

Synthesis and Tryptic Hydrolysis of *p*-Guanidinophenyl Esters Derived from Amino Acids and Peptides¹⁾

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A facile synthetic method for *p*-guanidinophenyl esters derived from a variety of amino acids and peptides, including D-amino acids, is presented. The kinetic behavior of trypsin towards these synthetic esters, inverse substrates, was analyzed. The spatial requirement of the enzyme active site for catalytic efficiency is discussed based on the steric characteristics of the substrates.

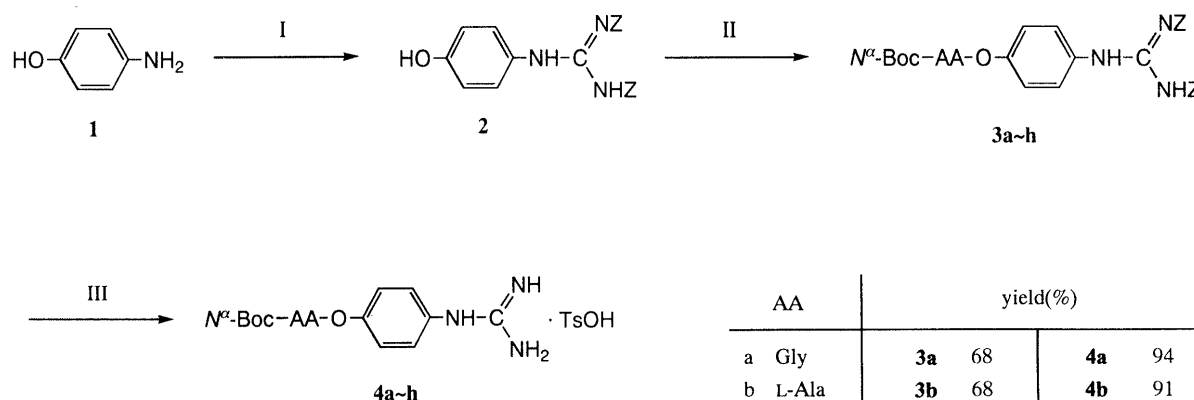
Key words tryptic hydrolysis; enzyme kinetics; *N*^α-(*tert*-butoxycarbonyl)amino acid *p*-guanidinophenyl ester; *N*^α-(*tert*-butoxycarbonyl)peptide *p*-guanidinophenyl ester; inverse substrate; trypsin

Previously we reported²⁾ that the *p*-guanidinophenyl esters behave as specific substrates for trypsin and trypsin-like enzymes. In these esters the site-specific group (a charged guanidinium) for the enzyme is included in the leaving group portion instead of being in the acyl moiety. Such a substrate is termed³⁾ an “inverse substrate.” Such 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 characteristic of conventional substrates. Thus, the enzymatic deacylation step can be analyzed separately from the acylation step. Moreover, we showed that inverse substrates were applicable to enzymatic peptide synthesis.⁴⁾ Therefore, a general method for the preparation of a variety of inverse substrates would be valuable. However, synthesis of these inverse substrates is usually difficult, because acylation reaction of the guanidinophenol hydroxy group fails due to the poor nucleophilicity of the hydroxy group. During the course of our investigation of alternative synthetic methods for inverse substrates,⁵⁾ we have succeeded in developing a practically useful method.

In this paper, an additional synthetic method for *N*^α-

Boc-amino acid *p*-guanidinophenyl esters was studied and the kinetic properties of inverse substrates in the trypsin-catalyzed reaction were determined.

Synthesis of Inverse Substrates For the synthesis of guanidinophenyl 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.⁵⁾ Another is to carry out the key step at the first stage, *i.e.*, *N*-blocked *p*-guanidinophenol is used as the starting material. In this study, the latter route was investigated, because it was considered that the intermediary *N*-blocked *p*-guanidinophenol should be sufficiently reactive to acylation reagents. We therefore tried amidination of *p*-aminophenol (**1**) with a newly developed reagent, 1-[*N,N'*-bis(*Z*)amidino]pyrazole, which was proposed as a specific tool for the preparation of *N*-blocked guanidine derivatives.⁶⁾ Compound **1** was reacted with 1-[*N,N'*-bis(*Z*)amidino]pyrazole. Pure *p*-[*N',N''*-bis(*Z*)guanidino]phenol (**2**) was easily obtained in 92% yield. Condensation of **2** with *N*-Boc-amino acids by using DCC and DMAP in DMF was successful. Reaction yields of the esters (**3a–h**) were 64–71%, as



