MPTP (Table I). The oxidation of these compounds was inhibited by pargyline, an inhibitor of monoamine oxidase (Table III). At the highest concentration of pargyline tested (2.5 μM), **3i** was noticeably, but inexplicably, insensitive to inhibition. Most substitutions on the phenyl ring do not significantly alter the substrate effectiveness of these homologues (Table I, **3a–g**, **31**). In addition, the replacement of the phenyl groups with a heterocyclic (**3j**) or naphthyl substituent (**3i**) yields better substrates than MPTP. However, hydrophilic substituents are poorly tolerated (**3m**). Furthermore, substitution at the benzylic methylene bridge is clearly disfavored (**3h**, **3k**). The latter observation is consistent with the reduced oxidative activity of MPTP relative to 4-homo-MPTP since the Cl′-position of MPTP may be regarded as a substituted benzylic carbon. Although the ethylene-bridged compounds **6a** and **6b** are fairly good substrates, the vinyl-bridged analogues **7a** and **7d** showed poor activity. Given that **6a** and **7a** are of similar length, this disparity, possibly a reflection of the ridigity of **7a**, suggests that **6a** may not bind to the enzyme in its linear conformation. This view is consistent with earlier reports which indicate that substitution along the long axis of MPTP (either at C4′ or at the *N*-methyl group) invariably leads to relatively poor substrates of MAO.[11,20] Alternatively, some unfavorable electronic effects may be involved.

In addition to increasing flexibility, the methylene bridge in **3a** and its congeners should further project the phenyl ring into regions of the substrate binding site which would otherwise be out of reach. In earlier studies of MAO,[11,20] MPTP displayed significant bulk tolerance at the meta position of the phenyl group. This observation suggests the existence of a hydrophobic pocket in this region. It is also noteworthy that, in these earlier studies, 3′-bromo-MPTP and **3a** were found to be the best substrates of monomaine oxidase B. By superimposing models of these two structures (data not shown), it becomes apparent that both the phenyl group of **3a** and the bromine atom of 3′-bromo-MPTP can occupy the same region on the enzyme. Thus, the increased oxidative activity of these flexible MPTP analogues may be attributed, in part, to their ability to bind to hydrophobic regions such as this. In addition, the data suggest that the optimum separation between the amine and the lipophilic moiety is defined by the 4-pyridylmethyl fragment.

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Supplementary Material Available: NMR data of all compounds synthesized (10 pages). Ordering information is given on any current masthead page.

Hemoglobin S Antigelation Agents Based on 5-Bromotryptophan with Potential for Sickle Cell Anemia

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5-Bromotryptophan (5-BrTrp) is the most potent amino acid derivative reported in the literature to inhibit the gelation of hemoglobin S (from sickle cell anemia patients). Trp-Trp is also more potent than Trp as an antigelation agent. Therefore, we have prepared a series of dipeptides containing 5-BrTrp and evaluated the antigelation activity. 5-BrTrp-5-BrTrp is the most potent, i.e., 5.9 times the activity of Trp, followed by 5-BrTrp-Trp and then Trp-5-BrTrp. This improved antigelation potency for 5-BrTrp-5-BrTrp and 5-BrTrp-Trp is very significant and will be pursued further as lead compounds with potential for sickle cell anemia.

Sickle cell anemia is a genetic disease that results from the substitution of a valine residue for glutamic acid at the β6-position in the hemoglobin (Hb) molecule. Deoxygenated hemoglobin S (HbS) molecules aggregate together to form long helical fibers that deform and rigidify the red blood cells.[1-3]

The basic unit of the sickle hemoglobin fiber appears to be a pair of monofilament strands, with the two strands approximately in half-register with each other.[4-6] The arrangement of the individual HbS molecules within these strands has been suggested by Love and co-workers from X-ray studies of HbS crystals and involves two axial and two lateral intermolecular contact regions within the deoxy-HbS double strand.[7,8] In the deoxy-HbS double-strand structure, the β6-Val residue from a hemoglobin molecule in one strand fits into a hydrophobic pocket formed by EF helices of a β-chain in a hemoglobin molecule from the neighboring strand. That molecule, in turn, has the β6-Val from its other β-chain inserted into the hydrophobic EF pocket in the next molecule up or down the chain in the original strand. Thus, each hemoglobin molecule contributes one β6-mutation site and one hydrophobic pocket to the double-strand structure.

