Regioselective Modification of Lysine's Amino Groups by Proteases

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The regioselectivity of six serine proteases for the amino groups of lysine was investigated. α-Chymotrypsin showed a preference for the α-amino group, although the selectivity can be varied 10-fold depending on the reaction medium. Subtilisin Carlsberg and other bacterial proteases were highly specific for the ε-amino group, regardless of the reaction medium: they were used as catalysts for the preparative synthesis of isopeptides in anhydrous *tert*-amyl alcohol.

Enzymatic peptide synthesis[1] has been recognized as a useful methodology for the production of biologically active peptides, including commercially important ones such as aspartame[2] and insulin.[3] One of the advantages of this approach over the chemical one is that it requires no or minimal protection of side-chain functional groups of amino acids due to proteases' high specificity. The ε-amino group of lysine, however, sometimes participates in a protease-catalyzed coupling reaction. A product of this side reaction, an ε-substituted lysine derivative (isopeptide) has several biological characteristics different from those of an α-isomer ("normal" peptide), especially high stability against proteases *in vivo*.[4] Consequently, it should be of definite interest to establish enzymatic reaction conditions with which one can synthesize specifically either α- or ε-substituted derivatives of the lysine moiety at will without laborious protection and deprotection processes.

Kullmann[5] reported synthesis of the α-isomer by the reaction between Boc-Tyr-OEt and Lys-N_2H_2Ph catalyzed by α-chymotrypsin. Aso[6] obtained the same result for an analogous reaction. However, both of them isolated the products with preparative HPLC and never mentioned whether the ε-isomer formation was also observed.

Recently, we[7] reported that a mixture of both regioisomers was obtained by the reaction between *N*-acetyl-L-phenylalanine ethyl ester (Ac-Phe-OEt) and L-lysine *tert*-butyl ester (Lys-O-*tert*-Bu) catalyzed by α-chymotrypsin, whereas subtilisin Carlsberg catalyzed the specific formation of the ε-isomer.

In this study, we investigated extensively the regioselectivity of a wide range of proteases and the dependency of their selectivity on the reaction medium, and optimal reaction conditions for the specific enzymatic synthesis of either α- or ε-substituted lysine derivatives were established.

Materials and Methods

Enzymes. α-Chymotrypsin (from bovine pancreas), elastase (from porcine pancreas), subtilisin Carlsberg (from *Bacillus licheniformis*), and subtilisin BPN′ (from *Bacillus amyloliquefaciens*) were obtained from the Sigma Chemical Co. Proteinase K (from *Tritirachium album*) was purchased from Merck, and alkaline protease (from *Streptomyces griseus*) was from Seikagaku Kogyo Co., Ltd.

For reactions in aqueous solution, all enzymes were used without further purification. For reactions in anhydrous organic solvents, subtilisin Carlsberg, subtilisin BPN′,

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proteinase K, and alkaline protease were lyophilized from aqueous solutions of pH 7.8, 7.8, 7.8 and 11.0, respectively, prior to use.[8]

Substrates. All amino acids used in this study have the L-configuration, unless otherwise specified. X-ε-Lys or Lys(X) depicts a lysine derivative, whose ε-amino group is substituted with group X.

Ac-Phe-OEt was obtained from the Sigma Chem. Co. Monochloroethyl ester of *N*-acetyl-L-phenylalanine (Ac-Phe-OEtCl) was synthesized according to a reported procedure.[9]

Lys-O-*tert*-Bu (**1**) was synthesized by catalytic hydrogenolysis of Lys(Z)-O-*tert*-Bu (**1a**) in the presence of 10% Pd/C. **1a** was synthesized from Lys(Z) (purchased from Kokusan Chemical Works, Ltd.) according to a slight modification of a reported procedure[10] and obtained in a 55% yield as a p-toluenesulfonic acid salt (mp 114–116°C. Found: C, 59.31; H, 7.16; N, 5.46. Calcd. for $C_{25}H_{36}O_7N_2S_2$: C, 59.04; H, 7.13; N, 5.51%). Hydrogenolysis of **1a** was carried out in methanol after desalting with a 1 N NaOH aqueous solution. After methanol was evaporated in a vacuum, **1** was obtained as a viscous liquid with a quantitative yield, and used for enzymatic reactions without further purification. D-Lys-O-*tert*-Bu (**2**) was synthesized from D-Lys(Z), according to the same procedure as that used for the synthesis of **1**.

