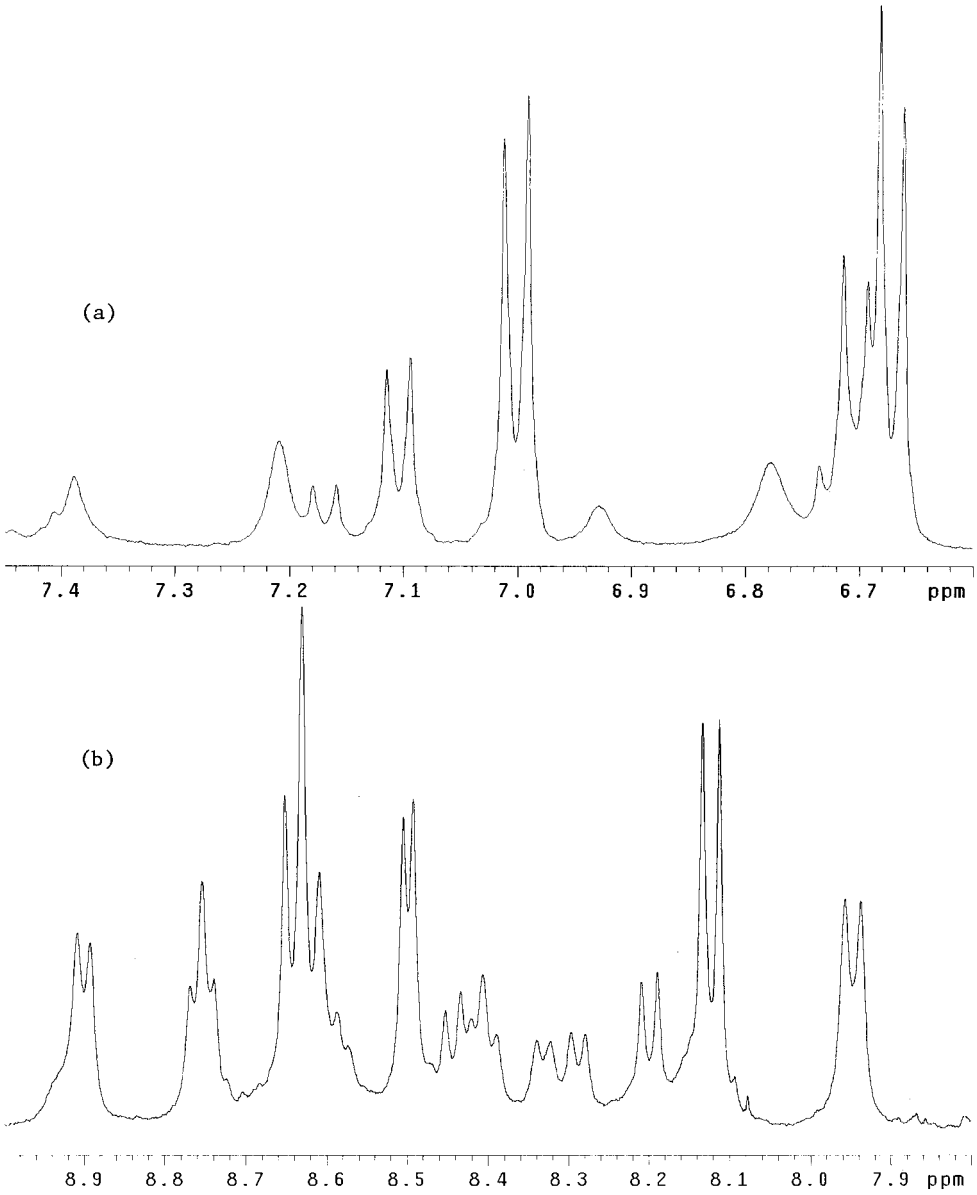


Figure 1. 400 MHz ^{1}H NMR spectrum of **1** in CD_3OH. All of the signals are tripled since the molecule exists as a 10:3.7:1 mixture of three conformers A, B and C in solution. The upper trace (a) depicts the 6.60–7.45 ppm region of the spectrum determined at 40°. The three doublets at 7.00. 7.10 and 7.17 ppm are assigned to the H-5/9 protons of the Tyr unit for conformers A, B and C, respectively. The three doublets at 6.67, 6.70 and 6.73 ppm are assigned to the H-6/8 protons of conformers A, B and C. The broad signals at 6.78 and 7.21 ppm are assigned to the 1° amide protons of the Asn unit for conformer A and the broad signals at 6.93 and 7.38 ppm are assigned to the 1° amide protons of the Asn unit for conformer B. Only one of the 1° amide proton signals is seen at 40° for conformer C (7.41 ppm). The lower trace (b) depicts the 7.80–9.00 ppm region of the spectrum determined at 0°C. The six doublets at 8.90, 8.64, 8.62, 8.50, 8.12, and 7.95 ppm and the triplet at 7.95 ppm are assigned to the seven 2° amide protons in conformer A. The six doublets at 8.44, 8.42, 8.40, 8.33, 8.29, and 8.20 ppm and the triplet at 8.59 ppm are assigned to the seven 2° amide protons in conformer B. None of the signals for the seven 2° amide protons in conformer C are seen at 0°.

1080 Da for **1**. Specifically the positive-ion FABMS displayed a MH^+ ion peak at *m/z* 1081, a MNa^+ ion peak at *m/z* 1103 and a MK^+ ion peak at *m/z* 1119, whereas the negative-ion FABMS exhibited a $[M-H]^-$ ion peak at *m/z* 1079. Inspection of the ^{1}H and ^{13}C NMR spectra suggested that **1** was peptidal, but determination of its molecular formula from the NMR data was hampered since nostopeptolide A1 existed as a complex mixture of conformers in solution. In CD_3OH, for example, a 10:3.7:1 mixture of three conformers (A, B and C) could be seen (Fig. 1).

