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Solid-phase peptide synthesis by fragment condensation: Coupling in swelling volume

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Summary

The condensation of short peptides to resin-bound fragments was examined with respect to high coupling yields with only a small molar excess of a peptide in the reaction solution. The best results were achieved by the addition of reactants (C-unprotected peptide, DIC, and HOBt) dissolved in a so-called swelling volume of an appropriate solvent to a dry resin with an attached N-deprotected peptide chain. Each coupling step was followed by the end-capping of unreacted resin-bound peptide with 2,4-dinitrofluorobenzene. The substituted dinitroaniline chromophore formed in this reaction made the detection and separation of deletion peptides easy. Both conventional and 'swelling volume' methods were compared on parallel syntheses of the HIV-1 protease C-terminal 78–99 fragment. The yields of the isolated heneicosapeptide were 21 and 81% in favor of the 'swelling volume' procedure.

Abbreviations: AA, amino acid; Abu, α-aminobutyric acid; AcOH, acetic acid; Bu^t, *tert*-butyl; DCM, dichloromethane; DIC, *N*,*N*′-diisopropylcarbodiimide; DMA, *N*,*N*′-dimethylacetamide; DNFB, 2,4-dinitrofluorobenzene; Dnp, 2,4-dinitrophenyl; EDT, ethanedithiol; FAB-MS, fast-atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; HPLC, high-performance liquid chromatography; MeOH, methanol; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; SPFC, solid-phase fragment condensation; TFA, trifluoroacetic acid; TFE, trifluoroethanol; Trt, trityl. Amino acid symbols denote the L-configurations. Abbreviations for amino acids and nomenclature of peptides follow the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [Eur. J. Biochem., 138 (1984) 9].

Introduction

Although the solid-phase fragment condensation (SPFC) synthesis of longer peptides has obvious drawbacks (unpredictable solubility and reactivity of protected fragments), it also has several advantages in comparison to a stepwise method (significant physicochemical differences between the target molecule and deletion side-products; the possibility of implementation of fragment condensation into a conventional stepwise synthesis to overcome a difficult sequence). The concept of a so-called 'convergent synthesis' and a complete methodological approach for the solid-phase fragment synthesis of peptides were investigated and discussed by the group of Albericio and Giralt [1]. Various solid-phase-based fragment condensation approaches were reviewed and compared by Benz [2]. However, a conventional SPFC protocol (2-chlorotrityl chloride resin [3], Fmoc/Bu^t; DIC/HOBt) adapted to the synthesis of e.g. HIV-1 protease and the V-3 loop of gp 120 did not give satisfactory results in our hands, even with a large excess of reagents. Coupling yields dramatically dropped in regions with difficult sequences, crude peptides were hard to purify and the overall yields of the desired peptides were low. In this communication, we describe an alternative method for SPFC which allows for high coupling yields without wasting expensive reagents.

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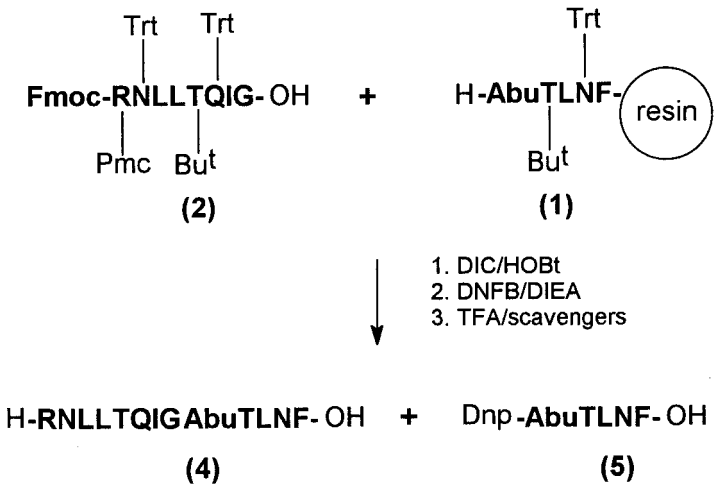

Figure 1. Synthetic scheme for condensation I.

