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Development of Selective Inhibitors against Plasma Kallikrein[1)]

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Specific plasma kallikrein inhibitors were designed and synthesized and their structure–activity relationship was studied. *trans*-4-Aminomethylcyclohexanecarbonyl(Tra)-lysyl-4-ethoxycarbonylanilide inhibited plasma kallikrein and plasmin with IC$_{50}$ values of 23 and 210 μM, respectively, indicating that this compound is fairly specific to plasma kallikrein. Tra-arginyl-4-ethoxycarbonylanilide inhibited plasma kallikrein and plasmin with IC$_{50}$ values of 16 and 480 μM, respectively. Tra-homoarginyl-4-carboxyanilide inhibited plasma kallikrein and plasmin with IC$_{50}$ values of 14 μM and 1 mM, respectively. Finally, Tra–Arg(Mts)-4-acetylanilide (ACA) exhibited potent and selective inhibitory activity against plasma kallikrein (IC$_{50}$ value for plasma kallikrein: 2 μM and for plasmin: 42 μM).

Keywords plasma kallikrein; competitive inhibitor; specific inhibitor; design; synthesis; structure–activity relationship

It is well known that the action of plasma kallikrein (PK) liberates bradykinin (BK) from high molecular weight kininogen.[2)] It was also reported that PK can activate factor XII,[3)] prourokinase[4)] and plasminogen[5)] and may enhance blood polymorphonuclear leukocyte chemotaxis.[6)] These results suggest that PK has a broad spectrum of activities, but as yet, detailed studies of the role of PK remain to be performed.

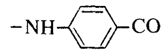
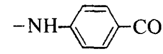
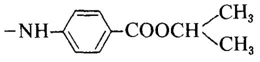
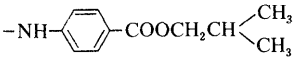
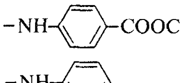
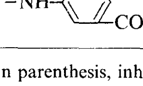
Aprotinin (Trazylol) is an inhibitor of both kallikrein and plasmin and it is widely used as a drug in the treatment of various diseases and as a tool for studying proteases.[7,8)] However, it should be noted that, though aprotinin is able to inhibit grandular kallikrein (GK), it is neither potent nor selective enough to inhibit PK.[8)] Soybean trypsin inhibitor (SBTI), a potent inhibitor of PK, can not inhibit GK. However, SBTI inhibits plasmin as well as PK, meaning that SBTI is not a selective inhibitor of PK.

Under these circumstances, our studies were directed to the synthesis of a specific plasma kallikrein inhibitor with the objective of obtaining a valuable and powerful tool in the study of the role of plasma kallikrein and of development of new types of clinical therapy. This paper deals with the synthesis of Lys, Arg and Hag derivatives, examination of their inhibitory activity against plasma kallikrein, plasmin, urokinase and thrombin and studies on structure–inhibitory activity relationship.

In our previous report,[9)] it was reported that *trans*-4-aminomethylcyclohexanecarbonyl(Tra)–Lys-4-methoxycarbonylanilide (**1**) inhibited plasma kallikrein with an IC$_{50}$ value of 50 μM and plasmin with an IC$_{50}$ value of >500 μM, indicating that this compound is ten fold more selective against plasma kallikrein. Thus, we synthesized Tra–Lys-4-methoxycarbonylanilide derivatives substituted methyl ester group by other alkyl ester groups and their inhibitory activities were examined as summarized in Table I.

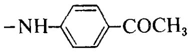
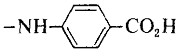
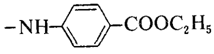
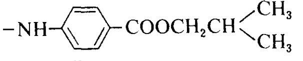
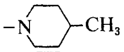
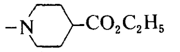
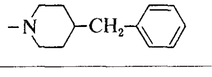
As can be seen in Table I, 3-ethoxycarbonylanilide derivative (**6**) as well as 4-ethoxycarbonylanilide derivative (**2**) exhibited inhibitory activity against plasma kallikrein rather than plasmin. The bulkiness of the alkyl ester group did not affect the inhibitory activity against plasma kallikrein and plasmin. All the above derivatives exhibited more potent inhibitory activity against plasma kallikrein than plasmin, indicating that an alkoxycarbonyl group on

TABLE I. IC$_{50}$ Values of NH_2CH_2–⟨H⟩⫶CO–Lys–R for Various Enzymes

	R	P. Kall	Pl	UK	TH
		S-2302	S-2251	S-2444	S-2238
1		50	>500 (17%)[a)]	310	>500 (0%)
2		23	210	110	ND
3		40	>200 (16%)	100	>400 (0%)
4		27	>500 (39%)	59	>1000 (0%)
5		50	>300 (11%)	110	>400 (0%)
6		60	>500 (31%)	150	>500 (0%)

a) In parenthesis, inhibition % at the concentration described (μM) is indicated.

TABLE II. IC$_{50}$ Values of NH_2CH_2–⟨H⟩⫶CO–Arg–R for Various Enzymes

	R	P. Kall	Pl	UK	TH
		S-2302	S-2251	S-2444	S-2238
7	–NHCH$_3$	>1000 (11%)[a)]	>1000 (12%)	ND	ND
8		36	420	110	>1000 (20%)
9		27	>1000 (38%)	340	>1000 (0%)
10		16	480	90	>1000 (20%)
11		15	370	50	>1000 (17%)
12		>1000 (32%)	>1000 (40%)	>1000 (28%)	>1000 (12%)
13		>1000 (42%)	>1000 (43%)	>1000 (34%)	>1000 (0%)
14		200	350	>500 (17%)	>1000 (27%)

a) In parenthesis, inhibition % at the concentration described (μM) is indicated.

the phenyl ring of the C-terminal anilide moiety is suitable for the interaction between inhibitor and plasma kallikrein.

It is well known that plasmin preferentially hydrolyzes the lysylpeptide bonds in physiological substrates, while thrombin, plasma kallikrein, urokinase and factor Xa preferentially hydrolyze the arginylpeptide bonds.[10] In fact, D-Ile–Phe–Lys–*p*NA is a good substrate for plasmin and D-Ile–Phe–Arg–*p*NA is for plasma kallikrein. D-Ile–Phe–Lys–CH_2Cl inhibited plasmin preferentially, while D-Ile–Phe–Arg–CH_2Cl inhibited plasma kallikrein preferentially.[11] So, Arg derivatives instead of Lys were synthesized in order to obtain plasma kallikrein inhibitors and their inhibitory activities are summarized in Table II.

