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Solid-phase Synthesis of MEN 11270, a New Cyclic Peptide Kinin B_2 Receptor Antagonist[1,2]

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Abstract: An efficient synthesis of the cyclic decapeptide MEN 11270 [H-DArg[1]-Arg[2]-Pro[3]-Hyp[4]-Gly[5]-Thi[6]-Dab[7]-DTic[8]-Oic[9]-Arg[10] c(7γ–10α)] was developed. Two three-dimensional orthogonal strategies were applied and compared: Fmoc/Tos/Boc (procedure A) and Fmoc/Pmc/Dde (procedure B). Both resulted in a 23-step strategy comprising the stepwise solid-phase chain assembly of the linear protected peptide, partial deprotection, solution-phase cyclization and final full deprotection. The stepwise assembly of the linear peptide was optimized by double coupling and acylation time prolongation for critical residues (Tic, Dab, Thi, Pro). *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium (HATU) was preferred as coupling reagent for Dab. In the cyclization step, the partial racemization of Arg[10] (31% using 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide/1-hydroxybenzotriazole (EDC/HOBt) as activation system) was reduced to 3% with HATU. The final deprotection was performed in the presence of dimethylsulfide (procedure A) and thiocresol (procedure B) as scavengers, to avoid the sulfation of Hyp side chain. The final compound and the main by-products were characterized by mass spectroscopy (MS), nuclear magnetic resonance (NMR) and racemization test. Procedure B produced operationally simpler and more efficient results than A (28% overall yield versus 4%). Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: cyclic peptides; solid-phase peptide synthesis; synthetic strategies

INTRODUCTION

In the past few years, several antagonists for kinin receptors have been developed, either peptide [1] or non-peptide [2] in nature. Among the peptides, HOE 140 or H-DArg[1]-Arg[2]-Pro[3]-Hyp[4]-Gly[5]-Thi[6]-Ser[7]-DTic[8]-Oic[9]-Arg[10]-OH [3] represents the prototype of a class of highly potent compounds that have been studied from the conformational point of view to determine the structural features for most favourable receptor–ligand interaction. Experimental evidence was provided that HOE 140 adopts a *C*-terminal β-turn due to the presence of the turn-structure inducing residues DTic[8] and Oic[9] [4].

A good expedient to induce a preferred conformation and impose conformational constraint is cyclization. Cyclic peptide antagonists have been demonstrated to be useful tools for structure-activity studies, especially for elucidation of bioactive

Abbreviations: CH_3CN, acetonitrile; Dab, 2,4-diaminobutyric acid; EDC, 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide; Et_2O, diethyl ether; iPrOH, isopropyl alcohol; Oic, octahydroindole-2-carboxylic acid; OPA-IBCL, *o*-phthaldialdehyde-*N*-isobutyryl-L-cysteine; Pfp, pentafluorophenyl; PEG-PS, polyethylene glycol-polystyrene; PIP, piperidine; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulphonyl; PTFE, polytetrafluoroethylene; PYBOP, 1-benzotriazolyloxy-tris-pyrrolidino-phosphonium; R_t, retention time; SPPS, solid-phase peptide synthesis.

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[1] Solid-phase synthesis of MEN 11270, a new cyclic peptide kinin B_2 receptor antagonist (preliminary account of present work), Sixth Solid Phase Synthesis Symposium, University of York, UK, 1999.

[2] Abbreviations used follow the nomenclature recommended by the IUPAC–IUB Joint Commission on Biochemical Nomenclature (*Eur. J. Biochem.* 1984; **138**: 9).

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conformation. On this basis, we designed a cyclized analog of HOE 140 which bears a 14-atom cycle in the *C*-terminal part. A 14-membered lactam cycle has been in fact proved useful to constrain a β-turn conformation in peptide sequences [5]. The resulted compound, MEN 11270 [H-DArg^1-Arg^2-Pro^3-Hyp^4-Gly^5-Thi^6-Dab^7-DTic^8-Oic^9-Arg^{10} c(7γ–10α)] **(1)**, (Figure 1), showed an activity comparable to that of the parent linear compound (pK_i = 10.3 and 10.6 for MEN 11270 and HOE 140, respectively, in the B_2 kinin receptor constitutively expressed by WI38 human fibroblasts) [6], a prolonged duration of action *in vivo* and a higher metabolic stability (manuscript in preparation).

In this paper, we describe the solid-phase synthesis of MEN 11270. The synthesis of homodetic cyclic peptides has been extensively described in the literature [7]. Depending on the sequence to cyclize, the appropriate protecting groups have to be chosen. In the synthesis described here, both the three-dimensional orthogonal strategies Fmoc/Tos/Boc (A) and Fmoc/Pmc/Dde (B) were used. Both strategies resulted in a 23-step synthetic pathway comprising the stepwise solid-phase chain assembly of the linear protected peptide, followed by partial deprotection, solution-phase cyclization and final deprotection. Strategies A and B are compared in this work.

MATERIALS AND METHODS

Chemicals

Protected amino acids, coupling reagents and resins were purchased from Bachem (Bubendorf, Switzerland) and Novabiochem (Läufelfingen, Switzerland). Solvents and reagents were all of analytical grade and used without further purification.

For amino acid analysis, the standards of Gly, Arg, Hyp and Pro were purchased from Hewlett Packard GmbH (Waldbronn, FRG), D-Tic, Oic, and Thi from Bachem AG (Bubendorf, Switzerland), D-Arg from Fluka (Buchs, Switzerland), Dab, norvaline and sarcosine from Sigma (St Louis, MO, USA). All high performance liquid chromatography (HPLC) eluents were from Baker (Deventer, Holland).

