

**DIRECT DETECTION OF HOMOCYSTEINE AND SYNTHESIS OF LANTHANIDE
COMPLEXES AS BIOSENSORS**

A Dissertation

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DEDICATION

To my parents, Wang Yongfu and Yin Xingjie. Thank you for giving me life, love, and support.

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LIST OF ABBREVIATIONS

amu	Atomic Mass Units
ATP	Adenosine triphosphate
ASFB	4-Aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole
BMF	Bromomethylfluorescein
BSA	Bovine serum albumin
Ca²⁺	Calcium ion
calcd	Calculated
CE	Capillary electrophoresis
CMPI	2-Chloro-1-methylpyridinium iodide
Co (II)	Cobalt ion
CQMT	2-Chloro-1-methylquinolinium tetrafluoroborate
Cys	Cysteine
DCM	Dimethylmethane
DHA	Dehydroascorbic acid
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
<i>e.g.</i>	<i>exempli gratia</i> (for example)
Eq.	Equation
EIA	Enzyme immunoassay

ELISA	Enzyme-linked immunosorbent assay
<i>et al.</i>	<i>et alii</i> (and others)
EtOAc	Ethyl acetate
Eu³⁺	Europium ion
EuCl₃	Europium chloride
FB	Fluorone black
FeCl₃	Ferric chloride
FITC	Fluorescein isothiocyanate
FMI	Fluorescein-5-maleimide
FPIA	Fluorescence polarization immunoassay
FT-IR	Fourier transform infrared
GC	Gas chromatography
GC-ECD	Gas chromatography-electron capture detector
GC-FPD	Gas chromatography-flame photometric detection
GC-MS	Gas chromatography-mass spectrometry
GSH	Glutathione
HCl	Hydrochloric acid
Hcy	Homocysteine
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HNO₃	Nitric acid
H₂O	Water
H₂O₂	Hydrogen peroxide
HPLC	High performance liquid chromatography

H₂S	Hydrogen sulfide
IAAF	6-Iodoacetamidofluorescein
IR	Infrared
K₂CO₃	Potassium carbonate
KOH	Potassium hydroxide
La³⁺	Lanthanum ion
LaCl₃	Lanthanum chloride
LC	Liquid chromatography
LC-MS	Liquid chromatography – mass spectrometry
MALDI	Matrix-Assisted Laser Desorption/Ionization
mBrB	Monobromobimane
MeOH	Methanol
MHz	Megahertz
min	Minute
Mn²⁺	Manganese ion
MS	Mass spectrometry
MV²⁺	Methyl viologen
MV^{•+}	Methyl viologen radical cation
m/z	Mass-to-charge ratio in mass spectrometry
NAD⁺	Nicotinamide adenine dinucleotide
NaHCO₃	Sodium bicarbonate (sodium hydrogen carbonate)
Na₂SO₄	Sodium sulphate
NH₃	Ammonia
nm	Nanometer

NMDA	N-methyl-D-aspartate
NMR	Nuclear Magnetic Resonance
NO	Nitric oxide
OPA	<i>O</i> -phthalaldehyde
O=PPh₃	Triphenylphosphine oxide
pH	Measure of the acidity of a solution
pKa	Measure of the strength of an acid
PPh₃	Triphenylphosphine
ppm	Parts per million
psi	Pounds per square inch
ROS	Reactive oxygen species
RSD	Relative standard deviation
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SBD-F	ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulphonate
SNOHO	S-nitrosohomocysteine
TCEP	Tris (2-carboxyethyl) phosphine
TOF	Time of flight
UO₂	Uranium dioxide
UV/Vis	Ultraviolet/visible
vs.	Versus

ABSTRACT

At elevated levels, homocysteine (Hcy) is a risk factor for cardiovascular diseases and Alzheimer's disease. It's also implicated in a number of other diseases such as neural tube defects, pregnancy complications, and renal failures. The monitoring of Hcy levels in plasma is of current concern. To date, the biochemical mechanism by which Hcy is involved in disease states is unclear. Herein, we describe highly selective colorimetric methods for the direct determination of Hcy. Inexpensive, commercially available materials are reported. The results show potential application for the detection of Hcy in human blood plasma. Additionally, new HPLC postcolumn detection methods for biological thiols are developed. The potential biomedical relevance of the chemical mechanisms involved in the detection of Hcy is discussed.

In addition to the detection of bioactive amino acids (such as Hcy and congeners), the detection of neutral sugars has been of great interest and ongoing for over a century. A big challenge remaining in this area is to achieve sensitive determination of specific saccharides at physiologically relevant pH. Inspired by calcium-dependent recognition of saccharides by C-type lectins, a water-soluble salophene-lanthanum complex was synthesized. It can be used in the detection of neutral sugars at physiological pH.

Anionic sugar detection is also of importance to biomedical research. For instance, an increase or decrease in total sialic acid levels in biological fluids or tissues can indicate the occurrence of certain cancers. In the general procedure for the determination of sialic acid, a hydrolysis step is typically required to release bound sialic acid residues from the glycoconjugates. Two problems are often encountered in the hydrolysis: i) destruction of analytes; ii) incomplete release of sialic acid residues. New effective sensing agents for the determination of sialic acid are thus needed. A salophene-europium complex was synthesized

and used in the selective detection of sialic acid-containing gangliosides under neutral conditions. Electrostatic interactions of Eu^{3+} with the carboxylate group of the sialic acid moiety as well as the secondary interactions of Eu^{3+} with proximal oligosaccharide hydroxyls play important roles in the signal transduction.

CHAPTER 1. INTRODUCTION

The detection of bioactive sugars and amino acids is of great current interest. Our program focuses on developing convenient methods for the selective detection of biologically relevant molecules. My research involves new directions in the sensing of amino acids, neutral sugars and anionic sugars.

1.1 Direct Detection of the Amino Acid Hcy Using Organic Sensing Agents

At elevated levels in blood plasma, the rare amino acid Hcy has been identified as a risk factor for Alzheimer's and cardiovascular diseases.^{1,2} It's also implicated in a number of other diseases such as neural tube defects, pregnancy complications and renal failures.³⁻⁵ The detection of Hcy has thus attracted great interest in both the medical and chemical fields. Many sensor technologies have been developed. However, most of the work has relied on separation techniques due to interference from structurally similar thiols. Immunoassays have been developed. However, immunoassay reagents are relatively fragile and expensive in general as compared to synthetic organic reagents.

The actual role of Hcy in disease is unclear. After many years of study, it is still not yet known if Hcy causes disease, is a consequence of disease or is simply a biomarker.⁶ There is a significant need to uncover the role of Hcy in disease.

1.2 Syntheses of Lanthanide Complexes as Biosensors for Sugars

Metallomacrocycles have recently been employed in the field of supramolecular chemistry.^{7,8} Most of these macrocycles are constructed with metal cation(s) incorporated into organic ligands. Lanthanides can extend their ligand coordination number via "ligand-sphere" extension, leading to highly coordinated complexes.⁹ Additionally, La³⁺ has been shown to exhibit relatively stronger affinity for sugars

compared to most other metal ions.¹⁰ Hence, we propose that the incorporation of La^{3+} into a salophene ligand may provide a useful receptor for the determination of saccharides.

Additionally, effective new sensing agents are needed for the determination of total sialic acid, which indicates the occurrence of certain cancers. A challenge is to achieve the selective detection of sialic acids without using a hydrolysis step to release bound sialic acid residues from gangliosides. Eu^{3+} has been reported to have higher affinity to GM1 than to free sialic acid.¹¹ Moreover, the smaller of the ionic radius of a lanthanide, the larger are the intramolecular interactions among its ligands. Eu^{3+} has a smaller ionic radius than that of La^{3+} , we thus hypothesize that a Eu^{3+} -salophene complex may function as a good receptor for gangliosides.

1.3 Research Goals

My research goal is to detect biologically relevant amino acids and sugars under physiological conditions, which mainly include (i) uncovering the differences in the organic chemistry of biological thiols; (ii) understanding the fundamental organic chemistry relevant to the involvement of Hcy in disease; (iii) creating simple new methods which lead to selective, straightforward detection of Hcy; (iv) synthesizing new sensing agents for neutral and anionic sugars.

The approach involves synthetic organic and organometallic sensing agents. Understanding the mechanism of binding and signal transduction is an important aspect of the work, particularly when it is relevant as a biomimetic process.

In the next chapter, the background on the aminothiol Hcy is presented.

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CHAPTER 2. BACKGROUND OF HCY

2.1 Discovery of Hcy

In 1932, Butz and du Vigneaud, at the University of Illinois, discovered a compound by heating methionine in sulfuric acid.¹ They found that this compound had chemical properties similar to those of cysteine (Cys) and cystine. After characterization, they named this compound “homocystine” since it’s the “next higher symmetrical homolog of cystine”. They also concluded that homocystine was the oxidized form of Hcy, which is formed during the hydrolysis of methionine.

Though Butz and du Vigneaud also pointed out that Hcy might support the growth of animals on Cys-deficient diets, little was known about the broader biomedical significance of this sulfur amino acid until the early 1960s, when children with mental retardation, dislocation of ocular lenses, seizures and skeletal abnormalities were found to have high concentrations of Hcy in the urine.² The discovery of homocystinuria (homocystine excreted in the urine) initiated an upsurge of research interest in Hcy.

2.2 Structures of Hcy and Other Biologically Related Thiols

As mentioned above, the structure of Hcy (**1**, Figure 2.1) is very close to that of Cys (**2**, Figure 2.1). It has one more carbon in the side chain compared to Cys. As its demethylation product, Hcy also resembles the essential amino acid methionine (**3**, Figure 2.1). All three amino acids, Hcy, Cys and methionine, are metabolically linked. Another structurally similar biological thiol is the tripeptide γ -glutamyl-cysteinyl-glycine, glutathione (GSH, **4**, Figure 2.1). Cys is the most abundant low-molecular-weight thiol in plasma whereas GSH is the most predominant in cells. The biological function of GSH is to remove harmful oxidizing agents and maintain the reducing environment inside cells.

