Supporting Information

Identification of a Small Molecule that Increases Hemoglobin Oxygen Affinity and Reduces SS Erythrocyte Sickling

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Supplemental Information contains

- Supplementary Methods
- Supplementary Results
- Supplementary Tables (Tables S1-S4)
- Supplementary Figures (Figures S1-S7)

Supplementary Methods

Reagents. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, β-nicotinamide adenine dinucleotide phosphate (NADP), ferredoxin, ferredoxin-NADP reductase, and catalase were purchased from Sigma-Aldrich.

Preparation of hemoglobin (HbA). Outdated donated human red blood cells (RBCs) were obtained from the Blood Bank of Massachusetts General Hospital. The use of the RBCs for obtaining hemoglobin was reviewed and approved by the Institutional Review Board (IRB) of Partners Human Research Committee. RBCs were centrifuged at 3,000 rpm for 15 min at 4°C. The supernatant was discarded, and the remaining RBCs were washed three times with an equal volume of 0.9% sodium chloride. Three equivalents of distilled water were added to the washed RBCs to lyse the cells, and the mixture was centrifuged at 20,000 g for 1 h at 4°C. The supernatant was collected and centrifuged again (same conditions), and the resulting supernatant was collected and dialyzed against Dulbecco's phosphate buffered saline (DPBS, pH 7.4) at 4°C. After dialysis, the sample was sterilized by passing it twice through 0.2-μm filters. The absorption spectrum of the hemoglobin solution was measured from 500–700 nm, fitted to a linear combination of pure oxygenated hemoglobin (oxyHb), deoxygenated hemoglobin (deoxyHb), and oxidized hemoglobin which cannot bind oxygen (metHb) (*1*) plus a baseline (*2-4*) (least-squared fitting) using a program (Solver in Excel 2007, Microsoft) to determine the total concentration of hemoglobin.

Identification of molecules that bind to hemoglobin using small molecule microarrays (SMMs). Isocyanate groups were introduced onto glass slides and compounds were affixed to the isocyanate-functionalized surfaces, as previously described (*5*). A total of nine unique SMM slides were used containing 38,700 compounds. Each slide contained 48 "blocks", and each block contained approximately 100 unique surface-immobilized small molecules. Compounds were printed in duplicates, and each slide was screened in duplicate, resulting in four replicates for any given compound. SMM slides were incubated in a TBS solution containing Tween 20 (0.01 vol%; TBS-T) containing bovine serum albumin (BSA, 0.1 wt%) for 30 min to prevent non-specific binding of hemoglobin to the SMMs. The slides were rinsed with TBS-T buffer for 2 min and incubated in a TBS-T solution

containing purified hemoglobin (1 μg mL^{-1} = 16 nM as hemoglobin tetramer) for 1 h. The hemoglobin-treated slides were rinsed with TBS-T buffer for 2 min and incubated with a TBS-T solution containing an antibody directed against human hemoglobin (mouse-IgG, 0.25 μg mL^{-1}, Catalog No. ab55081, Abcam) for 30 min. Antibody-treated slides were washed for 2 min with TBS-T buffer and then incubated with a secondary detection anti-mouse IgG antibody labeled with Cy5 fluorescent dye (0.2 μg mL^{-1}, Catalog No. A10524, Invitrogen) for 30 min, followed by washing with TBS-T (three times) and distilled water (once) for 2 min. All incubations were performed at room temperature and on a shaker set to the lowest setting. Slides were scanned using GenePix 4200A (Molecular Devices) with an excitation wavelength of 635 nm.

Using SMM Hits-Analyzer software (Broad Institute), the fluorescent signal of the foreground of a given spot (*F*) was divided by its local background (*B*) resulting in a calculated *F/B* ratio for each spot on which small molecules were affixed. The F/B ratios were fitted to a Cauchy distribution plot for every block on the slide, and the software determined which spots were considered "positive" using a P = 0.07 as a threshold. If all four “positive” replicates had P < 0.07, they were selected as “hit” molecules binding to hemoglobin. An antibody counter screen was performed to exclude false positives that bind antibodies used in the screen rather than the target molecule hemoglobin. The summary data of the screening results are reported in Table S1.

Sample preparation for the hemoglobin oxygen binding assay. A detailed scheme of the sample preparation is presented in Figure S2A. Compound plates were generated by adding the hemoglobin-binding compounds (0.75 μL, 10 mM in DMSO) to the central 240 wells (well IDs: 3C-22N) of two 384 deep well plates (Catalog No. 3347, Corning). IHP and NEM were added to plates as controls (0.75 μL, 10 mM in DPBS). To prevent oxidation of hemoglobin during the experiment, glucose-6-phosphate (0.4 mg mL^{-1}), glucose-6-phosphate dehydrogenase (1.0 U mL^{-1}), NADP (0.05 mg mL^{-1}), ferredoxin (0.01 mg mL^{-1}), ferredoxin-NADP reductase (0.01 U mL^{-1}), and catalase (2 x 10^3 U mL^{-1}) were added to hemoglobin in DPBS to produce the Hayashi reducing system (*6*). The DPBS solution containing hemoglobin (10 μM) with the Hayashi reducing system (125 μL/well) was dispensed into the compound plates, and the plates were shaken at 800 rpm for 3 min. Aliquots

of the reaction mixture were transferred to the central 240 wells of four 384-well assay plates (Catalog No. 262160, Thermo Scientific, 50 µL/well). The final concentration of hemoglobin, compound, and DMSO was 10 µM, 60 µM, and 5 vol%, respectively.

