A Facile Synthesis of *p*- and *m*-(Amidinomethyl)phenyl Esters Derived from Amino Acid and Tryptic Hydrolysis of These Synthetic Inverse Substrates

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A facile synthetic method for *p*- and *m*-(amidinomethyl)phenyl esters derived from a variety of amino acids is presented. We analyzed the kinetic behavior of trypsin towards these synthetic esters, which are inverse substrates. The substituent (*meta*- and *para*-isomers) and isosteric effects of (amidinomethyl)phenyl esters are discussed.

Key words inverse substrate; enzymatic kinetics; *N-tert*-butoxycarbonyl-amino acid; bovine trypsin

Previously we reported that the *p*-amidinophenyl esters behave as specific substrates for trypsin and trypsin-like enzymes.[1)] In these esters the site-specific group (charged amidinium) for the enzyme is included in the leaving group portion instead of being in the acyl moiety. Such a substrate is termed an "inverse substrate".[1)] Inverse substrates allow the specific introduction of an acyl group carrying a non-specific residue into the trypsin active site. The characteristic features of inverse substrates suggested that they are useful for enzymatic peptide synthesis. We demonstrated the successful application of inverse substrates for trypsin-catalyzed coupling.[2—7)] Thus development of general method for the preparation of a variety of inverse substrates would be valuable.

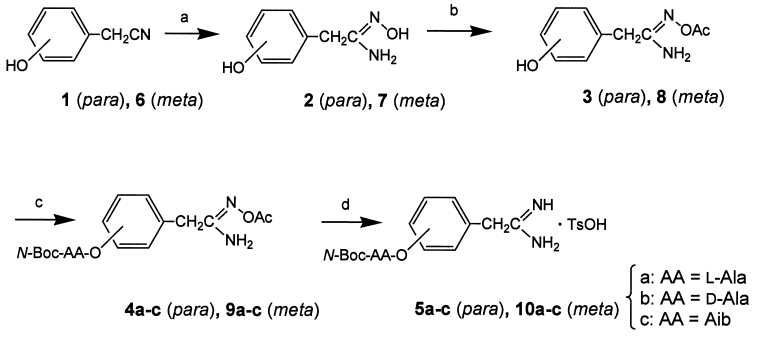
Herein, we designed and synthesized two series of a new type inverse substrates; *N-tert*-butoxycarbonyl (Boc)-amino acid *p*- and *m*-(amidinomethyl)phenyl ester, which are homoanalogous to amidinophenyl esters[2)] and isosteres of guanidinophenyl esters, respectively.[4)] We also studied the kinetic properties of these synthetic inverse substrates in the trypsin-catalyzed hydrolysis.

Synthesis of Inverse Substrates We synthesized the (amidinomethyl)phenyl esters (**5**, **10**) by using Brian's[8)] catalytic hydrogenation of {[(acetoxy)imino]-2-aminoethyl}-phenyl esters (**4**, **9**) as a key step. Hydroxybenzyl cyanide (**1**, **6**) was converted to {[(hydroxy)imino]-2-aminoethyl}phenol (**2**, **7**) with an excess amount of hydroxylamine hydrochloride in the presence of potassium carbonate in H_2O–EtOH and refluxed for 4 h to give a quantitative yield. Selected protection of the hydroxyl group of **2** and **7** with the equivalent of Ac_2O in pyridine at 0 °C and then room temperature afforded {[(acetoxy)imino]-2-aminoethyl}phenol (**3**, **8**) in 86 and 88% yield, respectively. Condensation of **3** and **8** with *N*-Boc-amino acids by using dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) in *N*,*N*-dimethylformamide (DMF)–EtOAc was successful. Reaction yields of the esters (**4a**—**c**, **9a**—**c**) were 58—88%. The next deprotection step was carried out by catalytic hydrogenation to give *N*-Boc-amino acid (amidinomethyl)phenyl esters (**5a**—**c**, **10a**—**c**) as *p*-toluenesulfonic acid (*p*-TsOH) salt in essentially quantitative yields, as shown in Chart 1.

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.

