PROTEASE-INHIBITORY ACTIVITIES OF LEUPEPTIN ANALOGUES†

TETSUSHI SAINO, TETSUYA SOMENO, SHIN-ICHI ISHII[a],
TAKAAKI AOYAGI[b] and HAMAO UMEZAWA[b,††]

Research Laboratories, Pharmaceuticals Group, Nippon Kayaku Co., Ltd.,
3-31-12 Shimo, Kita-ku, Tokyo 115, Japan
[a]Faculty of Pharmaceutical Science, Hokkaido University,
Kita-12, Nishi-6, Kita-ku, Sapporo 060, Japan
[b]Institute of Microbial Chemistry,
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

(Received for publication August 4, 1987)

Thirty analogues of leupeptin were synthesized and examined for their inhibitory activities against trypsin, papain, plasmin, kallikrein, thrombin and urokinase *in vitro*. Benzoyl- and α-naphthalenesulfonyl-L-leucyl-L-argininal were 8 times more inhibitory to papain, benzyloxycarbonyl-L-pyroglutamyl-L-leucyl-L-argininal 10 times more to trypsin and plasmin, and DL-2-pipecolyl-L-leucyl-L-argininal 25 times more to kallikrein than leupeptin. Against urokinase, only L-pyroglutamyl-L-leucyl-L-argininal exhibited a potent inhibitory activity. α-Naphthalenesulfonyl-, dansyl- and benzyloxycarbonyl-(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutyryl-L-leucyl-L-argininal were inhibitory to thrombin.

Leupeptin **1**, a microbial peptide with a structure of Ac-L-Leu-L-Leu-L-Argal, was reported as a strong inhibitor of trypsin-family proteases in 1969[1]. Since then **1** and its analogues have been chemically synthesized[2~4], but all the methods used were generally lacking in efficiency chiefly because of racemization of the argininal moiety and instability of the aldehyde products. In the preceding paper, we succeeded in exploring an efficient method to synthesize chemically and optically pure **1** containing an Ac-L-[^{14}C]-Leu moiety from H-L-Leu-L-Argal dibutylacetal that had been prepared by thermolysin digestion of **1** dibutylacetal[5]. In the present study, this method was used for the synthesis of more than 30 analogues having a general structure of acyl or sulfonyl (*R*)-L-Leu-L-Argal, and the synthesized analogues were examined for their inhibitory activity against trypsin-family proteases.

Materials and Methods

The analogues indicated in Tables 1 and 2 were synthesized as described previously[6] except that the R-OSu was replaced with R-Cl or R-OH plus diphenylphosphoryl azide (DPPA) in some cases. Racemization during the synthesis was followed by the use of HPLC that had been successful in indication of all the optically different forms of **1** in the previous work. The synthesized analogues were identified by using field desorption mass spectrometry (FD-MS) or fast atom bombardment mass spectrometry (FAB-MS) as described previously. Mass spectrometry was carried out on a Jeol-DX300 (for FD-MS) and Jeol-D300 (for FAB-MS) mass spectrometers equipped with a Jeol-JMS-2000S mass data analysis system, emitter current, 15~25 mA; acceleration voltage, 3 kV for FD-MS, and acceleration voltage, 3 kV using argon gas and glycerol matrix for FAB-MS. Specific optical rotations were measured on a Perkin-Elmer 241 polarimeter. Column chromatography was performed using Silica gel 60 (Merck) and TLC was done by a precoated Silica gel 60 F_{254} plate (Merck), visualizing with Sakaguchi and ninhydrin reagents and iodine. The physico-chemical data were shown in Table 2.

† Argininal derivatives used in this study were hydrochlorides. Following abbreviations were used: Argal; argininal, R-OSu; acid *N*-hydroxysuccinimide ester, R-Cl; acid chloride.

†† Deceased.

Table 1. Inhibitory activities of analogues against various proteases.