I) 1-[*N,N'*-bis(*Z*)amidino]pyrazole in THF

II) *N*^α-Boc-AA, DCC, 4-dimethylaminopyridine in DMF

III) H₂, 10% Pd-C, *p*-TsOH·H₂O in EtOH-Et₂O

AA		yield(%)			
a	Gly	3a	68	4a	94
b	L-Ala	3b	68	4b	91
c	D-Ala	3c	67	4c	90
d	L-Leu	3d	71	4d	92
e	D-Leu	3e	67	4e	92
f	L-Phe	3f	68	4f	93
g	D-Phe	3g	65	4g	92
h	β-Ala	3h	64	4h	86

Chart 1

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shown in Chart 1. The next deprotection step was carried out by catalytic hydrogenation to give *N*^α-Boc-amino acid *p*-guanidinophenyl esters (**4a—g**) as the TsOH salts in essentially quantitative yields. These results are summarized in Chart 1. The total yields of the inverse substrates by this method were approximately twice that by the previous method.⁵⁾ This improvement is due to the decrease in the number of reaction steps, *i.e.*, to three from eight in the previous method.⁵⁾

The proposed method will provide a variety of inverse substrates which are useful not only for the mechanistic analysis of tryptic catalysis, but also for enzymatic peptide synthesis.

Kinetic Parameters for Trypsin-Catalyzed Hydrolysis

The kinetics of tryptic hydrolysis of guanidinophenyl ester substrates was analyzed. The *N*^α-Boc-peptide *p*-guanidinophenyl esters (**4i—q**), prepared by the previous method,⁵⁾ were also subjected to kinetic analysis. Determination of kinetic parameters was carried out as described,^{3,5)} and the values obtained are listed in Table 1. All compounds were found to have strong affinity for trypsin, with *K_s* values in the range of 10⁻⁴—10⁻⁶ M. These *K_s* values are comparable to those of typical sub-

strates such as L-lysine and L-arginine derivatives. In terms of the acylation rate constant, *k*₂, D-amino acid ester is a less efficient substrate than the corresponding L-amino acid ester. However, esters of the D-amino acid series still qualify as specific substrates. The *m*-isomer (**4r**) showed a much slower acylation rate (*ca.* 1/1200 and 1/90), in contrast with the *p*-isomers (**4d** and **4e**, respectively), although there was little difference in binding affinity. This difference between *m*- and *p*-isomers is probably due to the strict stereochemical requirements of the active site, *i.e.*, inaccessibility of the unfavorably positioned carbonyl carbon of the *m*-isomer to the enzyme catalytic residue, even though the binding itself is tight and specific. This property is very similar to those of typical inverse substrates such as amidinophenyl and aminomethylphenyl esters.⁸⁾ The parameter, *k*₂/*K_s*, introduced by Brot and Bender⁹⁾ was used for the evaluation of the specificity of substrates. In terms of this *k*₂/*K_s* (or *k*_{cat}/*K_m*¹⁰⁾ value, compounds **4a—q** are nearly equivalent to the *p*-amidinophenyl ester series.¹¹⁾ The feasibility of analysis of the deacylation process for a wide variety of acyl groups is the most striking characteristic of inverse substrates.¹⁰⁾ Indeed, the deacylation rate constants have been successfully determined. In Table 1, the overall catalytic rate, *k*_{cat}, is included.¹¹⁾ The deacylation rate constants of *N*^α-amino acids derived from aliphatic α-amino acids (**4a**, **4b**, and **4d**) were of the order of 10⁻¹ s. The rate constant of the β-amino acid (**4h**) was shown to be of the same order of magnitude. This implies that the interaction of small alkyl groups with the active site in the form of the acyl-enzyme may not cause any significant perturbation. Elongation of the peptide chain did not markedly affect the deacylation (**4a**, **4i**, and **4j**). Substitution of aromatic side chain favored the deacylation (**4f**), and elongation of the peptide chain (**4f**, **4p**, and **4q**) had little influence on the deacylation efficiency. *N*^α-Boc-D-amino acid residues definitely underwent deacylation, though the rates were relatively slow: 1/3—1/165 of those of the

