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Design and Synthesis of *para*-Fluorophenylalanine Amide Derivatives as Thrombin Receptor Antagonists

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An antagonist specific for the thrombin receptor is expected to be a remedy for thrombosis. Structure-activity studies of thrombin receptor-tethered ligand SFLLRNP have revealed the importance of the Phe-2-phenyl group in receptor recognition and the replacement of the Phe-2 by *para*-fluorophenylalanine [(*p*-F)Phe] was found to enhance its activity [Nose, T. *et al.* (1993) *Biochem. Biophys. Res. Commun.* 193, 694-699]. In order to obtain a small sized antagonist, a series of (*p*-F)Phe derivatives was designed and synthesized novel structural elements essential for receptor interactions being introduced at both the N and C-termini. β-Mercaptopropionyl (βMp) or its derivative activated by *S*-3-nitro-2-pyridinesulphenyl (Npys) was introduced at the N-terminus, and phenylmethyl amines were coupled to the C-terminus. All compounds were inactive when assayed for human platelet aggregation, indicating that they are not agonists. β-Mercaptopropionyl derivatives were also inactive as antagonists. However, Npys-containing analogs were found to inhibit the agonist activity of SFLLRNP. In particular, SNpys-βMp-(*p*-F)Phe-NH-R [R=-CH(C_6H_5)$_2$ and -CH_2-CH-(C_6H_5)$_2$] potently suppressed platelet aggregation. The results suggested that (*p*-F)Phe can be used as a structural core to construct an effective antagonist conformation.

Key words: antagonist, *para*-fluorophenylalanine, platelet aggregation, *S*-3-nitro-2-pyridinesulphenyl group, tethered ligand peptide, thrombin receptor.

The serine protease, thrombin, plays a central role in blood coagulation by not only converting fibrinogen to fibrin, but also by inducing platelet aggregation. Thrombin is also a potent agonist for various cells evoking various biological activities (*1, 2*). Most of these biological activities of thrombin are mediated through its specific functional receptors, which have been cloned from cells such as human platelets and endothelial cells, rat vascular smooth muscle cells, and hamster lung fibroblasts (*3-5*). The thrombin receptor is classified as a novel type in the family of seven transmembrane domain receptors, since in the receptor protein the ligand segment is present in the receptor molecule itself. When the receptor is cleaved by the enzyme, thrombin, at the peptide bond between Arg-41 and Ser-42, a newly exposed N-terminal fragment acts as a tethered ligand to activate the receptor (*3*). To date, three other receptors have been identified as such enzyme-activated receptors (*6-9*).

A synthetic heptapeptide, SFLLRNP (one letter amino acid code), corresponding to the tethered ligand of the thrombin receptor can activate the receptor without thrombin (*3, 10*). Studies on the structure-activity relationships of SFLLRNP have been performed to clarify the structural elements important for receptor activation, and several essential elements were defined. For instance, the amino group of Ser-1 at the N-terminus is important for full receptor activation (*11-13*). Leu-3 behaves as a connecting unit to construct a bioactive conformation, while Leu-4 interacts directly with the receptor (*14*). The guanidino group of Arg-5 is also important for the electrostatic interaction with the receptor acidic groups (*15*). The role of Phe-2 is particularly interesting, since the hydrogen atoms of the Phe-2-phenyl group were found to be essential for receptor activation (*10-12*).

In general, a receptor antagonist will be a useful tool for elucidating the molecular mechanism of receptor-mediated signal transduction. For the thrombin receptor, one would also be a remedy for thrombosis, because disordered platelet aggregation is often critical for thrombosis (*16*). In the molecular design of antagonists, the idea of introducing an additional structural element into the ligand is often valid to obtain an inhibitor which interacts with a functional

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Abbreviations: βMp, β-mercaptopropionic acid; Boc, *tert*-butoxycarbonyl; Cha, cyclohexylalanine; CIP, 2-chloro-1,3-dimethylimidazolidinium hexafluorophosphate; DIEA, *N,N'*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; EDC·HCl, 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide hydrochloride; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, hydroxybenzotriazole; HPTLC, high-performance thin-layer chromatography; Npys, 3-nitro-2-pyridinesulphenyl; (*p*-F)Phe, *para*-fluorophenylalanine; PPP, platelet-poor plasma; PRP, platelet-rich plasma; SFLLRNP, Ser-Phe-Leu-Leu-Arg-Asn-Pro amide; TFA, trifluoroacetic acid; Trt, trityl.