As expected, Tra–Arg-4-ethoxycarbonylanilide (**10**) and Tra–Arg-4-isobutyloxycarbonylanilide (**11**) inhibited plasma kallikrein more strongly than plasmin.

As illustrated in Fig. 1, these compounds inhibited plasma kallikrein competitively. Tra–Arg-4-carboxyanilide (**9**) inhibited plasma kallikrein with an IC_{50} value similar to those of the corresponding ester derivatives, while its inhibitory activity against plasmin decreased compared with the corresponding alkyl ester compound, resulting in an increase of discrepancy between the inhibitory activity against plasma kallikrein and plasmin. The inhibitory activity of Tra–Arg–ACA (**8**) against plasma kallikrein and plasmin exhibited a similar tendency to that of ethoxycarbonylanilide derivatives (**10**). In Tra–Arg-piperidine amide derivatives, only Tra–Arg–BPP (**14**) slightly inhibited plasmin but did not inhibit thrombin, although N^{α}-protected-Arg-piperidine amide derivatives inhibited thrombin very strongly and selectively.[12] These results indicate that the combination of Tra and Arg is not suitable for thrombin inhibitors.

Next, homoarginine (Hag) derivatives were synthesized and their inhibitory activity against plasmin, plasma kallikrein, urokinase and thrombin were examined and the results are summarized in Table III. Tra–Hag-4-methoxycarbonylanilide (**18**) and Tra–Hag-4-carboxyanilide (**17**) inhibited plasma kallikrein with similar IC_{50} values (18 and 14 μM, respectively) and the latter inhibitor exhibited weaker inhibitory activity against plasmin than the former one (IC_{50} values: >1 mM and 610 μM, respectively). These phenomena are similar to those of Arg derivatives described above. Tra–Hag-piperidine amide derivatives (**19**—**21**) also did not inhibit thrombin. The combination of Tra and Hag is not suitable for thrombin inhibitors.

As stated above, in our inhibitor series, substitution of Lys by Arg [Tra–Lys-4-ethoxycarbonylanilide (**2**) and Tra–Arg-4-ethoxycarbonylanilide (**10**)] did not dramatically increase inhibitory activity against plasma kallikrein,

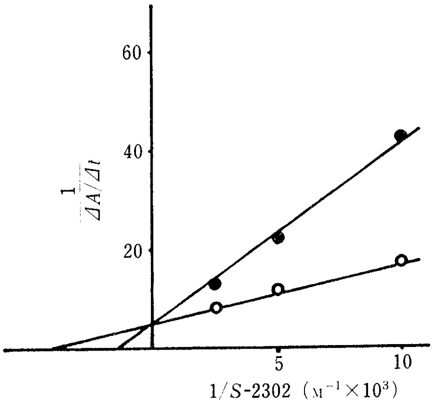
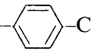
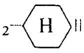
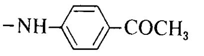
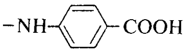
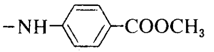
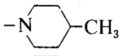
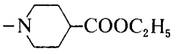
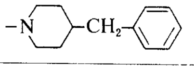


Fig. 1. Mode of Inhibition of Tra–Arg–NH––$COOC_2H_5$ (**10**) against Plasma Kallikrein

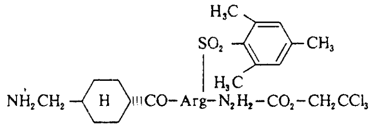
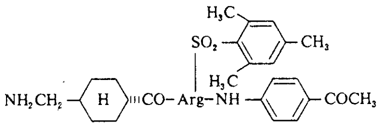
●: with inhibitor (20 μM); ○: without inhibitor.

TABLE III. IC_{50} Values of NH_2CH_2–CO–Hag–R for Various Enzymes

	R	P. Kall	Pl	UK	TH
		S-2302	S-2251	S-2444	S-2238
15	$-NHCH_3$	>1000 (24%)[a]	>1000 (13%)	580	>1000 (0%)
16		270	>1000 (8%)	>500 (15%)	>1000 (0%)
17		14	>1000 (48%)	95	>1000 (0%)
18		18	610	85	>1000 (0%)
19		>1000 (39%)	>1000 (27%)	880	>1000 (0%)
20		>1000 (47%)	>1000 (35%)	>1000 (48%)	>1000 (0%)
21		>500 (36%)	650	>250 (30%)	>500 (28%)

a) In parenthesis, inhibition % at the concentration described (μM) is indicated.

TABLE IV. IC_{50} Values of Arg Derivatives for Various Enzymes

	Compound	P. Kall	Pl	UK	TH
		S-2302	S-2251	S-2444	S-2238
22		38	4.7	600	230
23		2.0	42	370	>400 (21%)[a]

a) In parenthesis, inhibition % at the concentration described (μM) is indicated.

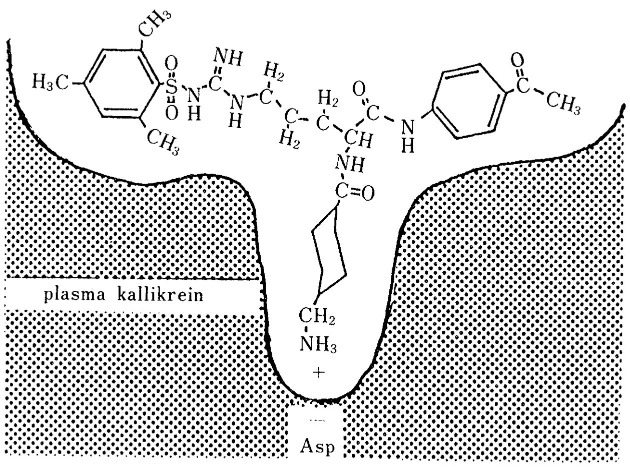


Fig. 2. Schematic Representation of Interaction of Tra–Arg(Mts)–ACA (**23**) with Enzyme