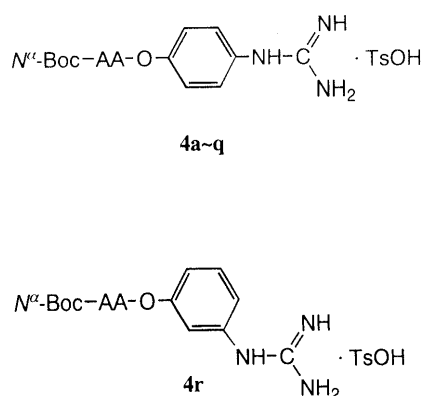


Fig. 1. Structures of Inverse Substrates

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

Substrate No.	<i>K_s</i> (<i>K_m</i>) (M)	<i>k</i> ₂ (s ⁻¹)	<i>k</i> ₃ (<i>k</i> _{cat}) (s ⁻¹)	<i>k</i> ₂ / <i>K_s</i> (<i>k</i> _{cat} / <i>K_m</i>) (s ⁻¹ M ⁻¹)	<i>k</i> ₃ (L)/ <i>k</i> ₃ (D)
<i>N</i> ^α -Boc-Gly-OGp (4a)	3.17 × 10 ⁻⁴	2.69 × 10 ²	3.55 × 10 ⁻¹	8.49 × 10 ⁵	
<i>N</i> ^α -Boc-Gly-Gly-OGp (4i) ^{a)}	3.23 × 10 ⁻⁵	1.99 × 10	3.10 × 10 ⁻¹	6.16 × 10 ⁵	
<i>N</i> ^α -Boc-Gly-Gly-Gly-OGp (4j) ^{a)}	3.76 × 10 ⁻⁴	8.72 × 10	1.27 × 10 ⁻¹	2.32 × 10 ⁵	
<i>N</i> ^α -Boc-L-Ala-Gly-OGp (4k) ^{a)}	4.93 × 10 ⁻⁶	1.40 × 10 ²	1.37 × 10 ⁻¹	2.84 × 10 ⁷	2.70
<i>N</i> ^α -Boc-D-Ala-Gly-OGp (4l) ^{a)}	5.90 × 10 ⁻⁵	7.13	5.07 × 10 ⁻²	1.21 × 10 ⁵	
<i>N</i> ^α -Boc-L-Ala-OGp (4b)	2.77 × 10 ⁻⁴	1.23 × 10 ²	4.09 × 10 ⁻¹	4.44 × 10 ⁵	11.7
<i>N</i> ^α -Boc-D-Ala-OGp (4c)	6.62 × 10 ⁻⁴	6.71 × 10	3.51 × 10 ⁻²	1.01 × 10 ⁵	
<i>N</i> ^α -Boc-L-Ala-L-Ala-OGp (4m) ^{a,b)}	(5.73 × 10 ⁻⁵)	—	(4.88 × 10 ⁻¹)	(8.52 × 10 ³)	16.1
<i>N</i> ^α -Boc-D-Ala-D-Ala-OGp (4n) ^{a)}	9.57 × 10 ⁻⁵	2.10 × 10	3.03 × 10 ⁻²	2.19 × 10 ⁵	
<i>N</i> ^α -Boc-L-Phe-D-Ala-OGp (4o) ^{c)}	2.44 × 10 ⁻⁵	6.37	2.80 × 10 ⁻²	2.61 × 10 ⁵	
<i>N</i> ^α -Boc-L-Leu-OGp (4d)	3.60 × 10 ⁻⁴	1.70 × 10 ²	6.19 × 10 ⁻¹	4.72 × 10 ⁵	6.83
<i>N</i> ^α -Boc-D-Leu-OGp (4e)	7.41 × 10 ⁻⁵	1.28 × 10	9.06 × 10 ⁻²	1.73 × 10 ⁵	
<i>N</i> ^α -Boc-L-Phe-OGp (4f) ^{b)}	(2.52 × 10 ⁻⁵)	—	(2.68 × 10)	(1.06 × 10 ⁶)	165
<i>N</i> ^α -Boc-D-Phe-OGp (4g)	1.74 × 10 ⁻⁴	3.72 × 10	1.62 × 10 ⁻¹	2.14 × 10 ⁵	
<i>N</i> ^α -Boc-L-Phe-L-Phe-OGp (4p) ^{a,b)}	(2.21 × 10 ⁻⁵)	—	(2.64)	(1.19 × 10 ⁵)	
<i>N</i> ^α -Boc-L-Tyr-Gly-Gly-L-Phe-OGp (4q) ^{b,c)}	(3.60 × 10 ⁻⁵)	—	(1.01 × 10)	(2.81 × 10 ⁵)	
<i>N</i> ^β -Boc-β-Ala-OGp (4h)	2.77 × 10 ⁻⁵	4.61 × 10	2.09 × 10 ⁻¹	1.66 × 10 ⁶	
<i>N</i> ^α -Boc-L-Leu-OGp (<i>meta</i> form) (4r) ^{b)}	1.36 × 10 ⁻⁴	(1.41 × 10 ⁻¹) ^{d)}	(1.15 × 10 ⁻¹)	8.46 × 10 ²	

