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Synthesis of Active Center-Directed Peptide Inhibitors of Plasmin[1)]

Yoshio Okada,*,[a] Yuko Tsuda,[a] Naoki Teno,[a] Keiko Wanaka,[b]
Miyako Bohgaki,[b] Akiko Hijikata-Okunomiya,[c]
Taketoshi Naito,[d] and Shosuke Okamoto[b]

Faculty of Pharmaceutical Sciences, Kobe-Gakuin University,[a] Nishi-ku, Kobe 673, Japan
Kobe Research Projects on Thrombosis and Haemostasis, Saiseikai Hyogo Hospital,[b]
Chuo-ku, Kobe 651, Japan, School of Allied Medical Sciences,
Kobe University,[c] Suma-ku, Kobe 654, Japan and Life
Science Research Lab., Showa Denkō Co., Ltd.,[d]
Ohta-ku, Tokyo 146, Japan

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Active center-directed peptide inhibitors of plasmin were designed based on the structure of specific substrates of plasmin and synthesized by a conventional solution method. Their effects on plasmin were examined and the structure–activity relationship was studied. D-Ile–Phe–Lys–BZA (4-benzoylanilide) inhibited plasmin activities toward S-2251 and fibrin (IC_{50}: 0.069 mM and 0.18 mM respectively) but D-Ile–Phe–Lys–BPP (4-benzylpiperidine amide) was not inhibitory. However D-Ile–Phe–Lys–BZA was cleaved by plasmin to release benzoylaniline, indicating that this type of peptide inhibitor is not stable to plasmin. It was found that Tos–Lys–*p*NA was not cleaved by palsmin and inhibited plasmin activity toward not only fibrin but also small peptide substrates and fibrinogen by blocking the active center of plasmin with some selectivity. In order to obtain potent and stable inhibitors of plasmin, it is recommended to design them with reference to the structures of Tos–Lys–*p*NA and the specific substrate, D-Ile–Phe–Lys–*p*NA.

Keywords——plasmin; competitive inhibitor; drug design; Lys derivative; synthesis; substrate-derived inhibitor; structure–activity relationship

It is well known that proteinases and their natural inhibitors regulate biological functions cooperatively to maintain homeostasis, and imbalances between proteinases and their natural inhibitors cause serious disorders. With regard to plasmin, an imbalance between plasmin and its natural inhibitors (α_2-macroglobulin, α_2-plasmin inhibitor, *etc.*) also causes serious syndromes, such as hyperfibrinolysis. At present, ε-aminocaproic acid (EACA)[2)] and *trans*-4-aminomethylcyclohexanecarboxylic acid (*t*-AMCHA)[3)] are employed clinically as plasmin inhibitors. These inhibitors showed fairly potent inhibition of fibrinolysis by plasmin, but very slight inhibition on amidolysis of small peptide substrates, because these inhibitors exhibit an inhibitory effect on plasmin by blocking the lysine binding site (LBS) of the enzyme, which is not the catalytic site.[4)]

Thus, our research goal was the synthesis of active center-directed inhibitors of plasmin, with the object of obtaining potent and selective inhibitors of plasmin toward not only fibrinolysis but also amidolysis, resulting in the inhibition of fibrinogenolysis by plasmin as reported elsewhere.[5)]

Previously, we reported that D-Ile–Phe–Lys–*p*NA was a selective substrate with a low K_m value for plasmin.[6)] Kiss *et al.* also reported independently that D-Ile–Phe–Lys–*p*NA was the best substrate for plasmin, considering the K_m value.[7)] Based on the structure of the substrate described above, it is deduced that D-Ile–Phe sequence has a very favorable three-dimensional structure for binding with the active center of plasmin. As shown in Table I, a series of substrates inhibited plasmin activity toward Boc–Val–Leu–Lys–MCA with IC_{50} values

Table I. Relationship between K_m and IC_{50} Values of Peptidyl-*p*-nitroanilides for Plasmin

Compound	K_m (mM)	IC_{50}[a] (mM)
D-Val–Leu–Lys–*p*NA[b]	0.30	0.26
D-Ile–Phe–Lys–*p*NA[c]	0.02	0.03
3-MV–Ile–Phe–Lys–*p*NA[c]	0.18	0.10
Ile–Phe–Lys–*p*NA[c]	0.33	0.19

a) Boc–Val–Leu–Lys–MCA was used as the substrate (final concentration: 0.1 mM). *b*) See ref. 7. *c*) See ref. 6.

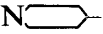
Table II. Values of *p*NA Derivatives for Plasmin Activities toward S-2251 and Fibrin

Compound	IC_{50} (mM)	
	S-2251	Fibrin
Tos–Lys–*p*NA (**1**)	0.70	0.78
Tos–Phe–Lys–*p*NA (**2**)	(K_m = 0.22)[c]	0.25
Dns–Phe–Lys–*p*NA (**3**)	(K_m = 0.17)	0.11
Bz–Phe–Lys–*p*NA (**4**)	(K_m = 0.17)	0.17
Ile–D-Phe–D-Lys–*p*NA (**5**)[a]	> 1.00	> 2.00
D-Ile–Phe–Lys–*p*NA (**6**)[b]	(K_m = 0.020)	0.18

a) See ref. 9. *b*) See ref. 6. *c*) K_m values instead of IC_{50} values are described in parentheses.

proportional to the corresponding K_m values. It is clear that these peptides inhibited plasmin by binding with the active center of the enzyme in competition with Boc–Val–Leu–Lys–MCA, which means that these peptides are active center-directed inhibitors. This paper deals with synthesis of peptide derivatives with a Lys residue at the P_1 position[8] and examination of their inhibitory effect on plasmin activities toward small peptide substrates and fibrin.