although in the case of chloromethylketone derivatives, Arg derivatives inhibited plasma kallikrein much more strongly than Lys derivatives.[11] Arg and Hag piperidine amide derivatives did not inhibit thrombin, indicating that these derivatives could manifest inhibitory activity against plasma kallikrein by quite a different mechanism from that of Arg derivatives reported previously.[12] In order to gain further insight into the inhibitory mechanism, Tra–Arg(Mts)–ACA (**23**) and Tra–Arg(Mts)–NHNH–Troc (**22**), in which basisity of Arg is decreased by protection of the guanidino group by Mts (mesitylenesulfonyl), were synthesized and their inhibitory effect on plasma kallikrein, plasmin, urokinase and thrombin was examined. As summarized in Table IV, Tra–Arg(Mts)–ACA (**23**) inhibited plasma kallikrein with an IC_{50} value of 2.0×10^{-6} M, while it inhibited plasmin with an IC_{50} value of 4.2×10^{-5} M. Tra–Arg(Mts)–NHNH–Troc (**22**) inhibited plasma kallikrein and plasmin with IC_{50} values of 3.8×10^{-5} and 4.8×10^{-6} M, respectively. From these results, it is deduced that the positively charged amino group of Tra moiety might interact with the negatively charged group of the active center of enzymes as illustrated in Fig. 2. Previously, Muramatsu and Fujii prepared various esters of ε-aminocaproic acid and *trans*-4-aminomethylcyclohexanecarboxylic acid, which exhibited inhibitory effects on trypsin, plasmin, plasma kallikrein and thrombin.[13] In these cases, an amino group of ε-aminocaproic acid or Tra also interacted with a negatively charged group of enzymes. In our previous report[9] about Lys derivatives, both Lys derivatives in which an ε-amino group of Lys or an amino group of Tra was protected, could inhibit plasmin. In the above Arg and Hag derivatives, it is deduced that either the guanidino group of Arg and Hag or the amino group of Tra can interact with the negatively charged group of the active center of enzymes and the amino group of Tra is more suitable for the interaction than the guanidino group of Arg and Hag.

These results provided us with a rational method for designing potent and specific inhibitor against not only plasma kallikrein but also plasmin and thrombin.

Experimental

The melting points are uncorrected. Optical rotations were measured with an automatic polarimeter, model DPI-360 (Japan Spectroscopic Co., Ltd.). On thin-layer chromatography (TLC) (Kieselgel G, Merck), Rf^1, Rf^2, Rf^3, Rf^4, Rf^5. Rf^6, Rf^7 and Rf^8 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), $CHCl_3$ and ether (4 : 1) and *n*-PrOH, H_2O, AcOEt and conc. NH_4OH (5 : 1 : 2 : 1), respectively.

General Procedure for Preparation of Boc–Lys(Z)–R (R: See Table V) A mixed anhydride [prepared from 3.0 g of Boc–Lys(Z)–OH (3.0 g, 7.9 mmol) and ethyl chloroformate (0.76 ml, 7.9 mmol)] in THF (80 ml) was added to an ice-cold solution of 4-alkoxycarbonylaniline [prepared from 4-alkoxycarbonylaniline hydrochloride (7.9 mmol) and Et_3N (1.1 ml, 7.9 mmol)] in *N*,*N*-dimethylformamide (DMF) (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 10% citric acid, 5% Na_2CO_3 and water, 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 petroleum ether. Yield, mp, $[\alpha]_D$ value, elemental analysis and *Rf* values are summarized in Table V.

General Procedure for Preparation of Z–Tra–Lys(Z)–R (R: See Table VI) An acid chloride [prepared from Z–Tra–OH (0.77 g, 3.5 mmol) and $SOCl_2$ (0.80 ml, 6.7 mmol)] in $CHCl_3$ (10 ml) was added to an ice-cold solution of H–Lys(Z)-4-alkoxycarbonylanilide·HCl [prepared from Boc–Lys(Z)-4-alkoxycarbonylanilide (0.4 mmol) and 5.6 N HCl/dioxane (0.70 ml)] in DMF (8 ml) containing Et_3N (0.11 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, AcOEt and water were added to the residue to afford crystals, which were collected by filtration and recrystallized from AcOEt. Yield, mp, $[\alpha]_D$ value, elemental analysis and *Rf* values are summarized in Table VI.

General Procedure for Preparation of H–Tra–Lys–R (R: See Table VII) Z–Tra–Lys(Z)-4-alkoxycarbonylanilide (0.03 mmol) in MeOH (4 ml) was hydrogenated over a Pd catalyst. After removal of Pd and the solvent, ether was added to the residue to afford crystals. Yield, mp, $[\alpha]_D$ value, elemental analysis and *Rf* values are summarized in Table VII.

General Procedure for Preparation of Z–Arg(NO_2)–R (R: See Table VIII) PCl_3 (0.12 ml, 1.35 mmol) was added to a solution of the corresponding amine component [prepared from corresponding hydrochloride (2.7 mmol) and Et_3N (0.38 ml, 2.7 mmol)] in dry pyridine (25 ml) cooled with ice-salt. The reaction mixture was stirred at the same temperature for 15 min and then at room temperature for 30 min. Z–Arg(NO_2)–OH (0.95 g, 2.7 mmol) was added to the above solution 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 1 N HCl, 5% Na_2CO_3 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 and reprecipitated from AcOEt and ether. Yield, mp, $[\alpha]_D$ value, elemental analysis and *Rf* values are summarized in Table VIII.

