POTENT INHIBITION OF THROMBIN BY THE NEWLY SYNTHESIZED

ARGININE DERIVATIVE No. 805.   THE IMPORTANCE OF STEREO-

STRUCTURE OF ITS HYDROPHOBIC CARBOXAMIDE PORTION.

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SUMMARY: Four stereoisomers of 4-methyl-1-[N^2-(3-methyl-1,2,3,4-tetrahydro-8-quinolinesulfonyl)-L-arginyl]-2-piperidinecarboxylic acid were synthesized and examined for the inhibitory effect on thrombin. The inhibitory potency varied largely with the stereoconfiguration of the 4-methyl-2-piperidinecarboxylic acid portion. The (2R,4R)-isomer was the most potent inhibitor with a Ki of 0.019 μM, while the (2R,4S) and (2S,4R)-isomers showed the values of Ki 0.24 and 1.9 μM, respectively. The least potent inhibitor, (2S,4S)-isomer, showed a Ki of 280 μM which is approximately 15,000 times that of (2R,4R)-isomer.

INTRODUCTION

Considering the important role of thrombin in thrombogenesis,

several groups have studied the compounds able to inhibit thrombin

action (1-4). We also have studied independently a series of

synthetic thrombin inhibitors. Our studies originating from simple

arginine derivatives have attained several hundred synthetic thrombin

inhibitors which bear three binding portions in their structures

like a tripod constituted by an arginine side chain having a posi-

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Abbreviations: 4MPE, ethyl 4-methyl-2-piperidinecarboxylate;
4MPA, 4-methyl-2-piperidinecarboxylic acid; MQPA, 4-methyl-
1-[N^2-(3-methyl-1,2,3,4-tetrahydro-8-quinolinesulfonyl)-L-arginyl]-2-piperidinecarboxylic acid.

tively charged guanidino group and two hydrophobic groups of an

aromatic N^2-substituent and a carboxamide portion (5-8). We obtained

4-methyl-1-[N^2-(3-methyl-1,2,3,4-tetrahydro-8-quinolinesulfonyl)-L-

arginyl]-2-piperidinecarboxylic acid (MQPA), which had the most

potent thrombin inhibitory effect. However, the synthesis and the

examination of thrombin inhibitory potency of each stereoisomer of

MQPA in 4-methyl-2-piperidinecarboxylic acid portion led to a marked

difference in a thrombin inhibitory effect among four stereoisomers.

The most potent isomer, NO. 805 (MCI-9038 or MD-805), had a Ki of

0.019 μM. The second and third isomers were, respectively, 10 and

100 times less inhibitory than No. 805, and the last isomer approxi-

mately 15,000 times less inhibitory than No. 805. It was an unex-

pected result that a change in the stereostructure of the hydropho-

bic binding portion composed of 4-methyl-2-piperidinecarboxylic acid,

which is only a partial structure of the thrombin inhibitory arginine

derivative, resulted in such a remarkable difference in a thrombin

inhibitory potency. A presumption of active site stereogeometry

of thrombin on the basis of these results strongly suggested that

a hydrophobic pocket to bind a hydrophobic carboxamide portion

exists in the vicinity of the active site of thrombin, in addition

to the binding pockets for a positively charged arginine side chain

and an aromatic group on the 2-nitrogen of arginine, and the size

of this pocket is relatively small.