Measurement of the oxygen dissociation curve (ODC) of hemoglobin. Hemoglobin (20 µM as tetramer) was diluted with DPBS, and antifoam (0.2 vol%, Catalog No. AFA-25, TCS Scientific Corporation) was added to prevent foaming of the sample. Compounds were dissolved in DMSO as stock solutions, which were added to hemoglobin. The final concentration of DMSO was adjusted to 5 vol%. In evaluating Bohr effect of hemoglobin, sodium phosphate buffer (0.1 M phosphate, pH 6, 6.5, 7, 7.5, or 8.0) was used instead of DPBS. The ODC of hemoglobin was measured with a HEMOX analyzer (TCS Scientific Corporation) at 37^{o}C.

Measurement of the ODC of whole blood. The use of blood for evaluating oxygen affinity was reviewed and approved by the IRB of Partners Human Research Committee. After obtaining informed consent from volunteers, blood was drawn from the volunteers into tubes containing EDTA. The concentration of hemoglobin of the blood was determined using a blood gas analyzer (ABL 800 FLEX, Radiometer Medical). The blood was diluted with HEMOX solution (TCS Scientific Corporation) containing N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES, 30 mM), sodium chloride (135 mM), and potassium chloride (5 mM) in water (pH 7.4), and antifoam (0.2 vol%), then mixed with compounds. The final concentration of DMSO in the diluted blood sample was 0.8 vol%. The ODC was measured with a HEMOX analyzer (TCS Scientific Corporation) at 37^{o}C.

Measurement of ODCs of hemolysates from SS RBCs. The use of blood from SCD patients for anti-sickling assays was reviewed and approved by the IRB at the Children's Hospital of Philadelphia. After obtaining informed consent from patients, their parents, or their legal guardians, blood was collected from patients with SCD into tubes containing EDTA. Blood suspensions (hematocrit~ 20%) were mixed with 0.5, 1.5, or 2 mM of TD-1 or without TD-1 and the mixture was hemolyzed. Corresponding clarified hemolysates from individual samples were subjected to ODC analyses in 0.1 M potassium phosphate buffer (pH 7.0) at 25^{o}C,

using the HEMOX Analyzer. ODC for each condition was measured using blood from one subject and P_{50} for each condition was determined from the ODCs measured using three different subjects.

Evaluation of anti-sickling effects of TD-1. The morphology of hypoxic sickled RBCs was evaluated using the previously reported method (*7, 8*). The collected blood from SCD patients was diluted using HEMOX buffer supplemented with glucose (10 mM) and BSA (0.2 wt%) to adjust hematocrit~ 20% of the suspensions. The suspensions were pre-incubated under air in the absence or presence of three different concentrations (0.5, 1.5, and 2 mM) of TD-1 at 37^{o}C for 1 hour. The suspensions were then incubated with a 4% O_2/96% N_2 gas mixture at 37^{o}C for 3 hours. Aliquots (5 µL) of each sample were collected without exposure to air into 2% glutaraldehyde solution for immediate fixation. Fixed cell suspensions were introduced into glass microslides (Fiber Optic Center) (*9*) and subjected to microscopic morphological analysis. The percentage of sickled cells for each condition was obtained using blood from one subject with a computer-assisted image analysis system, as described previously (*7, 10*).

Supplementary Results

Characterization of TD-1. The purity of TD-1 was 96%, as determined by an Agilent technologies, 1200 Infinity Series analytical HPLC system operating at a flow rate of 0.9 mL min^{-1}, using a linear gradient of 2-98 vol% acetonitrile in water (both solvents contain 0.1 vol% of ammonium hydroxide) over 2 min, on a Waters Acquity UPLC BEH C18 column (1.7 µm, 2.1 x 50 mm) set at 60°C. ^{1}H and ^{13}C-NMR spectra were recorded at 25^{o}C and 60°C (Figure S4 and S5) and at 25^{o}C (Figure S6) respectively using a Bruker Avance III (300 MHz for ^{1}H, 75 MHz for ^{13}C). Chemical shifts were referenced to the residual solvent peaks in the deuterated DMSO used. HR-MS spectra were taken on TOF-Agilent 6230 UHPLC/PDA/MS with ESI source and the difference between the measured ion mass and the expected ion mass was less than 5 ppm.

TD-1 (Di((5-(2,3-dihydro-1,4-benzodioxin-2-yl))-4H-1,2,4-triazol-3-yl)disulfide): Purity by analytical HPLC: 96%. ^{1}H NMR (DMSO-d_6, 300 MHz, 60^{o}C) δ 6.96–6.85 (m, 4 H), 5.47 (bs, 1 H), 4.54 (dd, *J* = 11.6, 2.6 Hz, 1 H), 4.35 (dd, *J* = 11.6, 7.2 Hz, 1 H); ^{13}C NMR (DMSO-d_6, 75 MHz, 25^{o}C) δ 160.6, 158.2, 154.4, 143.1, 142.7, 122.1, 117.6, 68.3, 65.7; HR-MS (ESI+): Calculated. for $C_{20}H_{17}N_6O_4S_2$ $[M+H]^+$ 469.0753, found 469.0748.

Binding of monomeric units (MUs) of TD-1 to hemoglobin. Figure S7 shows the detailed interactions between the eight MUs and the tetrameric hemoglobin. The two symmetry-related MU-1 molecules form disulfide bond with the two β-Cys93 residues, separated by 6.4 Å (Figure S7A). In addition, each MU-1 molecule forms hydrogen-bond and hydrophobic interactions with both β-subunits, which should help tie the two subunits together to stabilize the R-state.