General Procedure for Preparation of Z–Tra–Arg(NO_2)–R (R: See Table IX) An acid chloride [prepared from Z–Tra–OH (0.72 g, 3.3 mmol) and $SOCl_2$ (1.6 ml, 13.3 mmol)] in $CHCl_3$ (20 ml) was added to an ice-cold solution of H–Arg(NO_2)–R·HBr [prepared from the corresponding N^{α}–Z-protected compound (0.83 mmol) and 25% HBr–AcOH (8 ml, 2.5 mmol)] in DMF (15 ml) containing Et_3N (0.24 ml, 1.7 mmol). 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 water, dried over Na_2SO_4 and

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

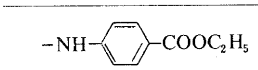
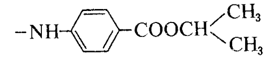
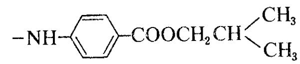
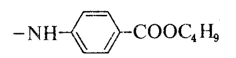
Compound R	Yield (%)	mp (°C)	$[\alpha]_D$ (Solvent)	Formula	Elemental analysis Calcd (Found) C	H	N	TLC Rf^1	Rf^2
	12.6	95—99	−1.8° (DMF)	$C_{28}H_{37}N_3O_7$	63.8 (63.9	7.02 7.12	7.97 8.23)	0.50	0.95
	73.9	Amorphous	−15.8° ($CHCl_3$)	$C_{29}H_{39}N_3O_7$	64.4 (64.6	7.21 7.33	7.76 7.68)	0.68	0.76
	51.4	Amorphous	−15.7° ($CHCl_3$)	$C_{30}H_{41}N_3O_7$	64.9 (65.1	7.58 7.62	7.56 7.35)	0.60	
	44.7	Amorphous	−25.9° ($CHCl_3$)	$C_{30}H_{41}N_3O_7$	64.9 (64.9	7.58 7.40	7.56 7.37)	0.61	
	21.6	Amorphous	−11.3° (MeOH)	$C_{28}H_{37}N_3O_7$ $\cdot 0.5H_2O$	62.7 (62.9	7.09 6.98	7.83 7.62)	0.60	0.67

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

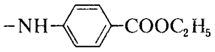
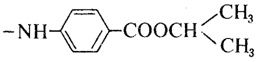
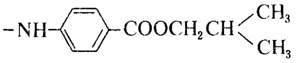
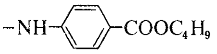
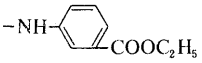
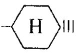
Compound R	Yield (%)	mp (°C)	$[\alpha]_D$ (Solvent)	Formula	Elemental analysis Calcd (Found) C	H	N	TLC Rf^1	Rf^2
	16.8	169—173	−1.4° (MeOH)	$C_{39}H_{48}N_4O_8\cdot H_2O$	65.2 (65.5	6.96 7.11	7.80 8.13)	0.70	
	31.0	163—171	−23.5° (CHCl$_3$)	$C_{40}H_{50}N_4O_8$	67.3 (67.0	7.00 7.13	7.84 7.89)	0.69	0.56
	30.7	136—141	−17.3° (CHCl$_3$)	$C_{41}H_{52}N_4O_8\cdot 3H_2O$	62.9 (62.9	7.41 7.40	7.16 7.29)	0.57	
	55.4	145—149	−24.5° (CHCl$_3$)	$C_{41}H_{52}N_4O_8\cdot H_2O$	66.0 (65.7	7.24 7.43	7.50 7.71)	0.56	0.82
	84.0	148—152	−0.7° (DMF)	$C_{39}H_{48}N_4O_8$	66.9 (66.8	6.85 7.03	8.00 8.04)	0.83	0.86

TABLE VII. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of NH_2CH_2–CO–Lys–R

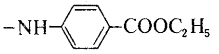
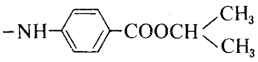
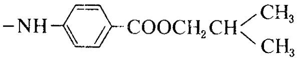
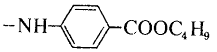
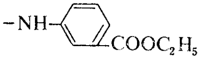
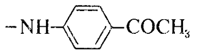
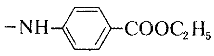
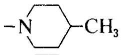
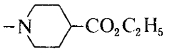
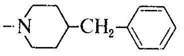
Compound R	Yield (%)	mp (°C)	$[\alpha]_D$ (Solvent)	Formula	Elemental analysis Calcd (Found) C	H	N	TLC Rf^5	Rf^6
2 	55.5	46—50	−7.3° (MeOH)	$C_{23}H_{36}N_4O_4\cdot 1.5H_2O$	60.2 (59.9	8.49 8.23	12.2 12.5)	0.54	
3 	60.7	52—54	−16.5° (MeOH)	$C_{24}H_{38}N_4O_4\cdot H_2O$	62.1 (61.7	8.62 8.33	12.1 12.1)	0.49	0.78
4 	83.4	128—135	−12.7° (MeOH)	$C_{25}H_{40}N_4O_4\cdot 5H_2O$	54.6 (54.6	9.38 9.29	9.80 10.1)	0.51	0.78
5 	68.7	Amorphous	−18.6° (MeOH)	$C_{25}H_{40}N_4O_4$	65.2 (65.5	8.75 8.76	12.2 12.1)	0.59	0.87
6 	66.3	Amorphous	−8.3° (MeOH)	$C_{23}H_{36}N_4O_4\cdot 2H_2O$	59.0 (59.1	8.54 8.33	12.0 12.1)	0.54	

TABLE VIII. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of Z–Arg(NO$_2$)–R

Compound R	Yield (%)	mp (°C)	$[\alpha]_D$ (Solvent)	Formula	Elemental analysis Calcd (Found) C	H	N	TLC Rf^1	Rf^2
–NHCH$_3$	10.7	Oil	−12.4° (CHCl$_3$)	$C_{15}H_{22}N_6O_5$	49.2 (49.0	6.07 6.21	22.9 22.6)	0.52	0.55
	65.2	150—151	+15.0° (DMF)	$C_{22}H_{26}N_6O_6$	56.2 (56.2	5.53 5.38	17.9 17.7)	0.60	0.62
	74.0	79—85	+12.4° (DMF)	$C_{23}H_{28}N_6O_7$	55.2 (55.5	5.60 5.60	16.8 16.5)		0.37
	63.5	99—104	+11.2° (DMF)	$C_{25}H_{32}N_6O_7$	56.9 (56.9	6.06 6.03	15.9 15.7)		0.47
	34.6	Oil	−36.9° (CHCl$_3$)	$C_{20}H_{30}N_6O_5$	55.3 (55.7	6.91 7.12	19.4 19.1)	0.51	0.58
	63.7	Oil	−27.0° (CHCl$_3$)	$C_{22}H_{32}N_6O_7$	53.7 (53.5	6.50 6.40	17.1 16.8)	0.50	0.51
	47.6	Oil	−30.8° (CHCl$_3$)	$C_{26}H_{34}N_6O_5$	61.2 (61.2	6.66 7.00	16.5 16.5)	0.46	0.48

concentrated to a small volume. Ether was added to the residue to give crystals, which were collected by filtration and reprecipitated from AcOEt and ether. Yield, mp, $[\alpha]_D$ value, elemental analysis and *Rf* values are summarized in Table IX.