GSH plays essential roles in antioxidant defense and regulation of cellular events such as protein synthesis, gene expression, immune response and cell proliferation and apoptosis. Other biologically relevant thiols include coenzyme A, dihydrolipoic acid and cysteamine.

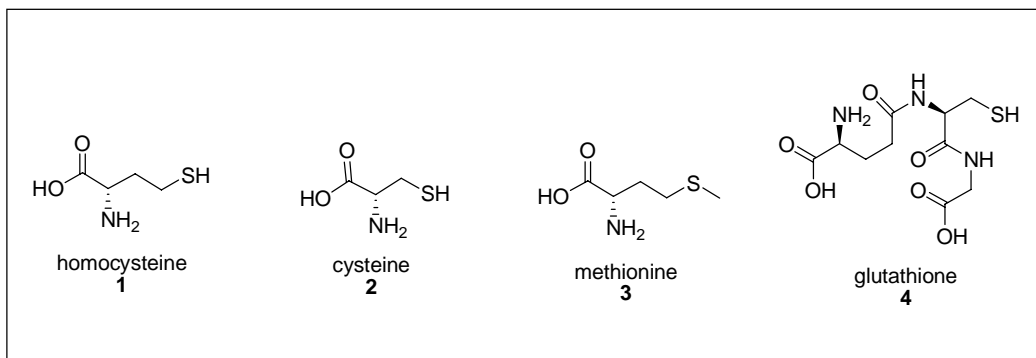


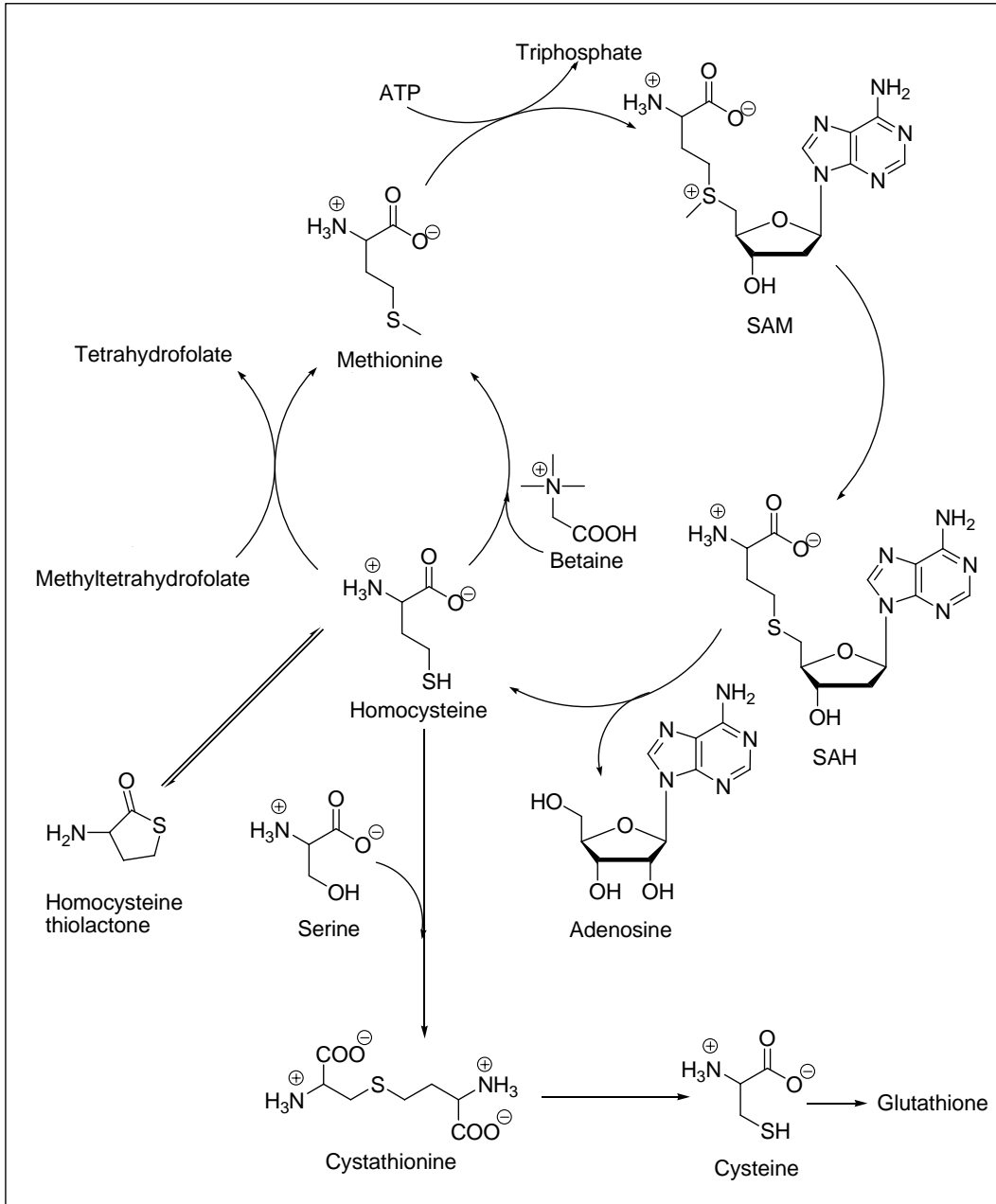
Figure 2.1. Structures of some biological thiols

2.3 Hcy Metabolism

Hcy is an intermediate metabolite in the metabolism of methionine (Scheme 2.1). In biological systems, S-adenosylmethionine (SAM) serves as the most widely used methyl donor for many reactions. Therefore, it's often called the universal methylating agent. SAM is synthesized from a reaction of L-methionine with ATP, catalyzed by enzyme methionine adenosyltransferase.³ In the presence of a methyl-transferase, SAM is then demethylated to give rise to S-adenosylhomocysteine (SAH).⁴ SAH is subsequently broken down to Hcy and adenosine by the enzyme S-adenosylhomocysteine hydrolyase.⁵ Nicotinamide adenine dinucleotide (NAD⁺) is required as a cofactor in this reaction.

The Hcy formed can enter two distinct metabolic pathways. First, it can be remethylated back to methionine and thus conserved within the methionine cycle. During the remethylation, Hcy receives a methyl group either from methyltetrahydrofolate or from betaine (trimethylglycine) to form methionine.⁶ Secondly, Hcy can be converted to

Cys via an irreversible transsulfuration pathway, in which Hcy reacts with serine in the presence of cystathionine- β -synthase (a vitamin B₆-dependent enzyme) to afford cystathionine.⁷



Scheme 2.1. Metabolism of Hcy

The cystathionine formed is then hydrolyzed to generate Cys with another enzyme, cystathionine γ -lyase.^{7,8} The irreversibility of this pathway indicates the inability of Cys to serve as a precursor for methionine. Once Cys is generated, about 70% is rapidly converted to the tripeptide GSH.⁹ The distribution of Hcy among these two competing pathways depends on two factors — the tissue content of the relevant enzymes and the kinetic properties of the enzymes.^{6, 10}

Beside the above mentioned two metabolic pathways, Hcy can also form an intramolecular thioester, homocysteine thiolactone. Homocysteine thiolactone is chemically reactive and can easily acylate the free amino groups of proteins.¹¹

2.4 Homeostasis of Hcy

The intake of Hcy from the diet is very small. The majority of Hcy is produced by the metabolism of methionine. Many factors affect the distribution of Hcy in the body resulting in a variety in physiological levels. Hcy metabolism depends on related enzymes and their proper function, adequate levels of substrates and associated amino acids, as well as efficient vitamin levels. Small metabolic changes will affect the distribution of Hcy among different tissues, cells, intracellular compartments and extracellular media. Moreover, intracellular import and export, cellular uptake and output as well as transport also influence the distribution of Hcy. Mechanistic understanding of the distribution and the transportation of Hcy in human body is of importance for understanding of its possible roles in disease.

2.5 Hcy in Plasma

Hcy is found in several forms after being released into plasma.¹²⁻¹⁴ Most Hcy (more than 70%) is conjugated to proteins. About 5-15% is present as homocystine.

Another 5-15% forms mixed disulfides with other low-molecular-weight thiols such as Cys and GSH. Hcy exists only in small amount as free, reduced form (<1%). Thus the term, total Hcy, is used to refer to the sum of the concentrations of all free plus disulfide bound Hcy.¹⁴

The oxidation of Hcy to disulfides in plasma is coupled to the reduction of O₂ giving rise to the formation of hydrogen peroxide (H₂O₂), which is thought to lead to oxidative stress.⁸ However, more recent evidence indicates that most of the oxidized Hcy (disulfides) arise primarily from disulfide exchange reactions. Only a small fraction of disulfides result from direct oxidation reactions.¹⁵

Hcy can also react with nitric oxide (NO), which is released by endothelial cells to form S-nitrosohomocysteine (SNOHO). SNOHO is a strong antiplatelet and vasodilator agent. The nitrosylation of Hcy, on one hand, lowers the availability of NO, which is a neurotransmitter and involved in muscle relaxation.¹⁶ On the other hand, the nitrosylation reduces the production of peroxide and therefore inhibits the formation of reactive oxygen species (ROS).¹⁷

The reduced form of Hcy can also undergo a reversible reaction to form homocysteine thiolactone (Scheme 2.1). However, the concentration of homocysteine thiolactone in plasma is very low (nanomolar) due to non-specific enzymatic hydrolysis. Homocysteine thiolactone can also form an amide bond readily with the epsilon nitrogen of lysine residues (*vide infra*).¹⁸

2.6 Relevance of Hcy to Public Health

When the metabolic pathway of Hcy to either methionine or Cys is disrupted due to genetic defects or nutritional deficiency, the export of Hcy from the cellular medium to

the extracellular medium will be imbalanced, resulting in the elevation of Hcy levels in blood plasma (hyperhomocysteinemia) or urine (homocystinuria). At elevated levels in plasma, Hcy has been confirmed as an independent risk factor for Alzheimer's and cardiovascular diseases.^{19,20} It's also implicated in a number of other diseases such as neural tube defects,²¹ pregnancy complications,²² and renal failures.²³

- **Alzheimer's Disease**

Alzheimer's disease is a disorder in the human brain. The disease was named after a German doctor, Alois Alzheimer. In 1906, Dr. Alzheimer first described the disease. He found abnormal clumps and tangled fibers in the brain tissue of a woman who had died of a mental illness. Today, these clumps and tangles, which are believed to disrupt the transmission of impulses amongst brain cells, are considered the signs of the disease.

