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Protease-Catalyzed Peptide Synthesis for the Site-Specific Incorporation of α-Fluoroalkyl Amino Acids into Peptides†

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Received September 18, 2002

Substitution of native amino acids by fluoroalkyl analogues represents a new approach for the design of biologically active peptides with increased metabolic stability as well as defined secondary structure and provides a powerful label for spectroscopic investigations. Here, we introduce a methodology for the incorporation of sterically demanding C^{α}-fluoroalkyl amino acids into the P_1 position of peptides catalyzed by the commercially available proteases trypsin and α-chymotrypsin. The combination of 4-guanidinophenyl ester of C^{α}-fluoroalkyl amino acids as substrate mimetics with frozen-state reaction conditions provided the most efficient strategy for protease-catalyzed site-specific introduction of this kind of nonnatural amino acids into peptide sequences. Consequently, a library of di-, tri-, and tetrapeptides containing α-methyl, α-difluoromethyl, and α-trifluoromethyl alanine, leucine, and phenylalanine in the P_1 position was synthesized catalyzed by trypsin as well as α-chymotrypsin. Trypsin was shown to be the more versatile protease.

Introduction

Extending the spectra of building blocks that can be used for protein engineering beyond the natural amino acids broadens the scope of proteins to areas such as materials science.[1] Strategic placement of highly functionalized nonnatural amino acid residues or biophysical probes, respectively, expands the repertoire of protein functions and facilitates detailed physical studies on this important class of organic compounds. Many functional groups such as, e.g., halides are rarely found in the natural amino acid pool. Especially, the incorporation of fluorine can have dramatic effects on protein stability, protein−protein interactions, and the physical properties of protein-based materials.[2] As a result of the unique electronic properties of fluorine, α-fluoroalkyl amino acids constitute an exciting class of $C^{\alpha,\alpha}$-disubstituted amino acids.[3] Furthermore, fluorine-containing peptides provide the opportunity of studying their conformational properties and of elucidating metabolic processes by ^{19}F NMR.[4] Therefore, incorporation of fluoroalkyl amino acids is a new approach to the design of biologically active peptides with increased metabolic stability [5] as well as defined secondary structure and provides a powerful label for spectroscopic investigations.

Several peptide and protein synthesis methods have been developed for the site-specific introduction of nonnatural amino acids into protein sequences. Stepwise solid-phase peptide synthesis is largely limited to a number of 50−60 couplings as resin-bound byproducts accumulate over time and the solubility decreases. The classic solution chemistry approach, i.e., the chemical condensation of fully protected peptide fragments in organic solvents, bears the risk of racemization.[6] Native chemical ligation has advanced as a technology for the synthesis of large polypeptides but is restricted to the coupling of very few amino acids because of its mechanism or provides a nonnatural functionality at the coupling site.[7] Protein semisynthesis, in which a synthetic peptide is ligated to a protein fragment to produce a full-length protein, has been used to incorporate nonnatural amino acids into proteins as well.[8] In vivo translation methods have been successfully applied for the site-specific introduction of nonnatural amino acids into a protein sequence, but these methods are still technically daunting.[9] As an additional strategy, enzymes

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† Dedicated to Prof. P. Welzel on the occasion of his 65th birthday.

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10.1021/jo020613p CCC: $25.00
Published on Web 02/27/2003

have been specifically engineered to catalyze the ligation of unprotected peptide fragments for the total chemical synthesis of proteins.[10]

The combination of chemical synthetic methods with enzymatic peptide bond formation using proteases has received increased attention as an attractive alternative to classical peptide chemistry since enzymes work generally racemization free, highly regio- and stereoselectively, under mild reaction conditions and require only minimal side chain protection.[11] However, there are some disadvantages to this approach. There is a permanent risk of undesired proteolytic side reactions due to the native amidase activity of proteases. Furthermore, the substrate specificity of available proteases restricts the number of residues between which a peptide bond can be synthesized. Recently, a powerful concept was established that overcomes these limitations of the classical enzymatic approach. The application of substrate mimetics allows the use of even noncoded amino acids as substrates. This concept first reported for trypsin-catalyzed peptide synthesis is based on the binding site specific 4-guanidinophenyl ester (OGp) functionality to mediate acceptance of nonspecific amino acid moieties in the specificity-determining S_1 position of the enzyme (notation according to Schechter and Berger [12]).[13] Other Arg-specific proteases, e.g., thrombin and clostripain, and the Phe-specific protease α-chymotrypsin react similarly with OGp esters.[14] This method, in combination with SPPS, has been successfully applied already for protease-catalyzed fragment condensation of larger peptide sequences.[15]

While di- and tripeptide methyl esters containing *N*-terminal α-trifluoromethyl amino acids (αTfm amino acids) are accepted as substrates by subtilisin, α-chymotrypsin, trypsin, and clostripain,[16] direct enzymatic coupling of α-fluoroalkyl amino acids has been unsuccessful. Even in the case of Z-(αTfm)Gly-OMe, which was shown to be a very specific substrate for subtilisin, protease-catalyzed peptide synthesis failed.[17] Applying the advantages of the substrate mimetic concept to fluoroalkyl amino acids, we have finally succeeded in incorporating these sterically demanding $C^{\alpha,\alpha}$-dialkyl amino acids into the P_1 position of peptides enzymatically. Although the serine proteases trypsin and α-chymotrypsin had previously been successfully applied for enzymatic coupling of 4-guanidinophenyl esters of unspecific as well as noncoded amino acids,[13d,14b] to the best of our knowledge nothing was known about how much the nature of the second substituent at the α-carbon atom influences individual enzyme−substrate interactions and, thus, the catalytic efficiency of these proteases. These effects are discussed here. α-Methyl amino acids were included in this study in order to distinguish between electronic effects of the fluorinated group and the steric demands of an alkyl substituent.

