Synthesis and Properties of N^{α}-(*tert*-Butyloxycarbonyl)peptide *p*-Guanidinophenyl Esters as Trypsin Substrates[1)]

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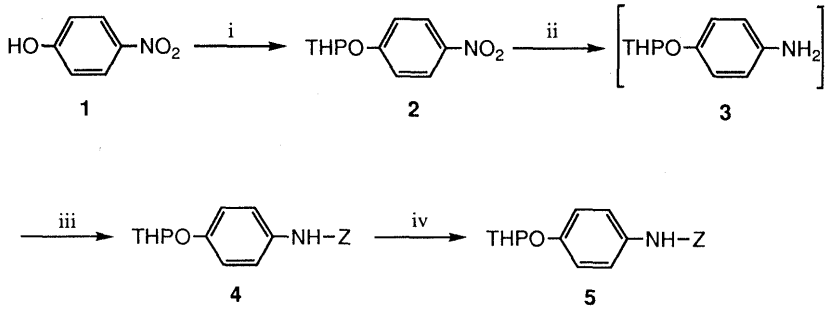
N^{α}-(*tert*-Butyloxycarbonyl)peptide *p*-guanidinophenyl esters were synthesized by amidination of N^{α}-(*tert*-butyloxycarbonyl)peptide *p*-aminophenyl esters with 1-[*N*,*N'*-bis(benzyloxycarbonyl)amidino]pyrazole, followed by deprotection by catalytic hydrogenation, in good total yields. These synthetic esters were characterized as specific substrates for trypsin, and kinetic parameters for the trypsin-catalyzed hydrolysis are presented.

Key words N^{α}-(*tert*-butyloxycarbonyl)peptide *p*-guanidinophenyl ester; 1-[*N*,*N'*-bis(benzyloxycarbonyl)amidino]pyrazole; inverse substrate; trypsin; tryptic hydrolysis; enzyme kinetics

In a previous paper,[2)] we reported that *p*-acetoxyphenylguanidine behaved as a specific substrate for trypsin and trypsin-like enzymes, in spite of the fact that the site-specific group (a charged guanidinium) for the enzyme is not included in the acyl moiety but in the leaving group portion. Such a substrate is termed[3)] an "inverse substrate" for trypsin and trypsin-like enzymes. Inverse substrates are useful for specific introduction of a wide variety of acyl groups into the trypsin active site,[4)] and such acyl enzymes are expected to be useful for the analysis of the active site structure of trypsin and trypsin-like enzymes. We therefore wished to develop a general procedure for the synthesis of N^{α}-Boc-peptide *p*-guanidinophenyl esters. Schellenberger *et al.*[5)] recently reported the synthesis of N^{α}-Z-L-alanine and N^{α}-Z-L-proline *p*-guanidinophenyl esters by condensation of *p*-guanidinophenol with N^{α}-Z-amino acid. We initially applied their procedure for the preparation of N^{α}-Boc-L-alanine *p*-guanidinophenyl ester, but only a small amount of N^{α}-Boc-L-alanine *p*-[*N*-(N^{α}-Boc-L-alanyl)guanidino]phenyl ester was obtained. In this paper, we report a general method for the synthesis of N^{α}-Boc-peptide *p*-guanidinophenyl esters. Their properties in relation to trypsin are also described.

Synthesis of Inverse Substrates *p*-*N*-Z-Aminophenol (**5**), a key compound in this study, was prepared from *p*-nitrophenol (**1**) as shown in Chart 1. Tetrahydropyranylation of *p*-nitrophenol (**1**) in the presence of a catalytic amount of TsOH in benzene at room temperature gave the *p*-tetrahydropyran-2-yloxynitrobenzene (**2**) in 74% yield. Compound **2** was converted to the *p*-aminophenol derivative **3** by catalytic hydrogenation over 10% palladium on carbon (10% Pd–C) in EtOH at room temperature and **3** was used for the next reaction without purification. Protection of the amino group of **3** with Z-ONSu in $CHCl_3$ afforded *p*-tetrahydropyran-2-yloxy-*N*-Z-aminobenzene (**4**) in 90% yield from **2**. Then, compound **4** was converted to *p*-*N*-Z-aminophenol (**5**) with TsOH in MeOH at 60 °C in quantitative yield.

N^{α}-Boc-Peptide *p*-*N*-Z-aminophenyl esters (**7a—d**) containing a glycine residue at the C-terminal position were synthesized by direct coupling of N^{α}-Boc-peptide (**6**) and compound **5**, as shown in Chart 2. Condensation of *p*-*N*-Z-aminophenol (**5**) with N^{α}-Boc-glycine derivatives (**6a—d**) by the use of DCC in the presence of a catalytic amount of DMAP in a mixture of DMF–CH_2Cl_2 (1 : 1) afforded compounds **7a—d** in 66—99% yields. On the other hand, N^{α}-Boc-peptide *p*-*N*-Z-aminophenyl esters (**7e—g**) containing an optically active amino acid residue at the C-terminal position were synthesized *via* N^{α}-Boc-amino acid *p*-*N*-Z-aminophenyl esters (**8e—g**) and subsequent elongation of the peptide chain in order to prevent racemization. N^{α}-Boc-Amino acid *p*-*N*-Z-aminophenyl esters (**8e—g**) were also obtained *via* the route used for the preparation of compounds **7a—d** in 82—96% yields. Selective removal of the N^{α}-Boc group from compounds



i) 3,4-dihydro-2*H*-pyran, *p*-TsOH·H_2O in benzene; ii) H_2, 10% Pd-C in EtOH
iii) *N*-(benzyloxycarbonyloxy)succinimide in $CHCl_3$; iv) *p*-TsOH·H_2O in MeOH

