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An Efficient Solid-Phase Strategy for the Construction of Chemokines[1]

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Abstract: Synthesis of chemokines *via* stepwise SPPS approaches has been shown to be a challenge. Herein, a complete study of different coupling methods, solvents and temperature combined with a continuous-flow synthesizer equipped with feedback monitoring was carried out. The results from this study indicate that this family of molecules can be prepared using an Fmoc/Bu^t chemical approach and provide a general method to apply for the elongation of other difficult sequences. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: chemokines; cleavage cocktails; coupling reagents; HATU; magic mixture; peptide synthesis; RANTES

INTRODUCTION

Chemokines, a subfamily of chemotactic cytokines, are a class of proteins that range in length from 65 to 90 amino acids (8–16 kDa) and contain one or two disulfide bridges. There are three main subgroups of chemokines, classified according to the location of the Cys residues within the molecule [1]. Recently, chemokines have received attention due to their role in leukocyte activation, inflammatory reactions, and chemoattraction [2], as well as potential roles in the treatment of both AIDS and cancer [3].

One of the most interesting molecules of this family is RANTES (regulated-upon-activation, normal T cell expressed and secreted), a β-subtype chemokine, which is a highly basic 8-kDa, 68 amino acid protein with four Cys residues and a pI = 9.5 [4]. For chemokine-mediated blockage of HIV infection, RANTES binds to two sites at the CCR5 receptor [5]. Site I binds to the outer surface of the receptor and addresses the sequence, while at Site II the *N*-terminal flexible tail attaches to the receptor to initiate a signal.

Synthesis of this class of molecules represents a challenge, primarily due to their size but also because of the presence of amino acids that are prone to undergoing side reactions during either the assembly or the final deprotection (7 Arg, Trp, Met),

Abbreviations: ;TOF-MS, atmospheric pressure ionization time-of-flight mass spectrometry; MPS, multiple peptide synthesis; NMP, *N*-methylpyrrolidinone; PAL, 5-[4-(aminomethyl)-3,5-dimethoxyphenoxy]valeric acid; Pbf, 2,2,4,6,7-pentamethyl-dihydrobenzylfuran-5-sulfonyl; PEG-PS, polyethylene glycol-polystyrene (graft resin support); PyAOP, 7-aza-benzotriazol-l-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate; RANTES, regulated-upon-activation, normal T cell expressed and secreted; TFFH, tetramethylfluoroformamidinium hexafluorophosphate; Amino acid symbols denote L-configuration unless indicated otherwise.

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and also due to sequences that may be considered problematic (Thr-Thr, Val-Val, Asn-Pro). Therefore, chemokines have been mainly assembled using chemical ligation techniques [6]. This strategy is based on the 'chemoselective reaction' in aqueous media of unprotected peptides bearing appropriate residues at the *C*- and the *N*-termini that have previously been assembled on solid-phase [7]. Unfortunately, these protocols require an expertise that it is not available in all research laboratories. Due to the emergence of more efficient coupling reagents and polymeric supports, as well as to engineering designs that build flexibility in instrumentation, a linear strategy for chemokine synthesis, based on the most flexible Fmoc/Bu^t strategy, was examined to determine the optimal method for the preparation of these medicinally important targets. Furthermore, conclusions from this study can be applied for the synthesis of other medium sized peptides or small proteins.

The RANTES sequence (Figure 1) with all Cys residues protected by the Acm group to prevent a premature disulfide bond formation was chosen as a model. Different coupling methods, such as TBTU, HATU, TFFH, and PyAOP [8], as well as the use of the 'magic mixture' [9] and/or high temperatures as a solvent, for both coupling and Fmoc removal steps were studied.

MATERIAL AND METHODS

Fmoc-PAL-PEG-PS (0. 18 mmol/g), Fmoc-amino acids, and TBTU, HATU, PyAOP, and TFFH were obtained from PE Biosystems (Framingham, MA, USA). The following side-chain protecting groups were used: Bu^t for Asp, Glu, Thr, Ser, and Tyr; Acm for Cys; Trt for His, Asn, and Gln; Pbf for Arg; and Boc for Lys and Trp. Solvents for peptide synthesis (DMF and CH_2Cl_2) were obtained from PE Biosystems, while acetonitrile for HPLC was obtained from EM Science (Gibbstown, NJ, USA).

