The Structural Requirements for an Inverse Substrate for Enzymatic Peptide Synthesis: Position Isomers of Guanidinonaphthyl Esters as the Acyl Donor Component[1)]

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Four series of inverse substrates, position isomers of guanidinonaphthyl esters derived from *N*-(*tert*-butyloxycarbonyl)amino acid, were prepared as acyl donor components for trypsin-catalyzed peptide synthesis. The kinetic behavior of these synthetic inverse substrates toward spontaneous and tryptic hydrolysis was analyzed. These substrates were found to readily couple with α-amino acid *p*-nitroanilide to produce peptide. 4-Guanidino-1-naphthyl esters, in which the guanidino group and the carbonyl group are aligned linearly on the shorter axis of the naphthalene ring, were the most efficient substrates for enzymatic peptide synthesis. The method was especially useful for the preparation of peptides containing α,α-dialkyl amino acids. The enzymatic hydrolysis of the resulting products was negligible.

Key words inverse substrate; enzymatic peptide synthesis; 4-guanidino-1-naphthyl ester; structural requirement; enzymatic kinetics

Considerable effort has been devoted to protease-catalyzed peptide synthesis.[2)] Enzymatic peptide synthesis is more advantageous than chemical synthesis since the enzymatic method is stereoselective and is secure from racemization.[2)] There are, however, several problems to be solved with this method. Secondary hydrolysis of the resulting peptide may arise from the inherent nature of the protease. Moreover, the method is limited to the use of amino acid derivatives which meet the enzymatic specificity as the coupling component.

In our previous work, it was shown that *p*-amidino- and *p*-guanidinophenyl esters behave as specific substrates for trypsin and trypsin-like enzymes.[3,4)] In these compounds the site-specific group for the enzyme, charged amidinium or guanidinum group, is not included in the acyl moiety but in the leaving group. Thus a new term, "inverse substrate", was proposed for these esters.[4)] The esters provided a novel method for the specific introduction of an acyl group for a wide variety of acyl groups into a trypsin active site, and such acyl enzymes have proven to be useful for the peptide coupling reaction.[5)] *p*-(Guanidinomethyl)phenyl esters have proved especially useful for the enzymatic coupling of hindered α-amino acids.[6)] The combination of the use of *m*-(guanidinomethyl)phenyl esters and *Streptomyces griseus* (SG) trypsin was also relevant for the coupling of some amino acids.[7)] Thus the design of a variety of inverse substrates can be one of the effective approaches for the development of trypsin-catalyzed peptide synthesis.

In this paper, we report the preparation of four new position isomers of guanidinonaphthyl esters (Fig. 1), their kinetic properties toward spontaneous and enzymatic hydrolysis, and their application to enzymatic peptide coupling.

Chemistry Aminonaphthol includes fourteen position isomers. Among them, four position isomers (4-amino-1-naphthol (**1**), 5-amino-1-naphthol (**6**), 5-amino-2-naphthol (**10**), 6-amino-1-naphthol (**14**)) were selected as the starting material for the preparation of four groups of guanidinonaphthyl esters (Fig. 1). The synthetic procedure for each guanidinonaphthyl ester is shown in Chart 1. Aminonaphthol was reacted with 1-[*N'*,*N''*-bis(Z)amidino]pyrazole.[8)] Pure [*N'*,*N''*-bis(Z)guanidino]naphthol was obtained in 58—98% yields. The physical and spectral data of the [*N'*,*N''*-bis(Z)guanidino]naphthols are listed in Table 1. Condensation of *N*-blocked guanidinonaphthol with *N*-Boc-amino acids by using DCC and DMAP in a mixture of dioxane and CH_2Cl_2 was successful. Reaction yields of the esters were 75—92%, as shown in Table 2. The next deprotection step was carried out by catalytic hydrogenation to give *N*-Boc amino acid guanidinonaphthyl esters as the TsOH salts essentially in quantita-

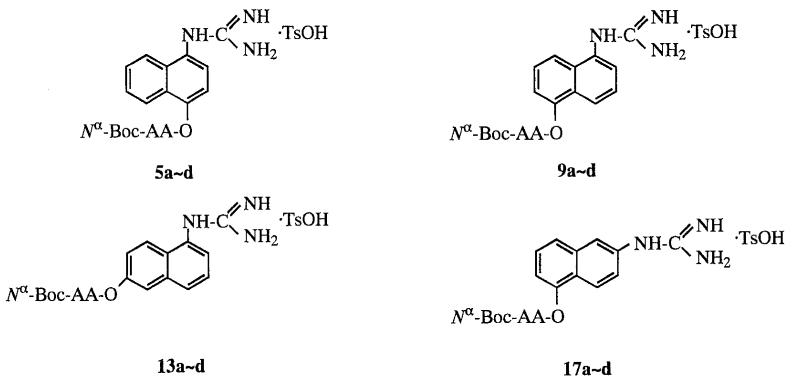

Fig. 1. Structure of Inverse Substrates

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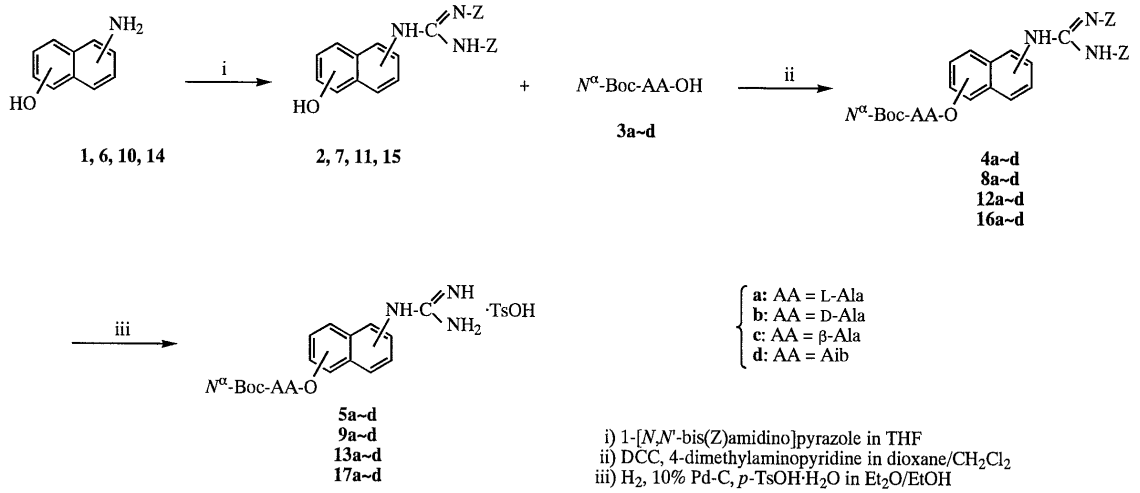


