Enzymatic Peptide Synthesis with *p*-Guanidinophenyl and *p*-(Guanidinomethyl)phenyl Esters as Acyl Donors[1)]

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Two series of "inverse substrates", *N*-Boc-amino acid *p*-guanidinophenyl and *p*-(guanidinomethyl)phenyl esters, were prepared as acyl donor components for enzymatic peptide synthesis. The kinetic behavior of these esters toward bovine and *Streptomyces griseus* (SG) trypsin was analyzed. The spatial requirement of the active site of these enzymes for catalytic efficiency is discussed based on the steric characteristics of the substrates. These substrates were found to couple readily with amino acid *p*-nitroanilides to produce peptides. SG trypsin was the most efficient catalyst among the enzymes tested (bovine, porcine, and SG trypsin).

Key words enzymatic peptide synthesis; *p*-guanidinophenyl ester; *p*-(guanidinomethyl)phenyl ester; bovine trypsin; porcine trypsin; *Streptomyces griseus* trypsin

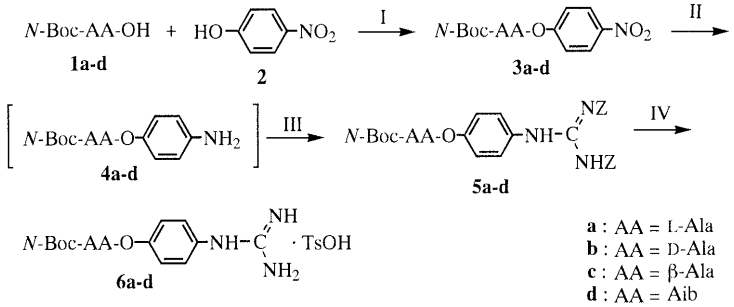
Peptide synthesis by protease-catalyzed reverse reaction has been extensively studied with a variety of amino acids and peptide derivatives as coupling compounds.[2)] It is known that enzymatic peptide synthesis is more advantageous than chemical synthesis in many respects; it is highly stereoselective and racemization-free, and requires minimal side-chain protection.[2)] The most serious defect of the enzymatic method, however, is the restricted substrate specificity. Thus, the application of proteases for peptide synthesis has been limited due to the specificity of the enzymes.

In a previous paper, we reported that the *p*-amidinophenyl and *p*-guanidinophenyl esters behave as specific substrates for trypsin and trypsin-like enzymes.[3,4)] In these esters the site-specific groups (charged amidinium and guanidinium) for the enzyme are included in the leaving-group portion instead of being in the acyl moiety. Such a substrate is termed an "inverse substrate".[5)] Inverse substrates allow the specific introduction of an acyl group carrying a non-specific residue into the trypsin active site without recourse to a cationic acyl moiety, which is a characteristic of conventional substrates. These acyl trypsin intermediates are expected to play a key role in trypsin-catalyzed peptide synthesis. We previously reported bovine trypsin-catalyzed peptide synthesis by using *N*-Boc-amino acid *p*-amidinophenyl and *p*-guanidinophenyl esters as acyl donors.[6)]

In the previous communication, we also reported that even α,α-diakyl amino acids could be used in *Streptomyces griseus* (SG) trypsin-catalyzed coupling reactions.[7)] Herein, we report the kinetic behavior of *N*-Boc–amino acid *p*-guanidinophenyl (*N*-Boc–AA–OG) and *p*-(guanidinomethyl)phenyl esters (*N*-Boc–AA–OMG) with bovine and SG trypsin, and we describe enzymatic peptide synthesis with these esters as acyl donor components. The applicability of three trypsins of different origins (bovine, porcine and SG trypsin) as catalysts for sterically less favorable reaction processes was also studied.

Synthesis of Inverse Substrates We synthesized two series of substrates, *N*-Boc–AA–OG (**6a—d**) and *N*-Boc–AA–OGM (**13a—d**), both having a guanidino moiety. For the synthesis of guanidinophenyl and (guanidinomethyl)phenyl esters, introduction of a guanidino group seems to be the key step. One possible approach is to carry out the key step at the final stage, as reported in our previous paper.[3)] Another is to carry out the key step at the first stage. In this study, synthesis of guanidinophenyl esters was carried out by applying the former procedure, while synthesis of (guanidinomethyl)phenyl esters employed the latter method, as shown in Charts 1 and 2.

