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Peptidyl α-Keto Thiazole as Potent Thrombin Inhibitors

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Abstract: We report the synthesis and evaluation of α-keto thiazole derivatives such as D-Phe-Pro-Arg-thiazole **9** as a novel type of thrombin inhibitor. Tripeptidyl α-keto thiazole **9** exhibited the inhibitory activity of thrombin at nanomolar levels and showed a more potent prolongation effect on clotting time than argatroban at a dose of 3 mg/kg intravenously.

Introduction

Thrombin (EC 3.4.21.5) is a serine protease and the key enzyme in the blood coagulation cascade.[1] Thrombin catalyzes the conversion of fibrinogen to fibrin, which then polymerizes to form a hemostatic plug. Thus, there has been considerable interest in research for thrombin inhibitors as anticoagulant agents.[2]

An approach to the design of the serine protease inhibitor has been the replacement of the scissile amide bond by an electron-withdrawing carbonyl group.[3] Edwards *et al.* originally reported that α-keto benzoxazole derivative was a mechanism-based elastase inhibitor in which the nitrogen atom of the benzoxazole interacted with the histidine residue of the catalytic triad in serine protease.[4] Recently, we elucidated that α-keto heterocyclic compounds possessed potent inhibitory activity for prolyl endopeptidase (PEP).[5] In the course of our PEP inhibitors study, we substituted the amino acid residue in the P_1 position of phenylbutanoyl-Pro-A-thiazole **1~5** (A: Pro, Ala, Val, Lys, and Arg) (Table 1). The basic residue of Arg or Lys was indispensable for the inhibitory activity of thrombin, but not PEP.

Table 1. Structure and inhibitory potencies of α-keto thiazole derivatives.

$Ph(CH_2)_3CO$-Pro-A-thiazole

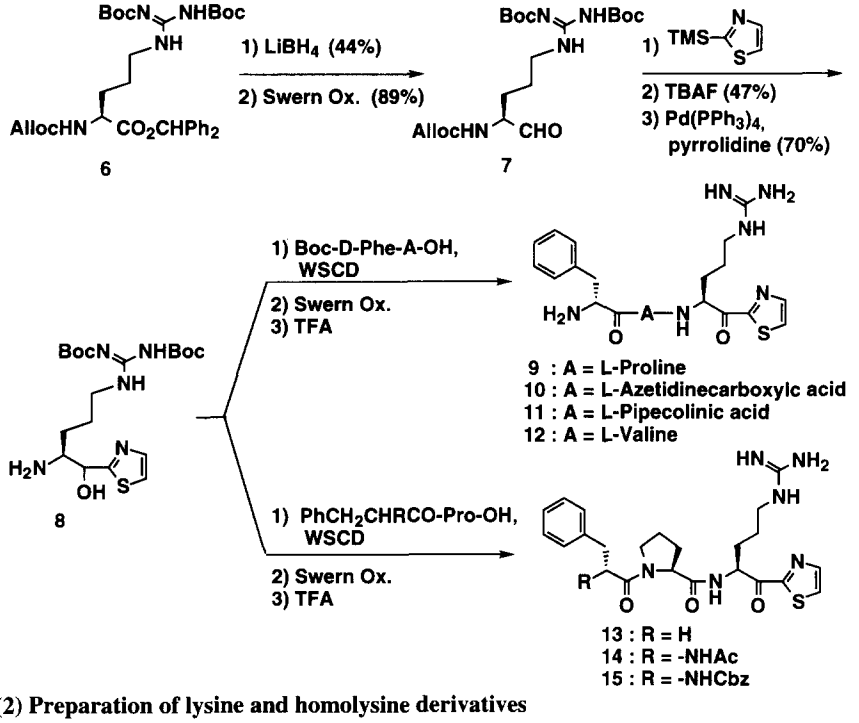
No	A (amino acid)	Inhibitory Activity IC_{50} (μM)			
		PEP	Elastase	Thrombin	Trypsin
1	Proline	0.0044	>1000	>1000	>1000
2	Alanine	0.0050	>1000	>1000	>1000
3	Valine	7.7	>1000	>1000	>1000
4	Lysine	7.8	Not determined	46	2.4
5	Arginine	28	>1000	2.0	0.23

These results also indicated that α-keto heterocyclic compound was useful for research on other serine protease inhibitors. Tripeptide aldehyde and chloromethyl ketone analogs of D-Phe-Pro-Arg sequence are reported to be high effective and reversible inhibitors of thrombin.[2] Considering these factors for inhibitor design, we examined the structure-activity relationship of the tripeptide α-keto thiazole compounds in the P_1, P_2 and P_3 positions.

