A New Strategy for the Synthesis of Cyclopeptides Containing Diaminoglutaric Acid

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Abstract: A new synthesis of orthogonally protected diaminoglutaric acid containing peptides using the Ugi four component condensation is presented. To demonstrate that this method is useful to replace cystine by diaminoglutaric acid in biologically interesting peptides, we built up two cyclic somatostatin analogues deriving from Sandostatin and from TT-232. A photolytically cleavable amine derivative of the nitroveratryl type is used for the Ugi four component condensation. Because of a racemic build up of the new stereocentre of the diaminoglutaric acid, and racemization of the isonitrile component, four diastereomeric peptides resulted that were separated by HPLC. The stereochemistry of the cyclopeptides could be easily and unambiguously assigned by chiral gas chromatography and a reference sample of enantiomerically pure (2S,4S)-diaminoglutaric acid. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: cyclopeptides; diaminoglutaric acid; photolytic cleavage; Somatostatin; Ugi four component condensation (4CC)

INTRODUCTION

Disulphide bridges play an important role in the stabilization of secondary and tertiary structures of proteins and peptides. We were interested in a drastical shortening of this bridge to introduce a stronger conformational constraint. Slightly shorter bridges with different stereochemistry are found in

lanthionine residues, in which the $C^\beta SSC'^\beta$ bridge is substituted by $C^\beta SC'^\beta$. A much shorter bridge could be constructed by using diaminoglutaric acid, which is equivalent to a substitution of the four atom disulphide bridge by only one methylene group. Sulphide bridges are found in nature in the lanthionine element [1] and has been used several times to constrain bioactive peptides [2,3]. On the other hand, diaminoglutaric acid occurs in nature only as a degradation product of streptamine [4] and as a precursor of the biosynthesis of diaminobutyric acid [5], and, to our knowledge, it has never been used as building block for peptide synthesis. One reason for this may result from the difficulty in differentiating between the two amino and the two carboxyl groups. Herein, we present a new approach to obtain the orthogonally protected diaminoglutaric acid building block. This strategy was then applied to the synthesis of the somatostatin analogues **1** and **2** shown in Figure 1. 4-H-D-Phe-c[(2S,4R/S)-Dag-D/L-Phe-D-Trp-Lys-Thr]-2-Thr(ol) **1** is a diaminoglutaric acid analogue of Octreotide (Sandostatin) (D-Phe-D-[Cys-Phe-D-Trp-Lys-Thr-Cys]-Thr(ol)) [6], 4-H-D-Phe-c[(2S,4R/S)-Dag-

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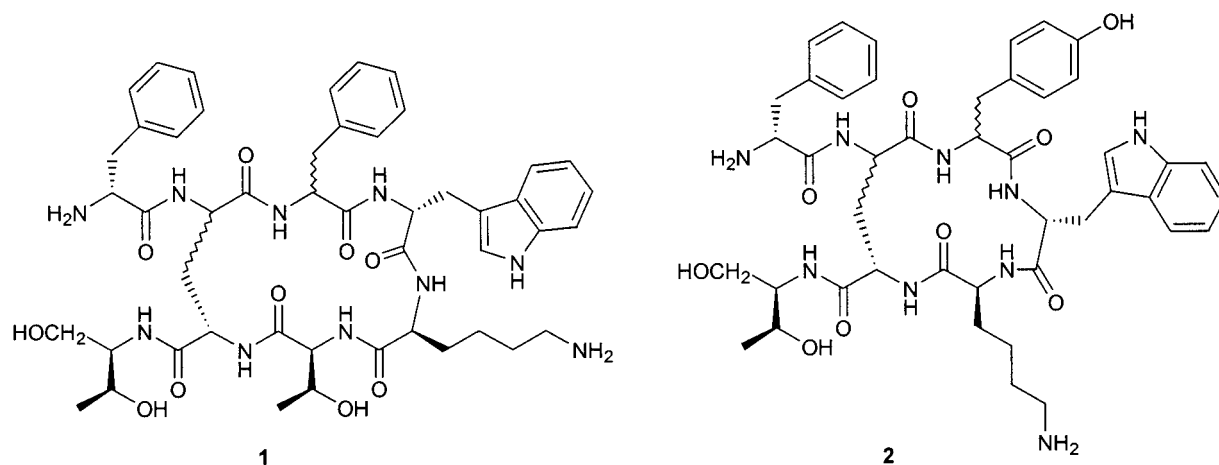
Figure 1   Diaminoglutaric acid analogues of Octreotide (**1**) and TT-232 (**2**).

D/L-Tyr-D-Trp-Lys]-2-Thr(ol) **2** derives from the shortened Sandostatin analogue TT-232 (D-Phe-c[Cys-Tyr-D-Trp-Lys-Cys]-Thr-NH$_2$) recently synthesized by Keri et al. [7–9].

Our approach uses the Ugi four component condensation (Ugi-4CC), which allowed us to introduce Fmoc and Boc protection, as well as allyl and benzyl protection simultaneously (Figure 2).

RESULTS AND DISCUSSION

The 4CC results in N-alkylated peptides in which the N-alkyl residue is very difficult to remove. Therefore, the key step in this procedure was the choice of a suitable amine component which allows the selective cleavage of the N-alkyl bond in the 4CC-product without interfering with all four C- and N-terminal protecting groups. The amine component of choice

is of the nitroveratryl type. Patchornik et al. have introduced the NVOC (6-nitroveratryloxycarbonyl)-protecting group [10] and Holmes described an o-nitrobenzyl photolabile linker for solid phase synthesis [11,12]. We applied this concept to a mild cleavable N-alkyl residue of this type.

Another problem encountered in using the Ugi-4CC is the creation of a new stereocentre in a non-selective way. In addition, the racemization of the isonitrile component yields in four diastereomers. It is known that using chiral auxiliaries the 4CC can be performed with stereochemical induction, but these auxiliaries are either difficult to cleave [13], or the enantioselectivity is not satisfying [14]. Because we were interested in obtaining all four stereoisomers, we performed the Ugi-4CC without stereochemical induction, and separated the four isomers at different stages of the synthesis by HPLC.
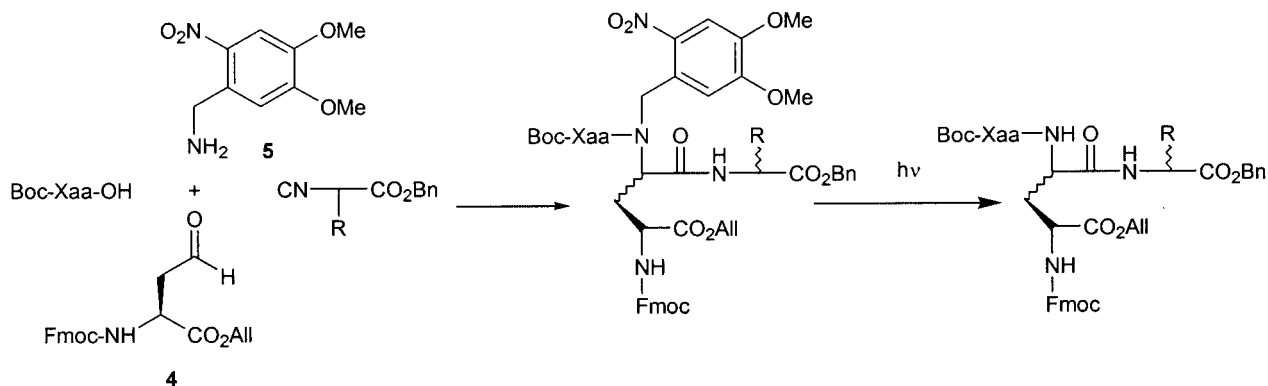

Figure 2   General scheme for the synthesis of orthogonally protected diaminoglutaric acid containing peptides by Ugi-4CC.