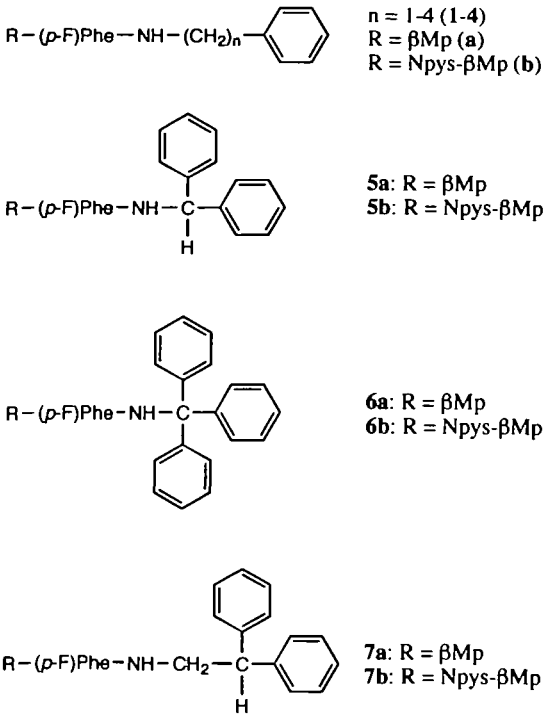

Fig. 1. **The structures of (*p*-F)Phe derivatives designed as antagonists for the thrombin receptor.**

group in the receptor molecule (*17-19*). If the interaction of such an element with the receptor is strong enough to prevent the conformational change required for receptor activation, then the molecule could be an antagonist. Recently, antagonist activity against the thrombin receptor was reported for β-mercaptopropionyl-Phe-Cha-Cha-Arg-Lys-Pro-Asn-Asp-Lys amide (*20, 21*), which was designed based on the structure of thrombin receptor-tethered ligand SFLLRNPNDK (residues 42-51). This β-mercaptopropionyl (βMp)-containing peptide inhibited, for example, the relaxation of pig coronary arteries with endothelium caused by SFLLRNP. The maximal relaxation caused by 100 μM SFLLRNP was suppressed 50-95% (*21*). Despite this, however, no systematic studies on thrombin receptor antagonists have been performed yet.

In our previous studies, the replacement of Phe-2 of SFLLRNP by *para*-fluorophenylalanine [(*p*-F)Phe] was found to enhance receptor activation several times (*10, 22*). In the present study, we designed and synthesized a series of derivatives of (*p*-F)Phe in which the N and C-termini were modified with β-mercaptopropionic acid and phenylmethyl amines, respectively (Fig. 1). Furthermore, the 3-nitro-2-pyridinesulphenyl (Npys) group was attached to the mercapto group of βMp, with the expectation that the resulting mixed disulfide, SNpys, interacts specifically with the receptor (*23*). The C-terminal phenylmethyl groups were expected to elicit hydrophobic interactions that prevent the receptor conformation from changing for activation. Here we describe the results of biological evaluation of these compounds through the human platelet aggregation assays.

MATERIALS AND METHODS

Materials—Benzylamine was purchased from Nacalai Tesque (Kyoto). Phenethylamine, 3-phenyl-1-propylamine, and 4-phenylbutylamine were purchased from Aldrich (Wisconsin, USA). Benzhydrylamine, triphenylmethylamine, and 2,2-diphenylethylamine were obtained from Tokyo Kasei (Tokyo). *N*-Hydroxybenzotriazole (HOBt), 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl), *N,N′*-diisopropylethylamine (DIEA), and 2-chloro-1,3-dimethyl-imidazolidinium hexafluorophosphate (CIP) were purchased from Watanabe (Hiroshima). 1-Hydroxy-7-azabenzotriazole (HOAt) was obtained from the Peptide Institute (Osaka). All other chemicals were of the best grade available.