Chart 1

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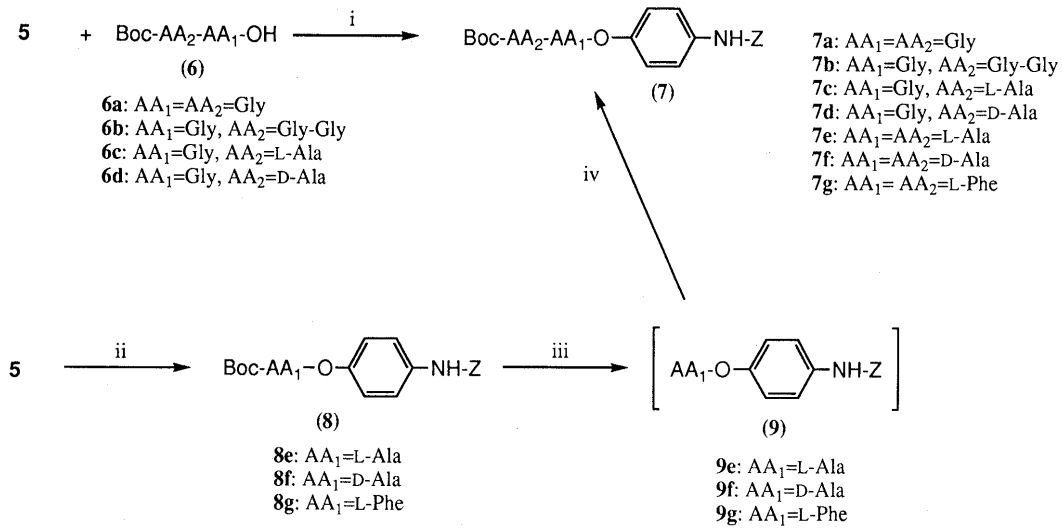


i) DCC, 4-dimethylaminopyridine in DMF/CH_2Cl_2
ii) Boc-AA_1-OH, DCC, 4-dimethylaminopyridine in DMF/CH_2Cl_2
iii) TFA-anisole
iv) Boc-AA_2-OH, DCC, 4-dimethylaminopyridine, diisopropylethylamine in DMF

Chart 2

Table 1. Yield, Physical and Spectral Data of N^{α}-(*tert*-Butyloxycarbonyl)peptide *p*-*N*-(Benzyloxycarbonyl)aminophenyl Esters

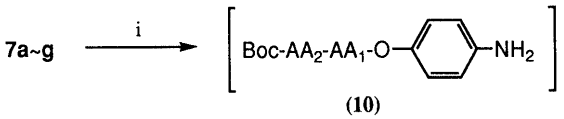
Product	Peptide	Yield (%)	mp (°C) (Recryst. solv.)	IR (KBr) ν (cm^{-1})	$[\alpha]_D^{25}$ ($c=1.0$, $CHCl_3$)	^{1}H-NMR ($CDCl_3$/TMS) δ, J (Hz)	Formula	Analysis (%) Calcd (Found) C	H	N
7a	Gly–Gly	66	160—162 (AcOEt)	3353, 1783, 1718, 1685, 1667, 1646, 1628	—	1.45 (9H, s), 3.87 (2H, d, 5.9), 4.30 (2H, d, 5.4), 5.12 (1H, br), 5.20 (2H, s), 6.68 (1H, m), 6.75 (1H, s), 7.04 (2H, d, 9.3), 7.35—7.41 (7H, m)	$C_{23}H_{27}N_3O_7$	60.39 (60.83)	5.95 (6.15)	9.19 (9.13)
7b[a]	Gly–Gly–Gly	75	176—178 (EtOH)	3312, 1779, 1698, 1681, 1668, 1611	—	1.45 (9H, s), 3.74 (2H, s), 3.95 (2H, s), 4.19 (2H, s), 5.17 (2H, s), 7.03 (2H, d, 8.8), 7.30—7.47 (7H, m)	$C_{25}H_{30}N_4O_8$	58.36 (58.51)	5.88 (5.95)	10.89 (10.90)
7c[b]	L-Ala–Gly	90	168—169 (EtOH)	3331, 1775, 1675, 1612	−10.4	1.38 (3H, d, 7.3), 1.44 (9H, s), 4.22—4.29 (3H, m), 4.97 (1H, br), 5.20 (2H, s), 6.77 (2H, br s), 7.04 (2H, d, 8.8), 7.34—7.40 (7H, m)	$C_{24}H_{29}N_3O_7$	61.14 (61.26)	6.20 (6.29)	8.91 (8.90)
7e[c]	L-Ala–L-Ala	82	147—149 (AcOEt–hexane)	3331, 1769, 1701, 1686, 1658	−40.4	1.37 (3H, d, 7.3), 1.44 (9H, s), 1.56 (3H, d, 7.3), 4.19 (1H, m), 4.78 (1H, q, 7.3), 4.97 (1H, m), 5.20 (2H, s), 6.66 (1H, m), 6.71 (1H, s), 7.04 (2H, d, 8.8), 7.32—7.40 (7H, m)	$C_{25}H_{31}N_3O_7$	61.84 (61.84)	6.44 (6.46)	8.65 (8.69)
7g	L-Phe–L-Phe	87	183—186 (EtOH)	3334, 1769, 1702, 1687, 1660	+6.4	1.39 (9H, s), 3.05 (2H, d, 6.4), 3.16 (2H, d, 6.4), 4.35 (1H, m), 4.97 (1H, m), 5.20 (2H, s), 6.35 (1H, m), 6.87 (2H, d, 8.8), 7.08 (2H, m), 7.12—7.41 (17H, m)	$C_{37}H_{39}N_3O_7$	69.73 (69.73)	6.16 (6.25)	6.59 (6.57)