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

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



Reagents: a) NH_2OH HCl, K_2CO_3 / H_2O-EtOH; b) Ac_2O / pyridine; c) *N*-Boc-AA-OH, DMAP, DCC / DMF-EtOAc
d) H_2, Pd-C / EtOH

Chart 1

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K_s and k_2 are useful for evaluating substrates. The former can provide information on the strength of the binding between the substrate and the enzyme, which is 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 inverse substrates was monitored spectrophotometrically, using the procedure described previously.[1,9] The kinetic parameters of compounds **5a**—**c** and **10a**—**c** were determined as shown in Table 4, and the parameters were compared with those of *p*-(**11**)[9,10] and *m*-guanidinophenyl acetate (**12**),[9,10] *N*-Boc-L-Ala *p*-(**13**)[7] and *m*-(guanidinomethyl)phenyl ester (**14**),[6] and *N*-Boc-L-Ala *p*-amidinophenyl ester (**15**).[11] Binding affinity of *p*-isomers (**5a**—**c**) for trypsin is slightly greater than that of the *m*-isomers (**10a**—**c**), though the result is opposite in the comparison of spatially small guanidinophenyl acetates (**11**, **12**). In general, all *N*-Boc-amino acid (amidinomethyl)phenyl esters used in this study moderate binding affinity for trypsin with K_m values in the range of 10^{-3}—10^{-4} M nearly comparable to those of the (guanidinomethyl)phenyl esters (**13**, **14**). Hydrolysis of inverse substrate has been shown to proceed by consecutive acylation and deacylation processes.[1] If the acylation is much faster than the subsequent deacylation process, each rate constant (k_2 and k_3) would be determined directly from the spectrometric trace of the presteady-state and steady-state parts of the hydrolysis, respectively. *p*-Guanidinophenyl acetate is a substrate of this case: k_2 and k_3 were determined in this manner.[9,10] The behavior of (amidinomethyl)phenyl esters were different from those of the *p*-guamidinophenyl acetate. In the case of (amidinomethyl)-phenyl esters, the acylation and deacylation rates are close to each other, accordingly, their rate constants are undeterminable. Thus the overall catalytic rate constant ($k_{cat.}$) and K_m were determined as listed in Table 4. At first, kinetic parameters of *N*-Boc-L-Ala-OAM (**5a**, **10a**) were compared with those for *N*-Boc-L-Ala-OGM (**13**, **14**), which were reported in our previous paper.[5,6] In these compounds, although the

Table 1. Yield, Physical and Spectral Data of {[(Hydroxy)imino]-2-aminoethyl}phenols (**2**, **7**) and {[(Acetoxy)imino]-2-aminoethyl}phenols (**3**, **8**)

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	92	171—173 (H_2O)	3450, 3382, 3208, 3096	3.11 (2H, s), 5.26 (2H, s), 6.65 (2H, d, 8.4), 7.04 (2H, d, 8.4), 8.81 (1H, s), 9.18 (1H, s)	$C_8H_{10}N_2O_2 \cdot H_2O$	52.16 (52.03	6.57 6.55	15.20 15.20)
7	67	153—156 (decomp.) (Acetone)	3497, 3380, 3202	3.14 (2H, s), 5.31 (2H, s), 6.65 (1H, dd, 2.4, 8.0), 6.66—6.68 (2H, m), 7.04 (1H, dd, 8.0, 8.0), 8.86 (1H, s), 9.27 (1H, s)	$C_8H_{10}N_2O_2$	57.82 (57.76	6.07 6.06	16.86 16.91)
3	86	156—157 (Hexane/EtOH)	3488, 3364, 1747, 1647	2.02 (3H, s), 3.20 (2H, s), 6.30 (2H, s), 6.67 (2H, d, 8.4), 7.10 (2H, d, 8.4), 9.24 (1H, s)	$C_{10}H_{12}N_2O_3$	57.69 (57.26	5.81 5.89	13.45 13.15)
8	88	143—147 (EtOH)	3471, 3358, 1752, 1647	2.03 (3H, s), 3.23 (2H, s), 6.36 (2H, s), 6.61 (1H, dd, 2.4, 8.0), 6.71—6.74 (2H, m), 7.07 (1H, dd, 8.0, 8,0), 9.32 (1H, s)	$C_{10}H_{12}N_2O_3$	57.69 (57.68	5.81 5.82	13.45 13.37)

Table 2. Yield, Physical and Spectral Data of (*tert*-Butoxycarbonyl)amino Acid {[(Acetoxy)imino]-2-aminoethyl}phenyl Esters