a) See reference 5. b) Kinetic parameter determined with a pH-stat. c) See reference 7. d) Calculated value using *k*₃ = 0.619 s⁻¹ for *N*^α-Boc-L-Leu-OGp (**4d**), with the following equation: *k*_{cat} = *k*₂*k*₃/(*k*₂ + *k*₃).

L-enantiomer. The enantiomeric preference ($k_3(\text{L})/k_3(\text{D})$) was more pronounced for more effective substrates. Accumulation of further observations should lead to a better understanding of the enzyme action at the molecular level.

Experimental

The melting points were measured on a Yanagimoto micro melting point apparatus. 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).

***p*-[*N*',*N*''-Bis(benzyloxycarbonyl)guanidino]phenol (2)** A solution of *p*-aminophenol (1) (2.18 g, 20 mmol) and 1-[*N*',*N*'-bis(*Z*)amidino]pyrazole (7.54 g, 20 mmol) in absolute THF was stirred at room temperature for 12 h in an atmosphere of nitrogen. The reaction mixture was diluted with benzene-AcOEt (6:1) and the solution was passed through a silica gel column. The benzene-AcOEt (6:1) eluate was evaporated to dryness *in vacuo* and the solid residue was recrystallized from EtOH to give **2** (7.71 g, 92%) as colorless needles. mp 145–146.5°C. *Anal.* Calcd for $\text{C}_{23}\text{H}_{21}\text{N}_3\text{O}_5$: C, 65.86; H, 5.05; N, 10.02. Found: C, 65.71; H, 4.95; N, 9.85.

***m*-[*N*',*N*''-Bis(benzyloxycarbonyl)guanidino]phenol (5)** Compound **5** (87%) was obtained from *m*-aminophenol by a procedure similar to that described for compound **2**. mp 148–149°C, colorless columns after recrystallization from EtOH. *Anal.* Calcd for $\text{C}_{23}\text{H}_{21}\text{N}_3\text{O}_5$: C, 65.86; H, 5.05; N, 10.02. Found: C, 65.97; H, 5.04; N, 9.97.

