Regioselective Covalent Modification of Hemoglobin in Search of Antisickling Agents

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Although the molecular defect in sickle hemoglobin that produces sickle cell disease has been known for decades, there is still no effective drug treatment that acts on hemoglobin itself. In this work, a series of diversely substituted isothiocyanates (R-NCS) were examined for their regioselective reaction with hemoglobin in an attempt to alter the solubility properties of sickle hemoglobin. Electrospray mass spectrometry, molecular modeling, X-ray crystallography, and conventional protein chemistry were used to study this regioselectivity and the resulting increase in solubility of the modified hemoglobin. Depending on the attached R-group, the isothiocyanates were found to react either with the Cysβ93 or the N-terminal amine of the α-chain. One of the most effective compounds in the series, 2-(*N*,*N*-dimethylamino)ethyl isothiocyanate, selectively reacts with the thiol of Cysβ93 which, in conjunction with the cationic group, was seen to perturb the local hemoglobin structure. This modified HbS shows an approximately 30% increase in solubility for the fully deoxygenated state, along with a significant increase in oxygen affinity. This compound and a related analogue appear to readily traverse the erythrocyte membrane. A discussion of the relation of these structural changes to inhibition of gelation is presented. The dual activities of increasing HbS oxygen affinity and directly inhibiting deoxy HbS polymerization, in conjunction with facile membrane traversal, suggest that these cationic isothiocyanates show substantial promise as lead compounds for development of therapeutic agents for sickle cell disease.

Introduction

Sickle cell anemia results from the substitution of Val for Glu at position 6 in the hemoglobin β-chain. This substitution results in abnormal rigidity of erythrocytes at low oxygen tension, due to intracellular polymerization of the sickle hemoglobin (HbS). In turn, this leads to occlusions in the microcirculation.[1] The polymerization of HbS involves both axial and lateral contacts,[2] with one mutant Valβ6 residue participating in a lateral intermolecular hydrophobic contact through the interaction between the donor Valβ6 and the acceptor Pheβ85 and Leuβ88.[2−4]

Hydroxyurea is currently the most useful therapy for reducing the symptoms of sickle cell disease, but patients exhibit variable responses, and it does not eliminate symptoms.[5] Thus, there is still interest in finding therapeutic agents that directly block HbS polymerization. Unlike the development of most drugs, researchers have adopted covalent modification as an acceptable approach in their search for antisickling agents.[1] This rationale centers on the very large amount of HbS that must be targeted. For noncovalent drugs with dissociation constants lower than 10^{-6} molar, nearly all of the drug would be bound to HbS because of the unusually high concentration of the target Hb protein,[6] i.e., approximately 2×10^{-3} molar Hb concentration in the blood at normal hematocrit. As it is generally accepted that at least 20% of the total hemoglobin must be altered to produce a clinically significant outcome,[4,7] this would require a minimum drug concentration of approximately 0.4 mM for the noncovalent case—a blood level that would be considered unacceptable. Furthermore, for the noncovalent case, the putative drug would be continuously excreted or metabolized, requiring chronic administration at high doses.

On the other hand, a covalent drug administered repeatedly and in low doses, could progressively modify the target macromolecules due to the typically longer half-life of the covalent complex. In the case of Hb, this half-life is on the order of 120 days,[8] and could translate into a blood-level advantage of greater than 2 orders of magnitude for the covalent approach. Although there can be toxicity problems with this approach to drug design, it should be noted that important drugs, such as aspirin and phenoxybenzamine, which act through covalent modification of the cyclooxygenase,[9] and the β-adrenergic receptor,[10] respectively, clearly demonstrate that such an approach can exhibit an acceptable therapeutic window.

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One approach to reduce the HbS polymerization has been to disrupt or to destabilize the intermolecular interactions of the deoxy (T) state conformation by direct covalent modification of the HbS protein. In essence, the effect of the pathologic point mutation is reversed by modification of an amino acid at another site in the protein. Several structurally unrelated compounds have been partially successful in this regard.[11] Any specificity of covalent reaction of agents on the Hb surface must ultimately result from differences in the reactant's structure interacting with the diverse topological features on the protein's surface. On the reactant side, these structural features can be arbitrarily divided into two categories: (1) the nature of the reactive functionality, and (2) the structure of the group attached to the reactive function. In the latter case, regioselectivity for a particular site on the molecule is expected to result from favorable or unfavorable interactions with the protein's surface, e.g., binding complementarity of the R group with the protein prior to reaction, or perhaps steric hindrance leading to exclusion from reaction at a particular site.

In the present work, we examine a series of isothiocyanates, in which only the R-group is changed, for their regioslective reactions on the Hb surface. The initial measure of selectivity involved determining whether the agents reacted covalently with the α- or the β-chain of the Hb tetramer. Selected isothiocyanates were tested for antisickling activity, and for those showing the greatest antisickling activity, the amino acid side chain responsible for the covalent modification was also determined. The ability to inhibit HbS polymerization was shown to correlate with both the site of modification and the structure of the R-group of the reacting isothiocyanate. In addition, the biological activity was correlated with the extent of HbS modification for one of the more active agents, a dimethylaminoethyl isothiocyanate. Molecular modeling also suggests that the R-group may be directing the isothiocyanate moiety in its reaction with the Hb surface. Finally, molecular modeling also suggests that the activity of the dimethylaminoethyl isothiocyanate in inhibiting deoxy HbS polymerization may result from perturbation of intermolecular electrostatic interactions within the HbS polymer fiber.

Chemistry

Isothiocyanate Preparations. Structurally diverse isothiocyanates (Figure 1) were required to evaluate their chemical reaction with the Hb surface. The starting amines for preparation of the isothiocyanates were chosen from a set of chemically diverse amines that were commercially available. The derivatives included both aryl and alkyl derivatives containing neutral, positive, and negatively charged functionality, as well as different structural variations such as cyclic and acyclic structures. For example, the aryl isothiocyanates **1**−**6** are composed of two acids (**5**, **6**), two bases (**3**, **4**), and two neutrals (**1**, **2**). Similarly, the alkyl isothiocyanates, **7**−**21**, are composed of an acid (**9**), seven neutrals (**8**, **10**−**12**, **15**−**17**), and seven bases (**7**, **13**, **14**, **18**−**21**).

The isothiocyanates shown in Figure 1 were synthesized by one of three different methods. Isothiocyanate **5** was formed from *p*-aminosalicylic acid via a procedure

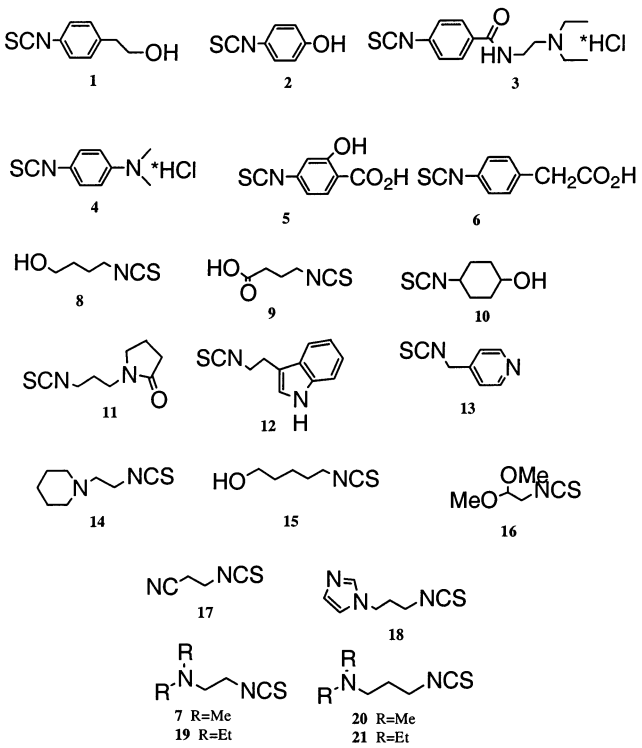
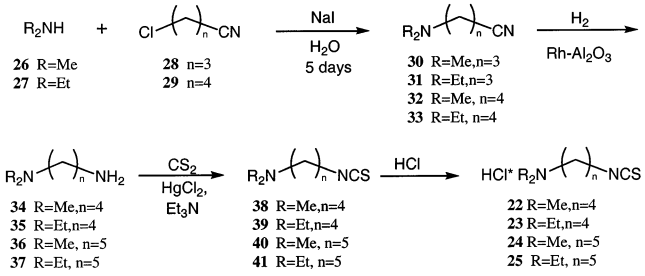


Figure 1. Isothiocyanates prepared for reaction with HbA.

Scheme 1



adapted from Seligman utilizing thiophosgene.[12] The syntheses of isothiocyanates **1**−**4**, **6**, **8**−**18** was accomplished starting from their respective primary amines, following a previously reported procedure[13] using di-2-pyridyl thionocarbonate. This procedure gave isothiocyanates **1**−**4**, **6**, **8**−**18** in fairly high yields, after either column chromatography or vacuum distillation. Dialkylaminoalkyl isothiocyanates **7** and **19**−**21** were prepared by a previously reported procedure from McElhinney,[14] using carbon disulfide and $HgCl_2$ in acetone. Removal of the mercury salts by filtration, followed by vacuum distillation, provided the isothiocyanates (**7** and **19**−**21**).

The synthesis of hydrochloride salts **22**−**25** is outlined in Scheme 1. Following a modified preparation,[15,16] the dialkylamine (**26** or **27**), chloronitrile (**28** or **29**), and sodium iodide, in water, were stirred for 5 days. Vacuum distillation yielded dialkylaminonitriles **30**−**33**. Catalytic hydrogenation over a rhodium−alumina catalyst afforded mixtures of primary (**34**−**37**) and the corresponding secondary amines. Unable to easily separate these amines, the mixture was used to prepare isothiocyanates **38**−**41**, as only the primary amine will form the isothiocyanate, which were then isolated by vacuum distillation. The white hydrochloride salts (**22**−**25**) were

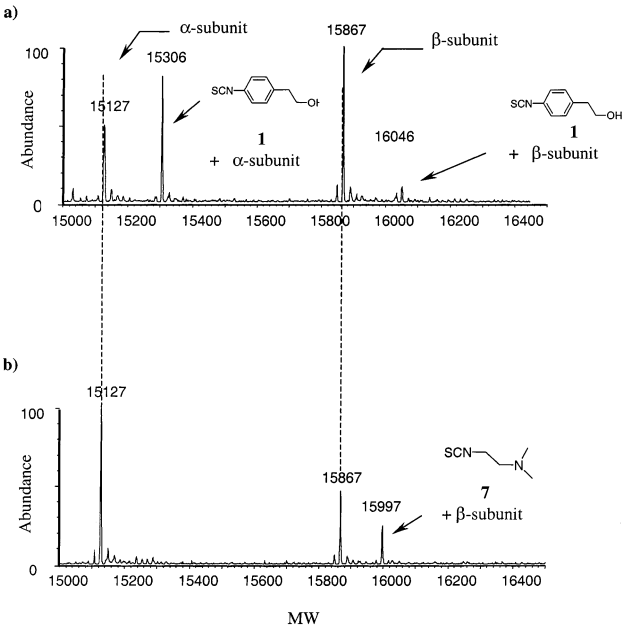


Figure 2. Mass shift analysis of covalently modified HbA processed by maximum entropy deconvolution. (a) HbA modified with isothiocyanate **1** (MW 179), (b) HbA modified with isothiocyanate **7** (MW 130).

formed by bubbling HCl gas through an ether solution of the crude isothiocyanate.

Selective Hb α- and *β*-Chain Modification by Isothiocyanates. Reactions of the various isothiocyanates with the α- and *β*-chains of HbA were analyzed by electrospray mass spectrometry (ESMS) as previously described.[17] Isothiocyanates **1**, **4**, and **7**−**9** were tested in triplicate. The remaining analyses were singular. The variability in triplicate experiments was about 10%, suggesting the general uncertainty expected for this type of analysis. As can be seen, there are clear differences between the reactivity of these derivatives and the α- and *β*-subunits of the HbA molecule, suggesting that the R-group of the isothiocyanate does, in fact, influence the course of the reaction.

For example, the ESMS of HbA modified with aryl isothiocyanate **1** and alkyl isothiocyanate **7** are shown in Figure 2. In electrophilic addition of the isothiocyanate with a nucleophile, there is no other side product, and the mass of the product is equal to the sum of the mass of the isothiocyanate and the nucleophile, here the Hb subunit. As may be seen in Figure 2a, there is a 15306 *m*/*z* ion corresponding to the modified α-subunit, and one at 16046 *m*/*z* for the *β*-subunit, indicating that both subunits react, at least to some extent, with the aryl isothiocyanate **1** (MW 179). There was no indication that either subunit was modified by more than one molecule of isothiocyanate. The latter would, of course, be indicated by a family of peaks separated by 179 amu. All of the reactions studied between the isothiocyanate and the two subunits showed only a one-to-one reaction under the conditions used. The selectivity of isothiocyanates **1**−**13**, **16**−**18**, **20**, **21** for the α- and *β*-subunit are listed in Table 1.

The clear preference for the aromatic alcohol **1** to react with the α- versus the *β*-subunit suggests selectivity of this reagent for the α-subunit (>7×). However, this property was not shared by all of the isothiocyanates.

Table 1. Competitive Reaction of Isothiocyanates for the α- and *β*-Subunits of Normal Hemoglobin[a]

compound (200 μM)	% of subunit modification (100 μM HbA) at 3 h	
	α-subunit	*β*-subunit
1	72	8
	66	9
	72	9
2	51	8
3	40	17
	36	16
	42	15
4	48	10
5	35	3
6	42	13
7	0	34
	0	40
	0	41
8	3	31
	2	36
	2	35
9	0	25
	0	28
	0	30
10	0	18
11	0	24
12	7	24
13	6	58
16	11	33
17	10	21
18	7	13
20	0	38
21	0	37

[a] Products were identified by the shift in mass of the corresponding covalently modified Hb subunits. Multiply charged ion spectra were processed using a maximum entropy deconvolution, and the yield was obtained from the equation: yield = height of modified/(height of unmodified + height of modified).

Thus, ESMS analysis of the reaction of alkyl isothiocyanate **7** (MW 130) with HbA indicated no detectable reaction with the α-subunit. Instead, the ions represented by the peak at 15997 *m*/*z* in Figure 2b indicate almost exclusive reaction with the *β*-subunit. Indeed, examination of the isothiocyanates tested revealed a general trend (Table 1). Isothiocyanates **1**−**6**, where the isothiocyanate is directly attached to an aromatic ring, showed selectivity for the α-subunit. On the other hand, alkyl isothiocyanates **7**−**13**, **16**−**18**, **20**, **21**, where the reactive moiety is attached to an sp^3 hybridized carbon, were found to preferentially modify the *β*-subunit.

However, this selectivity is not absolute. For example, aryl isothiocyanates **3** and **4** showed a 2-fold selectivity for the α-subunit relative to the *β*-subunit. Furthermore, we could not achieve complete, selective modification of the α-subunit over the *β*-subunit, using various conditions of reaction time and concentration, or reactant ratios. Alkyl isothiocyanates also showed varying degrees of selectivity depending on the structure of the substituent. For example, amine isothiocyanate **7** and acid isothiocyanate **9** showed a clear preference for the *β*-subunit. But heterocyclic isothiocyanates **12** and **13** had about a 10−20% selectivity for the α-subunit relative to the *β*-subunit. Taken together, these results strongly suggest that different isothiocyanates are reacting at different sites on the Hb molecule and with different degrees of regioselectivity as a function of the attached R-group. To better understand this selectivity, we determined the specific amino acid residues being covalently modified with selected isothiocyanates and

examined the reaction specificity using molecular modeling and X-ray crystallography.