First of all, according to the synthetic method described previously,[6] we prepared Lys–*p*NA derivatives with substitution at the N-terminus of the Lys residue and examined their effects on plasmin; the results are summarized in Table II. It is interesting that Tos–Lys–*p*NA (**1**) was not cleaved by plasmin but inhibited plasmin activities toward S-2251 (D-Val–Leu–Lys–*p*NA)[9] and fibrin as well (IC_{50}, 0.70 and 0.78 mM, respectively, and > 1.0 mM for thrombin, 2.8 mM for trypsin and > 1.0 mM for urokinase), whereas incorporation of a Phe residue converted **1** to fairly good substrates (**2**—**4**) of plasmin and inhibited the fibrinolytic activity of plasmin with IC_{50} values similar to the corresponding K_m values. Substitution of Tos, Dns and Bz residues by D-Ile gave a much more favorable substrate (**6**) of plasmin ($K_m = 2.0 \times 10^{-5}$ M). However, this compound inhibited fibrinolytic activity of plasmin with approximately the same IC_{50} value as the other substrates described above. The stereoisomer of the substrate, Ile–D-Phe–D-Lys–*p*NA (**5**)[10] did not show any effect on plasmin, although some stereoisomers of substrates exhibited inhibitory activity in the case of human leukocyte elastase, as reported by the authors.[11]

It is deduced that the negatively charged active center of plasmin interacts with the positively charged ε-amino group of lysine more favorably than the guanidino group, and the lysyl bond is easily cleaved by plasmin. In order to obtain potent and stable inhibitors, it is required that the lysyl bond of inhibitors should not be cleaved by plasmin. Thus, two kinds of inhibitors were designed. One is Lys derivatives with substitution at the N and/or C-terminus by a non-peptidic compound, because Tos–Lys–*p*NA (**1**) is not susceptible to plasmin but can inhibit the enzyme by blocking the active center. The other is peptide derivatives with substitution at the C-terminus of Lys (P_1) of a specific substrate by some compound so that the lysyl bond is not cleaved by plasmin. If the peptide bond is a tertiary amide, usual proteinases can not cleave the bond, and this idea led us to prepare lysylpiperidine amide derivatives. Among these derivatives, 4-benzylpiperidine amide derivatives are the most favorable for binding with the S_1' region of plasmin (Tos–Lys–N–R, R = CH_3, no inhibition of plasmin; R = C_6H_5, IC_{50} for plasmin 0.80 mM toward S-2251 and 0.50 mM toward fibrin, R = $CH_2C_6H_5$, IC_{50} for plasmin 0.30 mM toward S-2251 and 0.15 mM toward fibrin). Thus, we synthesized various kinds of lysyl-4-benzylpiperidine amide derivatives according to the route shown in Fig. 1 and examined their inhibitory effects on plasmin activity; the results

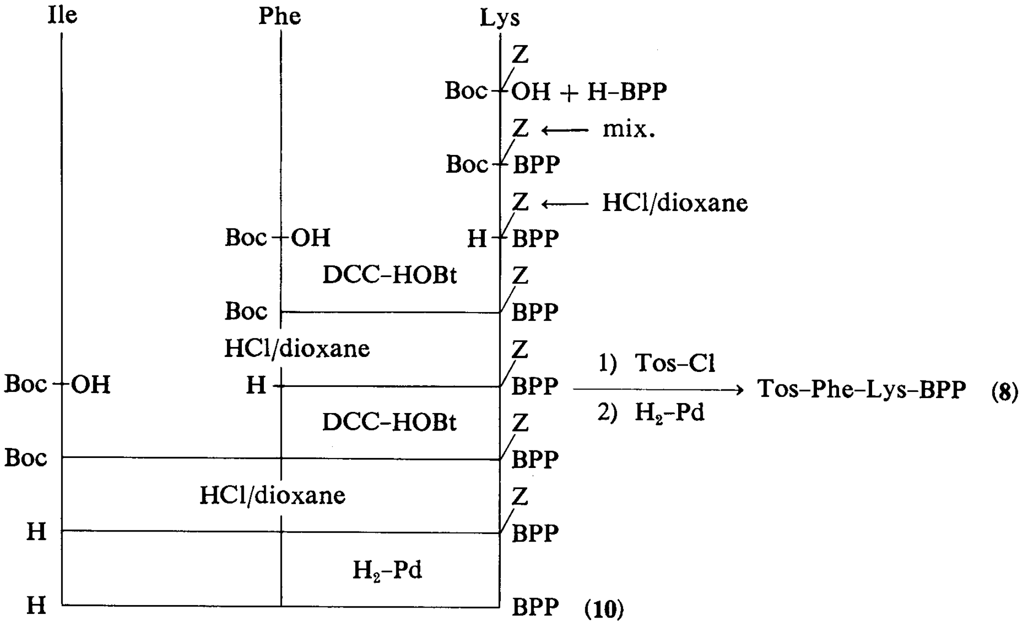


Fig. 1. Scheme of the Synthesis of Peptidyl-BPP Derivatives

Table III. IC_{50} Values of BPP Derivatives for Plasmin Activities toward S-2251 and Fibrin

Compound	IC_{50} (mM)	
	S-2251	Fibrin
Tos–Lys–BPP (**7**)	0.30	0.15
Tos–Phe–Lys–BPP (**8**)	0.21	0.10
3-MV–Phe–Lys–BPP (**9**)	2.5	1.5
Ile–Phe–Lys–BPP (**10**)	3.0	2.1
D,L-Ile–Phe–Lys–BPP (**11**)	1.7	0.95

Table IV. IC_{50} Values of BZA Derivatives for Plasmin Activities toward S-2251 and Fibrin

Compound	IC_{50} (mM)	
	S-2251	Fibrin
Tos–Lys–BZA (**12**)	0.14	0.15
Tos–Phe–Lys–BZA (**13**)	>0.10	>0.025
Dns–Phe–Lys–BZA (**14**)	0.20	0.10
D-Ile–Phe–Lys–BZA (**15**)	0.069	0.18
D-Ile–Phe–Lys–ACA (**16**)	0.11	0.19
D-Ile–Phe–Lys–DMBZA (**17**)	0.21	0.26

are summarized in Table III. Tos–Lys–BPP (**7**) and Tos–Phe–Lys–BPP (**8**) inhibited plasmin activity toward S-2251 with IC_{50} values of 0.30 mM and 0.21 mM, respectively. The tosyl group of **8** was replaced by bulky groups, 3-methylvaleroyl (3-MV), Ile and D,L-Ile residues. These compounds (**9**—**11**) inhibited plasmin activities toward S-2251 and fibrin more weakly than **7** and **8**. The presence of a 3-MV, Ile or D,L-Ile residue at the N-terminus and a BPP group at the C-terminus might prevent the positively charged ε-amino group of Lys residue from having access to the negatively charged part of the active center of plasmin due to the bulkiness of those groups.

