Development of Active Center-Directed Inhibitors against Plasmin[1)]

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Active center-directed inhibitors of plasmin were designed based on the structure of specific substrates of plasmin and then synthesized. Their effects on plasmin were examined and the structure–inhibitory activity relationship was studied. N^{α}-*trans*-4-Aminomethylcyclohexanecarbonyllysine 4-benzoylanilide (Tra–Lys–BZA) inhibited plasmin activities toward S-2251 and fibrin with IC_{50} values of 15 and 6.1 μM, respectively and N^{α}-*trans*-4-aminomethylcyclohexanecarbonyllysine 4-benzylpiperidine amide (Tra–Lys–BPP) did not show any detectable inhibitory activity. Moreover, it was revealed that Tra–Lys–4-methoxycarbonylanilide inhibited plasma kallikrein more potently than plasmin.

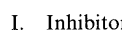
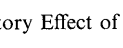
Keywords plasmin; competitive inhibitor; design; Lys derivative; synthesis; structure–activity relationship

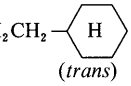
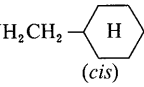
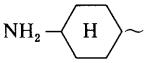
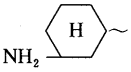
It is well known that proteinases and their natural inhibitors regulate biological functions cooperatively to maintain homeostasis, and imbalances between proteinases and their natural inhibitors cause serious disorders. With regard to plasmin, an imbalance between plasmin and its natural inhibitors (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 (*trans*-AMCHA)[3)] are employed clinically as plasmin inhibitors. These inhibitors showed fairly potent inhibition of fibrinolysis by plasmin, but very slight inhibition of the amidolysis of small peptide substrates and fibrinogenolysis by plasmin, because these inhibitors exhibit an inhibitory effect on plasmin by blocking the lysine binding site (LBS) of an enzyme, which is not the catalytic site.[4)]

Thus, our research goal was to synthesize active center-directed inhibitors of plasmin, with the objective of obtaining potent and selective inhibitors of plasmin toward not only fibrinolysis but also amidolysis and fibrinogenolysis. This report deals with the design and synthesis of active center-directed plasmin inhibitors.

Previously, we reported that D-Ile–Phe–Lys–*p*NA was a selective substrate with a low K_m value for plasmin,[5)] and that D-Ile–Phe–Lys–BZA inhibited plasmin activities toward S-2251 and fibrin with IC_{50} values of 69 and 180 μM, respectively, by blocking the active center of plasmin, but D-Ile–Phe–Lys–BPP was not inhibitory.[6)] It was also found that a free amino group of D-Ile at the P_3 position[7)] might contribute to an increment in affinity between the substrates or inhibitors and the enzyme.

First of all, we prepared Lys derivatives substituted for the D-Ile–Phe moiety of D-Ile–Phe–Lys–BZA using an amino-containing compound. As shown in Fig. 1, the desired compounds were prepared and their inhibitory activities against plasmin are summarized in Table I. From Table I, it was revealed that the ε-aminocaproyl (Eac) or *trans*-4-aminomethylcyclohexanecarbonyl (Tra) group is suitable for binding with plasmin, presumably because the position of the free amino group of Eac or Tra is similar to that of the free amino group of D-Ile–Phe moiety. The

TABLE I. Inhibitory Effect of R–Lys–NH–  –CO–  on Plasmin

	R	IC_{50} (μM)		
		S-2251	Fn	Fg
1	$NH_2(CH_2)_4CO$–	16	17	36
2	$NH_2(CH_2)_5CO$–	12	>10 (18%)[a)]	ND
3	NH_2CH_2–  –CO– (*trans*)	15	6.1	13
4	NH_2CH_2–  –CO– (*cis*)	400	260	ND
5	NH_2–  –CO–	>250 (0%)	>250 (6%)	ND
6	 –CO– (NH_2)	>200 (43%)	64	ND

a) In parentheses, inhibitory % at the concentration described is indicated. ND = not determined.

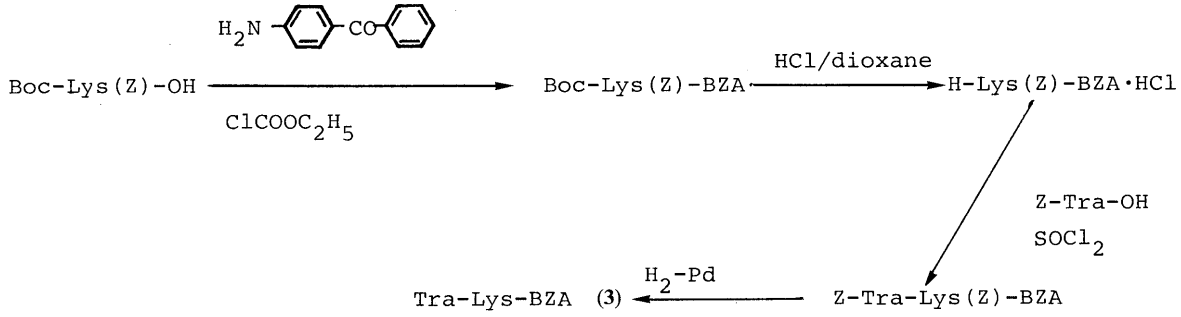


Fig. 1. Synthetic Route to Tra–Lys–BZA (3)

TABLE II. Inhibitory Effect of NH_2CH_2-⟨H⟩-CO-R-NH-⟨⟩-CO-⟨⟩ on Plasmin

	R	IC_{50} (μM)		
		S-2251	Fn	Fg
3	Lys	15	6.1	13
7	D-Lys	>200 (0%)[a]	>200 (0%)	ND
8	Orn	>300 (33%)	>300 (43%)	ND

a) In parentheses, inhibitory % at the concentration described is indicated.