The predicted pK_a value[8)] of *p*-(guanidinomethyl)phenol (8.91) is larger than that of the *p*-guanidinophenol (7.40). Therefore, *N*-Boc–AA–OGM (**13**) are conjugates derived from the less acidic phenol derivative and, thus are expected to be more resistant to spontaneous hydrolysis than the *N*-Boc–AA–OG (**6**). The resistance of substrates



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

Chart 1

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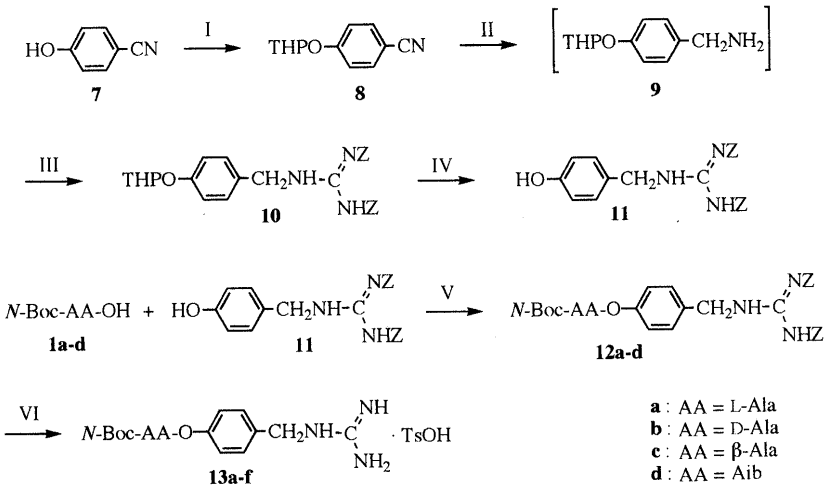

I, 3,4-dihydro-2*H*-pyran, *p*-TsOH·H_2O in benzene;
II, $LiAlH_4$ in THF;
III, 1-[*N*,*N*'-bis(Z)amidino]pyrazole in THF;
IV, *p*-TsOH·H_2O in MeOH;
V, DCC, DMAP in DMF-CH_2Cl_2;
VI, H_2, 10%Pd-C, *p*-TsOH·H_2O in EtOH-Et_2O

Chart 2

Table 1. Kinetic Parameters for the Bovine and SG Trypsin-Catalyzed Hydrolysis of Inverse Substrates

Substrate (No.)	K_s (K_m) (M)	k_2 (s^{-1})	k_3 ($k_{cat.}$) (s^{-1})	k_2/K_s ($k_{cat.}/K_m$) ($s^{-1}M^{-1}$)
Bovine trypsin				
N-Boc-L-Ala-OG (**6a**)[a]	2.77×10^{-4}	1.23×10^{2}	4.09×10^{-1}	4.44×10^{5}
N-Boc-D-Ala-OG (**6b**)[a]	6.62×10^{-4}	6.71×10	3.51×10^{-2}	1.01×10^{5}
N-Boc-Aib-OG (**6d**)	2.37×10^{-3}	1.57	2.58×10^{-3}	6.64×10^{2}
N-Boc-L-Ala-OMG (**13a**)	(4.31×10^{-4})	—	(7.60×10^{-1})	(1.76×10^{3})
N-Boc-Aib-OMG (**13d**)	(1.83×10^{-4})	(7.90×10^{-4})[b]	(6.05×10^{-4})	(3.31)
SG trypsin				
N-Boc-L-Ala-OG (**6a**)	1.28×10^{-4}	7.35×10	5.05	5.72×10^{5}
N-Boc-D-Ala-OG (**6b**)	1.66×10^{-4}	5.07×10	1.37×10^{-1}	3.05×10^{5}
N-Boc-Aib-OG (**6d**)	7.70×10^{-4}	7.88	4.18×10^{-2}	1.02×10^{4}
N-Boc-L-Ala-OMG (**13a**)	(1.13×10^{-4})	(2.52)[c]	(1.68)	(1.49×10^{4})
N-Boc-Aib-OMG (**13d**)	(1.99×10^{-4})	—	(7.24×10^{-2})	(3.63×10^{2})

a) See reference 4. *b*) Calculated value using $k_3=2.58\times10^{-3}\,s^{-1}$ for *N*-Boc-Aib-OG (**6d**) with the following equation: $k_{cat.}=k_2k_3/(k_2+k_3)$. *c*) Calculated value using $k_3=5.05\,s^{-1}$ for *N*-Boc-L-Ala-OG (**6a**) with the following equation: $k_{cat.}=k_2k_3/(k_2+k_3)$.

to spontaneous hydrolysis has significant effect on the peptide coupling yield, especially in the coupling of sterically hindered α-amino acids.