Chart 1

Table 1. Yield, Physical and Spectral Data of *p*-[*N'*,*N''*-Bis(benzyloxycarbonyl)guanidino]naphthols

Product	Yield (%)	mp (°C) (Recryst. solv.)	IR (KBr) ν (cm^{-1})	^{1}H-NMR (DMSO-d_6/TMS) δ, J (Hz)	Formula	Analysis (%) Calcd (Found)		
						C	H	N
2	62	184—186 (EtOH/dioxane/hexane)	3268, 1728, 1652 1622	4.90 (2H, s), 5.30 (2H, s), 6.87 (2H, d, 7.8), 7.13—7.32 (5H, br) 7.32—7.60 (8H, m), 8.19 (1H,d, 8.3), 10.07 (1H, s), 10.35 (1H, s), 11.68 (1H, s)	$C_{27}H_{23}N_3O_5$	69.07 (69.10	4.94 5.04	8.95 8.82)
7	58	167—168 (AcOEt)	3220, 1715, 1648 1624	4.94 (2H, s), 5.32 (2H, s), 6.92 (1H, dd, 2.9, 5.6), 7.25—7.50 (13H, m), 7.77 (1H, d, 7.3), 8.07 (1H, d, 8.3), 10.28 (1H, s), 10.31 (1H, s), 11.64 (1H,s)	$C_{27}H_{23}N_3O_5$	69.07 (69.17	4.94 5.00	8.95 8.85)
11	98	167—169 (EtOH/dioxane/benzene/hexane)	3247, 1721, 1656 1615, 1600	5.07 (2H, s), 5.27 (2H, s), 6.81 (1H, d, 8.3), 7.23—7.45 (12H, m), 7.56 (1H, d, 8.8), 8.02 (1H, s), 8.08 (1H, d, 9.3), 10.12 (1H, s), 10.18 (1H, s), 11.42 (1H, s)	$C_{27}H_{23}N_3O_5$	69.07 (69.20	4.94 5.01	8.95 8.82)
15	80	306—307 (dec.) (AcOEt/dioxane)	3253, 1720, 1652 1615, 1600	4.93 (2H, s), 5.31 (2H, s), 7.13—7.60 (15H, m), 7.81 (1H, d, 9.3), 9.87 (1H, s), 10.25 (1H, s), 11.62 (1H,s)	$C_{27}H_{23}N_3O_5$	69.07 (68.95	4.94 5.02	8.95 8.87)

tive yields, as shown in Table 3. Thus *N*-Boc-amino acid guanidinonaphthyl esters in each series were prepared after three reaction steps from commercial aminonaphthols.

Kinetic Parameters for Trypsin-Catalyzed Hydrolysis of Synthetic Inverse Substrates The kinetic constants for the trypsin-catalyzed hydrolysis were analyzed on the basis of the following scheme.

$$E+S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} EA \xrightarrow{k_3} E+P_2$$
$$\qquad\qquad\qquad\quad +$$
$$\qquad\qquad\qquad\quad P_1$$

In this scheme, the following symbols are used: E, enzyme; S, substrate; ES, enzyme–substrate complex; EA, acyl enzyme; P_1, alcohol component of the substrate; P_2, acid component of the substrate; K_s, dissociation constant of enzyme–substrate complex; k_2, rate constant of acylation step; k_3, rate constant of deacylation step. The kinetic parameters K_s and k_2 are useful in evaluating substrates. The former can provide information on the strength of the binding of the substrate to the enzyme, which is a characteristic of the enzymatic process, while the latter directly reflects the accessibility of the carbonyl function of the substrate molecule to the catalytic residue of the enzyme in the ES complex. The *N*-Boc-amino acid guanidinonaphthyl esters (**5a—d**, **9a**, **13a**) were subjected to kinetic analysis. Determination of kinetic parameters was carried out as previously described,[3b,4] and the values obtained are listed in Table 4. The parameters were also compared with those of *N*-Boc-amino acid *p*-guanidinophenyl esters previously reported.[3c,6] N^{α}-Boc-L-alanine 6-guanidino-1-naphthyl ester (N^{α}-Boc-L-Ala-OGN) (**17a**) was insoluble in the medium, and the kinetic parameters could not be determined. All compounds were found to have moderate affinity for trypsin with K_s (K_m) values in the range of 10^{-3}—10^{-4} M. These K_s (K_m) values were slightly larger than those of the corresponding *N*-Boc-amino acid *p*-guanidinophenyl esters as shown in Table 4. The K_s value of **5c** containing β-amino acid was two orders of magnitude larger than that of N^{β}-Boc-β-Ala-OG. In k_2 values, guanidinonaphthyl esters are less efficient substrates than the corresponding *p*-guanidinophenyl esters. However, naphthyl esters still qualified as specific substrates. The 1-guanidino-6-naphthyl ester (**13a**) showed a much slower acylation rate (*ca.* 1/1230 and 1/75), compared with the *p*-guanidinophenyl ester and 1-guanidino-4-naphthyl ester (**5a**), respectively. This difference between the 1,4- and 1,6-di-substituted isomers is similar to that between *p*- and *m*-isomers of guanidinophenyl ester as previously reported,[3c] and it is probably due to the stereochemical requirements of the active site, *i.e.*,

Table 2. Yield, Physical and Spectral Data for *N*-(*tert*-Butyloxycarbonyl)amino Acid *p*-[*N'*,*N''*-Bis(benzyloxycarbonyl)guanidino]naphthyl Esters