TABLE III. Inhibitory Effect of NH_2CH_2-⟨H⟩-CO-Lys-R on Plasmin

	R	IC_{50} (μM)	
		S-2251	Fn
9	-N(COOC$_2$H$_5$)	>1000 (0%)	>1000 (19%)[a]
10	-N-CH$_2$-⟨⟩	>500 (7%)	>500 (16%)
11	-N-	>500 (18%)	>500 (33%)
12	-NH-⟨⟩-NO$_2$	>500 (41%)	530
13	-NH-	>500 (9%)	>500 (31%)
14	-NH-⟨⟩-COOCH$_3$	>500 (17%)	330
15	-NH-⟨⟩-COCH$_3$	39	9.3
3	-NH-⟨⟩-CO-⟨⟩	15	6.1
16	-O-⟨⟩-CO-⟨⟩	24	170
17	-CH$_2$NH-⟨⟩-CO-⟨⟩	450	510
18	-CH$_2$O-⟨⟩-CO-⟨⟩	660	200

a) In parentheses, inhibitory % at the concentration described is indicated.

compound (**3**) exhibited inhibitory activity against plasmin toward S-2251 and fibrin with IC_{50} values of 15 and 6.1 μM, respectively.

As summarized in Table II, Tra–D-Lys–BZA and Tra–Orn–BZA did not show any detectable inhibitory activity against plasmin, indicating that Lys should be in an L-configuration, and that Orn instead of Lys is not suitable for the manifestation of inhibitory activity.

Next, the BZA moiety of Tra–Lys–BZA (**3**) was substituted by piperidine type compounds and aniline type compounds, and their inhibitory activities against plasmin were examined and the results are summarized in Table III.

Previously, it was reported that Tos–Lys–BPP inhibited plasmin toward S-2251 and fibrin with IC_{50} values of 300 and 150 μM, respectively. However, Tra–Lys–BPP (**10**)

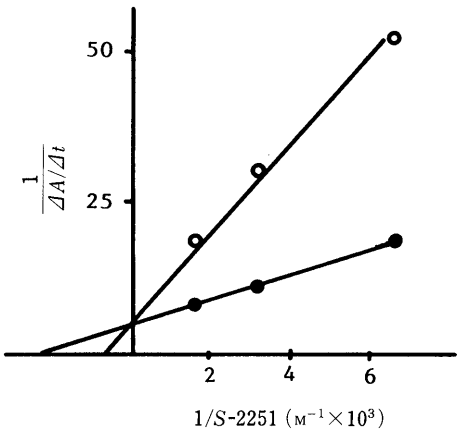


Fig. 2. Mode of Inhibition of Tra–Lys–BZA (**3**) toward Plasmin
○: Tra–Lys–BZA (**3**), ●: no inhibitor.

TABLE IV. Comparison of Inhibitory Activity against Plasmin

Compound	IC_{50} (μM)		
	S-2251	Fn	Fg
NH_2CH_2-⟨H⟩-COOH (*trans*-AMCHA)	75000	60	9500
CH$_3$-⟨⟩-SO$_2$-Lys-NH-⟨⟩-NO$_2$	700	780	900
CH$_3$-⟨⟩-SO$_2$-Lys-N-⟨⟩-CH$_2$-⟨⟩	300	150	ND
CH$_3$-⟨⟩-SO$_2$-Lys-NH-⟨⟩-CO-⟨⟩	140	150	200
D-Ile-Phe-Lys-NH-⟨⟩-CO-⟨⟩	69	180	ND
NH_2CH_2-⟨H⟩-CO-Lys-NH-⟨⟩-CO-⟨⟩	15	6.1	13

and other piperidine type derivatives did not show any detectable inhibitory activity against plasmin, as can be seen in Table III. Tra–Lys–piperidine amide derivatives are not suitable for the manifestation of inhibitory activity against plasmin. These facts are compatible with the fact that D-Ile–Phe–Lys–BPP did not inhibit plasmin.[6] Tra–Lys–*p*NA (**12**) weakly inhibited plasmin towards S-2251 and fibrin [IC_{50} = 500 μM (41% inhibition) and 530 μM, respectively], but *p*-nitroaniline was not liberated from Tra–Lys–*p*NA (**12**) by the action of plasmin, indicating that this type of compound is stable to the action of plasmin. Tra–Lys–ACA (**15**) inhibited plasmin activity toward S-2251 and fibrin with IC_{50} values of 39 and 9.3 μM, respectively. Tra–Lys–4-methoxycarbonylanilide (**14**) inhibited plasma kallikrein with an IC_{50} value of 50 μM[8] and plasmin toward S-2251 and fibrin with IC_{50} values of 500 μM (17% inhibition) and 330 μM, respectively, indicating that this compound (**14**) is a plasma kallikrein inhibitor rather than plasmin inhibitor.

Next, the –CONH– bond of Lys–BZA was modified with an ester bond (**16**), aminomethyl ketone, –$COCH_2NH$– (**17**) and oxymethyl ketone, –$COCH_2O$– (**18**). The inhibitory activity of these compounds against plasmin decreased compared with the corresponding amide bond derivative (**3**) as shown in Table III.

As stated above, the Lys–*p*NA bond of Tra–Lys–*p*NA (**12**) was not cleaved by plasmin; however, these com-

pounds, including Tra–Lys–BZA (**3**), inhibited plasmin competitively as shown in Fig. 2. This result posed the question; which amino group (Tra or Lys in **3**) interacts with S_1 position of plasmin[7]? In order to clarify this problem, Z–Tra–Lys–4-benzoylphenoxymethyl ketone (**19**) and Tra–Lys(Z)–4-benzoylphenoxymethyl ketone (**20**), which were derivatives of compound (**18**), were prepared, and their inhibitory activity against plasmin was examined. These derivatives of **18** were selected because the lysyl bond in **18** is stable to the action of plasmin. The fact that Tra–Lys(Z)–4-benzoylphenoxymethyl ketone (**20**) still exhibited weak inhibitory activity against plasmin toward S-2251 and fibrin with IC_{50} values of 200 and 60 μM, respectively, suggested the possibility that the amino group of Tra might interact with the S_1 position of plasmin. Moreover, it was deduced that the existence of an ε-amino group of Lys in compound (**18**) decreased the inhibitory activity of **18** against plasmin from the fact that compound (**20**) exhibited more potent inhibitory activity than the parent molecule (**18**). It is surprising that Tra–Lys derivatives obtained here interact with plasmin in quite a different manner from D-Ile–Phe–Lys derivatives, although Tra–Lys derivatives were designed based on the structure of D-Ile–Phe–Lys derivatives.