Peptide Synthesis

The syntheses of protected linear peptides were performed on a Milligen 9050 Plus automatic synthesizer (Millipore, Burlington, MA, USA), using Multiple Column Peptide Synthesis protocol supplied by the manufacturer for Fmoc strategy (users' manual) [8].

For selective removal of Boc or Dde protecting group from the linear protected precursor, a Labortec SP 640 semi-automatic synthesizer was used.

The starting resins were polyethylene glycol-polystyrene (PEG-PS) HCl (Millipore) for Fmoc/Tos/Boc strategy and Novasyn Tentagel Trityl (TGT) alcohol for Fmoc/Dde/Pmc strategy. Attachment of the first amino acid was carried out following reported methods [8]. Substitution level was estimated by photometric determination of the Fmoc group [8].

Analytical Methods

Synthetic peptides were purified by reverse-phase HPLC on a Waters Delta-Prep 3000 preparative

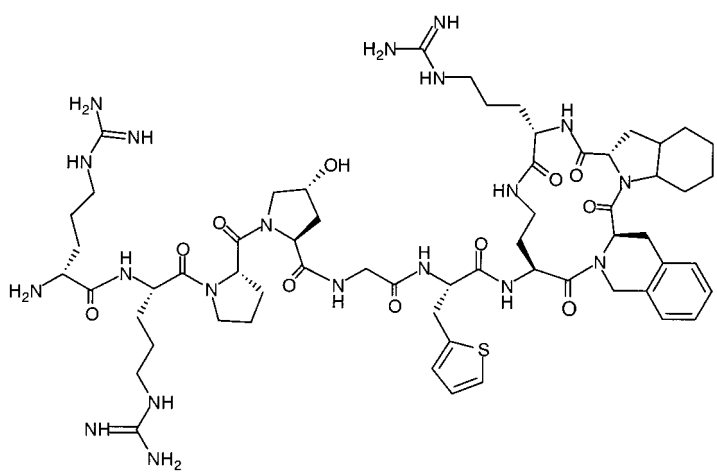

Figure 1 MEN 11270 (compound **1**) [H-DArg^1-Arg^2-Pro^3-Hyp^4-Gly^5-Thi^6-Dab^7-DTic^8-Oic^9-Arg^{10} c(7γ–10α)].

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chromatography apparatus (Waters, Milford, MA, USA). Columns and gradients used are reported in the experimental procedures for each compound. The effluent in all cases was monitored at 240 nm with a Waters 2487 detector.

Analytical HPLC characterization was performed on a Beckman System Gold apparatus, using a Vydac C18 218TP54 (4.6 × 250 min) column, with linear CH_3CN (0.1% trifluoroacetic acid (TFA)) gradient (20–80%, 26 min, 1 ml/min for protected linear peptides, 20–50%, 10 min, 1 ml/min for unprotected cyclic peptides) and detection at 210 nm.

Amino acid analysis on the final cyclized peptide was performed by hydrolysis in gaseous HCl 6 N + 0.1% phenol in vacuo at 150 °C for 1 h with a Waters Pico Tag Work Station. Several 20 µl aliquots of a MEN 11270 solution 2 mg/ml ca in H_2O/acetic acid (1:1) were dried in 6 min diameter glass tubes before undergoing the hydrolysis in a 40 ml reaction vial (Waters, Milford, MA). Inside the reaction vial 200 µl of HCl/phenol solution were placed and the atmosphere evacuated and flushed with nitrogen three times before applying the final vacuum. The quantitative determination of Gly, Arg, Thi, Hyp, Pro, was performed by OPA-Fmoc pre-column automated derivatization with a HP 1090 AminoQuant system. Dab was not quantitatively analysed for the possibility of a double derivatization. The hydrolysate was redried *under vacuum* and 20 µl of HCl 0.1 N containing 500 pmol/µl each of norvaline and sarcosine, as internal standards, were added, and the sample analysed with a HP 1090 AminoQuant system with ultraviolet (UV) detection [9]. D-Tic and Oic were analysed by pre-column manual Fmoc derivatization and subsequent reversed phase (RP) C8 HPLC analysis with an in-house developed method: to the redried hydrolysate were added 20 µl of HCl 0.1 N containing 1000 pmol/µl of sarcosine, as internal standard, 80 µl of 0.5 M pH 7.7 sodium borate buffer and 100 µl of Fmoc–Cl 2 mM in acetonitrile. The reaction proceeded for 10 min at room temperature (r.t.) and was stopped with 10 µl of 40 mM adamantaneamine in acetone/water 3:1. RP analysis was performed on a Symmetry Shield C8 3.9 × 150 min column (Waters, Milford, MA). A gradient from sodium acetate 100 mM pH 4.4 (eluent A) and acetonitrile (eluent B) was used at 1 ml/min: from 0% to 20% B in 5 min, to 60% B in 20 min. As a sample, 10 µl of the reaction solution were injected in HPLC (HP 1090 AminoQuant with Ultra Violet-Diode Array Detector) and detected at 263 nm. Sarcosine (IS), D-Tic and Oic, as Fmoc derivatives, eluted at 14.1, 20.2 and 21.4 min, respectively. The standard curve was done with three levels at 500, 1000 and 2000 pmol/µl of each standard injected in triplicate; calculated correlation coefficient R was 0.99989 for D-Tic and 0.99972 for Oic.

