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Development of Crosslinking Reagents, and Intra- and Intermolecular Multi-Linking Hemoglobin Molecules as Potential Blood Substitutes

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DEVELOPMENT OF CROSSLINKING REAGENTS, AND INTRA- AND INTERMOLECULAR MULTI-LINKING OF HEMOGLOBIN MOLECULES AS POTENTIAL BLOOD SUBSTITUTES

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To my late daughter:

Mary Zheng

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ABBREVIATIONS

AN	Acetonitrile
AS	Ammonium Sulfate
2,3-BPG (or BPG)	2,3-bisphosphoglycerate
DBCPA	Tris(3,5-dibromosalicyl) (3,5-dicarboxy phenoxy) acetate
DBEBE	Tetrakis(3,5-dibromosalicyl) 5,5'-triethyleneglycol dibenzoate ether
DBEIE	Tetrakis(3,5-dibromosalicyl) 5,5'-triethyleneglycol diisophthalate ether
DBPDB	Tetrakis(3,5-dibromosalicyl) 4,4'-(1,3-propanedioxy) dibenzoate
DBPDI	Tetrakis(3,5-dibromosalicyl) 5,5'-(1,3-propanedioxy) diisophthalate
DBSF	Bis(3,5-dibromosalicyl) fumarate
DBSTC	Tris(3,5-dibromosalicyl) tricarballylate
DBSTM	Tris(3,5-dibromosalicyl) trimesate
DCC	N,N'-dicyclohexylcarbodiimide
DI	De-ionized
DMF	N,N'-Dimethylformamide
DMSO	Dimethyl sulfoxide

FTIR	Fourier transform infrared
GC	Gas chromatography
Hb A	Hemoglobin A
Lys	Lysine
MOPS	4-(N-morpholino) propanesulfonic acid
NMR	Nuclear magnetic resonance
PBS	Phosphate buffer saline
PEG6000	Polyethylene glycol 6000
RBC	Red blood cell
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
SMPDI	Tetrakis(sodium methyl phosphate) 5,5'-(1,10-propanedioxy) diisophthalate
tBu	*Tert*-butyl group
TDBS	*Tert*-butyl 3,5-dibromosalicylate
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
Tris	Tris(hydroxymethyl) aminomethane hydrochloride
UV/VIS	Ultraviolet/visible
Val	Valine
XL	Crosslink
UNXL	Uncrosslink

ABSTRACT

Blood substitutes have been sought for several centuries and in many ways. Hemoglobin-based blood substitutes are the most interesting among those because of hemoglobin's unique structure and function to transport oxygen. The tetrameric hemoglobin in solution can dissociate into dimers which are readily removed by the kidney. To overcome the limitations to hemoglobin, it can be modified by biotechnology and/or chemistry. In chemistry, molecular modification requires crosslinking of hemoglobin to prevent formation of dimers. One of the most interesting small regions of the hemoglobin molecule is the β cleft of the BPG binding site. A series of different kinds of crosslinking reagents were synthesized and reacted only with Val1, Lys82 and Lys144 of the β-chain of hemoglobin. The negative charge from the phosphate or carboxyl groups of the reagents has an electrostatic interaction with positive charges from the residues in the BPG cleft.

For development of blood substitutes, a series of novel multi-(3,5-dibromosalicyl) linkers were designed and synthesized for modification of hemoglobin in this dissertation. In addition to electrostatic effects, selectivity

and efficiency can be enhanced by taking advantage of steric effects which play an important role in determining the regioselectivity of organic reagents. Computer modeling techniques were utilized to model 3-dimensional structures of hemoglobin A molecules and deoxyhemoglobin crystal unit cells, and design crosslinkers. In a manner similar to that used in a successful synthesis and purification of *tert*-butyl salicylate, *tert*-butyl 3,5-dibromosalicylate was prepared using DCC to couple *tert*-butyl alcohol and 3,5-dibromosalicylic acid. The crosslinking reagents successfully synthesized include: Two bis-, two tris- and two tetrakis(3,5-dibromosalicyl) reagents, and one tetrakis(sodium methyl phosphate) reagent.

Both oxy and deoxy hemoglobin were crosslinked with DBSTC. The denaturation transition (T_m) of the oxy crosslinked hemoglobin increased 14.5 °C and that of the deoxy crosslinked hemoglobin, 13.0 °C. The apparent rate constant (k_{app}) of autoxidation for the oxy crosslinked hemoglobin remained the same as native hemoglobin but that of the deoxy crosslinked hemoglobin increased by 34%. The higher oxygen affinity and lower cooperativity of the crosslinked proteins compared with native hemoglobin indicated that the crosslink shifted the conformation to the R state.

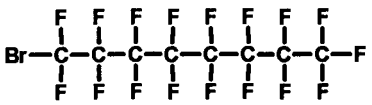
The major species from reaction of deoxyhemoglobin with DBCPA contained high yields of both bi- and tri-linked proteins. The denaturation transition (T_m) of both bi- and tri-linked hemoglobins increased 14.0 °C. The apparent rate constant (k_{app}) of autoxidation for the crosslinked hemoglobins

remained the same as native hemoglobin. The bi-linked species had a lower oxygen affinity and unchanged cooperativity compared with native hemoglobin, while the tri-linked proteins exhibited increased oxygen affinity and decreased cooperativity. SDS-PAGE analysis showed multi-bands in dimer range, indicating heterogeneous reaction of DBCPA with hemoglobin because of the asymmetrical structure of the reagent.

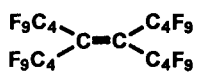
The elongated bi-linkers were designed to crosslink between Lys82β of two different hemoglobin tetramers. However, no octamers were isolated by gel filtration. The tetra-linkers were designed to crosslink between two hemoglobin tetramers with bi-linking of each hemoglobin between Lys82βs in one step. Besides dimeric bands, trimeric and tetrameric bands were shown by SDS-PAGE, corresponding to tri-linking and tetra-linking of hemoglobin with the tetra-linkers.

Chapter 1

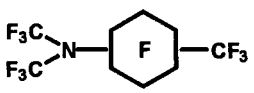
INTRODUCTION



F-acetylbromide

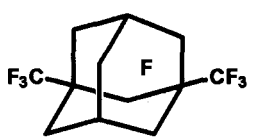


Bis(F-dibutyl) ethene



F-N,N-dimethylcyclo-hexylmethylamine

F-decalin



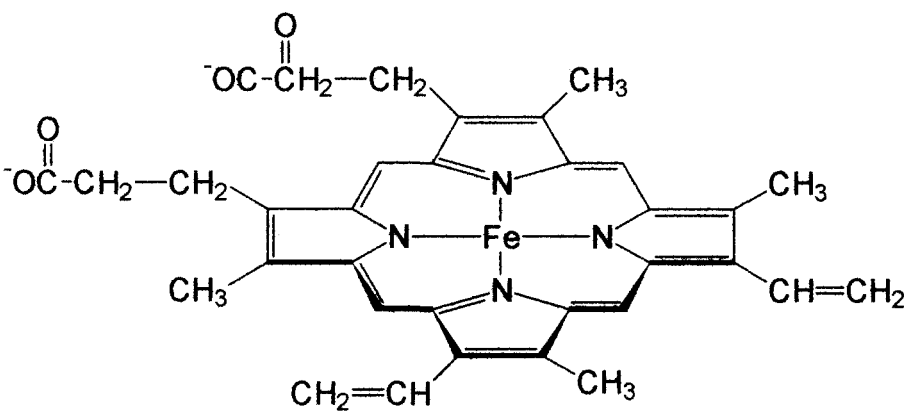
F-1,3-dimethyladamantane

The lack of donor blood and possible complications with transfusion blood have been accentuated recently because of problems related to AIDS. As a result, there is an increasing interest in developing blood substitutes for transfusions and other applications. Two major areas of blood substitutes have been developed: (1) the biotechnological approaches, using modified hemoglobin, synthetic heme, and other approaches, and (2) the chemical approaches, using perfluorochemicals. The emulsion of perfluorochemicals can dissolve oxygen up to 70% per unit volume [1-2]. Perfluorochemicals are not antigenic and their sizes are so small

comparing with that of RBC's that they could pass through occluded blood vessels. Unfortunately, perfluorochemicals accumulate in the liver and in the spleen to the point that these organs are unable to clear other foreign substitutes when they are transfused to patients [3]. Hemoglobin, because of its unique structure and function to transport oxygen, becomes the most interesting potential blood substitute [4-6].

Structure of Hemoglobin

Hemoglobin, the oxygen carrier of the blood, was the second protein whose structure was determined by X-ray crystallography [7, 8]. Myoglobin, the oxygen-storage protein of muscle was the first. Hemoglobin was also among



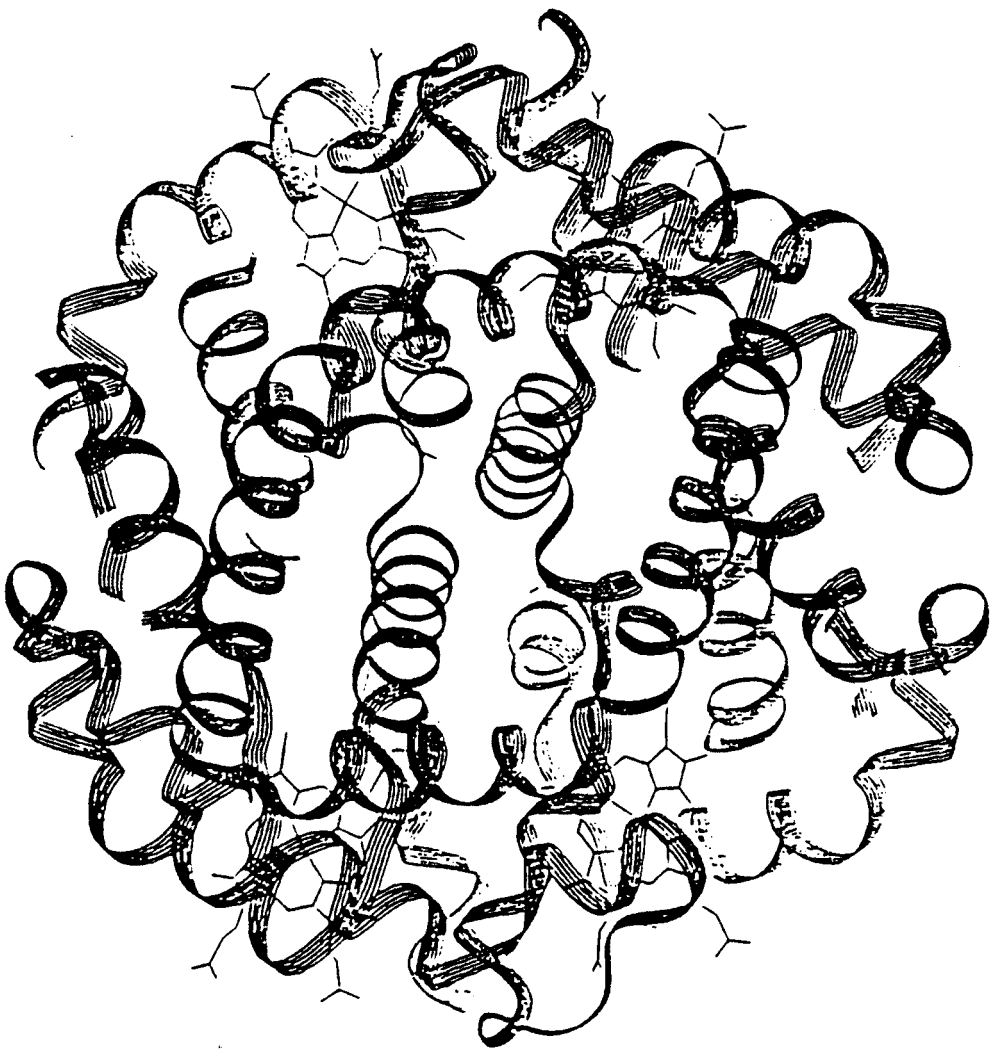
Heme (Fe-protoporphyrin IX)

the first to have its amino acid sequence determined. The protein is easily obtained in large quantities. Every milliliter of blood has approximately 5 billion erythrocytes or red cells, and each erythrocyte is packed with 280 million molecules of hemoglobin.

The capacity of hemoglobin to bind oxygen depends on the presence of a non-polypeptide unit, namely, a heme group. The heme also gives hemoglobin its distinctive red color. The heme consists of an organic part, protoporphyrin, and an iron atom. The iron atom in heme binds to the four nitrogens in the center of the protoporphyrin ring. The iron can form two additional bonds, one on either side of the heme plane. The iron atom can be in the ferrous (+2) or the ferric (+3) oxidation state, and the corresponding forms of hemoglobin are called ferrohemoglobin and ferrihemoglobin (also called methemoglobin). Only ferrohemoglobin, the +2 oxidation state, can bind oxygen.

Vertebrate hemoglobins consist of four polypeptide chains, two of one kind and two of another. The four chains are held together by noncovalent attractions. Each contains a heme group and a single oxygen-binding site. Hemoglobin A (**Figure 1**), the principal hemoglobin in adults, consists of two α chains with 141 amino acids and 15,126 molecular weight each, and two β chains with 146 residues and 15,867 molecular weight each. The heme adds another 616 daltons to each chain, so the total molecular weight of the adult hemoglobin tetramer is 64,450 daltons. The three-dimensional structures of myoglobin and the α and β chains of human hemoglobin are strikingly similar. About 75% of the main chain is in an α-helical conformation. The eight major helical segment, all right-handed, are referred to as A, B, C, ..., H. The first

Figure 1. Hemoglobin A in the deoxy form.

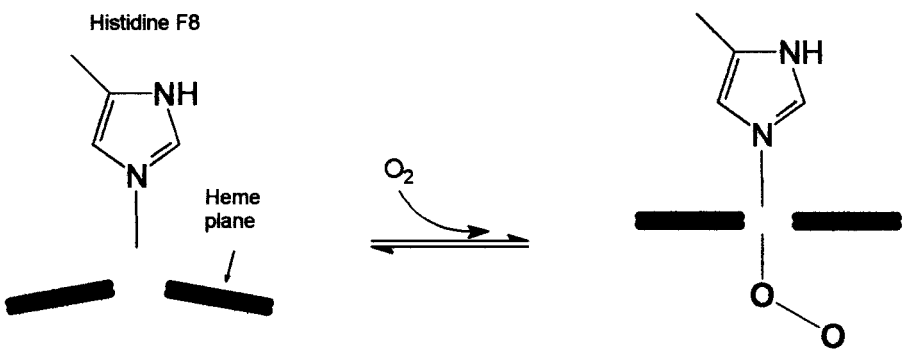


residue in helix A is designated A1, the second A2, and so forth. Adults also have a minor hemoglobin (~2% of the total hemoglobin) called hemoglobin A_2, which contains δ chains in place of the β chains of hemoglobin A. Thus, the subunit composition of hemoglobin A is $\alpha_2\beta_2$, and that of hemoglobin A_2 is $\alpha_2\delta_2$. The hemoglobin molecule is nearly spherical, with a diameter of 55 Å. The four chains are packed together in a tetrahedron. Each α chain is in contact with both β chains. In contrast, there are few interactions between the two α chains or between the two β chains.

The heme groups are located in crevices near the exterior of the molecule, one in each subunit. The four oxygen-binding sites are far apart; the distance between the two closest iron atoms is 25 Å. The highly polar propionate side chains of the heme are on the surface of a chain of hemoglobin. At physiological pH, these carboxylic acid groups are ionized. The rest of the heme is inside the chain of the hemoglobin, where it is surrounded by nonpolar residues except for two histidines. The iron atom of the heme is directly bonded to one of these histidines, namely, residue F8. This histidine, which occupies the fifth coordination position, is called the proximal histidine. The oxygen-binding site is on the other side of the heme plane, at the sixth coordination position. A second histidine residue (E7), termed the distal histidine, is near the heme but not bonded to it. In aqueous solution, oxygen very rapidly oxidizes a ferrous heme to ferric heme which can not bind oxygen. In hemoglobin, the heme groups are much less susceptible to oxidation because

two subunits or two hemoglobin molecules cannot readily associate to form a heme-O_2-heme complex. The heme is protected in a hydrophobic binding pocket.

The conformations of the three physiologically pertinent forms of hemoglobin — deoxy-, oxy- and ferri-forms (methemoglobin) — are very similar except at the sixth coordination position. In deoxyhemoglobin, it is empty; in oxyhemoglobin, it is occupied by O_2; in methemoglobin, it is occupied by water. The axis of the bound O_2 is at an angle to the iron-oxygen bond. The quaternary structure of deoxyhemoglobin is termed the T (tense or taut) form; that of oxyhemoglobin, the R (relaxed) form. In deoxyhemoglobin, the iron atom is

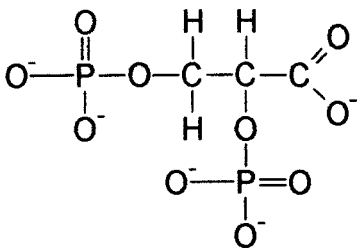


Oxygenation

about 0.4 Å out of the porphyrin plane toward the proximal histidine, so that the heme group is domed (convex) in the same direction. On oxygenation, the iron atom moves into the plane of the porphyrin to form a strong bond with O_2, and the heme becomes more planar. The iron atom pulls the proximal histidine with it when it moves into the plane of the porphyrin. This movement of histidine F8 shifts the F helix, the EF corner, and the FG corner. These conformational

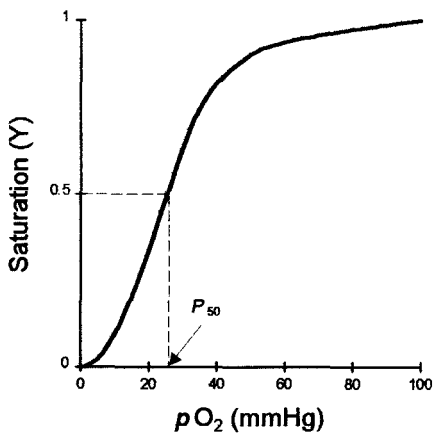
changes are in turn transmitted to the subunit interfaces where they rupture interchain salt links to switch the protein to the R form. Thus, a structural change (oxygenation) within a subunit is translated into structural changes at the interfaces between subunits. The binding of oxygen at one heme site is thereby communicated to parts of the molecule that are far away.

Oxygen-Binding of Hemoglobin



2,3-Bisphosphoglycerate (BPG)

Hemoglobin is an allosteric protein. The binding of O_2 to hemoglobin enhances the binding of additional O_2 to the same hemoglobin molecule [7, 8]. In other words, O_2 binds cooperatively to hemoglobin. The affinity of hemoglobin for oxygen depends on pH. The CO_2 molecule also affects the oxygen-binding characteristics of hemoglobin. Both H^+ and CO_2 promote the release of bound O_2. Reciprocally, O_2 promotes the release of bound H^+ and CO_2. The oxygen affinity of hemoglobin is further regulated by organic phosphates such as 2,3-bisphosphoglycerate (BPG) resulting in a low affinity for oxygen.



Oxygen dissociation curve of Hb

A plot of Y, the saturation, defined as the fractional occupancy of all the oxygen-binding sites in a solution, versus pO_2, the partial pressure of oxygen, is called an oxygen dissociation curve. Oxygen affinity can be characterized by a quantity P_{50}, which is the partial pressure of oxygen at which 50% of sites are filled (i.e., at which $Y = 0.5$). The oxygen dissociation curve of hemoglobin is sigmoidal, which agrees with the equation derived for the hypothetical equilibrium

$$Hb(O_2)_n \rightleftharpoons Hb + nO_2$$

This expression yields

$$Y = \frac{(pO_2)^n}{(pO_2)^n + (P_{50})^n}$$

which can be rearranged to give

$$\frac{Y}{1 - Y} = \left(\frac{pO_2}{P_{50}}\right)^n$$

This equation states that the ratio of oxyheme (Y) to deoxyheme ($1 - Y$) is equal to the nth power of the ratio of pO_2 to P_{50}. Taking the logarithms of both sides of the equation gives

$$\log \frac{Y}{1 - Y} = n \log pO_2 - n \log P_{50}$$

A plot of $\log [Y/(1 - Y)]$ versus $\log pO_2$, called a Hill plot, approximates a straight line. Its slope n at the midpoint of the binding ($Y = 0.5$) is called the Hill

coefficient. The value of *n* increases with the degree of cooperativity; the maximum possible value of *n* is equal to the number of binding sites.

The Hill coefficient of 2.8 for hemoglobin indicates that the binding of oxygen in hemoglobin is cooperative. Binding at one heme facilitates the binding of oxygen at the other hemes on the same tetramer. Conversely, the unloading of oxygen at one heme facilitates the unloading of oxygen at the others. In other words, the heme groups of a hemoglobin molecule communicate with each other. The cooperative binding of oxygen by hemoglobin enables it to deliver 1.83 times as much oxygen under typical physiological conditions as it would if the sites were independent.

The oxygen affinity of hemoglobin within red cells is lower than that of hemoglobin in free solution. BPG binds to hemoglobin and has a large effect on its affinity for oxygen. This highly anionic organic phosphate is present in human red cells at about the same molar concentration as hemoglobin. In the absence of BPG, the P_{50} of hemoglobin is 13-16 mmHg. In its presence, P_{50} became 26 mmHg. Thus, BPG lowers the oxygen affinity of hemoglobin by a factor of 2, which is essential in enabling hemoglobin to unload oxygen in tissue capillaries. BPG diminishes the oxygen affinity of hemoglobin by binding to deoxyhemoglobin but not to the oxygenated form. The binding site for BPG consists of multiple positively charged residues on each β chain: the α-amino group, His2, Lys82, and His143. These groups interact with the strongly negatively charged BPG which carries nearly four negative charges at

physiologic pH. On oxygenation, BPG is extruded because the central cavity becomes too small. Specifically, the gap between the H helices of the β chains becomes narrowed. Also, the distance between the α-amino groups increases from 16 to 20 Å, which prevents them from simultaneously binding the phosphates of a BPG molecule. The reason why BPG decreases oxygen affinity is now evident. BPG stabilizes the deoxyhemoglobin quaternary structure by non-covalently cross-linking the β chains. In other words, BPG shifts the equilibrium toward the T form. The carboxyl-terminal residues of deoxyhemoglobin form eight salt links that must be broken for oxygenation to occur. The binding of BPG contributes additional salt links that must be broken, and so the oxygen affinity of hemoglobin is diminished.

Denaturation of Hemoglobin

A hemoglobin molecule retains its biological activity only when it is in the native state. One of the requirements for a suitable blood substitute is its high thermal stability. The thermal stability of human hemoglobin is known to be fairly low and a tetrameric hemoglobin molecule is able to dissociate into its $\alpha\beta$ dimer subunits, even in mild solution conditions (e.g. neutral pH, low salt concentration, low temperature, etc.). Exposure to temperatures above 50 °C causes rapid (within minutes) irreversible denaturation with precipitation [9].

The analysis is based on a two-state model in which the protein is considered to be either in the native or denatured state [11-12]: N $\rightleftharpoons$ D,

where N and D represent the native state and the denatured state, respectively. This model assumes that the native protein structure unfolds in a cooperative manner: Any partial unfolding of the structure destabilizes the remaining structure, which must simultaneously collapse to the random coil [10]. The transition can be characterized at any stage by a single variable f_D, the fraction of molecules in the denatured state. If y represents the experimental variable being used to follow the transition, and y_N and y_D are the characteristic values of y for the initial and final states, we have the following equation:

$$y = y_N + f_D(y_D - y_N), \text{ or}$$

$$f_D = (y - y_N)/(y_D - y_N)$$

In the UV/Vis spectral method [13], absorbance A is used as the experimental variable. Therefore, the equation is converted to:

$$f_D = (A - A_N)/(A_D - A_N)$$

The thermal denaturation transition T_m can be obtained by the first-derivative method which calculates the slope of the absorbance versus temperature (dA/dT). The minima of the dA/dT versus temperature at which the absorbance is changing most rapidly are T_m's.

Autoxidation of Hemoglobin

A normal physiological function of hemoglobin is the reversible binding of oxygen which can only occur with the heme iron in the reduced state (Fe(II)). Although hemoglobin in red blood cells is slowly oxidized to methemoglobin

(Fe(III)) at a rate of about 3% per day, the level of methemoglobin in the body remains constant at 1% because whenever methemoglobin forms in red cells, it is readily reduced to ferrohemoglobin by an enzymatic system, methemoglobin reductase and NADH-cytochrome b_5 reductase [14-15]. Outside the erythrocytes, the amount of methemoglobin can not be minimized by enzymes. The oxidation of hemoglobin decreased significantly when anti-oxidant enzymes such as catalase and superoxide dismutase (SOD) were present with hemoglobin [16-17]. The autoxidation may result in hemichrome formation, protein denaturation, Heinz body formation and other changes [18-19].

The autoxidation rate of hemoglobin is influenced by many factors, such as pH value, oxygen pressure, temperature, and BPG concentration [20-22]. The fact that the autoxidation reaction is favored by BPG or inositol hexaphosphate (IHB) suggests that deoxyhemoglobin is faster to autoxidize than oxyhemoglobin.

The oxyhemoglobin and methemoglobin concentrations (mM) can be calculated using the following equations:

$$[\text{Oxyhemoglobin}] = 66A_{577} - 80A_{630}$$

$$[\text{Methemoglobin}] = 279A_{630} - 3A_{577}$$

However, in most cases, the reaction is more complicated. The autoxidation can cause other changes, such as the formation of hemichrome which is a common intermediate in various types of hemoglobin denaturation [23-25] or generation of other denatured derivatives, e. g., choleglobin. Subtracting A_{700} -

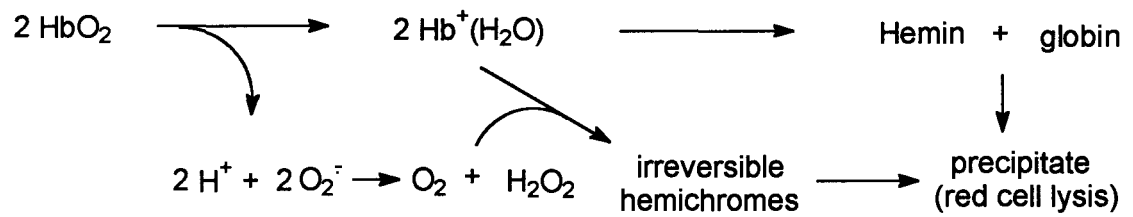
0.005, as the contribution of choleglobin, from each of the other absorbances, the concentrations of oxyhemoglobin, methemoglobin and hemichrome can be calculated using these equations:

$$[\text{Oxyhemoglobin}] = 119A_{577} - 39A_{630} - 89A_{560}$$

$$[\text{Methemoglobin}] = 28A_{577} + 307A_{630} - 55A_{560}$$

$$[\text{Hemichrome}] = -133A_{577} - 114A_{630} + 233A_{560}$$

The mechanism of the autoxidation reaction is a complex process. It is found that the autoxidation is a pseudo-first order reaction dependent upon the concentration of hemoglobin, ligand and period of time [26]. The most plausible reaction route for the autoxidation is described as following [27]:



The apparant rate constant of hemoglobin autoxidation, k_{app}, can be obtained by calculation of the slope of the logarithms of oxyhemoglobin concentration as a function of time. The half life time of oxyhemoglobin in autoxidation, $t_{1/2}$, is calculated as following:

$$t_{1/2} = \ln 2 / k_{app}$$

Hemoglobin-Based Blood Substitutes [28-29]

Hemoglobin in solution has the capability to transport oxygen and, theoretically, could be used as a substitutes for red cells. Since hemoglobin solutions are oncotically active, they can also expand plasma volume. These properties would be desirable for a resuscitation fluid when rapid initial treatment of hypovolemia and tissue hypoxia is required. As a resuscitation fluid, hemoglobin must be able to maintain tissue oxygenation for a specified period of time. There are no well-defined physiological criteria to help quantify the effects of an oxygen-transporting hemoglobin. Ideally, hemoglobin solution should have the same capability to maintain tissue oxygenation as blood.

The concept of using hemoglobin as a blood substitute is not new [30] but renal toxicity limited its development. In recent years, the interest in the development and evaluation of hemoglobin solutions as a blood substitutes has intensified [31]. With increasing knowledge and better understanding of hemoglobin, a clearer picture has been achieved concerning the capabilities and limitations of this potential oxygen-transporting fluid, as well as the manipulations that may overcome these limitations.

Some of the limitations of unmodified hemoglobin solution are easily defined. (1) Hemoglobin transports oxygen as a ligand that requires heme and globin. The reversible binding of oxygen requires interaction between four chains of hemoglobin, i.e. cooperativity, which results from the ability of the protein to exist as two different quaternary structure (relaxed and tense) that

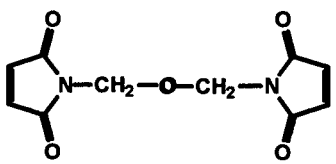
have different oxygen affinities [32]. Cooperativity allows hemoglobin to on-load oxygen when the oxygen tension is high (approximately 100 mmHg pO_2) and to off-load oxygen when the oxygen tension is low (approximately 40 mmHg pO_2) and gives rise to the sigmoidal shape of the oxygen-hemoglobin dissociation curve. Hemoglobin in solution has a higher oxygen affinity (lower P_{50}) than intraerythrocytic hemoglobin [33]. The tense state of hemoglobin in red cells is stabilized by the presence of organic phosphates such as BPG [34]; the tense state of hemoglobin is not stabilized since 2,3-BPG is absent and, therefore, hemoglobin in solution has a lower P_{50} (13-16 mmHg). (2) Aqueous hemoglobin exists in equilibrium between the tetrameric (MW 64,000) and dimeric (MW 32,000) forms [35]. The dimers are readily excreted by the kidneys and results in rapid intravascular elimination of hemoglobin solution (2-4 hour plasma half-disappearance). (3) Since hemoglobin is a colloidal-active protein, it in solution exerts a colloid oncotic pressure which limits it to a lower concentration (7 g/dL) of hemoglobin compared to the intra-erythrocytic concentration (30 g/dL) [36].

To overcome the limitations inherent to hemoglobin, it can be modified by biotechnology [37-38] and/or chemistry. In chemistry, two basic approaches have been used: (1) Molecular modification; (2) Environmental modification. Molecular modification requires the stabilization of hemoglobin to prevent dimer formation and maintenance of the tense conformational state. To do this, hemoglobin has to be crosslinked and this topic is discussed in detail in the next section on Crosslinking of Hemoglobin. Environmental modification requires

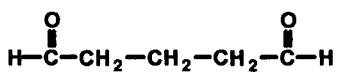
hemoglobin encapsulation to simulate erythrocytes. If hemoglobin and BPG could be encapsulated, then hemoglobin could be maintained in the tense conformational state. If the encapsulation material was biologically similar to erythrocytes, then longer intravascular retention than unmodified hemoglobin would occur. Encapsulation of hemoglobin was shown by Chang [39-43] to be feasible but the physicochemical properties of capsules did not allow the material to circulate. The studies by Djordjevich and Miller [44] and Hauser et al. [45] suggested that liposomal micelles may provide physicochemical properties that are biologically acceptable.

Crosslinking of Hemoglobin

There have been three approaches to crosslinking: (1) intermolecular (between two or more hemoglobins), (2) intramolecular (within one hemoglobin tetramer) and, (3) hemoglobin-macromolecular (hemoglobin to a non-excretable macromolecule, such as dextran [46-49] and polyethylene glycol [50-51]).



Bis(N-maleimidomethyl) ether

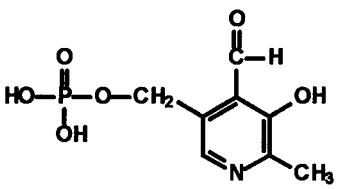


Glutaraldehyde

Crosslinking hemoglobin was shown, by Bunn and Jandl [35], to reduce renal elimination and increase intravascular retention. The reagent they used, bis(N-maleimidomethyl) ether, did allow conformational changes; their derivative had a high oxygen affinity (P_{50} = 3 mmHg). Most of the

crosslinking reagents used have reacted with amino groups of hemoglobin. Since the $\alpha\beta$ dimer has 22 primary amino groups which can react, the functional group specificity is insufficient to give homogeneity. Thus, using glutaraldehyde [52-57] or imidoesters [58] to crosslink hemoglobin produces heterogeneous products.

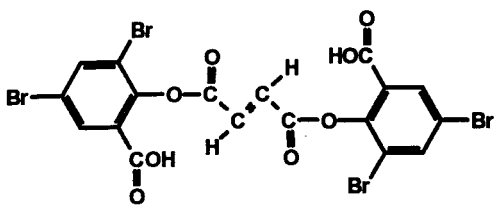
Benesch et al [59] showed that pyridoxal 5-phosphate had an analogous effect to BPG to lower affinity (P_{50} = 26-30 mmHg); it binds covalently to hemoglobin, unlike BPG, but does not act as a crosslinking reagent. The discovery of a specific hemoglobin crosslinker by Ruth and Reinhold Benesch [60] led to the description of a variety of modifications of hemoglobin which not only reduce their oxygen affinity but also stabilize the tetrameric structure so that their vascular retention can be prolonged. In 1975, Benesch et al. [61] used 2-nor-2-formylpyridoxal-5'-phosphate (NFPLP) to react with deoxyhemoglobin and, after reduction with sodium borohydride, to form a covalent bridge between β_1Val1 and β_2Lys82. The negative charge from the phosphate group of the pyridoxal compound has an electrostatic interaction with positive charges from the amino acid residues in the BPG cleft. This effect stabilized the T state which mimics closely the effect of BPG on hemoglobin [62-64]. In 1988, Benesch and Kwong [65-66] also developed a new class of bis-pyridoxal polyphosphate compounds



Pyridoxal 5-phosphate

to crosslink deoxyhemoglobin between the amino terminus of a β-chain and the lysine 82 of the other β-chain, as in the case using NFPLP.

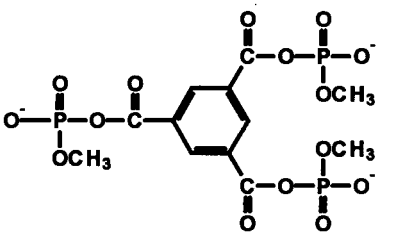
Klotz and his co-workers synthesized a series of diaspirin compounds, such as bis(3,5-dibromosalicyl) fumarate (DBSF), succinate (DBSS), glutarate (DBSG) and adipate (DBSA) during 1975 and 1979 [67-68]. The dibromosalicyl ether compounds are more specific in their reactions. Walder and his co-workers showed that DBSF reacts with oxyhemoglobin to crosslink between β_1Lys82 and β_2Lys82 [69]. However, DBSF crosslinks between the two Lys99 of the α chains when it reacts with deoxyhemoglobin [70]. White, Yang and Olsen [71-72] used DBSF to stabilize hemoglobin thermally. Huang and Olsen [73] used DBSS and DBSG to crosslink met-, oxy- and deoxyhemoglobin. The added flexibility of these reagents results in a more heterogeneous product but does not greatly affect the maximum thermal stability of the crosslinked hemoglobins.



Bis(3,5-dibromosalicyl) fumarate

Kluger et al. [74-77] synthesized a series of bis methyl acetyl phosphate (MAP) acetylation agents, and showed that the reagents only reacts with Val1, Lys82 and Lys144 of the β chains.

Kluger et al. synthesized two tri-linkers: trimesoyl tris(3,5-dibromosalicylate) (TTDS) [78] whose reaction with deoxyhemoglobin is highly selective for the βLys82 residues and the third active ester group reacts much more slowly, principally undergoing hydrolysis; and trimesoyl tris(methyl phosphate) (TTMP) [79] which is selective to react between β_1Val1 and β_2Lys82. The major species is tri-linked between Val1β_1, Lys82β_1 and Lys82β_2, and both tri- and bi-linked species produced from deoxyhemoglobin have a considerably lower oxygen affinity than does native hemoglobin while maintaining a high degree of cooperativity.



Trimesoyl tris(methyl phosphate)

Work of the Dissertation

For development of blood substitutes, a series of novel aspirin multi-linkers were designed and synthesized for modification of hemoglobin in this dissertation. Anionic acylating reagents show selectivity for certain amino groups within hemoglobin, particularly those located in the site which binds the polyanionic regulator BPG. In addition to electrostatic effects, selectivity and efficiency can be enhanced by taking advantage of steric effects which play an important role in determining the regioselectivity of organic reagents. Such effects can increase the selectivity of a protein reagent. According to this principle, computer modeling techniques [78] were utilized to model 3-

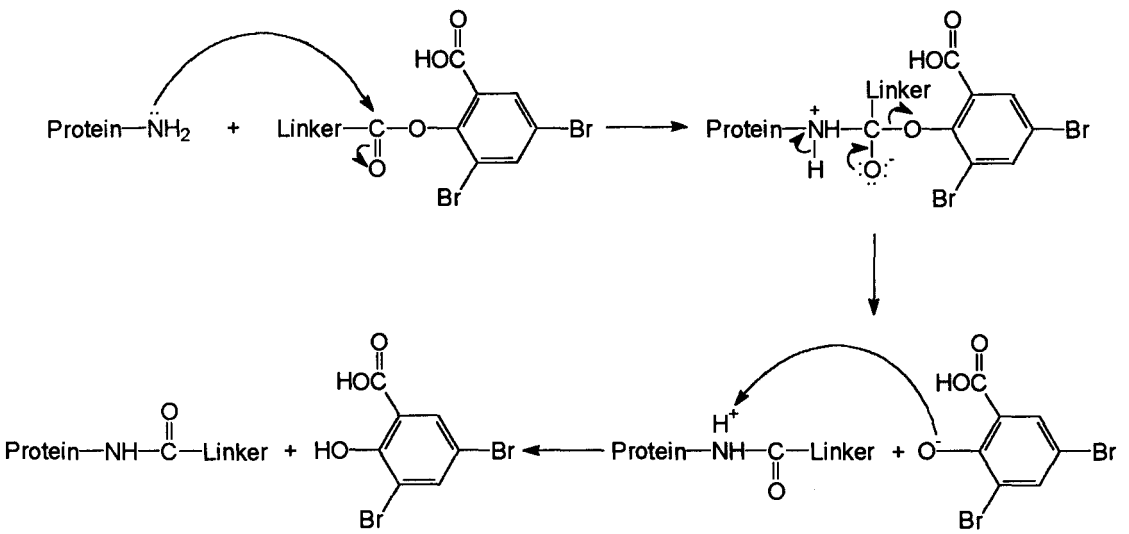
dimensional structures of hemoglobin A molecules and hemoglobin crystal unit cells, measure distances between amino groups of lysine or other residues, indicate possible sites of crosslinking reactions and design the reagents with sizes, shapes and polarities that fit the specific reactive sites in hemoglobin A.

Bulky multifunctional reagents possess the selectivity for reaction on specific-sites in a protein due to steric effects and electrostatic interaction. However, the reaction of 3,5-dibromosalicylic acid with aromatic carboxylic acid chlorides, or with acid chlorides expected to form bulky reagents, gives a very low yield of the desired products in the presence of a tertiary amine as the catalyst. The method developed by Klotz involving protection of the carboxyl group of 3,5-dibromosalicylic acid as the *tert*-butyl ester, coupling, and deprotection [81], was used successfully to produce a good yield of the desired products. However, the preparation of *tert*-butyl 3,5-dibromosalicylate has not been mentioned in any related publication. Based on the successful synthesis and purification of *tert*-butyl salicylate, *tert*-butyl 3,5-dibromosalicylate was made using N, N'-dicyclohexylcarbodiimide (DCC) to couple *tert*-butyl alcohol and 3, 5-dibromosalicylic acid.

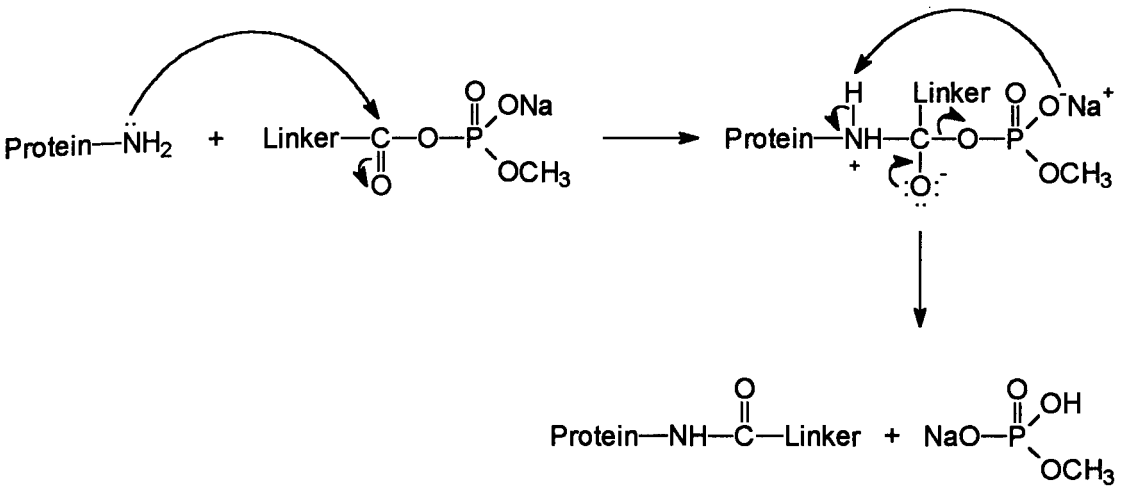
The reagents successfully synthesized for reaction with hemoglobin in the BPD binding site include: bis(3,5-dibromosalicyl) 4,4'-(1,3-propanedioxy) dibenzoate, bis(3,5-dibromosalicyl) 4,4'-triethyleneglycol dibenzoate ether, tris(3,5-dibromosalicyl) tricarballylate, tris(3,5-dibromosalicyl) (3,5-dicarboxy phenoxy) acetate, tetrakis(3,5-dibromosalicyl) 5,5'-(1,3-propanedioxy)

diisophthalate, tetrakis(3,5-dibromosalicyl) 5,5'-triethyleneglycol diisophthalate ether and tetrakis(methyl phosphate) 5,5'-(1,10-decanedioxy) diisophthalate. The tri-linkers were designed to get multi-linking of intrahemoglobin and the long bi- and tetra-linkers to crosslink interhemoglobins.

The reaction of hemoglobin with the tri-linkers gave both double and tri-linking as major products. Double-, tri- and tetra-crosslinking were found in reaction of hemoglobin with the tetra-linkers. The mechanism of crosslinking reaction of proteins with an aspirin crosslinker is as follows:



and that of crosslinking reaction of proteins with a methyl phosphate crosslinker as follows:



We also studied crosslinking in hemoglobin crystals. Oxygen-binding, denaturation and autoxidation of isolated crosslinked hemoglobins were measured and compared with those of native hemoglobin.

Chapter 2

MATERIALS AND EXPERIMENTS

Chemicals and Other Materials

Methanol, ethanol, tetrahydrofuran (THF), acetone, diethyl ether, hexane, *tert*-butyl alcohol, 3,5-dibromosalicylic acid, 1.3-dicyclohexylcarbodiimide (DCC), bromoacetic acid, 1,3-dibromopropane, 1,2-bis(2-iodoethoxy) ethane, 4-hydroxybenzoic acid, 5-hydroxyisophthalic acid, tricarballylic acid, trimethyl phosphate, thionyl chloride, N,N-dimethylformamide (DMF), trifluoroacetic acid (TFA), glacial acetic acid, silica gel, chloroform-d_3 ($CDCl_3$), dimethyl sulfoxide-d_6 (DMSO-d_6) were purchased from Aldrich Chemical Co., Inc.; inorganic chemicals: anhydrous magnesium sulfate, anhydrous sodium sulfate, calcium hydride, potassium metal, hydrochloric acid, sodium iodide from Stansi Scientific Co.; DEAE-Sephadex A-50 gel, SDS homogeneous gel, SDS low molecular weight markers, SDS buffer strips, Blue R, from Pharmacia Fine Chemicals; tris(hydroxymethyl aminomethane hydrochloride (Tris), 4-morpholinepropane

sulfonic acid (MOPS), Sephadex G-25 gel, from Sigma Chemical Co.; sodium cyanide, ammonium sulfate, urea, sodium phosphate, sodium hydroxide from J. T. Baker Chemical Co.; potassium ferricyanide, sodium chloride from Mallinckrodt, Inc.; guanidine hydrochloride, sodium dodecylsulfate, from Pierce Chemical Co.; β-mercaptoethanol from CalBiochem.; alkaline agarose gels from Corning Medical; O_2 Probe solution, electrode membrane from YSI Incorporated; TLC silica gel plates, filter paper from Whatman Ltd..

Instruments

Crosslinkers were synthesized using an electrical heating mantle, magnetic stirrer and BÜCHI rotary-evaporator. Identification of synthetic organic compounds was ascertained by recording NMR spectra using a Varian VXR 300 MHz NMR Spectrometer, infrared spectra using an ATI Mattson FTIR, and melting points were determined using a Mel-TEMP. Preparation of hemoglobin was carried out using a Sorvall RC-5B Refrigerated Superspeed Centrifuge, hemoglobin A and hemoglobin A_2 were separated through a Sephadex A-50 ion-exchange column with an ISCO UA-5 absorbance/fluorescence detector, ISCO Tris pump and ISCO fraction collector, and purity of hemoglobin was determined using Cornning ACI clinical gel. Reaction of hemoglobin with crosslinking reagents was carried out in water bath. Concentration, denaturation and autoxidation of native hemoglobin and crosslinked hemoglobins were tested

using a Hewlett-Packard (HP) 8452A UV/VIS Diode Array Spectrophotometer with a PC computer Model 386, Endocal Refrigerated Circulating Bath RTE-9, DCR-4 Digital Controller/Readout and Omega temperature indicator. Oxygen-binding of blood, hemoglobin and crosslinked hemoglobins was conducted using a Hemox-analyzer Model B with a plotter. Crosslinked hemoglobins were analyzed using a Pharmacia SDS Slab Gel Electrophoresis Unit SE250 with an ISCO Gel Scanner Model 1312. Buffer solutions were prepared using an Orion research digital pH meter. Hemoglobin was concentrated using an Amicon Microconcentrator. Computer modeling was done using an Iris Indigo Silicon Graphics with an Insight II/Discover software from Biosym.

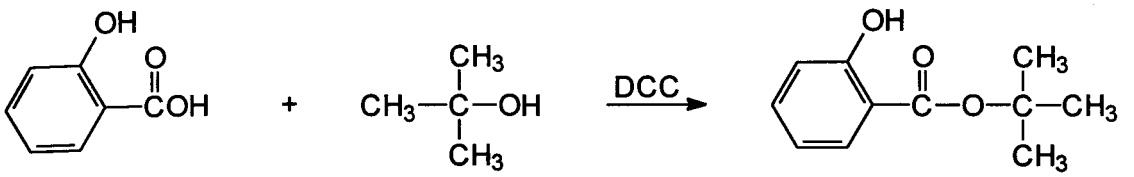
Experiments

Synthesis of *tert*-butyl salicylate (see **Scheme I**)

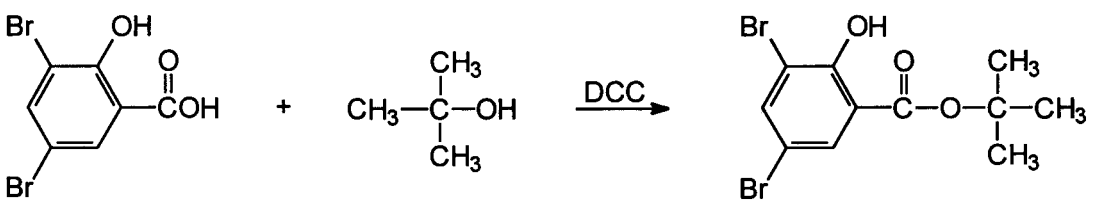
2.10 g (0.01 mol) of 1,3-dicyclohexylcarbodiimide (DCC) dissolved in 5 mL of *tert*-butyl alcohol was added slowly into the solution of 1.38 g (0.01 mol) of salicylic acid in 5 mL of *tert*-butyl alcohol. The reaction mixture was stirred at room temperature for 3 h. The white precipitate (dicyclohexylurea, DCU) was removed by suction filtration. The filtrate was concentrated by a rotary-evaporator. The residue was separated by a silica gel column with methylene chloride as eluent. The product obtained was a liquid with a strong fruity odor.

Synthesis of *tert*-butyl 3,3-dibromosalicylate (see **Scheme II**)

Scheme I.



Scheme II.



***Tert*-butyl 3,5-dibromosalicylate (TDBS)**

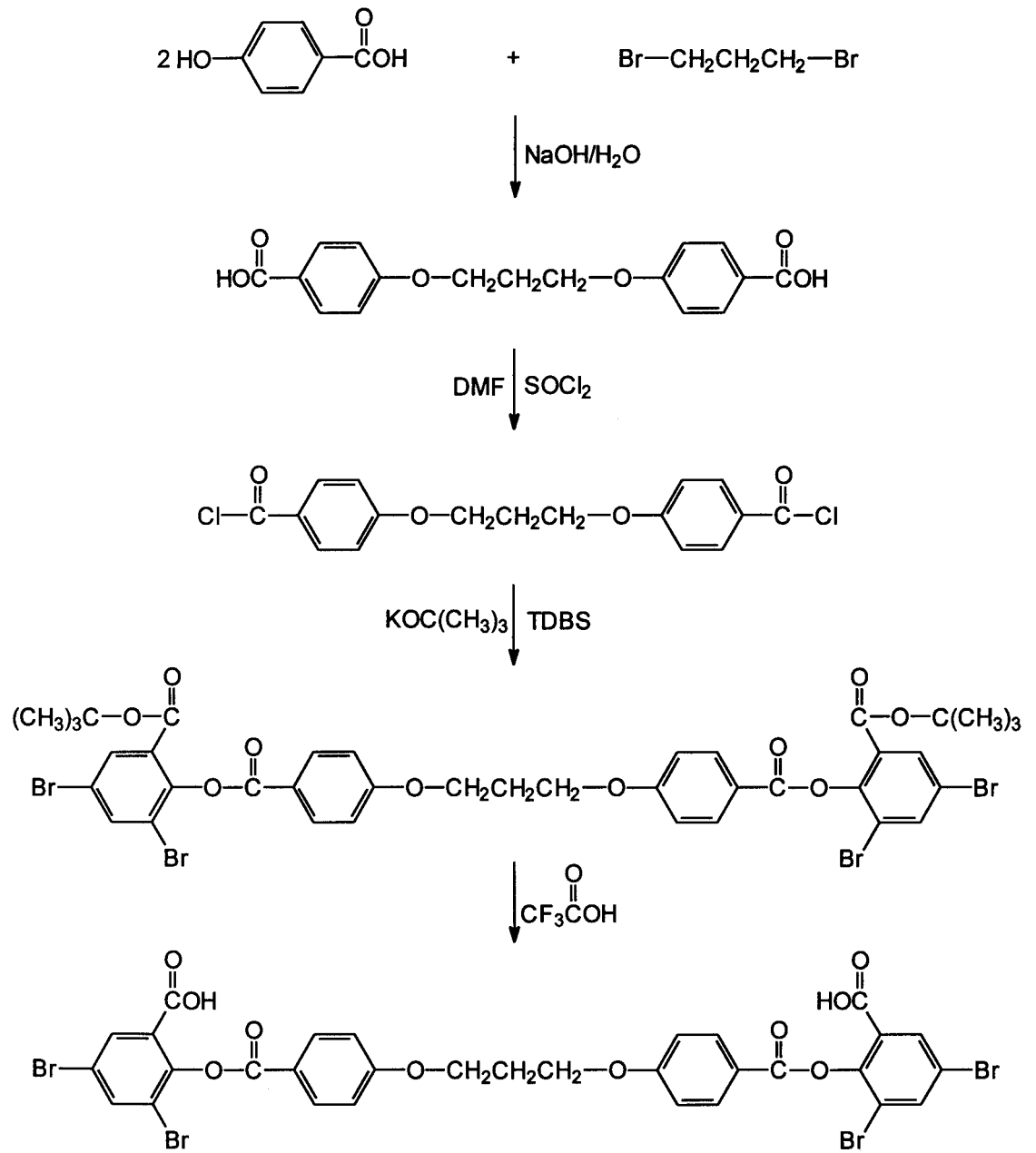
5.1 g (0.025 mol) of DCC in 50 mL of *tert*-butyl alcohol was added into the solution of 5.9 g (0.02 mol) of 1,3-dibromosalicylic acid in 50 mL of *tert*-butyl alcohol. The reaction mixture was stirred at room temperature for 5 h. The white precipitate (DCU) was filtered off by suction and the excess *tert*-butyl alcohol was removed by a rotary-evaporator. The residue was recrystallized from ethanol and 4.5 g of white needle crystals were obtained (yield: 63.9%).

Synthesis of bis(3,5-dibromosalicyl) 4,4'-(1,3-propanedioxy) dibenzoate
(see **Scheme III**)

4,4'-(1,3-Propanedioxy) dibenzoic acid. 2.02 g (0.01 mol) of 1,3-dibromopropane dissolved in 15 mL of methanol was added in the solution of 2.76 g (0.02 mol) of 4-hydroxybenzoic acid and 2.0 g (0.05 mol) of sodium hydroxide in 15 mL of water. The reaction mixture was refluxed overnight to get a homogeneous solution. The methanol was removed by a rotary-evaporator. The remaining aqueous solution was filtered and acidified with 6 N hydrochloric acid. The precipitate was collected by suction filtration and washed with water. 1.66 g of the product was obtained (yield: 52.5%).

4,4'-(1,3-Propanedioxy) dibenzoyl chloride. 0.5 g (1.58 mmol) of 4,4'-(1,3-propanedioxy) dibenzoic acid and then 1 drop of dimethylformamide (DMF) were added into 5 mL of thionyl chloride (distilled). The mixture was refluxed overnight with stirring and a drying trap. The excess thionyl chloride was removed by distillation and the residue was recrystallized from hexane to get white crystals (yield: 45.3%).

Scheme III.


