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# Selective Inhibition of Thrombin by (2*R*,4*R*)-4-Methyl-1-[$N^2$-[(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl]-L-arginyl)]-2-piperidinecarboxylic Acid†

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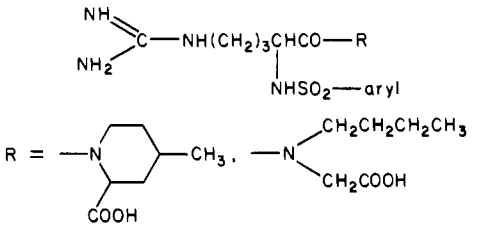
ABSTRACT: The potency of thrombin inhibition by 4-methyl-1-[$N^2$-[(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)-sulfonyl]-L-arginyl]-2-piperidinecarboxylic acid (MQPA) depended on the stereoconformation of the 2-piperidinecarboxylic acid moiety. $K_i$ values for bovine $\alpha$-thrombin were 0.019 $\mu$M with (2*R*,4*R*)-MQPA, 0.24 $\mu$M with (2*R*,4*S*)-MQPA, 1.9 $\mu$M with (2*S*,4*R*)-MQPA, and 280 $\mu$M with (2*S*,4*S*)-MQPA. (2*R*,4*R*)-MQPA of the four stereoisomers of MQPA was also the most potent inhibitor for other trypsin-like serine proteases with $K_i$ values of 5.0 $\mu$M for trypsin, 210 $\mu$M for factor Xa, 800 $\mu$M for plasmin, and 1500 $\mu$M for plasma kallikrein. Examination of the potency of thrombin inhibition by arginine derivatives related to MQPA in structure suggested the presence of a specific binding site for the carboxamide portion (C-terminal side). The relative inhibitory potency of the four stereoisomers of MQPA for trypsin was nearly identical with that for thrombin, suggesting that the specific binding site for the carboxamide portion is present in both enzymes. Modification of thrombin by phosphopyridoxylation or the presence of heparin did not significantly alter the binding of MQPA.

A series of $N^2$-substituted L-arginine ester and amide derivatives has been examined for inhibitory effects on thrombin. Potent inhibition of thrombin has been found with arginine derivatives having two hydrophobic moieties, an aromatic moiety such as the substituted naphthalenesulfonyl group as an $N^2$-substituent of arginine and alkylamines or cyclic amines such as butylamine or 4-methylpiperidine as an amine component of the carboxamide portion (Okamoto et al., 1980; Kikumoto et al., 1980a). On the basis of these observations, it has been suggested that thrombin possesses three binding sites corresponding to (1) the guanidino group, (2) the aromatic $N^2$-substituent, and (3) the hydrophobic carboxamide portion. A typical compound of this type of thrombin inhibitors, 1-($N^2$-dansyl-L-arginyl)-4-ethylpiperidine, exhibited a highly specific inhibition of thrombin among trypsin-like serine proteases (Hijikata et al., 1979; Nesheim et al., 1979). These inhibitors, however, were too toxic to be used as antithrombotic agents in man.

In an effort to obtain less toxic thrombin inhibitors, it has been found that introduction of a carboxyl group into the carboxamide portion of $N^2$-substituted L-arginine amide derivatives resulted in less toxic derivatives without a loss of inhibitory effect. A structure–activity study of the carboxyl-containing $N^2$-substituted L-arginine derivatives showed that the carboxyl group should be introduced at the carbon adjacent to the amide nitrogen, as shown in the following general formula, to provide maximum inhibition of thrombin (Kikumoto et al., 1980b).




The stereochemical study of one such inhibitor, 4-methyl-1-[$N^2$-[(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl]-L-arginyl]-2-piperidinecarboxylic acid (MQPA),[1] indicated that the stereoconformation of the 4-methyl-2-piperidinecarboxylic acid portion is important for its binding to thrombin, (2*R*,4*R*)-MQPA, the synthetic thrombin inhibitor No. 805, being the most potent thrombin inhibitor (Okamoto et al., 1981).[2] In this paper, the inhibitory effect of (2*R*,4*R*)-MQPA

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[1] Abbreviations: MQPA, 4-methyl-1-[$N^2$-[(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl]-L-arginyl]-2-piperidinecarboxylic acid; QPA, 1-[$N^2$-[(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl]-L-arginyl]-2-piperidinecarboxylic acid; MQP, 4-methyl-1-[$N^2$-[(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl]-L-arginyl]piperidine; QP, 1-[$N^2$-[(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl]-L-arginyl]piperidine; 4MPA, 4-methyl-2-piperidinecarboxylic acid; H-D-Phe-Pip-Arg-pNA, H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide; Bz-Ile-Glu-Gly-Arg-pNA, *N*-benzoyl-L-isoleucyl-L-glutamyl ($\gamma$-OH and $\gamma$-$OCH_3$)-glycyl-L-arginine-*p*-nitroanilide; H-D-Pro-Phe-Arg-pNA, H-D-prolyl-L-phenylalanyl-L-arginine-*p*-nitroanilide; H-D-Val-Leu-Lys-pNA, H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide; Tos-Gly-Pro-Arg-pNA, *N*-tosyl-glycyl-L-prolyl-L-arginine-*p*-nitroanilide; Tris, tris(hydroxymethyl)-aminomethane.

