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# Synthesis of α-fluoroalkyl substituted peptides via enzymatic fragment condensation

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**Abstract**

A peptide library consisting of di- and tripeptide esters and tripeptide amides, respectively, containing α-Tfm amino acids in different positions was synthesized and tested for enzymatic fragment condensations catalyzed by the proteases α-chymotrypsin, trypsin and clostripain. 

## 1. Introduction

Metabolic and conformational stabilization of pharmacologically active peptides can be achieved by incorporation of sterically hindered amino acids, e.g. $C^{\alpha,\alpha}$-disubstituted amino acids.[1–5] α-Trifluoromethyl substituted amino acids (α-Tfm amino acids, Scheme 1), a subclass of $C^{\alpha,\alpha}$-disubstituted amino acids, also fulfil these requirements while featuring additional properties based on the high electronegativity of fluorine. A trifluoromethyl group in the α-position of an amino acid exerts considerable polarization effects on the neighbouring substituents. This structural alteration influences the proteolytic stability of peptides containing Tfm amino acids, resulting in retarded degradation by peptidases[6,7] and, consequently, in prolonged intrinsic activity. Upon incorporation of α-Tfm amino acids, severe conformational restrictions are exerted on the peptide chain. Furthermore, due to the high electron density, the trifluoromethyl substituted peptides are capable of interacting with enzyme or receptor subsites in a manner impossible for the fluorine-free pendants. Therefore, α-Tfm amino acids are interesting building blocks for peptide synthesis.[8]

Chemical peptide syntheses, especially fragment condensations, often suffer from problems such as racemization and time consuming side-chain protection/deprotection strategies.[9] Therefore, enzymatic peptide coupling offers an exciting alternative to chemical methods for peptide synthesis. Proteolytic enzymes, which by definition catalyze the hydrolysis of peptide bonds, can effect high yielding peptide

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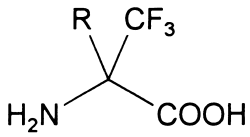


Scheme 1.

couplings under conditions which shift the equilibrium away from proteolysis.[10–12] This methodology combines the guarantee of a high stereo- and regiospecific racemization-free condensation under mild reaction conditions resulting in a high purity of the target peptide.

Dipeptide esters with N-terminal α-Tfm amino acid are accepted as substrates by subtilisin and α-chymotrypsin.[6,13] Using this strategy several tachykinin analogs containing α-Tfm amino acids have been synthesized by a combination of solution methods and enzymatic fragment condensation using α-chymotrypsin.[8,14] However, so far no systematic studies exist for enzymatic condensation of peptide fragments bearing α-Tfm amino acids in different positions. Therefore, the goal of this study was to investigate the utility of a broad spectrum of proteases for the incorporation of α-Tfm-substituted amino acids into longer peptide sequences via condensation of peptide fragments. Consequently, a peptide library of di- and tripeptide esters as acyl donors substituted by α-Tfm amino acids in $P_2$- and $P_3$-position (nomenclature according to Schechter and Berger[15]) and tripeptide amide acceptors containing α-Tfm amino acids in $P_2'$-position was synthesized. In the acyl donor esters, the $P_1$-position was occupied by Phe and Tyr according to the primary specificity of α-chymotrypsin for aromatic side-chains as well as by Lys for trypsin and clostripain which are specific for basic side-chains,[16,17] respectively.