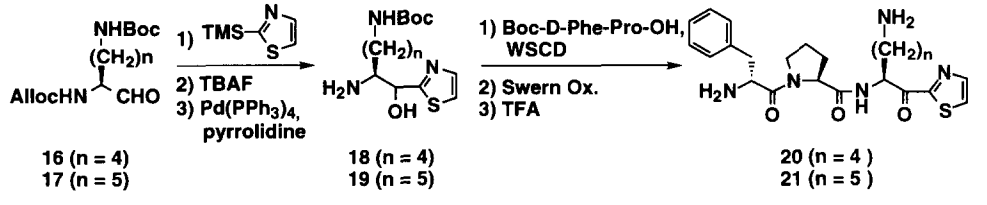
Chemistry: We synthesized a series of tripeptide arginine, lysine, and homolysine derivatives with α-keto thiazole moiety. All compounds were obtained in a convergent strategy as shown in Scheme I. The arginine aldehyde **7** was prepared by the reduction of N-Allyloxycarbonyl(Alloc) arginine ester **6** followed by Swern oxidation.[6] The reaction of aldehyde **7** with 2-(trimethylsilyl)thiazole gave the 2-thiazole derivative, which was converted to amino alcohol **8** by deprotection of the Alloc group. Condensation of the amino alcohol **8** with Boc-D-Phe-Pro-OH followed by Swern oxidation gave the tripeptide. The desired α-keto thiazole compound **9** was prepared by the TFA deprotection of the Boc protective group. In a similar manner, the corresponding arginine derivatives **10~15**, lysine derivative **20**, and homolysine derivative[7] **21** were synthesized.[6]

Scheme I

(1) Preparation of arginine derivatives



(2) Preparation of lysine and homolysine derivatives

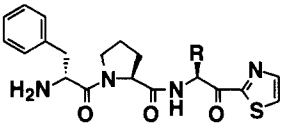


Enzyme assay: Human thrombin was purchased from Sigma Chemical Company Ltd. Thrombin and trypsin assays were performed as described by Kawabata.[8] PEP and elastase assays were carried out as described by Walter[9] and Bieth[10], respectively. Urokinase, plasmin, and plasma kallikrein assays were performed by the method used by Morita.[11] Experiments were conducted in 96-well plates, and the rates of hydrolysis were measured fluorometrically with excitation at 380 nm and emission at 440 nm or spectrophotometrically at 405 nm.

Ex vivo anticoagulant studies: Five minutes after intravenous administration of test compound **9** and argatroban[12], the prolonged activated partial thromboplastin time (APTT) and prothrombin time (PT) were determined by the method of Takamiya[13] and Suzuki[14], respectively.

Results & Discussion: Replacement of the arginine in **9** with a lysine or homolysine residue at the P_1 position resulted in a marked decrease in the inhibitory potency of thrombin, although the thrombin to trypsin ratios remained unchanged (Table 2). Unexpectedly, the homolysine derivative **21**, which possesses carbon chains of the same length as arginine, was less potent than lysine derivative **20**. These results showed that the arginine in the P_1 position was most suitable for the thrombin inhibitor. The α-keto thiazole inhibitor **9** exhibited a 40-fold increase in potency to inhibit thrombin as compared to argatroban.

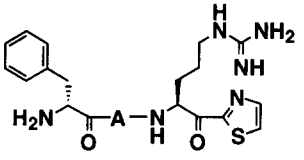
Table 2. Effect of modifications in the P_1 position on thrombin inhibitors

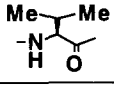


No	R	Inhibitory Activity IC_{50} (μM)		Selectivity
		Thrombin	Trypsin	Trypsin/Thrombin
9	$-(CH_2)_3-N(H)-C(=NH)-NH_2$	0.0015	0.0042	2.8
20	$-(CH_2)_4-NH_2$	0.26	1.2	4.6
21	$-(CH_2)_5-NH_2$	14	16	1.1
Argatroban		0.060	not determined	

The proline residue at the P_2 position in **9** was replaced by azetidine-2-carboxylic acid **10**, pipecolinic acid **11**, or valine **12** in order to investigate the influence of conformationally constrained amino acid (Table 3). While the azetidine-2-carboxylic acid derivative **10** maintained potency for the inhibition of thrombin, the pipecolinic acid derivative **11** was 480-times less active than **9**. Compound **10** exhibited the same selectivity for thrombin over trypsin as **9**. These results coincided with the results of Shuman et al.[15] Replacement of proline with valine did not significantly change the potency for the inhibition of thrombin, although the valine derivative **12** exhibited remarkable selectivity for thrombin over trypsin (trypsin/thrombin=21). This selectivity would be due to a conformational difference between the S_2 subsite of thrombin and trypsin.