Materials and methods

Reagents

2-Chlorotrityl chloride resin and Fmoc amino acid derivatives were purchased from Calbiochem-Novabiochem AG (Läufelfingen, Switzerland). Some derivatives were from Senn Chemicals AG (Dielsdorf, Switzerland). Solvents for peptide synthesis (DIC, DNFB, HOBt and DIEA) were obtained from Fluka (Buchs, Switzerland), Sigma (St. Louis, MO, U.S.A.) and Aldrich (Milwaukee, WI, U.S.A.). The molecular weights of protected fragments, the target peptide and side-products were determined using FAB-MS technique on a ZAB-EQ VG analytical spectrometer (Manchester, U.K.). Amino acid analyses of all products were determined after hydrolysis with 6 M HCl (110 °C, 20 h) using a Biochrom 20 (Pharmacia, Manchester, U.K.). Analytical RP-HPLC was performed on a Spectra-Physics module (pump SP 8800, UV-vis detector SP 8450, integrator SP 4290) with a Vydac C_{18} column (218TP54). Preparative HPLC was performed with a Vydac C_{18} column (218TP510). The solvent system for both analytical and preparative HPLC was as follows: A (0.05% TFA in water) and B (0.05% TFA in MeOH). The linear gradient for analytical HPLC (1 ml/min) was 0% A – 100% B over 60 min. The linear gradient (3 ml/min) 30% A – 100% B over 50 min was used for preparative HPLC. UV detection was done at 222 and/or 360 nm.

Preparation of protected fragments

The C-terminal heneicosapeptide segment of HIV-1 protease was divided into the following three fragments:

Fmoc-Abu-Thr(Bu^t)-Leu-Asn(Trt)-Phe-2-chlorotrityl resin – **1**

Fmoc-Arg(Pmc)-Asn(Trt)-Leu-Leu-Thr(Bu^t)-Gln-Ile-Gly-OH – **2**

Fmoc-Pro-Thr(Bu^t)-Pro-Val-Val-Asn-Ile-Ile-Gly-OH – **3**

All three fragments were manually synthesized on 2-chlorotrityl chloride resin in polypropylene syringes with Teflon filters. The first amino acid was bound to the resin according to Barlos et al. [3] with a loading of 0.12 mmol per gram for Fmoc-Phe-resin and 0.34 mmol per gram for Fmoc-Gly-resin. The following synthetic protocol was used:

(1) Fmoc deprotection (20% piperidine/DMF, 5 and 20 min);
(2) wash (6 × DMF);
(3) coupling (Fmoc-AA/DIC/HOBt = 3:3:3 equiv in DMF), recoupling after 90 min, Kaiser ninhydrin test; and
(4) wash (6 × DMF).

Protected fragments **2** and **3** were obtained after cleavage with a mixture of AcOH/TFE/DCM (1:1:8,