The Four Components

The synthesis of the amine component **5** was performed according to a method described by Holmes *et al.* [11,12]. It was obtained in a simple three step procedure, starting from veratrylamine.

The benzyl protected isonitrile components **6** and **17** were synthesized by dehydratization [15,16] of the N-formyl amino acid derivatives [17]. As previously described [16], this method results in racemization of this component.

The acid component was a commercially available Boc protected amino acid derivative.

The aldehyde component **4** was synthesized by

reduction of the Fmoc and allyl protected Weinreb amide [18] **3** of aspartic acid, with DIBAH in 63% yield over two steps (Figure 3).

Peptide Synthesis

The *N*-nitrobenzylated tripeptides **7** and **18** were built up by four component condensation, with the amine **5**, the aldehyde **4**, the acid and the isonitrile component **6** or **17** in nearly quantitative crude yields (Figure 4). To avoid the negative effects of the internal filter of the resulting nitrosoaromatics, the following photolytic cleavage was performed under high dilution (1 mg/mL). After workup, this results
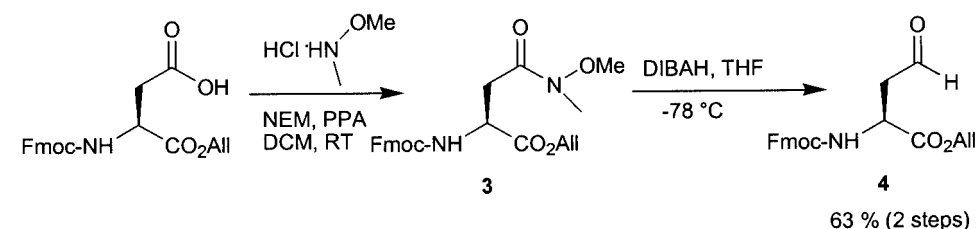

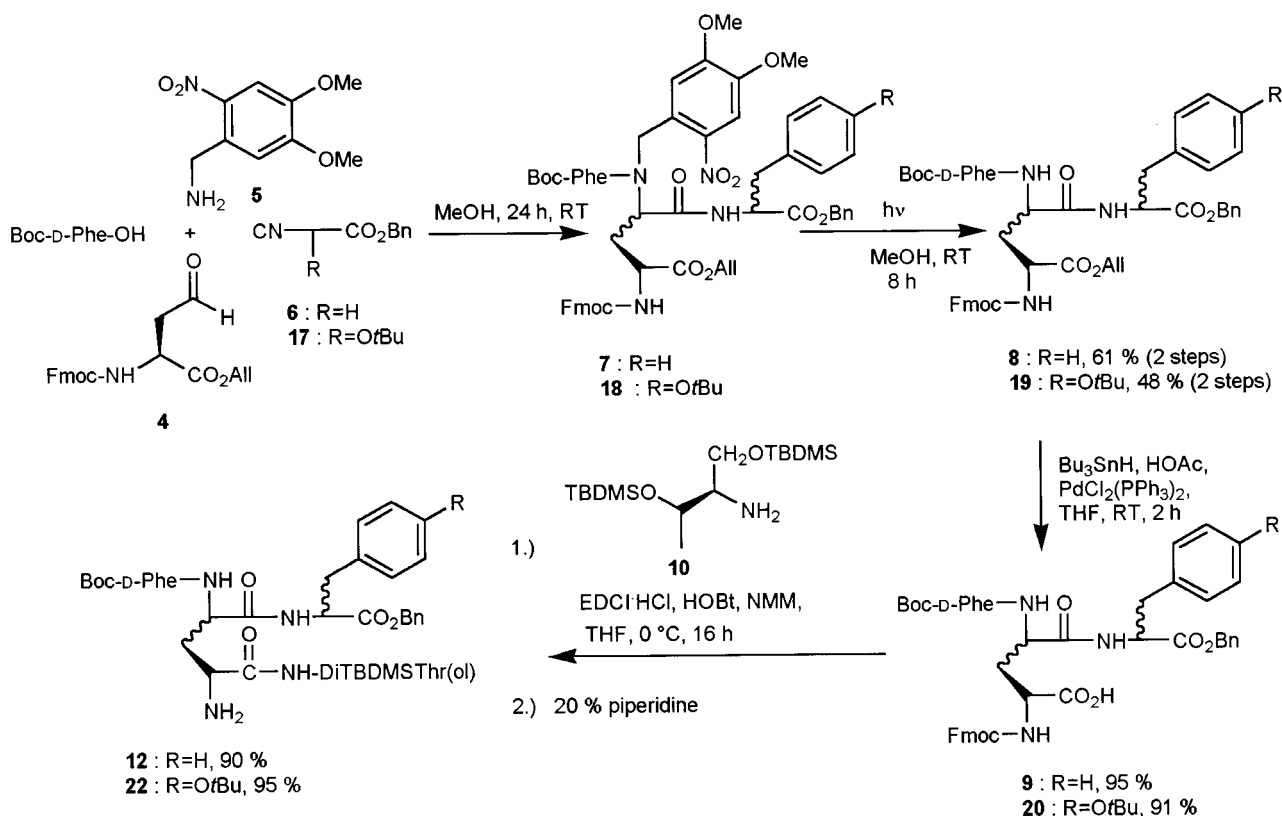Figure 3   Synthesis of the Fmoc and allyl protected aldehyde component **4**.

Figure 4   Synthesis of the *N*-deprotected tetrapeptide **12** and **22**.

in the fully orthogonally protected tripeptides **8** and **19** in 48–61% yield over two steps.

The allyl ester (**8** and **19**) is cleaved through palladium-catalysed hydrostannolysis [19], resulting in **9** and **20**.

Following Bauer *et al.* [6] and Wynanst *et al.* [20], the threoninol residue is essential for the activity of octreotide. We obtained this building block, **10**, by reduction of the Z-protected amino acid [21]. Experiments to protect the resulting diol as *t*-butyl ether resulted in very low yields. Therefore, we chose the TBDMS-group [22], which can be easily introduced and cleaved, together with the acid labile protecting groups.

