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DESIGN, SYNTHESIS, AND EVALUATION OF FIBRINOGEN INHIBITORS, ω-(p-AMIDINOPHENOXY)ALKANOYLASPARTIC ACID DERIVATIVES

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Abstract: Low molecular weight inhibitors of platelet aggregation are described. Compounds studied in this work were derived from the presumed active conformation of the adhesion tripeptide, ArgGlyAsp, using computer simulations. These studies led to the synthesis of a potent anti-platelet agent, 4-(4-amidinophenoxy)-butanoylaspartylvaline (**6**, FK633). Copyright © 1996 Elsevier Science Ltd

Uncontrolled platelet aggregation and platelet adhesion to the subendothelium of damaged blood vessels causes life-threatening diseases such as myocardial infarction,[1] transient ischemic attack,[1] and unstable angina.[2] Medicinal studies have elucidated both physiological mediators and synthetic inhibitors of the platelet aggregation cascade. One novel approach to thrombosis treatment and prevention arose from the remarkable finding that only a small amino acid sequence, ArgGlyAspSer (RGDS), mediates the von Willbrand factor-platelet interaction.[3] Subsequent studies have found that numerous adhesion reactions are RGD-dependent as well.[4] During platelet aggregation, the RGD sequence of fibrinogen binds activated heterodimer glycoprotein receptor GPIIb/IIIa on the surface of platelets.[5] This is thought to be the most critical step in platelet aggregation and several types of fibrinogen inhibitors have been reported in the past few years. Among them, GPIIb/IIIa antibody (c7E3) effectively enhances the rate of thrombolysis with tissue plasminogen activator (t-PA) and prevents reoccusion,[6] and reduces clinical restenosis after coronary angioplasty while non fibrinogen inhibitors have failed.[7] The general utility of this antibody, however, may be limited by its immunogenecity[8] and slow reversibility.[6] Hence several chemical approaches have also been studied to develop small fibrinogen inhibitors[9-12] which would inhibit the binding between the GPIIb/IIIa receptor and fibrinogen.

Conformation studies on the RGD moiety of several RGD containing peptides were carried out[13] to presume active conformation of the RGD moiety for rational design, showed that the RGD moiety constructs kind of turn like structure such as C7 turn,[13a] γ-turn,[13b] or β-turn[13c-e] in solution state or crystal state, which indicated their active conformation is near kind of turn structure.[14]

We here describe the design of ω-(p-amidinophenoxy)alkanoylaspartic acid derivatives using computer simulations, in which the active conformation of the RGD peptide was presumed to be a type II' β-turn, their synthesis, and their anti-platelet activity.

Design of Novel Fibrinogen Inhibitors: We initially designed an ideal compound (**1**) from the structures of reported fibrinogen inhibitors (Figure 1).[9-12] Compound **1** consists of three moieties: 1) two end groups vital for its activity (benzamidine and Asp), 2) a spacer moiety which correctly positions the two end groups, and 3) an amino acid derivative at the carboxy terminal. In order to select the length of the spacer moiety of compound **1** (m in figure 1), we used computer simulations. In our computer simulation,[15] each

compound **3** derivative, having m=2, 3(**1a**), 4(**1b**), or 5, was superimposed on the RGD peptide to position the two vital functional groups (amidino and carboxylic acid) of the both compounds (broken lines, left half, in Figure 1).

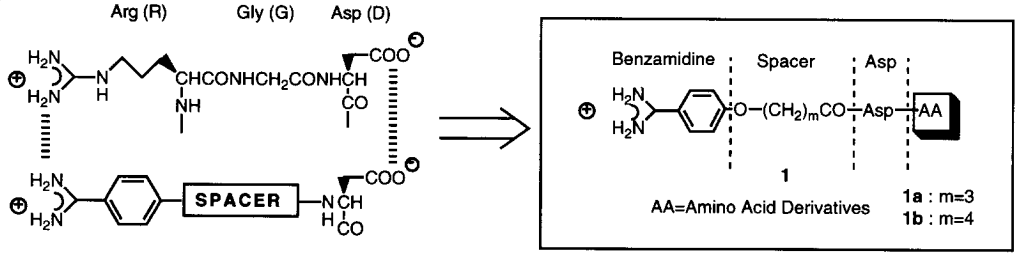


Figure 1. Tailor of a lead compound **1**

The conformation of the RGD peptide in this simulation was fixed to a type II' β-turn[17a] in which the Gly positions at (i+1)th[17b] according to the following two reasons: 1) an NMR study on a RGD protein, decorsin, recently showed that the solution structure of the RGD moiety is a distorted type II' β-turn[18] while the previous reports[13c-e] on the RGD conformation did not mention their subtype of β-turn. 2) Assuming a type II' β-turn as the active conformation of the RGD sequence, the evolutionary meaning of conservation of the Gly residue in the RGD sequence is clear; that is, Gly is conserved in a wide species range of adhesion proteins[4] in spite of the lack of any functional groups, in contrast with the other two conserved amino acids (Arg and Asp) which have functional groups that are able to interact with the receptor (GPIIb/IIIa) through strong hydrogen bonds. From this it can be thought that Gly plays an important role in maintaining the active conformation. While there are innumerable conformations, the type II' β-turn is thought to be the most suitable as the active conformation since it is well-known that Gly often appears in the (i+1)th position of type II' β-turn.[17c] Hence we decided to set the active conformation of the RGD sequence in our computer simulation studies to be a type II' β-turn in which Gly is positioned at (i+1)th.