The causes of Alzheimer's disease are still unclear. But research has shown that patients with Alzheimer's disease have significantly higher circulating levels of Hcy than healthy control subjects.²⁴ Oxidative stress may cause Alzheimer's disease. For instance, the over expression of glutathione peroxidase was found in Alzheimer patients, linking the disease to oxidative stress in the brain. Further evidence for the role of oxidative stress is that antioxidant supplements can delay the Alzheimer's-related disorders.

Recent research results by Lipton *et al.* indicate that Hcy is a neurotoxin.²⁵ Hcy can interact with the *N*-methyl-D-aspartate (NMDA) receptor and consequently cause excessive calcium influx and free radical production, resulting in neurotoxicity.

- **Cardiovascular Diseases**

Cardiovascular diseases include the dysfunctional conditions of heart and blood vessels, which supply oxygen and nutrition to the body. Hcy elevation has been identified

as an independent risk factor for vascular disease in the coronary, cerebral and peripheral circulation.²⁶ The first human study of Hcy in vascular disease was carried out in 1976. It showed that oral loading of methionine caused the increase of levels of homocystine and Hcy-Cys mixed disulfide in the plasma of patients with coronary heart disease.²⁷ It has been reported that 20%-30% of patients with coronary disease have moderately elevated Hcy levels either in the fasting state or after a methionine load.²⁸ In spite of a clear association of elevated Hcy with vascular disease, whether the excess Hcy itself confers vascular risk or it's just a secondary phenomenon still needs to be addressed. Additionally, increased levels of Hcy are strongly correlated to standard risk factors of vascular diseases such as smoking, gender, elevated lipids and aging. When determining the independent contribution of Hcy, these factors need to be considered.

- **Renal Disease**

Kidneys metabolize Hcy and are responsible for the clearance of plasma Hcy under physiological conditions. When the metabolism of Hcy in kidneys is interrupted, Hcy clearance will be reduced. Consequently, the concentration of Hcy in plasma will increase. It has been found that Hcy levels were significantly increased in patients with chronic renal failure and transplant recipients with impaired renal function.^{29,30}

- **Neural Tube Defects and Other Pregnancy Complications**

Experimental results from animal models indicate that elevated Hcy might be a teratogenic agent contributing to congenital defects of neural tubes.³¹ Hcy metabolism is involved in several biochemical pathways of the production of some nutrients, which are essential to the vascular, skeletal and nervous systems. Therefore, the disturbance of Hcy metabolism might be the underlying mechanism of the pathogenesis of neural tube

defects.³² Other pregnancy complications, such as spontaneous abortion, placental abruption, preterm delivery and low infant birth weight have also been associated with elevated Hcy levels.²²

2.7 Nutritional Intervention

Nutritional supplementation offers a way to prevent and treat hyperhomocysteinemia. In 1988, Kang *et al.* reported an inverse relationship between plasma Hcy and folate concentration.³³ Further studies confirmed that folate, vitamins and other nutrients can lower the concentration of Hcy in plasma.³⁴⁻³⁸ Selhub *et al.* studied the independent correlations between individual nutrients and plasma Hcy levels.³⁹ After controlling gender, age and other nutrients, they found that nonfasting plasma Hcy showed inverse relationships with folate, vitamin B12, B6, and pyridoxal-5'-phosphate. Among the studied population, two thirds of participants with hyperhomocysteinemia are attributed to vitamin deficiency.

2.8 Hcy's Role in Disease

After many years of intensive study and impressive progress, it is still not yet known if Hcy causes disease, is a consequence of it, or is simply a biomarker. There is an ongoing effort in the biomedical community involving the study of hyperhomocysteinemia and the role of Hcy in disease.

The role of Hcy in disease is controversial. Hcy has been reported to inhibit the oxidation of luminol and the dihydrorhodamine by strong oxidants.⁴⁰ Hcy also rapidly reduced ferrylmyoglobin to metmyoglobin.⁴⁰ In a comparative study of the ability of GSH, Cys and Hcy to reduce dehydroascorbic acid (DHA), Hcy promoted much more significant reduction of DHA than Cys and GSH. Furthermore, the reduction occurred at

Hcy concentrations that were over an order of magnitude smaller than those of GSH and Cys.⁴¹ Thus, Hcy may function as a relatively potent reducing agent and plays a protective role in diseases.

On the other hand, Hcy is considered as a causative factor. Initially, it was believed that Hcy promoted the generation of ROS due to auto-oxidation reactions.⁴² But recently, this hypothesis has been challenged. In human plasma, Cys is present in much higher concentrations (exceeding those of Hcy by 20-30 folds). Cys can undergo similar oxidative chemistry to that of Hcy. However, Cys has not typically been associated with oxidative stress. Additionally, another biologically relevant thiol, GSH, is typically associated with beneficial antioxidant activity. GSH scavenges free radicals and ROS (e.g. H₂O₂) via non-enzymatic or enzymatic reactions.^{43,44} It is believed that GSH deficiency, instead of elevation, plays a role in oxidative stress and contributes to aging and many diseases.

Some scientists believe that Hcy has a direct effect on vascular cells and tissues and causes cellular degeneration, damage to arterial intima, deposition of lipoproteins and so on.²⁷ Elevated levels of Hcy result in increased vascular accumulation of ROS, especially superoxide anion.⁴⁵⁻⁴⁷ Superoxide anion reacts rapidly with endothelium-derived NO to form peroxynitrite. This reduces the bioavailability of NO, an antiatherogenic molecule and contributes to decreased vasodilator capacity. In addition, peroxynitrite is highly reactive towards protein oxidation. However, the precise mechanism by which Hcy may induce the formation of ROS and peroxynitrite remains to be elusive.

Therefore, mechanistic understanding of the role of Hcy in disease is of great importance for developing effective biomedical strategies to improve health and to treat disease. The following chapter describes known methods for Hcy detection.

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CHAPTER 3. OVERVIEW OF ANALYTICAL METHODS FOR THE DETECTION OF HCY

Due to the important biological relevance of Hcy, the detection of Hcy in biological fluids has attracted high interest in both the medical and chemical fields. A large number of prospective methods for the detection of Hcy have been published in the literature. Most methods available can be divided into two categories: direct methods and indirect methods. Direct methods mainly refer to immunoassays such as enzyme immunoassay (EIA), fluorescence polarization immunoassay (FPIA) and enzyme-linked immunosorbent assay (ELISA). Most indirect methods are based on separation techniques including liquid chromatography (LC), high performance liquid chromatography (HPLC), capillary electrophoresis (CE) and gas chromatography (GC).

3.1 Sample Pretreatment

All methods for the detection of Hcy include a reduction step to release free Hcy from its –S-S- bound forms before the detection. Lots of reducing agents have been used. Usually, these agents are sulfhydryl (-SH) containing compounds or phosphines, such as dithiothreitol (DTT), mercaptoethanol, tri-*n*-butylphosphine and tris (2-carboxyethyl) phosphine (TCEP).¹⁻³

3.2 Immunoassays

Immunoassays combine the theories of chemistry and immunology to facilitate a specific and sensitive determination of a certain analyte. The crux of an immunoassay is the specificity of the antibody-antigen reaction. Several immunoassays have been developed.

In 1985, Refsum *et al.* reported a radioenzymic assay for the determination of Hcy in plasma and urine.⁴ Reduction of disulfides with the reducing agent DTT released

the free Hcy, which was consequently converted to S-[¹⁴C] adenosylhomocysteine. Quantification of S-[¹⁴C] adenosylhomocysteine by HPLC and liquid scintillation counting eventually gave the concentration of Hcy.

To avoid the use of radioisotopes, EIA and FPIA were introduced for the determination of Hcy.^{5,6} The general principle of these two assays is based on enzymatic conversion of free reduced Hcy to S-adenosylhomocysteine (SAH), followed by quantification of SAH with the use of a monoclonal anti-SAH antibody.

The most attractive feature of an immunoassay is its selectivity. However, radioimmunoassays often involve toxic substances. EIA and FPIA have high capacity but also show some imprecision.⁷

3.3 Liquid Chromatography – Mass Spectrometry (LC-MS)

The small sample demand and rapid processing time make the LC-MS an advantageous method with large capacity. In addition, its high accuracy also makes it a potential candidate for reference methods.^{8,9} However, it requires cumbersome and expensive equipment, which is not suitable for clinically diagnostic applications.

3.4 HPLC

HPLC has been largely used in the determination of Hcy as it can be readily coupled with various detection methodologies. Synchronous detection of different thiols in a same run is another advantage of the method. Furthermore, this technology can be easily automated.

- **HPLC with Fluorometric Detection**

Fluorescence spectroscopy is a widely used method due to its high sensitivity and reliability. Because Hcy itself is non-fluorescent, the determination of Hcy with

fluorescence relies heavily on a derivatization step, in which Hcy reacts with fluorophores to generate detectable species.

Several molecules have been utilized for the derivatization of Hcy. For instance, Araki and Sato have used ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulphonate (SBD-F, Figure 3.1) as a pre-column derivatizing agent.¹⁰ *o*-Phthalaldehyde (OPA, Figure 3.1) is a fast derivatizing agent for a wide range of amino thiols over an extensive pH range.¹¹ Monobromobimane (mBrB, Figure 3.1)¹²⁻¹⁴ and other derivatizing agents have also been utilized by several research groups. However, most of these reagents are unselective and have some drawbacks and limitations (Table 3.1).