Product	AA	Yield (%)	mp (°C) (Recryst. solv.)	IR (KBr) ν (cm^{-1})	$[\alpha]_D^{25}$ (c=1.0, $CHCl_3$)	^{1}H-NMR ($CDCl_3$/TMS) δ, J (Hz)	Formula	Analysis (%) Calcd (Found)		
								C	H	N
4a[a]	L-Ala	81	159—160 (AcOEt/hexane)	1752, 1727, 1678, 1637, 1615	−17.6°	1.49 (9H, s), 1.69 (3H, d, 7.3), 4.72 (1H, q, 7.3), 5.09 (2H, s), 5.14 (1H, d, 7.3), 5.31 (2H, s), 7.28—7.31 (6H, m), 7.40—7.43 (5H, m), 7.56—7.60 (2H, m), 7.93 (1H, d, 7.8), 7.99 (1H, d, 6.4), 8.02 (1H, dd, 6.4, 7.8), 10.61 (1H, s), 12.04 (1H, s)	$C_{35}H_{36}N_4O_8$	65.61 (65.55	5.66 5.64	8.74 8.59)
4c	β-Ala	87	119—121 (AcOEt/hexane)	1750, 1733, 1720, 1684, 1637	—	1.47 (9H, s), 2.99 (2H, t, 5.9), 3.58 (2H, dt, 5.9, 5.9), 5.09 (2H, s), 5.31 (2H, s), 7.27—7.36 (6H, m), 7.38—7.43 (5H, m), 7.54—7.62 (2H, m), 7.86 (1H, d, 7.8), 7.99 (1H, d, 6.4), 8.01 (1H, dd, 6.4, 7.8), 10.61 (1H, s), 12.04 (1H, s)	$C_{35}H_{36}N_4O_8$	65.61 (65.79	5.66 5.75	8.74 8.75)
4d	Aib	75	154—156 (AcOEt/hexane)	1761, 1729, 1718, 1652, 1647, 1637	—	1.48 (9H, s), 1.75 (6H, s), 5.08 (2H, s), 5.14 (1H, br s), 5.30 (2H, s), 7.27—7.30 (6H, m), 7.39—7.43 (5H, m), 7.35 (1H, ddd, 0.9, 6.8, 8.3), 7.58 (1H, ddd, 1.5, 6.8, 8.3), 7.98 (2H, d, 7.8), 8.02 (1H, d, 7.8), 10.58 (1H, s), 12.04 (1H, s)	$C_{36}H_{38}N_4O_8$	66.04 (66.19	5.85 5.85	8.56 8.54)
8a[b]	L-Ala	90	168—170 (benzene/hexane)	1756, 1725, 1679, 1640, 1608	−11.6°	1.49 (9H, s), 1.69 (3H, d, 7.3), 4.72 (1H, q, 7.3), 5.10 (2H, s), 5.13 (1H, br s), 5.31 (2H, s), 7.27—7.33 (6H, m), 7.39—7.53 (5H, m), 7.54 (1H, d, 8.3), 7.56 (1H, d, 7.8), 7.80 (1H, d, 8.3), 7.90 (1H, d, 8.8), 8.09 (1H, d, 7.8), 10.66 (1H, s), 12.04 (1H, s)	$C_{35}H_{36}N_4O_8$	65.6 (65.55	5.66 5.67	8.74 8.71)
8c	β-Ala	86	142—143 (benzene/hexane)	1760, 1729, 1684, 1636, 1608	—	1.47 (9H, s), 2.99 (2H, t, 5.9), 3.58 (2H, dt, 5.9, 5.9), 5.10 (2H, s), 5.15 (1H, br s), 5.31 (2H, s), 7.28—7.34 (6H, m), 7.38—7.43 (5H, m), 7.52 (1H, d, 7.8), 7.56 (1H, d, 8.8), 7.73 (1H, d, 8.3), 7.90 (1H, d, 8.8), 8.09 (1H, d, 7.8), 10.67 (1H, s), 12.05 (1H, s)	$C_{35}H_{36}N_4O_8$	65.61 (65.63	5.66 5.71	8.74 8.72)
8d	Aib	83	160—162 (benzene/hexane)	1757, 1736, 1694, 1628, 1608	—	1.47 (9H, s), 1.76 (6H, s), 5.10 (2H, s), 5.14 (1H, br s), 5.31 (2H, s), 7.28—7.33 (6H, m), 7.39—7.43 (5H, m), 7.50 (1H, d, 8.3), 7.52 (1H, d, 8.8), 7.88 (2H, d, 8.3), 8.06 (1H, d, 7.8), 10.66 (1H, s), 12.05 (1H, s)	$C_{36}H_{38}N_4O_8$	66.04 (66.13	5.85 5.90	8.56 8.47)
12a[c]	L-Ala	82	104—106 (benzene/hexane)	1768, 1762, 1720, 1689, 1647, 1635, 1624	−8.0°	1.48 (9H, s), 1.68 (3H, d, 7.3), 4.72 (1H, q, 7.3), 5.13 (1H, br s), 5.17 (2H, s), 5.27 (2H, s), 7.19 (1H, d, 7.8), 7.28—7.42 (10H, m), 7.45 (1H, d, 7.8), 7.66—7.71 (2H, dd, 2.0, 8.8), 7.87 (1H, d, 8.8), 8.20 (1H, d, 2.0), 10.46 (1H, s), 11.94 (1H, s)	$C_{35}H_{36}N_4O_8$	65.61 (65.75	5.66 5.73	8.74 8.68)
12c	β-Ala	86	106—108 (benzene/hexane)	1751, 1724, 1691, 1647, 1625	—	1.46 (9H, s), 2.97 (2H, t, 5.9), 3.57 (2H, dt, 5.9, 5.9), 5.08 (1H, t, 5.9), 5.17 (1H, br s), 5.27 (2H, s), 7.21 (1H, d, 7.8), 7.28—7.42 (10H, m), 7.45 (1H, d, 7.8), 7.69 (2H, dd, 2.0, 8.8), 7.80 (1H, d, 8.3), 8.19 (1H, d, 2.0), 10.46 (1H, s), 11.94 (1H, s)	$C_{35}H_{36}N_4O_8$	65.61 (65.68	5.66 5.69	8.74 8.69)
12d	Aib	86	80—82 (benzene/hexane)	1751, 1718, 1705, 1647, 1635	—	1.46 (9H, s), 1.75 (6H, s), 5.13 (2H, br s), 5.17 (2H, s), 5.27 (2H, s), 7.19 (1H, d, 7.8), 7.30—7.41 (10H, m), 7.44 (1H, d, 7.3), 7.67 (2H, dd, 2.0, 8.8), 8.18 (1H, d, 2.0), 10.45 (1H, s), 11.94 (1H, s)	$C_{36}H_{38}N_4O_8$	66.04 (66.21	5.85 5.94	8.56 8.59)
16a[d]	L-Ala	92	131—132 (benzene/hexane)	1778, 1730, 1686, 1636	−17.8°	1.48 (9H, s), 1.60 (3H, d, 6.8), 4.61 (1H, q, 6.8), 5.09 (2H, s), 5.24 (1H, br s), 5.30 (2H, s), 7.28—7.32 (6H, m), 7.37—7.42 (5H, m), 7.51 (1H, dd, 7.8, 7.8), 7.61 (1H, d, 2.4), 7.67 (1H, d, 8.3), 8.02 (2H, d, 7.3), 10.62 (1H, s), 12.04 (1H, s)	$C_{35}H_{36}N_4O_8$	65.61 (65.84	5.66 5.85	8.74 8.45)
16c	β-Ala	86	130—131 (benzene/hexane)	1758, 1717, 1692, 1647	—	1.46 (9H, s), 2.86 (2H, t, 5.9), 3.54 (2H, dd, 5.9, 5.9), 5.09 (3H, overlap s), 5.30 (2H, s), 7.26—7.31 (6H, m), 7.37—7.43 (5H, m), 7.51 (1H, dd, 7.8, 7.8), 7.61 (1H, d, 2.4), 7.68 (1H, d, 8.3), 8.00 (2H, d, 7.3), 10.63 (1H, s), 12.04 (1H, s)	$C_{35}H_{36}N_4O_8$	65.61 (65.78	5.66 5.78	8.74 8.65)
16d	Aib	91	146—148 (benzene/hexane)	1751, 1717, 1637,	—	1.47 (9H, s), 1.67 (6H, s), 5.01 (1H, br s), 5.09 (2H, s), 5.30 (2H, s), 7.28—7.35 (6H, m), 7.37—7.43 (5H, m), 7.49 (1H, dd, 7.8, 7.8), 7.59 (1H, d, 2.4), 7.66 (1H, d, 8.3), 7.98 (2H, d, 7.3), 10.62 (1H, s), 12.04 (1H, s)	$C_{36}H_{38}N_4O_8$	66.04 (66.09	5.85 5.96	8.56 8.41)