## 2. Results and discussion

At first, the influence of the α-Tfm functionality within the carboxy component on enzymatic catalysis was investigated by peptide fragment condensations. In the case of α-chymotrypsin, reactions of both in $P_2$- and $P_3$-position α-Tfm-substituted di- and tripeptide acyl donor esters, respectively, were performed using specific Lys- and Arg-containing nucleophilic pentapeptides. The results found for $P_3$-position α-Tfm-containing derivatives H-TfmXaa-Leu-Phe-OMe (Xaa=Leu, Phe) show that both diastereomers of each acyl donor were coupled efficiently (Table 1). As indicated by the similar high yields, neither the different side chains and configuration nor the α-Tfm substitution itself affect the yield determining deacylation of the acylenzyme by the pentapeptides. As shown in Table 2 a comparable situation for the $P_2$ α-Tfm-containing acyl donor H-TfmLeu-Tyr-OMe seems probable, too. In contrast to α-chymotrypsin, catalysis by clostripain and trypsin appears to be more affected by the α-Tfm substitution in $P_2$-position (Table 3). Within trypsin-mediated reactions the strongest effects were observed for the diastereomers of H-TfmPhe-Lys-OMe. In this case the α-Tfm group causes generally lower yields. Additionally, the *S*-configuration of the α-Tfm amino acid results in a further decrease. Remarkably, for the structurally similar diastereomers of H-TfmPhg-Lys-OMe, high yields were obtained indicating that only a weak influence of the α-Tfm amino acid on the enzymatic coupling reaction occurs. This result suggests a dependence of the α-Tfm effect both on the individual side chain and configuration of the α-Tfm-bearing amino acid residue. Analysing the data found for clostripain, the influence of the individual side chain of the α-Tfm-substituted amino acid seems to be lower. Nevertheless, clostripain also discriminates between the diastereomers of both acyl donors to a certain extent, indicated by the variation in product yields. However, in contrast to trypsin, clostripain catalyzes the coupling of the all-*S*-diastereomer of H-TfmPhe-Lys-OMe more effectively than of the *R*,*S*-derivative.

Table 1
Yields of α-chymotrypsin-catalyzed peptide syntheses using $P_3$ α-Tfm-substituted carboxy components[a]

| Acyl donor | Yield [%] | | |
|---|---|---|---|
| | Acyl acceptor | | |
| | RAAAG | KAAAG | AARAG |
| H-TfmLeu-Leu-Phe-OMe (DI) | 81.9 | 77.4 | 71.3 |
| H-TfmLeu-Leu-Phe-OMe (DII) | 93.2 | 80.1 | 73.6 |
| H-TfmPhe-Leu-Phe-OMe (DI) | 84.5 | 79.4 | 79.1 |
| H-TfmPhe-Leu-Phe-OMe (DII) | 75.8 | 72.3 | 66.0 |

[a] Conditions: 0.1M $Na_2CO_3$/$NaHCO_3$ buffer, pH 9.0, 0.2M NaCl, 20% DMSO, [acyl donor]: 2mM, [acyl acceptor]: 10mM, [α-chymotrypsin]: $2.0 \times 10^{-7}$ M.

Table 2
Yields of α-chymotrypsin-catalyzed peptide syntheses using $P_2$ α-Tfm-substituted carboxy components[a]

| Acyl donor | Yield [%] | | |
|---|---|---|---|
| | Acyl acceptor | | |
| | RAAAG | KAAAG | AARAG |
| H-TfmLeu-Tyr-OMe (DI; S,S) | 86.6 | 61.9 | 80.9 |
| H-TfmLeu-Tyr-OMe (DII; R,S) | 86.5 | 75.2 | 78.4 |

[a] Conditions: 0.1M $Na_2CO_3$/$NaHCO_3$ buffer, pH 9.0, 0.2M NaCl, 20% DMSO, [acyl donor]: 2mM, [acyl acceptor]: 10mM, [α-chymotrypsin]: $6.7 \times 10^{-7}$ M.

Table 3
Yields of trypsin- and clostripain-catalyzed peptide syntheses using $P_2$ α-Tfm-substituted carboxy components[a]

| Acyl donor | Yield [%] | | | |
|---|---|---|---|---|
| | Acyl acceptor | | | |
| | Trypsin | | Clostripain | |
| | LAAAG | MAAAG | LAAAG | MAAAG |
| H-TfmPhe-Lys-OMe (DI; R,S) | 38.3 | 60.9 | 47.1 | 27.6 |
| H-TfmPhe-Lys-OMe (DII; S,S) | 7.60 | 22.3 | 57.4 | 41.8 |
| H-TfmPhg-Lys-OMe (DI) | 69.0 | 87.5 | 74.6 | 65.2 |
| H-TfmPhg-Lys-OMe (DII) | 80.9 | 95.5 | 59.1 | 43.5 |

[a] Conditions: 0.1M $Na_2CO_3$/$NaHCO_3$ buffer, pH 9.0, 0.2M NaCl, 10% MeOH, [acyl donor]: 2mM, [acyl acceptor]: 10mM, [trypsin]: $1.7 \times 10^{-7}$ M, [clostripain] $3.7 \times 10^{-6}$M.