Boc-(*p*-F)Phe-OH was prepared from H-(*p*-F)Phe-OH, which was donated generously by Asahi Glass (Tokyo). *S*-Trityl-β-mercaptopropanoic acid (*S*-Trt-βMp-OH) was prepared from triphenylmethyl chloride and β-mercaptopropanoic acid (Tokyo Kasei) according to the method of Zervas and Photaki (*24*). *S*-3-Nitro-2-pyridinesulphenyl-β-mercaptopropanoic acid (SNpys-βMp-OH) was synthesized from 3-nitro-2-pyridinesulphenyl chloride (Kokusan Chemical Works, Tokyo) and β-mercaptopropanoic acid by the method of Yasunaga *et al.* (*23*). The purity of all the compounds prepared in the present study was verified by HPLC, elemental analysis, and ^{1}H-NMR.

Methods—High-performance thin-layer chromatography (HPTLC) was carried out on Silica Gel 60 (Merck, Frankfurt, Germany) with a solvent system of $CHCl_3$-MeOH-AcOH (95:5:1, v/v). Optical rotation was measured with a Union High Sensitivity Polarimeter PM-71 (Union Giken, Osaka). The melting points of compounds were uncorrected.

Syntheses of Boc-(p-F)Phe Amide Derivatives—Boc-(*p*-F)Phe-OH (283 mg, 1 mmol), HOBt (184 mg, 1.2 mmol), and EDC·HCl (211 mg, 1.1 mmol) were added to a solution of aryl amines (1 mmol each) in DMF (10 ml) at 0°C. These amines comprised benzylamine, phenethylamine, 3-phenyl-1-propylamine, 4-phenylbutylamine, benzhydrylamine, triphenylmethylamine, and 2,2-di-phenylethylamine. The reaction mixture was stirred for 3 h at 0°C and overnight at room temperature, and then evaporated *in vacuo*. The residue dissolved in EtOAc was washed with water, 4% $NaHCO_3$, and 5% $KHSO_4$, and then dried over Na_2SO_4. The organic solution was evaporated, and the residue was crystallized from EtOAc-ether-petroleum ether.

For preparation of Boc-(*p*-F)Phe-NH-C(C_6H_5)$_3$, DIEA (0.68 ml, 4 mmol), CIP (280 mg, 1 mmol), and triphenylmethylamine (285 mg, 1.1 mmol) were added to a solution of Boc-(*p*-F)Phe-OH (283 mg, 1 mmol) and HOAt (34 mg, 0.25 mmol) in CH_2Cl_2 (8 ml) at 0°C (*25-27*). The reaction mixture was stirred overnight at room temperature and then evaporated. The residue on a glass filter was washed with 4% $NaHCO_3$, 5% $KHSO_4$, and water. Purification was carried out on a silica gel column (2.2×60 cm) eluted with $CHCl_3$-EtOAc (40:1) to afford the desired compound in a yield of 22%.

Syntheses of S-Trt-βMp-(p-F)Phe Amide Derivatives—Boc-(*p*-F)Phe amide derivatives (0.2 mmol each) were dissolved in trifluoroacetic acid (TFA) (1.5 ml) and then the reaction mixture was stirred for 1 h at 0°C. After evapora-

tion, the residue was crystallized from ether as trifluoroacetates. The coupling reactions for *S*-Trt-βMp-OH and H-(*p*-F)Phe amide trifluoroacetate were carried out as described for the preparation of Boc-(*p*-F)Phe amide derivatives. Several compounds were further purified on a silica gel column (2.2×60 cm) eluted with $CHCl_3$-MeOH (20:1) or $CHCl_3$-EtOAc (40:1).

Syntheses of βMp-(p-F)Phe Amide Derivatives (1a-7a)—To obtain free thiol-containing βMp-(*p*-F)Phe amide derivatives, the *S*-Trt group of *S*-Trt-βMp-(*p*-F)Phe amide derivatives was removed by treatment with an equivalent amount of silver nitrate-pyridine in an alcohol solution (*28*). *S*-Trt-βMp-(*p*-F)Phe amides (0.1 mmol each) in DMF (2 ml) in a round-bottomed flask covered with aluminum foil were added to a solution of silver nitrate (51 mg, 0.3 mmol) and pyridine (0.03 ml, 0.3 mmol) in methanol (2 ml), and then the reaction mixture was stirred for 4 h at room temperature. The precipitate obtained on the addition of diethyl ether was collected by filtration, washed with ether, and dried *in vacuo*. The resultant solid was suspended in 2 ml of methanol-DMF (1:1), and then β-mercaptoethanol (0.1 ml, 5-fold excess) was added to the solution. The mixture was stirred gently for 1 h at room temperature. The yellow solid was filtered off and the filtrate was poured into deoxygenated water. The resulting precipitate was collected by filtration, washed with ether, and dried. Recrystallization from methanol-ether afforded the desired compounds as white solid.

