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# STRUCTURE-ACTIVITY RELATIONSHIPS OF DOUBLE-STRAND RGD PEPTIDES AS GPIIb/IIIa RECEPTOR ANTAGONISTS

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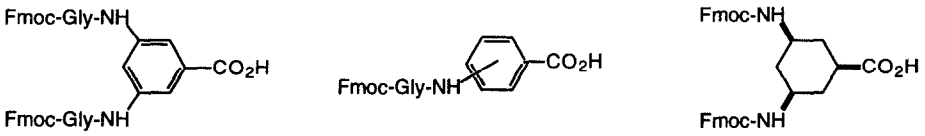
**Abstract:** A series of new double-strand RGD peptides are synthesized and their inhibitory activities evaluated for platelet aggregation. Substantial improvement in activity is observed with these novel RGD peptides in comparison with single-strand RGD peptides. The structure-activity relationships of these double-strand RGD peptides are discussed.

Improper platelet aggregation in pathological or surgical condition leads to occlusion of blood vessels causing serious damage to terminal tissue. The initial adhesion of platelets, to the injured vessel is promoted by GP IIb/IIIa receptors on platelet and adhesive glycoproteins, such as fibrinogen, vitronectin and von Willebrand factor, which exist in the plasma membrane.[1] GPIIb/IIIa is part of the superfamily of adhesion receptors, many of them share a common recognition site for adhesive proteins which containing the short amino acid sequence Arg-Gly-Asp (RGD).[2,3] GP IIb/IIIa receptor also be attached by the synthetic peptides with RGD residue. Those peptides may act as competitive, reversible inhibitors in assays of cellular adhesion. They compete directly for fibrinogen binding sites on platelets in a noncytotoxic manner. Since the binding of the platelet receptor with these proteins and peptides is competitive and reversible, the high affinity of the ligands toward the receptor may cause better antiplatelet aggregation capacity.[4] One of our approaches toward highly active peptide-based inhibitors of platelet aggregation is to incorporate multiple RGD units in one molecule by using appropriate linkers.

Various linear and cyclic RGD peptides have been synthesized for preventing platelet aggregation.[5] One of our approaches toward highly active peptide-based inhibition of platelet aggregation is to incorporate multi RGD units into a peptide by using appropriate linkers. Increase in the number of RGD sequence in a molecule which interacts with GPIIb/IIIa receptor should enhance its overall inhibitory activity, viz., a higher local concentration of the RGD sequence should provide better inhibitory activity because the probability that they would interact with the receptor should be increased. In fact, we have observed more than doubled activity in a series of novel double-strand RGD peptides in comparison with the corresponding single-strand RGD peptides. We describe here the design, synthesis and SAR study of novel double-strand RGD peptides.

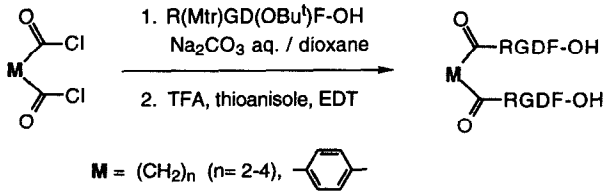
**Synthesis of double-strand RGD peptides.** We have synthesized a series of novel double-strand RGD peptides which are summarized in Table 1. Two RGD-containing peptide fragments were connected with linkers such as amino acids, diamino acids, and dicarboxylic acids. Peptides **1-4** were prepared by reacting two equivalents of R(Mtr)GD(OBu$^t$)F-OH and one equivalent of a diacid chloride, e.g., terephthaloyl chloride (*p*-Pht), succinyl chloride (Suc), glutaryl chloride (Pda), or adipoyl chloride (Bda), in a mixture of 0.7*N* $Na_2CO_3$ and dioxane (Scheme 1). All other peptides were obtained through solid-phase synthesis using *N*-Fmoc-amino acid on a Du Pont's RaMP™ system (Scheme 2). 3,5-Bis(Fmoc-glycylamino)benzoic acid ((Fmoc-Gly)$_2$Dab), 2-, 3-