a) ^{1}H-NMR spectrum was measured in MeOH-d_4. *b*) The enantiomer (**7d**) of this compound showed almost the same spectral data except for the optical rotation, $[\alpha]_D^{25} = +27.8$. *c*) The enantiomer (**7f**) of this compound showed almost the same spectral data except for the optical rotation, $[\alpha]_D^{25} = +45.8$.

8e—g was achieved by treatment with a mixture of TFA–anisole (9 : 1) at room temperature to give amino acid *p*-*N*-Z-aminophenyl esters (**9e—g**), and these esters **9e—g** were used for the next reaction without purification. Condensation of **9e—g** with N^{α}-Boc-amino acid by the use of DCC in DMF afforded N^{α}-Boc-peptide *p*-*N*-Z-aminophenyl esters (**7e—g**) in 73—87% yields from **8e—g**.

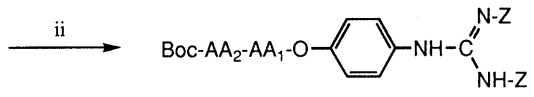
N^{α}-Boc-peptide *p*-guanidinophenyl esters as their *p*-toluenesulfonic acid salts (**12a—g**) were prepared from compounds **7a—g**, as shown in Chart 3. Deprotection of compounds **7a—g** by catalytic hydrogenation over 10% Pd–C in EtOH at room temperature afforded N^{α}-Boc-peptide *p*-aminophenyl esters (**10a—g**), and these products were used for the subsequent amidination without further purification. Amidination reagent, 1-[*N*,*N*′-bis(Z)amidino]pyrazole, was prepared according to the reported procedure.[6] N^{α}-Boc-Peptide *p*-aminophenyl esters (**10a—g**) were reacted with 1-[*N*,*N*′-bis(Z)-amidino]pyrazole in THF at room temperature to give N^{α}-Boc-peptide *p*-[*N*′,*N*″-bis(Z)guanidino]phenyl esters (**11a—g**) in 46—82% yields. These esters **11a—g** were completely cleaved by catalytic hydrogenation over 10% Pd–C in EtOH to give N^{α}-Boc-peptide *p*-guanidinophenyl esters (**12a—g**) as the TsOH salts in essentially quantitative yields.

In the previous paper,[2] we reported that *p*-acetoxyphenylguanidine and *p*-trimethylacetoxyphenylguanidine



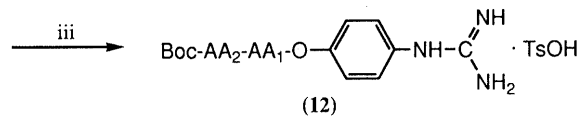
(10)

10a: AA_1=AA_2=Gly
10b: AA_1=Gly, AA_2=Gly-Gly
10c: AA_1=Gly, AA_2=L-Ala
10d: AA_1=Gly, AA_2=D-Ala
10e: AA_1=AA_2=L-Ala
10f: AA_1=AA_2=D-Ala
10g: AA_1= AA_2=L-Phe



(11)

11a: AA_1=AA_2=Gly
11b: AA_1=Gly, AA_2=Gly-Gly
11c: AA_1=Gly, AA_2=L-Ala
11d: AA_1=Gly, AA_2=D-Ala
11e: AA_1=AA_2=L-Ala
11f: AA_1=AA_2=D-Ala
11g: AA_1= AA_2=L-Phe



(12)

12a: AA_1=AA_2=Gly
12b: AA_1=Gly, AA_2=Gly-Gly
12c: AA_1=Gly, AA_2=L-Ala
12d: AA_1=Gly, AA_2=D-Ala
12e: AA_1=AA_2=L-Ala
12f: AA_1=AA_2=D-Ala
12g: AA_1= AA_2=L-Phe

i) H_2, 10% Pd-C in EtOH
ii) 1-[*N*,*N*'-bis(Z)amidino]pyrazole in THF
iii) H_2, 10% Pd-C, *p*-TsOH·H_2O in EtOH

Chart 3

behaved as specific substrates for trypsin and trypsin-like enzymes. However, one of the major problems was the difficulty in the synthesis of *p*-guanidinophenyl esters from α-amino acid and peptide derivatives. This problem was solved by the present procedure. In this study, the compounds (**12a—d**) were synthesized for comparison with the N^{α}-acetylpeptide *p*-amidinophenyl esters used in the previous studies.[4d] Compounds (**12e—g**) were also synthesized as examples of products with an optically active amino acid residue at the C-terminal position.