A variety of noncovalent inhibitors of deoxyhemoglobin aggregation, presumably acting by competitive binding at one of the important HbS–HbS intermolecular contact sites, have been investigated. In vitro studies utilizing

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amino acids,[9] various phenyl derivatives,[10] alkylureas,[11] small peptides,[12] aryl-substituted alanines,[13] halogenated aryl derivatives of oxy acids,[14] disubstituted benzoic acids,[15,16] and biaromatic phenylalanyl derivatives[17] suggest several structural correlations with antigelation activity. Bicyclic and biaromatic compounds appear to be more effective than monocyclic compounds, and aromatic moieties are more effective than aliphatic[16] ones. For aromatic compounds, the higher the hydrophobicity and ring polarizability, the higher will be the gelation inhibitory activity.[12] However, aromaticity alone is not a sufficient condition for inhibition of deoxy-HbS aggregation, as shown by the fact that several aromatic compounds also enhance gelation.[9]

A detailed knowledge of the binding site stereochemistry on the Hb molecule is also needed. ^{1}H NMR relaxation studies[17,18] have suggested that there is a binding site for aromatic compounds near the heme iron of Hb, but no detailed binding site has been proposed. This site would probably be consistent with inhibitor binding in the hydrophobic pocket at the lateral contact region that is complementary to the β6-mutation site within the deoxy-HbS polymer structure.[19] X-ray studies have shown that three pairs of clofibric acid molecules cocrystallize with deoxy-HbA at sites inside the internal cavity of the deoxy-Hb tetramer, and one pair cocrystallizes with COHb at a site near α14-Trp.[14] ^{1}H NMR studies on the interaction of phenylalanine (and tryptophan) with deoxy-Hb also suggest that phenylalanine binds to deoxy-HbS at locations close to the β-heme cleft and to the β2-His residues.[20] Binding studies of biaromatic peptides with deoxy-Hb have suggested the existence of one symmetric binding site with moderate affinity (i.e., two identical sites per Hb tetramer) and one or more sites with weaker affinity.[21] Recent ^{1}H NMR studies in our laboratory with SL-Phe, a spin-labeled (SL) analogue of phenylalanine, also suggest that there may be binding site(s) near the β-chain N- and C-termini of COHbS.[22] Collectively these results suggest that different inhibitors may bind at different locations and/or that there may be more than one binding site per Hb tetramer for some inhibitors.

5-Bromotryptophan (5-BrTrp) is the most potent inhibitor of HbS gelation reported to date which does not involve covalent binding to hemoglobin.[13] Previous studies by Poillon[13] have shown that placement of the bromo substituent at other ring locations, i.e., 4, 6, or 7, while still active, resulted in lowered potency in the C_{sat} assay. We now report the synthesis and evaluation of a series of dipeptides containing 5-BrTrp as inhibitors of HbS gelation. Subsequent studies will attempt to elucidate the binding site for these compounds, although it is anticipated that they could interact at the hydrophobic pocket or acceptor site on the β-chain which includes the β85-Phe and β88-Leu residues. It would appear that the bromo substituent on the indole ring provides an important orientation to the dipeptide at the binding site on the hemoglobin surface. This binding interaction could be through a hydrophobic interaction or a dipolar interaction.

Some preliminary studies (P. Thiyagarajan and M. E. Johnson, unpublished) with SL-Phe (SL-Phe-O*t*-Bu), a phenylalanine derivative containing a stable free radical as a spin label, have demonstrated that the erythrocyte membrane is permeable to amino acid analogues of this type. The concentration inside and outside the red cell was equal within about 20 min of incubation of 0.5 mM SL-Phe with washed red cells in phosphate-buffered saline. The electron paramagnetic resonance (EPR) signal demonstrated a virtually identical binding interaction for the SL derivative in the red-cell suspensions to that observed in membrane-free solutions of hemoglobin. Red cell lysis was not observed to increase over a 24-h period in the presence of SL-Phe compared to controls.