Coupling of the bis silylated threoninol **10** with HOBt/EDCI activation to this peptide and Fmoc deprotection results in the tetrapeptides **12** and **22** (Figure 5).

Under the same conditions, fragment condensation of Fmoc-D-Trp(Boc)-Lys(Boc)-Thr(*t*Bu)-OH to **12** (Figure 6) and of Fmoc-D-Trp(Boc)-Lys(Boc)-OH to **22** (Figure 7) was performed. This led to the fully protected linear precursors of the cyclic diaminoglu-

taric acid bridged somatostatin analogues **15** and **25**. After deprotection of the temporary protection groups, cyclization with HOAt/DIC [23] and acidic removal of the permanent protection groups the four diastereomers of each cyclic somatostatin analogue **1a–d** and **2a–d** are obtained by HPLC separation (Figures 8 and 9).

The stereochemistry of the amino acids in the four stereoisomeric peptides (**1a–d** and **2a–d**) was determined via chiral amino acid analysis by gas chromatography using LipodexE as chiral stationary phase [24]. The critical stereocentres are the newly created stereocentre of the diaminoglutaric acid residue, as well as the phenylalanine in the Octreotide analogue **1**, and the tyrosine in the TT-232 derived peptide **2** resulting from the racemic isonitrile components **6** and **17**. For the assignment of the stereochemistry of the diaminoglutaric acid, this building block was synthesized enantiomerically pure after a method described by Belokon *et al.* [25], and derivatized using standard procedures [26]. To prove that the diastereomers of diaminoglutaric acid were separable by GC, we performed
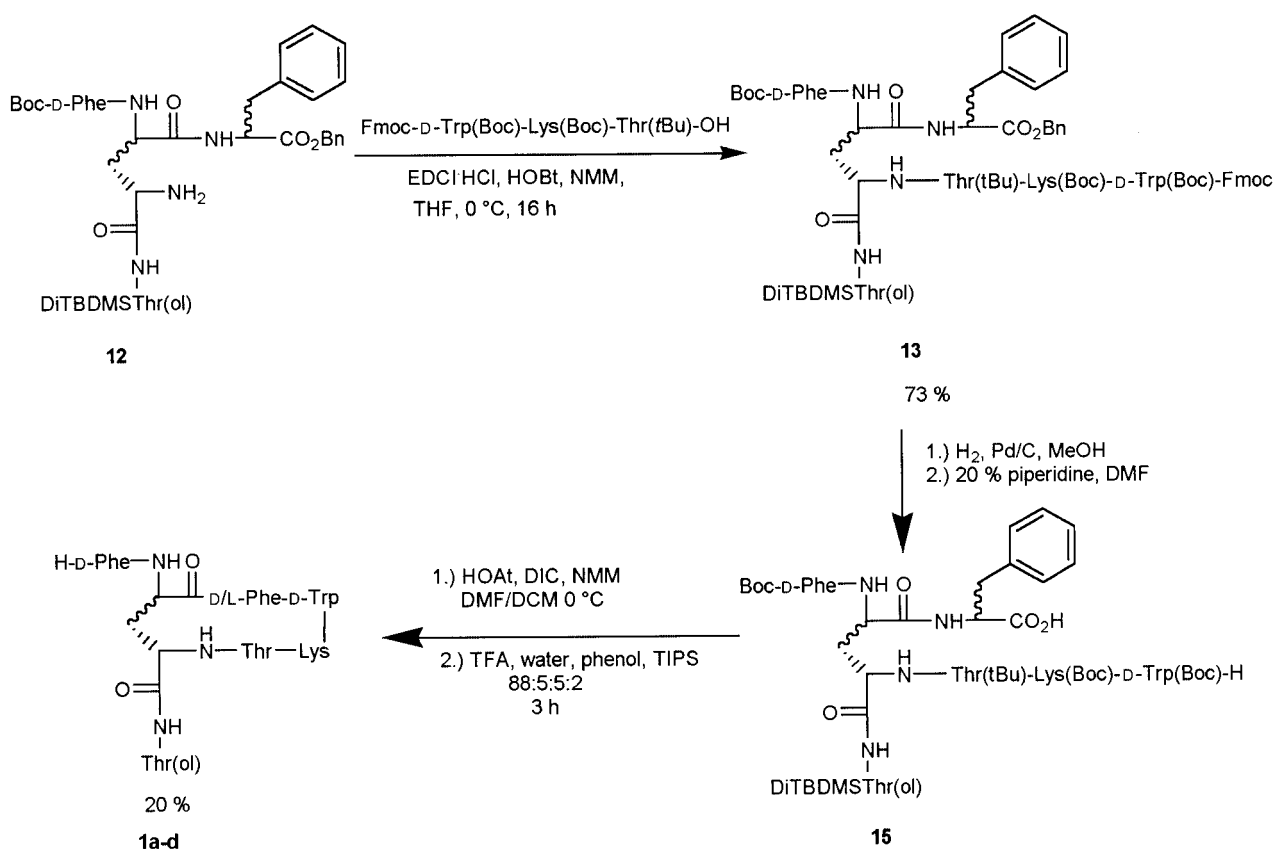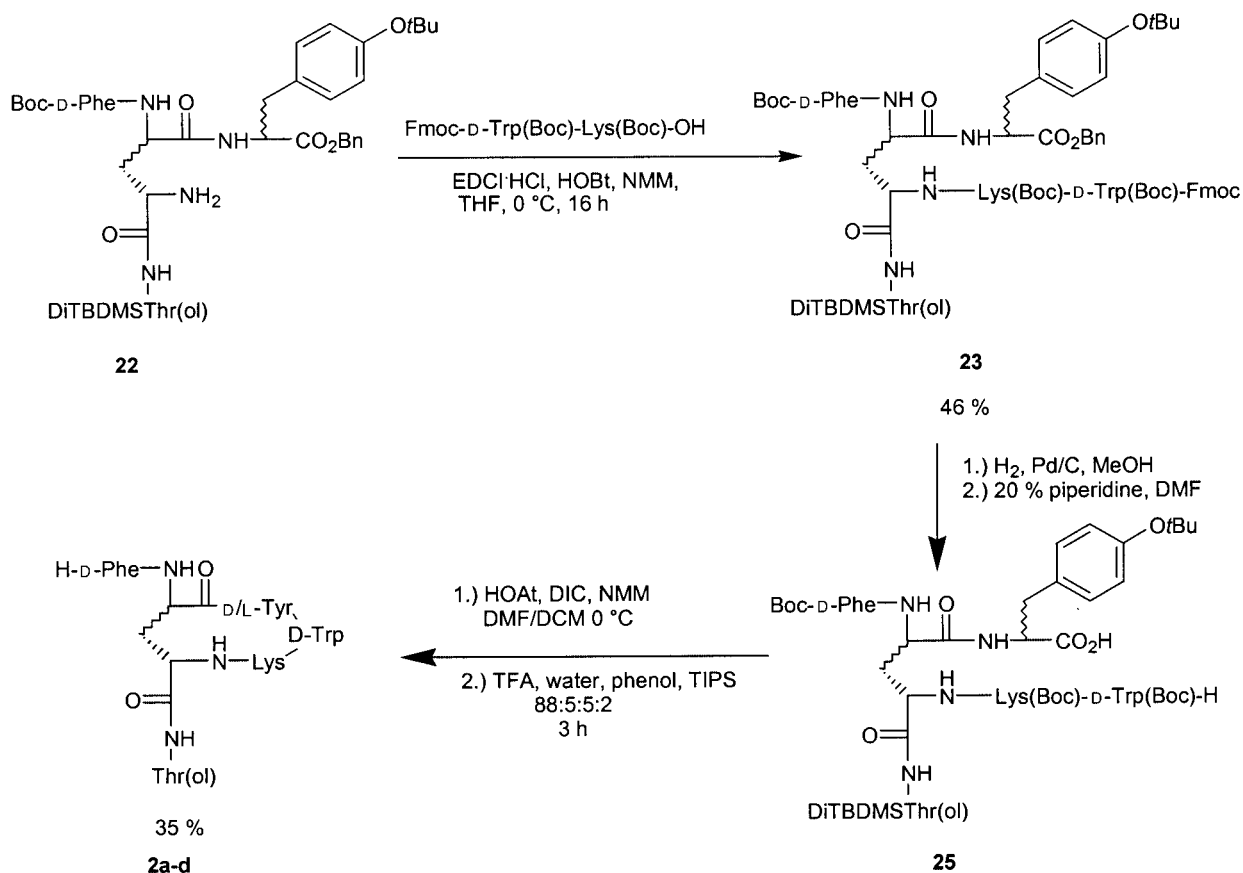