a—*d*) The enantiomer of these compounds (**4b**, **8b**, **12b**, **16b**) showed almost the same spectral data except for the optical rotation: *a*), **4b**, $[\alpha]_D^{25}$=+17.8°; *b*), **8b**, $[\alpha]_D^{25}$=+12.0°; *c*), **12b**, $[\alpha]_D^{25}$=+8.2°; *d*), **16b**, $[\alpha]_D^{25}$=+17.8°.

inaccessibility of the unfavorably positioned acyl moiety of the 1,6-di-substituted isomer to the enzyme catalytic residue, even though the binding itself is tight and specific. It was recognized that the deacylation rates of inverse substrates should not be dependent upon the leaving groups as shown for *p*-amidinophenol and *p*-guanidinophenol.[3a] The k_3 values

Table 3. Yield, Physical and Spectral Data of *N*-(*tert*-Butyloxycarbonyl)amino Acid *p*-Guanidinonaphthyl Esters as Their *p*-Toluensulfonic Acid Salts

Product	AA	Yield (%)	IR (KBr) ν (cm^{-1})	$[\alpha]_D^{25}$ (c=1.0, MeOH)	^{1}H-NMR (MeOH-d_4/TMS) δ, J (Hz)	FAB-MS m/z (M^++H)
5a[a]	L-Ala	94	3337, 3171, 1762, 1683, 1609	−34.0°	1.48 (9H, s), 1.63 (3H, d, 7.3), 2.35 (3H, s), 4.53 (1H, q, 7.3), 7.20 (2H, d, 8.3), 7.36 (1H, d, 7.8), 7.53 (1H, d, 8.3), 7.63—7.70 (4H, m), 7.97 (1H, d, 8.8), 8.11 (1H, d, 8.3)	373
5c	β-Ala	88	3358, 3186, 1765, 1751, 1720, 1698, 1684, 1654, 1637, 1609	—	1.49 (9H, s), 2.36 (3H, s), 3.00 (2H, t, 6.8), 3.53 (2H, t, 6.8), 7.21 (2H, d, 8.3), 7.39 (1H, d, 7.8), 7.53 (1H, d, 8.3), 7.66—7.70 (2H, m), 7.69 (2H, d, 8.3), 7.98 (1H, d, 8.8), 8.01 (1H, d, 8.8)	373
5d	Aib	96	3343, 3182, 1763, 1715, 1700, 1683, 1609	—	1.49 (9H, s), 1.68 (6H, s), 2.35 (3H, s), 7.20 (2H, d, 8.3), 7.34 (1H, d, 7.8), 7.53 (1H, d, 7.8), 7.59—7.70 (2H, m), 7.68 (2H, d, 8.3), 7.96 (1H, d, 7.8), 8.17 (1H, d, 8.3)	387
9a[b]	L-Ala	98	3337, 3179, 1772, 1765, 1753, 1700, 1684, 1675, 1609	−25.6°	1.48 (9H, s), 1.62 (3H, d, 7.3), 2.36 (3H, s), 4.52 (1H, q, 7.3), 7.21 (2H, d, 8.3), 7.37 (1H, d, 7.3), 7.57—7.62 (3H, m), 7.68 (2H, d, 8.3), 7.88 (1H, d, 8.8), 8.12 (1H, d, 8.3)	373
9c	β-Ala	90	3366, 3183, 1772, 1750, 1717, 1699, 1684, 1618	—	1.46 (9H, s), 2.35 (3H, s), 2.98 (2H, t, 6.8), 3.51 (2H, t, 6.8), 7.21 (2H, d, 8.3), 7.40 (1H, d, 7.3), 7.55 (1H, d, 7.3), 7.56—7.65 (2H, m), 7.69 (2H, d, 8.3), 7.87 (1H, d, 8.3), 8.02 (1H, d, 8.3)	373
9d	Aib	82	3337, 3179, 1763, 1700, 1683, 1610	—	1.49 (9H, s), 1.67 (6H, s), 2.36 (3H, s), 7.21 (2H, d, 8.3), 7.35 (1H, d, 7.8), 7.54—7.66 (3H, m), 7.69 (2H, d, 8.3), 7.86 (1H, d, 8.3), 8.20 (1H, d, 8.3)	387
13a[c]	L-Ala	85	3348, 3187, 1775, 1762, 1700, 1684, 1654, 1618	−17.6°	1.48 (9H, s), 1.61 (3H, d, 7.3), 2.35 (3H, s), 4.50 (1H, q, 7.3), 7.21 (2H, d, 8.3), 7.28 (1H, d, 7.8), 7.40 (1H, dd, 2.0, 8.8), 7.56 (1H, dd, 7.8, 7.8), 7.69 (2H, d, 8.3), 7.82 (1H, d, 8.8), 7.84 (1H, d, 2.0), 8.10 (1H, d, 8.8)	373
13c	β-Ala	86	3354, 3179, 1761, 1751, 1698, 1684, 1654, 1647, 1618	—	1.47 (9H, s), 2.35 (3H, s), 2.96 (2H, t, 6.8), 3.50 (2H, t, 6.8), 7.21 (2H, d, 8.3), 7.31 (1H, d, 7.8), 7.40 (1H, dd, 2.0, 8.8), 7.54 (1H, dd, 7.8, 7.8), 7.69 (2H, d, 8.3), 7.80 (1H, d, 8.3), 7.82 (1H, d, 2.0), 7.99 (1H, d, 8.8)	373
13d	Aib	85	3358, 3179, 1762, 1748, 1720, 1700, 1684, 1654, 1647, 1637, 1618	—	1.48 (9H, s), 1.66 (6H, s), 2.35 (3H, s), 7.21 (2H, d, 8.3), 7.29 (1H, d, 7.8), 7.37 (1H, dd, 2.0, 8.8), 7.55 (1H, dd, 7.8, 7.8), 7.70 (2H, d, 8.3), 7.79 (1H, d, 8.3), 7.81 (1H, d, 2.0), 8.17 (1H, d, 8.8)	387
17a[d]	L-Ala	96	3387, 3197, 1771, 1698, 1674, 1637, 1618	−41.0°	1.47 (9H, s), 1.55 (3H, d, 7.3), 2.35 (3H, s), 4.40 (1H, q, 7.3), 7.21 (2H, d, 8.3), 7.40 (1H, dd, 2.4, 8.8), 7.50 (1H, d, 8.8), 7.60 (1H, dd, 8.8, 8.8), 7.68 (2H, d, 8.3), 7.74 (1H, d, 2.4), 7.96 (1H, d,, 8.8), 7.98 (1H, d, 8.8)	[e]
17c	β-Ala	89	3393, 3180, 1749, 1706, 1687, 1675, 1609	—	1.45 (9H, s), 2.82 (2H, t, 6.8), 3.47 (2H, t, 6.8), 7.20 (2H, d, 8.3), 7.41 (1H, dd, 2.4, 8.8), 7.48 (1H, dd, 2.4, 8.8), 7.59 (1H, dd, 8.8, 8.8), 7.68 (2H, d, 8.3), 7.75 (1H, d, 2.4), 7.94 (1H, d, 8.8), 7.96 (1H, d, 8.8)	[f]
17d	Aib	93	3394, 3189, 1755, 1693, 1677, 1637, 1601	—	1.47 (9H, s), 1.59 (6H, s), 2.35 (3H, s), 7.21 (2H, d, 8.3), 7.41 (1H, dd, 2.4, 8.8) 7.49 (1H, dd, 2.4, 8.8), 7.60 (1H, dd, 8.8, 8.8), 7.68 (2H, d, 8.3), 7.70 (1H, d, 2.4) 7.94 (1H, d, 8.8), 7.97 (1H, d, 8.8)	[g]