All of the exchangeable proton signals (no correlation with ^{13}C signals in the HSQC spectrum) were found in the 6.6–9.0 ppm region of the ^{1}H NMR spectrum of **1** in CD_3OH. By carefully studying this region at several temperatures we were able to determine that nine amide NH protons were present in the molecule. At ambient temperature seven 2° amide-type NH signals were found at 8.70 (d), 8.61 (dd), 8.410 (d), 8.409 (d), 8.35 (d), 7.92 (d) and 7.89 (d) ppm and two 1° amide-type NH signals were found at 7.25 and 6.87 ppm for the major conformer A. These nine signals were the most intense ones and had the same relative intensities. At lower and higher temperatures the signals at 8.410 and 8.409 ppm clearly separated. Similar sets of nine NH

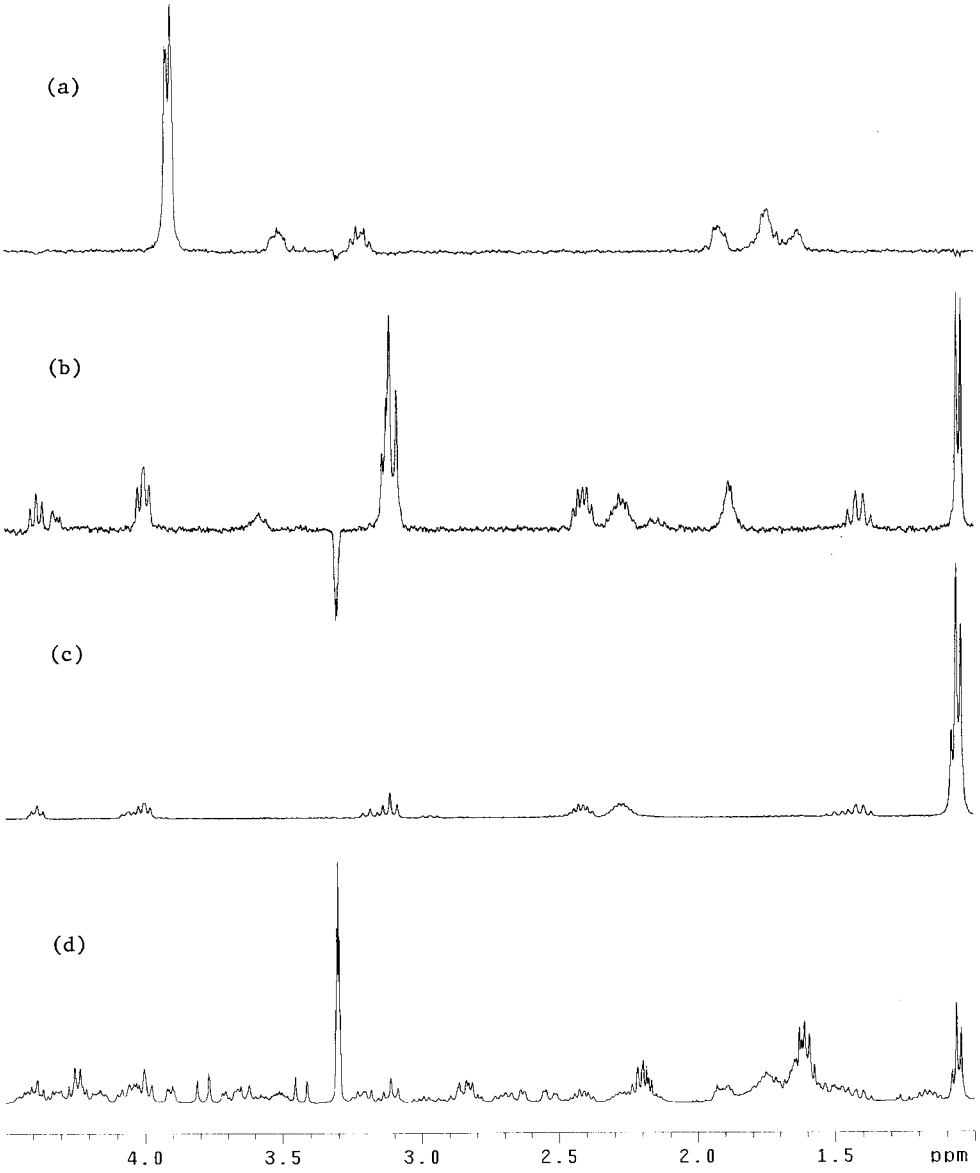


Figure 2. 400 MHz 1D TOCSY spectra of **1** in CD_3OH at 25°C resulting from excitation of (a) the broad doublet at δ 3.92 (Pro H-2, conformer A), (b) the triplet at δ 3.12 (MePro H-5proS, conformer A) which must have a multiplet underneath the triplet for one of the Pro H-5 protons in conformer B, and (c) the overlapping doublets at δ 1.06 and 1.08 (4-MePro 4-Me, conformers A and B, respectively). The chemical shifts and signal patterns seen in trace (a) are assigned to the Pro unit in conformer A. Trace (b) shows all of the proton signals for the MePro unit in conformer A and the Pro unit in conformer B (1.92, 2.14, 3.57, 4.31 ppm). Trace (c) shows all of the protons signals for the 4-MePro unit in conformers A and B. The 1.0–4.5 ppm region of the 400 MHz ^{1}H NMR spectrum of **1** in CD_3OH is shown in trace (d).

signals could be found for conformers B and C. At 0°C all nine NH signals for conformer B were separated from each other and from the ones for conformer A (Fig. 1).

One-dimensional TOCSY experiments[11] enabled us to identify the amino acids associated with the seven 2° amide NH signals. Excitation of each signal produced a spectrum in which all of the signals for the protons that were directly- or relay-coupled to the NH were observed. The chemical shifts and coupling constants associated with the various signal patterns that were found strongly suggested that the NH signals at 8.70 and 8.35 ppm belonged to two Leu units and the NH signals at 8.61, 8.410, 8.409, 7.92 and 7.89 ppm belonged to Gly, Ser, Tyr, Ile and Asp (or Asn) units, respectively. The observance of the two 1° amide NH signals at 7.25 and 6.87 ppm, however, was more consistent with Asn rather than Asp. Additional 1D TOCSY experiments allowed us to determine that Pro and 4-methylproline (MePro) units were also present (Fig. 2). COSY analysis confirmed that **1** was composed of the nine amino acid units found by the 1D TOCSY spectra.