Product	Amino acid	Yield (%)	mp (°C) (Recryst. solv.)	IR (KBr) ν (cm^{-1})	$[\alpha]_D^{25}$ (c=1.0, MeOH)	^{1}H-NMR (DMSO-d_6/TMS) δ, J (Hz)	Formula	Analysis (%) Calcd (Found)		
								C	H	N
4a	L-Ala	75	132—133 (Hexane/EtOAc)	3375, 1741, 1688	−46.4	1.39 (9H, s), 1.40 (3H, d, 7.3), 2.03 (3H, s), 3.34 (2H, s), 4.25 (1H, q, 7.3), 6.43 (br s, 2H), 7.01 (2H, d, 8.3), 7.16 (2H, d, 8.3), 7.53 (1H, d, 6.8), 7.75 (2H, d, 8.3)	$C_{18}H_{25}N_3O_6$	56.98 (56.76	6.64 6.49	11.08 11.21)
4b	D-Ala	88	134—136 (Hexane/EtOAc)	3376, 1740, 1688	+47.6	1.37 (3H, d, 7.3), 1.39 (9H, s), 2.03 (3H, s), 3.34 (2H, s), 4.20 (1H, q, 7.3), 6.43 (br s, 2H), 7.01 (2H, d, 8.3), 7.36 (2H, d, 8.3), 7.50 (1H, d, 7.9)	$C_{18}H_{25}N_3O_6$	56.98 (56.94	6.64 6.72	11.08 11.20)
4c	Aib	58	120—121 (Benzene)	3378, 1757, 1708	—	1.39 (9H, s), 1.42 (6H, s), 2.03 (3H, s), 3.34 (2H, s), 6.43 (2H, br s), 6.97 (2H, d, 7.8), 7.35 (2H, d, 7.8), 7.55 (1H, br s)	$C_{19}H_{27}N_3O_6$	58.00 (58.09	6.92 6.97	10.68 10.50)
9a	L-Ala	75	112—113 (Benzene/hexane)	3376, 3345, 1767, 1734, 1692	−52.8	1.38 (3H, d, 7.3), 1.40 (9H, s), 2.04 (3H, s), 3.37 (2H, s), 4.23 (1H, q, 7.3), 6.39 (2H, s), 6.96 (1H, d, 7.9), 7.06 (1H, s), 7.23 (1H, d, 7.9), 7.35 (1H, dd, 7.7, 7.7), 7.34 (1H, m)	$C_{18}H_{25}N_3O_6$	56.98 (57.06	6.64 6.58	11.08 11.00)
9b	D-Ala	73	112—113 (Benzene/hexane)	3377, 3347, 1766, 1735, 1692	+50.4	1.38 (3H, d, 7.3), .40 (9H, s), 2.04 (3H, s), 3.37 (2H, s), 4.23 (1H, q, 7.3), 6.40 (2H, s), 6.96 (1H, d, 7.9), 7.06 (1H, s), 7.23 (1H, d, 7.7), 7.36 (1H, dd, 7.9), 7.44 (1H, m)	$C_{18}H_{25}N_3O_6$	56.98 (57.09	6.64 6.71	11.08 10.93)
9c	Aib	68	77—79 (Benzene)	3377, 1747, 1699	—	1.40 (9H, s), 1.45 (6H, s), 2.04 (3H, s), 3.36 (2H, s), 6.40 (2H, s), 6.92 (1H, d, 7.9), 7.02 (1H, s), 7.23 (1H, d, 7,9), 7.32—7.37 (1H, m), 7.44 (1H, m)	$C_{19}H_{27}N_3O_6$	58.00 (58.13	6.92 6.96	10.68 10.57)

Table 3. Yield, Physical and Spectral Data of (*tert*-Butoxycarbonyl)amino Acid (Amidinomethyl)phenyl Esters *p*-Toluenesulfonic Acid Salt (**5a**—**c**, **10a**—**c**)