L-Phenylalanyl-L-lysine *tert*-butyl ester (Phe-Lys-O-*tert*-Bu; **3**) was synthesized by hydrogenolysis of Z-Phe-Lys(Z)-O-*tert*-Bu (**3a**), which was obtained by a coupling reaction between Z-Phe and **1a** in the presence of DCC in a 73% yield (mp 126–127°C. Found: C, 67.91; H, 6.91, N, 6.74. Calcd. for $C_{35}H_{43}O_7N_3$: C, 68.05; H, 7.02, N, 6.80%). **3** was obtained as a waxy residue with a quantitative yield after evaporating methanol in a vacuum, and used for the enzymatic synthesis without further purification. (See the synthesis of **7**).

N-Acetyl-L-phenylalanyl-L-lysine *tert*-butyl ester (Ac-Phe-Lys-O-*tert*-Bu, **4**; the α-isomer) was synthesized by hydrogenolysis of Ac-Phe-Lys(Z)-O-*tert*-Bu (**4a**) and purified as a HCl salt in an 82% yield (mp 192–193°C. Found: C, 58.90; H, 7.91; N, 9.64. Calcd. for $C_{21}H_{34}O_4N_3Cl$: C, 58.94, H, 8.01; N, 9.82%). **4a** was prepared via a coupling reaction between Ac-Phe and **1a** using DCC and 1-hydroxybenzotriazole as a coupling reagent in a 77% yield (mp 136–137°C. Found: C, 66.24; H, 7.51; N, 7.94. Calcd. for $C_{29}H_{39}O_6N_3$: C, 66.26; H, 7.48, N, 7.99%).

N^{ε}-(*N*-Acetyl-L-phenylalanyl)-L-lysine *tert*-butyl ester (Ac-Phe-ε-Lys-O-*tert*-Bu, **5**; the ε-isomer) was synthesized enzymatically as described below.

Enzymatic reactions in aqueous solution. A typical procedure was the following: the reaction mixture (1 ml) comprised of 0.1 M Ac-Phe-OEt, 0.2 M **1**, 0.5 mg/ml protease, 33% dimethylformamide, and 0.2 M carbonate buffer (pH 8.5) was incubated at 30°C. After a certain period of time (typically 5 min), 40 μl of the reaction mixture was withdrawn and diluted with 0.5 ml of the HPLC eluent. Five μl of the resultant solution was subjected to HPLC analysis.

HPLC analysis was carried out with a YMC-Pack ODS Column (AQ-312, 150 × 6 mm, YMC Inc.); acetonitrile–water–trifluoroacetic acid = 33 : 67 : 0.1 as the eluent at a flow rate of 1 ml/min. The eluent was monitored spectrophotometrically at 254 nm.

Enzymatic reactions in organic solvents (analytical). All solvents were dried by shaking with 3-A molecular sieves prior to use. The water content in each solvent was less than 0.02%, near the sensitivity limit of the classical Karl Fisher method.[8]

Lyophilized enzyme powder (5 mg/ml) was placed in a selected organic solvent (1 ml) containing 100 mM Ac-Phe-OEt and 200 mM **1**, and the resultant suspension was shaken at 45°C and 100 rpm for 24 hr. HPLC analysis of the products was performed in the same way as for the reaction in aqueous solution.

Enzymatic synthesis in organic solvents (preparative). Enzymatic synthesis of **5** was carried out as follows. Lyophilized subtilisin Carlsberg (90 mg) was placed in anhydrous *tert*-amyl alcohol (30 ml) containing 809 mg of Ac-Phe-OEtCl (3 mmol) and 727 mg (3.6 mmol) of **1**, and the resultant suspension was stirred at 45°C for 24 hr. The enzyme was then removed by filtration, and the filtrate was condensed under reduced pressure. The residue was diluted with methylene chloride, washed with a 4% Na_2CO_3 aqueous solution, and separated. Methylene chloride was evaporated in a vacuum after dried over $MgSO_4$, and the residue was recrystallized from a mixture of *n*-hexane and ethyl acetate (5 : 1). **5** (993 mg, 2.54 mmol) was obtained in an 85% yield (mp 86–88°C, Found: C, 64.66; H, 8.35; N, 10.67. Calcd. for $C_{21}H_{33}N_3O_4$: C, 64.42; H, 8.50; N, 10.73%).