a—*d*) The enantiomer of these compounds (**5b**, **9b**, **13b**, **17b**) showed almost the same spectral data except for the optical rotation: *a*), **5b**, $[\alpha]_D^{25}$=+29.8°; *b*), **9b**, $[\alpha]_D^{25}$=+24.4°; *c*), **13b**, $[\alpha]_D^{25}$=+16.8°; *d*), **17b**, $[\alpha]_D^{25}$=+37.4°. *e*) *Anal.* Calcd for $C_{26}H_{32}N_4O_7S$: C, 57.34; H, 5.92; N, 10.29; S, 5.89. Found: C, 57.51; H, 5.93; N, 10.13; S, 5.91. *f*) *Anal.* Calcd for $C_{26}H_{32}N_4O_7S$: C, 57.34; H, 5.92; N, 10.29; S, 5.89. Found: C, 57.33; H, 5.97; N, 10.03; S, 5.66. *g*) *Anal.* Calcd for $C_{27}H_{34}N_4O_7S$: C, 58.05; H, 6.13; N, 10.03; S, 5.74. Found: C, 58.21; H,6.16; N, 9.92; S, 5.64.

Table 4. Comparison of Kinetic Parameters for Trypsin-Catalyzed Hydrolysis of Guanidinophenyl Ester and Guanidinonaphthyl Ester

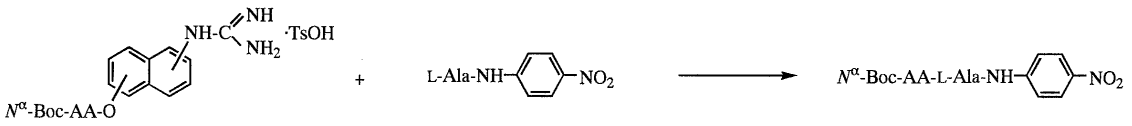
Substrate (No.)	K_s (K_m) (M)	k_2 (s^{-1})	k_3 (k_{cat}) (s^{-1})	k_2/K_s (k_{cat}/K_m) ($s^{-1}\cdot M^{-1}$)
N^{α}-Boc-L-Ala-OG[a]	2.77×10^{-4}	1.23×10^{2}	4.09×10^{-1}	4.44×10^{5}
N^{α}-Boc-D-Ala-OG[a]	6.62×10^{-4}	6.71×10	3.51×10^{-2}	1.01×10^{5}
N^{β}-Boc-β-Ala-OG[a]	2.77×10^{-5}	4.61×10	2.09×10^{-1}	1.66×10^{6}
N^{α}-Boc-Aib-OG[b]	2.37×10^{-3}	1.57	2.58×10^{-3}	6.64×10^{2}
N^{α}-Boc-L-Ala-OGN (**5a**)	1.69×10^{-4}	4.15×10	5.13	2.46×10^{5}
N^{α}-Boc-D-Ala-OGN (**5b**)	1.28×10^{-3}	3.80×10	1.79×10^{-1}	2.96×10^{4}
N^{β}-Boc-β-Ala-OGN (**5c**)	2.91×10^{-3}	2.68×10^{2}	4.84×10^{-1}	9.21×10^{4}
N^{α}-Boc-Aib-OGN (**5d**)	2.16×10^{-4}	3.36×10^{-1}	7.18×10^{-3}	1.55×10^{3}
N^{α}-Boc-L-Ala-OGN (**9a**)	(3.37×10^{-4})	$3.02\times10^{c)}$	(1.90)	(5.64×10^{3})
N^{α}-Boc-L-Ala-OGN (**13a**)	(1.28×10^{-3})	$5.56\times10^{-1,d)}$	(5.50×10^{-1})	(4.30×10^{2})

a) See reference 3*c*. *b*) See reference 6*b*. *c*) Calculated value using k_3=5.13 s^{-1} for N^{α}-Boc-L-Ala-OGN (**5a**), with the following equation: $k_{cat}=k_2k_3/(k_2+k_3)$. *d*) Calculated value using k_3=5.13 s^{-1} for N^{α}-Boc-L-Ala-OGN (**5a**), with the following equation: $k_{cat}=k_2k_3/(k_2+k_3)$.

of guanidinonaphthyl esters in the present case, however, were somewhat different. They were larger than those of the corresponding guanidinophenyl esters, possibly owing to a rate-enhancement caused by an interaction with the guanidinonaphthyl group.

The parameter, k_2/K_s (or k_{cat}/K_m[9]), introduced by Brot and Bender is informative for the evaluation of the substrates.[10] In the comparison of this k_2/K_s (or k_{cat}/K_m) value, 1-guanidino-4-naphthyl ester (**5a**) is much more specific than 1-guanidino-5-naphthyl ester (**9a**) and 1-guanidino-6-naphthyl ester (**13a**) to trypsin.

