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NON-PEPTIDE GLYCOPROTEIN IIb/IIIa INHIBITORS. 9. CENTRALLY CONSTRAINED ALPHA-SULFONAMIDES ARE USEFUL TOOLS FOR EXPLORING PLATELET RECEPTOR FUNCTION

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Abstract: Two fluorescent, centrally constrained fibrinogen receptor antagonists were prepared to probe ligand receptor interactions.

The binding of fibrinogen to platelet glycoprotein GPIIb/IIIa (fibrinogen receptor) represents a final pathway in both hemostasis and pathologically critical thrombosis,[1] and can be inhibited by proteins or peptides containing an RGD tripeptide or its synthetic mimetics.[2] The rational design of fibrinogen receptor antagonists requires effective methods for measurement of ligand binding to the receptor. A common approach is to evaluate the inhibition of binding of radiolabeled or biotinylated ligand.[3] Recently, the utility of high molecular weight fluorescent ligands for binding to GPIIb/IIIa on platelets[4] was demonstrated. We describe the synthesis of potent, low molecular weight fluorophore-containing fibrinogen receptor antagonists and the application of the compounds to the study of ligand binding to isolated GPIIb/IIIa and to GPIIb/IIIa on platelets.

In a recent paper, we described the preparation of potent fibrinogen receptor antagonists by incorporating an element of "central constraint"[5] that would appropriately direct the vectors of the N- and C-terminal chains. Compound L-709,780 (1) proved to be a potent, selective, and orally active fibrinogen receptor antagonist[5] (Figure 1, Table 1).

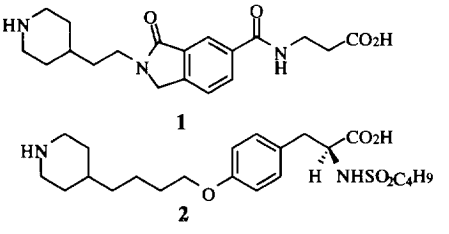
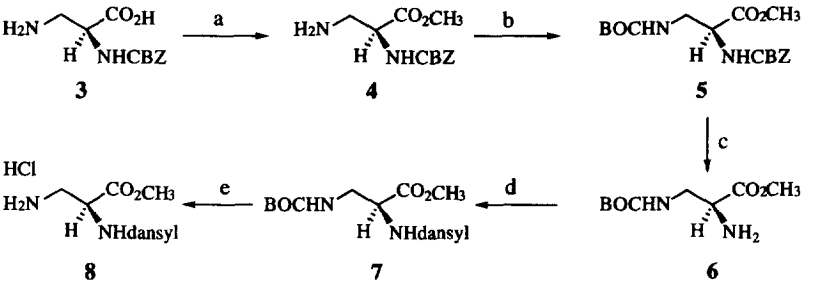


Figure 1. Small Molecule Fibrinogen Receptor Antagonists.

Compound **1** provided us with a useful, low-molecular weight tool for the exploration of receptor/ligand interactions. We therefore pursued a fluorescent analog of **1**, and chose dansyl as a fluorescent moiety. The dansyl fluorophore is a frequently-used reporting moeity in the study of proteins and ligand/receptor interactions because of the dependence of fluorescence on the polarity and fluidity of the fluorophore microenvironment.[6]

The key role that the alpha sulfonamide plays in the potency of Aggrastat™ (**2**)[7] (Figure 1, Table 1) suggested that a fluorophore could be most easily incorporated into the structure of **1** through an alpha sulfonamide linkage. The dansylated probe **10** was prepared as outlined in Schemes 1 and 2.

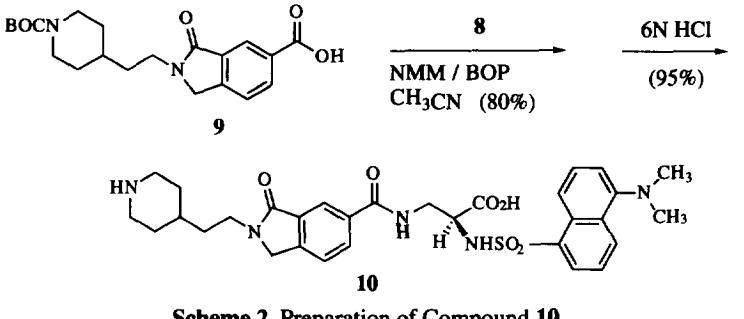


Scheme 1. Preparation of dansylated 2,3-diaminopropionic acid.

