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Enzymatic Coupling of α,α-Dialkyl Amino Acids using Inverse Substrates as Acyl Donors[1]

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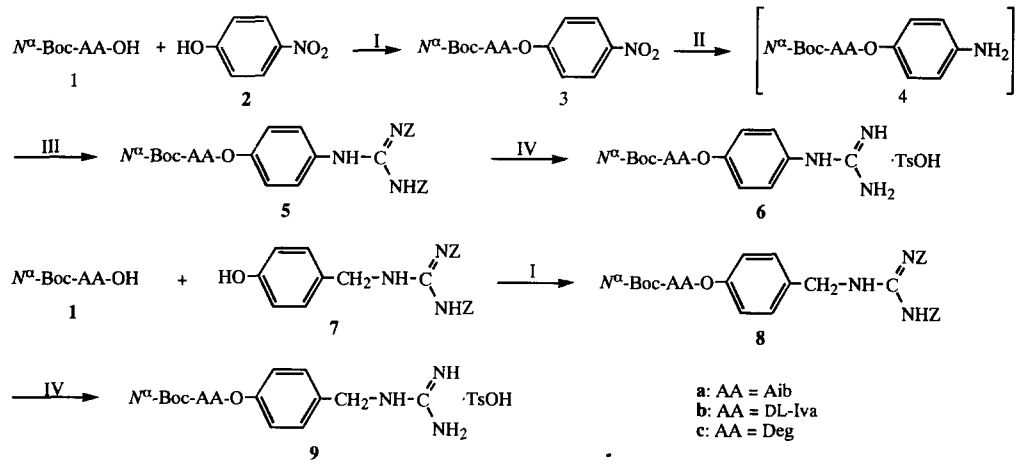
Abstract: Two series of inverse substrates, N^{α}-Boc-α,α-dialkyl amino acid *p*-guanidino- and *p*-(guanidinomethyl)phenyl esters, were prepared as acyl donor components for enzymatic peptide synthesis. They were found to be readily coupled with amino acid *p*-nitroanilide to produce peptide. *Streptomyces griseus* trypsin was a more efficient catalyst than bovine trypsin.

Sterically hindered α-amino acids such as the α-aminoisobutylic acid (Aib, 2-methylalanine) and isovaline (Iva, 2-ethylalanine, α-amino-α-methylbutyric acid) are constituents of naturally occurring peptide antibiotics.[2] Peptides containing these amino acids, especially achiral Aib and 2,2-diethylglycine (Deg, α-amino-α-ethylbutyric acid), are of interest as the model for conformational analysis of the peptide backbone (formation of α- or 3_{10}-helices, or β-turns).[3] However, the introduction of α,α-dialkyl amino acids into a peptide is difficult,[4] because the reactivity of sterically hindered α,α-disubstituted amino acids is much lower than that of typical α-amino acids. Kiso *et al.* recently developed a suitable coupling reagent, CIP, in the presence of an additive HOAt or HODhbt, for α,α-dialkyl amino acids,[5] and reported its use for the chemical synthesis of Alamethicin F-30 in solution.[6] It is known that enzymatic peptide synthesis is more advantageous than the chemical synthesis in many respects (highly stereoselective, racemization-free and requires minimal side-chain protection).[7] Thus, synthesis of peptides containing α,α-dialkyl amino acids by virtue of the enzymatic method seems promising. The most serious defect of the enzymatic method, however, is the discrimination of the amino acid residue due to the substrate specificity. Thus, the use of proteases in peptide synthesis has been limited by the specificity of the enzymes.[7]

Previously, we reported[8] that inverse substrates such as *p*-amidino- and *p*-guanidinophenyl esters behave as specific substrates for trypsin and trypsin-like enzymes and allow the specific introduction of an acyl group carrying a non-specific residue into the enzyme active site. The characteristic features of inverse substrates suggested that they are useful for enzyme-catalyzed peptide synthesis. In fact, we demonstrated successful application of inverse substrates for trypsin-catalyzed coupling.[9] Herein, we report the enzymatic coupling of less reactive substrates carrying hindered amino acid. Comparison was made at the applicability of two trypsins of different origin (bovine and *streptomyces griseus* (SG) trypsin) as the catalyst for the sterically less favorable

process.