Finally, the inhibitory activities obtained above are summarized in Table IV and compaired with the activity of other plasmin inhibitors. As can be seen in Table IV, Lys-derivatives (Tos–Lys–*p*NA, Tos–Lys–BPP, Tos–Lys–BZA *etc.*) exhibited more potent inhibitory activity against plasmin toward S-2251 and fibrinogenolysis. Finally, Tra–Lys–BZA (**3**) inhibited plasmin not only toward S-2251 and fibrinogenolysis but also toward fibrinolysis much more potently than *trans*-AMCHA by blocking the active center of plasmin. It was also revealed that Tra–Lys–4-methoxycarbonylanilide (**14**) inhibited plasma kallikrein more potently than plasmin.[8] These results provided us with some ideas for designing specific inhibitors against various enzymes, and further extensive studies are now proceeding in our laboratory in order to obtain more potent and selective inhibitors not only against plasmin but also plasma kallikrein.

Experimental

The melting points are uncorrected. Optical rotations were measured with an automatic polarimeter, model DIP-360 (Japan Spectroscopic Co., Ltd.). Amino acid compositions of acid hydrolysates (6 N HCl, 110 °C, 18 h) were determined with an amino acid analyzer (K-101AS, Kyowa Seimitsu). For thin-layer chromatography (TLC) (Kieselgel G, Merck), Rf^1, Rf^2, Rf^3, Rf^4, Rf^5, Rf^6 and Rf^7 values refer to the systems of $CHCl_3$, MeOH and AcOH (90:8:2), $CHCl_3$, MeOH and H_2O (89:10:1), $CHCl_3$, MeOH and H_2O (8:3:1, lower phase), *n*-BuOH, AcOH and H_2O (4:1:5, upper phase), *n*-BuOH, AcOH, pyridine and H_2O (4:1:1:2), *n*-BuOH, AcOH, pyridine and H_2O (1:1:1:1) and $CHCl_3$ and ether (4:1), respectively.

General Procedure for Synthesis of R–Lys(Z)–BZA [R: Z–NH(CH$_2$)$_4$CO–, Z–NH(CH$_2$)$_5$CO–, Z–NH–CH$_2$–(H)–CO– (*trans*), (*cis*), Z–NH–(H)–CO–, Z–NH–(H)–CO–] An acid chloride [prepared from the corresponding carboxylic acid derivative (2 mmol) and $SOCl_2$ (0.6 ml, 8 mmol) as usual] in $CHCl_3$ was added to an ice-cold solution of H–Lys(Z)–BZA·HCl [prepared from Boc–Lys(Z)–BZA[6] (0.70 g, 1.3 mmol) and 5.6 N HCl/dioxane (2.3 ml) as usual] in DMF (8 ml) containing Et_3N (0.36 ml). The reaction mixture was stirred at room temperature 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 H_2O, dried over Na_2SO_4, and concentrated to a small volume. The oily product in $CHCl_3$ (4 ml) was applied to a silica gel column (1.4 × 27 cm), equilibrated and eluted with $CHCl_3$. Individual fractions (100 ml each) were collected, and the solvent of the effluent (tube Nos. 8—10) was removed by evaporation. Petroleum ether was added to the residue to give crystals, which were collected by filtration. Yield, mp, $[\alpha]_D$ value, *Rf* values and elemental analysis are summarized in Table V.

General Procedure for Synthesis of R–Lys–BZA [R: NH$_2$(CH$_2$)$_4$CO–, NH$_2$(CH$_2$)$_5$CO–, NH$_2$CH$_2$–(H)–CO– (*trans*), (*cis*), NH$_2$–(H)–CO–, NH$_2$–(H)–CO–] The corresponding N^{α}-Z-derivative (1.0 mmol) in EtOH (8 ml) was hydrogenated over a Pd catalyst. After removal of Pd and the solvent, ether was added to the residue to afford an amorphous powder. Yield, mp, $[\alpha]_D$ value, *Rf* values and elemental analysis are summarized in Table VI.

Boc–D-Lys(Z)–BZA A mixed anhydride [prepared from Boc–D-Lys(Z)–OH (5.7 g, 15 mmol) and ethyl chloroformate (1.4 ml, 15 mmol) as usual] in THF (200 ml) was added to an ice-cold solution of 4-benzoylaniline (2.6 g, 15 mmol) in THF (50 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 1 N HCl, 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4 and evaporated down. The oily product in $CHCl_3$ (10 ml) was applied to a silica gel column (2.6 × 47.5 cm), equilibrated and eluted with $CHCl_3$. Individual fractions (150 ml each) were collected. The solvent of the effluent (tube Nos. 8—13)

TABLE V. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of R–Lys(Z)–BZA

Compound R	Yield (%)	mp (°C)	$[\alpha]_D$ (°) (Solvent)	Formula	Elemental analysis Calcd (Found) C	H	N	TLC Rf^1	Rf^2
Z–NH–(CH$_2$)$_4$CO–	64.7	120—124	−27.0 ($CHCl_3$)	$C_{40}H_{44}N_4O_7$	69.3 (69.2	6.41 6.53	8.08 8.08)	0.64	
Z–NH–(CH$_2$)$_5$CO–	73.1	95—98	−28.3 ($CHCl_3$)	$C_{41}N_{46}N_4O_7$	69.7 (69.5	6.57 6.48	7.92 7.68)	0.72	
Z–NH–CH$_2$–(H)–CO– (*trans*)	37.9	188—192	+2.7 (DMF)	$C_{43}H_{48}N_4O_7$	70.5 (70.2	6.55 6.62	7.65 7.78)	0.83	0.85
Z–NH–CH$_2$–(H)–CO– (*cis*)	50.9	78—85	−24.6 (MeOH)	$C_{43}H_{48}N_4O_7$	70.5 (70.5	6.55 6.57	7.65 7.57)	0.80	0.85
Z–NH–(H)–CO–	17.6	53—59	−36.6 (MeOH)	$C_{42}H_{46}N_4O_7$ 1.5H_2O	70.2 (70.3	6.45 6.83	7.79 7.72)	0.52	0.84
Z–NH–(H)–CO–	61.1	167—169	+2.9 (DMF)	$C_{42}H_{46}N_4O_7$	70.2 (70.4	6.45 6.45	7.79 7.81)	0.57	0.86