Table I. C_{sat} Assay of L-Tryptophan Derivatives with L-Trp as Standard

compound	slope, g/(dL M)[a]	R^b
L-tryptophan (L-Trp)	124 ± 4 (13)	1.0
Trp-Trp	235 ± 9 (6)	1.9 ± 0.1
Trp-5-BrTrp, **11**	240 ± 24 (5)	1.9 ± 0.2
5-BrTrp-Trp, **10**	334 ± 15 (6)	2.7 ± 0.2
5-BrTrp	356 ± 19 (5)	2.9 ± 0.2
5-BrTrp-5-BrTrp, **12**	726 ± 59 (6)	5.9 ± 0.5

[a] Slopes are calculated from the combined regression of the data from all assays used. Uncertainties listed are the standard errors of estimate. Number of assays are shown in parentheses. [b] The ratio of the slope for each derivative to that for unmodified L-Trp. Uncertainties are calculated from the uncertainties in the slopes. Poillon[13] has shown that Trp is about twice as active as Phe. Thus the ratios with respect to Phe would be about twice the values shown here.

Chemistry

The synthesis of the 5-BrTrp dipeptides was accomplished by an adaptation of the mixed anhydride procedure that has been used previously in our laboratory.[23] Commercially available 5-BrTrp was converted to the Boc derivative by the procedure of Keller et al. in 92% yield.[24] The resulting Boc-5-BrTrp was converted to the mixed anhydride intermediate and then coupled with Trp-OMe to provide the protected dipeptide **3**, in 74% yield after chromatographic purification. 5-BrTrp required an activation time of 12 min for optimal yields, which is similar to the activation time for Trp. Diazomethane was used to convert 5-BrTrp to the corresponding methyl ester. Boc-Trp and 5-BrTrp-OMe were also coupled by the mixed anhydride procedure to produce **4** in 76% yield, and correspondingly, Boc-5-BrTrp and 5-BrTrp-OMe were coupled to produce **5** in 61% yield.

Deprotection was accomplished stepwise for each of the dipeptides. The methyl esters were saponified with NaOH in aqueous MeOH in greater than 90% yield following

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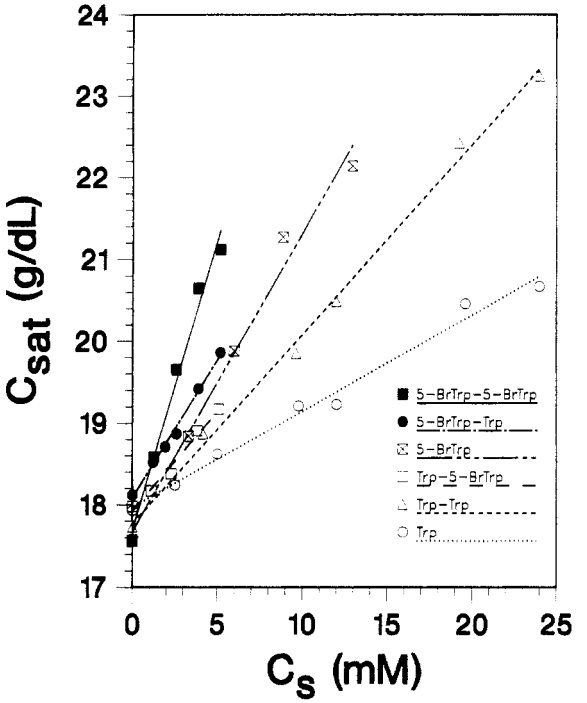

Figure 1. Solubility profiles for Trp derivatives in which the equilibrium solubility of deoxy-HbS, C_{sat}, has been plotted against the concentration of the additive, C_s. Data shown are averages from all of the assays for each compound. In all cases, HbS samples were equilibrated for 1 h and phase separation was obtained by ultracentrifugation for 1 h at 30 °C. The concentration of HbS in the supernatant phase corresponds to C_{sat}.

chromatographic purification. Because of the sensitivity of the indole nucleus to acid, the removal of the Boc group was done at −20 °C, using trifluoroacetic acid diluted with methylene chloride. ^{1}H NMR spectra, mass spectra, and elemental analyses confirmed the structures of the desired dipeptides 10–12.

Biological Evaluation

C_{sat} Assay. The 5-BrTrp-containing dipeptides were evaluated as inhibitors of the aggregation of deoxy-HbS by using a modification of the method of Mazhani et al.[25] The results were compared with those for Trp, which was included as a reference compound and are reported in Figure 1 and Table I.