and 4-(Fmoc-glycylamino)benzoic acids (Fmoc-Gly-2-Abz, Fmoc-Gly-3-Abz, and Fmoc-Gly-4-Abz) were prepared through the condensation of Fmoc-Gly-Cl and diamino- or aminobenzoic acid. (*Z,Z*)-3,5-Bis(Fmoc-amino)cyclohexanecarboxylic acid ((Fmoc)$_2$Dac) was prepared through hydrogenation of 3,5-diaminobenzoic acid on Rh-C in 86% yield, followed by Fmoc protection of amino groups with Fmoc-OSu. Acylations at the N-termini of the double-strand RGD peptides were carried out with *n*-hexanoic acid, benzoic acid and 4-guanidinobenzoic acid in the presence of DIC and HOBt in DMF, followed by the standard deprotection and cleavage from the resin using TFA in the presence of thioanisole and ethylenedithiol (EDT) (Scheme 2).

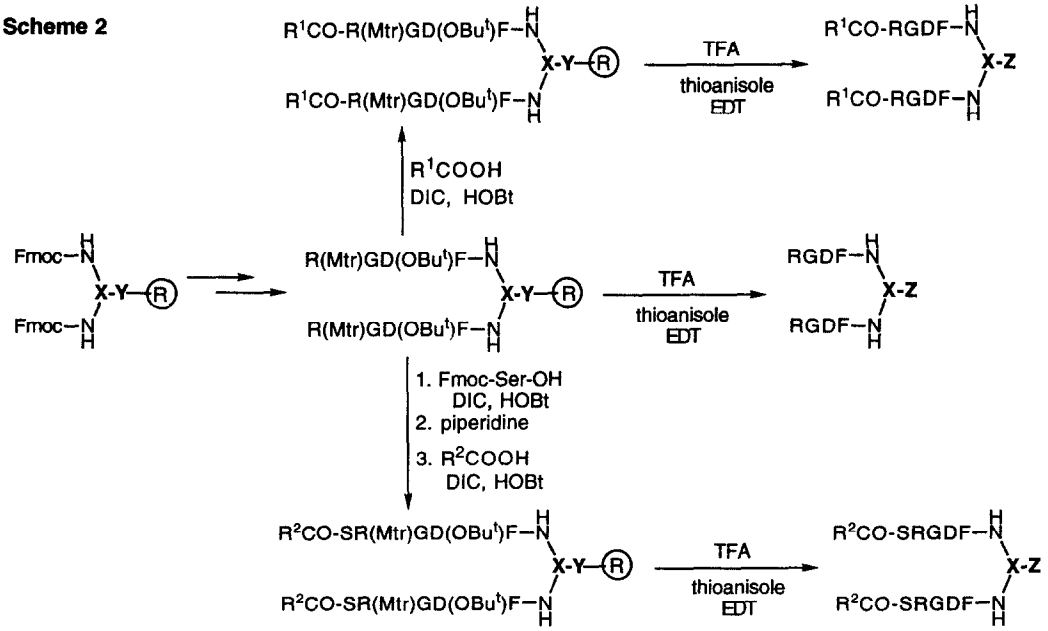



**(Fmoc-Gly)$_2$Dab** **Fmoc-Gly-Abz** **(Fmoc)$_2$Dac**

**Scheme 1**




**Scheme 2**




**X** = Lys, Orn, Dac, (Gly)$_2$Dab; **Y** = NH or (G)RGDF; **Z** = $NH_2$ or (G)RGDF-$NH_2$(-OH);
$R^1$ = *n*-pentyl, phenyl; $R^2$ = 4-guanidinophenyl