***N*-(*tert*-Butyloxycarbonyl)amino Acid *p*-[*N*',*N*''-Bis(benzyloxycarbonyl)guanidino]phenyl Ester (3)** General Procedure: A solution of *N*^α-Boc-amino acid (2.4 mmol), *p*-[*N*',*N*'-bis(*Z*)guanidino]phenol (**2**) (838 mg, 2 mmol) and DMAP (24.4 mg, 0.2 mmol) in DMF (6 ml) was treated with DCC (495 mg, 2.4 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*. The residue was diluted with benzene-AcOEt (6:1) and purified on a silica gel column. The pure compounds **3a–g** were obtained by recrystallization from EtOH. **3a**: Colorless fine needles. mp 125–126°C. *Anal.* Calcd for $\text{C}_{30}\text{H}_{32}\text{N}_4\text{O}_8$: C, 62.49; H, 5.59; N, 9.72. Found: C, 62.49; H, 5.64; N, 9.57. **3b**: Colorless fine needles. mp 132–133°C. $[\alpha]_{\text{D}}^{25}$ –28.0° ($c=1.0$, CH_3CN). *Anal.* Calcd for $\text{C}_{31}\text{H}_{34}\text{N}_4\text{O}_8$: C, 63.04; H, 5.80; N, 9.49. Found: C, 63.06; H, 5.82; N, 9.50. **3c**: Colorless needles. mp 133–134°C. $[\alpha]_{\text{D}}^{25}$ +27.2° ($c=1.0$, CH_3CN). *Anal.* Calcd for $\text{C}_{31}\text{H}_{34}\text{N}_4\text{O}_8$: C, 63.04; H, 5.80; N, 9.49. Found: C, 63.19; H, 5.91; N, 9.41. **3d**: Colorless needles. mp 128–129°C. $[\alpha]_{\text{D}}^{25}$ –25.0° ($c=1.0$, CH_3CN). *Anal.* Calcd for $\text{C}_{34}\text{H}_{40}\text{N}_4\text{O}_8$: C, 64.54; H, 6.37; N, 8.86. Found: C, 64.54; H, 6.51; N, 8.76. **3e**: Colorless needles. mp 128–129°C. $[\alpha]_{\text{D}}^{25}$ +24.8° ($c=1.0$, CH_3CN). *Anal.* Calcd for $\text{C}_{34}\text{H}_{40}\text{N}_4\text{O}_8$: C, 64.54; H, 6.37; N, 8.86. Found: C, 64.49; H, 6.49; N, 8.80. **3f**: Colorless needles. mp 157–159°C. $[\alpha]_{\text{D}}^{25}$ –11.6° ($c=1.0$, CH_3CN). *Anal.* Calcd for $\text{C}_{37}\text{H}_{38}\text{N}_4\text{O}_8$: C, 66.65; H, 5.75; N, 8.40. Found: C, 66.50; H, 5.88; N, 8.24. **3g**: Colorless fine needles. mp 157–158°C. $[\alpha]_{\text{D}}^{25}$ +11.4° ($c=1.0$, CH_3CN). *Anal.* Calcd for $\text{C}_{37}\text{H}_{38}\text{N}_4\text{O}_8$: C, 66.65; H, 5.75; N, 8.40. Found: C, 66.48; H, 5.61; N, 8.37. Compounds **3h** and **3r** were synthesized by the same procedure as employed for the *p*-compounds (**3a–g**). **3h**: Colorless powder. mp 79–80.5°C. *Anal.* Calcd for $\text{C}_{31}\text{H}_{34}\text{N}_4\text{O}_8$: C, 63.04; H, 5.80; N, 9.49. Found: C, 62.75; H, 5.84; N, 9.39. **3r**: Colorless needles. mp 128–129°C. $[\alpha]_{\text{D}}^{25}$ –21.6° ($c=1.0$, CH_3CN). *Anal.* Calcd for $\text{C}_{34}\text{H}_{40}\text{N}_4\text{O}_8$: C, 64.54; H, 6.37; N, 8.86. Found: C, 64.59; H, 6.50; N, 8.75.

***N*-(*tert*-Butyloxycarbonyl)amino Acid *p*-Guanidino]phenyl Ester *p*-Toluenesulfonic Acid Salt (4)** General Procedure: Catalytic 10% Pd on carbon (20 mg) was added to a solution of *N*^α-Boc-amino acid *p*-[*N*',*N*'-bis(*Z*)guanidino]phenyl ester (**3a–g**) (1 mmol) and *p*-toluenesulfonic acid monohydrate (1 mmol) in EtOH (10 ml) and Et₂O (10 ml), and the mixture was vigorously stirred in an atmosphere of hydrogen at room temperature for 12 h. The catalyst was filtered off, and the filtrate was evaporated to dryness *in vacuo*. The residue was washed with dry

Et₂O to give *N*^α-Boc-amino acid *p*-guanidinophenyl ester (**4a–g**) as the *p*-toluenesulfonic acid salt and a colorless amorphous solid. **4a**: FAB-MS m/z : 309 ($\text{M}+\text{H}$)⁺. **4b**: $[\alpha]_{\text{D}}^{25}$ –37.4° ($c=1.0$, MeOH). FAB-MS m/z : 323 ($\text{M}+\text{H}$)⁺. **4c**: $[\alpha]_{\text{D}}^{25}$ +36.0° ($c=1.0$, MeOH). FAB-MS m/z : 323 ($\text{M}+\text{H}$)⁺. **4d**: $[\alpha]_{\text{D}}^{25}$ –31.2° ($c=1.0$, MeOH). FAB-MS m/z : 365 ($\text{M}+\text{H}$)⁺. **4e**: $[\alpha]_{\text{D}}^{25}$ +28.0° ($c=1.0$, MeOH). FAB-MS m/z : 365 ($\text{M}+\text{H}$)⁺. **4f**: $[\alpha]_{\text{D}}^{25}$ –7.4° ($c=1.0$, MeOH). FAB-MS m/z : 399 ($\text{M}+\text{H}$)⁺. **4g**: $[\alpha]_{\text{D}}^{25}$ +6.8° ($c=1.0$, MeOH). FAB-MS m/z : 399 ($\text{M}+\text{H}$)⁺. Compounds **4h** and **4r** were synthesized by the same procedure as employed for the *p*-compounds (**4a–g**). **4h**: Colorless plates (EtOH–Et₂O). *Anal.* Calcd for $\text{C}_{22}\text{H}_{31}\text{N}_4\text{O}_{7.5}\text{S}$: C, 52.47; H, 6.20; N, 11.12; S, 6.37. Found: C, 52.59; H, 6.05; N, 11.11; S, 6.30. **4r**: Colorless amorphous solid. $[\alpha]_{\text{D}}^{25}$ –26.8° ($c=1.0$, MeOH). FAB-MS m/z : 365 ($\text{M}+\text{H}$)⁺.