Figure 5 Synthesis of the Octreotide analogue **1**.

Figure 6   Synthesis of the TT-232 derived peptide 2.

racemization tests with the enantiopure (S,S)-diaminoglutaric acid. After treating this amino acid with 20% ammonia, a second peak (Figure 9(b)) appeared in the GC-MS with mass selective detection ($m/z = 337$ [M-COOEt]$^+$).

Compounds **1a–d** were tested by the MTT assay [27], but turned out to be inactive.

CONCLUSION

We have demonstrated a new synthesis of orthogonally protected diaminoglutaric acid containing peptides. A photolytically cleavable amine component is used as ammonia synthon for the Ugi four component condensation. The method proceeds via nearly racemic build up of the new stereocentre of the diaminoglutaric acid and racemization of the isonitrile component. However, we were able to separate the resulting four diastereomers of both target peptides by HPLC. The stereochemistry could be easily and unambiguously assigned by chiral gas-

chromatography, and a reference sample of enantiomerically pure (2S,4S)-diaminoglutaric acid.

EXPERIMENTAL METHODS

Materials and Methods

NMR spectra were recorded using a Bruker AC250 (250 MHz) spectrometer. Chemical shifts are expressed in p.p.m (δ) relative to residual solvent. Multiplicities are reported as (s) singlet, (d) doublet, (t) triplet, (q) quartet and (m) multiplet. Mass spectra were taken with a Finnigan LCQ (ESI) and a Finnigan Model 8230 (EI/CI). TLC was performed on silica gel plates (Merck Silica 60 F254 sheets). Chiral amino acid determination was performed with a Hewlett Packard (GC-system 6890, mass selective detector 5973) machine with a LipodexE (25 m × 250 µm) column. Photolysis was performed by irradiation with a 150 W high pressure Hg lamp (TQ 150 Z2 from Heraeus).

Figure 7 (a) HPLC analysis of the four diastereomers of 4-H-D-Phe-c[(2S,4R/S)-Dag-D/L-Phe-D-Trp-Lys-Thr]-2-Thr(ol) **1a–1d**). (b) Stereochemical assignment of the four diastereomers.

