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MODIFIED THROMBIN RECEPTOR-AGONIST PEPTIDE LIGANDS. SYNTHESIS AND CONFORMATIONAL ANALYSIS OF ANALOGS OF THE N-TERMINAL TRIPEPTIDE REGION

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Abstract: Analogs **2-24** of the N-terminal tripeptide region of the tethered peptide ligand for human thrombin receptor have been synthesized for elucidation of the receptor bound conformation of the ligand peptide and the conformational analysis of model structures **25-30** of those peptide analogs suggested a plausible conformation for the receptor bound structure of the tripeptide region.

A serine protease thrombin activates a variety of cells such as platelets, endothelial cells and vascular smooth muscle through proteolytic cleavage of the extracellular N-terminus bond between Arg41 and Ser42 of human thrombin receptor.[1-3] The newly generated N-terminal region then functions as a "tethered ligand", probably interacting with an, as yet, unidentified site in the remaining extracellular sites or the transmembrane region of the receptor. In support of this activation model, antibodies which recognize the cleavage site have inhibited the effects of thrombin[4-7] and peptides having the N-terminal 14 residues have been shown to act as agonist ligands on the thrombin receptor.[1]

Since Vu *et al* showed the activity of the N-terminal tetradecapeptide as a ligand peptide for the thrombin receptor,[1] the minimal sequence requirement has been determined using fragments of this peptide[8] and essential groups for the activation of the receptor have been examined using substitutions or modifications of selected amino acids and "alanine scan" experiment.[9-11] In human platelet aggregation assay, the pentapeptide (SFLLR) showed a potent activity although the tetrapeptide (SFLL) still retained the activity slightly.[8] The alanine scan experiment has indicated that Phe2 is the most essential residue in the peptide and the residues of Leu4 and Arg5 are also important for the activity.

Structures of peptides around the N-terminal sequence (SFLLRN) have been investigated by NMR spectroscopy.[12] The five N-terminal residues of the tetradecapeptide (thrombin receptor 42-55) were unstructured in solution although a 3_{10} helix structure was observed for the five residues in a longer peptide (thrombin receptor 33-55) which covers the cleavage site. Crystallographic structure of thrombin receptor peptide(38-56) complexed with thrombin has exhibited a β-strand-like structure for the five N-terminal residues (SFLLR) with a kink at Leu3 (thrombin receptor 44) and a turn structure at Leu4-Pro7 (thrombin receptor 45-48) region.[13] Thus, the tethered ligand peptide appears to take a variety of conformations depending on conditions and then it is difficult to deduce a receptor bound conformation of the peptide from those structural data.

Herein we wish to report the synthesis of the modified peptides particularly around the Phe2 residue which is the most important residue in the thrombin receptor ligand of the hexapeptide (SFLLRN) and the

synthesis of the substituted peptides at position 3. We also report an examination of a plausible receptor bound conformation of the main chain of the N-terminal tripeptide (SFL), using model structures **25-30**.

All the peptides were prepared by methods of a standard solid-phase peptide synthesis on Rink resin using N^{α}-Fmoc protection. The N-terminus acyl groups (**1-6**), 1-tetrahydroisoquinolinecarboxylic acid (Tic) (**9** and **10**), aminoisobutyric acid (Aib) (**13**), and α- or β-methylphenylalanine (**20-24**) were coupled manually, using benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP)[14] as a coupling agent. N-Methyl-peptide bonds in compounds **7** and **8** were formed by use of bromo-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBroP)[15] as the coupling agent. N-Terminus alkyl groups (**16-19**) or pseudopeptide linkage (ψCH_2NH) were introduced to the N-terminus deblocked-peptide resin by the reductive alkylating method using N^{α}-*t*Boc amino aldehyde and $NaBH_3CN$ in DMF containing 1%AcOH. Crude peptides were purified by reversed-phase HPLC using linear gradient mode of H_2O-CH_3CN containing 0.1%TFA. Structures were identified by FAB-MS. A mixture of the diastereomeric peptides, having the racemic α-methylphenylalanine or β-methylphenylalanine (threo:erythro=2:1), were separated by reversed-phase HPLC. The configuration of the methylphenylalanines were determined by comparison of the authentic samples with HPLC after hydrolysis of the peptides.[16,17]

