Combined Solid Phase and Solution Synthesis of the Fully Protected Segment 74–99 of HIV 1-Protease with the Application of a New Trityl-Linker

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A new trityl-linker for the strategy of combined solid phase and solution synthesis has been used for the synthesis of the fully protected segment 74–99 of HIV 1-protease. The fully protected fragments 74–81, 82–99 and 90–99 have been synthesized on the polystyrene-polyethylenglykol resin TentaGel, split off with dilute acetic acid and assembled in solution without loss of side-chain protecting groups. After protection of the C-terminus of the fragment 90–99, the second fragment was coupled with TBTU/NMM. The couplings were monitored by IS-MS and HPLC. Racemization was checked by chiral gas-chromatography on a Chirasil-Val capillary.

Introduction

Despite recent developments in solid-phase peptide synthesis techniques which enable the stepwise preparation of large fragments in good yields and high purity [1], the total synthesis of small proteins or large peptides continues to be one of the major challenges for chemists. Among the numerous approaches described [2], the combined solid-phase and solution method proposed by Riniker et al. [3] seems to be one of the most promising. In this method the fully protected peptide fragments are prepared by solid phase techniques and the target proteins or peptides subsequenly assembled in solution by coupling the individual protected fragments. Two distinct advantages are obvious: 1) The synthetic intermediates can be purified before being coupled; 2) The fragment condensation of two protected fragments in solution is more readily monitored than the reaction on the resin. This strategy requires linkers for solid phase synthesis which allow the cleavage of the fully protected fragments under very mild conditions to prevent loss of side chain protection.

In order to test the efficiency of inhibition of HIV 1-protease by means of drugs, significant amounts of purified synthetic HIV 1-protease are required. The total synthesis of the Abu67-95-modified HIV 1-protease analog [4] using Boc strategy and results of the cysteine containing enzyme [5] have been reported. In the course of solid phase synthesis of HIV 1-protease with Fmoc-strategy we encountered difficulties in the coupling and de-protection steps 81–87 and 61–71 of the sequence [6]. To overcome these difficulties these regions can be divided into two pieces and be joined by fragment condensation. Recently we have developed a new trityl-linker (p-(diphenylhydroxyethyl)benzoic acid) for the rapid synthesis of fully protected fragments [6]. This linker can be introduced to any kind of amino functionalized polymer. The assembled protected fragments can be cleaved off with acetic acid or hexafluoroisopropanol, avoiding contact of the side-chain protecting groups with the more stronger acid, TFA.
Comparative tests [6] have shown the new trityl-linker to be superior to linkers introduced by Rini-ker [7] and Rink [8].

Barlos et al. introduced the 2-chlorotrityl chloride resin for the synthesis of protected peptide fragments [9]. This linker allows protected fragments to be cleaved from the resin by dilute acetic acid. However, its application is restricted to polystyrene (1% DVB) as polymer support, and cannot be employed with polystyrene-polyethylene glycol supports [10].

In this paper we report upon the preparation of the fragment 74–99 of HIV 1-protease by condensation of the fully protected peptides in solution. The investigation was carried out in order to test the concept of combined solid-phase and solution synthesis of small proteins and large peptides.

Experimental

Coupling of the first Fmoc amino acid to the linker

After coupling of the linker p-(diphenylhydroxymethyl) benzoic acid (available from PepChem, Goldammer & Clausen, Im Winkelrain 73, 72076 Tübingen) to the PS-PEG-NH₂-resin TentaGel (Rapp-Polymere, Ernst-Simon-Str. 9, 72072 Tübingen) using HOBr/DIC the trityl-linker carrying resin was chlorinated overnight employing a solution of 50% acetyl chloride in abs. DCM followed by washings with DCM. The resin was dried in vacuo. Subsequently the first Fmoc amino acid was coupled to the trityl-linker with a fourfold excess and with a fivefold of DIEA in DCM for 90 minutes. MeOH was added for the capping of the remaining trityl-Cl function. The resin was washed thoroughly with DCM, DMF, MeOH and finally diethylether and dried in vacuo. The determination of the loading by the method of Meienhofer [12] resulted in values of 90% for Fmoc-Gly and Fmoc-Pro.

Syntheses of the fragments 90–99, 82–89 and 74–81

The syntheses were carried out on the above mentioned resins using a fourfold excess of Fmoc amino acids and TBTU/NMM. Therefore the continous flow peptide synthesizer Milligen 9050 was employed. For the fragment 90–99 (Fmoc-Leu-Thr(tBu)-Gln(Tmob)-Ile-Gly-Cys(Trt)-Thr(tBu)-Leu-Asn(Tmob)-Phe-OH) coupling times were prolonged from 12 to 15 minutes and for Thr and Ile double coupleings were used, for the fragment 82–89 (Fmoc-Val-Asn(Trt)-Ile-Ile-Gly-Arg(Pmc)-Asn(Trt)-Leu-OH) at the positions 83 (Asn), 87 (Arg) and 88 (Asn) double coupleings were necessary, for the fragment 74–81 (Fmoc-Thr(tBu)-Val-Leu-Val-Gly-Pro-Thr(tBu)-Pro-OH) the coupling times were prolonged to 15 minutes and for Leu a double coupling was applied.