TABLE VI. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of R–Lys–BZA

Compound R		Yield (%)	mp (°C)	$[\alpha]_D$ (°) (Solvent)	Formula	Elemental analysis Calcd (Found) C	H	N	TLC *Rf*[5]	*Rf*[6]
$NH_2(CH_2)_4CO-$	(1)	82.0	Amorphous	−30.3 (MeOH)	$C_{24}H_{32}N_4O_3 \cdot 2HCl$	57.9 (57.6	6.90 6.98	11.3 11.0)	0.10	0.78
$NH_2(CH_2)_5CO-$	(2)	91.0	Amorphous	−35.1 (MeOH)	$C_{25}H_{34}N_4O_3 \cdot 2HCl$	58.7 (58.5	7.11 7.21	11.0 10.8)	0.12	0.88
NH_2CH_2–(*trans*)-H–CO–	(3)	91.3	89—92	−6.5 (MeOH)	$C_{27}H_{36}N_4O_3 \cdot 1.5H_2O$	66.0 (66.1	7.99 7.98	11.4 11.1)	0.56	
NH_2CH_2–(*cis*)-H–CO–	(4)	40.0	Amorphous	−44.0 (MeOH)	$C_{27}H_{36}N_4O_3 \cdot 1.5H_2O$	66.0 (66.3	7.99 8.28	11.4 11.3)	0.54	
NH_2–H–CO–	(5)	51.9	Amorphous	−68.3 (MeOH)	$C_{26}H_{34}N_4O_3 \cdot 1.5H_2O$	65.4 (65.6	7.75 7.44	11.7 11.5)	0.63	
NH_2–H–CO–	(6)	62.4	59—60	−25.7 (MeOH)	$C_{26}H_{34}N_4O_3 \cdot H_2O$	66.7 (66.5	7.69 7.68	12.0 11.7)	0.56	

was removed by evaporation. Petroleum ether was added to the residue to give crystals, yield 3.3 g (44.8%), mp 52—55 °C, $[\alpha]_D^{26}$ +9.8° (*c*=1.0, MeOH), *Rf*[1] 0.65, *Rf*[3] 0.92. *Anal.* Calcd for $C_{32}H_{37}N_3O_6$: C, 68.7; H, 6.66; N, 7.51. Found: C, 68.8; H, 6.71; N, 7.48.

Z-Tra-D-Lys(Z)-BZA An acid chloride [prepared from Z-Tra-OH (0.2 g, 0.86 mmol) and $SOCl_2$ (0.15 ml, 3.4 mmol) as usual] in $CHCl_3$ (10 ml) was added to an ice-cold solution of H-D-Lys(Z)-BZA·HCl [prepared from Boc-D-Lys(Z)-BZA (0.2 g, 0.39 mmol) and 5.6 N HCl/dioxane (0.7 ml) as usual] in DMF (54 ml) containing Et_3N (0.1 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvents, the residue was extracted with AcOEt. The extract was washed with 1 N HCl, 5% Na_2CO_3 and H_2O, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to give a precipitate, which was collected by filtration and recrystallized from AcOEt, yield 50 mg (17.5%), mp 133—135 °C, $[\alpha]_D^{26}$ −7.0° (*c*=0.1, DMF), *Rf*[2] 0.38, *Rf*[3] 0.61. *Anal.* Calcd for $C_{43}H_{48}N_4O_7$: C, 70.5; H, 6.55; N, 7.65. Found: C, 70.4; H, 6.58; N, 7.75.

H-Tra-D-Lys-BZA (7) Z-Tra-D-Lys(Z)-BZA (31.4 mg, 0.04 mmol) in MeOH (10 ml) was hydrogenated over a Pd catalyst. After removal of Pd and the solvent, ether was added to the residue to afford crystals, yield 10.0 mg (50.1%), mp 75—79 °C, $[\alpha]_D^{26}$ +8.5° (*c*=0.1, MeOH), *Rf*[5] 0.45, *Rf*[6] 0.79. *Anal.* Calcd for $C_{27}H_{36}N_4O_3 \cdot 1.5H_2O$: C, 66.0; H, 7.91; N, 11.4. Found: C, 66.0; H, 7.64; N, 11.0.

Boc-Orn(Z)-BZA The title compound was synthesized from a mixed anhydride [prepared from Boc-Orn(Z)-OH (1.2 g, 3.3 mmol) and ethyl chloroformate (0.32 ml, 3.3 mmol) as usual] and 4-benzoylaniline (0.65 g, 3.3 mmol) by the same method as described in the synthesis of Boc-D-Lys(Z)-BZA. Yield 0.95 g (52.5%), mp 97—99 °C, $[\alpha]_D^{26}$ −13.4° (*c*=0.9, MeOH), *Rf*[1] 0.69, *Rf*[2] 0.80. *Anal.* Calcd for $C_{31}H_{35}N_3O_6 \cdot 0.5H_2O$: C, 67.2; H, 6.50; N, 7.58. Found: C, 66.9; H, 6.44; N, 7.62.