General Procedure for Preparation of H–Tra–Arg–R (R: See Table X) Z–Tra–Arg(NO$_2$)–R (0.2 mmol) in AcOH–H$_2$O (5 ml–5 ml) was hydrogenated over a Pd catalyst. After removal of Pd and the solvent, water was added to the residue and the solution was lyophilized to give a hygroscopic powder. Yield, mp, $[\alpha]_D$ value, elemental analysis and *Rf* values are summarized in Table X.

Z–Arg(Mts)-4-Methoxycarbonylanilide PCl$_3$ (0.36 g, 4 mml) was added to a solution of 4-methoxycarbonylaniline (1.2 g, 8 mmol) in dry pyridine (40 ml) cooled with ice-salt. The reaction mixture was stirred at the same temperature for 15 min and at room temperature for 30 min. Z–Arg(Mts)–OH [prepared from Z–Arg(Mts)-OH·CHA (4.8 g, 8 mmol) and 1 N HCl (8 ml) as usual] was added to the above solution 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 1 N HCl, 5% Na$_2$CO$_3$ and water, dried over Na$_2$SO$_4$ and evaporated down. Ether was added to the residue to give a precipitate,

TABLE IX. Yield, Melting Point, Optical Rotation, Rf Values and Analytical Data of Z–Tra–Arg(NO_2)–R

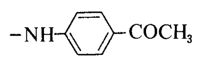
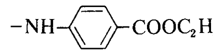
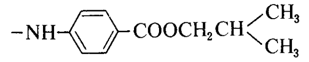
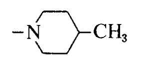
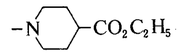
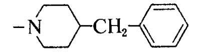
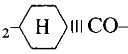
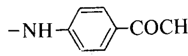
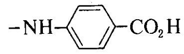
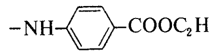
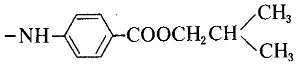
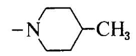
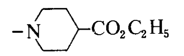
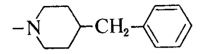
Compound R	Yield (%)	mp (°C)	$[\alpha]_D$ (Solvent)	Formula	Elemental analysis Calcd (Found)			TLC	
					C	H	N	Rf^1	Rf^2
$-NHCH_3$	44.3	115—119	−3.9° (DMF)	$C_{23}H_{33}N_7O_6$	54.9 (54.7	6.62 6.48	19.5 19.6)	0.60	0.62
	78.3	172—176	+2.7° (DMF)	$C_{30}H_{39}N_7O_7$	59.1 (58.9	6.40 6.48	16.1 15.9)	0.52	0.68
	50.9	95—100	−78.8° ($CHCl_3$)	$C_{31}H_{41}N_7O_8$ $\cdot 0.5H_2O$	57.4 (57.6	6.48 6.43	15.2 15.1)		0.52
	50.2	86—92	−20.5° ($CHCl_3$)	$C_{33}H_{45}N_7O_8$	59.4 (59.5	6.74 7.02	14.7 14.6)		0.46
	78.5	Oil	−40.9° ($CHCl_3$)	$C_{28}H_{43}N_7O_6$	58.7 (58.4	7.58 7.71	17.1 17.2)	0.53	0.62
	51.5	Oil	−30.8° ($CHCl_3$)	$C_{38}H_{45}N_7O_8$	57.1 (57.4	7.13 7.42	15.1 14.9)	0.58	0.55
	66.2	Oil	−35.4° ($CHCl_3$)	$C_{34}H_{47}N_7O_6$ $\cdot 3.5H_2O$	57.3 (57.2	7.63 7.38	13.8 14.0)	0.54	0.58

TABLE X. Yield, Melting Point, Optical Rotation, Rf Values and Analytical Data of NH_2CH_2-CO–Arg–R

Compound R	Yield (%)	mp (°C)	$[\alpha]_D$ (Solvent)	Formula	Elemental analysis Calcd (Found)			TLC	
					C	H	N	Rf^5	Rf^8
7 $-NHCH_3$	69.0	Amorphous	−7.0° (MeOH)	$C_{15}H_{30}N_6O_2$ $\cdot 2AcOH \cdot H_2O$	49.1 (49.3	8.67 8.81	18.1 17.8)	0.10	0.38
8 	59.1	Amorphous	−17.3° (MeOH)	$C_{22}H_{34}N_6O_3$ $\cdot 2AcOH \cdot H_2O$	54.9 (54.6	7.80 7.94	14.8 14.8)	0.10	0.51
9 	82.7	Amorphous	−11.4° (MeOH)	$C_{21}H_{32}N_6O_4$ $\cdot 2AcOH \cdot H_2O$	52.7 (52.7	7.44 7.28	14.7 14.6)	0.10	0.52
10 	50.9	Amorphous	−12.8° (MeOH)	$C_{23}H_{36}N_6O_4$ $\cdot 2AcOH \cdot 1.5H_2O$	53.4 (53.4	7.25 7.49	13.8 13.6)	0.45	
11 	89.4	Amorphous	−8.0° (MeOH)	$C_{25}H_{40}N_6O_4$ $\cdot 2AcOH \cdot 2H_2O$	54.1 (54.3	8.07 8.02	13.0 13.2)	0.52	
12 	75.6	Amorphous	−19.1° (MeOH)	$C_{20}H_{38}N_6O_2$ $\cdot 2AcOH \cdot H_2O$	54.1 (53.8	9.08 9.24	15.8 16.0)	0.10	0.36
13 	68.4	Amorphous	−23.2° (MeOH)	$C_{22}H_{40}N_6O_4$ $\cdot 2AcOH \cdot H_2O$	52.7 (52.5	8.84 8.82	14.2 14.2)	0.10	0.42
14 	78.2	Amorphous	−13.5° (MeOH)	$C_{26}H_{42}N_6O_2$ $\cdot 2AcOH \cdot 1.5H_2O$	62.9 (62.7	7.31 7.11	15.1 15.3)	0.10	0.38

which was collected by filtration and reprecipitated from AcOEt and petroleum ether, yield 4.4 g (88.2%), mp 87—89°C, $[\alpha]_D^{26}$ −14.5° (c=1.0, MeOH), Rf^1 0.54, Rf^2 0.45. *Anal.* Calcd for $C_{31}H_{37}N_5O_7S$: C, 59.7; H, 6.00; N, 11.2. Found: C, 59.6; H, 5.91; N, 11.5.