Peptide synthesis was carried out using a continuous-flow PerSeptive Biosystems Pioneer Peptide Synthesis Workstation with 5-[4-(9-fluorenylmethoxycarbonyl) 3,5-dimethoxy]valeric acid polyethylene glycol-polystyrene (Fmoc-PAL-PEG-PS). This system utilizes a UV detector to measure the absorbance of solutions flowing through it, which provides feedback monitoring of the synthetic process [10]. This modifies reaction times (Fmoc removal and coupling of the next amino acid) based on the efficiency of the Fmoc removal. Slow Fmoc removal may be attributed to poor accessibility to the growing peptide support and, thus, the subsequent attachment of the amino acid may be difficult; therefore, extended times can be beneficial. The following protocol was used. (i) Deprotection step with 10 column volumes of piperidine:DMF (1:4, v/v) for 5 min (Method A) or until the change in the detector value over a period of time falls below the *slope factor* value (extended deblock protocol). (ii) Wash with 10 column volumes of DMF at 30 ml/min. (iii) Coupling with Fmoc-amino acid: coupling reagent:DIEA (1:1:2) with 3 min of preactivation and a coupling time of either 30 min (Methods A–D, G–J), 1 h (Method F), or a time obtained after multiplying the default time (30 min) by a factor equal to the ratio of the extended deblock time to the deblock time in the protocol (extended couple protocol). The activated amino acid was prepared by dissolving 4 equivalents of Fmoc–amino acid in equal parts with a 0.5 M solution of coupling reagent in DMF and a 1.0 M DIEA solution in DMF. Additional DMF was added dependent on the synthesis scale to raise the solution quantity to a volume that the system could handle. The final concentration of activated amino acid ranged from 0.068 M (at a 0.1 mmol scale) to 0.16 M (1 mmol scale) [10]. (iv) Wash with 8 column volumes of DMF at 30 ml/min. Finally, (v) wash with neat DIEA for 1 min to remove any HOAt and HOBt that has bonded non-covalently to the resin (Methods A,B, D–J), and which could interfere with the on-line detection of the Fmoc peak [10]. The volume of the column is dependent on the scale of the synthesis and the void volume of the support (the void volume for PEG-PS is $\sim$4.4 ml/g). Elongation of the first 30 amino acids occurred at the 1 mmol scale. Completion of the syntheses then occurred at the 0.1 mmol scale. Multiple peptide synthesis (MPS) was carried out with the same instrument at the 50 μmol scale. Peptide synthesis transformations and washes were carried out at 25°C unless indicated otherwise.

Peptides were examined using HPLC on a Waters apparatus with a Model 600 solvent delivery system, a Wisp Model 712 automatic injector, a Model 490 programmable wavelength UV detector, and a

H-Ser-Pro-Tyr-Ser-Ser-Asp-Thr-Thr-Pro^{9}-Cys-Cys-Phe-Ala-Tyr-Ile-Ala-Arg-Pro-Leu^{19}-Pro-Arg-Ala-His-Ile-Lys-Glu-Tyr-Phe-Tyr^{29}-Thr-Ser-Gly-Lys-Cys-Ser-Asn-Pro-Ala-Val^{39}-Val-Phe-Val-Thr-Arg-Lys-Asn-Arg-Gln-Val-Cys-Ala-Asn-Pro-Glu-Lys-Lys-Trp-Val-Arg-Glu-Tyr-Ile-Asn-Ser-Leu-Glu-Met-Ser^{68}-NH_2

Figure 1 Amino acid sequence of RANTES (1–68).

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Networking Computer 860 to control system operation and collect data. Samples were dissolved in neat TFA and injected into the system [11]; they were then eluted using a C_{18} reversed-phase column (4.0 × 50 mm, 3 μm, 120 Å; YMC, Wilmington, NC, USA). Peptides were eluted with a linear gradient over 30 min of 0.1% TFA in H_2O and 0.1% TFA in CH_3CN from 4:1 to 2:3 at a flow rate of 1.0 ml/min at 50°C, and detection at 220 nm.