Product	Amino acid	Yield (%)	mp (°C) (Recryst. solv.)	IR (KBr) ν (cm^{-1})	$[\alpha]_D^{25}$ (c=1.0, MeOH)	^{1}H-NMR (DMSO-d_6/TMS) δ, J (Hz)	Formula	Analysis (%) Calcd (Found)			
								C	H	N	S
5a	L-Ala	92	176—177 (CH_3CN)	1777, 1705	−38.4	1.39 (9H, s), 1.40 (3H, d, 6.8), 2.28 (3H, s), 3.69 (2H, s), 4.21 (1H, q, 6.8), 7.12 (2H, d, 8.3), 7.41 (2H, d, 8.3), 4.21 (1H, q, 6.8), 7.12 (2H, d, 8.3), 7.41 (2H, d, 8.3), 8.99 (2H, m), 9.04 (2H, br s)	$C_{16}H_{23}N_3O_3$ $C_7H_8O_3S$	55.97 (56.76	6.33 6.49	8.51 8.37	6.50 6.54)
5b	D-Ala	92	176—177 (Hexane/EtOAc)	1777, 1698	+36.7	1.37 (3H, d, 7.1), 1.39 (9H, s), 2.28 (3H, s), 3.69 (2H, s), 4.20 (1H, q, 7.1), 7.07—7.12 (4H, m), 7.42—7.53 (4H, m), 8.59 (1H, br s), 9.10 (2H, br s)	$C_{16}H_{23}N_3O_3\cdot$ $C_7H_8O_3S$	55.97 (55.86	6.33 6.42	8.51 8.59	6.50 6.63)
5c	Aib	98	204—205 (Hexane/EtOH)	1764, 1709	—	1.40 (9H, s), 1.44 (6H, s), 2.29 (3H, s), 3.69 (2H, s), 7.06 (2H, d, 8.3), 7.12 (2H, d, 8.3), 7.44 (2H, d, 8.1), 7.50 (2H, d, 8.1), 7.59 (1H, s), 8.61 (2H, s), 9.11 (2H, s)	$C_{16}H_{23}N_3O_3\cdot$ $C_7H_8O_3S$	56.79 (56.60	6.55 6.67	8.28 7.98	6.32 6.26)
10a	L-Ala	94	154—155 (Benzene/EtOH)	1764, 1712	−32.7	1.40 (9H, s), 1.40 (3H, d, 7.2), 2.29 (3H, s), 3.72 (2H, s), 4.24 (1H, q, 7.2), 6.91—7.07 (1H, m), 7.09 (2H, d, 8.2), 7.16 (1H, s), 7.20 (1H, d, 7.7), 7.48 (2H, d, 8.2), 8.56 (2H, s), 9.07 (2H, s)	$C_{16}H_{23}N_3O_3\cdot$ $C_7H_8O_3S$	55.97 (55.92	6.33 6.27	8.51 8.43	6.50 6.46)
10b	D-Ala	92	153—154 (Benzene/EtOH)	1772, 1706	+32.0	1.40 (9H, s), 1.40 (3H, d, 7.2), 2.29 (3H, s), 3.72 (2H, s), 4.24 (1H, q, 7.2), 7.00—7.09 (1H, m), 7.14 (2H, d, 8.2), 7.28 (1H, s), 7.36 (1H, d, 7.7), 7.43 (1H, d, 7.7), 7.47 (2H, d, 8.2), 8.54 (2H, s), 9.07 (2H, s)	$C_{16}H_{23}N_3O_3\cdot$ $C_7H_8O_3S$	55.97 (56.13	6.33 6.41	8.51 8.36	6.50 6.61)
10c	Aib	89	164—165 (Benzene/EtOH)	1744, 1709	—	1.40 (9H, s), 1.45 (6H, s), 2.28 (3H, s), 3.71 (2H, s), 6.99—7.04 (1H, m), 7.09 (2H, d, 8.2), 7.11 (1H, s), 7.31 (1H, d, 7.7), 7.41 (1H, d, 7.7),7.47 (2H, d, 8.2), 8.52 (2H, s), 9.07 (2H, s)	$C_{16}H_{23}N_3O_3\cdot$ $C_7H_8O_3S$	56.79 (56.65	6.55 6.52	8.28 8.31	6.32 6.26)

Table 4. Kinetic Parameters for the Trypsin-Catalyzed Hydrolysis of Inverse Substrate