Analogues of leupeptin		IC_{50} ($\times 10^{-5}$ M)			
		Papain	Trypsin	Plasmin	Kallikrein
1	Ac-L-Leu-	0.086	0.22	1.0	1.0
2[b]	Bz-	0.012	3.4	4.4	0.37
3[a]	Benzenesulfonyl-	0.029	0.27	2.4	9.0
4[c]	*p*-CH_3-Bz-	0.021	2.6	3.3	1.8
5[a]	*p*-Toluenesulfonyl-	0.022	0.17	1.5	15
6[c]	*p*-CH_3O-Bz-	0.025	3.0	3.9	2.0
7[a]	*p*-NO_2-Bz-	0.024	3.2	3.1	0.70
8[c]	β-Naphthalenecarbonyl-	0.022	1.4	[d]	0.97
9[a]	α-Naphthalenesulfonyl-[e]	0.012	0.84	2.6	20
10[a]	Dansyl-[f]	0.026	0.16	2.3	7.4
11[a]	Phenylacetyl-	0.033	0.33	1.5	1.4
12[c]	DL-Mandelyl	0.068	0.16	0.34	3.2
13[a]	$CH_3(CH_2)_2CO$-	0.053	1.3	3.8	0.53
14[a]	$CH_3(CH_2)_4CO$-	0.037	0.37	1.5	7.4
15[a]	$CH_3(CH_2)_7CO$-	0.025	0.18	0.27	1.3
16[b]	Cyclopropanecarbonyl-	0.045	6.9	[d]	3.3
17[c]	Cyclohexanecarbonyl-	0.055	3.6	5.6	0.43
18[c]	Nicotinyl-	0.034	1.2	2.2	0.18
19[c]	*iso*-Nicotinyl-	0.075	3.9	8.1	0.61
20[c]	Pyridine-2-carbonyl-	0.09	10.0	[d]	1.9
21[a]	Ethoxycarbonyl-	0.11	20.0	[d]	17
22[l]	Phthalyl-	0.94	18.0	[d]	0.57
23[c]	*Z*-L-Leu-	0.027	0.14	[d]	1.3
24[c]	*Z*-L-Phe-	0.068	0.14	0.12	0.17
25[c]	*Z*-(2*S*,3*R*)-AHPA-[g,h]	0.076	0.11	0.23	0.53
26[c]	*Z*-L-Pyr-[i]	0.031	0.027	0.10	2.5
27	L-Pyr-[j]	0.18	0.12	0.12	0.26
28[c]	*Z*-L-Pro-	0.02	1.1	[d]	1.4
29	L-Pro-	0.15	2.5	[d]	0.10
30[c]	*Z*-DL-Pip-[k]	0.6	1.1	[d]	3.3
31	DL-Pip-	1.1	1.2	[d]	0.04

[a] Prepared by an acid chloride method, [b] prepared by an acid plus DPPA method, [c] prepared by an acid *N*-hydroxysuccinimide ester method, [d] not tested, [e] IC_{50} against thrombin, 9.0×10^{-5} M, [f] IC_{50} against thrombin, 1.5×10^{-5} M, [g] benzyloxycarbonyl-(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutyryl-, [h] IC_{50} against thrombin, 1.0×10^{-4} M, [i] benzyloxycarbonyl-L-pyroglutamyl-, [j] IC_{50} against urokinase, 1.1×10^{-5} M, [k] 2-pipecolyl, [l] phthalated with carboethoxy phthalimide.

Compounds **27**, **29**, and **31** were synthesized from the intermediates of **26**, **28**, and **30**, respectively, by catalytic hydrogenation followed by an acid hydrolysis.

Protease activities were conventionally assayed. Milk casein was used for assay of both papain and trypsin activities as the substrate, and Bz-L-Arg-OEt for assay of kallikrein and thrombin[6]. Fibrinogen, and glutaryl (Glt)-Gly-Arg-4-methylcumaryl-7-amide (MCA) were used for assay of plasmin[6] and urokinase[7] activities, respectively.

Inhibition was expressed in IC_{50}, the inhibitor concentration to give 50% inhibition of protease activity.

For details of the assay methods see refs 7 and 8.