Preliminary Toxicity Profile. The screening method first described by Irwin[26] and modified by Malone and Robichaud[27] was used to evaluate 5-BrTrp-Trp. 5-BrTrp and Trp-Trp were also included for comparison. Tests of central nervous system (CNS), autonomic, and neuromuscular effects were used to characterize the pharmacological activity profile in mice.

Locomotor Activity. Activity cages equipped with photoelectric cells were used to evaluate the sedative effects of 5-BrTrp-Trp and 5-BrTrp at a dose of 300 mg/kg in mice. The results are given in Table II.

Results and Discussion

The ability of compounds to inhibit the aggregation of deoxy-HbS has been used as an initial indication of whether the compounds have potential as antisickling agents. The assay data are shown in Figure 1 and are summarized in Table I and indicate that Trp-5-BrTrp is approximately equipotent to Trp-Trp in the C_{sat} assay. Both 5-BrTrp-5-BrTrp and 5-BrTrp-Trp are more potent than Trp-Trp, which indicates that the presence of the 5-BrTrp in the first position of the dipeptide makes an important contribution to the binding of the dipeptide to the binding site on the hemoglobin. It is also noteworthy that 5-BrTrp-5-BrTrp is even more potent than 5-BrTrp-Trp, and thus, the 5-BrTrp in the second position also makes a contribution to the binding interaction and thus the potency. Poillon has reported that Trp is approximately twice as potent as Phe. The potency of 5-BrTrp-5-BrTrp is about 6-fold more than that of Trp, which would make it about 12-fold more potent than Phe and would establish it as the most potent noncovalent inhibitor of deoxyhemoglobin S gelation reported so far. Sunshine et al.[28] have previously suggested that a potency of 10 times that of Phe would be estimated to reduce the clinical symptomology of sickle cell anemia to that of the less severe form of sickle cell disease, S/β^+-thalassemia. This has been considered as a minimum goal for the development of a clinically useful agent. Thus, the observation that 5-BrTrp-5-BrTrp has more than 10 times the potency of Phe is particularly significant because that threshold level has now been exceeded for the first time by a compound that binds to hemoglobin noncovalently and that can serve as a lead compound for further investigation and possible enhancement of potency.

Table II. Effects of Tryptophan Peptides on Locomotor Activity in Mice

	30-min counts			
	5-Br-Trp		5-Br-Trp-Trp	
group	before	after	before	after
1	495	94	428	142
2	463	149	469	131
3	229	141	321	87
4	573	162	239	74
⟨X⟩	440	136	364	108
SD	148	30	52	33
SEM	74	15	25	17
	$p = 0.007$ ($p < 0.01$)		$p = 0.003$ ($p < 0.01$)	

Since this series of compounds was the first to exhibit such a high level of potency, a preliminary assessment of potential toxicity was made. This preliminary evaluation of potential toxicity for these peptides showed that 5-BrTrp-Trp as well as 5-BrTrp produced a decrease in locomotor activity, apparently due to mild CNS depression associated with ip administration of high doses (300 and 700 mg/kg) in mice. Some degree of writhing movements and increased urination was also seen at the 700 mg/kg dose of these compounds. Trp-Trp was not different from the Gly-Gly controls at any dose level. Further evaluation of 5-BrTrp-Trp and 5-BrTrp in the locomotor activity cage test indicated that both compounds significantly ($p < 0.01$) decreased locomotor activity in mice at 300 mg/kg and were apparently equipotent as reported in Table II. The mechanism for the observed CNS depression is not known at this time, but it may be the result of direct or indirect serotonin agonist activity. Decarboxylation of 5-bromotryptophan would produce 5-bromotryptamine which is a structural analogue of serotonin. Serotonin is known to be associated with sedative or sleep-inducing activity.[29] Also, *p*-chlorophenylalanine, a tryptophan hydroxylase

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inhibitor, depletes the brain of serotonin in cats, and methysergide, a serotonin antagonist, produces an increase in wakefulness and produces insomnia in humans.[30,31]

Further attempts to increase the potency of 5-BrTrp peptides are currently underway in our laboratory. The structure–activity relationships associated with the observed CNS depression must also be understood and the structure modified accordingly in order to develop antisickling agents based on **12**. The substantial increases in potency observed to date have shown that this class of compounds can have potential for the development of a possible treatment for sickle cell anemia.