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.

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

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.

Trypsin-catalyzed hydrolysis of peptidyl inverse substrates was monitored spectrophotometrically, using the procedure described previously.[4d] The rates of the rapid acylation of trypsin were determined by the stopped-flow technique. Determination of kinetic parameters for trypsin-catalyzed hydrolysis was carried out by the thionine displacement method, under the conditions: $[S]_0 \gg [E]_0 < [\text{Thionine}]$, as described in the literature.[4d] The kinetic parameters of compounds **12a—d** were determined to be as shown in Table 4, and the parameters were compared with those of N^{α}-acetylpeptide *p*-amidinophenyl esters previously reported (Table 4).[4e] It was shown that N^{α}-Boc-peptide *p*-guanidinophenyl esters (**12a—d**) behave as specific substrates for trypsin, based on their k_2/K_s values. The parameter, k_2/K_s, has been introduced by Brot and Bender[7] for the evaluation of the specificity of substrates. The observed k_2/K_s values, in the range of 10^5—10^7 ($s^{-1} \cdot M^{-1}$), are sufficiently large for trypsin substrates. The parameters for trypsin-like enzymes such as human urokinase were up to 1.0×10^4 ($s^{-1} \cdot M^{-1}$). It has already been shown that inverse substrates are virtually unaffected by enzymes other than those of the trypsin family.[3] For chymotrypsin, the values were so small that they were not determinable.

In terms of this value, **12a—d** were nearly equivalent to the series of *p*-amidinophenyl esters as trypsin and trypsin-like enzyme substrates.[4a-f] Compound **12c** was the most specific substrate and its k_2/K_s value was even larger than that of the normal-type substrate, *p*-nitrophenyl *p*-guanidinobenzoate (NPGB).[8] It should be not that a large k_2/K_s value may be due to either small K_s or large k_2 values. Therefore, it seems likely that the subsite interaction of the enzyme active site with L-alanine residue is stronger than those with D-alanine and glycine.[9] The resulting tight ES complex is favarable for the following acylation process.

Our interest was centered on the deacylation process, which cannot be explored by using conventional substrates. The deacylation rates have been analyzed only for the normal-type substrate which possesses a cationic acyl group. An inverse substrate can reveal the deacylation rate for a noncationic acyl group which is not susceptible to the enzyme in a conventional substrate. It was recognized that the deacylation process was not dependent upon the leaving groups, such as *p*-amidinophenol and *p*-guanidinophenol. So, comparison of the deacylation rate for N^{α}-acetylpeptide *p*-amidinophenyl esters together with N^{α}-Boc-peptide *p*-guanidinophenyl esters is meaningful, *i.e.*, each rate reflects the intrinsic interaction of the acyl residue with the enzyme. Elongation of the peptide chain slightly decreased the efficiency of deacylation of N^{α}-

Table 2. Yield, Physical and Spectral Data of N^{α}-(*tert*-Butyloxycarbonyl)peptide *p*-[*N'*,*N''*-Bis(benzyloxycarbonyl)guanidino]phenyl Esters

Product	Peptide	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
11a	Gly–Gly	78	136—138 (EtOH)	3415, 1767, 1731, 1720, 1656, 1620	—	1.45 (9H, s), 3.87 (2H, d, 5.9), 4.31 (2H, d, 5.4), 5.10 (1H, br), 5.14 (2H, s), 5.24 (2H, s), 6.65 (1H, br), 7.08 (2H, d, 8.8), 7.29—7.35 (10H, m), 7.60 (2H, d, 9.3), 10.28 (1H, s), 11.88 (1H, s)	$C_{32}H_{35}N_5O_9$	60.66 (60.73)	5.57 (5.59)	11.05 (11.05)
11b	Gly–Gly–Gly	46	132—134 (EtOH)	3295, 1761, 1721, 1646	—	1.44 (9H, s), 3.80 (2H, d, 5.4), 4.02 (2H, d, 5.9), 4.25 (2H, d, 5.9), 5.14 (2H, s), 5.24 (2H, s), 7.07 (2H, d, 8.8), 7.29—7.35 (5H, m), 7.58 (2H, d, 8.8), 10.27 (1H, s), 11.88 (1H, s)	$C_{34}H_{38}N_6O_{10}$	59.12 (59.00	5.55 5.59	12.17 12.18)
11c[a]	L-Ala–Gly	82	146—148 (EtOH)	3344, 1771, 1731, 1691, 1667, 1640	−4.4	1.39 (3H, d, 7.3), 1.44 (9H, s), 4.22—4.30 (3H, m), 4.93 (1H, br), 5.15 (2H, s), 5.24 (2H, s), 6.76 (1H, br), 7.08 (2H, d, 8.8), 7.31—7.39 (5H, m), 7.59 (2H, d, 8.8)	$C_{33}H_{37}N_5O_9$	61.20 (61.05	5.76 5.75	10.81 10.84)
11e[b]	L-Ala–L-Ala	57	152—155 (EtOH)	3344, 1763, 1733, 1685, 1675, 1651, 1629	−31.6	1.37 (3H, d, 6.8), 1.44 (9H, s), 1.56 (3H, d, 7.3), 4.19 (1H, m), 4.78 (1H, q, 7.3), 5.14 (2H, s), 5.24 (2H, s), 6.64 (1H, m), 7.07 (2H, d, 8.8), 7.28—7.40 (10H, m), 7.59 (2H, d, 8.8), 10.27 (1H, s), 11.88 (1H, s)	$C_{34}H_{39}N_5O_9$	61.72 (61.61	5.94 6.05	10.58 10.62)
11g	L-Phe–L-Phe	76	184—186 (EtOH)	3343, 3311, 1757, 1727, 1683, 1665, 1655, 1629	0.0	1.39 (9H, s), 3.05 (2H, d, 6.3), 3.16 (2H, d, 6.3), 4.37 (1H, br), 4.97 (1H, m), 5.15 (2H, s), 5.40 (2H, s), 6.33 (1H, d, 6.8), 6.91 (2H, d, 8.8)	$C_{46}H_{48}N_5O_9$	67.80 (67.58	5.94 5.93	8.59 8.68)