Atmospheric pressure ionization mass analysis (API-TOF-MS) of crude peptide samples were performed on a Mariner Biospectrometry Workstation (PE Biosystems, Framingham, MA). Samples were diluted to 1 μM in 1% HOAc (JT Baker, Phillipsburg, NJ) in $CH_3CN–H_2O$ (1:1). Mass spectra were also recorded on a Voyager Elite-DE (PE Biosystems), operated in the reflector mode. The effective flight path was 3.0 m. Desorption/ionization was carried out via a nitrogen laser operating at 337 nm. Positive ion spectra were recorded by applying a +25 kV extraction voltage to the MALDI-TOF plate following a 75 ns delay from the laser pulse. A grid voltage of 65% of the extraction field was used in the analysis. Samples were prepared to a final concentration of ~0.5 μg/μl in the matrix by mixing the peptide (~1 mg/ml in 0.1% TFA in H_2O) with 10 mg/ml of α-cyano-4-hydroxycinnamic acid in a solution of 0.1% TFA in $CH_3CN:H_2O$ (1:1). Approximately, 1 μl of the sample was deposited on the MALDI-TOF plate and allowed to dry *in vacuo* prior to analysis.

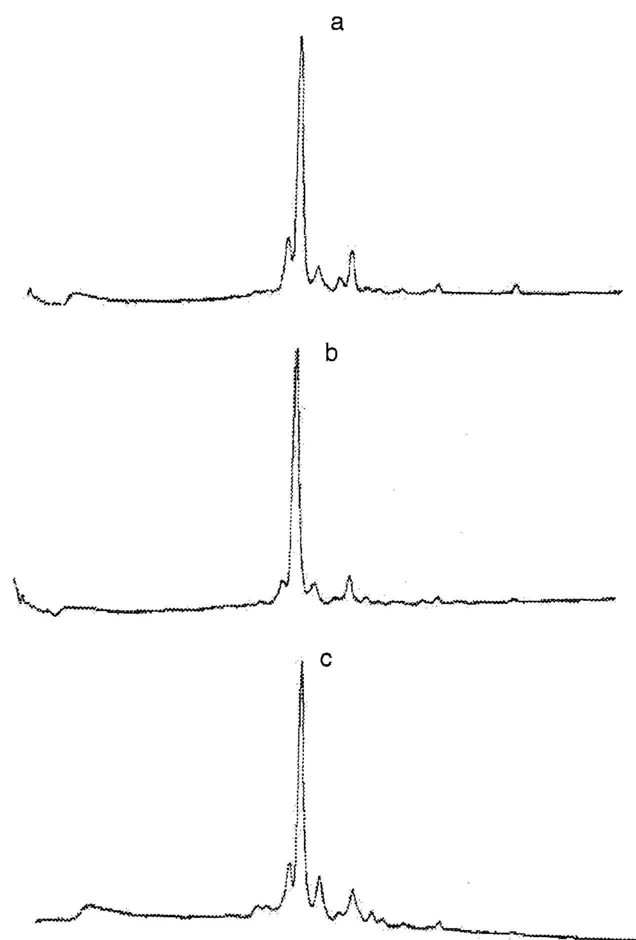

Figure 2 HPLC of crude RANTES (39–68) synthesized in a: (a) 0.1 mmol scale in the stand-alone synthesis mode; (b) 1.0 mmol scale in the stand-alone synthesis mode; and (c) 50 μmol in the MPS mode. See text for experimental conditions.

RESULTS AND DISCUSSIONS

Synthesis of RANTES (39–68)

To provide a resin for implementation in the studies discussed below, an initial large-scale synthesis (1.0 mmol) of RANTES (39–68) was carried out as outlined above. An aliquot of the resin was cleaved with Reagent R (TFA:thioanisole:1,2-ethanedithiol:anisole, 90:5:3:2) for 1 h at 25°C to yield the proper peptide (API-TOF-MS, calculated 3698.2; found 3696.8) in excellent purity, as shown by HPLC (Figure 2(b)). The synthesis was repeated at 0.1 mmol and 50 μmol in the stand-alone and MPS modes of the Peptide Synthesis Workstation, respectively (Figure 2). Interestingly, the best quality of the target peptide was obtained for the large-scale synthesis. For this 30 amino acid sequence, chain elongation using the MPS mode showed a quality similar to that obtained at the 0.1 mmol scale. These results indicate the reproducibility of the instrument to successfully assemble a 30-mer sequence at three variable modes: small- and large-scale as well as MPS.