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.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 **4m**, **4f**, **4p**, **4q**, and **4r** were analyzed potentiometrically using a pH-stat under steady-state conditions following the reported procedure.³ 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.

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References and Notes

- The following abbreviations are used: Boc = *tert*-butyloxycarbonyl, Z = benzyloxycarbonyl, DCC = *N*,*N*'-dicyclohexylcarbodiimide, DMF = *N*,*N*-dimethylformamide, DCUrea = dicyclohexylurea, DMAP = 4-dimethylaminopyridine, Gp = *p*-guanidinophenyl.
- Nozawa M., Tanizawa K., Kanaoka Y., *J. Pharmacobio-Dyn.*, **3**, 213–219 (1980).
- Tanizawa K., Kasaba Y., Kanaoka Y., *J. Am. Chem. Soc.*, **99**, 4485–4488 (1977).
- Itoh K., Sekizaki H., Toyota E., Fujiwara N., Tanizawa K., *Bioorg. Chem.*, **24**, 59–68 (1996); Schellenberger V., Jakubke H.-D., Zapevalova N. P., Mitin Y. V., *Biotechnol. Bioeng.*, **38**, 104–108 (1991); Schellenberger V., Schellenberger U., Jakubke H.-D., Zapevalova N. P., Mitin Y. V., *ibid.*, **38**, 319–321 (1991).
- Itoh K., Sekizaki H., Toyota E., Tanizawa K., *Chem. Pharm. Bull.*, **43**, 2082–2087 (1995).
- Bernatowicz M. S., Wu Y., Matsueda G. R., *Tetrahedron Lett.*, **34**, 3389–3392 (1993); Bernatowicz M. S., Wu Y., Matsueda G. R., *J. Org. Chem.*, **57**, 2497–2502 (1992).
- 4o**: Colorless amorphous solid, $[\alpha]_{\text{D}}^{25}$ +24.6° ($c=1.0$, MeOH), FAB-MS m/z : 470 ($\text{M}+\text{H}$)⁺; **4q**: Colorless amorphous solid, $[\alpha]_{\text{D}}^{25}$ +1.1° ($c=1.1$, MeOH), FAB-MS m/z : 676 ($\text{M}+\text{H}$)⁺.
- Nakano M., Tanizawa K., Nozawa M., Kanaoka Y., *Chem. Pharm. Bull.*, **28**, 2212–2216 (1980).
- Brot E. F., Bender M. L., *J. Am. Chem. Soc.*, **91**, 7187–7191 (1969).
- The k_{cat}/K_m value is equivalent to k_2/K_s where k_{cat} and K_m denote the overall catalytic rate constant and the Michaelis constant, respectively, as reported; Ryan J. T., Fenton W. J., II, Chang T.-I., Feinman D. R., *Biochemistry*, **15**, 1337–1341 (1976).
- Fujioka T., Tanizawa K., Kanaoka Y., *J. Biochem.*, **89**, 637–643 (1981).
- Catalytic rate constants, k_{cat} , determined from overall reaction rates are nearly equal to k_3 according to the equation; $k_{\text{cat}} = k_2k_3/(k_2 + k_3)$ ($k_2 \ll k_3$), as reported; Ryan J. T., Fenton W. J., II, Chang T.-I., Feinman D. R., *Biochemistry*, **15**, 1337–1341 (1976).
- Chase T., Jr., Shaw E., *Biochem. Biophys. Res. Commun.*, **29**, 508–514 (1967).
- Brandt K. G., Himoe A., Hess G. P., *J. Biol. Chem.*, **242**, 3973–3982 (1967); Nozawa M., Tanizawa K., Kanaoka Y., *J. Pharm. Dyn.*, **3**, 213–219 (1980).