Isothiocyanate Reaction Site on the α-Chain. Similar to the methods of Desiderio,[18] we used trypsin digestion with reversed phase HPLC/ESMS analysis of the resultant peptide fragments for identification of the isothiocyanate reaction site on the Hb α-chain. We selected two aryl isothiocyanates, **1** and **3**, as representative of this group for defining the site of reaction on the α-subunit. These two isothiocyanates provide a high degree of α-subunit modification with different structural characteristics (**1**, alcohol; **3**, amine). Samples of HbA·CO were first individually modified with aryl isothiocyanates **1** and **3**. These modified HbA molecules were stirred in acidic acetone to remove heme, and then the precipitated globins were digested with trypsin. The resultant peptide mixtures were analyzed by reversed-phase LC/MS, and the resulting chromatograms were compared to that of unmodified HbA·CO (data not shown).

Extracted ion chromatograms of the α-subunit were used to identify the residue responsible for the modification. Trypsin digestion of the α-subunit of hemoglobin should theoretically generate fourteen peptide fragments, since trypsin is specific for Lys and Arg residues. Thus, eleven cuts at Lys and three cuts at Arg residues, where one of the Arg residues is C-terminal, should occur. If the isothiocyanate modified a Lys, trypsin hydrolysis would be unlikely at that particular Lys residue. The molecular weight of such an unhydrolyzed fragment would be the sum of the molecular weight of the two theoretical fragments and that of the isothiocyanate minus water. The ESMS total ion chromatograms were scanned for these possible molecular weights. However, peaks corresponding to such fragments were not detected.

If the isothiocyanate modified an amino acid within a fragment, the molecular weight of the modified fragment would be the sum of the molecular weight of the unmodified fragment and the given isothiocyanate. The ESMS total ion chromatogram scanned for these masses allowed us to identify one new peak corresponding to the addition of the isothiocyanate to the fragment from the hydrolysis of the first Lys (α7); i.e., $m/z = 908$ from the alcohol-substituted isothiocyanate **1**, and $m/2z = 504$ and $m/z = 1006$ from the amine-substituted isothiocyanate **3**. For the α1−α7 fragment (sequence VLSPADK) there are two possible nucleophiles for reaction with an isothiocyanate: the N-terminal amine and the ϵ-amino group of Lysα7. However as mentioned, hydrolysis of a modified Lys residue is unlikely. Therefore, it was concluded that the reaction site on the α-subunit for the aryl isothiocyanates was the N-terminal residue.

Isothiocyanate Reaction Site on the *β*-Chain. In an attempt to determine the reaction site on the *β*-subunit for the alkyl isothiocyanates, trypsin digestion was again applied as in the aryl isothiocyanate case, expecting that the *β*-chain N-terminal amine would be similarly modified. The aliphatic isothiocyanates **7**−**9** were used for these analyses. Unexpectedly, the HPLC chromatograms of the digested peptide were the same as that for the unmodified HbA controls. One possible explanation for this could be that the functional group generated from modification by the alkyl isothiocyanates is unstable to the experimental conditions. Further, it was shown by ESMS that, although the covalent bond with the alkyl isothiocyanate was stable to the acidic acetone used to remove the heme, incubation in the slightly basic ammonium bicarbonate buffer (pH 8.5) that was used for the tryptic digestion, resulted in cleavage of the isothiocyanate bond with the protein for the adducts of **7**−**9**. Since the anticipated thiourea that would be formed by addition of the isothicyanate to the N-terminal amine should be stable under these conditions, this result suggested that modification of some other residue was occurring.

Chiancone has reported that the Cys*β*93 sulfhydryl group is solvent accessible and available for nucleophilic reactions.[19] To determine if this residue was the site of reaction, the ionic character of the HbA α-chain modified with alkyl isothiocyanates **7** (positive), **8** (neutral), and **9** (negative) was tested using electrophoresis. The sulfhydryl group is neutral under physiological conditions, and the new functional group formed from the reaction between the sulfhydryl group and the isothiocyanate is a dithiocarbamate that is also neutral under physiological conditions. On the other hand, if the reaction site is positively charged, for example an ϵ-amino group of Lys, this charge would be removed upon reaction with the isothiocyanate via the formation of a thiourea. Thus, the charge-state of the modified protein would be reduced by one. The electrophoretic pattern of HbA modified with the three different alkyl isothiocyanates, **7**, **8**, and **9**, matched that expected when a sulfhydryl group was the site of reaction (data not shown).

Additional evidence that the aliphatic isothiocyanates react with the *β*93Cys residue was obtained by titration of free sulfhydryl groups in the modified Hb using the *p*-chloromercuribenzoic acid method of Boyer.[20] HbA has 6 Cys residues, two from the two α-subunits, and four from the two *β*-subunits. Among these, only the two Cys*β*93s are solvent accessible and reactive.[19] Given the above work, we expected two free sulfhydryl groups from HbA modified with the aryl isothiocyanates that react with the N-terminus of the α-subunit and no free sulfhydryl group from HbA modified with the alkyl derivatives.

HbA was modified with aryl isothiocyanate **5** and alkyl isothiocyanates **7**, **8**, and **9**, and then treated with 2 equiv of *p*-chloromercuribenzoic acid. The UV absorbance for these reaction products was then compared with a standard curve that was prepared with unmodified HbA treated with *p*-chloromercuribenzoic acid. The results of this titration indicate that HbA modified by the aryl isothiocyanate **5** has two free sulfhydryl groups, but HbA modified by alkyl isothiocyanates **7**, **8**, and **9** did not have any free sulfhydryl groups, as expected (data not shown). On the basis of this evidence and the electrophoresis data, it was concluded that Cys*β*93 was the reaction site for the alkyl isothiocyanates. This conclusion was verified by X-ray crystallographic analysis of deoxy Hb modified with 2-(*N*,*N*-dimethylamino)-ethyl isothiocyanate **7**, as discussed below.

Erythrocyte Permeability to Isothiocyanates. For these compounds to be therapeutically useful, they must be taken up by erythrocytes, and covalently react

with the intracellular Hb. To evaluate their potential for uptake, we have incubated **7** and **24** with washed erythrocytes, washed the cells to remove excess compound, and then measured Hb modification by mass spectrometry. We find that both compounds produce significant Hb modification, with **24** exhibiting about 2-fold higher modification than **7**, as might be expected from their relative cLogP values (1.0 and 2.5 for **7** and **24**, respectively). Thus, uptake and modification of intracellular Hb is clearly feasible, with optimization of cLogP probably the most important parameter for maximizing uptake.

Autodock Analysis of the Reaction of 2-(*N*,*N*-Dimethylamino)ethyl Isothiocyanate (7) with the Cysβ93 Site of Deoxy-HbS. The original premise of this work was that if one screened a series of reactive agents (e.g., substituted isothiocyanates), one might find agents which would covalently react with specific sites on a macromolecule. Further, specific alteration in the chemical structure of the protein could lead to specific changes in the biological activity of the protein, in this case Hb. The above experimental results suggest that such site-specific reactions on a protein surface are possible. Thus, whereas the alkyl isothiocyanates tend to react with the Cysβ93 residue, the aryl isothiocyanates tend to react preferentially with the N-terminus of the α-chain and, to a lesser extent, with the N-terminus of the β-chain.

To better understand the topological interactions underlying this regioselectivity, we have used Autodock[21] to analyze the binding of the 2-(*N*,*N*-dimethylamino)ethyl isothiocyanate ligand **7** to deoxy HbS (pdb code: 2HBS),[2] based on the assumption that initial noncovalent interaction with the protein influences the final covalent reaction. The results indicate that the isothiocyanate ligand binds preferentially to the β93 site. The lowest energy result indicates that the isothiocyanate unit is approximately 3.5 Å away from the sulfur of the cysteine. The orientation of the molecule is such that the isothiocyanate moiety is pushed into the pocket first, with the *N*,*N*-dimethylamino group sticking slightly out, near the surface of the HbS molecule. Visual analysis of the electrostatic maps show the Cysβ93 site to be a rather deep pocket that is dominated by high electronegativity in the area in and around the Cysβ93. This electronegativity could be expected to stabilize the positively charged amino-isothiocyanate ligand in this region. Further, the positively charged *N*,*N*-dimethylamino group shows an attraction for the nearby Aspβ94 carboxyl group on the perimeter surface of the pocket. The isothiocyanate ligand is positioned between two surface histidines, Hisβ97 and Hisβ146, which are approximately 4−6 Å away. These two histidines are separated on one side by the Aspβ94, and on the other side of the pocket by Proα37, Lysα40, and Thrα41. The Cysβ93 residue is actually underneath the Aspβ94, but has the reacting sulfur protruding slightly into the pocket so it is easily accessible to the incoming alkyl isothiocyanate unit.

Since this particular isothiocyanate is not bulky compared to the size of the pocket, it easily orients so the isothiocyanate can react with the sulfur of Cysβ93, while positioning the positively charged amino end toward the Aspβ94. This provides one explanation for the attraction of the isothiocyanate to this region. Inspection of the rest of the HbS molecular surface suggests that there are other regions of high electronegativity, e.g., around heme groups. However, there is no other region of high electronegativity which contains a pocket into which small ligands can "fall".

A similar Autodock analysis with aryl isothiocyanate **4** indicates that it also prefers Cysβ93 as its lowest energy binding site. Further, since it is known that aryl isothiocyanates are more reactive than alkyl isothiocyanates toward nucleophilic attack,[22] one would anticipate that the aryl isothiocyanates would also react with the thiol of Cysβ93. However as we have shown, the preferred reaction site is the N-terminus of the α-chain. A visual comparison of these two sites provides a probable explanation. The α-N-terminal amines are located near the α-entrance to the center cavity of the hemoglobin, where there is sufficient space for numerous (at least two) isothiocyanates, thus resulting in an unhindered reaction path for the isothiocyanates to the N-terminal amine. In contrast, the Cysβ93 site is a relatively small pocket (8 Å wide and deep). The nucleophilic thiol group of interest is located approximately midway down into the pocket, tucked under the carbonyl of the surface Aspβ94 (i.e., the thiol is not on the surface of the protein and is only accessible from one side). Molecular docking of the aryl and alkyl isothiocyanates into the Cysβ93 pocket shows the association of the thiol and isothiocyanate groups prior to the nucleophilic attack, indicating steric and electrostatic effects. Docking **7** into this site positions the isothiocyanate approximately 3.5 Å away from the thiol, with the alkyl chain bending away from the thiol group. Docking of the aryl isothiocyanate **4** positions the isothiocyanate group approximately 4 Å from the thiol and has the phenyl ring positioned between the two surface histidines, which are on either side of the Aspβ94. For the nucleophilic attack to actually occur, these isothiocyanate groups must move closer to the thiol. In the alkyl case, this is easily accomplished due to the negligible steric interactions with the neighboring residues and the alkyl chain flexibility. In the aryl case, the phenyl ring is favorably sandwiched between two histidines and is already in close proximity (less than 3 Å) to the carbonyl of the Aspβ94, prior to the reaction occurring. Due to the phenyl group's bulk and rigidity, it is sterically prevented from moving closer to the thiol by this Aspβ94, and instead, rotates itself away, so that the isothiocyanate directly attached to the ring is unable to get close enough to the thiol for the nucleophilic attack to take place. Therefore, we postulate that the aryl isothiocyanates are sterically prevented from reacting at the Cysβ93 site and are driven to react with the less reactive N-terminal amine group, while the smaller *N*,*N*-alkyl derivative reacts at the β93 residue.

Biological Activity and Covalent Modification. Ariens[23] and Stephenson[24] introduced the concept of affinity and efficacy to account for the alteration of protein function by noncovalent ligand−receptor interactions. It may be of value to examine similar concepts in discussing biological activity in the context of covalent modification. For example, in the present work, aryl isothiocyanates tend to modify the N-terminus of the α-subunit, while alkyl isothiocyanates preferentially

react with the Cysβ93 residue. These specificities of the covalent reactant can be equated to differences in "affinity" of the reactant for these two modification sites.

Covalent modification of a protein receptor by a ligand can be described by the following relationship:

$$P + L \leftrightarrow P{\cdot}L \rightarrow P{-}L \rightarrow \text{functional change}$$

where P is the protein, L is the covalent modifier, P·L is a noncovalent complex, and P−L is the covalently modified protein leading to a given functional change. In this scenario, the covalent modifier interacts with the protein to form a reversible complex, which then covalently modifies the protein to alter its biological function. The on/off rate of the reversible P·L complex is likely to be much faster than the covalent bond forming reaction. In this case, the noncovalent interaction with the protein, as a function of its K_d, could effectively increase the concentration of L at a specific site on the protein relative to other solvent accessible sites—one source of regioselectivity. Under proper conditions, the R-group of the incoming ligand may provide proper orbital alignment for enhanced reaction at a given site on the protein enhancing reaction at that given site.

It should also be recognized that reactants that react at the same site on a protein can change the structure of the protein in different ways depending on the attached R-group. In turn, this can lead to possible structure/function changes in the protein. The latter would be comparable to different drugs having different "efficacy" in the case of noncovalent receptor binding. After sufficient time for the chemical reaction to reach completion, one would expect that any biological activity caused by alteration in the protein's structure would show a linear relationship with respect to the concentration of the P−L covalent complex so formed, and that the intrinsic efficacy of the reacting ligand would be represented by the slope of this line.

With these thoughts in mind, we tested the antisickling activity of several isothiocyanates to identify which covalent site gave the greater antisickling activity. In addition, we examined the relationship of activity with respect to the amount of covalently modified protein and evaluated the concept of "efficacy" of structurally different isothiocyanates reacting at the Cysβ93 site.

Antisickling Activity of Sickle Hemoglobin Covalently Modified with Various Isothiocyanates. HbS modified with two aryl (**1**, **5**) and fifteen alkyl isothiocyanates (**7**−**9**, **14**−**25**) were screened for the inhibition of HbS aggregation using the standard saturation solubility, or C_{sat}, assay.[25] As shown in Table 2, all compounds showed varying degrees of antisickling activity regardless of the reaction site, i.e., Cysβ93 or the amino terminus of the α-subunit. However, in general, alkyl isothiocyanates tended to have better antisickling activity than aryl isothiocyanates. This suggests that reaction at the Cysβ93 residue provides greater antisickling activity than reaction at the N-terminus of the α-subunit for the compounds studied. HbS modified with alkyl isothiocyanates **7**−**9**, **14**−**25** which all reacted at the Cysβ93 residue, also showed varying degrees of antisickling activity. This result implies that the substituent on the reactive functional group plays a role in the inhibition of polymerization similar to different drugs reacting noncovalently at a single receptor site, as has been suggested above.

The alkyl isothiocyanates used for the evaluation of antisickling activity are all simple molecules, yet they have differing charge states: **7**, **14**, **18**−**25** are positively charged amines; **8**, **15**−**17** are neutral compounds; **9** is negatively charged under the physiological conditions tested. Among them, amines **7**, **14**, **19**−**21**, **22**−**25** had the most potent antisickling activity. This suggests that the positive charge ($-N^+$ at physiological pH) now near the Cysβ93 residue destabilizes the deoxy HbS polymer fiber structure.

Relationship between Antisickling Activity and the Amount of Covalently Modified HbS. The anticipated linear relationship between the amount of protein modification and biological activity was tested using a reconstitution experiment with HbS modified with *N*,*N*-dimethyl isothiocycante **7**. HbS was completely modified with isothiocyanate **7** and then purified by gel filtration to remove any unreacted isothiocyanate and its byproducts. The resulting modified HbS was then mixed with unmodified HbS to prepare 10%, 50%, 80%, and 90% modified HbS of known concentration.

All samples were screened for antisickling activity following the standard C_{sat} assay and the data graphically presented in a plot of solubility increase of HbS over control against percentage of modification. This plot is shown in Figure 3 and indicates that an approximately linear relationship exists between the percentage of covalent modification and antisickling activity. Drugs acting through covalent modification should show such a relationship as predicted above. More specifically, the observed behavior suggests that **7**-modified HbS is not excluded from the polymer, but is incorporated into the polymer in a manner that partially destabilizes fiber formation.

Mechanism for the Antisickling Activity of Isothiocyanate-Modified HbS. It is not completely clear how the alkyl isothiocyanates reacting at the Cysβ93 residue inhibit polymerization of HbS, although it appears to occur via more than one mechanism. Both the X-ray structure of the covalently modified HbS and molecular modeling studies were used to clarify the mechanism(s) of antisickling.