Z–Tra–Arg(Mts)-4-Methoxycarbonylanilide The title compound was prepared by the same way as described in the synthesis of Z–Tra–Arg(NO_2)–R from an acid chloride [prepared from Z–Tra–OH (1.1 g, 5.2 mmol) and $SOCl_2$ (2.4 ml, 5.2 mmol)] and H–Arg(Mts)-4-methoxycarbonylanilide·HCl [prepared from the corresponding N^α-Z-derivative (1.2 g, 2 mmol) in MeOH (100 ml) by catalytic hydrogenation in the presence of 1 N HCl (2 ml)]. The crude product in $CHCl_3$ (3 ml) was applied to a silica gel column (2.2×17 cm), equilibrated and eluted with $CHCl_3$ (350 ml), followed by 1% MeOH in $CHCl_3$. Individual fractions (50 ml each) were collected. The solvent of the effluent (tube Nos. 13—29) was removed by evaporation. Ether was added to the residue to give a precipitate, which was collected by filtration, yield 0.8 g (60%), mp 114—116 °C, $[\alpha]_D^{26}$ −5.6° (c=1.1, MeOH), Rf^1 0.64, Rf^2 0.56. *Anal.* Calcd for $C_{39}H_{50}N_6O_8S \cdot 0.5H_2O$: C, 60.7; H, 6.61; N, 10.9. Found: C, 60.9; H, 6.53; N, 11.0.

Z–Tra–Arg(Mts)-4-Carboxyanilide A solution of Z–Tra–Arg(Mts)-4-methoxycarbonylanilide (0.38 g, 0.5 mmol) in MeOH (40 ml) containing 1 N NaOH (1.5 ml) was stirred at room temperature for 30 min and at room temperature for 2 h. The pH of the reaction mixture was adjusted to 7 with AcOH. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% AcOH and water, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to give a precipitate, which was collected by filtration, yield 0.33 g (88.2%), mp 134—135 °C, $[\alpha]_D^{26}$ −5.0° (c=1.0, MeOH), Rf^1 0.44, Rf^2 0.33. *Anal.* Calcd for $C_{38}H_{48}N_6O_8S \cdot 0.75H_2O$: C, 59.9; H, 6.49; N, 11.0. Found: C, 60.0; H, 6.49; N, 11.0.

H–Tra–Arg-4-Carboxyanilide (9) Z–Tra–Arg(Mts)-4-carboxyanilide (0.26 g, 0.35 mmol) was treated with 1 M TFMSA–thioanisole in TFA (2 ml) in the presence of *m*-cresol (0.38 ml, 10 eq) in the ice-bath for 90 min and ether was added to the solution to give a precipitate, which was collected by centrifugation and dried over KOH pellets *in vacuo*. The resulting powder in H_2O (3 ml) was treated with Amberlite IRA 45 (acetate form). The solution was applied to a column of Sephadex G-25 (1.4×140 cm), equilibrated and eluted with 3% AcOH. Individual fractions (5 g each) were collected and the solvent of the effluent (tube Nos. 50—55) was removed by lyophilization to afford hygroscopic powder. Yield, mp, $[\alpha]_D$ value, elemental analysis and Rf values are summarized in Table X.

General Procedure for Preparation of Z–Hag(NO_2)–R (R: See Table XI) PCl_3 (0.23 ml, 2.5 mmol) was added to a solution of the corresponding amine component (5 mmol) in dry pyridine (20 ml) cooled with ice-salt. The reaction mixture was stirred at the same temperature for 15 min and at room temperature for 30 min. Z–Hag(NO_2)–OH (1.8 g, 5 mmol) was added to the above solution and the reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was

TABLE XI. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of Z- or Boc–Hag(NO_2)–R

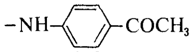
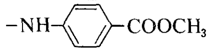
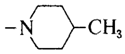
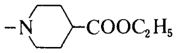
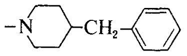
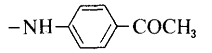
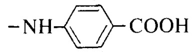
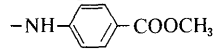
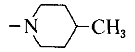
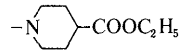
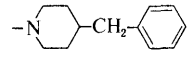
Compound		Yield (%)	mp (°C)	$[\alpha]_D$ (Solvent)	Formula	Elemental analysis Calcd (Found)			TLC	
N^α	R					C	H	N	*Rf*^{1}	*Rf*^{2}
Z	–$NHCH_3$	34.5	Amorphous	−10.8° ($CHCl_3$)	$C_{16}H_{24}N_6O_5 \cdot 2H_2O$	46.1 (46.3	6.79 6.58	20.2 20.1)	0.33	
Z		24.2	Amorphous	−11.6° ($CHCl_3$)	$C_{23}H_{28}N_6O_6 \cdot 2H_2O$	53.1 (53.0	6.21 6.19	16.1 16.3)	0.25	
Z		20.5	Amorphous	−20.6° ($CHCl_3$)	$C_{23}H_{28}N_6O_7 \cdot H_2O$	53.3 (53.5	5.84 5.85	16.2 16.0)	0.26	
Boc		20.1	Amorphous	−8.6° ($CHCl_3$)	$C_{18}H_{34}N_6O_5$	52.2 (52.1	8.29 8.11	20.3 20.5)	0.32	0.52
Boc		15.5	Amorphous	−10.2° ($CHCl_3$)	$C_{20}H_{36}N_6O_7$	50.9 (50.7	7.70 7.56	17.8 17.9)	0.40	0.58
Z		20.5	Amorphous	−21.7° ($CHCl_3$)	$C_{27}H_{36}N_6O_5 \cdot 1.5H_2O$	58.8 (58.6	7.14 7.15	15.2 15.3)	0.38	