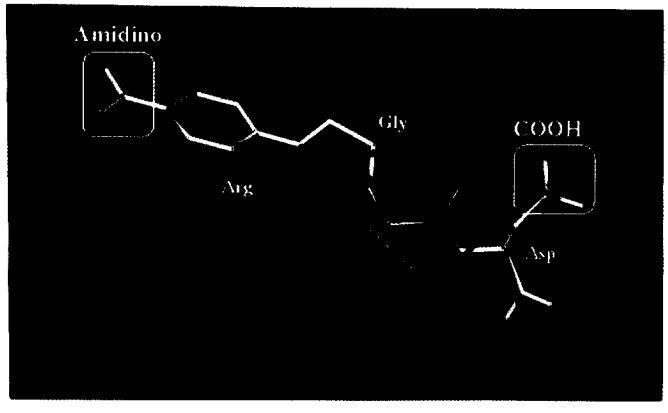


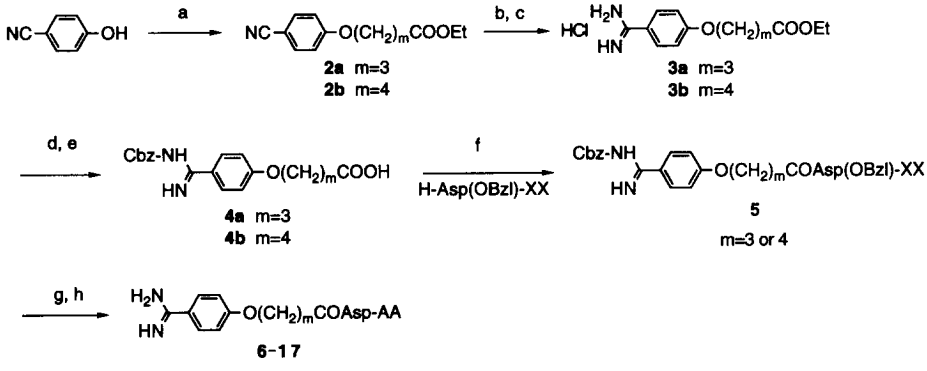
Figure 2. Two energetically indistinguishable conformations of compound **1** (m=3 (**1a**); cyan, m=4 (**1b**); yellow) which were obtained by means of superimposition studies with type II' β-turn of the RGD sequence (pink). The Gly positions at the (i+1)th of type II' β-turn (see text). AA moiety in compound **1** is not shown.

Computer simulation studies provided two energetically indistinguishable answers: m=3 and 4 (Figure 2), in which the two vital moieties position at the same place with reasonable conformations (energy loss of these conformation is less than 5 kcal/mol compared with the their global minimum conformation). Hence we decided to synthesize 4-(4-amidinophenoxy)butanoylaspartic acid (m=3: **1a**) and 3-(4-amidinophenoxy)pentanoyl-aspartic acid derivatives (m=4: **1b**), and modified only C-terminal moiety of these compounds (AA in Figure 2) because the C-terminal moiety is known to be important for potent inhibitory activity.[19]

Synthesis: The general preparation of the desired derivatives **6–17** is outlined in Scheme 1. We utilized t-butoxycarbonyl (Boc) as a temporary protecting group and benzyloxycarbonyl (Cbz) and benzyl (Bzl) ester for the other protecting groups.

Alkylation of 4-cyanophenol with ethyl ω-bromoalkylcarboxylate in the presence of K_2CO_3 afforded 4-(ω-ethoxycarbonylalkoxyl)benzonitriles (**2**). Imidation of **2** with HCl in ethanol and subsequent coupling with ammonia in refluxing ethanol gave 4-amidinophenyl derivatives (**3**). Introduction of the Cbz group onto the amidino moiety and hydrolysis of the ethyl ester provided acids **4**. Coupling of **4** with protected Asp derivatives by 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide (EDC) and 1-hydroxybenzotriazole (HOBt) gave **5**, which were deprotected via hydrogenation using 10 % palladium on charcoal to give crude desired compounds (**6–17**), which were purified *via* reverse phase HPLC for bioassays.[20]

Scheme 1.