In Table 2, hemoglobin modified with the three alkyl isothiocyanates **7**, **8**, and **9** showed increased oxygen affinity compared with unmodified hemoglobin. This conclusion was based on their P_{50} values, defined as the oxygen pressure at which hemoglobin is 50% oxygenated. It has been reported that Cysβ93 is connected with Hisβ146 through an ionic interaction that is part of a salt bridge that stabilizes the deoxy conformation of hemoglobin, and most covalent modifications at Cysβ93 have shown increased oxygen affinity.[26] The β93Cys residue is also located adjacent to the proximal Hisβ92 which interacts directly with the heme iron. Thus, it is reasonable that modification of Cysβ93 could influence the oxygen affinity of hemoglobin.[3] The X-ray analysis of HbS covalently modified with the *N*,*N*-dimethylamino isothiocyanate **7** supports this contention (see below).

On the other hand, in the C_{sat} assay, HbS is first completely deoxygenated through the chemical reaction with sodium dithionite and then in the deoxy state, incubated to generate the polymer. Under these condi-

Table 2. Inhibition of HbS Polymerization, Oxygen Affinity, and Hill Coefficients for Selected Isothiocyanate-Modified Hemoglobins

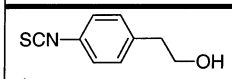
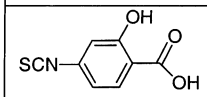
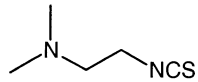
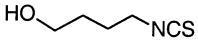
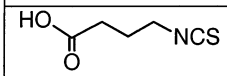
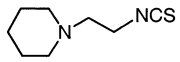
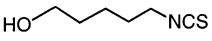
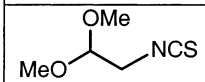
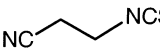
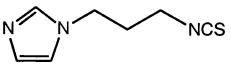
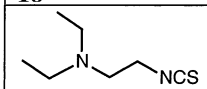
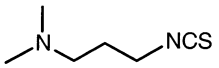
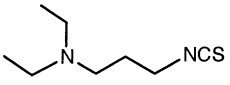
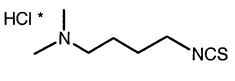
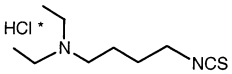
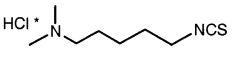
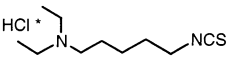
Compound	C_o (gm/dL)	C_{sat} (gm/dL)	C_{sat}/C_o	$(C_{sat}-C_o)/C_o$ (×100)	$\Delta\log P_{50}$[a]	Hill Coefficient[b]
 **1**	16.98	18.72	1.10	10.3		
 **4**	16.98	18.22	1.07	7.3		
 **7**	16.47 15.54	21.19 19.76	1.29 1.27	28.7 27.2	 -0.17	 2.1
 **8**	16.80 16.36	17.97 18.00	1.07 1.10	7.0 10.0	-0.27	1.9
 **9**	16.47 15.54	18.09 17.03	1.10 1.10	9.8 9.6	 -0.27	 2.0
 **14**	16.80 16.36 16.47	20.85 20.05 20.46	1.24 1.23 1.24	24.1 22.6 24.2		
 **15**	16.98	18.76	1.11	10.0		
 **16**	16.97 16.18 16.54	18.24 17.38 17.27	1.07 1.07 1.04	7.5 7.4 4.4		
 **17**	16.07 16.27 16.21	17.36 17.84 17.68	1.08 1.10 1.09	8.0 9.6 9.1		
 **18**	16.97 16.18 16.54	20.55 18.62 19.55	1.21 1.15 1.18	21.1 15.1 18.2		
 **19**	16.97 16.18 16.54	20.96 19.79 20.05	1.24 1.22 1.22	23.5 22.3 21.2		
 **20**	16.07 16.27 16.21	19.19 19.93 19.26	1.19 1.22 1.19	19.4 22.5 18.8	 -0.08	
 **21**	16.07 16.27 16.21	19.87 19.60 19.70	1.24 1.20 1.22	23.6 20.5 21.5		
 **22**	15.30 15.63 15.10	18.79 19.09 19.15	1.23 1.22 1.27	22.8 22.1 26.8		

Table 2 (Continued)

Compound	C_o (gm/dL)	C_{sat} (gm/dL)	C_{sat}/C_o	$(C_{sat}-C_o)/C_o$ (×100)	$\Delta\log P_{50}$[a]	Hill Coefficient[b]
 **23**	15.92	20.69	1.30	30.0		
	15.00	19.08	1.27	27.2		
	15.13	19.73	1.30	30.4		
 **24**	15.92	21.26	1.34	33.5		
	15.00	20.23	1.35	34.9		
	15.13	20.67	1.37	36.6		
 **25**	15.92	21.13	1.33	32.7		
	15.00	20.40	1.36	36.0		
	15.13	20.38	1.35	34.7		

[a] $\Delta\log P_{50}$ of of modified vs unmodified HbA; negative sign indicates that P_{50} of modified Hb is lower than that of unmodified Hb. [b] Hill coefficient of unmodified HbA: 3.16 lit. from Bonaventura.[27]

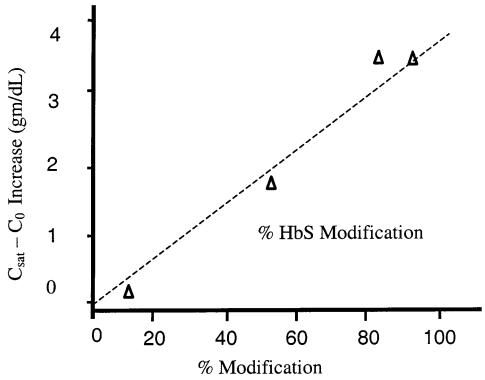


Figure 3. Inhibition of HbS polymerization vs percent modification of HbS with *N*,*N*-dimethyl isothiocyanate **7**.

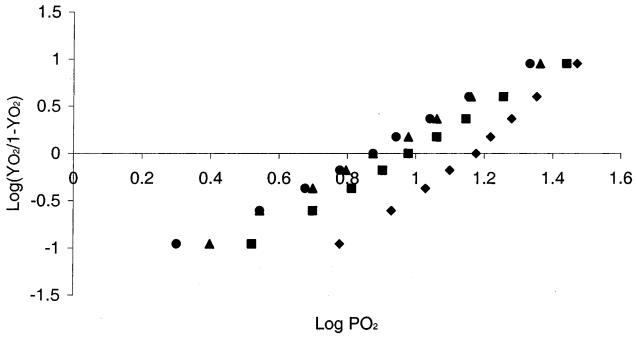


Figure 4. Hill plot of HbA modified with various isothiocyanates. ◆ Unmodified HbA, ● HbA modified with **8**, ▼ HbA modified with **9**, ■ HbA modified with **7**.

tions, the C_{sat} assay detects only the direct inhibition of HbS polymer formation, and the effect of the increased oxygen affinity or destabilization of the T state in the inhibition of HbS polymerization is not involved. Accordingly, the C_{sat} results listed above indicate that alkyl isothiocyanates covalently bound to HbS inhibit the polymerization of HbS not only by increasing the oxygen affinity, but also by direct inhibition of intermolecular contacts in the polymer. It is thus likely that the shift to the R state will be the initial effect of these inhibitors, with direct inhibition of polymerization coming into play only under conditions of extensive deoxygenation.

HbA modified with **7**, **8**, and **9** showed smaller Hill coefficients relative to unmodified HbA (see Figure 4 and Table 2), and $\Delta\log P_{50}$ decreases of ~0.17 (**7**) and ~0.27 (**8** and **9**). The Hill coefficients are equivalent to those observed for Cysβ93 S-nitrosated Hb, and the $\Delta\log P_{50}$s bracket the $\Delta\log P_{50}$ of 0.211 observed for Cysβ93 S-nitrosated Hb.[27] The functional changes for HbA modified at Cysβ93 by **7**, **8**, or **9** are very similar to the effect of NO adduct formation at Cysβ93, suggesting a common mechanism for functional changes. The Cysβ93 is located close to $\alpha_1\beta_1$ and $\alpha_2\beta_2$ contacts within the tetramer.[28] Thus, covalent modification of Cysβ93 with the alkyl isothiocyanate could also disturb the dimer−dimer interaction and therefore decrease the cooperativity.

X-ray and Molecular Modeling Analysis of Deoxy-HbS Modified by 2-(*N*,*N*-Dimethylamino)ethyl Isothiocyanate (7) at Cysβ93. The X-ray crystal structure of native deoxyHbA, covalently modified with the 2-(*N*,*N*-dimethylamino)ethyl isothiocyanate **7**, was determined to 1.9 Å resolution and to *R*-factors of 17.9% (R_{cryst}) and 21.3% (R_{free}), respectively. A summary of the X-ray data collection and refinement statistics is presented in Table 3. The final model consists of one $(\alpha\beta)_2$ tetramer per asymmetric unit, with 574 amino acid residues in four chains, four Fe-heme groups, and 239 water molecules. Analysis of $F_o - F_c$ difference electron density maps in the vicinity of Cysβ93, in both β chains of the tetramer, revealed interpretable density for 2-(*N*,*N*-dimethylamino)ethyl isothiocyanate modified cysteine residues (Lcy93 in Figure 5**)**. The modified cysteines were built into electron density and then refined to their final positions as shown in Figure 5.

Covalent modification of Cysβ93 by the *N*,*N*-dimethylamino isothiocyanate **7** wedges the modified side-chain between residues Aspβ94 and Hisβ146 and consequently breaks the salt bridge between these two residues. As previously discussed, this salt bridge is important for stabilization of the T-state conformation of hemoglobin. In the T-state, the side-chains of Aspβ94 and Hisβ146

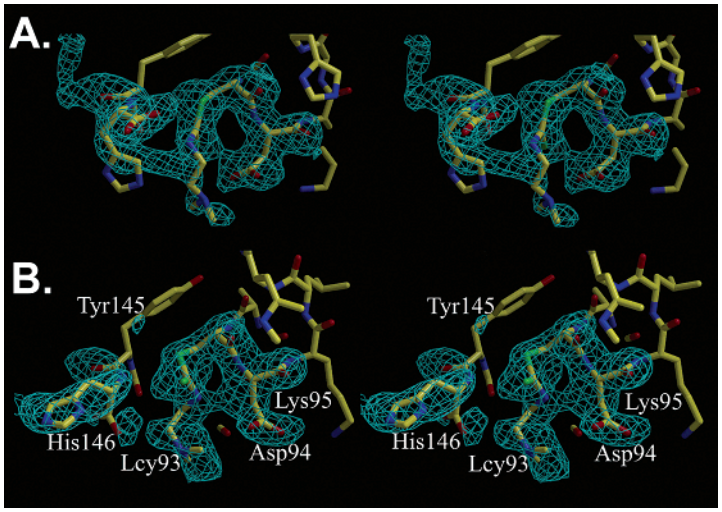


Figure 5. Stereoviews of omit ($F_o - F_c$) difference Fourier electron density maps, contoured at 2.5σ, for deoxy HbA modified with 2-(*N*,*N*-dimethylamino)ethyl isothiocyanate (**7**). Residues Cysβ93, Aspβ94, and Hisβ146 were omited from Fourier calculations. The β-subunit B-chain is shown in panel A, and the β-subunit D-chain is shown in panel B in approximately the same orientation. Differences between the subunits near the modified Cysβ93 (Lcy93) are clearly observable.

Table 3. X-ray Data Collection and Statistics for HbA Modified with Isothiocyanate 7

Data Collection	
unit cell dimensions	$a = 63.618$, $b = 83.792$, $c = 112.259$ Å
space group	$P2_12_12_1$ (#19)
resolution	50.0−1.90 (1.97−1.90)
observations	275074 (1133 rejected)
unique, averaged reflections	44466
redundancy	6.2 (≅6)
completeness	92.5 (92.4) %
$I/\sigma(I)$	16.0 (2.1)
R(merge)	0.090 (0.527)
χ^2	1.290 (0.863)

Refinement	
resolution	20.0−1.90 (1.91−1.90)
reflections included (88.0%)	42192 (900)
test set (4.7%)	2231 (60)
R(cryst)	0.179 (0.293)
R(free)	0.213 (0.328)
FOM	0.862
RMSD Lengths	0.009
RMSD Angles	1.252
water molecules	239

Average *B*-Values (Å^2)

	all chain atoms	all Fe(porphyrin) atoms
A	29.6	28.3
B	26.8	23.0
C	23.6	22.0
D	30.1	28.6

Chain RMSD Comparisons

	C(α) only	all atoms
A/C	0.186 (141)	0.521 (1069)
B/D	0.288 (146)	0.815 (1132)

are separated by approximately 2.8 Å. This distance is increased to approximately 7.0 Å in the covalently modified HbA, and to 9.1 Å in R-state hemoglobin structures. Similar shifts in the positions of Aspβ94 and Hisβ146 have been observed in molecular modeling studies using glutathione as a covalent modifier of Cysβ93.[28] The electron density surrounding Aspβ94 extends toward the nitrogen of the ($N^+(CH_3)_2$) group in in both the B- and D-chains of the β-subunit (Figure 5). In addition, the polar group of the isothiocyanate ligand's ($N^+(CH_3)_2$) group comes within 4.0 Å of the side chain of Aspβ94 in the B-chain β-subunit, suggesting the potential formation of a salt bridge (Figure 5a).

The average side-chain B-factor values for Aspβ94 (36 Å^2) and Hisβ146 (36 Å^2) in the covalently modified version of HbA lie between the values observed for the R-state (Aspβ94 = 50 Å^2 and Hisβ146 = 79 Å^2) and T-state (Aspβ94 = 27 Å^2 and Hisβ146 = 29 Å^2). The increased *B*-factor values suggest that an increase in the motion of these residues occurs upon modification of Cysβ93. The electron density surrounding residue Hisβ146 in both the B- and D-chains of the β subunit is partially disordered, with slightly more disorder observed in the D-chain, suggesting an increase in the mobility of the Hisβ146 residue compared to the T-state. Increased motion of these residues is also consistent with a previous study in which modification of Cysβ93 by NO produced complete disappearance of the electron density for the Hisβ146 residue.[29] Together, these observations strongly suggest that the covalent modification disrupts the Hisβ146-Aspβ94 salt bridge normally observed in the T-state, producing increased oxygen affinity, reduced cooperativity, and a shift toward the R-state.

The covalently modified, deoxyHbA structure was superimposed with the native deoxyHbA structure using the program LSQMAN.[30] Analysis of the overall structure of covalently modified deoxyHbA indicates that the main conformational changes that occur in the tertiary and quaternary structures are localized near the Hisβ146 and Aspβ94 side chains. Therefore, upon covalent modification, the R-state character of the protein increases, which may explain the observed increase in oxygen affinity. It is worth noting that the R-state of hemoglobin does not form a gel, and for this reason, a HbA conformation with increased R-state character would be less prone to gelation.[29] The salt bridge between Hisβ146-Aspβ94 has been suggested to account for about half of the overall Bohr effect.[31,32] Thus, disruption of this interaction may explain the observed increased oxygen affinity of the modified hemoglobin.