In order to investigate the influence of the α-Tfm functionality within the amino component on enzymatic C–N couplings, model reactions using H-TfmAla-$NH_2$ and H-Xaa-TfmAla-Ala-$NH_2$ (Xaa=Ala, Lys) with unmodified acyl donors have been performed. The results found for the individual enzymes are summarized in Table 4. As shown by these data none of the enzymes used are capable of accepting H–TfmAla–$NH_2$ as an amino component. This is in agreement with the failed acceptance of various proteases for the $C^{\alpha,\alpha}$-dialkylated analog H–Aib–$NH_2$.[18] The shift of the α-Tfm amino acid into the $P_2'$-position enables clostripain to catalyze the formation of peptide bonds. As indicated by the comparative yields, the discrimination of clostripain between the various diastereomers seems to be only low, a fact which was already found for peptides containing coded amino acids.[19,20] In contrast, neither α-chymotrypsin nor trypsin catalyses the coupling of $P_2'$ α-Tfm-substituted amino components. This lack of acceptance may be the result of disturbing effects of the α-Tfm group on the interaction between the enzyme and the backbone of the amino component which is one of the most important contact domains of both enzymes with the nucleophile.[21,22] Interestingly, even the presence of highly S′ subsite specific amino acids within the amino component; e.g. Lys in $P_1'$-position in the case of α-chymotrypsin,[23] cannot overcome these unfavourable α-Tfm effect. Obviously, clostripain seems not to form such highly important contacts to the backbone of amino components illustrated by the acceptance of $P_2'$ α-Tfm-containing nucleophiles.

Therefore, only clostripain may be a suitable biocatalyst for coupling both α-Tfm-containing carboxy and amino components. In order to verify this assumption, model reactions using the already mentioned $P_2'$ α-Tfm-modified acyl donors and the always higher specific diastereomers of Xaa-TfmAla-Ala-$NH_2$ (Xaa=Ala, Lys) were performed. As shown by the results listed in Table 5, in all reactions clostripain mediates the formation of the appropriate peptide product as predicted by the single experiments. Analyzing the efficiency of catalysis, the highest yields were found for reactions using H-TfmPhg-Lys-OMe (DII) as acyl donors, which were even slightly higher than those found for the standard acyl donor Z-Arg-OMe (Table 4). In all other cases somewhat lower yields were obtained indicating a rather unfavourable influence of the $P_2'$ α-Tfm modification on the product yield. Assuming this, the shift of the α-Tfm group from the $P_2'$- to the $P_3'$-position might decrease this effect resulting in generally higher efficiencies of the enzymatic reactions.

Table 4
Coupling of unmodified acyl donors with α-Tfm-substituted amino components by clostripain, trypsin, and α-chymotrypsin[a]

| Acyl acceptor | Yield [%] | | |
|---|---|---|---|
| | Enzyme | | |
| | Clostripain [b] | Trypsin [c] | α-Chymotrypsin [d] |
| H-TfmAla-$NH_2$ (DI; DII) | n.s. | n.s. | n.s. |
| H-Ala-TfmAla-Ala-$NH_2$ (DI) | 42.4 | n.s. | n.s. |
| H-Ala-TfmAla-Ala-$NH_2$ (DII) | 58.3 | n.s. | n.s. |
| H-Lys-TfmAla-Ala-$NH_2$ (DI) | 32.7 | n.s. | n.s. |
| H-Lys-TfmAla-Ala-$NH_2$ (DII) | 50.5 | n.s. | n.s. |

[a] Conditions: 0.1M $Na_2CO_3$/$NaHCO_3$ buffer, pH 9.0, 0.2M NaCl, 20% MeOH, [acyl donor]: 2mM, [acyl acceptor]: 10mM. [b] acyl donor: Z-Arg-OMe, [clostripain]: $1.0\times10^{-7}$M. [c] acyl donor: Z-Arg-OMe, [trypsin]: $1.25\times10^{-7}$M. [d] acyl donor: Mal-Tyr-OMe, [α-chymotrypsin]: $1.8\times10^{-7}$M. n.s.: no synthesis.