Synthesis

Bz-L-Leu-L-Argal Hydrochloride (Compound **2**)

Benzoic acid (146 mg, 1.2 mmol) and 437 mg (1 mmol) of L-Leu-L-Argal dibutylacetal hydrochloride were dissolved in 5 ml of DMF, and the resulting solution was chilled in an ice bath. To the

Table 2. Physico-chemical data on analogues.

Compound No.[a]	Yield (%)	$[\alpha]^b$	Rf[c]	MF (−HCl)	MW (*m/z*)	MS[d], *m/z* (relative intensity, %)			
						Method	$(M+H)^+$	$(M-18+H)^+$	Other
1			0.41～0.28						
2	38.2	−13.2	0.59～0.40	$C_{19}H_{29}O_3N_5$	375.46	FD	376 (100)	358 (13)	
3	23.7	−30.7	0.55～0.39	$C_{18}H_{29}O_4N_5S$	411.27	FAB	412 (100)	394 (58)	
4	38.2	−1.6	0.56～0.43	$C_{20}H_{31}O_3N_5$	389.29	FAB	390 (31)	372 (28)	119 ((methylbenzoyl), 100)
5	30.3	−35.6	0.54～0.40	$C_{19}H_{31}O_4N_5S$	425.43	FD	426 (100)	408 (7)	
6	47.0	+3.2	0.51～0.37	$C_{20}H_{31}O_4N_5$	405.29	FAB	406 (76)	388 (100)	248 ((M−Argal), 24), 135 ((methoxybenzoyl), 100)
7	39.6	+6.7	0.58～0.45	$C_{19}H_{28}O_5N_6$	420.27	FAB	421 (51)	403 (100)	150 ((nitrobenzoyl), 99)
8	48.7	+24.6	0.53～0.42	$C_{23}H_{31}O_3N_5$	425.29	FAB	426 (83)	408 (53)	268 ((M−Argal), 13), 155 ($(C_{12}H_7CO+H)$, 100)
9	57.8	−62.6	0.58～0.44	$C_{22}H_{31}O_4N_5S$	461.29	FAB	462 (64)	444 (100)	
10	43.4	+20.9	0.57～0.43	$C_{24}H_{36}O_4N_6S$	504.34	FD	505 (100)		
11	30.8	−42.7	0.58～0.38	$C_{20}H_{31}O_3N_5$	389.29	FD	390 (100)	372 (18)	
12	38.3	−63.2	0.50～0.38	$C_{20}H_{31}O_4N_5$	405.29	FD	406 (100)		
13	70.2	−44.2	0.41～0.33	$C_{16}H_{31}O_3N_5$	341.29	FAB	342 (100)	324 (98)	
14	70.9	−42.5	0.56～0.44	$C_{18}H_{35}O_3N_5$	369.32	FAB	370 (100)	352 (51)	212 ((M−Argal), 9)
15	56.4	−37.8	0.64～0.46	$C_{21}H_{41}O_3N_5$	411.37	FD	412 (100)		384 ((M−CO+H), 9)
16	32.2	−47.1	0.48～0.34	$C_{16}H_{29}O_3N_5$	339.27	FAB	340 (100)	322 (87)	182 ((M−Argal), 22)
17	43.1	−45.1	0.54～0.40	$C_{19}H_{35}O_3N_5$	381.32	FD	382 (100)		
18	43.4	−14.0	0.40～0.19	$C_{18}H_{28}O_3N_6$	376.27	FD	377 (28)	359 (100)	
19	42.4	−7.3	0.30～0.20	$C_{18}H_{28}O_3N_6$	376.27	FD	377 (100)	359 (23)	
20	63.6	−5.4	0.37～0.29	$C_{18}H_{28}O_3N_6$	376.27	FAB	377 (44)	359 (100)	191 ((pyridinecarbonyl-Leu-CO), 100)
21	65.9	+11.3	0.42～0.32	$C_{15}H_{29}O_4N_5$	343.27	FAB	344 (39)	326 (100)	158 ((Argal+H), 53)
22	36.8	−0.9	0.51～0.40	$C_{20}H_{27}O_4N_5$	401.33	FAB	402 (68)	384 (73)	160 (100)
23	40.5	−37.9	0.56～0.47	$C_{26}H_{42}O_5N_6$	518.53	FD	519 (100)	501 (22)	
24	36.5	−24.7	0.67～0.51	$C_{29}H_{40}O_5N_6$	552.55	FD	553 (100)	535 (7)	525 ((M−CO+H), 20)
25	21.0	−17.2	0.58～0.39	$C_{30}H_{42}O_6N_6$	582.57	FD	583 (100)		555 ((M−CO+H), 14)
26	29.8	−46.3	0.36～0.20	$C_{25}H_{36}O_6N_6$	516.42	FD	517 (100)		489 ((M−CO+H), 19)
27	27.7	−48.6	0.28～0.13	$C_{17}H_{30}O_4N_6$	382.29	FAB	383 (100)	365 (10)	243 ((Pyr-Leu-OH+H), 22)
28	35.5	−76.0	0.43～0.30	$C_{25}H_{38}O_5N_6$	502.49	FAB	503 (54)	485 (100)	232 ((*Z*-Pro), 17)
29	39.9	−51.3	0.10～0.01	$C_{17}H_{32}O_3N_6$	368.36	FAB	369 (37)	351 (100)	
30	30.5	−40.0	0.53～0.44	$C_{26}H_{40}O_5N_6$	516.47	FAB	517 (100)	499 (32)	246 ((*Z*-DL-Pip), 11)
31	23.1	−36.2	0.09～0.02	$C_{18}H_{34}O_3N_6$	382.38	FD		365 (100)	