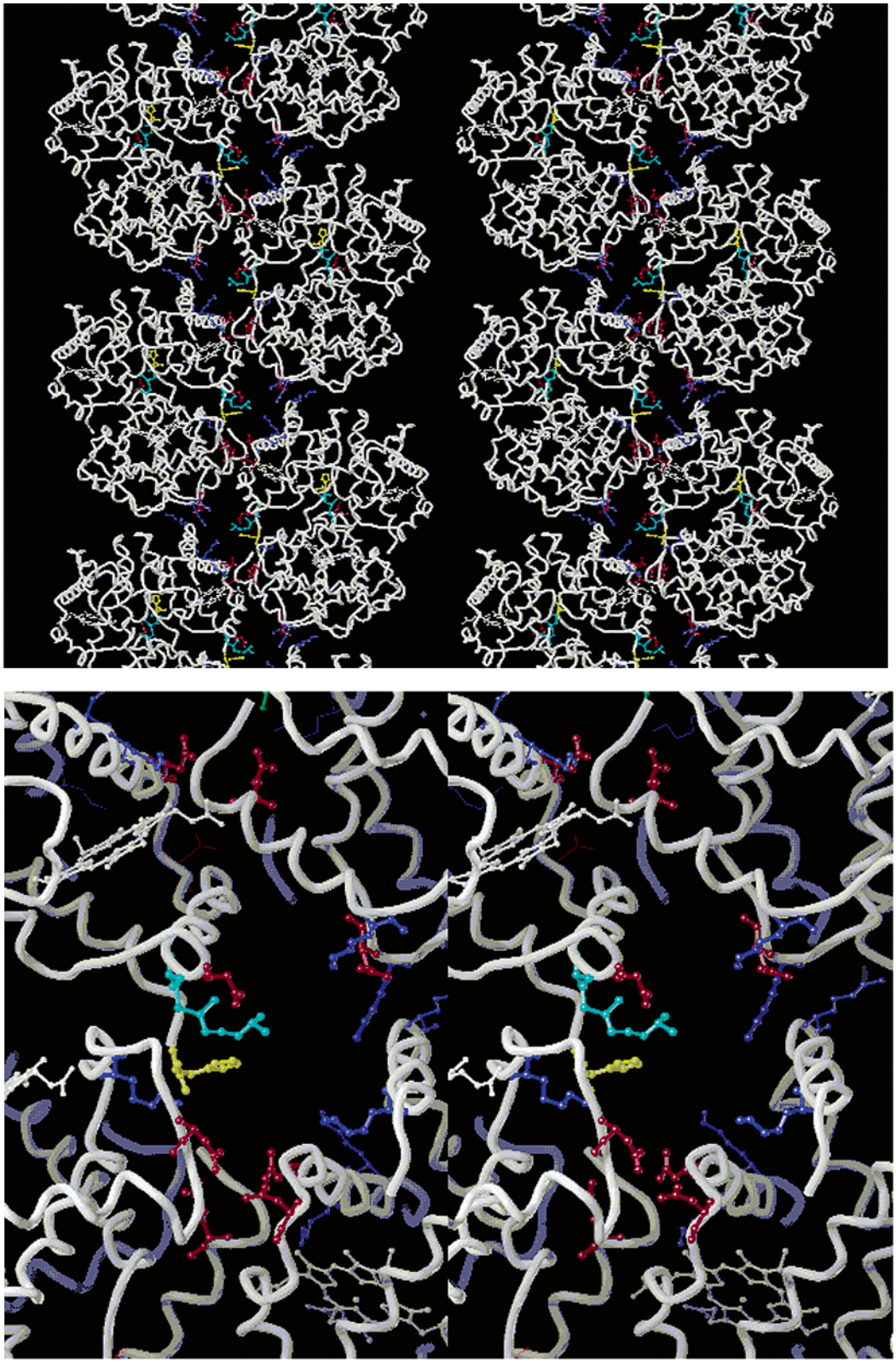


Figure 6. (top) Stereoview of the X-ray crystal structure of deoxy hemoglobin HbA covalently modified with 2-(*N*,*N*-dimethylamino)-ethyl isothiocyanate (**7**), superimposed into the double strand structure of deoxy HbS[2]. Lysine (dark blue), glutamate, and aspartate (both red) residues are within 15 Å from the covalently modified Cys β 93 (light blue) site. (bottom) Stereo close up of the substitution region.

The covalently modified deoxy HbA was also superimposed onto the deoxy HbS structure with the Gluβ6Val substitution. The resulting superposition is shown in Figure 6a in an orientation depicting the double strand of hemoglobin found in the deoxyhemoglobin S crystal.[2] A close up of the region of interest is shown in Figure 6b, where the insertion of a cationic group at Cysβ93 occurs in a "gap" region where the overall electrostatic potential would be dominated by four Lys residues on neighboring molecules (Lysβ8, Lysβ120, and two

Lysβ17's), as well as two other Lys residues slightly further away from the Cysβ93 modification (Lysβ65 and Lysβ66). Besides these Lys residues on the neighboring molecules of the adjacent strand(s), there are three Lys residues near the covalently modified Cysβ93 (Lysα40, Lysβ95, and Lysβ144 which are not shown due to their close proximity to Cysβ93) for the native HbS polymer fiber. Some of these Lys residues are neutralized locally by nearby Glu or Asp residues. This is the case for the Gluβ121 next to Lysβ120, as well as the Gluβ90 (also not shown in Figure 6) near Lysβ144, and Lysβ95 on the other side of Aspβ94. There are a few other Asp and Glu residues nearby that are unlikely to contribute significantly toward the effect of the Lys residues' positive potential (Aspβ73, Aspβ79, Gluβ7, and Aspα47). This is due to the participation of these Asp and Glu residues in the intermolecular lateral contact site, and hence influence the Lys residues in this "gap" region very little.

In the native structure Aspβ94 forms a salt bridge with Hisβ146, effectively neutralizing the charge for both residues. Upon adduct formation with **7** at Cysβ93, the imidazole ring of Hisβ146 is displaced, inserting a weakly basic (cationic) group approximately into the center of the positive electrostatic potential generated by these Lys residues. Local charge neutralization of Aspβ94 appears to be maintained by the cationic group of **7** (Figure 5), as well as a likely hydrogen bond formation between the protonated amine of **7** and the carboxylate group of Aspβ94 (**7** to Aspβ94 N- - -O distances are ~2.5 Å and 3.3 Å). The change in electric field vectors can be seen in Figure 7, which shows that, in the presence of the positively charged **7**, the largest change in electric field occurs adjacent to the side chain imidazole of Hisβ146 and the Aspβ94 COO^- group. The change of the field surrounding the Hisβ146 is more likely due to the movement of the imidazole ring being pushed down from the Aspβ94 and minimally due to the nearby positively charged ligand. The change of the field surrounding the Aspβ94, however, from one which is pointed away from the Asp's COO^- group in the unbound Hb to one where a portion of the field has been eliminated in the liganded Hb is likely due to the additional positive charge of the ligand influencing the electric field vectors in and around this COO^-.

For the anionic **9** (COO^- group) and neutral **8** (OH), inhibitory activities are likely because of the disruption of the Aspβ94-Hisβ146 salt bridge. However, they would not produce charge neutralization of the Aspβ94 side chain, which accounts for their lower inhibitory activities. The fact that they show essentially equivalent activities may be accountable by two effects. The resulting anionic group of **9** (which is farther into this "gap" region due to the longer chains of **9** compared to **7**) may interact favorably with the Lys-generated electrostatic potential—in effect, partially compensating the attraction−repulsion effects between the Aspβ94 carboxylate/Cysβ93-adduct and the displaced Hisβ146 imidazole. Although **8** lacks a charge, it does have a hydrogen available for hydrogen bonding interactions with the COO^- of Aspβ94. This would imply that both charge effects and hydrogen bonding effects at the Aspβ94 may influence the degree of inhibitory activity of these molecules. To test this hypothesis, a longer alkyl chain dimethylamine could be designed to see if inhibitory effects are less, due to the protonated amine being too far from the COO^- of the Aspβ94, but yet effective due to the influence on the electric field.

Summary

In this work, we have shown that design of agents for regioselective modification of Hb is possible through selection of an appropriate group for molecular recognition, coupled with a group that permits covalent reaction at the recognition site. Although it is well-known that isothiocyanates can react with both free amines and thiol groups, we have shown that for the Hb system, the isothiocyanate reactive functionality, coupled to aromatic groups, reacts selectively with the N-terminal amino groups, with a preference for the α-chain. In contrast, the isothiocyanate functionality, coupled to aliphatic groups, reacts exclusively with the Cysβ93 thiol when added at stoichiometric or sub-stoichiometric levels.

Although this represents only a low resolution case for reaction specificity, it implies that design of a molecule with a precise fit on the protein surface might provide a molecular orientation which could greatly enhance covalent reactivity at one site over another on a protein surface. The ability to produce such a specific covalent modification could represent a unique approach to altering protein structure/function in pharmaceutical design.

Thus in the present case, compounds **7**, **8**, and **9**, with cationic, neutral hydroxyl, and anionic R-groups, respectively, provide intriguing contrasts in their functional effects. All three show nearly identical effects on the oxygen transport function of Hb, with similar reductions in both the Hill coefficients and the P_{50} values, indicating similar shifts from T- toward R-state for all three modified Hbs. In this respect, the effects are similar to a variety of compounds including glutathione,[28] *N*-ethylmaleimide,[28,33] iodoacetamide,[33] and NO.[27] There is a striking difference in their effect on HbS polymerization, however, with the cationic **7** (and its analogues **23**−**25**) approximately three times more effective at inhibition of HbS polymerization than the anionic **9** or the neutral hydroxylic **8**, which both exhibit inhibitory activities similar to glutathione,[28] NO,[27] and other SH-modifying reagents.

As discussed above, we speculate that introduction of a cationic group at Cysβ93 and the consequent displacement of the Hisβ146 side chain imidazole combine to produce a subtle modification of the electrostatic field that opposes the field from residues in neighboring molecules within the fiber. In addition, increasing the oxygen affinity of HbS is known to decrease HbS polymerization within the red cell under conditions of partial deoxygenation, and to reduce the red cell morphological changes associated with sickle cell disease.[34] Regardless of the mechanism of action, in the present work the substantial increase in inhibitory activity against HbS polymerization, combined with the effect of increasing Hb oxygen affinity and relatively facile traversal of the erythrocyte membrane suggest that the cationic isothiocyanates **7** and **23**−**25** show substantial promise as lead compounds for the development of therapeutic agents for sickle cell disease.

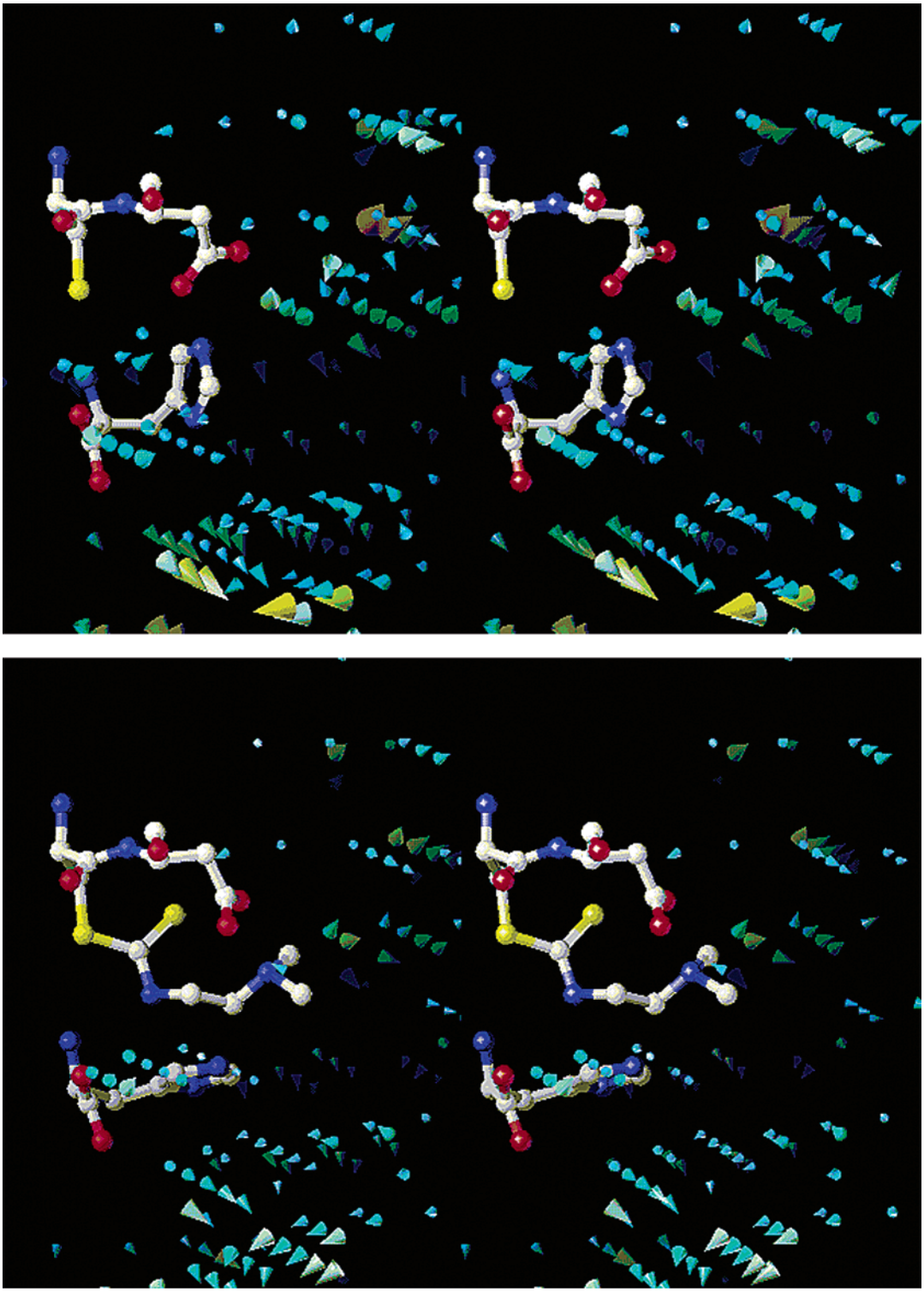


Figure 7. Electric field vectors adjacent to residues Lcyβ93, Aspβ94, and Hisβ146. (Top) Field for native Hb. (Bottom) Field for modified Hb.

Experimental Section

General. All nuclear magnetic resonance (NMR) were obtained using Bruker Avance 300 and 500 MHz FT NMR spectrometers. All absorptions are expressed in parts per million relative to tetramethylsilane as the internal standard. All infrared (IR) spectra were obtained using a Jasco V550 FT/IR-410 spectrophotometer. All elemental analyses were performed by Midwest Microlab of Indianapolis, IN, and were within $\pm$0.4% of the theoretical values. Elemental analyses were obtained for all isothiocyanates tested biologically, but not for those used only to examine regiospecific reactions on the Hb surface. Mass spectra were recorded with a MAT 90 for CI, Finnigan LCQ for APCI and routine ESMS, and a Micromass Quattro II for Hb analyses and HRMS. UV absorbances were recorded on a Jasco V550 spectrophotometer. Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. High-performance liquid chromatography (HPLC) was performed on a Waters 510 liquid chromatograph. Thin-layer chromatography (TLC) was carried out on 0.25 mm precoated Whatman SILG/UV silica plates. Routine flash column chromatography was effected on silica gel 60 purchased from Aldrich. All solvents used were either

HPLC grade (Fisher Scientific) or anhydrous (Aldrich). All other chemicals were purchased from either Aldrich or Fisher Scientific.

General Procedure for the Synthesis of Isothiocyanates 1−4, 6, 8−18. These compounds were prepared by a previously reported procedure.[13] To a stirred solution of the primary amine in CH_2Cl_2 was added di-2-pyridyl thionocarbonate (1.0 equiv) at 25 °C. The reaction mixture was stirred for 24 h, unless otherwise noted. The solvent was removed by rotary evaporation. The crude product was purified by either column chromatography on silica gel or vacuum distillation.

4-(2-Hydroxyethyl)phenylisothiocyanate (1). 4-(2-Hydroxyethyl)aniline (0.14 g, 1.02 mmol) was dissolved in CH_2Cl_2 (20 mL). To this mixture was added di-2-pyridyl thionocarbonate (0.23 g, 1.02 mmol). Column chromatography (1:1 hexane/EtOAc) yielded a yellow oil (**1**) (0.16 g, 0.89 mmol, 87% yield), which crystallized in the freezer upon solvent removal; mp 42−44 °C. IR (neat): 3313, 2945, 2869, 2172, 2092 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.35−7.11 (m, 4H), 3.83 (t, J = 6.6 Hz, 2H), 2.85 (t, J = 6.6 Hz, 2H). ^{13}C NMR ($CDCl_3$): δ 138.68, 135.26, 130.57, 129.78, 126.24, 63.66, 39.09. CIMS m/z 180([M + H]$^+$, 100), 162([M − OH]$^+$, 43). Anal. (C_9H_9NOS) C, H, N.