Next, in order to remove the steric hindrance of BPP group, the BPP group was replaced by aniline derivatives because *p*-nitroanilide derivatives exhibited good affinity for the active center of plasmin. 4-Benzoylaniline, which has another aromatic ring on the anilide moiety, was used instead of the *p*NA group, and the inhibitory effect on plasmin is summarized in Table IV. Tos–Lys–BZA (**12**) inhibited plasmin activities toward S-2251 and fibrin with IC_{50} values of 0.14 and 0.15 mM, respectively. It is not clear why, although Dns–Phe–Lys–BZA (**14**) inhibited plasmin activities toward S-2251 and fibrin with IC_{50} values of 0.20 and 0.10 mM, respectively, 0.1 mM Tos–Phe–Lys–BZA (**13**) exhibited only a weak inhibitory effect on plasmin activity toward S-2251 (7% inhibition). D-Ile–Phe–Lys–BZA (**15**) inhibited plasmin activities toward S-2251 and fibrin with IC_{50} values of 0.069 and 0.18 mM, respectively. The

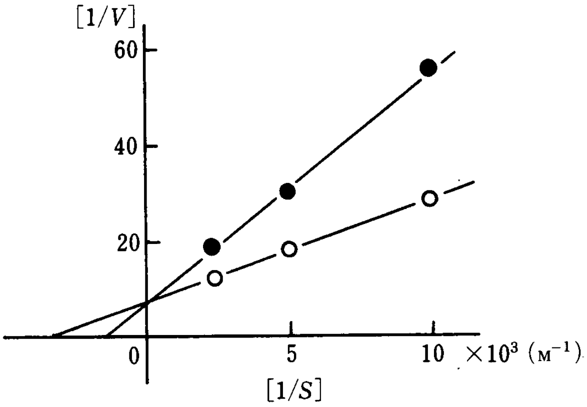


Fig. 2. Kinetics of Inactivation of Plasmin by Tos–Lys–*p*NA (**1**)

Substrate, D-Val–Leu–Lys–*p*NA; ●—●, with Tos–Lys–*p*NA (0.5 mM); ○—○, without Tos–Lys–*p*NA.

TABLE V. Plasmin-Inhibitory Activities of *t*-AMCHA and Lys Derivatives

Compound	IC$_{50}$ (mM)		
	S-2251	Fibrin	Fibrinogen
t-AMCHA	75	0.06	9.5
Tos–Lys–*p*NA (**1**)	0.70	0.78	0.90
Tos–Lys–BPP (**7**)	0.30	0.15	—[a]
Tos–Lys–BZA (**12**)	0.14	0.15	0.20

a) Not determined.

BZA group at the P$_1'$ position may be suitable to bind with the S$_1'$ site of plasmin, like the *p*NA moiety. D-Ile–Phe–Lys–ACA (**16**) and D-Ile–Phe–Lys–DMBZA (**17**) did not show more potent inhibitory activity than D-Ile–Phe–Lys–BZA (**15**).

It is possible that compounds **15**, **16** and **17** might be substrates for plasmin because these compounds are anilide derivatives. Thus, cleavage of the Lys–BZA bond by plasmin was examined. It was found that this bond of D-Ile–Phe–Lys–BZA (**15**) was cleaved by plasmin. Although **15** was a substrate for plasmin, the K_m value could not be calculated because of the non-linearity of the Lineweaver–Burk plot.[12] On the other hand, the Lys–BZA bond of Tos–Lys–BZA (**12**) was not cleaved by plasmin, as expected from the case of Tos–Lys–*p*NA (**1**). Thus, Tos–Lys–*p*NA type inhibitors are recommendable as stable inhibitors. In order to confirm that compounds of this type inhibit plasmin in a competitive manner as active center-directed inhibitors, the amidolytic activity of plasmin was assayed using S-2251 as a substrate in the absence and presence of Tos–Lys–*p*NA (**1**) and the results were plotted according to the method of Lineweaver and Burk[12] as shown in Fig. 2. This plot confirmed that Tos–Lys–*p*NA inhibited plasmin by blocking the active center. It was also found that this type of inhibitor inhibited plasmin activity toward not only fibrin but also S-2251 and fibrinogen, as summarized in Table V in comparison with the inhibitory activity of *t*-AMCHA. The inhibitory activities of our novel type compounds (**1**, **7** and **12**) on plasmin were 100—500 times as potent as that of *t*-AMCHA toward S-2251 and 10—50 times toward fibrinogen. In order to obtain more potent and selective inhibitors of plasmin, further research is under way in our laboratory.

Experimental

The melting points are uncorrected. Optical rotations were measured with an automatic polarimeter, model DIP-180 (Japan Spectroscopic Co., Ltd.). Amino acid compositions of acid hydrolysates (6 N HCl, 110 °C, 18 or 40 h) were determined with an amino acid analyzer (K-101AS, Kyowa Seimitsu). For column chromatography, a Toyo SF-160K fraction collector was used. For thin-layer chromatography (TLC) (Kieselgel G, Merck), Rf^1, Rf^2, Rf^3, Rf^4, Rf^5 and Rf^6 values refer to the systems of $CHCl_3$, MeOH and AcOH (90 : 8 : 2), $CHCl_3$, MeOH and H_2O (8 : 3 : 1, lower phase), $CHCl_3$, MeOH and H_2O (8 : 5 : 1), $CHCl_3$, MeOH and H_2O (89 : 10 : 1), *n*-BuOH, AcOH and H_2O (4 : 1 : 5, upper phase) and *n*-BuOH, AcOH, pyridine and H_2O (4 : 1 : 1 : 2), respectively.

Tos–Lys(Z)–*p*NA——*p*-Nitroaniline (1.4 g) was dissolved in pyridine (20 ml) and PCl_3 (0.45 ml) was added to the solution at −15 °C. The reaction mixture was stirred at the same temperature for 15 min,[13,14] Tos–Lys(Z)–OH[15] (4.3 g) was added to the above solution and the reaction mixture was stirred at 60 °C for 3 h. After removal of the solvent, the residue was dissolved in AcOEt. This solution was washed with 5% Na_2CO_3, 1 N HCl and H_2O, dried over Na_2SO_4 and concentrated to a small volume. Petroleum ether was added to the residue to give a crystalline material, which was recrystallized from AcOEt–ether, yield 4.5 g (81%), mp 100—102 °C, $[\alpha]_D^{25}$ $-32.1°$ (c=0.5, MeOH), Rf^1 0.44, Rf^2 0.83.