Synthesis of RANTES (1–68)

Assembling study. Completion of RANTES (1–68) was conducted (0.1 mmol scale) starting with peptide resin containing RANTES (39–68) using the different methods shown in Table 1. All methods (except A) incorporated the extended deblock protocol, which continues N^{α}-Fmoc removal when UV data determines a difficult deprotection [12]. In Methods A–C, HATU, TBTU/HOBt, and TFFH were incorporated as the coupling reagents, respectively. Method D

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Table 1 Compilation of Methods used to Assemble RANTES (1–68) Starting with RANTES (39–68)-PAL-PEG-PS

Method	Deblocking	Coupling	Time
A	Piperidine:DMF (1:4)	HATU/DIEA/DMF	
B	Piperidine:DMF (1:4)	TBTU/HOBt/DIEA/DMF	Extended deblocking
C	Piperidine:DMF (1:4)	TFFH/DIEA/DMF	Extended deblocking
D	Piperidine:DMF (1:4)	1. HATU/DIEA/DMF 2. PyAOP/DIEA/DMF	Extended deblocking
E	Piperidine:DMF (1:4)	HATU/DIEA/DMF	Extended deblocking/coupling
F	Piperidine:DMF (1:4)	HATU/DIEA/DMF	Extended deblocking, 1-h coupling
G	1% Triton X 100 in piperidine:DMF:NMP (1:2:2)	HATU/DIEA/DMF	Extended deblocking
H	1% Triton X 100 in piperidine:DMF:NMP (1:2:2)	HATU/DIEA in 1% Triton X 100 and 2 N ethylene carbonate in CH_2Cl_2:DMF:NMP (3:3:1)	Extended deblocking
I	1% Triton X 100 in piperidine:DMF:NMP (1:2:2), 55°C	HATU/DIEA in 1% Triton X 100 and 2 N ethylene carbonate in CH_2Cl_2:DMF:NMP (3:3:1), 55°C	Extended deblocking
J	Piperidine:DMF (1:4), 55°C	HATU/DIEA in 1% Triton X 100 and 2 N ethylene carbonate in CH_2Cl_2:DMF:NMP (3:3:1), 55°C	Extended deblocking

involved a first coupling with HATU followed by an additional treatment (double coupling) with PyAOP. Methods E and F used the extended coupling protocol (analogous to extended deblock protocol) and 1 h coupling for all amino acids, respectively. In Methods A–F, all deprotections and couplings were carried out with DMF as the solvent. In the next series of methods (G–J), 'magic mixture' was used as the solvent for the deprotection and/or the coupling step. 'Magic mixture', which was first described by Zhang *et al.* [9], contains ethylene carbonate, a non-ionic detergent Triton X 100, ethylene carbonate, and NMP for breaking the secondary structure as well as the interchain interaction of the peptidyl-resin, and DMF and CH_2Cl_2 for reducing the viscosity of the mixture. In Methods I and J, all processes were carried out at 55°C.

The quality of the syntheses was examined following the incorporation of every five residues *via* HPLC and API-TOF-MS of the crude product obtained by cleavage of an aliquot of peptidyl-resin with Reagent R. Analysis of the data indicated that HATU-mediated coupling was superior to TBTU (A versus B) and Asn[36] was not incorporated with TFFH-mediated coupling (C). The latter result corroborates the previous observation, which suggests that the coupling of protected Asn with TFFH must be carried out in the presence of an equivalent of HOBt or preferably HOAt [13]. Double coupling and increasing the acylation times did not significantly improve the quality of the synthesis (D–F). The extended coupling protocol gave similar results to increased (1 h) coupling times (E versus F). This result indicates that magic mixture in conjunction with column heating for both coupling and deblocking clearly gave optimal results (H–J versus G) for RANTES (9–68), with no detrimental consequences to the synthesis.

Unfortunately, HPLC analysis of the final crude products could not readily distinguish the improved results achieved by using magic mixture and column heating. However, careful analysis of the HPLCs of the crude products following the addition of every five residues revealed the differences in the various methods, particularly in the 40–60 residue region. Figures 3–5 show the HPLC corresponding to the

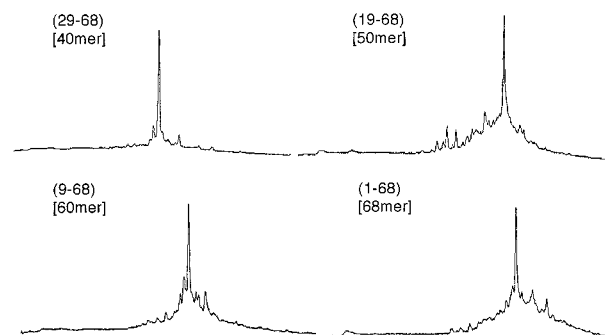

Figure 3 HPLC of crude RANTES (1–68) synthesized following Method A. See text for experimental conditions.