The synthesis of Ac-Phe-ε-D-Lys-O-*tert*-Bu (**6**) was carried out by a similar procedure using **2** instead of **1**, with a yield of 82% (mp 109–110°C, HR-EIMS Found: 391.2515 (M^+). Calcd. for $C_{21}H_{33}N_3O_4$: 391.2471).

The synthesis of Phe-Lys(Ac-Phe)-O-*tert*-Bu (**7**) was conducted as follows. One g of **3a** (1.62 mmol) was hydrogenated in the presence of 10% palladium carbon (100 mg) in methanol (30 ml) for 4 hr. After the catalyst was removed by filtration, the filtrate was evaporated in a vacuum, and 567 mg (1.6 mmol) of **3** was obtained as a waxy residue in a quantitative yield. Completion of the deprotection was confirmed by ^1H NMR (see Table V).

To the residue, 377 mg (1.48 mmol) of Ac-Phe-OEt, 28 ml of dried *tert*-amyl alcohol, and 84 mg of lyophilized subtilisin Carlsberg was added, and the resultant suspension was stirred at 45°C for 38 hr. After the enzyme was filtered, the filtrate was evaporated under vacuum. The residue was purified by silica-gel chromatography (chloroform–methanol = 10 : 1), and 581 mg (1.08 mmmol)

of 7 was obtained in a 77% yield (amorphous powder, Lys 1.00 (1); Phe 2.06 (2)).

Structure elucidation. The structure of compounds **4**–**7**, namely, the position of substitution of the lysine moiety, was confirmed by ^{1}H NMR analysis, the details of which are described in Results and Discussion.

Enzymatic hydrolysis of **4** *and* **5**. A 0.1 M phosphate buffer solution (pH 7.6) containing 10% acetonitrile, 10 mM **4** or **5**, and 0.2 mg/ml protease was incubated at 30°C. After a certain period of time (typically one hour), aliquots were removed and subjected to HPLC analysis.

Results and Discussion

Regioselectivity of serine proteases

The enzymatic coupling reaction between Ac-Phe-OEt and Lys-O-*tert*-Bu (**1**) was selected as a model reaction in the present study. Ac-Phe-OEt, the two isomeric peptidic products **4** and **5**, and *N*-acetyl-L-phenylalanine (Ac-Phe, resulting from the unwanted enzymatic hydrolysis of Ac-Phe-OEt) were found to have the retention times of 14.5 min, 8.4 min, 6.1 min, and 5.5 min, respectively, under the HPLC conditions described in Materials and Methods. The complete separation of these components made it possible to quantitate the product ratio of the enzymatic reaction by HPLC analysis.

Table I. Protease-catalyzed Coupling Reaction between Ac-Phe-OEt and Lys-O-*tert*-Bu[a]

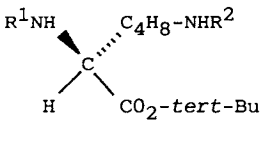
Enzyme	Conversion (%)[b]	4(%)	5(%)
α-Chymotrypsin	44	21	8.4
Subtilisin Carlsberg	97	0	33
Elastase	19	6.1	2.9
Subtilisin BPN′	70	0	21
Proteinase K	96	0	25
Alkaline protease	99	0	28

[a] For conditions, see Materials and Methods.
[b] Consumption of Ac-Phe-OEt.

Initially, we investigated the regioselectivity of six proteolytic enzymes in Table I, all of which are serine proteases and commercially available. Table I shows that on the basis of their regioselectivity these enzymes can be classified into two categories; group 1 which consists of α-chymotrypsin and elastase, and group 2 including the other bacterial proteases. The two proteases in group 1, both of mammalian origin, have a preference for the α-amino group as a nucleophile, as expected from the inherent specificity of a protease. However, formation of a considerable amount of the ε-isomer (**5**) was also observed; the ratio between the two isomers was about 2.5.

In contrast, the four proteases belonging to group 2 catalyze the specific formation of **5**. From independent experiments, the rate of the protease-catalyzed hydrolysis of the α-isomer (**4**) was found to be much slower than that of the formation of **5** from Ac-Phe-OEt. The ε-specificity revealed in Table I is therefore assumed to be kinetically controlled.