Kinetic Parameters for Enzymatic Hydrolysis The kinetics of bovine and SG trypsin-catalyzed hydrolyses of typical inverse substrates were analyzed.[9] Determination of kinetic parameters was carried out as described,[3,5] and the values obtained are listed in Table 1. Kinetic parameters for *N*-Boc-L-Ala-OMG (**13a**) were compared with those for *N*-Boc-L-Ala-OG (**6a**), which have been reported in our previous paper.[4] The K_m and $k_{cat.}$ values of **13a** are nearly equivalent to K_s and k_3 of **6a**. The parameter, k_2/K_s (or $k_{cat.}/K_m$[10]), introduced by Brot and Bender is informative for the evaluation of the specificity of substrates.[11] This k_2/K_s (or $k_{cat.}/K_m$) value of **13a** is two orders of magnitude smaller than that of **6a**. It could be considered that the less specific character of **13a** arises from the greater distance between the carbonyl carbon atom and the guanidino group. The kinetics of *N*-Boc-α-isobutyric acid *p*-guanidinophenyl ester (*N*-Boc-Aib-OG) (**6d**) were also compared with those of *N*-Boc-L-Ala-OG (**6a**). The values of all parameters of **6d** were one—three orders of magnitude smaller than those of **6a**, as shown in Table 1. This result indicated that the bulky α,α-dialkyl substituent impedes the placement of **6d** in juxtaposition with the active site of trypsin. The $k_{cat.}/K_m$ value for *N*-Boc-Aib-OMG (**13d**) showed a much less favorable interaction.

In our previous work, the 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 than bovine trypsin.[12] Therefore, SG trypsin could be expected to exhibit specific interaction even with bulky substrates such as **6d** and **13d**. The results in Table 1 showed that catalytic hydrolysis of **6d** and **13d** is more efficient with SG trypsin than with bovine trypsin.

Strict stereo-specificity is one of the characteristic features of enzyme-catalyzed reactions. This stereo-specific-

Table 2. Yield of Enzymatic Peptide Synthesis[a]