a) The enantiomer (**11d**) of this compound showed almost the same spectral data except for the optical rotation, $[\alpha]_D^{25} = +18.8$. *b*) The enantiomer (**11f**) of this compound showed almost the same spectral data except for the optical rotation, $[\alpha]_D^{25} = +38.8$.

Table 3. Yield, Physical and Spectral Data of N^{α}-(*tert*-Butyloxycarbonyl)peptide *p*-Guanidinophenyl Esters as Their *p*-Toluenesulfonic Acid Salts

Product	Peptide	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 ($M^+ + H$)
12a	Gly–Gly	98	3336, 1772, 1675	—	1.45 (9H, s), 2.36 (3H, s), 3.78 (2H, s), 4.22 (2H, s), 7.21—7.30 (6H, m), 7.69 (2H, d, 8.3)	366
12b	Gly–Gly–Gly	94	3342, 1767, 1674	—	1.45 (9H, s), 2.36 (3H, s), 3.75 (2H, s), 3.95 (2H, s), 4.22 (2H, s), 7.21—7.30 (6H, m), 7.69 (2H, d, 8.3)	423
12c[a]	L-Ala–Gly	92	3336, 1769, 1678	−18.8	1.33 (3H, d, 7.3), 1.44 (9H, s), 2.36 (3H, s), 4.12—4.26 (3H, m), 7.22—7.25 (4H, m), 7.33 (2H, d, 8.8), 7.70 (2H, d, 8.3)	380
12e[b]	L-Ala–L-Ala	91	3332, 1767, 1676	−48.0	1.31 (3H, d, 6.8), 1.43 (9H, s), 1.55 (3H, d, 7.3), 2.36 (3H, s), 4.11 (1H, q, 7.3), 4.55—4.62 (1H, m), 7.22 (4H, dd, 2.9 and 8.8), 7.31 (2H, d, 8.3), 7.70 (2H, d, 8.3)	394
12g	L-Phe–L-Phe	94	3332, 1765, 1678	−10.0	1.34 (9H, s), 2.36 (3H, s), 2.76 (1H, dd, 9.3 and 13.2), 3.04 (1H, dd, 5.4 and 13.2), 3.21—3.28 (2H, m), 4.32—4.35 (1H, m), 4.80—4.93 (1H, overlap with DOH), 7.05 (2H, d, 8.3), 7.21—7.32 (14H, m), 7.70 (2H, d, 7.8)	546

a) The enantiomer (**12d**) of this compound showed almost the same spectral data except for the optical rotation, $[\alpha]_D^{25} = +17.2$. *b*) The enantiomer (**12f**) of this compound showed almost the same spectral data except for the optical rotation, $[\alpha]_D^{25} = +50.2$.

Boc-glycine derivatives. The deacylation rate for N^{α}-Boc-glycylglycylglycine is 40% of that for N^{α}-Boc-glycylglycine. This result is the reverse of that for N^{α}-acetylglycine derivatives (Table 4). This difference seems to be due to the N^{α}-protecting group in the acyl component. The N^{α}-Boc-D-alanylglycine residue underwent deacylation, and the rate was 1/2.7 of that of the N^{α}-Boc-L-alanylglycine residue. The enantiomeric preference is more prominent for the acetyl peptide, as shown in Table 4. The deacylation rate of N^{α}-Boc-peptides (**12a—d**) was one order of magnitude less than that of the corresponding N^{α}-acetylpeptides. Therefore, it can be assumed that N^{α}-Boc-peptide, involving a sterically bulky *N*-protecting group, causes some distortion of the enzyme active site and this results in a decrease of the catalytic activity.[2)]

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. Chemical shifts are quoted in parts per million (ppm) with tetramethylsilane (TMS) as an internal standard. Coupling constants (J) are given in Hz. The following abbreviations are used: s, singlet; d, doublet; t, triplet; q, quartet; br s, broad singlet; dd, doublet of doublets; m, multiplet. Optical rotations were measured with a JASCO DIP-360 digital polarimeter in a 5 cm length cell. TLC was performed on Merck Silica gel 60F-254. Column chromatography was carried out on silica gel (Kanto Chemicals, over 100 mesh). Kinetic parameters were determined with a Union Giken RA-401 stopped-flow spectrometer and a Hitachi U-2000 UV spectrophotometer. Bovine pancreas trypsin (EC 3.4.21.4) was purchased from Worthington Biochemical Corp. (twice recrystallized, lot TRL).