MATERIALS AND METHODS

Preparation of (2R,4R) and (2S,4S) ethyl 4-methyl-2-piperidine-
carboxylates: Ethyl 4-methyl-2-piperidinecarboxylate (4MPE) was
prepared according to the modified procedure of Bonnett et al. (9).
Trans isomer (mixture of the (2R,4R) and (2S,4S) isomers) of 4MPE
was separated by fractional distillation in vacuo. The boiling
point of trans isomer was 83-85°/7 mmHg and that of cis isomer
107-108°/5 mmHg. Trans-4MPE was hydrolyzed by boiling with conc.
HCl for 4 h to give trans-4MPA HCl salt. After desalting of this
HCl salt by cation exchange resin, trans-4MPA (14.3 g) and L-

tartaric acid (15.0 g) was dissolved in boiling EtOH (290 ml).
Upon cooling, the precipitate was filtered off, washed with cold
EtOH and recrystallized from 90% EtOH (100 ml) to give (2R,4R)-
4MPA·L-tartaric acid salt (14.6 g), mp 183.9-185.0°, $[\alpha]_D^{26}$ +4.4°
(c 10, H_2O). The absolute configuration of the crystals was estab-
lished by X-ray analysis. (2R,4R)-4MPA·L-tartaric acid salt was
desalted by cation exchange resin and the eluate was evaporated
to dryness to give powderly crystals. Recrystallization from EtOH-
H_2O yielded (2R,4R)-4MPA (6.3 g), mp 275-277°, $[\alpha]_D^{18}$ -18° (c 10,
2N HCl). (2S,4S)-4MPA was obtained from trans-4MPA and D-tartaric
acid as described for (2R,4R)-4MPA. (2R,4R)- and (2S,4S)-4MPA
were esterified with EtOH and thionylchloride to give (2R,4R)-
4MPE, bp 83-85°/7 mmHg, $[\alpha]_D^{22}$ -24.0° (c 5, EtOH) and (2S,4S)-4MPE,
bp 83-85°/7 mmHg, $[\alpha]_D^{22}$ +24.1° (c 5, EtOH), respectively.

Preparation of (2S,4R) and (2R,4S) ethyl 4-methyl-2-piperidine-
carboxylates: A solution of (2R,4R)-4MPE (2.0 g) in EtOH (50 ml)
was heated for epimerization at 150° in a sealed tube for 5 h.
After the evaporation of the solvent, the residue was fractionally
distilled in vacuo to give (2S,4R)-4MPE (1.16 g), bp 107-108°/5 mmHg,
$[\alpha]_D^{24}$ -12.5° (c 5, EtOH). (2R,4S)-4MPE was obtained by epimeriza-
tion of (2S,4S)-4MPE as described above, bp 107-108°/5 mmHg,
$[\alpha]_D^{24}$ +11.5° (c 5, EtOH).

Preparation of stereoisomers of 4-methyl-1-[N^2-(3-methyl-1,2,3,4-
tetrahydro-8-quinolinesulfonyl)-L-arginyl]-2-piperidinecarboxylic
acid (MQPA): N^2-(tert-Butoxycarbonyl)-N^G-nitro-L-arginine was
condensed with each stereoisomer of 4MPE by the mixed anhydride
method using isobutyl chloroformate and triethylamine at -20° to
give N^2-(tert-butoxycarbonyl)-N^G-nitro-L-arginyl-4MPE, which was
purified by silica gel column chromatography. After removal of
tert-butoxycarbonyl group by HCl-AcOEt, N^G-nitro-L-arginyl-4MPE
was sulfonylated with 3-methyl-8-quinolinesulfonyl chloride to give
N^2-(3-methyl-8-quinolinesulfonyl)-N^G-nitro-L-arginyl-4MPE, which
was also purified by silica gel column chromatography. Hydrolysis
of this compound by NaOH, followed by hydrogenolysis of the nitro
group and hydrogenation of the pyridine ring by Pd-C/H_2, gave MQPA,
which was recrystallized from EtOH-H_2O. (2R,4R)-MQPA monohydrate
had mp 176-180°, $[\alpha]_D^{27}$ +76.1° (c 1, 0.2N HCl); (2S,4S)-MQPA, mp
270-272°, $[\alpha]_D^{25}$ +57° (c 1, 0.2N HCl); (2R,4S)-MQPA monohydrate,
mp 174-176°, $[\alpha]_D^{25}$ +43° (c 1, 0.2N HCl); and (2S,4R)-MQPA mono-
hydrate, mp 229-232°, $[\alpha]_D^{25}$ +35° (c 1, 0.2N HCl).