4-Isothiocyanatophenol (2). 4-Aminophenol (0.11 g, 1.01 mmol) was dissolved in CH_2Cl_2 (20 mL). To this mixture was added di-2-pyridyl thionocarbonate (0.23 g, 1.01 mmol). Column chromatography (1:2 hexane/EtOAc) yielded a pale yellow solid (**2**) (0.11 g, 0.073 mmol, 73% yield); mp 42−44 °C. IR (KBr): 3397, 2173, 2119 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.15−7.09 (m, 2H), 6.82−6.76 (m, 2H), 5.75 (s, 1H). ^{13}C NMR ($CDCl_3$): δ 155.15, 134.17, 127.62, 124.10, 116.78. APCIMS m/z 150([M − H]$^-$, 100).

4-(2-(*N*,*N*-Diethylamino)ethylaminocarbonyl)phenyl Isothiocyanate Monohydrochloride Salt (3). Procainamide HCl (0.2 g, 0.74 mmol) in CH_2Cl_2 (20 mL) was cooled to 0 °C in an ice bath. To this were added 1 mL of H_2O and $NaHCO_3$ (500 mg). This was then followed by the addition of di-2-pyridyl thionocarbonate (0.17 g, 0.74 mmol). After 30 min, the solution was diluted with CH_2Cl_2 (50 mL) and washed with water. The organic layer was dried over $MgSO_4$ and evaporated. The residue was diluted with ethyl acetate (30 mL), and then HCl was bubbled through the solution for an additional 5 min at 0 °C. The mixture was warmed to room temperature and stirred for 30 min. The precipitate that formed was filtered and washed with ethyl acetate. Recrystallization from acetone afforded a yellow solid (**3**) (0.072 g, 0.023 mmol, 31% yield); mp 178−180 °C. IR (KBr): 3238, 2603, 2188, 2133, 1655 cm^{-1}. ^1H NMR ($CDCl_3$): δ 11.65 (bs, 1H), 9.11 (bs, 1H), 8.17−8.11 (m, 2H), 7.32−7.26 (m, 2H), 3.96−3.87 (m, 2H), 3.29−3.23 (m, 2H), 3.23−3.12 (m, 4H), 1.44 (t, J = 7.5 Hz, 6H). ^{13}C NMR ($CDCl_3$): δ 166.78, 134.91, 132.03, 129.63, 126.16, 53.47, 48.82, 35.76, 9.04. APCIMS m/z 278([M + H]$^+$, 100).

4-Isothiocyanato-*N*,*N*-dimethylaniline Monohydrochloride Salt (4). 4-Amino-*N*,*N*-dimethylaniline HCl (0.10 g, 0.58 mmol) in CH_2Cl_2 (20 mL) was cooled to 0 °C in an ice bath. To this were added 1 mL of H_2O and $NaHCO_3$ (500 mg). This was then followed by the addition of di-2-pyridyl thionocarbonate (0.134 g, 0.58 mmol). After 30 min, the solution was diluted with CH_2Cl_2 (50 mL) and washed with water. The organic layer was dried over $MgSO_4$ and evaporated. The residue was diluted with ethyl acetate (30 mL), and then HCl was bubbled through the solution for an additional 5 min at 0 °C. The mixture was warmed to room temperature and stirred for 30 min. The precipitate that formed was filtered and washed with diethyl ether. Column chromatography (4:1 hexane/EtOAc) afforded a white solid (**4**) (0.080 g, 0.037 mmol, 78% yield); mp 144−146 °C. IR (KBr): 2408, 2200, 2143, 1506 cm^{-1}. ^1H NMR (DMSO-d_6): δ 7.38 (d, J = 8.4 Hz, 2H), 7.06 (bs, 2H), 6.20 (bs, 1H), 2.99 (s, 2H). ^{13}C NMR (DMSO-d_6): δ 146.96, 127.98, 118.21, 43.35. APCIMS m/z 179([M + H]$^+$, 100).

4-Isothiocyanatophenylacetic Acid (6). 4-Aminophenylacetic acid (0.10 g, 0.66 mmol) was dissolved in CH_2Cl_2 (20 mL). To this mixture was added di-2-pyridyl thionocarbonate (0.16 g, 0.66 mmol), which was stirred for 4 h at 25 °C. The mixture was diluted with CH_2Cl_2 (50 mL), washed with 3 N HCl (50 mL), and then extracted twice with 10% aqueous Na_2CO_3 (50 mL). The combined aqueous solutions were washed with CH_2Cl_2 (50 mL), acidified to pH 1.0 with concentrated HCl, and extracted twice with CH_2Cl_2 (50 mL). The combined organic layers were dried over $MgSO_4$. Column chromatography (1:1 hexane/EtOAc) yielded a white solid (6) (0.10 g, 0.52 mmol, 83% yield); mp 142−144 °C. IR (KBr): 2194, 2138, 1695 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.30−7.15 (m, 4H), 3.66 (s, 2H). ^{13}C NMR ($CDCl_3$): δ 177.31, 132.87, 131.05, 130.92, 126.33, 126.30, 40.87. APCIMS m/z 192([M − H]$^-$, 56), 148([M − CO_2]$^-$, 100).

4-Isothiocyanatobutanol (8). 4-Aminobutanol (0.09 g, 1.0 mmol) was dissolved in CH_2Cl_2 (20 mL). To this mixture was added di-2-pyridyl thionocarbonate (0.23 g, 1.0 mmol). Column chromatography (1:2 hexane/EtOAc) yielded a pale yellow oil (**8**) (0.12 g, 0.92 mmol, 91% yield). IR (neat): 3347, 2946, 2186, 2108 cm^{-1}. ^1H NMR ($CDCl_3$): δ 3.71 (t, J = 6.6 Hz, 2H), 3.60 (t, J = 6.6 Hz, 2H), 1.99 (bs, 1H), 1.88−1.76 (m, 2H), 1.76−1.64 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 62.21, 45.36, 29.88, 26.99. CIMS m/z 132([M + H]$^+$, 95), 114([M − OH]$^+$, 100). Anal. (C_5H_9NOS) C, H, N.

4-Isothiocyanatobutyric Acid (9). 4-Aminobutyric acid (0.20 g, 1.94 mmol) was dissolved in CH_2Cl_2 (20 mL). To this mixture was added di-2-pyridyl thionocarbonate (0.45 g, 1.94 mmol). Column chromatography (1:1 hexane/EtOAc) yielded a clear oil (**9**) (0.21 g, 1.45 mmol, 75% yield). IR (neat): 2191, 2111, 1709 cm^{-1}. ^1H NMR ($CDCl_3$): δ 10.75 (bs, 1H), 3.65 (t, J = 7.2 Hz, 2H), 2.55 (t, J = 7.2 Hz, 2H), 2.03 (q, J = 7.2 Hz, 2H). ^{13}C NMR ($CDCl_3$): δ 179.05, 44.59, 31.06, 25.29. CIMS m/z 146([M + H]$^+$, 62), 128([M − OH]$^+$, 100), 87([M − NCS]$^+$, 50). Anal. ($C_5H_7NO_2S$) C, H, N.

4-Isothiocyanatocyclohexanol (10). To 4-aminocyclohexanol HCl (0.10 g, 0.66 mmol) in CH_2Cl_2 (20 mL) and 10% aqueous Na_2CO_3 (5 mL) was added di-2-pyridyl- thionocarbonate (150 mg, 0.66 mmol) at room temperature. After 5 h, the solution was diluted with CH_2Cl_2 (50 mL) and washed with water (30 mL) and 3 N HCl (30 mL). The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure. Column chromatography (1:2 hexane/EtOAc) afforded a colorless oil (**10**) (0.060 g, 0.39 mmol, 47% yield). IR (neat): 3347, 2940, 2150, 2097 cm^{-1}. ^1H NMR ($CDCl_3$): δ 3.86−3.72 (m, 1H), 3.72−3.55 (m, 1H), 2.22−2.05 (m, 2H), 2.10−1.75 (m, 3H), 1.71−1.55 (m, 2H), 1.52−1.20 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 130.76, 68.05, 55.01, 31.87, 30.28. CIMS m/z 158 ([M + H]$^+$, 52), 99([M − NCS]$^+$, 69), 81([M − NSC − H_2O]$^+$, 100).

***N*-(3-Isothiocyanatopropyl)-γ-butyrolactam (11).** *N*-(3-Aminopropyl)-γ-butyrolactam (0.20 g, 1.41 mmol) was dissolved in CH_2Cl_2 (20 mL). To this mixture was added di-2-pyridyl thionocarbonate (0.33 g, 1.41 mmol). Column chromatography (EtOAc) yielded a yellow oil (**11**) (0.08 g, 0.43 mmol, 43% yield). IR (neat): 2187, 2111, 1682 cm^{-1}. ^1H NMR ($CDCl_3$): δ 3.59 (t, J = 6.6 Hz, 2H), 3.47−3.32 (m, 4H), 2.43 (t, J = 7.8 Hz, 2H), 2.14−2.03 (m, 2H), 2.02−1.85 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 176.11, 48.03, 43.39, 40.60, 31.23, 28.19, 18.34. CIMS m/z 185([M + H]$^+$, 100), 126([M − NCS]$^+$, 79).

2-(Indol-3-yl)ethyl Isothiocyanate (12). 6-(2-Aminoethyl)indole (0.20 g, 1.25 mmol) was dissolved in CH_2Cl_2 (20 mL). To this mixture was added di-2-pyridyl thionocarbonate (0.29 g, 1.25 mmol). Column chromatography (5:1 hexane/EtOAc) yielded a white solid (**12**) (0.183 g, 0.91 mmol, 91% yield); mp 46−47 °C. IR (KBr): 3398, 2185, 2097, 1456 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.04 (bs, 1H), 7.54 (d, J = 7.8 Hz, 1H), 7.35 (d, J = 7.8 Hz, 1H), 7.26−7.12 (m, 2H), 7.04 (d, J = 2.4 Hz, 1H), 3.72 (t, J = 6.9 Hz, 2H), 3.12 (t, J = 6.9 Hz, 2H). ^{13}C NMR ($CDCl_3$): δ 136.68, 130.07, 127.22, 123.46, 122.77, 120.12, 118.71, 111.90, 111.59, 46.09, 26.90. APCIMS m/z 203([M + H]$^+$, 100), 144([M − HNCS]$^+$, 36).

(4-Isothiocyanatomethyl)pyridine (13). 4-Aminomethylpyridine (0.15 g, 1.39 mmol) was dissolved in CH_2Cl_2 (20 mL). To this mixture was added di-2-pyridyl thionocarbonate (0.32 g, 1.39 mmol). After following the generalized procedure above, column chromatography (1:2 hexane/EtOAc) yielded a brown oil (**13**) (0.20 g, 1.33 mmol, 96% yield). IR (neat): 2198, 2095

cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 8.65 (d, J = 4.5 Hz, 2H), 7.28 (d, J = 4.5 Hz, 2H), 4.79 (s, 2H). ^{13}C NMR ($CDCl_3$): δ 150.75, 143.59, 134.82, 121.62, 47.97. APCIMS m/z 151([M + H]$^+$, 100), 93([M − NCS]$^+$, 13).

1-(2-Isothiocyanatoethyl)piperidine (14). 1-(2-Aminoethyl)piperidine (0.13 g, 1.0 mmol) was dissolved in CH_2Cl_2 (10 mL). To this mixture was added di-2-pyridyl thionocarbonate (0.23 g, 1.0 mmol). After following the generalized procedure above, column chromatography (1:2 hexane/EtOAc) yielded a clear oil (**14**) (0.082 g, 0.048 mmol, 47% yield). IR (neat): 2936, 2190, 2096 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 3.59 (t, J = 6.6 Hz, 2H), 2.64 (t, J = 6.6 Hz, 2H), 2.55−2.35 (m, 4H), 1.68−1.48 (m, 4H), 1.49−1.38 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 58.42, 54.81, 43.49, 26.33, 24.53. APCIMS m/z 171([M + H]$^+$, 100), 112([M − NCS]$^+$, 23). Anal. ($C_8H_{15}N_2SCl$) C, H, N.

5-Isothiocyanatopentanol (15). 5-Aminopentanol (0.10 g, 1.0 mmol) was dissolved in CH_2Cl_2 (20 mL). To this mixture was added di-2-pyridyl thionocarbonate (0.23 g, 1.0 mmol). Column chromatography (1:2 hexane/EtOAc) yielded a clear oil (**15**) (0.10 g, 0.69 mmol, 71% yield). IR (neat): 3347, 2940, 2182, 2106 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 3.68 (t, J = 6.3 Hz, 2H), 3.54 (t, J = 6.3 Hz, 2H), 1.82−1.70 (m, 2H), 1.67−1.45 (m, 4H). ^{13}C NMR ($CDCl_3$): δ 62.88, 45.41, 32.20, 30.17, 23.35. APCIMS m/z 146([M + H]$^+$, 63), 128([M − OH]$^+$, 100). Anal. (C_6H_{11}NOS) C, H, N.

2,2-Dimethoxyethyl Isothiocyanate (16). Aminoacetaldehyde dimethyl acetal (0.20 g, 1.90 mmol) was dissolved in CH_2Cl_2 (20 mL). To this mixture was added di-2-pyridyl thionocarbonate (0.44 g, 1.90 mmol). The product, obtained by vacuum distillation (bp 105−106 °C/15 mmHg), was a clear liquid (**16**) (0.34 g, 0.231 mmol, 12% yield) after following the generalized procedure above. This compound matched the previously reported boiling point,[35,36] as well as IR, mass spectrometry, and proton NMR data.[35] ^{13}C NMR ($CDCl_3$): δ 130.98, 101.53, 54.18, 54.17, 46.51. Anal. ($C_5H_9NO_2S$) C, H, N.

1-Isothiocyanatopropyl Nitrile (17). 3-Aminopropionitrile fumarate salt (0.30 g, 2.34 mmol) in CH_2Cl_2 (30 mL) was cooled to 0 °C in an ice bath. To this were added 1 mL of H_2O and a small spatula full of $NaHCO_3$. This was then followed by the addition of di-2-pyridyl thionocarbonate (0.54 g, 2.34 mmol). The ice bath was removed, and the reaction mixture was allowed to stir for 24 h at 25 °C. This was followed by an extraction with saturated $NaHCO_3$ solution (2 × 30 mL) and saturated NaCl solution (2 × 30 mL) to remove starting materials and byproducts. The organic layer was dried with $MgSO_4$, and the solvent was removed by rotary evaporation. Column chromatography (1:1 hexane/EtOAc) yielded a clear oil (**17**) (0.149 g, 1.33 mmol, 57% yield). IR (NaCl): 2208, 2112, 2086 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 3.88 (t, J = 13.1 Hz, 2H), 2.78 (t, J = 13.1 Hz, 2H). ^{13}C NMR ($CDCl_3$): δ 130.89, 115.98, 41.04, 19.05. Anal. ($C_4H_4N_2S$) C, H, N.

1-(3-Isothiocyanatopropyl)imidazole (18). 1-(3-Aminopropyl)imidazole (0.1 g, 0.80 mmol) was dissolved in CH_2Cl_2 (10 mL). To this mixture was added di-2-pyridyl thionocarbonate (0.19 g, 0.80 mmol). After following the generalized procedure above, column chromatography (1:1 hexane/EtOAc) yielded an orange oil (**18**) (0.08 g, 0.48 mmol, 60% yield). IR (NaCl): 2201, 2115, 1653, 1508, 1135 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.50 (s, 1H), 7.07 (d, J = 1.1 Hz, 2H), 6.94 (d, J = 1.3 Hz, 1H), 4.12 (dt, J = 6.6, 2.0 Hz, 2H), 3.51 (dt, J = 5.7, 1.7 Hz, 2H), 2.19−2.09 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 137.09, 131.92, 130.01, 118.67, 43.37, 41.76, 30.08. Anal. ($C_7H_9N_3S$) C, H, N.