Enzyme	Entry No.	Acyl donor (No.)	Reaction time (h)	Product (No.)	Yield (%)
Bovine trypsin	1	*N*-Boc-L-Ala-OG (**6a**)	0.2	*N*-Boc-L-Ala-L-Ala-*p*NA (**16a**)	64[b]
	2	*N*-Boc-L-Ala-OMG (**13a**)	5	*N*-Boc-L-Ala-L-Ala-*p*NA (**16a**)	95
	3	*N*-Boc-D-Ala-OG (**6b**)	0.3	*N*-Boc-D-Ala-L-Ala-*p*NA (**16b**)	74[b]
	4	*N*-Boc-D-Ala-OMG (**13b**)	24	*N*-Boc-D-Ala-L-Ala-*p*NA (**16b**)	63
	5	*N*-Boc-β-Ala-OG (**6c**)	0.5	*N*-Boc-β-Ala-L-Ala-*p*NA (**16c**)	54
	6	*N*-Boc-β-Ala-OMG (**13c**)	5	*N*-Boc-β-Ala-L-Ala-*p*NA (**16c**)	44
	7	*N*-Boc-Aib-OG (**6d**)	0.5	*N*-Boc-Aib-L-Ala-*p*NA (**16d**)	55[c]
	8	*N*-Boc-Aib-OMG (**13d**)	24	*N*-Boc-Aib-L-Ala-*p*NA (**16d**)	23[c]
	13	*N*-Boc-L-Ala-OMG (**13a**)	5	*N*-Boc-L-Ala-D-Ala-*p*NA (**17a**)	20
	14	*N*-Boc-D-Ala-OMG (**13b**)	24	*N*-Boc-D-Ala-D-Ala-*p*NA (**17b**)	14
Porcine trypsin	15	*N*-Boc-L-Ala-OG (**6a**)	0.5	*N*-Boc-L-Ala-L-Ala-*p*NA (**16a**)	60
	16	*N*-Boc-L-Ala-OMG (**13a**)	5	*N*-Boc-L-Ala-L-Ala-*p*NA (**16a**)	74
	17	*N*-Boc-D-Ala-OG (**6b**)	0.5	*N*-Boc-D-Ala-L-Ala-*p*NA (**16b**)	76
	18	*N*-Boc-D-Ala-OMG (**13b**)	24	*N*-Boc-D-Ala-L-Ala-*p*NA (**16b**)	71
	19	*N*-Boc-β-Ala-OG (**6c**)	0.5	*N*-Boc-β-Ala-L-Ala-*p*NA (**16c**)	41
	20	*N*-Boc-β-Ala-OMG (**13c**)	10	*N*-Boc-β-Ala-L-Ala-*p*NA (**16c**)	50
	21	*N*-Boc-Aib-OG (**6d**)	1	*N*-Boc-Aib-L-Ala-*p*NA (**16d**)	56
	22	*N*-Boc-Aib-OMG (**13d**)	24	*N*-Boc-Aib-L-Ala-*p*NA (**16d**)	36
	23	*N*-Boc-L-Ala-OMG (**13a**)	5	*N*-Boc-L-Ala-D-Ala-*p*NA (**17a**)	12
	24	*N*-Boc-D-Ala-OMG (**13b**)	24	*N*-Boc-D-Ala-D-Ala-*p*NA (**17b**)	15
SG trypsin	25	*N*-Boc-L-Ala-OG (**6a**)	0.5	*N*-Boc-L-Ala-L-Ala-*p*NA (**16a**)	73
	26	*N*-Boc-L-Ala-OMG (**13a**)	5	*N*-Boc-L-Ala-L-Ala-*p*NA (**16a**)	89
	27	*N*-Boc-D-Ala-OG (**6b**)	0.5	*N*-Boc-D-Ala-L-Ala-*p*NA (**16b**)	78
	28	*N*-Boc-D-Ala-OMG (**13b**)	24	*N*-Boc-D-Ala-L-Ala-*p*NA (**16b**)	97
	29	*N*-Boc-β-Ala-OG (**6c**)	0.5	*N*-Boc-β-Ala-L-Ala-*p*NA (**16c**)	61
	30	*N*-Boc-β-Ala-OMG (**13c**)	24	*N*-Boc-β-Ala-L-Ala-*p*NA (**16c**)	73
	31	*N*-Boc-Aib-OG (**6d**)	0.5	*N*-Boc-Aib-L-Ala-*p*NA (**16d**)	57[c]
	32	*N*-Boc-Aib-OMG (**13d**)	24	*N*-Boc-Aib-L-Ala-*p*NA (**16d**)	96[c]
	33	*N*-Boc-L-Ala-OMG (**13a**)	5	*N*-Boc-L-Ala-D-Ala-*p*NA (**17a**)	13
	34	*N*-Boc-D-Ala-OMG (**13b**)	24	*N*-Boc-D-Ala-D-Ala-*p*NA (**17b**)	16

a) Conditions: acyl donor, 1 mM; acyl acceptor (L-Ala-*p*NA), 20 mM; enzyme, 10 μM; 50% DMSO–MOPS (50 mM, pH 8.0 containing 20 mM $CaCl_2$); 25 °C. *b*) See reference 5*b*. *c*) See reference 7.

ity is exhibited toward typical conventional substrates for each enzyme. The inverse substrate, however, has a unique structure which makes it difficult for the enzyme to discriminate the enantiomers of the substrate. An achiral site-specific guanidinium group which is recognized by the binding site of the enzyme was included in the leaving portion of the substrate. Therefore, the asymmetric acyl group plays a minor role in the interaction with the enzyme binding site. Enantiomers **6a** and **6b** did not greatly differ in their interactions with bovine or SG trypsin. SG trypsin is rather preferable for the hydrolysis of **6b**.

Enzymatic Peptide Synthesis Enzymatic peptide coupling reaction was carried out by incubating an inverse substrate (acyl donor, 1 mM) with L- or D-Ala-*p*NA (acyl acceptor, 20 mM) and enzyme (10 μM) in a mixture (1 : 1) of MOPS buffer (50 mM, pH 8.0, containing 20 mM $CaCl_2$) and DMSO at 25 °C. The progress of the coupling reaction was monitored by HPLC. Elution peaks were correlated to those of authentic samples obtained by chemical synthesis.[13] The coupling yield was determined for reaction times up to 72 h. In Table 2, the coupling reactions are listed with the reaction period required for the attainment of the maximum yield. Coupling reactions of *N*-Boc–AA–OG (**6**) and *N*-Boc–AA–OGM (**13**) with three catalysts, bovine, porcine, and SG trypsin, were compared.

