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Discovery of carboxypeptidase Y as a catalyst for the incorporation of sterically demanding α-fluoroalkyl amino acids into peptides

Sven Thust and Beate Koksch*

Department of Organic Chemistry, Faculty of Chemistry and Mineralogy, University of Leipzig, Johannisallee 29, D-04103 Leipzig, Germany

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Dedicated to Prof. Dr. H.-D. Jakubke on the occasion of his 70th birthday

Abstract—The first example of a direct enzymatic coupling of two different, sterically demanding C^{α}-fluoroalkyl amino acids to amino acid nucleophiles is reported. N-Protected Ala methyl ester derivatives bearing a methyl-, difluoromethyl-, or trifluoromethyl group, respectively, instead of the α-proton were accepted as substrates by carboxypeptidase Y and could, therefore, be coupled directly to various nucleophiles.

Effective site-specific incorporation of a wide variety of non-natural amino acids into peptides and proteins remains a topic of high interest. Highly functionalized amino acid residues can serve as valuable tools to be used as biophysical probes for investigation of structure–function relationships or for the construction of tailor-made biomolecules. Incorporation of fluorine usually influences the physical properties of amino acids and proteins dramatically.[1–3] Increased resistance toward proteolysis as well as the stabilization of secondary structures are mentioned here amongst others.[4] Moreover, strategic placement of fluoroalkyl groups within peptides or proteins, respectively, provides the opportunity for studying conformational properties, peptide/protein-membrane interactions, or metabolic processes by ^{19}F NMR.[5,6]

In our attempt to provide a broad variety of methods for the fast and simple incorporation of fluorinated amino acids into peptides, a commercially available protease has been found that, for the first time, makes possible the direct enzymatic coupling of the sterically demanding α-fluoroalkyl alanine derivatives.

Enzymatic peptide coupling represents an attractive alternative to chemical peptide synthesis methods as the latter often suffer from problems such as racemization and expensive, time consuming side-chain protection/deprotection strategies. We could already show the applicability of several proteases for the synthesis of peptides containing α-fluoroalkyl amino acids as long as the α-fluoroalkyl amino acids do not appear in positions P_1 and P'_1 (notation according to Schechter and Berger[7]).[8–11] Recently, we succeeded in incorporating α-methyl as well as α-fluoroalkyl amino acids into the P_1 position of peptides applying the substrate mimetic concept.[12] However, direct enzymatic coupling of α-fluoroalkyl amino acids has failed so far.

Carboxypeptidase Y (CPY) is known to possess high catalytic activity and enantioselectivity for kinetic resolutions of many α-tertiary substituted carboxylic acid esters.[13] In contrast to the above mentioned proteases, CPY has been shown to be provided with a broad S_1 and S'_1 specificity while hydrophobic enzyme–substrate interactions are of high importance.[14–17]

Protease catalyzed peptide synthesis can be considered only on condition that substrate esters are hydrolyzed by the protease within a reasonable period of time. Therefore, several N-protected alanine methyl esters bearing a methyl- (Me), difluoromethyl- (Dfm), or trifluoromethyl- (Tfm) group, respectively, instead of the α-proton were incubated with CPY. α-Amino isobutyric

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* Corresponding author. Tel.: +49-341-9736532; fax: +49-341-9736-599; e-mail: koksch@chemie.uni-leipzig.de

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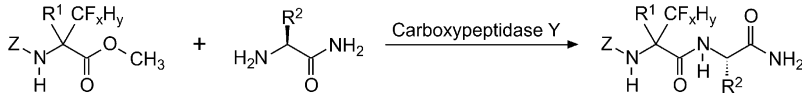

Scheme 1. General model of the CPY-catalyzed peptide synthesis using C^{α}-alkylated alanine methyl ester and different nucleophilic amino components. R^1: CH_3; R^2: H, CH_3, $CH(CH_3)_2$, $CH_2CH(CH_3)_2$; x: 2, 3; y: 0, 1, 3.