These findings suggest that group 1 enzymes may be suitable catalysts for a selective synthesis of **4**, and group 2 enzymes for that of **5**. The effort to achieve the former purpose was focused on how to increase the selectivity from 2.5 to the preparatively useful level, *i.e.*, more than ten. In order to attain the latter purpose, we sought a way to avoid the unwanted side reaction, enzymatic hydrolysis of the ester substrate, which made the yield of **5** only 30% when more than 90% of Ac-Phe-OEt was exhausted.



compound	R^1	R^2
1	H	H
4	Ac-Phe-	H
5	H	Ac-Phe-

Ac-Phe- : N-acetylphenylalanyl-

Fig. 1. Structures of Compounds **1**, **4**, and **5**.

Table II. EFFECTS OF THE REACTION MEDIUM ON α-CHYMOTRYPSIN'S SELECTIVITY AND REACTIVITY[a]

pH	Conversion (%)[b]	**4**(%)	**5**(%)	α/ε
8.5[c]	44	21	8.4	2.5
7.0[d]	62	28	10	2.8
6.0[d]	94	48	7.2	6.7
5.0[e]	58	28	9.5	2.9

[a] Containing 33% DMF; for other conditions, see Materials and Methods.
[b] Consumption of Ac-Phe-OEt.
[c] Carbonate buffer.
[d] Phosphate buffer.
[e] Acetate buffer.

Table III. EFFECTS OF COSOLVENT ON α-CHYMOTRYPSIN'S SELECTIVITY AND REACTIVITY[a]

Cosolvent[b]	Conversion (%)[c]	**4**(%)	**5**(%)	α/ε
DMF	44	21	8.4	2.5
Dioxane	29	11	4.2	2.6
DMSO	99	41	12	3.4
Acetonitrile	5	1.6	0.7	2.3

[a] For conditions, see Materials and Methods.
[b] 33% in carbonate buffer solution (pH 8.5).
[c] Consumption of Ac-Phe-OEt.

Effects of the reaction medium on α-chymotrypsin's selectivity

The enzymatic reaction shown in Table I was performed in a pH 8.5 aqueous buffer solution containing 33% dimethylformamide (DMF), *i.e.*, under conditions analogous to those reported by Kullmann[5] or Aso.[6] To make α-chymotrypsin more regioselective, we addressed the question of whether the regioselectivity can be altered by changing the reaction medium.

Tables II and III depict the effects of pH and cosolvent, respectively, on the peptide synthesis selectivity. Table II reveals that the optimal pH for regioselectivity is 6, while dimethylsulfoxide (DMSO) was found to be the best cosolvent in pH 8.5 buffer solution as seen in Table III. Interestingly, the enzymatic activity, as reflected by consumption of Ac-Phe-OEt, also became the highest at pH 6 (Table II) or with DMSO (Table III).

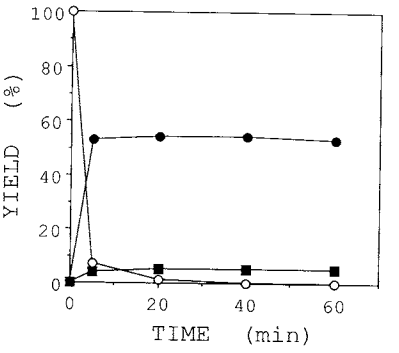


Fig. 2. Time Course of the α-Chymotrypsin-catalyzed Coupling Reaction between Ac-Phe-OEt and Lys-O-*tert*-Bu in a pH 8.5 Aqueous Buffer Solution Containing 33% DMF.

Ac-Phe-OEt, (○); **4**, (●); **5**, (■). For conditions, see Materials and Methods.

Figure 2 shows the time course of an α-chymotrypsin-catalyzed reaction at pH 6.0 in the presence of 33% DMSO (a combination of the optimal conditions). For this case, the concentration of the enzyme was reduced 5-fold because of its high reactivity. Under these conditions, the yield of **4** exceeded 50%, and the selectivity was indeed improved to 12. Moreover, the enzymatic hydrolysis of the product peptide, which is often a serious drawback of the enzymatic method, was found to be almost negligible.

Morihara and Oka[11] reported that with L-leucine amide (Leu-NH_2) as a nucleophile, α-chymotrypsin exhibits high catalytic activity for peptide synthesis under alkaline conditions (pH > 9). Deprotonation of the α-amino group of Leu-NH_2 at high pH should be responsible for this phenomenon. In the present study, however, the yield of **4** became the highest at pH 6, although a lower fraction of α-amino groups of **1** is deprotonated at this pH than pH 8.5. Consequently, at least in the present case, the regioselectivity seems to be controlled by the ionization state of the enzyme, and not by the protonation state of the substrate.