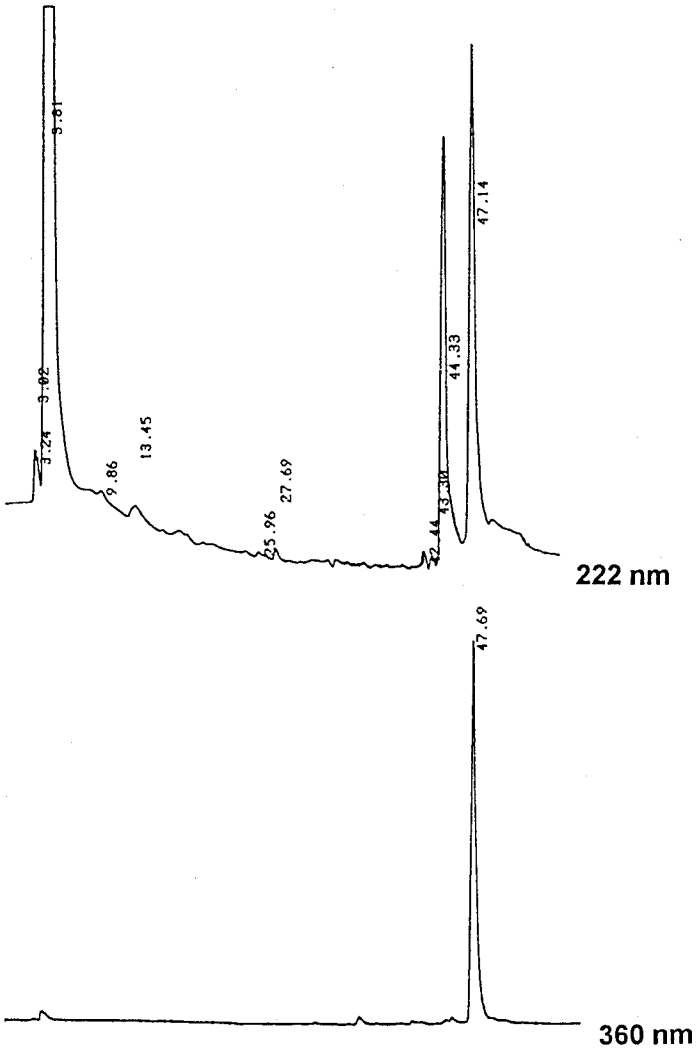

Figure 2. HPLC of the crude product of condensation I in a conventional volume.

5 ml/100 mg of resin, 30 min) [3]. The resin was thoroughly washed with DCM. Pooled extracts were evaporated and lyophilized from aqueous AcOH. Products were characterized and used for fragment couplings without further purification. The resin-bound fragment **1** was characterized in protected form after mild cleavage from a small sample of the resin (according to the method above, including N-terminal Fmoc deprotection) and then deprotected in a strong acid mixture (TFA/triisopropylsilane/water = 90:5:5, precipitated with diethyl ether, dried and lyophilized from aqueous AcOH) for additional characterization.

Fragment **1**

Molecular weight of the protected form: found 1099.4; calculated 1099.6 ($M+H^+$); for deprotected peptide 579.6 and 579.3 ($M+H^+$), respectively. Amino acid analysis: Asn (1) 1.0; Thr (1) 1.1; Leu (1) 1.0; Phe (1) 1.1; α-Abu (1) 1.0.

Fragment **2**

Molecular weight: found 1944.5; calculated 1944.2 ($M+H^+$). Amino acid analysis: Asn (1) 1.1; Thr (1) 1.2; Gln (1) 1.0; Gly (1) 1.0; Ile (1) 0.9; Leu (2) 2.0; Arg (1) 0.9. The yield after cleavage was 67%. Purity was estimated to be 92% from HPLC.

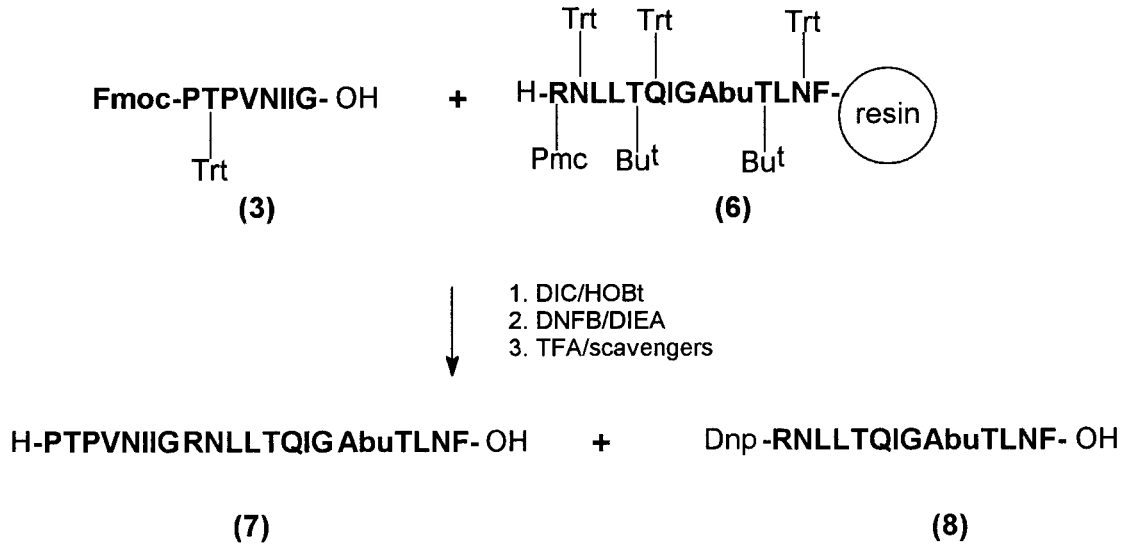

Figure 3. Synthetic scheme for condensation II.