The other six MU molecules are located in the central water cavity, each making contact with the protein and at least with one other MU molecule that tie all four subunits together to also stabilize the relaxed structure. MU-2 and MU-2' form disulfide bond with the two β-Cys112 residues. Each molecule makes hydrogen-bond and/or hydrophobic interactions with the α- and β-subunits, as well as hydrophobic interaction with the adjacent non-covalently bound MU-3 and MU-3' molecules (Figure S7B). Each of the two MU-3 molecules also makes hydrogen-bond and/or hydrophobic interactions with three of the four hemoglobin subunits (Figure S7C). In addition, MU-3/MU-3' makes hydrogen-bond and/or hydrophobic

interactions with three other MU molecules, including MU-2, MU-4 and MU-4’ (Figure S7C). MU-4 makes extensive hydrophobic interactions with its symmetry-related partner MU-4’, as well as with both MU-3 and MU-3’. It also forms several protein interactions with the α-subunits (Figure S7D).

Table S1. Screening method for the identification of small molecules that bind to hemoglobin using small molecule microarrays (SMMs).

Category	Parameter	Description
Assay	Type of assay	Binding assay on small molecule micro arrays
	Target	Human hemoglobin
	Primary measurement	Fluorescence
	Reagents and Assay protocol	See Supplementary Methods section
Library	Library size	38,700
	Library composition	Bioactives, Diversity oriented synthesis (DOS), commercially available collection
	Source	Broad Institute
	Additional comments	The DOS library is specific to the Broad Institute.
Screen	Format	Biochemical with a secondary antibody fluorescent detection
	Concentration(s) tested	1 μg ml^{-1} Hemoglobin
	Plate controls	2,3-DPG
	Reagent/compound dispensing system	Aushon 2470 Arrayer
	Detection instrument and software	GenePix 4300A (Molecular Devices), GenePix Pro7, SMM Analysis software
	Assay validation/QC	Postitive control = 2,3-DPG
	Correction factors	N/A
	Normalization	Non-bound compounds in each block (See Supplementary Methods section)
Post-HTS analysis	Hit criteria	P value < 7%
	Hit rate	1.1%
	Additional assay(s)	Detection antibody only counter screen

Table S2. Screening method for the identification of compounds that alter hemoglobin's oxygen affinity from amongst the 427 molecules binding to hemoglobin using a High-throughput assay.

Category	Parameter	Description
Assay	Type of assay	Absorption spectra (500-700 nm)
	Target	Oxygen binding of hemoglobin
	Assay protocol	The fraction of oxygenated hemoglobin is evaluated in the presence and absence of the compounds (Details are in METHODS section).
Library	Library size	427 compounds
	Library composition	Compounds identified to bind to hemoglobin by small molecule microarrays (See Table S1)
	Source	Broad Institute
Screen	Format	384 well format
	Concentrations tested	0 and 60 µM
	Detection instrument and software	MultiSkan Go and Microsoft Excel
	Assay validation/QC	Use of known allosteric effectors of hemoglobin; IHP (decrease of oxyHb%) and NEM (increase of oxyHb%)
	Calibration factors	Adjustment factor was introduced to control the uneven distribution of oxygenation values (See Methods section)
	Normalization	Z score (See METHODS section)
Post-HTS analysis	Hit criteria	Z_{oxy} is > 2.5 or <2.5 and Z_{met} is < 2.5 (See METHODS section)
	Hit rate	1.4%
	Additional assay(s)	Measurement of oxygen dissociation curve of hemoglobin

Table S3. Z-scores (Z_{oxy}) of six hit molecules measured at three different saturation conditions of oxygenated hemoglobin. The six hit compounds (Compound 1-6) showed (1) both Z_{oxy}> 2.5 (increase of oxyHb%) and Z_{met}< 2.5 or (2) both Z_{oxy}< -2.5 (decrease of oxyHb%) and Z_{met}< 2.5. Some portion of Compound 1 would be partially oxidized to disulfide (TD-1) when Compound 1 was identified as a molecule altering P_{50}.

	Z_{oxy}		
Compound	**High oxyHb%$_{ctrl}$**	**Medium oxyHb%$_{ctrl}$**	**Low oxyHb%$_{ctrl}$**
1	0.20	1.6	2.8
2	-0.88	1.5	3.8
3	-1.3	1.3	3.4
4	0.33	2.3	4.9
5	0.33	2.8	6.2
6	-4.6	-6.0	-3.0
NEM	3.5	15	23
IHP	-29	-21	-9.0

Table S4. Crystallographic data and refinement statistics for COHb in complex with TD-1. Numbers in parentheses are for the highest resolution shell. All reflections were used in the refinement.

Data Collection	
Space group	$P4_122$
Unit-cell *a,b,c* (Å)	62.1, 62.1, 173.9
Resolution (Å)	29.2–2.15 (2.18–2.15)
Unique reflections	19122
Redundancy	10.8 (11.2)
Completeness (%)	98.6 (97.9)
Average I/σ(I)	14.9 (5.4)
R_{merge} (%)	9.5 (45.0)
Refinement	
No. of reflections	19071
Resolution (Å)	18.6–2.15 (2.23–2.15)
R_{work} (%; 95% of data)	22.8 (39.2)
R_{free} (%; 5% of data)	25.9 (37.6)
R.m.s.d. bonds (Å)	0.013
R.m.s.d. angles (°)	2.0
Dihedral angles	
Most favored (%)	87.1
Allowed (%)	12.8
Average B (Å^2) / atoms	
Protein	45.7/2192
Heme	45.9/90
Effector	73.9/64
Solvent	53.3/158
PDB ID code	**4NI0**

$R_{merge} = \Sigma_{hkl}\Sigma_i|I_{hkli} - \langle I_{hkli}\rangle|/\Sigma_{hkl}\Sigma_i\langle I_{hkli}\rangle$. R_{free} calculated with 5% of reflections excluded throughout the refinement.