The cosolvent concentration was also found to affect the regioselectivity. When the concentration of DMF was reduced from 33% to 20% (aqueous buffer, pH 6), the yield of **5** was suppressed to 2%, as seen in Fig. 3.

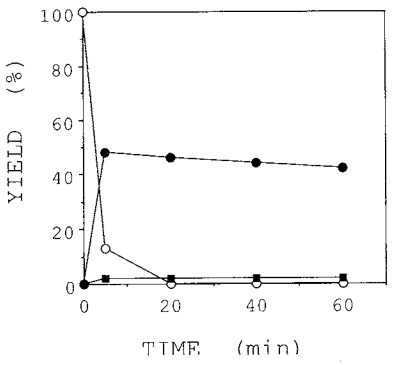


Fig. 3. Time Course of the α-Chymotrypsin-catalyzed Coupling Reaction between Ac-Phe-OEt and Lys-O-*tert*-Bu in a pH 6.0 Aqueous Buffer Solution Containing 20% DMF.

Ac-Phe-OEt, (○); **4**, (●); **5**, (■). For conditions, see Materials and Methods.

Although the yield of **4** never exceeded 50% (presumably due to a greater fraction of water), the ratio between the two isomers after 5 min reached 27, which is 10-fold higher than the selectivity in a pH 8.5 buffer solution with 33% of DMF.

Recently Rubio *et al.*[12] have reported that the regioselectivity of *P. cepacia* lipase, when used as a catalyst of a transesterification reaction in anhydrous organic solvents, depends on the hydrophobicity of the reaction medium. The present study reveals that in aqueous solution it is also possible to significantly alter the enzyme's regioselectivity by changing medium conditions.

Specific synthesis of the ε-isomer in an organic solvent

Table I indicates that the four bacterial proteases catalyze the specific formation of the ε-isomer of a dipeptide (**5**). Changing pH or cosolvent did not affect regioselectivity: only the ε-isomer formation was observed, although the yield was about 30% or less because of competitive enzymatic hydrolysis of the ester substrate.

Enzymatic reactions in anhydrous organic solvent[13] have emerged as a powerful tool for the production of a variety of compounds, including peptides.[14,15] This approach seemed to be an ideal way to avoid the present side reaction.

Table IV. Ppotease-catalyzed Coupling Reaction between Ac-Phe-OEt and Lys-O-*tert*-Bu[a] in Anhydrous *tert*-Amyl Alcohol

Enzyme	Conversion (%)[b]	**4**(%)	**5**(%)
Subtilisin Carlsberg	93	0.8	88
Subtilisin BPN′	51	0.3	49
Proteinase K	77	0.4	75
Alkaline protease	75	2.2	66

[a] For conditions, see Materials and Methods.
[b] Consumption of Ac-Phe-OEt.

Table V. ^{1}H NMR Chemical Shift (δ value from TMS) of Lysine's α-Methine and ε-Methylene Protons in Compounds **1**–**7**[a]

Compound	Chemical Shift (ppm)		Solvent
	α-Methine	ε-Methylene	
1	3.31	2.74	CD_3OD
2	3.32	2.71	$CDCl_3$
3	3.61	2.66	CD_3OD
4	4.27	2.85	CD_3OD
5	3.28	3.08	CD_3OD
6	3.26	3.10	$CDCl_3$
7	3.62	3.05	CD_3OD

[a] For conditions, see Materials and Methods.

We tested more than ten anhydrous solvents as reaction media for the present enzymatic reaction, and found that *tert*-amyl alcohol, which has been successfully used for peptide syntheses,[7,15,16] is the best medium for enzymatic activity. (α-Chymotrypsin and elastase did not exhibit any appreciable catalytic activities in the tested anhydrous solvents.) As one can see in Table IV, the ε-selectivity of the enzymes was maintained even in the anhydrous solvent, and the side reaction was indeed greatly diminished to give a satisfactory yield of **5**. With the enzyme giving the highest yield, subtilisin Carlsberg, we succeeded in the specific preparative synthesis of **5** with an 85% yield without protection–deprotection processes.