Results and Discussion

Substrate Mimetic Synthesis. Several α-difluoromethyl (αDfm), αTfm, and α-methyl (αMe) substituted amino acids were esterified as previously described with 4-[*N*′,*N*″-bis(*tert*-butyloxycarbonyl)guanidino]phenol using TBTU as coupling reagent.[18] Boc-protected 4-guanidinophenyl esters of Z-Aib-OH, Z-D,L-(αDfm)Ala-OH, and Z-D,L-(αTfm)Ala-OH were successfully synthesized in good yields (Scheme 1). In contrast to the Ala derivatives, activation of the carboxyl group of C^{α}-fluoroalkyl Leu and Phe by TBTU results in the formation of stable oxazolones while the formation of 4-guanidinophenyl esters is not observed. The sterically highly demanding situation at the α-carbon atom induced by the isopropyl and benzyl residue, respectively, in combination with the fluoroalkyl substituent induces this unwanted site reaction. To prevent the oxazolone formation, C^{α}-fluoroalkyl Leu and Phe derivatives were activated using DIC/HOAt since with this coupling reagent an active ester intermediate is formed.[19] Additionally, the nucleophilicity of the hydroxyl group of 4-[*N*′,*N*″-bis(*tert*-butyloxycarbonyl)guanidino]phenol had to be increased by reaction with *n*-butyllithium to provide the lithium salt of the phenol. Via this strategy, Z-L-(αMe)Phe-OGp, Z-D-(αMe)Phe-OGp, Z-D,L-(αDfm)Leu-OGp, and Z-D,L-(αTfm)Leu-OGp, Z-D,L-(αDfm)Phe-OGp, and Z-D,L-(αTfm)Phe-OGp were obtained in very good yields. Finally, deprotection of the guanidino funtionality can be achieved by treatment with TFA in combination with ultrasound. No hydrolysis of the substrate mimetics was observed even under these drastic reaction conditions (Scheme 1).

Trypsin-Catalyzed Peptide Bond Formation. Before coupling C^{α}-fluoroalkyl and C^{α}-methyl amino acids to several amino acid amides as well as small peptide sequences in the presence of trypsin, we proved that the new substrate mimetics Z-Aib-OGp, Z-D,L-(αDfm)Ala-OGp, Z-D,L-(αTfm)Ala-OGp, Z-L-(αMe)Phe-OGp, Z-D-(αMe)Phe-OGp, Z-D,L-(αDfm)Phe-OGp, Z-D,L-(αTfm)Phe-OGp, Z-D,L-(αDfm)Leu-OGp, and Z-D,L-(αTfm)Phe-OGp were accepted by this protease. All 4-guanidinophenyl

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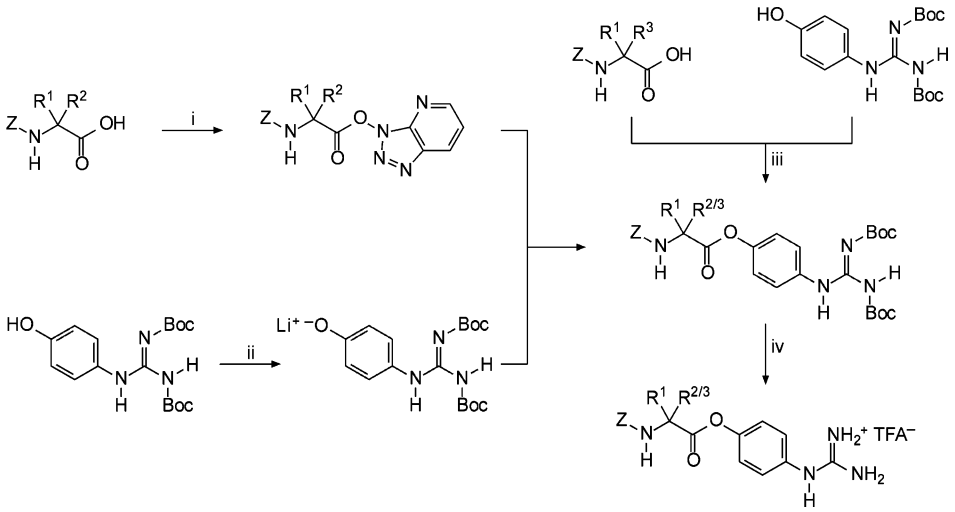
SCHEME 1. Synthesis of $C^{\alpha,\alpha}$-Dialkyl Amino Acid-4-guanidinophenyl Esters[a]



[a] R^1: CH_3, CF_2H, CF_3; R^2: $CH_2C_6H_5$, $CH_2CH(CH_3)_2$; R^3: CH_3. (i) DIC, HOAt, THF; (ii) *n*-butyllithium, THF; (iii) TBTU, DIEA, DMF; (iv) TFA, ultrasound.

TABLE 1. Yields (%) of Trypsin-Catalyzed Peptide Synthesis Using Substrate Mimetics of $C^{\alpha,\alpha}$-Dialkylated Amino Acids[a]

	acyl donor								
	Z-$X_{C\alpha}$Ala-OGp, $X_{C\alpha}$ =			Z-$X_{C\alpha}$Phe-OGp, $X_{C\alpha}$ =				Z-$X_{C\alpha}$Leu-OGp, $X_{C\alpha}$ =	
acyl acceptor	Me	Dfm	Tfm	L-Me	D-Me	Dfm	Tfm	Dfm	Tfm
H-Gly-NH_2	18	35	14	16	50	47	40	58	13
H-Leu-NH_2	19	51	18	23	53	62	46	64	27
H-Met-NH_2	35	70	27	45	82	72	53	88	36
H-Ala-Ala-OH	20	65	22	41	68	87	63	90	40
H-Ala-Met-OH	25	62	25	28	56	82	47	88	48
H-Ala-Arg-OH	50	83	33	63	88	92	71	94	54
H-Ala-Ala-Lys-OH	22	51	17	47	77	79	53	83	37
H-Ala-Ala-Ala-OH	22	54	19	43	79	86	61	88	45
H-Ala-Ala-Pro-OH	37	64	28	54	86	87	61	90	41

[a] Condition: 0.1 M HEPES, 0.2 M NaCl, 0.02 M $CaCl_2$, pH 8.0, 10−40% DMSO, 25 °C. [acyl donor] 4 mM, [acyl acceptor] 20 mM, [trypsin] 3.0×10^{-5} to 1.2×10^{-4} M; all errors are less than 5%.

esters were hydrolyzed by trypsin within short reaction times to provide the free *N*-protected amino acids.[20]