Measurement of inhibition of the clotting activity of thrombin
and determination of inhibition constants: Bovine α-thrombin was
purified from topical thrombin purchased from Mochida Pharmaceu-
tical according to Lundblad (10). It had a specific activity of
2300 NIH units/mg.
    The clotting activity of thrombin was measured at 37° in the
presence of the varying amount of inhibitors in the reaction mixture
containing 0.1 M borate-saline buffer, pH 7.4, 0.1% fibrinogen
(Sigma) and 0.4 NIH unit of thrombin in a total volume of 0.5 ml
and I-50 values of inhibitors for the clotting activity were
determined, which were defined as the concentration at which the
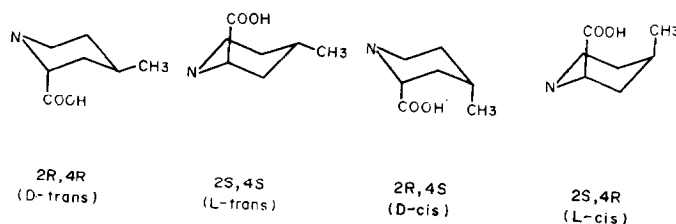clotting time was prolonged by twice that of the control.

<u>Fig. 1.</u>   The stereostructures of 4MPA portion of MQPA.

The inhibition constants, Ki, were determined by measuring the effect of inhibitors on the activity of hydrolysis of a peptide substrate by thrombin.   The reaction mixture containing 0.1 M Tris-HCl buffer, pH 8.0, and varying amount of H-D-Phe-Pip-Arg-p-nitro-aniline (S-2238) (Kabi Diagnostica) in a total volume of 3 ml was incubated at 37° and the reaction was started by the addition of 10 μl of 6.6 NIH units/ml of thrombin.   The initial velocity of the increase in absorbance at 405 nm was recorded by Hitachi 200 record-ing spectrophotometer.   Reaction cells were coated with silicone to prevent adsorption of thrombin to glass.   The data were analyzed by Lineweaver-Burk plot.

<center>RESULTS</center>

The stereostructure of 4MPA portion of MQPA are illustrated

in Fig. 1.   Since preliminary X-ray crystallographical study of

the (2R,4R)-isomer showed that the COOH group of 4MPA took on an

axial configuration, all the isomers were illustrated tentatively

with the axial COOH group.   Since, in the (2R,4R)-isomer, the ste-

reoconfiguration at the 2 position is D form and the 2-COOH group

is trans to the 4-CH$_3$ group, (2R,4R)-isomer may also be called

the (D-trans)-isomer.   The 4MPA portion of (2S,4S)-isomer is the

antipode of that of (2R,4R)-isomer and therefore the (2S,4S)-isomer

is inevitably the (L-trans)-isomer.

All four stereoisomers inhibited bovine α-thrombin competi-

tively with respect to the synthetic substrate S-2238 as illustrated

for the (2R,4R)-isomer, No. 805, in Fig. 2, but the inhibitory

potency of each isomer varied largely with stereostructure of the

4MPA portion (Table 1).   The (2R,4R)-isomer showed the most potent

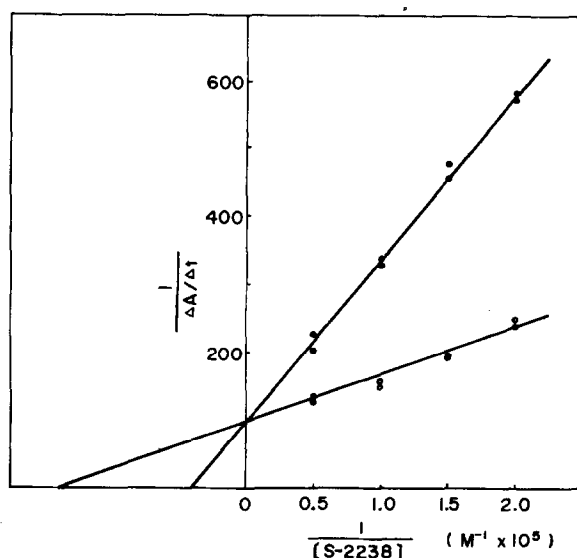inhibition with a Ki of 0.019 μM, while the (2S,4S)-isomer was the