Table V depicts ^{1}H NMR chemical shift data of α-methine and ε-methylene protons of the

lysine moiety in compounds **1**–**7**. By comparing the data for **5** with those for **1**, one can conclude that only the ε-protons of **5** show a downfield shift from 2.74 to 3.08 ppm, while no significant change in the chemical shift is observed in the α-methine proton. Consequently, **5** is identified as a derivative of **1**, the ε-amino group of which is acylated (the ε-substituted derivative). By analyzing the data of **4** and **1** in the same way, **4** can be identified as the α-substituted derivative.

Since the selectivity expressed by subtilisin in *tert*-amyl alcohol is quite unique and of synthetic usefulness, we next examined its applicability to the analogues of **1**: D-Lys-O-*tert*-Bu (**2**) and Phe-Lys-O-*tert*-Bu (**3**).

D-Amino acids are generally poor substrates of proteases. However, we expected that the ε-amino group of D-lysine might still serve as a substrate for subtilisin because of its remoteness from the asymmetric center. Under the same conditions as those for the synthesis of **5**, we succeeded in the specific synthesis of Ac-Phe-ε-D-Lys-O-*tert*-Bu (**6**) with an 83% yield: the structure was confirmed by analyzing the ^{1}H NMR data of **2** and **6** in Table V.

Phenylalaninamide was reported to be a good substrate for subtilisin-catalyzed peptide synthesis in *tert*-amyl alcohol.[14] Since the present study demonstrates that subtilisin exhibits ε-specificity for lysine, **3** should be an interesting substrate to see whether subtilisin can distinguish between the two reactive amino groups: the α-amino group of phenylalanine and the ε-amino group of lysine. The single product of the reaction between Ac-Phe-OEtCl and **3** in *tert*-amyl alcohol catalyzed by subtilisin Carlsberg and isolated with a 77% yield, was identified as the ε-substituted derivative Phe-Lys(Ac-Phe)-O-*tert*-Bu **7** (by comparing the ^{1}H NMR data for **7** to those for **3** in Table V). No formation of Ac-Phe-Phe-Lys-O-*tert*-Bu was observed by HPLC, which implies that the α-amino group of **3** was completely untouched in spite of its inherent high reactivity. If the regioselectivity shown here is generally applicable, the subtilisin-catalyzed coupling in *tert*-amyl alchol can provide a new method for a selective modification of peptides, which should be very interesting for peptide chemists.

Table VI. PROTEASE-CATALYZED HYDROLYSIS OF **4** AND **5**[a]

Enzyme	Hydrolyzed portion (%)	
	4	**5**
α-Chymotrypsin	100	0
Subtilisin Carlsberg	94	0
Elastase	100	0
Subtilisin BPN′	90	0
Proteinase K	78	0
Alkaline protease	53	0

[a] For conditions, see Materials and Methods.

Enzymatic hydrolysis of α- and ε-isomers

One of the expected characteristics of an ε-substituted (isopeptide) lysine derivative is its high stability against protease-catalyzed hydrolysis. Table VI shows that no appreciable hydrolysis of **5** was observed in the presence of any of the proteases, while **4** (a "normal" peptide) was readily degraded by each of them.

Our data have established that the four bacterial proteases employed in this study are highly specific catalysts for the formation of **5** which they cannot hydrolyze and are inactive in the synthesis of **4** which they cleave efficiently. These conclusions may seem to be contradictory to the principle of microscopic reversibility.

Note, however, that enzymatic peptide synthesis from an ester substrate as the acyl donor, so called kinetically controlled synthesis,[1] is not the reverse reaction of enzymatic peptide hydrolysis. Consequently, the reaction paths are distinct to and from the transition state of these two reactions, and this difference is apparently sufficient to allow the enzyme to exhibit opposite selectivities in the closely related reactions.

In summary, our study showed that the specific modification of the amino groups of lysine is feasible by protease catalysis. α-Chymotrypsin is a good catalyst for the synthesis of the α-substituted lysine derivative

("normal peptides), although its reactivity and selectivity strongly depend on the medium conditions. Subtilisin and other bacterial proteases are excellent catalysts for the production of ε-substituted lysine derivatives (isopeptides), especially when used in anhydrous *tert*-amyl alcohol. We believe that the latter reaction system will be a general method for the regioselective modification of polyamines*[1] and are conducting further investigations in this direction.

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*[1] Our preliminary data have shown that subtilisin Carlsberg exhibits δ-selectivity to ornithine derivatives.