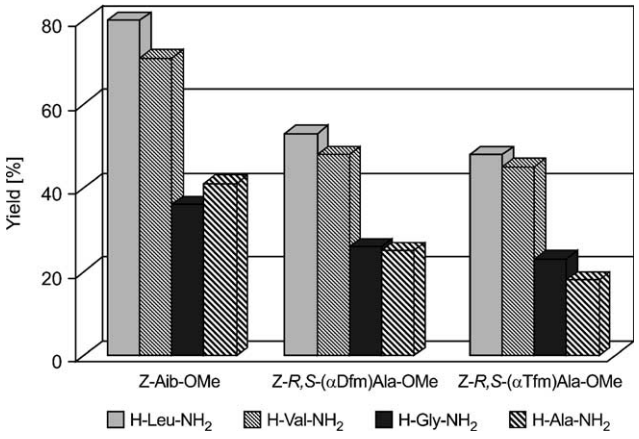

Figure 1. Carboxypeptidase Y catalyzed peptide synthesis of and different amino acid amides. Reagents and conditions: 0.1 M HEPES buffer (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid), 0.2 M NaCl, 0.02 M $CaCl_2$, 20% DMSO, pH 8.0, 37 °C; [acyl donor]: 4 mM, [acyl acceptor]: 40 mM, [CPY]: $1.6–3.2\times10^{-6}$ M, all errors are less than 5%.

acid (Aib) was included in this study as a point of reference to be able to distinguish between electronic effects of the fluorinated group and steric demands of a second α-alkyl substituent. All derivatives were hydrolyzed by CPY within short reaction times to give the free N-protected amino acids.

Based on these results, the suitability of the C^{α}-fluoroalkyl alanine methyl ester to serve as acyl donors for CPY-catalyzed peptide bond formation was studied. Acyl acceptors were chosen following the known P_1' specificity of CPY for hydrophobic amino acid amides.[18] Therefore, H-Leu-NH_2, H-Val-NH_2, H-Ala-NH_2, and H-Gly-NH_2, were used as nucleophilic components (Scheme 1). The results are summarized in Figure 1. CPY catalyzes the acyl transfer to all of the four amino acid amides to give the desired N-protected dipeptide amides. However, the extent of peptide bond formation was considerably influenced by the second substituent at the C^{α}-atom. In general, CPY was found to be considerably more efficient in catalyzing peptide bond formation in the case of the Aib substrate. Highest peptide yields (80% and 70%, respectively) were obtained for reactions using nucleophiles with bulky residues (Leu and Val, respectively). The efficiency of acyl transfer was substantially diminished in the case of H-Ala-NH_2 (40%) and even lower for H-Gly-NH_2 (35%). This result is in agreement with the known specificity of the protease for hydrophobic residues within both binding sites.[14–17] The same tendency was observed for both of the corresponding C^{α}-fluoroalkyl amino acid esters. Here, the highest peptide yields (50%) could be obtained for reaction of Z-*R*,*S*-(αDfm)Ala-OMe with H-Leu-NH_2 and reaction of Z-*R*,*S*-(αTfm)Ala-OMe with H-Leu-NH_2 (45%), respectively.

For the interpretation of the differences in product yields between the Aib substrate and both of the α-fluoroalkyl substituted amino acid esters, the use of racemic mixtures in the case of the latter ones has to be taken into account. Therefore, the influence of the absolute configuration of the α-fluoroalkyl amino acid on the efficiency of peptide bond formation by CPY was studied for the Tfm derivative in more detail. Separation of the diastereomeric products of the enzymatic reaction would have been the easiest way to do so. Unfortunately, separation of the diastereomeric product mixtures by HPLC failed. Therefore, the dipeptide methyl ester Z-Phe-*R*,*S*-(αTfm)Ala-OMe was chemically synthesized from Z-Phe-OH and racemic H-(αTfm)Ala-OMe. Diastereomers of this dipeptide derivative could be separated by flash chromatography (diastereomer I was identified as the product, which was eluted first from the column, diastereomer II was eluted as the second product). Both diastereomeric dipeptide esters were reacted separately with all four nucleophilic amino acid amides under CPY catalysis to give eight different N-protected tripeptide amides. As an example, both diastereomers of the tripeptide Z-Phe-(αTfm)Ala-Ala-NH_2 were synthesized in semi-preparative scale to enable ^1H as well as ^{19}F NMR characterization. The absolute configuration of (αTfm)Ala within this tripeptide sequence could be assigned based on ^{19}F NMR analysis in combination with an earlier published X-ray structure analyses.[9] According to these data, diastereomer I of the tripeptide Z-Phe-(αTfm)Ala-Ala-NH_2 refers to the *all-S* form while diastereomer II bears the *R*-enantiomer of (αTfm)Ala.