Coupling yields of peptides were fundamentally equivalent for the reactions using **13** and **6** though the reactions with **13** were slow. Bovine and porcine trypsin catalyzed the reaction in a similar manner, but SG trypsin showed different behavior (Table 2). The coupling reaction involving a D-acyl acceptor was not favorable (Entries 13, 14, 23, 24, 33 and 34). In contrast to the acyl acceptor, the configuration of the acyl donor did not markedly affect the reaction yield. The reaction rate for the acyl donor derived from D-amino acid was more or less slower, but the final coupling yield was nearly equal to that of the corresponding L-enantiomer. This was also the case for the acyl donors other than the D-alanine derivative (**6b**). The final coupling yield for the reaction using an inverse substrate such as *N*-Boc-D-Phe-OG or *N*-Boc-D-Ala-D-Ala-OG as the acyl donor is as good as that of the corresponding acyl donor derived from the L-enantiomer.[5b] In the case of product **16a**, the best yield was obtained by bovine typsin-catalyzed coupling of *N*-Boc-L-Ala-OMG (**13a**) with **14**, while the coupling product **16b** was obtained quantitatively by means of the SG trypsin-catalyzed reaction (Entry 28 in Table 2). The peptide (**16c**) containing a β-amino acid was also obtained in satisfactory yield by using the combination of a *p*-(guanidinomethyl)phenyl derivative (**13c**) with SG trypsin.

In the case of **13d** as an acyl donor, the coupling

yields strongly depended on the catalyst, and an almost quantitative coupling yield was obtained in the SG trypsin-catalyzed reaction. In any event, bovine, porcine, and SG trypsins are useful for the synthesis of hindered peptides such as *N*-Boc–Aib–L-Ala–*p*NA (**16d**).

SG trypsin is still effective with *N*-Boc–AA–OGM (**13**), even though the distance between the positive charge and the carbonyl carbon (8.378 Å) is longer than that in the case of *N*-Boc–AA–OG (**6**) (7.726 Å). The differences in the coupling rates and yields shown in Table 2 might mainly arise from 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 a characteristic feature of inverse substrates.[5] 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** ($k_2 = 73.5\,s^{-1}$) with **13a** ($k_2 = 2.52\,s^{-1}$) for SG trypsin, and **6d** ($k_2 = 1.57\,s^{-1}$) with **13d** ($k_2 = 0.00079\,s^{-1}$) for bovine trypsin, only the acylation rate constant was different (*ca.* 30 and 2000 times, respectively) though the binding affinity and deacylation rate constant were shown to be comparable (Table 1). Thus, the acylation process (especially for sterically hindered substrates) is considered to be the critical determinant of the coupling rate and yield. Substrate **6d** underwent rapid acylation, resulting in accumulation of the acyl enzyme. The coupling yield was, nevertheless, unsatisfactory. This is possibly due to inherent susceptibility of the acyl enzyme to hydrolysis, because no acyl donor (**6d**) remained after 0.5 h.

It appears that secondary hydrolysis of the coupling product can be disregarded in our enzymatic procedure, since the coupling product is not decreased even after 72 h reaction time.

Experimental

Melting points were determined on a Yanagimoto melting point apparatus and are uncorrected. IR spectra were taken on a JASCO VALOR-III. ^{1}H-NMR spectra were recorded on a JEOL EX-400 spectrometer. The optical rotations were measured with a JASCO DIP-360 digital polarimeter in a 5 cm cell. The FAB-MS were taken with a JEOL JMS-HX-110 spectrometer. Kinetic parameters were determined with a Union Giken RA-401 stopped-flow spectrometer, a Hitachi U-2000 UV spectrophotometer, and a Radiometer TTT-80 pH-stat. Bovine pancreas trypsin (EC 3.4.21.4) was purchased from Worthington Biochemical Corp. (twice crystallized, lot TRL). Porcine pancreas trypsin (EC 3.4.21.4) was purchased from Sigma Chemical Co. (lyophilized, salt-free, lot IX). *Streptomyces griseus* trypsin was prepared according to the reported procedures.[14]

***N*-(*tert*-Butyloxycarbonyl)-α-aminoisobutyric Acid *p*-Guanidinophenyl Ester *p*-Toluenesulfonate (6d)** This compound was synthesized according to the reported procedure.[3] Colorless amorphous solid. FAB-MS *m/z*: 337$(M+H)^+$.