Investigation of the chiral integrity of the peptides was carried out by means of amino acids analysis. The peptides were hydrolysed with gaseous 6 N HCl + phenol 0.1% at 150 °C for 1 h and analysed using a Hewlett Packard 1090 HPLC AminoQuant for the determination of their D- and L-primary amino acids as *o*-phthaldialdehyde-*N*-isobutyrul-L-cysteine (OPA-IBLC) derivative [10].

Electrospray mass spectrometry (ES-MS) spectra were recorded on a Finnigan LCQ ion trap mass spectrometer, introducing the samples by infusion via a built-in syringe pump. Unless indicated differently, the observed molecular weights are intended as mono-isotopic. Identity of products and by-products was also assessed by tandem MS of their singly- or doubly-charged quasi-molecular ions. When necessary, samples were also analysed by on-line HPLC-MS using a Hewlett Packard (series 1100) HPLC apparatus under the same chromatographic conditions described above.

Proton magnetic resonance (PMR) spectra were acquired at 500 MHz on a Bruker Avance instrument, using dimethylsulphoxide (DMSO)-*d*6 as solvent, at a temperature of 300 K. Subsequent processing was carried on under SwaN-MR [11,12]. For assignment purposes the following two-dimensional spectra were acquired: correlated spectroscopy (COSY), total correlation spectroscopy (TOCSY) and nuclear Overhauser enhanced spectroscopy (NOESY) (the latter with a mixing time of 0.5 s). TOCSY and NOESY spectra were phase-sensitive implementing the States-Time Proportional Phase Increments method. The size of all these spectra was equal to 1024 complex points along t_2 and 512 increments along t_1. Phase-sensitive spectra were prolonged in t_1 by linear prediction up to 1024 complex points.

Synthesis of Compound 1 with Fmoc/Tos/Boc Strategy (Procedure A)

Fmoc - DArg(Tos) - Arg(Tos) - Pro - Hyp(tBu) - Gly - Thi-Dab(Boc)-DTic-Oic-Arg(Tos)-resin (2). The *C*-terminal Arg(Tos) was coupled via symmetrical anhydride (4-dimethylaminopyridine (DMAP) 0.1%) to PEG-PS (Millipore) resin previously loaded with the linker *p*-hydroxymethyl-phenoxyacetic acid (HMPA), obtaining a substitution level of 0.19 mmol/g (79% with respect to the initial value of 0.24

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mmol/g). Peptide syntheses were typically performed on 1 g samples of preloaded resin (recycle flow rate was normally 5 ml/min and increased to 10 ml/min in repeated cycles). Fmoc-amino acids were coupled using a 2.5-fold excess of amino acid activated by 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium/1-hydroxybenzotriazole (TBTU/HOBt) system. Couplings of DTic Dab(Boc), Thi and Pro were repeated twice. Pro was used as Fmoc-Pro-OPfp derivative in the second acylation. Recycle times were prolonged from 30 to 45 min for all amino acids derivatives.

Fmoc-DArg(Tos)-Arg(Tos)-Pro-Hyp-Gly-Thi-Dab-DTic-Oic-Arg(Tos)-OH (3). Selective removal of *tert*-butyloxycarbonyl (Boc) and *tert*-butyl (tBu) protecting groups from Dab and Hyp side chains, respectively, and cleavage of the peptide from the peptide-resin **(2)** were concomitantly performed by treating the protected peptide resin with TFA/PhOH/Et_3SiH/H_2O 88:5:2:5 (r.t., 7 h). The resin was filtered and washed several times with TFA, dichloromethane (DCM) and MeOH. The filtrate was added to an ice-cold mixture of light petroleum ether/Et_2O 4:1 and freezed overnight. The crude peptide was filtered through a Millipore polytetrafluoroethylene (PTFE) (0.45 μm) filter, washed with cold Et_2O, collected from the filter by dissolution with AcOH, diluted with water and lyophilized. RP-HPLC purification, performed on a Vydac C18 218TP1022, 10–15 μm, 250 × 22 min using a linear CH_3CN (0.1% TFA) gradient (40–70%, 60 min, 60 ml/min), led to the desired product (R_t = 17.3 min, 28% yield). ES-MS: $[M + 2H]^{2+}$ m/z 1002.2, found average MW 2002.4 (theor. 2002.4).

H-DArg(Tos)-Arg(Tos)-Pro-Hyp-Gly-Thi-Dab-DTic-Oic-Arg(Tos) c(7γ–10α) (4). Cyclization was performed by adding 1-benzotriazolyloxy-tris-pyrrolidino-phosphonium (PYBOP) (1.2 eq) and diisopropylethylamine (DIPEA) (2.4 eq) to an ice-cold 1 mM *N*,*N*-dimethylformamide (DMF) solution of the linear protected peptide **(3)**. The reaction mixture was allowed to warm to r.t. and stirred for 4 h. After concentration *under vacuum* the residue was treated with 10% Et_2NH in THF at r.t. for 2 h in order to remove the *N*-terminal Fmoc protection, and then dried *under vacuum*. Purification via RP-HPLC was performed using a Waters Delta Pack WAT011799 column, 18–100 A, 19000 min and a linear CH_3CN (0.1% TFA) gradient was used (35–65%, 60 min, 20 ml/min). Compound **(4)** (R_t = 15.38 min) was obtained with a 28% overall yield. ES-MS: $[M + 2H]^{2+}$, m/z 782.5, found average MW 1762.0 (theor. 1762.1).