[a] The numbering of analogues is defined in Table 1, [b] 578 nm, (*c* 0.5～1.5, AcOH), [c] leupeptin gave three spots on silica gel TLC using a solvent system of BuOH - AcOH - H_2O (4 : 2 : 1) because of its structural isomerism[1]. All the other analogues here also carried the isomerism.

[d] FD-MS; acceleration voltage, 3 kV, emitter current, 15～25 mA. FAB-MS; argon gas, acceleration voltage, 3 kV, matrix, glycerol.

solution were added 215 μl of DPPA and 120 μl of triethylamine, and the resulting solution was stirred for 8 hours at room temp. The reaction mixture was concentrated to dryness. The residue was subjected to silica gel column chromatography (1.5×40 cm) using a mixed solvent of BuOH, *n*-BuOAc, AcOH and H_2O in a ratio of 4:2:1:1 as a eluting solvent. The fractions having the Rf value of 0.7, and showing a positive reaction with Sakaguchi reagent and negative with ninhydrin reagent were collected and concentrated to dryness. The resulting residue was treated with a mixture of 1 N HCl and acetonitrile in a ratio of 1:2 for 15 hours at room temp. After the reaction mixture was diluted with 50 ml of water, it was neutralized with a weakly basic resin Dowex WGR (OH^- type). The resulting aqueous solution was freeze-dried to yield 157 mg of compound **2**. Physicochemical data were shown in Table 2.

DL-Mandelyl-L-Leu-L-Argal Hydrochloride (Compound **12**)

DL-Mandelic acid *N*-hydroxysuccinimide ester (500 mg, 2.02 mmol) and 437 mg (1 mmol) of L-Leu-L-Argal dibutylacetal hydrochloride were dissolved in 5 ml of DMF under ice cooling. Then 110 μl of *N*-ethylmorpholine was added and the resulting solution was stirred for 8 hours at room temp. The reaction mixture was treated in a same manner as the preparation of compound **2** to give 169 mg of compound **12**.

p-Toluenesulfonyl-L-Leu-L-Argal Hydrochloride (Compound **5**)

p-Toluenesulfonyl chloride (191 mg, 1 mmol) and 437 mg (1 mmol) of L-Leu-L-Argal dibutylacetal hydrochloride were dissolved in 5 ml of chloroform under ice cooling. Then 140 μl of triethylamine was added and the resulting solution was stirred for 8 hours at room temp. The reaction mixture was treated in a same manner as the preparation of compound **2** to give 140 mg of compound **5**. Other analogues were prepared in similar manners as compound **2**, compound **12** or compound **5**.