Figure S1. The small molecule microarray assay used to detect small molecules that bind to hemoglobin was validated by confirming the binding of hemoglobin to 2,3-DPG affixed on the microarray. (A) Fluorescence image of small molecule microarrays on a glass slide. (B) Magnified fluorescence image of small molecule microarrays. A red spot indicates binding of hemoglobin to 2,3-DPG affixed on the arrays.

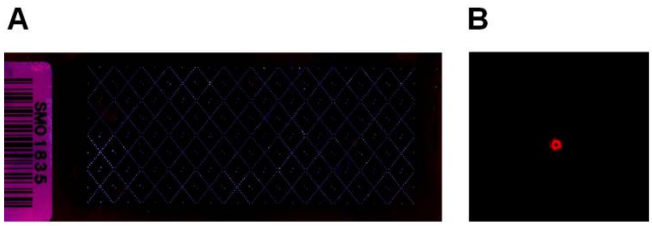


Figure S2. Experimental setup for high-throughput hemoglobin-oxygen binding assay to evaluate oxyHb% in the absence and the presence of the compounds that bind to hemoglobin. (A) Scheme to combine hemoglobin with test compounds for the high-throughput hemoglobin oxygen binding assay and (B) Setup of the chamber to evaluate oxyHb% at the three oxygen saturation conditions.

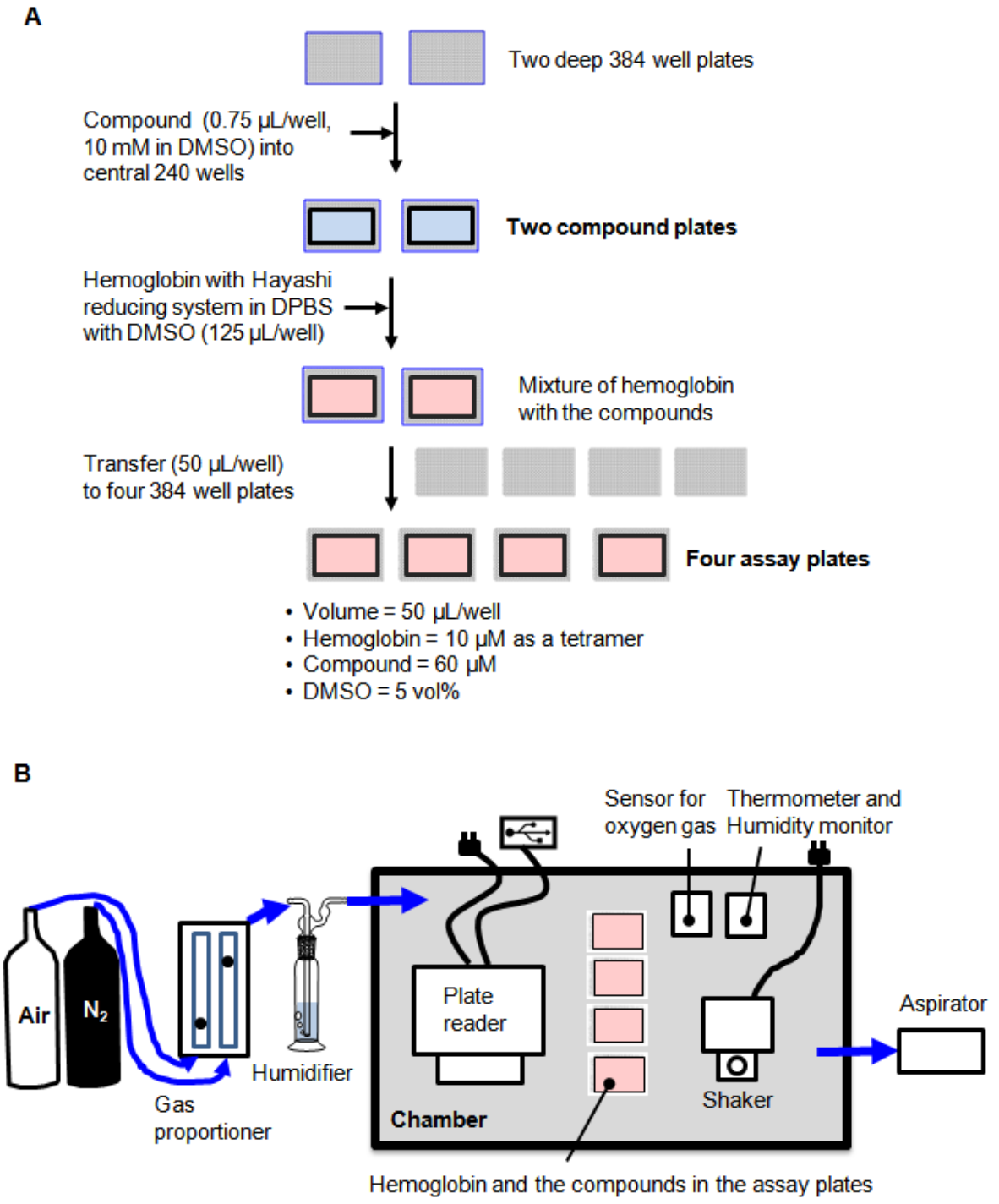


Figure S3. Electron density map of COHb structure in complex with MUs. (A) Initial Fo-Fc map (contoured at 2.6α) of MU-1 , (B) Final 2Fo-Fc map (contoured at 0.9 α) of MU-1, and (C) Illustration of MU-1 bound to β-Cys93. (D) Initial Fo-Fc map (contoured at 2.6α) of MU-2/MU-3/MU-4, (E) Final 2Fo-Fc map (contoured at 0.9α) of MU-2/MU-3/MU-4, and (F) Illustration of MU-2/MU-3/MU-4.