We designed and synthesized two series of inverse substrates, N^{α}-Boc-α,α-dialkyl amino acid *p*-guanidino- and *p*-(guanidinomethyl)phenyl esters, both having a guanidine moiety. Synthetic procedures are shown in Scheme 1. A series of *p*-guanidinophenyl esters (**6a-c**)[10] was prepared by condensation of N^{α}-Boc-amino acids (**1**) with *p*-nitrophenol (**2**), reduction of the nitro group, amidination and final deprotection. Total yields are



I, DCC, DMAP in AcOEt; II, H_2, 10%Pd-C in EtOH; III, 1-[*N*,*N*'-bis(Z)amidino]pyrazole in THF; IV, H_2, 10%Pd-C, *p*-TsOH·H_2O in EtOH-Et_2O

Scheme 1. Synthetic route for inverse substrates (**6a-c** and **9a-c**)

66% for **6a**, 69% for **6b** and 53% for **6c**, respectively. The other series of compounds was *p*-(guanidinomethyl)phenyl esters (**9a-c**), which was newly designed as inverse substrates. The guanidino group was not directly substituted on the phenyl ring; therefore, they were to be expected more resistant toward spontaneous hydrolysis than the *p*-guanidinophenyl esters (**6**). A *p*-[*N*',*N*"-bis(Z)-guanidinomethyl]phenol (**7**) was prepared from *p*-cyano- phenol through 4 steps in 39% total yield. N^{α}-Boc-amino acid *p*-(guanidinomethyl)phenyl esters (**9a-c**)[10] were prepared by condensation of **1a-c** with phenol derivative (**7**) and subsequent deprotection. Total yields are 59% for **9a**, 64% for **9b** and 39% for **9c**, respectively.

Enzymatic peptide coupling reaction was carried out by incubating an acyl donor (1 mM) with an acyl acceptor (L-Ala-*p*NA) (**10**) (20 mM) and enzyme (10 μM) in a mixture of MOPS buffer (50 mM, pH 8.0, containing 20 mM $CaCl_2$) and DMSO (1:1) at 25°C (Scheme 2). The progress of the coupling reaction was monitored by HPLC.[11] Elution peaks were correlated to those of authentic samples which were chemically synthesized.[12] The coupling yield was determined during the period of the reaction time which was prolonged to 72h. In Table 1, coupling reactions are summarized with the reaction period required for the attainment of the maximum yield.

6a-c or **9a-c** + L-Ala-*p*NA (**10**) $\xrightarrow{\text{enzyme}}$ N^{α}-Boc-AA-L-Ala-*p*NA (**11a-c**)

Scheme 2. Enzymatic coupling of inverse substrates (**6a-c** and **9a-c**)

Coupling reactions of *p*-guanidinophenyl ester (**6**) and *p*-(guanidinomethyl)phenyl ester (**9**) with L-alanine *p*-nitroanilide (L-Ala-*p*NA) (**10**) were compared in the case of each catalyst, bovine and SG trypsin. Bovine and

SG trypsin achieved almost the same efficiency in the coupling reaction of **6a** (Entry 1 and 2 in Table 1). In the case of **9a**, however, the coupling yields strongly depend on the catalyst, and the reactions were slower. Almost quantitative coupling yield was obtained in the SG trypsin catalyzed reaction. The difference between **6a** and **9a** in the coupling rates seemed to be due to the difference in acyl-enzyme formation rates. In any event, both bovine and SG trypsins are useful for the synthesis of hindered peptides such as N^{α}-Boc-Aib-L-Ala-*p*NA (**11a**).

Table 1. Peptide coupling by use of an inverse substrate as the acyl donor component

Entry No.	Acyl donor (No.)	Reaction time(h)	Enzyme	Product (No.)	Yield(%)
1	N^{α}-Boc-Aib-OG (**6a**)	0.5	bovine trypsin	N^{α}-Boc-Aib-L-Ala-*p*NA (**11a**)	55
2	N^{α}-Boc-Aib-OG (**6a**)	0.5	SG trypsin	N^{α}-Boc-Aib-L-Ala-*p*NA (**11a**)	57
3	N^{α}-Boc-Aib-OGM (**9a**)	24	bovine trypsin	N^{α}-Boc-Aib-L-Ala-*p*NA (**11a**)	23
4	N^{α}-Boc-Aib-OGM (**9a**)	24	SG trypsin	N^{α}-Boc-Aib-L-Ala-*p*NA (**11a**)	96
5	N^{α}-Boc-DL-Iva-OG (**6b**)	10	bovine trypsin	N^{α}-Boc-DL-Iva-L-Ala-*p*NA (**11b**)	78
6	N^{α}-Boc-DL-Iva-OG (**6b**)	5	SG trypsin	N^{α}-Boc-DL-Iva-L-Ala-*p*NA (**11b**)	80
7	N^{α}-Boc-DL-Iva-OGM (**9b**)	48	bovine trypsin	N^{α}-Boc-DL-Iva-L-Ala-*p*NA (**11b**)	11
8	N^{α}-Boc-DL-Iva-OGM (**9b**)	48	SG trypsin	N^{α}-Boc-DL-Iva-L-Ala-*p*NA (**11b**)	95
9	N^{α}-Boc-Deg-OG (**6c**)	48	bovine trypsin	N^{α}-Boc-Deg-L-Ala-*p*NA (**11c**)	16
10	N^{α}-Boc-Deg-OG (**6c**)	48	SG trypsin	N^{α}-Boc-Deg-L-Ala-*p*NA (**11c**)	35
11	N^{α}-Boc-Deg-OGM (**9c**)	48	bovine trypsin	N^{α}-Boc-Deg-L-Ala-*p*NA (**11c**)	n.d.*
12	N^{α}-Boc-Deg-OGM (**9c**)	48	SG trypsin	N^{α}-Boc-Deg-L-Ala-*p*NA (**11c**)	n.d.*