(a) MeOH/7 equiv $SOCl_2$, (95%); (b) BOC_2O/CH_2Cl_2/sat. $NaHCO_3$/1 N NaOH (95%); (c) H_2 (balloon)/10% Pd/C/EtOH (95%); (d) pyridine, $CHCl_3$, dansyl chloride (50–75%); (e) HCl/EtOAc −78° – 0 °C (95%).

The appropriately derivatized 2(S)-sulfonylamino-3-aminopropionate **8** was prepared as described in Scheme 1. The commercially available Nα-Z-L-2,3-diaminopropionic acid (**3**) (Fluka) was esterified using MeOH/$SOCl_2$ to give ester **4**. The free amine was protected with a BOC group, the CBZ group removed, the revealed amine sulfonylated, and the BOC group cleaved to give **8**. The amine **6** could also be prepared via selective BOC protection of methyl 2(S),3-diaminopropanoate using previously described methods.[8]

The dansylated C-terminus was coupled using BOP to the previously described isoindoline acid **9**.[5] The N-terminal BOC and the methyl ester were removed in one step using 6 N HCl to give L-736,622 (**10**).[9] Acidic rather than basic conditions were optimal for ester cleavage without racemization.



Scheme 2. Preparation of Compound **10**.

Despite the addition of the bulky dansylsulfonamido group, **10** proved to be a more potent inhibitor of platelet aggregation and had a higher affinity for binding to purified GPIIb/IIIa (See Table 2) than **1**. The optimal excitation wavelength for **10** is ~ 340 nm with extinction coefficient 4650 $cm^{-1}M^{-1}$ (H_2O, pH 7.4).

Table 1. Inhibition of Platelet Aggregation, IC_{50}, and Binding to Purified Receptor GPIIb/IIIa, ED_{50}.

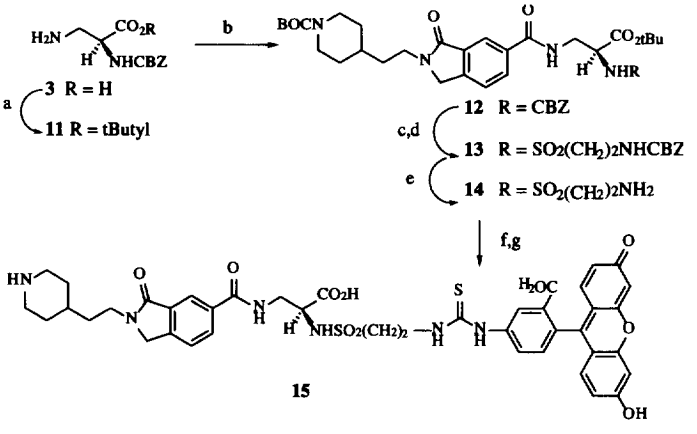
Compound	*IC_{50} (nM)	**ED_{50} (nM)
1	27	12
2	18	0.9
10	12	2.1
15	31	1.9

* Inhibition of human gel filtered platelet aggregation stimulated by ADP (10 mM).[10]

** Measured by competition with (^{125}I)-L-692,884 for binding to purified GPIIb/IIIa coated onto yttrium silicate Scintillation Proximity Assay Fluomicrospheres.[11]

These characteristics made **10** appropriate for binding studies using the purified receptor, however binding measurements on platelets in platelet rich plasma or in whole blood were problematic because of interference from protein absorbance and the low concentration of receptors. A fluorescein analog appeared to be an alternative from among the available fluorophores that have absorbance above 450 nm, as its excitation wavelength is suitable for common flow cytometers. We chose to prepare a sulfonamide with a short side chain as a linker between the antagonist and the fluorescent group as this approach may enhance the affinity of the final compound for GPIIb/IIIa.