4-Isothiocyanosalicylic Acid (5). This procedure was adapted from that of Seligman.[12] *p*-Aminosalicylic acid (1.53 g, 11.2 mmol) was suspended in water (32 mL) and concentrated HCl (3.4 mL). To this suspension, thiophosgene (1.35 g, 11.7 mmol) was added in one portion, and the reaction mixture was stirred for 3 h at 25 °C. The solid product was filtered, washed with water (100 mL), and dried under vacuum over P_2O_5. The resulting solid was recrystallized from benzene to yield a white solid (**5**) (1.1 g, 6.1 mmol, 55% yield); mp 185−187 °C. IR (KBr): 3433, 2077, 1665 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 11.45 (bs, 2H), 7.83 (d, J = 8.4 Hz, 1H), 7.02−6.94 (m, 2H). ^{13}C NMR (DMSO-d_6): δ 171.79, 162.56, 136.77, 136.53, 132.75, 118.06, 114.81, 113.45. APCIMS m/z 194([M − H]$^-$, 100), 150([M − CO_2]$^-$, 12). Anal. ($C_8H_5NO_3S$) C, H, N.

General Procedure for the Synthesis of Isothiocyanates 7, 19−21. These compounds were all prepared by a previously reported procedure.[14] The primary amine in acetone (5 mL) was cooled to −10 °C in a dry ice/acetone bath. Carbon disulfide (1.1 equiv) in acetone (5 mL) was added dropwise via an addition funnel over 15 min. Then, over 45 min, the temperature was allowed to slowly rise to 10 °C. The reaction mixture was then cooled to −15 °C. Over 45 min, a solution of $HgCl_2$ (1.0 equiv) in acetone (15 mL) was added, keeping the temperature below −10 °C. After warming to 0 °C over 15 min, Et_3N (2.2 equiv) was added slowly by syringe. Finally, the reaction mixture was refluxed for 1 h, becoming black. Upon cooling, the salts were filtered through Celite, and washed subsequently with acetone (1 × 50 mL) and ether (2 × 50 mL). The filtrate was treated with $MgSO_4$. Solvent was removed by rotary evaporation. To the residue was added ether (75 mL), and this was filtered. The filtrate was concentrated and vacuum distillation yielded the isothiocyanates (**7**, **19**−**21**).

2-(*N*,*N*-Dimethylamino)ethyl Isothiocyanate (7). This compound has been previously prepared and spectroscopically characterized.[37] Anal. ($C_5H_{11}N_2SCl$) C, H, N.

2-(*N*,*N*-Diethylamino)ethyl Isothiocyanate (19). 2-(Diethylamino)ethylamine (2.0 g, 17.2 mmol) was dissolved in 15 mL of acetone. After following the procedure outlined above, vacuum distillation (bp 118−122 °C/12 mmHg) yielded a clear liquid (**19**) (1.57 g, 9.94 mmol, 58% yield). This compound matched the previously reported boiling point and spectral data.[38] ^{13}C NMR ($CDCl_3$): δ 131.98, 52.53, 47.44, 44.01, 12.10. HRMS ESI+ (m/z) calcd. for (M + H)$^+$ $C_7H_{15}N_2S$, 159.0956; found 159.0961. Anal. ($C_7H_{15}N_2SCl$) C, H, N.

3-(*N*,*N*-Dimethylamino)propyl Isothiocyanate (20). 3-(Dimethylamino) propylamine (1.0 g, 9.80 mmol) was dissolved in 10 mL acetone. After following the procedure outlined above, vacuum distillation (bp 110−111 °C/10 mmHg) yielded a clear liquid (**20**) (0.99 g, 6.88 mmol, 70% yield). This compound matched the previously reported boiling point and spectral data.[38] ^{13}C NMR ($CDCl_3$): δ 131.08, 55.92, 45.34, 42.91, 27.89. Anal. ($C_6H_{12}N_2S$) C, H, N.

3-(*N*,*N*-Diethylamino)propyl Isothiocyanate (21). 3-(Diethylamino)propylamine (1.0 g, 7.69 mmol) was dissolved in 10 mL of acetone. After following the procedure outlined above, vacuum distillation (bp 129−131°C/15 mmHg) yielded a clear liquid (**21**) (0.85 g, 4.94 mmol, 65% yield). This compound matched the previously reported boiling point and spectral data.[38] ^{13}C NMR ($CDCl_3$): δ 131.23, 49.21, 46.86, 43.03, 27.88, 11.74. HRMS ESI+ (m/z) calcd. for (M + H)$^+$ $C_8H_{17}N_2S$, 173.1112; found 173.1114. Anal. ($C_8H_{17}N_2SCl$) C, H, N.

4-(*N*,*N*-Dimethylamino)butyl Isothiocyanate Monohydrochloride Salt (22). Following a procedure similar to Kupchan's,[15,16] 4-chlorobutyronitrile (**28**) (10.0 g, 97.1 mmol), dimethylamine (**26**) (73.0 mL, 582.5 mmol), and sodium iodide (1.46 g, 9.71 mmol) were stirred together for 5 d at 25 °C. Vacuum distillation (78−80 °C/15 mmHg) yielded a clear liquid, 4-dimethylaminobutyronitrile (**30**) (3.15 g, 28.1 mmol, 29% yield). This compound matched the previously reported boiling point.[16,39,40] IR (NaCl): 2944, 2248, 1148, 1040 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 2.44−2.36 (m, 4H), 2.22 (s, 6H), 1.82−1.78 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 119.55, 57.34, 45.07, 23.27, 14.52. HRMS ESI+ (m/z) calcd. for (M + H)$^+$ $C_6H_{13}N_2$, 113.1079; found 113.1043. Compound **30** (1.7 g, 15.2 mmol) and the Rh−Al_2O_3 catalyst (0.47 g) were dissolved in MeOH (40 mL). Hydrogenation was carried out in a Parr shaker under H_2 at 37 psi of pressure for 5 h. When hydrogen uptake was complete, the solution was filtered through Celite to remove the catalyst. The filtrate was concentrated by rotary evaporation. The crude product contained a mixture of primary (*N*,*N*-dimethylbutane-1,4-diamine, **34**) and secondary amines, 2:1, respectively, which were not separated. IR (NaCl): 3291, 1466, 1308, 1041 cm^{-1}. ^{1}H NMR ($CDCl_3$) (peaks for **34** only): δ 2.71 (t, J = 6.7 Hz, 2H), 2.61 (t, J = 6.7 Hz, 2H), 2.22 (s, 6H), 1.51−1.46 (m, 4H). ^{13}C NMR ($CDCl_3$): δ 49.87, 45.43,

42.09, 25.50, 25.02. The mixture of primary (**34**) and secondary amines (1.4 g, 12.1 mmol/molecular weight of **34**) was dissolved in 20 mL of acetone. After following the procedure used for compounds **7**, **19**−**21**, the resulting crude product was a yellow liquid, 4-(*N*,*N*-dimethylamino)butylisothiocyanate (**38**). IR (NaCl): 2942, 2173, 2103, 1264, 1041 cm^{-1}. ^1H NMR ($CDCl_3$): δ 3.55 (t, J = 6.5 Hz, 2H), 2.29 (t, J = 7.1 Hz, 2H), 2.22 (s, 6H), 1.75−1.72 (m, 2H), 1.61−1.56 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 129.80, 58.60, 45.39, 44.33, 27.88, 24.54. Crude isothiocyanate (**38**) (1.1 g, 6.96 mmol) was dissolved in diethyl ether (30 mL), and HCl gas was bubbled through the solution. The white precipitate (**22**) so formed was very hygroscopic and could not be filtered. The product was washed with ether (3 × 30 mL) and decanted to yield a yellow-orange gum. It was then dried under vacuum (0.91 g, 4.69 mmol, 67% yield, 9% overall yield). ^1H NMR (D_2O): δ 3.66 (t, J = 6.1 Hz, 2H), 3.19 (t, J = 6.6 Hz, 2H), 2.89 (s, 6H), 1.90−1.73 (m, 4H). ^{13}C NMR (D_2O): δ 57.24, 44.53, 43.00, 26.19, 21.75. Anal. ($C_7H_{14}N_2SCl$) C, H, N.

4-(*N*,*N*-Diethylamino)butyl isothiocyanate Monohydrochloride Salt (23). Following a procedure similar to Kupchan's,[15,16] 4-chlorobutyronitrile (**28**) (10.0 g, 97.1 mmol), diethylamine (**27**) (60.1 mL, 582.5 mmol), and sodium iodide (1.46 g, 9.71 mmol) in H_2O (50 mL) were stirred together for 5 d at 25 °C. Vacuum distillation (98−103 °C/15 mmHg) yielded a clear liquid, 4-diethylaminobutyronitrile (**31**) (4.54 g, 32.4 mmol, 33% yield). This compound matched the previously reported boiling point.[15,40] IR (NaCl): 2969, 2247, 1200, 1073 cm^{-1}. ^1H NMR ($CDCl_3$): δ 2.50 (q, J = 7.2 Hz, 4H), 2.42 (t, J = 7.1 Hz, 4H), 1.81−1.72 (m, 2H), 1.01 (t, J = 7.2 Hz, 6H). ^{13}C NMR ($CDCl_3$): δ 119.93, 50.94, 46.77, 23.52, 14.72, 11.75. HRMS ESI+ (*m*/*z*) calcd. for $(M + H)^+$ $C_8H_{17}N_2$, 141.1392; found 141.1390. Compound **31** (0.5 g, 3.57 mmol) and the Rh−Al_2O_3 catalyst (0.11 g) were dissolved in MeOH (20 mL). Hydrogenation was carried out in a Parr shaker under H_2 at 40 psi of pressure for 2.5 d. When hydrogen uptake was complete, the solution was filtered through Celite to remove the catalyst. The filtrate was concentrated by rotary evaporation. The crude product contained a mixture of primary (*N*,*N*-diethylbutane-1,4-diamine, **35**) and secondary amines, 1:2, respectively, which were not separated. IR (NaCl): 3292, 1466, 1306, 1041 cm^{-1}. ^1H NMR ($CDCl_3$) (peaks for **35** only): δ 2.62 (t, J = 7.2 Hz, 2H), 2.52 (q, J = 7.2 Hz, 4H), 2.42 (t, J = 7.2 Hz, 2H), 1.49−1.42 (m, 4H), 1.02 (t, J = 7.2 Hz, 6H). ^{13}C NMR ($CDCl_3$): δ 52.56, 46.58, 41.96, 24.64, 24.19, 11.41. The mixture of primary (**35**) and secondary amines (0.51 g, 3.54 mmol/molecular weight of **35**) was dissolved in 15 mL of acetone. After following the procedure used for compounds **7**, **11**−**21**, the resulting crude product was a yellow liquid, 4-(*N*,*N*-diethylamino)butyl isothiocyanate (**39**). IR (NaCl): 2938, 2179, 2100, 1270, 1042 cm^{-1}. ^1H NMR ($CDCl_3$): δ 3.55 (t, J = 6.5 Hz, 2H), 2.51 (q, J = 7.1 Hz, 4H), 2.44 (t, J = 7.2 Hz, 2H), 1.77−1.68 (m, 2H), 1.61−1.51 (m, 2H), 1.01 (t, J = 7.1 Hz, 6H). ^{13}C NMR ($CDCl_3$): δ 129.89, 51.77, 46.65, 44.92, 28.00, 24.00, 11.55. Crude isothiocyanate (**39**) (0.10 g, 0.54 mmol) was dissolved in diethyl ether (30 mL) and HCl gas was bubbled through the solution. The white precipitate (**23**) so formed was filtered, washed with ether (3 × 30 mL), and dried under vacuum (0.08 g, 0.36 mmol, 67% yield, 3% overall yield); mp 71−73 °C. ^1H NMR (D_2O): δ 3.66 (t, J = 5.9 Hz, 2H), 3.24 (q, J = 7.1 Hz, 4H), 3.18 (t, J = 7.2 Hz, 2H), 1.90−1.72 (m, 4H), 1.29 (t, J = 7.2 Hz, 6H). ^{13}C NMR (D_2O): δ 51.47, 48.00, 44.86, 26.66, 21.44, 8.88. Anal. ($C_9H_{19}N_2SCl$) C, H, N.

5-(*N*,*N*-Dimethylamino)pentyl Isothiocyanate Monohydrochloride Salt (24). Following a procedure similar to Kupchan's,[15,16] 5-chlorovaleronitrile (**29**) (1.0 g, 8.55 mmol), dimethylamine (**26**) (6.4 mL, 51.3 mmol), and sodium iodide (0.128 g, 0.855 mmol) were stirred together for 5 d at 25 °C. Vacuum distillation (98−100 °C/15 mmHg) yielded a clear liquid, 5-dimethylaminovaleronitrile (**32**) (0.54 g, 4.29 mmol, 50% yield). This compound matched the previously reported boiling point.[15,39] IR (NaCl): 2944, 2247, 1262, 1040 cm^{-1}. ^1H NMR ($CDCl_3$): δ 2.39 (t, J = 6.8 Hz, 2H), 2.29 (t, J = 6.8 Hz, 2H), 2.21 (s, 6H), 1.73−1.59 (m, 4H). ^{13}C NMR ($CDCl_3$): δ 119.58, 58.41, 45.30, 26.41, 23.17, 16.94. HRMS ESI+ (*m*/*z*) calcd for $(M + H)^+$ $C_7H_{15}N_2$, 127.1235; found 127.1235. Compound **32** (1.0 g, 7.94 mmol) and the Rh−Al_2O_3 catalyst (0.25 g) were dissolved in MeOH (40 mL). Hydrogenation was carried out in a Parr shaker under H_2 at 37 psi of pressure for 5 h. When hydrogen uptake was complete, the solution was filtered through Celite to remove the catalyst. The filtrate was concentrated by rotary evaporation. The crude product contained a mixture of primary (*N*,*N*-dimethylpentane-1,5-diamine, **36**) and secondary amines, 1:1, respectively, which were not separated. IR (NaCl): 3293, 1465, 1307, 1041 cm^{-1}. ^1H NMR ($CDCl_3$) (peaks for **36** only): δ 2.69 (t, J = 6.9 Hz, 2H), 2.52 (t, J = 7.2 Hz, 2H), 2.22 (s, 6H), 1.50−1.42 (m, 4H), 1.38−1.30 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 50.03, 45.49, 42.15, 27.66, 25.26, 24.75. The mixture of primary (**36**) and secondary amines (1.0 g, 7.69 mmol/molecular weight of **36**) was dissolved in 15 mL of acetone. After following the procedure used for compounds **7**, **19**−**21**, the resulting crude product was a yellow liquid, 5-(*N*,*N*-dimethylamino)pentyl isothiocyanate (**40**). IR (NaCl): 2939, 2180, 2105, 1272, 1041 cm^{-1}. ^1H NMR ($CDCl_3$): δ 3.53 (t, J = 6.7 Hz, 2H), 2.29 (t, J = 7.4 Hz, 2H), 2.21 (s, 6H), 1.75−1.70 (m, 2H), 1.50−1.43 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 129.86, 59.22, 45.28, 44.83, 29.72, 26.76, 24.27. Crude isothiocyanate (**40**) (0.60 g, 3.49 mmol) was dissolved in diethyl ether (30 mL), and HCl gas was bubbled through the solution. The white precipitate (**24**) so formed was filtered, washed with ether (3 × 30 mL), and dried under vacuum (0.53 g, 2.55 mmol, 73% yield, 16% overall yield); mp 93−94 °C. ^1H NMR (D_2O): δ 3.61 (t, J = 6.4 Hz, 2H), 3.15 (t, J = 8.1 Hz, 2H), 2.87 (s, 6H), 1.82−1.71 (m, 4H), 1.53−1.46 (m, 2H). ^{13}C NMR (D_2O): δ 57.89, 44.76, 42.93, 28.62, 23.60, 23.16. Anal. ($C_8H_{17}N_2SCl$) C, H, N.