Table 3.1. Several commonly used fluorophores and their limitations

Derivatizing agent	Limitations
monobromobimane labeling	(i) Unstable at room temperature and in H ₂ O; (ii) batch to batch varying impurity levels; (iii) fluorescent hydrolysis products produced upon labeling (iv) gradient and relatively complex elution chromatography needed.
iodoacetamide labeling	(i) Cross-reactivity with His, Tyr and Met; (ii) promotes loss of NH ₃ upon reaction with Cys residues; (iii) weakens mass spectrometric sensitivity.
<i>o</i> -phthalaldehyde labeling	(i) Highly pH sensitive reaction; (ii) thiol adduct exhibits high photo-instability; (iii) cross reactivity with many other amino acids
maleimide labeling	(i) Hydrolysis peaks are encountered at the beginning and end of chromatographic elution; (ii) cross linking to amines; (iii) unwanted rearrangements of conjugates
hexaiodoplatinate labeling	Broad cross-reactivity with interferences including thioethers, thiazolidines and ascorbic acid

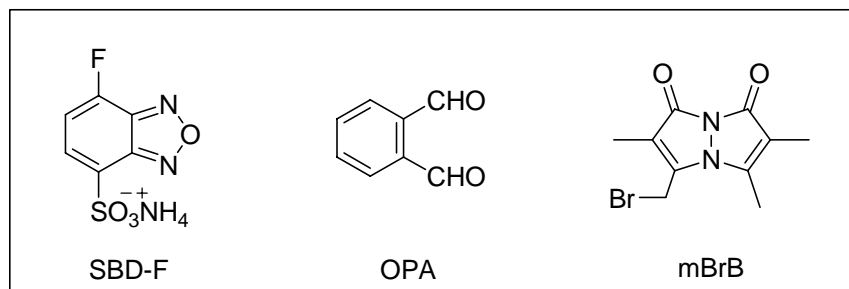


Figure 3.1. Examples of fluorescent derivatizing agents

- **HPLC with UV/Vis Detection**

UV/Vis detectors have also been used in the detection of Hcy. In order to develop a useful and efficient technique, a derivatization step is needed. The derivatizing agents must be able to react rapidly with Hcy to form stable products with sufficient UV/Vis absorption. 2-Chloro-1-methylpyridinium iodide (CMPI, Figure 3.2) is a commonly used derivatizing agent.¹⁵⁻¹⁶ 2-chloro-1-methylquinolinium tetrafluoroborate (CQMT, Figure 3.2), a derivative of CMPI, has also been used.¹⁷

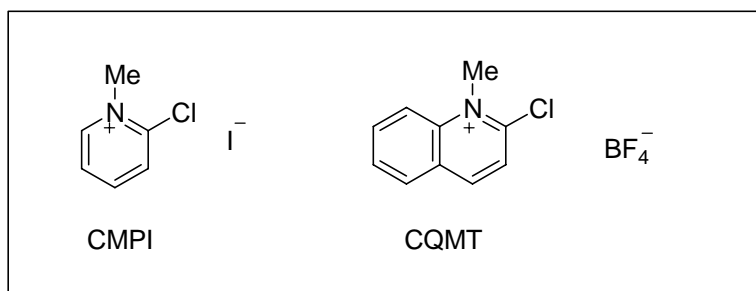


Figure 3.2. Examples of UV/Vis derivatizing agents

- **HPLC with Electrochemical Detection**

The redox chemistry of -SH group in Hcy makes it suited for electrochemical detection. Hcy can be chemically oxidized to homocystine at bare electrodes is the sensing root in this detection methodology. As the measurement can be directly carried out at the electrode surface, no derivatization is needed prior to the chromatographic

separation. A number of electrode substrates, including carbon,^{18,19} glassy carbon,^{20,21} graphite,²² platinum,²³ gold,²⁴⁻²⁷ and silver²⁸ have been exploited. To improve poor voltammetric responses with these common substrates, the use of bismuth-doped lead dioxide²⁹ and boron doped diamond electrodes³⁰ have been employed. Solid electrodes coupled with electron transfer mediators also have been utilized.³¹⁻⁴¹ In order to overcome the leaching of mediators from the surface of electrodes, carbon paste electrodes, into which the mediator can be mixed, have been used.⁴² Though the shorter run time is a major advantage of electrochemical detection over the aforementioned two methods, it still suffers from problems such as low reproducibility and high imprecision, which are due to the deterioration of measurement cells.¹

3.5 CE

As a commonly used separation technique, CE has been utilized in the detection of Hcy. Similar to HPLC, CE is often combined with different detection strategies, for instance, electrochemical detection, fluorescence detection and UV/Vis spectroscopy. CE coupled with different detection methods has been a complementary technique to HPLC.

- **CE with Electrochemical Detection**

Normally, direct detection of Hcy on common solid electrodes is very difficult due to the slow electron-transfer kinetics. In addition, electrode alignment and isolation of the detector from the separation voltage makes the CE-electrochemical detection more complicated. There are several sensitive and selective techniques that have been presented with modified electrodes. O'Shea *et al.* have developed a thiol-specific method with a gold/mercury-amalgamated electrode.⁴³ Pasas *et al.* described a microchip using a CE-electrochemical detection format for the determination of Hcy.⁴⁴ Amongst the

modified electrodes, the one chemically modified with pyrroloquinone quinone shows potential.⁴⁵

- **CE with Fluorescence Detection**

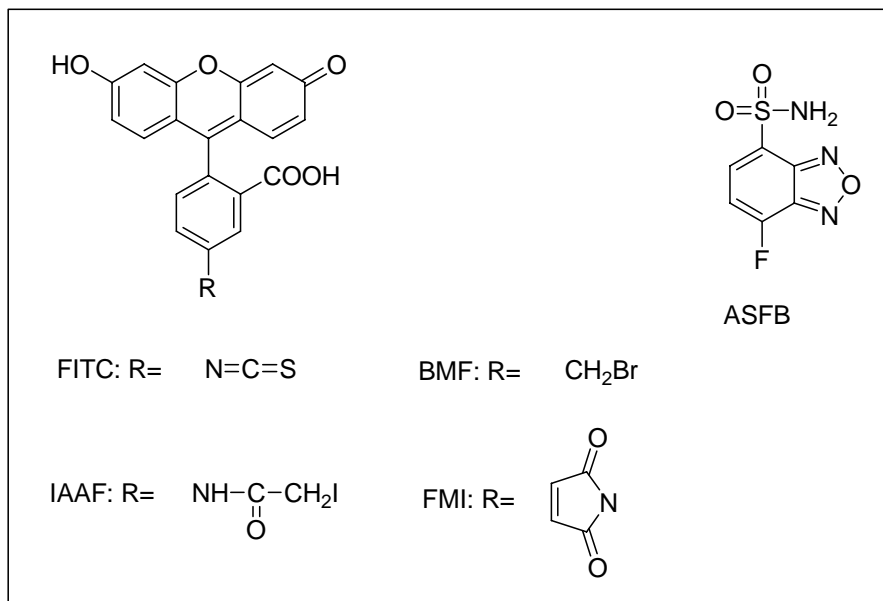


Figure 3.3. Commonly used fluorophores for CE

Just as in HPLC-fluorescence detection, CE with fluorescence detection also requires a derivatization step. Several fluorogenic agents including fluorescein isothiocyanate (FITC),⁴⁶ bromomethylfluorescein (BMF),⁴⁷ 6-iodoacetamidofluorescein (IAAF),⁴⁸ 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ASF),^{49,51} and fluorescein-5-maleimide (FMI),⁵⁰ have been used for the derivatization (Figure 3.3).

- **CE with UV/Vis Detection**

Compared to electrochemical and fluorescence detection, UV/Vis detection is rarely used due to its lack of sensitivity.

3.6 GC

GC is seldom used in the detection of Hcy because of the incompatibility of thiols with the GC system.⁵² Only a few assays have been reported to date. Outlined below is a summary of currently available methods using GC for the detection of Hcy.

- **GC with Mass Spectrometry (GC-MS)**

Derivatization of Hcy with ethyl chloroformate to generate a volatile amino acid ester has been described by Pietzsch *et al.*⁵³ Another derivatizing agent *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide has also been introduced.^{54,55} Although the derivatization processes are time-consuming, the assay developed is very reliable and sensitive and has been considered as a reference method.

- **GC with Flame Photometric Detection (GC-FPD)**

A selective method for the detection of total blood plasma Hcy has been reported by a research group at Okayama University in Japan.⁵⁶ After reduction, the free reduced Hcy was converted to its *N,S*-diisopropoxycarbonyl methyl ester derivative, which was then detected by flame photometric detection.

- **GC with Electron Capture Detector (GC-ECD)**

Myung *et al.* have developed a selective and sensitive method for the detection of Hcy and related biological thiols by GC-ECD.⁵⁷ The analytes were first reduced to their free thiol forms, which were consequently converted to their *N* (O, S)-ethylcarbonyl pentafluoropropyl esters. After extracted with chloroform and hexane, the derivatized analytes were determined by GC-ECD.

3.7 Conclusion

Due to interferences from the biological matrix, the determination of Hcy in biological fluids is often carried out in conjunction with separation techniques such as LC,

GC, HPLC and CE. Several detection protocols including electrochemical detection, UV/Vis and fluorescence spectroscopy have been reported. All of these techniques have shown different advantages and limitations.

The highly useful commercial immunoassays monitor one analyte at a time and utilize relatively fragile biological materials. In addition, immunoassay reagents are relatively expensive compared to common chemical reagents.

Furthermore, as stated by Refsum *et al.* in their comprehensive 2004 review co-authored by a group of the world's leading experts in this field,⁵⁸ many analytical methods afford results that do not fulfill baseline bias and imprecision criteria. Interlaboratory, inter and inpatient errors in the determination of Hcy and other biological thiols persist. There is a need for reference methods and/or standardized methods, which should provide high selectivity, sensitivity together with high efficiency and precision.

In following two chapters, a new colorimetric method and a new automatic postcoulmn HPLC method developed in our lab for the detection of Hcy will be described.

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CHAPTER 4. COLORIMETRIC DETECTION OF HCY*

Methods for the direct detection of Hcy are hampered due to interference from common, structurally related biological thiols such as Cys and GSH. Hcy analyses are thus often performed in conjunction with separation techniques. As part of our program for the selective detection of colorless biomolecules,¹ we develop highly selective methods for the determination of Hcy by using inexpensive, commercially available materials, such as methyl viologen (MV^{2+}) and 9-phenyl 2,3,7-trihydroxy-6-fluorone (fluorone black, FB). The chemical mechanism involved in the detection promotes the understanding of the unique organic and bioorganic chemistry of Hcy and may also help understand the role of Hcy in disease.