***C*$^{\alpha,\alpha}$-Dialkyl Alanine Derivatives.** On the basis of these results, the suitability of the new substrate mimetics for serving as acyl donors in trypsin-catalyzed peptide bond formations was investigated. Acyl acceptor sequences were chosen to ensure coverage of the broad specificity range of trypsin according to published studies about substrate mimetics of proteinogenic L- or noncoded D-amino acids.[21] Therefore, the nucleophile library comprised amino acid amides as well as di- and tripeptides having Met, Leu, Gly, Gln, Ala in P_1'-; Arg, Met, Ala, in P_2'-; and Pro, Ala, Lys in P_3'-position. Results are summarized in Table 1. Remarkable differences in product yields between the (αDfm)Ala derivative and both Aib and (αTfm)Ala substrate mimetics indicate a significant influence of the second α-substituent at the acyl donor on individual enzyme−substrate interactions. The efficiency of peptide synthesis using Z-(αDfm)Ala-OGp as the acyl component is in most cases at least twice as high as for the corresponding Aib and (αTfm)Ala derivatives, respectively, while product yields for both of the latter were found within the same range. The S′-specificity profile of the amino acid amides and di- and tripeptides remains unchanged despite the use of a variety of substituted amino acid esters. H-Met-NH_2, H-Ala-Arg-OH, and H-Ala-Ala-Pro-OH still remain the most specific nucleophiles, while H-Gly-NH_2 and H-Ala-Ala-OH function as unspecific acyl acceptors.[21]

Before comparing the differences in product yields between (αDfm)Ala derivatives and Aib and (αTfm)Ala substrate mimetics, respectively, the use of racemic mixtures in the case of Z-protected (αDfm)Ala and (αTfm)Ala esters unlike for the Aib derivative has to be taken into account. A significant influence of the absolute configuration of the dialkyl amino acids on enzyme−substrate interactions within the active site of trypsin seems to be a more likely reason for that difference than the steric demand of the second substituent (alkyl or fluoroalkyl, respectively) at the C^{α}-atom. To prove this assumption, enzymatic peptide synthesis of Z-(αDfm)Ala-Met-NH_2 and Z-(αTfm)Ala-Met-NH_2 was carried out in a semipreparative scale. Diastereomers were separated by HPLC and characterized by ^{19}F NMR. In case of the αTfm-substituted peptide a diastereomer ratio of 1:3 was found, whereas for the corresponding αDfm-substituted

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peptide the ratio was 1:1. Obviously, both enantiomers of the Z-(αDfm)Ala-enzyme complexes are deacylated by the nucleophile at the same rate, which gives identical product yields. In contrast, one enantiomer of the Z-(αTfm)Ala-enzyme complex appears to be hydrolyzed faster by water than aminolyzed by the nucleophile, resulting in the formation of the amino acid instead of peptide bond formation. An important feature of the Dfm group is its ability to function as a hydrogen bond donor.[22] Unlike the Tfm group, the αDfm amino acid could interact with the S′ region of the enzyme stabilizing the acyl enzyme intermediate. This would lead to a delayed reaction of at least one of the enantiomers with any kind of nucleophile, resulting in a simultaneous aminolysis.

***C*$^{\alpha,\alpha}$-Dialkyl Leucine and Phenylalanine Derivatives.** After *C*$^{\alpha}$-dialkyl alanine derivatives were successfully incorporated into the P_1 position of peptides by trypsin, acyl transfer reactions of αDfm- and αTfm-substituted leucine and phenylalanine substrate mimetics, as well as (αMe)Phe to nine different nucleophiles varying in length and sequence, were studied. Surprisingly, in all cases trypsin catalyzed peptide bond formation using the sterically higher demanding amino acid derivatives was working with higher efficiency than obtained for the corresponding alanine derivatives (Table 1). The highest difference was observed for P_1 Ala and Phe derivatives. The efficiency of the acyl transfer increases as follows: (αTfm)Leu < (αDfm)Leu and L-(αMe-)Phe < (αTfm)Phe < D-(αMe)Phe < (αDfm)Phe, respectively. The stereochemistry clearly influences the individual substrate−enzyme interaction. Interestingly, as already observed for the Ala substrate mimetics, also in case of α-fluoroalkyl Leu and Phe the αDfm derivatives give higher peptide yields than the αTfm-substituted amino acids (Figure 1). This fact again indicates the difference in properties and reaction behavior of a αDfm- versus a αTfm-substituted amino acid, which certainly influences enzyme−substrate interactions. Principally, all of the studied sterically demanding *C*$^{\alpha}$-fluoroalkyl amino acid derivatives could be incorporated enzymatically into the P_1 position of peptides for the first time by applying the substrate mimetic concept.

Medium Engineering. Using the advantage of substrate mimetics in combination with reactions in the frozen aqueous system was already described as a powerful concept for an irreversible and efficient protease-catalyzed peptide synthesis independently on the primary protease specificity as well as without the risk of proteolytic side reactions.[23] As Aib- and αTfm-substituted Ala derivatives gave only unsatisfying peptide yields at room temperature, the applicability of the freezing technology to *C*$^{\alpha,\alpha}$-dialkyl amino acids was investigated. Therefore, acyl transfer reactions of Z-Aib-OGp as well as Z-(αTfm)Ala-OGp to eight different amino acid amides and di- and tripeptides chosen out of the already described nucleophile library were studied at −15°C and compared with the results obtained at room temperature (Figure 2). In every case studied here, the freezing effect shifts the ratio between hydrolysis and aminolysis toward