Trypsin-Catalyzed Peptide Coupling Reaction Trypsin-



5a~d, 9a~d, 13a~d, 17a~d **18** **19a~d**

Chart 2

Table 5. Spontaneous Hydrolysis of Various Guanidinonaphthyl Esters[a)]

Substrate No.	k_{hyd} ($\times 10^6 \cdot s^{-1}$)	$t_{1/2}$ (h)	Substrate No.	k_{hyd} ($\times 10^6 \cdot s^{-1}$)	$t_{1/2}$ (h)	Substrate No.	k_{hyd} ($\times 10^6 \cdot s^{-1}$)	$t_{1/2}$ (h)	Substrate No.	k_{hyd} ($\times 10^6 \cdot s^{-1}$)	$t_{1/2}$ (h)
5a	7.6	25	**9a**	3.1	63	**13a**	2.2	86	**17a**	2.5	77
5b	4.2	46	**9b**	3.0	64	**13b**	1.7	113	**17b**	3.8	51
5c	2.9	66	**9c**	2.9	66	**13c**	1.25	160	**17c**	3.15	62
5d	no reaction		**9d**	no reaction		**13d**	no reaction		**17d**	no reaction	

a) Conditions: substrate, 1 mM; L-Ala-*p*NA, 20 mM; 50% DMSO/MOPS (50 mM, pH 8.0, containing 10 mM $CaCl_2$); 25 °C.

Table 6. Yield of Trypsin-Catalyzed Peptide Coupling Reaction[a)]

Entry No.	Acyl donor (No.)	Reaction time (h)	Product (No.)	Yield (%)
1	N^{α}-Boc-L-Ala-OGN (**5a**)	1	N^{α}-Boc-L-Ala-L-Ala-*p*NA (**19a**)	79
2	N^{α}-Boc-L-Ala-OGN (**9a**)	2	N^{α}-Boc-L-Ala-L-Ala-*p*NA (**19a**)	72
3	N^{α}-Boc-L-Ala-OGN (**13a**)	12	N^{α}-Boc-L-Ala-L-Ala-*p*NA (**19a**)	75
4	N^{α}-Boc-L-Ala-OGN (**17a**)	12	N^{α}-Boc-L-Ala-L-Ala-*p*NA (**19a**)	88
5	N^{α}-Boc-D-Ala-OGN (**5b**)	1	N^{α}-Boc-D-Ala-L-Ala-*p*NA (**19b**)	75
6	N^{α}-Boc-D-Ala-OGN (**9b**)	6	N^{α}-Boc-D-Ala-L-Ala-*p*NA (**19b**)	74
7	N^{α}-Boc-D-Ala-OGN (**13b**)	24	N^{α}-Boc-D-Ala-L-Ala-*p*NA (**19b**)	46
8	N^{α}-Boc-D-Ala-OGN (**17b**)	24	N^{α}-Boc-D-Ala-L-Ala-*p*NA (**19b**)	39
9	N^{β}-Boc-β-Ala-OGN (**5c**)	0.5	N^{β}-Boc-β-Ala-L-Ala-*p*NA (**19c**)	34
10	N^{β}-Boc-β-Ala-OGN (**9c**)	2	N^{β}-Boc-β-Ala-L-Ala-*p*NA (**19c**)	35
11	N^{β}-Boc-β-Ala-OGN (**13c**)	1	N^{β}-Boc-β-Ala-L-Ala-*p*NA (**19c**)	24
12	N^{β}-Boc-β-Ala-OGN (**17c**)	8	N^{β}-Boc-β-Ala-L-Ala-*p*NA (**19c**)	32
13	N^{α}-Boc-Aib-OGN (**5d**)	10	N^{α}-B0c-Aib-L-Ala-*p*NA (**19d**)	82
14	N^{α}-Boc-Aib-OGN (**9d**)	24	N^{α}-Boc-Aib-L-Ala-*p*NA (**19d**)	42 (66)[b)]
15	N^{α}-Boc-Aib-OGN (**13d**)	48[c)]	N^{α}-Boc-Aib-L-Ala-*p*NA (**19d**)	—
16	N^{α}-Boc-Aib-OGN (**17d**)	48[c)]	N^{α}-Boc-Aib-L-Ala-*p*NA (**19d**)	—

a) Conditions: acyl donor, 1 mM; acyl acceptor, 20 mM; trypsin, 10 μM; 50% DMSO-MOPS (50 mM, pH 8.0, containing 10 mM $CaCl_2$); 25 °C. *b*) The value in parenthesis is yield after 96 h. *c*) The starting materials are completely intact even at 48 h.

catalyzed peptide coupling reaction (Chart 2) was carried out as described in the Experimental. The progress of the coupling reaction was monitored by HPLC. Elution peaks were correlated to those of authentic samples which were chemically synthesized according to the reported procedure.[5d,6a,11)] The reaction period was determined by HPLC when the peak of starting *N*-Boc-AA-OGN completely disappeared, and further progress of coupling product was not observed on the HPLC elution diagram. Control experiments in the absence of trypsin showed that hydrolysis of *N*-Boc-AA-OGN was so slow that no appreciable amount of the esters was consumed during the period that enzymatic reaction was completed. The rate constants (k_{hyd}) for the spontaneous hydrolysis were determined as described in the experimental and the results are listed in Table 5. All compounds were found to have small k_{hyd} values in the range of $1.25—7.6\times10^{-6}\,s^{-1}$ which is equivalent to a half-life time of 160—25 h. Consequently, the spontaneous hydrolysis of acyl donor during enzymatic peptide coupling is negligible. Thus the enzymatic hydrolysis of acyl donor is the only reaction competitive to the peptide synthesis. Therefore, the residual part of the coupling yield shown in Table 6 could be nearly equal to the part of hydrolysis of acyl enzyme except entry 15 and 16. All guanidinonaphthyl esters derived from N^{α}-Boc-α-aminoisobutylic acid (N^{α}-Boc-Aib) were resistant to spontaneous hydrolysis. It is note worthy that **13d** and **17d** are susceptible to enzymatic hydrolysis though **5a** and **9a** are not susceptible.

The yields of the trypsin-catalyzed peptide coupling reaction are summarized in Table 6. Toward all inverse substrates derived from N^{α}-Boc-L-Ala, trypsin behaved as a moderately effective catalyst for the synthesis of the peptides (entry 1—4 in Table 6). The difference among N^{α}-Boc-L-Ala-OGN (**5a**, **9a**, **13a**, **17a**) in the coupling rates seemed to be due to the difference in acyl-enzyme formation rates. The coupling reaction involving D-amino acid strongly depends on the structure of acyl donor. A moderate yield was obtained with **5b** and **9b,** and their reaction rate is slightly slower than that of the corresponding N^{α}-Boc-L-Ala derivatives (**5a**, **9a**). On the other hand, the response of **13b** and **17b** is considerably different from that of L-Ala (**13a**, **17a**). The coupling reaction of inverse substrates containing a β-amino acid is not favorable even though the reaction is fast (entry 9—12 in Table 6). The coupling reaction of inverse substrates derived from Aib strongly depends on the structure. The reaction yield is ex-

ceedingly high in the case of 1-guanidino-4-naphthyl ester (**5d**). The behavior of compounds (**13d, 17d**) was noted since they were insensitive to tryptic catalysis for either hydrolysis and aminolysis. Compound **13d** and **17d** are exceptional esters which remained unchanged in the presence of trypsin even after a long period of incubation (*ca.* 48 h).