4.1 Experimental Section

- **General**

All chemicals were purchased from Sigma-Aldrich or Cambridge Isotope Labs and used without further purification. UV/Vis data were obtained using a Spectramax Plus 384 spectrophotometer (Molecular Devices). Fluorescence spectra were recorded using a spectrofluorimeter SPEX Fluorolog-3 equipped with double excitation and emission monochromators and a 400 W Xe lamp. All spectroscopic data were recorded at

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room temperature. Matrix Assisted Laser Desorption Ionization Mass spectra were acquired using a Bruker Proflex III MALDI mass spectrometer with and without matrix (anthracene or dithranol). NMR spectra were obtained in 70% CD₃OD, 30% D₂O on a Bruker DPX-250 or DPX-300 spectrometer, unless otherwise indicated.

- **Detection of Hcy with MV²⁺**

Equimolar amounts of Hcy, Cys and GSH are dissolved in three 1mL solutions of MV²⁺ (4.0 mM) in tris buffer (0.1M, pH 7.5), respectively, to furnish solutions with final concentration of thiol at 17 mM. 1mL of the same MV²⁺ solution containing no thiol is a control. All samples are gently refluxed on a hot plate until a blue color forms in the Hcy-containing solution (5 min).

- **Detection of Hcy in Human Blood Plasma with MV²⁺**

Commercial lyophilized human blood plasma is reconstituted with distilled H₂O. Three 0.5 mL aliquots of reconstituted plasma are spiked with Hcy, Cys and GSH, respectively. The concentration of added thiols in plasma is 800 μM. 0.5 mL of the same reconstituted plasma containing no thiol is used as a control. Each of the above plasma samples is mixed with 0.5 mL of the solution of MV²⁺ (800 μM) in tris buffer (0.5 M, pH 7.5). All samples are refluxed on a hot plate until a blue color forms in the Hcy-containing plasma sample.

- **Detection of Hcy with FB**

Hcy, Cys or GSH is dissolved in a 0.005 M, pH 7.3 phosphate buffer, respectively, to make a solution with the final concentration at 1.0×10^{-3} M. FB is dissolved in methanol to make a solution of 1.44×10^{-5} M. 0.1mL of each thiol solution is mixed with 0.2mL of 0.005 M, pH 7.3 phosphate buffer and 0.7mL of FB solution. 0.3mL of 0.005

M, pH 7.3 phosphate buffer mixed with 0.7mL of FB solution is taken as the control. All samples are stirred for 5 min at room temperature before UV/Vis spectra are acquired.

- **Detection of Hcy with FB in the Presence of Triphenylphosphine (PPh₃)**

Hcy, Cys or GSH is dissolved in a 0.005 M, pH 7.3 phosphate buffer, respectively, to afford a solution with a final concentration at 1.0×10^{-3} M. FB is dissolved in methanol to make a solution of 2.5×10^{-5} M. PPh₃ is dissolved in methanol to make a solution with the concentration of 1.5×10^{-3} M. 0.1mL of each thiol solution is mixed with 0.2mL of 0.005 M, pH 7.3 phosphate buffer, 0.3mL of PPh₃ solution and 0.4mL of FB solution. 0.3mL of 0.005 M, pH 7.3 phosphate buffer mixed with 0.3mL of PPh₃ solution and 0.4mL of FB solution is used as a control. All samples are stirred for 5 min at room temperature before UV/Vis spectra are acquired.

- **Detection of Hcy in Human Blood Plasma with FB**

Commercial lyophilized human blood plasma is reconstituted with distilled H₂O. Disulfide-bound thiols are liberated from proteins by stirring the reconstituted plasma in a commercial disulfide reducing agent, TCEP gel. This is followed by deproteinization upon addition of MeOH which also contains PPh₃ (1.5×10^{-3} M). After centrifugation (5.0 min, 3000 g), the supernatant is filtered through a 0.45 mm filter.

For percent (%) recovery determinations, known amounts of Hcy are added to reconstituted plasma samples before reduction and deproteinization steps. After the reduction and deproteinization, the spiked plasma samples are mixed with FB solutions. UV/Vis spectra are then obtained. The absorbance difference with respect to the unspiked sample is correlated with the concentration of added Hcy. The calibration curve is thus constructed. The Hcy concentration present in the commercial blood plasma

sample is determined from the calibration curve by subtracting the absorbance of a non-reduced plasma solution containing FB and PPh₃ from the absorbance of a reduced plasma sample containing FB and PPh₃.

- **Effect of EDTA on the Detection of Hcy with FB**

A 0.2 mL aliquot of a solution of EDTA (1.0×10^{-3} M) in 0.05 M, pH 7.3 phosphate buffer is added to 0.1 mL of a solution of Hcy (1.0×10^{-3} M) in the same phosphate buffer. A mixture of 0.2mL of the same phosphate buffer and 0.1mL of Hcy solution is taken as the control. The mixtures are stirred for 5 min at room temperature. The UV/Vis spectra are acquired after the addition of 0.7 mL of FB solution (1.44×10^{-5} M) in MeOH to the above samples.

- **Effect of Fe³⁺ on the Detection of Hcy with FB**

To 0.3mL of a solution of Hcy (3.33×10^{-4} M) in 0.05 M, pH 7.3 phosphate buffer, is added 0.1 mL of a solution of FeCl₃ (1.0×10^{-4} M) in MeOH. After the addition of 0.6 mL of FB solution (1.67×10^{-5} M) in MeOH to the above solution containing Hcy and FeCl₃, the UV/Vis spectrum is acquired. A solution containing same amounts of Hcy and FB except for FeCl₃ is taken as the control.

4.2 Results and Discussion

- **Redox Chemistry of Biological Thiols**

Biological thiols can be oxidized in nature to form thiyl radicals. The formation of thiyl radicals in biological systems mainly includes three routes as follows:²

- (i) “Repair” reaction

This reaction often refers to the hydrogen donation process of GSH to carbon-centered radicals in DNA or proteins.

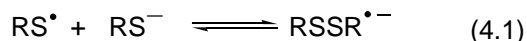
(ii) Oxidation by reactive oxygen/nitrogen species

Some reactive oxygen/nitrogen species such as hydroxyl radical ($\cdot\text{OH}$), nitrogen dioxide radical ($\text{NO}_2\cdot$), and peroxy radical ($\text{ROO}\cdot$) can often react with thiols to generate thiyl radicals.

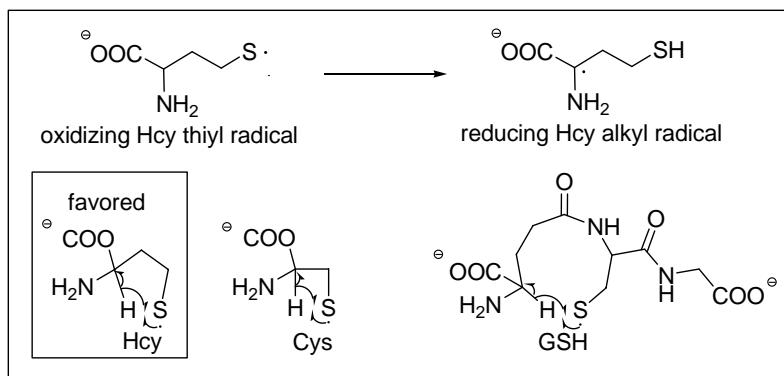
(iii) Enzymatic oxidation reaction

Enzymes that can catalyze the oxidation of thiols are mainly peroxidase, such as horseradish peroxidase, lactoperoxidase, and myeloperoxidase.

The ensuing fate of these oxidizing radicals is of concern to public health. Two of the many characteristic reactions of thiyl radicals include the formation of a reducing disulfide radical anion (Eq. 4.1) and the formation of a reducing α -amino carbon-centered radical (Eq. 4.2). Eq. 4.2 is an analogous process of “repairing” reactions.



The formation of the α -amino alkyl radical of Cys was reported in 1971 by Neta.³ It was later described that the α -amino alkyl radical of GSH could be formed intramolecularly via the thiyl radical (as in Eq. 4.2).^{4,5} Zhao *et al.* reported in 1994 that intramolecular formation of captodatively-stabilized α -amino alkyl radical in Hcy should be particularly favorable.^{5a} This was attributed to an intramolecular hydrogen abstraction mechanism, which involves a favored five-membered ring transition state (Scheme 4.1).^{5a} Armstrong and co-workers have also made recent contributions concerning proton abstraction of thiyl radicals. They state that during biological repair processes, glutathione thiyl radical can undergo either an inter- or an intra-molecular process to form a carbon-centered radical with relatively equal ability.⁶



Scheme 4.1. Intra-molecular proton abstraction of thiyl radicals leading to the formation of the α -aminoalkyl radicals

- **Selective Detection of Hcy with MV^{2+}**

The dication MV^{2+} was used by Zhao *et al.* during thorough investigations of the equilibrium kinetics of the reducing disulfide (Eq. 4.1) and the reducing α -amino carbon-centered radicals (Eq. 4.2), derived from the Hcy, Cys and GSH thiyl radicals.⁵ The formation of reducing radicals was monitored via changes in the UV/Vis spectra indicating the production of the methyl viologen radical cation ($MV^{\cdot+}$).^{5a} Under the basic conditions used (pH 10.5), no colorimetric selectivity between GSH, Cys and Hcy was observed.

At neutral solution pH, thiolate anion concentration is only a fraction of that of protonated thiol. Thus, at neutral pH, reducing disulfide radical anion formation should be diminished (Eq. 4.1). The intra-molecular proton-coupled electron transfer process (Eq. 4.2) which is most favored for Hcy (Scheme 4.1), should become more predominant at physiological pH. Additionally, zwitterionic amino acids cannot captodatively stabilize α -amino carbon-centered radicals. When the ammonium moiety is deprotonated under alkaline conditions, rearrangement to captodatively-stabilized carbon-centered radicals occurs much more readily and hence less selectively. We therefore reason that the

formation of the reducing α -amino alkyl radical should occur more readily in the case of Hcy compared to all other thiols, at neutral, rather than alkaline pH.