***p*-Tetrahydropyran-2-yloxynitrobenzene (2)** $TsOH \cdot H_2O$ (15 mg) was added to a suspension of *p*-nitrophenol (**1**) (13.9 g, 0.1 mol) and

Table 4. Kinetic Parameters for the Trypsin-Catalyzed Hydrolysis of Inverse Substrates and Related Compounds at pH 8.0

Compound	K_s (M)	k_2 (s^{-1})	k_3 (s^{-1})	k_2/K_s ($s^{-1} \cdot M^{-1}$)	Reference
Boc–Gly–Gly–OGp (**12a**)	3.23×10^{-5}	19.9	3.10×10^{-1}	6.16×10^{5}	This work
Boc–Gly–Gly–Gly–OGp (**12b**)	3.76×10^{-5}	87.2	1.27×10^{-1}	2.32×10^{5}	This work
Boc–L-Ala–Gly–OGp (**12c**)	4.93×10^{-6}	140	1.37×10^{-1}	2.84×10^{7}	This work
Boc–D-Ala–Gly–OGp (**12d**)	5.90×10^{-5}	7.13	5.07×10^{-2}	1.21×10^{5}	This work
Ac–OGp[a]	8.84×10^{-4}	81.6	1.25×10^{-2}	9.23×10^{4}	4*e*
NPGB[b]	3.65×10^{-5}	269	4.1×10^{-5}	7.37×10^{6}	8
Ac–Gly–Gly–OAm	2.74×10^{-5}	20.9	1.26	7.63×10^{5}	4*e*
Ac–Gly–Gly–Gly–OAm	4.65×10^{-5}	14.9	1.46	3.20×10^{5}	4*e*
Ac–L-Ala–Gly–OAm	3.64×10^{-6}	9.55	1.83	2.62×10^{5}	4*e*
Ac–D-Ala–Gly–OAm	1.92×10^{-5}	6.56	2.76×10^{-1}	3.41×10^{5}	4*e*
Ac–OAm[c]	3.87×10^{-5}	17.0	9.26×10^{-3}	4.39×10^{5}	4*e*
NPAB[d]	5.03×10^{-6}	30.4	6.53×10^{-2}	6.04×10^{6}	3

a) *p*-Acetoxyphenylguanidine. *b*) *p*-Nitrophenyl *p'*-guanidinobenzoate; kinetic parameters of this compound were determined at pH 7.8. *c*) *p*-Acetoxyphenylamidine. *d*) *p*-Nitrophenyl *p'*-amidinobenzoate.

3,4-dihydro-2*H*-pyran (12.6 g, 0.15 mol) in dry benzene (50 ml). An exothermic reaction occurred immediately after the addition, and the suspension changed to a clear solution. After the exothermic reaction stopped, the reaction mixture was warmed at 60 °C for 30 min. It was diluted with AcOEt (50 ml), washed successively with 2 M aqueous NaOH, water and saturated brine, dried (Na_2SO_4), and evaporated to give the crude product. Pure **2** (17.74 g, 74%) was obtained by recrystallization from hexane as colorless plates. mp 54—55 °C. IR (KBr): 1515, 1342, 1205, 1177, 1111, 1052, 1023 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.60—1.65 (1H, m), 1.67—1.79 (2H, m), 1.88—1.94 (2H, m), 1.96—2.06 (1H, m), 3.62—3.67 (1H, m), 3.79—3.85 (1H, m), 5.54 (1H, dd, J=2.9, 2.9 Hz), 7.12 (2H, d, J=9.3 Hz), 8.18 (2H, d, J=9.3 Hz). *Anal.* Calcd for $C_{11}H_{13}NO_4$: C, 59.19; H, 5.87; N, 6.27. Found: C, 59.19; H, 5.95; N, 6.22.

***p*-Tetrahydropyran-2-yloxyaminobenzene (3)** A solution of **2** (11.15 g, 50 mmol) in EtOH (250 ml) containing 10% Pd–C (250 mg) was vigorously stirred in an atmosphere of hydrogen at room temperature for 20 h. The catalyst was filtered off and the filtrate was evaporated to dryness *in vacuo*. The crude residue was used for the next reaction without purification.

***p*-Tetrahydropyran-2-yloxy-*N*-(benzyloxycarbonyl)aminobenzene (4)** A solution of crude **3** (from **2**, 50 mmol) in $CHCl_3$ (100 ml) was treated with Z-ONSu (12.45 g, 50 mmol). The reaction mixture was stirred at room temperature for 2 h, then the solution was concentrated to 20 ml *in vacuo*. The residue was diluted with AcOEt (150 ml) and the solution was washed with saturated brine, dried (Na_2SO_4), and evaporated to give the crude product. Pure **4** (14.8 g, 90% from **2**) was obtained by recrystallization from AcOEt–benzene as colorless fine needles. mp 123—125 °C. IR (KBr): 3247, 1719, 1220 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.58—1.72 (3H, m), 1.83—1.89 (2H, m), 1.95—2.03 (1H, m), 3.56—3.61 (1H, m), 3.87—3.93 (1H, m), 5.19 (2H, s), 5.35 (1H, dd, J=2.9, 2.9 Hz), 6.55 (1H, br), 7.00 (2H, d, J=8.8 Hz), 7.26—7.29 (2H, overlap with $CHCl_3$), 7.29—7.41 (5H, m). *Anal.* Calcd for $C_{19}H_{21}NO_4$: C, 69.71; H, 6.47; N, 4.28. Found: C, 69.75; H, 6.56; N, 4.28.