5-(*N*,*N*-Diethylamino)pentyl Isothiocyanate Monohydrochloride Salt (25). Following a procedure similar to Kupchan's,[15,16] 5-chlorovaleronitrile (**29**) (10.0 g, 85.5 mmol), diethylamine (**27**) (53.0 mL, 512.8 mmol), and sodium iodide (1.28 g, 8.55 mmol) in H_2O (50 mL) were stirred together for 5 d at 25 °C. Vacuum distillation (110−120 °C/15 mmHg) yielded a clear liquid, 5-diethylamino valeronitrile (**33**) (7.15 g, 46.4 mmol, 54% yield). This compound matched the previously reported boiling point.[41,42] IR (NaCl): 2968, 2247, 1204, 1071 cm^{-1}. ^1H NMR ($CDCl_3$): δ 2.50 (q, J = 7.2 Hz, 4H), 2.44 (t, J = 7.3 Hz, 2H), 2.39 (t, J = 7.0 Hz, 2H), 1.74−1.54 (m, 4H), 1.01 (t, J = 7.2 Hz, 6H). ^{13}C NMR ($CDCl_3$): δ 119.68, 51.72, 46.71, 26.08, 23.44, 16.99, 11.62. Anal. ($C_9H_{18}N_2$) C, H, N. Compound **33** (1.6 g, 10.4 mmol) and the Rh−Al_2O_3 catalyst (0.327 g) were dissolved in MeOH (40 mL). Hydrogenation was carried out in a Parr shaker under H_2 at 40 psi of pressure for 2.5 d. When hydrogen uptake was complete, the solution was filtered through Celite to remove the catalyst. The filtrate was concentrated by rotary evaporation. The crude product contained a mixture of primary (*N*,*N*-diethylpentane-1,5-diamine, **37**) and secondary amines, 1:2, respectively, which were not separated. IR (NaCl): 3291, 1464, 1308, 1040 cm^{-1}. ^1H NMR ($CDCl_3$) (peaks for **37** only): δ 2.69 (t, J = 7.8 Hz, 2H), 2.54 (q, J = 7.0 Hz, 4H), 2.42 (t, J = 7.8 Hz, 2H), 1.54−1.42 (m, 4H), 1.35−1.27 (m, 2H), 1.03 (t, J = 7.1 Hz, 6H). ^{13}C NMR ($CDCl_3$): δ 52.71, 46.69, 41.97, 26.72, 25.34, 24.83, 11.45. The mixture of primary (**37**) and secondary amines (1.6 g, 10.1 mmol/molecular weight of **37**) was dissolved in 5 mL acetone. After following the procedure used for compounds **7**, **19**−**21**, the resulting crude product was a yellow liquid, 5-(*N*,*N*-diethylamino)pentyl isothiocyanate (**41**). IR (NaCl): 2941, 2175, 2102, 1270, 1040 cm^{-1}. ^1H NMR ($CDCl_3$): δ 3.52 (t, J = 6.6 Hz, 2H), 2.53 (q, J = 7.1 Hz, 4H), 2.43 (t, J = 7.8 Hz, 2H), 1.77−1.68 (m, 2H), 1.49−1.42 (m, 4H), 1.03 (t, J = 7.1 Hz, 6H). ^{13}C NMR ($CDCl_3$): δ 128.97, 52.52, 46.76, 44.94, 29.86, 26.29, 24.58, 11.50. Crude isothiocyanate (**41**) (0.66 g, 3.30 mmol) was dissolved in diethyl ether (30 mL) and HCl gas was bubbled through the solution. The white precipitate (**25**) so formed was filtered, washed with ether (3 × 30 mL), and dried under vacuum (0.41 g, 1.74 mmol, 53% yield, 9% overall yield); mp 90−92 °C. ^1H NMR (D_2O): δ 3.61 (t, J = 6.2 Hz, 2H), 3.22 (q, J = 7.2 Hz, 4H), 3.15 (t, J = 8.4 Hz, 2H), 1.81−1.69 (m, 4H),

1.56−1.44 (m, 2H), 1.28 (t, J = 7.3 Hz, 6H). ^{13}C NMR (D_2O): δ 51.98, 47.82, 44.97, 28.86, 23.60, 23.19, 8.71. Anal. ($C_{10}H_{21}N_2SCl$) C, H, N.

Biology. Preparation of Normal and Sickle Hemoglobin and C_{sat} Assay. Preparation of stripped hemoglobins (HbA from normal adult human donors and HbS from sickle homozygous patients) and their purification have previously been described.[43] Packed red blood cells (26.5 mL) in distilled water (53.5 mL), which had been washed three times with isotonic NaCl solution, were lysed in distilled water by first stirring for 30 min in a cold room. To this solution was added saturated ammonium sulfate solution (pH 7.00 adjusted with NaOH, 20 mL) and then stirred for another 30 min in a cold room. The Hb solution was separated from the precipitate by centrifugation at 2500*g* in a Sorvall RC-5B centrifuge for 30 min. Carbon monoxide was bubbled through the Hb solution for 30 min while on ice. Salt was removed from the Hb by gel filtration on a Sephadex G-25 column, preequilibrated with phosphate buffer (5 mM, pH 7.2) at 2−4 °C. Eluates containing Hb were washed with phosphate buffer (5 mM, pH 7.0, 1 L), followed by sodium azide solution (0.02%, 1 L). The resulting solution was concentrated to 15 g/dL by ultrafiltration at 2500 g in a Sorvall RC-5B centrifuge and the concentrated Hb stored in liquid nitrogen. When ESMS analyses were required, ammonium acetate buffer (50 mM, pH 7.0) was used in place of the phosphate buffer.

The saturation concentration (C_{sat}) of deoxy HbS was determined in 50 mM phosphate buffer at pH 7.0 following our previously published methods.[25] Briefly, CO was removed by photodissociation under an oxygen atmosphere, then HbS or inhibitor-modified HbS was deoxygenated by adding 20 μL of a 0.9 M sodium dithionite solution to 330 μL of 35 g/dL $HbSO_2$ in SW55 centrifuge tubes on ice, and the resulting solution then centrifuged for 1 h at 30 °C. After centrifugation, the supernatant phase was separated and the concentration determined spectrophotometrically. Aliquots for mass spectrometry were also obtained. Further details are in De Croos et al.[25]

Chemical Modification of Hb with Various Isothiocyanates. Modification of HbA·CO (all stated Hb concentrations are in terms of the $\alpha_2\beta_2$ tetramer) with isothiocyanates **1**−**13**, **16**−**18**, **20**−**21** was carried out at pH 7.3 with each isothiocyanate in a molar ratio of compound:Hb = 2:1 at a 100 μM final concentration of HbA·CO. The isothiocyanate was dissolved in ammonium acetate buffer (50 mM, pH 7.3) containing dimethyl sulfoxide (2%) to give a concentration of 1 mg/mL. For **3**, **7**, and **9** only buffer was used without dimethyl sulfoxide. HbA·CO solution was added to isothiocyanate, and then the resulting mixture was incubated at 37 °C for 3 h. An aliquot (10 μL) of the reaction mixture was diluted with acetonitrile/water (1/1 = v/v, 1 mL) containing formic acid (0.2%) and analyzed using the Micromass Quattro II mass spectrometer; cone voltage = 30 V, source temperature = 70 °C, flow rate = 10 μL/min, capillary potential = 3.5 kV. The mass spectrometer was scanned from m/z 600−1300 every 10 s for 3 min. For the study, the mass scale was calibrated using horse heart myoglobin (Mr = 16951.5). From the multiply charged peaks envelopes in the ESMS, the molecular weights and peak intensities of the α-, β-, and modified subunits were obtained using maximum entropy deconvolution software (Masslynx, Micromass Boston MA) based on peak width at half-height of base peak and m/z of each of the multiply charged peaks. Representative processed spectrums are shown in Figure 2. The reaction yields were obtained from peak height normalized by (height of modified)/(height of modified + height of unmodified).

Identification of Isothiocyanate Reaction Site on Normal Hemoglobin α-Subunit Using Trypsin Digestion. Isothiocyanates **1** and **3** were used as representative aryl isothiocyanates in the identification of the reaction site on the α-subunit. Modification of HbA·CO with isothiocyanates **1** and **3** was carried out at pH 7.3 with each isothiocyanate in a molar ratio of compound:Hb = 3:1 at 1 mM final concentration of HbA·CO. The isothiocyanate was dissolved in ammonium acetate buffer (50 mM, pH 7.3) containing dimethyl sulfoxide (2%) for **1** or in buffer only for **3** to give a concentration of 1 and 2 mg/mL, respectively. The solution for **1** (268 μL) was diluted with ammonium acetate buffer (116 μL, 50 mM, pH 7.3), and the solution for **3** (235 μL) was diluted with ammonium acetate buffer (149 μL, 50 mM, pH 7.3). HbA·CO solution (116 μL 27.9%) was added to each isothiocyanate solution, and then the resulting mixtures were incubated at 37 °C for 3 h.

Modified and unmodified globins were isolated from the heme according to the method of Marta.[43] Briefly, hemoglobin solution (1 mM, 300 μL) was added dropwise, with stirring, to a 1.5% v/v solution of concentrated HCl in acetone (15 mL) cooled below 0 °C. This caused the globin to precipitate leaving the heme in solution. The precipitate was washed three times by suspending in acetone, centrifuged, and dried under vacuum.

Trypsin stock solution was prepared by dissolving l-1-tosylamido-2-phenylethylchloromethyl-ketone-treated (TPCK) trypsin (1 mg) in ammonium bicarbonate buffer (1 mL, 50 mM, pH 8.0). Globin (1 mg) was suspended in ammonium bicarbonate buffer (490 μL, 50 mM, pH 8.0) and trypsin stock solution (10 μL). The resulting mixture was incubated overnight at 37 °C. The tryptic peptide mixture was ultrafiltered (10000 MW cut off, Amicon, Bedford, MA). The filtrate (20 μL) was injected into a Finnigan HPLC system equipped with a Vydac C18 reversed-phase column (2.1 × 250 mm, Chrom Tech, Inc. Apple Valley, MN) with Finnigan LCQ ESMS detection (molecular weight scan range: 350−2000 m/z). The mobile phase consisted of solution A (water containing 0.1% formic acid) and solution B (acetonitrile containing 0.1% formic acid): a linear gradient from 0 to 60% solution B was run over a period of 45 min at a flow rate of 200 μL/min. Peaks were assigned based on molecular weights of expected fragments (data not shown).

Identification of Reaction Site on β-Subunit Using Gel Electrophoresis. HbA·CO (1mM) was modified with compound **7**, **8**, or **9** (3 equiv) for 3 h as described above. ESMS confirmed at least 95% of the β-subunit had been modified with less than 5% of α-subunit modification. Electrophoresis was done as previously described[44] with minor modification. Briefly, the three modified and unmodified HbA molecules were applied to a Titan III cellulose acetate plate (Helena Laboratories, Beaumont, TX) which was soaked in buffer (0.1 M Tris, 2 mM EDTA, 50 mM boric acid, pH 8.4) just before the electrophoresis. The electrophoresis was carried out for 15 min at 300 V in the same buffer above. The resulting plate was dried by standing for 1 h and stained in Ponceau S (Helena Laboratories, Beaumont, TX) in 3% aqueous trichloroacetic acid for 15 min, followed by 3% aqueous acetic acid twice before rinsing with distilled water. The resulting gels are not shown.

Identification of Reaction Site on Normal Hemoglobin β-Subunit by Titration of Cysβ93 Sulfhydryl Group with *p*-Chloromercuribenzoic Acid. The free sulfhydryl groups in HbA modified with compounds **5**, **7**, **8**, and **9** were titrated with *p*-chloromercuribenzoic acid (PMB) in ammonium acetate buffer (50 mM, pH 7.3), according to the method of Boyer.[20] Briefly, PMB (1 mg) was dissolved in aqueous NaOH (0.01 N, 1 mL), and a 100 μL aliquot was diluted to 1 mL with ammonium acetate buffer (50 mM, pH 7.0). The final concentration of this stock solution was determined from UV absorbance at 232 nm using the extinction coefficient $\epsilon = 1.69 \times 10^4\ M^{-1}\ cm^{-1}$.[20] PMB stock solution (100 μL) was diluted with ammonium acetate buffer (50 mM, pH 7.0, 900 μL). The UV absorbance of this solution relative to the ammonium acetate buffer (50 mM, pH 7.0) was measured at 250 nm.

A standard curve for the increase in UV absorbance from the reaction between PMB and the free sulfhydryl group in unmodified HbA was prepared. PMB stock solution (100 μL) was added to 0.125, 0.25, 0.375, 0.5, and 0.625 equiv of unmodified HbA·CO, and the final volumes were adjusted to 1 mL with ammonium acetate buffer (50 mM, pH 7.0). The references, which were used for the double beam UV spectrophotometer, were prepared the same as above but with ammonium acetate buffer (50 mM, pH 7.0) used in the place of the PMB. The UV absorbances of the solutions of PMB and unmodified HbA·CO mixtures relative to the reference were

measured at 250 nm. An increase in UV absorbance due to mercaptide formation from the reaction between PMB and the free sulfhydryl group in unmodified HbA relative to PMB was obtained by subtraction of the UV absorbance of diluted PMB stock solution (measured above) from that of the solutions of PMB and HbA·CO mixtures. The increase in UV absorbance of samples was plotted against the equivalents of free sulfhydryl in the unmodified HbA·CO. As HbA·CO has two β-subunits, the 0.125, 0.25, 0.375, 0.5, and 0.625 equiv of unmodified HbA·CO have 0.25, 0.5, 0.75, 1.0, and 1.5 equiv of free sulfhydryl, respectively, for reaction with PMB.

In a separate experiment, HbA·CO was modified with isothiocyanates **5**, **7**, **8**, and **9**, as previously described. The modified HbA·CO (0.5 equiv or 1 equiv of sulfhydryl) was added to PMB stock solution (100 μL), and the final volume was adjusted to 1 mL with ammonium acetate buffer (50 mM, pH 7.0). The UV absorbance of each modified HbA solution was measured at 250 nm. The increase in UV absorbance due to mercaptide formation was obtained from subtraction of the UV absorbance of diluted PMB stock solution from that of the solutions of PMB and modified HbA·CO mixtures.

The number of equivalents of free sulfhydryl in HbA·CO modified with isothiocyanates was obtained by comparing the increase in UV absorbance of modified HbA·CO with that in the standard curve (data not shown).

Cell Permeability Measurements for Compounds 7 and 24. Human packed red blood cells obtained from Blood Bank (UIC hospital) were washed three times with 4 volumes of phosphate buffered saline (pH 8.0). Washed cells were incubated with equal volumes of compound at a 4:1 molar ratio of each isothiocyanate to Hb (tetramer) in phosphate-buffered saline (50 mM phosphate, pH 7.4) on a tube rocker for 6 h at room temprature. After incubation, the cells were washed three times with phosphate-buffered saline to remove the excess label. To release hemoglobin from the erythrocytes, cells were mixed with 2 volumes of deionized distilled water and stirred gently for 30 min in the cold. The resulting hemoglobin solution, which also contained cell membranes, was mixed and stirred for 30 min with 1/4 volume of saturated ammonium sulfate solution (pH 7.0) in the cold. The mixture was centrifuged at 12000 rpm for 30 min (at 4 °C), and the supernatant was collected and concentrated to 5% using an Amicon Centriflow Membrane Cone (MWCO 2500) (4 °C, 3500 rpm). The concentrated hemoglobin solution was desalted using Micro DispoDialyzer (Spectrum Lab) (MWCO 25 kD) and deionized distilled water(8 °C, 24 h) and concentrated to 1.8%. An aliquot of modified hemoglobin was submitted for mass spectrometry analysis as described above.