Z-Tra-Orn(Z)-BZA The title compound was prepared from an acid chloride [prepared from Z-Tra-OH (0.1 g, 0.43 mmol) and $SOCl_2$ (0.08 ml, 1.7 mmol)] and H-Orn-BZA·HCl [prepared from Boc-Orn(Z)-BZA (0.1 g, 0.19 mmol) and 5.6 N HCl/dioxane (0.4 ml) as usual] by the same method as described in the synthesis of Z-Tra-D-Lys(Z)-BZA. Yield 0.10 g (65.3%), mp 152—160 °C, $[\alpha]_D^{26}$ −11.6° (*c*=0.3, DMF), *Rf*[1] 0.63, *Rf*[2] 0.56. *Anal.* Calcd for $C_{42}H_{46}N_4O_7$: C, 70.4; H, 7.38; N, 8.21. Found: C, 70.3; H, 7.74; N, 8.07.

H-Tra-Orn-BZA (8) Z-Tra-Orn(Z)-BZA (60.0 mg, 0.08 mmol) in DMF (12 ml) was hydrogenated over a Pd catalyst. Yield 29.9 mg (79.1%), mp 99—104 °C, $[\alpha]_D^{26}$ −11.3° (*c*=0.5, DMSO), *Rf*[5] 0.47. *Anal.* Calcd for $C_{26}H_{34}N_4O_3 \cdot 2.5H_2O$: C, 63.1; H, 7.88; N, 11.0. Found: C, 63.1; H, 7.90; N, 11.3.

General Procedure for Synthesis of Boc-Lys(Z)-R [R: -N(-COOC$_2$H$_5$)-CH$_2$-, -N(-CH$_2$-)-, -NH-C$_6$H$_4$-NO$_2$, -NH-C$_6$H$_4$-COOCH$_3$] A mixed anhydride [prepared from Boc-Lys(Z)-OH (3.8 g, 10 mmol) and ethyl chloroformate (0.96 ml, 10 mmol) as usual] in THF (60 ml) was added to an ice-cold solution of the corresponding amino component (0.01 mol) in THF (30 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 1 N HCl, 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4 and evaporated down. The oily product in $CHCl_3$ (10 ml) was applied to a silica gel column (2.6 × 47.5 cm), equilibrated and eluted with $CHCl_3$. Individual fractions (150 ml each) were collected. The solvent of the effluent (tube Nos. 5—10) was removed by evaporation. Petroleum ether was added to the residue to give crystals. Yield, mp, $[\alpha]_D^{26}$ value, *Rf* values and elemental analysis are summarized in Table VII.

General Procedure for Synthesis of Z-Tra-Lys(Z)-R [R: -N(-COO-C$_2$H$_5$)-CH$_2$-, -N(-CH$_2$-)-, -NH-C$_6$H$_4$-NO$_2$, -NH-C$_6$H$_4$-COOCH$_3$, -NH-C$_6$H$_4$-COCH$_3$] An acid chloride [prepared from Z-Tra-OH (1.5 g, 6.8 mmol) and $SOCl_2$ (1.2 ml, 27.2 mmol)] in $CHCl_3$ (10 ml) was added to an ice-cold solution of H-Lys(Z)-R·HCl [prepared from the corresponding Boc-Lys(Z)-R (2.3 mmol) and 5.5 N HCl/dioxane (4.2 ml) as usual] in DMF (10 ml) containing Et_3N (0.6 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, AcOEt and H_2O were added to the residue to give crystals, which were collected by filtration and recrystallized from MeOH. Yield, mp, $[\alpha]_D$ value, *Rf* values and elemental analysis are summarized in Table VIII.

General Procedure for Synthesis of Tra-Lys-R [R: -N(-COOC$_2$H$_5$)-CH$_2$-, -N(-CH$_2$-)-, -NH-C$_6$H$_4$-NO$_2$, -NH-C$_6$H$_4$-, -NH-C$_6$H$_4$-COOCH$_3$, -NH-C$_6$H$_4$-COCH$_3$] The corresponding protected compound (34.8 mg, 0.05 mmol) in DMF (6 ml) was hydrogenated over a Pd catalyst. After removal of Pd and the solvent, ether was added to the residue to afford an amorphous powder. In the case of the *p*NA derivative, a Z group was removed by HBr/AcOH. Yield, mp, $[\alpha]_D$ value, *Rf* values and elemental analysis are summarized in Table IX.

Z-Tra-Lys(Z)-4-Benzoylphenyl Ester An acid chloride [prepared from Z-Tra-OH (1.2 g, 5.5 mmol) and $SOCl_2$ (0.9 ml, 22 mmol)] in $CHCl_3$ (10 ml) was added to an ice-cold solution of H-Lys(Z)-4-benzoylphenyl ester·HCl [prepared from the corresponding N^{α}-Boc-derivative (0.3 g, 0.54 mmol) and 5.6 N HCl/dioxane (1 ml)] in DMF (5 ml) containing Et_3N (0.15 ml). The reaction mixture was stirred at room temperature 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 H_2O, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to give a precipitate, which was collected by filtration and reprecipitated from AcOEt and ether, yield 0.16 g (40.4%), mp 109—113 °C, $[\alpha]_D^{26}$

TABLE VII. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of Boc–Lys(Z)–R

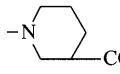
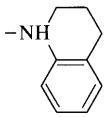
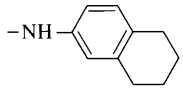
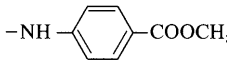
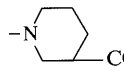
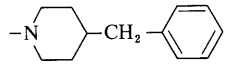
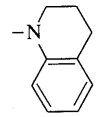
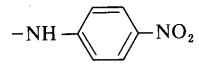
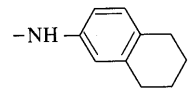
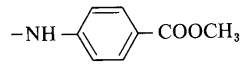
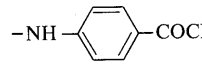
Compound R	Yield (%)	mp (°C)	$[\alpha]_D$ (°) (Solvent)	Formula	Elemental analysis Calcd (Found) C	H	N	TLC Rf^1	Rf^2
	43.2	Oil	−6.6 (MeOH)	$C_{27}H_{41}N_3O_7 \cdot H_2O$	66.7 (66.6	9.25 9.52	13.0 12.9)	0.54	0.67
	27.8	Oil	−4.1 (MeOH)					0.64	0.76
	64.8	95—96	−13.4 (MeOH)	$C_{29}H_{39}N_3O_5$	68.3 (68.3	7.71 7.84	8.25 8.22)	0.69	0.80
	39.0	118—119	−25.7 ($CHCl_3$)	$C_{27}H_{35}N_3O_7 \cdot 1/2H_2O$	62.1 (62.4	6.89 6.89	8.04 8.03)	0.60	0.65