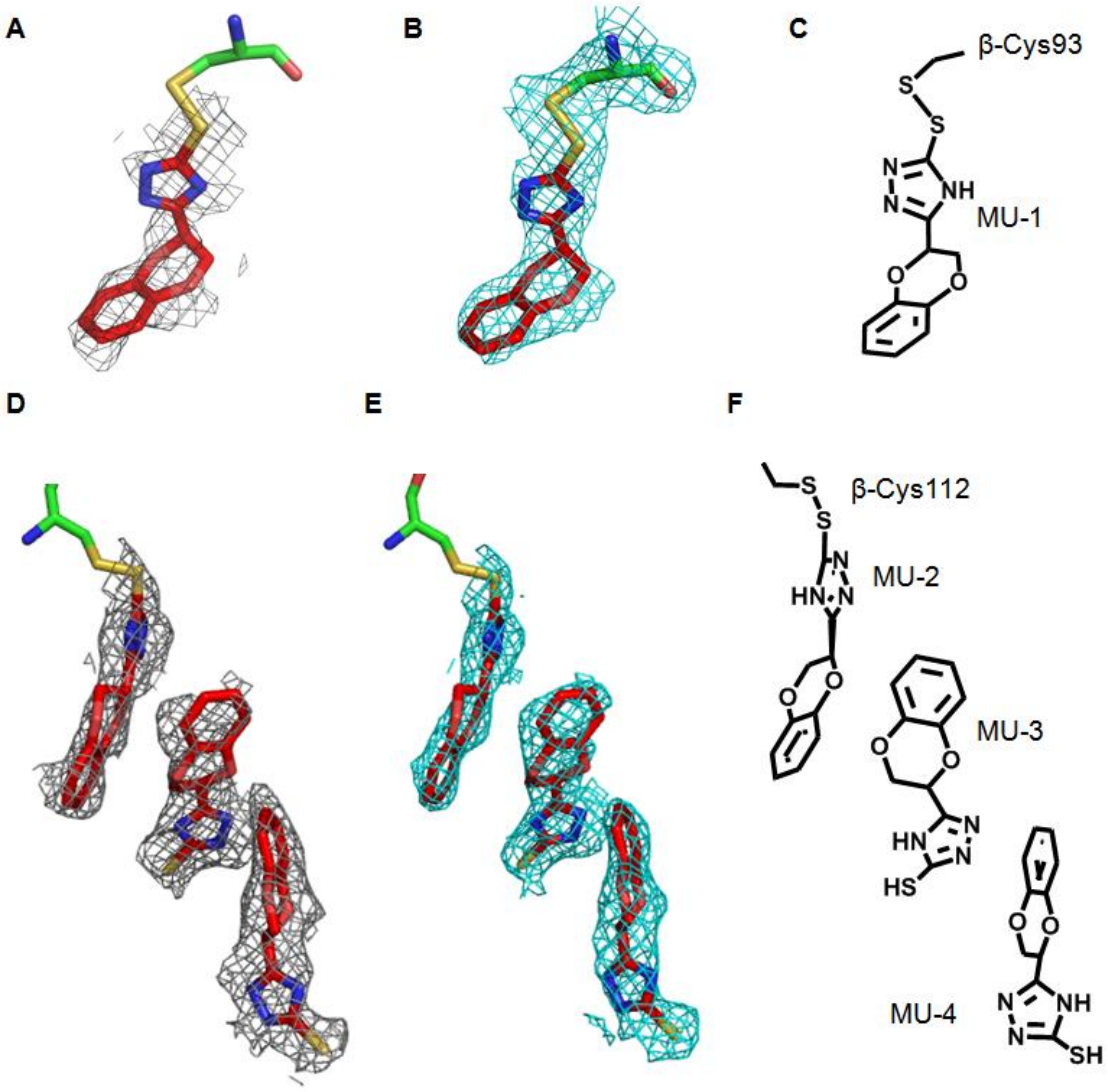


Figure S4. ^{1}H NMR (DMSO-d_6, 300 MHz, 25^{o}C) of TD-1.