Further examination of the ^{1}H and ^{13}C NMR spectra indicated that **1** also possessed a butyryl group (δ_H 2.22, 1.61, 0.92), a ketone carbonyl (δ_C 206.0), and an isolated methylene adjacent to two π-bonds (δ_H 3.44 and 3.80, $J_{gem}=-18$ Hz). The latter two groups suggested that an aceto unit was inserted in the peptide chain. This further suggested that the aceto group was acylated by one of the amino acids and that the C-1 carbonyl of the amino acid had

Table 1. NMR data for nostopeptolide A1 (**1**) in CD_3OH at 25°C

C/H no.	δ_H (*J* in Hz)	δ_C[a]	HMBC[b]	ROESY[c]
Butyryl				
1	–	176.1, s	H-2, H-3, Ile NH, Ile H-2	
2	2.22, t (7.4)	38.8, t	H-3, H-4	Ile NH
3	1.61, hex (7.4)	20.4, t	H-2, H-4	
4	0.92, t (7.4)	14.1, q	H-2, H-3	
Ile				
1	–	174.2, s	H-2, Ser NH	
2	4.25, t (7.9)	59.2, d	NH, 3-Me	Ser NH
3	1.79, m	38.2. d	H-2, 3-Me, H-4b, H-5	
3-Me	0.88, d (7.0)	16.0, q	H-2, H-3b	
4a	1.48, m	26.0, t	H-2, 3-Me, H-5	
4b	1.16, m	–		
5	0.87, t (7.4)	11.4, q	–	
NH	7.92, d (7.9)	–		Butyryl H-2
Ser				
1	–	168.8, s	H-2	
2	4.97, ddd (10.4, 7.7, 2.2)	51.8, d	H-3b	4-MePro H-5a
3a	4.54, dd (12.3, 2.2)	66.3, t	H-2	
3b	4.00, dd (12.3, 10,4)	–		
NH	8.410, d (7.7)	–		Ile H-2
4-MePro				
1	–	174.7, s	H-2, H-3*proR*, LeuAc NH	
2	4.38, t (8.6)	61.8, d	H-5*proR*	H-3*proS*, LeuAc NH
3*proS*	2.41, ddd (12.6, 8.6, 6.9)	38.7, t	H-2, H-5*proR*, 4-Me	H-2, H-4
3*proR*	1.41, ddd (12.6, 10.9, 8.6)	–		4-Me
4	2.28, m	35.2, d	H-3*proS*, H-5*proS*, 4-Me	H-3*proS*, H-5*proR*
4-Me	1.06, d (6.4)	16.7, q	H-5*proS*	H-3*proR*, H-5*proS*
5*proR*	4.00, dd (10.2, 7.5)	56.0, t	H-3*proS*, 4-Me	H-4, H-Ser H-2
5*proS*	3.12, t (10.2)	–		4-Me
LeuAc				
1	–	170.4, s	H-2a/b, Leu NH	
2a	3.80, d (18.0)	48.1, t	–	Leu NH
2b	3.44, d (18.0)	–		H-4, Leu NH
3	–	206.0, s	H-2a/b, H-5b	
4	4.16, m	59.0, d	H-5b	H-2b
5a	1.64, m	38.3, t	6-Me, H-7	
5b	1.54, m			
6	1.64, m	26.1, d	6-Me, H-7	
7	0.91, d (6.0)	21.7, q	H-6, 6-Me	
6-Me	0.94, d (6.4)	23.8, q	H-6, H-7	
NH	8.70, d (6.6)	–		4-MePro H-2
Leu				
1	–	175.3, s	H-2, Gly NH, Gly H-2a/b	
2	4.23, m	54.9, d	NH, H-3a/b	Gly NH
3	1.60, m	41.1, t	H-2, 4-Me, H-5	
4	1.71, m	25.9, d	H-3, 4-Me, H-5	
5	0.93, d (6.0)	22.1, q	H-3, 4-Me	
4-Me	0.97, d (6.6)	23.4, q	H-3, H-5	
NH	8.35, d (5.3)	–		LeuAc H-2a/b
Gly				
1	–	171.5, s	H-2a/b, Asn NH	
2a	4.07, dd (17.1, 7.4)	43.8, t	–	
2b	3.69, dd (17.1, 4.9)	–		
NH	8.61, dd (7.4, 4.9)	–		Leu H-2
Asn				
1	–	172.3, s	H-3a/b, Tyr NH	
2	4.82, ddd (9.2, 8.3, 3.7)	51.4, d	–	
3a	2.70, dd (14.5, 9.2)	39.6, t	–	
3b	2.53, dd (14.5, 3.7)	–		
NH	7.89, d (8.3)	–		
$CONH_2$	6.87, br s, 7.25, br s	175.0, s	H-3a/b	
Tyr				
1	–	172.1, s	H-2, H-3a/b	
2	4.63, m	54.4, d	H-3a/b	Pro H-2
3a	2.87, m	39.9, t	H-5/9	
3b	2.81, m	–		
4	–	128.4, s	H-3a/b, H-5/9	
5/9	7.00, d (8.3)	131.3, d	H-3a/b, H-9/5	Pro H-2
6/8	6.67, d (8.3)	116.4, d	H-5/9, H-8/6	
7	–	157.8, s	H-5/9, H-6/8	
NH	8.409, d (7.7)			

Table 1 (*continued*)

C/H no.	δ_H (*J* in Hz)	δ_C[a]	HMBC[b]	ROESY[c]
Pro				
1	–	173.3, s	H-2, Ser H-3a/b	
2	3.92, brd (7.4)	60.7, d	–	Tyr H-2, Tyr 5/9
3a	1.92, m	31.5, t	H-2	
3b	1.75, m	–		
4a	1.75, m	23.4, t	H-2	
4b	1.62, m	–		
5a	3.51, m	47.4, t	–	
5b	3.22, m	–		

[a] Multiplicity deduced by DEPT spectroscopy.
[b] Correlations observed were optimized for $^nJ_{CH}$=7 Hz.
[c] Only key cross-peaks are listed.

become the ketone group. The presence of a C–CO–CH_2–CO–N fragment was supported by the facile exchange of the methylene protons at 3.44 and 3.80 ppm in CD_3OD and a 0.8 ppm upfield shift of the methylene carbon signal (48.1 ppm). Since **1** exhibited an ester carbonyl band (1729 cm^{-1}) in its IR spectrum and behaved like a neutral compound, the peptide chain had to have the butyryl group attached to the N-terminus and the Ser unit attached as an ester to the C-terminus. Addition of the elemental compositions for the nine amino acid units and the aceto and butyryl groups, minus the OH proton from Ser, led to the molecular formula $C_{53}H_{80}N_{10}O_{14}$ for **1**, in agreement with the MS data.