H-DArg-Arg-Pro-Hyp-Gly-Thi-Dab-DTic-Oic-Arg c(7γ–10α) (1). Full deprotection of **(4)** was accomplished in HF/Me_2S (9/1, −70–0 °C, 6 h). After HF evaporation, the residue was collected by dissolution with AcOH, diluted with H_2O and lyophilized. RP-HPLC purification, performed using a Vydac C18 218TP1520 (50 × 250 mm) column and linear CH_3CN (0.1% TFA) gradient (22–40%, 60 min, 60 ml/min), gave the desired product (R_t = 8.8 min, 4% overall yield). ES-MS: $[M + H]^+$, m/z 1299.7 (theor. 1299.7). As a by-product, 2% of Hyp-sulfated product was found (R_t = 8.7 min). ES-MS: $[M + H]^+$, m/z 1379.5 (theor. 1379.6).

Synthesis of Compound 1 with Fmoc/Pmc/Dde Strategy (Procedure B)

Boc-DArg(Pmc)-Arg(Pmc)-Pro-Hyp(tBu)-Gly-Thi-Dab(Dde)-DTic-Oic-Arg(Pmc)-resin (5). Attachment of the *C*-terminal residue to NovaSyn TGT alcohol resin was accomplished via the known two-step procedure. The chloruration step was carried out preferentially with $SOCl_2$ (2 ml/g, anhydrous toluene, 60–70 °C, 3 h, under N_2) than with AcCl. The moisture-sensitive chloride resin was immediately treated with the previously dried Fmoc-Arg(Pmc)-OH (3 eq) and DIPEA (12 eq) in anhydrous DCM (r.t., 2 h, under N_2), leading to a substitution level of 0.20 mmol/g (71% with respect to the initial value). Reproducible results were obtained when the alcohol resin was washed with anhydrous DCM and dried *under vacuum* over KOH for several hours.

Fmoc-Dab(Dde)-OH was synthesized from the commercial Fmoc-Dab-OH by reaction with Dde-OH and DIPEA in EtOH/AcOH/DCM 1:1:1 (r.t., 7 d). After removal of the solvent at reduced pressure, AcOEt was added, the organic phase extracted with HCl 0.5 N and the acidic aqueous solution was extracted with AcOEt.

The combined organic phases were washed with H_2O, dried over Na_2SO_4, filtered and concentrated *in vacuum*. The oily residue was triturated with light petroleum and converted into a solid (94% yield). ^{1}H-NMR (500 MHz, DMSO-*d*6): 8 (ppm) 13.30 (m, 1H), 7.90 (d, 2H), 7.7 (m, 2H), 7.43–7.31 (dt, 4H), 4.32 (d, 2H), 4.23 (t, 1H), 4.00 (m, 1H), 3.48 (m, 2H), 2.46 (s, 3H), 2.26 (s, 4H), 2.03–1.91 (m, 2H), 0.94 (s, 6H). ES-MS: $[M + H]^+$, m/z 505.2 (theor. 505.2).

The syntheses of **(5)** were performed on 1–3.8 g samples of preloaded resin (recycle flow rate was from 5 to 20 ml/min). A two-fold excess of Fmoc-amino acid and Boc-DArg(Pmc)-OH was used. *C*-Activation was achieved by the HOBt/TBTU method, except for Fmoc-Dab(Dde)-OH, which required HATU

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activation. The couplings of DTic, Dab(Dde), Thi, Pro, Arg(Pmc), DArg(Pmc) were repeated twice. Pro was used as Fmoc-Pro-OPfp derivative in the second acylation. Recycle times were prolonged from 30 to 45 min for all amino acids derivatives.

Boc - DArg(Pmc) - Arg(Pmc) - Pro - Hyp(tBu) - Gly - Thi-Dab-DTic-Oic-Arg(Pmc)-OH (6). Selective removal of Dde protecting group from Dab side chain amino function was performed with 2% hydrazine in DMF (r.t., 3 × 5 min) The cleavage of the partially protected peptide from the resin was accomplished by AcOH/DCM/MeOH 20:16:4 (r.t., 1 h). The mixture was then concentrated *in vacuum*, diluted with water and lyophilized. The crude protected linear peptide was purified via RP-HPLC using a Vydac C18 218TP1520 (50 × 250 min) column and linear CH_3CN (0.1% TFA) gradient (50–80%, 60 min, 60 ml/min), obtaining the desired product with a 66% overall yield ($R_t = 26.3$ min). ES-MS: $[M + 2H]^{2+}$, m/z 1136.5, found average MW 2271.0 (theor. 2272.1).

H- DArg- Arg- Pro- Hyp- Gly- Thi- Dab- DTic- Oic- Arg c(7γ– 10α) (1). Cyclization of **(6)** was performed by adding HATU (1.2 eq) and DIPEA (2.4 eq) to a 1 mM DMF solution of the linear protected peptide (r.t., 1 h). After concentration *under vacuum*, the residue was treated with TFA/thiocresol 90:10 and stirred (r.t., 2 h). The reaction mixture was added to an ice-cold mixture of light petroleum/Et_2O 4:1 and freezed overnight. The crude peptide was filtered through a Millipore PTFE (0.45 μm) filter, washed with cold Et_2O, collected by dissolution with AcOH, diluted with water and lyophilized. Purification via RP-HPLC, performed on a Vydac C18 218TP2022 (22 × 250 mm) column and using a linear CH_3CN (0.1% TFA) gradient (17–30%, 52 min, 20 ml/min), gave **1** ($R_t = 8.8$ min) with a 28% overall yield. ES-MS: $[M + H]^+$, m/z 1299.6 (theor. 1299.7). As a by-product, an isomer of **1**, **1a** (*vide infra*) was isolated in 0.9% yield ($R_t = 9.3$.min), ES-MS: $[M + H]^+$, m/z 1299.6 (theor. 1299.7).