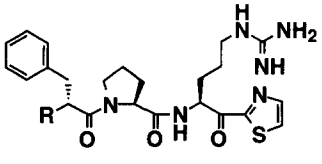
Table 3. Effect of modifications in the P_2 position on thrombin inhibitors



No	A	Inhibitory Activity IC_{50} (μM)		Selectivity
		Thrombin	Trypsin	Trypsin/Thrombin
10		0.003	0.009	3.0
9		0.0015	0.0042	2.8
11		0.73	41	56
12		0.0086	0.18	21

The substitution of the D-phenylalanine residue in **9** changed the inhibitory potency of thrombin (Table 4). Deletion of an amino group at the P_3 position showed the marked decrease in potency (**9** vs **13**). The importance of amino group was further investigated by acylation of compound **9**. A difference in inhibitory activity between the acetyl and benzyloxycarbonyl(Cbz) derivatives (**14** vs **15**) was observed. The acetyl compound **14** exhibited a 1000-fold loss in potency for the inhibition of thrombin as compared to compound **15**. The addition of the acetyl group in **9** led to a 20-fold decrease in selectivity. In this study, the modification at the P_3 position demonstrated no improvement in selectivity for thrombin over trypsin. On the other hand, the aldehyde derivative incorporating lactam sulfonamide moiety are reported to display potent and selective inhibitor.[16] The sulfonamide at the P_3 position might be a useful moiety for thrombin inhibitor.

Table 4. Effect of modifications in the P_3 position on thrombin inhibitors



No	R	Inhibitory Activity IC_{50} (μM)		Selectivity
		Thrombin	Trypsin	Trypsin/Thrombin
13	-H	0.82	0.23	0.3
9	$-NH_2$	0.0015	0.0042	2.8
14	-NHAc	2.3	0.23	0.1
15	-NHCbz	0.002	0.0016	0.8

Recently, Costanzo et al. reported that α-keto benzothiazole inhibitor displays selectivity for thrombin compared with trypsin or plasmin.[17] The moiety of α-keto benzothiazole in the $P_{1'}$ position would also provide a novel interaction with thrombin, but not trypsin.

The compound **9** with D-Phe-Pro-Arg motif showed the most potent inhibitory activity for thrombin (IC_{50} value: 1.5 nM). IC_{50} values (μM) for other serine proteases were as follows: trypsin, 0.0042; urokinase, 15.3; plasmin, >100; plasma kallikrein, >100.[12] These results suggested that the selectivity for thrombin over other proteases could be attributed to the different recognition of a D-Phe-Pro-Arg motif.

Finally, we evaluated the antithrombotic potency of compound **9** in comparison with argatroban for the ability to prolong prothrombin time (PT) and activated partial thromboplastin time (APTT) in rats (Table 5). Five minutes after intravenous administration of compound **9**, PT was prolonged 1.5 fold over the control value at 1 mg/kg and 12 fold at 3 mg/kg. A similar inhibitory potency was observed following intravenous administration of 1 mg/kg and 3 mg/kg with 2.0 fold and 8.3 fold APTT elevation, respectively. Although the α-keto thiazole compound **9** showed a 40-fold increase in potency to inhibit the amidolytic activity of thrombin as compared to argatroban, the antithrombotic activity of compound **9** was about 2-fold more potent than that of argatroban. A possible explanation for the decreased activity of compound **9** in coagulation assays is that α-keto thiazole compound might be a slow binding inhibitor and/or was less stable in vivo than argatroban.

Table 5. Antithrombotic effect of compound 9 on intravenous administration

Compound	Dose (mg/kg)	PT inhibitor / PT control	APPT inhibitor / APPT control
9	1	1.5	2.0
9	3	12	8.3
Argatroban	3	6.2	4.8

Conclusion: We extended the utility of α-keto heterocyclic compound to thrombin inhibitors. Tripeptidyl α-keto thiazoles exhibited the inhibitory activity of thrombin at nanomolar levels. The modification at the P_2 position altered the selectivity for thrombin over trypsin. Compound **9** exhibited a more potent prolongation effect on the clotting time than argatroban at a dose of 3mg/kg intravenously. The tripeptide derivative with α-keto heterocycle represented a promising new thrombin inhibitor.

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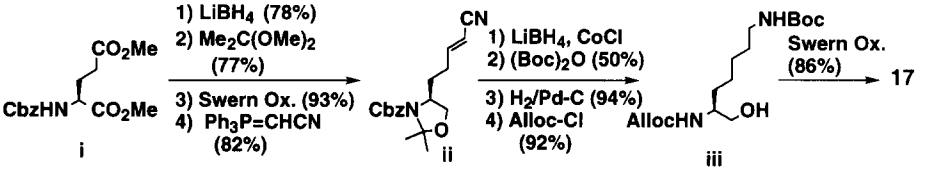
7. We prepared **17** from the glutamic acid ester **i** by using the methods shown below.



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