All of the $^1J_{CH}$ correlations could now be made with complete confidence from the HSQC spectrum. A DEPT experiment established which of the protonated ^{13}C signals belonged to methine, methylene or methyl carbons. A HMBC experiment then allowed us to assign all of the signals for the 11 carbonyl carbons of the nine amino acid units, the butyryl group and the aceto group (Table 1). HMBC cross peaks were clearly seen for $^2J_{CH}$-coupling between the H-2 proton(s) and the carbonyl carbon (C-1) of the butyryl, Ile, Ser, MePro, Gly, Tyr, Pro, and one of the Leu units (the one showing its NH signal at 8.35 ppm). $^2J_{CH}$-Cross peaks were also seen in the HMBC spectrum between the methylene protons (H-2a/b of LeuAc) and the amide (C-1) and ketone (C-3) carbonyls of the acylated aceto unit. Although HMBC cross peaks could not be observed between H-4 and C-3 in the amino acid derived acyl group, $^3J_{CH}$-cross peaks were visible between one of the β-protons in the second Leu unit (1.54 ppm; H-5b in LeuAc) and the ketone carbonyl. This meant that the carbonyl of the second Leu unit was connected to methylene of the aceto unit. $^2J_{CH}$-Cross peaks were not seen between H-2 and C-1 of the Asn unit, but could be seen between the H-3 protons and C-4 (1° amide carbonyl). Nevertheless $^3J_{CH}$-cross peaks were visible between the H-3 protons and the C-1 carbonyl of Asn.

Further examination of the HMBC spectrum enabled us to sequence all of the partial structures into two expanded structures which essentially represented two moieties of **1**. All seven 2° amide NH protons showed $^2J_{CH}$-cross peaks to the adjacent amide carbonyl carbons and these connectivities generated two sequences Butyryl-Ile-Ser and MePro-LeuAc-Leu-Gly-Asn-Tyr. $^3J_{CH}$-Cross peaks from the H-3 protons of Ser to C-1 of Pro indicated that the last amino acid unit Pro was connected to Ser by an ester bond. The resulting two moieties could only be connected together in one way to complete the gross structure, i.e. by attaching Ser to MePro and Tyr to Pro by amide bonds. ROESY correlations (Pro H-2 to Tyr H-2 and H_2-5/9 for conformer A and Pro H_2-5 to Tyr H-3 for conformer B) confirmed the gross structure.

The gross structures of **2** and **3** were elucidated using similar arguments. FABMS indicated that the molecular formula of **2** differed from **1** in having one less methylene. The NH signals for the major conformer A of **2** had essentially the same ^1H chemical shifts (Table 2); however, the 1D TOCSY spectrum generated by excitation of the NH doublet at 7.90 ppm showed signals that were consistent with a Val unit rather than an Ile unit. FABMS indicated that **3** had the same molecular formula as **1**. Comparison of ^1H NMR spectra suggested that **1** and **3** had the same gross structures, but differed in stereochemistry in the LeuAc unit.

Relative and absolute stereochemistry

Acid hydrolysis of **1**, **2** or **3** in 6N HCl at 110°C for 24 h led to a mixture of eight amino acids that could be separated by HPLC on a chiral column (see Table 3 for conditions). The amino acids in the acid hydrolyzates of **1** and **3** were shown to be L-Pro (t_R 5.99 min), Gly (7.45), L-Ser (7.82), L-Asp (14.55), L-Tyr (20.85), (2*S*,4*S*)-4-methylproline (**4**) (21.00), L-Leu (21.41), and L-Ile (2*S*,3*S*) (37.23) by co-elution with authentic standards. The HPLC chromatogram for the acid hydrolyzate of **2** was almost the same; however, the L-Ile peak was replaced by a L-Val peak (t_R 14.77 min).

As expected leucylacetic acid (LeuAcOH) was not found in any of the acid hydrolyzates. L- (**5**) and D-LeuAcOH (**6**) would have rapidly decarboxylated to *S*- or *R*-3-amino-5-methylhexan-2-one under the reaction conditions and therefore would not have been detected in the HPLC assay described above. When **3** was treated with 1N NaOH in 1:1 DME/water prior to acid hydrolysis, however, a small peak for D-Leu was observed in the HPLC chromatogram. A similar treatment of **1** did not lead to D-Leu. Presumably a retro-Claisen reaction had partially occurred in the LeuAc unit when **3** was treated with base. Acid hydrolysis of the fragmented **3** then led to D-Leu. The failure of **1** to give D-Leu under the same reaction conditions implied that

Table 2. ^{1}H and ^{13}C NMR data for nostopeptolide A2 (**2**) and A3 (**3**) in CD_3OH (^{13}C NMR of (**2**) acquired in CD_3OD; **2** and **3** exist as 10:3.7:1 and 2:1:1 mixtures of three conformers A, B and C in CD_3OH solution, respectively) at 25°C