HPLC was performed on different systems: (I): Beckmann (solvent delivery module 110 B; controller 420; Knauer variable wavelength detector (uvicord)) (II): Waters (solvent delivery module 510; programmable multi wavelength detector 490E; controller 490E) (III): Pharmacia Basic 10 A; analytical column: YMC-ODSA (5 μm, 250 mm 4 mm; flow rate 1 mL/min); preparative column: YMC-ODSA (5 μm, 250 mm, 20 mm; flow rate 6 mL/min). The eluant was 0.1% TFA in acetonitrile-water. Different linear gradients were employed for the analytical runs.
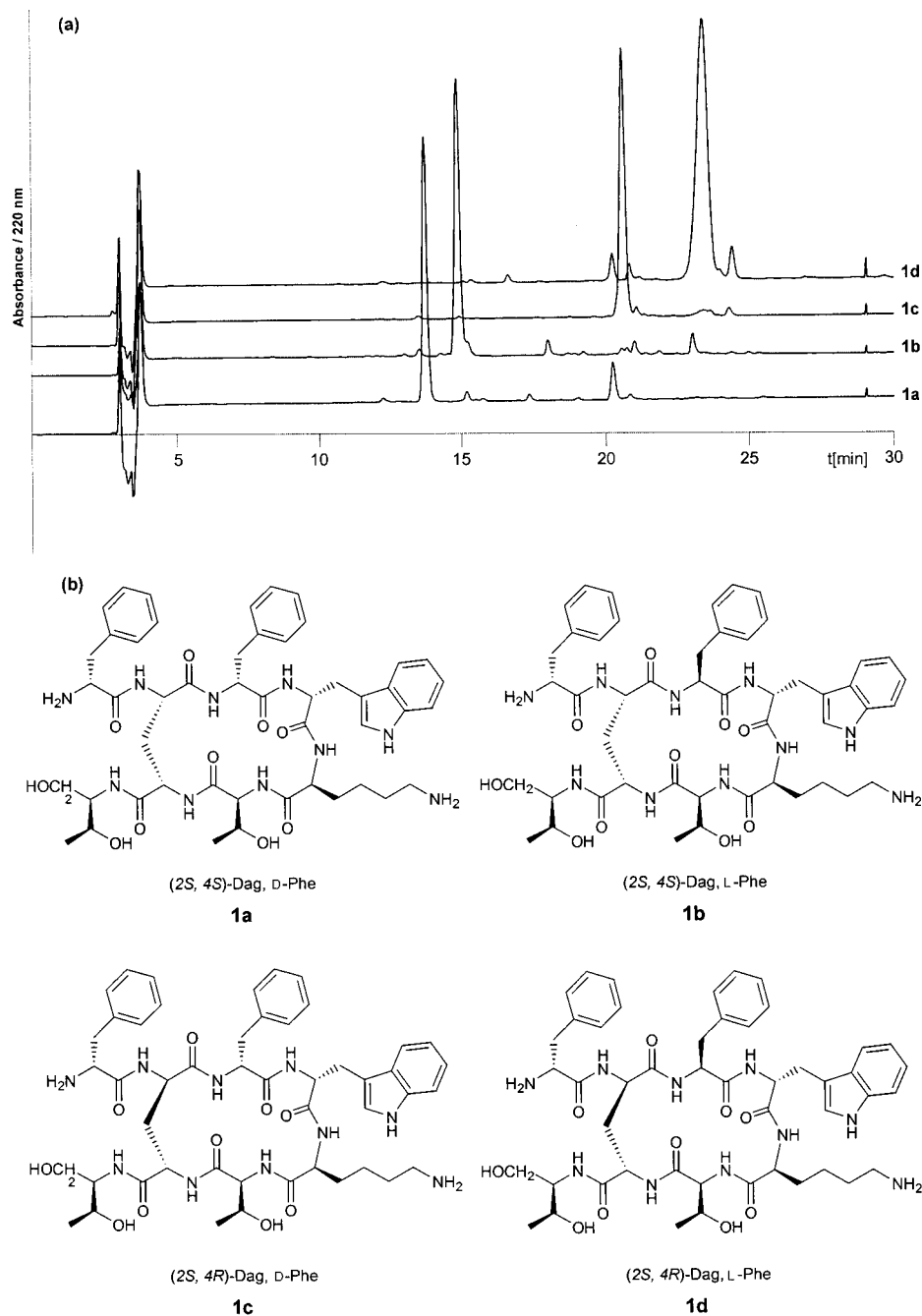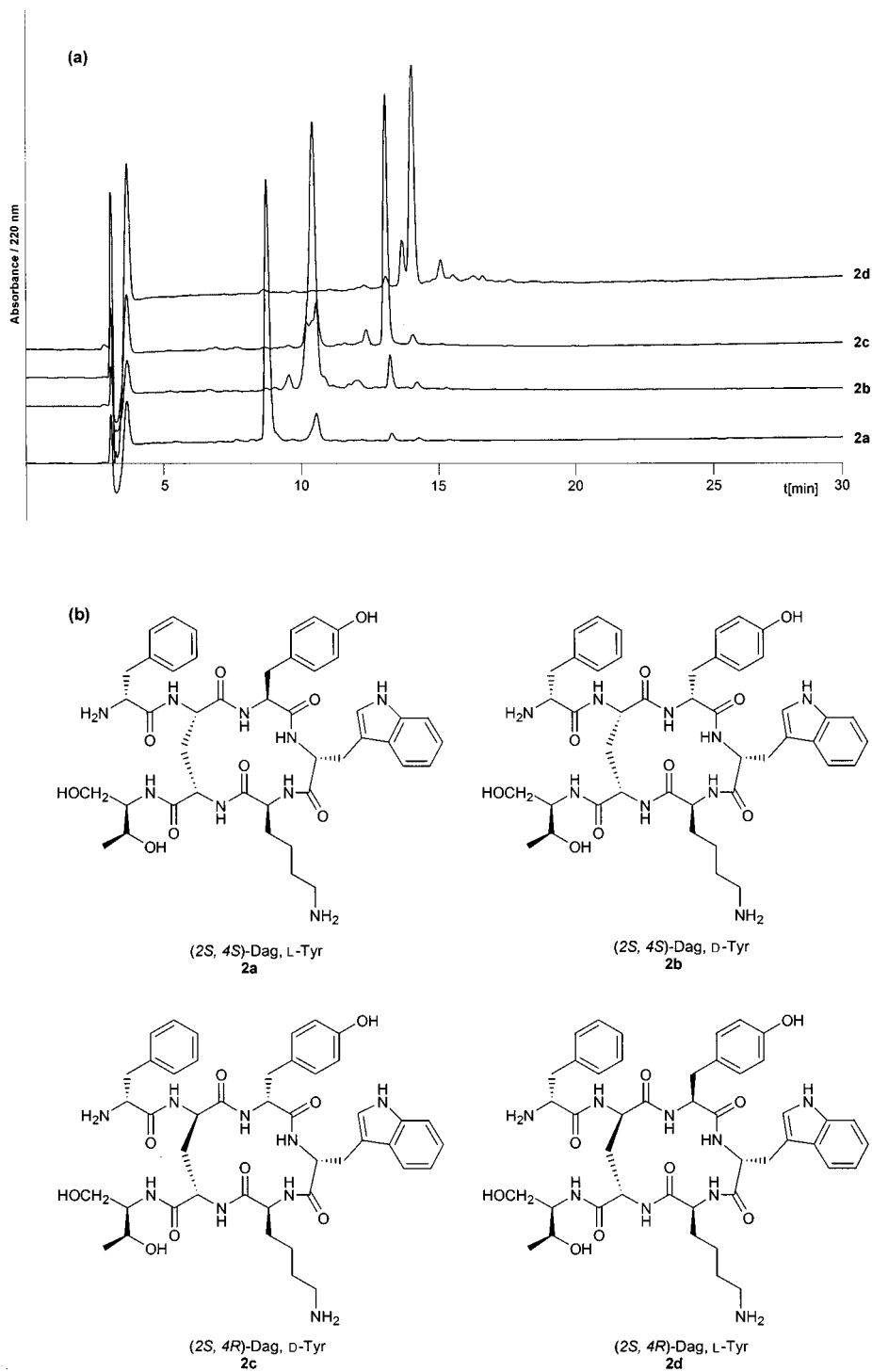
Figure 8 (a) HPLC analysis of the four diastereomers of 4-H-D-Phe-c[(2S,4R/S)-Dag-D/L-Tyr-D-Trp-Lys]-2-Thr(ol) **2a–d**. (b) Stereochemical assignment of the four diastereomers.
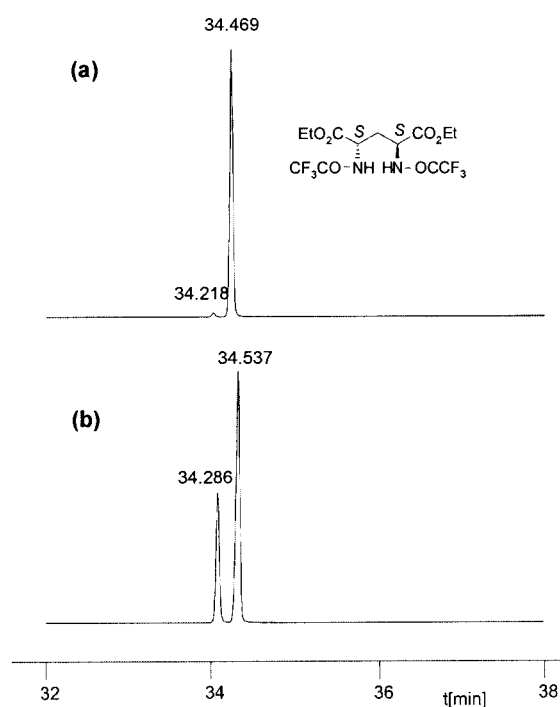
Figure 9 GC on LipodexE with mass selective detection ($m/z = 337$ [M-CO$_2$Et]$^+$); (a) (2S,4S) diaminoglutaric acid after derivatization; (b) (2S,4S) diaminoglutaric after racemization with 20% ammonia and derivatization.