Fragment **3**

FAB-MS afforded two signals (1088.8 and 1110.8) which correspond to calculated values 1088.6 ($M+H^+$) and 1110.6 ($M+Na^+$). Amino acid analysis: Asn (1) 1.2; Thr (1) 1.1; Pro (2) 2.0; Gly (1) 1.1; Val (1) 1.0; Ile (2) 1.7. The yield after cleavage was 88%. HPLC purity was estimated to be 96%.

Fragment condensations

Condensation I in a conventional volume

Condensation I of fragments **1** and **2** (see Figure 1) was carried out on 70 mg of resin-bound fragment **1** (8.4 μmol). First, the N-terminal Fmoc group was removed with 20% piperidine in DMF (5 and 20 min). After washing the resin with DMF (no drying!), the coupling was accomplished in 2 ml of DMA solution containing fragment **2** (12.6 μmol), DIC and HOBt (1.5:1.5:1.5 equiv) for 18 h. Then the resin was washed with DMA and recoupled with 1 equiv of the reactants for another 18 h (the Kaiser ninhydrin test was still positive). After condensation and washing, again with DMA, the unreacted amino groups were end-capped with DNFB/DIEA (10:5 equiv) in DMA/DCM (1:1) for 60 min [4]. Twenty mg of the resin-bound product was removed for an additional condensation step and the remaining portion of the resin was deprotected with 20% piperidine in DMF (5 and 20 min), washed with DMF followed by DCM, and dried. The final cleavage was done in 1 ml of TFA/EDT/triisopropylsilane/water (87.5:2.5:5:5) for 2 h in a polypropylene syringe with a Teflon filter. The cleavage mixture was filtered into a round-bottom flask, the resin was washed with TFA and all the extracts were collected and evaporated. The product was precipitated from diethyl ether, dried and lyophilized from aqueous AcOH. Product **4** and by-product **5** were detected in crude peptide material by HPLC (Figure 2). Product **5** was identified at 360 nm and separated by preparative HPLC.

Peptide **4** Molecular weight: found 1475.2; calculated 1474.8 ($M+H^+$). Amino acid analysis: Arg (1) 0.9; Asn (2) 2.0; Thr (2) 2.0; Gln (1) 0.9; Gly (1) 0.9; Ile (1) 0.9; Leu (3) 2.9; Phe (1) 1.1; Abu (1) 1.2. The yield of the product after HPLC preparative separation was 3.5 mg (40%).

Peptide **5** Molecular weight: found 745.3; calculated 745.3 ($M+H^+$). Amino acid analysis: Asn (1) 0.9; Thr (1) 1.0; Ile (1) 1.0; Phe (1) 1.2. The yield of the HPLC separated product was 1.3 mg (29%).

Condensation II in a conventional volume

Condensation II (Figure 3) in a conventional volume was performed in an analogous manner as condensation I. A sample (20 mg) of the resin-bound product of condensation I (conventional conditions, see above) was coupled with 1.5 equiv (3.6 μmol) of fragment **3**, DIC and HOBt in 0.6 ml of DMA for 18 h. Then the

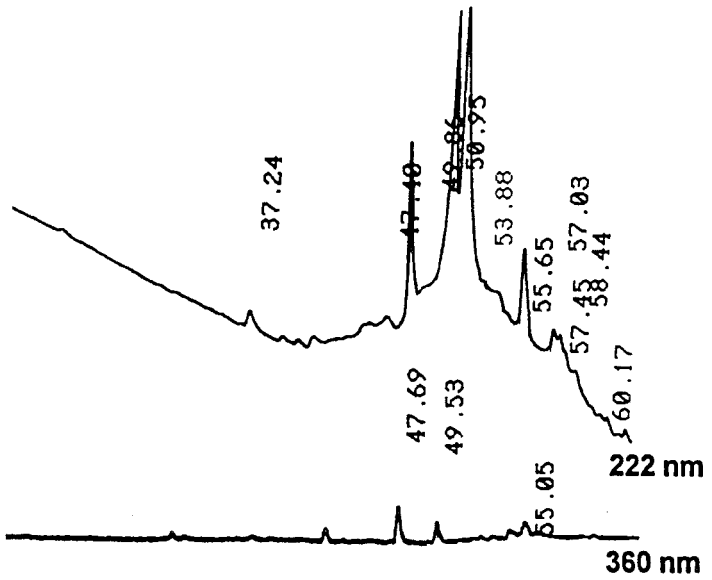

Figure 4. HPLC of the crude product of condensation II in a conventional volume.