Syntheses of Npys-βMp-(p-F)Phe Amide Derivatives (1b-7b)—Boc-(*p*-F)Phe amide derivatives were treated with TFA for removal of the Boc group. The coupling reactions for Npys-βMp-OH (0.18 mmol) and H-(*p*-F)Phe amide trifluoroacetate (0.18 mmol) were carried out as described for the Boc-(*p*-F)Phe amide derivatives. In some cases, purification was carried out on a silica gel column (2.2×60 cm) eluted with $CHCl_3$-MeOH (20:1).

Synthesis of the Reference Antagonist Peptide βMp-Phe-Cha-Cha-Arg-Lys-Pro-Asn-Asp-Lys Amide (8)—Peptide synthesis was performed with an automated peptide synthesizer ABI 430A (Applied Biosystems, California, USA) with the Fmoc synthetic strategy using Fmoc-SAL resin (0.1 mmol scale). The peptide liberated by Reagent K (82.5% TFA, 5% phenol, 5% water, 2.5% ethanedithiol, and 5% thioanisole) was first purified by gel filtration (Sephadex G-10) with 30% AcOH and dithiothreitol (37 mg, 0.2 mmol), and then by preparative reversed-phase HPLC on a pre-packed column [LiChrospher RP-18 (e) 5 μm, 25×250 mm (Merck)] with a linear gradient of 0.1% TFA and 80% acetonitrile.

Human Platelet Aggregation Assay—Blood was obtained from healthy donors who had not taken any medication for the previous one week. The collected blood was anticoagulated with citrate (1 part 3.8% sodium citrate to 9 parts blood), and platelet-rich plasma (PRP) was obtained by centrifugation at 80×*g* for 10 min at room temperature. Platelet-poor plasma (PPP) was prepared by centrifugation at 2,000×*g* for 10 min at room temperature. The prepared PRP was used within 4 h, and the aggregation test was carried out at 37°C by the standard turbidimetric procedure using NKK hema tracer PAT-4M (MC Medical, Tokyo), PPP being used as a reference. The peptide concentration required for half-maximal platelet aggregation was obtained by three determinations. For antagonist activity in platelet aggregation, peptides were examined for suppression of the aggregation activity of SFLLRNP. Prior to addition of the agonist, SFLLRNP, a test compound was incubated with PRP for 3 min, and then the extent of aggregation was assessed with and without the test compound.

RESULTS AND DISCUSSION

Synthesis of (p-F)Phe Amide Derivatives—A series of (*p*-F)Phe amides (Fig. 1) was prepared by the two-step coupling procedure using water-soluble carbodiimide (EDC) in the presence of an additive, HOBt. As shown in Fig. 2, Boc-(*p*-F)Phe-OH was first coupled with various amines. In most cases, the corresponding Boc-(*p*-F)Phe amides were obtained in reasonable yields. However, when triphenylmethylamine, NH_2-$C(C_6H_5)_3$, was utilized as an amine, no coupling occurred with the EDC/HOBt method. This difficulty in coupling was assumed to be due to the steric hindrance of triphenylmethylamine. When steric hindrance affects the amide formation, 2-chloro-1,3-dimethylimidazolidinium hexafluorophosphate (CIP) is useful as a coupling reagent, as reported by Akaji *et al.* (*25, 27*). They also recommended the use of 1-hydroxy-7-azabenzotriazole (HOAt) as an additive instead of HOBt. Eventually, the target compound, Boc-(*p*-F)Phe-NH-C-$(C_6H_5)_3$, was obtained in a reasonable yield (22%).