RESULTS AND DISCUSSION

For the synthesis of the cyclic decapeptide MEN 11270 **(1)** two three-dimensional orthogonal strategies have been applied and compared (Figure 2): Fmoc/Tos/Boc (procedure A) and Fmoc/Pmc/Dde (procedure B). The structural analysis of **1** was accomplished by MS (Figure 3, panel A), NMR (Table 2A) and amino acid analysis (Gly = 1.00, Arg = 2.97, Hyp = 0.87, Oic = 0.88, Pro = 0.95, Thi = 0.90, Tic = 1.12).

In the solid-phase peptide synthesis (SPPS) of the linear precursor, the amount of deletion products was minimized with prolonged acylation times and selected repeated couplings. Particular attention was paid to the Dab residue, which can be considered the most critical point of the sequence assembly. The final Fmoc-Dab(Dde)-OH could be purified by flash chromatography, but eluants containing AcOH should be avoided, as if adsorbed by the solid final product, acetylation of H-DTic^8-Oic^9-Arg^{10}(Pmc)-resin peptide fragment occurs to a high extent. The difficult coupling of amino acids with Tic derivatives, as part of conformationally constrained peptides, was already reported [13]. In our sequence, the more efficient HATU activation method, besides prolonged reaction times, was necessary to improve the coupling yield of the constrained dipeptide DTic-Oic with the bulky Fmoc-Dab(Dde)-OH [14]. Increased time for the deprotection of DTic, reported as beneficial to achieve a complete Fmoc removal [14], provided no advantages.

The orthogonal deprotection of the Dab side chain amino group can be achieved in strategy A (PEG-PS resin) during the cleavage with TFA (Figure 2). This procedure releases also the protecting group of the potentially reactive hydroxyl group on Hyp side chain. In strategy B on NovaSyn TGT, two consecutive reactions are required, namely the removal of Dde on resin, followed by the cleavage from the solid support (Figure 2). Selective removal of Dde protecting group from Dab side-chain amino function was carried out on the resin, immediately after completion of the SPPS protocol. The subsequent cleavage from the solid support was accomplished with diluted AcOH, thus preventing the premature peptide full deprotection.

RP-HPLC purification of the partially protected linear peptide was performed to achieve a cleaner cyclization reaction and to avoid the undesired activation of AcOH, included in the crude material either as traces or as acetate counter ion. This problem occurred in one of our synthetic procedures, where the Dab-acetylated linear compound H-DArg-Arg-Pro-Hyp-Gly-Thi-Dab(Ac)-DTic-Oic-Arg-OH (Figure 3, panel B) was isolated after final deprotection in a 11% overall yield.

In the solution phase cyclization, different activation systems were tested (Table 1). It is known that HOAt-derived coupling reagents resulted highly effective in cyclization processes in terms of reactivity [15]. In the linear precursor of **1**, the presence of turn inducing amino acids such as Oic and DTic favours intramolecular cyclization. Actually, no

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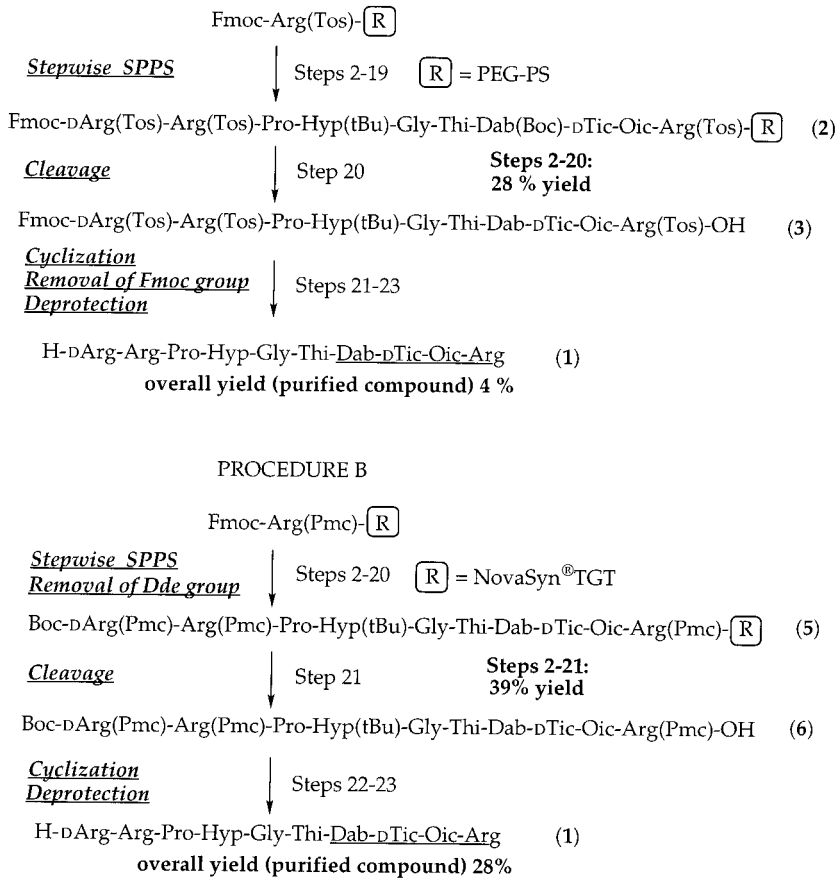