The cleavage of the above mentioned fragments from the resin was carried out by adding a mixture of 50% acetic acid, 45% DCM and 5% methanol as scavenger to the resin. After 2 h the solution of the fully protected fragment was separated from the resin by filtration and heptane was added causing a precipitation of the product. After evaporation to dryness heptane was added again to remove the acetic acid acetotropically. The crude products were analysed by HPLC using a Nucleosil 5 µm ODS column (Grom Analytik + HPLC GmbH, Herrenberger Str. 54, 71083 Herrenberg) and a water acetonitril gradient as eluent. Mass spectra were measured with the Sciex Taga 6000e Triple Quadrupol mass spectrometer.

Esterification of Fmoc-90-99-OH to the tert-butyl ester

500 mg of the fully protected fragment Fmoc-90-99-OH (0.24 mmol) were dissolved in 20 ml of abs. 1,2-dichloroethane and additional 5 ml (28 mmol) trichloro-tert-butyl aceticidmate added. This mixture was heated to 60°C for 6 h and after cooling evaporated to dryness. The remainder was treated with a mixture of abs. diethylether and lgin (30/50). The precipitated product was centrifuged and dried. Further purification was run on a silicagel column which was 10 cm of length and had a diameter of 2 cm. As eluent a mixture of CHCl₃:lgin (80:20):MeOH:HAc=80:10:10:1 was applied. After already 5 min the non-polar product elutes from the column in a yield of 80%.

Fmoc-deprotection from Fmoc-90-99-OrBu

400 mg Fmoc-90-99-OrBu (0.19 mmol) were treated with 20% piperidine in DMF for 30 min. After evaporation of the solvents the working up was done as already mentioned in the esterification procedure. The product H-90-99-OrBu was obtained in 95% yield (338 mg=0.18 mmol).

Coupling of Fmoc-82-89-OH to H-90-99-OrBu

481 mg Fmoc-82-89-OH (0.26 mmol) were dissolved in 3 ml of DMF. 82,2 mg TBTU (0.26 mmol) and 28,2 µl NMM (0.26 mmol) were added. After a preactivation time of 15 min this mixture was poured on 320 mg H-90-99-OrBu (0.17 mmol) in 2 ml DMF. The reaction time was 2 h. The vola-
tiles then were evaporated, the residue taken up in a mixture of DCM and MeOH and loaded on a Sephadex LH 20 column containing the same solvent system. The thus purified product Fmoc-82-99-OrBu yielded in 55.9% (358 mg=0.095 mmol).

Separation of the pair of diastereomers of the fully protected 18mer Fmoc-82-99-OrBu

The racemized 18mer (358 mg) was loaded on a silicagel column using CHCl₃:MeOH:HAc=35:5:0.5 as eluent. The two fractions obtained were treated with a mixture of diethylether and heptane causing precipitation. The complete removal of acetic acid was performed by another addition of heptane by forming an aceotropic mixture. Checking the racemization shows the second fragment to contain the all-L-fragment with a yield of 177 mg (0.047 mmol).

Coupling of Fmoc-74-81-OH to H-82-99-OrBu

67 mg Fmoc-74-81-OH (0.06 mmol) were solved in 1 ml DCM and 0.5 ml DMF. 19.3 mg TBTU (0.06 mmol) and 13.2 µl NMM (0.12 mmol) were added and stirred for 15 min for preactivation with a subsequent addition of 141 mg H-82-99-OrBu (0.04 mmol) in 3 ml DMF. After 2 h reaction time the working up is done as already mentioned for the coupling of Fmoc-82-89-OH to H-90-99-OrBu giving 53 mg Fmoc-74-99-OrBu (0.011 mmol = 27.5%).

Determination of the racemization

For the determination of the degree of racemization of the single amino acids in a peptide the peptide was hydrolysed using 6 N HCl at 110°C for 12 h. Then the amino acids were derivatized employing HCl/m ethanol for the C-terminus and trifluoro acetic anhydride for the N-terminus. After the addition of a mixture of D-amino acids as inner standard the rate of racemization was determined by chiral gas-chromatography on a Chirasil-Val-capillary [13].