After deprotection of the Boc group, the liberated (*p*-F)Phe amides were coupled with Npys-βMp-OH or Trt-βMp-OH by the EDC/HOBt method (43-65% yield). The resulting Npys-βMp-(*p*-F)Phe amides were utilized directly for the assays to evaluate the ability to cause the aggregation of platelets. For the preparation of βMp-(*p*-F)Phe amides having a free thiol, the Trt group of Trt-βMp-(*p*-F)Phe amides was removed by treatment with $AgNO_3$/pyridine followed by β-mercaptoethanol. SFLLRNP was synthesized by the standard Boc strategy of the manual solid phase method using Boc-amino acids and *p*-methylbenzhydrylamine resin, whereas a reference antagonist, βMp-Phe-Cha-Cha-Arg-Lys-Pro-Asn-Asp-Lys amide (**8**), was prepared by the Fmoc strategy of the automated solid phase method using Fmoc-SAL resin.

Evaluation of Agonist Activities of (p-F)Phe Amide

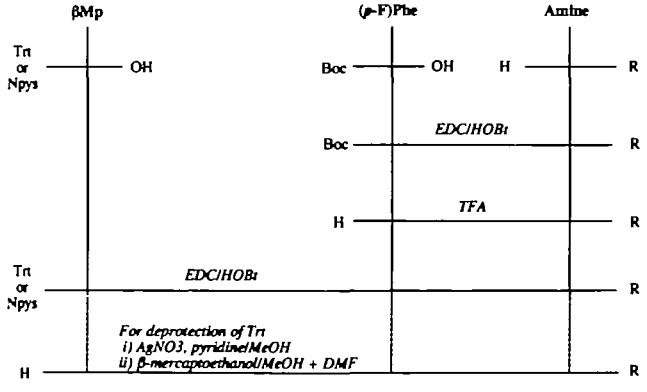

Fig. 2. **Synthetic schemes for (p-F)Phe amide derivatives.** Npys-βMp-(*p*-F)Phe amides were the final compounds in the synthesis, while Trt-βMp-(*p*-F)Phe amides were further treated for deprotection of the Trt group, as shown by broken lines.

Derivatives—Platelet aggregation was monitored by recording the change in transmittance, which was taken as 100% when platelet-poor plasma (PPP) was tested and 0% when platelet-rich plasma (PRP) was tested. Due to the poor solubility of compounds in an aqueous solution, they were dissolved in dimethyl sulfoxide (DMSO) and directly injected into PRP in the assay cuvette. No influence of DMSO on the platelet aggregation was confirmed for the activity of SFLLRNP. None of the analogs exhibited any agonist activity even at 100 μM. They did not cause the aggregation of platelets at all.

Antagonist Activities of (p-F)Phe Amide Derivatives—Because of the inactivity of (*p*-F)Phe amide derivatives in the human platelet aggregation assay, they were expected to exhibit antagonist activity. Thus, all (*p*-F)Phe amide derivatives were examined to determine to what extent they can suppress the platelet aggregation induced by agonist peptide SFLLRNP. A series of (*p*-F)Phe amide derivatives (100 μM) was incubated first with PRP for 3 min, and then the agonist, SFLLRNP (10 μM), was added. It was found that all compounds having a free mercapto group, βMp-(*p*-F)Phe amides, caused no aggregation of platelets. For example, as shown in Fig. 3A, compound βMp-(*p*-F)Phe-NH-CH_2-$CH(C_6H_5)_2$ (**7a**) did not affect the agonist activity of SFLLRNP at all.

In contrast, the platelet aggregation caused by SFLLRNP was suppressed completely by Npys-βMp-(*p*-F)Phe-NH-CH_2-$CH(C_6H_5)_2$ (**7b**) (Fig. 3B). Even several repeated injections of SFLLRNP did not elicit any platelet aggregation after treatment of PRP with compound **7b**. It should be noted that almost all the Npys analogs showed distinct suppression of aggregation (Fig. 4). Clearly, the Npys-containing derivatives are pure antagonists in this platelet aggregation assay, since they were unable to induce platelet aggregation by themselves. When the two strongest analogs, **5b** and **7b**, were examined in detail, they showed dose-dependent inhibition of the aggregation by SFLLRNP (Fig. 5). They were almost equipotent, with IC_{50} values of 52 ± 5.0 and 54 ± 5.0 μM, respectively.