General Procedures

1. Fragment condensation. A solution of 1.2 equivalents of *C*-deprotected peptide, 1.2 equivalents of HOBt and 1.0 equivalents of *N*-deprotected fragment diluted in abs. THF (~20 mL/mmol) were stirred at 0°C. Then, 1.2 equivalents of EDCI·HCl and 2.4 equivalents of NMM were added to the solution. The pH was controlled between 8 and 8.5. This mixture was stirred for 4 h at 0°C, and 16 h at room temperature. After evaporation of the solvent at reduced pressure, the residue was diluted in EtOAc, and the organic layer was extracted with 5% NaHSO$_4$, 5% NaHCO$_3$ and water. The EtOAc layer was dried over MgSO$_4$. After evaporation, the resulting crude peptide can either be purified by flash chromatography or immediately be used in the next step.

2. Removal of the benzyl group. 10 mg 10% Pd/C were added to a solution of 0.1 mmol peptide or amino acid derivative in 20 mL methanol under argon atmosphere. The argon was exchanged with hydrogen and the suspension was stirred until no benzyl ester could be detected by TLC. The mixture was filtered through celite®, and

the filtrate concentrated under reduced pressure to yield an oil. The product was used without further purification.

3. Removal of the Fmoc protecting group in solution. 0.2 mmol Fmoc protected peptide fragment were stirred at room temperature in 20 mL 20% piperidine/DMF for 20 min. After evaporation of the solvent *in vacuo*, the product was purified by flash chromatography.

Aldehyde Component

Fmoc - Aspartic - acid - (β - N - methyl - N - methoxy-amide)-allyl ester 3. 5.1 g (12.8 mmol) Fmoc-Asp-OAll, 1.8 g *N,O*-dimethylhydroxylamine and 10.7 mL (6.6 equivalents) *N*-ethylmorpholine were diluted in 40 mL dry DCM and cooled to − 10°C. To this solution, 12.7 mL (5.1 equivalents) PPA (50% in DMF) were added. The reaction mixture was then stirred for 90 min at 0°C, and 2 h at room temperature before being concentrated under reduced pressure. The residue was diluted in EtOAc and the organic layer was washed with 5% KHSO$_4$, 2.5% NaHCO$_3$ and water. After drying over MgSO$_4$ and evaporation of the solvent, the product was obtained in nearly quantitative yield without further purification.

R_f (EtOAc:hexane:1:1): 0.30; ^{1}H-NMR (250 MHz, d_6-DMSO, 300 K): $\delta = 7.88$ (d, 2H, H-Ar), 7.75–7.68 (m, 3H, H-Ar, NH), 7.44–7.28 (m, 4H, H-Ar), 5.92–5.79 (m, 1H, H-allyl), 5.35–5.25 (dd, 1H, H-allyl-*trans*), 5.20–5.15 (dd, 1H, H-allyl-*cis*), 4.57–4.47 (m, 3H, Hα, CH$_2$-allyl), 4.33–4.19 (m, 3H, CH$_2$-CH-(Fmoc)), 2.97–2.72 (m, 2H, Hβ); ^{13}C-NMR (62.5 MHz, d_6-DMSO, 300 K): $\delta = 171.1$ ((*C*=O)-Fmoc), 169.9 ((*C*=O)-amide), 155.8 ((*C*=O)-allyl ester), 143.7, 140.7 (C-Ar), 132.3 (CH-allyl), 127.6, 127.0, 125.1, 120.1 (C-Ar), 117.5 (CH$_2$=CH), 65.7, 65.0 (CH$_2$=CH–CH$_2$, CH$_2$-Fmoc), 61.0 (–N–O–CH$_3$), 50.0, 46.6 (CH-Fmoc, Cα), 33.5, 31.8 (Cβ, –N–CH$_3$); mass (ESI): $m/z = 438.9$ [M + H]$^+$.

Fmoc-L-Aspartic acid semialdehyde allyl ester 4. To a cooled solution (− 78°C) of 2.9 g (6.4 mmol) Fmoc-aspartic-acid-(β-*N*-methyl-*N*-methoxyamide)-allyl ester **3** in 80 mL dry THF 7.7 mL (7.8 mmol) fresh DIBAH (1 M in THF) were added dropwise over 1 h. After stirring for 2 h, additional 5.1 mL, and after 3 h, 2 mL DIBAH were added. After a total reaction time of 7 h, the solution was poured into 100 mL ice cold 5% KHSO$_4$. The aqueous layer was extracted three times with EtOAc. Precipitated aluminates were then centrifugated and removed by centrifugation. After evaporation of the solvent, the crude material was

purified by flash chromatography and the product was obtained as colourless solid.

Yield: 1.53 g (63%); m.p.: 106°C; R_f (EtOAc:hexane 1:1): 0.69; $[\alpha]_D^{25} = +16.1$ ($c = 0.14$, CHCl$_3$); ^{1}H-NMR (250 MHz, d_6-DMSO, 300 K): $\delta = 9.61$ (s, 1H, H-aldehyde), 7.89–7.84 (m, 3H, NH, 2H-Ar), 7.68, (d, 2H, H-Ar), 7.44–7.28 (m, 4H, H-Ar), 5.91–5.80 (m, 1H, H-allyl), 5.32–5.23 (dd, 1H, H-allyl-*trans*), 5.21–5.15 (dd, 1H, H-allyl-*cis*), 4.64–4.53 (m, 3H, Hα, CH$_2$-allyl), 4.35–4.19 (m, 3H, CH$_2$–CH (Fmoc)), 2.94–2.76 (m, 2H, Hβ); ^{13}C-NMR (62.5 MHz, d_6-DMSO, 300 K): $\delta = 204.9$ (C-aldehyde), 171.3 ((C=O)-Fmoc), 155.8 ((C=O)-allyl ester), 144.3, 141.9 (C-Ar), 132.9 (CH-allyl), 127.6, 127.0, 125.0, 120.6 (C-Ar), 118.6 (CH$_2$=CH), 65.7, 65.1 (CH$_2$=CH–CH$_2$, CH$_2$-Fmoc), 48.5, 46.5 (CH-Fmoc, Cα), 44.0 (Cβ); mass (ESI): $m/z = 402.5$ [M + Na]$^+$.

2-Fmoc-4-Boc-ᴅ-Phe-(2S,4R/S)-Dag-2-allyl-4-ᴅ/ʟ-Phe-OBn 8 and 2-Fmoc-4-Boc-ᴅ-Phe-(2S,4R/S)-Dag-2-allyl-4-ᴅ/ʟ-Tyr(tBu)-OBn 19.