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Table I: Melting Points and Specific Rotations of Synthetic Compounds

| compd | mp (°C) | specific rotation |
|---|---|---|
| (2*R*,4*R*)-MQPA monohydrate | 176–180 | $[\alpha]^{27}_D$ +76.1° (*c* 1, 0.2 N HCl) |
| (2*R*,4*S*)-MQPA monohydrate | 174–176 | $[\alpha]^{25}_D$ +43° (*c* 1, 0.2 N HCl) |
| (2*S*,4*R*)-MQPA monohydrate | 229–232 | $[\alpha]^{25}_D$ +35° (*c* 1, 0.2 N HCl) |
| (2*S*,4*S*)-MQPA | 270–272 | $[\alpha]^{25}_D$ +57° (*c* 1, 0.2 N HCl) |
| (2*R*,4*R*)-MQPA-Me | amorphous | $[\alpha]^{27}_D$ +109° (*c* 1, MeOH) |
| (2*R*)-QPA | 181–183 | $[\alpha]^{25}_D$ +175.3° (*c* 1, MeOH) |
| (2*S*)-QPA | amorphous | $[\alpha]^{25}_D$ +114.2° (*c* 1, MeOH) |
| MQP | 196–198 | $[\alpha]^{25}_D$ +106.2° (*c* 1, MeOH) |
| QP | 206 | $[\alpha]^{25}_D$ +103.1° (*c* 1, MeOH) |

on thrombin is compared with that on trypsin, plasmin, plasma kallikrein, and factor Xa to examine the specificity of inhibition. The other three stereoisomers of MQPA were also examined for their inhibitory effect on trypsin-like serine proteases other than thrombin. Further, the arginine derivatives related to MQPA in structure with respect to the 4-methyl-2-piperidinecarboxylic acid portion were examined for thrombin inhibitory effects. The present investigation shows that (2*R*,4*R*)-MQPA selectively inhibits thrombin via the firm binding to the hydrophobic binding pocket for its carboxamide portion. This study also suggests that other trypsin-like proteases possess a hydrophobic binding pocket for the 4-methyl-2-piperidinecarboxylic acid portion of MQPA.

## Experimental Procedures

*Materials.* Four stereoisomers of MQPA, (2*R*,4*R*)-MQPA, (2*R*,4*S*)-MQPA, (2*S*,4*R*)-MQPA, and (2*S*,4*S*)-MQPA, were prepared as described previously (Okamoto et al., 1981). Briefly, the 4MPA or 4MPA ethyl ester of the synthetic material was first resolved into four stereoisomers to synthesize each stereoisomer. The absolute configuration of each isomer of 4MPA or its ethyl ester was established by X-ray analysis. Each isomer of 4MPA ethyl ester was condensed with $N^2$-(*tert*-butoxycarbonyl)-$N^G$-nitro-L-arginine to finally obtain each stereoisomer of MQPA. X-ray crystallographical analysis of the stereoisomers of MQPA confirmed that the stereoconfiguration of the 4MPA portion of MQPA retained that of the 4MPA ethyl ester used as the starting material. (2*R*,4*R*)-MQPA-Me was prepared by esterification of (2*R*,4*R*)-MQPA with $SOCl_2$ and MeOH and reprecipitated several times from MeOH–ethyl ether to give an amorphous solid.