Structure-Activity Relationships of Thrombin Receptor Antagonists—The amino group of Ser-1 of a thrombin receptor-tethered ligand peptide is one of the most important structural elements for eliciting agonist activity. In order to design an antagonist, Scarborough *et al.* (*20*) first eliminated this amino group of the tethered peptide, SFLLRNPNDK (residues 42–51). The resulting desamino-Ser derivative (Fig. 6) exhibited weak agonist activity in a platelet aggregation assay, but showed no inhibition of the agonist activity of SFLLRNP. Then, they replaced the

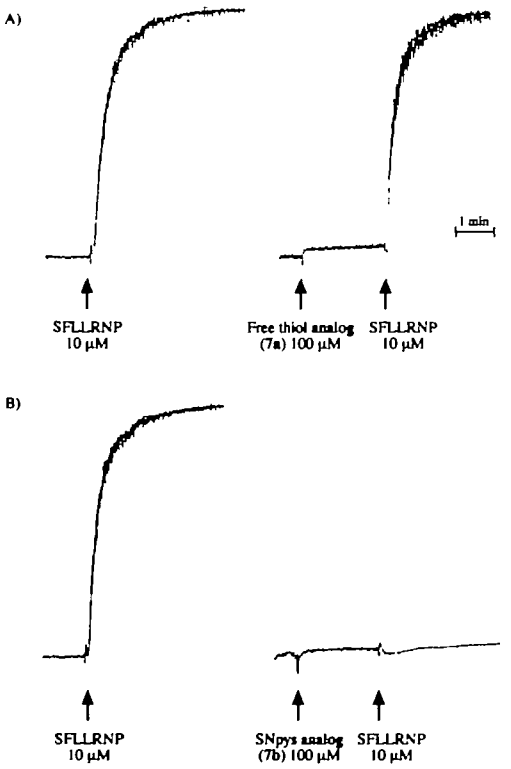

Fig. 3. **Inhibitory activity of (*p*-F)Phe amide derivatives against the agonist activity of thrombin receptor-tethered ligand peptide SFLLRNP in platelet aggregation.** (A) βMp-(*p*-F)Phe-NH-CH_2-$CH(C_6H_5)_2$ (**7a**), and (B) Npys-βMp-(*p*-F)Phe-NH-CH_2-$CH(C_6H_5)_2$ (**7b**).

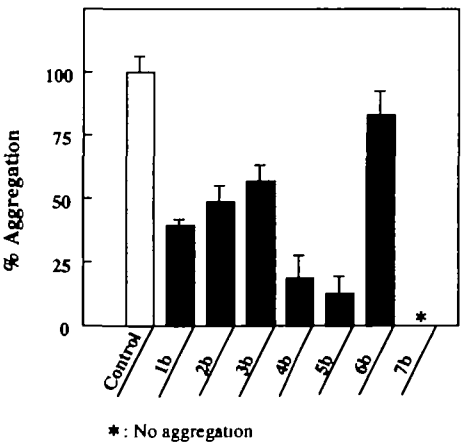

Fig. 4. **Relative inhibitory activity of Npys-βMp-(*p*-F)Phe amide derivatives.** The concentration of the test compounds was 100 μM, and that of agonist SFLLRNP was 10 μM. Control, full aggregation activity induced by 10 μM SFLLRNP.

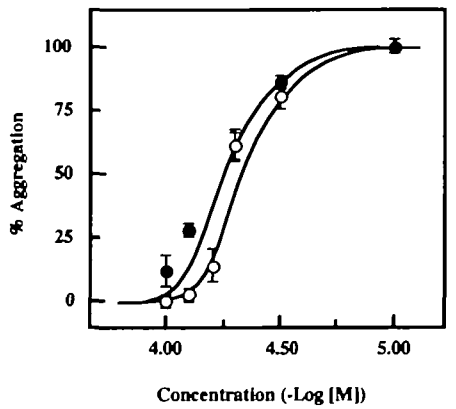

Fig. 5. **Dose-dependent inhibition of human platelet aggregation by Npys-βMp-(*p*-F)Phe-NH-$CH(C_6H_5)_2$ (5b) and Npys-βMp-(*p*-F)Phe-NH-CH_2-$CH(C_6H_5)_2$ (7b).** The lines connecting (●) and (○) indicate the curves for compounds **5b** and **7b**, respectively. The agonist tested was 10 μM SFLLRNP.