Reagents: (a) Br-$(CH_2)_n$-COOEt, K_2CO_3/DMF, 40–45 ℃, 4 h; (b) HCl/EtOH, -5 ℃ to rt, overnight; (c) NH_3/EtOH, reflux, 2.5 h; (d) Cbz-Cl, NaOH, H_2O, at pH=8.5–9.0 (Schotten-Baumann); (e) conc. HCl, rt, 2.5 h; (f) HOBt, EDC/DMF, rt, 6 h; (g) H_2, 10 % Pd–C/THF, 1 N HCl, rt, 4.5 h; (h) purification *via* RP-HPLC. Cbz=benzyloxycarbonyl; XX=protected amino acid derivatives; AA= amino acid derivatives.

Biological Activity: The inhibitory activities of these compounds against human platelet aggregation induced by ADP were measured first.[21] The potent compounds from the first screening were then tested in an inhibition study of fibrinogen attachment to platelets to confirm that the anti-platelet activity of these compounds was due to inhibition of fibrinogen binding[22] (Table 1).

We first synthesized compound **13** in which m=4 and AA=Ser in Figure 1 (**1b**). The reason for the selection of Ser as the AA moiety is that RGDS peptide, the original active peptide, has a Ser residue at the same position.[3] Compound **13** showed an IC_{50} = 12 μM for inhibition of platelet aggregation induced by ADP, which is six times stronger activity than that of the RGDS peptide (IC_{50} = 75 μM) as shown in Table 1. This result

showed that replacement of the ArgGly moiety of the RGDS peptide to a 4-amidinophenoxyalkanoyl moiety is effective as indicated by computer simulation. Replacement of Ser, a hydrophilic amino acid, to hydrophobic amino acids such as Val or Tyr provided **14** (IC_{50}=0.23 μM) and **15** (IC_{50}=0.64 μM), respectively. These inhibitory activities are 52-fold and 19-fold higher in potency compared with **13**. A similar effect on the C-terminal moiety on anti-platelet activity has been observed in structure-activity relationships studies on the RGDS peptide.[18] These results indicated that Ser is not vital, and hydrophobic amino acids are more suitable as the AA moiety, which encouraged further modifications of the AA moiety. Methylation of phenolic hydroxy of compound **15** was carried out to increase its hydrophobicity, which provided compound **16** with a 2-fold increase in potency. Esterification of the C terminal of compound **15** resulted in much decreased potency (**17**, IC_{50}=4.5 μM).

Next, removal of one methylene unit in compound **14** was carried out to give 4-(4-amidinophenoxy)-butanoylaspartylvaline (**6**), and resulted in an IC_{50}=0.10 μM activity. The activity of compound **6** is 2-fold more potent than that of **14**. Similarly, shortening the spacer moiety in compound **15** increased the potency 4 times (**12**, IC_{50}=0.16 μM). Replacement of Val with other aliphatic amino acids (**7**–**10**) whose hydrophobic property may be similar to that of Val gave similar results in potency. The potency of compounds whose m=3 (**1a**, **6**–**12**) is similar to that of compounds whose m=4 (**1b**, **13**–**17**). These results are consistent with the results of the our computer simulation, that is, compounds whose m=3 or 4 are indistinguishable.

Table 1. Structures and their Inhibitory Activities

compd.	m[a]	AA[a]	Inhibitory Activities[b] IC_{50} (μM) Platelet Aggregation[21]	fibrinogen binding[22]
6	3	ValOH	0.10±0.01	0.088±0.01
7	3	NleOH	0.076±0.07	
8	3	IleOH	0.14±0.02	
9	3	LeuOH	0.13±0.02	
10	3	γ-Me-LeuOH	0.23±0.04	
11	3	Tyr(Me)OH	0.12±0.06	
12	3	TyrOH	0.16±0.01	
13	4	SerOH	12±0.09	
14	4	ValOH	0.23±0.05	0.23±0.05
15	4	TyrOH	0.64±0.13	0.64±0.01
16	4	Tyr(Me)OH	0.27±0.08	0.27±0.08
17	4	TyrOMe	4.5±0.9	
RGDS			75±12	95±10

[a]See Figure 1. [b]Three determinations are at least made for each compound.

Compounds (**6**, **14**-**15**) which showed potent inhibitory activity in the above screening were assayed in fibrinogen binding assay (Table 1). The inhibitory activity of these compounds is similar to that in the first screening, which indicated that the anti-platelet activity of these compounds is due to inhibition of fibrinogen binding to GPIIb/IIIa on the surface of platelets. Among these compounds, compound **6** (FK633) was selected as an clinical compound because of its potent anti-platelet activity based on fibrinogen binding inhibition, low cost to synthesize, low toxicity, absence of side effects *in vivo*, high solubility, etc. Compound **7** which showed the most potent inhibitory activity in vitro was not selected for further investigation due to its high cost. Further structure-activity relationship studies on FK633 (**6**) are under way, and will be reported in the future.