Tos–Lys–*p*NA (1)——A solution of Tos–Lys(Z)–*p*NA (1.2 g) in 25% HBr/AcOH (2.1 ml) was stored at 0 °C for 5 min and at room temperature for 50 min. Ether was added to the solution to give a precipitate, which was collected by filtration and dried over KOH pellets *in vacuo*. This crude material in water was washed with ether and the water layer was treated with Amberlite IRA-45 (acetate form). Lyophilization of the above solution gave an amorphous powder. This compound in $CHCl_3$ (3 ml) was applied to a silica gel column (1.8 × 27 cm) equilibrated and eluted with $CHCl_3$ (400 ml), followed by 3% MeOH in $CHCl_3$ (400 ml), 5% MeOH in $CHCl_3$ (1600 ml) and 7% MeOH in $CHCl_3$ (1400 ml). With 7% MeOH in $CHCl_3$ (200—1400 ml), the desired compound was obtained and converted to the corresponding hydrochloride, yield 0.38 g (39%), mp 215—218 °C, $[\alpha]_D^{25}$ $-21.1°$ (c=0.9, MeOH), Rf^3 0.81, Rf^5 0.37. *Anal.* Calcd for $C_{19}H_{24}N_4O_5S \cdot HCl$: C, 49.9; H, 5.41; N, 12.3. Found: C, 49.7; H, 5.71; N, 12.2.

General Procedure for X–Phe–Lys(Z)–*p*NA [X = Tos, Dns, Bz]——H-Phe–Lys(Z)–*p*NA·HCl (0.18 g, 0.41 mmol) [prepared from Boc–Phe–Lys(Z)–*p*NA[6] and 3.0 N HCl/dioxane] was dissolved in DMF (10 ml) containing Et_3N (0.82 ml). X–Cl (X = Tos, Dns, Bz) (0.41 mmol) was added to the above solution at 0 °C. The reaction mixture was stirred at the same temperature for 2 h. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 0.1 N HCl and water, dried over Na_2SO_4 and concentrated to a small volume. Ether was added to the residue to give crystals, which were collected by filtration. The yield, mp, $[\alpha]_D$ values, analytical data and Rf values are summarized in Table VI.

General Procedure for Preparation of X–Phe–Lys–*p*NA [X = Tos(2), Dns(3), Bz(4)]——A solution of X–Phe–Lys(Z)–*p*NA (0.2 mmol) and anisole (0.1 ml) in 25% HBr/AcOH (1.0 ml) was stored at room temperature for 30 min. Ether was added to the solution to give a white precipitate, which was collected by filtration and dried over KOH pellets *in vacuo*. The crude Dns–Phe–Lys–*p*NA in 5% AcOH (3 ml) was applied to a Sephadex G-25 column (2.5 × 90 cm), equilibrated and eluted with 5% AcOH. Individual fractions (3 g each) were collected and the eluate (tube Nos. 77—84) was lyophilized to give a purified material. The yield, mp, $[\alpha]_D$ value, analytical data and Rf values are summarized in Table VII.

General Procedure for Preparation of Boc–Lys(Z)–Y [Y = BPP, BZA, ACA, DMBZA]——As an example, the method of preparation of Boc–Lys(Z)–BPP is described. A mixed anhydride [prepared from Boc–Lys(Z)–OH (5.7 g, 15 mmol), ethyl chloroformate (1.4 ml, 15 mmol) and Et_3N (2.1 ml, 15 mmol) in THF (200 ml) as usual] was added to the solution of 4-benzylpiperidine (2.6 g, 15 mmol) in THF (50 ml) at 0 °C. The reaction mixture was stirred at 4 °C for

TABLE VI. Yields, Melting Points, $[\alpha]_D$ Values, Elemental Analysis and Rf Values of X–Phe–Lys(Z)–*p*NA

Compound (X)	Yield (%)	mp (°C)	$[\alpha]_D^{25}$ (DMF)	Formula	Elemental analysis Calcd (Found)			TLC	
					C	H	N	Rf^1	Rf^2
Tos	38	210—215	$-5.6°$ (c=0.4)	$C_{36}H_{39}N_5O_8S$	61.6 (61.3	5.60 5.41	9.97 9.90)	0.36	
Dns	66	185—190	$-17.2°$ (c=0.5)	$C_{41}H_{44}N_6O_8S$	63.0 (62.9	5.61 5.55	10.8 10.8)	0.72	
Bz	83	197—200	$-2.5°$ (c=0.4)	$C_{36}H_{37}N_5O_7$	66.3 (66.1	5.72 5.65	10.7 10.6)	0.54	0.56

TABLE VII. Yields, Melting Points, $[\alpha]_D$ Values, Elemental Analyses and Rf Values of X–Phe–Lys–*p*NA

Compound (X)	Yield (%)	mp (°C)	$[\alpha]_D^{25}$ (MeOH)	Formula	Elemental analysis Calcd (Found)			TLC		
					C	H	N	Rf^2	Rf^5	Rf^6
Tos (**2**)	69	143—146	$-70.4°$ (c=0.1)	$C_{28}H_{33}N_5O_6S \cdot$ $HBr \cdot H_2O$	50.4 (49.9	5.44 5.28	10.5 10.3)	0.30	0.47	0.71
Dns (**3**)	38	Amorphous	$-110°$ (c=0.2)	$C_{33}H_{38}N_6O_6S \cdot$ $HBr \cdot 2H_2O$	51.9 (51.6	5.67 5.51	11.0 10.8)	0.30	0.47	0.71
Bz (**4**)	42	Amorphous	$-45.1°$ (c=0.8)	$C_{28}H_{31}N_5O_5 \cdot$ $CH_3COOH \cdot 2H_2O$	58.7 (58.8	6.41 6.21	11.4 11.6)	0.32	0.53	0.74

TABLE VIII. Yields, Melting Points, $[\alpha]_D$ Values, Elemental Analyses and *Rf* Values of Boc–Lys(Z)–Y