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})
N-Boc-L-Ala-O*p*AM (**5a**)	(2.26×10^{-3})		(1.37)	(6.08×10^{3})
N-Boc-D-Ala-O*p*AM (**5b**)	(9.42×10^{-4})		(9.71×10^{-2})	(1.03×10^{2})
N-Boc-Aib-O*p*AM (**5c**)	(3.63×10^{-4})		(3.91×10^{-2})	(1.08×10^{2})
Ac-*p*OG (**11**)[a]	8.84×10^{-4}	81.6	1.25×10^{-2}	9.23×10^{4}
N-Boc-L-Ala-O*p*GM (**13**)[b]	(4.31×10^{-4})		(7.60×10^{-1})	(1.76×10^{3})
N-Boc-L-Ala-O*p*Am (**15**)[c]	1.55×10^{-6}	15.2	1.55	1.02×10^{6}
N-Boc-L-Ala-O*m*AM (**10a**)	(6.67×10^{-3})		(9.75×10^{-1})	(1.47×10^{2})
N-Boc-D-Ala-O*m*AM (**10b**)	(2.52×10^{-3})		(6.72×10^{-3})	(2.67)
N-Boc-Aib-O*m*AM (**10c**)	(7.88×10^{-4})		(5.87×10^{-4})	(7.44×10^{-1})
Ac-*m*OG (**12**)[a]	8.83×10^{-5}		1.25×10^{-2}[d]	3.06×10^{2}
N-Boc-L-Ala-O*m*GM (**14**)[d]	(1.75×10^{-3})	3.03×10^{-3}[e]	(1.85×10^{-1})	(1.06×10^{2})

a) See refs. 11, 12. *b*) See ref. 5. *c*) See ref. 8. *d*) See ref. 6. *e*) Theoretically deduced. O*p*AM: *p*-(amidinomethyl)phenyl; O*p*GM: *p*-(guanidinomethyl)phenyl; O*p*Am: *p*-amidinophenyl; O*m*AM: *m*-(amidinomethyl)phenyl; O*m*GM: *m*-(guanidinomethyl)phenyl.

K_m value of **13** is one order of magnitude larger than the those for **5a**, **10a** and **14**, other parameters approximate to each others. This result showed that behavior of (amidinomethyl)- and (guanidinomethyl)phenyl esters for trypsin are almost same.

Next kinetic parameters for *N*-Boc-L-Ala-OAM (**5a**, **10a**) were compared with those for *N*-Boc-L-Ala-O*p*Am (**15**), which were reported in our previous paper.[9] The $k_{cat.}$ values of **5a** and **10a** approximate to k_3 of **15**, but the K_m values of **5a** and **10a** are three orders of magnitude larger than the K_s value of **15**. The parameter k_2/K_s (or $k_{cat.}/K_m$[12]) introduced by Brot and Bender is informative for the evaluation of the specificity of substrates.[13] These k_2/K_s (or $k_{cat.}/K_m$) values of **5a** and **10a** are three to four orders of magnitude smaller than that of **15**. The less specific character of **5a** and **10a** are likely to arise from the different acidity of phenol and basicity of the amidino group owing to the nonresonant amidino group and aromatic ring, and the considerably low affinity of **5a** and **10a**.

The kinetic parameters for *N*-Boc-D-Ala containing inverse substrates (**5b**, **10b**) were compared with those for *N*-Boc-L-Ala containing inverse substrates (**5a**, **10a**). Although the K_m values of all compounds showed nearly same values, the $k_{cat.}$ values are variance. Thus $k_{cat.}/K_m$ value of **10b** is two

or three orders of magnitude smaller than those for other (**5a**, **5b**, **10a**).

The kinetic parameters for *N*-Boc-Aib-OAM (**5c**, **10c**) were also compared with those for *N*-Boc-L-Ala-O*p*Am (**15**). The K_s (K_m) value for **5c** and **10c** was two orders of magnitude larger than those for **15**, respectively, as shown in Table 4. The k_3 ($k_{cat.}$) value of **5c** and **10c** was two orders to four orders of magnitude smaller than that of **15**, respectively. This result indicated that the bulky α,α-dialkyl substituent impedes the placement of **5c** and **10c** in juxtaposition with the active site of trypsin. The $k_{cat.}/K_m$ value for **10c** showed a much less favorable interaction. However, we previously reported that even α,α-dialkyl amino acid *m*-(guanidinomethyl)phenyl ester could be used in a *Streptomyces griseus* trypsin-catalyzed coupling reaction.[14] We conclude that all new synthetic inverse substrates examined in this study could be expected as acyl donor components in trypsin-catalyzed peptide synthesis.