***N*-(*tert*-Butyloxycarbonyl)amino Acid *p*-(Guanidinomethyl)phenyl Ester *p*-Toluenesulfonates (13)** These compounds (**13a—d**) were synthesized according to the previously reported procedure.[3] **13a**: Obtained in 95% yield as colorless fine plates (EtOH), mp 185—187 °C, $[\alpha]_D^{25}$ −38.8° ($c = 1.0$, MeOH). *Anal.* Calcd for $C_{23}H_{32}N_4O_7S$: C, 54.32; H, 6.34; N, 11.02; S, 6.30. Found: C, 54.20; H, 6.46; N, 10.77; S, 6.24. **13b**: Obtained in 92% yield as colorless needles. This compound showed almost the same physical and spectral data as the enantiomer (**13a**) except for the optical rotation, $[\alpha]_D^{25}$ +34.6° ($c = 1.0$, MeOH). **13c**: Obtained in 92% yield as colorless plates (EtOH–hexane), mp 185—187 °C. *Anal.* Calcd for $C_{23}H_{32}N_4O_7S$: C, 54.32; H, 6.34; N, 11.02; S, 6.30. Found: C, 54.19; H, 6.44; N, 10.94; S, 6.39. **13d**: Obtained in 82% yield as colorless plates (EtOH), mp 185—187 °C. *Anal.* Calcd for $C_{24}H_{34}N_4O_7S$: C, 55.16; H, 6.56; N, 10.72; S, 6.14. Found: C, 54.99; H, 6.73; N, 10.50; S, 6.05.

Kinetic Measurements The kinetic parameters, K_s, k_2 and k_3 for the hydrolysis of guanidinophenyl esters (**6a, b, d**), were determined by means of the thionine displacement method using a stopped-flow technique. The reaction was carried out in 50 mM Tris–HCl buffer (pH 8.0, containing 20 mM $CaCl_2$) according to the reported method.[6] The kinetic parameters, K_m and $k_{cat.}$, for (guanidinomethyl)phenyl esters (**13a, d**) were determined by using a pH-stat. The reaction was carried out in 0.1 M KCl (containing 20 mM $CaCl_2$), and the titrant used for the analysis was 10 mM NaOH.

Enzymatic Peptide Coupling Reaction Peptide coupling reaction was carried out at 25 °C in 50% DMSO–MOPS buffer (pH 8.0, containing 20 mM $CaCl_2$). Concentrations of acyl donors (**6a—d** and **13a—d**), acyl acceptor (**14** and **15**), and enzyme were 1, 20 mM, and 10 μM, respectively. The progress of the peptide coupling reaction was monitored by HPLC under the following conditions: column (4.6 × 250 mm, Wakosil 5C18-200), isocratic elution at 1 ml/min, 0.1% trifluoroacetic acid/acetonitrile. An aliquot of the reaction mixture was injected and peaks were detected at 310 nm (*p*-nitroanilide moiety).

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References and Notes

1) The following abbreviations are used: Boc = *tert*-butyloxycarbonyl, DCC = *N,N'*-dicyclohexylcarbodiimide, DMAP = 4-dimethylaminopyridine, Z = benzyloxycarbonyl, MOPS = 3-morpholino-1-propanesulfonate, OG = *p*-guanidinophenyl, OMG = *p*-(guanidinomethyl)phenyl.
2) For example: Tsuzuki H., Oka T., Morihara K., *J. Biochem.* (*Tokyo*), **88**, 669—675 (1980); Nakatuka T., Sasaki T., Kaiser E. T., *J. Am. Chem. Soc.*, **99**, 4485—4488(1987); Wong C.-H., Science, **244**, 1145—1152 (1989); Schellenberger V., Jakubke H.-D., *Angew. Chem. Int. Ed. Engl.*, **30**, 1437—1449 (1991).
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8) The calculation of pK_a values was performed using the pKalc program (CompuDrug Chemistry Ltd.).
9) The following symbols are used: K_s, dissociation constant of enzyme-substrate complex; k_2, rate constant of acylation step; k_3, rate constant of deacylation step; K_m, Michaelis constant; $k_{cat.}$ overall catalytic rate constant.
10) The $k_{cat.}/K_m$ value is equivalent to k_2/K_s as reported; Ryan J. T., Fenton W. J., II, Chang T.-I., Feinman D. R., *Biochemistry*, **15**, 1337—1341 (1976).
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