Compound (Y)	Yield (%)	mp (°C)	$[\alpha]_D^{25}$ (MeOH)	Formula	Elemental analysis Calcd (Found) C	H	N	TLC *Rf*[1]	*Rf*[2]	*Rf*[3]
BPP	77	Oil	−2.76°	$C_{31}H_{43}N_3O_5$	69.3	8.00	7.82	0.67		
			(*c*=1.6)		(69.3	8.27	7.54)			
BZA	44	116—119	−10.9°	$C_{32}H_{37}N_3O_6$	68.7	6.66	7.51	0.65	0.92	
			(*c*=0.6)		(68.9	6.72	7.62)			
ACA	33	126—127	−9.72°	$C_{27}H_{35}N_3O_6$	65.2	7.09	8.45	0.58		0.51
			(*c*=1.1)		(64.9	7.15	8.33)			
DMBZA	69	68—71	−7.45°	$C_{34}H_{42}N_4O_6$	67.8	7.02	9.30	0.49	0.78	0.44
			(*c*=0.9)		(67.5	7.10	9.60)			

TABLE IX. Yields, Melting Points, $[\alpha]_D$ Values, Elemental Analyses and *Rf* Values of Boc–Phe–Lys(Z)–Y

Compound (Y)	Yield (%)	mp (°C)	$[\alpha]_D^{25}$ (MeOH)	Formula	Elemental analysis Calcd (Found) C	H	N	TLC *Rf*[1]	*Rf*[2]	*Rf*[3]
BPP	36	Amorphous	−9.54°	$C_{40}H_{52}N_4O_6$	70.2	8.18	8.18	0.66		
			(*c*=0.7)	$2H_2O$	(70.3	7.89	8.33)			
BZA	63	100—103	−18.0°	$C_{41}H_{46}N_4O_7$	69.7	6.51	7.92	0.65	0.92	
			(*c*=0.5)		(69.4	6.81	7.96)			
ACA	78	174—176	−9.65°	$C_{36}H_{44}N_4O_7$	67.1	6.88	8.69	0.67		0.55
			(*c*=1.0)		(67.3	6.96	8.73)			
DMBZA	66	91—100	−13.6°	$C_{43}H_{51}N_5O_6$	68.9	6.85	9.33	0.60		0.72
			(*c*=0.4)		(69.0	7.03	9.05)			

18 h. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and H_2O, dried over Na_2SO_4 and concentrated to a small volume. Petroleum ether was added to the residue to give an oily material. The other Boc–Lys(Z)–Ys were prepared in the same manner as described above. Boc–Lys(Z)–BZA and Boc–Lys(Z)–ACA were recrystallized from AcOEt and ether. Boc–Lys(Z)–DMBZA was purified by silica gel column chromatography. The yield, mp, $[\alpha]_D$ value, analytical data and *Rf* values are summarized in Table VIII.

Tos–Lys–BPP (7)——Tos–Lys(Z)–OSu (1.06 g, 2.1 mmol) [prepared from 2.2 g of Tos–Lys(Z)–OH[15] and 0.58 g of HOSu by the DCC method as usual] and 4-benzylpiperidine (0.35 g, 2.0 mmol) were dissolved in dioxane (15 ml) and the reaction mixture was stirred at 10 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 1 N HCl, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to give Tos–Lys(Z)–BPP as a crystalline material (0.82 g, 67%). This product (0.82 g) in 30% HBr/AcOH (1.5 ml) was stored at room temperature for 20 min. Ether was added to the solution to afford a precipitate as the HBr salt. This was converted to the corresponding free amine by treatment with 5% $NaHCO_3$ and extraction with $CHCl_3$ to give the title compound as an oily material, yeild 0.59 g (94%). This compound was converted to the corresponding hydrochloride as follows. The oily material (0.59 g) was dissolved in 9% HCl/EtOH (1 ml). After removal of HCl and EtOH, ether was added to the residue to give crystals, which were recrystallized from EtOH and ether, yeild 0.41 g (64.1%), mp 82—86 °C, $[\alpha]_D^{25}$ +53.6° (*c*=0.1, MeOH), *Rf*[5] 0.33. *Anal.* Calcd for $C_{25}H_{33}N_3O_3S \cdot HCl$: C, 65.0; H, 7.80; N, 9.10. Found: C, 64.8: H, 7.94; N, 8.95.

General Procedure for Preparation of Boc–Phe–Lys(Z)–Y [Y = BPP, BZA, ACA, DMBZA]——Boc–Phe–OH (15 mmol), HOBt (17 mmol) and H–Lys(Z)–Y · HCl [prepared from Boc–Lys(Z)–Y (15 mmol) and 2.8 N HCl/dioxane] were dissolved in DMF (100 ml) containing Et_3N (15 mmol). DCC (17 mmol) was added to the above cold solution and the reaction mixture was stirred at −15 °C for 1 h and at 4 °C for 18 h. After removal of the dicyclohexylurea and the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3, 10% citric acid and H_2O, dried over Na_2SO_4 and concentrated to a small volume. Petroleum ether was added to the residue to give a desired material. Boc–Phe–Lys(Z)–BZA and Boc–Phe–Lys(Z)–ACA were recrystallized from AcOEt and ether and AcOEt, respectively. Boc–Phe–Lys(Z)–BPP was purified by silica gel chromatography. The yield, mp, $[\alpha]_D$ value, analytical data and *Rf* values are summarized in Table IX.

Tos–Phe–Lys(Z)–BPP——The title compound was prepared from H–Phe–Lys(Z)–BPP·HCl [prepared from 0.3 g (0.56 mmol) of Boc–Phe–Lys(Z)–BPP and 5.5 N HCl in dioxane] and Tos–Cl (0.21 g, 1.1 mmol) in the same manner as described for the preparation of Tos–Phe–Lys(Z)–*p*NA and recrystallized from AcOEt and ether, yield 0.2 g (47.4%), mp 132—136 °C, $[\alpha]_D^{25}$ −24.7° (c=0.5, MeOH), Rf^1 0.51. *Anal.* Calcd for $C_{42}H_{50}N_4O_6S \cdot H_2O$: C, 66.7; H, 6.87; N, 7.40. Found: C, 66.7; H, 6.92; N, 7.64.