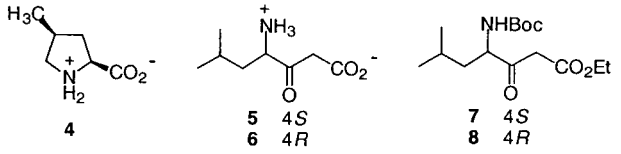
C/H no.	Nostopeptolide A2		Nostopeptolide A3	
	δ_H (J in Hz)	δ_C[a]	δ_H (J in Hz)	δ_C[b]
Butyrate				
1	–	176.1, s	–	176.0, s
2	2.21, m	38.7, t	2.22, m	38.8, t
3	1.62, m	20.4, t	1.61, m	20.4, t
4	0.93, m	14.1, q	0.92, m	14.1, q
Val/Ile				
1	–	174.1, s	–	174.3, s
2	4.22, dd (8.0, 6.9)	59.9, d	4.22, t (7.9)	59.2, d
3	2.03, m	31.9. d	1.79, m	38.3, d
3-Me	0.91, d (6.2)	18.5, q	0.88, d (7.2)	16.1, q
4a	0.91, d (6.2)	19.8, q	1.48, m	25.9, t
4b	–	–	1.14, m	–
5	–	–	0.86, t (7.2)	11.5, q
NH	7.90, d (8.0)	–	7.96, d (7.9)	–
Ser				
1	–	168.8, s	–	168.7, s
2	4.96, ddd (10.4, 7.4, 2.5)	51.7, d	4.94, ddd (10.5, 7.7, 2.7)	51.8, d
3a	4.52, dd (12.0, 2.5)	66.1, t	4.47, dd (12.2, 2.7)	65.9, t
3b	4.03, dd (12.0, 10.4)	–	4.09, dd (12.2, 10.5)	–
NH	8.40, d (7.4)	–	8.40, d (7.7)	–
4-MePro				
1	–	174.6, s	–	175.6, s
2	4.38, t (8.6)	61.8, d	4.39, t (8.3)	61.9, d
3*proS*	2.42, m	38.9, t	2.34, m	38.7, t
3*proR*	1.41, m	–	1.44, m	–
4	2.28, m	35.1, d	2.28, m	35.3, d
4-Me	1.06, d (6.3)	16.8, q	1.07, d (6.3)	16.6, q
5*proR*	4.01, dd (10.2, 7.0)	55.9, t	4.03, dd (10.2, 7.1)	55.8, t
5*proS*	3.12, dd (10.2)	–	3.12, dd (10.2)	–
LeuAc				
1	–	170.4, s	–	170.4, s
2a	3.79, d (17.0)	47.3, t	3.92, d (17.3)	46.6, t
2b	3.47, d (17.0)	–	3.51, d (17.3)	–
3	–	206.0, s	–	208.4, s
4	4.13, ddd (10.7, 6.4, 3.0)	59.0, d	4.15, m	60.0, d
5a	1.64, m	38.2, t	1.56, m	39.8, t
5b	1.54, m		1.56, m	
6	1.64, m	26.1, d	1.66, m	25.87, d
7	0.91, d (6.8)	21.7, q	0.89, d (6.3)	21.84, q
6-Me	0.93, d (6.6)	23.8, q	0.96, d (5.8)	23.5, q
NH	8.70, d (6.4)	–	8.77, d (6.3)	–
Leu				
1	–	175.3, s	–	175.3, s
2	4.24, dd (9.1, 5.5)	54.6, d	4.32, m	54.3, d
3	1.61, m	41.0, t	1.63, m	41.1, t
4	1.73, m	25.8, d	1.74, m	25.86, d
5	0.93, m	21.9, q	0.93, d (6.5)	21.87, q
4-Me	0.97, d (6.3)	23.4, q	0.96, d (7.0)	23.3, q
NH	–	–	8.33, d (6.3)	–
Gly				
1	–	171.4, s	–	171.2, s
2a	4.04, dd (17.2, 6.6)	43.6 t	3.99, dd (17.2, 6.5)	44.0, t
2b	3.69, dd (17.2, 5.5)	–	3.77, dd (17.2, 5.9)	–
NH	8.61, dd (6.6, 5.5)	–	8.56, dd (6.5, 5.9)	–
Asn				
1	–	172.2, s	–	171.9, s
2	4.82, ddd (8.4, 7.6, 4.8)	51.7, d	4.82, m	50.9, d
3a	2.67, dd (15.5, 8.4)	39.8, t	2.61, m	39.1, t
3b	2.55, dd (15.5, 4.8)	–	2.56, m	–
NH	7.88, d (7.6)	–	7.99, d (8.9)	–
$CONH_2$	6.86, br s, 7.27, br s	174.8, s	6.89, br s, 7.44, br s	175.0, s
Tyr				
1	–	172.1, s	–	172.2, s
2	4.63, m	54.3, d	4.70, m	54.1, d
3a	2.85, m	39.8, t	2.87, m	39.9, t
3b	2.85, m	–	2.83, m	–
4	–	128.4, s	–	128.4, s
5/9	7.01, d (8.2)	131.3, d	7.00, d (8.4)	131.4, d
6/8	6.68, d (8.2)	116.3, d	6.66, d (8.4)	116.4, d

Table 2 (*continued*)

C/H no.	Nostopeptolide A2		Nostopeptolide A3	
	δ_H (*J* in Hz)	δ_C[a]	δ_H (*J* in Hz)	δ_C[b]
7	–	157.6, s	–	157.8, s
NH	8.38, d (7.4)		8.39, d (8.4)	
Pro				
1	–	173.3, s	–	173.1, s
2	3.89, dd (8.2, 2.7)	60.6, d	3.94, m	60.7, d
3a	1.92, m	31.5, t	1.93, m	31.4, t
3b	1.72, m	–	1.74, m	–
4a	1.77, m	23.3, t	1.80, m	23.2, t
4b	1.67, m	–	1.64, m	–
5a	3.51, m	47.3, t	3.50, m	47.3, t
5b	3.23, m	–	3.23, m	–

[a] Multiplicity deduced by DEPT spectroscopy.
[b] Multiplicity based on HSQC data.

epimerization at C-4 in the LeuAc unit was not occurring during the degradation.[12]



The relative stereochemistry of the MePro unit in **1**–**3** was determined to be cis by ROESY correlations. In the ROESY spectrum of **1** (Table 1), for example, MePro H-3*proS* showed a correlation to 4-Me, but not to H-2, whereas MePro H-3*proR* showed correlations to both H-2 and H-4. All four 4-methylprolines were synthesized from L- and D-glutamic acid[13] (Scheme 1) for use as standards in the HPLC analysis.

Experimental

General procedures

NMR spectra were recorded in $CDCl_3$, CD_3OH or CD_3OD at 25°C on spectrometers operating at 300, 400 and 500 MHz for ^1H and 75, 100 and 125 MHz for ^{13}C, using solvent signals as internal references. All 2-D NMR experiments were performed on a Varian 400 Varian UN174 INOVA spectrometer. HSQC experiments were optimized for $^1J_{CH}$=140 Hz and HMBC experiments for $^nJ_{CH}$=7 Hz.

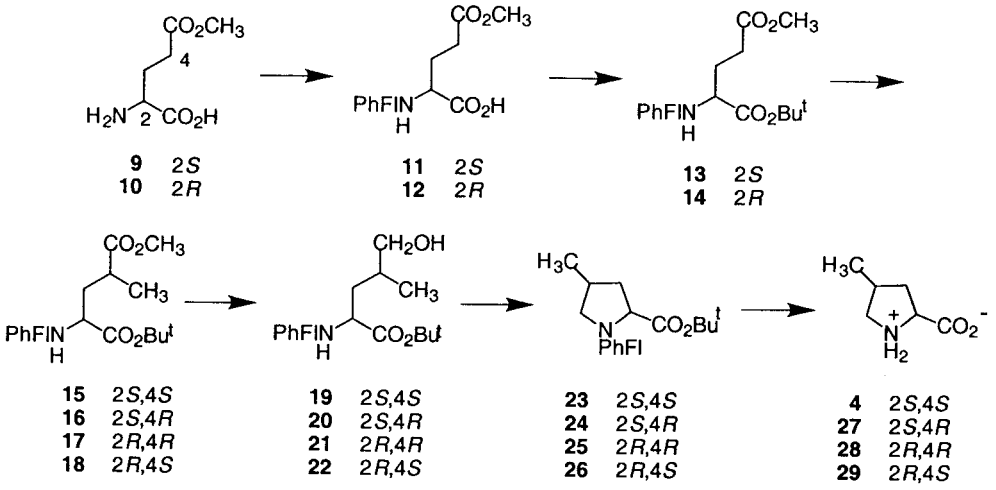
Isolation

Freeze dried *Nostoc* sp. GSV224 (250.0 g) was extracted with CH_3CN/CH_2Cl_2 (4:1) at room temperature for 48 h and then filtered. The filtrate was evaporated in vacuo and the dark green residue (2.0 g) was applied to an ODS-coated