It can be presumed from a previous report[6] that the coupling yield and the reaction rate are sensitive to the linear distance between the positive charged carbon atom and the carbonyl carbon of the ester. The distances[12] in the natural substrate's arginine is 6.526 Å, and that of artificial "inverse substrate", *p*-amidinophenyl ester, which is also an efficient substrate for enzymatic peptide synthesis, is almost the same (6.597 Å). The distance for another efficient inverse substrate, *p*-guanidinophenyl ester, was analyzed as 7.726 Å, though that of *p*-(guanidinomethyl)phenyl ester which is a less efficient substrate for trypsin was analyzed to be 8.378 Å. Therefore a linear distance shorter than 7.8 Å seems to be a necessary requirement. On the contrary, both of the *meta* isomers guanidinophenyl ester and (guanidinomethyl)-phenyl ester have almost the same distance as that of *p*-guanidinophenyl ester (7.352 and 7.731 Å, respectively), but neither of them were effective acyl donors for trypsin-catalyzed peptide synthesis. It can be considered that they do not meet the steric requirements of the active site with respect to their relative spatial arrangement. The bond angle and/or direction between carbonyl group and cationic substituent might be another important factor.

In the case of the guanidinonaphthyl esters, the bond angle or direction seems more important than the distance. The distances were 7.553 Å for **5**, 8.545 Å for **9**, 8.187 Å for **13**, and 7.838 Å for **17**, respectively. The distance of **5** and **17** was almost the same as that of *p*-guanidinophenyl ester (7.726 Å), and both of them were expected to be efficient substrates. Compound (**17**), however, was shown to be very slow to react, in a similar manner to *m*-guanidinophenyl ester. Both the compounds (**9**, **13**) have a much longer distance than 7.8 Å, but only compound (**9**) behaved as a moderately efficient substrate. It was considered that the spatial arrangement of the 1,4- (**5**) and 1,5-isomer (**9**) for guanidinonaphthyl esters, in which the two substituents are aligned along with the shorter axis of the naphthalene ring, are greatly preferred for tryptic catalysis.

In any event, 1-guanidino-4-naphthyl esters (**5a—d**) universally result in good peptide coupling yield with a variety of acyl groups. From these results, the structural preference of the substrate as acyl donor was supposed such that the guanidino group and acyl group are aligned linearly on the shorter axis of the naphthalene ring

It must be emphasized that the coupling yields in the present method were not decreased by a longer period of incubation. This result suggests that secondary hydrolysis of the resulting products is negligible.

Experimental

Melting points were measured on a Yanaco MP-500 micro melting point apparatus and are uncorrected. IR spectra were recorded on a JASCO VALOR-III FT-IR spectrometer. ^{1}H-NMR spectra were recorded on a JEOL JNM-FX-400 spectrometer. Optical rotations were measured with a JASCO DIP-360 digital polarimeter in a 5 cm cell. 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. HPLC was performed on a Shimadzu LC-6A pump system equipped with a Shimadzu SPD-6AV UV-VIS spectrophotometric detector. Bovine pancreas trypsin (EC 3.4.21.4) was purchased from Worthington Biochemical Corp. (twice crystallized, lot TRL).

4-[*N'*,*N''*-Bis(benzyloxycarbonyl)guanidino]-1-naphthol (2a) A suspension of 4-amino-1-naphthol hydrochloride (**1**) (1.95 g, 10 mmol) and 1-[*N*,*N'*-bis(benzyloxycarbonyl)amidino]pyrazole (3.77 g, 10 mmol) in absolute THF (4 ml) containing *N*,*N*-diisopropylethylamine (1,29 g, 10 mmol) was stirred overnight at room temperature under an atmosphere of nitrogen. The reaction mixture was diluted with benzene–dioxane–AcOEt (6 : 5 : 1) and passed through a silica gel column (i.d. 5×60 cm). The eluate was evaporated to dryness *in vacuo* and the solid residue was recrystallized from EtOH–benzene to give **2**. Compounds **7**, **11**, and **15** were synthesized from **6**, **10**, and **14** following a similar procedure as described above. Yields, physical properties and spectral data for **2**, **7**, **11**, **15** are given in Table 1.

***N*-(*tert*-Butyloxycarbonyl)amino Acid [*N'*,*N''*-Bis(benzyloxycarbonyl)-guanidino]naphthyl Ester** A solution of [*N'*,*N''*-bis(Z)guanidino]naphthol (703.5 mg, 1.5 mmol), **3** (1.5 mmol), and DMAP (18.3 mg, 0.15 mmol) in a mixture of dioxane (4 ml) and CH_2Cl_2 (1 ml) was treated with DCC (340 mg, 1.65 mmol) at 0 °C. The reaction mixture was stirred for 1 h at the same temperature, then warmed to room temperature, and stirring was continued for 12 h. The resulting precipitate of DCUrea was filtered off and the filtrate was concentrated to dryness *in vacuo*. The pure *N*-Boc-amino acid [*N'*,*N''*-bis(Z)guanidino]naphthyl ester was obtained by recrystallization. Yields, physical properties and spectral data of *N*-Boc-amino acid [*N'*,*N''*-bis(Z)guanidino]naphthyl esters are given in Table 2.

***N*-(*tert*-Butyloxycarbonyl)amino Acid Guanidinonaphthyl Ester *p*-Toluenesulfonate** A suspension of *N*-Boc-amino acid [*N'*,*N''*-bis(Z)-guanidino]naphthyl ester (1 mmol) and *p*-TsOH·H_2O (190 mg, 1 mmol) in a mixture of EtOH (14 ml) and Et_2O (14 ml) containing 10% Pd–C (35 mg) was vigorously stirred overnight under an atmosphere of hydrogen at room temperature. The catalyst was filtered off, and the filtrate was evaporated to dryness *in vacuo*. The amorphous residue was washed with dry ether to give *N*-Boc-amino acid guanidinonaphthyl ester *p*-toluenesulfonate except **17**. Pure compounds **17a—d** were obtained by recrystallization. Yields, physical properties and spectral data of *N*-Boc-amino acid guanidinonaphthyl esters *p*-toluenesulfonate are given in Table 3.