(2*R*)- and (2*S*)-1-[$N^2$-[(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl]-L-arginyl]-2-piperidinecarboxylic acids (QPA) were prepared as follows. D-2-Piperidinecarboxylic acid was prepared from racemic 2-piperidinecarboxylic acid (Aldrich) by the use of L-tartaric acid as a resolving agent according to the modified procedure of Leithe (1932). Similarly, L-2-piperidinecarboxylic acid was obtained by the use of D-tartaric acid as a resolving agent. D- and L-2-piperidinecarboxylic acids were esterified with EtOH and $SOCl_2$ to give ethyl D-2-piperidinecarboxylate [bp 69–71 °C (3 mmHg); $[\alpha]^{24}_D$ +13.8° (*c* 4.08, EtOH)] and ethyl L-2-piperidinecarboxylate [bp 69–70 °C (3 mmHg); $[\alpha]^{24}_D$ −14.1° (*c* 4.01, EtOH)]. These esters were used as the starting materials to obtain (2*R*)- and (2*S*)-QPA by the previously described method (Okamoto et al., 1981). 1-[$N^2$-[(3-Methyl-1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl]-L-arginyl]-piperidine (QP) was prepared as follows. $N^2$-(*tert*-Butoxycarbonyl)-$N^G$-nitro-L-arginine was condensed with piperidine by the mixed anhydride method with isobutyl chloroformate and triethylamine in tetrahydrofuran at −20 °C to give 1-[$N^2$-(*tert*-butoxycarbonyl)-$N^G$-nitro-L-arginyl]piperidine, which was purified by silica gel column chromatography with chloroform–MeOH (97:3) as solvent. After removal of the *tert*-butoxycarbonyl group by HCl–AcOEt, 1-($N^G$-nitro-L-arginyl)piperidine was sulfonylated with 3-methyl-8-quinolinesulfonyl chloride to give 1-[$N^2$-[(3-methyl-8-quinolinyl)-sulfonyl]-$N^G$-nitro-L-arginyl]piperidine, which was also purified by silica gel column chromatography in the same solvent. Hydrogenolysis of the nitro group and hydrogenation of the pyridine ring by Pd–C/$H_2$ gave QP, which was recrystallized from $H_2O$–EtOH. 4-Methyl-1-[$N^2$-[(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl]-L-arginyl]piperidine (MQP) was prepared by a similar method. The melting points and specific rotations for these compounds were summarized in Table I.

H-D-Phe-Pip-Arg-pNA (S-2238), Bz-Ile-Glu-Gly-Arg-pNA (S-2222), H-D-Val-Leu-Lys-pNA (S-2251), and H-D-Pro-Phe-Arg-pNA (S-2302) were products of Kabi Diagnostica, Stockholm. Heparin was obtained from Sigma Chemical Co., St. Louis, MO. Bovine α-thrombin was purified from commercial topical thrombin (Mochida Pharmaceutical, Tokyo) according to Lundblad (1971) with sulfopropyl-Sephadex C-50. The specific activity of α-thrombin was 2300–2500 NIH units/mg. Lyophilized human α-thrombin was obtained from Sigma. Bovine plasminogen was obtained from Daiichi Chemicals, Tokyo, and activated to plasmin by urokinase before use. Twice-crystallized, lyophilized trypsin from bovine pancreas was obtained from Sigma. Factor X was purified from bovine plasma according to Bajaj & Mann (1973) and activated to factor Xa by Russell's viper venom obtained from Sigma. Plasma kallikrein was partially purified from bovine plasma up to the first CM-cellulose column chromatography step of the method of Colman & Bagdasarian (1976). Phosphopyridoxylated thrombin was obtained according to the method of Griffith (1979) by reacting bovine α-thrombin (50 NIH units) with pyridoxal 5′-phosphate (50 mM) for 20 min followed by reduction with $NaBH_4$ and desalting by gel filtration through a Sephadex G-25 column.

*Enzyme Assay and Measurement of Kinetic Constants.* The rate of increase in absorbance at 405 nm due to hydrolysis of synthetic peptide substrates was measured in the presence and absence of inhibitors with a Hitachi 200 spectrophotometer at 37 °C in the following reaction system: 5–20 μM H-D-Phe-Pip-Arg-pNA and 0.1 M Tris-HCl buffer (pH 8.0) for thrombin, 25–100 μM Bz-Ile-Glu-Gly-Arg-pNA and 0.1 M Tris-HCl buffer (pH 8.0) for trypsin, 50–200 μM H-D-Pro-Phe-Arg-pNA and 0.1 M Tris-HCl buffer (pH 8.8) for plasma kallikrein, 250–1000 μM H-D-Val-Leu-Lys-pNA and 0.1 M Tris-HCl buffer (pH 7.4) for plasmin, and 160–400 μM Bz-Ile-Glu-Gly-Arg-pNA, 0.1 M Tris-HCl buffer (pH 8.0), 50 mM NaCl, and 10 mM $CaCl_2$ for factor Xa. H-D-Phe-Pip-Arg-pNA was used as the substrate for phosphopyridoxylated thrombin. The enzyme assay (final volume of 3 mL) was

[2] The selective thrombin inhibitor (2*R*,4*R*)-MQPA (MCI-9038) is being developed for clinical use as a thrombin inhibitory antithrombotic in Japan under Development Number MD-805.