Table 5
Yields of clostripain-catalyzed coupling of α-Tfm-containing carboxy and amino components[a]

| Acyl donor | Yield [%] | |
|---|---|---|
| | Acyl acceptor | |
| | H-Ala-TfmAla-Ala-$NH_2$ (DII) | H-Lys-TfmAla-Ala-$NH_2$ (DII) |
| H-TfmPhe-Lys-OMe (DI; R,S) | 25.4 | 34.7 |
| H-TfmPhe-Lys-OMe (DII; S,S) | 39.7 | 38.8 |
| H-TfmPhg-Lys-OMe (DI) | 33.2 | 28.0 |
| H-TfmPhg-Lys-OMe (DII) | 61.9 | 68.8 |

[a] Conditions: 0.1M $Na_2CO_3$/$NaHCO_3$ buffer, pH 9.0, 0.2M NaCl, 30% MeOH, [acyl donor]: 2mM, [acyl acceptor]: 10mM, [clostripain] $3.7x10^{-6}$M.

## 3. Conclusion

The advantages of proteases as catalysts for highly selective peptide bond formations are strictly dependent on specific substrate–enzyme interactions. However, our results show that in addition to peptides containing exclusively proteinogenic amino acid residues, which usually fulfil these requirements, peptide fragments with non-natural α-Tfm amino acids were also accepted. In this context, only the coupling of α-Tfm-containing carboxy components appear to be less restricted. Generally, all proteases used were capable of reacting with $P_3$ and $P_2$ α-Tfm-modified acyl donors forming the appropriate peptide bond to model amino components. Besides that, reactions catalyzed by α-chymotrypsin were affected only insignificantly, whereas the higher specific proteases clostripain and especially trypsin show a somewhat more sensitive behaviour. In contrast, the coupling of $P_1'$ and $P_2'$ α-Tfm-substituted amino components is more problematic and depends decisively on the enzyme used. While the presence of the α-Tfm group at these positions causes general loss of acceptance by α-chymotrypsin and trypsin, $P_2'$ α-Tfm-containing amino components were successfully coupled by clostripain. As a result, clostripain can be used as the biocatalyst for coupling peptide fragments containing α-Tfm amino acids in the carboxy as well as in the amino moiety. Using this catalytic potential the limited spectrum of suitable methods for incorporating α-Tfm-containing fragments into peptides can be considerably expanded.

## 4. Experimental

### 4.1. Materials

Amino acid derivatives, *N*,*N*′-diisopropylcarbodiimide (DIC), *N*-methylmorpholine (NMM), isobutylchloroformate, palladium on charcoal (Pd/C) were purchased from commercial suppliers. All reagents were of the highest available purity. α-Tfm amino acids and their protected derivatives were obtained as racemates via known procedures.[24,25] Solvents were purified and dried by the usual methods. Flash chromatography was performed using silica gel (0.032–0.064 μm) from Riedel de Haën (Germany) with $CHCl_3$:methanol and ethyl acetate:hexanes as eluent, respectively. Mass spectra were recorded using thermospray ionization with a Hewlett Packard 5989 A instrument.

TLCK ($N^{\alpha}$-*p*-tosyl-L-lysine chloromethyl ketone) treated bovine α-chymotrypsin (EC 3.4.21.1) and TPCK ($N^{\alpha}$-*p*-tosyl-L-phenylalanine chloromethyl ketone) treated bovine trypsin (EC 3.4.21.4) were

products of Fluka Chemie AG (Switzerland) and Sigma (Germany), respectively, and were used without further purification. Clostripain (EC 3.4.22.8) was obtained from Hoechst AG (Germany) and purified and characterized as described previously.[26] Its activity was checked spectrophotometrically on a Shimadzu UV-160A spectrophotometer (Japan). The enzyme was activated before any applications for 2–3 h in the presence of 1.0 mM $CaCl_2$ containing 2.5 mM DL-dithiothreitol at a specific activity of 400 nkat/ml.