Experimental

General The melting points were measured on a Yanaco micro melting point apparatus. IR spectra were taken on a JASCO VALOR-III FT-IR spectrometer. ^{1}H-NMR spectra were recorded on a JEOL JNM-FX-400 FT NMR 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. The optical rotations were measured with a JASCO DIP-360 digital polarimeter in a 5 cm cell. Kinetic parameters were determined with a Radiometer TTT-80 pH-stat. Flash column chromatography was performed using Silica Gel 60N (Kanto Chemical Co., Inc.) as a solid support in the immobile phase. Kieselgel 60 F-254 plates (Merck) were used for thin-layer chromatography (TLC). Bovine pancreas trypsin (EC 3.4.21.4) was purchased from Worthington Biochemical Corp. (twice crystallized, lot TRL).

***p*-{[(Hydroxy)imino]-2-aminoethyl}phenol (2)** To a solution of 4-hydroxybenzyl cyanide (**1**) (01.65 g, 80 mmol) in EtOH (80 ml) was added to a solution of hydroxylamine hydrochloride (19.46 g, 0.28 mol) and potassium carbonate (19.34 g, 0.14 mol) in H_2O (120 ml), and the mixture was refluxed for 4 h. The mixture was concentrated to half volume and then cooled to room temperature. The formed precipitate was collected by filtration to give **2** (13.17 g, 99%) as brownish needles. Physical properties and spectral data are given in Table 1.

***p*-{[(Acetoxy)imino]-2-aminoethyl}phenol (3)** *p*-{[(Hydroxy)imino]-2-aminoethyl}phenol (**2**) (1.66 g, 10 mmol) was soluble in hot pyridine (20 ml), and to a solution was added dropwise acetic anhydride (946 ml) at 0 °C, and the mixture was stirred at room temperature for 1 h. To the mixture was added heptane, and azeotropic mixtures were concentrated *in vacuo*. The resulting residue was recrystallized from hexane/EtOH to give **3** (1.79 g, 86%) as colorless needles. Physical properties and spectral data are given in Table 1.

(*tert*-Butoxycarbonyl)amino Acid *p*-{[(Acetoxy)imino]-2-aminoethyl}-phenyl Esters (4a—c) General Procedure: To a solution of *p*-{[(acetoxy)imino]-2-aminoethyl}phenol (**3**) (312 mg, 1.5 mmol), *N*-Boc-amino acid (1.7 mmol), and DMAP (24 mg, 0.2 mmol) in DMF (5 ml) and EtOAc (5 ml) was added DCC (351 mg, 1.7 mmol) at 0 °C, and the mixture was stirred at the same temperature for 1 h. Then the mixture was stirred at room temperature for overnight and concentrated. The resulting residue was chromatographed on a silica gel column (benzene/EtOAc=1 : 3). The benzene/EtOAc=1 : 3 elute was evaporated to dryness *in vacuo*, and the solid was recrystallized to give **4**. Physical properties and spectral data are given in Table 2.

(*tert*-Butoxycarbonyl)amino Acid *p*-(Amidinomethyl)phenyl Esters *p*-Toluenesulfonic Acid Salt (5a—c) General Procedure: A solution of (*tert*-butoxycarbonyl)amino acid *p*-{[(acetoxy)imino]-2-aninoethyl}phenyl ester (**4**) (1.0 mmol) and *p*-toluenesulfonic acid monohydrate (1 mmol) in EtOH (10 ml) was hydrogenated over 10% Pd–C (10 mg) at room temperature for overnight with vigorous stirring. The catalyst was filtered away, and the filtrate was concentrated *in vacuo*. The residue was washed with absolute ether, and the solid was recrystallized to give **5** as a colorless solid. Physical properties and spectral data are given in Table 3.

3-Hydroxybenzyl Cyanide (6) This compound was prepared from (3-methoxyphenyl)acetonitrile according to the reported procedure.[15]

***meta*-Series Compounds** These compounds were synthesized by a procedure similar to that employed for the corresponding *p*-compounds (**5a**—**c**). Physical properties and spectral data are given in Tables 1—3.

Kinetic Measurements Enzyme concentration was determined by active site titration using *p*-nitrophenyl *p'*-guanidinobenzoate.[16] Analysis of kinetic parameters was carried out by potentiometrically using a pH-stat under steady-state conditions following the reported procedure.[1,9] Determination of k_{cat} and K_m were 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.

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References and Notes

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