TABLE VIII. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of Z–Tra–Lys(Z)–R

Compound R	Yield (%)	mp (°C)	$[\alpha]_D$ (°) (Solvent)	Formula	Elemental analysis Calcd (Found) C	H	N	TLC Rf^1	Rf^2
	28.3	Oil	+28.3 (MeOH)	$C_{38}H_{52}N_4O_8$	65.9 (65.5	7.57 7.38	8.09 8.03)	0.52	0.50
	47.3	Oil	+2.7 (MeOH)	$C_{42}H_{54}N_4O_6$	71.0 (70.9	7.66 7.66	7.88 7.92)	0.60	0.64
	28.3	94—96	+42.9 (DMF)	$C_{39}H_{48}N_4O_6$	70.0 (70.1	7.23 7.18	8.38 8.13)	0.45	0.94
	61.3	199—205	+5.9 (MeOH)	$C_{36}H_{43}N_5O_8$	64.2 (64.3	6.43 6.50	10.4 10.4)	0.81	0.86
	60.5	210—213	−6.0 (DMF)	$C_{40}N_{50}N_4O_6$	70.4 (70.3	7.38 7.48	8.21 8.07)	0.74	0.80
	68.4	203—204	−33.4 ($CHCl_3$)	$C_{38}H_{46}N_4O_8$	66.5 (66.2	6.70 6.91	8.16 8.06)	0.70	
	71.2	202—204	−4.0 (DMF)	$C_{38}H_{46}N_4O_7$	68.1 (67.8	6.86 6.86	8.35 8.19)	0.63	0.78

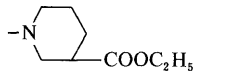
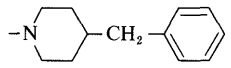
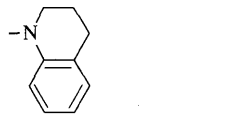
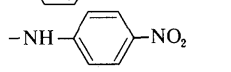
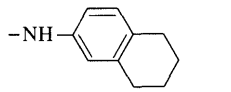
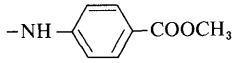
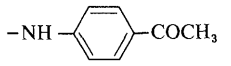
−20.3° (c=0.4, $CHCl_3$), Rf^1 0.65, Rf^2 0.83. *Anal.* Calcd for $C_{43}H_{47}N_3O_8$: C, 70.4; H, 6.47; N, 5.73. Found: C, 70.2; H, 6.41; N, 5.74.

H–Tra–Lys–4-Benzoylphenyl Ester (16) Z–Tra–Lys(Z)–4-Benzoylphenyl ester (0.077 g, 0.1 mmol) was dissolved in 25% HBr/AcOH (0.2 ml, 0.6 mmol). The reaction mixture was stored at room temperature for 30 min. Ether was added to the solution to yield a white precipitate, which was collected by filtration and dried over KOH pellets *in vacuo*. The resulting HBr salt in EtOH (4 ml) containing Et_3N (0.28 ml, 0.2 mmol) was kept at room temperature for 10 min and purified by column chromatography. Individual fractions (3 g each) were collected. After removal of the solvent of the effluent (tube Nos. 45—56), ether was added to the residue to give crystals, yield 0.026 g (55.9%), mp 75—78 °C, $[\alpha]_D^{26}$ −11.5° (c=0.1, MeOH), Rf^5 0.42, Rf^6 0.88. *Anal.* Calcd for $C_{27}H_{35}N_3O_4 \cdot 2H_2O$: C, 67.1; H, 8.15; N, 8.69. Found: C, 66.9; H, 8.38; N, 8.54.

Z–Tra–Lys(Z)–4-Benzoylanilinomethyl Ketone 4-Benzoylaniline (0.23 g, 1.2 mmol), $NaHCO_3$ (0.1 g, 1.2 mmol), NaI (0.18 g, 1.2 mmol) and Boc–Lys(Z)–CH_2Cl (0.48 g, 1.2 mmol) were dissolved in DMF (15 ml). The reaction mixture was stirred at 45 °C for 2 d. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% $NaHCO_3$ and H_2O, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to give Boc–Lys(Z)–4-benzoylanilinomethyl ketone as an amorphous powder [yield 0.24 g (35.3%), $[\alpha]_D^{26}$ −21.2° (c=1.2, $CHCl_3$)]. An acid chloride [prepared from Z–Tra–OH (0.39 g, 1.7 mmol) and $SOCl_2$ (1.2 ml, 7 mmol)] in $CHCl_3$ (10 ml) was added to an ice-cold solution of H–Lys(Z)–4-benzoylanilinomethyl ketone·HCl [prepared from the corresponding N^{α}-Boc-derivative (0.25 g, 0.44 mmol) obtained above and 5.6 N HCl/dioxane (0.81 ml)] in DMF (8 ml) containing Et_3N (0.12 ml). The reaction mixture was stirred at room temperature 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 H_2O, dried over Na_2SO_4 and evaporated down. The oily product in $CHCl_3$ (3 ml) was applied to a silica gel column (2.4 × 32 cm), equilibrated and eluted with $CHCl_3$. Individual fractions (50 ml each) were collected. The solvent of the effluent (tube Nos. 2—7) was removed by evaporation. Petroleum ether was added to the residue to afford an amorphous powder, yield 0.1 g (31.1%), $[\alpha]_D^{26}$ −2.5° (c=0.9, $CHCl_3$), Rf^1 0.50, Rf^2 0.76. *Anal.* Calcd for $C_{44}H_{50}N_4O_9 \cdot H_2O$: C, 69.1; H, 6.80; N, 7.33. Found: C, 69.3; H, 6.60; N, 7.06.