Figure 4.1. Selective color change in response to Hcy in solutions of MV^{2+} . (Left to right: no added analyte, Cys, Hcy, GSH)

Upon heating respective colorless solutions of MV^{2+} (4.0 mM, in pH 7.5, 0.5 M tris buffer) containing specific biological thiols (17 mM) at reflux for 5 min, without protection from ambient air or light, visual signaling occurs selectively for Hcy. The solution containing Hcy can be readily seen to turn blue, from colorless. Solutions of MV^{2+} containing Cys or GSH remain completely colorless upon heating (Figure 4.1). The color formation in the Hcy-containing solution can be monitored via the appearance of absorptions at 398 nm and 605 nm (Figure 4.2).

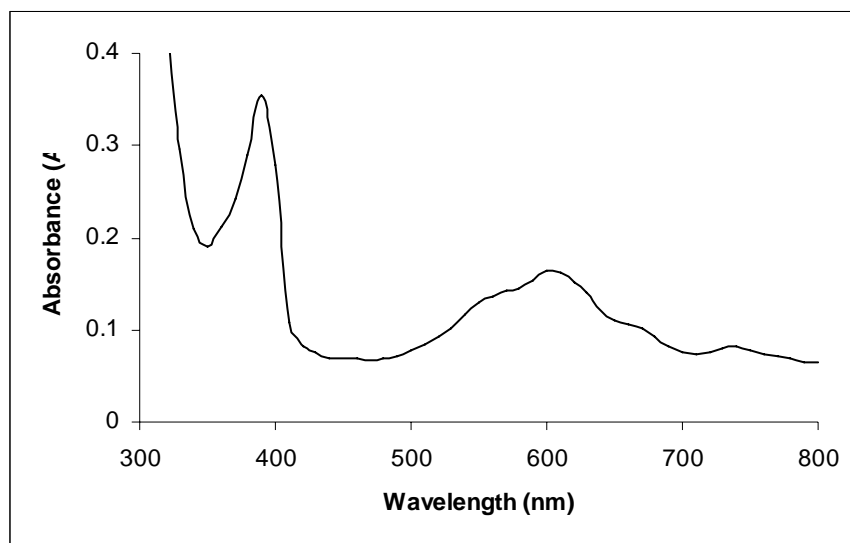


Figure 4.2. UV/Vis spectrum of a MV^{2+} solution (4.0 mM) in the presence of Hcy (17 mM) in tris buffer (0.1 M, pH 7.5) after gentle reflux for 5 min.

- **Selective Detection of Hcy in Human Blood Plasma with MV^{2+}**



Figure 4.3. Selective detection of Hcy in human plasma without prior deproteinization. (added thiol left to right: none, Hcy, Cys, GSH. Inset: samples before heating.)

Common thiol determinations in plasma often require a deproteinization step after the reduction of disulfides to release the free thiols for analysis. Under our conditions, we find that this process may not be necessary. Upon heating in the presence of MV^{2+} , commercial plasma samples spiked with Hcy, Cys and GSH afford selective blue color formation only corresponding to the presence of Hcy (Figure 4.3). This result is completely consistent with the prior results in buffer solutions. It demonstrates that deproteinization procedures such as centrifugation may not be necessary in this determination technique, thereby potentially allowing for relatively facile sample pretreatment. Work on optimizing detection limit and sensitivity is continuing.

- **Detection of Hcy with FB**

In order to extend the scope and generality of selective Hcy detection, we use electron-accepting fluorone black (Figure 4.4), a commercially available xanthene dye, to confirm the Hcy selectivity observed in the presence of MV^{2+} .

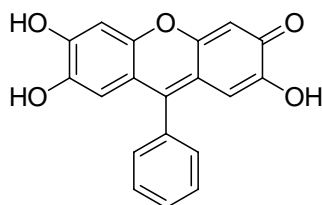


Figure 4.4. The structure of FB

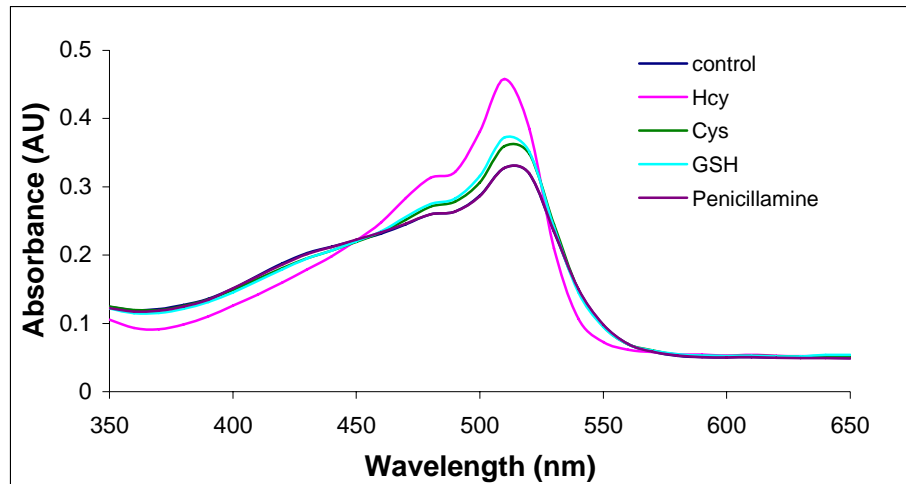


Figure 4.5. UV/Vis spectra of solutions containing FB (1.0×10^{-5} M) and different biological thiols (1.0×10^{-4} M).

Upon addition of 10 equivalent Hcy (1.0×10^{-4} M final concentration, in 0.015M, pH 7.3 phosphate buffer) to a solution of FB (1.0×10^{-5} M final concentration, in MeOH), an increase in absorbance at 510 nm is observed at room temperature. A similar, but relatively smaller absorbance change is observed for Cys, GSH and penicillamine (Figure 4.5). Other amino acids (glycine, alanine, serine, methionine, glutamine, lysine, arginine, threonine) and the disulfide homocystine do not promote a detectable absorbance change (Figure A1).

- **Selective Detection of Hcy with FB in the Presence of PPh₃**

The disulfide reduction in biological samples is often accomplished with a reducing agent such as a phosphine. We investigate the effect of PPh₃ on this process, initially in order to mimic the conditions used to prepare biological samples for analysis via disulfide reduction. When PPh₃ (45 equivalent to dye) is present, an absorbance change only occurs in the presence of Hcy. No change is observed for Cys or other biological thiols (Figure A2).

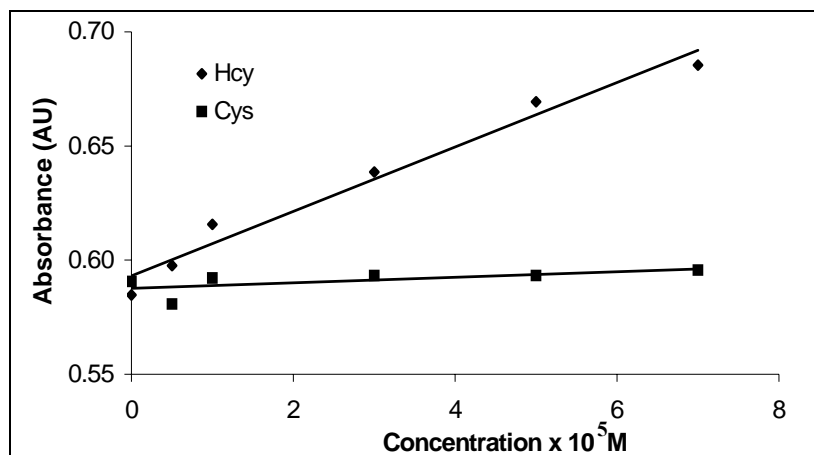


Figure 4.6. Absorbance at 510 nm vs. concentrations of Cys or Hcy in the presence of FB (1.0×10^{-5} M) and PPh_3 (4.5×10^{-4} M).

Concentration-dependent dye absorbance increases are thus observed only for Hcy. No response is observed for Cys (Figure 4.6). In addition, we observe selective fluorescence emission increases corresponding to the presence of Hcy under these conditions (Figure A3).

- **Investigation of the Function of PPh_3**

To investigate the function of PPh_3 in the selective determination of Hcy, a series of experiments have been carried out. The UV/Vis spectrum of a buffered solution of FB with only added PPh_3 exhibits absorbance increases in a similar fashion as it does when Hcy is added. This suggests that the phosphine may act as a competing reducing agent.

^{31}P NMR spectroscopy shows that FB enhances the formation of triphenylphosphine oxide ($\text{O}=\text{PPh}_3$) in a solution of FB and PPh_3 (Figure A4). After 19 h, the formation of $\text{O}=\text{PPh}_3$ is not observed in the PPh_3 solution which doesn't contain FB. Whereas in the PPh_3 solution which contains FB, the formation of $\text{O}=\text{PPh}_3$ is clearly observed. The formation of $\text{O}=\text{PPh}_3$ is well known to proceed via the PPh_3 radical cation.

Thus, PPh_3 inhibits the reduction of FB with thiols other than Hcy by acting as a competing reducing agent.

Hcy thus serves as the best reducing agent among the biological thiols. At least 45 equivalent PPh_3 (to dye) is needed to suppress the interactions of other thiols with FB. This is the threshold level of competing reducing agent, PPh_3 , which allows for only Hcy-generated spectrophotometric changes in solutions containing FB and PPh_3 . If a 30-fold molar excess of Cys (to Hcy) is added to a solution of Hcy (1.0×10^{-5} M, approximating both Hcy and Cys levels in plasma) and FB (0.5×10^{-5} M) in the presence of PPh_3 (5.0×10^{-4} M), no spectral change is observed compared to the original Hcy-FB solution (Figure 4.7).