**TABLE I. Inhibition of Platelet Aggregation[a]**

| Peptide Number | Structure[b] | Type | FAB-MS *m/z* $(M+1)^+$ | $IC_{50}$ (μM)[c] |
|---|---|---|---|---|
| **1** | $Pht(RGDF\text{-}OH)_2$ | I | 1117 | 13 |
| **2** | $Suc(RGDF\text{-}OH)_2$ | I | 1069 | 15 |
| **3** | $Pda(RGDF\text{-}OH)_2$ | I | 1083 | 15 |
| **4** | $Bda(RGDF\text{-}OH)_2$ | I | 1097 | 14 |
| **5** | GRGDFG-(2-Abz)-GRGDF-OH | II | 1259 | 63 |
| **6** | GRGDFG-(3-Abz)-GRGDF-OH | II | 1259 | 19 |
| **7** | GRGDFG-(4-Abz)-GRGDF-OH | II | 1259 | 35 |
| **8** | $(RGDF)_2Dac\text{-}NH_2$ | III | 1108 | 50 |
| **9** | $(RGDF)_2K\text{-}NH_2$ | III | 1097 | 35 |
| **10** | $(Bz\text{-}RGDF)_2K\text{-}NH_2$ | IIIa | 1293 | 2.6 |
| **11** | $(Hex\text{-}RGDF)_2K\text{-}NH_2$ | IIIa | 1305 | 2.3 |
| **12** | $(RGDFPG)_2Dab\text{-}NH_2$ | III | 1411 | 13 |
| **13** | $(Hex\text{-}RGDFPG)_2Dab\text{-}NH_2$ | IIIa | 1488 | 0.35 |
| **14** | $(Ac\text{-}RGDFP)_2K\text{-}NH_2$ | IIIa | 1375 | 4.1 |
| **15** | $(Bz\text{-}RGDFP)_2K\text{-}NH_2$ | IIIa | 1499 | 2.2 |
| **16** | $(Hex\text{-}RGDFP)_2K\text{-}NH_2$ | IIIa | 1488 | 1.2 |
| **17** | $(Coum\text{-}RGDFP)_2K\text{-}NH_2$ | IIIa | 1636 | 2.0 |
| **18** | $(GuBz\text{-}S\text{-}RGDFP)_2K\text{-}NH_2$ | IIIa | 1787 | 0.087 |
| **19** | $(GRGDFG)_2Dab\text{-}GRGDF\text{-}NH_2$ | IV | 1863 | 33 |
| **20** | $(RGDF)_2Dac\text{-}RGDF\text{-}OH$ | IV | 1585 | 22 |
| **21** | $(Bz\text{-}RGDF)_2Dac\text{-}RGDF\text{-}OH$ | IVa | 1793 | 7.4 |

[a]Human platelet aggregation was induced by ADP. [b]Abbreviations are as follows: Abz = Aminobenzoyl; Ac = acetyl; Bda = Butanedicarbonyl; Bz = benzoyl; Coum = Coumarin-3-carbonyl; Dab = 3,5-Diaminobenzoyl; Dac = 3,5-Diaminocyclohexanoyl; GuBz = 4-guanidinobenzoyl; Hex = *n*-Hexanoyl; Pda = Propanedicarbonyl; Pht = *p*-Phthalyl; Suc = Succinyl. [c]Normalized value based on the $IC_{50}$ of $RGDF\text{-}NH_2$ as 50 μM. Each value is the average of three runs and experimental error is within 15%.

**Bioassay *in vitro*.** The platelet aggregation inhibitory activity of the new double-strand RGD peptides thus synthesized were evaluated by *in vitro* assay using platelet-rich plasma (PRP) and ADP as the activator.[6] In order to normalize the deviation in each set of experiments, $RGDF\text{-}NH_2$ was used as the standard ($IC_{50}$ = 50 μM). Typical procedure is as follows. Fresh blood is taken from volunteers and placed in test tubes containing trisodium citrate. Platelet-rich plasma (PRP) is prepared by centrifugation and adjusted to 300,000/μL with platelet poor plasma (PPP). This PRP (450 μL) is placed in a siliconized cuvette equipped with a magnetic stirrer. Various concentrations of the peptide solution (36 μL) are added and incubated for 1 min, then platelet aggregation

is induced by addition of ADP (54 μL, 100 μM). The extent of platelet aggregation is determined by a change in light transmission through the PRP on an aggregometer. The ability of the peptides to inhibit platelet aggregation is evaluated and the $IC_{50}$ is determined as the concentration of peptide required to produce 50% inhibition of the response to ADP in the presence of the TRIS buffer. Results are summarized in Table 1.