Figure 2 Procedures A and B.

traces of cyclodimer as side product were detected. However, when the activating system was not effective (see HOBt/EDC, Table 1) the process resulted quite slow and only small amounts of product were observed after 48 h in the reaction mixture. Moreover, a significant amount of the isomeric (as determined by MS) by-product **1a** was found (Table 1). The chiral identity of compounds **1** and **1a** were determined via racemization test and NMR. The enantiomeric ratio for the Arg residues was L-Arg/D-Arg 64.1/35.9 for **1** and L-Arg/D-Arg 38.3/61.7 for **1a** (calculated from the OPA-IBLC HPLC analysis area %), in agreement with the inversion of one of the two L-Arg residues. NMR data for both compounds are reported in Table 2. The ^{1}H spectrum of the minor isomer **1a** was assigned first, due to its relative simplicity. The spectroscopic patterns of the single residues were already known from the previously characterized model compounds Ac-Dab-DTic-Oic-Arg c(1γ–4α) and Ac-Dab-DTic-Oic-DArg c(1γ–4α). The chemical shifts of Dab, D-Tic, Oic and Arg^{10} residues in the isomer of **1** and in the model peptide containing D-Arg were the same. We concluded that **1a** underwent epimerization in the Arg^{10} residue. The spectrum of **1** is more complicated, because two distinct conformers are present. The assignment of all peaks was facilitated by the knowledge acquired with its epimer. The two conformers have been characterized as having a different conformation of the Dab-DTic peptide bond. In the *trans* conformer, we can observe a nOe effect between the α hydrogen of Dab and the ε hydrogens of D-Tic. In the *cis* conformer, we observe the nOe effect between the two α hydrogens of the same amino acids. By means of the nOe contacts, we deduced that the conformation of the Dab-DTic amide bond in **1a** is only *cis*. The global conformation of the *cis* conformer of **1** must be similar to that of **1a**; in fact, the chemical shifts and the coupling constants are almost the same.

The extent of epimerization at the *C*-terminal residue during the cyclization step was minimized using HATU, which provided the fastest reaction time and also proved more effective than PYBOP in terms of yield (Table 1).

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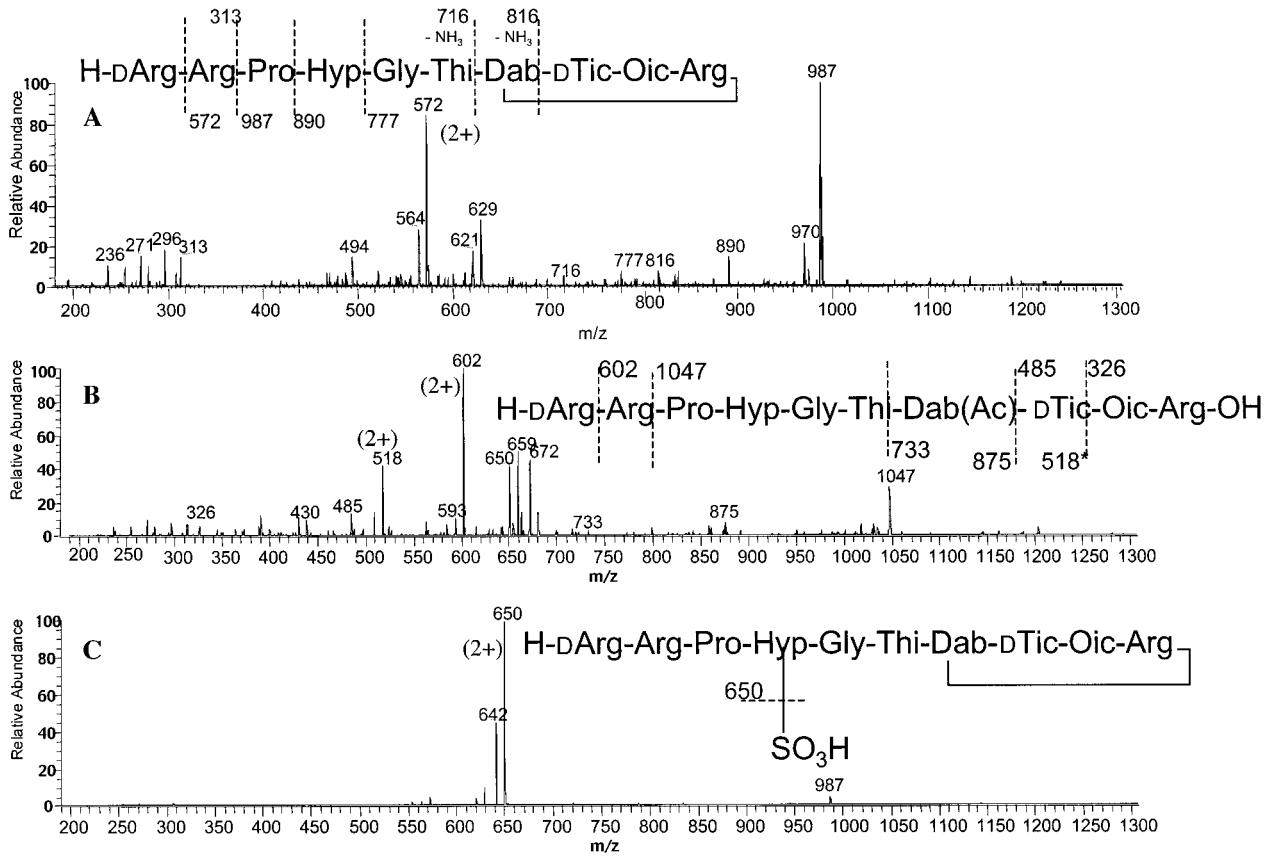