Table 1. Agonist Activity of the N-Terminal-Hexapeptide Derivatives in Human Platelet Aggregation†

A_1-Phe-Leu-Leu-Arg-Asn

compd.	A_1	ED_{50}(μM)
1.	Ser	0.92± 0.14
2.	Formyl	62.4 ±11.0
3.	Acetyl	84.5 ±4.56
4.	Propionyl	35.0 ±0.23
5.	*n*-Butyryl	75.1 ±3.58
6.	Lactyl	49.0 ±8.98
16.	N-Me	>1000
17.	N,N-Me_2	>1000
18.	N-Et	>1000
19.	N,N-Et_2	>1000

Ser-A_2-Leu-Leu-Arg-Asn

compd.	A_2	ED_{50}(μM)
7.	N-MePhe	>1000
9.	Tic	>1000
14.	ψ-Phe(a)	382 ±148
20.	α-MePhe	169 ±49
21.	βt-MePhe(b)	2.00± 1.14
22.	βe-MePhe(c)	27.0 ±4.50

Ser-A_2-Pro-Leu-Arg-Asn

compd.	A_2	ED_{50}(μM)
23.	βt-MePhe(b)	1.60± 0.4
24.	βe-MePhe(c)	216 ±2.0

Ser-Phe-A_3-Leu-Arg-Asn

compd.	A_3	ED_{50}(μM)
8.	N-MeLeu	70.0 ±75
10.	Tic	4.60± 1.50
11.	Pro	5.30± 1.70
12.	Gly	14.8 ±2.90
13.	Aib	>1000
15.	ψ-Leu(d)	>1000

a: $\psi[CH_2NH]$-Phe
b: β-threo-MePhe
c: β-erythro-MePhe
d: $\psi[CH_2NH]$-Leu

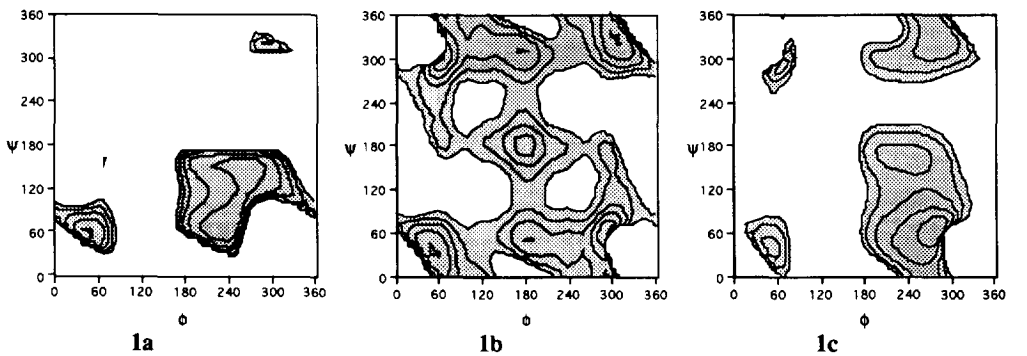
† A minimum of three determinations was performed for each experiment.

Table **1** shows a list of the modified peptides and the potency of those peptides in human platelet aggregation assay.[18] Peptides **2-5** having acyl groups such as formyl, acetyl, propionyl and *n*-butyryl substituted for Ser1 retained the activity. Substitution of lactyl group **6** for Ser1 was also effective in the platelet aggregation. It has been suggested that N-terminal amino group is essential for the activity.[19] However, elimination of the amino group or substitution of hydroxyl group for the amino group was still

effective in the activity though those derivatives **2-6** reduced their potency 40-90 times. This result was consistent with those reported previously.[20,21] The dramatic reduction of the activity due to modification of the peptide bond by N-alkylation (compounds **7** and **9**) and reduction of the carbonyl group(pseudopeptide analogs **14** and **16-19**) indicates that the peptide bond between Ser1 and Phe2 should be involved in an essential interaction with the receptor or another part of the ligand peptide for the activity.

