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PREPARATION AND PROPERTIES OF A FIBRINOGEN RECEPTOR ANTAGONIST CONTAINING THE ARG-GLY-ASP SEQUENCE AND NITROXIDE RADICALS

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Abstract: A nitroxide spin-labelled compound derived from the potent fibrinogen receptor antagonist 2-mercaptobenzoyl-Nα-methyl-Arg-Gly-Asp-2-mercaptoanilide cyclic disulfide has been prepared. Its physical and biological properties are reported.

The tripeptide Arg-Gly-Asp (RGD) has been identified as a common recognition sequence in many adhesive proteins involved in a variety of biological processes (1). For example, the binding of the RGD-containing plasma protein fibrinogen to its platelet receptor GPIIb-IIIa constitutes a critical step in platelet aggregation and, consequently, formation of thrombus. There is considerable effort in the development of small RGD peptide and peptide mimetic fibrinogen receptor antagonists as potential antithrombotic agents (2). 2-Mercaptobenzoyl-Nα-methyl-Arg-Gly-Asp-2-mercapto-anilide cyclic disulfide (SKF-107260, Compound 1) is a potent fibrinogen receptor antagonist and is very effective in the inhibition of platelet aggregation (3). The probable solution conformations and crystal structure of this highly active antagonist of the platelet GPIIb-IIIa receptor have been examined by NMR and X-ray crystallography (4). We describe here a chemical strategy to derivatize SKF-107260 with nitroxide free radicals. An RGD-containing nitroxide-labeled peptide may be useful as a spin probe for fibrinogen receptor in EPR (electron paramagnetic resonance) studies (5,6), or as a selective contrast agent in MRI (magnetic resonance imaging) applications (7,8). The results of radioligand binding, inhibition of platelet aggregation and EPR spectrometric study are presented for this RGD-nitroxide peptide hybrid.

The preparation of an RGD nitroxide compound could have additional significance. Recently nitroxide spin labels have been shown to possess superoxide dismutase mimicing activity (9,10), to inhibit lipid peroxidation in biomembranes (11) and to prevent cardiac reperfusion damage (12). On the other hand, the leukocyte integrin Mac-1 (CD11b/CD18), which also contains the RGD recognition sequence, has been shown to mediate adherence-dependent

hydrogen peroxide (13) and superoxide (14) production by neutrophils. The incorporation of an RGD binding motif with an antioxidant nitroxide in the same molecule represents a new concept which could have pharmacological significance in the inhibition of neutrophil-mediated, oxygen radical-induced tissue injuries.

The disulfide linkage in 1 engages this peptide in a more restricted conformation (4). The cyclic peptide is quite stable by itself in aqueous and organic solvents. During our circular dichroism (CD) investigation of the secondary structures of cyclic and linear RGD peptides, we discovered that the disulfide bond in 1 is easily cleaved by reduction with dithiothreitol (DTT) in pH 7.4 phosphate buffer. As indicated from the CD spectra in Figure 1, the reduction is essentially complete at slightly over 1:1 DTT:peptide ratio. The large negative molar ellipticity from 240 nm to 380 nm in the absence of DTT is attributable to the diaryl disulfide exciton coupling. The reduced form 2 is devoid of any CD response in this region. The CD responses observed in the 190 to 240 nm region is attributable to the conformational change in the peptide backbone amides.

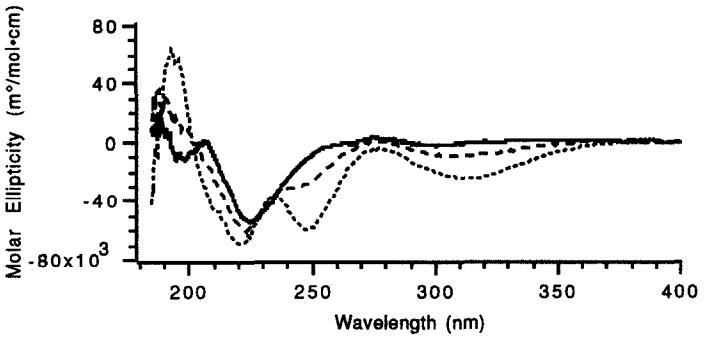
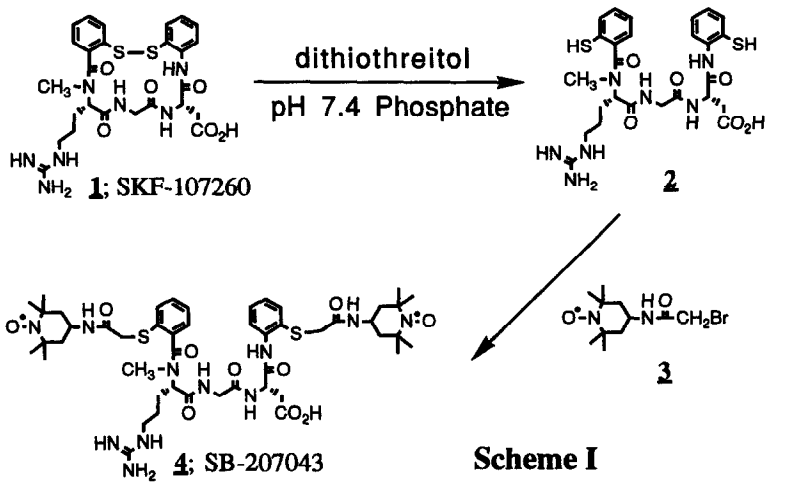