1.0 equivalent of aldehyde **4**, and 1.2 equivalents of 5-nitro-3,4-dimethoxybenzylamine **5** were stirred for 1 h at room temperature in abs. MeOH (5 mL/mmol). To this solution, 1.2 equivalents of acid component Boc-ᴅ-Phe-OH and 1.2 equivalents of either isonitrile **6** or **17** were added and stirred for 16 h, or until no aldehyde component could be detected by TLC. After evaporation of the solvent at reduced pressure, the residue was dissolved in EtOAc and extracted with 5% NaHSO$_4$, 5% NaHCO$_3$ and water. After drying over MgSO$_4$ and evaporation of the solvent, the corresponding *N*-alkylated peptide (**7** or **18**) was photolysed without further purification.

7: R_f (EtOAc:hexane 1:2): 0.28; RP-HPLC: $t_R = $ 25.9, 26.1, 26.9; (60–100% ACN; 30 min, (II)); mass (ESI): $m/z = 1126$ [M + Na]$^+$.

18: R_f (CHCl$_3$:MeOH:HOAc 100:2:1): 0.42, RP-HPLC: $t_R = $ 26.60, 26.90, 28.4, 28.70; (60–100% ACN; 30 min, (II)); mass (HPLC-MS): $m/z = 1176.1$ [M + H]$^+$, 1198.3 [M + Na]$^+$.

The crude product of the four component condensation was diluted in methanol (1 mg/mL) and photolysed for 12 h. The deep yellow solution was evaporated to dryness and the brown residue purified by flash chromatography resulting in **8** and **19**.

8: Yield after chromatographic work-up: 2.12 g (61% referring to the aldehyde component [4]; R_f (EtOAc:hexane 1:2): 0.11; RP-HPLC: $t_R = $ 0.4, 21.6, 21.8; (60–100% ACN; 30 min, (II)); mass (HPLC-MS): $m/z = 909.2$ [M + H]$^+$, 931.3 [M + Na]$^+$.

19: Yield after chromatographic work-up: 614 mg (48% referring to the aldehyde component **4**); R_f

(EtOAc:hexane 1:1): 0.29; RP-HPLC: $t_R = 17.20$, 17.60, 18.30, 18.60; (70–100% ACN; 30 min, (I)); mass (HPLC-MS): $m/z = 980.9$ [M + H]$^+$, 1003.1 [M + Na]$^+$.

2-Fmoc-4-Boc-ᴅ-Phe-(2S,4R/S)-Dag-2-OH-4-ᴅ/ʟ-Phe-OBn 9 and 2-Fmoc-4-Boc-ᴅ-Phe-(2S,4R/S)-Dag-2-OH-4-ᴅ/ʟ-Tyr(tBu)-OBn 20.

0.5 mmol allyl ester, 0.05 equivalent of PdCl$_2$(PPh$_3$)$_2$ and 1.5 equivalents of acetic acid were diluted in 25 mL THF. 1.0 equivalent of Bu$_3$SnH was added dropwise, and the mixture was stirred at room temperature for 2 h. The solution was evaporated to dryness and the residue purified by flash chromatotgraphy.

9: Yield after chromatographic work-up: 197 mg (95%); R_f (CHCl$_3$:MeOH:HOAc 100:1:1): 0.12; RP-HPLC: $t_R = 16.60$, 16.85, 17.77, 18.30; (60–100% ACN; 30 min, (II)); mass (HPLC-MS): $m/z = 869.1$ [M + H]$^+$, 891.3 [M + Na]$^+$.

20: Yield after chromatographic work-up: 535 mg (91%); R_f (CHCl$_3$:MeOH:HOAc 9:1:0.1): 0.7; RP-HPLC: $t_R = 10.6$, 11.30, 12.10, 12.80; (70–100% ACN; 30 min, (I)); mass (HPLC-MS): $m/z = 940.8$ [M + H]$^+$, 963.1 [M + Na]$^+$.

2-Fmoc-4-Boc-ᴅ-Phe-(2S,4R/S)-Dag-2-DiTBDMS-Thr(ol)-4-ᴅ/ʟ-Phe-OBn 11 and 2-Fmoc-4-Boc-ᴅ-Phe-(2S,4R/S)-Dag-2-DiTBDMS-Thr(ol)-4-ᴅ/ʟ-Tyr(tBu)-OBn 21.

Coupling of the threoninol building block **10** to the peptides **9** and **20** was performed according to general procedure [1]. The crude product was Fmoc deprotected (general procedure [3]) resulting in **12** and **22**.

11: R_f (EtOAc:hexane 1:2): 0.49, (CHCl$_3$:MeOH 100:1): 0.27; RP-HPLC: $t_R = 26.53$, 27.33, 28.62; (100% ACN; 30 min, (C)); mass (ESI): $m/z = 1184.1$ [M + H]$^+$, 1206.2 [M + Na]$^+$.

21: R_f (EtOAc:hexane 1:2): 0.37; mass (ESI): $m/z = 1256.1$ [M + H]$^+$, 1278.3 [M + Na]$^+$.

2-H-4-Boc-ᴅ-Phe-(2S,4R/S)-Dag-2-DiTBDMS-Thr(ol)-4-ᴅ/ʟ-Phe-OBn 12.

Yield: 304 mg (90%); R_f (EtOAc:hexane 1:2): 0.14, (CHCl$_3$:MeOH 8:1): 0.64; RP-HPLC: $t_R = 29.28$, 29.88, 30.77, 31.05; (60–100% ACN; 30 min, (C)); mass (ESI): $m/z = 962.2$ [M + H]$^+$.

2-H-4-Boc-ᴅ-Phe-(2S,4R/S)-Dag-2-DiTBDMS-Thr(ol)-4-ᴅ/ʟ-Tyr(tBu)-OBn 22.

Yield: 511 mg (95%); R_f (CHCl$_3$:MeOH 8:1): 0.22; mass (HPLC-MS): $m/z = 1034.2$ [M + H]$^+$, 1056.2 [M + Na]$^+$.

2-Fmoc-ᴅ-Trp(Boc)-Lys(Boc)-Thr(tBu)-4-Boc-ᴅ-Phe-(2S,4R/S)-Dag-2-DiTBDMS-Thr(ol)-4-ᴅ/ʟ-Phe-OBn 13 and 2-Fmoc-ᴅ-Trp(Boc)-Lys(Boc)-4-Boc-ᴅ-Phe-(2S,4R/S)-Dag-2-DiTBDMS-Thr(ol)-4-ᴅ/ʟ-Tyr(tBu)-

OBn **23**. Fmoc-D-Trp(Boc)-Lys(Boc)-Thr(*t*Bu)-OH and Fmoc-D-Trp(Boc)-Lys(Boc)-OH were synthesized by standard Fmoc-coupling protocols, e.g. with HOBt/TBTU/DIPEA, using TCP resin [28,29].