2 HO
COH
+
Br—CH2CH2CH2—Br
NaOH/H2O
HOC
O—CH2CH2CH2—O
COH
DMF
SOCl2
Cl—C
O—CH2CH2CH2—O
C—Cl
KOC(CH3)3
TDBS
(CH3)3C—O—C
C—O—C(CH3)3
Br
CF3COH
COH
HOC

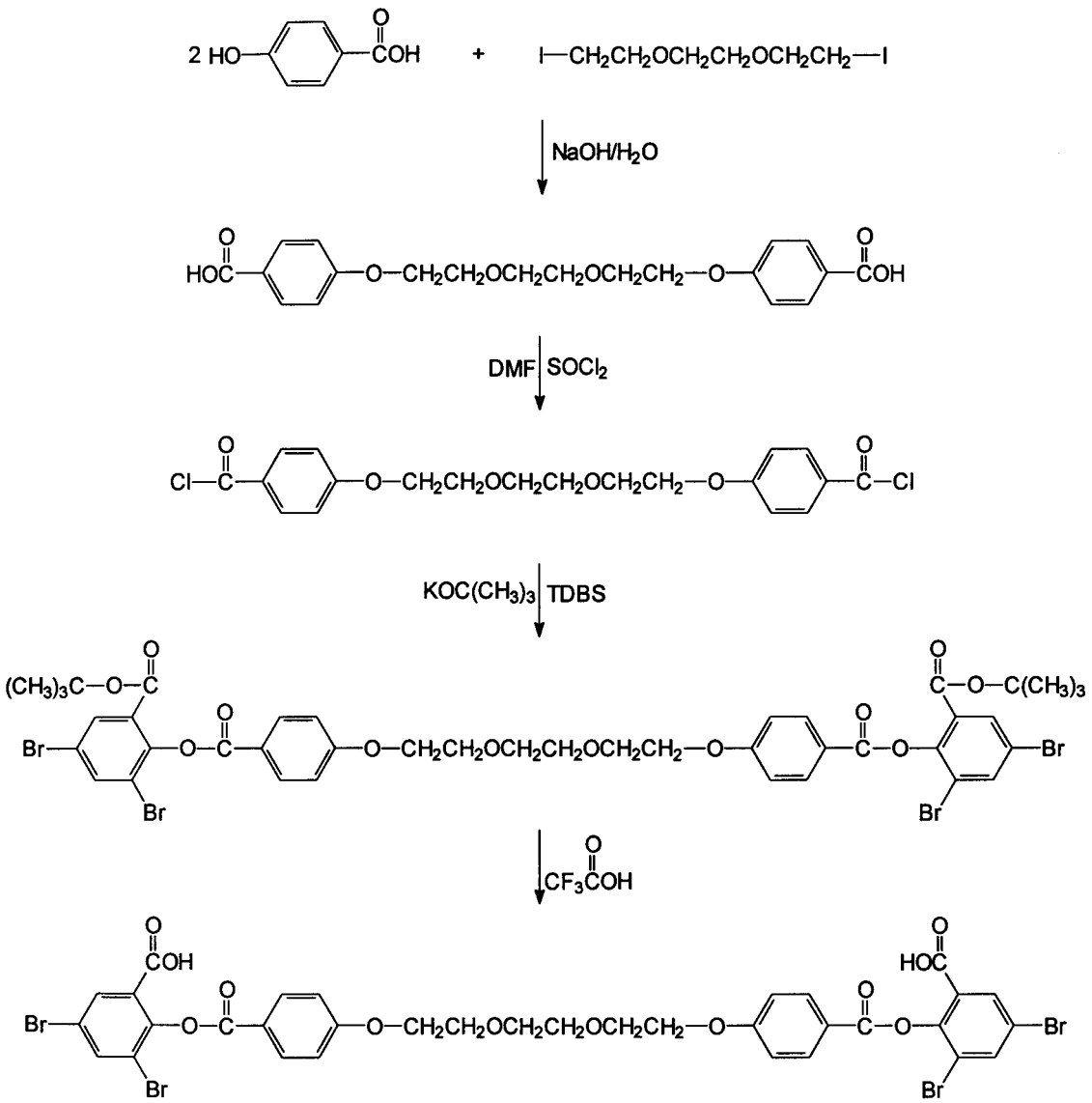
Bis(*tert*-butyl 3,5-dibromosalicylate) 4,4'(1,3-propanedioxy) dibenzoate. 1.10 g (3.12 mmol) of *tert*-butyl 3,5-dibromosalicylate and 0.35 g (3.12 mmol) of potassium *tert*-butoxide were stirred in 30 mL of tetrahydrofuran (THF) (distilled over calcium hydride) for 30 min at room temperature. 0.61 g (1.58 mmol) of 4,4'-(1,3-propanedioxy) dibenzoyl chloride dissolved in 10 mL of THF was added into the mixture and stirred overnight at room temperature. The THF was removed by a rotary-evaporator. Diethyl ether was added for extraction and the ether was subsequently washed with water. The organic layer was dried over anhydrous Na_2SO_4 and concentrated by a rotary-evaporator. The residue was recrystallized from ethanol (yield: 51.2%).

Bis(3,5-dibromosalicyl) 4,4'-(1,3-propanedioxy) dibenzoate. 0.10 g of bis(*tert*-butyl 3,5-dibromosalicylate) 4,4'-(1,3-propanedioxy) dibenzoate was dissolved in 2 mL of trifluoroacetic acid (distilled) and kept in an ice bath for 1 h. 2 mL of diethyl ether (distilled over CaH_2) was added in the solution and the mixture was kept at 4°C overnight. The precipitate was filtered and dried by suction and 0.05 g of white powder product was obtained (yield: 58.1%).

Synthesis of bis(3,5-dibromosalicyl) 4,4'-triethyleneglycol dibenzoate ether (see **Scheme IV**)

4,4'-Triethyleneglycol dibenzoic acid ether. 3.07 g (0.01 mol) of 1,2-bis(2-iodoethoxy) ethane in 10 mL of methanol were added into the solution of 2.76 g (0.02 mol) of 4-hydroxybenzoic acid and 2.0 g (0.05 mol) sodium hydroxide dissolved in 10 mL of water. The reaction mixture was refluxed overnight to get a

Scheme IV.


2 HO
COH
+
I—CH2CH2OCH2CH2OCH2CH2—I
NaOH/H2O
HOC
O—CH2CH2OCH2CH2OCH2CH2—O
COH
DMF
SOCl2
Cl—C
O—CH2CH2OCH2CH2OCH2CH2—O
C—Cl
KOC(CH3)3
TDBS
(CH3)3C—O—C
C—O—C(CH3)3
Br
CF3COH
COH
HOC

homogeneous solution. The methanol was removed by a rotary-evaporator. The remaining aqueous solution was filtered and acidified with 6 N hydrochloric acid. The precipitate was collected by suction filtration and washed with water. 1.60 g of product was obtained (yield: 41.0%).

4,4'-Triethyleneglycol dibenzoyl ether chloride. 0.3 g (0.768 mmol) of 4,4'-triethyleneglycol dibenzoic acid ether and 1 drop of DMF were added in 4 mL of thionyl chloride (distilled). The mixture was refluxed overnight with stirring and a drying trap. The excess thionyl chloride was removed by distillation and the residue was dried in a fume hood (yield: 43.5%).

Bis(*tert*-butyl 3,5-dibromosalicylate) 4,4'-triethyleneglycol dibenzoate ether. 0.49 g (1.39 mmol) of *tert*-butyl 3,5-dibromosalicylate and 0.15 g (1.34 mmol) of potassium *tert*-butoxide in 8 mL of THF (distilled over calcium hydride) were stirred for 30 min at room temperature. 0.298 g (0.65 mmol) of 4,4'-triethyleneglycol dibenzoyl chloride ether in 10 mL of THF was added into the mixture and stirred overnight at room temperature. The THF was removed by a rotary-evaporator. Diethyl ether was added to extract and the ether layer was subsequently washed with water. The organic layer was dried over anhydrous Na_2SO_4 and concentrated by a rotary-evaporator. The residue was recrystallized from ethanol (yield: 47.9%).

Bis(3,5-dibromosalicyl) 4,4'-triethyleneglycol dibenzoate ether. 0.1 g of bis(*tert*-butyl 3,5-dibromosalicyl) 4,4'-triethyleneglycol dibenzoate ether was dissolved in 2 mL of trifluoroacetic acid and kept in an ice bath for 1 h. 2 mL of diethyl ether was added to the solution and the mixture was kept at 4 °C overnight. The precipitate

was filtered and dried by suction and 0.04 g of white powder was obtained (yield: 45.8%).

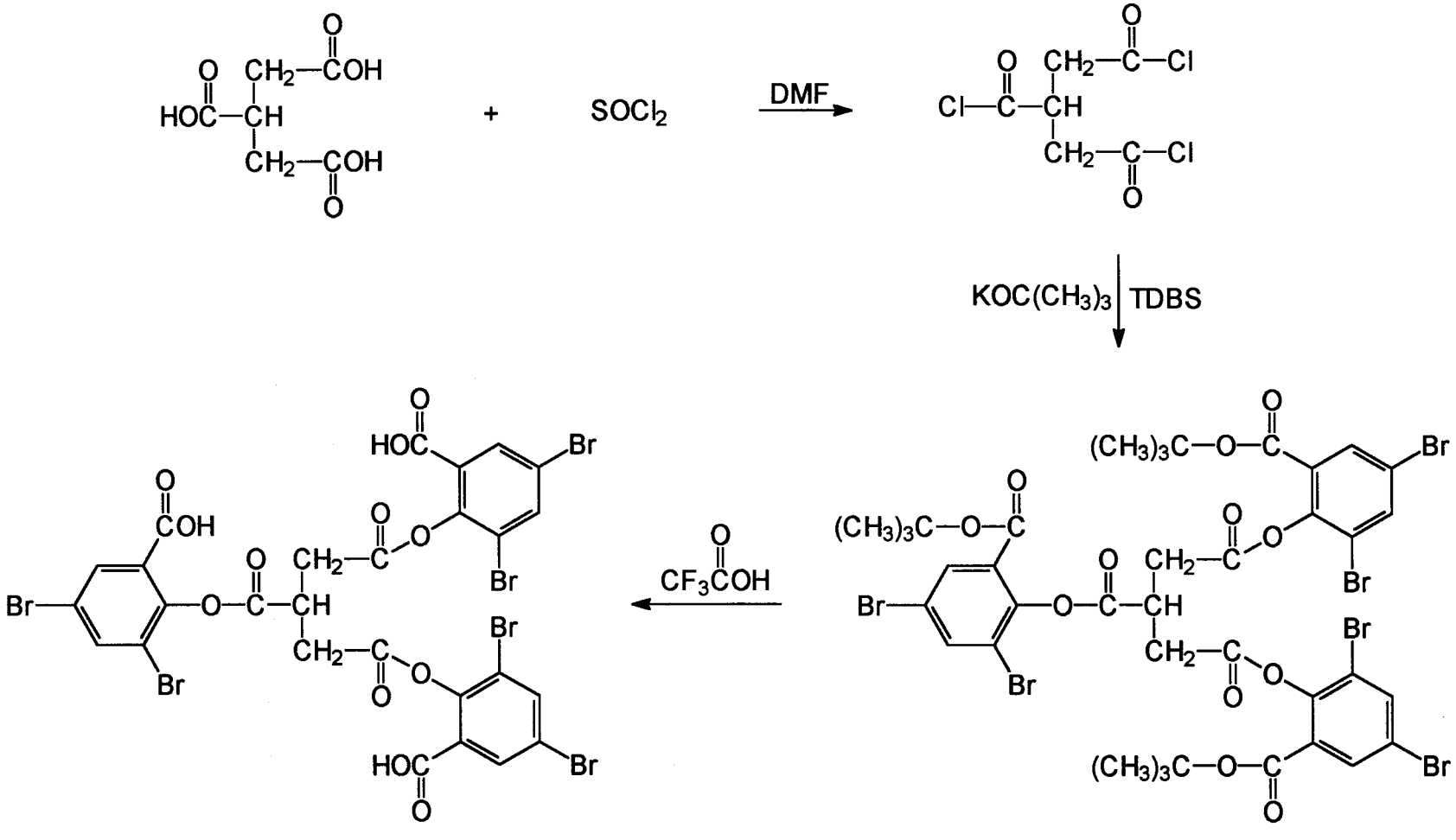
Synthesis of tris(3,5-dibromosalicyl) tricarballylate (see **Scheme V**)

Tricarballylyl trichloride. 2.2 g (0.0125 mol) of tricarballylic acid and 5 drops of DMF were added into 25 mL of thionyl chloride (distilled). The mixture was refluxed overnight with stirring and a drying trap. The excess thionyl chloride was removed by distillation. The residue was purified by vacuum distillation at 117-119°C (4.2-4.3 mmHg) to give 2.1 g of liquid product (72.6% yield).

Tris(*tert*-butyl 3,5-dibromosalicylate) tricarballylate. 0.92 g (2.60 mmol) of *tert*-butyl 3,5-dibromosalicylate and 0.25 g (2.61 mmol) of potassium *tert*-butoxide in 10 mL of THF (distilled over CaH_2) were stirred for 30 min at room temperature. 0.20 g (0.65 mmol) of tricarballylyl trichloride in 5 mL of THF was added to the mixture, which was subsequently stirred overnight at room temperature. The THF was removed with a rotary-evaporator. The residue was extracted by diethyl ether and the ether layer was then washed with water. The organic layer was dried over anhydrous Na_2SO_4 and concentrated with a rotary-evaporator. The residue was recrystallized from ethanol (yield: 50.7%).

Tris(3,5-dibromosalicyl) tricarballylate. 0.1 g of tris(*tert*-butyl 3,5-dibromosalicyl) tricarballylate was dissolved in 2 mL of trifluoroacetic acid (distilled) and kept in an ice bath for 1 h. 2 mL of diethyl ether was added to the solution and the mixture was kept at 4 °C overnight. The precipitate was filtered and dried by suction and 0.07 g of white powder was obtained (yield: 80%).

Scheme V.



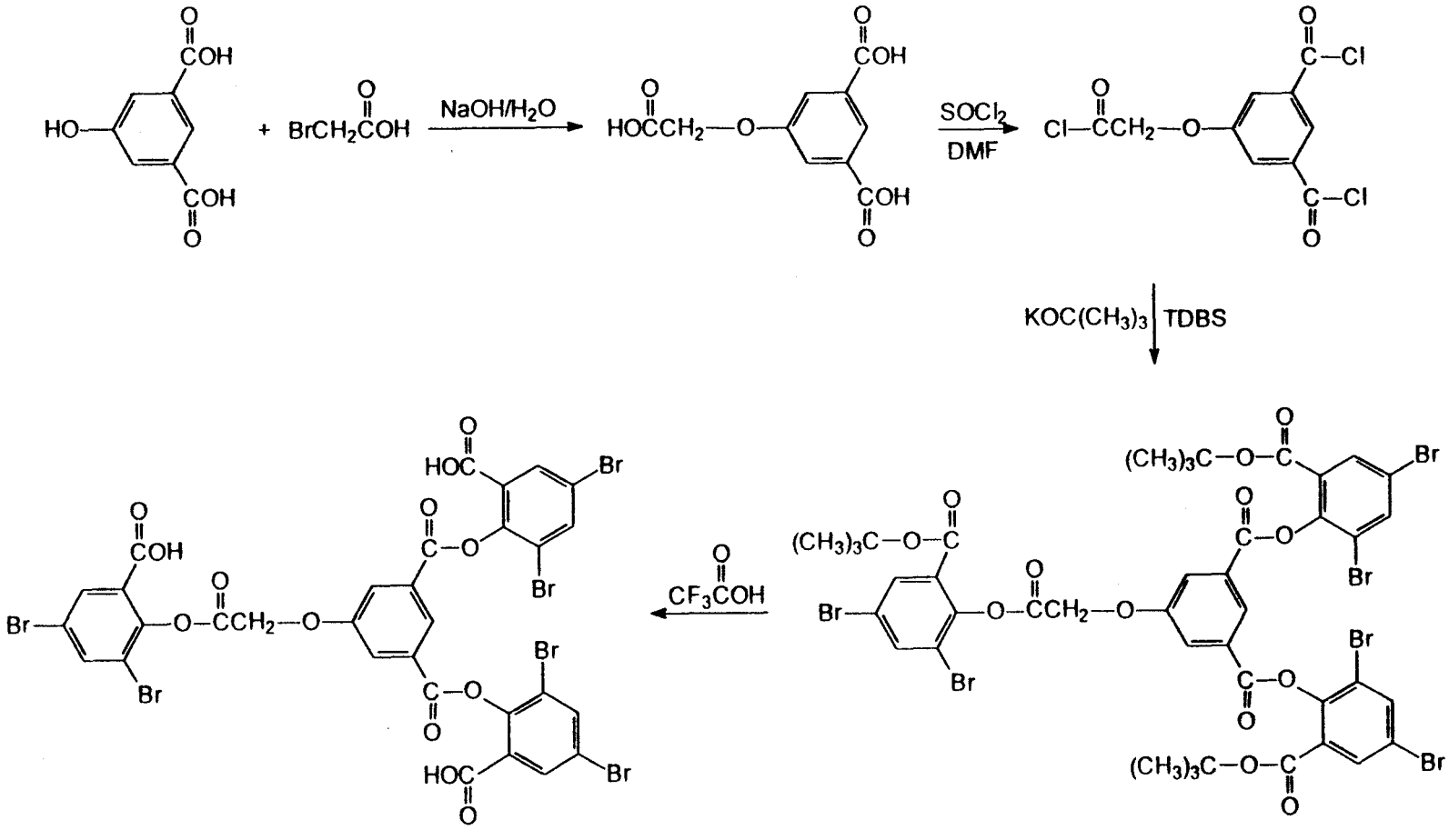
Synthesis of Tris(3, 5-dibromosalicyl) 5-acetate isophthalate ether
(see **Scheme VI**)

(3,5-Dicarboxy phenoxy) acetic acid. 3.64 g (0.02 mol) of 5-hydroxyisophthalic acid, 2.78 g (0.02 mol) of bromoacetic acid and 4.0 g (0.10 mol) of sodium hydroxide were dissolved in 50 mL of water and the mixture was then refluxed overnight. The reaction mixture was filtered and acidified with 6 N hydrochloric acid. The precipitate was collected by suction filtration and washed in succession with water and acetone. 3.0 g of the product was obtained (yield: 62.5%).

(3,5-Dicarboxy phenoxy) acetyl trichloride. 0.5 g (2.08 mmol) of (3,5-dicarboxy phenoxy) acetic acid and 2 drops of DMF were added to 10 mL of thionyl chloride (distilled) and the mixture was refluxed with stirring and a drying trap overnight. The excess $SOCl_2$ was removed by distillation. The residue was recrystallized from hexane and the product was yielded 0.27 g of white crystals (43.9% yield).

Tris(*tert*-butyl 3, 5-dibromosalicylate) (3,5-dicarboxy phenoxy) acetate. 0.54 g (1.5 mmol) of *tert*-butyl 3,5-dibromosalicylate and 0.36 g (1.5 mmol) of potassium *tert*-butoxide were stirred in dry THF (distilled over CaH_2) for 30 min at room temperature. 0.15 g (0.5 mmol) of (3,5-dicarboxy phenoxy) acetyl trichloride dissolved in 4 mL of THF was added into the mixture, which was subsequently stirred overnight at room temperature. The THF was removed with a rotary-evaporator. The residue was extracted with diethyl ether and was washed

Scheme VI.



with water. The organic layer was dried over anhydrous Na_2SO_4 and concentrated by a rotary-evaporator. The residue was recrystallized from ethanol (0.31 g, 49.2% yield).

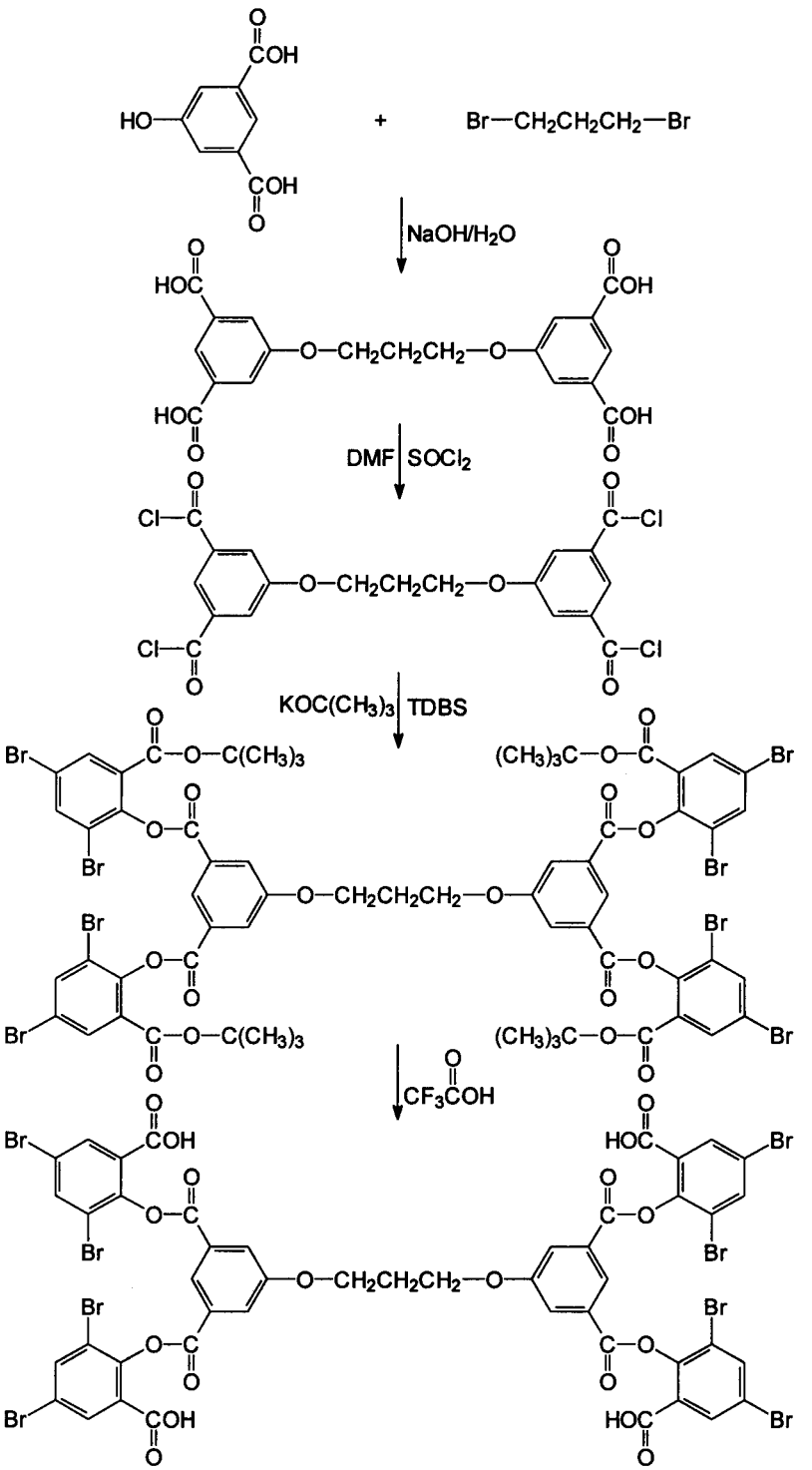
Tris(3, 5-dibromosalicyl) (3,5-dicarboxy phenoxy) acetate. 0.3 g of tris(*tert*-butyl 3,5-dibromosalicyl) (3,5-dicarboxy phenoxy) acetate was dissolved in 2 mL of trifluoroacetic acid (distilled) and kept in an ice bath for 1 h. 2 mL of diethyl ether was added to the solution and the mixture was kept at 4°C overnight. The precipitate was collected by suction filtration and 0.22 g of product was obtained (84.8% yield).

Synthesis of tetrakis(3,5-dibromosalicyl) 5,5'-(1,3-propanedioxy) diisophthalate (see **Scheme VII**)

5,5'-(1,3-Propanedioxy) diisophthalic acid. 2.01 g (0.01 mol) of 1,3-dibromopropane in 15 mL of methanol was added to the solution of 3.64 g (0.02 mol) of 5-hydroxyisophthalic acid and 3 g (0.075 mol) of sodium hydroxide in 15 mL of water. The reaction mixture was refluxed overnight to get a homogeneous solution. The methanol was removed with a rotary-evaporator. The remaining aqueous solution was filtered and acidified with 6 N hydrochloric acid. The precipitate was collected by suction filtration and washed with water. 1.88 g of product was obtained (46.5% yield).

5,5'-(1,3-Propanedioxy) diisophthalyl tetrachloride. 0.25 g (0.618 mol) of 5,5'-(1,3-propanedioxy) diisophthalic acid and 1 drop of DMF were added to 3 mL thionyl chloride (distilled) and the mixture was refluxed with stirring and a drying trap

Scheme VII.


HO
COH
O
+
Br—CH2CH2CH2—Br
NaOH/H2O
HOC
O—CH2CH2CH2—O
DMF
SOCl2
Cl—C
C—Cl
KOC(CH3)3
TDBS
Br
O—C(CH3)3
(CH3)3C—O
CF3COH

overnight. The excess thionyl chloride was removed by distillation and the residue was recrystallized from hexane to get white crystals (48.6% yield).

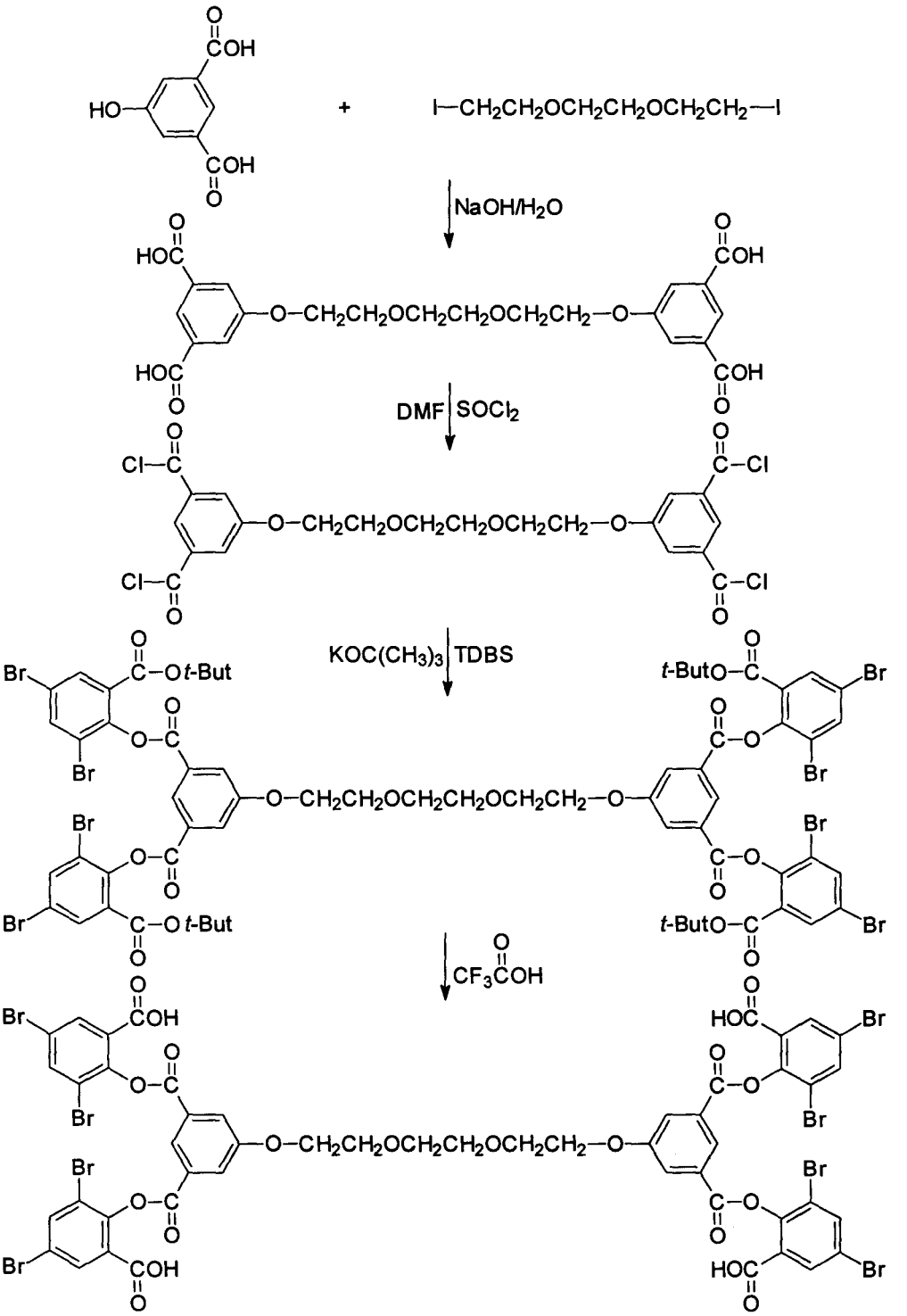
Tetrakis(*tert*-butyl 3,5-dibromosalicylate) 5,5'-(1,3-propanedioxy) diisophthalate. 0.6 g (1.6 mmol) of *tert*-butyl 3,5-dibromosalicylate and 0.18 g (1.6 mmol) of potassium *tert*-butoxide in 25 mL of THF (distilled over calcium hydride) were stirred for 30 min at room temperature. 0.27 g (0.40 mol) of 5,5'-(1,3-propanedioxy) diisophthalyl tetrachloride in 50 mL of THF was added into the mixture and stirred overnight at room temperature. The THF was removed with a rotary-evaporator. The residue was extracted with diethyl ether. The ether layer was washed with water and dried over anhydrous Na_2SO_4 and concentrated by a rotary-evaporator. The residue was recrystallized from ethanol (50.3% yield).

Tetrakis(3,5-dibromosalicyl) 5,5'-(1,3-propanedioxy) diisophthalate. 0.1 g of tetras(*tert*-butyl 1,3-dibromosalicylate) 5,5'-(1,3-propanedioxy) diisophthalate was dissolved in 2 mL of trifluoroacetic acid (distilled) and kept in an ice bath for 1 h. 2 mL of diethyl ether was added to the solution and the mixture was kept at 4 °C overnight. The precipitate was filtered and dried by suction and 0.07 g of white powder product was obtained (83.5% yield).

Synthesis of tetrakis(3,5-dibromosalicyl) 5,5'-triethyleneglycol diisophthalate ether(see Scheme VIII)

5,5'-Triethyleneglycol diisophthalic acid ether. 3.70 g (0.01 mol) of 1,2-bis(2-iodoethoxy) ethane in 15 mL of methanol was added into the solution of 3.64 g (0.02 mol) of 5-hydroxyisophthalic acid (0.02 mol) and 3.0 g (0.075 mol) of sodium

Scheme VIII.


HO
COH
O
+
I—CH2CH2OCH2CH2OCH2CH2—I
NaOH/H2O
HOC
COH
O—CH2CH2OCH2CH2OCH2CH2—O
DMF SOCl2
Cl—C
C—Cl
KOC(CH3)3 TDBS
Br
t-But
t-ButO—C
CF3COH

hydroxide in 15 mL of water. The reaction mixture was refluxed overnight to get a homogeneous solution. The methanol was removed with a rotary-evaporator. The remaining aqueous solution was filtered and acidified with 6 N hydrochloric acid. The precipitate was collected by suction filtration and washed with water. 1.75 g of the product was obtained (36.6% yield).

5,5'-Triethyleneglycol diisophthalyl ether tetrachloride. 0.2 g (0.418 mol) of 5,5'-triethyleneglycol diisophthalic acid ether and 1 drop of DMF were added into 4 mL of thionyl chloride (distilled). The mixture was refluxed overnight with stirring and a drying trap. The excess thionyl chloride was removed by distillation and the residue was recrystallized from hexane to obtain white crystals.

Tetrakis(*tert*-butyl 3,5-dibromosalicylate) 5,5'-triethyleneglycol diisophthalate ether. 0.69 g (1.96 mmol) of *tert*-butyl 3,5-dibromosalicylate and 0.21 g (1.96 mmol) of potassium *tert*-butoxide in 10 mL of THF (distilled over calcium hydride) were stirred for 30 min at room temperature. 0.237 g (0.475 mmol) of 5,5'-triethyleneglycol diisophthalyl ether tetrachloride in 10 mL of THF was added into the mixture, which was then stirred overnight at room temperature. The THF was removed with a rotary-evaporator. Diethyl ether was added for extraction. The ether layer was washed with water and dried over anhydrous Na_2SO_4 and concentrated by a rotary-evaporator. The residue was recrystallized from ethanol.

Tetrakis(3,5-dibromosalicyl) 5,5'-triethyleneglycol diisophthalate ether. 0.1 g of tetrakis(*t*-butyl 3,5-dibromosalicylate) 5,5'-triethyleneglycol diisophthalate ether was

dissolved in 2 mL of trifluoroacetic acid (distilled) and kept in an ice bath for 1 h. 2 mL of diethyl ether was added to the solution and the mixture was kept at 4 °C overnight. The precipitate was filtered and dried by suction and 0.7 g of white powder product was obtained (58.1% yield).

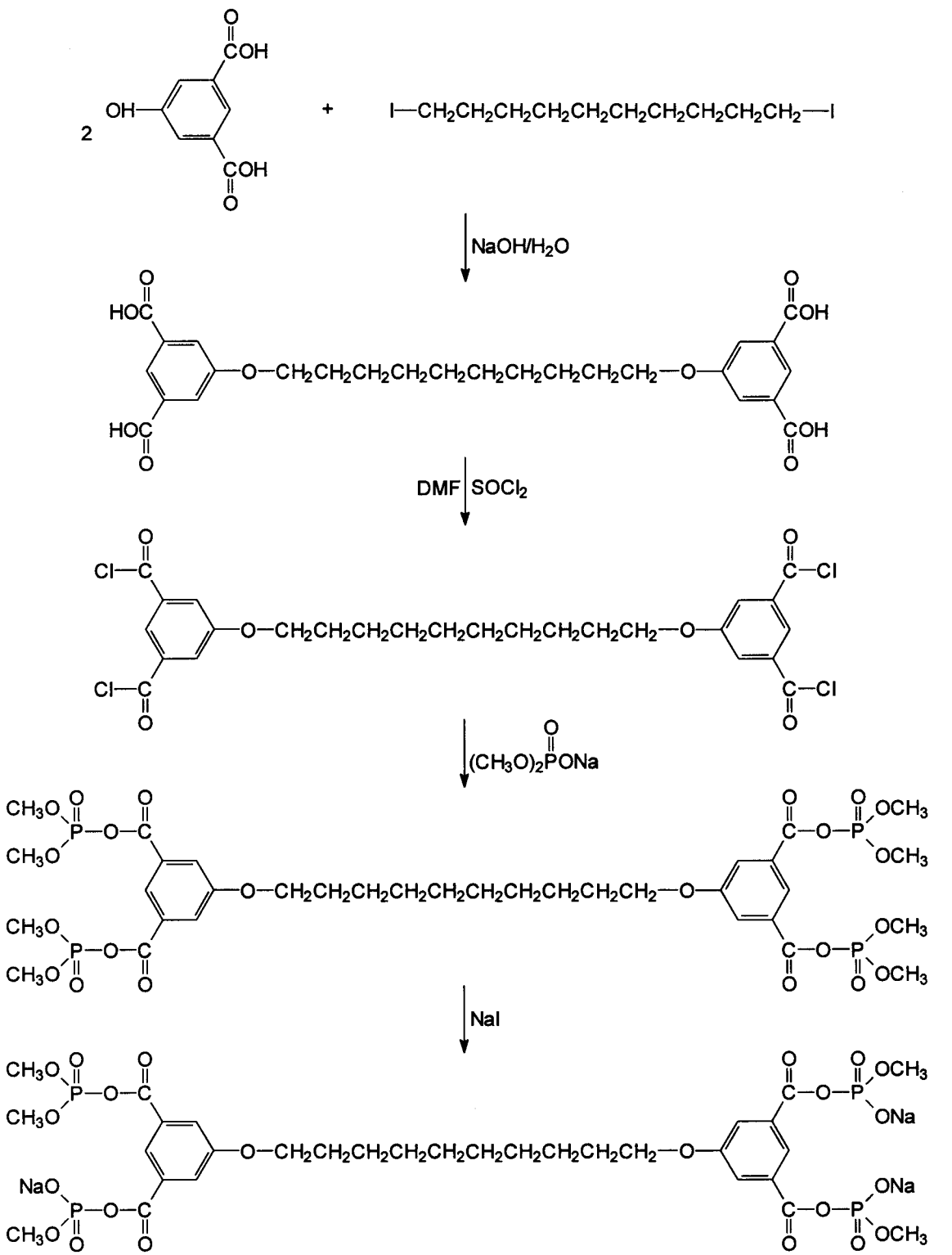
Synthesis of tetrakis(methyl phosphate) 5,5'-(1,10-decanedioxy) diisophthalate (see **Scheme IX**)

5,5'-(1,10-decanedioxy) diisophthalic acid. 3.94 g (0.01 mol) of 1,10-diiododecane in 10 mL of dimethyl sulfoxide (DMSO) was added into 3.64 g (0.02 mol) of 5-hydroxyisophthalic acid and 3 g (0.075 mol) of sodium hydroxide dissolved in 10 mL of water. The mixture was stirred and refluxed overnight. 50 mL of water was added to dissolve the mixture. The solution was filtered and acidified with 6 N hydrochloric acid. The precipitate was collected by suction filtration and washed with hot water. 2.5 g of the desired product was obtained (50 % yield).

5,5'-(1,10-decanedioxy) diisophthalate tetrachloride. The mixture of 0.5 g of 5,5'-(1,10-decanedioxy) diisophthalic acid and 5 mL of thionyl chloride was stirred and refluxed in the presence of 1 drop of DMF for 2 days. The excess $SOCl_2$ was removed by distillation and the residue was recrystallized from dry acetone which yielded 0.23 g (40 % yield).

Tetrakis(dimethyl phosphate) 5,5'-(1,10-decanedioxy) diisophthalate. 0.30 g of 5,5'-(1,10-decanedioxy) diisophthalate tetrachloride in 2 mL of dry THF was added to the mixture of 0.34 g of sodium dimethyl phosphate in 3 mL of dry THF. The mixture was stirred in an ice bath for 3 h. The precipitate was removed by suction

Scheme IX.


2
OH
COH
COH
O
+
I—CH2CH2CH2CH2CH2CH2CH2CH2CH2CH2—I
NaOH/H2O
HOC
HOC
COH
COH
O—CH2CH2CH2CH2CH2CH2CH2CH2CH2CH2—O
DMF
SOCl2
Cl—C
Cl—C
C—Cl
C—Cl
(CH3O)2P(=O)ONa
CH3O
CH3O
OCH3
OCH3
NaI
NaO
ONa

filtration and the filtrate was concentrated with a rotary-evaporator to obtain a yellowish oil.

Tetrakis(sodium methyl phosphate) 5,5'-(1,10-decanedioxy) diisophthalate. 0.35 g of sodium iodide dissolved in 2.5 mL of dry acetone was added to the oily residue dissolved in 2.0 mL of dry acetone. The mixture was stirred at room temperature overnight with protection from light. The precipitate was collected by suction filtration and washed with hot acetone. 0.23 g of product was obtained.

Preparation of hemoglobin

Hemoglobin A was isolated from packed red blood cells (RBC's), obtained from Life Source, following the procedure of Dozy et al. [82]. 80 mL of ice cold phosphate buffer saline (PBS: 150 mM NaCl in 5 mM phosphate buffer, pH 8.0) was added to 20 mL of red blood cells (RBC's). The mixture was stirred gently to be well mixed and centrifuged at 3000 rpm (1075 g) for 10 min with a Sorvall RC5B refrigerated superspeed centrifuge using an SS-34 rotor. The supernatant and buffy coat were removed by aspiration. The washing of the RBC's with PBS was repeated another two times to make sure that the RBC's were free of unwanted plasma materials. All these steps were performed at 4 °C. The washed erythrocytes were lysed in 40 mL of ice cold deionized water with gentle stirring for 30 min in an ice bath. Then 20 mL of ice cold neutral saturated ammonium sulfate was added to the mixture and stirred for 2 h in an ice bath. Then the mixture was centrifuged at 12,000 rpm (17,210 g) for 10 min. The hemoglobin in the supernatant

was collected and the precipitate, containing membranes and other residues, was discarded.

The hemolysate was desalted by dialysis against Tris buffer (0.05 M Tris, 1 mM NaCN, pH 8.5). The completion of desalting was checked by adding 1 M $BaCl_2$ to the dialysis buffer solution to test if $BaSO_4$ precipitate was able to form. After desalting was complete, the dialysate was ready for ion-exchange chromatography.

Ion-exchange chromatography was performed according to the methods of Huisman and Dozy [83], and Dozy et al. [82]. DEAE-Sephadex A-50 was swollen in deionized water. The column was packed with the Sephadex gel and equilibrated with a buffer composed of 0.05 M Tris, 1 mM NaCN, pH 8.5, at a flow rate of approximately 20 mL/h at 4 °C. When the pH value of the eluent buffer reached 8.5, the dialyzed hemolysate was applied to the top of the column. Proteins were separated with a linear pH gradient from 8.5 to 7.2 with 0.05 M Tris, 1 mM NaCN. The elution was detected at 280 nm by a ISCO UA-5 absorbance/fluorescence detector.

The purity of hemoglobin A was tested using clinical alkaline agarose gel electrophoresis (Corning Medical). The hemoglobin was concentrated by ultrafiltration over an Amico PM 10 membrane to the desired concentration. The concentration of hemoglobin and percentages of oxyhemoglobin, methemoglobin and hemichrome were determined spectrophotometrically on a HP 8452A UV/VIS diode array spectrophotometer with a program written in QUICK BASIC version 4.5 with QB LIBRARY revision 2.01 (see **Appendix I (a)**): The concentration of

hemoglobin is determined by using the extinction coefficient, $\varepsilon_{mM,}$ of 13.8 per heme at 542 nm [84] and the percentages of the components are obtained by calculating concentrations of oxyhemoglobin, methemoglobin and hemichrome [85]. The concentration of catalase is determined by using ε_{mM} of 420 per tetramer at 405 nm [86].

Crystallization of hemoglobin

In high salt concentration. Crystals of deoxyhemoglobin were grown by the method of Perutz [87]. The hemoglobin was dialyzed against 0.01 M ammonium phosphate buffer of pH 7.0 (8.5 mL of 2 M $(NH_4)_2HPO_4$, 1.5 mL of 2 M $NH_4H_2PO_4$ and 2 L deionized water). Crystallization was carried out at room temperature at a series of salt concentrations which pass through the critical point where crystallization begins. The optimum pH, buffer and hemoglobin concentrations are given in **Table 1**.

Solution C, pH = 6.5, was made by mixing 0.8 volumes of 4 M $(NH_4)_2SO_4$ plus 0.05 volumes of 2 M $(NH_4)H_2PO_4$ plus 0.15 volumes of 2 M $(NH_4)_2HPO_4$. Hemoglobin was at 6% (60 mg/mL HbA) to give a final concentration of 1% in the crystallization setup. Sodium dithionite concentration was 1.25 M (5.44 g in 25 mL of deionized water made under nitrogen).

The mixing of all solutions above was carried out in a glove bag filled with nitrogen gas. Crystallization setup was by the batch method. The vials with the

Table 1. Crystallization setup for high salt conditions.

Sample	Solution C* (μL)	DI H_2O (μL)	1.25 M $Na_2S_2O_4$ (μL)	6% Hb A (μL)
1	698	37	15	150
2	675	60	15	150
3	653	82	15	150
4	623	112	15	150
5	600	135	15	150
6	578	157	15	150
7	563	172	15	150

* See page 46.

above crystallization solutions were stored in nitrogen-filled desiccator jars sealed with greased ground glass lids. The crystals took over a week to grow.

In low salt concentration. Crystals of deoxyhemoglobin were grown by the method of Ward et al. [88] and Arnone et al. [89]. Crystallization was from solutions that were 1% (10 mg/mL) in hemoglobin, 10 mM potassium chloride, 4 mM sodium dithionite and over a range of 9.5 to 10.75% PEG-6000. The actual volumes of the different solutions that were mixed are shown in **Table 2**.

The mixing of all solutions above was carried out in a glove bag filled with nitrogen gas. Crystallization setup was by the batch method. The vials with the above crystallization solutions were stored in nitrogen-filled desiccator jars sealed with greased ground glass lids. The crystals took over two weeks to grow.

Computer modeling

The modeling of deoxyhemoglobin crystals and design of reagents were carried out using INSIGHT II software. For the modeling of deoxyhemoglobin crystals, the coordinate file of deoxyhemoglobin (2HHB) from the Protein DataBase (PDB) was converted into fit of the $P2_1$ space group by using a program written for the VAX-VMS 8530 main-frame (see **Appendix II**). A crystal unit of deoxyhemoglobin was modeled by using CELL, CELL_DISPLAY and MACRO_CELL in the ASSEMBLY command of INSIGHT. Another program was written for the VAX-VMS 8530 main-frame to make a model of deoxyhemoglobin crystal composed of six tetramers in INSIGHT II (see **Appendix III**).

Table 2. Crystallization setup for low salt conditions.

Sample	0.1 M KCl (μL)	1.25 M $Na_2S_2O_4$ (μL)	25% PEG-6000 (μL)	2 M PO_4^{3-} buffer (μL)	6% Hb A (μL)
1	100	5	380	315	200
2	100	5	390	305	200
3	100	5	400	295	200
4	100	5	410	285	200
5	100	5	420	275	200
6	100	5	430	265	200

Reagents were designed by using EDIT, 3D-STRUCTURE AND OPTIMIZE in BUILDER module of INSIGHT II.

Crosslinking reaction of hemoglobin

With tris(3,5-dibromosalicyl) tricarballylate. The reaction with tris(3,5-dibromosalicyl) tricarballylate (DBSTC) was carried out in 0.01 M MOPS, 1 mM NaCN, at pH 7.0, essentially according to the method of Walder et al. [68] for oxyhemoglobin and of Chatterjee et al. [70] for deoxyhemoglobin. The concentration of hemoglobin was 0.55 mM and the molar ratio of hemoglobin tetramer to DBSTC was kept at 1 to 1.1 for all the experiments.

The reaction with oxyhemoglobin was done by adding the calculated amount of DBSTC (powder) to the hemoglobin solution. The mixture was gently swirled every 30 min for a total of 3 h at 37 °C. Afterward, the reaction solution was submerged in an ice bath for 30 min to terminate the reaction. The hemoglobin solution was dialyzed in Tris buffer (pH 8.5) to get rid of small molecules and isolated through a DEAE-Sephadex A-50 column chromatography with a linear gradient of 0.05 M Tris, 1 mM NaCN, pH 8.5 to 7.2.

For the deoxyhemoglobin crosslinking reaction, the oxyhemoglobin solution was purged with a wet stream of nitrogen gas for at least 3 h in an ice bath until the solution was purplish. Then the solution was transferred to a water bath already equilibrated at 37 °C. The reagent, DBSTC , suspended in the buffer of 0.01 M MOPS, 1mM NaCN, pH 7.0 was injected into the hemoglobin solution. The

nitrogen purging was maintained during the reaction. The other conditions of reaction and isolation of the crosslinked hemoglobins were the same as that of oxyhemoglobin.

With tris(3,5-dibromosalicyl) (3,5-dicarboxy phenoxy) acetate. The reaction of deoxyhemoglobin with tris(3,5-dibromosalicyl) 5-acetate isophthalate ether (DBCPA) was performed in 0.01 M MOPS, 1 mM NaCN, pH 7.2 , essentially according to the method of Kluger et al. [77-78]. The concentration of hemoglobin tetramer was 0.55 mM and the molar ratio of hemoglobin to the reagent was 1 to 1.1.

The oxyhemoglobin solution was purged with a wet stream of nitrogen gas for at least 3 h in an ice bath until the solution was purplish. Then the solution was transferred in a water bath already equilibrated at 37 °C. DBCPA, suspended in the buffer of 0.01 M MOPS, 1 mM NaCN, pH 7.2, was injected into the deoxyhemoglobin solution while purging with nitrogen. The nitrogen purging was maintained during the reaction which lasted for 3 h at 37 °C. The reaction solution was submerged in an ice bath for 30 min to terminate the reaction. The hemoglobin solution was dialyzed against 0.05 M Tris, 1mM NaCN, pH 8.5, to remove small molecules and isolated through a DEAE-Sephadex A-50 column with a linear gradient of 0.05 M Tris, 1 mM NaCN, pH 8.5 to 7.2.

With the elongated bi-linkers and tetra-linkers. The reaction of oxyhemoglobin with the bi-linkers and tetra-linkers was performed in 0.01 M MOPS, 1 mM NaCN and pH 7.2. The concentration of hemoglobin was 3.5-5 mM and the molar ratio of hemoglobin tetramer to the reagent was 2-2 to 1. The reaction of oxyhemoglobin

was done by adding the calculated amount of one of the bi-linkers and tetra-linkers to the hemoglobin solution. The mixture was gently swirled every 30 min for a total of 3 h at 37 °C. The reaction solution was submerged in an ice bath for 30 min to terminate the reaction. The hemoglobin solution was dialyzed in Tris buffer (pH 8.5) to remove small molecules and tested by SDS-PAGE.

With bis(3,5-dibromosalicyl) fumarate in crystallization solutions. Bis(3,5-dibromosalicyl) fumarate (DBSF) was synthesized according to the method of Zaugg et al. [67]. The reaction of oxyhemoglobin with DBSF was done in several crystallization solutions at room temperature for 2 days. The solutions were prepared according the crystallization conditions of Silva et al. [90] with both high salt and low salt conditions. The concentration of hemoglobin was at 1% and the molar ratio of hemoglobin to DBSF was 1 to 1.1. After reaction, the hemoglobin solutions were dialyzed against deionized water to remove small molecules.

In crystal forms. The calculated amount of tris(3,5-dibromosalicyl) tricarballylate (DBSTC) was suspended in the high salt solution of crystallization condition. In a glove bag filled with nitrogen gas, crystals from high salt condition were transferred into the solution in which DBSTC was added. The molar ratio of the reagent to hemoglobin was 1.5 to 1. The sample was stored in a nitrogen-filled desiccator sealed with a ground and greased lid at room temperature. After a week, the crystals were dissolved in a small amount of deionized water. The hemoglobin solution was dialyzed against deionized water to take off small molecules. Crystals from low salt

condition were treated in the same way except that DBSTC was suspended in low salt solution.

Bis(3,5-dibromosalicyl) fumarate was used to react with crystal hemoglobin in the same conditions as DBSTC in both high salt and low salt solutions.

Oxygen-binding of hemoglobin

Oxygen-binding experiments were performed at 37 °C and 10 psi gas outlet for both air and nitrogen on the Hemox-analyzer with an automate plotter for recording oxygen equilibrium curves (TCS Products). A blank-run with water was made to determine if the membrane of the electrode of oxygen pressure was in proper condition in which the minimum reading was between 2.0 and 3.0 mmHg. A sample was prepared with 4 mL of Hemox-solution (0.05 M potassium phosphate, 135 mM NaCl, pH 7.4), 10 μL anti-foaming agent (Sigma) and a calculated volume of hemoglobin which produce a final concentration higher than 55 μM per heme. The sample was oxygenated with air until equilibrium was reached and the potentiometer was adjusted to a meter reading of 150 mmHg, which is the oxygen partial pressure under atmospheric conditions. Then the flow of nitrogen was started and the deoxygenation was continued until the oxygen partial pressure (pO_2) reading went below 3 mmHg. Finally, the association curve was recorded while the sample was oxygenated again.

P_{50}, a value that describes oxygen affinity, was obtained corresponding to the oxygen partial pressure (pO_2) at 50% of oxygen saturation (Y). The Hill

coefficient (n) was obtained by calculation of the slope of $\log(Y/(1-Y))$ as a function of $\log(pO_2)$ near $\log(Y/(1-Y)) = 0$ [7-8, 10].

Autoxidation and denaturation of hemoglobin

The autoxidation and denaturation experiments were conducted using an HP 8452A UV/VIS spectrophotometer, temperature control system and PC computer using a program written in QUICK BASIC version 4.5 with QB LIBRARY revision 2.01 (see **Appendices IV** and **V**).

For the autoxidation experiments, the method of Winterbourn [85] was followed. The hemoglobins were freshly purified within a few days of use and the samples were dialyzed in 0.01 M MOPS, pH 7.0 (CN^- free). Seven cuvettes were used, the first one for the reference and the other six for samples. The concentration of the sample in each cuvette was adjusted to approximately 14 mM per heme, corresponding to an absorbance of 2 at 416 nm. The data were recorded every 30 min for each sample at 560, 576, 630 and 700 nm for the total time of 17 h.

There are two common methods to determine the denaturation of hemoglobin: (1) measuring the amount of precipitate of hemoglobin as a function of time at a particular temperature [91] or (2) determine the unfolding of hemoglobin from the absorbance changes as a function of increasing temperature, which was developed by White and Olsen [71] and Yang and Olsen [72], and used in this dissertation as well. The experiment was performed in 0.01 M MOPS, pH 7.0 (CN^- free), in the presence of 0.9 M guanidine to keep the hemoglobins from precipitating

after the samples were denatured. Seven cuvettes were used and the first one was for the reference and the other six for the samples of three native hemoglobin and three crosslinked hemoglobin. A concentration of 7.0 mM per heme for each sample was adjusted, corresponding to an absorbance of 1.0 at 416 nm. Before the denaturation experiment, the oxyhemoglobin was converted into methemoglobin by oxidizing it with 9.7 × 10 μM potassium ferricyanide for 15 min. The program was set up to measure each sample at an interval of about 122 sec at 280, 406, 410, 418, 542, 576 and 630 nm for a total time of 210 min.

The other experiments

Nuclear magnetic resonance (NMR). The ^{1}H and ^{13}C NMR spectra were recorded in deuterated solvents (DMSO-d_6 or $CDCl_3$) in 5 mm diameter tubes. For the ^{1}H NMR spectra, the frequency was 300 MHz, the nucleus was set at 1.250 with a spectral width of 4000 Hz, the acquisition time was 3.752 sec, the pulse width was 20 msec, the delay time was 0, and the number of transients was 16. For the ^{13}C spectra, the frequency was 75 MHz, the nucleus was set at 13,500 with a spectral width of 16501.7 Hz, the acquisition time was 1,639 sec, the pulse width was 8.7 msec, no delay time, and the transient number was 1024.