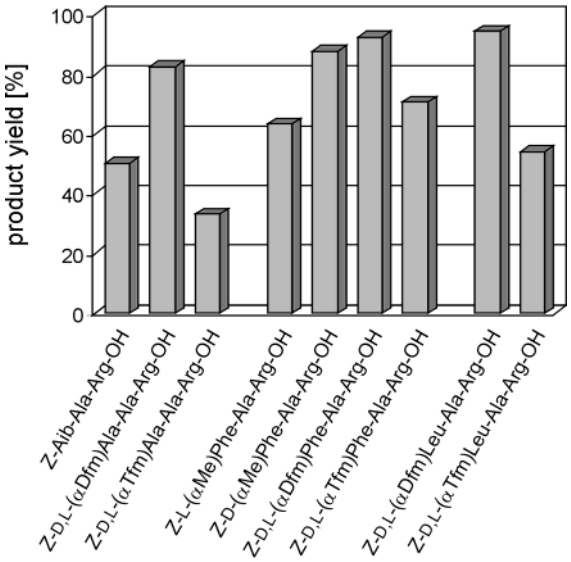

FIGURE 1. Comparison of the acyl transfer rates of *C*$^{\alpha,\alpha}$-dialkylated substrate mimetics with the amino acceptor H-Ala-Arg-OH. Condition: 0.1 M HEPES, 0.2 M NaCl, 0.02 M $CaCl_2$, pH 8.0, 10%−40% DMSO, 25 °C. [acyl donor] 4 mM, [H-Ala-Arg-OH] 20 mM, [trypsin] 3.0×10^{-5} to 1.2×10^{-4} M; all errors are less than 5%.

peptide bond formation. The Z-Aib-trypsin complex is slightly better deacylated at 25°C compared to the corresponding αTfmAla-enzyme intermediate. Decreasing the temperature does not change this tendency. Moreover, the difference between Aib and (αTfm)Ala is more significant at −15°C. Again, the results for (αTfm)-Ala have to be interpreted as the total of both enantiomers. Obviously, the influence of the stereochemistry is of higher importance for substrate−enzyme interactions in the frozen state; a fact that is in agreement with data published elsewhere.[24] Applying freezing reaction conditions increases the peptide yield, dependent on the nucleophile used, up to 90% for Aib and up to 72% for (αTfm)Ala, respectively. Summarizing these results clearly shows that applying the substrate mimetic strategy or a combination of substrate- and medium engineering, respectively, makes possible the trypsin-catalyzed incorporation of *C*$^{\alpha}$-methyl- and *C*$^{\alpha}$-fluoroalkyl-substituted amino acids into the P_1 position of peptides with high yields.

α-Chymotrypsin-Catalyzed Peptide Bond Formation. Motivated by the efficient linking of nonnatural as well as nonpeptidic acyl residues with amino acid and dipeptide amides by α-chymotrypsin we tried to apply this protease for the incorporation of *C*$^{\alpha}$-fluoroalkyl amino acids into the P_1 position of peptides. At first, 4-guanidinophenyl esters of all *C*$^{\alpha}$-fluoroalkyl as well as *C*$^{\alpha}$-dialkyl amino acids were checked for their applicability as substrates for α-chymotrypsin. Results summarized in Table 2 show that now only 4-guanidinophenyl esters of Ala derivatives were hydrolyzed by the protease within short reaction times to give the free *N*-protected amino acids. In contrast to trypsin, α-chymotrypsin obviously does not accept *C*$^{\alpha}$-dialkyl amino acids, which carry a sterically higher demanding amino acid side chain at the

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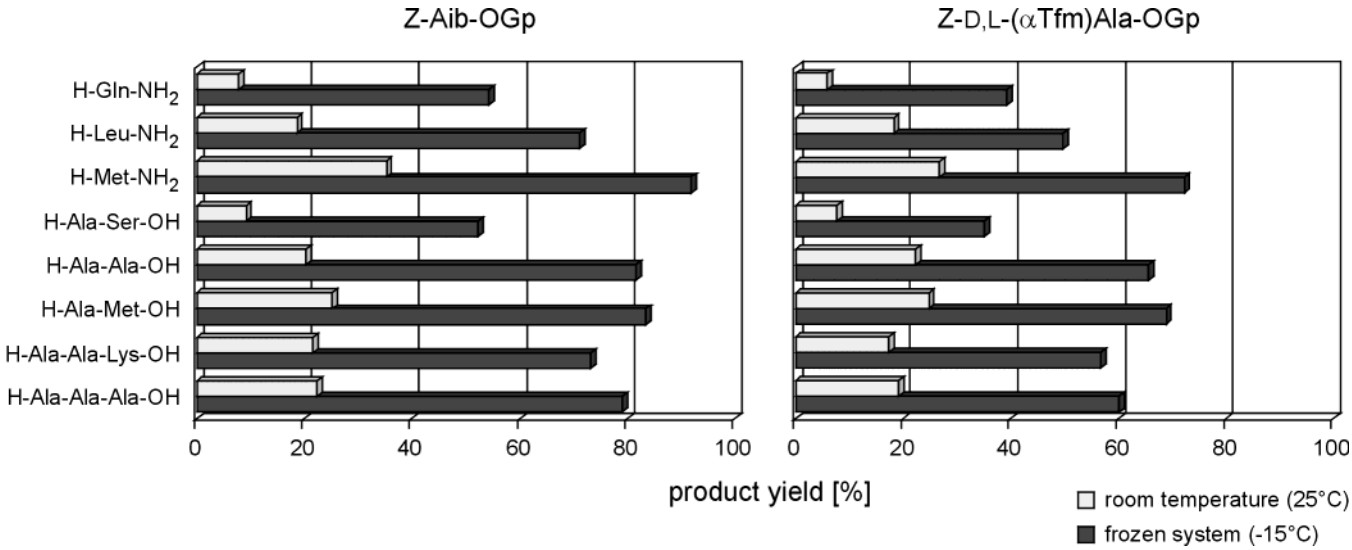
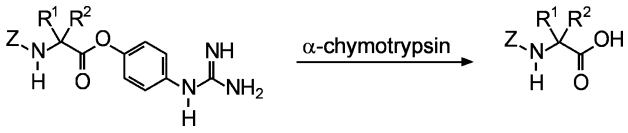


FIGURE 2. Product yields of trypsin-catalyzed substrate mimetics mediated peptide synthesis at room temperature and in the frozen aqueous system. Condition: 0.1 M HEPES, 0.2 M NaCl, 0.02 M $CaCl_2$, pH 8.0, 10% DMSO, 25 °C/H_2O, pH 8.0, 10% DMSO, −15 °C. [acyl donor] 4 mM, [acyl acceptor] 20 mM, [trypsin] 3.0×10^{-5} M, 25 °C/7.5×10^{-5} M, −15 °C; reaction time 2 h, 25 °C/24 h −15 °C; all errors are less than 5%.