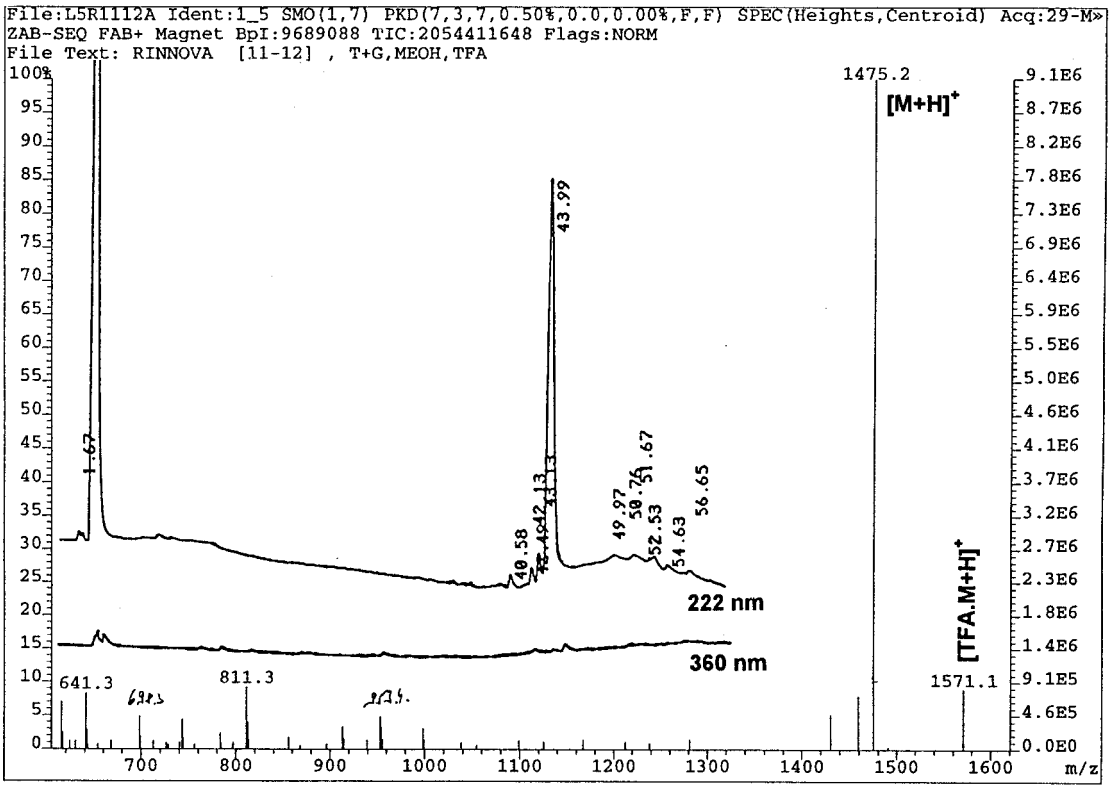

Figure 5. HPLC and FAB-MS of the crude product of condensation I in a swelling volume.