Results and Discussion

Inhibition of Papain

Effect of the analogues of **1** on papain activity was examined. As shown in Table 1, arylacyl and arylsulfonyl analogues, **2** to **10**, strongly inhibited the activity. On an average, these inhibitions were about 4 times stronger than that by **1**. Among the nine analogues, benzoyl (**2**) and α-naphthalenesulfonyl (**9**) derivatives showed the highest inhibition. The dansyl derivative (**10**) was about half as active as **2** and **9**, but was useful as a fluorescent affinity-labeling reagent for trypsin-family proteases (H. SAWADA; unpublished data). Among three analogues with pyridine carbonyl substituents (**18**, **19** and **20**), the nicotinyl derivative was the most inhibitory but 3 times less inhibitory than **2** and **9**. Introduction of a prolyl residue as the P_3 site residue[8] leading to **29** and that of benzyloxycarbonylamino acids to a region occupied with P_3 and P_4 residues (leading to **23**, **24**, **26** and **28**) caused slightly improvement in the inhibitory activity of **1**. Most of the other analogues were similar to or slightly stronger than **1** in the activity. This may be compatible with the broad substrate specificity of papain.

Inhibition of Trypsin

Arylsulfonyl derivatives (**3**, **5**, **9** and **10**) were inhibitory as **1** (Table 1). Arylacyl derivatives (**2**, **4**, **6**, **7** and **8**) were much less inhibitory. However, insertion of one methylene unit between the phenyl and the carbonyl group of the arylacyl moiety brought about increase in inhibitory activity. Thus, phenylacetyl derivatives (**11** and **12**) were as inhibitory as arylsulfonyl ones. These results may be an indication that trypsin has a stricter substrate specificity than papain. Insertion of the longer chain endowed the alkylacyl derivatives (**13** to **15**) with the markedly stronger inhibitory activity. Introduction of pyroglutamyl (Pyr) residue as the P_3 residue and that of benzyloxycarbonylamino acids

to a region consisting of the P_3 and P_4 residue resulted in further increase of the activity, except in the case of **28**. *Z*-L-Pyr-L-Leu-L-Argal (**26**) was the strongest inhibitor among all the derivatives examined here.

Inhibition of Plasmin

Almost all of the inhibitions observed here were very similar in degree to the corresponding inhibitions observed with trypsin (Table 1). Inhibitions by arylacyl and arylsulfonyl derivatives (**2** to **10**) were less than that by **1**, but that by an arylalkylacyl derivative (**12**) was 3 times more. The nonanoyl derivative (**15**), an alkylacyl derivative with the highest lipophilicity, was as active as **12**. Notably, the benzyloxycarbonylaminoacyl derivatives were 5 to 10 times more inhibitory, and **26** that was the strongest inhibitor of trypsin was again the most inhibitory to plasmin. For an effective inhibitor against both plasmin and trypsin activities, the P_3 site is seemingly necessary to be occupied by an amino acid residue.

Inhibition of Kallikrein

Arylacyl analogues (**2**, **4**, **6**, **7** and **8**) were inhibitory against kallikrein similarly to **1** (Table 1). The inhibitions of *n*-butanoyl (**13**), cyclohexanecarbonyl (**17**), nicotinyl (**18**), *iso*-nicotinyl (**19**) and phthalyl (**22**) derivatives were 2 to 5 times stronger than **1**, though comparatively weak against trypsin and plasmin.

Thus these derivatives, especially **22**, are considered to be highly specific inhibitors of kallikrein. Among all the derivatives examined, DL-2-pipecolyl (**31**) derivative showed the strongest inhibition that was also highly specific to kallikrein.

Inhibition of Thrombin and Urokinase

1 and most of the analogues were not inhibitory to both thrombin and urokinase activities (Table 1). Only α-naphthalenesulfonyl (**9**), dansyl (**10**) and benzyloxycarbonyl-(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutyryl (**25**) derivatives were weakly inhibitory against thrombin as shown in the legends for Table 1.