TABLE 2. α-Chymotrypsin-Catalyzed Hydrolysis (%) of $C^{\alpha,\alpha}$-Dialkyl-Substituted Amino Acid-4-guanidinophenyl Esters[a]



	R²		
R¹	CH_3 (Ala)	CH_2Ph (Phe)	$CH_2CH(CH_3)_2$ (Leu)
CH_3 (Me)	100	*b*	*b*
CF_2H (Dfm)	100	15	17
CF_3 (Tfm)	100	10	9

[a] Condition: 0.1 M HEPES, 0.2 M NaCl, 0.02 M $CaCl_2$, pH 8.0, 10−30% DMSO, 25 °C, 48 h. [substrate] 4 mM, [α-chymotrypsin] 4.8×10^{-5} to 2.4×10^{-4} M; all errors are less than 5%. [a] Not determined.

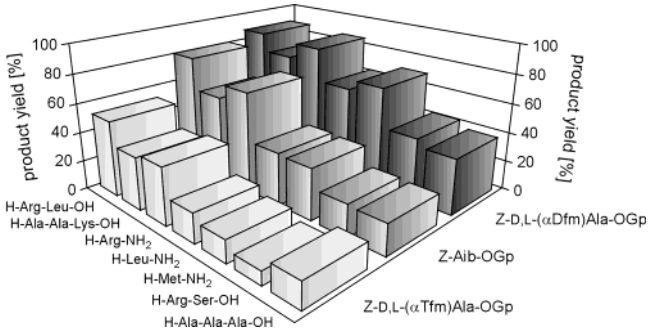


FIGURE 3. Yields (%) of chymotrypsin-catalyzed peptide synthesis using substrate mimetics of $C^{\alpha,\alpha}$-dialkyl alanine derivatives. Condition: 0.1 M HEPES, 0.2 M NaCl, 0.02 M $CaCl_2$, pH 8.0, 10% DMSO, 25 °C. [acyl donor] 4 mM, [acyl acceptor] 20 mM, [chymotrypsin] 4.8×10^{-5}M; all errors are less than 5%.

α-carbon atom than a methyl group. A phenyl or isopropyl residue in combination with an α-fluoroalkyl or α-methyl substituent creates a sterical demand that seems to be too high, reducing drastically the interaction between substrates and the active site of α-chymotrypsin. Therefore, only the Ala-derived substrate mimetics Z-Aib-OGp, Z-(αDfm)Ala-OGp, and Z-(αTfm)Ala-OGp were reacted with a small library of amino acid amides and di- and tripeptides catalyzed by α-chymotrypsin. The results convincingly show that again, the αDfm-substituted derivative acts as the more efficient acyl donor out of the fluoroalkyl derivatives, acylating the specific nucleophiles H-Arg-NH_2, H-Arg-Leu-OH, and H-Ala-Ala-Lys-OH with yields between 55% and 80% (Figure 3). However, in contrast to trypsin, α-chymotrypsin was found to be even more efficient, catalyzing the deacylation of the Z-Aib-enzyme complex. Remarkably, α-chymotrypsin efficiently catalyzes the peptide bond formation for both enantiomers of (αDfm)Ala with the same rate, while in case of (αTfm)Ala one of the enantiomers is preferably incorporated into peptides. This finding is in close agreement to the results found for trypsin catalyzed reactions.

Conclusions

Effective site-specific incorporation of a wide variety of nonnatural amino acids into peptides and proteins remains a topic of high interest as it provides the opportunity for a more detailed understanding of protein structure and function. A method has been developed that, for the first time, makes possible the specific incorporation of sterically demanding α-fluoroalkyl as well as α-methyl amino acids into the P_1 position of peptides using commercially available proteases. This concept, first reported for trypsin-catalyzed peptide synthesis, is based on the binding site specific 4-guanidinophenyl ester functionality to mediate acceptance of nonspecific amino acid moieties in the specificity-determining S_1 position of the enzyme.

4-Guanidinophenylester of C^{α}-fluoroalkyl Ala derivatives or Aib, respectively, can be easily prepared by

reaction of the *N*-protected amino acids with 4-[*N*′,*N*″-bis(*tert*-butyloxycarbonyl)guanidino]phenol using TBTU as coupling reagent. The sterically higher demanding C^{α}-fluoroalkyl or methyl Leu and Phe derivatives, respectively, can be synthesized in high yields as well but have to be activated with DIC/HOAt and reacted with the lithium salt of the guanidinophenol.

In general, all of the C^{α}-dialkyl amino acids studied here can be coupled directly to various nucleophiles, different in sequence and length, by trypsin, whereas α-chymotrypsin only catalyzes acyl transfer reactions of Ala-derived acyl donors. Remarkably, in all cases the effeciency of peptide synthesis is much higher in the case of αDfm-substituted amino acid derivatives compared to αTfm- as well as α-methyl-substituted ester. A significant influence of the absolute configuration of the dialkyl amino acids on enzyme−substrate interactions within the active site of trypsin could be shown to more likely be the reason for that difference than the steric demand of the second substituent (alkyl or fluoroalkyl, respectively) at the C^{α}-atom. Moreover, the ability of the Dfm group to function as a hydrogen bond donor compared to the Tfm functionality certainly contributes to the observed difference in the interaction pattern between the fluoroalkyl amino acids and the S′ region of the enzyme. The influence of the stereochemistry in the case of αTfm-substituted Ala derivatives for substrate−enzyme interactions is of even higher importance in the frozen state. Application of freezing reaction conditions in combination with the substrate mimetic strategy succeeded in an increase of peptide yield, dependent on the nucleophile used, up to 72% for the α-TfmAla derivative.

Experimental Section

Materials. TPCK-treated bovine trypsin (EC 3.4.21.4) and TLCK-treated bovine α-chymotrypsin (EC 3.4.21.1) were obtained from Sigma (Germany). Both proteases were used without further purification. Amino acid derivatives, peptides, 4-aminophenol, benzyl chloroformate (Z-chloride), Boc anhydride, *n*-BuLi, DIC, DIEA, HOAt, *S*-methylisothiourea, and TBTU were purchased from common suppliers. αTfm and αDfm amino acids and their *N*-protected derivatives were obtained as racemates via procedures published elsewhere.[25] If not otherwise stated, all reagents were of the highest available commercial purity. Solvents were purified and dried by common methods. Flash chromatography was performed using silica gel (0.032−0.064 μm) with petroleum ether/ethyl acetate as eluent. Mass spectra were recorded using thermospray ionization.