Fourier transient infrared (FTIR). The samples were loaded on KBr plates and scanned from 4000 to 500 cm^{-1}. The background noise was measured and deducted from the sample results. The scan number was 20.

Alkaline Agarose Gel Electrophoresis. It was carried out by using commercially available alkaline hemoglobin gel (Corning Medical) at 240 volts for 20 min in an alkaline hemoglobin buffer (16.7 g of sodium barbital and 2.5 g of barbital in 2 L of deionized water, pH 8.6). The gel was stained with the stain solution (1.7 g Amino Black 10B in 1 L of 5% acetic acid) for 10 min, immersed in the first 5% acetic acid solution for 30 sec, dried in an oven at 65 °C, cooled to room temperature, rinsed in the first 5% acetic acid solution for 1 min and in the second 5% acetic acid solution until all excess stain was removed, and finally rinsed with deionized water for 2 min and dried in a heating oven.

Sodium dodecylsulfate polyacrylamide Electrophoresis (SDS-PAGE). The samples were denatured for 15 min at 100 °C with a denaturing solution (0.25 M Tris (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.05% bromophenol blue). The separation of the samples was carried out using a PHAST system (Pharmacia) at a constant current of 10 mA (250 V) and the development was done by staining with 0.1% of PhastGel blue R solution and destaining with a solution (30% methanol, 10% of acetic acid and 60% deionized water). The resulting SDS gel was scanned on the ISCO gel scanner and the percentage of crosslinking can be calculated by using the gel scanning program ISCO.

Chapter 3

TERT-BUTYL PROTECTED SALICYLATES AND ELONGATED BI-LINKERS

RESULTS

The first section of this chapter summarizes the analytical results for each of the compounds synthesized. The spectra are given at the end of the chapter.

Tert-butyl salicylate

^{1}H NMR (DMSO-d_6) (see **Figure 2 (a)**): δ (ppm) 10.76 (s, 1H, OH), 7.73 (d, 1H, ArH), 7.50 (t, 1H, ArH), 6.93 (m, 2H, ArH), 1.57 (s, 9H, tBu).

^{13}C NMR (DMSO-d_6) (see **Figure 2 (b)**): δ (ppm) 168.87 (s, 1C, C=O), 160.50 (s, 1C, O-ArC), 135.48 (s, 1C, ArC), 130.05 (s, 1C, ArC), 119.24 (s, 1C, ArC), 117.29 (s, 1C, ArC), 113.72 (s, 1C, ArC), 82.78 (s, 1C, O-C), 27.74 (s, 3C, tBu).

FTIR (KBr) (see **Figure 3**): 3145.2 cm^{-1} (aromatic C-H), 2984.9 cm^{-1} (-CH_3), 1672.3 cm^{-1} (C=O).

Tert-butyl 3,5-dibromosalicylate

Melting point: 108-109 °C.

^{1}H NMR ($CDCl_3$) (see **Figure 4 (a)**): δ (ppm) 11.71 (s, 1H, OH), 7.83 (d, 1H, ArH), 7.80 (d, 1H, ArH), 1.61 (s, 9H, tBu).

^{13}C NMR ($CDCl_3$) (see **Figure 4 (b)**): δ (ppm) 168.24 (s, 1C, C=O), 157.54 (s, 1C, O-ArC), 140.34 (s, 1C, ArC), 131.67 (s, 1C, ArC), 115.91 (s, 1C, ArC), 112.20 (s, 1C, ArC), 110.37 (s, 1C, ArC), 84.68 (s, 1C, O-C), 28.08 (s, 3C, tBu).

4,4'-(1,3-Propanedioxy) dibenzoic acid

Decomposition: 200 °C.

^{1}H NMR (DMSO-d_6) (see **Figure 5 (a)**): δ (ppm) 7.87 (d, 4H, ArH), 7.02 (d, 4H, ArH), 4.19 (t, 4H, O-CH_2), 2.20 (m, 2H, CH_2).

^{13}C NMR (DMSO-d_6) (see **Figure 5 (b)**): δ (ppm) 168.98 (s, 2C, C=O), 162.05 (s, 2C, O-ArC), 131.37 (s, 4C, ArC), 123.02 (s, 2C, ArC), 114.27 (s, 4C, ArC), 64.49 (s, 4C, O-CH_2), 28.38 (s, 1C, CH_2).

4,4'-(1,3-Propanedioxy) dibenzoyl dichloride

^{1}H NMR ($CDCl_3$) (see **Figure 6 (a)**): δ (ppm) 8.07 (d, 4H, ArH), 6.99 (d, 4H, ArH), 4.27 (t, 4H, O-CH_2), 2.35 (m, 2H, CH_2).

^{13}C NMR ($CDCl_3$) (see **Figure 6 (b)**): δ (ppm) 167.09 (s, 2C, C=O), 164.44 (s, 2C, O-ArC), 134.01 (s, 4C, ArC), 125.67 (s, 2C, ArC), 114.62 (s, 4C, ArC), 64.52 (s, 4C, O-CH_2), 28.81 (s, 1C, CH_2).

Bis(*tert*-butyl 3,5-dibromosalicyl) 4,4'-(1,3-propanedioxy) dibenzoate

Melting point: 81-85 °C.

^{1}H NMR ($CDCl_3$) (see **Figure 7 (a)**): δ (ppm) 8.18 (d, 4H, ArH), 7.98 (d, 2H, ArH), 7.89 (d, 2H, ArH), 7.01 (d, 4H, ArH), 4.27 (t, 4H, O-CH_2), 2.35 (m, 2H, CH_2), 1.35 (s, 18H, tBu).

^{13}C NMR ($CDCl_3$) (see **Figure 7 (b)**): δ (ppm) 163.69 (s, 2C, C=O), 163.42 (s, 2C, C=O), 162.63 (s, 2C, O-ArC), 147.00 (s, 2C, O-ArC), 138.86 (s, 2C, ArC), 133.75 (s, 2C, ArC), 133.00 (s, 4H, ArC), 129.83 (s, 2C, ArC), 121.57 (s, 2C, ArC), 119.66 (s, 2C, ArC), 119.39 (s, 2C, ArC), 114.75 (s, 4C, ArC), 83.31 (s, 2C, O-C), 64.81 (s, 2C, O-CH_2), 29.33 (s, 1C, CH_2), 28.17 (s, 6C, tBu).

Bis(3,5-dibromosalicyl) 4,4'-(1,3-propanedioxy) dibenzoate

Melting point: 135-140 °C.

^1H NMR (DMSO-d_6) (see **Figure 8 (a)**): δ (ppm) 8.29 (s, 2H, ArH), 8.09 (s, 2H, ArH), 8.06 (s, 4H, ArH), 7.16 (d, 4H, ArH), 4.29 (t, 4H, O-CH_2), 2.26 (m, 2H, CH_2).

^{13}C NMR (DMSO-d_6) (see **Figure 8 (b)**): δ (ppm) 163.61 (s, 2C, C=O), 163.23 (s, 2C, C=O), 162.88 (s, 2C, O-ArC), 146.89 (s, 2C, O-ArC), 138.65 (s, 2C, ArC), 133.19 (s, 2C, ArC), 132.32 (s, 4C, ArC), 128.20 (s, 2C, ArC), 120.31 (s, 2C, ArC), 119.53 (s, 2C, ArC), 118.84 (s, 2C, ArC), 64.80 (s, 2C, O-CH_2), 28.10 (s, 1C, CH_2).

4,4'-Triethyleneglycol dibenzoic acid ether

Decomposition: 300 °C.

^1H NMR ($CDCl_3$) (see **Figure 9 (a)**): δ (ppm) 7.88 (d, 4H, ArH), 7.01 (d, 4H, ArH), 4.14 (t, 4H, O-CH_2), 3.75 (t, 4H, O-CH_2), 2.49 (s, 4H, O-CH_2).

^{13}C NMR ($CDCl_3$) (see **Figure 9 (b)**): δ (ppm) 167.00 (s, 2C, C=O), 162.09 (s, 2C, O-ArC), 131.35 (s, 4C, ArC), 123.02 (s, 2C, ArC), 111.28 (s, 4C, ArC), 69.96 (s, 2C, O-CH_2), 68.81 (s, 2C, O-CH_2), 67.43 (s, 2C, O-CH_2).

4,4'-Triethyleneglycol diisophthalyl ether dichloride

^{1}H NMR (DMSO-d_6) (see **Figure 10 (a)**): δ (ppm) 8.05 (d, 4H, ArH), 6.96 (d, 4H, ArH), 4.20 (t, 4H, O-CH_2), 3.89(t, 4H, O-CH_2), 3,74(s, 4H, O-CH_2).

^{13}C NMR (DMSO-d_6) (see **Figure 10 (b)**): δ (ppm) 167.09 (s, 2C, C=O), 164.58 (s, 2C, O-ArC), 133.96 (s, 4C, ArC), 125.62 (s, 2C, ArC), 114.76 (s, 4C, ArC), 70.95 (s, 2C, O-CH_2), 69.47 (s, 2C, O-CH_2), 67.92 (s, 2C, O-CH_2).

Bis(*tert*-butyl 3,5-dibromosalicyl) 4,4'-triethyleneglycol dibenzoate ether

Melting point: 115-130 °C.

^{1}H NMR (DMSO-d_6) (see **Figure 11 (a)**): δ (ppm) 8.16 (d, 4H, ArH), 7.99 (d, 2H, ArH), 7.90 (d, 2H, ArH), 7.01 (d, 4H, ArH), 4.23 (t, 4H, O-CH_2), 3.91 (t, 4H, O-CH_2), 3.78 (s, 4H, O-CH_2), 1.35 (s, 18H, CH_3).

^{13}C NMR (DMSO-d_6) (see **Figure 11 (b)**): δ (ppm) 163.41 (s, 2C, C=O), 163.12 (s, 2C, C=O), 162.35 (s, 2C, O-ArC), 146.64 (s, 2C, O-ArC), 138.53 (s, 2C, ArC), 133.44 (s, 2C, ArC), 132.61 (s, 4C, ArC), 129.51 (s, 2C, ArC), 121.21 (s, 2C, ArC), 119.34 (s, 2C, ArC), 119.06 (s, 2C, ArC), 114.55 (s, 4C, ArC), 83.02 (s, 2C, O-C), 70.94 (s, 2C, O-CH_2), 69.59 (s, 2C, O-CH_2), 67.69 (s, 2C, O-CH_2), 27.83 (s, 6C, CH_2).

Bis(3,5-dibromosalicyl) 4,4'-triethyleneglycol dibenzoate ether

Melting point: 157-161 °C.

^1H NMR ($CDCl_3$) (see **Figure 12 (a)**): δ (ppm) 8.28 (d, 2H, ArH), 8.08 (s, 2H, ArH), 8.05 (d, 4H, ArH), 7.14 (d, 4H, ArH), 4.22 (t, 4H, O-CH_2), 3.78 (t, 4H, O-CH_2), 3.63 (s, 4H, O-CH_2).

^{13}C NMR ($CDCl_3$) (see **Figure 12 (b)**): δ (ppm) 163.59 (s, 2C, ArC=O), 163.25 (s, 2C, ArC=O), 162.86 (s, 2C, O-ArC), 146.87 (s, 2C, O-ArC), 138.63 (s, 2C, ArC), 133.17 (s, 2C, ArC), 132.26 (s, 4C, ArC), 128.16 (s, 2C, ArC), 120.25 (s, 2C, ArH), 119.51 (s, 2C, ArC), 118.62 (s, 2C, ArC), 114.85 (s, 4C, ArC), 69.94 (s, 2C, O-CH_2), 68.71 (s, 2C, O-CH_2), 67.69 (s, 2C, O-CH_2).

Crosslinking reaction

DBPDB crosslinked hemoglobin gave 5% dimers, and DBEBE crosslinked hemoglobin, 35% dimers in SDS-PAGE analysis (**Table 3**).

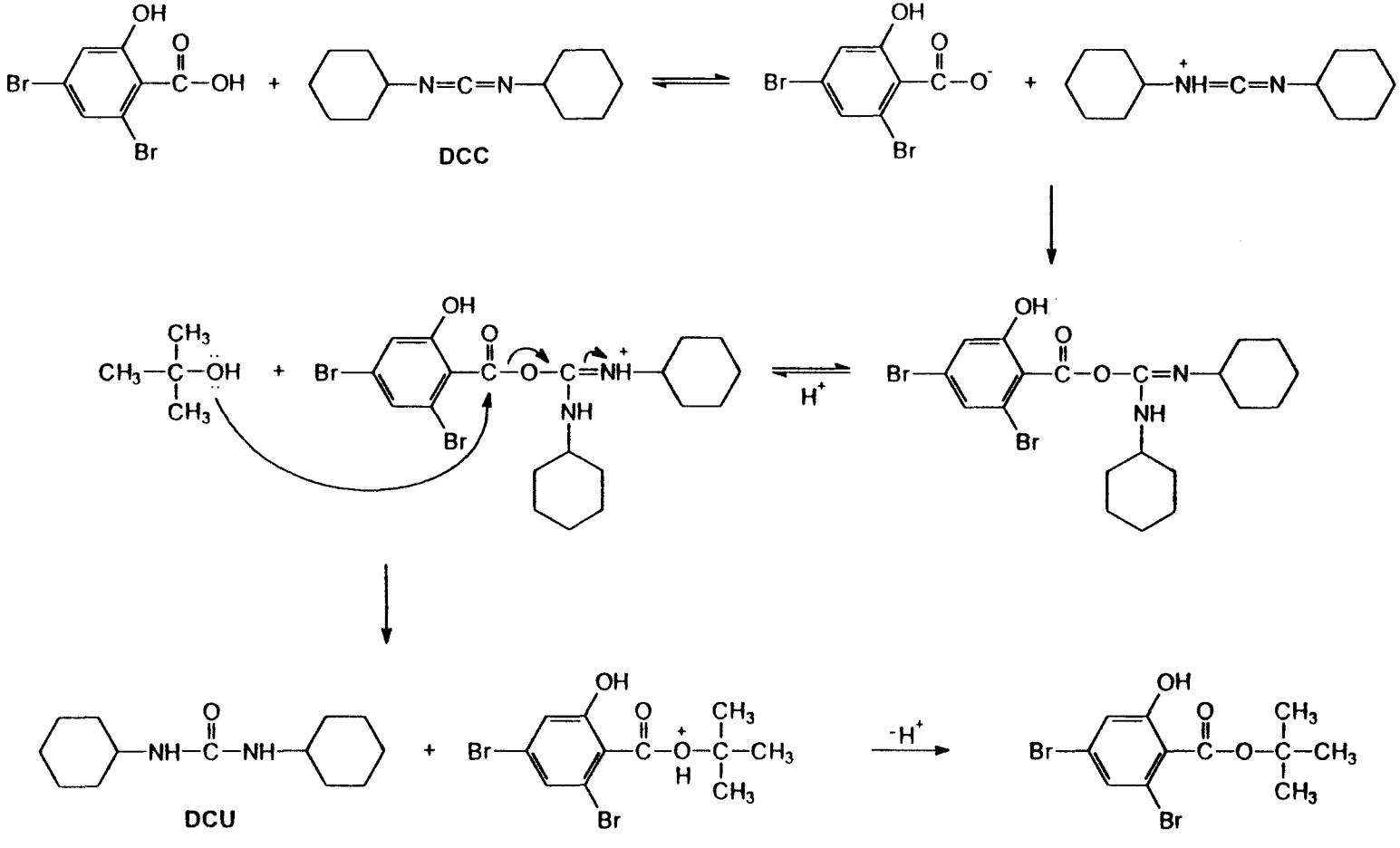
DISCUSSION

***Tert*-butyl protection of 3,5-dibromosalicylic acid.** *Tert*-butyl 3,5-dibromosalicylate was used to prepare aspirin crosslinking reagents with unreactive acid chlorides in previous work [78, 81, 92]. In this study, *tert*-butyl 3,5-dibromosalicylate was synthesized by coupling 3,5-dibromosalicylic acid with *tert*-butyl alcohol in the present of DCC as a coupling reagent (**Scheme X**). To minimize the self-reaction of carboxyl group with phenol hydroxyl of the salicylic acid, a large amount of *tert*-butyl alcohol was used in the reaction as solvent and reactant. The desired product is white needle crystals with a melting point of

Table 3. SDS-PAGE analysis of crosslinked hemoglobins by the elongated bi-linkers.

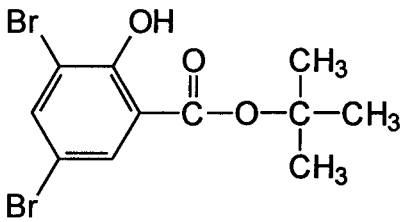
Hb sample	% Monomer	% Dimer
DBPDB-XL	95	5
DBEBE-XL	65	35

Scheme X.


OH
O
Br
C–OH
Br
+
N=C=N
DCC
OH
O
Br
C–O⁻
Br
+
+
NH=C=N
CH₃
CH₃–C–OH
CH₃
+
OH
O
Br
C–O–C=NH
NH
Br
H⁺
OH
O
Br
C–O–C=N
NH
Br
O
NH–C–NH
DCU
+
OH
O
Br
C–O–C–CH₃
H
CH₃
Br
-H⁺
OH
O
Br
C–O–C–CH₃
CH₃
Br

108-109 °C after recrystallization from ethanol. It is stable in alkaline media but hydrolyzed in strong acidic media.

Tert-butyl salicylate was characterized by ^{1}H and ^{13}C NMR (**Figure 2**): the resonance at chemical shift δ 10.76 ppm is due to the hydroxyl Hydrogen, the four peaks between δ 7.75 - 6.89 are due to the aryl hydrogens and the nine peaks at δ 1.57 are due to the methyl groups; the peak at δ 168.87 is due to the carbonyl carbon, the six peaks at δ 160.50 - 113.72 are due to the aryl carbons, the peak at δ 82.78 is due to the methine carbon and the peak at δ 27.74 is due to the methyl's carbons. *Tert*-butyl 3,5-dibromosalicylate was characterized by ^{1}H and ^{13}C NMR (**Figure 4**): the peak at δ 11.71 is due to the hydroxyl hydrogen, the two resonances at δ 7.83 and 7.80 are due to the aryl hydrogens and the peak at δ 1.61 is due to the methyl groups; the peak at δ 168.24 is due to the carbonyl carbon, the six peaks at δ 157.54 - 110.37 are due to the aryl carbons, the peak at δ 84.68 is due to the methine carbon and the peak at δ 28.08 is due to the methyl carbon. The *tert*-butyl protection of the salicylic acids was successful.



***Tert*-butyl 3,5-dibromosalicylate**

To elongate the bridging lengths of crosslinkers, amide-containing chains of different lengths were first considered as bridging structures at the first design

because of its hydrophilic character. However, the amide bond reacts with thionyl chloride and is cleaved during the preparation of the acid chloride:

$$RNHCOR' + SOCl_2 \rightarrow RCl + R'CN + SO_2 + HCl$$

We found that ether linkages were very stable to refluxing with thionyl chloride. The introduction of hydrophilic multi-ethyleneglycol into the crosslinkers can improve the solubility in aqueous solution. This is important because the hydrophilic bridging structure prevents its folding in aqueous solution which makes its bridging size much smaller than expected. The acid chloride compounds were prepared by reflux with thionyl chloride in the presence of DMF. The bis(3,5-dibromosalicyl) esters were synthesized successfully according to the method developed by Klotz et al. [81]. The bridging lengths of the reagents were determined by computer modeling and are summarized in **Table 4**.

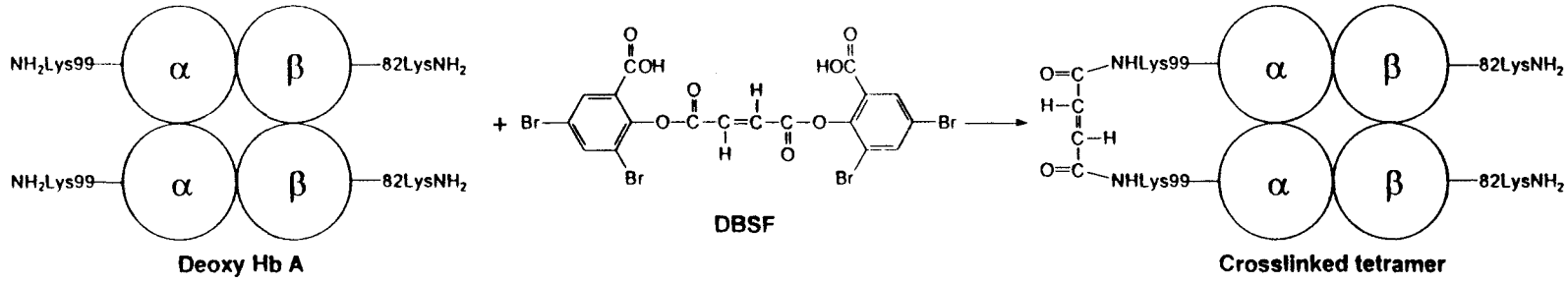
The bis(3,5-dibromosalicyl) linkers, DBPDB and DBEBE, were designed to crosslink between two intramolecular tetrameric hemoglobins as shown in **Scheme XI**. In the first step, deoxyhemoglobin is crosslinked with bis(3,5-dibromosalicyl) fumarate (DBSF) between Lys99's of the α chains [70]. In the second step, the two intramolecularly crosslinked hemoglobins would be crosslinked intermolecularly between Lys82β in different tetramers to form an octameric hemoglobin.

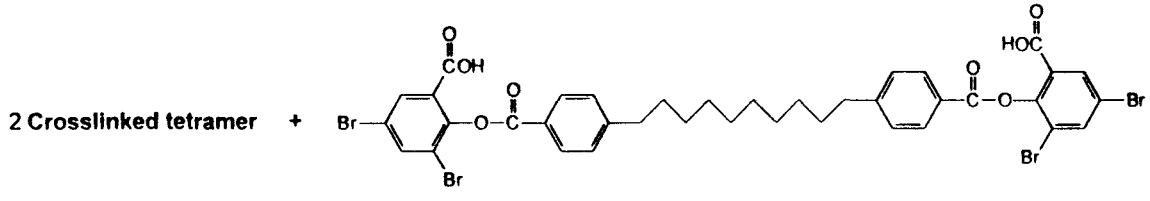
The syntheses of bis(3,5-dibromosalicyl) 4,4'-(1,3-propanedioxy) dibenzoate and bis(3,5-dibromosalicyl) 4,4'-triethyleneglycol diisophthalate ether failed when the

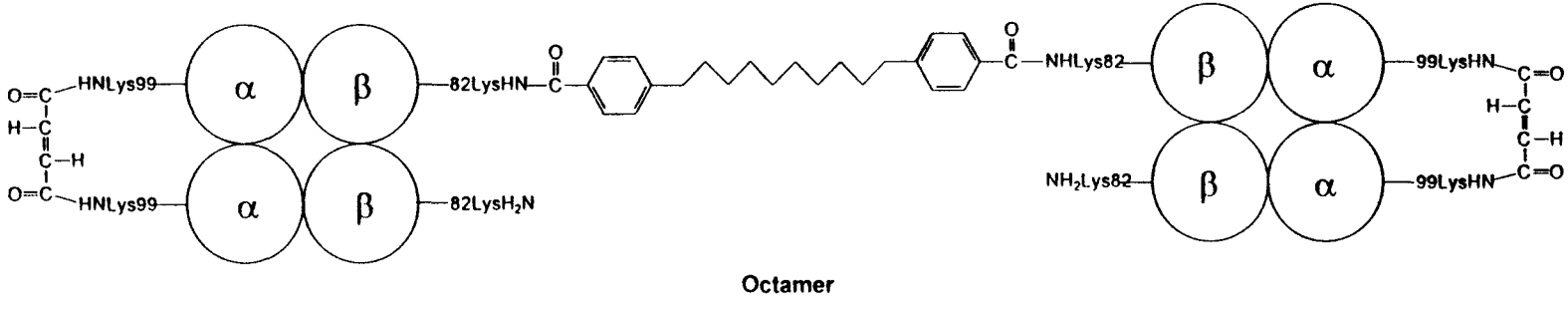
Table 4. Bridging lengths between carbonyl carbons of the crosslinkers.

Compound	Bridging length (Å)
DBPDB	14.3
DBEBE	19.6

Scheme XI

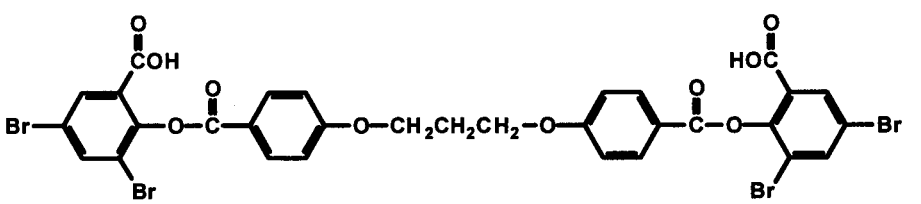

NH2Lys99
α
β
82LysNH2
Deoxy Hb A
DBSF
NHLys99
Crosslinked tetramer


2 Crosslinked tetramer
+


HNLys99
82LysHN
NHLys82
99LysHN
NH2Lys82
82LysH2N
Octamer

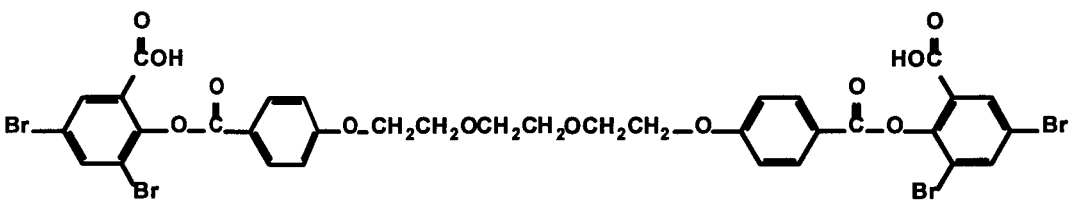
dibenzoyl chlorides did not react with 3,5-dibromosalicylic acid in the present of triethylamine. The crosslinkers were synthesized in this dissertation in four main steps. (1) The production of dibenzoic acids in a Williamson ether synthesis. (2) The production of the corresponding acid chlorides with thionyl chloride using DMF catalysis. (3) The production of the corresponding bis(*tert*-butyl 3.5-dibromosalicylate) dibenzoate esters. And (4) the deprotection of *tert*-butyl esters by triflouroacetic acid to form the corresponding bis(3,5-dibromosalicyl) dibenzoate ester.

Bis(3,5-dibromosalicyl) 4,4'-(1,3-propanedioxy) dibenzoate (DBPDB) was characterized by ^{1}H and ^{13}C NMR (**Figure 8**): The resonances at δ 8.29 and 8.09 are due to the four aryl hydrogens (outer rings), the resonances at δ 8.06 and 7.16 are due to the eight aryl hydrogens (inner rings) and the peaks at δ 4.29 and 2.26 are due to the six methylene hydrogens; the two peaks at δ 163.61 and 163.23 are due to the carbonyl carbons on the aspirin and benzoate aromatic rings, the ten peaks at δ 162.88 - 114.67 are due to the aspirin and benzoate aryl carbons and the two peaks at δ 64.80 and 28.10 are due to the methylene carbons.



Bis(3,5-dibromosalicyl) 4,4'-(1,3-propanedioxy) dibenzoate

Bis(3,5-dibromosalicyl) 4,4'-triethyleneglycol dibenzoate ether was characterized by ^{1}H and ^{13}C NMR (see **Figure 12**): The resonances at δ 8.28 and 8.08 are due to the four aryl hydrogens (outer rings), the resonances at δ 8.05 and 7.14 are due to the eight aryl hydrogens (inner rings), and the resonances at δ 4.22, 3.78 and 3.63 are due to the twelve methylene hydrogens; the two peaks at δ 163.59 and 163.25 are due to the carbonyl carbons on the aspirin and benzoate benzenes, the ten peaks at δ 162.86 - 114.85 are due to the aspirin and benzoate benzene carbons and the three peaks at δ 69.94, 68.71 and 67.69 are due to the methylene carbons. The syntheses of the two aspirin double linkers were successful.

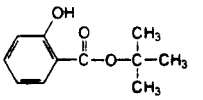


Bis(3,5-dibromosalicyl) 4,4'-triethyleneglycol dibenzoate ether

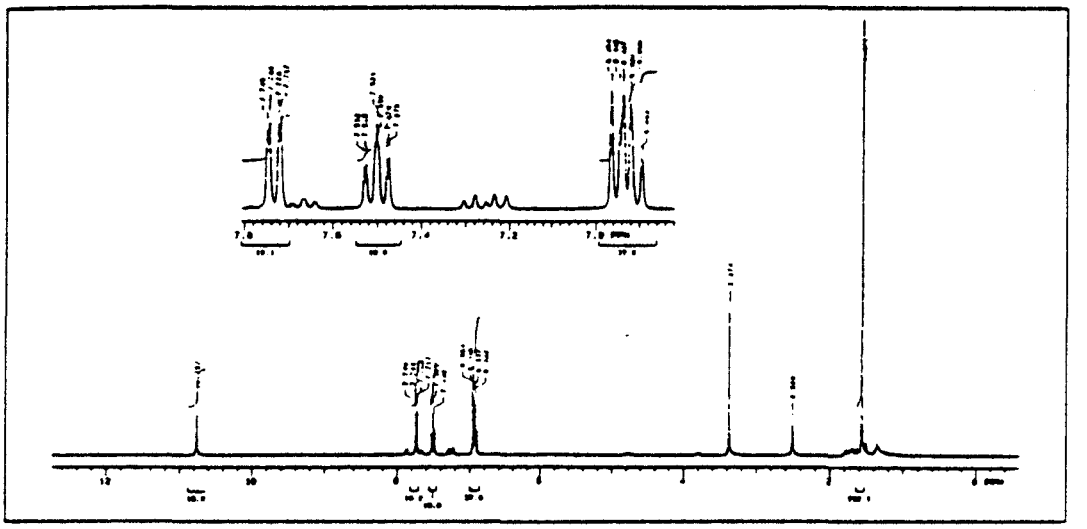
Reaction of hemoglobin with DBPDB had much fewer dimers shown in SDS-PAGE analysis than with DBEBE (**Table 3**). For DBEBE crosslinked proteins, 35% dimers by SDS-PAGE infers that there was intramolecular rather than intermolecular crosslinking since the intermolecular reaction should give only 25% dimers. No octamer was isolated from DBEBE crosslinked proteins by G-150 (Fine) gel filtration. The DBEBE crosslinking of hemoglobin must be between two subunits of the same molecule (**Figure 13 (A)**) and some

crosslinking at the same subunit (**Figure 13 (B)**). The cleft between the two β chains is too small for the elongated bi-linkers to enter and react with the Lys82's. Moreover, the mono ester group at each end of the bi-linkers may not be as sterospecific as DBSF and may react with other amino groups on the surface of hemoglobin. The other end of the bi-linkers with a mono ester group may bend backward to the same hemoglobin and react with an amino group at the same or different subunit. DBPDB, due to its relatively short structure, mainly crosslinked within the same subunit (**Figure 13 (B)**).

Figure 2. ^{1}H and ^{13}C NMR of *tert*-butyl salicylate (DMSO-d_6).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

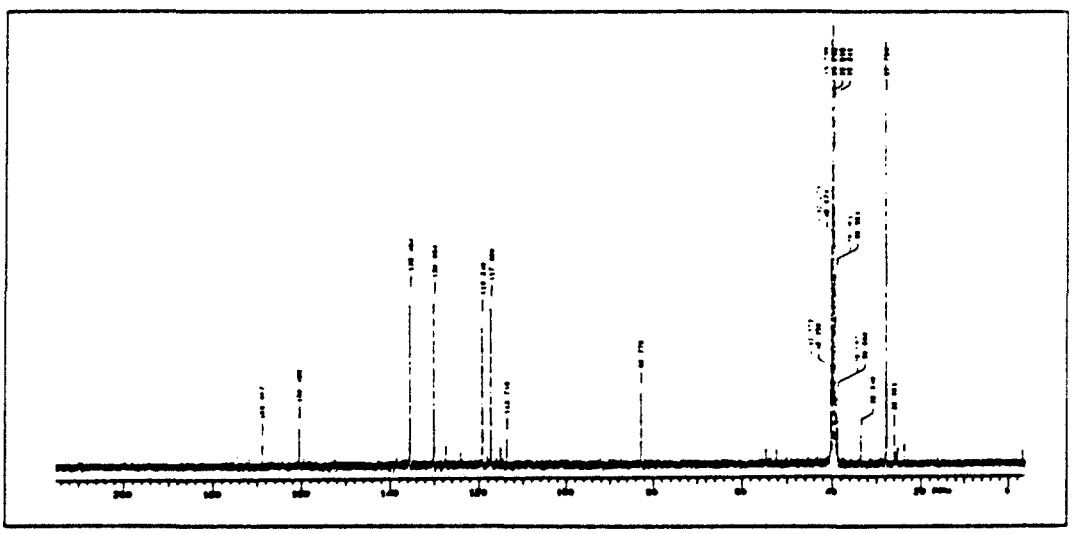


Figure 3. FTIR of *tert*-butyl salicylate (KBr).

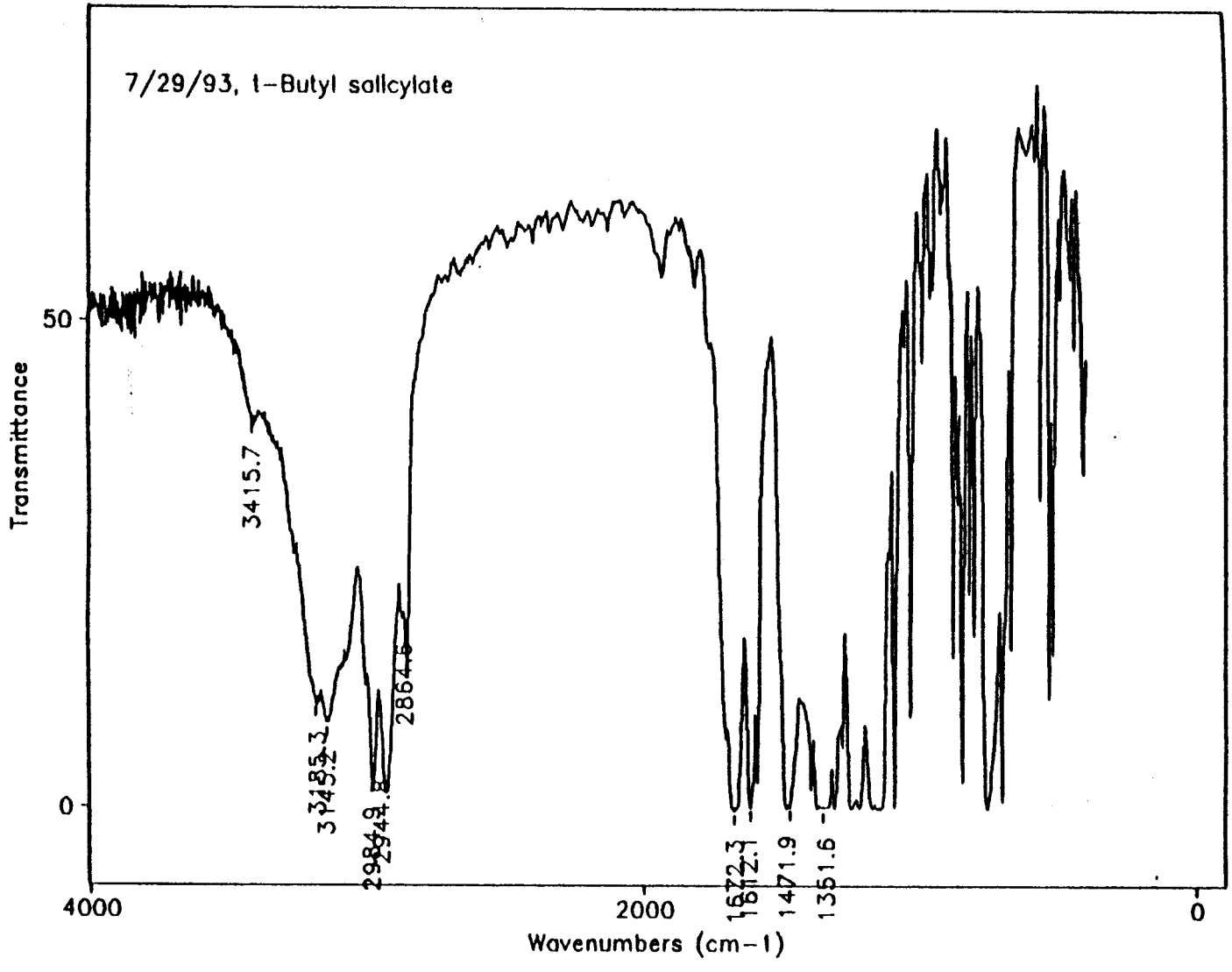
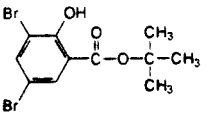
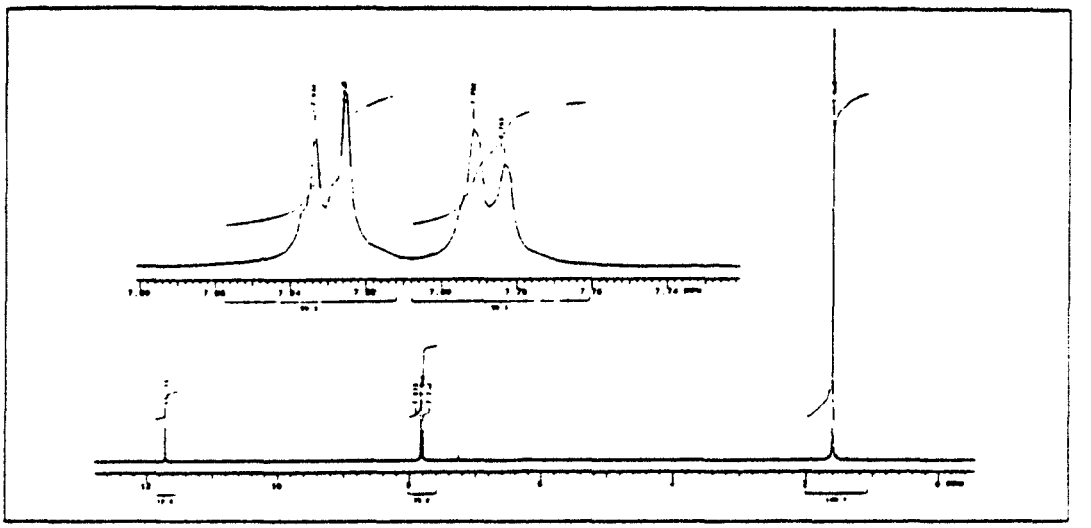

7/29/93, t-Butyl salicylate
Transmittance
50
0
3415.7
2944.8
2864.6
1672.3
1612.1
1471.9
1351.6
4000
2000
0
Wavenumbers (cm-1)

Figure 4. ^{1}H and ^{13}C NMR of *tert*-butyl 3,5-dibromosalicylate ($CDCl_3$).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

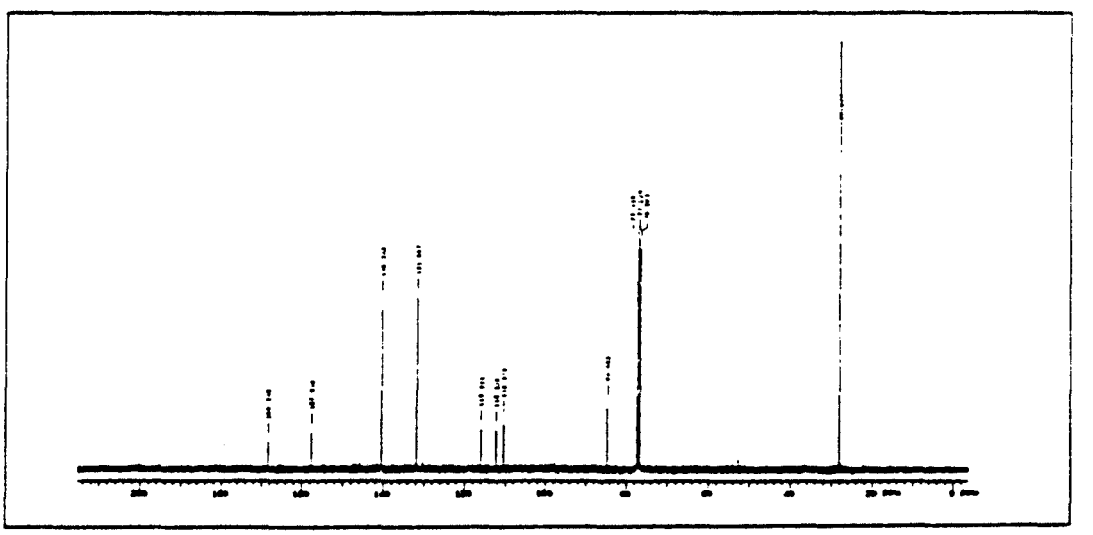
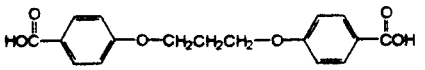
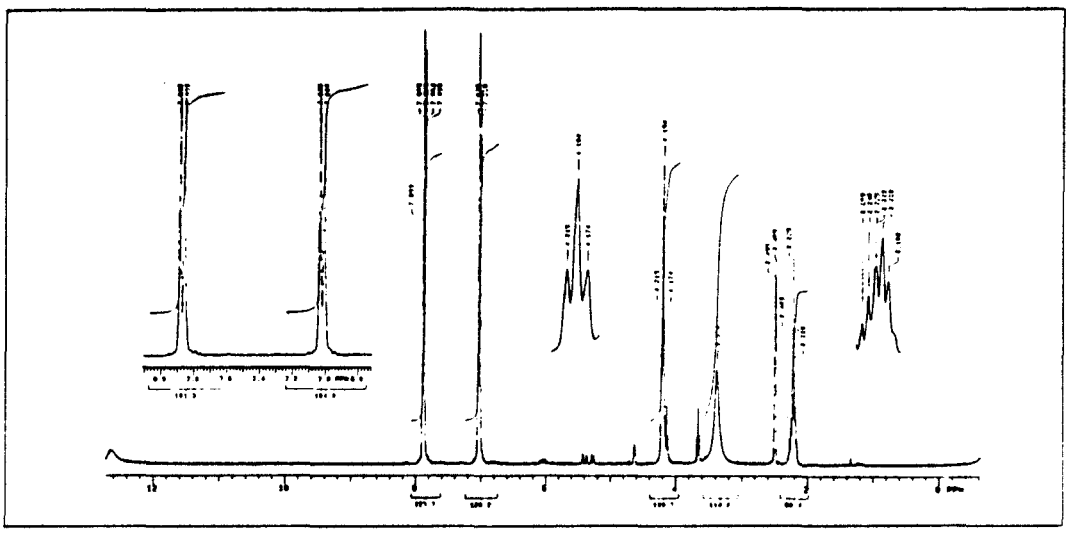


Figure 5. ^{1}H and ^{13}C NMR of 4,4'-(1,3-propanedioxy) dibenzoic acid (DMSO-d_6).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

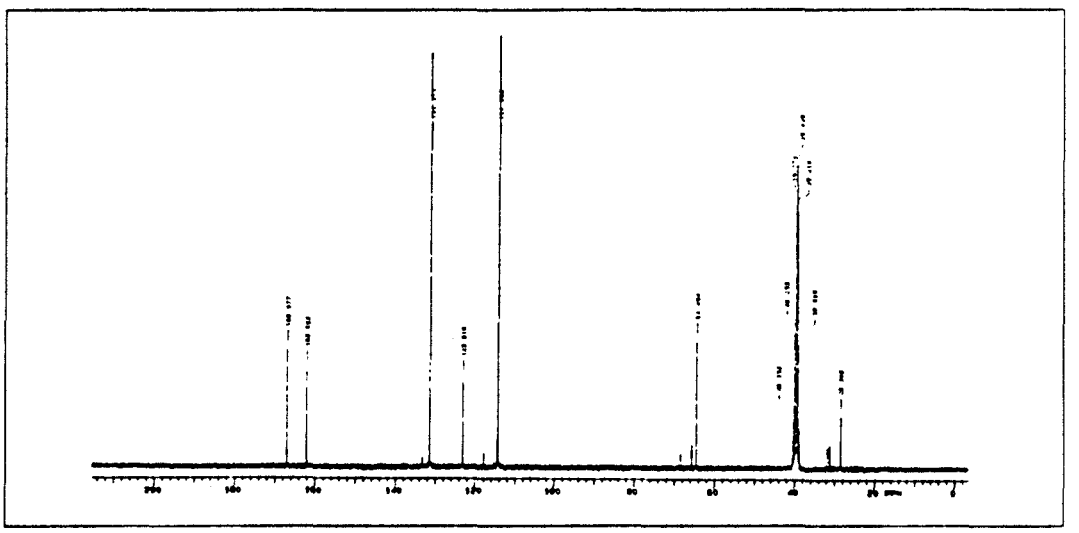
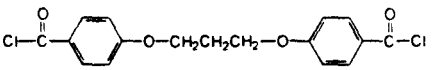
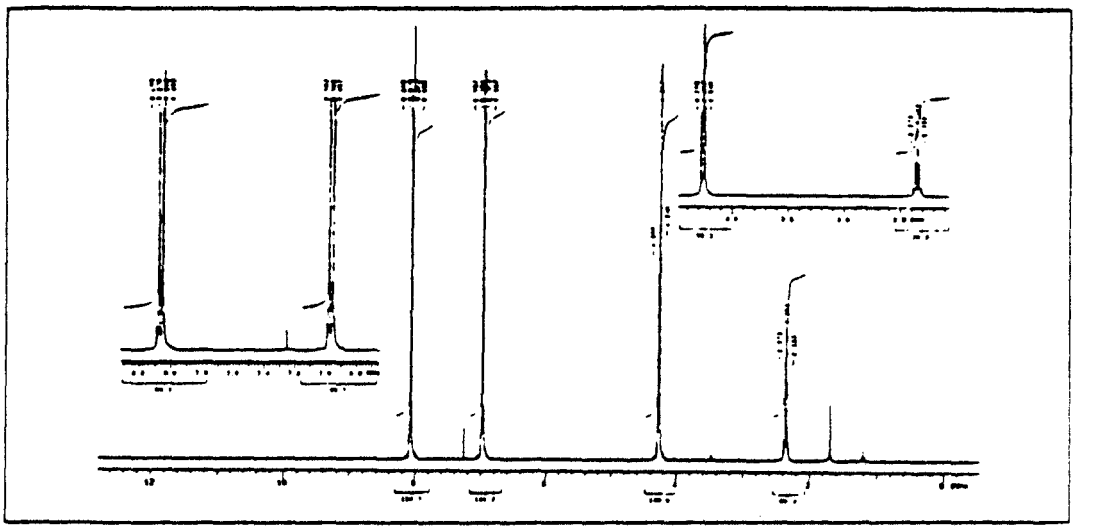


Figure 6. ^{1}H and ^{13}C NMR of 4,4'-(1,3-propanedioxy) dibenzoyl dichloride ($CDCl_3$).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

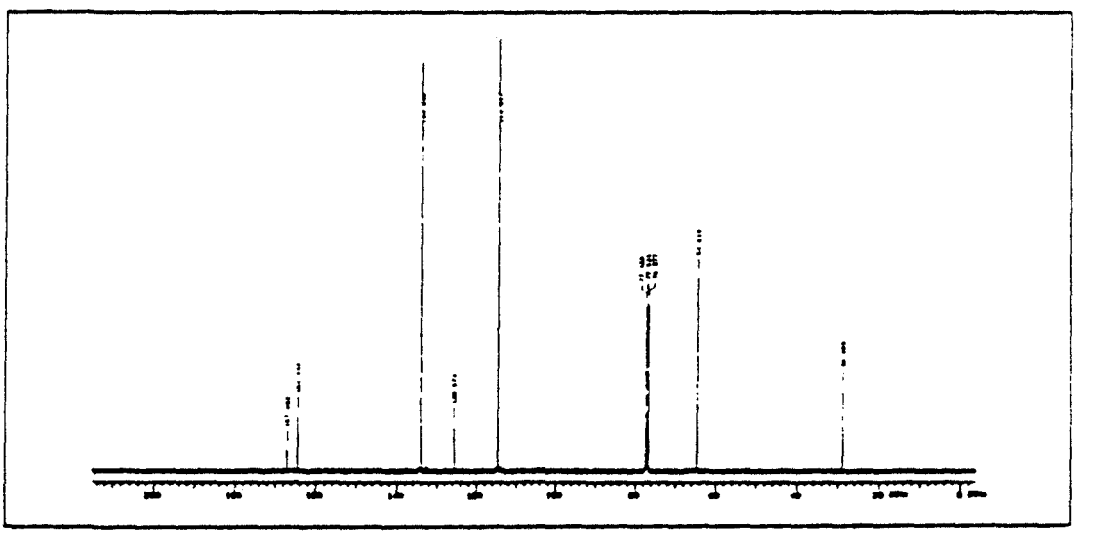
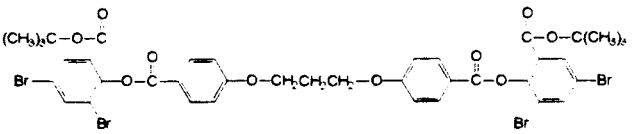
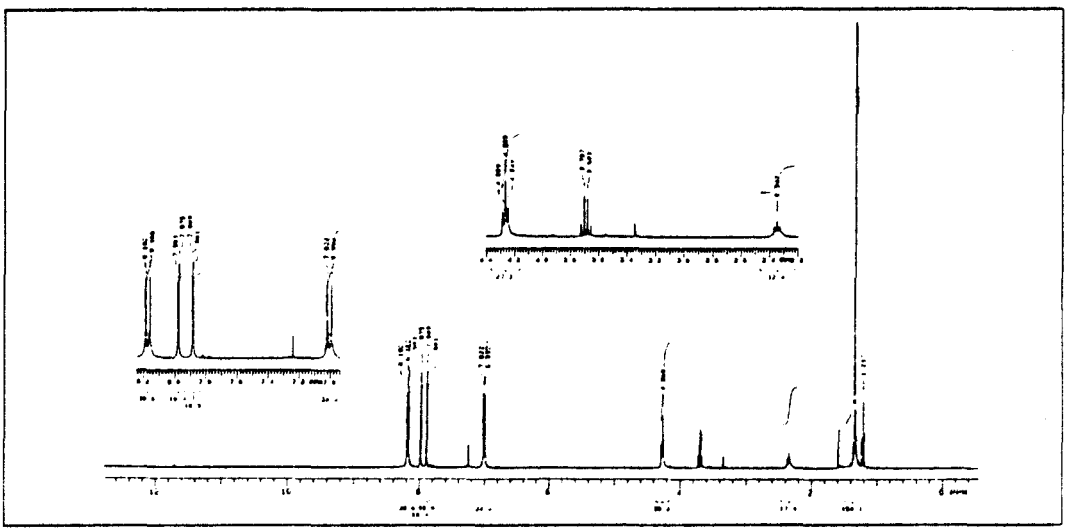


Figure 7. ^{1}H and ^{13}C NMR of bis(*tert*-butyl 3,5-dibromosalicyl) 4,4'-(1,3-propanedioxy) dibenzoate ($CDCl_3$).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

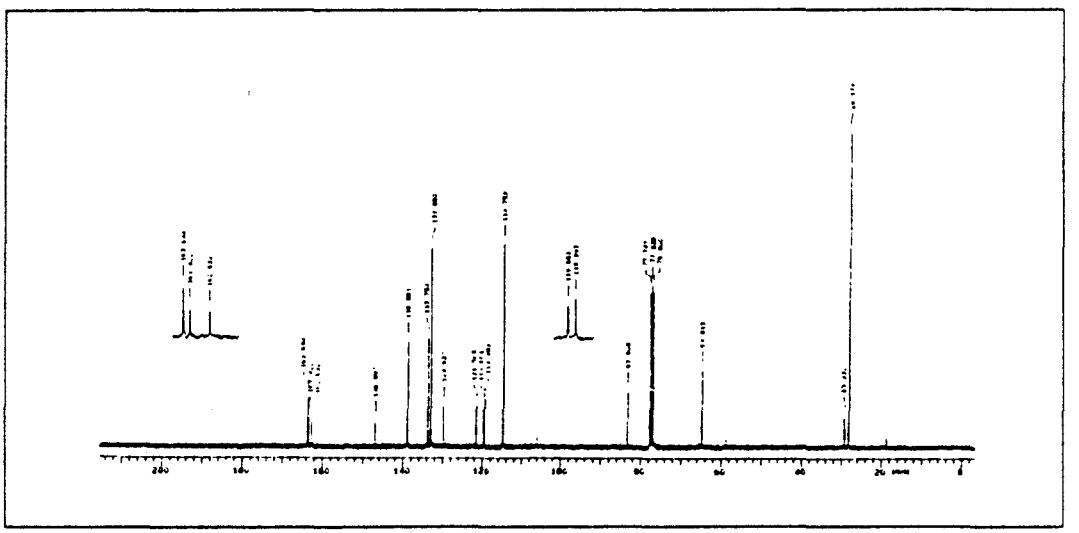
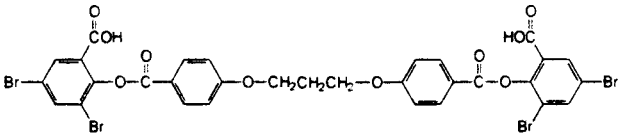
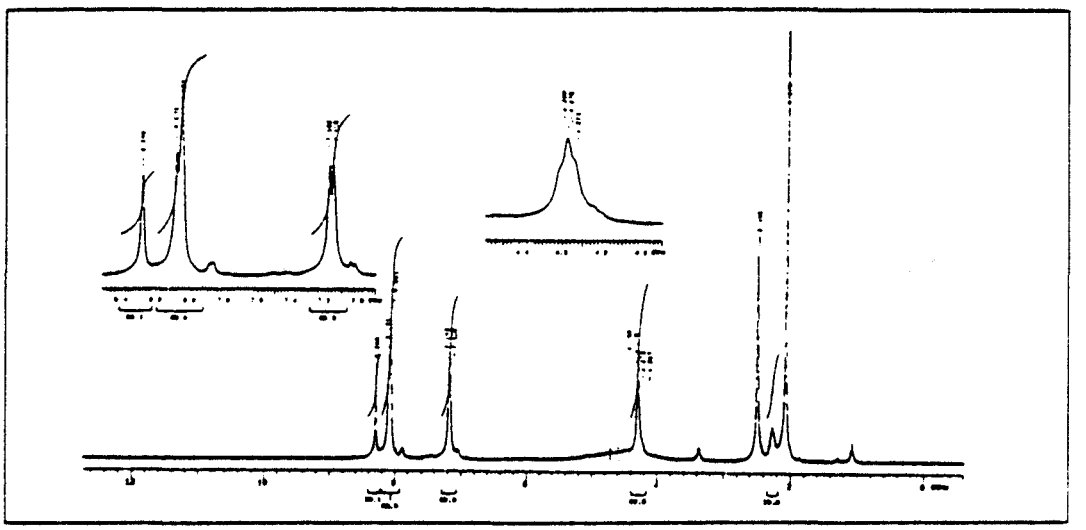


Figure 8. **^{1}H and ^{13}C NMR of bis(3,5-dibromosalicyl) 4,4'-(1,3-propanedioxy) dibenzoate (DMSO-d_6).**



(a) ^{1}H NMR.