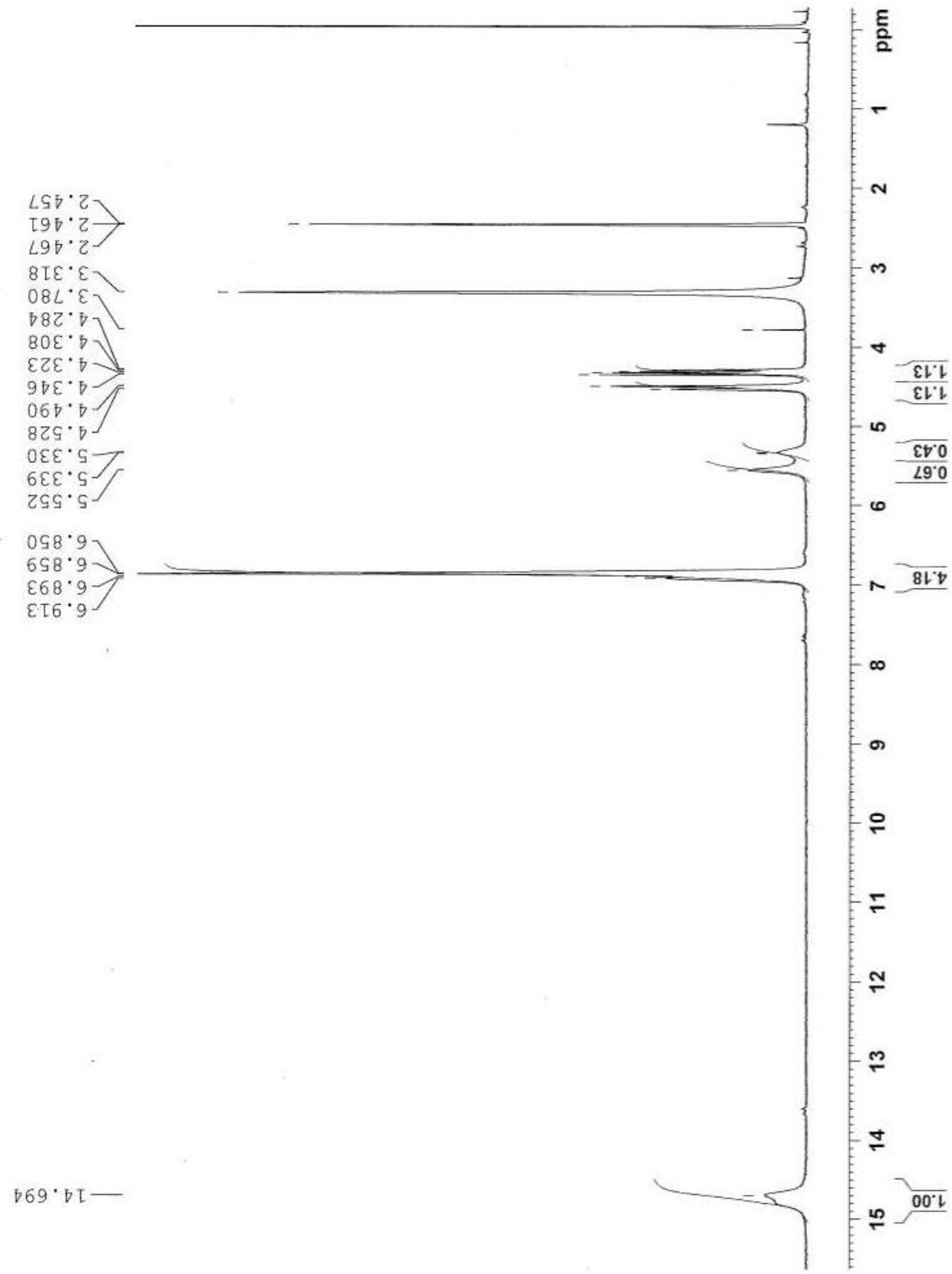


Figure S5. ^{1}H NMR (DMSO-d_6, 300 MHz, 60^{o}C) of TD-1.