Figure 3 Panel A: tandem mass spectrum of the doubly-charged quasi-molecular ion of **1**, *m*/*z* 650.4; panel B: tandem mass spectrum of the doubly-charged quasi-molecular ion of the linear Dab-acetylated by-product, *m*/*z* 680.5; panel C: tandem mass spectrum of the doubly-charged quasi-molecular ion of **1** sulphate, *m*/*z* 690.3.

Table 1 Yields and Extent of Epimerization in Different Cyclization Conditions of the Protected Linear Peptide

Activation method	Reaction conditions	MEN 11270 **(1)** (yield %)	Epimer **(1a)** (%)[a]
PYBOP	PYBOP (2.4 eq)/DIPEA (4.8 eq); 0 °C, 4 h	30	10
EDC/HOBt	EDC (1.8 eq)/HOBt (4.5 eq); r.t., 48 h	11[b]	31
HATU	HATU (1.1 eq)/DIPEA (3 eq); r.t., 2 h	73	3

[a] Area percentage (analytical HPLC analysis).
[b] HPLC of the reaction mixture after 48 h.

Final deprotection was performed with HF in procedure A and TFA in procedure B. It is apparent that harsh conditions used in procedure A determined a fall in the final yield (4%), while treatment with moderate strength acid in procedure B proved more appropriate for the substrate (overall yield 28%). The deprotection in procedure B was carried out directly on the crude cyclization mixture, after concentration *under vacuum*, as RP-HPLC purification of the protected cyclic peptide was found to give no improvement in the yield. Using neat TFA as cleavage agent, 61% of Hyp-sulfated peptide was obtained. This side reaction, already described for Ser and Thr side-chains [16], occurs during the removal by TFA of the Pmc protecting group from Arg residues. As the relative R_t of **1** and of its sulfated analogue were quite close, they could not be separated via HPLC. The latter could anyway be

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Table 2 ^{1}H Chemical Shifts in DMSO-*d*6 (300 K) of Compound **1** and its Epimer **1a**

H	NH	α	β	γ	δ	Others
A: Compound ***1***						
DArg^1	8.16	3.82	1.69, 1.73	1.50	3.11	ε, 7.83; 6.8–7.5
Arg^2 *trans**	8.77	4.53	1.51, 1.71	1.52	3.08	ε, 7.67; 6.8–7.5
Arg^2 *cis**	8.76	=	=	=	=	=
Pro^3	–	4.52	2.09	1.82, 1.86	3.48, 3.72	–
Hyp^4 *trans*	–	4.33	1.90, 1.99	4.37	3.47, 3.66	OH, 5.19
Hyp^4 *cis*	–	=	=	=	=	OH, 5.18
Gly^5 *trans*	8.17	3.71	–	–	–	–
Gly^5 *cis*	8.09	3.61, 3.69	–	–	–	–
Thi^6 *trans*	8.19	4.57	2.97, 3.16	–	–	6.87, 7.25; 7.29
Thi^6 *cis*	8.02	4.45	2.99, 3.08	–	–	=
Dab^7 *trans*	8.54	4.25	2.09	2.93, 3.71	6.68	–
Dab^7 *cis*	8.17	3.85	1.70, 1.78	2.37, 3.46	7.51	–
DTic^8 *trans*	–	4.52	2.81, 3.30	–	–	ε, 4.37, 4.63; 7.2–7.5
DTic^8 *cis*	–	5.69	3.27	–	–	=
Oic^9 *trans*	–	4.28	1.82, 2.23	2.45	4.12	1.2–1.95
Oic^9 *cis*	–	4.15	1.88, 1.92	2.40	4.06	=
Arg^{10} *trans*	7.07	4.31	1.50	1.45	3.13	ε, 7.65
Arg^{10} *cis*	8.47	3.54	1.78	1.38	3.06	ε, 7.60
B: Compound ***1a***						
DArg^1	8.14	3.81	1.69	1.49	3.11	ε, 7.71; 6.7–7.5
Arg^2	8.76	4.54	1.51, 1.71	1.52	3.08	ε, 7.58; 6.7–7.5
Pro^3	–	4.53	2.10	1.85	3.50, 3.73	–
Hyp^4	–	4.33	1.90, 1.98	4.37	3.46, 3.66	OH, 5.17
Gly^5	8.06	3.60, 3.72	–	–	–	–
Thi^6	8.04	4.45	2.98, 3.09	–	–	6.82, 6.88, 7.27
Dab^7	8.17	3.70	1.60, 1.79	2.35, 3.46	7.77	–
DTic^8	–	5.60	3.25	–	-	ε, 4.69, 4.45; 7.2–7.32
Oic^9	–	4.33	1.73, 1.98	2.40	4.04	1.16–2.06
DArg^{10}	8.80	3.82	1.68	1.48	3.03	ε, 7.50; 6.7–7.5

cis and *trans* refers to the two observable conformers with different conformation of the Dab-DTic peptide bond. = Means same value as in the trans conformer.

identified via MS (Figure 3, panel C). Attempts to desulfate the final mixture with H_2SO_4 solutions following described procedures [17] failed, giving rise to product degradation. To limit the extent of this side reaction, 10% of thiocresol was added as the appropriate scavenger for Pmc protecting groups [18], obtaining 2% of the undesired product.