***p*-*N*-(Benzyloxycarbonyl)aminophenol (5)** A suspension of **4** (9.08 g, 40 mmol) in MeOH (120 ml) was treated with $TsOH \cdot H_2O$ (760 mg, 4 mmol). The reaction mixture was refluxed for 1 h, then evaporated to dryness *in vacuo*. The solid residue was dissolved in AcOEt (200 ml) and the solution was washed with saturated brine, dried (Na_2SO_4), and evaporated to give the crude product. Pure **5** (9.40 g, 97%) was obtained by recrystallization from benzene–AcOEt as colorless needles. mp 160—162 °C. IR (KBr): 3306, 1709 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 4.72 (1H, s), 5.19 (2H, s), 6.52 (1H, br), 6.78 (2H, d, J=8.8 Hz), 7.23 (2H, d, J=8.8 Hz), 7.32—7.41 (5H, m). *Anal.* Calcd for $C_{14}H_{13}NO_3$: C, 69.13; H, 5.39; N, 5.76. Found: C, 69.23; H, 5.49; N, 5.76.

N^{α}-(*tert*-Butyloxycarbonyl)glycylglycine (**6a**), N^{α}-(*tert*-butyloxycarbonyl)glycylglycylglycine (**6b**), N^{α}-(*tert*-butyloxycarbonyl)-L-alanylglycine (**6c**) and N^{α}-(*tert*-butyloxycarbonyl)-D-alanylglycine (**6d**) were prepared by the reported procedure.[10]

N^{α}-(*tert*-Butyloxycarbonyl)glycylglycine *p*-*N*-(Benzyloxycarbonyl)aminophenyl Ester (7a) A solution of **5** (970 mg, 4 mmol), **6a** (928 mg, 4 mmol), and DMAP (48.8 mg, 0.4 mmol) in a mixture of DMF (15 ml) and CH_2Cl_2 (15 ml) was treated with DCC (960 mg, 4.4 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 ester **7a** (1.206 g, 66%) was obtained by recrystallization from AcOEt as a colorless powder. Compounds **7b—d** were synthesized from **6b—d** using the same procedure as described above. Yields, physical properties and spectral data of **7a—d** are given in Table 1.

N^{α}-(*tert*-Butyloxycarbonyl)-L-alanine *p*-*N*-(Benzyloxycarbonyl)aminophenyl Ester (8e) Compound **8e** (5.47 g, 66%) was obtained from **5** (4.86 g, 20 mmol) and N^{α}-Boc-L-alanine (3.78 g, 20 mmol) by a procedure similar to that described for **7a**. mp 153—155 °C, colorless needles after recrystallization from benzene. IR (KBr): 1766, 1708, 1688 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.46 (9H, s), 1.54 (3H, d, J=6.8 Hz), 4.53 (1H, m), 5.20 (2H, s), 6.72 (1H, br s), 7.04 (2H, d, J=9.3 Hz), 7.33—7.40 (7H, m). *Anal.* Calcd for $C_{22}H_{26}N_2O_6$: C, 63.76; H, 6.32; N, 6.76. Found: C, 63.85; H, 6.33; N, 6.78.

N^{α}-(*tert*-Butyloxycarbonyl)-D-alanine *p*-*N*-(Benzyloxycarbonyl)aminophenyl Ester (8f) Compound **8f** (7.96 g, 96%) was obtained from **5** (4.86 g, 20 mmol) and N^{α}-Boc-D-alanine (3.78 g, 20 mmol) by a procedure similar to that described for **7a**. mp 154—155 °C, colorless needles after recrystallization from benzene. IR (KBr): 1764, 1712, 1686 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ: 1.46 (9H, s), 1.54 (3H, d, J=6.8 Hz), 4.53 (1H, m), 5.07 (1H, m), 5.20 (2H, s), 6.69 (1H, br s), 7.04 (2H, d, J= 9.3 Hz), 7.34—7.40 (7H, m). *Anal.* Calcd for $C_{22}H_{26}N_2O_6$: C, 36.76; H, 6.32; N, 6.76. Found: C, 63.93; H, 6.29; N, 6.72.

N^{α}-(*tert*-Butyloxycarbonyl)-L-phenylalanine *p*-*N*-(Benzyloxycarbonyl)-aminophenyl Ester (8g) Compound **8g** (8.14 g, 83%) was obtained from **5** (4.86 g, 20 mmol) and N^{α}-Boc-L-phenylalanine (5.12 g, 20 mmol) by a procedure similar to that described for **7a**. mp 152—154 °C, colorless needles after recrystallization from benzene. IR (KBr): 1767, 1698 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.44 (9H, s), 3.22 (2H, d, J=6.4 Hz), 4.79 (1H, m), 5.20 (2H, s), 6.68 (1H, br s), 6.92 (2H, d, J=8.8 Hz), 7.22—7.41 (12H, m). *Anal.* Calcd for $C_{28}H_{30}N_2O_6$: C, 68.56; H, 6.16; N, 5.71. Found: C, 68.75, H, 6.26; N, 5.74.