(b) ^{13}C NMR.

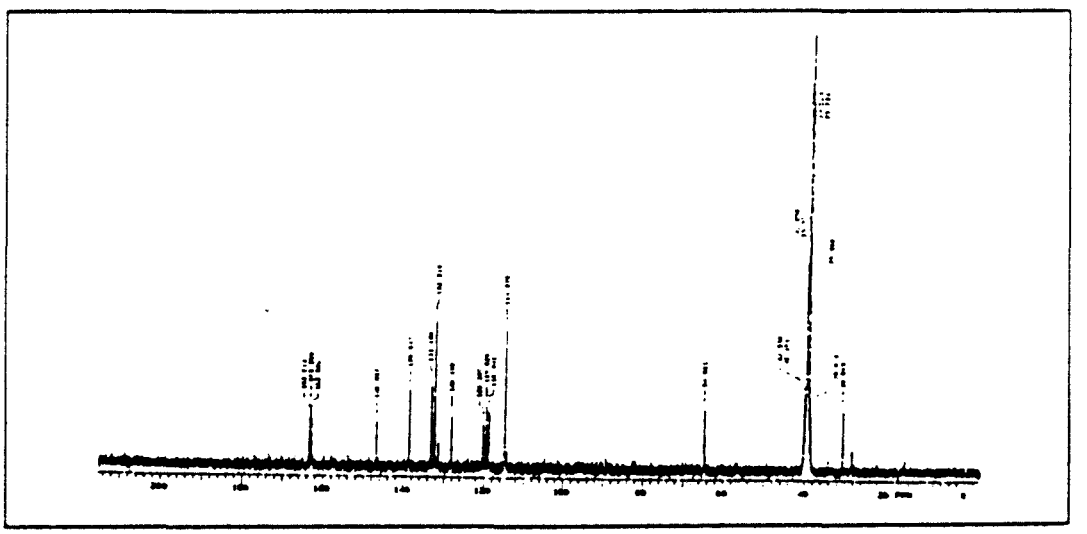
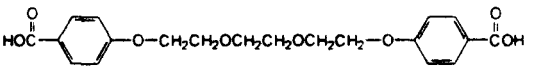
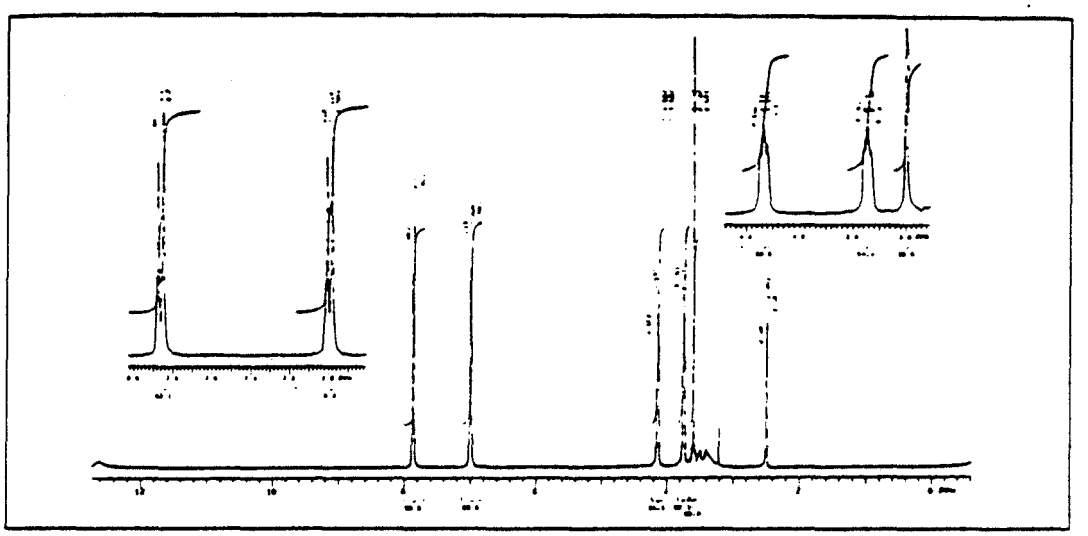


Figure 9. ^{1}H and ^{13}C NMR of 4,4'-triethyleneglycol dibenzoyl acid ether (DMSO-d_6).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

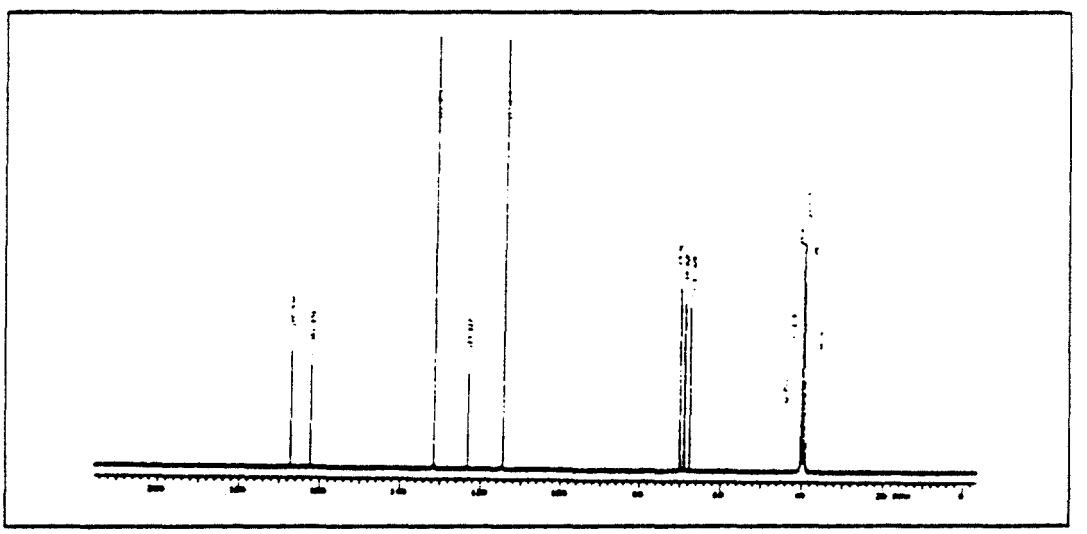
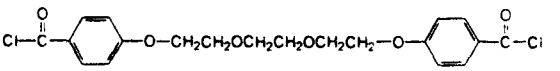
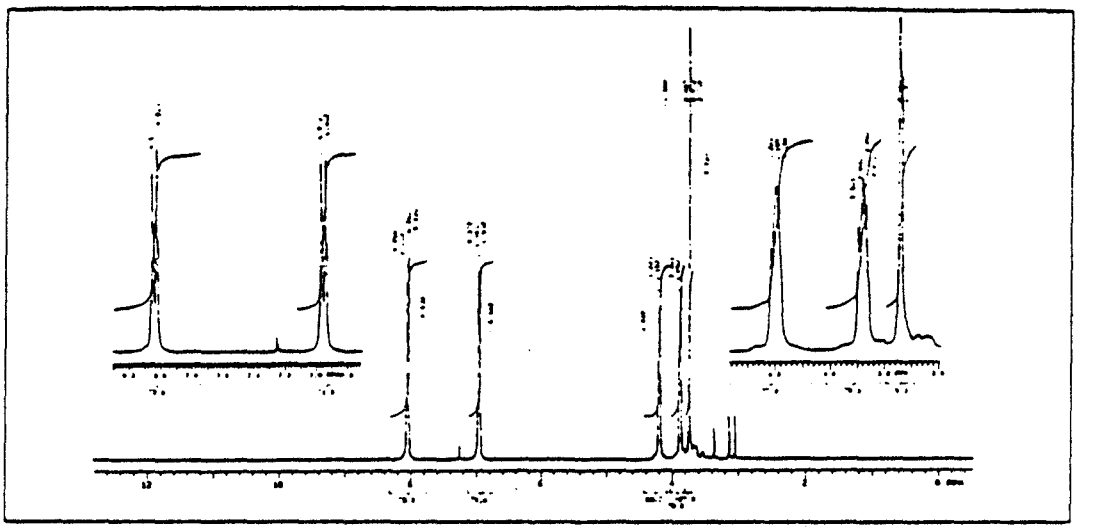


Figure 10. ^{1}H and ^{13}C NMR of 4,4'-triethyleneglycol dibenzoyl ether dichloride ($CDCl_3$).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

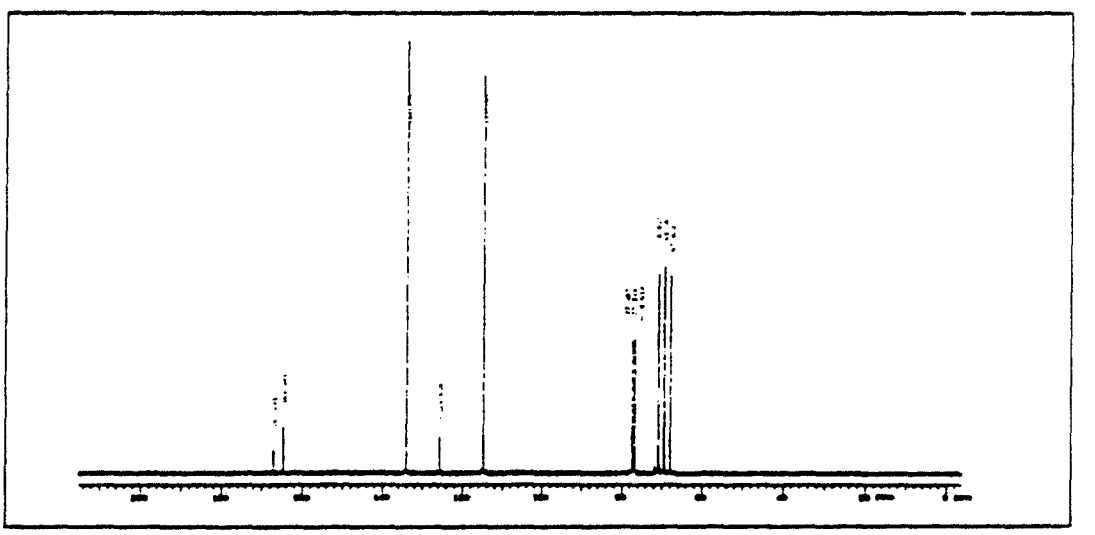
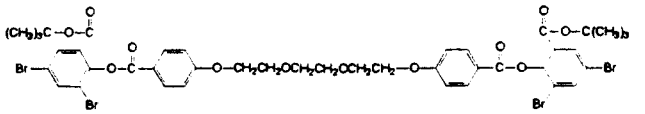
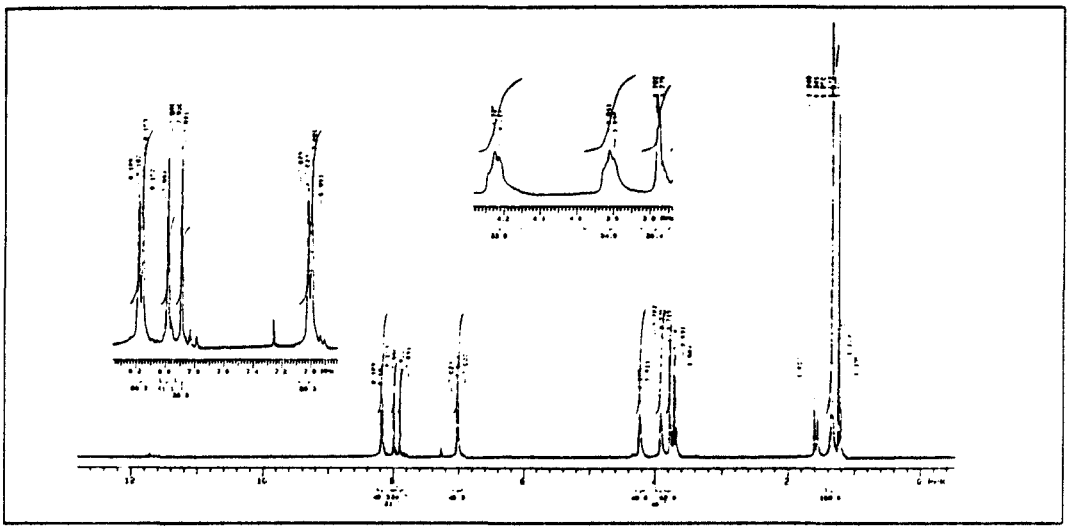


Figure 11. ^{1}H and ^{13}C NMR of bis(*tert*-butyl 3,5-dibromosalicyl) 4,4'-triethyleneglycol dibenzoate ether ($CDCl_3$).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

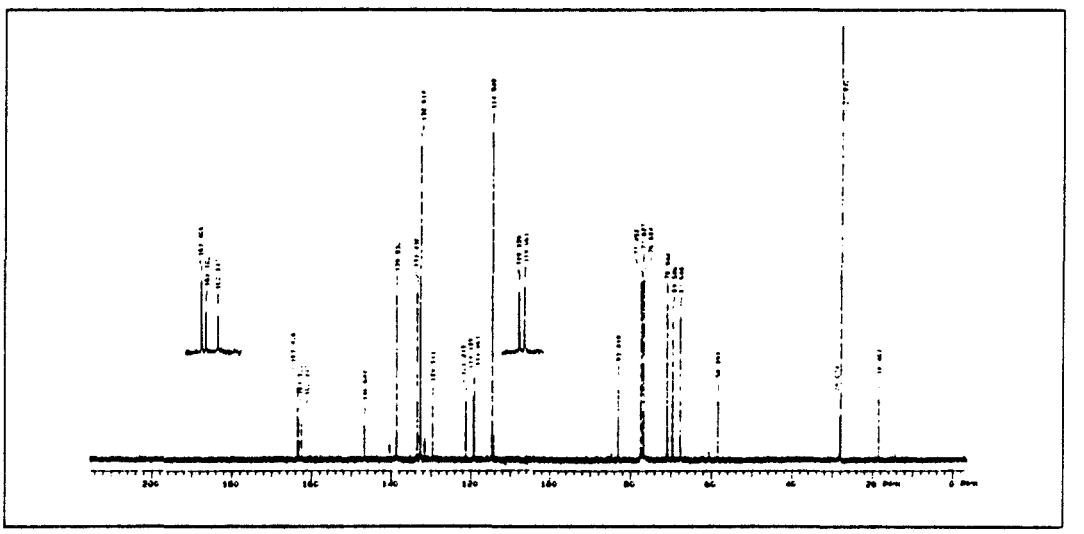
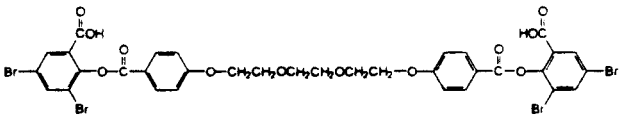
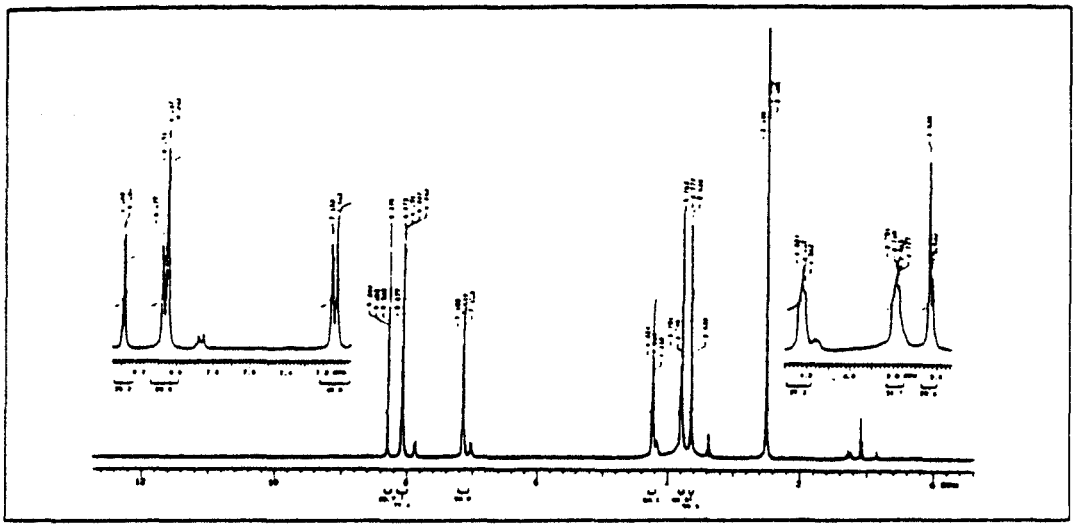


Figure 12. ^{1}H and ^{13}C NMR of bis(3,5-dibromosalicyl) 4,4'-triethyleneglycol dibenzoate ether (DMSO-d_6).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

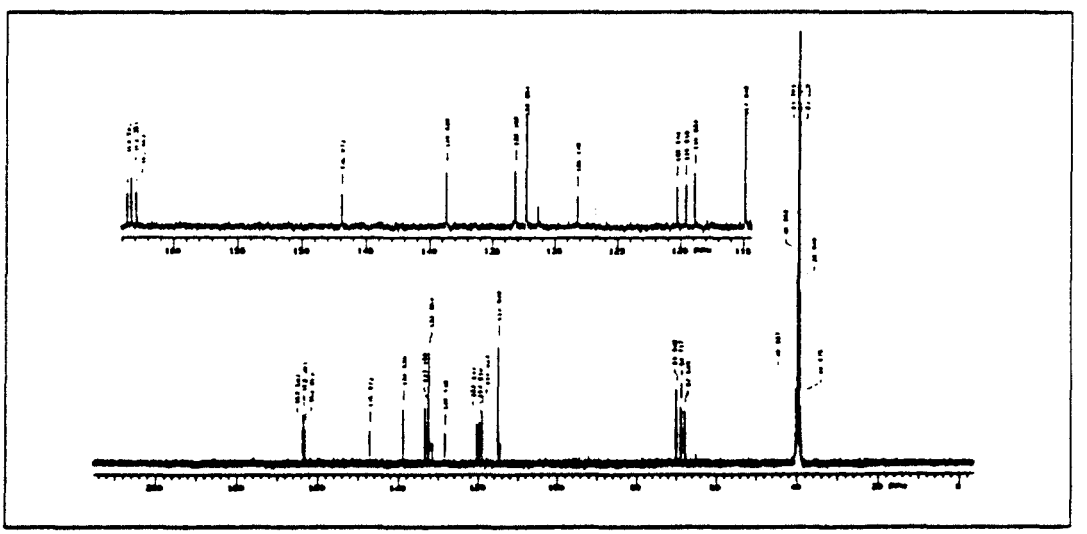
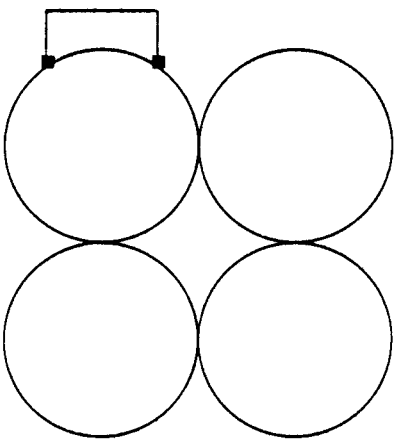
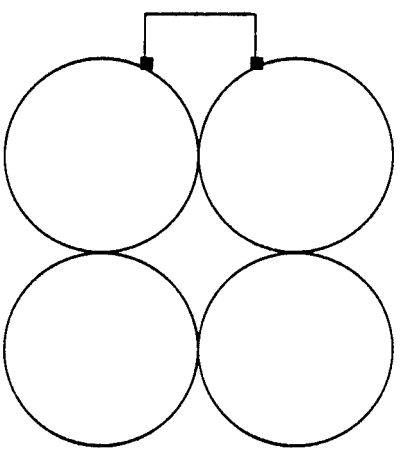


Figure 13. Possible intra and inter-subunit crosslinking of hemoglobin with the elongate bi-linkers.



(B)



(A)

Chapter 4

TRIS(3,5-DIBROMOSALICYL) TRICARBALLYLATE AND CROSSLINKING OF HEMOGLOBIN WITH THE TRILINKER

RESULTS

The first section of this chapter summarizes the analytical results for each of the compounds synthesized. The spectra are given at the end of the chapter.

Tricarballylic 1-acid chloride 2,3-anhydride

^{1}H NMR (DMSO-d_6) (see **Figure 14 (a)**): δ (ppm) 3.52-3.48 (m, 2H, CH_2-C=O), 3.46-3.39 (m, 1H, CH-C=O), 3.30-2.77 (m, 2H, CH_2-C=O).

^{13}C NMR (DMSO-d_6) (see **Figure 14 (b)**): δ (ppm) 172.30 (s, 1C, C=O), 171.35 (s, 1C, C=O), 168.11 (s, 1C, C=O), 46.11 (s, 1C, C*-C=O), 37.20 (s, 1C, C*-C=O), 33.40 (s, 1C, C*-C=O).

Tricarballylyl trichloride

Boiling point: 117-119 °C at 4.2-4.3 mmHg.

^{1}H NMR ($CDCl_3$,) (see **Figure 15 (a)**): δ 3.65-3.57(m, 1H, CH-C=O), 3.56-3.28 (m, 4H, CH_2-C=O).

^{13}C NMR ($CDCl_3$) (see **Figure 15 (b)**): δ (ppm) 172.34 (s, 1C, C=O), 171.51 (s, 2C, C=O), 48.37 (s, 1C, C*H-C=O), 46.34 (s, 2C, C*H_2-C=O).

FTIR (KBr) (see **Figure 16**): 2934.0 (d), 1788.1 (s), 970.9 (m) cm^{-1}.

Tris(*tert*-butyl 3,5-dibromosalicyl) tricarballylate

Melting point: 66-70 °C.

^1H NMR ($CDCl_3$) (see **Figure 17 (a)**): δ (ppm) 7.94-7.81 (m, 6H, ArH), 3.95 (m, 1H, CH-C=O), 3.61-3.22 (m, 4H, CH_2-C=O), 1.56 (d, 27H, tBu).

^{13}C NMR ($CDCl_3$) (see **Figure 17 (b)**): δ (ppm) 169.51-168.38 (s, 3C, C=O), 161.44 (s, 3C, C=O), 146.91 (d, 3C, O-ArC), 138.74 (d, 3C, ArC), 133.12 (d, 3C, ArC), 128,26 (d, 3C, ArC), 119.42 (d, 3C, ArC), 119.15 (d, 3C, ArC), 83.04 (d, 3C, O-C), 36.92 (s, 1C, C*H-C=O), 34.60 (s, 2C, C*H_2-C=O), 28.01 (s, 9C, tBu).

Tris(3,5-dibromosalicyl) tricarballylate

Melting point: 147-151 °C.

^1H NMR (DMSO-d_6) (see **Figure 18 (a)**): δ (ppm) 8.26 (m, 3H, ArH), 8.04 (m, 3H, ArH), 3.73 (m, 1H, CH-C=O), 3.45-3.10 (m, 4H, CH_2-C=O).

^{13}C NMR (DMSO-d_6) (see **Figure 18 (b)**): δ (ppm) 169.16-168.30 (s, 3C, C=O), 163.50 (d, 3C, As-C=O), 146.50 (d, 3C, O-ArC), 138.89 (d, 3C, ArC), 133.27 (d, 3C, ArC), 127.48 (d, 3C, ArC), 119.16 (d, 3C, ArC), 119.07 (d, 3C, ArC), 36.39 (s, 1C, C*H-C=O), 34.10 (s, 2C, C*H_2-C=O).

Isolation of the crosslinked proteins

Using ion-exchange chromatography, three components were isolated from the reaction of oxyhemoglobin (**Figure 19**) and four components from the reaction of deoxyhemoglobin (**Figure 20**) with tris(3,5-dibromosalicyl) tricarballylate. SDS-PAGE exhibited that the first peaks of both oxy and deoxy reactions were uncrosslinked hemoglobin and the other peaks were bi-crosslinked hemoglobins (**Table 5**); the second peaks were the major products from both oxy and deoxy reactions.

Denaturation

The effect of the tricarballylate crosslink on the thermal stability of hemoglobin was remarkable as shown in **Figure 21**. Native hemoglobin and the components from the second peaks of both oxy and deoxy reactions were tested. The denaturation transition (T_m) of native hemoglobin is 41.0°C and those of the oxy and deoxy crosslinked proteins 55.5 °C and 54.0 °C, increasing 14.5 and 13.0°C, respectively.

Autoxidation

Autoxidation of native hemoglobin and the components from the second peaks of oxy and deoxy reactions were tested (see **Figure 22**). The deoxy crosslinked protein (k_{app} = 8.70 × 10^{-2} ± 4.15 × 10^{-7}) was found to be more susceptible to autoxidation than native protein (k_{app} = 6.49 × 10^{-2} ± 2.32 × 10^{-7}). The autoxidation rate of the oxy crosslinked protein (k_{app} = 6.89 × 10^{-2} ± 2.75 × 10^{-7}) however, remained unchanged after crosslinking, as we desired.

Oxygen-binding

Table 5. SDS-PAGE Results for Isolated Crosslinked Hemoglobins.

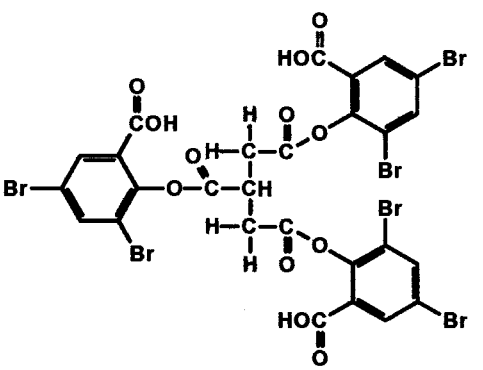
Sample	% Monomer	% Dimer
Hb A	100	0
Oxy XL-Hb A		
Peak 1	100	0
Peak 2	50	50
Peak 3	56	44
Deoxy XL-Hb A		
Peak 1	100	0
Peak 2	50	50
Peak 3	59	41
Peak 4	57	43

Figure 23 shows the oxygenation curves of fresh blood (P_{50} = 20.2 mmHg, n = 2.8), native hemoglobin (P_{50} = 11.7 mmHg, n = 2.0) and the components of the second peaks from the oxy (P_{50} = 3.6 mmHg, n = 1.2) and deoxy (P_{50} = 7.7 mmHg, n = 1.2) crosslinking. Obviously, in both oxy and deoxy crosslinking reactions the oxygen affinity of the hemoglobins increased and the Hill coefficient decreased.

The relevant data of denaturation, autoxidation and oxygen-binding are summarized in **Table 6**.

DISCUSSION

The synthesis of tris(3,5-dibromosalicyl) triscarballylate used the method of Bucci et al. [92] but the production of tricarballylyl trichloride differred. Instead of phosphorus pentachloride (PCl_5), we used $SOCl_2$ with DMF catalysis to obtain the acid chloride. However, formation of the acid chloride is dependent on the amount of DMF. Tricarballylic 1-acid chloride 2,3-anhydride (crystals) (**Figure 14**) or tricarballylyl trichloride (liquid) (**Figure 15**) was obtained if less or a greater amount of DMF was applied, respectively. The desired trilinker was characterized by ^{1}H and ^{13}C NMR spectroscopy (**Figure 18**): the six



Tris(3,5-dibromosalicyl) tricarballylate

Table 6. Comparison of Functional Properties of Crosslinked Hemoglobins.

Sample	T_m (°C)	$k_{app}\times100$ (h^{-1})	$t_{1/2}$ (h)	P_{50}	n
Fresh RBC	NA	NA	NA	20.2	2.8
Hb A	41.0	$6.49\pm2.32\times10^{-5}$	10.7	11.7	2.0
Deoxy-XL-Hb A	54.0	$8.70\pm4.15\times10^{-5}$	7.97	7.7	1.2
Oxy-XL-Hb A	55.5	$6.89\pm2.75\times10^{-5}$	10.06	3.6	1.2

hydrogen resonances at chemical shift δ 8.26 and 8.04 ppm are due to the three aspirin's aryl hydrogens, the one resonance at δ 3.73 is due to the methine, and because of the stereo structure, two hydrogen resonances, one due to each of the two methylenes are at δ 3.45-3.38 and the other two are at δ 3.18-3.10; the two peaks at δ 169.16 and 168.30 are assigned to the tricarballylate carbonyl carbons; a peak at δ 163.50, to the carbonyl carbons on the aspirin aromatic rings; six peaks at δ 146.49 - 119.07, to the carbons of aspirin aromatic rings; a peak at δ 36.39, to the methine carbon and a peak at δ 34.10, to the methylene carbons. The synthesis of the reagent was successful.

The isolations of the DBSTC crosslinked derivatives (**Figures 19 a** and **20 a**) show only one major product corresponding to the bi-linked species from both oxy and deoxyhemoglobin reactions. It is likely that the third 3,5-dibromosalicyl ester group is hydrolyzed and does not react with another hemoglobin residue. This type of reaction has been observed previously with tris(3,5-dibromosalicyl) trimesate [78]. SDS-PAGE gels showed the expected 1:1 ratio of dimers to monomers for a protein with a single inter-subunit crosslink (**Figures 19 b** and **20 b** and **Table 5**). The intramolecular crosslink improved the thermal stability.

According to the previous work [69, 78], the most likely possibility of the crosslinking of hemoglobin by the tri-linker was at the specific site between Lys$\beta_1$82 and Lys$\beta_2$82, which is consistant with these results. The crosslinking of intra-hemoglobin covalently between two β chains apparently improved the

thermal stability (**Figure 21** and **Table 6**). The crosslinking of both oxy and deoxyhemoglobin increased the T_m by 14.5 and 13.0 °C (Figure 4), respectively. The increase in T_m was identical to that observed to similar length crosslinks between Lys82β_1 and Lys82β_2 [71-73].

The autoxidation of hemoglobin is a pseudo first-order reaction within 17 h (**Figure 22**). The faster rate of autoxidation of the deoxy crosslinked hemoglobin than those of native hemoglobin and the oxy crosslinked hemoglobin (**Figure 22** and **Table 6**) resulted from the different quaternary structures after crosslinking. Deoxyhemoglobin (T state) autoxidizes faster than oxyhemoglobin (R state) [26, 94]. Yang and Olsen [95] showed that the apparent autoxidation rate of fumarate-crosslinked hemoglobins increased 78% for the α99 Lys crosslinked species that favors the T state conformation but only 22% for the β82 Lys crosslinked protein that prefers the R state. The 34% increase observed here for the tricarballylate crosslinked hemoglobin is similar to the earlier results for the β82 Lys fumarate-crosslinked hemoglobin. The two β-chains are closer in the R state than in the T state [96-97]. Thus, the Lys β82's are closer in oxyhemoglobin than in deoxyhemoglobin. The size of DBSTC prevents it from entering the cavity between the α-chains. Therefore, the most probable site for crosslinking by this reagent is between the two Lys β82's under either oxy or deoxy reactions. Due to the length of the reagent, both oxy and deoxy reaction products would favor the R state quaternary structure. The slight decrease in

oxygen affinity of the product produced under deoxy conditions corresponds to an increase in autoxidation rate. This effect is more dramatic in some other crosslinked hemoglobins, such as the α99 fumarate crosslinked species [95]. However, results from some crosslinked hemoglobins are difficult to give a reasonable explanation using conformation theory. Generally, crosslinking of hemoglobin at the BPG binding-site effects the autoxidation rate less than at some where else. This will be discussed more in Chapter 5.

It was known that in the oxy state the two β chains were closer to each other than those in deoxy state [96-97], and therefore the Lysβ82's in the R state would be closer to each other than those in the T state.

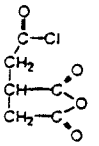
Although the Hill coefficient *n* decreased due to DBSTC crosslinking, the *n* values of the crosslinked proteins are still above 1.0, that is the oxygen binding remains slightly cooperative. Compared to native hemoglobin, the oxygen affinities of oxy crosslinked hemoglobins increased significantly (**Figure 23 and Table 6**) due to the R state conformation of the crosslinked proteins. The oxygen affinity of the deoxy crosslinked hemoglobin however, was not as low as we expected (**Figure 23** and **Table 6**). The crosslinking of deoxyhemoglobin by the short crosslinker would pull the Lysβ82's closer, resulting in the conformation to shift to R-state in some degree and an increase in the oxygen affinity. The distances between the three DBSTC ester carbonyl carbons are different due to the asymmetrical structure (**Table 7**). Therefore, deoxyhemoglobin would favor the longer side of DBSTC to crosslink, and oxyhemoglobin, the shorter side. It is

Table 7. Distances between DBSTC ester carbonyl carbons.

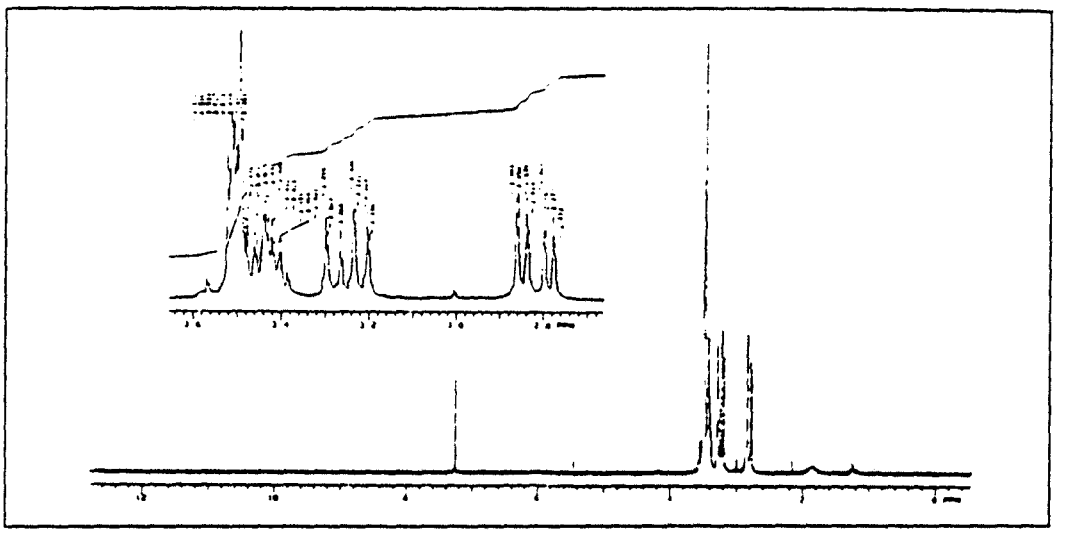
Tri-linler	Bridging distance (Å)		
DBSTC	5.31	5.75	6.19

consistant with Kluger's work [77] that oxygen affinity increases with decrease of bridging length.

Figure 14. ^{1}H and ^{13}C NMR of tricarballylic 1-acid chloride 2,3-anhydride (DMSO-d_6).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

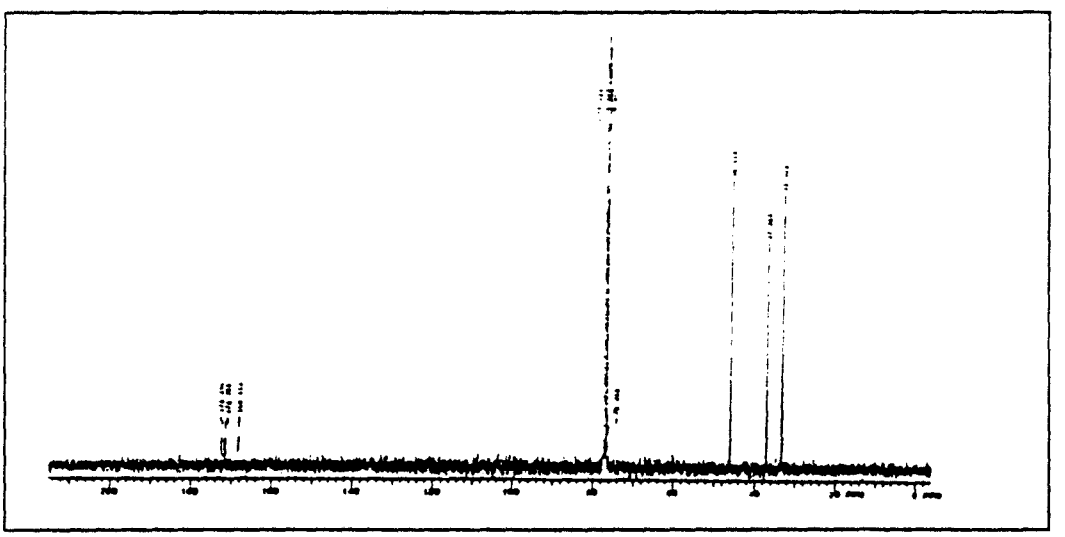
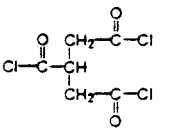
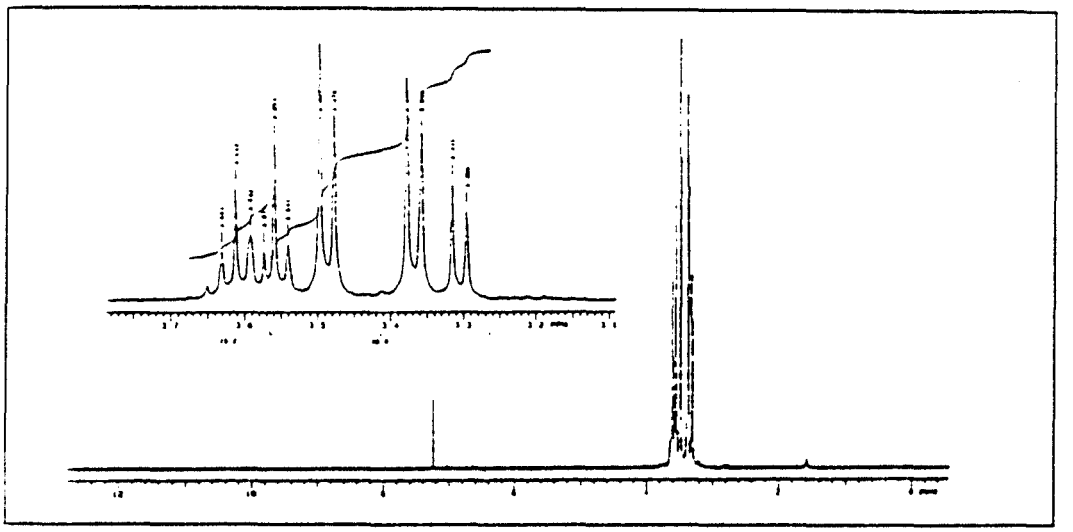


Figure 15. ^{1}H and ^{13}C NMR of tricarballylyl trichloride ($CDCl_3$).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

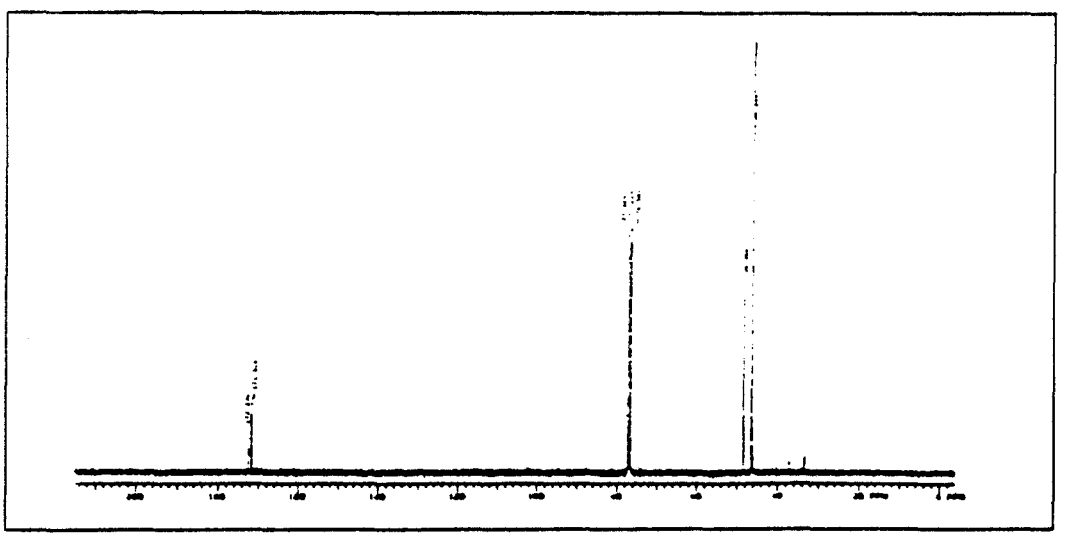


Figure 16. FTIR of tricarballylyl trichloride.

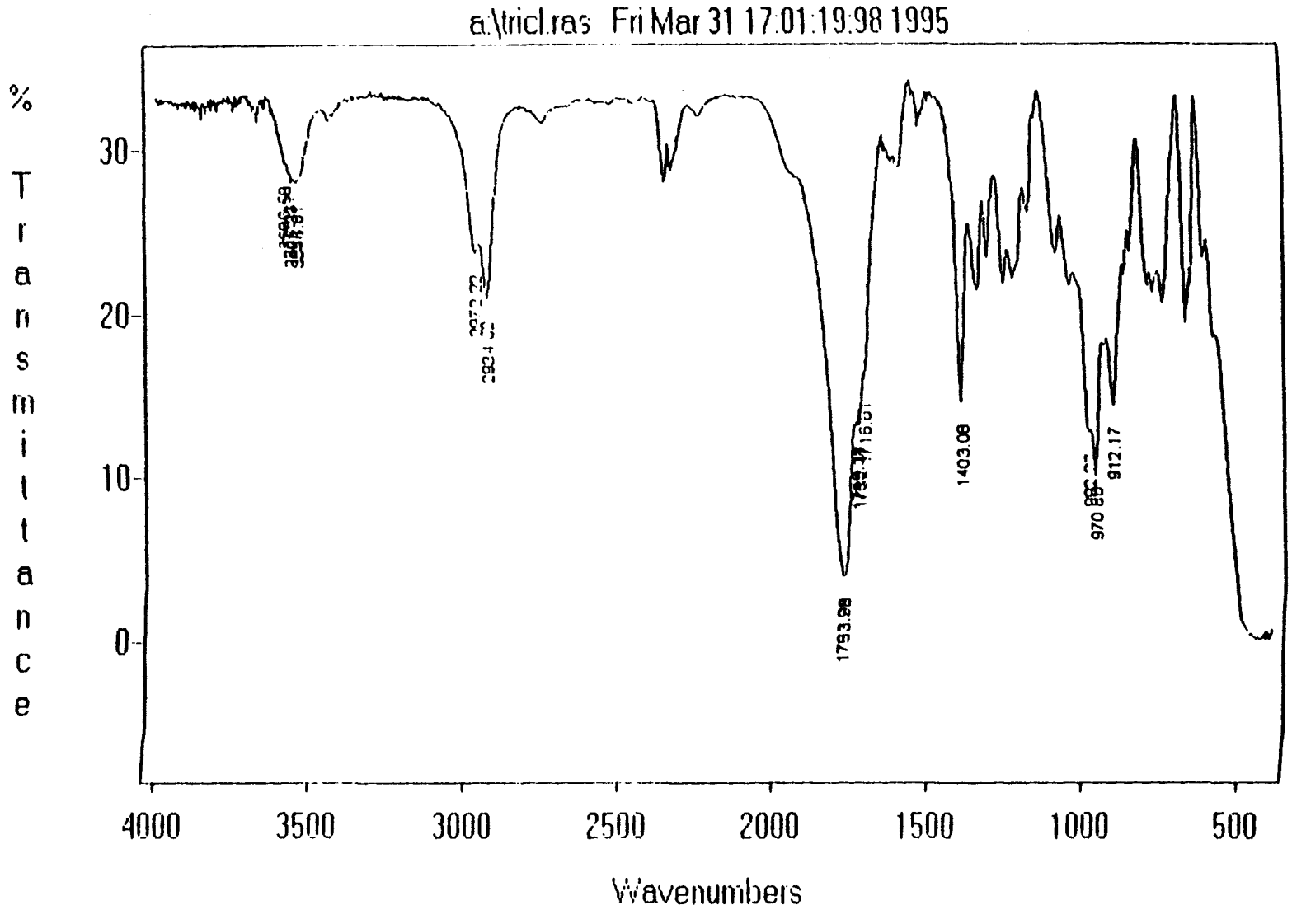
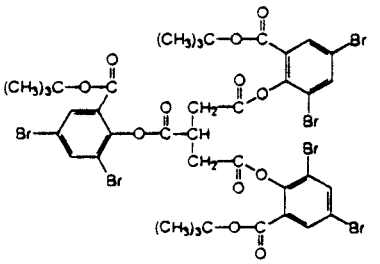
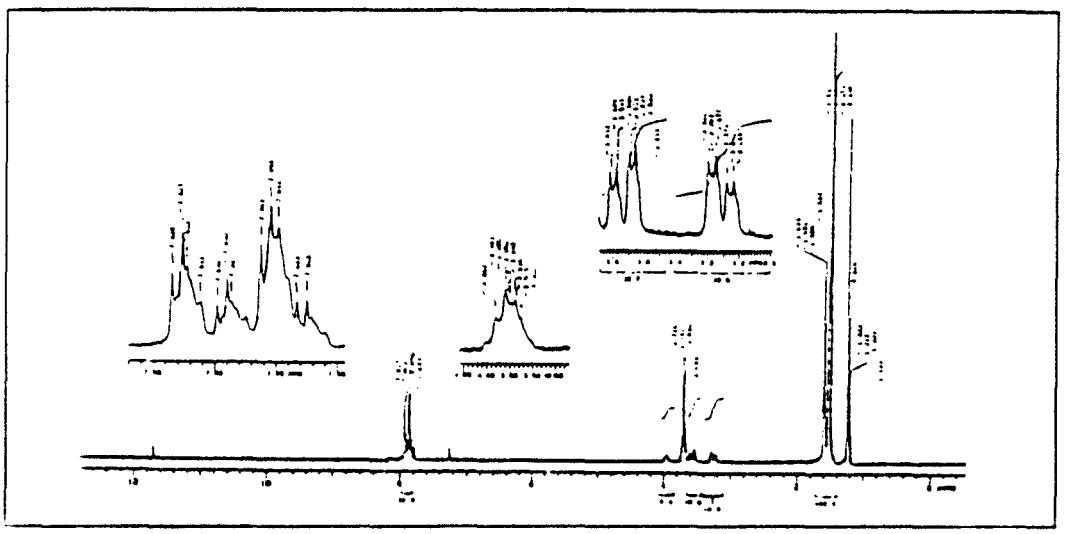

a:\tricl.ras Fri Mar 31 17:01:19:98 1995
% Transmittance
30
20
10
0
1793.98
1403.08
912.17
4000
3500
3000
2500
2000
1500
1000
500
Wavenumbers

Figure 17. ^{1}H and ^{13}C NMR of tris(*tert*-butyl 3,5-dibromosalicyl) tricarballylate ($CDCl_3$).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

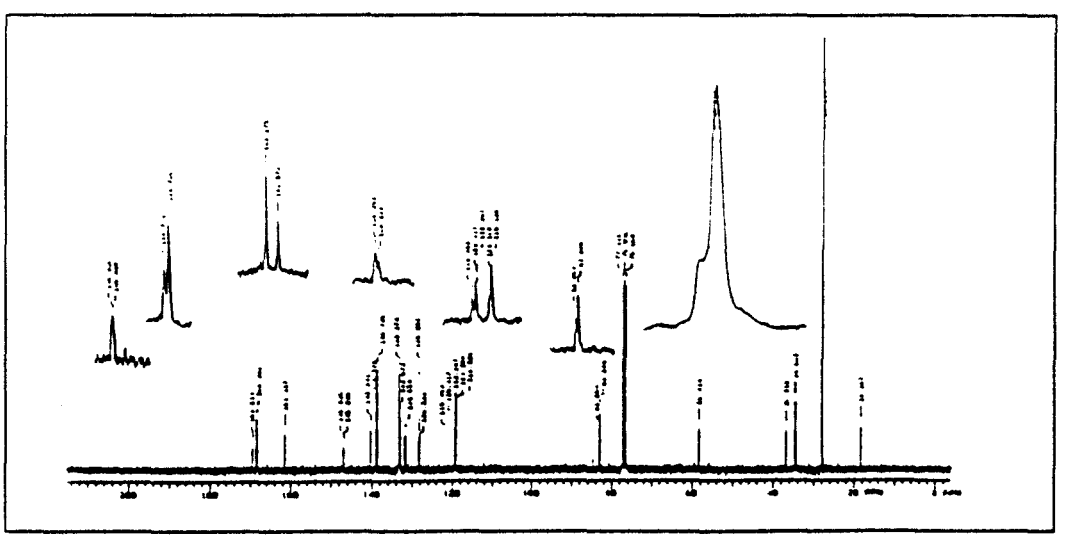
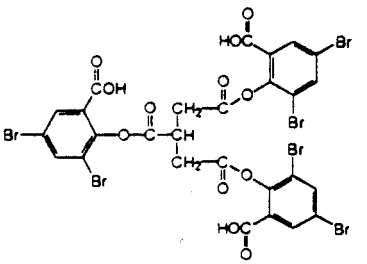
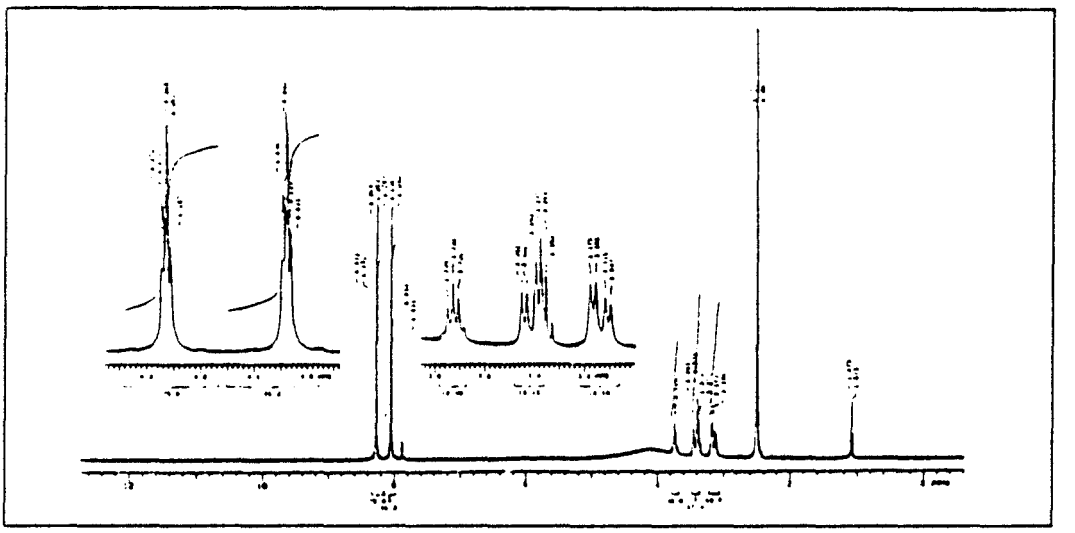


Figure 18. ^{1}H and ^{13}C NMR of tris(3,5-dibromosalicyl) tricarballylate (DMSO-d_6).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

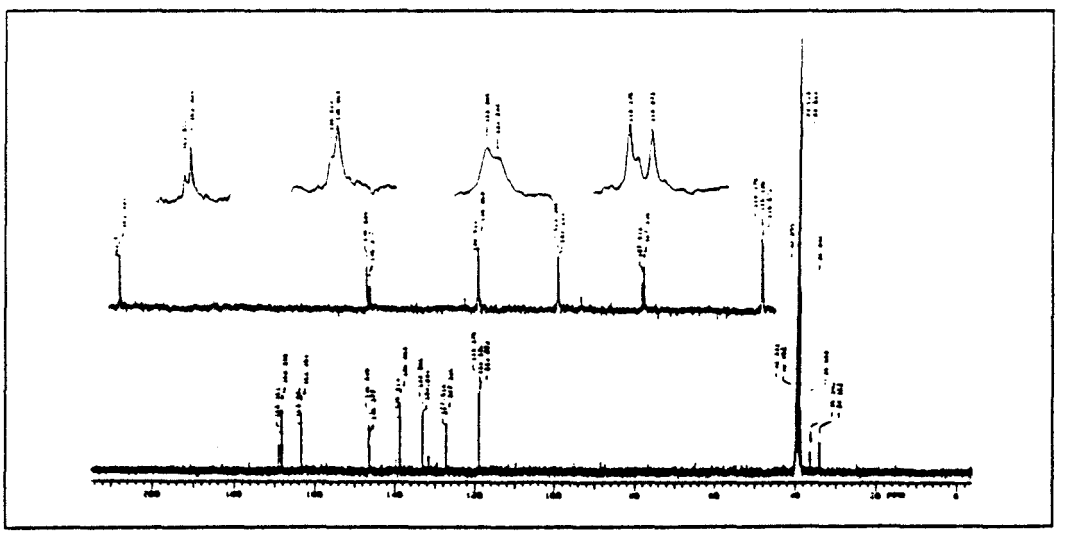
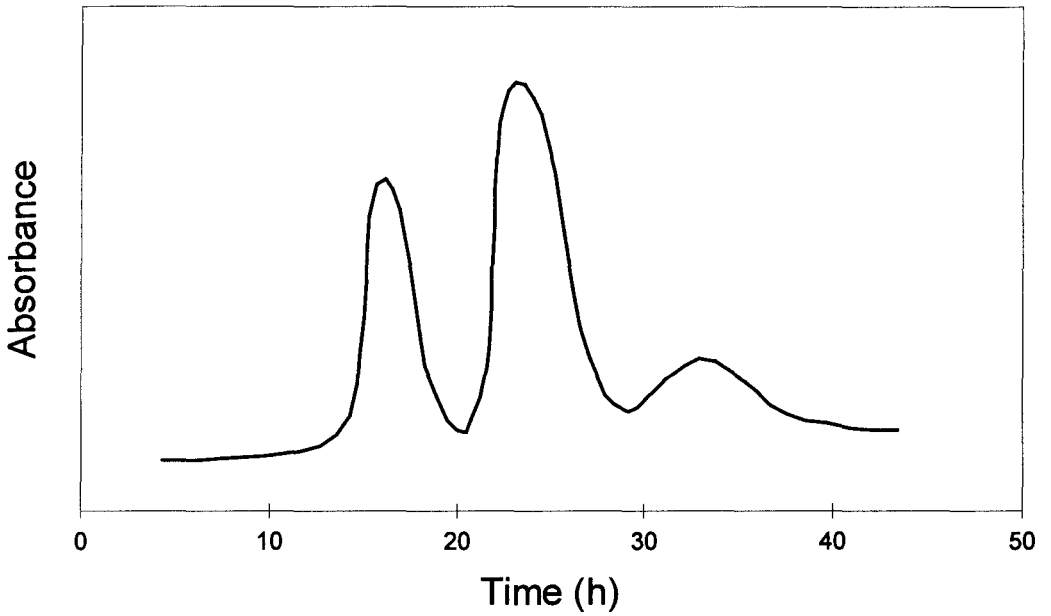


Figure 19. Anion-exchange chromatography and SDS-PAGE of hemoglobin crosslinked by DBSTC under oxy conditions.