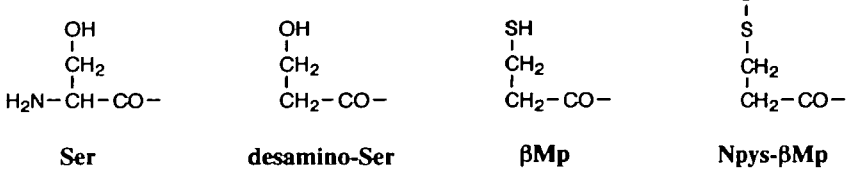

Fig. 6. **The N-terminal structures of thrombin receptor-tethered ligand peptides for designing the antagonists.**

hydroxyl (OH) group with a mercapto (SH) group, and detected weak antagonist activity for the resulting βMp-containing peptide. With further chemical modifications, Scarborough *et al.* obtained βMp-Phe-Cha-Cha-Arg-Lys-Pro-Asn-Asp-Lys amide (**8**) as a relatively potent antagonist (*20*). In this peptide, the parent Leu-3 and 4 residues are replaced by cyclohexylalanine (Cha) and Asn-5 by Lys.

We synthesized compound **8** as a reference and tested it in the human platelet aggregation assay. However, it was found that in our assay system compound **8** is extremely weak, not fully suppressing the agonist activity of SFLLR-NP. In addition to the report of antagonist activity in the platelet aggregation test by Scarborough *et al.* (*20*), Tesfamariam (*21*) found antagonist activity of compound **8** in an assay system for measuring the relaxation of pig coronary arteries with endothelium. The reason for the discrepancy in the assay results is not clear.

Our original idea to for obtaining a novel type of antagonist in the present study was to replace the C-terminal octapeptide sequence of this peptide, **8**, with structurally simple hydrophobic amide groups. This would give a very small sized antagonist with the structure of βMp-Phe amide. Substituting Phe with (*p*-F)Phe, we first prepared βMp-(*p*-F)Phe benzylamide (**1**). Compound **1** was inactive as an agonist and also inactive as an antagonist. An increase in the number of phenyl groups in the amide portion of **1**, creating a diphenylmethyl amide or triphenyl methyl amide, did not elicit antagonist activity. All βMp-(*p*-F)Phe amides were found to be inactive as both agonists and antagonists. Apparently, it is hard to expect specific receptor interactions for the β-mercapto (SH) group. In order to activate or modify the mercapto group, we took advantage of the structure of mixed disulfide bond SNpys group, which is able to react with a free mercapto group.

Eventually, all derivatives having the Npys-βMp group were found to function as antagonists. The results clearly indicate that one of the major forces that induces antagonism is the presence of this Npys-βMp group. The Npys group thus likely participates in specific interactions with particular receptor groups. The Npys group has a pyridine ring connected to a β-mercaptoyl group by a disulfide bond (Fig. 6). Two different kinds of interactions are feasible between the Npys group and the receptor groups. One is the formation of a disulfide bond with a free thiol group of the receptor. The Npys group attached to the mercapto group, namely, a mixed disulfide bond, only reacts with a free mercapto group *via* the thiol-disulfide exchange reaction (*23, 29*). The other is an hydrophobic interaction based on the hydrophobicity of Npys itself. The aromatic π-characteristic pyridyl group would participate in two different types of interactions; *i.e.*, π-π and alkyl-π interactions. These interactions might be strong enough to hold Npys-βMp-(*p*-F)Phe amides on the receptor, resulting in exclusion of an agonist from the binding sites. Although the antagonist activity reported for N-terminal *trans*-cinnamoyl peptides suggested the existence of a hydrophobic interaction between the peptide ligand and the receptor (*30*), what type of interaction is dominant in the antagonist activities of Npys-βMp-(*p*-F)Phe amides is not clear at this moment.

Potent antagonists, **5b** and **7b**, found in the present study possess a biphenylmethyl group, $CH(C_6H_5)_2$, at their C-terminis. A C-terminal biphenylmethyl group was also found recently by Rockwell *et al.* (*31*) for an enzyme inhibitor in a combinatorial chemistry study. The compound having the biphenylmethyl group inhibited strongly human stromelysin, a matrix metalloproteinase, and two phenyls of the biphenylmethyl group were found to each bind to different enzyme binding sites. Similarly, it is likely that the C-terminal two phenyl groups in compounds **5b** and **7b** interact specifically with different sites on the receptor.

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