Table II: Inhibition Constants of Four Stereoisomers of MQPA for Trypsin-like Proteases

| stereoisomers of MQPA | $K_i$ (μM) | | | | |
|---|---|---|---|---|---|
| | thrombin[a] | trypsin | factor Xa | plasmin | kallikrein[b] |
| (2*R*,4*R*)-MQPA | 0.019 | 5.0 | 210 | 800 | 1500 |
| (2*R*,4*S*)-MQPA | 0.24 | 30 | >500 | >500 | >500 |
| (2*S*,4*R*)-MQPA | 1.9 | 690 | >1500 | >1500 | >1500 |
| (2*S*,4*S*)-MQPA | 280 | >500 | >500 | >500 | >500 |

[a] Bovine α-thrombin was used. [b] Bovine plasma kallikrein was used.

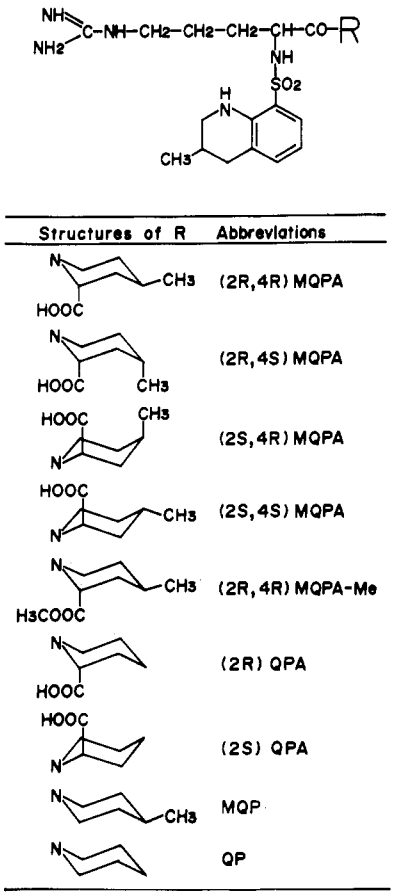


FIGURE 1: Stereochemical structures of arginine derivatives used in this study.

started by the addition of enzyme. Reaction cells were coated with silicon to prevent adsorption of thrombin to glass. The data were analyzed with the COMP program of Cleland (1979).

## Results and Discussion

*Stereochemical Structure of MQPA and Related Compounds.* The chemical structures of MQPA and related compounds are shown in Figure 1. MQPA has four stereoisomers having different stereostructure at the 4MPA portion. In (2*R*,4*R*)-MQPA, the stereoconfiguration at the 2-position is D form and the 2-carboxyl group is trans to the 4-methyl group, therefore (2*R*,4*R*)-MQPA is also named D-*trans*-MQPA. The other isomers, (2*R*,4*S*)-, (2*S*,4*R*)-, and (2*S*,4*S*)-MQPA are named likewise D-*cis*-, L-*cis*-, and L-*trans*-MQPA, respectively. Likewise, (2*R*)- and (2*S*)-QPA are D- and L-QPA, respectively. Axial configuration of the carboxyl group at the 2-position of the 4MPA portion of (2*R*,4*R*)-MQPA has been established by the X-ray crystallographical study (unpublished results). The carboxyl group of the 2-piperidinecarboxylic acid portion is considered not to take an equatorial configuration, because the steric interaction of the carboxyl group with the peptide carbonyl group would

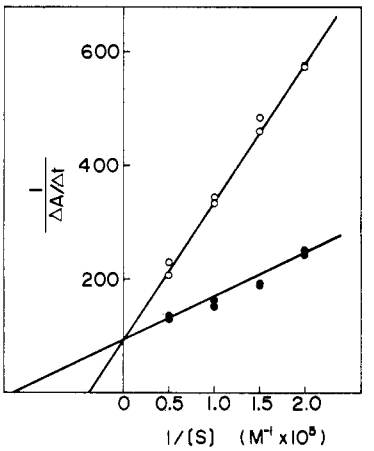


FIGURE 2: Competitive inhibition of thrombin by (2*R*,4*R*)-MQPA. Plots of reciprocal velocity ($\Delta A_{405nm}$/min) vs. reciprocal H-D-Phe-Pip-Arg-pNA concentration in the presence of 0 (●) and 0.05 μM (○) (2*R*,4*R*)-MQPA.