(a) Ion-exchange chromatography.



(b) SDS-PAGE.

2 — Unisolated hemoglobin

3 — The first peak

4 — The second peak

5 — The third peak

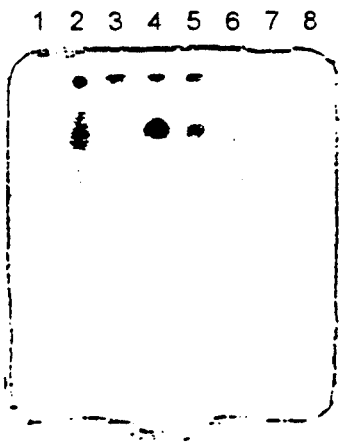
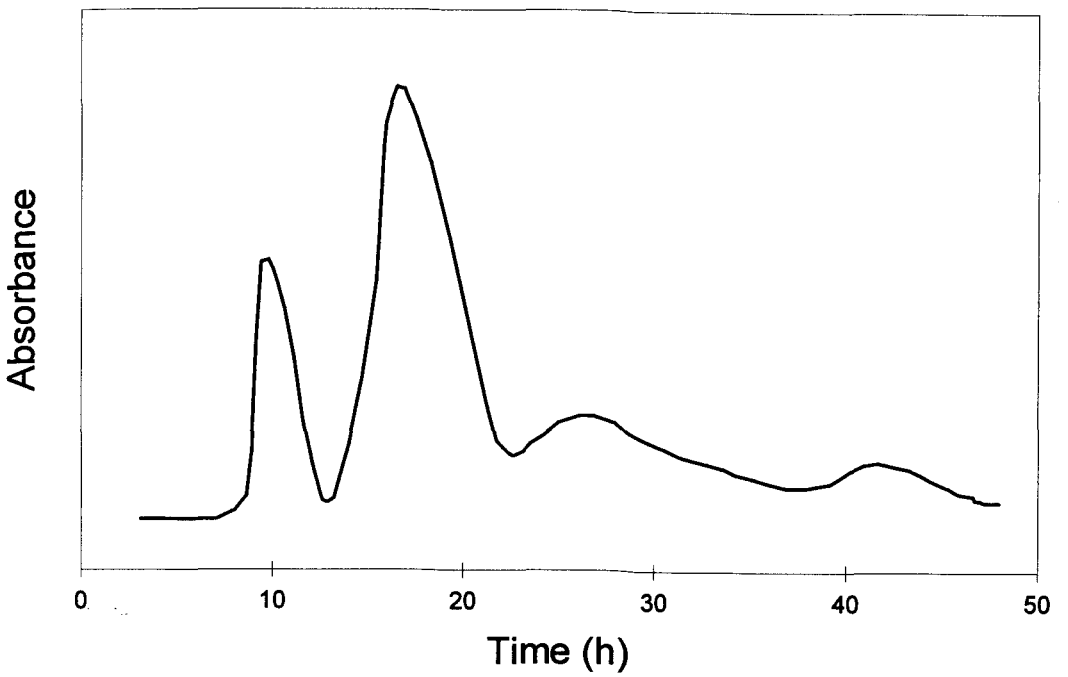


Figure 20. Anion-exchange chromatography and SDS-PAGE of hemoglobin crosslinked by DBSTC under deoxy conditions.

(a) Ion-exchange chromatography.



(b) SDS-PAGE.

1 — Protein markers

2 — Unisolated hemoglobin

3 — The first peak

4 — The second peak

5 — The third peak

6 —The 4th peak

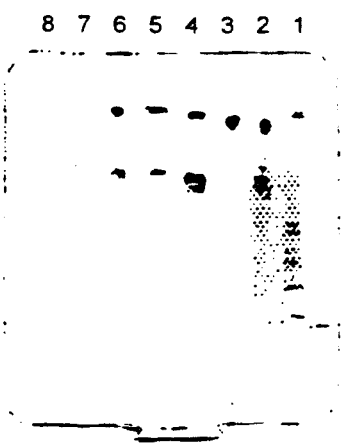


Figure 21. Denaturation of native and DBSTC crosslinked hemoglobins.

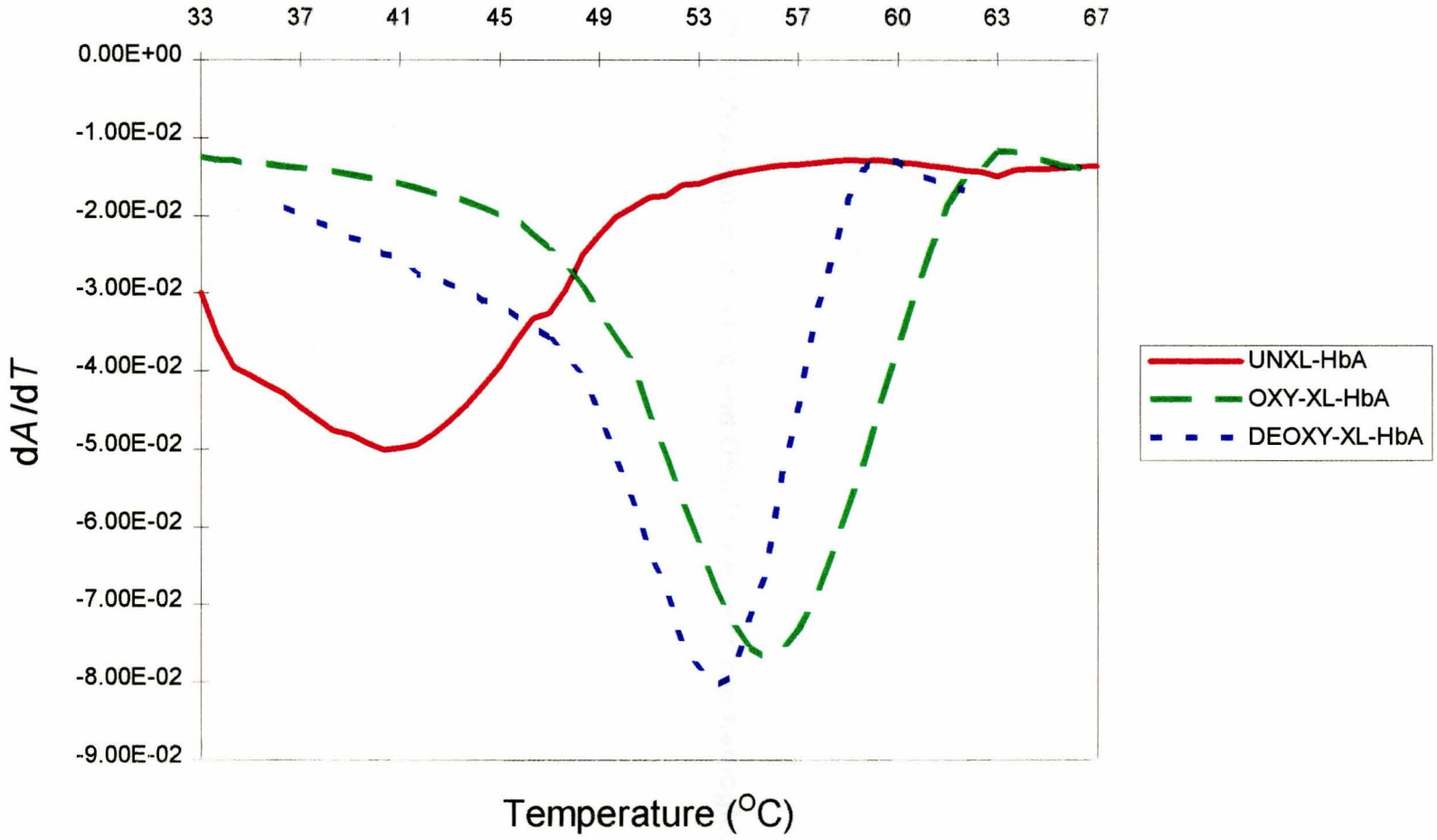

33
37
41
45
49
53
57
60
63
67
0.00E+00
-1.00E-02
-2.00E-02
-3.00E-02
-4.00E-02
-5.00E-02
-6.00E-02
-7.00E-02
-8.00E-02
-9.00E-02
dA/dT
Temperature (°C)
UNXL-HbA
OXY-XL-HbA
DEOXY-XL-HbA

Figure 22. Autoxidation of native and DBSTC crosslinked hemoglobins.

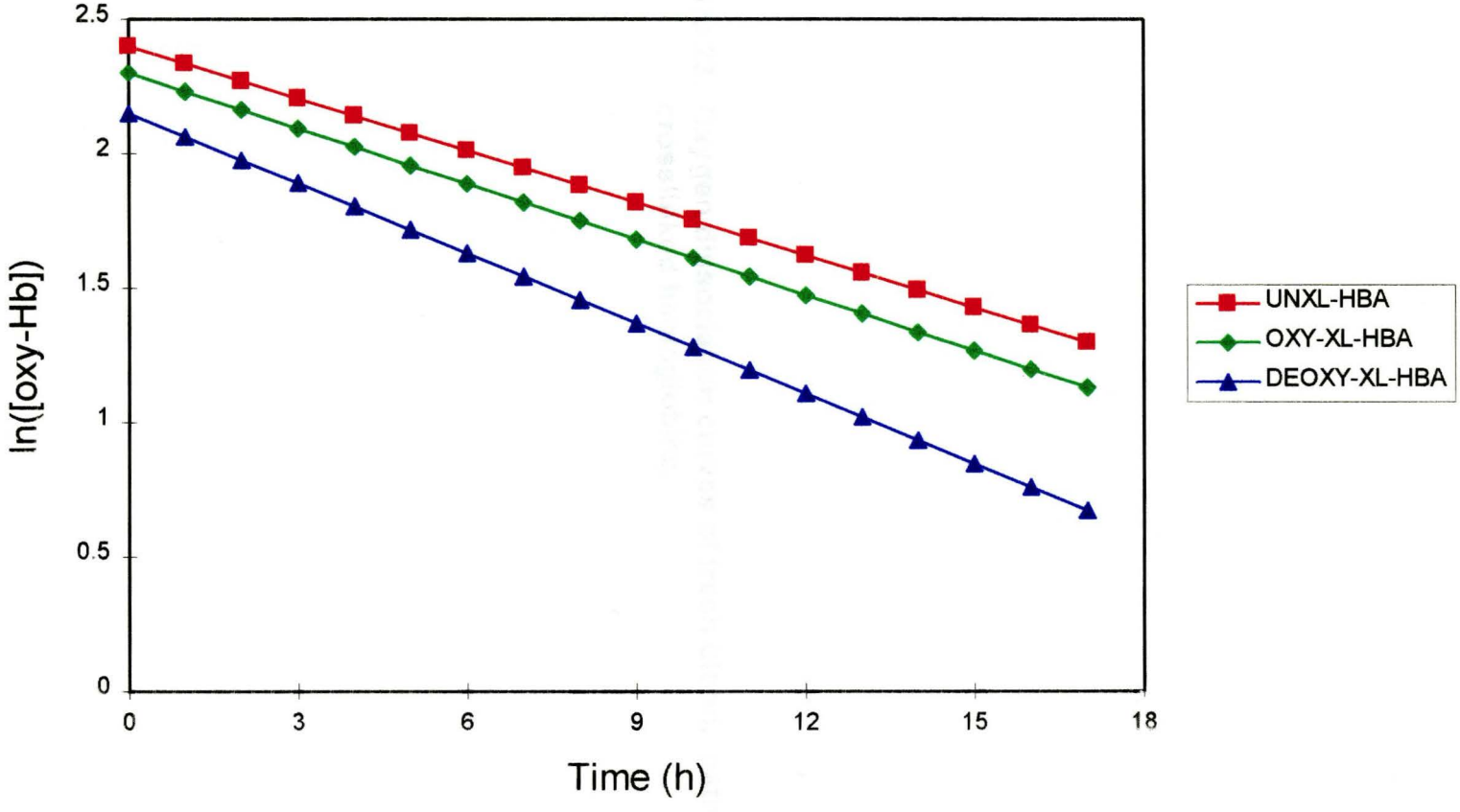
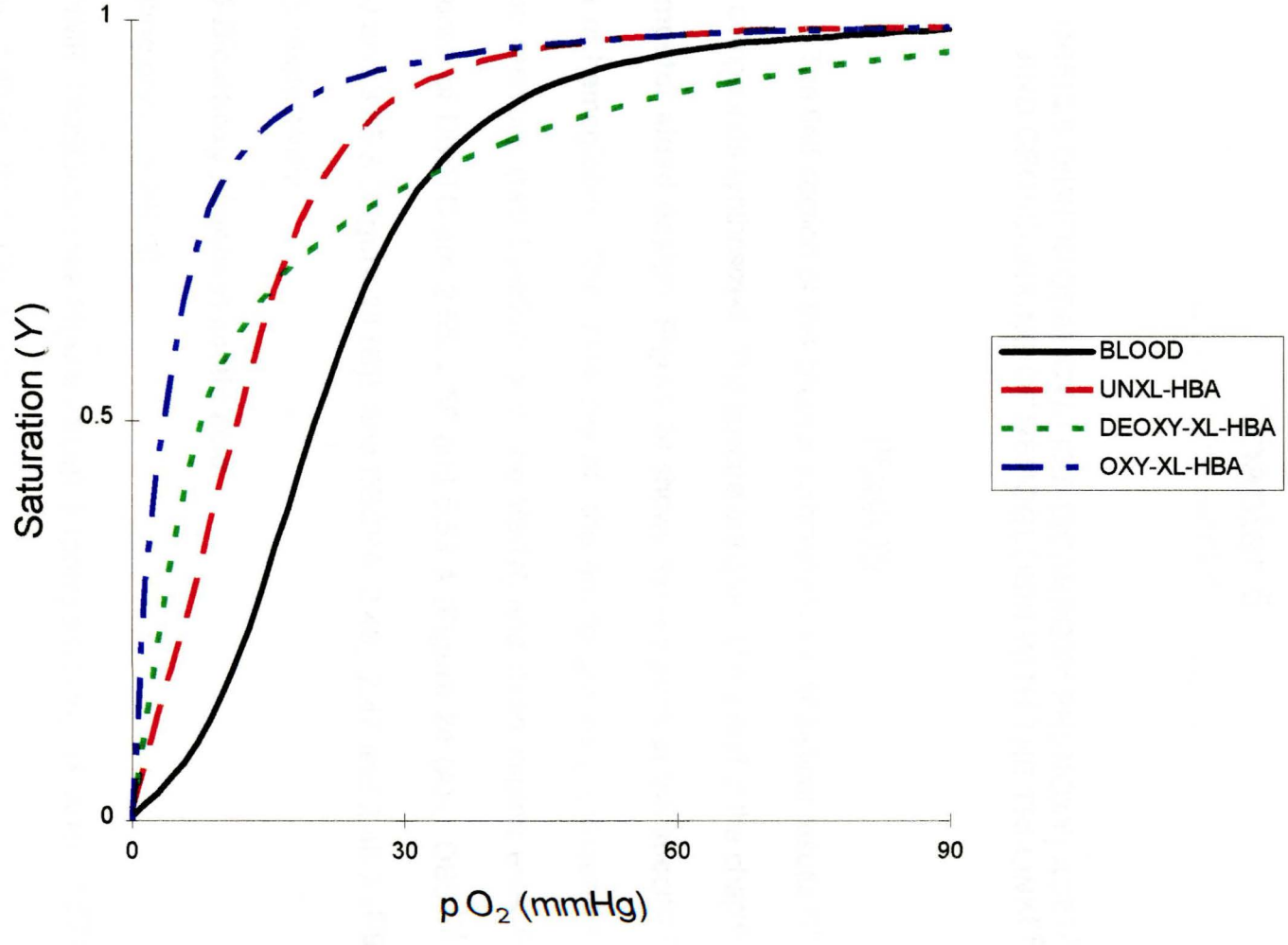

2.5
2
1.5
1
0.5
0
ln([oxy-Hb])
0
3
6
9
12
15
18
Time (h)
UNXL-HBA
OXY-XL-HBA
DEOXY-XL-HBA

Figure 23. Oxygen dissociation curves of fresh blood, native and DBSTC crosslinked hemoglobins.


1
0.5
0
Saturation (Y)
0
30
60
90
p O2 (mmHg)
BLOOD
UNXL-HBA
DEOXY-XL-HBA
OXY-XL-HBA

Chapter 5

TRIS(3,5-DIBROMOSALICYL) (3,5-DICARBOXY PHENOXY) ACETATE AND CROSSLINKING OF HEMOGLOBIN WITH THE TRI-LINKER

RESULTS

The first section of this chapter summarizes the analytical results for each of the compounds synthesized. The spectra are given at the end of the chapter.

Computer aided design. Figure 24 shows the reagents at the specific reaction site of hemoglobin. The distances of the amino groups (nucleophiles) of the three residues (two Lys82βs and one Val1β) and three aspirin ester carbonyl carbons of DBSTC are 2.55, 2.58 and 5.53 Å (**Figure 24 (A)**); DBSTM, 2.47, 2.50 and 3.95 Å (**Figure 24 (B)**); and DBCPA, 2.48, 2.47 and 2.48 Å (**Figure 24 (C)**), respectively.

(3,5-Dicarboxy phenoxy) acetic acid

Melting point: > 360°C.

^{1}H NMR (DMSO-d_6) (see **Figure 25 (a)**): δ (ppm) 8.04 (m, 1H, ArH), 7.53 (m, 2H, ArH), 4.49 (s, 2H, O-CH_2-C=O).

^{13}C NMR (DMSO-d_6) (see **Figure 25 (b)**): δ (ppm) 170.55 (s, 2C, C=O), 167.41 (s, 1C, C=O), 158.26 (s, 1C, O-ArC), 134.37 (s, 2C, ArC*-C=O), 122.30 (s, 1C, ArC), 118.36 (s, 2C, ArC), 66.40 (s, 2C, O-C*H_2-C=O).

(3,5-Dicarboxy phenoxy) acetyl trichloride

Melting point: 60.5-62°C.

^1H NMR ($CDCl_3$) (see **Figure 26 (a)**): δ (ppm) 8.55 (m, 1H, ArH), 7.87 (m, 2H, ArH), 5.09 (s, 2H, O-CH_2-C=O).

^{13}C NMR ($CDCl_3$) (see **Figure 26 (b)**): δ (ppm) 169.05 (s, 1C, C=O), 166.77 (s, 2C, C=O), 157.44 (s, 1C, ArC), 135.81 (s, 1C, ArC), 127.81 (s, 2C, ArC), 122.82 (s, 2C, ArC), 72.36 (s, 2C, O-C*H_2-C=O).

Tris(*tert*-butyl 3, 5-dibromosalicylate) (3,5-dicarboxy phenoxy) acetate

Melting point: 104-107°C.

^1H NMR ($CDCl_3$) (see **Figure 27 (a)**): δ (ppm) 8.76 (m, 1H, ArH), 8.18 (m, 2H, ArH), 8.03 (m, 2H, ArH), 7.98 (m, 1H, ArH), 7.94 (m, 2H, ArH), 7.88 (m, 1H, ArH), 5.19 (s, 2H, O-CH_2-C=O), 1.55 (s, 9H, CH_3), 1.39 (s, 18H, CH_3).

^{13}C NMR ($CDCl_3$) (see **Figure 27 (b)**): δ (ppm) 165.12 (s, 1C, C=O), 162.04 (s, 2C, C=O), 161.63 (s, 2C, C=O), 160.99 (s, 1C, C=O), 157.97-119.09 (16s, 24C, ArC & ArC), 83.33 (s, 1C, O-C), 83.17 (s, 2C, O-C), 65.29 (s, 2C, O-C*H_2-C=O), 28.06 (s, 3C, CH_3), 27.97 (s, 6C, CH_3),

Tris(3, 5-dibromosalicyl) (3,5-dicarboxy phenoxy) acetate

Melting point: 150-152°C.

^{1}H NMR (DMSO-d_6) (see **Figure 28 (a)**): δ (ppm) 8.43 (s, 1H, ArH), 8.26 (d, 2H, ArH), 8.20 (d, 1H, ArH), 8.06 (s, 4H, ArH), 8.01 (d, 1H, ArH), 5.39 (s, 2H, O-CH_2-C=O).

^{13}C NMR (DMSO-d_6) (see **Figure 28 (b)**): δ (ppm) 166.16 (s, 1C, C=O), 163.50 (s, 2C, C=O), 163.47 (s, 1C, C=O), 162.21 (s, 2C, C=O), 158.15-119.18 (16s, 24H, ArC & ArC), 65.36 (s, 2C, O-C*H_2-C=O).

Isolation of the crosslinked proteins

Anion-exchange chromatography produced three components from reaction of hemoglobin with tris(3,5-dibromosalicyl) (3,5-dicarboxy phenoxy) acetate (DBCPA) (**Figure 29 a**). The yield of crosslinked proteins of the third peak was high too.

Electrophoresis

The SDS-PAGE showed that the first peak was uncrosslinked hemoglobin with only a monomeric band, and the others are crosslinked hemoglobins with both monomeric (M_r 16,000) and dimeric (M_r 32,000) bands **(Figure 29 b and Table 8**). SDS-PAGE also showed multiple bands at dimer ranges of both the second and third peaks.

Denaturation

Figure 30 shows the denaturation of native hemoglobin A and the crosslinked hemoglobins. The curves of the slope of the Soret absorbance versus temperature have a single broad peak for each. The T_m was determined by measuring the temperature corresponding to the minimum of the

Table 8. SDS-PAGE Results for Isolated Crosslinked Hemoglobins.

Sample	% Monomer	% Dimer
Hb A	100	0
The 1st peak	100	0
The 2nd peak	51	49
The 3rd peak	50	50

peak. The T_m of the crosslinked hemoglobins was 55.0 °C for both the second and third peaks, 14.0 °C (ΔT_m) higher than that of native hemoglobin (41.0 °C) .

Autoxidation

Figure 31 shows the autoxidation of native and the DBCPA crosslinked hemoglobins. The hemoglobin concentrations decreased linearly at a pseudo first-order rate. The k_{app} obtained by calculation of the slopes of the regression lines showed that the autoxidation of the crosslinked hemoglobins of both the second and third peaks remained unchanged at approximately 7.0×10^{-2} h^{-1} corresponding to the half-life time 10.0 h, compared with that of native hemoglobin.

Oxygen-binding

Figure 32 shows oxygen equilibria of native and the DBCPA crosslinked hemoglobins. The DBCPA bi-linking of hemoglobin caused a decrease in oxygen affinity, raising the P_{50} value from 11.6 to 15.9 mmHg. The tri-linked proteins had a higher oxygen affinity with P_{50} 0.86 mmHg. Comparing with that of native hemoglobin (n = 2.5), the Hill coefficient of the bi-linking of hemoglobin remained almost unchanged (n = 2.4), while that of the tri-linking of hemoglobin was half of the pre-crosslinked (n = 1.2).

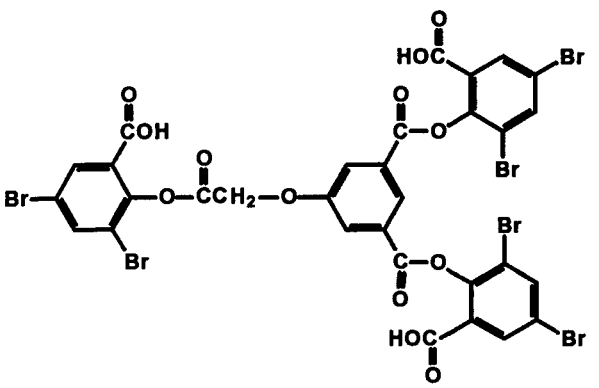
The relevant data of denaturation, autoxidation and oxygen-binding were summarized in **Table 9**.

Table 9. Comparison of Functional Properties of Crosslinked Hemoglobins.

Sample	T_m (°C)	ΔT_m (°C)	$k_{app} \times 100$ (h^{-1})	$t_{1/2}$ (h)	P_{50} (mm)	n
Hb A	41.0	0.0	$6.99 \pm 3.02 \times 10^{-5}$	9.92	11.6	2.5
The 2nd peak	55.0	14.0	$6.80 \pm 3.00 \times 10^{-5}$	10.2	15.9	2.4
The 3rd peak	55.0	14.0	$6.99 \pm 2.90 \times 10^{-5}$	9.92	8.6	1.3

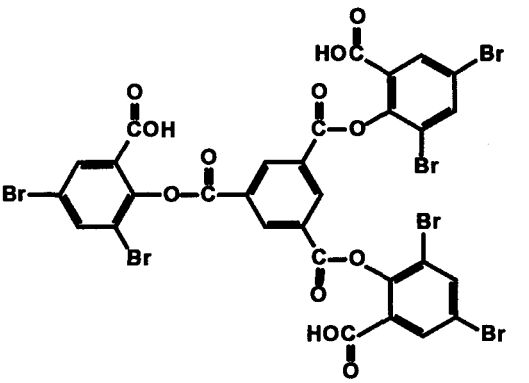
DISCUSSION

The synthesis of tris(3,5-dibromosalicyl) (3,5-dicarboxy phenoxy) acetate contains four steps: The first step is production of a (3,5-dicarboxy phenoxy) acetic acid; the second step is production of the corresponding tri(acid chloride); the third step is production of the corresponding tris(*tert*-butyl 3,5-dibromosalicyl) ester; the fourth step is the *tert*-butyl deprotection by triflouroacetic acid to form the corresponding tris(3,5-dibromosalicylate) ester. The desired product was characterized by ^{1}H and ^{13}C NMR (**Figure 28**): The three hydrogen resonances at chemical shift δ 8.43 and 8.03 are due to the isophthalate benzene groups, the six hydrogen peaks at δ 8.26, 8.20, 8.06 and 8.01 ppm are due to the aspirin's benzenes, the two hydrogen resonances at δ 5.39 are attributed to the methylene groups; the two peaks at δ 166.16 and 163.50 are assigned to the carbonyl carbons on the isophthalate aromatic ring, the two peaks at δ 163.47 and 162.21 to the carbonyl carbons on the aspirin aromatic rings, the multiple peaks at δ 158.15 - 119.18 to the isophthalate and aspirin aryl carbons and a peak at δ 65.36 to the methylene carbon. The synthesis of the reagent was successful.



Tris(3,5-dibromosalicyl) (3,5-dicarboxy phenoxy) acetate

It was confirmed that the crosslinking reaction of hemoglobin with tris(3,5-dibromosalicyl) trimesate DBSTM was at Lys82βs and few tri-linking involved Val1β; the third 3,5-dibromosalicylate ester principally undergoes hydrolysis instead of reaction with the α-amino group of a Valβ1 [78]. The tri-linking reaction of hemoglobin with DBCPA, whose structure is similar to DBSTM, was studied at the specific site of Lys82βs and Val1β, using computer modeling. As shown in **Figure 24**, DBCPA functional groups is closest to the amino groups of the residues of which the specific site comprises. This reaction can be understood in terms of a molecular model. The distance from the reactive carbonyl carbons to the α-amino group is 1.45 Å longer than to either of the εN's of the two Lys82βs. DBCPA provides the possible tri-linking at the BPG site in hemoglobin because its longer middle arm is able to reach a Valβ1 to react. The good fit of DBCPA at the specific site needs less activation energy for reaction of hemoglobin with the reagent to favor tri-linking of hemoglobin than those of DBSTC and DBSTM (**Table 10**).



Tris(3,5-dibromosalicyl) trimesate

The ion-exchange chromatography of the crosslinked derivatives with tris(3,5-dibromosalicyl) (3,5-dicarboxy phenoxy) acetate (**Figure 29a**) isolated

Table 10. Distances between ester carbonyl carbons of DBSTC, DBSTM and DBCPA.

Crosslinker	Distance (Å)		
DBSTC	3.3	3.8	4.6
SMPTM	5.1	5.1	5.2
DBSTM	5.1	5.1	5.1
DBCPA	5.1	5.5	6.6
Amide bond	2.35		

two major products from deoxyhemoglobin reaction. The first peak eluted from the column contained only Hb A. The component of the second peak is bi-linked hemoglobin having a dimeric band (32,000 MW) on SDS-PAGE (**Figure 29b** and **Table 8**). The multiple dimeric bands indicate that the crosslinking reaction was heterogeneous because of the asymmetry of the reagent. The component of the third peak is probably tri-linked hemoglobin, because it has lost additional positively charged groups as shown by its mobility on both anionic-exchange chromatography (**Figure 29 a**) and alkaline agarose gel electrophoresis. The dimeric bands of SDS-PAGE corresponding to tri-linking indicated that the tri-link was between two β subunits, being consistent with the reaction site of Lys82's and Val1 as we expected. The SDS-PAGE also shows multiple bands at the dimeric range. This is due to the fact that DBCPA has two isophthalate aspirin ester (IAE1 and IAE2) and one acetate aspirin ester (AAE). The symmetry of DBCPA structure is point group C_{2h} instead of C_{3h} as the symmetry of DBSTM is. Therefore, the crosslinking of hemoglobin with DBCPA is a heterogeneous reaction which can go: (1) IAE1 $\rightarrow \beta_1 82$, IAE2 $\rightarrow \beta_2 82$, and AAE $\rightarrow \beta 1$; (2) IAE1 $\rightarrow \beta_1 82$, IAE2 $\rightarrow \beta 1$, and AAE $\rightarrow \beta_2 82$; (3) IAE1 $\rightarrow \beta_1 82$, AAE $\rightarrow \beta_2 82$, and IAE2 $\rightarrow \beta 1$: (4) AAE $\rightarrow \beta_1 82$, IAE1 $\rightarrow \beta_2 82$, and IAE2 $\rightarrow \beta 1$; (5) AAE $\rightarrow \beta_1 82$, IAE $\rightarrow \beta 1$, and AAE $\rightarrow \beta_2 82$; ... The possible bi- and tri-linking are shown in **Figure 33**.

The DBCPA crosslinking of inter-subunit of hemoglobin improved the thermal stability significantly (**Figure 30** and **Table 9**). The broad transition of

the hemoglobins was expected due to the presumed multi-state nature of the denaturation. When hemoglobin is thermally denatured, the most likely first step is the dissociation of the $[\alpha\beta]^2$ tetramers into $\alpha\beta$ dimers followed by further dissociation into α and β monomers, and finally unfolding of the individual chains [71]. The covalent connection between the β chains of the DBCPA crosslinked proteins prevented tetramers from dissociation into $\alpha\beta$ dimers. The denaturation of the crosslinked hemoglobins underwent dissociation of tetramers into α monomers and β-β dimers as the first step which had to surmount a high energy barrier thermodynamically to overcome inter-subunit forces from two β chains and one α chain, while the denaturation of uncrosslinked hemoglobin surmounts low energy barriers to dissociate tetramers into $\alpha\beta$ dimers, and the dimers dissociates into monomers overcoming a inter-subunit force from only one β chain. The DBCPA crosslinking of hemoglobins resulted in a higher denaturation transition, ΔT_m = 14.0 °C, than native hemoglobin. However, the tri-linked hemoglobins did not exhibit more thermally stable than the bi-linked hemoglobins, indicating that inter-subunit tri-linked proteins have the same mechanism of denaturation as inter-subunit bi-linked proteins and have an equivalent dissociation energy to the later.

Although autoxidation of hemoglobin (Hb-Fe(II) $\rightarrow$ Hb-Fe(III)) is a very complex reaction, the tests showed the autoxidation of native and the DBCPA crosslinked hemoglobins was linear within 17 h and precise to 10^{-7} h^{-1} order (**Figure 31** and **Table 9**). Comparison of the autoxidation results of native and

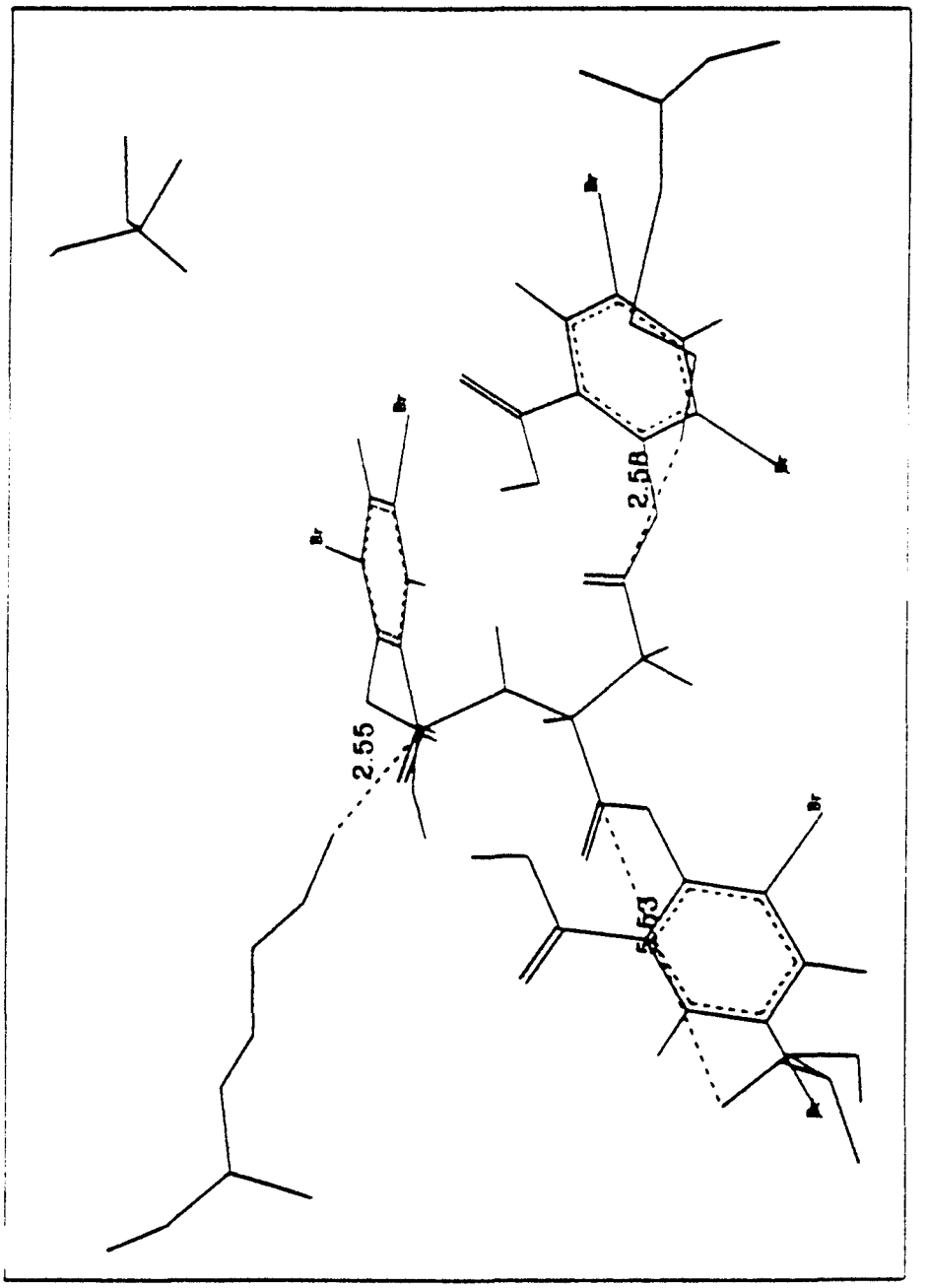
the deoxy crosslinked hemoglobins indicates that the autoxidation rate of the crosslinked hemoglobins remained unchanged with the k_{app} at approximately 7.0 $\times 10^{-2}$ h^{-1}. According to this study and the previous work, we can found that crosslinking at the BPG binding site of hemoglobin does not change the autoxidation rate constant as much as crosslinking at other sites of hemoglobin [95]. It was reported that fumarate crosslinking at Lys82βs of oxyhemoglobin had a smaller k_{app} than crosslinking at Lys99α's of deoxyhemoglobin [95]. It can be thought that DBCPA crosslinking of deoxyhemoglobin holds the two β chains closer than they should be at the T state so that the R state quaternary structure was favored [96-97]. Hemoglobin in the R state is less susceptible to autoxidation than in the T state [26]. Unlike DBSF crosslinked hemoglobins [95], the rate of autoxidation is not correlated with oxygen affinity for the DBCPA modified hemoglobins.

The DBCPA bi-linked hemoglobins had a lower oxygen affinity than native hemoglobin, while the tri-linked hemoglobins exhibit a higher oxygen affinity (**Figure 32** and **Table 9**). Many studies showed that crosslinking at BPG binding site of hemoglobin changed the oxygen affinity of hemoglobin significantly [14, 65, 69-70, 77, 79, 98-100]. When crosslinking of hemoglobin with reagents with small sizes, oxygen affinity usually increased [69, 77]. When crosslinking of deoxyhemoglobin with reagents with large sizes and rigid structures, oxygen affinity decreased [77, 79]. This is due to the fact that small reagents pull the two β chains, shifting (or keeping) the conformation of the hemoglobins in the R state

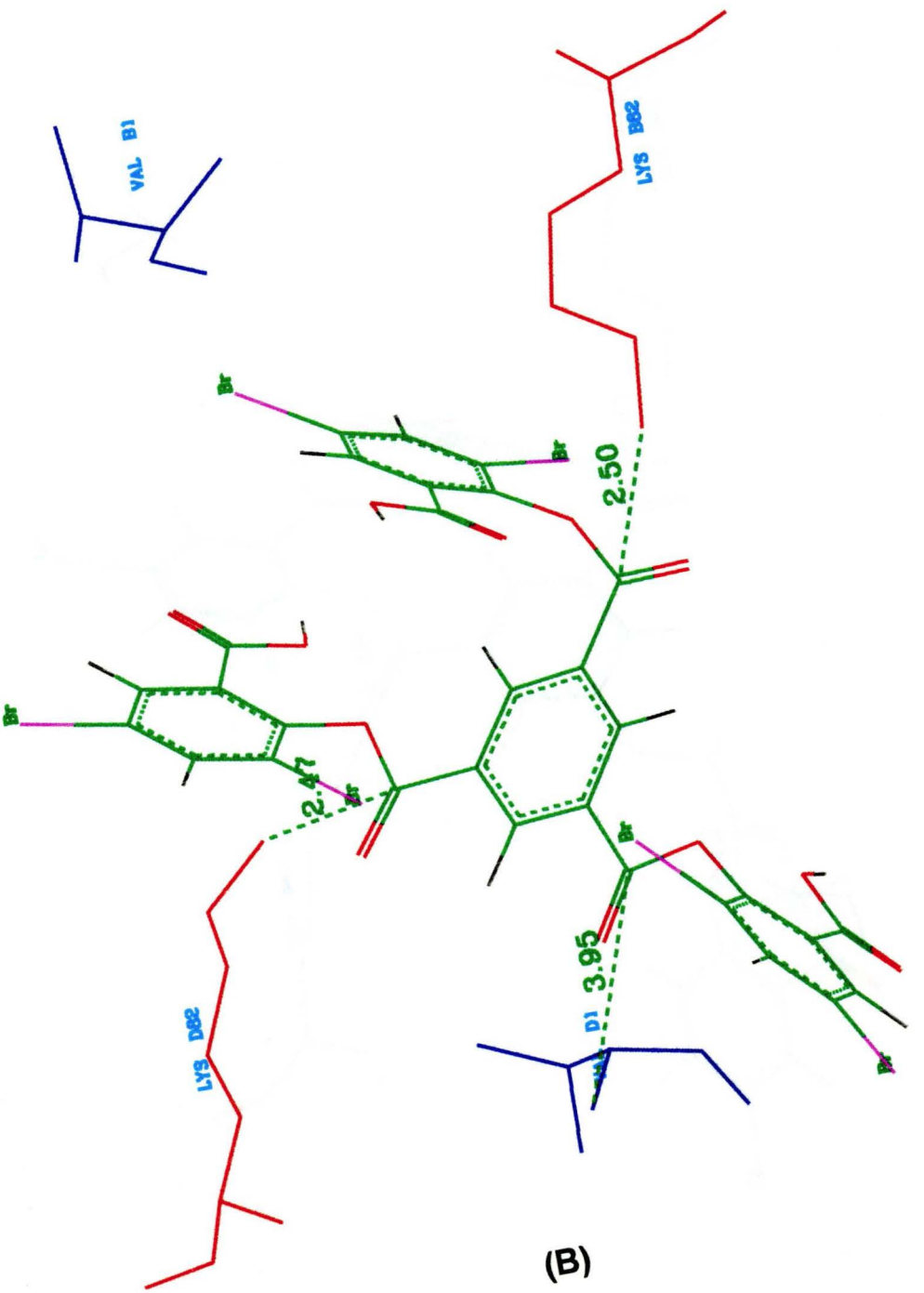
which favor oxygenation, while larger and rigid reagents prevent the β chains from coming close, holding the conformation of hemoglobin in the T state which favors deoxygenation. The decreased oxygen affinity of DBCPA bi-linked hemoglobins is consistent with the result of the bi-linked hemoglobins with tris(sodium methyl phosphate) trimesate (SMPTM) [79]. However, the increased oxygen affinity of the DBCPA tri-linked proteins is inconsistent with that of the SMPTM tri-linked hemoglobin [79]. According to Kluger's systematic studies in which different sizes of crosslinkers were used including bis(methyl phosphate) fumarate, isophthalate, terephthalate, *trans*-stilbene 3,3'-dicarboxylic acid, and *trans*-stilbene 4,4'-dicarboxylic acid, oxygen affinity decreased with increase of bridging distance between Lys82βs of hemoglobin; oxygen affinity increased with increase of bridging distance between Lys82β_1 and Val1β_2 of hemoglobin [77]. The SMPTM crosslinking distance between Lys82β_1 and Val1β_2 is 7.8 Å and that of DBCPA can be up to 9.3 Å (**Table 10**). Therefore, in tri-linking of hemoglobin, the bridging distance between Lys82β_1 and Val1β_2 is still a factor to affect the oxygen affinity of hemoglobin. The DBCPA tri-linked proteins had a half-fold *n* compared to native hemoglobin, indicating that the multi-linking of hemoglobin made the conformation of the hemoglobins less flexible, therefore less cooperative, than native hemoglobin.

The low oxygen affinity of DBCPA bi-linked hemoglobins also indicates the possibility that the crosslinking of hemoglobin was between Val1β_1 and Lys82β_2 with 3,5-dicarboxy of the tri-linker.

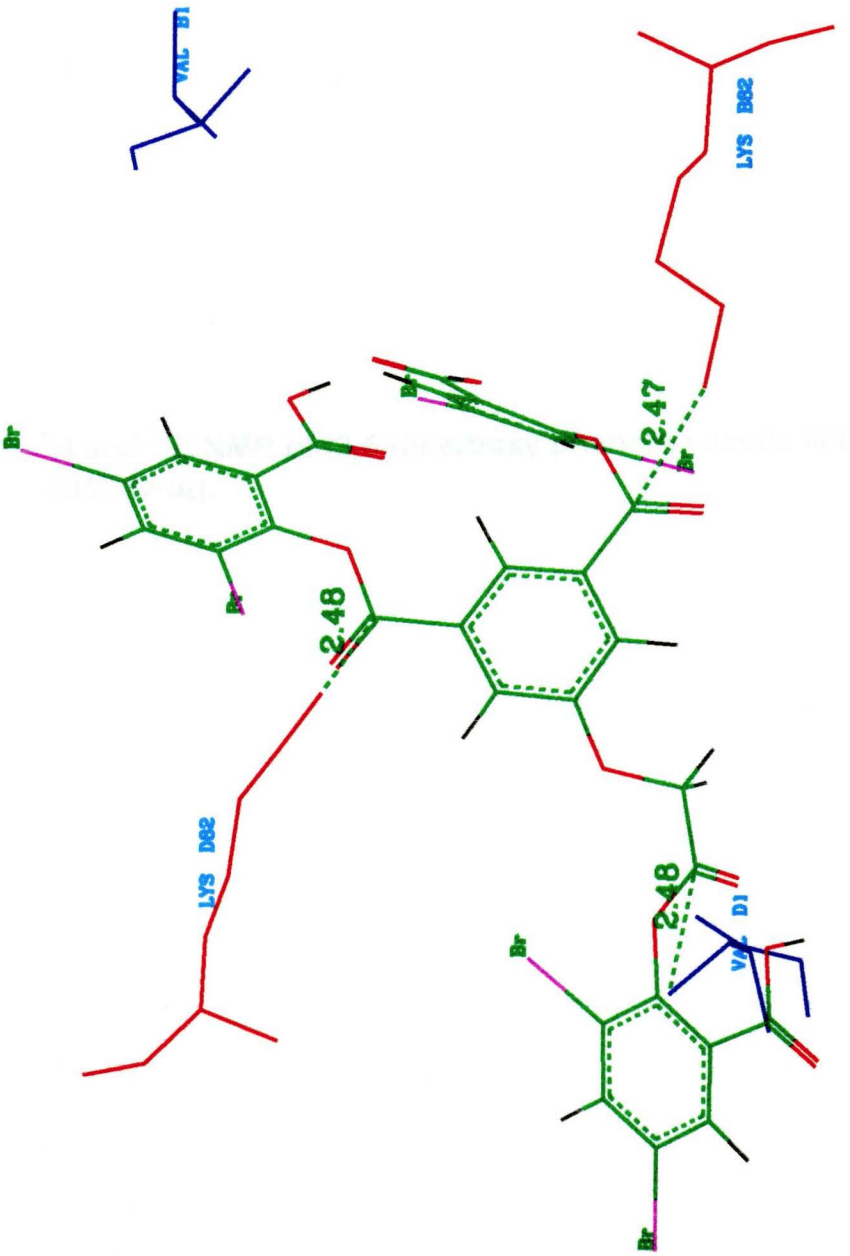
Figure 24. (A) DBSTC, (B) DBSTM and (C) DBCPA in the BPG binding site.