Results and Discussion

Synthesis of the fully protected fragments

In the course of the synthesis of HIV 1-protease on the PS-PEG-resin TentaGel using Fmoc-strategy difficulties are experienced in the coupling and deprotection steps 88-82 and 74-64 of the sequence. The couplings of Asn, Arg and Gly (positions 88-86) were particularly difficult. Reaction was not complete even after several couplings and deprotection was also hampered as indicated by UV-monitoring of a preview synthesis (Fig. 1).

It therefore seems rational to divide the sequence 74-99 of HIV 1-protease into three fragments (Fig. 2). The possibility of elevated racemization of the C-terminal Leu-residue of fragment Fmoc-82-89-OH during condensation must be born in mind.

Preliminary results from the synthesis of fully protected fragments with other linkers were not satisfactory. When using the the Riniker-linker (4-(4'-hydroxymethyl-3-methoxy-phenoxy) butyric acid) [7] 1% TFA in DCM was required to cleave
a protected fragment from the resin. Even brief contact with this reagent was found to lead to loss of the Boc protecting group of Lys and more so the Trt protecting group of His. HPLC assays indicated that Fmoc-His(Trt)-OH is stable in 50% acetic acid in DCM for up to 4 hours. The Rink-linker [8] releases the peptide with 50% acetic acid and was therefore tested. The cesium salt of 2,4-dimethoxy-4'-hydroxybenzophenone was added to brominated PS-PEG in DMF and shaken for 18 h at 50°C. Reduction of the ketone resin was carried out similarly to that described by Rink [8]. While the purity of fully protected fragments could thus be significantly improved, the yield was unsatisfactory because a part of the hydroxy function obtained from the ketone reduction is reduced to the methylene compound.

Both purity and yield of the fully protected fragments were dramatically increased when the new trityl-linker p-(diphenylhydroxymethyl) benzoic acid [6] was employed. This linker was coupled to the amino resin TentaGel by means of HOBr/DIC. The tertiary C-atom was chlorinated with acetyl chloride in DCM. The first Fmoc amino acid was applied in a fourfold excess with a fivefold excess of DIEA in DCM, leading to a loading in excess of 90%. Fragments were synthesized by continuous-flow solid-phase techniques employing the standard TBTU/NMM procedure [11] with a fourfold excess of the amino acid in each coupling step. The assembled fragments were finally cleaved off by treatment for 2–3 h with 50% acetic acid, 45% DCM containing 5% methanol as scavenger. After filtration the solution of the protected fragment was diluted with hexane or heptane causing precipitation. Volatiles were then removed by rotary evaporation and hexane or heptane added once again. This procedure was repeated until acetic acid was no longer present in the product. The fact that hexane or heptane form an aceotropic mixture with acetic acid protects the fully protected fragment from contact with concentrated acetic acid which could lead to deprotection of the side chains. Alternatively, the fragment may be cleaved from the resin within 20 min with 20% hexafluoroisopropanol in DCM. Purification was performed with hexane and heptane as described above.

The results of the syntheses are shown in Table I. HPLC and IS-MS confirmed the identity and the high purity of the fragments obtained. During the synthesis no diketopiperazine formation was observed, as confirmed by the high yields of the fragment after cleavage from the resin.

**Esterification of the fragment Fmoc-90-99-OH**

The esterification was accomplished by a modified procedure reported by Riniker [3]. TBTA was added to a solution of 2% Fmoc-90-99-OH in DCE, to give a final concentration of 25% and this mixture heated to 60°C. Progress of the reaction was monitored by HPLC. After 6 h, 84% of the starting material was converted to its t-butylester form (Fig. 3). Prolonged reaction time resulted in the formation of more polar by-products, attributed to the cleavage of trityl protecting groups during the esterification process. For this reason we used the Tmob-protecting-group for Asn and Gln rather than the Trt-group.

The purification of the crude product was performed on a silicagel column (1.5 x 10 cm) with

![Fig. 3. Kinetics of the esterification of Fmoc-90-99-OH to the tert-butylester.](image)
Table II. Percentage of D-enantiomers incurred by esterification. Gln and Asn were hydrolized to Glu and Asp during the hydrolyzing procedure.

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Leu</th>
<th>Thr</th>
<th>Gln(Glu)</th>
<th>Asn(Asp)</th>
<th>Ile</th>
<th>Phe</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Enantiomers (%)</td>
<td>3.9</td>
<td>&lt;0.1</td>
<td>4.2</td>
<td>3.8</td>
<td>&lt;0.1</td>
<td>3.7</td>
</tr>
</tbody>
</table>

CHCl₃:methanol:ligroin:acetic acid (80:10:10:1) as eluent. The purified product was investigated by HPLC and IS-MS (Fig. 4 and 5).

Racemization of some amino acids was detected by GC on a Chirasil-Val capillary (Table II). The high temperature used for the reaction is most probably responsible for the elevated racemization. The aspect of racemization of the amino acids and in particular of the C-terminal amino acid has been rather neglected to date.