Chemical Syntheses. Synthesis of Benzyloxycarbonyl-α-methyl Phenylalanine. According to the procedure described in ref 26.

Synthesis of 4-[*N*′,*N*″-Bis(*tert*-butyloxycarbonyl)guanidino]phenol. A solution of *S*-methylisothiourea and Boc anhydride in absolute dioxane was stirred at room temperature and pH 7.0 for 24 h. After complete consumption of *S*-methylisothiourea the solution was evaporated to half of the volume and adjusted to pH 3.0 using a saturated solution of $KHSO_4$. The reaction mixture was extracted with ethyl acetate several times, washed with water, and evaporated to dryness in vacuo. The resulting *N*′,*N*″-bis(*tert*-butyloxycarbonyl)-*S*-methylisothiourea was recrystallized from ethyl acetate/petroleum ether. Furthermore, to a solution of Boc-protected *S*-methylisothiourea was added 1.5 equiv of 4-aminophenol in dry THF, and the mixture was stirred at room temperature for 5−6 days. After evaporation of the organic phase to dryness in vacuo and several washing steps with methanol 4-[*N*′,*N*″-bis(Boc)guanidino]phenol could be isolated.

***N*′,*N*″-Bis(*tert*-butyloxycarbonyl)-*S*-methylisothiourea.** ^{1}H NMR (300.08 MHz, DMSO) δ 1.38/1.43 (s/s, 18H), 2.28 (s, 3H), 11.00 (s, 1H). Anal. Calcd for $C_{12}H_{22}N_2O_4S$: C, 49.64; H, 7.64; N, 9.65. Found: C, 49.30; H, 7.86; N, 9.38. MS (*m*/*z*) 291 $(M + H)^+$.

4-[*N*′,*N*″-Bis(Boc)guanidino]phenol. ^{1}H NMR (300.08 MHz, DMSO) δ 1.37/1.50 (s/s, 18H), 6.72/7.25 (m/m, 4H), 9.41 (s, 1H), 9.77 (s, 1H), 11.48 (s, 1H). Anal. Calcd for $C_{17}H_{25}N_3O_5$: C, 58.11; H, 7.17; N, 11.96. Found: C, 58.10; H, 7.08; N, 12.10. MS (*m*/*z*) 350 $(M - H)^+$.

General Procedure for the Synthesis of 4-Guanidinophenyl Esters of Z-Protected Alanine Derivatives. N^{α}-Z-protected amino acids were activated as 1-benzotriazolyl esters. Activation was performed by reaction of Z-(αTfm)Ala-OH, Z-(αDfm)Ala-OH, or Z-Aib-OH with TBTU (1 equiv) and DIEA (2 equiv) in DMF (20 mL) for 30 min at room temperature. A solution of 4-[*N*′,*N*″-bis(Boc)guanidino]phenol (1 equiv) dissolved in DMF (10 mL) was slowly added to the reaction mixture. After the mixture stirred overnight at room temperature, a high excess of distilled water was added to the solution. After sedimentation the precipitate was filtered, washed with water, and purified by flash column chromatography (eluent, petroleum ether/ethyl acetate). A final deprotection of the guanidino functionality of the bis(Boc)-protected compounds with trifluoroacetic acid and ultrasound resulted in the 4-*N*′,*N*″-deprotected 4-guanidinophenyl esters.

General Procedure for the Synthesis of 4-Guanidinophenyl Esters of Z-Protected Leucine and Phenylalaninee Derivatives. The *N*-protected α-fluoroalkyl amino acid derivatives Z-(αTfm)Phe-OH, Z-(αDfm)Phe-OH, Z-L-(αMe)-Phe-OH, Z-D-(αPhe)Me-OH, Z-(αTfm)Leu-OH, or Z-(αDfm)Leu-OH are dissolved in tetrahydrofuran (20 mL) and DIC (1 equiv) and HOAt (1 equiv) are added. Reaction mixtures are stirred at room temperature for 30 min.

A solution of *n*-BuLi (1.6 M in hexane) was added dropwise over 5 min to a stirred solution of 4-[*N*′,*N*″-bis(Boc)guanidino]-phenol in dry tetrahydrofuran (20 mL) at room temperature and under an argon atmosphere, and the reaction mixture was stirred for 30 min. The resulting 4-guanidinophenolate solution was added to the activated N^{α}-Z-protected amino acid derivative. The mixture was stirred for another 2 h at room temperature, and then citric acid (5 g/100 mL, 50 mL) was added. The organic solvent was removed under vacuum, and the aqueous solution was extracted with ethyl acetate, dried over magnesium sulfate, and concentrated to give a yellow oil. The crude compounds were purified by flash column chromatography on silica gel using petroleum ether/ethyl acetate as eluent. The 4-guanidinophenyl esters were obtained by deprotection of the corresponding bis(Boc)-protected derivatives with trifluoroacetic acid and ultrasound.

4-Guanidinophenyl-*N*-(benzyloxycarbonyl)-(α-trifluoromethyl)-alanineate, Trifluoroacetate. ^{1}H NMR (300.08 MHz, DMSO) δ (ppm) 1.73 (s, 3H), 5.13 (s, 2H), 7.14/7.31 (m/m, 4H), 7.36 (m, 5H), 7.62 (s, 3H), 8.83 (s, 1H), 10,04 (s, 1H); ^{13}C NMR (75.46 MHz, DMSO) δ (ppm) 18.84, 66.35, 122.54, 125.76, 127.96, 128.06, 128.36, 133.42, 147.83, 155.99, 165.40; ^{19}F (282.33 MHz, DMSO) δ (ppm) 3.12 (s, 3F), 4.36 (s, 3F, TFA). Anal. Calcd for $C_{21}H_{20}F_6N_4O_6$: C, 46.85; H, 3.74; N, 10.41. Found: C, 46.61; H, 3.53; N, 10.30. MS (*m*/*z*) 425 $(M + H)^+$.