H–Tra–Lys–4-Benzoylanilinomethyl Ketone (17) Z–Tra–Lys(Z)–4-ben-

TABLE IX. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of H–Tra–Lys–R

Compound R		Yield (%)	mp (°C)	$[\alpha]_D$ (°) (Solvent)	Formula	Elemental analysis Calcd (Found) C	H	N	TLC Rf^5	Rf^6
	(**9**)	59.2	Amorphous	−23.3 (MeOH)	$C_{22}H_{40}N_4O_4 \cdot H_2O$	66.7 (66.6	9.25 9.52	13.0 12.9)	0.56	
	(**10**)	51.4	Amorphous	−8.0 (MeOH)	$C_{26}H_{42}N_4O_2 \cdot 3.5H_2O$	61.8 (61.8	9.76 9.53	11.1 10.9)	0.55	
	(**11**)	59.2	Amorphous	−9.8 (MeOH)	$C_{23}H_{36}N_4O_2 \cdot H_2O$	67.4 (67.5	7.33 7.18	8.05 8.03)	0.56	
	(**12**)	61.3	Amorphous	−10.4 (MeOH)	$C_{20}H_{31}N_5O_4 \cdot 2HBr \cdot 4H_2O$	38.2 (37.9	6.52 6.33	11.1 10.9)	0.62	
	(**13**)	59.2	Amorphous	−8.8 (MeOH)	$C_{24}H_{38}N_4O_2 \cdot H_2O$	66.7 (66.6	9.25 9.52	13.0 12.9)	0.56	
	(**14**)	56.6	48—50	−9.3 ($CHCl_3$)	$C_{22}H_{34}N_4O_4 \cdot 3H_2O$	56.0 (56.3	7.47 7.69	10.9 10.6)	0.39	0.71
	(**15**)	66.3	164 (dec.)	−10.6 (DMF)	$C_{22}H_{34}N_4O_3 \cdot H_2O$	62.9 (63.1	8.57 8.35	13.3 13.0)	0.39	

zoylanilinomethyl ketone (0.046 g, 0.061 mmol) was dissolved in 25% HBr/AcOH (0.1 ml, 0.37 mmol) and the reaction mixture was stored at room temperature for 30 min. Ether was added to the solution to afford a white precipitate, which was collected by filtration and dried over KOH pellets *in vacuo*. A solution of the corresponding hydrobromide in EtOH (1 ml) containing Et_3N (0.17 ml, 0.12 mmol) was allowed to stand at room temperature for 10 min and applied to a column of Sephadex LH-20 (3.2 × 53 cm), equilibrated and eluted with EtOH. Individual fractions (3 g each) were collected. After removal of the solvent of the effluent (tube Nos. 47—58), ether was added to the residue to give an amorphous powder, yield 0.11 g (37%), $[\alpha]_D^{26}$ −0.38° (c=0.5, MeOH), Rf^5 0.47, Rf^3 0.70. *Anal.* Calcd for $C_{28}H_{38}N_4O_3 \cdot HBr \cdot 3H_2O$: C, 54.9; H, 7.34; N, 9.13. Found: C, 54.6; H, 7.69; N, 9.44.

Z–Tra–Lys(Z)–4-Benzoylphenoxymethyl Ketone 4-Benzoylphenol (0.26 g, 1.3 mmol), $NaHCO_3$ (0.11 g, 1.3 mmol), NaI (0.039 g, 1.3 mmol) and Boc–Lys(Z)–CH_2Cl (0.53 g, 1.3 mmol) were dissolved in DMF (10 ml), and the reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to give the title compound as an amorphous powder [yield 0.59 g (80.4%), $[\alpha]_D^{26}$ +4.47° (c=0.8, $CHCl_3$)]. An acid chloride [prepared from Z–Tra–OH (0.9 g, 4.1 mmol) and $SOCl_2$ (1.9 ml, 16 mmol)] in $CHCl_3$ (10 ml) was added to an ice-cold solution of H–Lys(Z)–4-benzoylphenoxymethyl ketone·HCl [prepared from the corresponding N^{α}-Boc-derivative (0.7 g, 1.2 mmol) and 5.6 N HCl/dioxane (2 ml)] in DMF (5 ml) containing Et_3N (0.34 ml). The reaction mixture was stirred at room temperature 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 H_2O, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to afford a precipitate, which was collected by filtration and recrystallized from AcOEt, yield 0.17 g (19.0%), mp 147—149 °C, $[\alpha]_D^{26}$ −38.0° (c=1.0, DMSO), Rf^1 0.80, Rf^2 0.85. *Anal.* Calcd for $C_{44}H_{49}N_3O_8$: C, 70.7; H, 6.56; N, 5.62. Found: C, 70.4; H, 6.75; N, 5.79.

H–Tra–Lys–4-Benzoylphenoxymethyl Ketone (18) Z–Tra–Lys(Z)–4-benzoylphenoxymethyl ketone (0.08 g, 0.1 mmol) was dissolved in 25% HBr/AcOH (0.2 ml, 0.6 mmol) and the solution was kept at room temperature for 30 min. Ether was added to the solution to yield a white precipitate, which was collected by filtration and dried over KOH pellets *in vacuo*. A solution of the resulting hydrobromide in EtOH (2 ml) containing Et_3N (0.028 ml, 0.2 mmol) was applied to a column of Sephadex LH-20 (3.2 × 53 cm), equilibrated and eluted with EtOH. Individual fractions (3 g each) were collected. After removal of the solvent of the effluent (tube Nos. 44—60), ether was added to the residue to give an amorphous powder, yield 0.045 g (93.9%), $[\alpha]_D^{26}$ −15.6° (c=0.8, MeOH), Rf^5 0.37, Rf^6 0.68. *Anal.* Calcd for $C_{28}H_{37}N_3O_4 \cdot HBr \cdot 3H_2O$: C, 54.8; H, 7.16; N, 6.84. Found: C, 54.3; H, 7.25; N, 6.67.