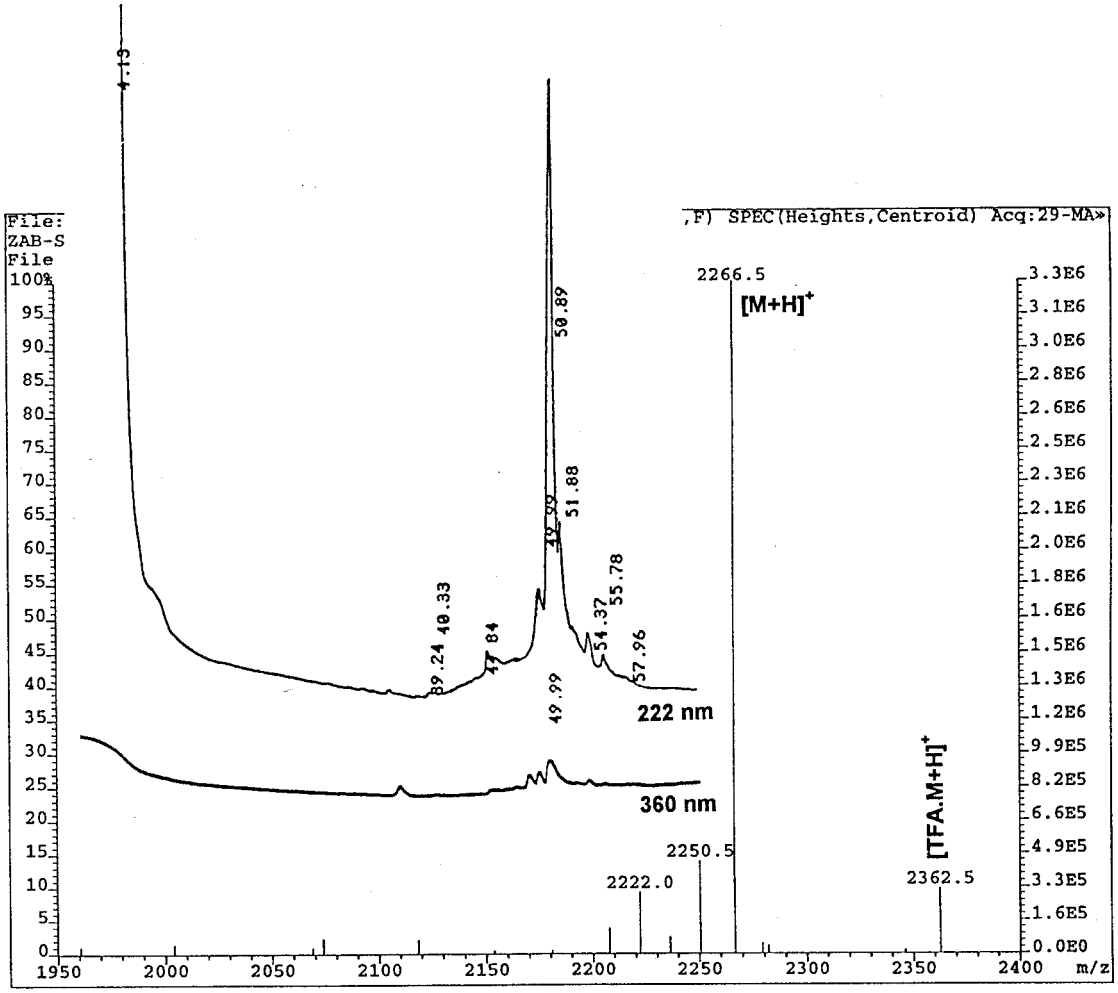

Figure 6. HPLC and FAB-MS of the crude product of condensation II in a swelling volume.

peptide on a support was recoupled with 1 equiv of the reactants for another 18 h (the Kaiser ninhydrin test was still positive), washed and dinitrophenylated. The cleaved product was analyzed by HPLC (Figure 4), and the mixture obtained was not separated. Target peptide **7** was accompanied by a number of side-products and was not isolated. According to HPLC, the yield of the peptide was about 21% (relative to the standard).

Condensation I in a swelling volume

Condensation I between fragments **1** and **2** in a swelling volume was accomplished on 100 mg (12 μmol) of resin-bound fragment **1**. The Fmoc group of the fragment was deprotected with 20% piperidine in DMF (5 and 20 min), and the resin was washed (DMF followed by DCM) and dried in vacuo. One and a half equiv (18 μmol) of fragment **2** was dissolved in 600 μl of DMA with 1.5 equiv of HOBt. Then an equimolar amount of DIC was added and the thoroughly dried resin was soaked with this solution. After 18 h, the Kaiser ninhydrin test was negative, and the resin was washed with DMA and dinitrophenylated (as described above). Fifty mg of the resin was removed, cleaved (as above) and analyzed. HPLC and FAB-MS of the crude product of the synthesis are displayed in Figure 5. The main product provided analytical characteristics identical with product **4**. The overall yield of the crude lyophilized peptide after cleavage was 8.2 mg (93%).