(A)

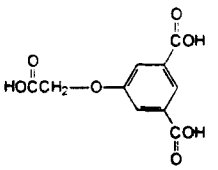


(B)

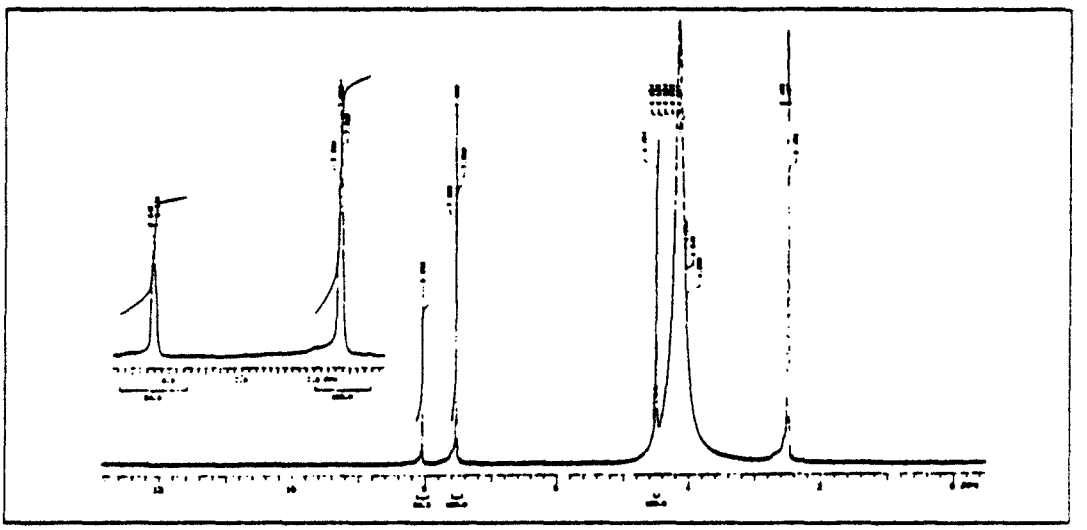


(C)

Figure 25. ^{1}H and ^{13}C NMR of (3,5-dicarboxy phenoxy) acetic acid (DMSO-d_6).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

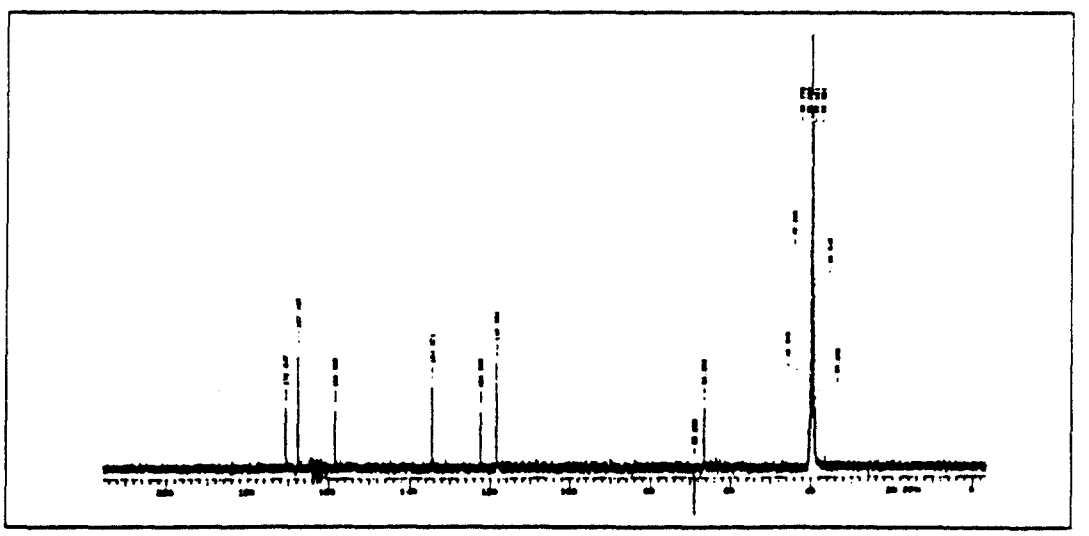
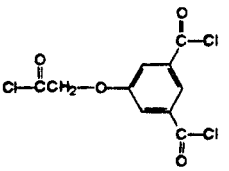
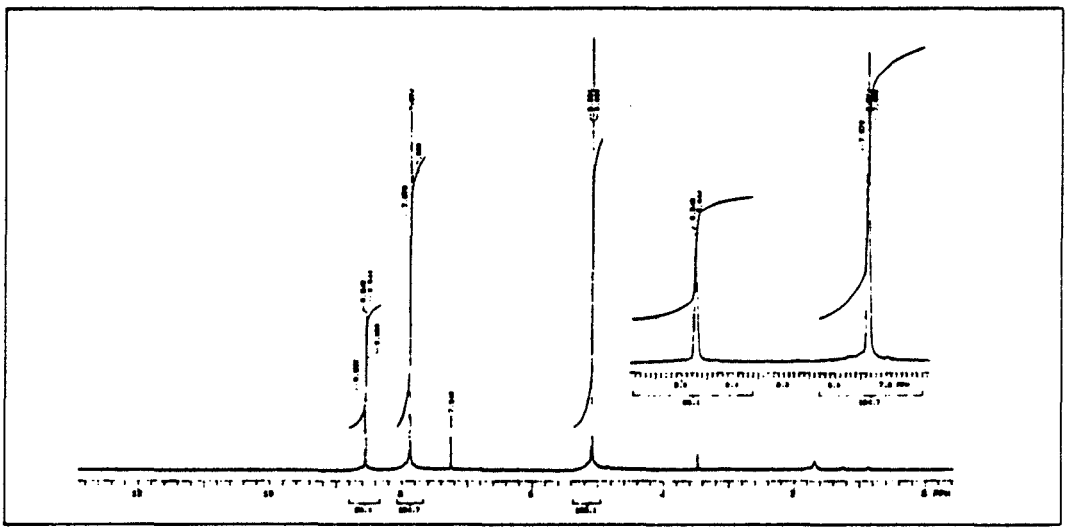


Figure 26. ^{1}H and ^{13}C NMR of (3,5-dicarboxy phenoxy) acetyl trichloride ($CDCl_3$).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

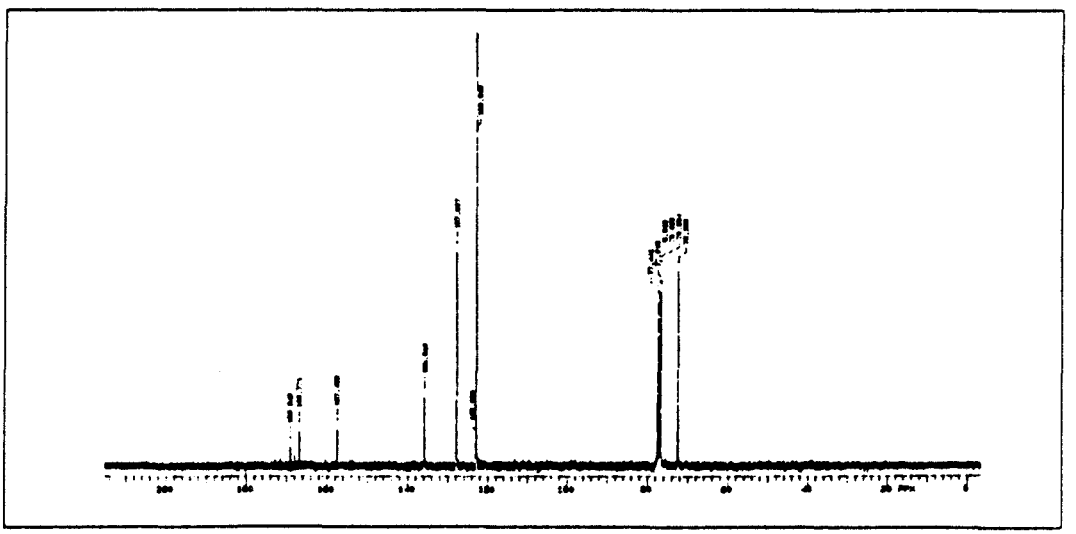
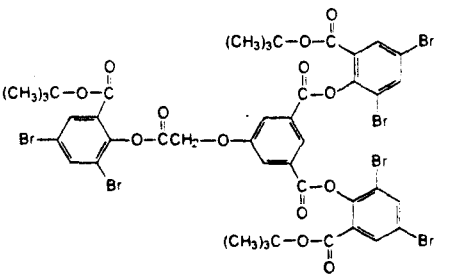
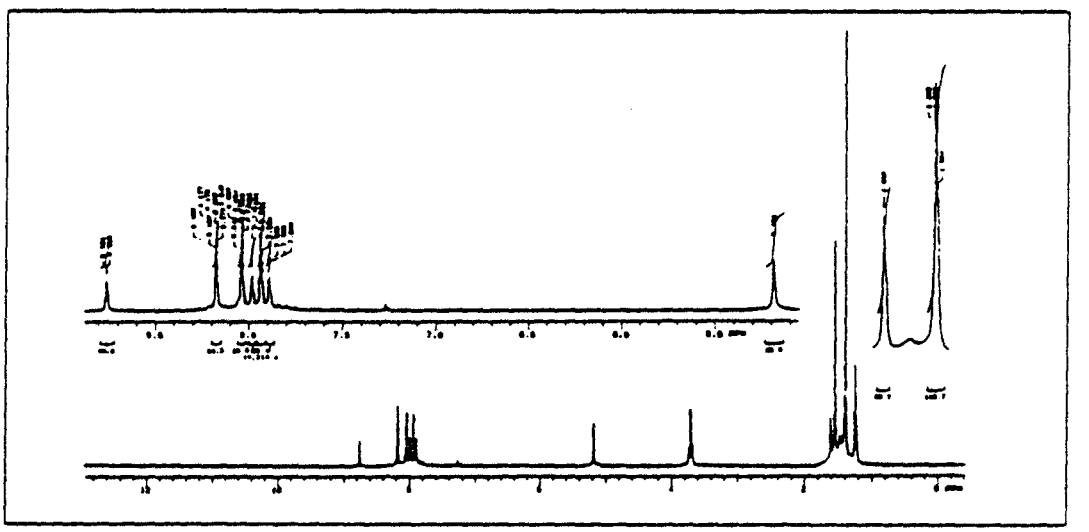


Figure 27. ^{1}H and ^{13}C NMR of tris(*tert*-butyl 3, 5-dibromosalicylate) (3,5-dicarboxy phenoxy) acetate ($CDCl_3$).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

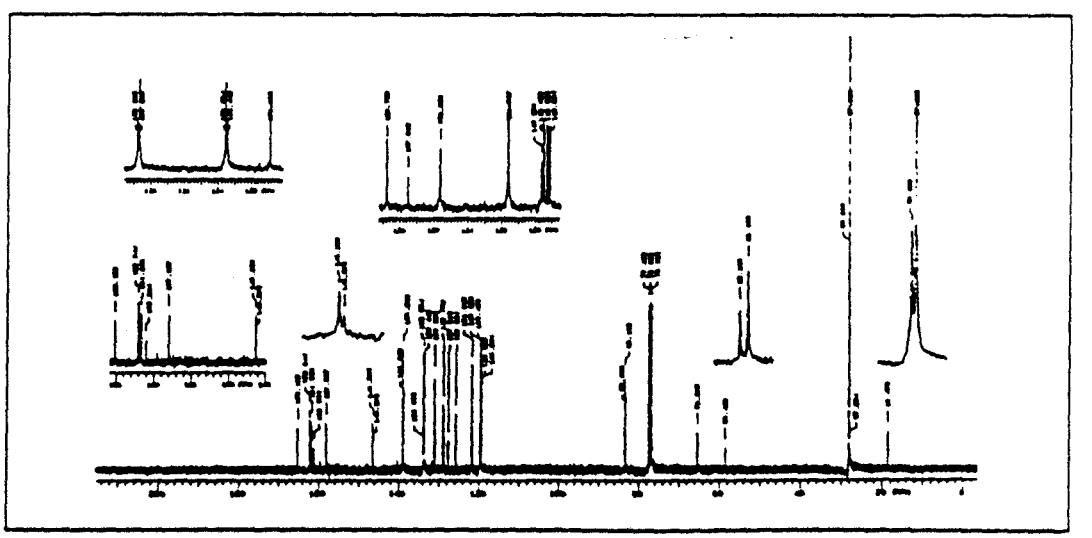
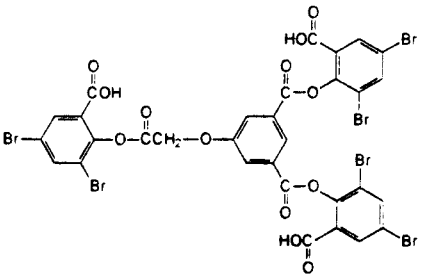
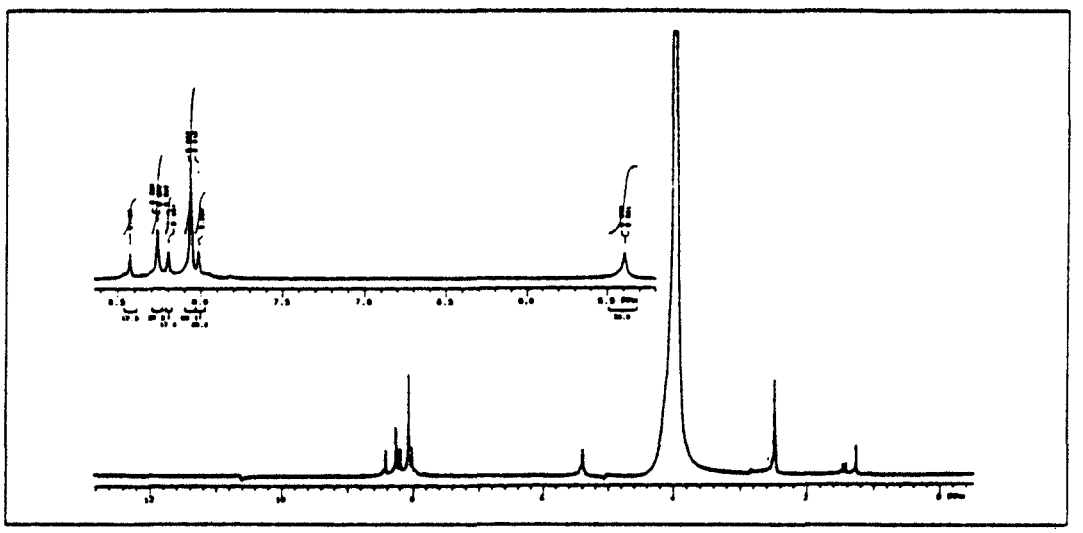


Figure 28. ^{1}H and ^{13}C NMR of tris(3, 5-dibromosalicyl) (3,5-dicarboxy phenoxy) acetate (DMSO-d_6).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

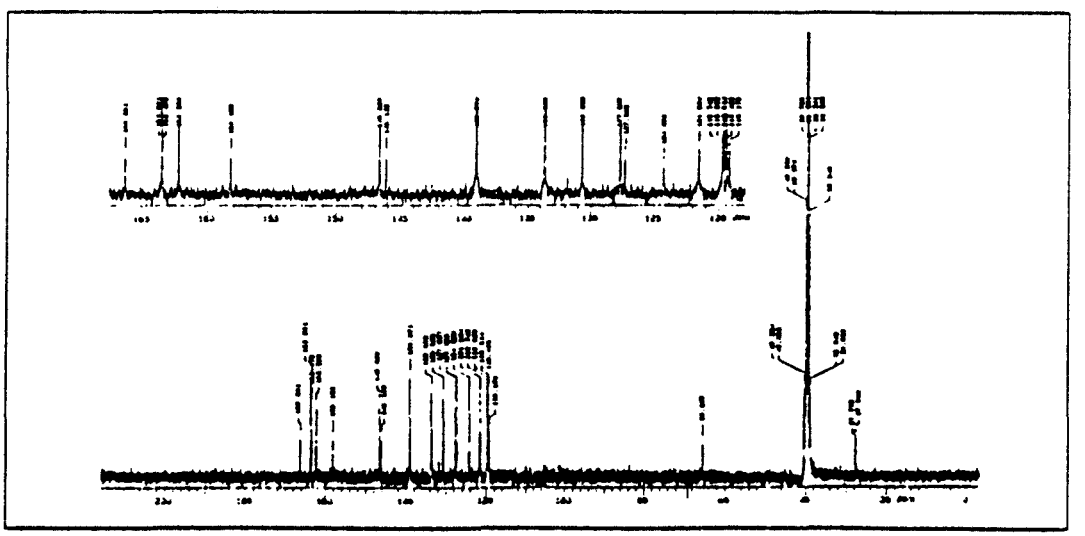
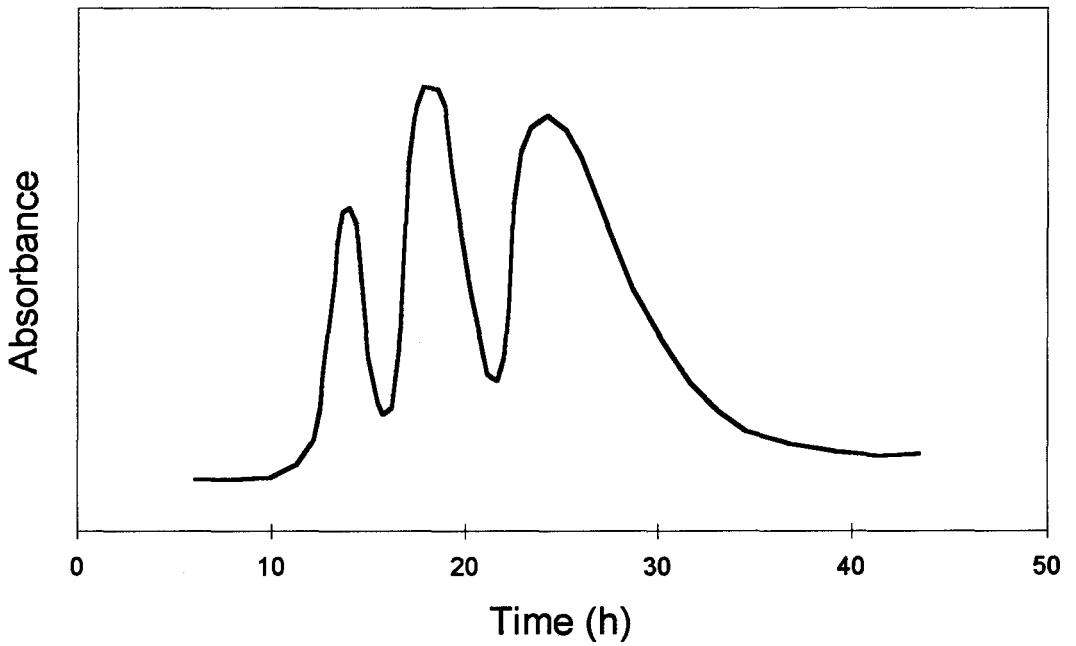


Figure 29. Anion-exchange chromatography and SDS-PAGE of hemoglobin crosslinked by DBCPA.

(a) Ion-exchange chromatography.



(b) SDS-PAGE.

1, 6 — Protein markers

2 — Unisolated hemoglobin

3 — The first peak

4 — The second peak

5 — The third peak

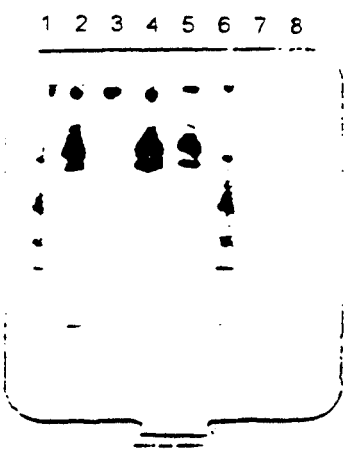


Figure 30. Denaturation of native and DBCPA crosslinked hemoglobins.

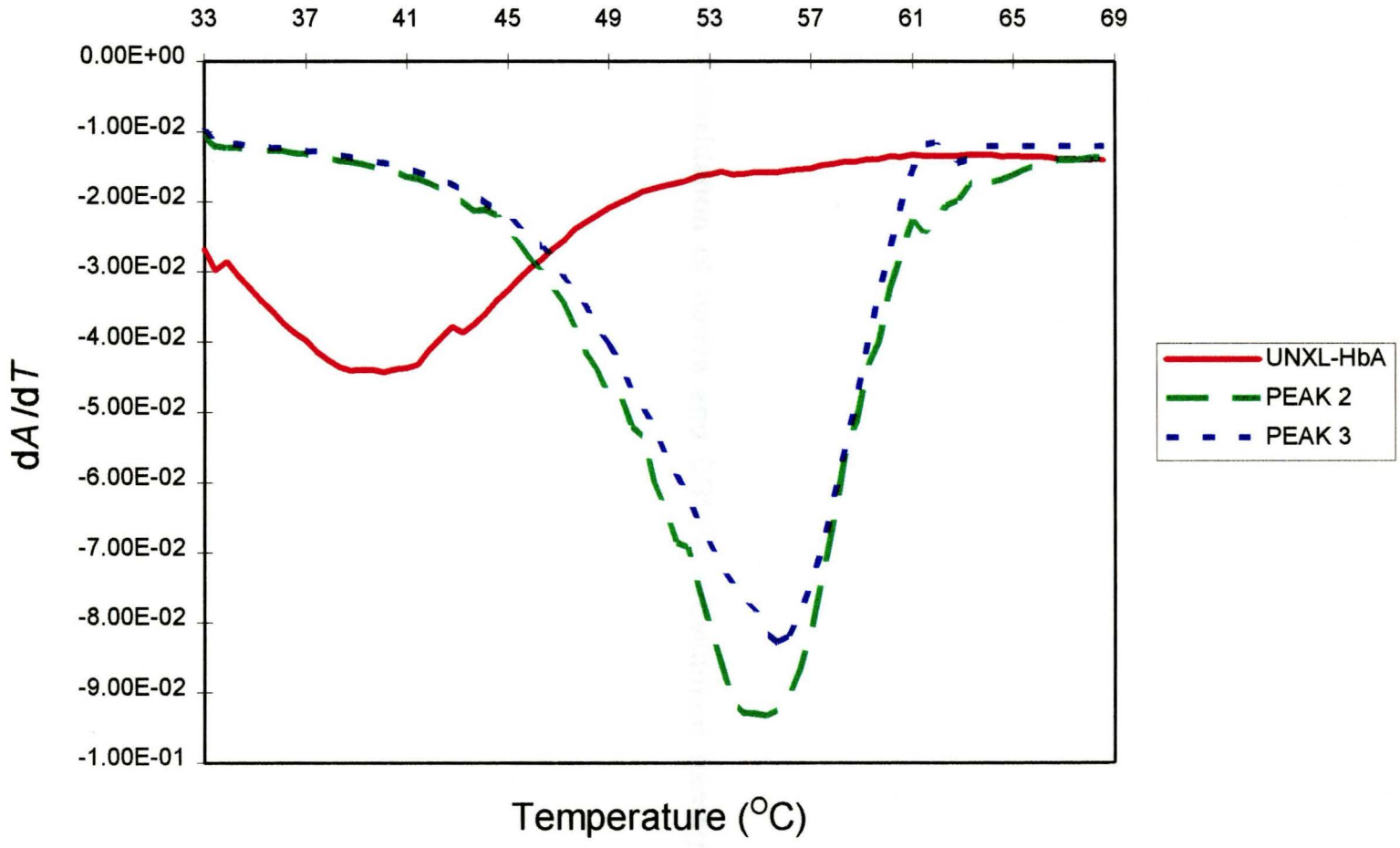

33
37
41
45
49
53
57
61
65
69
0.00E+00
-1.00E-02
-2.00E-02
-3.00E-02
-4.00E-02
-5.00E-02
-6.00E-02
-7.00E-02
-8.00E-02
-9.00E-02
-1.00E-01
dA/dT
Temperature (°C)
UNXL-HbA
PEAK 2
PEAK 3

Figure 31. Autoxidation of native and DBCPA crosslinked hemoglobins.

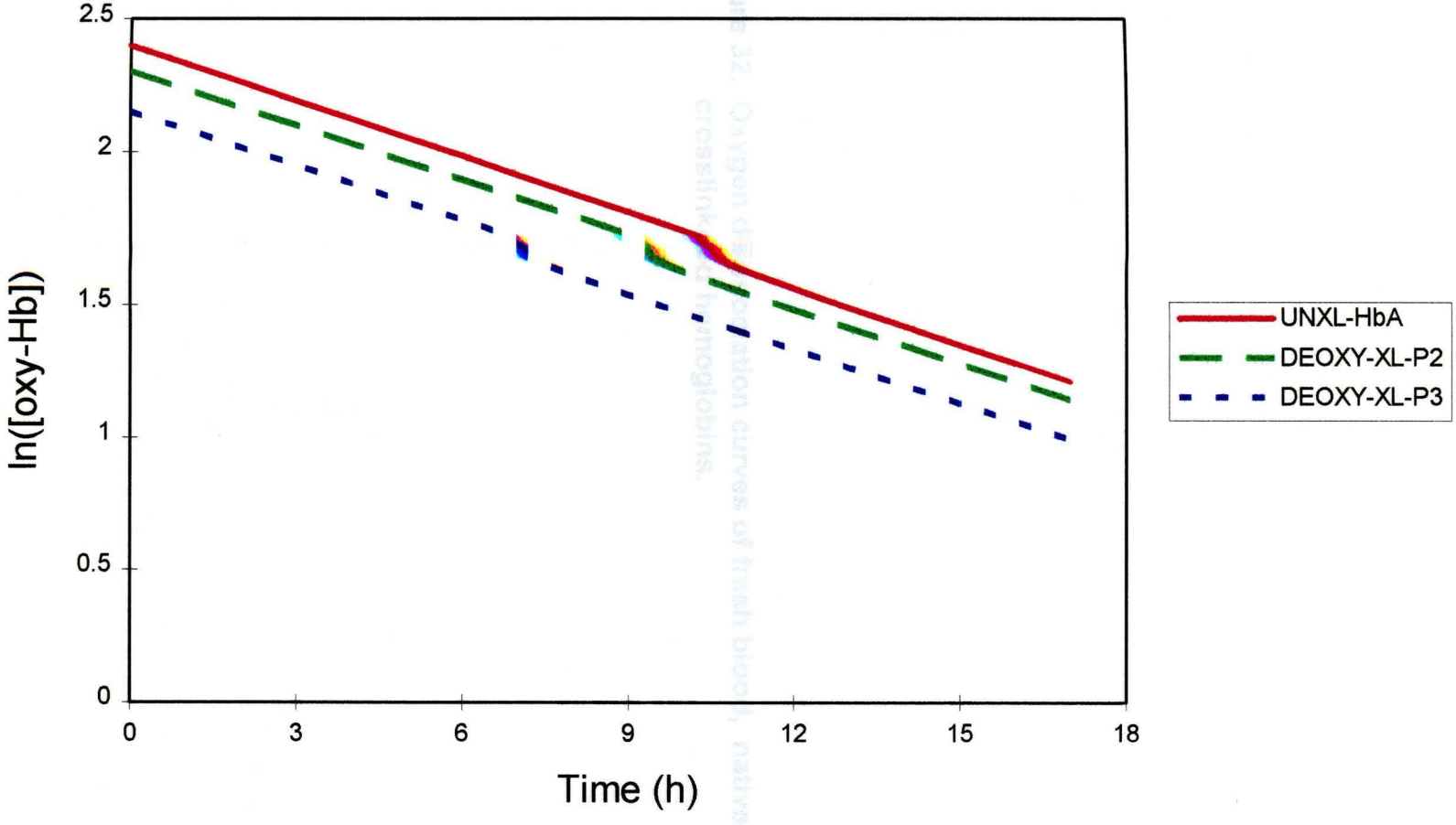

2.5
2
1.5
1
0.5
0
ln([oxy-Hb])
0
3
6
9
12
15
18
Time (h)
UNXL-HbA
DEOXY-XL-P2
DEOXY-XL-P3

Figure 32. Oxygen dissociation curves of fresh blood, native and DBCPA crosslinked hemoglobins.

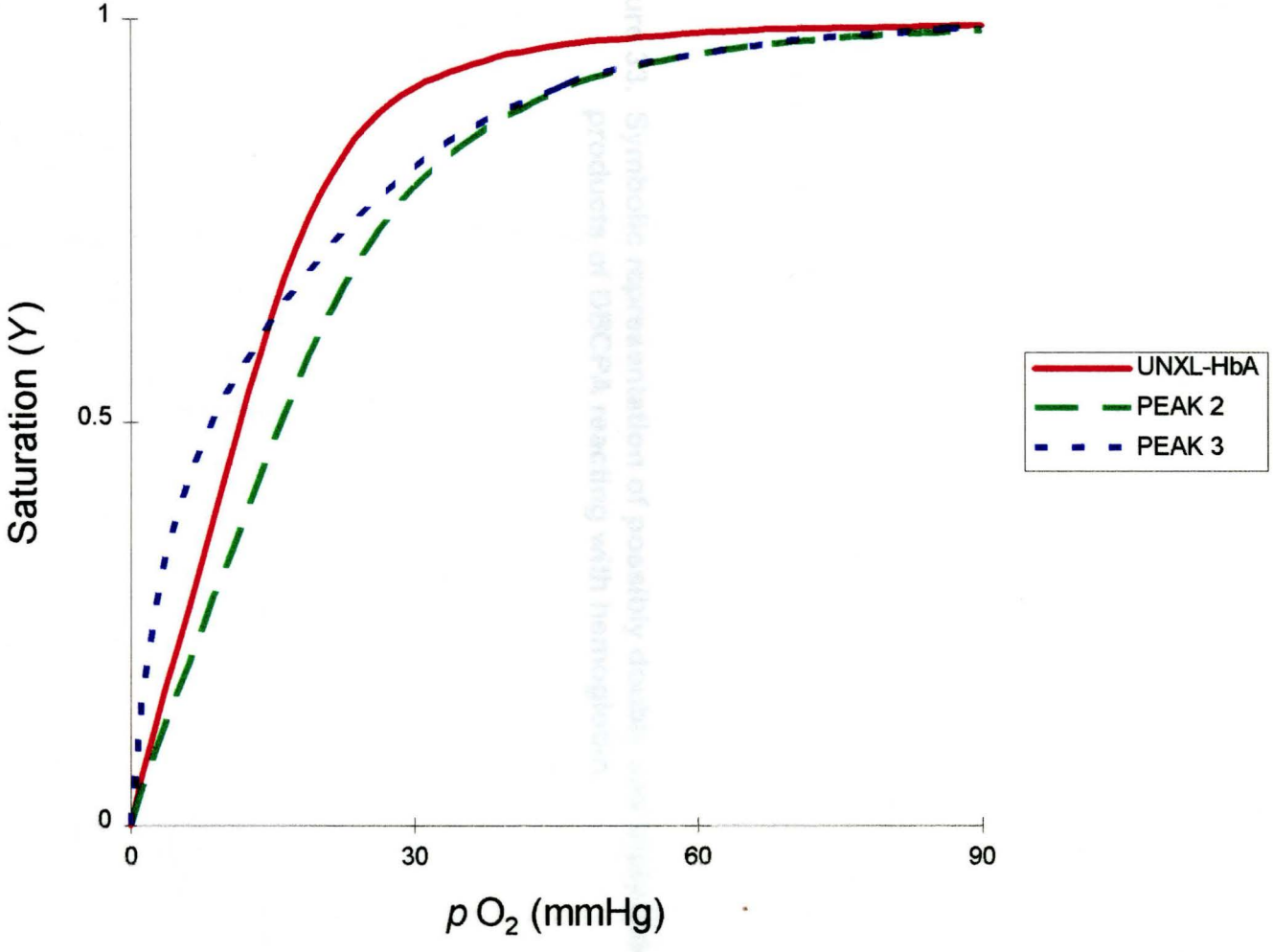
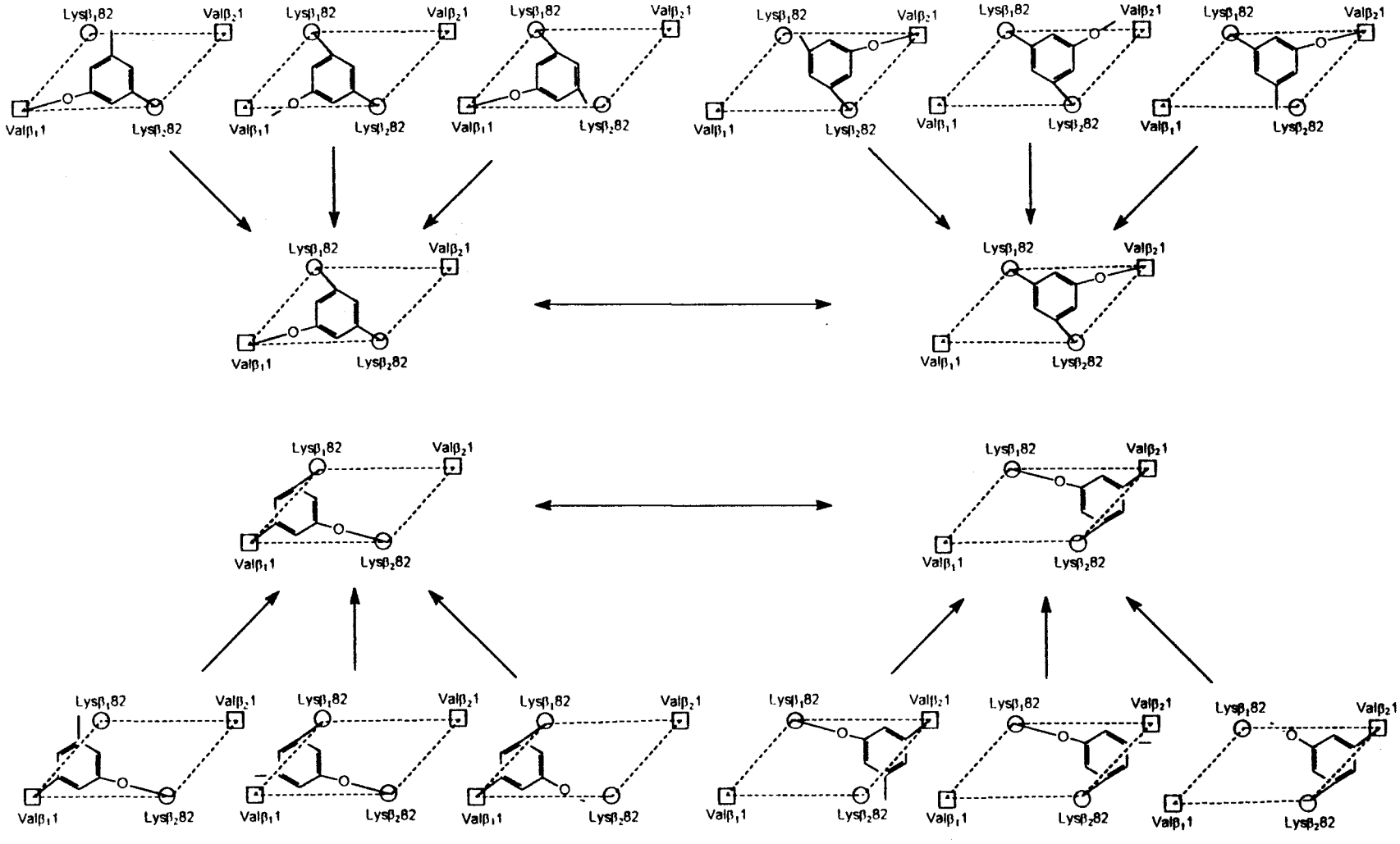

1
0.5
0
Saturation (Y)
0
30
60
90
p O2 (mmHg)
UNXL-HbA
PEAK 2
PEAK 3

Figure 33. Symbolic representation of possibly doubly and triply linked products of DBCPA reacting with hemoglobin.


Lysβ1 82
Valβ2 1
Valβ1 1
Lysβ2 82

Chapter 6

ELONGATED TETRA-LINKERS AND INTERMOLECULAR CROSSLINKING OF HEMOGLOBIN

RESULTS

The first section of this chapter summarizes the analytical results for each of the compounds synthesized. The spectra are given at the end of the chapter.

5,5'-(1,3-Propanedioxy) diisophthalic acid

Decomposition: 300 °C.

^{1}H NMR (DMSO-d_6) (see **Figure 34 (a)**): δ (ppm) 8.06 (t, 2H, ArH), 7.66 (d, 4H, ArH), 4.25 (t, 4H, O-CH_2), 2.22 (m, 2H, CH_2)

^{13}C NMR (DMSO-d_6) (see **Figure 34 (b)**): δ (ppm) 166.44 (s, 4C, C=O), 158.63 (s, 2C, O-ArC), 132.70 (s, 4C, ArC), 122.31 (s, 2C, ArC), 119.07 (s, 4C, ArC), 64.84 (s, 2C, O-CH_2), 28.40 (s, 1C, CH_2).

5,5'-(1,3-Propanedioxy) diisophthalyl tetrachloride

Melting point: 104.5-105.5 °C.

^{1}H NMR ($CDCl_3$) (see **Figure 35 (a)**): δ (ppm) 8.44 (t, 2H, ArH), 7.90 (d, 4H, ArH), 4.33 (t, 4H, O-CH_2), 2.41 (m, 2H, CH_2).

^{13}C NMR ($CDCl_3$) (see **Figure 35 (b)**): δ (ppm) 167.08 (s, 2C, C=O), 159.32 (s, 2C, O-ArC), 135.43 (s, 2C, ArC), 126.27 (s, 4C, ArC), 122.63 (s, 4C, ArC), 65.08 (s, 2C, O-CH_2), 28.73 (s, 1C, CH_2).

Tetrakis(*tert*-butyl 3,5-dibromosalicyl) 5,5'-(1,3-propanedioxy) diisophthalate

Melting point: 127-130 °C.

^1H NMR ($CDCl_3$) (see **Figure 36 (a)**): δ (ppm) 8.68 (s, 2H, ArH), 8.04 (d, 4H, ArH), 8.02 (d, 4H, ArH), 7.92 (d, 4H, ArH), 4.36 (t, 4H, O-CH_2), 2.42 (m, 2H, CH_2), 1.39 (s, 36H, tBu).

^{13}C NMR ($CDCl_3$) (see **Figure 36 (b)**): δ (ppm) 162.76 (s, 4C, C=O), 162.15 (s, 2C, C=O), 159.54 (s, 2C, O-ArC), 146.84 (s, 4C, O-ArH), 139.09 (s, 4C, ArH), 133.94 (s, 4C, ArH), 131.19 (s, 4C, ArH), 129,26 (s, 4C, ArC), 125.09 (s, 2C, ArC), 121.65 (s, 4C, ArC), 119.86 (s, 4C, ArC), 119.50 (s, 2C, ArC), 83.44 (s, 4C, O-C), 65.29 (s, 2C, O-CH_2), 29.60 (s, 1C, CH_2), 28.25 (s, 12C, tBu).

Tetrakis(3,5-dibromosalicyl) 5,5'-(1,3-propanedioxy) diisophthalate

Melting point: 175-180 °C.

^1H NMR (DMSO-d_6) (see **Figure 37 (a)**): δ (ppm) 8.38 (s, 2H, ArH), 8.31 (s, 4H, ArH), 8.09 (s, 4H, ArH), 8.02 (s, 4H, ArH), 4.41 (t, 4H, O-CH_2), 2.30 (m, 2H, CH_2).

^{13}C NMR (DMSO-d_6) (see **Figure 37 (b)**): δ (ppm) 163.40 (s, 4C, C=O), 162.26 (s, 2C, C=O), 159.28 (s, 2C, O-ArC), 146.66 (s, 4C, O-ArC), 138.90 (s, 4C, ArC), 133.43 (s, 4H, ArC), 130.44 (s, 4C, ArC), 127.60 (s, 4C, ArC), 123.00 (s, 2C,

ArC), 121.06 (s, 4C, ArC), 119.39 (s, 4C, ArC), 119.29 (s, 4C, ArC), 65.48 (s, 2C, O-CH_2), 27.37 (s, 1C, CH_2).

5,5'-Triethyleneglycol diisophthalic acid ether

Decomposition: 302 °C.

^{1}H NMR (DMSO-d_6) (see **Figure 38 (a)**): δ (ppm) 8.05 (t, 2H, ArH), 7.63 (d, 4H, ArH), 4.19 (t, 4H, O-CH_2), 3.77 (t, 4H, O-CH_2), 3.62 (s, 4H, O-CH_2).

^{13}C NMR (DMSO-d_6) (see **Figure 38 (b)**): δ (ppm) 166.40 (s, 2C, C=O), 158.66 (s, 2C, O-ArC), 132.61 (s, 4H, ArC), 122.35 (s, 2C, ArC), 70.02 (s, 2C, O-CH_2), 68.88 (s, 2C, O-CH_2), 67.85 (s, 2C, O-CH_2).

5,5'-Triethyleneglycol diisophthalyl ether tetrachloride

^{1}H NMR ($CDCl_3$) (see **Figure 39 (a)**): δ (ppm) 8.41 (t, 2H, ArH), 7.88 (d, 4H, ArH), 4.24 (t, 4H, O-CH_2), 3.90 (t, 4H, O-CH_2), 3.75 (s, 4H, O-CH_2).

^{13}C NMR ($CDCl_3$) (see **Figure 39 (b)**): δ (ppm) 167.10 (s, 2C, C=O), 159.52 (s, 2C, O-CH_2), 135.28 (s, 2C, ArC), 126.19 (s, 4C, ArC), 122.94 (s, 4C, ArC), 70.96 (s, 2C, O-CH_2), 69.53 (s, 2C, O-CH_2), 68.63 (s, 2C, O-CH_2).

Tetrakis(*tert*-3,5-dibromosalicyl) 5,5'-triethyleneglycol diisophthalate ether

Melting point: 95-98 °C.

^{1}H NMR ($CDCl_3$) (see **Figure 40 (a)**): δ (ppm) 8.66 (t, 2H, ArH), 8.03 (d, 4H, ArH), 8.00 (d, 4H, ArH), 7.90 (d, 4H, ArH), 4.28 (t, 4H, O-CH_2), 3.92 (t, 4H, O-CH_2), 3.78 (s, 4H, O-CH_2), 1.37 (s, 36H, tBu);

^{13}C NMR ($CDCl_3$) (see **Figure 40 (b)**): δ (ppm) 162.44 (s, 4C, C=O), 161.84 (s, 2C, C=O), 159.29 (s, 2C, O-ArC), 146.49 (s, 4C, O-ArC), 138.76 (s, 4C, ArC),

133.62 (s, 4C, ArC), 130.76 (s, 4C, ArC), 128.94 (s, 4C, ArC), 124.77 (s, 2C, ArC), 121.53 (s, 4C, ArC), 119.52 (s, 4C, ArC), 119.15 (s, 4C, ArC), 83.13 (s, 4C, O-C), 70.98 (s, 2C, O-CH_2), 69.59 (s, 2C, O-CH_2), 68.29 (s, 2C, O-CH_2), 27.92 (s, 12C, tBu).

Tetrakis(3,5-dibromosalicyl) 5,5-triethyleneglycol diisophthalate ether

Melting point: 157-160 °C.

^{1}H NMR (DMSO-d_6) (see **Figure 41 (a)**): δ (ppm) 8.37 (s, 2H, ArH), 8.32 (d, 4H, ArH), 8.09 (d, 4H, ArH), 7.99 (s, 4H, ArH), 4.32 (m, 4H, O-CH_2), 3.79 (m, 4H, O-CH_2), 3.64 (s, 4H, O-CH_2).

^{13}C NMR (DMSO-d_6) (see **Figure 41 (b)**): δ (ppm) 163.41 (s, 4C, ArC=O), 162.25 (s, 2C, ArC=O), 159.34 (s, 2C, O-ArC), 146.55 (s, 4C, O-ArC), 139.91 (s, 4C, ArC), 133.43 (s, 4C, ArC), 130.44 (s, 4C, ArC), 127.62 (s, 4C, ArC), 123.01 (s, 2C, ArC), 121.17 (s, 4C, ArC), 119.34 (s, 4C, ArC), 119.28 (s, 4C, ArC), 69.99 (s, 2C, O-CH_2), 68.85 (s, 2C, O-CH_2), 68.41 (s, 2C, O-CH_2).

5,5'-(1,10-decanedioxy) diisophthalic acid

^{1}H NMR (DMSO-d_6) (see **Figure 42 (a)**): δ (ppm) 8.05 (s, 2H, ArH), 7.61 (s, 4H, ArH), 4.04 (t, 4H, O-CH_2), 1.71 (m, 4H, CH_2), 1.38 (m, 4H, CH_2), 1.27(m, 8H, CH_2).

^{13}C NMR (DMSO-d_6) (see **Figure 42 (b)**): δ (ppm) 166.11 (s, 4C, C=O), 158.56 (s, 2C, ArC), 132.43 (s, 4C, ArC), 121.94 (s, 2C, ArC), 118.86 (s, 4C, ArC), 68.05 (s, 2C, O-CH_2), 28.92 (s, 2C, CH_2), 28.69 (s, 2C, CH_2), 28.48 (s, 2C, CH_2), 25.37 (s, 2C, CH_2).

5,5'-(1,10-decanedioxy) diisophthalate tetrachloride

^{1}H NMR (DMSO-d_6) (see **Figure 43 (a)**): δ (ppm) 8.41 (s, 2H, ArH), 7.85 (s, 4H, ArH), 4.06 (t, 4H, O-CH_2), 1.83 (m, 4H, CH_2), 1.48 (m, 4H, CH_2), 1.34 (m, 8H, CH_2).

^{13}C NMR (DMSO-d_6) (see **Figure 43 (b)**): δ (ppm) 167.01 (s, 4C, C=O), 159.73 (s, 2C, ArC), 135.29 (s, 2C, ArC), 125.76 (s, 4C, ArC), 122.65 (s, 4C, ArC), 69.30 (s, 2C, O-CH_2), 29.50 (s, 2C, CH_2), 29.34 (s, 2C, CH_2), 29.00 (s, 2C, CH_2), 25.99 (s, 2C, CH_2).

SDS-PAGE of the crosslinked hemoglobins

SDS-PAGE analysis (**Figure 44** and **45**) of the crosslinked hemoglobins exhibits 46% dimer, 2% trimer and 1% tetramer for crosslinking by tetrakis(3,5-dibromosalicyl) 5,5'-(1,3-propanedioxy) diisophthalate; 44% dimer, 12% trimer and 5% tetramer for crosslinking by tetrakis(3,5-dibromosalicyl) 5,5-triethyleneglycol diisophthalate ether; and 43% dimer, 6% trimer and 1% tetramer for crosslinking by tetrakis(sodium methyl phosphate) 5,5'-(1,10-decanedioxy) diisophthalate (**Table 11**).

Table 11. SDS-PAGE analysis of the DBPDI, DBTDI and SMPDI crosslinked hemoglobins by gel scanner.

Sample	% Dimer	% Trimer	% Tetramer
DBPDI-XL	46	2	1
DBTDE-XL	44	12	5
SMPDI-XL	43	6	1

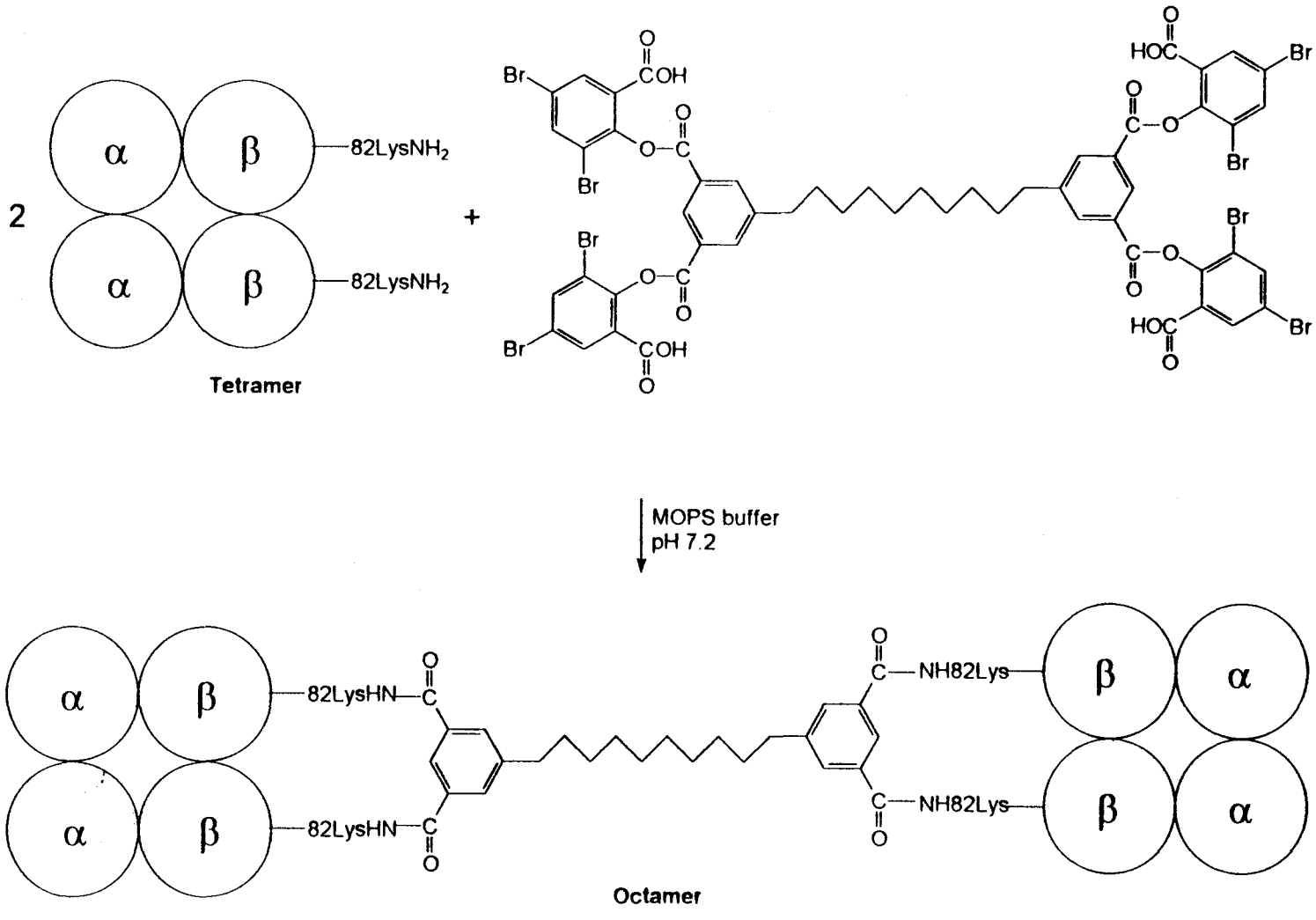
DISCUSSION

The tetrakis(3,5-dibromosalicyl) linkers, DBPDI, DBEIE and SMPDD, were designed to tetra-link between two tetrameric hemoglobins and between Lys82βs of each tetramer to form a octamer in one step (**Scheme XII**). Previously described crosslinkers with two meta 3,5-dibromosalicyl ester groups [77-78] or sodium methyl phosphate groups [79] showed high selectivity for reaction at the site between Lys82βs of hemoglobin. In the structures of the elongated tetra-linkers, each end contains two meta 3,5-dibromosalicyl ester groups which are sterospecific to crosslink between Lys82βs of one hemoglobin molecule. Reaction of the tetra-linkers with hemoglobin in one step simplifies the process of synthesis of a specifically modified octameric hemoglobin.

The syntheses of both tetrakis(3,5-dibromosalicyl) 5,5-(1,3-propanedioxy) diisophthalate (DBPDI) and tetrakis(3,5-dibromosalicyl) 5,5-triethyleneglycol diisophthalate ether (DBEIE) have four steps. The first step is the production of diisophthalic acids; the second step is the production of the corresponding acid chlorides; the third step is the production of the corresponding tetrakis(3,5-dibromosalicylate) esters; the fourth step is the *tert*-butyl deprotection by trifluoroacetic acid to form the corresponding tetrakis(3,5-dibromosalicyl)esters.

The tetrakis(3,5-dibromosalicyl) 5,5'-(1,3-propanedioxy) diisophthalate was characterized by ^{1}H and ^{13}C NMR (**Figure 37**): The six hydrogen resonances at chemical shifts δ 8.38 and 8.02 ppm are assigned to the isophthalate aryl

Scheme XII.


2
α
β
α
β
82LysNH2
82LysNH2
Tetramer
+
Br
COH
O
Br
Br
COH
O
HOC
Br
Br
HOC
Br
MOPS buffer
pH 7.2
82LysHN
82LysHN
NH82Lys
NH82Lys
Octamer

hydrogens, the eight hydrogen resonances at δ 8.31 and 8.09 ppm are the aspirin benzenes and the six hydrogen resonances at δ 4.41 and 2.300 ppm to the methylene groups; the two peaks at δ 163.40 and 162.26 ppm are assigned to the carbonyl carbons on the aspirin and isophthalate aromatic rings, the ten peaks at δ 159.28 - 119.29 ppm to the aspirin and isophthalate aryl carbons and two peaks at δ 65.48 - 27.37 ppm to the methylene carbons. The synthesis of the reagent was successful.

The desired product for tetrakis(3,5-dibromosalicyl) 5,5-triethyleneglycol isophthalate ether was characterized by ^{1}H and ^{13}C NMR (**Figure 41**): The six hydrogen resonances at chemical shifts δ 8.37 and 7.99 ppm are assigned to the isophthalate benzenes, the eight hydrogen resonances at δ 8.32 and 8.09 ppm to the aspirin benzenes and the twelve hydrogen resonances at δ 4.32, 3.79 and 3.64 ppm to the ethyleneglycol groups; the two peaks at δ 163.41 and 162.25 ppm are assigned to the carboxyl carbons on the aspirin and isophthalate aromatic rings, the ten peaks at δ 159.34 - 119.28 ppm to the aspirin and isophthalate aryl carbons and the three peaks at δ 69.99, 68.85 and 68.41 ppm are the ethyleneglycol carbons. The synthesis of the reagent was successful.

The preparation of tetrakis(sodium methyl phosphate) 5,5-(1,10-decanedioxy) diisophthalate (SMPDI) has four steps. The first step is the production of diisophthalic acids; the second step is the production of the corresponding acid chlorides; the third step is the production of the corresponding tetrakis(dimethyl phosphate); the fourth step is the production of the corresponding tetrakis(sodium

methyl phosphate). The product is soluble in water. The results of NMR characterization of tetrakis(sodium methyl phosphate) 5,5-(1,10-decanedioxy) diisophthalate were not satisfactory because of the low solubility of the compound in deuterium oxide. ^{1}H NMR shows multiple peaks around δ 4 ppm corresponding to the methoxy of phosphate and δ 1.5 ppm to decylene.

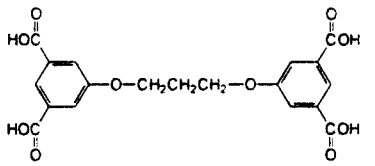
The results of SDS-PAGE in **Figure 44** show that DBPDI and DBEIE can intramolecularly crosslink very effectively, but intermolecular crosslinking is slight. The amount of triply and quadruply crosslinked species demonstrates the difficulties in having a second reaction once one end of the reagent has reacted with a BPG binding site. In addition, it is not certain that the trimers and tetramers observed upon SDS-PAGE actually represent intermolecular crosslinking. It is possible that there is little reaction with the α-chains of crosslinked tetramer. DBEIE gave higher yields of trimer and tetramer because of its longer size and greater solubility in aqueous solution than DBPDI (**Tables 11** and **12**). The reaction of hemoglobin with another kind of tetralinker, SMPDI, also produced double, triple and tetra-linking (**Figure 45** and **Table 11**), but it was less effective than DBEIE. While the methyl phosphate groups should provide water solubility for the termini of SMPDI, the ten methylene groups in the connecting region are more hydrophobic than the bridging structure of DBEIE and it folds in aqueous solution making the bridging length of the reagent much shorter than expected (**Table 12**).

Table 12. Bridging lengths between ester carbonyl carbons of the tetra-linkers

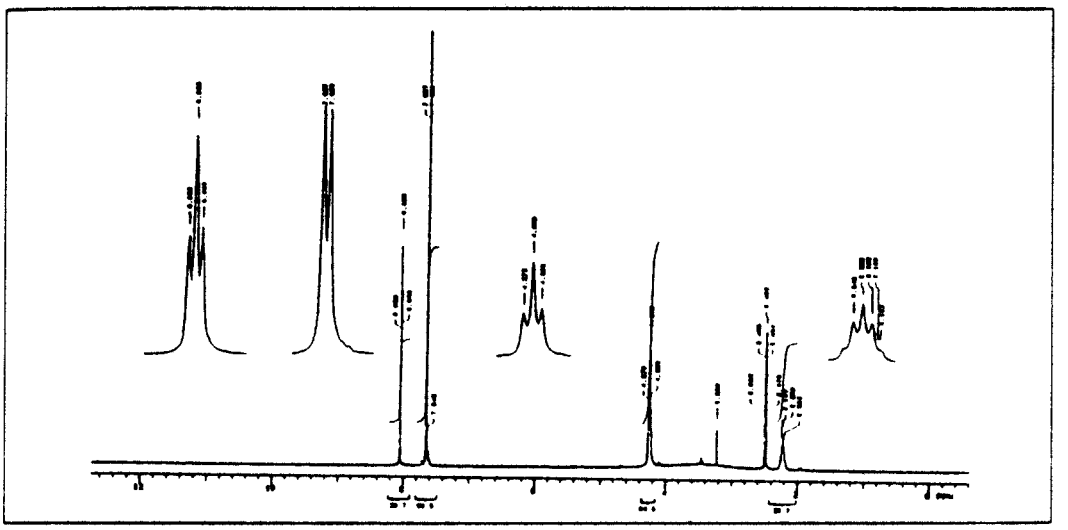
Compound	Bridging length (Å)					
DBPDI	5.1	5.2	10.7	10.7	13.3	13.7
DBEIE	5.1	5.1	15.4	16.9	18.1	20.1
SMPDI	5.2	5.2	18.8	20.0	21.3	23.0

The successful tetra-linking of hemoglobin would provide a new method to crosslink hemoglobin intramolecularly between two β chains and intermolecularly between two hemoglobin molecules at one step to produce a novel blood substitute, thermally stable octameric hemoglobin (**Scheme XII**). DBEIE appears to be the best of the three reagents developed here, but better reaction conditions need to be developed before it can be used to efficiently produce hemoglobin octamers.