Table 3. Chiral HPLC analysis of acid hydrolysates of nostopeptolides A1, A2 and A3

Amino acid	Retention time of standard in minutes		Amino acids found by co-elution		
	Eluant A[a]	Eluant B[b]	A1	A2	A3
Glycine	7.5		√	√	√
L-Serine	7.8		√	√	√
D-Serine	8.3				
L-Proline		6.0	√	√	√
D-Proline		10.3			
(2*S*,4*S*)-4-MePro	21.0		√	√	√
(2*S*,4*R*)-4-MePro	18.5				
(2*R*,4*R*)-4-MePro	42.0				
(2*R*,4*S*)-4-MePro	36.0				
L-Aspartic acid		14.6	√	√	√
D-Aspartic acid		19.1			
L-Tyrosine		20.9	√	√	√
D-Tyrosine		21.5			
L-Leucine		21.4	√	√	√
D-Leucine		23.4			√[c]
L-Isoleucine	37.2		√		√
L-Alloisoleucine	31.6				
D-Isoleucine	47.9				
D-Alloisoleucine	39.6				
L-Valine	14.8			√	
D-Valine	19.4				

[a] Mobile phase: 1.9 mM $CuSO_4$ in 5:95 MeCN/H_2O, flow rate 1.0 mL/min.
[b] Mobile phase: 1.7 mM $CuSO_4$ in 14:86 MeCN/H_2O, flow rate 0.8 mL/min.
[c] Only seen when **3** is treated with 1N NaOH prior to acid hydrolysis.



Scheme 1. Synthesis of the 4-methylprolines as described by Koskinen and Rapoport.[13]

silica column (60 g, 7×5 cm^2) and subjected to flash chromatography using successively 3:1 H_2O/CH_3CN (0.8 L), 1:1 H_2O/CH_3CN (0.8 L), 35:65 H_2O/CH_3CN (1 L), MeOH (0.8 L) and CH_2Cl_2 (0.5 L). The fraction that was eluted with 1:1 H_2O/CH_3CN was subjected to reversed phase HPLC (Econosil C18, 10 μm, 250 mm×22 mm, 10 μm, UV detection at 210 nm, flow rate 6.0 mL/min) using 0.02N trifluoroacetic acid in 65% H_2O/CH_3CN as the eluant. The fractions that emerged from the Econosil C18 column at 57.1 and 84 min were evaporated to give nostopeptolide A2 (4 mg) and nostopeptolide A1 (80 mg), respectively. Another fraction that eluted from the column at 96.2 min was evaporated and the residue (20.4 mg) was repeatedly subjected to reversed phase HPLC (YMC-Pack ODS-AQ, 250×10 mm, 5 μm, 3 mL/min) using a gradient of 30–50% CH_3CN in 0.02N CH_3COONH_4 for the first 20 min, then 50–80% CH_3CN for the next 20 min, and finally 80–100% CH_3CN for the last 5 min. The fraction that eluted at 21.2 min from the YMC-ODS column yielded nostopeptolide A3 (5 mg).

Nostopeptolide A1 (1). $[\alpha]_D^{25}=-42°$ (CH_3OH c=2.25); UV λ_{max} (ϵ) 203 (130125), 220 (70265 nm); IR (film) ν_{max} 3287, 1729 (ester), sh 1670 (amide), 1644 cm^{-1}; positive ion FABMS m/z (rel. intensity) 1119 MK^+, 30), 1103 (MNa^+, 100), 1081 (MH^+, 35), 898 (9); negative ion FABMS m/z (rel. intensity) 1079 ($[M-H]^-$, 100), 686 (3), 587 (5), 561 (7), 436 (5), 266 (19); HRFABMS m/z 1103.5826 (MNa^+, calcd for $C_{53}H_{80}N_{10}NaO_{14}$, −7.3 mmu error). ^1H NMR (400 MHz) and ^{13}C NMR (100 Hz) data in CD_3OH are given in Table 1.

Nostopeptolide A2 (2). $[\alpha]_D^{25}=-21°$ (CH_3OH c=2.0); UV λ_{max} (ϵ) 203 (115237), 220 (54468 nm); IR (film) ν_{max} 3293, 1730, 1671, 1645 cm^{-1}; FABMS m/z (rel. intensity) 1105 (MK^+, 7), 1089 (MNa^+, 27), 1067 (MH^+, 3); HRFABMS m/z 1089.5606 (MNa^+, calcd for $C_{52}H_{78}N_{10}NaO_{14}$, −0.9 mmu error). ^1H NMR (400 MHz) and ^{13}C NMR (100 Hz) data in CD_3OH are given in Table 2.

Nostopeptolide A3 (3). $[\alpha]_D^{25}=-46°$ (CH_3OH c=2.67); UV λ_{max} (ϵ) 200 (70626), 222 (16215 nm); IR (film) ν_{max} 3275, 1738, 1638 cm^{-1}; FABMS m/z (rel. intensity) 1103 (MNa^+, 58), 1081 (MH^+, 70), 898 (31); HRFABMS m/z 1081.5898 (MH^+, calcd for $C_{53}H_{81}N_{10}O_{14}$, +3.6 mmu error). ^1H NMR (400 MHz) and ^{13}C NMR (100 Hz) data in CD_3OH are given in Table 2.

Acid hydrolysis of nostopeptolides

Each nostopeptolide (0.5–1 mg) in 0.5–1 mL of 6N HCl was heated in a glass tube sealed under vacuum at 110°C for 12 hrs. The hydrolyzates were evaporated under a nitrogen stream and dried under vacuum. The individual mixtures were applied to a small C18 column and eluted with MeOH/H_2O (10:90). The eluates were dried under vacuum and reconstituted with 0.2–0.5 mL of H_2O prior to injection. The hydrolyzates were analysed by HPLC on a Phenomenex, Chirex D-pencillamine column using one of the two following mobile phase conditions, (A) 1.9 mM $CuSO_4$ in 5:95 CH_3CN/H_2O at a flow rate of 1.0 mL/min or (B) 1.7 mM $CuSO_4$ in 14:86 CH_3CN/H_2O at a flow rate of 0.8 mL/min, with UV detection at 245 nm for both systems. The amino acids in the acid hydrolyzates were identified by comparing the retention times with those of authentic standards, both alone and by co-injection. The results are summarized in Table 3.