Comparison of product yields show that, in general, CPY accepts both of the diastereomeric dipeptides while certainly always preferring the *S*-configured (αTfm)Ala in P_1 position (Fig. 2). The strongest discrimination between both diastereomeric substrates was observed for the synthesis of Z-Phe-(αTfm)Ala-Ala-NH_2. Reaction of the diastereomeric peptide containing the *S*-enantiomer of the fluorinated amino acid in P_1 position gave 55% yield while product formation dropped to 20% when *S*-(αTfm)Ala was replaced by the *R*-enantiomer. Therefore, product formation increased up to 40% by applying the pure diastereomer of Z-Phe-*S*-(αTfm)Ala-OMe and is now even slightly higher than in the case of the corresponding Aib peptide. The highest peptide yields out of the eight reactions could be obtained for the synthesis of Z-Phe-*S*-(αTfm)Ala-Leu-NH_2 (75%). Interestingly, peptide yields for reactions of Z-Phe-*S*-(αTfm)Ala-OMe with H-Gly-NH_2 and H-Ala-

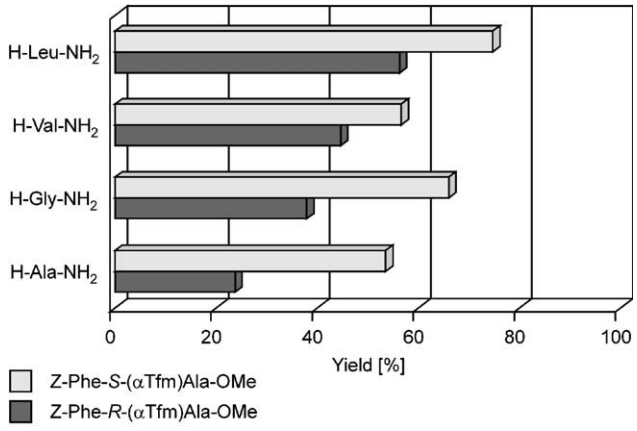

Figure 2. Influence of the absolute configuration of (αTfm)Ala on the efficiency of CPY-catalyzed peptide synthesis. Reagents and conditions: 0.1 M HEPES buffer, 0.2 M NaCl, 0.02 M $CaCl_2$, 40% DMSO, pH 8.0, 37 °C; [acyl donor]: 4 mM, [acyl acceptor]: 40 mM, [CPY]: 3.0×10^{-7}–1.6×10^{-6} M, all errors are less than 5%.

NH_2, respectively, are much higher now than observed when reacting each of the nucleophiles with Z-Aib-OMe (Fig. 1). These results imply that substitution of a methyl group for a Tfm group at the C^{α}-atom of Aib can result, depending on the absolute configuration, in an improved binding of the substrate within the active site of CPY. Remarkably, in the case of the α-Tfm analogues, reaction of the diastereomer possessing the fluorinated, bulkier side chain in the same position as it would be for the side chain in the case of a natural amino acid results in a lower peptide yield. Surprisingly, CPY accepts the bulky Tfm group within the binding site for the α-proton. The assumption can be made that the α-Dfm substituted Ala derivative will show a similar reaction behavior, however, this has to be further investigated.

In conclusion, we present here the first example of a direct enzymatic coupling of two different, sterically demanding C^{α}-fluoroalkyl amino acids to amino acid nucleophiles. N-Protected Ala methyl ester derivatives bearing a methyl-, difluoromethyl-, or trifluoromethyl group, respectively, instead of the α-proton were accepted as substrates by CPY and could, therefore, be coupled directly to various nucleophiles. Peptide yields between 20% and 75% were obtained depending on the nucleophilic amino acid derivative and the enantiomer of the fluoroalkyl amino acid. To our knowledge, this is the first time that sterically demanding C^{α}-dialkylated amino acids have been coupled as substrates directly with amino acid nucleophiles without any further activation of the electrophilic substrate and without any medium or enzyme engineering. The synthetic strategy introduced here, extends the scope of methods available for site-specific peptide and protein modification by fluorinated amino acids using a simple and environmentally attractive route. In this study, amino acid amides were used as nucleophiles. The application of amino acids[19] as nucleophiles will open up the possibility of incorporating a variety of fluorinated residues into biologically relevant peptides by enzymatic fragment condensation.

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