* not detected.

In the case of Iva, racemate was used. The potencies of the acyl donor were not different within the enantiomeric pair as shown in the previous paper.[9] In fact, two diastereomeric peptides were formed in 1:1 ratio when N^{α}-Boc-DL-Iva (**6b**) was reacted with **10**. N^{α}-Boc-DL-Iva (**6b**) gave the coupling product in satisfactory yield with bovine and SG trypsin, respectively, even though **6b** is less reactive than **6a**. *p*-(Guanidinomethyl)phenyl ester (**9b**) as well as **9a** exhibited remarkably different responses toward bovine and SG trypsin (Entry 7 and 8 in Table 1). On the other hand, Deg derivatives (**6c** and **9c**) apparently are ineffective substrates for either enzymatic coupling reaction (Entry 9-12 in Table 1). Possibly this is attributable to the steric hindrance due to the α,α-diethyl group of the substrate. It should be emphasized that the guanidinomethyl group discriminates between SG and bovine trypsin as shown in Table 1 (Entry 3 and 4, 7 and 8). In our previous work, spatial structure of the binding pocket was compared within the trypsin family, and it was deduced that SG trypsin has a spatially less restricted binding pocket compared to bovine trypsin.[13] SG trypsin is still effective with *p*-(guanidinomethyl)phenyl esters (**9**) even though the distance between the positive charge and the carbonyl carbon (8.378 Å) is longer than that in the case of *p*-guanidinophenyl esters (**6**)(7.726 Å). Differences in the coupling rates and yields shown in Table 1 might be responsible mainly for the acylation step. High accessibility of the carbonyl carbon of the acyl donor to the catalytic serine residue of the trypsin, i.e., efficient acylation, is the characteristic feature of inverse substrates.[8] For sterically hindered inverse substrates,

however, the efficiency at the acylation step will be decreased, and the diminished acylation rate will strongly affect the coupling rate and yield. Versatility of inverse substrates in the peptide coupling reaction is predictable from their kinetic parameters for trypsin-catalyzed hydrolysis. In the comparison of **6a** and **9a**, only the acylation rate constant was different (ca. 2000 times) though binding affinity and deacylation rate constant were shown to be comparable (unpublished data).[14] Thus, the acylation process (especially for sterically hindered substrates) is considered as the major and critical determinant of the coupling rate and yield. Substrates (**6a**) underwent rapid acylation, and complete accumulation of the acyl enzyme resulted. The coupling yield was, nevertheless, unsatisfactory. This is possibly due to inherent susceptibility of the resulted acyl enzyme to the hydrolysis, because no acyl donor (**6a**) remained after 0.5 h.

In conclusion, it can be proposed that trypsin-catalyzed peptide bond formation is achievable with both N^{α}-Boc-α,α-dialkyl amino acid *p*-guanidino- and *p*-(guanidinomethyl)phenyl esters as acyl donors. The SG trypsin is a more efficient catalyst than the bovine trypsin. The coupling reaction of N^{α}-Boc-Deg derivative (**9c**) was difficult even using SG trypsin. It is also proposed that the secondary hydrolysis of the coupling product can be disregarded in our enzymatic procedure, since the coupling product is not decreased even after 72h reaction time.

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Abbreviations

CIP=2-chloro-1,3-dimethylimidazolidium hexafluorophosphate, HOAt=1-hydroxy-7-azabenzotriazole, HODhbt =3-hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine, Boc=*tert*-butyloxycarbonyl, DCC=*N*,*N*'-dicyclohexylcarbodiimide, DMAP=4-dimethylaminopyridine, Z=benzyloxycarbonyl, MOPS=3-morpholino-1-propanesulfonate.

References and Notes

1. This paper is dedicated to Professor Yoshito Kishi on the occasion of his 60th birthday.
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