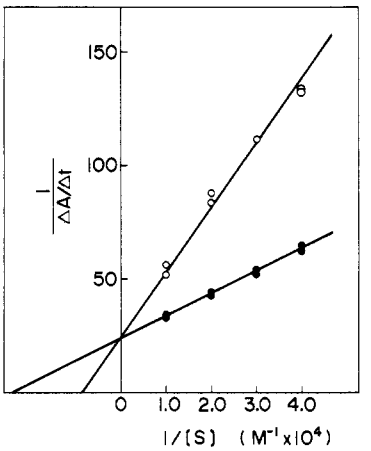


FIGURE 3: Competitive inhibition of trypsin by (2*R*,4*R*)-MQPA. Plots of reciprocal velocity ($\Delta A_{405nm}$/min) vs. reciprocal Bz-Ile-Glu-Gly-Arg-pNA concentration in the presence of 0 (●) and 10 μM (○) (2*R*,4*R*)-MQPA.

prevent the carboxyl group from taking the equatorial configuration. Therefore, it was assumed that the carboxyl group of the 2-piperidinecarboxylic acid portion takes an axial configuration in all other isomers.

*Stereospecificity for Inhibitory Effect of MQPA on Thrombin and Other Trypsin-like Serine Proteases.* The stereoisomers of MQPA were examined for inhibitory effect on thrombin, trypsin, plasma kallikrein, plasmin, and factor Xa. All the isomers showed a different inhibitory potency on each enzyme. The mode of the inhibition was competitive with respect to the synthetic peptide substrate in all enzymes tested. Figures 2–3 present the Lineweaver–Burk analysis for the inhibition of thrombin and trypsin by (2*R*,4*R*)-MQPA. The inhibitory potency of (2*R*,4*R*)-MQPA was not reduced by preincubation with thrombin or trypsin (data not shown). Further, it has been shown in our previous report that $N^2$-

substituted arginine amides having secondary amines such as *N*-*n*-butyl-*N*-methylamine and piperidine as the component of amides are not substrates for either thrombin or trypsin (Kikumoto et al., 1980a). Therefore, it seems likely that inhibitors presented in this paper are not serving as substrates for these enzymes.

The apparent $K_i$ values of the isomers for several trypsin-like proteases were shown in Table II. With thrombin, (2*R*,4*R*)-MQPA was the most potent inhibitor with an extremely low apparent $K_i$ of 0.019 $\mu$M, whereas (2*S*,4*S*)-MQPA, which is the antipode of (2*R*,4*R*)-MQPA, was the poorest inhibitor with an apparent $K_i$ of 280 $\mu$M. The apparent $K_i$ values of (2*R*,4*S*)- and (2*S*,4*R*)-MQPA were, respectively, about 10 and 100 times higher than that of (2*R*,4*R*)-MQPA. (2*R*,4*R*)-MQPA was also the most potent inhibitor, and the other isomers showed weak inhibition in clotting activity of thrombin with fibrinogen as substrate comparable to those with H-D-Phe-Pip-Arg-pNA as substrate (Okamoto et al., 1981). (2*R*,4*R*)-MQPA also exhibited potent inhibition of human $\alpha$-thrombin with an apparent $K_i$ of 0.039 $\mu$M, which is a little higher than that of 0.019 $\mu$M for bovine $\alpha$-thrombin as shown in Table V.

With trypsin, (2*R*,4*R*)-MQPA again was the most potent inhibitor, but its apparent $K_i$ value for trypsin was about 100 times higher than that for thrombin. Surprisingly, the relative inhibitory potency of the four stereoisomers of MQPA for trypsin was nearly identical with that for thrombin, the ratio of $K_i$ for trypsin to $K_i$ for thrombin of each isomer being 260 for (2*R*,4*R*)-MQPA, 125 for (2*R*,4*S*)-MQPA, and 360 for (2*S*,4*R*)-MQPA. (2*S*,4*S*)-MQPA scarcely inhibited trypsin at 500 $\mu$M, and an apparent $K_i$ value could not be determined because of the low solubility.

Only (2*R*,4*R*)-MQPA exhibited measurable inhibition of factor Xa, plasmin, and plasma kallikrein with high $K_i$ values of 210, 800, and 1500 $\mu$M, respectively. Low solubility did not permit study above 500 $\mu$M for (2*R*,4*S*)- and (2*S*,4*S*)-MQPA and above 1500 $\mu$M for (2*S*,4*R*)-MQPA. These three isomers exhibited no significant inhibitory effect on factor Xa, plasmin, and plasma kallikrein at the maximum possible concentration. Comparison of the inhibitory potency of (2*R*,4*R*)-MQPA among the proteases tested clearly showed that (2*R*,4*R*)-MQPA inhibited thrombin most potently with a $K_i$ lower by 2 orders of magnitude than that for trypsin. Inhibition of factor Xa, plasmin, and plasma kallikrein by (2*R*,4*R*)-MQPA was much less than that of thrombin with $K_i$ values of (2*R*,4*R*)-MQPA over 10000 times higher than that for thrombin.