### 4.2. Chemical syntheses

Mal–Tyr–OMe (Mal:maleyl) were synthesized by maleylation of H–Tyr–OMe according to standard methods.[27] The unmodified pentapeptides were synthesized with a semiautomatic batch peptide synthesizer SP 650 (Labortech AG, Switzerland) using *p*-alkoxybenzylalcohol resin, prepared according to Wang[28] and standard Fmoc chemistry. The peptides were precipitated with dry diethyl-, diisopropylether or mixtures of hexane:ethyl acetate. The syntheses of the α-Tfm-substituted model peptides were carried out in solution using standard procedures starting from the C-terminus.[29] The activation of the Z-protected natural amino acids was achieved via mixed anhydrides while in the case of the Z-protected Aib and α-Tfm amino acids the activation via DIC proved to be the more favourable method.[30] The Z-protecting group of all intermediates was cleaved by hydrogenolysis. The purification of all protected intermediates and of the final model peptide fragments was carried out by flash chromatography and recrystallization. Generally, the diastereomer of the α-Tfm-substituted model peptides first eluated was denominated as 'DI' and the second eluated peptide as 'DII', respectively. The identity and purity of all peptides and acyl donor esters were characterized by analytical HPLC, NMR, thermospray mass spectroscopy and elementary analysis. In all cases satisfactory analytical data (±0.4% for C, H, N) were found.

### 4.3. Enzymatic syntheses

The reactions were performed at 25°C in a total volume of 100 μl 0.1 M $Na_2CO_3$:$NaHCO_3$ buffer, pH 9.0, 0.2 M NaCl, containing 10 or 20% dimethyl sulfoxide or MeOH. Stock solutions of α-Tfm-modified acyl donors (4 mM) were prepared in distilled water containing 20% MeOH in the case of $P_1$ Lys-esters and 40% dimethyl sulfoxide for $P_1$ Phe/Tyr-esters. Stock solutions of Z–Arg–OMe and Mal–Tyr–OMe (4 mM) did not contain any organic solvents. Stock solutions of α-Tfm-modified acyl acceptors (20 mM) were prepared in 0.5 M $Na_2CO_3$:$NaHCO_3$ buffer, pH 9.0, 0.5 M NaCl containing 40% MeOH. After thermal equilibration of assay mixtures the reactions were initiated by addition of 9 μl of the respective enzyme stock solution. Reaction times of 3–60 min led to an ester consumption of approximately 90%. For the HPLC analysis, aliquots of 80 μl were withdrawn and diluted with a stop solution of 50% aqueous methanol containing 8% trifluoroacetic acid. The product yields were determined from three independent experiments for each reaction. In the same way for each acyl donor and acceptor an experiment without enzyme was carried out for determining the extent of nonenzymatic ester hydrolysis which was strictly less than 5%. Based on these control experiments nonenzymatic aminolysis could be ruled out. The identity of the formed peptide products was established by MALDI–ToF mass spectroscopy.

### 4.4. HPLC analyses

Samples were analyzed by analytical reversed phase HPLC using a SpectraSystem P 2000 system (ThermoSeparationProducts, USA) on C18 polymer coated columns (Vydac 218TP54, 5 μm, 300 Å,

[25x0.4 cm] The Separations Group, USA and Grom Capcell, 5 μm, 300 Å, [25x0.4 cm] Shiseido, Japan) which were thermostated at 25°C and eluted with various mixtures of water:acetonitrile containing 0.1% trifluoroacetic acid under gradient conditions. The measurements were detected at 254 nm (Phe- and Z-group-containing derivatives) and 280 nm (Mal-Tyr-OMe). The chromatograms were analyzed using the software Hyperdata Chromsoft (Vers. 2.15, Autochrom Inc., USA).

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