Fragment condensation of Fmoc-D-Trp(Boc)-Lys-(Boc)-Thr(*t*Bu)-OH to **12** and Fmoc-D-Trp(Boc)-Lys-(Boc)-OH to **22** according to general procedure [1] resulted in the fully protected and linear precursors **13** and **23** of the cyclic somatostatin analogues.

13: Yield after flash chromatography: 430 mg (73%); R_f (CHCl$_3$:MeOH:HOAc 9:1:0.1): 0.65; mass (ESI): $m/z = 1855.2$ [M + H]$^+$, 1878.4 [M + Na]$^+$.

23: Yield after flash chromatography: 395 mg (46%); R_f (EtOAc:hexane 1:2): 0.25; mass (ESI): $m/z = 1771.1$ [M + H]$^+$, 1793.3 [M + Na]$^+$.

N- and C-terminal deprotection of 13 and 23. Compounds **13** and **23** were hydrogenated according to general procedure [2] resulting in the *C*-terminal deprotected peptides **14** and **24**.

2-Fmoc-D-Trp(Boc)-Lys(Boc)-Thr(tBu)-4-Boc-D-Phe-(2S,4R/S)-Dag-2-DiTBDMS-Thr(ol)-4-D/L-Phe-OH 14. Yield: 392 mg (96%); R_f (CHCl$_3$:MeOH:HOAc 20:1:0.1): 0.38; mass (ESI): $m/z = 1765.1$ [M + H]$^+$, 1788.3 [M + Na]$^+$.

2-Fmoc-D-Trp(Boc)-Lys(Boc)-4-Boc-D-Phe-(2S,4R/S)-Dag-2-DiTBDMS-Thr(ol)-4-D/L-Tyr(tBu)-OH 24. Yield: 314 mg (85%); R_f (CHCl$_3$:MeOH:HOAc 20:1:0.1): 0.37; mass (ESI): $m/z = 1680.0$ [M + H]$^+$, 1702.3 [M + Na]$^+$.

Compounds **14** and **24** were Fmoc deprotected according to general procedure [3].

2-H-D-Trp(Boc)-Lys(Boc)-Thr(tBu)-4-Boc-D-Phe-(2S,4R/S)-Dag-2-DiTBDMS-Thr(ol)-4-D/L-Phe-OH 15. Yield after flash chromatography: 326 mg (96%); R_f (CHCl$_3$:MeOH:HOAc 20:1:0.1): 0.29; mass (ESI): $m/z = 1543.2$ [M + H]$^+$, 1565.3 [M + Na]$^+$.

2-H-D-Trp(Boc)-Lys(Boc)-4-Boc-D-Phe-(2S,4R/S)-Dag-2-DiTBDMS-Thr(ol)-4-D/L-Tyr(tBu)-OH 25. Yield after flash chromatography: 165 mg (61%); R_f (CHCl$_3$:MeOH:HOAc 10:1:0.1): 0.38; RP-HPLC: $t_R = 21.2$; (80–100% ACN; 30 min, (I)); mass (ESI): $m/z = 1458.3$ [M + H]$^+$, 1480.3 [M + Na]$^+$.

4-Boc-D-Phe-c((2S,4R/S)-Dag-D/L-Phe-D-Trp(Boc)-Lys(Boc)-Thr(tBu))-2-DiTBDMS-Thr(ol) 16 and 4-Boc-D-Phe-c((2S,4R/S)-Dag-D/L-Tyr(tBu)-D-Trp(Boc)-Lys-(Boc))-DiTBDMS-Thr(ol) 26. To a 5×10^{-3} M solution of the linear peptide in DCM:DMF (1:1) 1.0 equivalent of HOAt, 3.0 equivalents of *N*-methylmorpholine and 10 equivalents of DIC were added at 0°C, and the mixture was stirred at this temperature for 16 h.

After evaporation of the solvent *in vacuo*, the crude product was diluted in EtOAc and the organic phase extracted with 5% NaHSO$_4$, 5% NaHCO$_3$ and water.

16: mass (ESI): $m/z = 1525.1$ [M + H]$^+$, 1547.5 [M + Na]$^+$.

26: mass (ESI): $m/z = 1440.1$ [M + H]$^+$, 1463.4 [M + Na]$^+$.

The crude product **16** and **26** was deprotected resulting in four diastereomers **1a–d** and **2a–d** which were separated by HPLC.

Removal of the Acid Labile Protecting Groups *t*Bu, Boc and TBDMS

The peptide (**16** or **26**) was stirred in a mixture of 88% TFA, 5% phenol, 5% water and 2% triisopropylsilane at room temperature for 1 h. After evaporation of the solvent at reduced pressure, the peptide was precipitated with ice-cold diethyl ether. The precipitate was washed three times with diethyl ether, and the crude product purified by preparative HPLC to separate the four diastereomers.

The stereochemistry of the four diastereomers was determined gas-chromatographically on a LipodexE [24] column. For GC analysis, the peptide was hydrolysed using 6 N HCl at 110°C. Then the amino acids were derivatizised employing HCl/*n*-propanol for the *C*-terminus and trifluoro acetic anhydride for the *N*-terminus [26]. Enantiopure (*S,S*)-diaminoglutaric acid, D-Tyr, L-Tyr, D-Phe and L-Phe was used as internal standard.

4-H-D-Phe-c((2S,4R/S)-Dag-D/L-Phe-D-Trp-Lys-Thr)-2-Thr(ol) 1a–d. Yield: 27 mg (20%) after HPLC; RP-HPLC: $t_R = 13.68$ **1a**, 14.83 **1b**, 20.62 **1c**, 23.44 **1d**; (20–40% ACN; 30 min, (III)); mass (ESI): $m/z = 941.3$ [M + H]$^+$.

Stereochemistry:

1a: D-Phe, (2*S*,2*S*)-Dag.
1b: L-Phe, (2*S*,2*S*)-Dag.
1c: D-Phe, (2*S*,4*R*)-Dag.
1d: L-Phe, (2*S*,4*R*)-Dag.

4-H-D-Phe-c((2S,4R/S)-Dag-D/L-Tyr-D-Trp-Lys)-2-Thr(ol) 2a–d. Yield: 31 mg (35%) after HPLC; RP-HPLC: $t_R = 8.83$ **2a**, 10.52 **2b**, 13.20 **2c**, 14.19 **2d**; (20–40 ACN; 30 min, (III)); mass (ESI): $m/z = 856.3$ [M + H]$^+$, 878.4 [M + Na]$^+$.

Stereochemistry:

2a: L-Tyr, (2*S*,4*S*)-Dag.
2b: L-Tyr, (2*S*,4*S*)-Dag.
2c: D-Tyr, (2*S*,4*R*)-Dag.
2d: L-Tyr, (2*S*,4*R*)-Dag.

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