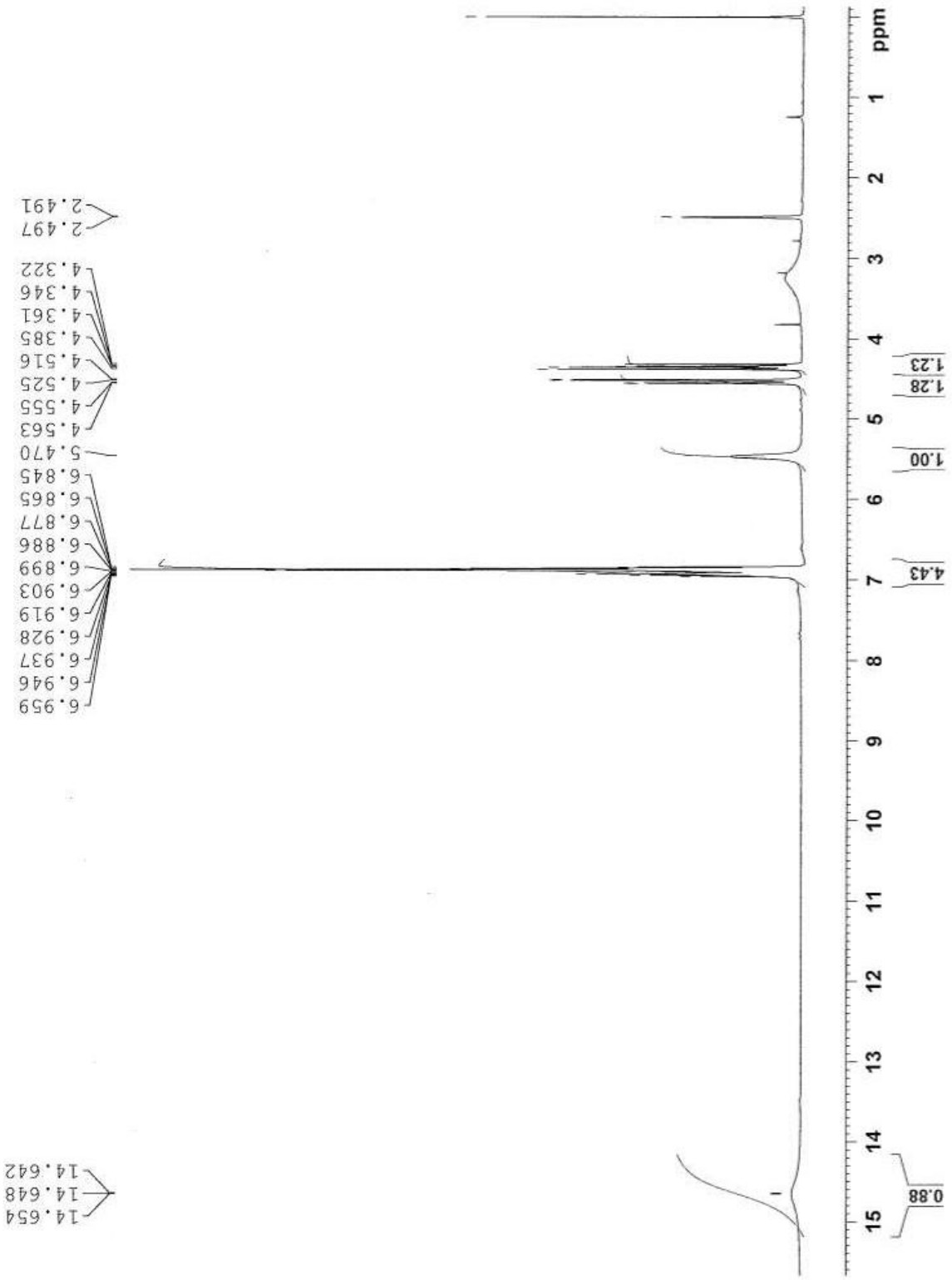


Figure S6. ^{13}C NMR (DMSO-d_6, 75 MHz, 60^{o}C) of TD-1.

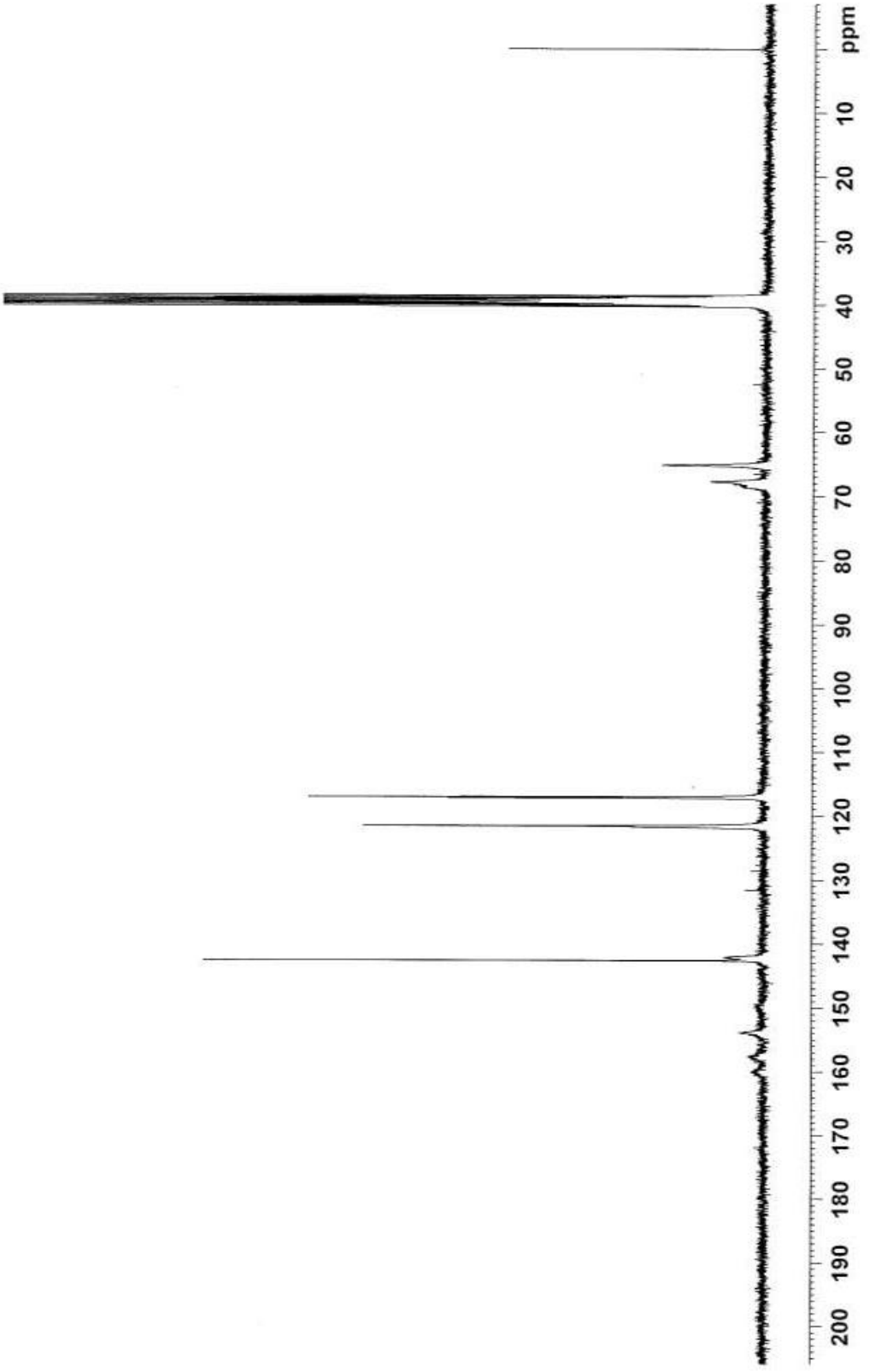
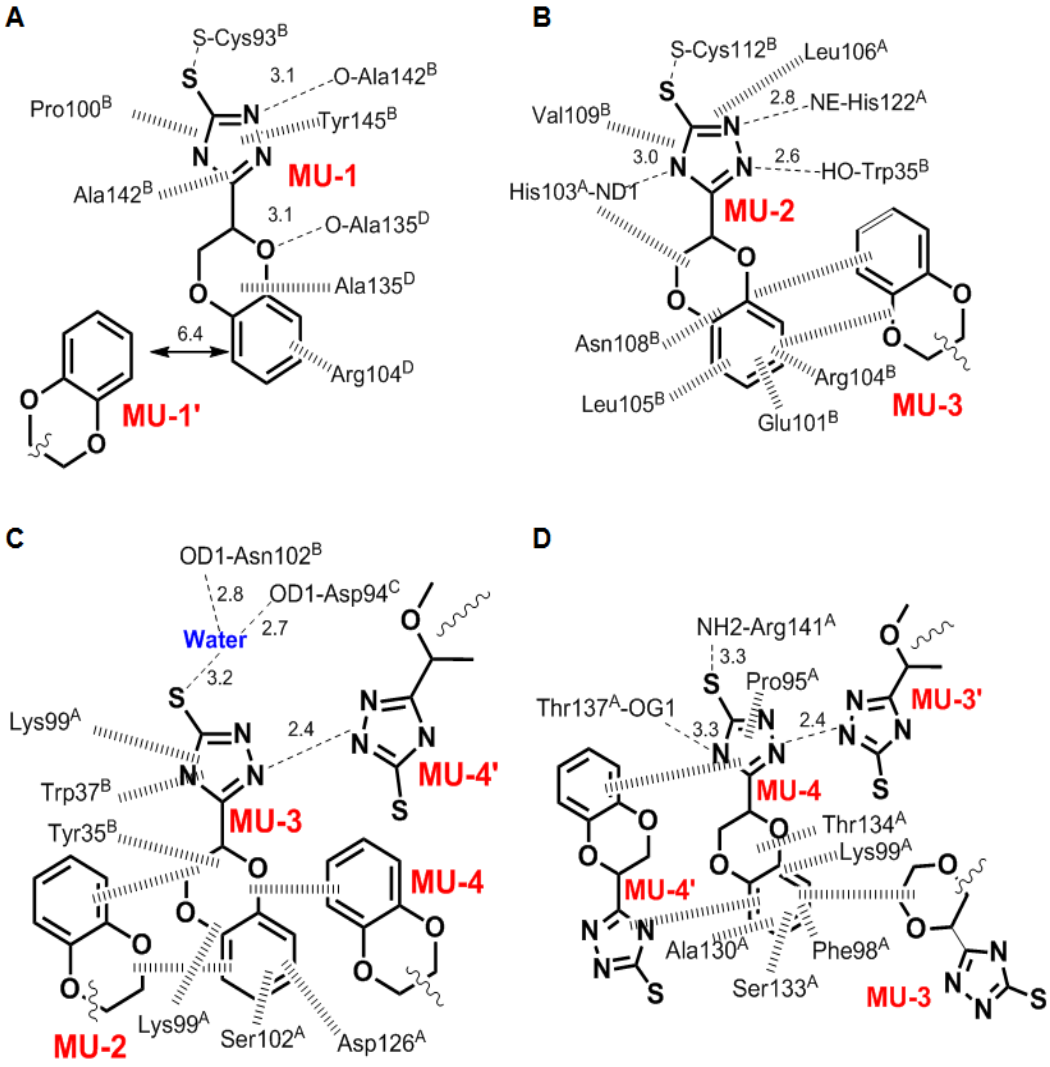