CONCLUSIONS

In conclusion, we developed an efficient, continuous flow, solid-phase synthesis of compound **1**. Fmoc/Tos/Boc strategy (procedure A), applied first as a preliminary approach, was replaced by procedure B (Fmoc/Dde/Pmc), which was developed and optimized. Compared to A, procedure B proved operationally simpler and more efficient (28% overall yield versus 4%). The optimization of SPPS protocol allowed an enhancement of the overall yield of the process. Further efforts are in progress to improve the final cyclization/deprotection step and to scale up the synthesis.

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REFERENCES

1. Kyle DJ. Structure-based drug design: progress toward the discovery of the elusive bradykinin receptor antagonists. *Curr. Pharmaceut. Design* 1995; **1**: 233–254.
2. Altamura M, Meini S, Quartara L, Maggi CA. Nonpeptide antagonists for kinin receptors. *Regul. Pept.* 1999; **80**: 13–26.
3. Lembeck F, Griesbacher T, Eckhardt M, Henke S, Breipohl G, Knolle J. New, long-acting, potent bradykinin antagonists. *Br. J. Pharmacol.* 1991; **102**: 297–304.
4. Guba W, Haessner R, Breiphol G, Henke S, Knolle J, Santagada V, Kessler H. Combined approach of NMR and molecular dynamics within a biphasic membrane mimetic: conformation and orientation of the bradykinin antagonist HOE 140. *J. Am. Chem. Soc.* 1994; **116**: 7532–7540.
5. Pavone V, Lombardi A, Nastri F, Saviano M, Maglio O, D'Auria G, Quartara L, Maggi CA, Pedone C. Design and structure of a novel Neurokinin A receptor antagonist cyclo(-Met[1]-Asp[2]-Trp[3]-Phe[4]-Dap[5]-Leu[6]-)cyclo(2β–5β). *J. Chem. Soc. Perkin Trans.* 1995; **2**: 987–993.
6. Meini S, Quartara L, Rizzi A, Patacchini R, Cucchi P, Giolitti A, Calò G, Regoli D, Criscuoli M, Maggi CA. MEN 11270, a novel selective constrained peptide antagonist with high affinity at the human kinin B_2 receptor. *J. Pharmacol. Exp. Therap.* 1999; **289**: 1250–1256.
7. Cavallaro V, Thompson P, Heran M. Solid phase synthesis of cyclic peptides: model studies involving $i-(i+4)$ side chain-to-side chain cyclization. *J. Peptide Sci.* 1998; **4**: 335–343.
8. Millipore. 9050 Plus PepSynthesizer User's Guide, Revision 1.0, 1992.
9. HP AminoQuant Series II — Operators Handbook, HP part 01090–90025.
10. Bruckner H, Westhauser T, Godel H. Liquid chromatographic determination of D- and L-amino acids by derivatization with o-phthalaldehyde and Nisobutyryl-L-cysteine. *J. Chromatogr.* 1995; **A711**: 201–215.
11. Balacco G. SwaN-MR: A complete and expansible NMR software for the Macintosh. *J. Chem. Inf. Comput. Sci.* 1994; **34**: 1235–1241.
12. Balacco G. SwaN-MR: from infancy to maturity. *Mol. Biol. Today* 2000; **1**: 23–28.
13. Ötvös F, Murphy RF, Lovas S. Coupling difficulty following replacement of Tyr with HOTic during synthesis of an analog of EGF B-loop fragment. *J. Peptide Res.* 1999; **53**: 302–307.
14. Albericio F, Bofill JM, El-Faham A, Kates SA. Use of onium salt-based coupling reagents in peptide synthesis. *J. Org. Chem.* 1998; **63**: 9678–9683.
15. Ehrich A, Heyne HU, Winter R, Beyermann M, Haber H, Carpino LA, Bienert M. Cyclization of all-L-pentapeptides by means of 1-hydroxy-7-azabenzotriazole-derived uronium and phosphonium reagents. *J. Org. Chem.* 1996; **61**: 8831–8838.
16. Jaeger E, Remmer HA, Jung G, Metzger J, Oberthür W, Rücknagel PK, Schäfer W, Sonnenbichler J. Zed I *O*-Sulfonierung von Serin und Threonin während der Abspaltung der Pmc- und Mtr-Schutzgruppen von Argininresten bei Fmoc-Festphasen-Synthesen. *Biol. Chem. Hoppe-Seyler* 1993; **374**: 349–362.
17. Goren BG, Kochansky ME. The stringent requirements for electrophiles in the facile solvolytic hydrolysis of neutral sulfate ester salts. *J. Org. Chem.* 1973; **38**: 3510–3513.
18. Beck-Sickinger AG, Schnorrenberg G, Metzger J, Jung G. Sulfonation of arginine residues as side reaction in Fmoc-peptide synthesis. *Int. J. Peptide Res.* 1991; **38**: 25–31.

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