A synthetic scheme allowing for the late introduction of the fluorescent group was explored. A *t*-butyl ester was chosen as the C-terminal protecting group to allow for a one-step final deprotection. Thus, **11** was prepared from **3** using standard conditions, then coupled to the isoindoline acid **9** to give **12**. The CBZ group was removed and the amine sulfonylated with 2-CBZ-aminoethane sulfonyl chloride.[12] The CBZ group was removed from **13** and the resulting amine **14** condensed with commercially available fluorescein isothiocyanate. Removal of the BOC and *t*-butyl ester protecting groups was accomplished in one step with $TFA/CHCl_3$ to give L-762,745 (15). As was observed for **10**, the bulky nature of the fluorescein functional group does not impair the potency of the fluorescein derivative **15** (IC_{50} = 31 nM).



Scheme 3. Preparation of Fluorescent Probe **15**.

(a) isobutylene, H_2SO_4, dioxane (64%); (b) BOP/NMM/DMF (93%); (c) H_2/10% Pd/C/EtOH(90%); (d) $CHCl_3$/NMM/$ClSO_2(CH_2)_2NHCBZ$ (41%); (e)H_2/10% Pd/C/20% H_2O in EtOH 65 psi (85%); (f) Fluorescein isocyanate/NMM/DMF (89%); (g)$TFA/CHCl_3$ (62%)

The use of fluorescent antagonists **10** and **15** to characterize the binding affinity of nonfluorescent antagonists to inactive[13] isolated GPIIb/IIIa and to GPIIb/IIIa on platelets is demonstrated in Figure 2. Equilibrium binding constants, K_D, for binding of **2** to either purified inactive form of GPIIb/IIIa solubilized in Triton X-100 micelles (Figure 2, Panel A) or resting human gel filtered platelets (Figure 2, Panel B) were obtained in competitive binding measurements with **10** (Panel A) and **15** (Panel B). The average value of K_D = 13 ± 4 nM[15] (n = 7) for binding of **2** to inactive GPIIb/IIIa suspended in Triton X-100 micelles agrees well with K_D = 14 ± 3 nM[15] (n = 5) obtained in flow cytometric measurements on resting platelets.[14] This indicates that the binding constants obtained on isolated inactive GPIIb/IIIa represent well the binding characteristics of the receptors on resting platelets, and supports the use of **10** and isolated inactive GPIIb/IIIa as a rapid, efficient surrogate for the characterization of inhibitor affinity to resting platelets. The low yield of activated GPIIb/IIIa obtained in purification[13] prohibits its routine use as a screening tool. However, we found that the competitive binding measurement of **2**, using **10**, on active, isolated GPIIb/IIIa K_D =1.9 ± 0.7 nM[15] (n = 3) (data not shown) agrees well with the ED_{50} (See Table 1) obtained in competition with (^{125}I)- L-692,884 for binding to the more readily available purified inactive GPIIb/IIIa coated onto Fluomicrospheres.[11] These results support the use of the SPA assay[11] as a tool for characterization of inhibitor binding to an active form of receptors. Thus, the fluorescence probes **10** and **15** can be used in the screening measurements of fibrinogen receptor antagonists. Future publications will describe how the fluorescent probes may be useful in the study of receptor conformations, number of receptors on platelets or other cells and receptor signaling.

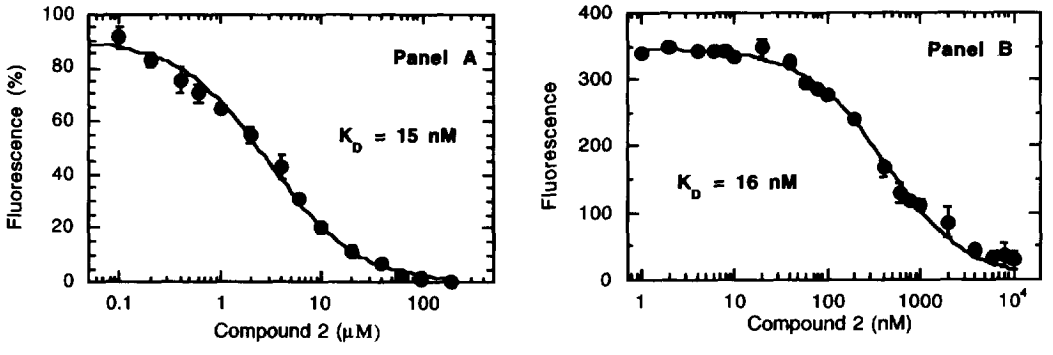


Figure 2. Panel A: Competitive binding (single measurement)[13]of **10** and **2** on purified inactive GPIIb/IIIa solubilized in Triton X-100 micelles. **Panel B**: Flow cytometric measurements (single measurement)[14] of competitive binding between **15** and **2** on resting human gel filtered platelets. Calculations of K_d values are based on K_D = 3.7 nM for **10** on purified GPIIb/IIIa[13] and K_D = 20 nM for **15** on resting platelets.[14]