In summary, computer simulation studies were carried out to generate novel anti-platelet agents based on fibrinogen inhibition. In our simulation, the active conformation of the reference peptide, ArgGlyAsp (RGD), was set to be a type II' β-turn according to experimental results and consideration on an evolutionary meaning of conservation of the Gly in the RGD sequence. These studies led to the synthesis of 4-(4-amidinophenoxy)-butanoylaspartylvaline **6** which showed potent inhibitory activities of platelet aggregation and fibrinogen binding.

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14. The active conformation is not always the same as its solution or crystal structure. Thus, these experimental reports on the RGD conformation are not evidence for the hypothesis that the active conformation of the RGD sequence is a type II' β-turn.
15. a) We used an in-house program, FIT,[16] which can impose a flexible compound on a target compound while rotating flexible dihedral angles. In the FIT program, the variable torsion angles are optimized to minimize the error function E, which is defined in the following equation:

$$E=\Sigma w_i d_i+A \quad (1)$$

where d_i is the distance between i-th corresponding atomic pair which is selected for superimposing the molecules, and w_i is a weight factor on i-th atomic pair. A in equation (1) is the conformational energy term adopted to avoid unfavorable conformations. The value of A is defined as a Lennard-Jones type

non-bonded potential function which is used in the SYBYL default force field (see ref. 15b):

$$A=\Sigma[k_{ab}x(1.0/L^{12}-2.0/L^{6})]$$

where k_{ab} is the geometric mean of the k constants associated between atom a and atom b, and L is the distance between the two atoms divided by the sum of their radii. b) Clark, M.; Cramer III, R. D.; Opdenbosch, N. V. *J. Comput. Chem.*, **10**, 982 (1989).

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17. a) A β-turn is usually classified under four types, namely type I, I', II, and II'. b) See ref. 17c about the number of amino acid such as i, (i+1) or (i+2)th. c) Wilmot, C. M.; Thornton, J. M. *J. Mol. Biol.*, **203**, 221 (1988). d) Crawford, J. L.; Lipscomb, W. N.; Schellman, C. G. *Proc. Nat. Acad. Sci. USA*, **70**, 538 (1973).
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20. A typical experimental procedure for compounds for biological assays (**14**). To a mixture of **4b** (0.61 g, 1.65 mmol), H-Asp(OBzl)Val-OBzl hydrochloride (0.61 g, 1.65 mmol), and 1-hydroxy-1 *H*-benzotriazole (0.22 g, 1.65 mmol) in DMF (5 ml) was added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (0.25 ml, 1.65 mmol), and was stirred at room temperature for 2 h. The reaction mixture was poured into a mixture of water and ethyl acetate, and was adjusted to pH 9.5 with 4 N NaOH. The separated organic phase was washed with saturated aq. $NaHCO_3$, water, brine, and dried over $MgSO_4$. After filtration, the filtrate was evaporated *in vacuo* to give a crude intermediate (m=4, XX=ValOBzl in compound **5**, 1.08 g, 95.2 %) as an oil.

    A mixture of the above crude intermediate (1.00 g, 1.46 mmol) and 10 % Pd-C (0.6 g) in a mixture of 1 N HCl (5 ml) and THF (10 ml) was stirred under H_2 atmosphere at room temperature for 6 h. After filtration, the filtrate was evaporated in vacuo. The resulting oil was subjected to preparative HPLC under the below conditions to give **14** (0.37 g, 44.9 %): mp 147 ℃ (dec.); IR(Nujol) 3300, 3080, 1660, 1600, 1530 cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ 0.84 (d *J*=6.7 Hz, 6 H), 1.5-1.8 (m, 4 H), 2.03 (m, 1 H), 2.19 (t like, 2 H), 2.4-2.8 (m, 2 H), 4.1 (m, 3 H), 4.7 (m, 1 H), 7.15 (d, *J*=8.9 Hz, 2 H), 7.76 (d *J*=7.9 Hz, 1 H), 7.81 (d, *J*=8.9 Hz, 2 H), 8.25 (d, *J*=7.8 Hz, 1 H), 9.05 (s, 2 H), 9.14 (s, 2 H); mass spectrum *m/e* 451 ($M+H^+$). HPLC conditions: Column, YMC-PACK R-ODS-15 S-15 120A ODS (YMC Co., Ltd.), 50ϕ x 250 mm; Elution, 25 % CH_3CN in 0.1 % TFAaq.; Flow, 118 ml/min; Detection, 254 nm; Retention time,8.0 min.

    Compounds **6**–**13** and **15**–**17** were prepared in a manner similar to that of **14**.
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