Experimental Section

Melting points were obtained on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Samples were dried overnight in vacuo prior to melting point determination or elemental analysis. Solvents and other reagents were reagent grade or better and used without further purification, except anhydrous methylene chloride, which was prepared by distillation from P_2O_5. 5-Bromo-DL-tryptophan and other organic intermediates were obtained from Aldrich. ^1H NMR spectra were measured on a Varian XL-300 (300-MHz) instrument. Chemical shifts are reported as δ values (ppm) relative to Me_4Si as internal standard. Mass spectra (CI and FAB) were recorded on a MAT-90 (Finnegan MAT) double-focusing mass spectrometer. Elemental analyses were performed by Midwest Micro Lab, Inc. (Indianapolis, IN). Column chromatography was performed on silica gel 60, 60 A (Merck).

***N*-(*tert*-Butyloxycarbonyl)-DL-5-bromotryptophan (2).** To a stirred solution of 5-bromo-DL-tryptophan (1; 0.5 g, 1.76 mmol) in 0.25 M NaOH (7 mL, 1.75 mmol) was added a solution of di-*tert*-butyldicarbonate (0.5 g, 2.3 mmol) in dioxane (5 mL) in one portion and the mixture was stirred for 2.5 h at ambient temperature.[24] The reaction mixture was then washed with ether (10 mL) and the aqueous layer was acidified with 0.1 M HCl. The precipitate formed was filtered, and the aqueous phase was extracted with ethyl acetate (3 × 10 mL). The combined organic layers were washed with brine (10 mL) and dried ($MgSO_4$). Evaporation of the solvent gave a white solid which was combined with the precipitate and dried to give 625 mg (92%) of pure product: mp 162–163 °C; ^1H NMR (CD_3COCD_3) δ 1.38 (s, 9 H), 3.15–3.36 (m, 2 H), 4.45–4.55 (dd, 1 H), 6.9 (d, 1 H), 7.2 (d, 1 H, 8 Hz), 7.28 (s, 1 H), 7.35 (d, 1 H, 8 Hz), 7.78 (s, 1 H); MS-CI (NH_3) 402, 400 (M + NH_4^+). Anal. ($C_{16}H_{19}BrN_2O_4$) C, H, Br, N.

DL-5-Bromotryptophan Methyl Ester Hydrochloride (3). To a stirred solution of 5-BrTrp (0.5 g, 1.7 mmol) in ether (50 mL) and methanol (10 mL) was added a solution of diazomethane (excess) in ether. Anhydrous HCl_g (1.87 mmol) in anhydrous tetrahydrofuran was added at 0 °C, and the mixture was allowed to stand for 5 min and evaporated to dryness to yield 5-BrTrpOMe·HCl, (3; 0.58 g, 100%): ^1H NMR ($CDCl_3$) (free base) δ 3.02 (dd, 1 H), 3.19 (dd, 1 H), 3.8 (m, 1 H), 7.02 (d, 1 H), 7.15–7.20 (m, 2 H), 7.71 (d, 1 H), 8.47 (br s, 1 H).

***N*-(*tert*-Butyloxycarbonyl)-5-bromo-DL-tryptophanyl-L-tryptophan Methyl Ester (4).** To a stirred solution of **2** (300 mg, 0.78 mmol) and *N*-methylpiperidine (81 mg, 0.1 mL, 0.82 mmol) in anhydrous methylene chloride (5 mL) was added isobutyl choroformate (105 mg, 0.1 mL, 0.77 mmol) dropwise at −15 °C. After 12 min, a solution of tryptophan methyl ester hydrochloride (200 mg, 0.78 mmol) and *N*-methylpiperidine (81 mg, 0.1 mL, 0.82 mmol) in anhydrous methylene chloride (5 mL) was added. The reaction mixture was stirred at −10 °C for 2 h, allowed to warm to room temperature, and stirred overnight. The mixture was washed with 5% $NaHCO_3$ (3 × 10 mL), 0.1 M HCl (3 × 10 mL), brine (10 mL), dried ($MgSO_4$), and evaporated to dryness (0.51 g). Chromatographic purification on silica gel and gradient elution with ethyl acetate and hexane (1:9) to ethyl acetate and hexane (7:3) yielded 339 mg (74%) of **4** as a white solid: mp 127–131 °C; ^1H NMR ($CDCl_3$) δ 1.39 (s, 9 H), 3.05–3.2 (m, 4 H), 3.58 (s, 3 H), 4.4 (s br, 1 H), 4.8 (s br, 1 H), 5.05–5.1 (d br, 1 H), 6.4–6.8 (m, 3 H), 7.0–7.45 (m, 6 H), 7.7 (dd, 1 H), 8.2–8.34 (br m, 2 H). Anal. ($C_{28}H_{31}BrN_4O_5$) C, H, N.