L-Alanine *p*-*N*-(Benzyloxycarbonyl)aminophenyl Ester Trifluoroacetate (9e) A mixture of **8a** (1.656 g, 4 mmol) and TFA–anisole (9 : 1, v/v) (10 ml) was stirred at room temperature for 1 h, then evaporated to dryness *in vacuo*. The solid residue of **9e** was used for the next reaction without purification. Compounds **9f—g** were synthesized in a similar manner.

N^{α}-(*tert*-Butyloxycarbonyl)-L-alanyl-L-alanine *p*-*N*-(Benzyloxycarbonyl)aminophenyl Ester (7e) A solution of **9e** (4 mmol from **8a**), N^{α}-Boc-L-alanine (756 mg, 4 mmol), DMAP (48.8 mg, 0.4 mmol) and diisopropylethylamine (516 mg, 4 mmol) in DMF (8 ml) was treated with DCC (824 mg, 4 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 20 h. The resulting precipitate of DCUrea was filtered off, and the filtrate was evaporated to dryness *in vacuo*. Pure **7e** (1.591 g, 82% from **11a**) was obtained as colorless needles by recrystallization from AcOEt–hexane. Compounds **7f—g** were synthesized from **9f—g** using the same procedure as described above. Yields, physical properties and spectral data of **7e—g** are given in Table 1.

N^{α}-(*tert*-Butyloxycarbonyl)glycylglycine *p*-Aminophenyl Ester (10a)

A suspension of **7a** (914 mg, 2 mmol) in EtOH (40 ml) containing 10% Pd–C was vigorously stirred overnight in an atmosphere of hydrogen at room temperature. The catalyst was filtered off and the filtrate was evaporated to dryness *in vacuo*. The crude residue of **10a** was used for the next reaction without purification. Compounds **10b—g** were similarly synthesized from **7b—g**.

***N*$^{\alpha}$-(*tert*-Butyloxycarbonyl)glycylglycine *p*-[*N'*,*N''*-Bis(benzyloxycarbonyl)guanidino]phenyl Ester (11a)** A solution of **10a** (2 mmol from **7a**) and 1-[*N*,*N'*-bis(benzyloxycarbonyl)amidino]pyrazole (754 mg, 2 mmol) in absolute THF (0.8 ml) was stirred overnight at room temperature an atmosphere of nitrogen. The reaction mixture was diluted with benzene–AcOEt (1 : 1) and passed through a short silica gel column (i.d. 3 × 30 cm). The eluate was evaporated to dryness *in vacuo* and the solid residue was recrystallized from EtOH to give **11a** (988 mg, 78% from **7a**) as colorless needles. Compounds **11b—g** were synthesized from **10b—g** using the same procedure as described above. Yields, physical properties and spectral data of **11a—g** are given in Table 2.

***N*$^{\alpha}$-(*tert*-Butyloxycarbonyl)glycylglycine *p*-Guanidinophenyl Ester *p*-Toluenesulfonic Acid Salt (12a)** A suspension of **11a** (633 mg, 1 mmol) and TsOH·H_2O (190 mg, 1 mmol) in EtOH (50 ml) containing 10% Pd–C (35 mg) was vigorously stirred overnight in 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 **12a** (526 mg, 98%). Compounds **12b—g** were synthesized from **11b—g** using the same procedure as described above. Yields and spectral data of **12a—g** are given in Table 3.

Kinetic Measurements Enzyme concentration was determined from active site titration using *p*-nitrophenyl *p'*-guanidinobenzoate.[11] Spectrophotometric analysis for determination of kinetic parameters was conducted on solutions in the concentration ranges of 2.40×10^{-5}—1.04×10^{-3} M substrate, 3.94×10^{-6} M enzyme and 2.50×10^{-5} M thionine. Determinations of k_2 and K_s were carried out in 0.05 M Tris buffer containing 0.02 M $CaCl_2$ and 0.8% EtOH (pH 8.0) at 25 °C. The observed first-order rate constants, k_{obsd}, were determined from the slope of the linear plots of $\log[(OD_{620})_t - (OD_{620})_0]$ *vs.* time. The values of k_2 and K_s were then determined by Lineweaver–Burk plots of $1/k_{obsd}$ against $1/[S]_0$. Steady-state catalytic rates, which are determined by the deacylation rate constant, k_3, were evaluated spectrophotometrically at pH 8.0 under the condition $[S]_0 \gg [E]_0$ as reported previously.[4d]

References and Notes

1) The following abbreviations are used: Boc = *tert*-butyloxycarbonyl, Z = benzyloxycarbonyl, THP = tetrahydropyran, DCC = *N*,*N'*-dicyclohexylcarbodiimide, DMF = *N*,*N*-dimethylformamide, DMAP = 4-dimethylaminopyridine, TFA = trifluoroacetic acid, TsOH = *p*-toluenesulfonic acid, Z-ONSu = *N*-(benzyloxycarbonyloxy)succinimide, DCUrea = dicyclohexylurea.
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