Boc–Tra–Lys(Z)–4-Benzoylphenoxymethyl Ketone The title compound was prepared by the essentially same method as described in the synthesis of Z–Tra–Lys(Z)–4-benzoylphenoxymethyl ketone from a mixed anhydride [prepared from Boc–Tra–OH (0.15 g, 0.58 mmol) and ethyl chloroformate (0.056 ml, 0.58 mmol)] and H–Lys(Z)–4-benzoylphenoxymethyl ketone·HCl [prepared from the corresponding N^{α}-Boc-derivative (0.2 g, 0.42 mmol) and 5.6 N HCl/dioxane (0.74 ml)]. Yield 0.067 g (22.4%), mp 99—102 °C, $[\alpha]_D^{26}$ −6.5° (c=0.2, $CHCl_3$), Rf^1 0.63, Rf^2 0.97. *Anal.* Calcd for $C_{41}H_{51}N_3O_8 \cdot 0.5H_2O$: C, 68.2; H, 7.20; N, 5.81. Found: C, 68.1; H, 6.95; N, 5.87.

H–Tra–Lys(Z)–4-Benzoylphenoxymethyl Ketone (19) Boc–Tra–Lys(Z)–4-benzoylphenoxymethyl ketone (0.052 g, 0.075 mmol) was dissolved in 5.6 N HCl/dioxane (0.1 ml, 0.38 mmol). After 1 h at room temperature, ether was added to the solution to afford a white precipitate, which was collected by filtration and dried over KOH pellets *in vacuo*. A solution of the resulting hydrochloride in EtOH (1 ml) containing a saturated Na_2CO_3 solution (1 ml) was applied to a column of Sephadex LH-20 (3.2 × 53 cm), equilibrated and eluted with EtOH. Individual fractions (3 g each) were collected. After removal of the solvent of the effluent (tube Nos. 19—26), ether was added to the residue to give crystals, yield 0.03 g (66.5%), mp 109—114 °C, $[\alpha]_D^{26}$ −2.7° (c=0.2, MeOH), Rf^4 0.42, Rf^5 0.70. *Anal.* Calcd for $C_{36}H_{43}N_3O_6 \cdot 2.5H_2O$: C, 65.7; H, 7.29; N, 6.38. Found: C, 65.4; H, 7.58; N, 6.29.

Z–Tra–Lys(Boc)–4-Benzoylphenoxymethyl Ketone 4-Benzoylphenol (0.73 g, 3.7 mmol), $NaHCO_3$ (0.31 g, 3.7 mmol), NaI (0.55 g, 3.7 mmol) and Z–Lys(Boc)–CH_2Cl (1.5 g, 3.7 mmol) were dissolved in DMF (15 ml) and the reaction mixture was stirred at 45 °C for 2 d. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to give Z–Lys(Boc)–4-benzoylphenoxymethyl ketone as an oily product (yield 70.6%), $[\alpha]_D^{26}$ −11.5° (c=1.1, $CHCl_3$)]. An acid chloride [prepared from Z–Tra–OH (0.17 g, 0.57 mmol) and $SOCl_2$ (0.27 ml, 2.3 mmol)] in $CHCl_3$ (10 ml) was added to an ice-cold solution of H–Lys(Boc)–4-benzoylphenoxymethyl ketone·HCl [prepared from the corresponding N^{α}-Z-derivative (0.22 g, 0.38 mmol) in MeOH (10 ml) containing 1 N HCl (0.38 ml) by catalytic hydrogenation] in DMF (10 ml) containing Et_3N (0.11 ml). The reaction mixture was stirred at room temperature overnight. 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 evaporated down. Ether was added to the residue to afford a precipitate, which was collected by filtration and recrystallized from AcOEt, yield 0.064 g (23.4%), mp 85—87 °C, $[\alpha]_D^{26}$ +0.8° (c=0.5, $CHCl_3$), Rf^1 0.71, Rf^2 0.72. *Anal.* Calcd for $C_{41}H_{51}N_3O_8$: C, 69.0; H, 7.15; N, 5.89. Found: C, 68.8; H, 7.35; N, 6.00.

Z-Tra-Lys-4-Benzoylphenoxymethyl Ketone (20) The title compound was prepared by the essentially same method as described in the synthesis of **19** from Z-Tra-Lys(Boc)-4-benzoylphenoxymethyl ketone (0.054 g, 0.075 mmol) and 5.6 N HCl/dioxane (0.1 ml). Yield 0.021 g (44.6%), mp 75—77 °C, $[\alpha]_D^{26}$ −10.0° (c=0.5 MeOH), Rf^4 0.31, Rf^5 0.61. *Anal.* Calcd for $C_{36}H_{43}N_3O_6 \cdot 3H_2O$: C, 64.8; H, 7.34; N, 6.29. Found: C, 65.1; H, 7.52; N, 6.39.

Assay Procedure IC_{50} values were determined according to the procedure described in the previous report.[6]

References and Notes

1) The customary L indication for amino acids is omitted; only D isomers are indicated. Standard abbreviations for amino acids and their derivatives are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 2584 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972). Other abbreviations used are: Z, benzyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; Tra, *trans*-4-aminomethylcyclohexanecarbonyl; Eac, ε-aminocaproyl; *p*NA, *p*-nitroanilide; BZA, 4-benzoylanilide; ACA, 4-acetylanilide; BPP, 4-benzylpiperidine amide; AcOH, acetic acid; DMF, *N*,*N*-dimethylformamide; AcOEt, ethyl acetate; THF, tetrahydrofuran; S-2251, H-D-Val-Leu-Lys-*p*NA; Fn, fibrin; Fg, fibrinogen; Tos, tosyl; Orn, ornithine.
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