Tos–Phe–Lys–BPP (8)——Tos–Phe–Lys(Z)–BPP (0.13 g, 0.2 mmol) in MeOH (4 ml) containing 1 N HCl (0.2 ml, 0.2 mmol) was hydrogenated over a Pd catalyst. After removal of Pd and the solvent, ether was added to the residue to afford an amorphous powder, yeild 0.11 g (82.5%), $[\alpha]_D^{25}$ −55.3° (c=0.2, MeOH), Rf^5 0.53. *Anal.* Calcd for $C_{34}H_{44}N_4O_4S \cdot HCl \cdot 2.5H_2O$: C, 59.6; H, 7.30; N, 8.18. Found: C, 59.4; H, 7.02; N, 8.12.

3-MV–Phe–Lys–(Z)–BPP——The title compound was prepared from 3-MV–OH (0.1 ml, 0.8 mmol) and H–Phe–Lys(Z)–BPP·HCl (500 mg, 0.8 mmol) [prepared from Boc–Phe–Lys(Z)–BPP and 3 N HCl/dioxane] by the DCC method in the same manner as described for the preparation of Boc–Phe–Lys(Z)–Y. The crude material in $CHCl_3$ (5 ml) was applied to a silica gel column (1.8 × 31.5 cm) equilibrated and eluted with $CHCl_3$. The solvent of the effluent (800—1200 ml) was removed by evaporation. Petroleum ether was added to the residue to give an oily product, yield 0.34 g (70%), $[\alpha]_D^{25}$ −7.2° (c=0.8, MeOH), Rf^5 0.58.

3-MV–Phe–Lys–BPP (9)——3-MV–Phe–Lys(Z)–BPP (0.33 g, 0.56 mmol) was dissolved in 60% AcOH (5 ml) and hydrogenated over a Pd catalyst. After removal of Pd and the solvent, petroleum ether was added to the residue to give an oily product, yield 0.26 g (82%), $[\alpha]_D^{25}$ −6.9° (c=0.7, MeOH), Rf^1 0.13, Rf^2 0.60, Rf^6 0.59. *Anal.* Calcd for $C_{33}H_{48}N_4O_3 \cdot CH_3COOH$: C, 69.1; H, 8.60; N, 9.20. Found: C, 68.9; H, 8.80; N, 9.41.

Boc–Ile–Phe–Lys(Z)–BPP——The title compound was prepared from Boc–Ile–OH (290 mg, 1.3 mmol) and H–Phe–Lys(Z)–BPP·HCl (780 mg, 1.3 mmol) [prepared from Boc–Phe–Lys(Z)–BPP and HCl/dioxane] by DCC method in the same manner as described for the preparation of Boc–Phe–Lys(Z)–Y. The crude material in $CHCl_3$ (5 ml) was applied to a silica gel column (1.8 × 31 cm) equilibrated and eluted with $CHCl_3$. The solvent of the effluent (400—1000 ml) was removed by evaporation. Ether and petroleum ether were added to the residue to give a powder, yield 500 mg (50%), mp 77—80 °C, $[\alpha]_D^{25}$ −22.2° (c=1.0, MeOH), Rf^1 0.49.

H–Ile–Phe–Lys–BPP (10)——Boc–Ile–Phe–Lys(Z)–BPP (130 mg, 0.16 mmol) was dissolved in 25% HBr/AcOH (0.16 ml, 0.48 mmol). The reaction mixture was stirred at 0 °C for 5 min and at room temperature for 50 min. Ether was added to the solution to yield a precipitate, which was collected by filtration and dried over KOH pellets *in vacuo*. This crude material in H_2O (10 ml) was washed with $CHCl_3$ and treated with Amberlite IRA-45 (acetate form). After addition of 1 N HCl (0.32 ml) to the above water layer, lyophilization was carried out to give an amorphous powder, yield 60 mg (60%), $[\alpha]_D^{25}$ +3.2° (c=0.9, MeOH), Rf^5 0.28, Rf^6 0.63. *Anal.* Calcd for $C_{33}H_{49}N_5O_3 \cdot 2HCl \cdot H_2O$: C, 60.5; H, 8.15; N, 10.7. Found: C, 60.2; H, 8.07; N, 10.4.

Boc–D,L-Ile–Phe–Lys(Z)–BPP——The title compound was prepared from Boc–D,L-Ile–OH (370 mg, 1.6 mmol) and H–Phe–Lys(Z)–BPP·HCl (1.0 g, 1.6 mmol) [prepared from Boc–Phe–Lys(Z)–BPP and HCl/dioxane] by the DCC method in the same manner as described for the preparation of Boc–Phe–Lys(Z)–Y. The crude material in $CHCl_3$ (5 ml) was applied to a silica gel column (2 × 30 cm) equilibrated and eluted with $CHCl_3$. The solvent of the effluent (500—1000 ml) was removed by evaporation. Petroleum ether was added to the residue to give a white powder, yield 0.60 g (57%), mp 82—85 °C, $[\alpha]_D^{25}$ −13.3° (c=1.0, MeOH), Rf^1 0.40.

D,L-H–Ile–Phe–Lys–BPP (11)——The title compound was prepared from Boc–D,L-Ile–Phe–Lys(Z)–BPP (150 mg, 0.18 mmol) and 25% HBr/AcOH in the same manner as described for the preparation of Tos–Lys–*p*NA (1). The crude material in 5% AcOH (5 ml) was subjected to gel-filtration on a Sephadex G-25 column (2.3 × 90 cm), equilibrated and eluted with 5% AcOH. Individual fractions (5 g each) were collected and the eluate (tube Nos. 52—58) was lyophilized to give an amorphous powder, yield 90 mg (91%), $[\alpha]_D^{25}$ −1.9° (c=0.6, MeOH), Rf^1 0.08, Rf^2 0.28. *Anal.* Calcd for $C_{33}H_{49}N_5O_3 \cdot 2HBr \cdot H_2O$: C, 53.3; H, 7.18; N, 9.41. Found: C, 53.0; H, 7.23; N, 9.39.