4-Guanidinophenyl-*N*-(benzyloxycarbonyl)-(α-methyl)-alanineate, Trifluoroacetate. ^{1}H NMR (300.08 MHz, DMSO) δ (ppm) 1.50 (s, 6H), 5.08 (s, 2H), 7.06/7.27 (m/m, 4H), 7.35 (m, 5H), 7.61 (m, 3H), 8.05 (s, 1H), 10,02 (s, 1H); ^{13}C NMR (75.46 MHz, DMSO) δ (ppm) 24.91, 55.65, 65.44, 122.67, 125.74, 127.81, 128.33, 132.80, 136.88, 148.86, 156.02, 173.03.

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Anal. Calcd for $C_{21}H_{23}F_3N_4O_6$: C, 52.07; H, 4.79; N, 11.57. Found: C, 52.30; H, 4.83; N, 11.70. MS (*m*/*z*) 371 $(M + H)^+$.

4-Guanidinophenyl-*N*-(benzyloxycarbonyl)-(α-difluoromethyl)-alanineate, Trifluoroacetate. ^{1}H NMR (300.08 MHz, DMSO) δ (ppm) 1.54 (s, 3H), 5.11 (s, 2H), 6.41 (t, J = 55.4 Hz, 1H), 7.09/7.29 (m/m, 4H), 7.36 (m, 5H), 7.42 (m, 3H), 8.57 (s, 1H), 9.68 (s, 1H); ^{13}C NMR (75.46 MHz, DMSO) δ (ppm) 18.01, 66.17, 122.55, 125.79, 127.98, 128.39, 133.31, 136.40, 148.00, 156.01, 164.21; ^{19}F (282.33 MHz, DMSO) δ (ppm) −52.68 (d/d, J_{FF} = 279.4 Hz, J_{FH} = 55.6 Hz, 1F), −49.61 (d/d, J_{FF} = 279.4 Hz, J_{FH} = 55.6 Hz, 1F), 4,70 (s, 3F, TFA). Anal. Calcd for $C_{21}H_{21}F_5N_4O_6$: C, 48.47; H, 4.07; N, 10.77. Found: C, 48.16; H, 3.83; N, 10.56. MS (*m*/*z*) 407 $(M + H)^+$.

4-Guanidinophenyl-*N*-(benzyloxycarbonyl)-(α-difluoromethyl)-phenylalaninate, Trifluoroacetate. ^{1}H NMR (300.08 MHz, DMSO) δ (ppm) 3.27−3.37 (m, 2H), 5.14 (s, 2H), 6.24 (t, J = 54,3 Hz, 1H), 7.00/7.29 (m/m, 4H), 7.32−7.41 (m, 10H), 7.61 (m, 3H), 8.55 (s, 1H), 10.00 (s, 1H); ^{13}C NMR (75.46 MHz, DMSO) δ (ppm) 63.99, 66.14, 122.39, 125.82, 127.45, 128.03, 128.40, 130.37, 133.31, 136.43, 147.94, 155.96, 166.45; ^{19}F (282.33 MHz, DMSO) δ (ppm) −52.13 to −51,83 (m, 2F), 4.35 (s, 3F, TFA). Anal. Calcd for $C_{27}H_{25}F_5N_4O_6$: C, 54.37; H, 4.22; N, 9.39. Found: C, 54.09; H, 4.19; N, 9.15. MS (*m*/*z*) 483 $(M + H)^+$.

4-Guanidinophenyl-*N*-(benzyloxycarbonyl)-(α-trifluoromethyl)-leucinate, Trifluoroacetate. ^{1}H NMR (300.08 MHz, DMSO) δ (ppm) 0.94−1.01 (m, 6H), 1.91−2.10 (m, 3H), 5.12 (s, 2H), 7.16/7.32 (m/m, 4H), 7.37 (m, 5H), 7.57 (m, 3H), 8.62 (s, 1H), 9.95 (s, 1H); ^{13}C NMR (75.46 MHz, DMSO) δ (ppm) 22.61, 23.37, 24.26, 41.15, 66.28, 122.38, 125.97, 127.93, 128.07, 128.39, 133.36, 136.30, 147.80, 155.95, 164.68; ^{19}F (282.33 MHz, DMSO) δ (ppm) 4.26 (s, 3F, TFA), 5.81 (s, 3F). Anal. Calcd for $C_{24}H_{26}F_6N_4O_6$: C, 49.66; H, 4.51; N, 9.65. Found: C, 49.50; H, 4.42; N, 9.44. MS (*m*/*z*) 467 $(M + H)^+$.

4-Guanidinophenyl-*N*-(benzyloxycarbonyl)-(α-difluoromethyl)-leucinate, Trifluoroacetate. ^{1}H NMR (300.08 MHz, DMSO) δ (ppm) 0.91−0.97 (m, 6H), 1.86 (m, 3H), 5.09 (s, 2H), 6.49 (t, J = 53,8 Hz, 1H), 7.11/7.29 (m/m, 4H), 7.33 (m, 5H), 7.59 (m, 3H), 8.29 (s, 1H), 10.00 (s, 1H); ^{13}C NMR (75.46 MHz, DMSO) δ (ppm) 23.06, 23.31, 24.13, 62.86, 66.03, 122.44, 125.98, 127.93, 128.36, 133.25, 136.49, 148.00, 155.99, 167.52; ^{19}F (282.33 MHz, DMSO) δ (ppm) −51.62 (m, 1F), −51.42 (m, 1F), 4.25 (s, 3F, TFA). Anal. Calcd for $C_{24}H_{27}F_5N_4O_6$: C, 51.25; H, 4.84; N, 9.96. Found: C, 51.09; H, 4.90; N, 9.73. MS (*m*/*z*) 449 $(M + H)^+$.