Figure S7. Two dimensional contacts between TD-1 as monomeric unit (MU) and COHb structure. Small dashed lines indicate hydrogen-bond interactions and broad dashed lines indicate hydrophobic contacts. Note that each of the MU molecules, with the exception of MU-1, makes contact with another MU molecule that ties all four subunits together.



References

1. Zijlstra, W. G., Buursma, A., and Assendelft, O. W. v. (2000) *Visible and near infrared absorption spectra of human and animal haemoglobin : determination and application*, VSP, Utrecht ;Boston.
2. Ronda, L., Bruno, S., Faggiano, S., Bettati, S., and Mozzarelli, A. (2008) Oxygen binding to heme proteins in solution, encapsulated in silica gels, and in the crystalline state, *Methods Enzymol. 437*, 311-328.
3. Rivetti, C., Mozzarelli, A., Rossi, G. L., Henry, E. R., and Eaton, W. A. (1993) Oxygen binding by single crystals of hemoglobin, *Biochemistry 32*, 2888-2906.
4. Bettati, S., and Mozzarelli, A. (1997) T state hemoglobin binds oxygen noncooperatively with allosteric effects of protons, inositol hexaphosphate, and chloride, *J. Biol. Chem. 272*, 32050-32055.
5. Casalena, D. E., Wassaf, D., and Koehler, A. N. (2012) Ligand discovery using small-molecule microarrays, *Methods Mol. Biol. 803*, 249-263.
6. Hayashi, A., Suzuki, T., and Shin, M. (1973) An enzymic reduction system for metmyoglobin and methemoglobin, and its application to functional studies of oxygen carriers, *Biochim. Biophys. Acta 310*, 309-316.
7. Abdulmalik, O., Safo, M. K., Chen, Q., Yang, J., Brugnara, C., Ohene-Frempong, K., Abraham, D. J., and Asakura, T. (2005) 5-hydroxymethyl-2-furfural modifies intracellular sickle haemoglobin and inhibits sickling of red blood cells, *Br. J. Haematol. 128*, 552-561.
8. Hijiya, N., Horiuchi, K., and Asakura, T. (1991) Morphology of sickle cells produced in solutions of varying osmolarities, *J. Clin. Lab. Med. 117*, 60-66.
9. Asakura, T., and Mayberry, J. (1984) Relationship between morphologic characteristics of sickle cells and method of deoxygenation, *J. Clin. Lab. Med. 104*, 987-994.
10. Horiuchi, K., Ohata, J., Hirano, Y., and Asakura, T. (1990) Morphologic studies of sickle erythrocytes by image analysis, *J. Clin. Lab. Med. 115*, 613-620.