Figure 34. ^{1}H and ^{13}C NMR of 5,5'-(1,3-propanedioxy) diisophthalic acid (DMSO-d_6).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

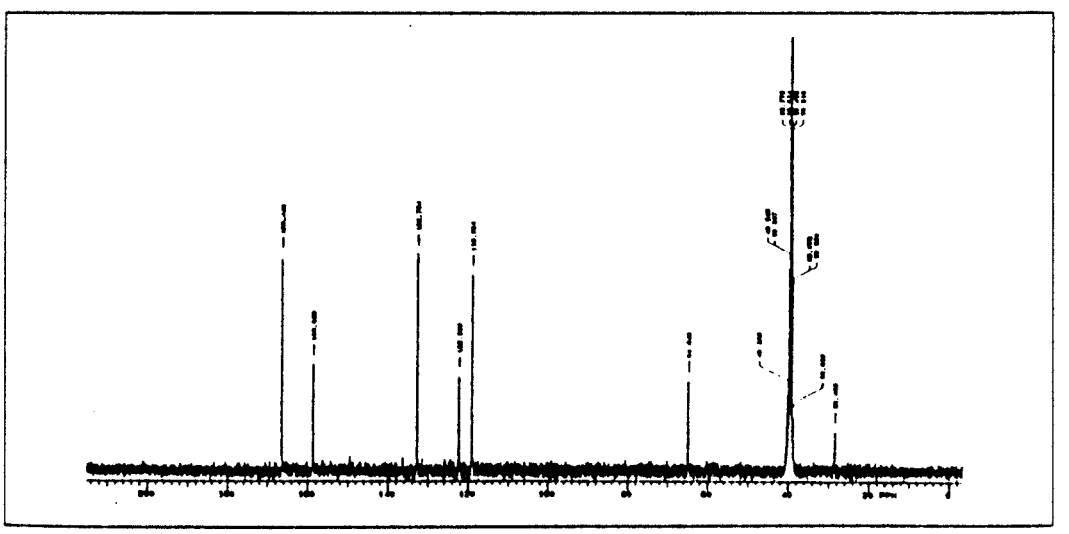
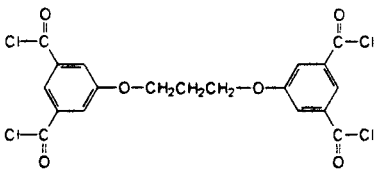
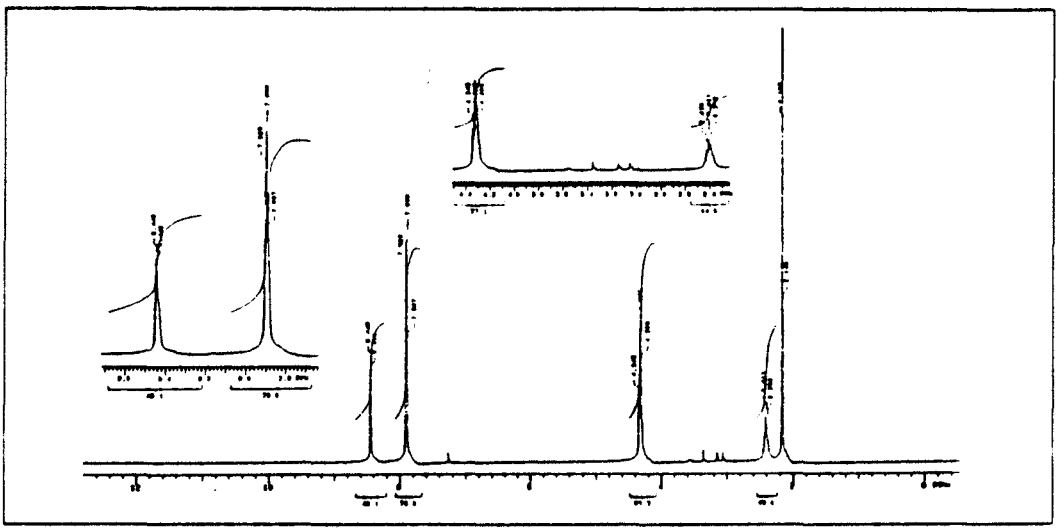


Figure 35. ^{1}H and ^{13}C NMR of 5,5'-(1,3-propanedioxy) diisophthalyl tetrachloride ($CDCl_3$).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

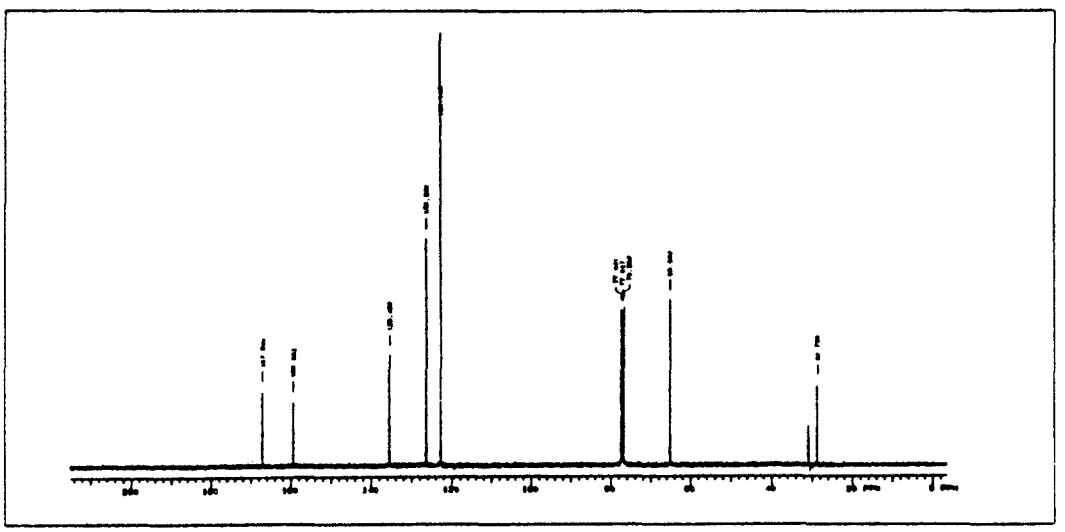
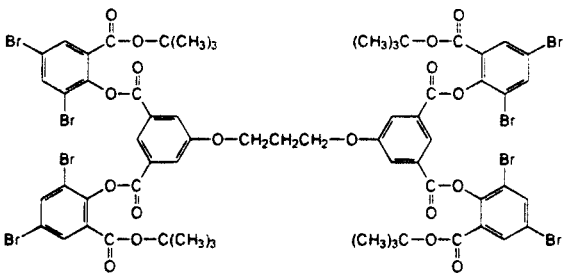
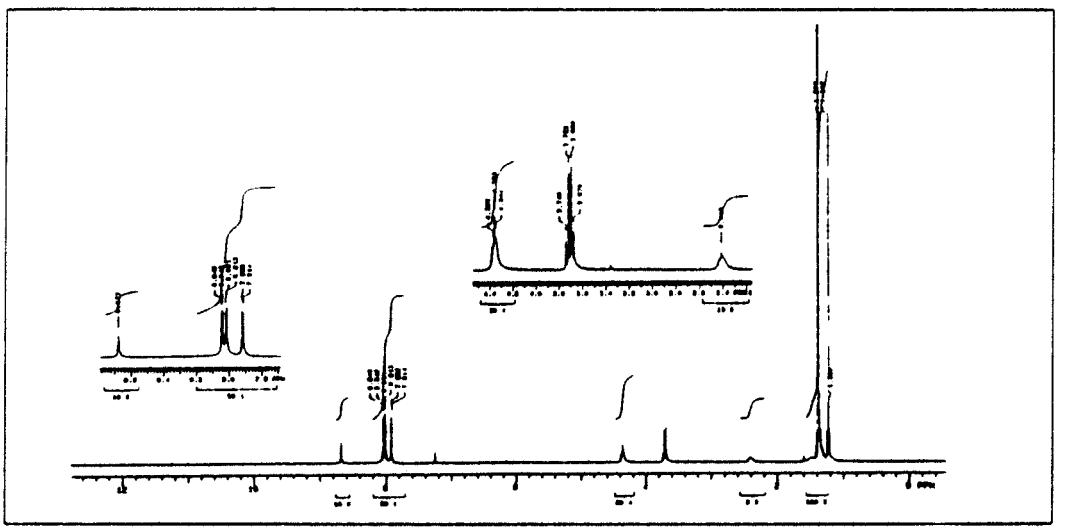


Figure 36. ^{1}H and ^{13}C NMR of tetrakis(*tert*-butyl 3,5-dibromosalicyl) 5,5'-(1,3-propanedioxy) diisophthalate ($CDCl_3$).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

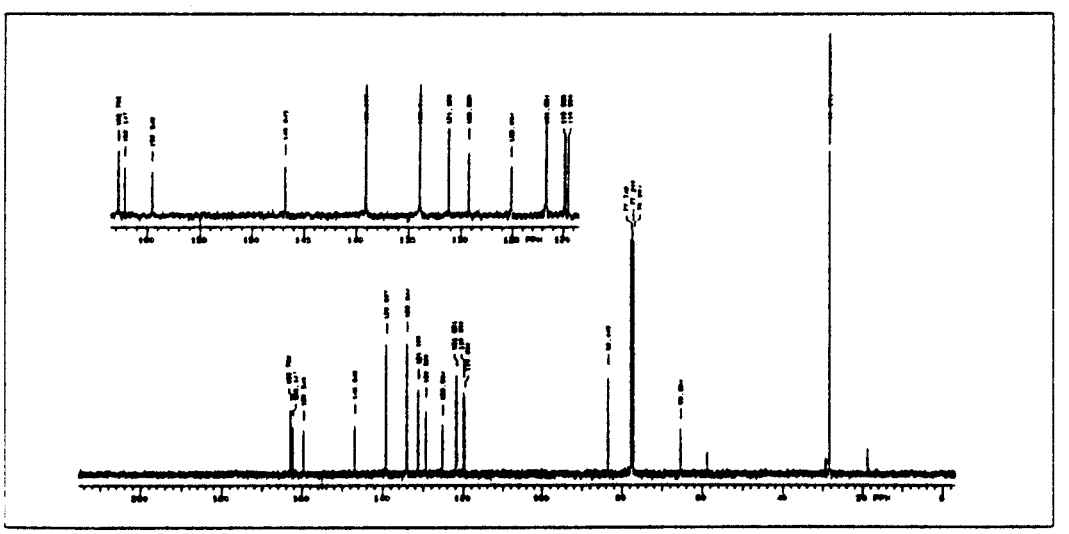
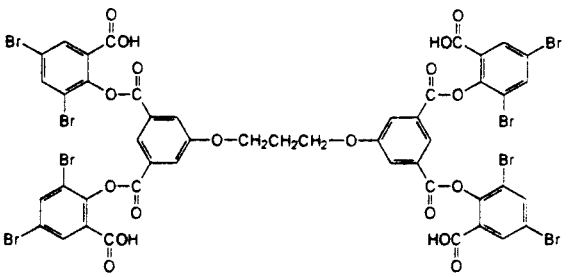
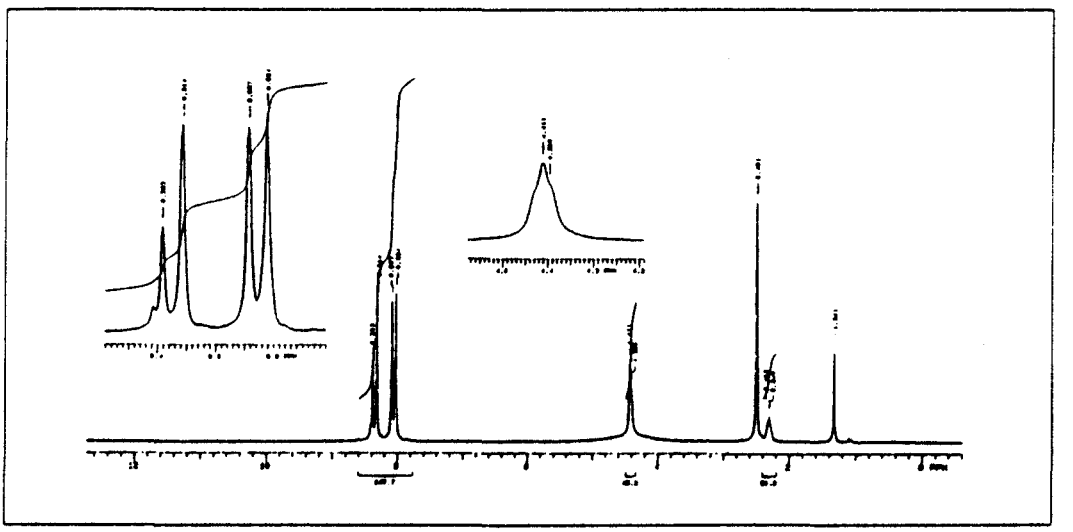


Figure 37. ^{1}H and ^{13}C NMR of tetrakis(3,5-dibromosalicyl) 5,5'-(1,3-propanedioxy) diisophthalate (DMSO-d_3).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

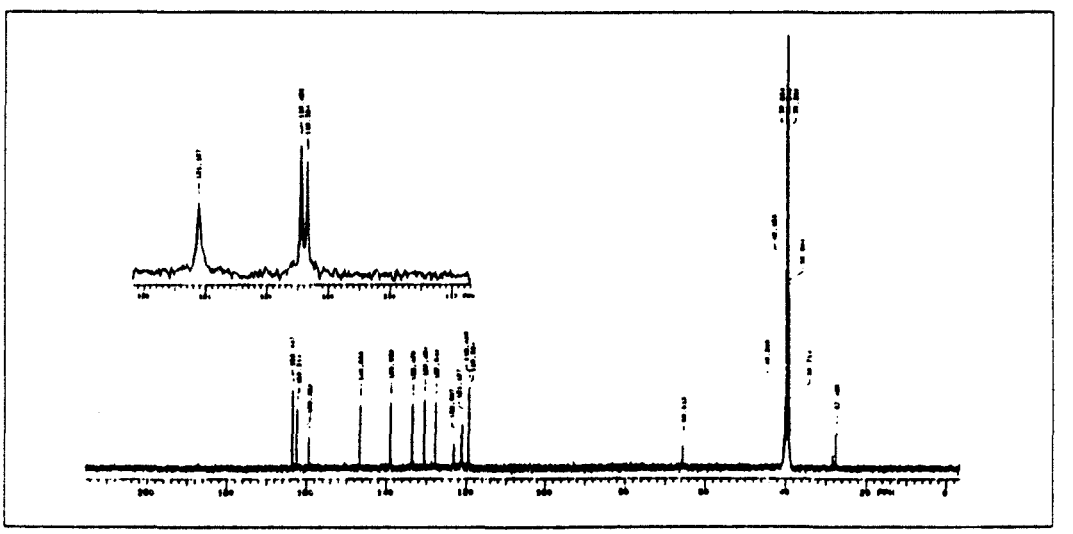
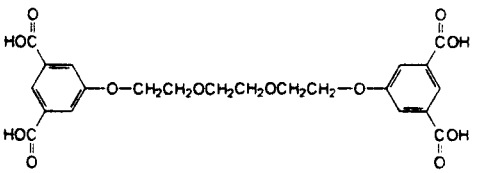
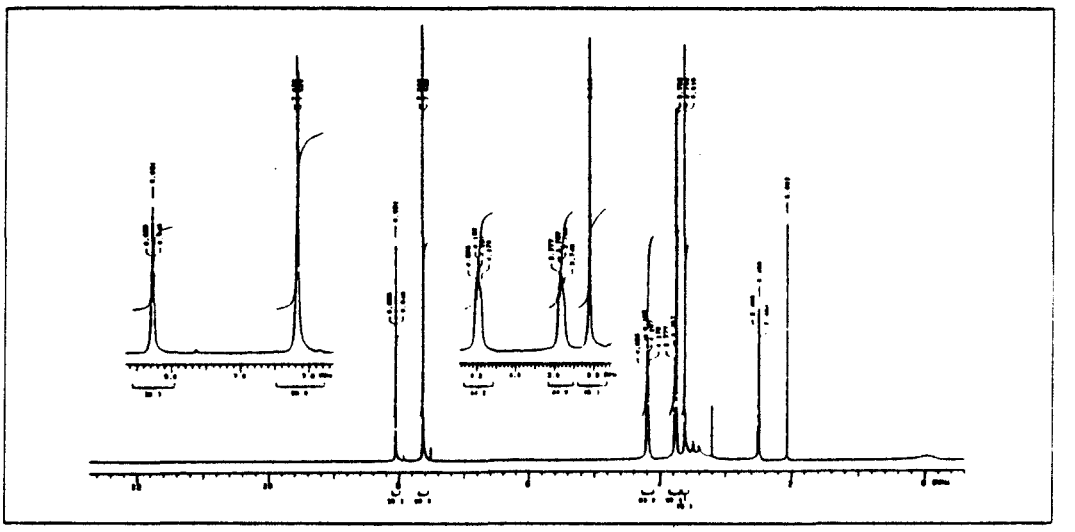


Figure 38. ^{1}H and ^{13}C NMR of 5,5'-triethyleneglycol diisophthalic acid ether (DMSO-d_6).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

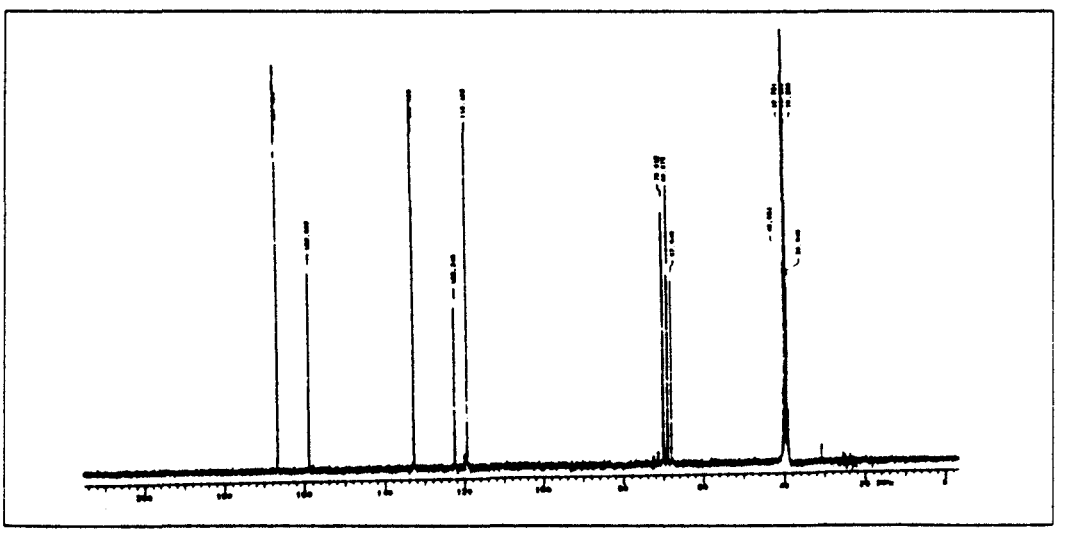
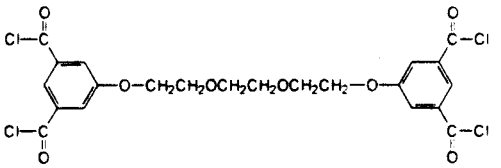
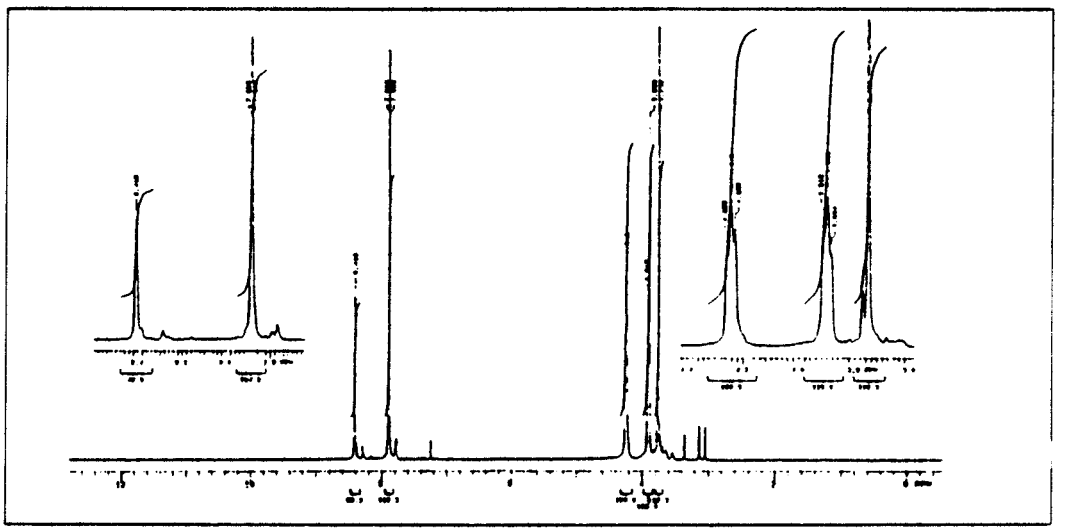


Figure 39. ^{1}H and ^{13}C NMR of 5,5'-triethyleneglycol diisophthalyl ether tetrachloride ($CDCl_3$).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

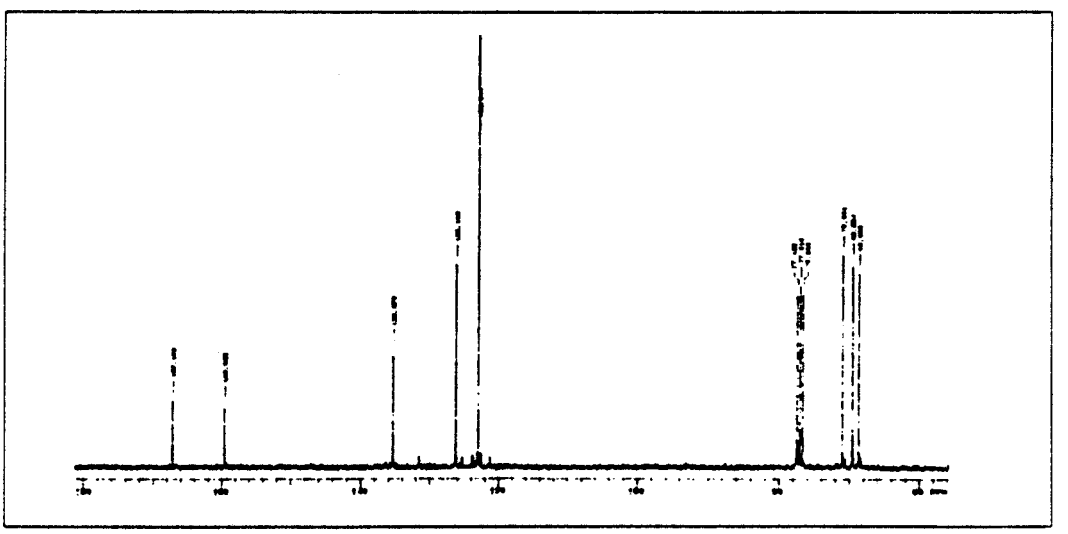
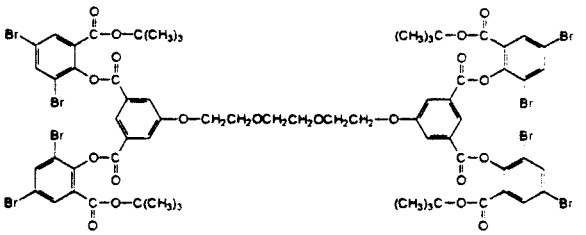
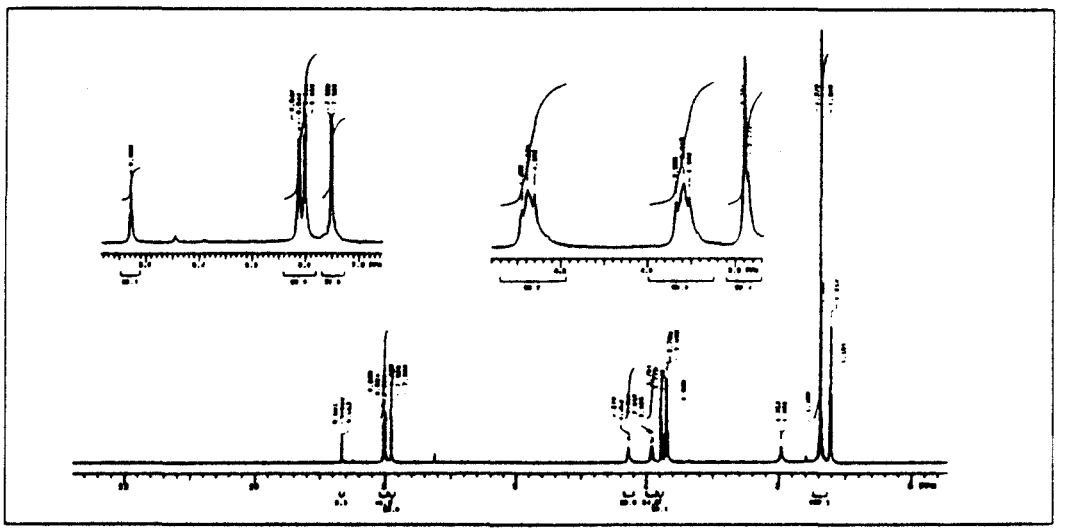


Figure 40. ^{1}H and ^{13}C NMR of tetrakis(*tert*-3,5-dibromosalicyl) 5,5'-triethyleneglycol diisophthalate ether ($CDCl_3$).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

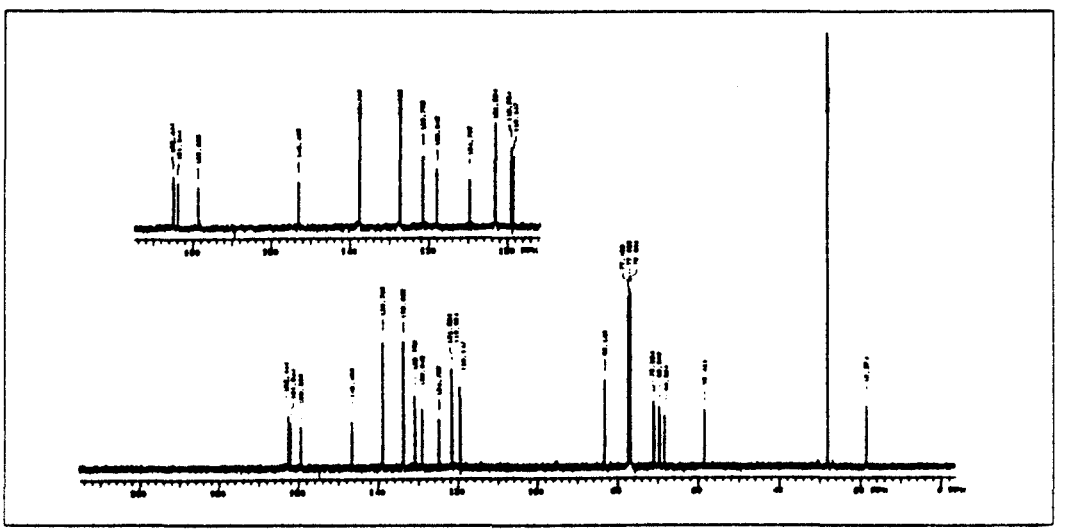
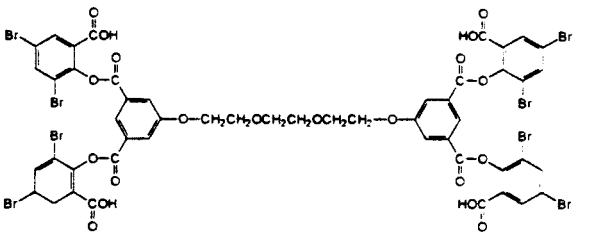
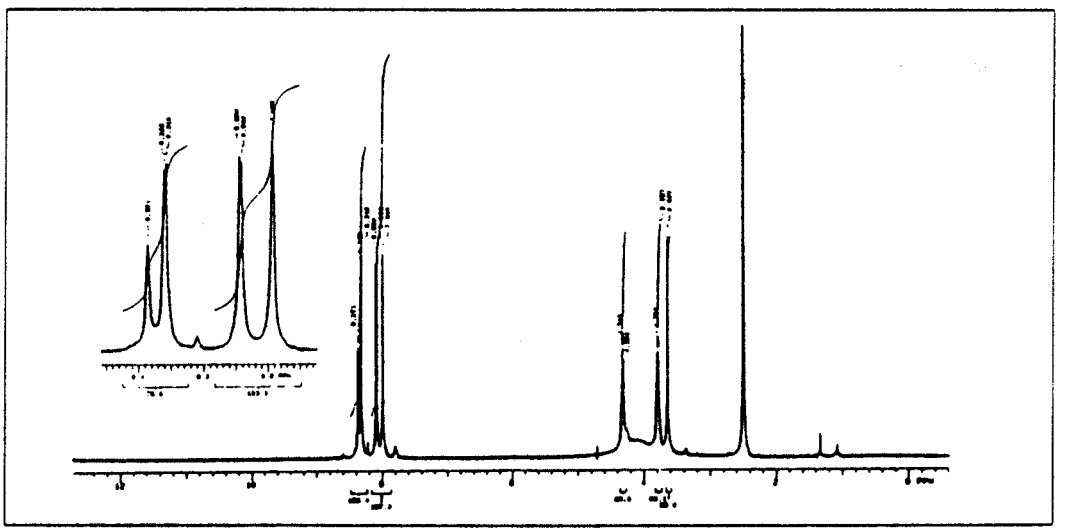


Figure 41. ^{1}H and ^{13}C NMR of tetrakis(3,5-dibromosalicyl) 5,5-triethyleneglycol diisophthalate ether (DMSO-d_6).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

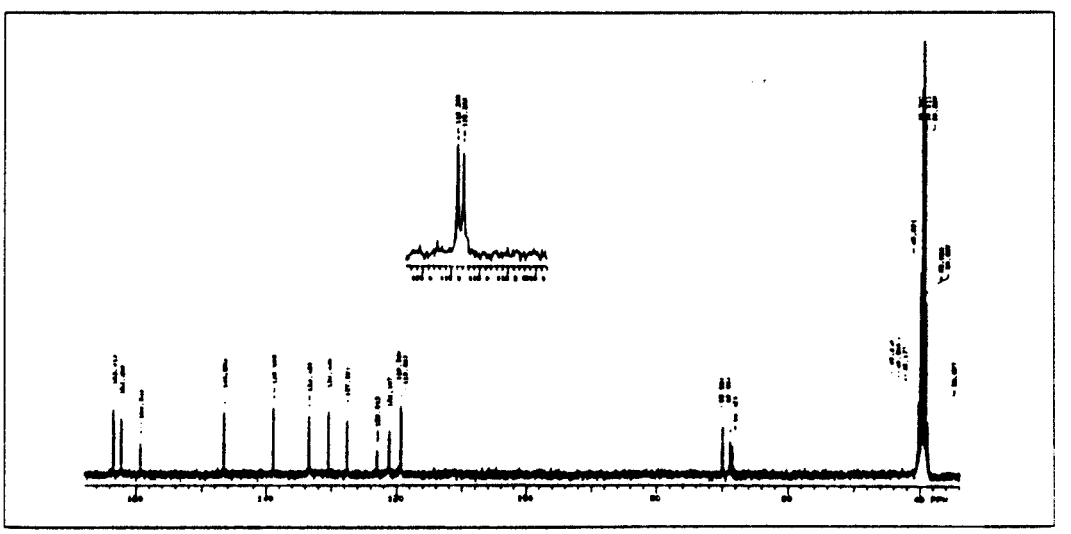
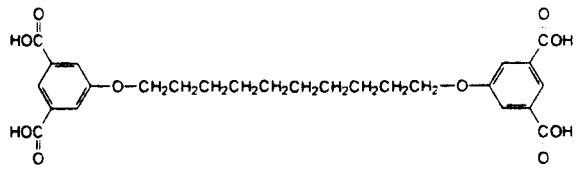
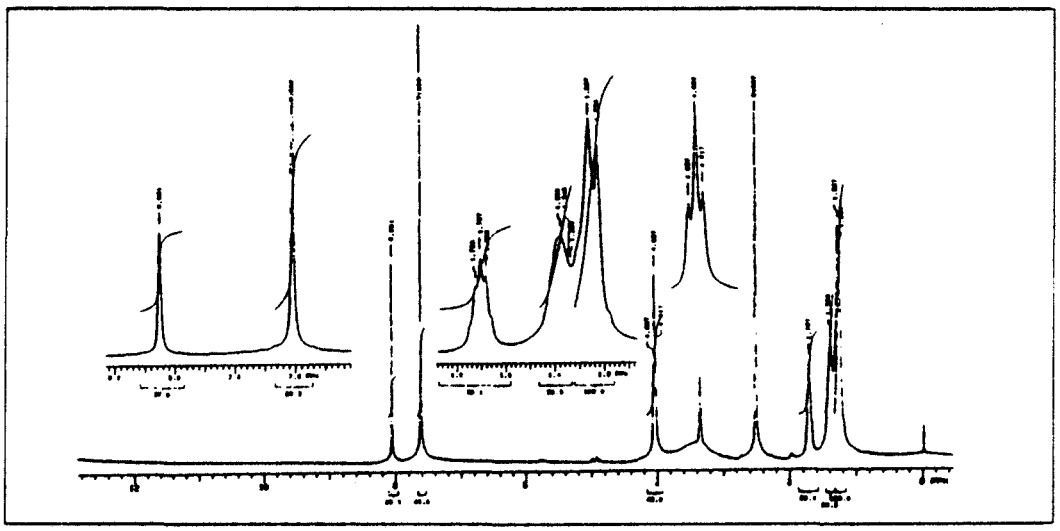


Figure 42. ^{1}H and ^{13}C NMR of 5,5'-(1,10-decanedioxy) diisophthalic acid (DMSO-d_6).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

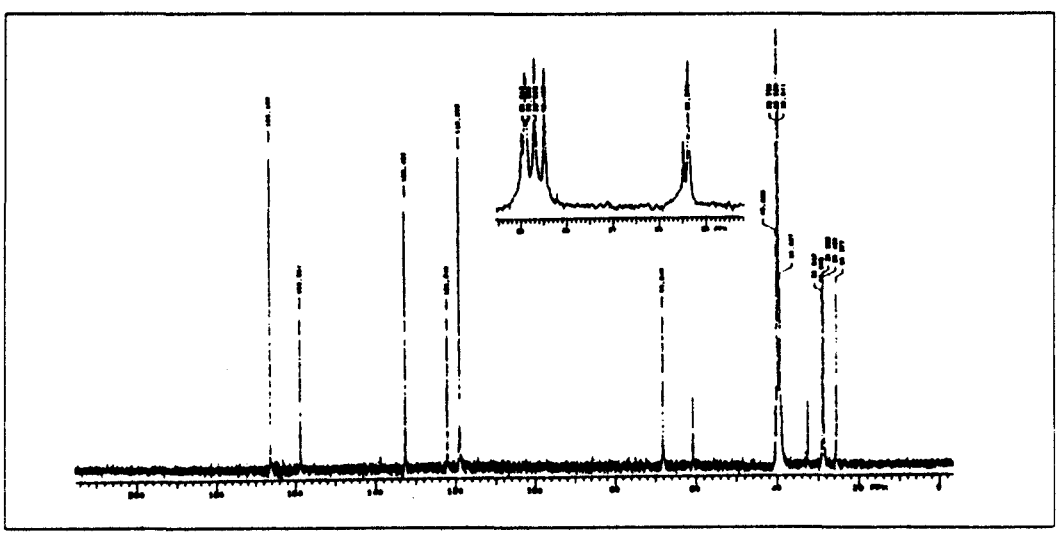
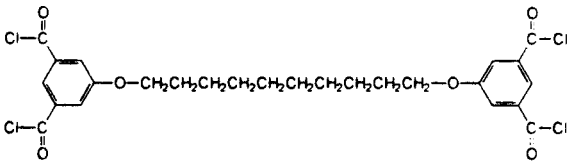
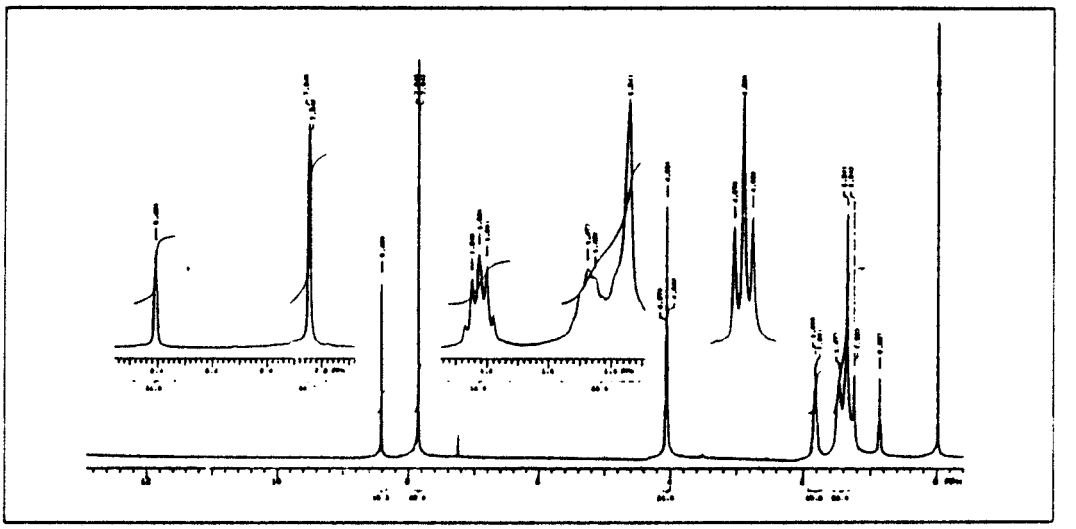


Figure 43. ^{1}H and ^{13}C NMR of 5,5'-(1,10-propanedioxy) diisophthalate tetrachloride ($CDCl_3$).



(a) ^{1}H NMR.



(b) ^{13}C NMR.

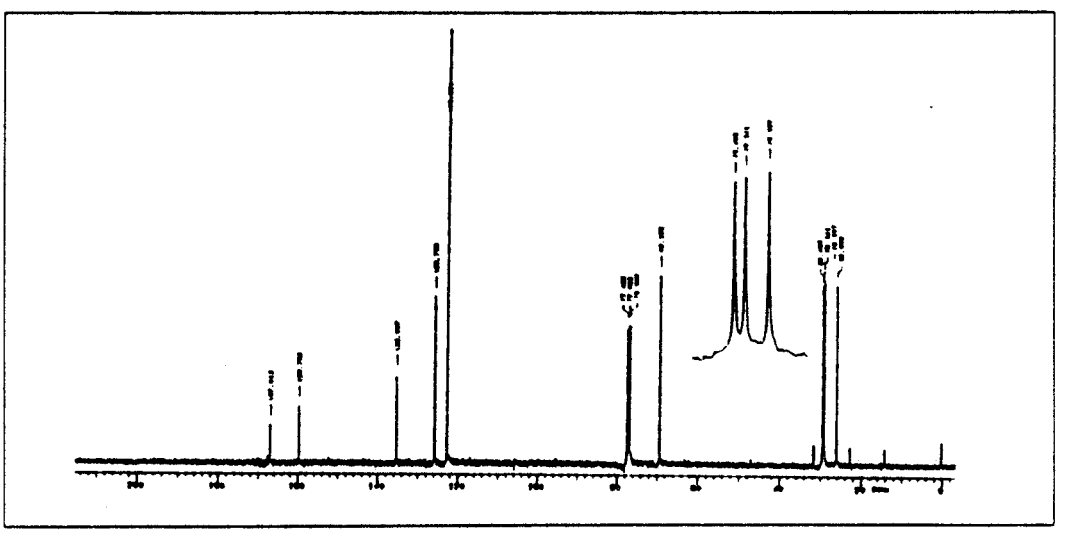


Figure 44. SDS-PAGE of the DBPDI and DBTDE crosslinked hemoglobins.

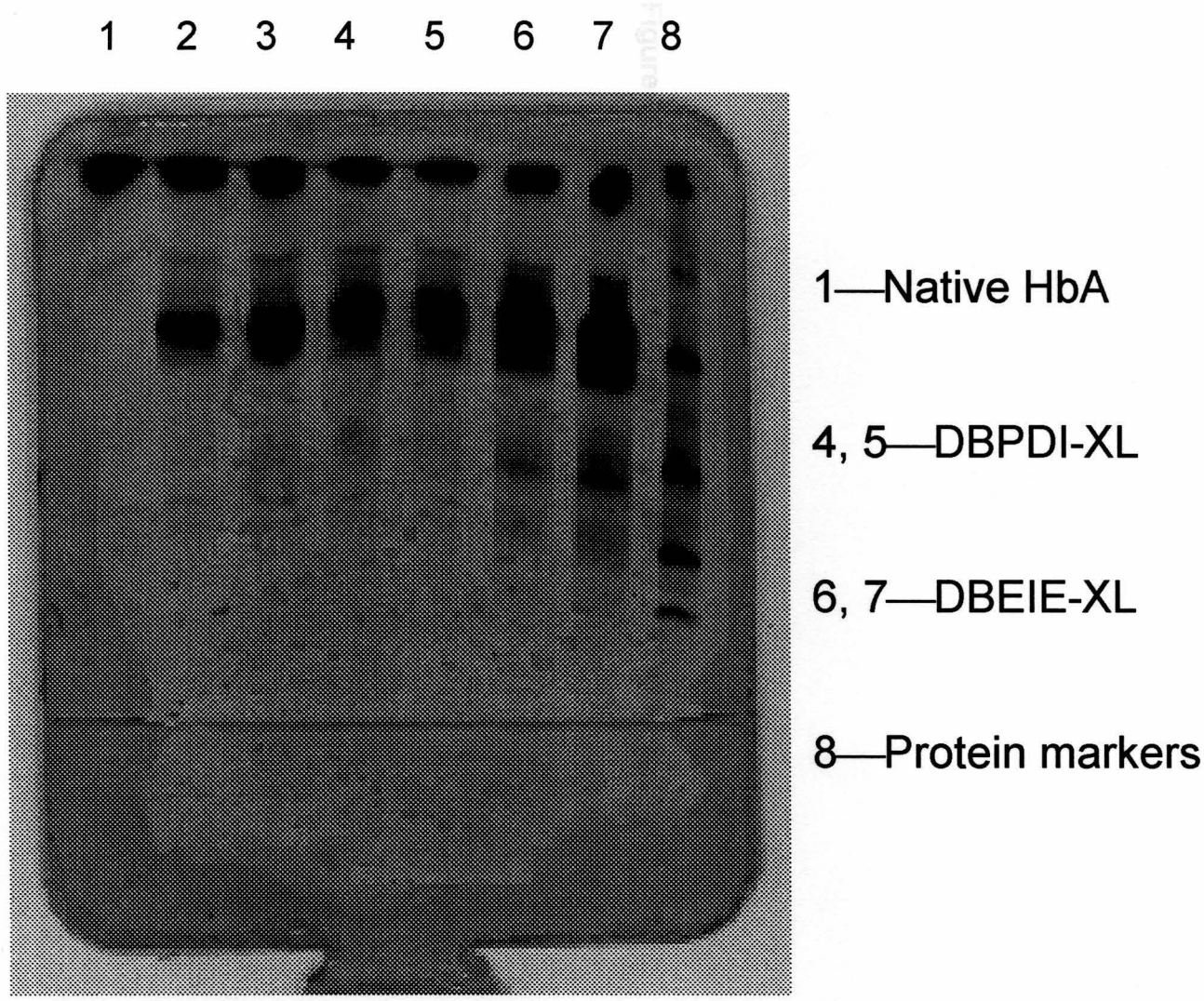
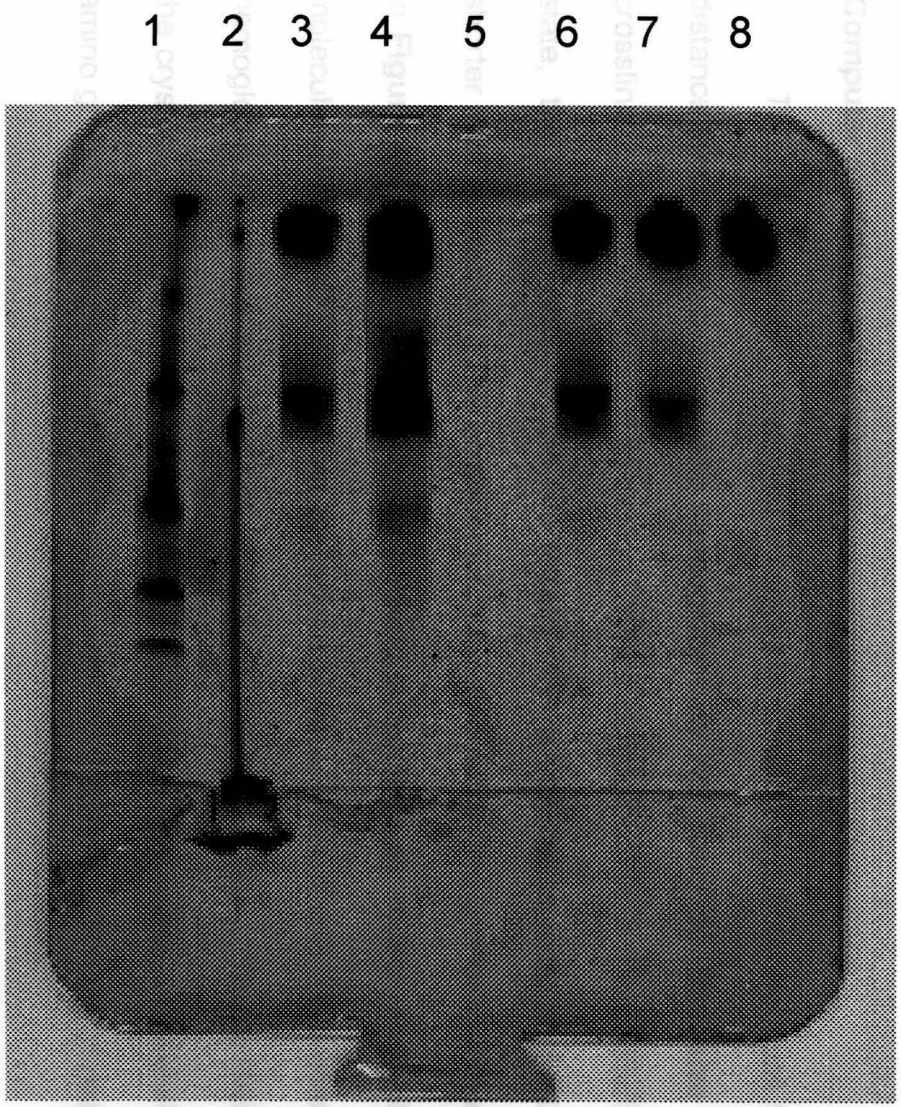

1 2 3 4 5 6 7 8
1—Native HbA
4, 5—DBPDI-XL
6, 7—DBEIE-XL
8—Protein markers

Figure 45. SDS-PAGE of the SMPDI crosslinked hemoglobins.



1—Protein markers

4—SMPDI-XL

8—Native HbA

Chapter 7

STUDY ON THE POSSIBILITY OF CROSSLINKING IN HEMOGLOBIN CRYSTALS

RESULTS AND DISCUSSION

Computer modeling and design

The production of crosslinked octamers in solution is difficult because the distance between two tetrameric hemoglobins is not fixed so that size of crosslinking reagent is difficult to design. When hemoglobin is in the crystalline state, the distance between the two tetramers in a unit cell is fixed and much shorter than in solution. The arrangement of molecules in the unit cell is shown in **Figure 46**. A model of the crystal packing comprising six deoxyhemoglobin molecules (**Figure 47**) shows that the space filled with water [87-89] between hemoglobin molecules provides pathways for small molecules to diffuse inside the crystals. In these channels there are possible sites composed of several ε-amino groups of lysines with which crosslinking reagents would be able to react.

Figure 46. A stereo view of the crystal unit of deoxyhemoglobin in $P2_1$ space group.

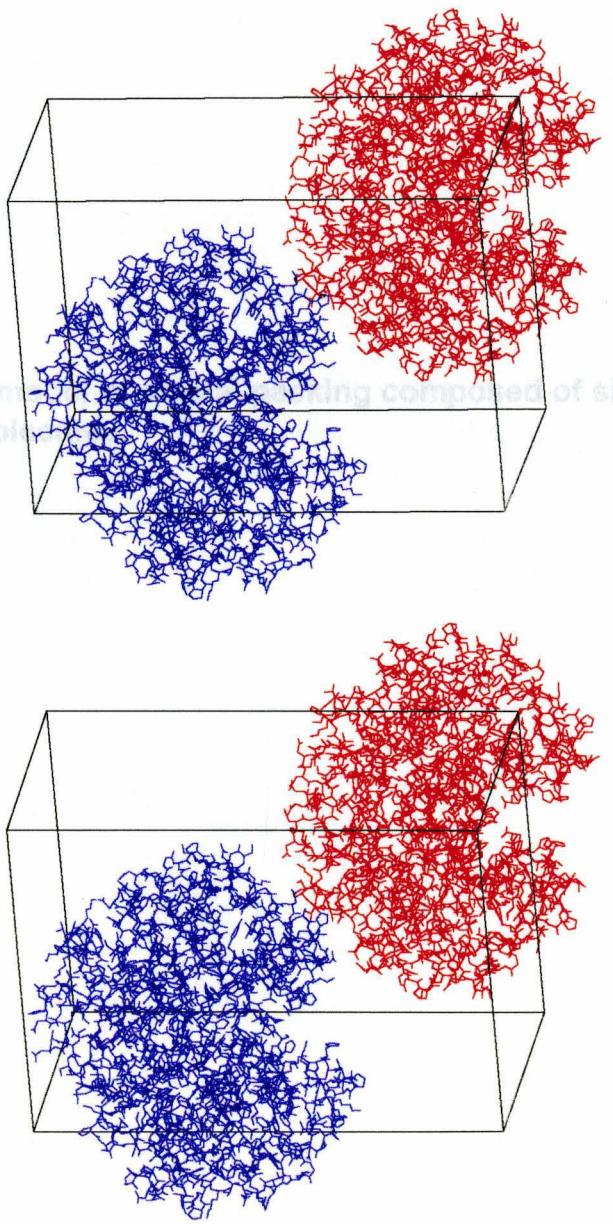
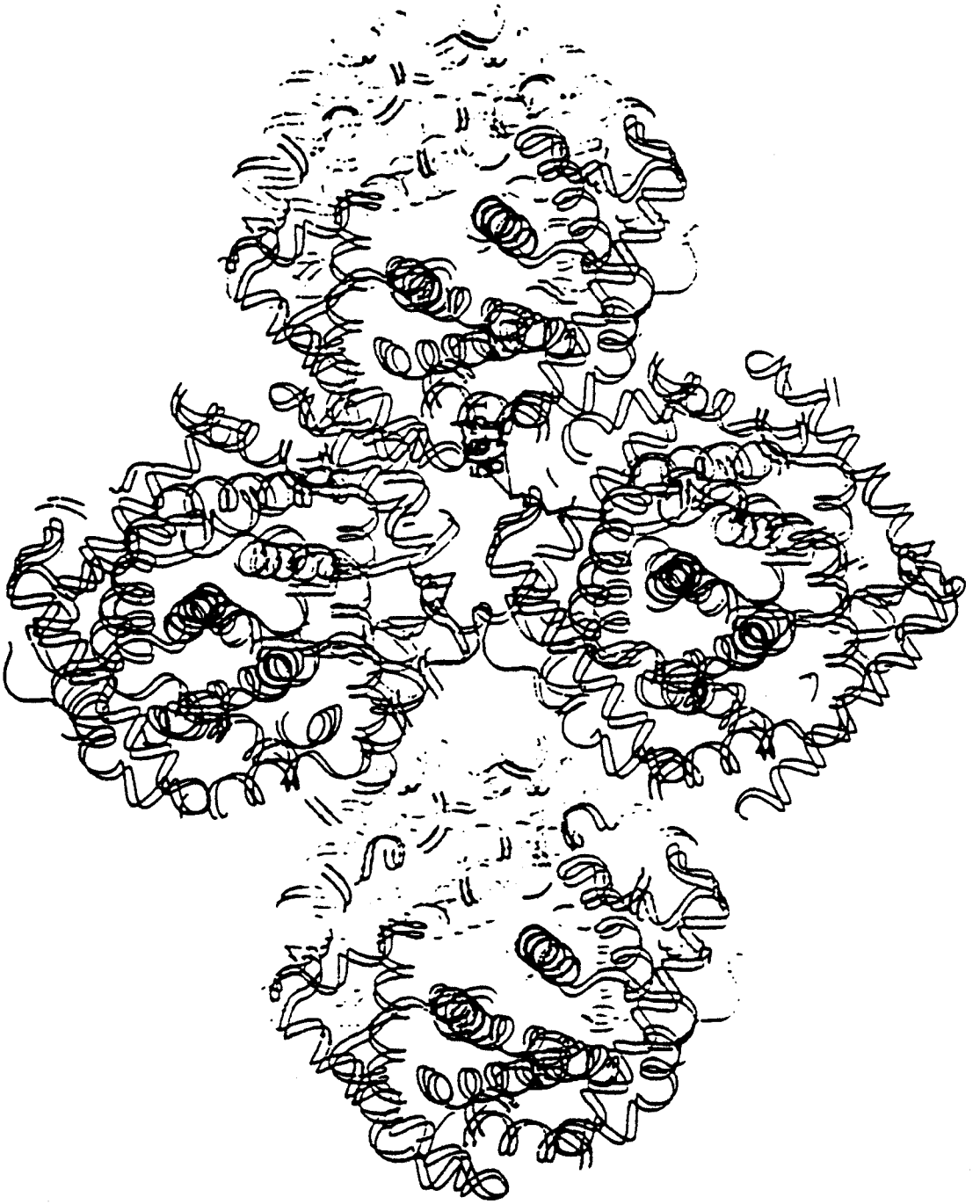


Figure 47. A model of crystal packing composed of six deoxyhemoglobin molecules.



Lysα7, and Lysα11 on one tetramer and Lysβ66 on another could be a possible specific site for crosslinking between the two tetramers (**Figure 48**). The tri-linker, tris(3,5-dibromosalicyl) tricarballylate, was designed with the suitable size to react at this site (**Figure 49**).

Reaction of hemoglobin with bis(3,5-dibromosalicyl) fumarate in crystallization conditions

Reaction of hemoglobin with bis(3,5-dibromosalicyl) fumarate (DBSF) has been well studied [69-72]. To determine if this type of reagents can be used to modify crystallized hemoglobin, the reaction was done under crystallization conditions [90]. The analysis of SDS-PAGE given in **Table 13** indicates that DBSF crosslinked between two chains of hemoglobin under both high salt (sample 1 and 2) and low salt (sample 3 and 4) crystallization conditions. The yields of crosslink in the high salt concentrations, however, were less than in the low salt concentrations. This result may be due to reaction of ammonium ions in the high salt buffer with the DBSF.

Reaction of crystallized hemoglobin with tris(3,5-dibromosalicyl) tricarballylate and bis(3,5-dibromosalicyl) fumarate

We used tris(3,5-dibromosalicyl) tricarballylate to react with crystallized hemoglobin from the high-salt condition. The result of SDS-PAGE did not show any dimer formation. The same reagent reacted with crystallized hemoglobin from the low-salted condition and also had no reaction. No crosslinking was obtained either when DBSF was used to react with crystallized hemoglobin from both high-salt and low-salt conditions.

Figure 48. Expanded view of the crystal model at the possible specific-site.