These results suggest that trypsin and other serine proteases possess a hydrophobic binding pocket that can interact more or less with the 4-methyl-2-piperidinecarboxylic acid portion of MQPA and that the structure of their binding pocket is at least partly of common nature in these serine proteases. Since the binding force of the molecule is the sum of the binding force of all binding portions of the molecule, it is difficult to conclude which portion of the (2*R*,4*R*)-MQPA molecule contributes to the selective inhibition of thrombin. The common nature of the binding pocket for the carboxyl terminal group among trypsin-like proteases, as suggested above, indicates that the (2*R*,4*R*)-4-methyl-2-piperidinecarboxylic acid portion of MQPA mainly contributes to the reinforcement of the binding affinity and the (1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl group portion contributes to the selective hydrophobic interaction with thrombin. Numerous compounds having amidinophenyl groups have been reported to inhibit trypsin-like proteases (Geratz et al., 1976; Parrish et al., 1978; Walsmann et al., 1974, 1979). These amidino compounds bind tightly to thrombin but bind also to trypsin and other trypsin-like proteases as effectively as to thrombin. This lower specificity of these compounds might reflect the lack of a second hydrophobic group corresponding to the (1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl group.

Table III: Inhibition Constants of Arginine Derivatives Related to MQPA for Bovine $\alpha$-Thrombin

| compd | $K_i$ for thrombin ($\mu$M) |
|---|---|
| (2*R*)-QPA | 0.31 |
| (2*S*)-QPA | 57 |
| (2*R*,4*R*)-MQPA-Me | 0.014 |
| MQP | 0.030 |
| QP | 0.13 |

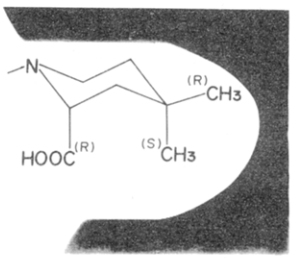


FIGURE 4: Proposed binding situation of 4MPA portion of (2*R*,4*R*)- and (2*R*,4*S*)-MQPA at binding site of thrombin. The structure of (2*R*,4*R*)-MQPA overlaps with that of (2*R*,4*S*)-MQPA with the exception of the 4-$CH_3$ group.

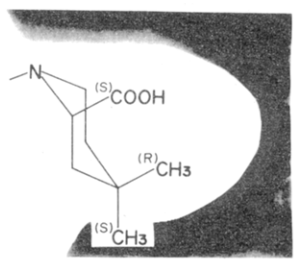


FIGURE 5: Proposed binding situation of 4MPA portion of (2*S*,4*R*)- and (2*S*,4*S*)-MQPA at binding site of thrombin. The structure of (2*S*,4*R*)-MQPA overlaps with that of (2*S*,4*S*)-MQPA with the exception of the 4-$CH_3$ group.

*Inhibition of Thrombin by Arginine Derivatives Related to MQPA and Proposal for the Conformation of the Binding Site of Thrombin.* Since (2*R*,4*R*)-MQPA was shown to be a specific and potent thrombin inhibitor, other arginine derivatives having various amine components related to the 4MPA portion of MQPA at the carboxamide portion were examined to study the binding mode of the carboxamide portion of (2*R*,4*R*)-MQPA. All the compounds tested showed competitive inhibition of thrombin, and apparent $K_i$ values are shown in Table III. The 2*R* isomer of the two stereoisomers of 2-piperidinecarboxylic acid derivative was about 100 times as potent as (2*S*)-QPA. The apparent $K_i$ of (2*R*)-QPA, 0.36 $\mu$M, was almost equal to that of QP, 0.12 $\mu$M. Furthermore, MQP showed a $K_i$ similar to that of (2*R*,4*R*)-MQPA shown in Table II.

The differences in the thrombin inhibitory potencies of the four stereoisomers of MQPA and related compounds made it possible to construct a stereogeometrical model for the binding of the 4MPA portion of MQPA to thrombin as shown in Figures 4 and 5. The binding interaction of two isomers with 2*R* configuration was shown in Figure 4 and that of other two isomers with 2*S* configuration in Figure 5. In (2*R*,4*R*)-MQPA, the most potent inhibitor in the present