**Fmoc-deprotection from Fmoc-90-99-OrBu**

Originally Fmoc-deprotection was achieved by treating the protected fragment with 4% piperidine in THF/DMA (5:1) for 4 h according to Riniker et al. [3]. In order to accelerate the cleavage reaction we used 20% piperidine in DMF for the deprotection. The reaction was indeed much faster and was complete within 30 min according to TLC. After evaporation to dryness the residue was taken up in DCM/methanol (1:1) and applied to a Sephadex LH-20 column (Fig. 6). Identity of the product was confirmed by IS-MS (Fig. 7).

**Fig. 4.** HPLC of the purified fully protected peptide Fmoc-90-99-OrBu; puffer A=0.1% TFA in water, puffer B=0.08% TFA in CH₂CN; 10–80% B in 30 min, 100% B in 35 min, 100–10% B in 40 min, 220 nm.

**Fig. 6.** Gelchromatographic purification of fragment H-90-99-OrBu after Fmoc-deprotection.

**Fig. 5.** IS-MS of the purified fully protected peptide Fmoc-90-99-OrBu.

**Fig. 7.** IS-MS of the N-terminal deprotected fragment H-90-99-OrBu.
Coupling of fragment Fmoc-82-89-OH to H-90-99-OrBu

The C-terminus of the fragment Fmoc-82-89-OH, present in a 1.5fold excess, was activated with TBTU and NMM. After a preactivation time of about 15 min in DMF, the fragment H-90-99-OrBu was added and reaction monitored via RP-HPLC. After 2 h no further change in the composition of the coupling mixture could be detected according to HPLC. The yield of the protected 18mer was 70%. The mixture was separated on a Sephadex LH-20 column (Fig. 8) with DCM/MeOH as eluent.

According to IS-MS, the first fraction from the Sephadex column contains the nearly pure 18mer. The mass \( m/z = 1872 \) of the second fraction corresponds to the fragment Fmoc-82-89-OH. The third fraction shows this mass very clearly (Fig. 9).

In both the first and second fractions, ions of the mass \( m/z = 1924 \) are present. This arises from the acylated compound H-90-99-OrBu, as indicated by MS-MS investigations.

Purity control of the protected 18mer by RP-HPLC indicated the presence of two components of similar concentration. Better separation of these fully protected and thus non-polar peptides can be achieved in a much shorter time by means of normal phase HPLC on silicagel with CHCl₃:MeOH:HAc=50:3:0.5 (Fig. 10 and 11).

Preparative separation of the two substances was performed by silicagel chromatography using a solvent system consisting of CHCl₃:CH₃OH:acetic acid = 35:5:0.5. The separated substances have the same IS-MS, suggesting that they are diastereomers formed during the coupling reaction.
This is confirmed by chiral gas chromatography after hydrolysis and derivatisation of the two fractions collected. The proportion of the L-enantiomer of leucine in the two fractions was measured as 67% and 98% respectively. If we assume that only the C-terminal leucine of fragment Fmoc-82-89-OH underwent racemisation, then the first fraction must contain 100% D-leucine at the C-terminal position of this fragment while in the second peak, leucine is practically exclusively in the L-form.

**Fmoc-deprotection from Fmoc-82-99-OtBu**

Again the N-terminal deprotection was performed with 20% piperidine in DMF for 30 min. After separation of the Fmoc-piperidine adduct on a Sephadex LH-20 column, the main fraction was analysed by IS-MS (Fig. 12).

**Coupling of fragment Fmoc-74-81-OH to H-82-99-OtBu**

Coupling was performed analogous to the coupling of the first two fragments. After evaporation of the solvents DCM and DMF the residue was dissolved in a mixture of DCM and methanol and separated on a Sephadex LH-20 column. Special attention was paid to the first major peak which was examined by IS-MS (Fig. 13). The width of the peak group at m/z=(M+3H)^+ results from a combination of protonated Fmoc-74-99-OtBu together with its sodium and potassium adducts, rather than from impurities. Despite the full protection of the 26mer, 4-fold protonated molecular ions were found. This can only be explained by protonation of the amide nitrogens under the conditions employed in the mass spectrometric measurements.

**Conclusions**

Fragment condensation in solution has been employed to synthesize a fully protected 26mer as part of a protein. Further couplings were difficult to achieve because of the poor solubility of the 26mer. The concept of treating the 26mer as a polymer bound peptide, with subsequent fragment condensation to a suspension of the 26mer proved nonfeasible. This method suffers from the solvation of the growing peptide chain, a problem also encountered during fragment condensation on the solid phase or during normal solid phase peptide synthesis. Nevertheless this method can be recommended for the synthesis of small peptides of up to about 30 amino acids or more depending on the sequence.

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