Tos–Lys–BZA (12)——4-Benzoylaniline (0.39 g, 2.0 mmol) was added to a solution of Tos–Lys(Z)–Cl (prepared from 0.87 g, 2.2 mmol of Tos–Lys(Z)–OH[15] and 0.46 g of PCl_5 as usual) in dioxane (15 ml). After 1 h, the solvent was removed by evaporation. The residue was neutralized with 5% $NaHCO_3$ and extracted with $CHCl_3$. The extract was washed with 1 N HCl and water, dried over Na_2SO_4 and evaporated down to give an oily material. This crude product was applied to a silica gel column (2 × 30 cm), equilibrated with *n*-hexane and eluted with *n*-hexane (300 ml), ether (500 ml) and then $CHCl_3$ (400 ml). After removal of the $CHCl_3$, Tos–Lys(Z)–BZA was obtained as crystals (yield 0.36 g, 30.4%). This material (0.36 g) in 30% HBr/AcOH (1 ml) was allowed to stand at room temperature for 15 min. Ether was added to the above solution to give a precipitate, which was collected by filtration and washed with ether. This hydrobromide was converted to the corresponding free amine by treatment with 5% $NaHCO_3$ and extraction with $CHCl_3$. This crude material was applied to a silica gel column (2.5 × 30 cm) equilibrated with $CHCl_3$ and eluted with $CHCl_3$ (500 ml) and 10% EtOH/$CHCl_3$ (300 ml). The solvent of the latter effluent was removed by evaporation to give the title compound, yield 0.24 g (86.3%), mp 72—75 °C, $[\alpha]_D^{25}$ −45.0° (c=0.1, MeOH), Rf^5 0.40. *Anal.* Calcd for $C_{25}H_{35}N_3O_3S$: C, 65.2; H, 6.05; N, 8.76. Found: C, 64.9; H, 6.14; N, 8.50.

Tos–Phe–Lys(Z)–BZA——The title compound was prepared from H–Phe–Lys(Z)–BZA·HCl [prepared from Boc–Phe–Lys(Z)–BZA (0.2 g, 0.31 mmol) and 5.5 N HCl in dioxane (0.56 ml, 3.1 mmol)] and Tos-Cl (0.12 g, 0.62 mmol) in the same manner as described for the preparation of Tos–Phe–Lys(Z)–*p*NA, yield 0.16 g (72.0%), mp 206—210 °C, $[\alpha]_D^{25}$ −45.2 ° (c=0.8, MeOH), Rf^1 0.50. *Anal.* Calcd for $C_{43}H_{44}N_4O_7S$: C, 67.9; H, 5.78; N, 7.36. Found: C, 67.8; H, 6.00; N, 7.27.

Dns–Phe–Lys(Z)–BZA——The title compound was prepared from H–Phe–Lys(Z)–BZA · HCl [prepared from Boc–Phe–Lys(Z)–BZA (0.21 g, 0.32 mmol) and 5.5 N HCl in dioxane (0.58 ml, 3.2 mmol)] and Dns-Cl (0.2 g, 0.64 mmol) in $CHCl_3$ (30 ml) in the same manner as described for the preparation of Dns–Phe–Lys(Z)–*p*NA and recrystallized from AcOEt and ether, yield 0.2 g (75.9%), mp 193—197 °C, $[\alpha]_D^{25}$ −26.5 ° (c=0.8, MeOH), Rf^1 0.54. *Anal.* Calcd for $C_{48}H_{49}N_5O_7S$: C, 68.7; H, 5.83; N, 8.28. Found: C, 68.3; H, 5.99; N, 8.33.

Tos–Phe–Lys–BZA (13)——Tos–Phe–Lys(Z)–BZA (0.11 g, 0.15 mmol) in DMF (5 ml) and MeOH (2 ml) containing 1 N HCl (0.15 ml, 0.15 mmol) was hydrogenated over a Pd catalyst. After removal of Pd and the solvents, ether was added to the residue to afford crystals, yield 0.093 g (82.6%), mp 150—151 °C, $[\alpha]_D^{25}$ −46.4 ° (c=0.33, MeOH), Rf^5 0.73. *Anal.* Calcd for $C_{35}H_{38}N_4O_5S \cdot HCl \cdot 4H_2O$: C, 57.2; H, 6.40; N, 7.62. Found: C, 57.6; H, 6.56; N, 7.54.

Dns–Phe–Lys–BZA (14)——The title compound was prepared from Dns–Phe–Lys(Z)–BZA (1.1 g, 1.3 mmol) and 25% HBr/AcOH (1.2 ml, 3.9 mmol) in the usual manner, yield 0.95 g (92.6%), amorphous, $[\alpha]_D^{25}$ −88.1 ° (c=0.8, MeOH), Rf^6 0.64. *Anal.* Calcd for $C_{40}H_{43}N_5O_5S \cdot HBr \cdot 2H_2O$: C, 58.5; H, 5.84; N, 8.52. Found: C, 58.3; H, 6.01; N, 8.89.

Z-D-Ile–Phe–Lys(Z)–BZA——The title compound was prepared from Z–D-Ile–OH (630 mg, 2.4 mmol) and H–Phe–Lys(A)–BZA · HCl [prepared from Boc–Phe–Lys(Z)–BZA (900 mg, 1.3 mmol) and 2.7 N HCl/dioxane (4.8 ml, 13 mmol)] by the DCC method in the same manner as described for the preparation of Boc–Phe–Lys(Z)–Y. The crude material was recrystallized from MeOH, yield 670 mg (68%), mp 222—225 °C, $[\alpha]_D^{25}$ +7.8 ° (c=0.5, DMF), Rf^1 0.80, Rf^2 0.75, Rf^5 0.81. *Anal.* Calcd for $C_{50}H_{55}N_5O_8$: C, 70.3; H, 6.49; N, 8.20. Found: C, 70.3; H, 6.56; N, 8.31.

D-Ile–Phe–Lys–BZA (15)——The title compound was prepared from Z–D-Ile–Phe–Lys(Z)–BZA (20 mg, 0.024 mmol) by hydrogenation over a Pd catalyst in the same manner as described for the preparation of 3-MV–Phe–Lys–BPP, yield 10 mg (80%), $[\alpha]_D^{25}$ −21.0 ° (c=0.1, MeOH), Rf^5 0.16, Rf^6 0.73. *Anal.* Calcd for $C_{34}H_{43}N_5O_4 \cdot 2CH_3COOH$: C, 64.7; H, 7.28; N, 9.92. Found: C, 64.8; H, 7.59; N, 10.4.