Figure 1 CD spectra of SKF-107260 (200 μM) titrated by dithiothreitol in pH 7.4, phosphate buffer. (·····), no DTT; (---), 0.57 X of DTT; (——), 1.14 X of DTT.

The free sulfhydryl groups provided convenient anchoring points for the nitroxide spin labels. Several types of alkylating spin label reagents, obtained from Aldrich, were tried. 4-(2-Bromoacetamido) TEMPO, compound 3, provided the best result based on the crude yield of the doubly labeled product as determined by HPLC. The general reaction scheme is shown in Scheme 1. 4-(2-Iodoacetamido) TEMPO yielded comparable result.



Scheme I

20 mg of 1 was dissolved in 20 ml of 10 mM pH 7.4 phosphate buffer, and 8.7 mg of DTT (2X excess) was added. The solution was allowed to stand for 20 minutes before 23 mg of 3 (20% excess) in 20 ml of the same buffer was added. After another 30 minutes, the crude products were fractionated using a semi-preparative HPLC procedure (Waters μ-Bondapak C18 column, mobile phase acetonitrile/water 275/725 with 0.05 % trifluroacetic acid) followed by lyophilization of the product-containing fractions. The major product is a white solid, m.p. 176° - 178° C. Mass spectrometry (electrospray) indicated a MH^+ at 1026, consistent with the molecular weight of compound 4 (SB-207043). The elemental analysis (CHN) result was satisfactory. The CD spectrum of 4 in pH 7.4 phosphate buffer, shown in Figure 2, indicated that this compound is in a similar linear peptide conformational distribution to the free sulfhydryl compound 2.

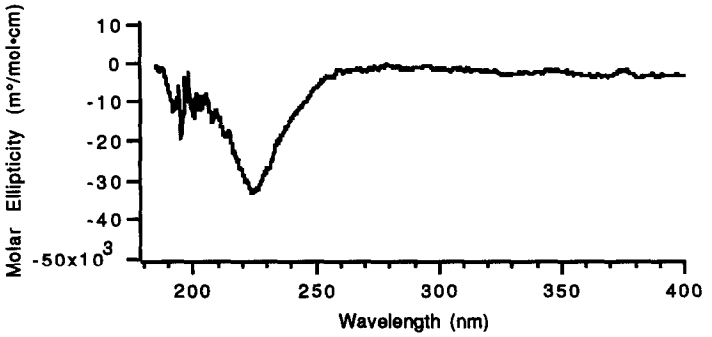


Figure 2 CD spectrum of the RGD spin probe SB-207043 in pH 7.4 phosphate buffer.

The EPR spectrum of 4 (Figure 3 a and b, dotted line) shows the typical 3-line pattern for a dilute aqueous solution of nitroxide spin label (g = 2.002; hyperfine coupling constant a_N = 17.1 G at the X-band frequency of 9.74 GHz). The high field peak height is smaller, so as the peak widths are broader than those for the spin label compound 3. This is expected for a slower tumbling of nitroxide in solution due to the larger molecular weight. It should be noted that, although 4 is a bi-radical compound, there is no spin-spin coupling between the two radicals because of the long distance separation between the two terminal nitroxide groups. Cyclic voltammetry of 4 in acetonitrile indicated that its electrochemical behavior is similar to other nitroxide spin labels (15). The two nitroxide groups in 4 are indistinguishable electrochemically or by EPR. The nitroxides are quickly reduced by ascorbate in pH 7 buffer, as indicated by the rapid disappearance of EPR signal.