***N*-(*tert*-Butyloxycarbonyl)-L-tryptophanyl-5-bromo-DL-tryptophan Methyl Ester (5).** *t*-Boc-L-Trp (250 mg, 0.82 mmol) was activated with isobutyl chloroformate (112 mg, 0.11 mL, 0.82 mmol) and *N*-methylpiperidine (81 mg, 0.82 mmol) in methylene chloride (5 mL) at −12 °C for 12 min. A solution of 5-bromo-DL-tryptophan methyl ester hydrochloride (**3**; 250 mg, 0.74 mmol) and *N*-methylpiperidine (73 mg, 0.1 mL, 0.74 mmol) was added dropwise. The workup and purification by chromatography were accomplished as for **4** to give 333 mg (76%) of pure product: mp 123–127 °C; ^1H NMR ($CDCl_3$) δ 1.40 (s, 9 H), 3.0–3.25 (m, 4 H), 3.60 (s, 3 H), 4.42 (br m, 1 H), 4.75 (br m, 1 H), 5.2 (br s, 1 H), 6.25–6.4 (m, 1 H), 6.45–6.75 (m, 1 H), 6.4–6.7 (m, 8 H), 8.1–8.25 (br m, 2 H). Anal. ($C_{28}H_{31}BrN_4O_5$) H, Br, N, C: calcd, 57.64; found, 58.19.

***N*-(*tert*-Butyloxycarbonyl)-5-bromo-DL-tryptophanyl-5-bromo-DL-tryptophan Methyl Ester (6).** *t*-Boc-5-Br-DL-Trp (**2**; 250 mg, 0.65 mmol) was activated with isobutyl chloroformate (88 mg, 0.09 mL, 0.65 mmol) and *N*-methylpiperidine (64 mg, 0.09 mL, 0.65 mmol) and coupled with 5-Br-TrpOMe·HCl (**3**; 217 mg, 0.65 mmol) according to the mixed anhydride procedure described above for **4** to produce 265 mg (61%) of **6**: mp 119–122 °C; ^1H NMR ($CDCl_3$) δ 1.41 (s, 9 H), 2.9–3.2 (m, 4 H), 3.65 (s, 3 H), 4.4 (br m, 1 H), 4.8 (br m, 1 H), 6.4–6.9 (m, 4 H), 7.15–7.3 (m, 4 H), 7.45–7.75 (m, 2 H), 8.3 (2 H, m). Anal. ($C_{28}H_{30}BrN_4O_5$) N, C: calcd, 50.77; found, 51.61; H: calcd, 4.57; found, 5.16.

***N*-(*tert*-Butyloxycarbonyl)-5-bromo-DL-tryptophanyl-L-tryptophan (7).** To a stirred solution of **5** (200 mg, 0.34 mmol) in methanol (5 mL) was added 3% NaOH (5 mL) at ambient temperature. After stirring for 20 min, the aqueous solution was washed with ether (10 mL) and acidified with 0.1 M HCl at 0 °C. The milky solution was extracted with ethyl acetate (3 × 10 mL); the organic layer was dried ($MgSO_4$) and evaporated to give a pale yellow solid. Chromatographic purification on silica gel and gradient elution with 30% ethyl acetate and 2% acetic acid in hexane to 70% ethyl acetate and 2% acetic acid in hexane yielded 180 mg (92%) of **7** as a white solid: mp 160–165 °C dec; ^1H NMR ($CDCl_3$) δ 1.34 (s, 9 H), 2.9–3.2 (m, 4 H), 4.35 (br m, 1 H), 4.72 (br m, 1 H), 6.4–6.9 (m, 4 H), 7.0–7.45 (m, 5 H), 7.55–7.7 (m, 2 H), 8.2–8.5 (m, 3 H). Anal. ($C_{22}H_{29}BrN_4O_5$) C, H, N.