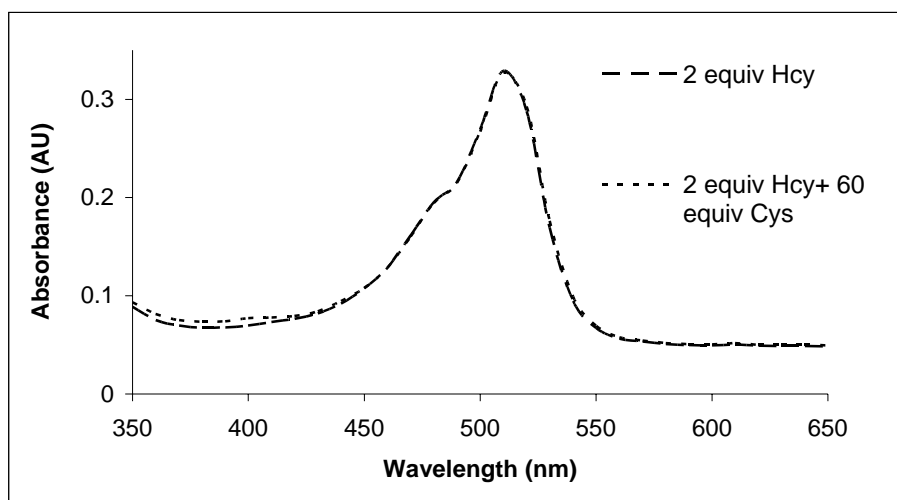


Figure 4.7. UV/Vis spectra of solutions containing FB (0.5×10^{-5} M), Hcy (1.0×10^{-5} M) and PPh_3 (5.0×10^{-4} M) in 30 % phosphate buffer (0.015 M, pH 7.3) and 70 % MeOH in the absence/presence of 30 equivalent Cys (to Hcy). No absorbance change is observed due to the added excess Cys.

- **The Study of the Detection Mechanism**

Solutions of fluorescein (Figure 4.8), unlike solutions of FB, do not exhibit spectral changes in response to Hcy or other thiols. (Figure 4.9).

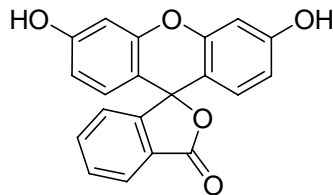


Figure 4.8. The structure of fluorescein

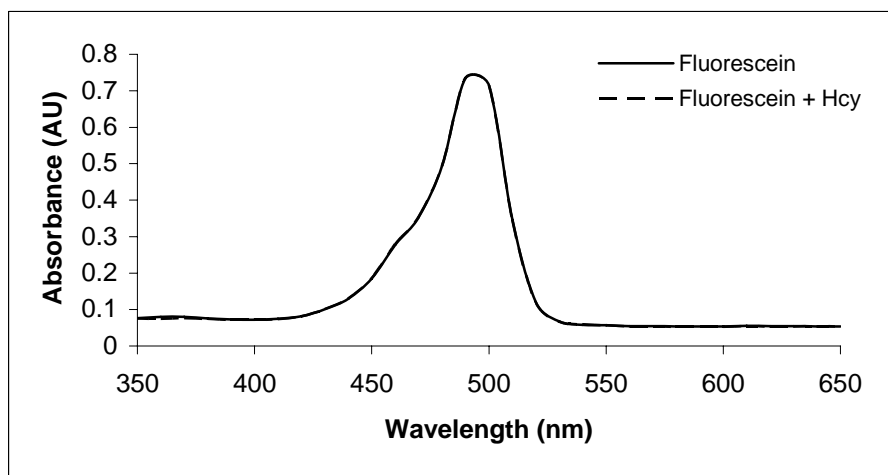


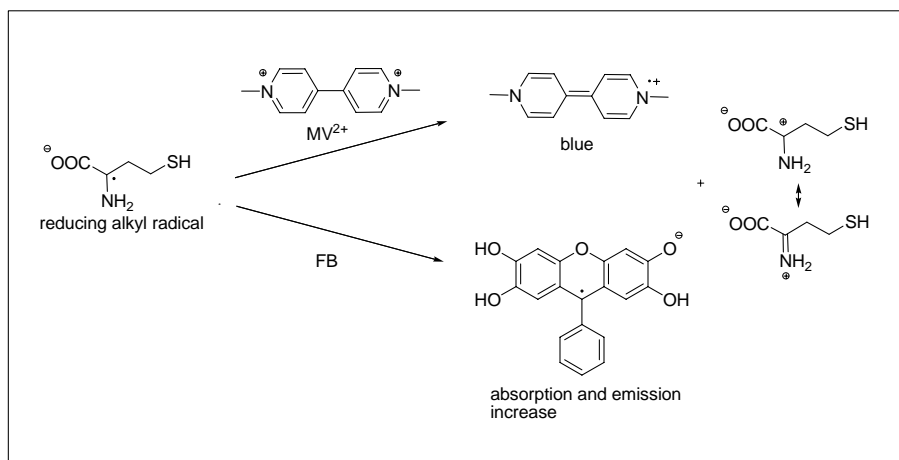
Figure 4.9. Detection of Hcy (1.0×10^{-4} M) with fluorescein (1.0×10^{-5} M) in 0.015 M phosphate buffer pH 7.3.

We thus propose that the formation of semiquinone radicals in FB is responsible for the signal changes observed (Scheme 4.2). Electron transfer results in raising the pK_a of FB. As in the case of the addition of a base, the absorbance of the dye at 510 nm increases. Similarly, electron transfer from Hcy carbon-centered radicals to MV^{2+} gives rise to the formation of MV^+ , which shows a blue color (Scheme 4.2).

- **Detection of Hcy in Human Blood Plasma with FB**

We successfully use FB to determine Hcy in a commercial human blood plasma sample via the standard addition method. A calibration curve derived from the solutions containing added Hcy standards is shown in Figure 4.10. It exhibits linearity in the working range from 0 to 15 μ M, which is inclusive of the upper limit of healthy Hcy

concentration. The percent recovery of Hcy is $102.9 \pm 7.3 \%$. The relative standard deviation (RSD) is 7.1% ($n = 3$).



Scheme 4.2. Electron transfer from Hcy carbon-centered radical to MV^{2+} or FB.

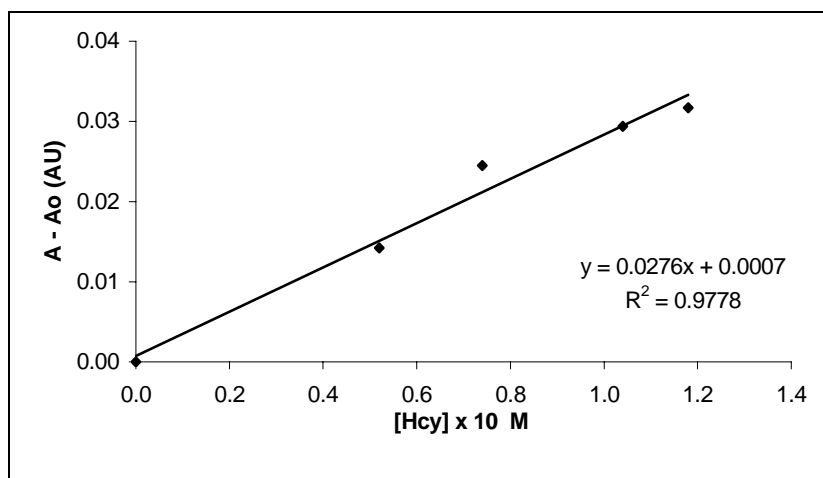


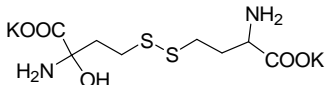
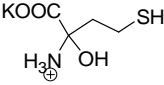
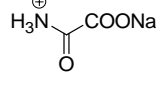
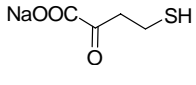
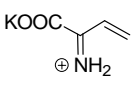
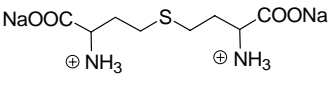
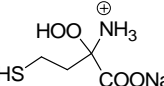
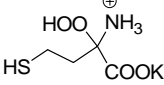
Figure 4.10. Calibration curve for the determination of Hcy in human plasma in the presence of PPh_3 and FB. (A is the absorbance of samples with added Hcy at 510nm; A_0 is the absorbance of samples without added standards at 510nm).

- **The Fate of the α -amino Carbon-centered Radical of Hcy**

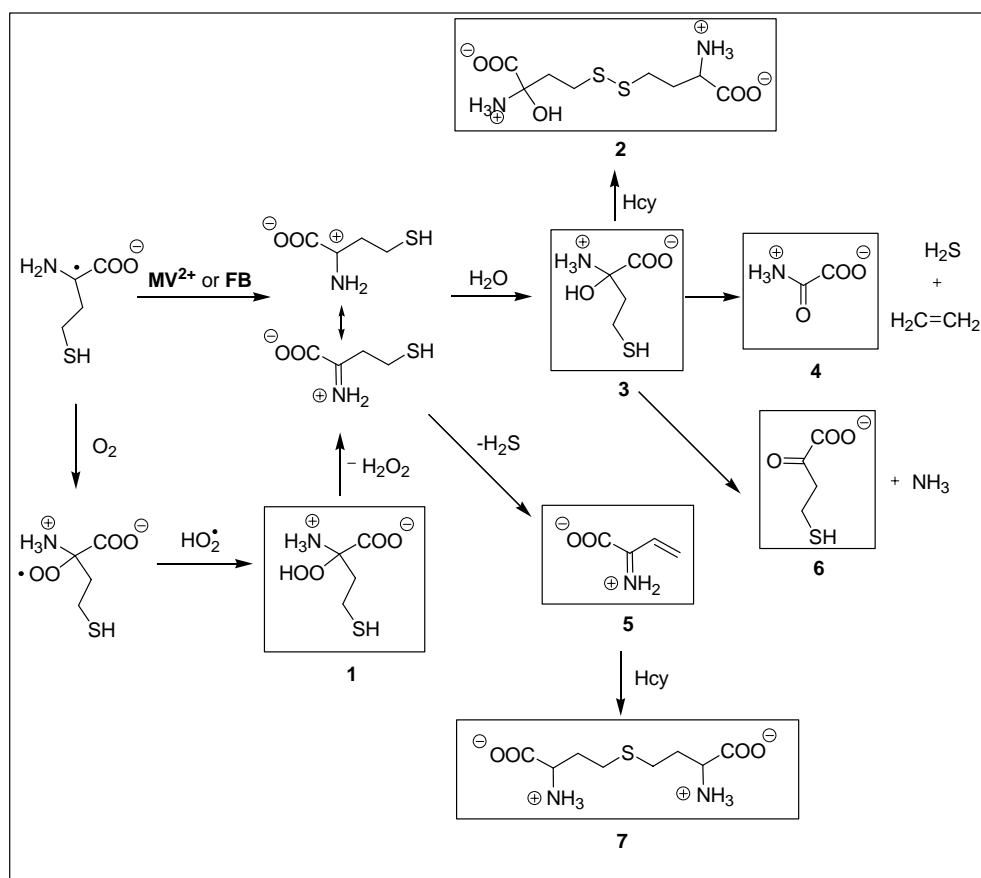
There is growing interest in the study of the metabolites of hyperhomocysteinemia.⁷ Amino acid-derived free radicals are often implicated in DNA

