Vol. 101, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 30, 1981 Pages 440-446

POTENT INHIBITION OF THROMBIN BY THE NEWLY SYNTHESIZED ARGININE DERIVATIVE No. 805. THE IMPORTANCE OF STEREO-STRUCTURE OF ITS HYDROPHOBIC CARBOXAMIDE PORTION.

Shosuke Okamoto and Akiko Hijikata

Department of Physiology,
Kobe University School of Medicine, Kobe, Japan

Ryoji Kikumoto*, Shinji Tonomura, Hiroto Hara, Kunihiro Ninomiya, Akira Maruyama, Mamoru Sugano and Yoshikuni Tamao

Central Research Laboratories,
Mitsubishi Chemical Industries Ltd.,
Midori-ku, Yokohama, Japan

Received June 10, 1981

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 stereo-configuration 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-

---

* Please address reprint requests to this author.
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.

0006-291X/81/140440-07$01.00/0

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 approximately 15,000 times less inhibitory than No. 805. It was an unexpected result that a change in the stereostructure of the hydrophobic 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-piperidinecarboxylates: 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 established 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-piperidinecarboxylates: 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 epimerization 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 monohydrate, 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 Pharmaceutical 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.

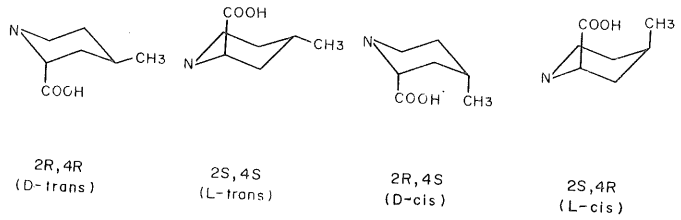


Fig. 1. The stereostructures of 4MPA portion of MQPA.

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-nitroaniline (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 recording spectrophotometer. Reaction cells were coated with silicone to prevent adsorption of thrombin to glass. The data were analyzed by Lineweaver-Burk plot.

RESULTS

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 stereoconfiguration 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 competitively 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

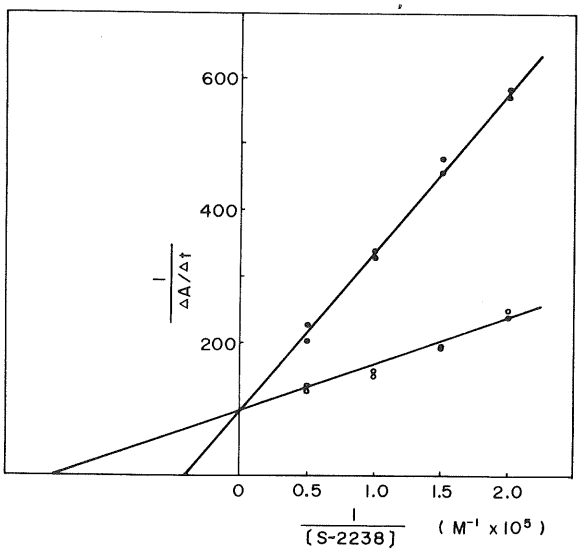
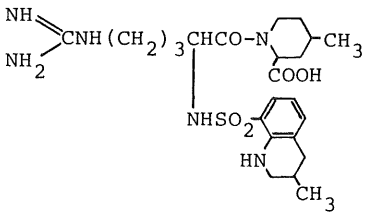


Fig. 2. Lineweaver-Burk plot of inhibition of bovine α-thrombin-catalyzed hydrolysis of S-2238.
o——o; In the absence of inhibitors.
●——●; In the presence of 0.05 μM (2R,4R)-MQPA.

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.

MQPA:



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

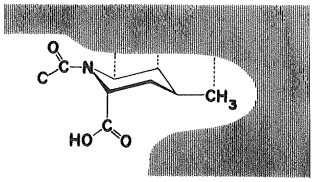


Fig. 3. Binding of (2R,4R)-MQPA to thrombin.

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 illustrated 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 interacting 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 stereoconfiguration of 4MPA portion of (2S,4S)-isomer would interfer probably sterically with binding of this portion to the narrow hydrophobic 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

The authors wish to thank Dr. S. Hattori, General Manager of Biosciences Laboratory, Central Research Laboratories, Mitsubishi Chemical Industries Ltd., for his valuable advice and encouragement throughout the work. We thank also Mrs. K. Sugano, Miss K. Okada, Mrs. K. Ikezawa and Mrs. E. Mori for their technical assistance.

REFERENCES

1. Tidwell, R. R., Geratz, J. D., Dann, O., Volz, G., Zeh, D., and Loewe, H. (1978) J. Med. Chem. 21, 613-623.
2. Glover, G., Wang, C., and Shaw, E. (1973) J. Med. Chem. 16, 262-266.
3. Hauptmann, J., Markwardt, F., and Walsmann, P. (1978) Thromb. Res. 12, 735-744.
4. Weinstein, M. J., and Doolittle, R. F. (1972) Biochim. Biophys. Acta 258, 577-590.
5. Okamoto, S., Hijikata, A., Kinjo, K., Kikumoto, R., Ohkubo, K., Tonomura, S., and Tamao, Y. (1975) Kobe J. Med. Sci. 21, 43-51.
6. Okamoto, S., Kinjo, K., Hijikata, A., Kikumoto, R., Tamao, Y., Ohkubo, K., and Tonomura, S. (1980) J. Med. Chem. 23, 827-830.
7. Kikumoto, R., Tamao, Y., Ohkubo, K., Tezuka, T., Tonomura, S., Okamoto, S., Funahara, Y., and Hijikata, A. (1980) J. Med. Chem. 23, 830-836.
8. Kikumoto, R., Tamao, Y., Ohkubo, K., Tezuka, T., Tonomura, S., Okamoto, S., and Hijikata, A. (1980) J. Med. Chem. 23, 1293-1299.
9. Bonnett, R., Clark, V. M., Giddey, A., and Todd, A. (1959) J. Chem. Soc. 2087-2093.
10. Lundblad, R. L. (1971) Biochemistry 10, 2501-2506.
11. Hijikata, A., Okamoto, S., Kikumoto, R., and Tamao, Y. (1979) Thrombos. Haemosts. (Stuttg.) 42, 1039-1045.