Z–D-Ile–Phe–Lys(Z)–ACA——The title compound was prepared from Z–D-Ile–OH (190 mg, 0.70 mmol) and H–Phe–Lys(Z)–ACA [prepared from Boc–Phe–Lys(Z)–ACA (520 mg, 0.8 mmol) and 2.4 N HCl/dioxane (3.3 ml, 8 mmol)] by the DCC method in the same manner as described for the preparation of Boc–Phe–Lys(Z)–Y. The crude material was submitted to gel-filtration on a Sephadex LH-20 column (2.5 × 70 cm) equilibrated and eluted with DMF. Individual fractions (5 g each) were collected and the eluate (tube Nos. 12—16) was concentrated. Ether was added to the residue to give a white precipitate, which was collected by filtration, yield 380 mg (60%), mp 182—186 °C, $[\alpha]_D^{25}$ −8.5 ° (c=1.1, DMF), Rf^1 0.55, Rf^2 0.70. *Anal.* Calcd for $C_{45}H_{53}N_5O_8$: C, 68.2; H, 6.74; N, 8.84. Found: C, 68.3; H, 6.80; N, 8.96.

H–D-Ile–Phe–Lys–ACA (16)——Z–D-Ile–Phe–Lys(Z)–ACA (100 mg, 0.13 mmol) was suspended in MeOH (15 ml) and hydrogenated over a Pd catalyst. After removal of Pd and the solvent, the residue was dissolved in H_2O followed by lyophilization to give an amorphous powder, yield 40 mg (47%), $[\alpha]_D^{25}$ −29.7 ° (c=0.7, MeOH), Rf^5 0.25, Rf^6 0.63, *Anal.* Calcd for $C_{29}H_{41}N_5O_4 \cdot 4H_2O$: C, 58.5; H, 8.29; N, 11.8. Found: C, 58.1; H, 8.03; N, 11.6.

Boc–D-Ile–Phe–Lys(Z)–DMBZA——A solution of Boc–Phe–Lys(Z)–DMBZA (400 mg, 0.55 mmol) in TFA (2.0 ml) containing anisole (0.2 ml) was kept at room temperature for 90 min. Ether was added to the solution to form a precipitate, which was collected by filtration and dried over KOH pellets *in vacuo*. Boc–D-Ile–OH (180 mg, 0.8 mmol) and the resultant H–Phe–Lys(Z)–DMBZA were coupled by the DCC method in the same manner as described for the preparation of Boc–Phe–Lys(Z)–Y. The crude material in $CHCl_3$ (5 ml) was applied to a silica gel column (2 × 28 cm) equilibrated and eluted with $CHCl_3$. The solvent of the effluent (1100—1800 ml) was removed by evaporation. Ether was added to the residue to give a solid mass, yield 180 mg (38%), mp 114—119 °C, $[\alpha]_D^{25}$ −15.7 ° (c=0.2, DMF), Rf^1 0.64, Rf^3 0.71. *Anal.* Calcd for $C_{49}H_{62}N_6O_8 \cdot H_2O$: C, 66.8; H, 7.39; N, 9.53. Found: C, 66.8; H, 7.06; N, 9.73.

D-Ile–Phe–Lys–DMBZA (17)——Boc–D-Ile–Phe–Lys(Z)–DMBZA (120 mg, 0.14 mmol) was dissolved in 25% HBr/AcOH (0.5 ml, 0.51 mmol) containing anisole (0.05 ml) and the solution was kept at room temperature for 1 h. Ether was added to the solution to form a precipitate, which was collected by filtration and dried over KOH pellets *in vacuo*, yield 35 mg (29%), $[\alpha]_D^{25}$ −11.9 ° (c=0.7, MeOH), Rf^5 0.14, Rf^6 0.52. *Anal.* Calcd for $C_{36}H_{48}N_6O_4 \cdot 3HBr$: C, 49.6; H, 5.89; N, 9.64. Found: C, 49.4; H, 5.91; N, 9.51.

Assay Procedure——1) Antifibrinolytic Assay: To a borate saline buffer (pH 7.4) solution of various concentrations of a peptide to be tested (0.6 ml), 0.2% bovine fibrinogen (plasminogen free) in the above buffer (0.2 ml), human plasmin solution (0.1 ml) and bovine thrombin 50 U/ml (0.1 ml) were added. Assay was carried out at 37 °C and the required time for complete lysis of the formed fibrin clot was measured. The IC_{50} value was taken as the concentration of inhibitor which prolonged the complete lysis time twofold in comparison with that in the case without inhibitor.

2) Antiamidolytic Assay: To a solution of various concentration of a peptide to be tested in Tris/HCl buffer (0.05 M, pH 7.4, 0.4 ml), 3 mM peptide substrate (S-2251) dissolved in the same buffer (0.05 ml) and the plasmin

solution (0.05 ml) were added. The reaction mixture was immediately incubated at 37 °C. The reaction was stopped after 4 min by adding 50% AcOH (0.05 ml) and the *p*NA released was determined by measuring the absorbancy at 405 nm. The IC_{50} value was taken as concentration of inhibitor which decreased the absorbancy at 405 nm by 50% compared with the absorbancy measured under the same conditions without inhibitor. When Boc–Val–Leu–Lys–MCA instead of S-2251 was used as a substrate, AMC (7-amino-4-methylcoumarin) released by plasmin was determined by measuring the fluorescence. Activation and emission wavelengths were 380 and 460 nm, respectively.16)

References and Notes

1) All amino acid residues are of L-configuration unless otherwise indicated. Standard abbreviations for amino acids and their derivatives are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972). Other abbreviations used are: Z, benzyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; Tos, tosyl; Dns, dansyl; Bz, benzoyl; 3-MV, 3-methylvaleroyl; *p*NA, *p*-nitroanilide; MCA, 4-methylcoumaryl-7-amide; BPP, 4-benzylpiperidine amide; BZA, 4-benzoylanilide; ACA, 4-acetylanilide; DMBZA, 3-(4-dimethylaminobenzoyl)-anilide; HOSu, *N*-hydroxysuccinimide; OSu, *N*-hydroxysuccinimide ester; Et_3N, triethylamine; AcOH, acetic acid; DDC, *N,N'*-dicyclohexylcarbodiimide; DMF, dimethylformamide; AcOEt, ethyl acetate; THF, tetrahydrofuran; *n*-BuOH, *n*-butanol.
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