TABLE XII. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of Z–Tra–Hag(NO_2)–R

Compound R	Yield (%)	mp (°C)	$[\alpha]_D$ (Solvent)	Formula	Elemental analysis Calcd (Found)			TLC	
					C	H	N	*Rf*^{1}	*Rf*^{2}
–$NHCH_3$	32.6	Amorphous	−15.2° ($CHCl_3$)	$C_{24}H_{37}N_7O_6$	55.5 (55.2	7.19 7.08	18.9 18.8)	0.52	
	75.2	76—81	−26.2° ($CHCl_3$)	$C_{31}H_{41}N_7O_7$	59.7 (59.7	6.64 6.65	15.7 15.9)	0.28	
	36.0	195—198	−31.8° (MeOH)	$C_{30}H_{39}N_7O_8$	57.6 (57.6	6.30 6.18	15.7 15.8)	0.29	0.10
	46.9	142—145	−12.5° (MeOH)	$C_{31}H_{41}N_7O_8$	58.2 (58.0	6.47 6.62	15.3 15.5)	0.56	0.41
	92.1	86—94	−5.2° ($CHCl_3$)	$C_{29}H_{45}N_7O_6$	59.3 (59.5	7.73 7.75	16.7 16.8)	0.70	0.63
	47.7	Amorphous	−8.5° ($CHCl_3$)	$C_{31}H_{47}N_7O_8$	57.7 (57.9	7.35 7.38	15.2 15.6)	0.41	
	56.6	Amorphous	−26.5° ($CHCl_3$)	$C_{35}H_{49}N_7O_6$	63.3 (63.6	7.46 7.45	14.8 14.7)	0.25	

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. The crude product in $CHCl_3$ (5 ml) was applied to a silica gel column (3.6 × 20 cm), equilibrated and eluted with $CHCl_3$ (200 ml), followed by 1% MeOH in $CHCl_3$ (200 ml), 1.5% MeOH in $CHCl_3$ (200 ml) and 2% MeOH in $CHCl_3$ (500 ml). The solvent of the 2% MeOH in $CHCl_3$ effluent was removed by evaporation. Petroleum ether was added to the residue to give an amorphous powder. Yield, mp, $[\alpha]_D$ value, elemental analysis and *Rf* values are summarized in Table XI.

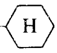
General Procedure for Preparation of Boc–Hag(NO_2)–R (R: See Table XI) A mixed anhydride [prepared from Boc–Hag(NO_2)–OH (1.0 g, 3 mmol) and ethyl chloroformate (0.3 ml, 3 mmol) as usual] in THF (20 ml) was added to an ice-cold solution of the corresponding amine component (3 mmol) in THF (20 ml). After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to afford an amorphous powder. Yield, mp, $[\alpha]_D$ value, elemental analysis and *Rf* values are summarized in Table XI.

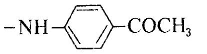
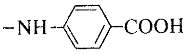
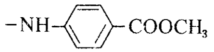
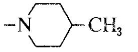
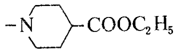
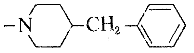
General Procedure for Preparation of Z–Tra–Hag(NO_2)–R (R: See Table XII) An acid chloride [prepared from Z–Tra–OH (1.8 g, 6 mmol) and $SOCl_2$ (2.8 ml, 6 mmol)] in $CHCl_3$ (5 ml) was added to an ice-cold solution of H–Hag(NO_2)–R·HBr or ·HCl [prepared from the corresponding N^α-Z- or Boc-derivative (3 mmol) by 25% HBr–AcOH or 5.6 N HCl–dioxane as usual] in DMF (20 ml) containing Et_3N (0.84 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 water, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to yield a precipitate, which was collected by filtration and recrystallized from EtOH. Yield, mp, $[\alpha]_D$ value, elemental analysis and *Rf* values are summarized in Table XII.

Z–Tra–Hag(NO_2)-4-Carboxyanilide Z–Hag(NO_2)-4-methoxycarbonylanilide (0.51 g, 0.8 mmol) was dissolved in MeOH (50 ml) containing 1 N NaOH (8 ml, 8 mmol) under cooling with ice. The reaction mixture was stirred at the same temperature for 30 min and at room temperature for 3 h. The pH of the reaction mixture was adjusted to 7 with AcOH. After removal of the solvent, the residue was extraced with AcOEt. The extract was washed with 3% AcOH and water, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to give a precipitate, which was collected by filtration. Yield, mp, $[\alpha]_D$ value, elemental analysis and *Rf* value was summarized in Table XII.

General Procedure for Preparation of H–Tra–Hag–R (R: See Table XIII) The corresponding protected compound (0.16 mmol) in AcOH–H_2O (8 ml—8 ml) was hydrogenated over a Pd catalyst. After removal of Pd and the solvent, the residue was dissolved in water and lyophilized to give a hygroscopic powder. Yield, mp, $[\alpha]_D$ value, elemental analysis and *Rf* values are summarized in Table XIII.

Boc–Tra–Arg(Mts)–NHNH–Troc A mixed anhydride [prepared from Boc–Tra–OH (0.39 g, 1.5 mmol) and ethyl chloroformate (0.14 ml, 1.5 mmol) as usual] in THF (20 ml) was added to an ice-cold solution of H–Arg(Mts)–NHNH–Troc·HCl [prepared from Z–Arg(Mts)–NHNH–Troc[14)] (0.51 g, 0.75 mmol) by catalytic hydrogenation] in DMF (20 ml) containing Et_3N (0.11 ml, 0.75 mmol). The reaction mixture was stirred at 4 °C 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 water, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to afford a precipitate, which was collected by filtration. The crude product in $CHCl_3$ (2 ml) was applied to a column of silica gel (1.6 × 10 cm), equilibrated and eluted with $CHCl_3$ (300 ml), followed by 1% MeOH in $CHCl_3$ (250 ml) and 3% MeOH in $CHCl_3$ (350 ml). The

TABLE XIII. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of NH$_2$CH$_2$-CO-Hag-R