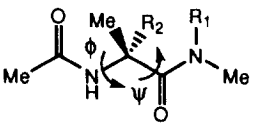
The Tic3 and Pro3 peptides **10** and **11** substituted for Leu3 retained high potencies, indicating that the main-chain conformations of Pro and Tic meet the requirement for the torsion angle of Leu3 for the receptor binding. Although presence of the *cis* peptide bond in **11** was suggested from its NMR spectrum (*cis*/*trans*=1/9), it is more plausible that the peptide bond between Phe2 and Pro3 for the receptor binding has *trans* conformation since the Pro3 analog **11** showed a slightly reduced potency. Since the N-methyl peptide **8** was about 80 times less potent than the parent peptide **1**, the intramolecularly alkylated back-bone structure is necessary for the receptor binding structure. The substitution of glycine residue for Leu3 afforded an active peptide **12** whereas the peptide **13** substituted by α,α-dimethylglycine (α-amino isobutyric acid, Aib) for Leu3 was inactive. The pseudopeptide **15** also completely lost the activity.

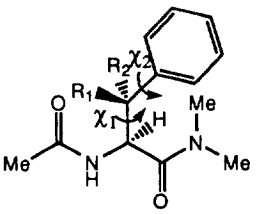
Introduction of methyl group at C_α or C_β position of Phe2 reduced their activity. L-α-MePhe peptide **20** was 200 times less active while C_β-methylated peptides **21** and **22** retained better potency than the C_α-methylated peptide **20**. Of the two C_β-methylated peptides, L-threo-β-MePhe peptide **21** showed a similar potency to the parent peptide **1** although L-erythro-β-MePhe peptide **22** was about 30 times less active. The substitution of Pro for the Leu3 residue of the threo-β-MePhe peptide gave an equi-potent peptide **23** with the parent peptide **1** while the same substitution in the erythro-β-MePhe peptide **24** reduced the potency about 200 times. All of the related peptides having D-isomers at position 2 or 3 were devoid of the activity (data not shown). The large decrease of the potency by introduction of methyl group at C_α of Phe2 would be due to perturbation of the main chain conformation. Of the four β-MePhe-peptides, preference of the threo-peptides **21** and **23** to the erythro-peptides **22** and **24** by ten to 100 times suggests that the phenylalanine residue favors a specific side-chain conformation in the receptor binding. Thus, the "methyl walk" on the Phe2 residue (Compounds **7**, **8**, **20**, **21**, **22**, **23**, and **24**) afforded the intriguing structure-activity information for elucidation of the receptor bound conformation of the phenylalanine of the peptide **1**.



Figures 1a-c. Energy Maps for the Main-Chain Conformations for the Model Fragments **25**, **26** and **27**.[23]

A torsional mobility of the main chain of the phenylalanine residue was represented by the low-energy areas in the conformational energy map for N-acetyl-N',N'-dimethylalaninamide (**25**) where N',N'-dimethylamine part represents the Pro3 residue. This model neglects an influence of the aromatic group on the main-chain conformation by omitting the group. Main-chain conformations of N-acetyl-N'-methylalaninamide (**27**) representing the Phe residue of the parent peptide **1** should share some low-energy areas with those of **25**. Besides, the low-energy conformations of N-acetyl-N'-methyl-dimethylglycinamide (**26**), representing the α-MePhe main chain should be unfavored for the receptor binding (Figure **1a-c**). Common areas of the low-energy conformations of the two model compounds **25** and **26** were then omitted as unfavored receptor bound conformations. Hence, we found a conformation at a common local energy minimum for **25** and **27** with torsion angles (ϕ=250° and ψ=160°) as a plausible receptor bound conformation of the Phe2 main chain. The main-chain conformation of Phe2 is commonly found in extended structures of proteins.