**Results and discussion.** Different types of double-strand RGD peptides, **I** - **IIIa**, and triple-strand RGD peptides, **IV** and **IVa**, have been investigated for SAR study. In the Type I peptides **1–4**, two RGDF fragments are linked at the *N*-termini by *p*-phthalic acid (Pht), succinic acid (Suc), propanedicarboxylic acid (Pda), or butanedicarboxylic acid (Bda). As Table 1 shows, their inhibitory activities for platelet aggregation ($IC_{50}$ = 13-15 μM) are 3-4 fold higher than RGDF-$NH_2$ ($IC_{50}$ = 50 μM). It should be noted that the activity *per RGD unit* of these Type I double-strand RGD peptides is 1.6-2 times better than that of RGDF-$NH_2$.

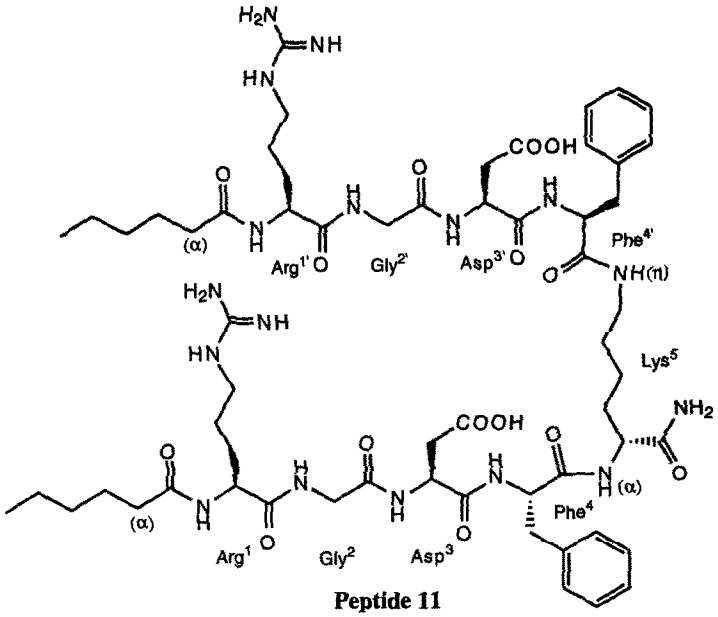
The Type II peptides **5** - **7** have 2-, 3-, and 4-aminobenzoic acid (Abz) as the linker of two GRGDF. In the Type II peptides, considerable difference in activity is observed between three regioisomers, i.e., the peptide **6** bearing 3-Abz as the linker is ca. two times better than **7** and ca. three time better than **5** in its activity, and only **6** has a better activity per RGD unit than RGDF-$NH_2$.

In the Type III peptides **8** and **9**, two RGDF fragments are connected by lysinamide and 3,5-diaminocyclohexanecarboxamide (Dac-$NH_2$), respectively. Peptide **9** shows better activity than **8** as well as RGDF-$NH_2$, but both of them possess lower per RGD unit activities than RGDF-$NH_2$. When RGDFPG sequence is employed, i.e., peptide **12**, the activity is substantially improved ($IC_{50}$ = 13 μM).