Fig. 2. Lineweaver-Burk plot of inhibition of bovine α-thrombin-catalyzed hydrolysis of S-2238.
o——o ; In the absence of inhibitors.
●——● ; In the presence of 0.05 μM (2R,4R)-MQPA.

least potent inhibitor with a Ki of 280 μM. The values of Ki of

the (2R,4S) and (2S,4R)-isomers were 0.24 and 1.9 μM, respectively.

In inhibition of bovine α-thrombin with fibrinogen as substrate,

Table 1. Inhibition constants and I-50 values of stereoisomers
of MQPA for thrombin.
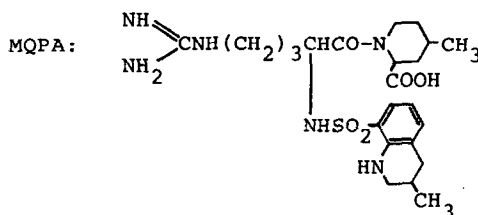
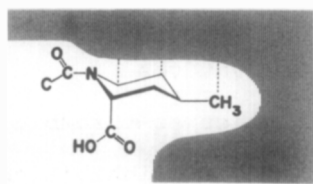| Configuration of 4MPA portion	Ki (μM)	I-50 (μM)
2R,4R	0.019	0.032
2R,4S	0.24	0.28
2S,4R	1.9	3.6
2S,4S	280	>150

Fig. 3. Binding of (2R,4R)-MQPA to thrombin.

the (2R,4R)-isomer was also the most potent inhibitor and the
other isomers showed weak inhibition comparable to those with
S-2238 as substrate. Thus, the inhibitory potency of MQPA was
shown to be highly related to the stereoconfiguration of both
the 2-COOH and 4-CH$_3$ groups of 4MPA.

DISCUSSION

The most potent inhibition by the (2R,4R)-isomer suggests
that the stereoconfiguration of the 2-COOH and 4-CH$_3$ groups in
the (2R,4R)-isomer is the best fitted to the binding site of
thrombin. The binding situation of the 4MPA portion of the (2R,4R)-
isomer at the binding site of thrombin may speculatively be illust-
rated in Fig. 3. Hydrophobic portions are considered to be securely
situated within the hydrophobic pocket constituted by some amino
acid residues of the thrombin molecule so as to make a hydrophobic
interaction firmer. The carboxyl group may be situated in the
direction to the open space, and possibly capable of interacting
with solvent water. In the (2R,4S) and (2S,4R)-isomers, the inter-
acting force of 4MPA portion would be partially reduced due to
the different stereoconfiguration at the 2 and 4 positions in
comparison with the (2R,4R)-isomer and especially the stereocon-
figuration of 4MPA portion of (2S,4S)-isomer would interfer pro-
bably sterically with binding of this portion to the narrow hydro-
phobic binding pocket. Thus, the present data clearly indicate
that the very narrow hydrophobic pocket exists in the vicinity of

the active site of thrombin to bind the carboxamide portion of N^2-substituted L-arginine derivatives.

One of the characteristics of thrombin inhibitory L-arginine derivatives was an highly selective inhibition of thrombin as reported earlier (11). No. 805, the (2R,4R)-isomer, also showed a selective inhibition of thrombin and detailed experiments are in progress and will be reported later.

ACKNOWLEDGMENTS

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