study, the hydrophobic portion of 4MPA is best fitted to a pocket constituted from hydrophobic amino acid residues of the thrombin molecule, the hydrophobic piperidine ring skeleton interacting hydrophobically with this pocket. The 4-methyl group of (2*R*,4*R*)-MQPA is considered to interact hydrophobically with the pocket, because (2*R*,4*R*)-MQPA possessed a $K_i$ one-tenth that of (2*R*)-QPA (with no 4-methyl group). (2*R*,4*S*)-MQPA was as inhibitory as (2*R*)-QPA, suggesting that the 4-methyl group with the axial configuration of (2*R*,4*S*)-MQPA no longer interacts with the hydrophobic pocket. The direct contribution of the 4-methyl group of (2*R*,4*R*)-MQPA to the hydrophobic interaction and no contribution of the 4-methyl group of (2*R*,4*S*)-MQPA are consistent with the observation that MQP (with no carboxyl group), in which the 4-methyl group takes an equatorial configuration as a stable form, inhibited thrombin more potently than QP (with no carboxyl and no methyl group). The 4-methylpiperidine portion of MQP probably binds to thrombin in a situation similar to that of (2*R*,4*R*)-MQPA shown in Figure 4, resulting in no difference in the binding affinity between (2*R*,4*R*)-MQPA and MQP. The 2-carboxyl group of *R* configuration seems not to bind directly to the binding site through the ionic interaction from the facts that the apparent $K_i$ values do not differ significantly between (2*R*,4*R*)-MQPA and -MQP and between (2*R*)-QPA and -QP. This concept is supported by the observation that the esterification of the 2-carboxyl group of (2*R*,4*R*)-MQPA did not significantly affect the binding affinity of (2*R*,4*R*)-MQPA. The 2-carboxyl group of *R* configuration seems to face the site opposite the hydrophobic protein surface. This site provides an isolated space too large to allow the interaction with it. Thus, the carboxyl group of the derivatives with 2*R* configuration neither contributes to nor interferes with the binding to thrombin.

On the other hand, the 2-carboxyl groups of the (2*S*)-2-piperidinecarboxylic acid portion face to the hydrophobic binding pocket as shown in Figure 5. Figure 5 was developed on the assumption that other parts of the inhibitors with 2*S* configuration bind to thrombin exactly like those of the compounds with 2*R* configuration and the 2-carboxyl group keeps on axial configuration. Thus, the 2-carboxyl group with *S* configuration interferes with the putative hydrophobic interaction, resulting in an apparent $K_i$ of (2*S*)-QPA 100 times as high as that of (2*R*)-QPA. The 4-methyl group of (2*S*,4*R*)-MQPA is probably involved in the hydrophobic interaction, because (2*S*,4*R*)-MQPA possessed an apparent $K_i$ lower than that of (2*S*)-QPA. Steric hindrance by the 4-methyl group of (2*S*,4*S*)-MQPA is strongly suggested by the fact that (2*S*,4*S*)-MQPA exhibited extremely weak inhibition of thrombin with an apparent $K_i$ higher than that of (2*S*)-QPA.

As mentioned above, thrombin possesses a hydrophobic pocket to which the (2*R*,4*R*)-4MPA portion of (2*R*,4*R*)-MQPA binds tightly. Meinwald et al. (1980) studied the hydrolysis of the Arg–Gly bond of the A$\alpha$ fibrinogen-like oligopeptides and found that these peptides do not bind to thrombin very well, whereas the A$\alpha$ and B$\beta$ chains of fibrinogen bind firmly to thrombin with $K_m$ values of 9.2 and 11.3 $\mu$M, respectively (Martinell & Scheraga, 1980). Our present results strongly suggest that a hydrophobic amino acid residue in the C-terminal side in native fibrinogen could be spatially close to the Arg-Gly moiety in such a way that the phenylalanine residue distant nine peptide sequence from the Arg–Gly bond should be close to Arg-Gly segment in the N-terminal side (Blombäck et al., 1969). The much higher binding affinity of (2*R*,4*R*)-MQPA than fibrinogen seems to result from the closer interaction of the hydrophobic groups of (2*R*,4*R*)-MQPA with thrombin, due to the smaller size of its molecule.

Table IV: Inhibition of Phosphopyridoxylated Thrombin by (2*R*,4*R*)-MQPA

| modified thrombin | $I_{50}$ ($\mu$M)[a] | fibrinogen clotting activity (NIH units/mg)[b] |
|---|---|---|
| phosphopyridoxylated thrombin[c] | 0.065 | <23 |
| thrombin phosphopyridylated in the presence of (2*R*,4*R*)-MQPA[d] | 0.063 | <23 |
| no modification (control) | 0.050 | 2300 |

[a] Concentration to inhibit a hydrolysis rate of 10 $\mu$M H-D-Phe-Pip-Arg-pNA by 50% was determined by measuring the rates in the presence of varying concentrations of (2*R*,4*R*)-MQPA. [b] Fibrinogen clotting activity was measured as described previously (Okamoto et al., 1981). [c] Thrombin was phosphopyridoxylated as described under Experimental Procedures. [d] Thrombin was phosphopyridoxylated in the presence of 10 $\mu$M (2*R*,4*R*)-MQPA.