Synthesis of ethyl *N*-Boc-L-leucylacetate (7) and ethyl *N*-Boc-D-leucylacetate (8). Compound **7** was synthesized from *N*-Boc-L-Leu in 44% yield using a procedure described for the synthesis of ethyl (4*R*,5*S*)-4-[(*tert*-butoxycarbonyl)amino-5-methyl-3-oxoheptanoate from *N*-Boc-D-Ile.[14] ^1H NMR ($CDCl_3$, 300 MHz) δ 0.90 (H_3-7, d, J=6.4 Hz), 0.92 (6-CH_3, J=6.1 Hz), 1.3–1.5 (1H, m), 1.42 (*t*-butyl protons, s), 1.5–1.7 (2H, m), 3.50/3.57 (H_2-2, AB quartet, J=17 Hz), 4.18 (OCH_2CH_3), 4.32 (H-4, td, J=9 and 3.5 Hz), 4.97 (NH, d, J=9). Compound **8** was synthesized from *N*-Boc-D-Leu by the same procedure and exhibited an identical ^1H NMR spectrum.

Degradation of compound 7 to L-leucine and compound 8 to D-leucine. Compound **7** (0.4 g) was dissolved in 2.5 mL of DME and treated with 2.5 mL of 2N NaOH at room temperature. After 2 h, the reaction mixture was acidified to pH 4 and extracted with EtOAc (3×20 mL). The extract

was washed with brine, dried over $mgSO_4$ and evaporated. The residue was subjected to flash chromatography on silica gel using eluants starting from 10% EtOAc to 50% EtOAc in hexane to obtain *N*-Boc-L-leucylacetic acid (0.35 g). The Boc protected acid was dissolved in TFA (2 mL) and stirred at room temperature. After 1 h, the solvent was evaporated and the residue was analyzed by HPLC (see Table 3 for conditions). Only L-Leu was observed in the HPLC chromatogram. Using the same procedure compound **8** was converted to D-Leu.

Synthesis of 4-methylprolines. A. Glutamic acid γ-methylester. A pre-cooled solution of 2.9 mL of acetyl chloride (40.8 mmol) in 20 mL of methanol was added dropwise to a suspension of L- or D-glutamic acid (5 g, 34 mmol) in 20 mL of methanol at 5°C. After 4 h the mixture was allowed to warm to RT and stirring was continued for 12 h. Pyridine (4 mL) was added and after 48 h, the product, a white solid, was collected and washed with ethanol and ether (4.65 g, 85%). ^{1}H NMR (CD_3OD, 300 MHz) of L- (**9**) or D-glutamic acid γ-methylester (**10**) δ 3.57 (H-2, t; *J*=6.5 Hz), 2.01–2.20 (H_2-3, m), 2.60 (H_2-4, t; *J*=7.4 Hz), 3.68 (γ-COOCH_3, s).

B. *N*-(9-(9-Phenylfluorenyl))glutamic acid γ-methyl ester. Compound **9** or **10** was converted to L- (**11**) or D-*N*-(9-(9-phenylfluorenyl))glutamic acid γ-methyl ester (**12**) as previously described.[13] ^{1}H NMR ($CDCl_3$, 300 MHz) of **11** or **12** δ 2.64 (H-2, dd; *J*=7.2 and 4.8 Hz), 1.66–1.76 (H-3, m), 1.79–1.91 (H-3, m), 2.25–2.45 (H_2-4, m), 3.67 (γ-COOCH_3, s), 7.19–7.73 (*N*-PhFl, 13H, m).

C. α-*tert*-Butyl γ-methyl *N*-(9-(9-phenylfluorenyl))glutamate. Compound **11** or **12** was converted to L- (**13**) or D-α-*tert*-butyl γ-methyl *N*-(9-(9-phenylfluorenyl))glutamate (**14**) as previously described.[13] ^{1}H NMR ($CDCl_3$, 300 MHz) of **13** or **14** δ 1.17 (α-*tert*-butyl ester, 9H, s), 2.51 (H-2, m), 1.65–1.73 (H_2-3, m), 2.27–2.51 (H_2-4, m), 3.67 (γ-COOCH_3, s), 7.18–7.71 (*N*-PhFl, 13H, m).

D. (2*S*,4*S*)-α-*tert*-Butyl γ-methyl *N*-(9-(9-phenylfluorenyl))-4-methylglutamate (15) and (2*S*,4*R*)-α-*tert*-butyl γ-methyl *N*-(9-(9-phenylfluorenyl))-4-methylglutamate (16). Compound **13** (2.62 g, 5.74 mmol) was converted to a 3:1 mixture of **15** and **16** (2.63 g, 97%) as previously described.[13] ^{1}H NMR ($CDCl_3$, 300 MHz) of **15** δ 1.14 (α-*tert*-butyl ester, 9H, s), 2.47 (2, dd; *J*=10.5 and 3.9 Hz), 1.28–1.37 (H-3, m), 1.83–1.93 (H-3, m), 2.66–2.77 (H-4, m), 0.79 (4-CH_3, d; *J*=7.2 Hz), 3.69 (γ-COOCH_3, s), 7.16–7.71 (*N*-PhFl, 13H, m). ^{1}H NMR ($CDCl_3$, 300 MHz) of 16 δ 1.18 (α-*tert*-butyl ester, 9H, s), 2.56 (H-2, dd; *J*=7.6 and 5.1 Hz), 1.35–1.44 (H-3, m), 1.81–1.91 (H-3, m), 2.69–2.80 (H-4, m), 1.07 (4-CH_3, d; *J*=7.1 Hz), 3.57 (γ-COOCH_3, s), 7.16–7.71 (*N*-PhFl, 13H, m).

(2*R*,4*R*)-α-*tert*-Butyl γ-methyl *N*-(9-(9-phenylfluorenyl))-4-methylglutamate (17) and (2*R*,4*S*)-α-*tert*-butyl γ-methyl *N*-(9-(9-phenylfluorenyl))-4-methylglutamate (18). Using the same procedure compound **14** (2.07 g, 4.52 mmol) was converted to a 3:1 mixture of **17** and **18** (2.03 g, 95%). The ^{1}H NMR spectra of **17** and **18** were identical with those of **15** and **16**, respectively.