X-ray Crystallography. Crystallization. Native, carbonmonoxyhemoglobin was first isolated from packed human RBC according to the procedure described by Perutz.[45] The protein was further purified using a DEAE-Sephadex column by gradient elution from 50 mM Tris buffer (pH 8.3 to pH 7.3).[46] The purified protein was then covalently modified with 2-(*N*,*N*-dimethylamino)ethyl isothiocyanate as described above and subsequently desalted on a Sephadex G25 column to remove free reagents and products. The modification and purity of the protein were checked by electrophoresis and ESMS. Prior to the crystallization, the sample was first oxygenated to remove bound CO and then deoxygenated using sodium dithionite in an anaerobic environment. Crystals were grown by the batch crystallization method at room temperature from a solution containing 0.75% protein, 19% PEGMME 2000, 5 mM sodium dithionite, 100 mM sodium citrate, 200 mM ammonium acetate at pH 6.6. Crystals suitable for X-ray data collection grew within a week to crystal sizes of approximately 0.5 × 0.4 × 0.2 mm. A single crystal was mounted in a 1 mm quartz glass capillary (Hampton Research, Laguna Niguel, CA), which was then sealed with epoxy. All manipulations were performed in a glovebag filled with nitrogen.

Data Collection and Structure Determination. X-ray diffraction data were collected at room temperature (298 K) on a R-AXIS−IIC imaging plate area detector equipped with a Rigaku RU-200 rotating anode with a Cu target (Kα, λ = 1.5418 Å) X-ray operating at 50 kV and 100 mA. A full data set was collected on a single crystal to 1.9 Å resolution. The data were processed with DENZO (v1.9.6),[47] assuming a mosaic spread of 0.3°, which refined to 0.225°. The data were scaled with SCALEPACK[47] and showed a maximum decay of approximately 25%. Covalently modified HbA crystallized in space group $P2_12_12_1$ with unit cell dimension of a = 63.65 Å, b = 83.76 Å, c = 112.23 Å, and with one $(\alpha\beta)_2$ tetramer in the asymmetric unit.

The structure was solved by molecular replacement using the program EPMR.[48] The complete, deoxyhemoglobin tetrameric structure 1A0Z[49] was used as the initial model for the molecular replacement search. Model refinement was carried out using the program CNS (v1.0) using a bulk-solvent correction.[50] A random selection of 5% of the total number of reflections were excluded from the refinement and used to calculate the free R-factor values. Manual model building was carried out with O[51] and with CCP4.[52]

Molecular Modeling. Autodock calculations were performed using AutoDock 3.0.[21] The deoxy-HbS crystal structure, 2HbS, was used. All water molecules were removed from the crystal structure, as were chains E−H, since the 2HbS crystal is a dimer, and only one HbS molecule (chains A−D) is needed for modeling. QUANTA, a program known to handle heme groups properly, was used to add polar hydrogens and charges to the HbS molecule. This molecule was then used for generating the atomic energy grids for autodock. Due to the size of the HbS molecule, and the desire for fine grids, the HbS molecule was divided into a 3 × 3 × 3 cube, resulting in a total of 27 grids. Each grid was approximately 34 × 30 × 30 Å, with an overlap of ~10 Å, to ensure that any region of the molecule would not be overlooked. The number of points per grid was 90 × 80 × 80, with 0.375 Å between points. The isothiocyanate ligands were created and minimized within Sybyl 6.7 (Tripos, Inc., St. Louis, MO) and Gasteiger-Marsili charges assigned to all atoms. These isothiocyanate ligand initial structures were then docked using all 27 grids, and not just those grids containing the β93 or α-N-terminus. Reasons for this were 2-fold: (1) since it is known that HbS has a number of possible "binding" sites, which are in part dependent upon the type of ligand, we wanted to have a complete picture as to where these ligands could theoretically interact, and (2), we wanted to verify whether the β93 and/or α-N-terminus were indeed the lowest energy binding site(s) for these ligands over the whole HbS molecule. Flexible ligand dockings were performed using the defaults of the Genetic Algorithm plus pseudo-Solis & Wets, with the exceptions of using a population size of 50, and 25000 energy evaluations, along with 300 iterations of S&W local search. A total of 30 GA runs were done with a cluster analysis.

To create the double-strand covalent-Hb structure in Figure 6, we began with the crystal structure containing the covalent ligand. The covalent-Hb dimer was first created by the symmetry translation of the monomer. Then, using moleman2,[52] the dimer was translated in the 'a' direction by −2, −1, 1, and 2 units. The final structure shown in Figure 6 was created using Sybyl 6.8 (Tripos, Inc., St. Louis, MO). Sybyl 6.8 was also used to generate the electric fields of this double-strand structure.

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Note Added after ASAP Posting

The Supporting Information paragraph in the version of the manuscript posted February 7, 2003, has been removed. The *Journal of Medicinal Chemistry* does not publish elemental analyses data. The corrected version of the manuscript was posted February 25, 2003.

References

(1) Manning, J. M. Covalent inhibitors of the gelation of sickle cell hemoglobin and their effects on function. *Advances in Enzymology and Related Areas of molecular Biology*; John Wiley & Sons: New York, 1991; pp 55−91.

(2) Harrington, D. J.; Adachi, K.; Royer, W. E., Jr. The high-resolution crystal structure of deoxyhemoglobin S. *J. Mol. Biol.* **1997**, *272*, 398−407.

(3) Bunn, H. F.; Forget, B. G. *Hemoglobin*: *molecular, genetic and clinical aspects*; W. B. Saunders: Philadelphia, 1986.

(4) Eaton, W. A.; Hofrichter, J. Hemoglobin S gelation and sickle cell disease. *Blood* **1987**, *70*, 1245−1266.

(5) Charache, S. Mechanism of action of hydroxyurea in the management of sickle cell anemia in adults. *Semin. Hematol.* **1997**, *34*, 15−21.

(6) Beddell, C. R.; Goodford, P. J.; Kneen, G.; White, R. D.; Wilkinson, S. et al. Substituted benzaldehydes designed to increase the oxygen affinity of human haemoglobin and inhibit the sickling of sickle erythrocytes. *Br. J. Pharmacol.* **1984**, *82*, 397−407.

(7) Sunshine, H. R.; Hofrichter, J.; Eaton, W. A. Requirement for therapeutic inhibition of sickle haemoglobin gelation. *Nature* **1978**, *275*, 238−240.

(8) Guyton, A. C. *Medical Physiology*; W. B. Saunders: Philadelphia, 1986.

(9) Vain, J. R. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature-New Biol.* **1971**, *231*, 231−235.

(10) Nickerson, M.; Hollenberg, N. K. *Blockade of alpha-adrenergic receptors*; Academic Press: New York, 1967; 243−305.

(11) Garel, M. C.; Domenget, C.; Galacteros, F.; Martin-Caburi, J.; Beuzard, Y. Inhibition of erythrocyte sickling by thiol reagents. *Mol. Pharm.* **1984**, *26*, 559−565.

(12) Seligman, R. B.; Bost, R. W.; McKee, R. L. Derivatives of p-aminosalicylic acid. *J. Am. Chem. Soc.* **1953**, *75*, 6334−6335.

(13) Kim, S.; Yi, K. Y. Di-2-pyridyl thionocarbonate. A new reagent for the preparation of isothiocyanates and carbodiimides. *Tetrahedron Lett.* **1985**, *26*, 1661−1664.

(14) McElhinney, R. S. Derivatives of thiocarbamic acid. Part I. Preparation of 4-substituted thiosemicarbazides. *J. Chem. Soc. C* **1966**, 950−955.

(15) Kupchan, S. M.; Bondesson, G.; Davies, A. P. Tumor inhibitors. 70. Structure-cytotoxicity relationships among N-acyltriamines related to solapalmitine. *J. Med. Chem.* **1972**, *15*, 65−68.

(16) Price, C. C.; Kabas, G.; Nakata, I. Some amino and ammonio nitrogen mustard analogues. *J. Med. Chem.* **1965**, *8*, 650−655.

(17) Park, S.; Johnson, M. E.; Venton, D. L. Regioselective chemical reaction on a protein surface. Presented ath the *217th National Meeting of the American Chemical Society*; Anaheim, CA, 1999.

(18) Desiderio, D., M. *Mass spectrometry*; Plenum Press: New York, 1994.

(19) Chiancone, E.; Currell, D. L.; Vecchini, P.; Antonini, E.; Wyman, J. Kinetics of the reaction of the "Masked" and "Free" sulfhydryl groups of human hemoglobin with p-Mercuribenzoate. *J. Biol. Chem.* **1970**, *245*, 4105−4111.

(20) Boyer, P. D. Spectrophotometric study of the reaction of protein sulfhydryl groups with organic mercurials. *J. Am. Chem. Soc* **1954**, *76*, 4331−4337.

(21) Morris, G. M.; Goodsell, D. S.; Halliday, R. S.; Huey, R.; Hart, W. E. et al. Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. *J. Comput. Chem.* **1998**, *19*, 1639−1662.

(22) Drobnica, L.; Kristian, P.; Augustin, J. The chemistry of the isothiocyanato group. *The Chemistry of Cyanates and Their Thio Derivatives*; Wiley: Chichester, England, 1977; pp 1003−1221.

(23) van Rossum, J. M.; Ariens, E. J. Receptor-reserve and threshold-phenomena. II. Theories on drug-action and a quantitative approach to spare receptors and threshold values. *Arch. Int. Pharmacodyn. Ther.* **1962**, *136*, 385−413.

(24) Stephenson, R. P. A modification of receptor theory. *Br. J. Pharmacol. Chemother.* **1956**, *11*, 379−393.

(25) De Croos, P. Z.; Sangdee, P.; Stockwell, B. L.; Kar, L.; Thompson, E. B. et al. Hemoglobin S antigelation agents based on 5-bromotryptophan with potential for sickle cell anemia. *J. Med. Chem.* **1990**, *33*, 3138−3142.

(26) Moffat, J. K.; Simon, S. R.; Konigsberg, W. H. Structure and functional properties of chemically modified horse hemoglobin. 3. Functional consequences of structural alterations and their implications for the molecular basis of cooperativity. *J. Mol. Biol.* **1971**, *58*, 89−101.

(27) Bonaventura, C.; Ferruzzi, G.; Tesh, S.; Stevens, R. D. Effects of S-nitrosation on oxygen binding by normal and sickle cell hemoglobin. *J. Biol. Chem.* **1999**, *274*, 24742−24748.

(28) Garel, M.-C.; Donemget, C.; Caburi-Martin, J.; Prehu, C.; Galacteros, F. et al. Covalent binding of glutathione to hemoglobin. *J. Biol. Chem.* **1986**, *261*, 14704−14709.

(29) Chan, N. L.; Rogers, P. H.; Arnone, A. Crystal structure of the S-nitroso form of liganded human hemoglobin. *Biochemistry* **1998**, *37*, 16459−16464.

(30) Kleywegt, G. J. Use of noncrystallographic symmetry in protein structure refinement. *Acta Cryst.* **1996**, *D52*, 842−857.

(31) Garel, M. C.; Caburi-Martin, J.; Domenget, C.; Kister, J.; Craescu, C. T. et al. Changes of polymerization and conformation of hemoglobin S induced by thiol reagents. *Biochim. Biophys. Acta* **1990**, *1041*, 133−140.

(32) Perutz, M. F.; Muirhead, H.; Mazzarella, L.; Crowther, R. A.; Greer, J. et al. Identification of residues responsible for the alkaline Bohr effect in haemoglobin. *Nature* **1969**, *222*, 1240−1243.

(33) Domenget, C.; Garel, M. C.; Rhoda, M. D.; Caburi-Martin, J.; Galacteros, F. et al. Kinetics of polymerization of hemoglobin S modified by thiol reagents and by oxidation. *Biochim. Biophys. Acta* **1985**, *830*, 71−79.

(34) Eaton, W. A.; Hofrichter, J. Sickle cell hemoglobin polymerization. *Adv. Protein Chem.* **1990**, *40*, 63−279.

(35) Jochims, J. C.; Seeliger, A. A new synthesis of aliphatic isothiocyanates. *Angew. Chem., Int. Ed. Engl.* **1967**, *6*, 174−175.

(36) Yamasaki, T.; Kawaminami, E.; Uchimura, F.; Okamoto, Y.; Okawara, T. et al. Preparation of novel tricyclic ring systems containing the pyridazinone ring. *J. Heterocycl. Chem.* **1992**, *29*, 825−829.

(37) Park, S.; Wanna, L.; Johnson, M. E.; Venton, D. L. A mass spectrometry screening method for antiaggregatory activity of proteins covalently modified by combinatorial library members: application to sickle hemoglobin. *J. Comb. Chem.* **2000**, *2*, 314−317.

(38) Siatra-Papastaikoudi, T.; Tsotinis, A.; Raptopoulou, C. P.; Sambani, C.; Thomou, H. Synthesis of new alkylaminoalkyl thiosemicarbazones of 3-acetylindole and their effect on DNA synthesis and cell proliferation. *Eur. J. Med. Chem.* **1995**, *30*, 107−114.

(39) Short, J. H.; Biermacher, U.; Dunnigan, D. A.; Leth, T. D. Sympathetic nervous system blocking agents. Derivatives of guanidine and related compounds. *J. Med. Chem.* **1963**, *6*, 275−283.

(40) Huber, W.; Clinton, R. O.; Boehme, W.; Jackman, M. Some dialkylaminoalkylamines. *J. Am. Chem. Soc.* **1945**, *67*, 1618−1619.

(41) Stevenson, G. W.; Williamson, D. Base strengths of cyanoamines. *J. Am. Chem. Soc.* **1958**, *80*, 5943−5947.

(42) Kurtz, P. Formation of nitriles. I. *Justus Liebigs Ann. Chem.* **1951**, *572*, 23−82.

(43) Marta, M.; Patamia, M.; Lupi, A.; Antenucci, M.; Io, M. D. Bovine Hemoglobin Cross-Linked through the beta Chains Functional and structural aspects. *J. Biol. Chem.* **1996**, *271*, 7473−7478.

(44) D'Avino, R.; Di Prisco, G. Hemoglobin from the Antarctic fish Notothenia coriiceps neglecta. 1. Purification and characterisation. *Eur. J. Biochem.* **1989**, *179*, 699−705.

(45) Bolton, W.; Cox, J. M.; Perutz, M. F. Structure and function of haemoglobin. IV. A three-dimensional Fourier synthesis of horse deoxyhaemoglobin at 5.5 A resolution. *J. Mol. Biol.* **1968**, *33*, 283−297.

(46) Adachi, K.; Asakura, T. Demonstration of a delay time during aggregation of diluted solutions of deoxyhemoglobin S and hemoglobin CHarlem in concentrated phosphate buffer. *J. Biol. Chem.* **1978**, *253*, 6641−6643.

(47) Otwinowski, Z.; Minor, W. Processing of X-ray Diffraction Data Collected in Oscillation Mode. *Methods in Enzymology*; Academic Press: New York, 1997; Part A, pp 307−326.

(48) Kissinger, C. R.; Gehlhaar, D. K.; Fogel, D. B. Rapid automated molecular replacement by evolutionary search. *Acta Crystallogr. D Biol. Crystallogr.* **1999**, *55*, 484−491.

(49) Kavanaugh, J. S.; Weydert, J. A.; Rogers, P. H.; Arnone, A. High-resolution crystal structures of human hemoglobin with mutations at tryptophan 37beta: structural basis for a high-affinity T-state. *Biochemistry* **1998**, *37*, 4358−4373.

(50) Rice, L. M.; Earnest, T. N.; Brunger, A. T. Single-wavelength anomalous diffraction phasing revisited. *Acta Crystallogr. D Biol. Crystallogr.* **2000**, *56*, 1413−1420.

(51) Jones, T. A.; Zou, J. Y.; Cowan, S. W.; Kjeldgaard, M. Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr.* **1991**, *A47*, 110−119.

(52) Bailey, S. The CCP4 suite: programs for protein crystallography. *Acta Crystallogr., Sect. D: Biol.Crystallogr.* **1994**, *D50*, 760−763.

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