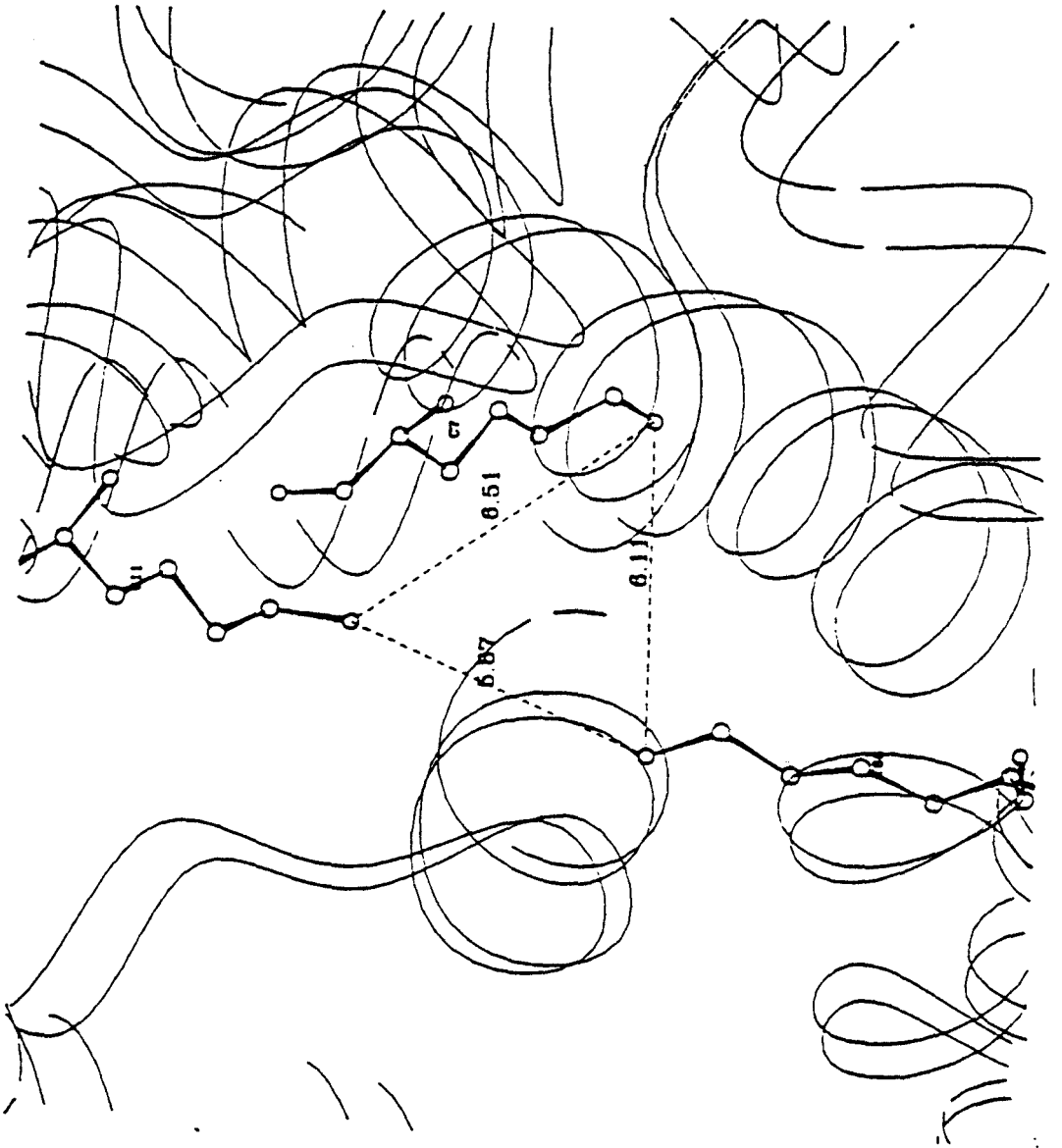

6.51
6.87
6.11

Figure 49. The size of tris(3,5-dibromosalicyl) tricarballylate.

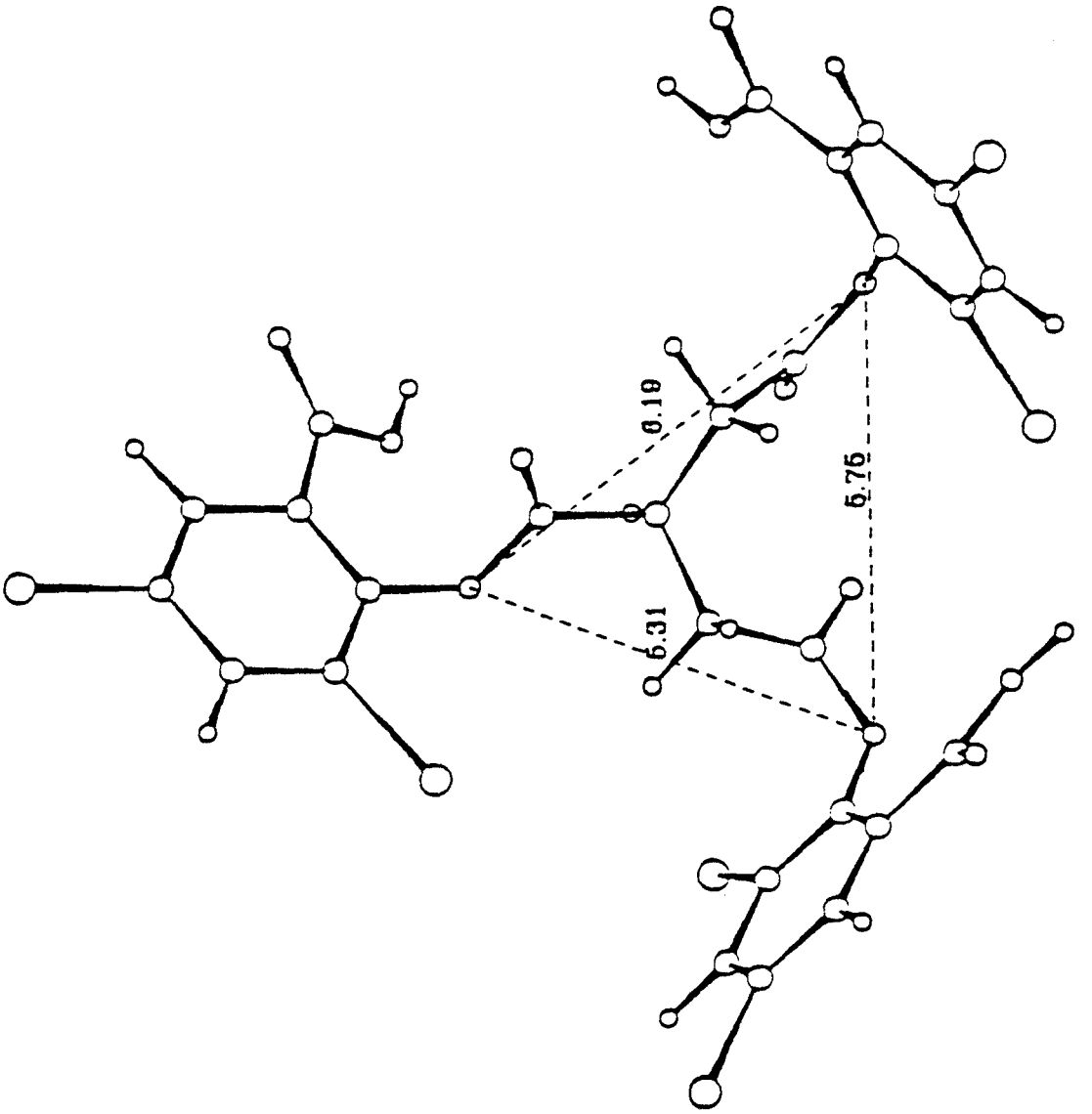

6.10
6.76
6.31

Table 13. SDS-PAGE analysis of DBSF crosslinking reaction under crystallization conditions.

Sample	Condition	% Monomer	% Dimer
1	2.3 M $(NH_4)_2SO_4$ 0.3 M $(NH_4)_2PO_4$	65	35
	Controlled test w/o crosslinker	100	0
2	1.5 M $(NH_4)_2SO_4$ 0.4 M $(NH_4)_2PO_4$	59	41
3	8% PEG-6000* 100 mM KCl 10 mM K_2HPO_4	46	54
	Controlled test w/o crosslinker	100	0
4	16% PEG-600 100 mM Na cacodylate 75 mM NaCl	50	50

* PEG-6000: Polyethylene glycol 6000.

The failure of crosslinking of crystallized hemoglobin might be due to one or more of the following causes:

(1) The reagents with the aspirin leaving groups are too large to diffuse in the channels between hemoglobin molecules.

(2) The solubility of the reagents in aqueous solution is too low, especially under high salt conditions, to make the high gradient of the reagents to drive them into the crystals.

(3) The transportation and crosslinking of the reagents in the crystals is much more complicated than we expected.

If the failure is a result of (1) and/or (2), crosslinking of crystallized hemoglobin would be still possible if a smaller reagent can be found which is soluble in aqueous solution and specific for the reaction site. Alternatively, forming smaller aggregates of hemoglobin under near crystallization conditions may be a better approach to solve this problem.

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Appendix I (a).

To use this program, click the icon with a name of **[HbA]&[Catalase]** in Window 3.1 and use soft keys to perform the measurement. Press the soft key of F1 for input of user's name, date, sample name and selection of hemoglobin or catalase, F2 for reference scanning, F3 for sample scanning, F9 for printing results with data and a spectrum (see **Appendix I (b)**) and F10 for termination of the program.

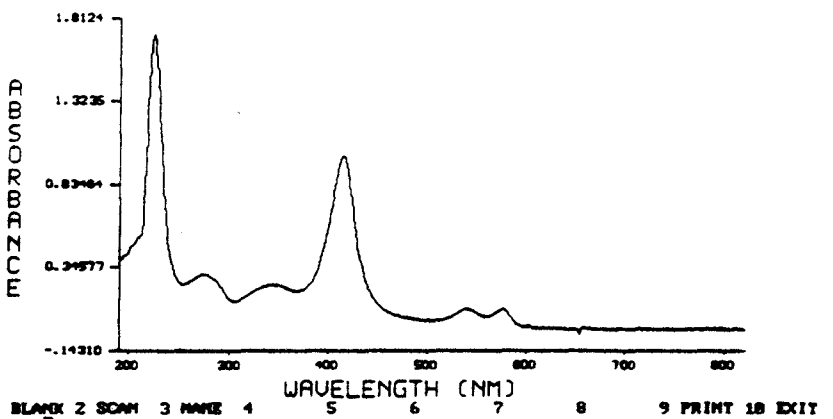
```
'$INCLUDE: '52SETUP.BAS'
'YAGUO ZHENG, 1995
'TO CALCULATE CONCENTRATION OF HEMOGLOBIN,
'PERCENTAGES OF METHEMOGLOBIN AND HEMICHROME.
CLS 0
KEY 1, " NAME"
KEY 2, " BLANK"
KEY 3, " SCAN"
KEY 9' " PRINT"
KEY 10, " EXIT"
KEY ON
PRINT "TURN ON 'HP 8452A'"

KEY(1) ON
ON KEY(1) GOSUB NAMING
KEY(2) ON
ON KEY(2) GOSUB REFFERE
KEY(3) ON
ON KEY(3) GOSUB SCANNIG
KEY(9) ON
ON KEY(9) GOSUB OUTCOPY
KEY(10) ON
ON KEY(10) GOSUB ENDPROG
IDLE: GOTO IDLE

REFFERE:
   CLS 0
   INPUT "ENTER THE AMOUNT (uL) OF BUFFER ADDED IN THE CELL"; A
   CLS 0
   PRINT "BLANKING"
   blank
   CLS 0
RETURN

SCANNIG:
   CLS 0
   INPUT "ENTER THE AMOUT (uL) OF SAMPLE ADDED IN THE CELL"; B
   CLS 0
   measure
```

```
   initplot RAW()
   plot RAW(), 15
RETURN

NAMING:
   CLS 0
   INPUT "ENTER USER NAME"; U$
   INPUT "ENTER DATE"; D$
   INPUT "ENTER SAMPLE NAME"; S$
   CLS 0
   PRINT "Select:": PRINT
   COLOR 15, 0: PRINT "  H";
   COLOR 7, 0: PRINT "emoglobin"
   COLOR 15, 0: PRINT "  C";
   COLOR 7, 0: PRINT "atalase"
   INPUT Sel$
   CLS 0
RETURN

OUTCOPY:
   LPRINT
   LPRINT "*****************************************"
   LPRINT "      USER: "; U$
   LPRINT "      DATE: "; D$
   LPRINT "      SAMPLE: "; S$
   LPRINT "*****************************************"
   LPRINT
   LPRINT "BUFFER ="; A; "uL": LPRINT
   LPRINT "SAMPLE ="; B; "uL"
   LPRINT
   IF Sel$ = "H" OR Sel$ = "h" THEN
      HbA = (RAW(177, 2) * (A + B)) / (B * 13.8 * 4)
      D = RAW(256, 2) - .005
      IF D < 0 THEN D = 0
      B1 = RAW(186, 2) - D
      B2 = RAW(194, 2) - D
      B3 = RAW(221, 2) - D
      IF B1 < O THEN B1 = 0
      IF B2 < 0 THEN B2 = 0
      IF B3 < 0 THEN B3 = 0
      OXYHBA = -89 * B1 + 119 *B2 -39 * B3
      MET = -55 * B1 + 28 *B2 + 307 * B3
      HEMI = 233 *B1 -133 * B2 -114 * B3
      IF MET < 0 THEN MET = 0
      IF HEMI < 0 THEN HEMI = 0
      METPT = 100 * MET / (OXYHBA + MET + HEMI)
      HEMIPT = 100 * HEMI / (OXYHBA + MET + HEMI)
      METHEMI = 100 * (MET + HEMI) / (OXYHBA + MET + HEMI)
      LPRINT RAW(177, 1); RAW(177,2)
      LPRINT RAW(186, 1); RAW(186, 2)
      LPRINT RAW(194, 1); RAW(194, 2)
      LPRINT RAW(221, 1); RAW(221, 2)
      LPRINT RAW(256, 1); RAW(256, 2)
      LPRINT
      LPRINT "CONCENTRATION OF HBA ="L HbA; "mM": LPRINT
      LPRINT "METHBA% ="; METPT; "%": LPRINT
      LPRINT "HEMICHROME% ="; HEMIPT; "%": LPRINT
      LPRINT "(METHBA+HEMICHRONE)% ="; METHEMI; "%": LPRINT
```

```
   ELSEIF Sel$ = "C" OR Sel$ = "c" THEN
      MAX = 0

      FOR I% = 106 TO 121
      IF MAX < RAW(I%, 2) THEN
      MAX = RAW(I%, 2)
      MAXA = RAW(I%, 1)
      END IF

      CAT = MAX * (A + B) / (B * 420)
      LPRINT MAXA; MAX: LPRINT
      LPRINT "CONCENTRATION OF CATALASE ="; CAT; "mM": LPRINT
      END IF
GETOUT:
   hardcopy 0, 344
RETURN

ENDPROG:
   CLS 0
   INPUT "EXIT (Y/N)"; A$
   IF A$ = "Y" OR A$ = "y" THEN
      CLS 0
      END
   END IF
RETURN

HANDLER:
   LPRINT "DATA OVERFLOW"
RESUME GETOUT
```

Appendix I (b). Results of hemoglobin and catalase.

```
**********************************
       USER: Yaguo Zheng
       DATE: 12/11/1995
       SAMPLE: New HbA
**********************************

 542   .1019135
 560   5.897522E-02
 576   .1030884
 630  -1.104736E-02
 700  -1.348877E-02

BUFFER = 495 uL

HbA = 5 uL

CONCENTRATION OF HBA = .1846258 mM

METHBA% = 0 %

HEMICHROME% = .4322735 %

(METHBA+HEMICHRONE)% = .4322735 %
```



****

USER: Yaguo Zheng
DATE: 1/25/1996
SAMPLE: Catalase

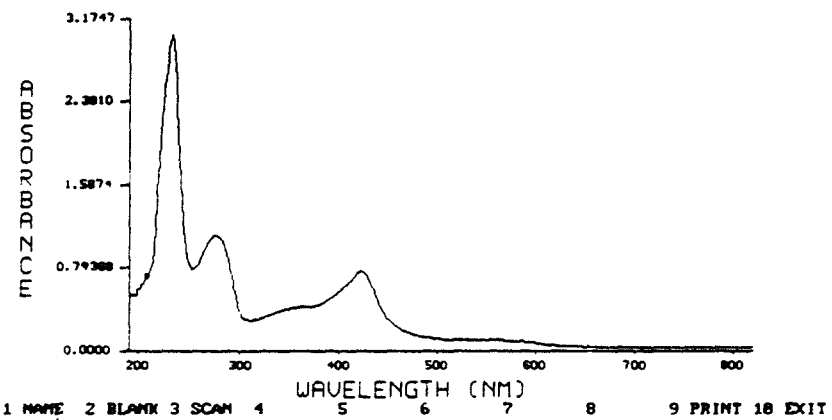
****

BUFFER = 495 uL

SAMPLE = 5 uL

422 .7640839

CONCENTRATION OF CATALASE = .1819247 mM



Appendix II.

```
C     PROGRAM TEST
C     YAGUO ZHENG
C     1993
      CHARACTER*1  AT(6), AN(4), RES(3), CH(1), K(6), L(4), S(80)
      INTEGER      I, IN, FT
      N=0
 20   N=N+1
      IF (N.GE.204.AND.N.LE.4986) THEN
      READ(5,  10) AT,I,AN,RES,CH,IN,X1,Y1,Z1,O,B,FT,K,L
 10   FORMAT(6A1,I5,2X,4A1,3A1,1X,A1,I4,4X,3F8.3,2F6.2,1X,I3,6A1,4A1)
C     TRANSFORM INTO UNIT CELL COORDINATES
      X2=(0.015462*X1+0.002192*Y1+0.003698*Z1+0.26656)+0.5
      Y2=(-0.001902*X1+0.011771*Y1+0.000974*Z1+0.16413)
      Z2=(-0.001061*X1-0.001721*Y1+0.018728*Z1+0.75059)
C     ORTHOGONALIZE COOPERATES
      X3=63.150*X2-8.731*Z2
      Y3=83.590*Y2
      Z3=53.087*Z2
      WRITE(6,10) AT,I,AN,RES,CH,IN,X3,Y3,Z3,O,B,FT,K,L
      ELSE
      READ(5,15,END=100) S
 15   FORMAT(80A1)
      WRITE(6,15) S
      END IF
      GOTO 20
 100  STOP
      END
```

Appendix III.

```
C      PROGRAM MODEL
C      YAGUO ZHENG, 1993
       INTEGER I,IN,FT,M,N,J,K,U,V
       DIMENSION (54,4783),Y(54,4783),Z(54,4783),AN(4783),CH(4783)
      1I(4783),IN(4783),O(4783),B(4783),L(4783),RES(4783),AT1(4783),
      2AT2(4783)
       M=0
 20    M=M+1
       READ (5,10,END=100) AT1(M),AT2(M),I(M),AN(M),RES(M),CH(M),
      3IN(M),X1,Y1,Z1,O(M),B(M),S,L(M)
 10    FORMAT (2A3,I5,2X,A4,A3,1X,A1,I4,4X,3F8.3,2F6.2,6X,A4,I4)
C      TRANSFORM INTO UNIT CELL COORDINATES
C      MAKE #1 HEMOGLOBIN
       X(1,M)=0.015462*X1+0.002192*Y1+0.003698*Z1+0.26656
       Y(1,M)=-0.001902*X1+0.011771*Y1+0.000974*Z1+0.16413
       Z(1,M)=-0.001062*X1+0.001721*Y1+0.018728*Z1+0.75059
C      MAKE #2 HEMOGLOBIN
       X(2,M)=X(1,M)+1.000
       Y(2,M)=Y(1,M)
       Z(2,M)=Z(1,M)
C      MAKE #3 HEMOGLOBIN
       X(3,M)=X(1,M)
       Y(3,M)=Y(1,M)
       Z(3,M)=Z(1,M)+1.000
C      MAKE #4 HEMOGLOBIN
       X(4,M)=X(1,M)+1.00
       Y(4,M)=Y(1,M)
       Z(4,M)=Z(1,M)+1.000
C      MAKE #5 HEMOGLOBIN
       X(5,M)=1.000-X(1,M)
       Y(5,M)=Y(1,M)+0.500
       Z(5,M)=2.000-Z(1,M)
C      MAKE #6 HEMOGLOBIN
       X(6,M)=1.000-X(1,M)
       Y(6,M)=Y(1,M)-0.500
       Z(6,M)=2.000-Z(1,M)
       GOTO 20
C      ORTHOGONALIZE COORDINATES
 100   DO 300,J=1,6
       DO 300,K=1,4783
       Z(J,K)=53.800*Z(J,K)-10.249*X(J,K)
       X(J,K)=62.313*X(J,K)
       Y(J,K)=83.590*Y(J,K)
C      PRINT #1-6 HEMOGLOBIN
       WRITE (6,10) AT1(K),AT2(K),I(K),AN(K),RES(K),CH(K),IN(K),
      4X(J,K),Y(J,K),Z(J,K),O(K),B(K),S,L(K)
 300   CONTINUE
       STOP
       END
```

Appendix IV.

```
DECLARE SUB PAUSE (Q$)
'$INCLUDE:  '52SETUP.BAS'

REM          ***********************************************
REM          ***                                         ***
REM          ***                YAGUO ZHENG              ***
REM          ***                   1995                  ***
REM          ***                                         ***
REM          ***********************************************

REM MAIN MANR
'TO PROVIDE A MANU TO SELECT DESIRED WORK

DIM C(1 TO 1) AS INTEGER, BS(1 TO 7) AS INTEGER, F(1 TO 1) AS INTEGER
DIM A(1 TO 103), B(1 TO 103, 1 TO 2), D(1 TO 103), T(1 TO 103)

DO
   CLS
   SCREEN 1
   CIRCLE (40, 42), 10, 2: PAINT (40, 42), 2
   CIRCLE (60, 42), 10, 2: PAINT (60, 42), 2
   CIRCLE (40, 58), 10, 2: PAINT (40, 58), 2
   CIRCLE (60, 58), 10, 2: PAINT (60, 58), 2
   LINE (38, 40)-(62, 60), 1, B
   LINE (39, 41)-(61, 59), 1, B
   LINE (41, 43)-(59, 57), 1, B
   LINE (42, 44)-(58, 56), 1, B
   CIRCLE (50, 50), 1, 3
   CIRCLE (260, 42), 10, 2: PAINT (260, 42), 2
   CIRCLE (280, 42), 10, 2: PAINT (280, 42), 2
   CIRCLE (260, 58), 10, 2: PAINT (260, 58), 2
   CIRCLE (280, 58), 10, 2: PAINT (280, 58), 2
   LINE (258, 40)-(282, 60), 1, B
   LINE (259, 41)-(281, 59), 1, B
   LINE (261, 43)-(279, 57), 1, B
   LINE (262, 44)-(278, 56), 1, B
   CIRCLE (270, 50), 1, 3
   LINE (80, 20)-(240, 80), 1, B
   LINE (81, 21)-(239, 79), 1, B
   LINE (83, 23)-(237, 77), 1, B
   LINE (118, 66)-(193, 66), 1
   LOCATE 5, 15: PRINT "AUTOXIDATION"
   LOCATE 6, 14: PRINT "& DENATURATION"
   LOCATE 8, 16: PRINT "Olsen Lab"
   LINE (20, 98)-(150, 118), 2, B
   LINE (170, 98)-(300, 118), 2, B
   LINE (20, 130)-(150, 150), 2, B
   LINE (170, 130)-(300, 150), 2, B
   LINE (20, 161)-(150, 181), 2, B
   LINE (170, 161)-(300, 181), 2, B
   LOCATE 14, 4: PRINT "(P)reparation"
   LOCATE 14, 23: PRINT "(B)eststep"
   LOCATE 18, 4: PRINT "(A)utoxidation"
```

```
    LOCATE 18, 23; PRINT "(D)enaturation"
    LOCATE 22, 4: PRINT "(R)esults"
    LOCATE 22, 23: PRINT "(E)xit"

    DO
       Q$ = UCASE$(INPUT$(1))
    LOOP WHILE INSTR("RBDPAE", Q$) = 0
    CLS
    SELECT CASE Q$
       CASE IS = "P"
          GOSUB CONCENTRATN
       CASE IS = "B"
          GOSUB BESTSTEP
       CASE IS = "D"
          GOSUB DENATURATION
       CASE IS = "R"
          GOSUB PRINTG
       CASE IS = "A"
          GOSUB AUTOXIDATION
       CASE ELSE
    END SELECT
LOOP UNTIL Q$ = "E"
END

BESTSTEP:
    REM BESTSTEP
    'TO OBTAIN THE OPTIMAL POSITION FOR EACH CELL
    SCREEN 9

    CLS
    PRINT "BESTSTEP SECTION": PRINT
    PRINT "INSERT A FORMATED HD DISK IN DRIVE B."
    PAUSSE B$
    IF B$ = "Q" THEN RETURN

    NUMWAVES = 1
    WAVELIST(1) = 416
    INTMODE = 2
    WAVEMODE = 1
    GAIN = 0

    OPEN "O", 1, "B:\BESTSTEP"
    CLS

    FOR I% = 1 TO 7
       XLABEL$ = "STEP NUMBER"
       YLABEL$ = "COUNT"
       PRINT "MEASURING CELL #"; I%
       MINSTEP% = (I% - 1) * 1075
       MAXSTEP% = MINSTEP% + 400
       MAXINTENT! = 0
       DRAWAXES CSNG(MINSTEP%), CSNG(MAXSTEP%), 500, 3000
       FOR J% = MINSTEP% TO MAXSTEP% STEP 4
          TRANSPORTSTEP J%
          measure
          COLOR 15, 0
          IF J% = MINSTEP% THEN
             PRESEP (J%, RAW(1, 2))
```

```
            ELSE
               LINE - (J%, RAW(1, 2))
            END IF
            IF RAW(1, 2) > MAXINTENT THEN
               MAXINTENT! = RAW(1, 2)
               MAXSTEP% = J%
            END IF
         NEXT J%
         PRINT #1, I%, MAXSTEP%
         CMD$ = "CEL" + STR$(I%) + STR$(MAXSTEP)
         SEND8452 CMD$
         CLS 0
      NEXT I%
      CLOSE #1

      PRINT
      PRINT "THE BESTSTEP DATA ARE SAVED IN THE FILE NAMED 'BESTSTEP'."
      PRINT
      OPEN "I", 1, "B:\BESTSTEP"
      FOR I% = 1 TO 7
         INPUT #1, I%, A
         PRINT I%, A
      NEXT I%
      CLOSE #1

      PRINT
      PRINT "THE BESTSTEP IS OVER."
      PRINT "PRESS ANY KEY TO GO BACK THE MAIN MENU."
      WHILE INKEY$ = "": WEND
RETURN

CONCENTRATN:
      REM $DYNAMIC
      SCREEN 9
      REM CONCENTRATION
      'TO ADJUST HEMOGLOBIN CONCENTRATION OF SAMPLES

      CLS
      PRINT "PREPARATION OF CONCENTRATION OF Hb SAMPLES."
      PRINT
      PAUSE B$
      IF B$ = "Q" THEN RETURN

      DO
         PRINT "SELECT:": PRINT
         PRINT "DENATURATION-PRISS 1 THEN <ENTER>."
         INPUT "AUTOXIDATION-PRESS 2 THEN <ENTER>."
         CLS
      LOOP WHILE A < 1 OR A > 2

      PRINT "ADD 500 ul OF THE BUFFER TO EACH OF 7 CELLS."
      PAUSE B$
      IF B$ = "Q" THEN RETURN

      PRINT "A. INSERT ALL THE 7 CELLS."
      PRINT "B. CLOSE THE DOOR."
      PAUSE B$
      IF B$ = "Q" THEN RETURN
```

```
    FOR I% = 2 TO 7
       HOME
       CELL I%
       blank
       PRINT "ADD STARTING AMOUNT (ul) OF Hb SAMPLE"; I% - 1
       PRINT "TO CELL"; I%; "."
       PAUSE B$
       IF B$ = "Q" THEN RETURN
       DO
          NUMWAVES = 1
          INTMODE = 0
          WAVEMODE = 1
          WAVELIST(1) = 416
          measure

          IF RAW(1, 2) < A THEN
             PRINT "THE ABSORBANCE AT"; RAW(1, 1); "IS"; RAW(1, 2); "."
             PRINT "ADD MORE Hb SAMPLE IN CELL #"; I%; "."
          ELSEIF  RAW(1, 2) > A + .1 THEN
             PRINT "THE ABSORBANCE AT"; RAW(1, 1); "IS"; RAW(1, 2); "."
             PRINT "THERE IS TOO MUCH SAMPLE IN CELL"; I%; "."
             PRINT "ADJUST Hb CONCENTRATION AGAIN."
          ELSE
             PRINT "THE ABSORBANCE AT"; RAW(1, 1); "IS"; RAW(1, 2); "."
             PRINT "THE CONCENTRATION IS FINE, GO ON."
          END IF
          PAUSE B$
          IF B$ = "Q" THEN RETURN
       LOOP WHILE RAW(1, 2)< A OR RAW(1, 2)= A + .1 OR RAW(1, 2) > A + .1
    NEXT I%

    IF A = 1 THEN
       A$ = "DENATURATION."
       PRINT "ADD 5 ul OF 9.7 x 100 uM K3Fe(CN)6 SOLUTION TO EACH CELL."
    ELSE
       A$ = "AUTOXIDATION."
    END IF
    PRINT : PRINT "TIGHTEN DOWN THE CELL CHANGER."
    PRINT : PRINT "THE SAMPLES ARE READY FOR "; A$
    PRINT : PRINT "PRESS ANY KEY TO GO BACK THE MAIN MANU."
    WHILE INKEY$ = "": WEND
    CLS
RETURN

DENATURATION:
    REM DENATRUATION

    SCREEN 9

    PRINT : PRINT "DENATURATION SECTION"
    PAUSE B$
    IF B$ = "Q" THEN RETURN

    INTMODE = 0
    WAVEMODE = 1
    NUMWAVES = 7
    WAVELIST(1) = 280
```

```
   WAVELIST(2) = 406
   WAVELIST(3) = 410
   WAVELIST(4) = 418
   WAVELIST(5) = 542
   WAVELIST(6) = 576
   WAVELIST(7) = 630
   C(1) = 0
   TIMECOUNT = 0

   PRINT "SEVEN WAVELENTHES ARE USED IN THE TEST:": PRINT
   FOR I% = 1 TO 7
   PRINT "LAMDA"; I%; "="; WAVELIST(I%)
   NEXT I%
   PAUSE B$
   IF B$ = "Q" THEN RETURN

   ON ERROR GOTO BIND
   OPEN "I", 1, "B:\BESTSTEP"
   FOR I% = 1 TO 7
      INPUT #1, I%, BS(I%)
   NEXT I%
   CLOSE #1

   CLS
   FOR I% = 2 TO 7
FORWARD:
         PRINT "ENTER FILE NAME FOR SAMPLE #"; I% - 1; "IN CELL #"; I%
         INPUT N$
         ON ERROR GOTO GETFILE
         OPEN "O", I%, "B:" + N$
   NEXT I%
   PAUSE B$
   IF B$ = "Q" THEN RETURN

   CLS
   PRINT "SET UP TEMPERATURN CONTROL SYSTEN.": PRINT
   PRINT "A, BATH DIAL = 80 ON 'RTE-9'."
   PRINT : PRINT "B. TEMPERATURE DIAL = 0 ON 'ETP-3'."
   PRINT : PRINT "C. PROGRAM = HOLD ON 'ETP-3'."
   PRINT : PRINT "D. BATH SWITCH = -20 TO +35 DEG C ON 'RTE-9'."
   PIRNT : PRINT "E, ACCESSORY INPUT = ENABLE ON 'DCR-4'."
   PAUSE B$
   IF B$ = "Q" THEN RETURN
   PRINT : PRINT "F. PRESS & HOLD SETPOINT/SENSOR TEMP BOTTON AND"
   PRINT : PRINT " ADJUST SETPOINT = 29 DEG C ON 'DCR-4'."
   PRINT : PRINT "G. PROGRAM END = HOLD, HEAT/COOL = HEAT & FWD/REV"
   PRINT : PRINT " = REV ON 'ETP-3."
   PRINT : PRINT "H. PRESS START/RESET BUTTON ON 'ETP-3'(NOTE: PROGRAM"
   PRINT : PRINT " END LIGHT IS OFF & METER IS AT 1)."
   PRINT : PRINT "I. TIME = 210 min ON 'ETP-3'."
   PRINT : PRINT "J. PRESS & HOLD BUTTON ON 'SET PROGRAM' ADAPTER CABLE"
   PRINT : PRINT " BOX AND ADJUST TEMPERATURE DIAL = 50.0 ON 'SET-3'"
   PRINT : PRINT " THEN RELEASE BOTTON."
   PAUSE B$
   IF B$ = "Q" THEN RETURN
   PRINT : PRINT "K. FWD/REV = FWD ON 'ETP-3'."
   PIRNT : PRINT "L. PRESS START/RESET BUTTON (NOTE: PROGRAM END LIGHE"
```

```
    PRINT : PRINT " IS OFF AND METER IS AT 0)."
    PRINT : PRINT "M. ON 'SET PROGRAM' ADAPTER CABLE BOX, ENABLE/DISABLE"
    PRINT : PRINT " = ENABLE."
    PRINT : PRINT "N. BATH SWITCH = +35 TO +100 DEG C ON 'RTE-9'."
    PRINT : PRINT "WHEN READY FOR DENATURATION, PROGRAM = RUN ON 'ETP-3'"
    PRINT : PRINT " AND CONTINUE."
    PAUSE B$
    IF B$ = "Q' THEN RETURN

    CLS
    INPUT "INPUT THE CURRENT TEMPERATURE"; T
    CLS
    PRINT "THE DENATURATION PROGRAM IS WORKING."
    PRINT : PRINT T: PRINT
    F(1) = CINT(T * 10)
    HOME
    FOR I% = 1 TO 103
       TRANSPORTSTEP BS(1)
       blank
       FOR J% = 2 TO 7
          START! = TIMER
          TIMER ON
          ON TIMER(15) GOSUB DENAT
STAY: GOTO STAY
THERE:
       FINISH! = TIMER
       TIMECOUNT = TIMECOUNT + (FINISH! - START!) / 60
       PRINT "TIME"; C(1); "="; FINISH! - START!; "sec"
       PRINT "TOTAL TIME: "; TIMECOUNT; "min": PRINT
    NEXT J%
    NEXT I%
    TIMER OFF
    FOR I% = 2 TO 7
       CLOSE #I%
    NEXT I%

    PRINT "DENATURATION IS OVER."
    PRINT : PRINT "TURN OFF THE TEMPERATURE CONTROL SYSTEM."
    PRINT "PRESS ANY KEY BACK THE MAIN MANU."
    WHILE INKEY$ = "": WEND
MERROR:
    RETURN

DENAT:
    BEEP
    INPUT "DELTA TEMPERATURE"; A
    F(1) = F(1) + A
    T = F(1) * .1
    PRINT T
    ON ERROR GOTO TERROR
    PRINT #J%, T
    TRANSPORTSTEP BS(J%)
    measure
    FOR K% = 1 TO 7
       PRINT #J%, RAW(K%, 2)
    NEXT K%
    C(1) = C(1) + 1
    RETURN THERE
```

```
GETFILE:
   PRINT : PRINT "DISK ERROR OR DRIVE B IS EMPTY!": PRINT
   RESUME FORWARD

BIND:
   PRINT "FILE NAMED BESTSTEP CAN NOT BE FOUND."
   PRINT "PLEASE MAKE SURE IT IS IN YOUE DISK."
   PRINT "PRESS ANY KEY TO CONTINUE.:
   WHILE INKEY$ = "": WEND
   RESUME

TERROR:
   PRINT : PRINT "ERROR! PLEASE WAIT.": PRINT
   RESUME MERROR

AUTOXIDATION:
   REM AUTOXIDATION

   SCREEN 9
   CLS
   PRINT "AUTOXIDATION SECTION": PRINT
   PAUSE B$
   IF B$ = "Q" THEN RETURN

   INTMODE = 0
   WAVEMODE = 1
   NUMWAVES = 4
   WAVELIST(1) = 560
   WAVELIST(2) = 576
   WAVELIST(3) = 630
   WAVELIST(4) = 700

   CLS
   PRINT "SET TEMPERATURE ON 'DCR-4' AT 39 DEG C."
   PAUSE B$
   IF B$ = "Q" THEN RETURN

   ON ERROR GOTO FIND
   OPEN "I", 1, "B:\BESTSTEP"
   FOR I% = 1 TO 7
      INPUT #1, I%, BS(I%)
   NEXT I%
   CLOSE #1

   CLS
   FOR I% = 2 TO 7
STRAIGHT:
         PRINT "ENTER FILE NAME FOR SAMPLE #"; I% - 1; "IN CELL #"; I%
         INPUT N$
         ON ERROR GOTO SETFILE
         OPEN "O", I%, "B:" + N$
   NEXT I%
   PAUSE B$
   IF B$ = 'Q" THEN RETURN

   CLS
   PRINT "ADJUST AND WAIT UNTIL THE TEMPERATURE IS"
```

```
    PRINT "STABLE AT 37 DEG C NO OMEGA DIGICATOR."
    PAUSE B$
    IF B$ = "Q" THEN RETURN

    CLS
    PRINT "THE AUTOXIDATION PROGRAM IS WORKING.": PRINT
    HOME
    FOR J% = 1 TO 7
       IF J% = 1 THEN
          TRANSPORTSTEP BS(1)
          blank
       ELSE
          TRANSPORTSTEP BS(J%)
          measure
          FOR K% = 1 TO 4
             PRINT #(J%), RAW(K%, 2)
          NEXT K%
       END IF
    NEXT J%

    FOR I% = 1 TO 34
       START! = TIMER
       HOME
       TIMER ON
       ON TIMER(1775.2) COSUB AUTOX
IDLE: GOTO IDLE
HERE:
       FINISH! = TIMER
       PRINT "TIME"; I%; "="; (FINISH! - START!) / 60; "min",
    NEXT I%
    TIMER OFF
    FOR I% = 2 TO 7
       CLOSE #I%
    NEXT I%

    PRINT : PRINT : PRINT "AUTOXIDATION IS OVER."
    PRINT "PRESS ANY KEY BACK THE MAIN MANU."
    WHILE INKEY$ = "": WEND
    RETURN

AUTOX:
    FOR J% = 1 TO 7
       IF J% = 1 THEN
          TRANSPORTSTEP BS(1)
          blank
       ELSE
          TRANSPORTSTEP BS(J%)
          measure
          FOR K% = 1 TO 4
             PRINT #(J%), RAW(K%, 2)
          NEXT K%
          EMD IF
    NEXT J%
    RETURN HERE

SETFILE:
    PRINT : PRINT "DISK ERROR OR DRIVE B IS EMPTY!": PRINT
    RESUMR STRAIGHT
```

```
FIND:
   PRINT "FILE NAMED BESTSTEP CAN NOT BE FOUND."
   PRINT "PLEASE MAKE SURE IT IS IN YOUR DISK."
   PAUSE B$
   IF B$ = "Q' THEN RETURN
   RESUME

PRINTG:
   REM PRINTING
   'THIS PART OF PROGRAM IS TO PRINT OUT
   'AUTOXIDEATION AND/OR DENATURATION RESULTS

   SCREEN 9
   CLS
   PRINT "DATA ANALYSIS"
   PAUSE B$
   IF B$ = "Q" THEN RETURN

   C(1) = 0

   DO
      PRINT "PRINT:": PRINT
      PRINT "  DENATURATION-PRESS 1 THEN <ENTER>"
      INPUT "  AUTOXIDATION-PRESS 2 THEN <ENTER>"; A
      CLS
   LOOP WHILE A < 1 OR A > 2

   IF A = 1 THEN
      GOSUB PDENAT
   ELSE
      GOSUB PAUTOX
   END IF
RETURN

PDENAT:
   CLS 0
   PRINT "DATA ANALYSIS OF DENATRATION": PRINT
   PAUSE B$
   IF B$ = "Q" THEN RETURN

   DO
      INPUT "ENTER FILE NAME"; N$
      ON ERROR GOTO FILENAME
      OPEN "I", 1, "B:" + N$

      INPUT "ENTER WAVELENTH #"; W

      INPUT "ENTER A TITLE FOR THE PLOT."; T$
      CLS 0
      FOR I% = 1 TO 103
         ON ERROR GOTO DATAHDL
         INPUT #1, T(I%)
         FOR J% = 1 TO 7
            IF J% = W THEN
               INPUT #1, A(I%)
            ELSE
               INPUT #1, C
```

```
            END IF
         NEXT J%
      NEXT I%
      m = 102

FLOPPING:
      CLOSE #1

      FOR I% = 1 TO m
         TEMPER1 = A(I% + 1) - A(I%)
         TEMPER2 = T(I% + 1) - T(I%)
         ON ERROR GOTO DATACUT
         D(I%) = TEMPER1 / TEMPER2
      NEXT I%
      N = 102

SKIPPING:
      FOR I% = 11 TO N - 10
         TEMP1 = 329 * D(I%) + 324 * (D(I% + 1) + D(I% - 1))
         TEMP2 = 309*(D(I%+2) + D(I%-2))+ 284 * (D(I% + 3) + D(I% - 3))
         TEMP3 = 249*(D(I%+4) + D(I%-4))+ 204 * (D(I% + 5) * D(I% - 5))
         TEMP4 = 149*(D(I%+6) + D(I%-6)) + 84 * (D(I% + 7) + D(I% - 7))
         TEMP5 = 9 * (D(I%+8) + D(I%-8)) - 76 * (D(I% + 9) + D(I% - 9))
         TEMP6 = -(171 * (D(I% + 10) + D(I% - 10)))
         B(I% - 10, 2) = (TEMP1 +TEMP2 +TEMP3 +TEMP4 +TEMP5 +TEMP6)/3059
         B(I% - 10, 1) = (T(I% + 1) + T(I%)) / 2
      NEXT I%

      TITLE$ = T$
      YLABEL$ = "dA/dT"
      XLABEL$ = "TEMPERATURE (DEG C)"
      YAUTOSCALE B(), 80, YMIN!, YMAX!
      DRAWAXES 28!, 70!, YMIN!, YMAX!

      COLOR 15, 0
      FOR I% = 1 TO N - 21
         IF I% = 1 THEN
            PRESET (B(I%, 1), B(I%, 2))
         ELSE
            LINE - (B(I%, 1), B(I%, 2))
         END IF
      NEXT I%

      INPUT "SAVE THE DATA (Y/N)"; A$
      CLS 0
      COLOR 7, 0
      IF A$ = "Y" OR A$ = "y" THEN
      W$ = LTRIM$(STR$(W))
      OPEN "O", 1, "B:\" + N$ + "W" + W$
      PRINT "THE DATA ARE SAVED IN FILE "; N$ + "W" + W$
      FOR I% = 1 TO N - 21
         PRINT B(I%, 1); B(I%, 2),
         PRINT #1, B(I%, 1)
      NEXT I%
      FOR I% = 1 TO N - 21
         PRINT #1, B(I%, 2)
      NEXT I%
      CLOSE #1
```

```
      END IF

      PRINT : INPUT "DO MORE FILES (Y/N)"; A$
      CLS 0
   LOOP WHILE A$ = "Y" OR A$ = "y"
   RETURN

PAUTOX:
   CLS 0
   PRINT "DATA ANALYSIS OF AUTOXIDATION": PRINT
   PAUSE B$
   IF B$ = "Q" THEN RETURN
   DO
      INPUT "ENTER FILE NAME"; N$
      ON ERROR GOTO FILENAME
      OPEN "I", 1, "B:" + N$

      INPUT "ENTER A TITLE FOR THE PLOT"; T$
      CLS 0
      TITLE$ = T$
      YLABEL$ = "LN[OXYHbA]"
      XLABEL$ = "TIME (HOUR)"
      DRAWAXES 0, 18, 0, 3

      X = 0
      Y = 0
      XSQU = 0
      YSQU = 0
      XY = 0

      FOR I% = 1 TO 35
         FOR J% = 1 TO 4
            INPUT #1, A(J%)
         NEXT J%
         HBA = 119 * A(2) - 39 * A(3) - 89 * A(1) + 9 * A(4) - .045
         B(I%, 2) = LOG(HBA)
         B(I%, 1) = (I% - 1) / 2
         COLOR 15, 0
         LINE (B(I%,1)-.1, B(I%,2)-.02)-(B(I%,1)+.1, B(I%,2)+.02), , BF
         x = x + B(I%, 1)
         XSQU = XSQU + B(I%, 1) * B(I%, 1)
         Y = Y + B(I%, 2)
         YSQU = YSQU + B(I%, 2) * B(I%, 2)
         XY = XY + B(I%, 1) * B(I%, 2)
      NEXT I%
      CLOSE #1

      LXX = XSQU - (x *x) / 35
      LYY = YSQU - (Y * Y) / 35
      LXY = XY - (x *Y) / 35
      slope! = LXY / LXX
      intercept! = (Y - slope! * x) / 35
      coeff! = ABS(LXY / (LXX * LYY) ^ .5)

      FOR I% = 1 TO 35
         B(I%, 2) = intercept! + slope! * B(I%, 1)
         IF I% = 1 THEN
            PRESET (B(I%, 1), B(I%, 2)), red
```

```
         ELSE
            LINE - (B(I%, 1), B(I%, 2)), red
         END IF
      NEXT I%

      'HARDCOPY 0, 344

      PRINT "PRESS ANY KEY TO CONTINUE."
      WHILE INKEY$ = "": WEND
      CLS 0

      OPEN "O", 1, "B:\" + N$ + "DAT"
      PRINT "THE DATA ARE SAVED IN FILE "; N$ + "DAT.": PRINT
      PRINT #1, "SLOPE ": PRINT #1, slope!
      PRINT #1, "INTERCEPT ": PRINT #1, intercept!
      PRINT #1, "R ": PRINT #1, coeff!
      PRINT "SLOPE "; slope!, "INTERCEPT "; intercept!, "R "; coeff!
      PRINT #1, : PRINT
      FOR I% = 1 TO 35
         PRINT #1, B(I%, 2)
         PRINT B(I%, 1); B(I%, 2),
      NEXT I%
      CLOSE #1

      PRINT
      INPUT "DO MORE FILES (Y/N)"; A$
      CLS 0
   LOOP WHILE A$ = "Y" OR A$ = "y"
   RETURN

FILENAME:
   PRINT "FILE NAMED "; N$; " CAN NOT BE FOUND."
   INPUT "ENTER FILE NAME"; N$
   CLS 0
   RESUME

DATACUT:
   N = I% - 1
   RESUME SKIPPING

DATAHDL;
   m = I% - 2
   RESUME FLOPPING

DEFINT A-Z
REM $DYNAMIC
SUB PAUSE (Q$)
   PRINT
   PRINT "SELECT:": PRINT
   COLOR 15, 0: PRINT "  C";
   COLOR 7, 0: PRINT "ontinue"
   COLOR 15, 0: PRINT "  Q";
   COLOR 7, 0: PRINT "uit  (BACK TO THE MAIN MANU)"
   DO
      Q$ = UCASE$(INPUT$(1))
   LOOP WHILE INSTR("CQ", Q$) = 0
   CLS
END SUB
```

Appendix V.

The following describes how to run the denaturation and autoxidation program. When the icon with a name of **autox&denat** in Window 3.1 was clicked, a graphic menu was displayed on the screen. The experiment can follow the procedure: **Preparation** → **Beststep** → **Autoxidation** (or **Denaturation**) → **Results** → **Exit**.

Preparation.

Press "P" or "p" to prepare samples. The screen displays:

PREPARATION OF CONCENTRATION OF SAMPLES

Select:

Continue
Quit (go back the main menu)

Press "C" or "c",

(Screen): Select:

DENATURATION-PRESS 1 THEN <ENTER>.
AUTOXIDATION-PRESS 2 THEN <ENTER>.?

Press "1",

(Screen): ADD 500 ul OF THE BUFFER TO EACH OF 7 CELLS

Press "C" or "c",

(Screen): A. INSERT ALL THE 7 CELLS.

B. CLOSE THE COVER.

Press "C" or "c",

(Screen): ADD STARTING AMOUNT (ul) OF Hb SAMPLE 1 TO CELL 2.

Press "C" or "c", if

(Screen): THE ABSORBANCE AT 416 IS x.xxxxxx.
ADD MORE SAMPLE IN CELL 2.

please add more sample in the cell and press "C" or "c". If

(Screen): THE ABSORBANCE AT 416 IS x.xxxxxx.
THERE IS TOO MUCH SAMPLE IN CELL 2.
ADJUST Hb CONCENTRATION AGAIN.

please add one or few drops of the buffer in the cell and press "C" or "c". If

(Screen): THE ABSORBANCE AT 416 IS x.xxxxxx.
THE CONCENTRATION IS FINE, GO ON..

press "C" or "c".

(Screen): ADD 5 ul OF 9.7 x 100 uM K3Fe(CN)6 SOLUTION TO EACH CELL.
TIGHTEN DOWN THE CELL CHANGER.
THE SAMPLES ARE READY FOR DENATURATION.
PRESS ANY KEY TO GO BACK THE MAIN MENU.

If press 2 for autoxidation, the same procedures will be followed without addition of $K_3Fe(CN)_6$.

Press any key to return to the main menu.

Beststep.

Press "B" or "b" and the screen displays:

BESTSTEP SECTION

INSERT A FORMATTED HD DISK IN DRIVE B.

After inserting a formatted high density disk in the B drive, press "C" or "c".

(Screen): *Beststep spectra.*

(Screen): THE BESTSTEP DATA ARE SAVED IN THE FILE NAMED 'BESTSTEP'.

Beststep data.

THE BESTSTEP IS OVER.

PRESS ANY KEY TO GO BACK THE MAIN MENU.

Press any key to return to the main menu.

Autoxidation.

Press "A" or "a",

(Screen): AUTOXIDATION SECTION

Press "C" or "c",

(Screen): SET TEMPERATURE ON 'DCR-4' AT 39 DEG C.

Press "C" or "c". If it is a wrong disk or the B drive is empty,

(Screen): FILE NAMED BESTSTEP CAN NOT BE FOUND.
PLEASE MAKE SURE IT IS IN YOUR DISK.

Press "C" or "c" after checking the disk.

(Screen): ENTER FILE NAME FOR SAMPLE # 1 IN CELL # 2
?

After repeating inputting six file names, press "C" or "c".

(Screen): ADJUST AND WAIT UNTIL THE TEMPERATURE IS
STABLE AT 37 DEG C ON OMEGA DIGICATOR.

Press "C" or "c",

(Screen): THE AUTOXIDATION PROGRAM IS WORKING.

TIME 1 = 30 MIN TIME 2 = 30 min .....TIME 15 = 30 min

AUTOXIDATION IS OVER.
PRESS ANY KEY TO GO BACK THE MAIN MENU.

Press any key to return to the main menu.

Denaturation.

Press "D" or "d",

(screen): DENATURATION SECTION

Press "C" or "c",

(Screen): SEVEN WAVELENGTHS ARE USED IN THE TEST:

Seven wavelengths.

Press "C" or "c",

(Screen): ENTER FILE NAME FOR SAMPLE # 1 IN CELL # 2
?

Type in six file names and press "C" and "c",

(Screen): *Instruction of temperature control system.*

Press "C" or "c",

(Screen): INPUT THE CURRENT TEMPERATURE ?

Type the temperature (e.g.: 28.0) showed on the OMEGA DIGICATOR.

(Screen): THE DENATURATION PROGRAM IS WORKING.

28.0

DELTA TEMPERATURE ?

Input the difference of temperature (e.g.: 1),

(Screen): 28.1
TIME 1 = 19.9092 sec
TOTAL TIME: 0.3318 min

Continue inputting temperatures until 618 points are added.

(Screen): DENATURATION IS OVER.

TURN OFF THE TEMPERATURE CONTROL SYSTEM.
PRESS ANY KEY TO GO BACK THE MAIN MENU.

Press any key to return to the main menu.

Results.

Press "R" or "r",

(Screen): DATA ANALYSIS

Press "C" or "c",

(Screen): RESULT OF
DENATURATION-PRESS 1 THEN <ENTER>
AUTOXIDATION-PRESS 2 THEN <ENTER> ?

Type 1 for denaturation results.

(Screen): DATA ANALYSIS OF DENATURATION

Press "C" or "c".

(Screen): ENTER FILE NAME ?

Type a file name (e.g.: dena1) saved in the disk.

(Screen): ENTER WAVELENGTH # ?

Type a number (e.g.: 2).

(screen): SAVE THE DATA (Y/N) ?

A denaturation diagram.

If press "Y" or "y",

(Screen): THE DATA ARE SAVED IN FILE DENA1W2

Denaturation data.

DO MORE FILE (Y/N) ?

Press "Y" or "y" to repeat data analysis or press another key to go back the main menu.

Type 2 for autoxidation results.

(Screen): DATA ANALYSIS OF AUTOXIDATION

Press "C" or "c".

(Screen): ENTER FILE NAME ?

Type a file name (e.g.: auto1) saved in the disk.

(Screen): PRESS ANY KEY TO CONTINUE.

An autoxidation diagram.

Continue.

(Screen): THE DATA ARE SAVED IN FILE AUTO1DAT

Autoxidation data.

DO MORE FILES (Y/N) ?

Press "Y" or "y" to continue or press another key to return to the main menu.

Exit. Press "E" or "e" to terminate the program.

VITA

The author, Yaguo Zheng, was born on April 23, 1958 in Shanghai, the People's Republic of China. After completion of his primary education in Shanghai, he entered Hongkou Technical School, Shanghai, P. R. China. He began to work in Shanghai Physiology Institute as a technician in 1977. He entered China Textile University, Shanghai, P. R. China IN 1980. After completion of his B. S. and M. S. in Textile Chemistry in 1987, he worked as a lecturer in Textile Chemical Engineering Department and a research associate in Applied Chemistry Institute, China Textile University, Shanghai, P. R. China. He entered the Department of Chemistry, Loyola University of Chicago, Chicago, U. S. A. in the fall semester of 1991 as a Ph.D. student and was rewarded a graduate assistantship for the academic year 1991-1995. He was rewarded a teaching fellowship for the year 1995-1996 by Graduate School, Loyola University of Chicago. He has been researching synthesis of crosslinking reagents and intra- and intermolecular multi-linking of hemoglobin as potential blood substitutes under the guidance of Professor Kenneth W. Olsen for a Ph. D. degree.

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8/23/96
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