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9. All compounds were characterized by NMR, TLC, HPLC, high-resolution mass spectroscopy and H, C, N analysis.
10. Platelet aggregation was measured in a functional assay which monitors the increase in light transmittance which occurs when platelets aggregate. Human gel-filtered platelets were adjusted to a concentration of 2 × 108/mL and mixed with 0.1 mg/mL human fibrinogen, 1 mM $CaCl_2$ and the compound of interest. Aggregation was then initiated by addition of the agonist (10 μM adenosine diphosphate (ADP)). Inhibition of platelet aggregation was determined by comparison of light transmittance values for the control and subject samples. The IC_{50} was determined as the concentration necessary to inhibit the change in light transmittance by 50%. At least two determinations were made for each compound and the IC_{50} calculated by fitting to a four parameter equation. The average standard error of the IC_{50} determinations was ±20%.
11. Purified GP IIb/IIIa was coated onto yttrium silicate Scintillation Proximity Assay Fluomicrospheres (Amersham RPN 143) and is abbreviated IIb/IIIa/SPA. The binding of the RGD-containing heptapeptide [^{125}I]-L-692,884 (New England Nuclear, NEX-330) to the yttrium silicate containing IIb/IIIa/SPA is detectable, without the necessity of separation of bound from free, in a Top Count Scintillation Counter. The ED_{50} for a nonradiolabeled compound was determined by competition with the binding of [^{125}I]-L-692,884 to IIb/IIIa/SPA at pH 7.5 (20 mM HEPES, 0.15 M NaCl, room

temperature) with ~0.3 nM of IIb/IIIa/SPA, ~0.3 nM of [^{125}I]-L-692,884 and a wide range of concentrations of the competing non-radiolabeled compounds. After equilibration, the bound CPM are measured and the ED_{50} value determined by non-linear least square fit to CPM = (Bmax-Bmin)/(1+ $(I/ED_{50})^B$) + Bmin, where **I** is the concentration of the test compound, **B** is the Hill slope, **Bmax** is the maximum binding observed without the test compound, and **Bmin** is the non-specific binding signal. The average standard error of mean for ED_{50} determinations was ±20%.

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13. GPIIb/IIIa was purified from resting human outdated platelets as described in Kouns, W. C.; Hadvary, P.; Haering, P.; Steiner, B. *J. Biol. Chem.* **1992**, *267*, 18844. Competitive fluorescent displacement binding measurements were done with both an inactive and an active form of GPIIb/IIIa (typically 0.6 μM) solubilized in Triton X-100 buffer (0.1% Triton X-100, 20 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, 1mM $MgCl_2$, pH 7.4) and 1 μM of **10** at 20 °C. Changes in the fluorescence of the solution upon addition of nonfluorescent fibrinogen receptor antagonist were recorded and, after correction for the fluorescence of GPIIb/IIIa and subtraction of the fluorescence of **10** in the buffer, were plotted as % of fluorescence of the initial solution (no nonfluorescent ligand present). The values of K_D were calculated from displacement measurements (see Figure 2, Panel A) using the K_D value of **10** 3.7 nM obtained in stopped-flow measurements. A single measurement is shown in Figure 2, Panel A.
14. Flow cytometric competitive binding measurements were done with human gel-filtered platelets (typically 2 × 106 cell/mL) incubated with 400 nM of **15** and different concentrations of nonfluorescent ligands for 1 h at a laboratory temperature in the dark. A fluorescence signal of platelet suspension was recorded using FACScan (Becton & Dickinson, USA) flow cytometer. The values of mean fluorescence per platelet of at least 10,000 cells gated by forward and side scattering were plotted vs. concentrations of a nonfluorescent ligand (see Figure 2, Panel B) and K_D values were calculated using the K_D value of **15** (20 nM) obtained in equilibrium binding measurements using the same platelet suspension. A single measurement is shown in Figure 2, Panel B.
15. All results are expressed as the mean value ± standard deviation. (Figure 2 shows typical experiments for each type of measurements.)

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