In order to inhibit degradation of peptides by aminopeptidases, we have introduced acyl groups to the N-termini of the Type III peptides, forming the Type IIIa peptides **10**, **11**, **13–18**. As Table 1 shows, more than one order of magnitude increase in potency ($IC_{50}$ = 2.3 - 2.6 μM) is observed when benzoyl (Bz) (**10**) or *n*-hexanoyl (Hex) (**11**) group is introduced to **9**. Marked enhancement of activity (37-fold) is observed when two *n*-hexanoyl groups are attached to **12** ($IC_{50}$ = 0.35 μM). Since it is clear that *N*-acylation is extremely effective to improve potency, we have looked at the SAR of the Type IIIa peptides **13–18** using different acyl groups, i.e., acetyl, benzoyl, *n*-hexanoyl, and coumarin-3-carbonyl, for $(RGDFP)_2K$-$NH_2$. Among these acyl groups, *n*-hexanoyl gives the best result (**16**, $IC_{50}$ =1.2 μM). Remarkable increase in potency (402-fold!) is achieved when 4-guanidinobenzoylserine (GuBz-S) residues are introduced the same peptide, $(RGDFP)_2K$-$NH_2$, i.e., peptide **18** possesses extremely strong inhibitory activity for platelet aggregation ($IC_{50}$ = 0.087 μM). The single strand version of **13**, Hex-RGDFPG-$NH_2$ ($IC_{50}$ = 3.1 μM) possesses one order of magnitude weaker activity than **13**, and GuBz-S-RGDFP-GABA-$NH_2$ (GABA = γ-aminobutanoic acid residue), a single strand version of **18**, shows only weak activity (IC50 = >50 μM).

In the Type IV peptides **19** and **20**, two GRGDF and two RGDF fragments are connected by Dab-$NH_2$ and Dac-$NH_2$, respectively. When *N*-benzoyl groups are attached to **20**, 3-fold increase in potency is observed (**21**, $IC_{50}$ = 7.4 μM). However, it is apparent that the introduction of third RGD unit to form triple-strand RGD peptides does not have any advantage over the double-strand RGD peptides.

**Conformational study by 2D NMR (ROESY).** Conformational study of peptide **11** was performed on the basis of ROESY spectroscopy and $^1$H NMR amide temperature coefficient measurements on a 600 MHz NMR combined with computational methods such as constrained distance geometry search and molecular dynamics using SYBYL 6.0 program on a Silicon Graphics Iris Workstation. No rOe's between amide protons are observed, which suggests that formation of a turn structure is unlikely.[7] Furthermore, temperature coefficients of amide protons, -4.4 to -5.4 ppb/K, indicate no notable intramolecular H-bonding formation, which rules out a possibility to form stable turn structure.[8] Strong rOe's are observed between an α-proton and an NH proton in the neighboring (i+1) amino acid residue, i.e., Hex(Hα)-Arg$^1$(NH), Hex'(Hα)-Arg$^{1'}$(NH), Arg$^1$(Hα)-Gly$^2$(NH), Arg$^{1'}$(Hα)-Gly$^{2'}$(NH), Gly$^2$(Hα)-Asp$^3$(NH), Gly$^{2'}$(Hα)-Asp$^{3'}$(NH), Asp$^3$(Hα)-Phe$^4$(NH), Asp$^{3'}$(Hα)-Phe$^{4'}$(NH), Phe$^4$(Hα)-Lys$^5$(NH), Phe$^{4'}$(Hα)-Lys$^5$(ηNH). The result indicates that the backbone of **8** is predominantly a β-sheet (extended) structure in DMSO.[9]




**Peptide 11**

**Conclusion.** We have found that double-strand RGD peptides have substantially better inhibitory activity for platelet aggregation than their single-strand counterparts. It is worthy of note that the activity of the double-strand RGD peptides *per RGD unit* is even better than that of the corresponding single-strand peptides, which indicates that increase in local RGD concentration is more effective than simply increasing overall RGD concentration. The understanding of this interesting synergistic effect needs further investigation. The effects of *N*-acyl groups on the potency of double-strand RGD peptides are very remarkable. *n*-Hexanoyl group is found to be more effective than benzoyl and coumarin-3-carbonyl groups. 4-Guanidinobenzoylseryl group has been identified as the most effective *N*-acyl group in this series of peptides so far examined to date, which has led to the finding of extremely active peptide **18** whose $IC_{50}$ is 0.087 μM (575 times better than RGDF-$NH_2$). 2D NMR (ROESY) study and

$^1$H NMR amide temperature coefficient measurements for an *N*-acylated double-strand RGD peptide **11** indicate an extended backbone structure.

Further studies on the SAR of new RGD peptides and their peptidomimetics are actively underway.

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