Kinetic Measurements Enzyme concentration was determined by active site titration using *p*-nitrophenyl *p'*-guanidinobenzoate.[13] Analysis of kinetic parameters was carried out by the thionine displacement method.[3c,14] Optical density changes at 620 nm were monitored with a stopped-flow spectrophotometer. The determination of k_2 and K_s was carried out in 0.05 M Tris–HCl buffer, pH 8.0, containing 0.02 M $CaCl_2$ at 25 °C. In these experiments, the concentrations were: enzyme, 3.31×10^{-6}—7.83×10^{-6} M; substrate, 2.40×10^{-5}—1.04×10^{-3} M; thionine; 2.50×10^{-5} M. Compounds **9a** and **13a** were analyzed potentiometrically using pH-stat conditions following the reported procedure.[3a] Determination of k_{cat} and K_m was carried out in 0.1 M KCl, pH 8.0, containing 0.02 M $CaCl_2$ at 25 °C. In these experiments the enzyme concentration was 1.95×10^{-8}—1.50×10^{-6} M, and the substrate concentration was 1.76×10^{-5}—1.07×10^{-4} M.

Spontaneous Hydrolysis A mixture of 50 μl of acyl donor stock solution (10 mM DMSO solution of **5a—d**, **9a—d**, **13a—d**, **17a—d**), 50 μl of internal standard stock solution (10 mM DMSO solution of 1-naphthol), 50 μl of acyl acceptor stock solution (200 mM DMSO solution of **18**), 250 μl of 50 mM MOPS buffer (containing 20 mM of $CaCl_2$, pH 8.0), and 100 μl of DMSO was incubated at 25 °C. The decrease of the acyl donor was monitored by HPLC under the following conditions: column i.d. 4.0×250 mm Wakosil 5C 18-200, isocratic elution at 1 ml/min, 0.1% trifluoroacetic acid/acetonitrile. An aliquot of the reaction mixture was injected and peaks were detected at 280 nm (guanidinonaphthyl ester and 1-naphthol moiety).

Trypsin-Catalyzed Peptide Coupling Reaction A mixture of 50 μl of acyl donor stock solution (10 mM DMSO solution of **5a—d**, **9a—d**, **13a—d**, **17a—d**), 50 μl of acyl acceptor stock solution (200 mM DMSO solution of **18**), 240 μl of 50 mM MOPS buffer (containing 20 mM of $CaCl_2$, pH 8.0), 150 μl of DMSO, and 10 μl of trypsin stock solution (1 mM solution in 1 mM HCl) was incubated at 25 °C. The progress of the coupling reaction was monitored by HPLC under the following conditions: column i.d. 4.0×250 mm Wakosil 5C 18-200, isocratic elution at 1 ml/min, 0.1% trifluoroacetic acid/acetonitrile. An aliquot of the reaction mixture was injected and peaks were detected at 280 nm (guanidinonaphthyl ester and *p*-nitroanilide moiety) or 310 nm (*p*-nitroanilide moiety). Peak identification was made by correlation with authentic samples which were chemically synthesized.[5d,6a,11] Peak intensities were used to calculate relative concentration.

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References and Notes

1) The following abbreviations are used: Boc=*tert*-butyloxycarbonyl, Z=benzyloxycarbonyl, AA=amino acid, DCC=*N*,*N*′-dicyclohexylcarbodiimide, DCUrea=*N*,*N*′-dicyclohexylurea, DMAP=4-dimethylaminopyridine, DMF=*N*,*N*-dimethylformamide, DMSO=dimethyl sulfoxide, THF=tetrahydrofuran, MOPS=3-morpholino-1-propanesulfonate, G=guanidinophenyl, GN=guanidinonaphthyl, *p*NA=*p*-nitroanilide.
2) For example: *a*) Tsuzuki H., Oka T., Morihara K., *J. Biochem.*, **88**, 669—675 (1980); *b*) Nakatuka T., Sasaki T., Kaiser E. T., *J. Am. Chem. Soc.*, **109**, 3808—3810 (1987); *c*) Wong C.-H., *Science*, **244**, 1145—1152 (1989); *d*) Schellenberger V., Jakubke H.-D., *Angew. Chem. Int. Ed. Engl.*, **30**, 1437—1449 (1991).
3) *a*) Nozawa M., Tanizawa K., Kanaoka Y., *J. Pharmacobio-Dyn.*, **3**, 213—219 (1980); *b*) Itoh K., Sekizaki H., Toyota E., Tanizawa K., *Chem. Pharm. Bull.*, **43**, 2082—,2087 (1995); *c*) Sekizaki H., Itoh K., Toyota E., Tanizawa K., *ibid.*, **44**, 1577—1579 (1996).
4) Tanizawa K., Kasaba Y., Kanaoka Y., *J. Am. Chem. Soc.*, **99**, 4485—4488 (1977).
5) *a*) Schellenberger V., Jakubke H.-D., Zapevalova N. P., Mitin Y. Y., *Biotechnol. Bioeng.*, **38**, 104—108 (1991); *b*) Schellenberger V., Schellenberger U., Jakubke H.-D., Zapevalova N. P., Mitin Y. Y., *ibid.*, **38**, 319—321 (1991); *c*) Itoh K., Sekizaki H., Toyota E., Fujiwara N., Tanizawa K., *Bioorg. Chem.*, **24**, 59—68 (1996); *d*) Sekizaki H., Itoh K., Toyota E., Tanizawa K., *Chem. Pharm. Bull.*, **44**, 1585—1587 (1996).
6) *a*) Sekizaki H., Itoh K., Toyota E., Tanizawa K., *Tetrahedron Lett.*, **38**, 1777—1780 (1997); *b*) *Idem*, *Chem. Pharm. Bull.*, **46**, 846—849 (1998).
7) Sekizaki H., Itoh K., Toyota E., Tanizawa K., *Amino Acids*, in press.
8) Bernatowicz M. S., Wu Y., Matsueda G. R., *Tetrahedron Lett.*, **34**, 3389—3392 (1993); *idem*, *J. Org. Chem.*, **57**, 2497—2502 (1992).
9) 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).
10) Brot E. F., Bender M. L., *J. Am. Chem. Soc.*, **91**, 7187—7191 (1969).
11) Kawai M., Nagai U., *Bull. Chem. Soc. Jpn.*, **55**, 1327—1328 (1982); Okada Y., Tsuda Y., Hirata A., Nagamatsu Y., Okamoto U., *Chem. Pharm. Bull.*, **30**, 4060—4068 (1982); Bieth J., Weremath C. G., *Biochem. Biophys. Res. Commun.*, **53**, 383—390 (1970).
12) All calculations were performed using the CSC Chem3D program for Macintosh Version 3.0 (Cambridge Scientific Computing, Inc.).
13) Chase T., Jr., Shaw E., *Biochem. Biophys. Res. Commun.*, **29**, 508—514 (1967).
14) Brandt K. G., Himoe A., Hess G. P., *J. Biol. Chem.*, **242**, 3973—3982 (1967).