Urokinase catalyses the conversion of plasminogen to plasmin by cleaving the Arg-Val linkage in a Pro-Gly-Arg-Val sequence of plasminogen. Considering this fact, IPSEN and CHRISTENSEN selected Pyr-Gly-Arg-4-nitroanilide as a suitable model substrate for urokinase[9]. Pyr-L-Leu-L-Argal (**27**) that is similar in structure to this substrate was included here in the analogues to be examined for inhibitory activity. As expected, **27** inhibited urokinase activity.

In the present study, we found that some leupeptin analogues inhibited proteases more strongly and more specifically than leupeptin. These selected inhibitors have already shown high utilities in the wide field of protease studies *in vitro* or *in vivo*[10,11]. Preparation of affinity adsorbents for the proteases is also being studied as an application. One of the successful result has been published[12].

References

1) AOYAGI, T.; T. TAKEUCHI, A. MATSUZAKI, K. KAWAMURA, S. KONDO, M. HAMADA, K. MAEDA & H. UMEZAWA: Leupeptins, new protease inhibitors from actinomycetes. J. Antibiotics 22: 283~286, 1969
2) SHIMIZU, B.; A. SAITO, A. ITO, K. TOKAWA, K. MAEDA & H. UMEZAWA: Synthetic studies on leupeptins and their analogs. J. Antibiotics 25: 515~523, 1972
3) SAEKI, H.; Y. SHIMADA, N. KAWAKITA, B. SHIMIZU, E. OHKI, K. MAEDA & H. UMEZAWA: An alternative synthesis of leupeptins and their analogs. Chem. Pharm. Bull. 21: 163~170, 1973

4) BORIN, G.; G. CHESSA, G. GAVAGGION, F. MARCHIORI & W. MULLER-ESTERL: Synthesis of leupeptins and inhibition of proteases I. Inhibition of acrosin and trypsin. Hoppe Seylers Z. Physiol. Chem. 362: 1435~1445, 1981
5) SAINO, T.; T. SOMENO, H. MIYAZAKI & S. ISHII: Semisynthesis of ^{14}C-labelled leupeptin. Chem. Pharm. Bull. 21: 2319~2325, 1982
6) AOYAGI, T.; S. MIYATA, M. NANBO, F. KOJIMA, M. MATSUZAKI, M. ISHIZUKA, T. TAKEUCHI & H. UMEZAWA: Biological activities of leupeptins. J. Antibiotics 22: 558~568, 1969
7) MORITA, T.; H. KATO, S. IWANAGA, K. TAKADA, T. KIMURA & S. SAKAKIBARA: New fluorogenic substrate for thrombin, factor Xa, kallikreins, and urokinase. J. Biochem. 82: 1495~1498, 1977
8) SCHECHIER, I. & A. BERGER: On the size of the active site in proteases. I. Papain. Biochem. Biophys. Res. Commun. 27: 157~162, 1967
9) IPSEN, H. H. & U. CHRISTENSEN: Kinetic studies of urokinase-catalysed hydrolysis of 5-oxo-L-prolylglycyl-L-arginine 4-nitroanilide. Biochem. Biophys. Acta. 613: 476~481, 1980
10) SAWADA, H.; M. KAWAHIGASHI, H. YOKOSAWA & S. ISHII: Trypsin-like enzyme from eggs of the ascidian (protochordate), *Halocynthia roretzi*. J. Biol. Chem. 260: 15694~15698, 1985
11) SAWADA, H.; H. YOKOSAWA, T. SOMENO, T. SAINO & S. ISHII: Evidence for the participation of two sperm proteases, spermosin and acrosin, in fertilization of the ascidian, *Halocynthia roretzi*: Inhibitory effects of leupeptin analogs on enzyme activities and fertilization. Dev. Biol. 105: 246~249, 1984
12) SOMENO, T.; T. SAINO, K. KATOH, H. MIYAZAKI & S. ISHII: Affinity chromatography of urokinase on an agarose derivative coupled with pyroglutamyl-lysyl-leucyl-argininal. J. Biochem. 97: 1493~1500, 1985