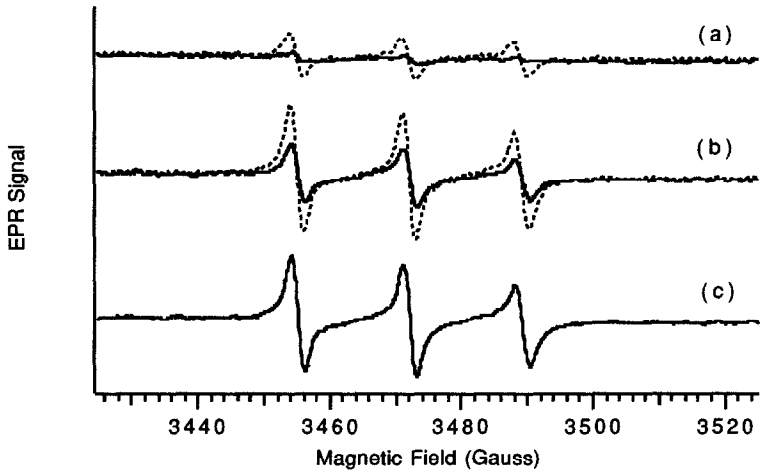


Figure 3 EPR spectra of SB-207043 in the presence or absence of purified GPIIb-IIIa in 20 mM Tris, pH 7.4, 140 mM NaCl, 2 mM $CaCl_2$ and 1% octylglucoside.
(a) (—): 0.9 μM SB-207043 + 1.5 mg/ml GPIIb-IIIa; (·····): 0.9 μM ligand only
(b) (—): 2.3 μM SB-207043 + 1.3 mg/ml GPIIb-IIIa; (·····), 2.3 μM ligand only
(c) same solution as (b) except that 5.2 μM of SKF-107260 had been added

An immediate question was how the cleavage of the diaryl disulfide linkage and the attachment of the two rather bulky acetamido TEMPO groups to 1 would affect the receptor binding property of the peptide. Figure 3a and 3b show that, upon addition of the purified GPIIb-IIIa, the EPR signal diminished in a concentration-dependent manner. The loss of EPR signal is

attributable to the severe line-broadening effect from the protein-bound spin probe. Addition of 1 to 5 mg/ml bovine serum albumin to 4 had no effect on the EPR signal (data not shown). In addition, the binding of compound 4 to GPIIb-IIIa is fully reversible as evidenced by the almost complete resumption of the EPR signal (compare Figure 3b and 3c) upon the addition of an excess amount of the antagonist SKF-107260. This ruled out the possibility that the decrease of EPR signal in the presence of GPIIb-IIIa protein is due to chemical reaction of the nitroxide.

The binding of 4 to GPIIb-IIIa was also studied in a radioligand competition assay using purified GPIIb-IIIa isolated from human platelets and reconstituted in liposomes (2). The dissociation constants (K_i) for 4, the parent compound SKF-107260 and the prototype nitroxide compound TEMPO are listed in Table 1. The chemical modifications to the potent antagonist SKF-107260 did induce about two orders of magnitude loss in binding affinity, while TEMPO was completely inactive. The receptor binding potency of 4, however, is comparable to many RGD-containing fibrinogen receptor antagonists previously reported (2,3). We have also examined the platelet aggregatory activities of these compounds. 4 exhibited an IC_{50} of about 1 μM in the in vitro inhibition of ADP-induced aggregation of human platelet-rich plasma (2).

Table 1. Binding Affinity for GPIIb-IIIa and Inhibition of Platelet Aggregation

	IIb-IIIa Affinity K_i (μM) ± SEM	Antiaggregatory IC_{50} (μM) ± SEM
SKF-107260	0.0045 ± 0.0002	0.057 ± 0.011
SB-207043	0.11 ± 0.003	1.02 ± 0.21
TEMPO	> 1000	> 200

Although nitroxide compounds have been extensively used in EPR studies for the past 20 years (5,6), only recently have their free radical biology and therapeutic implications begun to attract significant interest in biomedical research (9-12). We have prepared and characterized an RGD-containing nitroxide compound. It exhibits reasonably good affinity for fibrinogen GPIIb-IIIa receptor and moderate in vitro potency for inhibition of platelet aggregation. It would be interesting to investigate whether this compound has a synergistic effect on the inhibition of adherence-dependent reactive oxygen species production by neutrophils (13-14).

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