***N*-(*tert*-Butyloxycarbonyl)-L-tryptophanyl-5-bromo-DL-tryptophan (8).** *t*-Boc-L-Trp-5-DL-TrpOMe (200 mg, 0.35 mmol) was hydrolyzed with a 1:1 mixture of methanol and 3% NaOH with workup and purification as described above for **7** to give 185 mg (95%) of pure **8**: mp 168–172 °C; ^1H NMR ($CDCl_3$) δ 1.30 (s, 9 H), 2.8–3.3 (m, 4 H), 4.45 (br m, 1 H), 4.68 (br m, 1 H), 6.4–6.95 (m, 4 H), 7.0–7.35 (m, 5 H), 7.5–7.7 (m, 2 H), 8.1–8.6 (m, 3 H). Anal. ($C_{22}H_{29}BrN_4O_5$) C, H, Br, N.

***N*-(*tert*-Butyloxycarbonyl)-5-bromo-DL-tryptophanyl-5-bromo-DL-tryptophan (9).** Hydrolysis of *t*-Boc-5-Br-DL-Trp-5-Br-DL-TrpOMe (200 mg, 0.3 mmol) with 3% NaOH and methanol as described above for **7** gave 180 mg (92% yield) of pure **9** after chromatography: mp 154–157 °C dec; ^1H NMR ($CDCl_3$) δ 1.40 (s, 9 H), 2.9–3.3 (m, 4 H), 4.4 (br m, 1 H), 4.8 (br m, 1 H), 6.4–6.95 (m, 4 H), 7.15–7.35 (m, 4 H), 7.45–7.75 (m, 2 H), 8.2–8.35 (m, 2 H). Anal. ($C_{22}H_{28}Br_2N_4O_5$) C, H, N.

5-Bromo-DL-tryptophanyl-L-tryptophan (10). To a stirred solution of *t*-Boc-5-Br-DL-Trp-L-Trp (420 mg, 0.73 mmol) in anhydrous methylene chloride (2 mL) was added anhydrous trifluoroacetic acid (4 mL) dropwise at −20 °C over 2 h and the mixture was allowed to warm up to ambient temperature. When the reaction was complete (TLC), hexane was added to precipitate the product and the precipitate was washed with anhydrous ether (3 × 10 mL). To this solid was added 0.1% NH_4OH (0.5 mL) and the solution was freeze dried. Water (0.5 mL) was added, and the ammonium trifluoroacetate and water were removed by freeze drying. After repetition of this procedure (5x), the solid was purified by recrystallization from water and ethanol to give **10** as a white solid: total yield 285 mg (82%); mp 182 °C dec; ^1H NMR (CD_3OD) δ 2.75–3.33 (m, 4 H), 3.82–3.9 (m, 1 H), 4.58 (m, 1 H), 4.8 (br s, 6 H), 6.68–7.76 (br m, 1 H), 6.80 (s, 1 H), 6.89–6.94 (m, 1 H), 6.98–7.06 (m, 2 H), 7.15–7.29 (m, 3 H), 7.42–7.54 (m, 1 H); MS-FAB 471, 469 (M + 1). Anal. ($C_{22}H_{21}BrN_4O_3 \cdot H_2O$) C, H, N.

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L-Tryptophanyl-5-bromo-DL-tryptophan (11). *t*-Boc-L-Trp-5-Br-DL-Trp (100 mg, 0.17 mmol) was deprotected according to the same procedure as for **10** to yield 48 mg (58%) of pure **11**: mp 165 °C dec; ^{1}H NMR (CD_3OD) δ 2.9–3.3 (m, 4 H), 4.05 (m, 1 H), 4.6 (m, 1 H), 6.9–7.25 (m, 5 H), 7.3–7.8 (m, 4 H); MS-FAB 471, 469 (M + 1). Anal. ($C_{22}H_{21}BrN_4O_3 \cdot 0.5H_2O$) H, Br, N, C: calcd, 55.24; found, 55.79.

5-Bromo-DL-tryptophanyl-5-bromo-DL-tryptophan (12). *t*-Boc-5-Br-DL-Trp-5-Br-DL-Trp (150 mg, 0.23 mmol) was deprotected according to the same procedure as for **10** to yield 68 mg (54%) of pure **12**: mp 175 °C dec; ^{1}H NMR (CD_3OD) δ 2.9–3.3 (m, 4 H), 4.45 (m, 1 H), 4.8 (m, 1 H), 6.4–6.95 (m, 2 H), 7.1–7.3 (m, 4 H), 7.4–7.7 (m, 2 H); MS-FAB 551, 549, 547 (M + 1). Anal. ($C_{22}H_{20}Br_2N_4O_3 \cdot 1.5H_2O$) C, H, Br, N.