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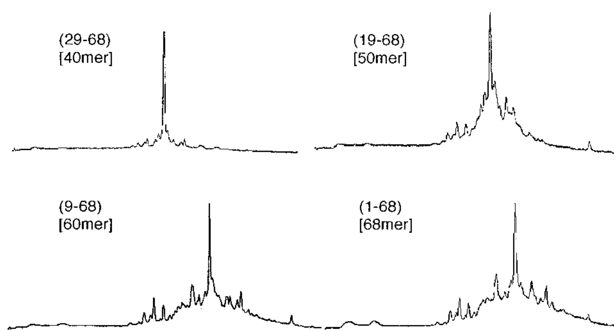

Figure 4 HPLC of crude RANTES (1–68) synthesized following Method B. See text for experimental conditions.

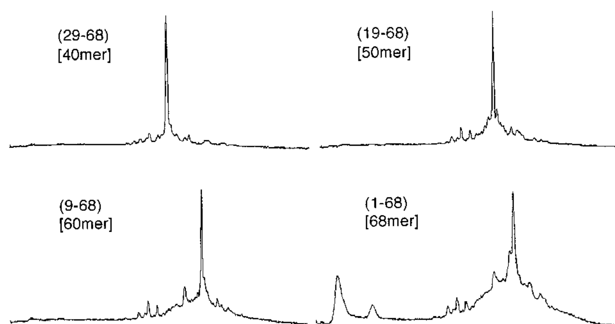

Figure 5 HPLC of crude RANTES (1–68) synthesized following Method I. See text for experimental conditions.

Table 2 API-TOF-MS Analysis of Crude Peptides (Method I)

Peptide	Theoretical mass	Experimental mass
RANTES (39–68)	3698.2	3696.79
RANTES (34–68)	4240.3	4240.91
RANTES (19–68)	6032.9	6032.96
RANTES (9–68)	7299.3	7298.54
RANTES (4–68)	7790.7	7790.10
RANTES (1–68)	8138.1	8137.37

synthesis progression of RANTES (1–68) following Methods A, B, and I. Table 2 shows the API-TOF-MS analysis of intermediate crude peptides synthesized following Method I.

Preparative HPLC was carried out on crude material (Method A) and all components were identified using MALDI-TOF MS. Analysis of the mass spectra indicated that acetylation presumably from acetate salts in DIEA during the coupling reaction [14] and trifluoroacetylation [15] of the side-chain hydroxyl function of the *N*-terminal Ser *via* an $N \rightarrow O$ acyl shift were prevalent.

Final cleavage and deprotection study. To reduce the amount of trifluoroacetylation, various cleavage cocktails were examined to determine their impact on the quality of recovered product [16]. RANTES (1–68) resins (Method A) were treated with: Reagent R for 2 and 4 h; Reagent N (TFA:thioanisole:anisole, 93:5:2) for 2 h; Reagent R^+ (TFA:thioanisole: 1,2-ethanedithiol:anisole:methanesulfonic acid, 87.5:5:3:2:2.5) for 10 min; Reagent B^+ (TFA:*m*-cresol:H_2O:triethylsilane, 88:5:5:2) for 1 and 2 h; and Reagent K (TFA:phenol:H_2O:thioanisole:1,2-ethanedithiol, 82.5:5:5:5:2.5) for 1 and 2 h. The quality of the crude materials varies depending on the cleavage cocktail and the time used for the manipulation. The simplest cocktail, Reagent N, does not lead to any appreciable amount of correct product. Extended times are detrimental for Reagent R. The highest purity of crude product was obtained with Reagent K for 2 h (Figure 6). In addition, trifluoroacetylation was eliminated by protecting the *N*-terminal amino group with Fmoc during cleavage followed by removal with a piperidine:DMF (1:4) solution.