Condensation II in a swelling volume
Fifty mg (6 μmol) of resin **6** (Figure 3) (obtained from condensation I in a swelling volume and Fmoc deprotection) was washed (DMF followed by DCM) and dried in vacuo. One and a half equiv (9 μmol) of fragment **3** was dissolved in 300 μl of DMA together with 1.5 equiv of HOBt. An equimolar amount of DIC was added and dry resin **6** was swollen in the reaction solution. The condensation was finished in 18 h (the Kaiser ninhydrin test was negative) and the resin was washed. Then DNFB end-capping was done, and the resin was washed, dried, cleaved and analyzed according to the procedures described in the preceding experiments. The synthesis resulted in product **7** (Figure 6) with a trace of Dnp-peptide **8**.

Peptide **7** Molecular weight: found 2266.5; calculated 2266.3 ($M+H^+$). Amino acid analysis: Arg (1) 0.9; Asn (3) 2.8; Thr (3) 3.0; Gln (1) 1.2; Pro (2) 2.0; Gly (4) 3.6; Val (1) 0.9; Ile (3) 2.8; Leu (3) 3.2; Phe (1) 1.2; Abu (1) 1.1. The overall yield of the crude product after cleavage was 11 mg (81%).

Peptide **8** Molecular weight: found 1640.6; calculated 1640.8 ($M+H^+$). Since only small amounts of peptide **8** were obtained, amino acid analysis was not performed.

Results and discussion

The fact that not an excess but the actual concentration of an activated component is important for a high yield of a coupling reaction is often ignored. Several authors have focused on the utilization of a small condensation volume in solid-phase peptide synthesis in the past. Ragnarsson et al. in 1974 [5], who first recognized in a comparative study the usefulness of a small reaction volume, recommended using only about 7 ml of solvent and 4 equiv of reactants per gram of resin to achieve high coupling yields. Recently, Eichler et al. [6] carried out automated stepwise solid-phase synthesis in a so-called 'inclusion volume'; swelled resin was condensed in a minimal volume of 0.3 M solution of reaction components (5 equiv reactants in about 0.5 ml of the solution per 100 mg of resin) which provided higher yields in comparison to the commonly used solution volume (1.5–3 ml/100 mg resin). Another inclusion volume concept was established by Frank [7] in his 'spot synthesis' on cellulose membranes.

We investigated in detail the assembly of the C-terminal sequence (21 amino acids) of the HIV-1 protease from three fragments using the SPFC approach. Fragments **1, 2** and **3** were designed according to the positions of glycine to avoid racemization on the C-terminus of a condensed fragment. Side-chain protection was chosen according to the standard Fmoc/Bu^t strategy. For the preparation of fragments **1** and **2**, Trt protection of asparagine was found necessary to prevent formation of the β-cyanoalanine [8]. A peptide fragment and coupling reagents were dissolved in a minimal (experimentally found) volume of DMA needed to swell the resin. The reaction volume was then equal to the swelling volume of the resin and represented the highest possible concentration of reaction components. The condensation reaction was then accomplished by soaking a resin-bound fragment with this solution. This experimental procedure made it possible to achieve high yields of condensation product using only 1.5 equiv molar excess of the protected fragment at a final concentration of 30 mM. In comparison to a condensation in a conventional volume, under the same conditions, the concentration in a swelling volume was approximately 5 times higher when the same molar excess was used. In spite of repeating condensation I in a conventional volume, the overall yield of the target peptide was only 40%. This is in contrast with the single condensation in a swelling volume that proceeded up to 93%. The final product of two consecutive condensations (I and II) in a swelling volume was obtained at 81% yield in comparison to 21% for conventional conditions. Unreacted amino groups were end-capped after each fragment condensation with 2,4-dinitrofluorobenzene which allowed direct identification of Dnp labelled deletion side-products (HPLC monitored at 360 nm) and thus superior separation of the desired peptide.

Conclusions

We have demonstrated that protected peptide fragments can be successfully condensed on a solid support in a so-called swelling volume (minimal volume in which a dry resin is completely swelled) in the solvent of choice. This experimental arrangement enabled us to obtain high yields of fragment condensations even though a low molar excess of reactants was used. We believe this method is a prospective tool for the microscale synthesis of longer peptides and/or for overcoming synthetically difficult regions of peptide

sequences. Limitations of the approach include the solubility of activated fragments used and the steric demands of the protecting groups which may critically influence the reaction yields.

Acknowledgements

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