E. α-*tert*-Butyl (2*S*,4*S*)-5-hydroxy-4-methyl-2-*N*-(9-(9-phenylfluorenyl))amino)pentanoate (19) and α-*tert*-butyl (2*S*,4*R*)-5-hydroxy-4-methyl-2-*N*-(9-(9-phenylfluorenyl))amino)pentanoate (20). The diastereomeric mixture of **15** and **16** (1.35 g, 2.86 mmol) was converted and separated into **19** (0.81 g, 64%) and **20** (0.26 g, 21%) as previously described.[13] ^{1}H NMR ($CDCl_3$, 300 MHz) of **19** δ 1.16 (α-*tert*-butyl ester, 9H, s), 2.49 (H-2, dd; *J*=10.2 and 3.7 Hz), 1.20–1.28 (H-3, m), 1.48–1.58 (H-3, m), 1.70–1.84 (H-4, m), 0.61 (4-CH_3, d; *J*=6.8 Hz), 3.32 (5-CH_2OH, dd; *J*=10.5 and 8.0 Hz), 3.48 (5-CH_2OH, dd; *J*=10.5 and 4.9 Hz), 7.17–7.71 (*N*-PhFl, 13 H, m). ^{1}H NMR ($CDCl_3$, 300 MHz) of 20 δ 1.25 (α-*tert*-butyl ester, 9H, s), 2.60 (H-2, dd; *J*=5.0 and 5.0 Hz), 1.28–1.37 (H-3, m), 1.43–1.50 (H-3, m), 1.65–1.77 (H-4, m), 0.80 (4-CH_3, d; *J*=6.8 Hz), 3.23 (5-CH_2OH, dd; *J*=10.7 and 7.1 Hz), 3.47 (5-CH_2OH, dd; *J*=10.7 and 4.6 Hz), 7.15–7.73 (*N*-PhFl, 13H, m).

α-*tert*-Butyl (2*R*,4*R*)-5-hydroxy-4-methyl-2-*N*-(9-(9-phenylfluorenyl))amino)pentanoate (21) and α-*tert*-butyl-(2*R*,4*S*)-5-hydroxy-4-methyl-2-N-(9-(9-phenylfluorenyl))amino)-pentanoate (22). As described above the mixture **17** and **18** (1.893 g, 4.01 mmol) was converted and separated into **21** (1.16 g, 65%) and **22** (0.38 g, 21%). The ^{1}H NMR spectra of **21** and **22** were identical with those of **19** and **20**, respectively.

F. (2*S*,4*S*)-4-Methyl-*N*-(9-(9-phenylfluorenyl))proline *tert*-butyl ester (23). Compound **19** (0.47 g, 1.06 mmol) was converted into **23** (0.41 g, 90%) as previously described.[13] ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.21 (α-*tert*-butyl ester, 9H, s), 2.98 (H-2, dd; *J*=8.3 and 8.3), 1.30 (H-3, m), 2.04 (H-3, m), 1.97 (H-4, m), 0.94 (4-CH_3, d; *J*=6.3 Hz), 3.01 (H-5, dd; *J*=11.2 and 9.4 Hz), 3.31 (H-5, dd; *J*=11.2 and 7.0 Hz), 7.10–7.71 (*N*-PhFl, 13H, m).

(2*S*,4*R*)-4-Methyl-*N*-(9-(9-phenylfluorenyl))proline *tert*-butyl ester (24). As described above for **19**, 130 mg (0.29 mmol) of **20** was transformed into 107 mg (86%) of **24**. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.28 (α-*tert*-butyl ester, 9H, s), 3.25 (H-2, m), 1.22–1.40 (H-3, m), 1.66 (H-3, m), 2.32–2.46 (H-4, m), 0.86 (4-CH_3, d; *J*=6.1 Hz), 3.16 (H-5, dd; *J*=10.0 and 1.0 Hz), 3.26 (H-5, m), 7.10–7.71 (*N*-PhFl, 13H, m).

(2*R*,4*R*)-4-Methyl-*N*-(9-(9-phenylfluorenyl))proline *tert*-butyl ester (25). As described above for **19**, 0.7 g (1.58 mmol) of **21** was transformed into 0.593 g (88%) of **25**. The ^{1}H NMR spectrum of **25** was identical with the one for **23**.

(2*R*,4*S*)-4-Methyl-*N*-(9-(9-phenylfluorenyl))proline *tert*-butyl ester (26). As described above for **19**, 177 mg (0.4 mmol) of **22** was transformed into 146 mg (86%) of **26**. The ^{1}H NMR spectrum of **26** was identical with the one for **24**.

G. (2*S*,4*S*)-4-Methylproline (4). Compound **23** (0.40 g, 0.94 mmol) was converted into 0.11 g, of **4** (91%) as previously described.[13] ^{1}H NMR (CD_3OD 300 MHz) δ 4.40 (H-2, dd; *J*=9.8 and 8.0 Hz), 1.69 (H-3, dt; *J*=−12.7 and 9.8 Hz), 2.60 (H-3, m), 2.49 (H-4, m), 1.12 (4-CH_3, d;

J=6.6 Hz), 2.89 (H-5, dd; J=−11.2 and 9.5 Hz), 3.49 (H-5, dd; J=−11.2 and 7.7 Hz).

(2*S*,4*R*)-4-Methylproline (27). As described above for **4**, 0.11 g of **24** (0.26 mmol) was converted into 28 mg of **27** (85%). ^{1}H NMR (CD_3OD 300 MHz) δ 4.36 (H-2, dd J=9.8 and 4.6 Hz), 2.00 (H-3, m), 2.27–2.47 (H-3 and H-4, m), 1.15 (4-CH_3, d; J=6.3 Hz), 2.84 (H-5, dd; J=−11.2 and 8.8 Hz), 3.53 (H-5, dd; J=−11.2 and 7.8 Hz).

(2*R*,4*R*)-4-Methylproline (28). As described above for **4**, 0.58 g of **25** (1.4 mmol) was converted into 0.155 g of **28** (88%). The ^{1}H NMR spectrum of **28** was identical with the one for **4**.

(2*R*,4*S*)-4-Methylproline (29). As described above for **4**, 0.15 g of **26** (0.35 mmol) was converted into 36 mg of **29** (79%). The ^{1}H NMR spectrum of **29** was identical with the one for **27**.

Acknowledgements

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