CONCLUSIONS

These results demonstrate that chemokines may be constructed using linear assembly in conjunction with Fmoc/Bu^t-based methods, continuous-flow instrumentation, PEG-PS resins, and HATU. In order to assess the quality of the various methods, following the addition of every five residues, HPLC analysis was carried out. Strong differences in HPLC purity were observed for the different methods during the assembly of RANTES containing the first 40–60 residues. Unfortunately, HPLC analysis of RANTES (1–68) for the different coupling and deprotection procedures did not provide a definitive optimal method. A key aspect to HPLC analysis was dissolving the crude product in neat TFA and carrying out the elution gradient at an elevated temperature (50°C).

The use of 'magic mixture' as a solvent and elevated temperatures was clearly beneficial for both the coupling and the deprotection reactions. The feedback monitoring system of the instrument gave similar quality to double coupling and increased acylation times. Thus, it is a useful tool that may be implemented to shorten the time to complete a

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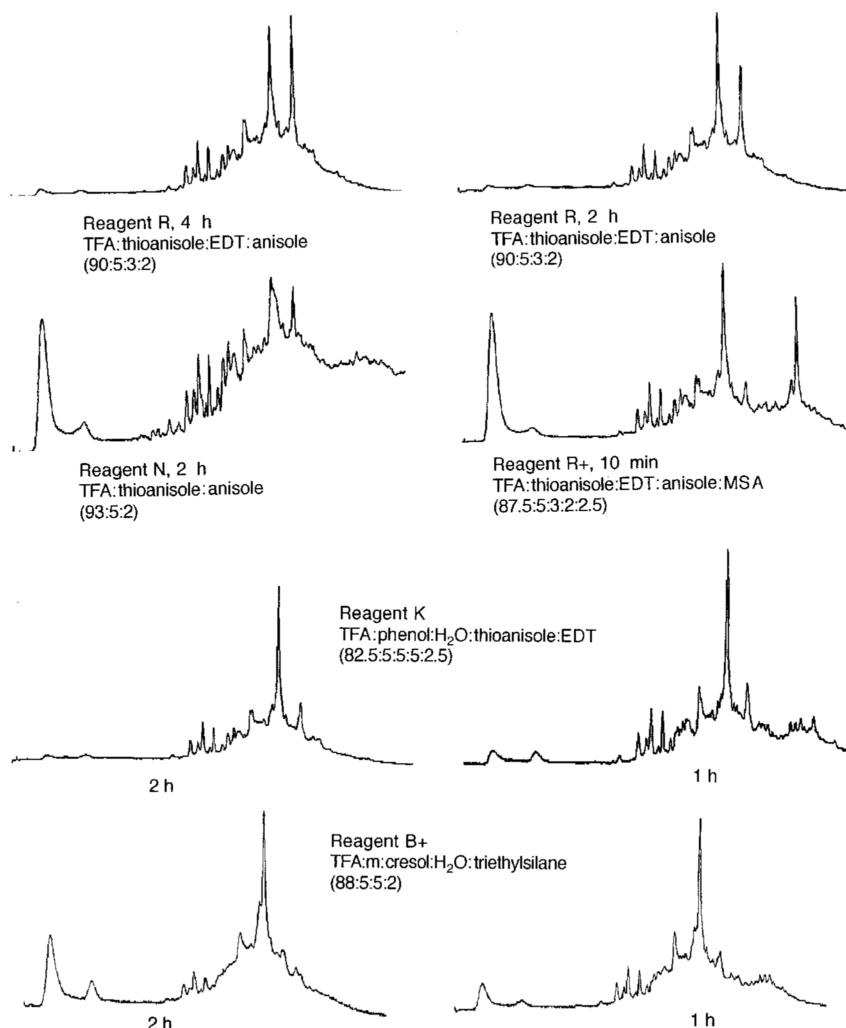

Figure 6 HPLC analysis of crude RANTES (1–68) using different cleavage cocktails. See text for experimental conditions.

synthesis. For coupling reagents that require DIEA, such as onium-based HATU, PyAOP, TBTU, and TFFH, the quality of the tertiary base is critical to prevent terminated sequences by undesired acetylation. The use of DIEA distilled from ninhydrin is highly recommended. Finally, different cleavage cocktails and reaction times may give crude products of variable purity. In this work, the best conditions for final cleavage of RANTES (1–68) were obtained with Reagent K for 2 h.

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