Preparation of HbS and C_{sat} Assay. Venous blood, anticoagulated with ethylenediaminetetraacetate (EDTA), was drawn from homozygous sickle cell anemia patients. The presence of sickle hemoglobin (SS) as opposed to SA or SC types was confirmed by electrophoresis using commercial Titan III cellulose acetate plates and $AFSA_2$ hemoglobin as a control (Helena Laboratories, TX). The HbF content of HbS was determined to be less than 5%. Membrane-free HbS was prepared according to the method of Rossi-Fanelli et al.,[32] except that 0.05 M phosphate-buffered saline (PBS) was used instead of NaCl. Ammonium sulfate was removed by passing a 15% HbS solution through a Sephadex G-25 column[33] which was eluted with 0.05 M phosphate buffer at pH 7.0. HbS was concentrated with cone ultrafiltration (Amicon) and stored as HbSCO under liquid nitrogen for later use.

The saturation concentration (C_{sat}) of deoxy-HbS by ultracentrifugation was determined according to the method described by Mazhani et al.[25] with some modifications as described below. Prior to deoxygenation, HbSCO was oxygenated by flushing O_2 slowly into a round-bottom flask containing HbSCO on a rotary evaporator under a flood lamp. Oxygenation was confirmed by an optical spectrum measurement. At 4 °C, 250 μL of 35% HbS-O_2 was added to 5 × 41 mm Beckman ultraclear centrifuge tubes containing 80 μL of a suitable concentration of the test substance. Each assay was run with six tubes, including one with no inhibitor and one containing Trp, as controls. Deoxygenation of HbS occurred upon addition of 20 μL of a 0.9 M sodium dithionite solution. Each tube was mixed thoroughly and incubated in an ice bath for 20 min. After 10 min, the tubes were mixed again to ensure a homogeneous solution. These tubes were then incubated in a refrigerated circulating bath under anaerobic conditions for 2 h at 30 °C. Gelled samples of 350 μL total volume were centrifuged for 1 h at 20400*g* in a SW 55Ti rotor at 30 °C. After centrifugation, the supernatant phase (monomeric HbS) was separated and converted to cyanomet-Hb with an excess of Drabkin's solution. The concentration of cyanomet-Hb was determined spectrophotometrically with the millimolar extinction coefficient at 540 nm.[34] An average of three absorbances per point was used to determine the concentrations of cyanomet-Hb. Solubility in grams per deciliter was plotted as a function of millimolar concentrations of the test compounds. Slopes were then compared to that of L-Trp as the standard. The initial concentration of HbS was between 24 and 26 g/dL.

Determination of Toxicity Profile. Pharmacological profile and evidence of toxicity were evaluated by using the method of Irwin[26] as modified by Malone and Robichaud.[27] Briefly, the method uses a battery of tests to determine the effects on CNS, autonomic, and neuromuscular systems. Solutions of 5-BrTrp (pH 9.9), 5-BrTrp-Trp (pH 8.3), and Trp-Trp (pH 7.1) were prepared in deionized, distilled water using 0.1 N NaOH or 0.1 N HCl as needed to aid solubility. The final concentrations were 30–40 mM. All solutions of test compounds were prepared fresh on the day of the experiment and were administered intraperitoneally (ip). A solution of Gly-Gly (pH 7.35) was administered to control animals. Male Swiss-Webster mice (20–30 g) were divided into four groups of five mice per group for a total of 20 animals for each compound tested. Doses of 100, 300, and 700 mg/kg were administered. Only one compound was tested per set of animals. No animal received more than one dose or more than one compound.

Following the administration of each compound, the mice were observed in individual cages for a period of 3 h for any pharmacological effects or lethality. The volume of solution injected did not exceed 0.7 mL.

Locomotor Activity. Activity cages equipped with photoelectric cells were used to evaluate the extent of the observed decrease in locomotor activity. A total of 20 mice were used for each compound. Four cages containing five mice per cage were used. Movements were recorded digitally for 30 min prior to ip administration of a 300 mg/kg dose of either 5-BrTrp or **10**. After a delay of 20 min, movements were again recorded for a 30-min period.

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