4-Guanidinophenyl-*N*-(benzyloxycarbonyl)-(α-trifluoromethyl)-phenylalanineate, Trifluoroacetate. ^{1}H NMR (300.08 MHz, DMSO) δ (ppm) 3.45−3.47 (m, 2H), 5.14 (s, 2H), 6.98/7.29 (m/m, 4H), 7.33−7.39 (m, 10H), 7.55 (m, 3H), 8.91 (s, 1H), 9.91 (s, 1H); ^{13}C NMR (75.46 MHz, DMSO) δ (ppm) 37.81, 66.40, 122.30, 125.88, 127.61, 128.06, 128.20, 128.41, 130.52, 133.13, 136.22, 147.72, 155.91, 163.85; ^{19}F (282.33 MHz, DMSO) δ (ppm) 4.13 (s, 3F, TFA), 6.88 (s, 3F). Anal. Calcd for $C_{27}H_{24}F_6N_4O_6$: C, 52.77; H, 3.94; N, 9.12. Found: C, 52.40; H, 3.83; N, 8.97. MS (*m*/*z*) 501 $(M + H)^+$.

4-Guanidinophenyl-*N*-(benzyloxycarbonyl)-(α-methyl)-D-phenylalaninate, Trifluoroacetate. ^{1}H NMR (300.08 MHz, DMSO) δ (ppm) 1.62 (s, 3H), 3.35−3.60 (m, 2H), 5.39 (m, 2H), 7.18/7.39 (m/m, 4H), 7.52−7.64 (m, 10H), 7.74 (m, 3H), 8.21 (s, 1H), 10.06 (s, 1H); ^{13}C NMR (75.46 MHz, DMSO) δ (ppm) 38.11, 59.13, 65.56, 122.69, 125.91, 126.75, 127.97, 128.35, 130.65, 132.82, 135.74, 136.96, 148.89, 155.95, 172.36. Anal. Calcd for $C_{27}H_{27}F_3N_4O_6$: C, 57.86; H, 4.86; N, 10.00. Found: C, 57.52; H, 4.83; N, 9.87. MS (*m*/*z*) 447 $(M + H)^+$.

4-Guanidinophenyl-*N*-(benzyloxycarbonyl)-(α-methyl)-L-phenylalaninate, trifluoroacetate. ^{1}H NMR (300.08 MHz, DMSO) δ (ppm) 1.34 (s, 3H), 3.02−3.33 (m, 2H), 5.08−5.16 (m, 2H), 7.03/7.21 (m/m, 4H), 7.24−7.40 (m, 10H), 7.52 (m, 3H), 7.91 (s, 1H), 9.88 (s, 1H); ^{13}C NMR (75.46 MHz, DMSO) δ (ppm) 38.68, 59.11, 65.54, 122.66, 125.85, 127.94, 128.36, 130.62, 132.83, 135.73, 148.84, 155.97, 172.34. Anal. Calcd for $C_{27}H_{27}F_3N_4O_6$: C, 57.86; H, 4.86; N, 10.00. Found: C, 57.60; H, 5.05; N, 9.75. MS (*m*/*z*) 447 $(M + H)^+$.

Enzymatic Syntheses. Trypsin- and α-chymotrypsin-catalyzed reactions were performed in a total volume of 60 μL containing 0.1 M HEPES buffer, pH 8.0, 0.2 M NaCl, and 0.02 M $CaCl_2$ at 25°C or distilled water at −15°C, respectively. Stock solutions of acyl donor esters (8 mM) were prepared in water. To guarantee a complete solubility of the ester derivatives 10−40% DMSO was added as cosolvent. Stock solutions of amino components were prepared in 0.2 M HEPES buffer, pH 8.0 containing 0.4 M NaCl and 0.04 M $CaCl_2$. Appropriate equivalents of NaOH were added to the stock solutions of nucleophiles in order to neutralize hydrochlorides and acetates. The final concentrations of acyl donors and acyl acceptors were 4 and 20 mM, respectively. The latter was calculated as free, N^α-unprotonated nucleophile concentration $[HN]_0$ according to the formalism of Henderson−Hasselbalch $[HN]_0 = [N]_0/(1 + 10^{pK-pH})$. The p$K$ values of the α-amino group of the nucleophilic amino components were determined by inflection point titration on a video-titrator. Measurements were carried out at a nucleophile concentration of 6.7 mM using 0.1 M HCl as titrant. The pK_α values were calculated by linear interpolation from the slope minimum range. Standard deviation was estimated to be pK_α ± 0.01. After thermal equilibration of the assay mixtures, the enzymatic coupling reactions were initiated by the addition of trypsin or chymotrypsin, leading to active enzyme concentrations of 30−120 μM and stirred at 25°C. Reaction times of 1−24 h led to a complete ester consumption. Reactions in frozen medium were performed analogous to the following procedure: The tube containing the appropriate acyl donor ester as well as the nucleophilic component was cooled to 0°C. Five microliters of the enzyme stock was added, the reaction mixture was rapidly shaken, and the tube was stored in liquid nitrogen for 20 s. After the shock freezing procedure, the tube was transferred into a freezer and incubated at −15°C. After varying reaction times, aliquots of 50 μL were withdrawn of the stirred and frozen reaction mixtures, diluted with 70 μL of aqueous stop solution containing 50% methanol, 25% DMSO and 3% trifluoroacetic acid, and stored at −20°C until HPLC analyses. For each substrate and nucleophile an experiment without enzyme was carried out to determine the extent of spontaneous ester hydrolysis, which was strictly less than 5%. On the basis of the same control experiments, nonenzymatic aminolysis of the acyl donor esters was investigated and could be ruled out. The data reported here represent the average of at least three independent experiments. The identity of the formed peptide products was established by thermospray mass spectroscopy.

HPLC Analyses. HPLC measurements were performed by analytical reversed phase HPLC on a C4 polymer coated column (10 μm, 250 mm × 4 mm) and a C8 reversed phase column (5 μm, 250 mm × 4.6 mm). Samples were eluted with various mixtures of water/acetonitril containing 0.1% trifluoroacetic acid under gradient conditions. Detection was carried out at 254 nm to monitor the aromatic chromophores of the acyl donors. The reaction rates and product yields were calculated from the peak areas of the substrate esters and the hydrolysis and aminolysis products, respectively. The chromatograms were analyzed using the software Mc DAcq (version 1.39, Bischoff Chromatography, Germany).

Acknowledgment. This work was supported by the Deutsche Forschungsgemeinschaft (Innovationskolleg "Chemisches Signal und biologische Antwort").

Supporting Information Available: Characterization of intermediate chemical products and HPLC retention times and MS data of enzymatic products. This material is available free of charge via the Internet at http://pubs.acs.org.

JO020613P