*Inhibitory Effect of MQPA on Thrombin Modified at a Binding Site Region.* It has been shown that thrombin reacts with various reagents to give modified thrombin with a reactivity to fibrinogen or synthetic substrates different from that of native thrombin. However, it has not been definitely shown how such a modification gives changes in conformation around the thrombin active site. As described above, stereoconformation of the 4MPA portion of MQPA has a great influence on its binding to thrombin. It is therefore of interest to use such a specific thrombin inhibitor as one described in this paper to study possible conformational change of the active site of modified thrombin. In this portion of the study, the inhibitory potency of MQPA for phosphopyridoxylated thrombin was compared to that for native thrombin. In addition, the effect of heparin on the inhibition of thrombin by (2*R*,4*R*)-MQPA was examined.

Griffith (1979) reported that two lysyl residues of human $\alpha$-thrombin are phosphopyridoxylated after reduction of pyridoxal 5′-phosphate/thrombin reaction solution with sodium borohydride and phosphopyridoxylation of the first site is accompanied by a loss in the fibrinogen clotting activity and that of the second site results in a decrease in the sensitivity to heparin in the antithrombin III/thrombin reaction. Since phosphopyridoxylation of thrombin results in little effect on the hydrolysis of synthetic substrates, the inhibitory effect of (2*R*,4*R*)-MQPA on the hydrolysis of H-D-Phe-Pip-Arg-pNA by phosphopyridoxylated bovine thrombin was examined. (2*R*,4*R*)-MQPA inhibited phosphopyridoxylated thrombin as effectively as native thrombin, as shown in Table IV. Further, phosphopyridoxylation in the presence of a large excess of (2*R*,4*R*)-MQPA did not affect the loss of fibrinogen clotting activity. These results suggest that the 2-carboxyl group of *R* configuration need not interact with the lysine residue supposed to be essential for the fibrinogen binding (Griffith, 1979), which is considered to be spatially apart from the active site.

Heparin binds to thrombin, enhancing the apparent binding affinity of thrombin for the synthetic substrate Tos-Gly-Pro-Arg-pNA and the anti-thrombin III, suggesting a conformational change around the binding site of thrombin (Griffith et al., 1979). (2*R*,4*R*)-MQPA inhibited thrombin in the presence of heparin more potently than in the absence of

Table V: Inhibition of Human α-Thrombin in the Presence or Absence of Heparin by (2*R*,4*R*)-MQPA

| conditions | $K_i$ of (2*R*,4*R*)-MQPA (μM) | $K_m$ of substrate (μM)[a] |
|---|---|---|
| with heparin[b] | 0.016 | 4.3 |
| without heparin | 0.039 | 15 |

[a] H-D-Phe-Pip-Arg-pNA was used as substrate. [b] Heparin of 2 μg/mL was added to the reaction mixture described under Experimental Procedures.

heparin, the apparent $K_i$'s being 0.016 μM and 0.039 μM, respectively, as shown in Table V. Similarly, a $K_m$ of H-D-Phe-Pip-Arg-pNA for thrombin was found to be smaller in the presence of heparin. Thus, heparin enhanced binding affinity for both the inhibitor and substrate. In spite of the differences in the structures of the substrate and the inhibitor with respect to the existence of the carboxyl group at the carboxamide portion, no significant differences were detected in the enhanced binding to heparin-bound thrombin.

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**Registry No.** MQPA, 74863-84-6; QPA, 74874-21-8; MQPA-Me, 87804-12-4; MQP, 87804-13-5; QP, 87804-14-6; thrombin, 9002-04-4; trypsin, 9002-07-7; factor Xa, 9002-05-5; plasmin, 9001-90-5; kallikrein, 9001-01-8; D-2-piperidinecarboxylic acid, 1723-00-8; L-2-piperidinecarboxylic acid, 3105-95-1; ethyl D-2-piperidinecarboxylate, 22328-77-4; ethyl L-2-piperidinecarboxylate, 22328-78-5; $N^2$-(*tert*-butoxycarbonyl)-$N^G$-nitro-L-arginine, 2188-18-3; piperidine, 110-89-4; 1-[$N^2$-(*tert*-butoxycarbonyl)-$N^G$-nitro-L-arginyl]piperidine, 87804-15-7; 1-($N^G$-nitro-L-arginyl)piperidine, 87804-16-8; 1-[$N^2$-[(3-methyl-8-quinolinyl)sulfonyl]-$N^G$-nitro-L-arginyl]piperidine, 87804-17-9.

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