	Compound R	Yield (%)	mp (°C)	[α]$_D$ (Solvent)	Formula	Elemental analysis Calcd (Found)			TLC
						C	H	N	*Rf*^{8}
15	-NHCH$_3$	91.7	Amorphous	−19.6° (MeOH)	$C_{16}H_{32}N_6O_2$ $\cdot 2CH_3COOH$	52.2 (52.1	8.75 8.68	18.2 18.0)	0.37
16		39.2	Amorphous	−10.5° (MeOH)	$C_{23}H_{36}N_6O_3$ $\cdot 2CH_3COOH$	57.4 (57.1	7.85 7.91	14.9 14.7)	0.47
17		80.3	Amorphous	−11.8° (MeOH)	$C_{22}H_{34}N_6O_4$ $\cdot 2CH_3COOH$	55.2 (55.2	7.49 7.18	14.8 14.8)	0.47
18		55.2	Amorphous	−21.5° (MeOH)	$C_{23}H_{36}N_6O_4$ $\cdot 2CH_3COOH \cdot 1.5H_2O$	53.4 (53.5	7.82 7.74	13.8 13.9)	0.45
19		50.8	Amorphous	−12.8° (MeOH)	$C_{21}H_{40}N_6O_2$ $\cdot 2CH_3COOH$	56.8 (56.5	9.15 9.25	15.9 15.7)	0.42
20		73.3	Amorphous	−15.7° (MeOH)	$C_{23}H_{42}N_6O_4$ $\cdot 2CH_3COOH$	55.3 (55.1	8.59 8.68	14.3 14.1)	0.42
21		67.6	Amorphous	−15.3° (MeOH)	$C_{27}H_{44}N_6O_2$ $\cdot 2CH_3COOH$	61.6 (61.5	8.67 8.81	13.9 13.6)	0.47

solvent of the 3% MeOH in CHCl$_3$ effluent was removed by evaporation. Ether was added to the residue to afford a precipitate, which was collected by filtration, yield 0.2 g (34.0%), mp, 71—74 °C, $[\alpha]_D^{26}$ −18.0° (c=1.0, CHCl$_3$), *Rf*^{1} 0.22, *Rf*^{3} 0.78. *Anal.* Calcd for $C_{31}H_{48}Cl_3N_7O_8S \cdot H_2O$: C, 46.4; H, 6.23; N, 12.2. Found: C, 46.5; H, 6.06; N, 11.9.

H-Tra-Arg(Mts)-NHNH-Troc (22) Boc-Tra-Arg(Mts)-NHNH-Troc (0.14 g, 0.18 mmol) was dissolved in 7.3 N HCl-dioxane (0.25 ml, 1.8 mmol). After 5 min, dioxane (0.25 ml) was added to the solution. The reaction mixture was stirred at room temperature for 60 min. Ether was added to the solution to yield a white precipitate, which was collected by filtration and dried over KOH pellets *in vacuo*, yield 0.11 g (82.4%), amorphous powder, $[\alpha]_D^{26}$ −13.0° (c=1.3, MeOH), *Rf*^{4} 0.46, *Rf*^{5} 0.85. *Anal.* Calcd for $C_{26}H_{40}Cl_3N_7O_6S \cdot HCl \cdot H_2O$: C, 42.3; H, 5.82; N, 13.3. Found: C, 42.3; H, 5.71; N, 13.5.

Z-Tra-Arg(Mts)-ACA Z-Arg(Mts)-ACA was first prepared by the same way as described in the synthesis of Z-Arg(Mts)-4-methoxycarbonylanilide from PCl$_3$ (0.02 g, 0.27 mmol), 4-acetylaniline (0.07 g, 0.54 mmol) and Z-Arg(Mts)-OH [prepared from Z-Arg(Mts)-OH·CHA (0.32 g, 0.54 mmol) and 1 N HCl (0.54 ml, 0.54 mmol)], yield 0.17 g (52.0%), mp 162—164 °C. Reaction of an acid chloride [prepared from Z-Tra-OH (0.6 g, 2.8 mmol) and SOCl$_2$ (1.3 g, 11.2 mmol)] and H-Arg(Mts)-ACA ·HBr [prepared from the corresponding N$^\alpha$-Z-derivative (0.43 g, 0.7 mmol) obtained above and 25% HBr-AcOH (0.7 ml, 2.1 mmol)] gave the title compound, yield 0.19 g (37.8%), mp 136—138 °C, $[\alpha]_D^{26}$ +8.0° (c=1.0, DMF), *Rf*^{1} 0.49, *Rf*^{2} 0.25. *Anal.* Calcd for $C_{39}H_{50}N_6O_7S$: C, 62.0; H, 6.75; N, 11.1. Found: C, 61.9; H, 6.54; N, 10.8.

H-Tra-Arg(Mts)-ACA (23) Z-Tra-(Mts)-ACA (0.08 g, 0.11 mmol) was dissolved in 25% HBr-AcOH (0.11 ml, 0.33 mmol). After 5 min, AcOH (0.11 ml) was added to the solution. The reaction mixture was stirred at room temperature for 40 min. Ether was added to the solution to yield a white precipitate, which was collected by filtration and dried over KOH pellets *in vacuo*, yield 0.068 g (90.1%), amorphous, $[\alpha]_D^{26}$ −3.7° (c=1.3, MeOH), *Rf*^{4} 0.18, *Rf*^{5} 0.54. *Anal.* Calcd for $C_{44}H_{44}N_6O_5S \cdot HBr \cdot 2.5H_2O$: 50.4; H, 6.84; N, 11.4. Found: C, 50.2; H, 6.59; N, 11.5.

Assay Procedure Amidolytic activity of enzymes was measured according to the procedure described previously.[12] The IC$_{50}$ value was taken as the concentration of the inhibitor which decreased the absorbance at 405 nm by 50% compared with the absorbance without an inhibitor. (P. Kall, plasma kallikrein; Pl, plasmin; UK, urokinase; TH, thrombin).

References and Notes

1) The customary L-configuration for amino acid residues 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**, 2485 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972). Other abbreviations used are Z, benzyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; Mts, mesitylenesulfonyl; CHA, cyclohexylamine; ACA, 4-acetylanilide; BPP, 4-benzylpiperidine; Hag, homoarginine, TFMSA, trifluoromethanesulphonic acid; AcOH, acetic acid; *n*-BuOH, *n*-butanol; AcOEt, ethyl acetate; Troc, 2,2,2-trichloroethyloxycarbonyl.
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