25. R_1=Me, R_2=H
26. R_1=H, R_2=Me
27. R_1=H, R_2=H

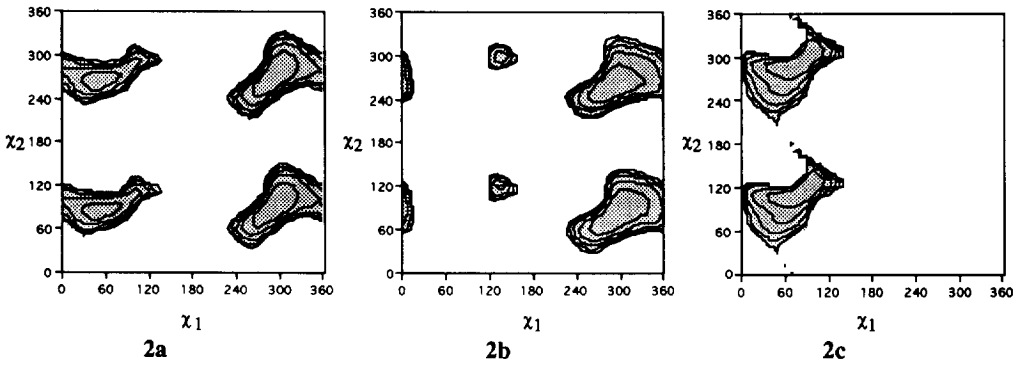
28. R_1=H, R_2=H
29. R_1=H, R_2=Me
30. R_1=Me, R_2=H

N-Acetyl-N'-methyl-dimethylglycinamide (**26**) was used as the model structure for the Aib3 in the inactive peptide **13**. The approximate observed range (ϕ=270°~300°) of torsion angles in proline-containing structures from the Cambridge structural Database[22] was adapted for the Pro3 of the active peptide **11**. A pair of candidate-torsion angles (ϕ=270° and ψ=120°) in a low-energy area of **27**, a Leu3 model, was selected comparing the energy ranges for those model structures **26** and **27** (Figure **1b** and **1c**) and the torsion angle range for the Pro3. The torsion angle (ψ=120°) belongs to that for an extended structure whereas the other torsion angle (ϕ=270°) implies a bent conformation at this site.

A low-energy conformation of the threo-β-MePhe2 peptides (**21** and **23**) should adapt to the receptor bound structure of the parent peptide **1**. On the other hand, the conformation close to the receptor bound structure would be unfavorable for the erythro derivatives (**22** and **24**) due to a steric interaction by the Cβ-methyl group. Thus, low-energy conformations of the threo peptide **29** at local minima unoverlapped with those of the erythro peptide **30** would be candidates for the receptor bound structure.

Figure **2a-c** shows the low-energy areas of the conformational-energy maps for N-acetyl-N',N'-dimethyl phenylalaninamide (**28**) and for N-acetyl-N'N'-dimethyl-(threo and erythro)-β-methylphenylalaninamide (**29** and **30**) at the favored main-chain conformation of Phe2 (ϕ=250° and ψ=160°). Those models (**29** and **30**) correspond to the Ser-threo-β-MePhe-Pro fragment of the highly active peptide **23** and the Ser-erythro-β-MePhe-Pro fragment of the less active peptide **24**, respectively. The energy maps in the Figures **2a-c** imply that a conformation lying in the area where a local energy minimum is located at χ_1=290° and χ_2=90° (270°) is a favored receptor bound conformation for the side chain of the Phe2 residue. It has been demonstrated that

conformation of the threo-β-MePhe residue incorporated in a bioactive peptide equilibrates between *trans* (χ_1=180°) and *gauche*(-) (χ_1=300°) in solution.[25] The present result indicates that the χ_1 for the Phe2 in **11** is within the area for the threo-β-MePhe. Since the model structures **28-30** have the N,N-dimethylamide moiety, the aromatic group will have a severe steric interaction with this moiety to result in a *gauche* conformational area (χ_1=~300°).



Figures 2a-c. Energy Maps for the Side-Chain Conformations for the Model Fragments **28, 29** and **30.** [23]

In conclusion, we elucidated the plausible conformation of the N-terminal tripeptide region for the receptor binding of the tethered ligand. The N-terminal Ser residue could be replaced by a simple acyl moiety such as formyl and acetyl moieties. The Phe2 residue has the limited conformation upon binding to the receptor. In particular, its main-chain torsion angles would be within those of an extended conformation. The Leu3 residue would bind to the receptor, having a similar torsion angle (ϕ) to that of proline and the other torsion angle (ψ) close to that of a β-structure. Trans configuration was assumed to the two peptide bonds based on the activity of the peptide analogs with modified-amide bond such as the N-methyl amino acids, proline, or Tic. Further elucidation of the conformation of the remaining part of the tethered peptide region would reveal a whole structural requirement for the receptor binding.

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