damage and the pathogenesis of disease. The readily-formed Hcy alkyl radical might be a significant site of free radical damage initiation or propagation. We thus extend our work in the study of byproducts formed from the Hcy carbon-centered radical under ambient conditions. It has been reported that α -amino acid alkyl radicals form NH_3 and several other radical termination and disproportionation products under ionizing radiation and relatively harsh oxidizing conditions.^{5,8} Several byproducts of Hcy alkyl radical reactions are identified under our assay conditions by using MALDI TOF MS (Table 4.1) with/without different matrices (Figure A5-A7).

Table 4.1. Byproducts of Hcy carbon-centered radicals

Product	Calcd.	Found	MALDI Matrix
	360.53	359.02	none
	190.28	190.05 191.01	Anthracene Dithranol
	112.04	112.52	none
	156.14	156.68 155.28	None Dithranol
	138.19	138.23	Dithranol
	282.06	283.33	Anthracene
	190.17	190.05 191.01	Anthracene Dithranol
	206.28	205.20	Anthracene

Scheme 4.3 shows a proposed mechanism of the formation of these products, which are generated under mild conditions: neutral pH and room temperature. Interestingly, compounds **2** and **7** have been found in the urine of patients with homocystinuria.⁹ Thus, this study may provide a starting point as a chemical model for the reactions of the α -amino carbon-centered radical derived from Hcy in the human body under non-radiative conditions.



Scheme 4.3. Proposed mechanism for the formation of byproducts from Hcy α -amino carbon-centered radical.

- **Evidence for H_2S Production from Hcy Carbon-centered Radicals**

In a separate experiment, the extent of formation of H_2S was evaluated for reactions of Hcy, Cys and GSH with MV^{2+} using hydrogen sulfide test strips. Figure

4.11 qualitatively shows that the sample containing Hcy produces a significantly higher amount of H₂S. Should Hcy produce H₂S more readily than GSH and Cys? It may have significant implications for Hcy-based toxicity compared to GSH and Cys.



Figure 4.11. H₂S test strips after contact with vapors produced by heated solutions containing equimolar amounts of GSH (left), Cys (center) and Hcy (right) in the presence of MV²⁺.

- **Effect of Transition Metals on the Detection of Hcy**

The oxidation of amino acids is generally catalyzed by transition metals. The mechanism of this process involves the formation of hydroxyl radicals (Fenton reaction). Therefore, studies of the oxidation of amino acids can be complicated by the presence of metal impurities in buffers and reagents. We thus investigate the effects of a chelating agent (EDTA) and an added transition metal (Fe³⁺) under our conditions, for completeness.

EDTA is a commonly used metal-chelating agent in studies of metal-catalyzed amino acid oxidation reactions. In a study of Fenton chemistry, Stadtman *et al.* showed that the oxidation of amino acids could be either stimulated or inhibited by various metal chelating agents, depending on the actual ratios of chelating agents to iron salts.⁸ The varying amino acid oxidation or inhibition of oxidation corresponding to changes in the

ratios of chelating agents to iron salts was attributed to variations in the composition, concentration and redox potentials of the different complexes.

Upon addition of EDTA (20 equivalent to FB) in our fluorone black assay, no differences in the UV/Vis spectra are observed as compared to those without EDTA (Figure 4.12).

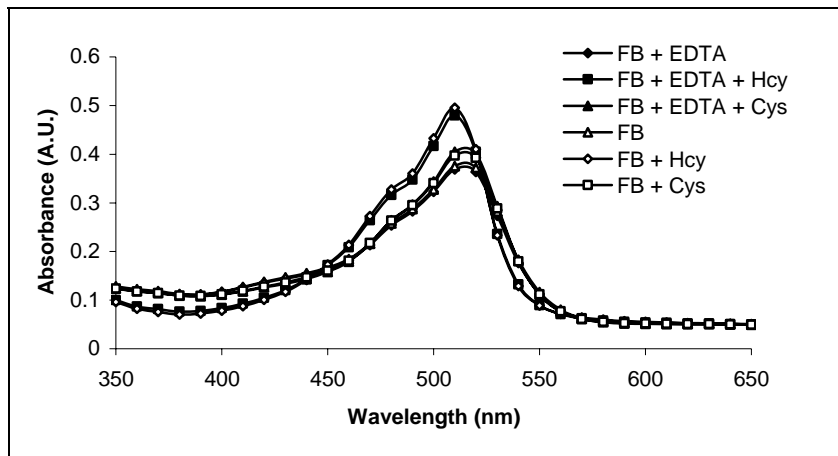


Figure 4.12. UV/Vis spectra of solutions containing FB (1.0×10^{-5} M) and Hcy or Cys (1.0×10^{-4} M) in the presence/ absence of EDTA (2.0×10^{-4} M).

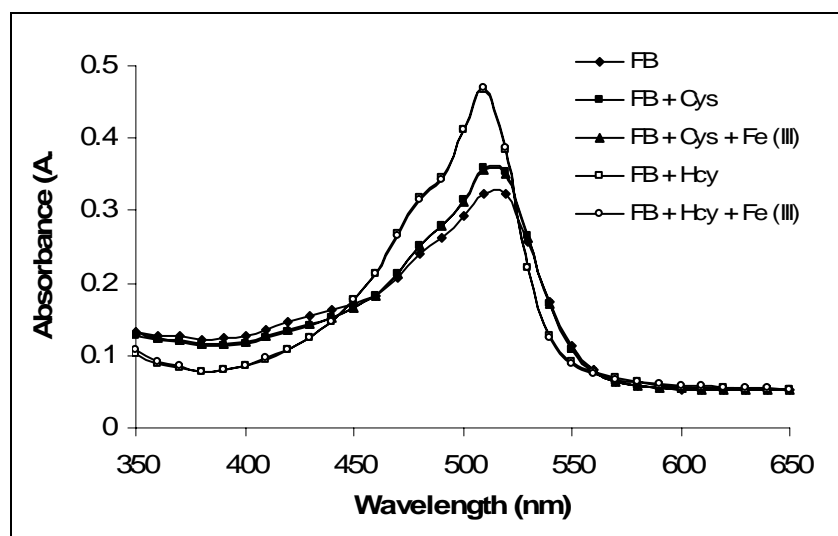


Figure 4.13. UV/Vis spectra of solutions containing FB (1.0×10^{-5} M) and Hcy or Cys (1.0×10^{-4} M) in the presence/ absence of FeCl_3 (1.0×10^{-5} M).

In addition, no spectral changes are observed upon addition of FeCl₃ (1.0×10^{-5} M) under the same conditions (Figure 4.13). The conclusion is that the mechanism shown in Scheme 4.1 best accounts for the observed Hcy selectivity in our assays, rather than Fenton chemistry.

4.3 Conclusion

In conclusion, the unique ability of Hcy to form reducing carbon-centered radicals has led to the discovery of the first Hcy-selective probes, MV²⁺ and FB. By using these two compounds, we have developed colorimetric methods for the selective detection of Hcy at neutral pH. MV²⁺ solutions turn color selectively corresponding to the presence of Hcy upon heating. We also selectively detect Hcy by utilizing FB and PPh₃ via UV/Vis spectroscopy at room temperature. The latter technique shows great potential for directly determining Hcy levels in human blood plasma.

The fundamental organic chemistry of Hcy, Cys and GSH should have broader biomedical implications. Rare metabolites such as **2**, found in the urine of homocystinurics, have been detected via the mild oxidation of Hcy at room temperature. The identification of such byproducts derived from Hcy carbon-centered radicals may help biomedical researchers in understanding the fate of Hcy under physiological conditions and its biological relevance to disease.

The mechanism showed in Scheme 3.1, rather than Fenton chemistry best accounts for the observed Hcy selectivity in our assays.

4.4 References

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CHAPTER 5. POSTCOLUMN HPLC DETECTION OF HCY*

The determination of Hcy is often carried out in conjunction with separations.¹ HPLC determination of Hcy and related thiols is currently used in large scale population studies on the international scene. Refsum, Ueland and co-workers have made pioneering advances in developing HPLC assays towards Hcy and related biothiol analysis, mainly using pre-column derivatization with fluorophores.¹

In pre-column HPLC detection of Hcy, a derivatizing step is often used. The derivatizing reagents typically contain universal electrophilic alkylating groups. There are nearly 50 thiol-reactive reagents currently sold by Molecular Probes, Inc.² There is thus great interest in developing agents for detecting Hcy and other biothiols.

In our laboratory, we have developed a simple HPLC post-column detection system for the analysis of mono- and oligosaccharides based on a boronic acid-derived chemosensor.³ Postcolumn detection allows for automation of the derivatization step and minimizes sample processing prior to analysis. Major concerns with introducing any new post-column techniques include (i) attaining appropriate reaction kinetics for generating an observable signal as well as (ii) diminishing background interference.

Recently, we demonstrate the use of MV²⁺ and FB in an automated HPLC postcolumn system for the determination of Hcy and other biological thiols. Both of the compounds have shown great potentials as postcolumn detection reagents.

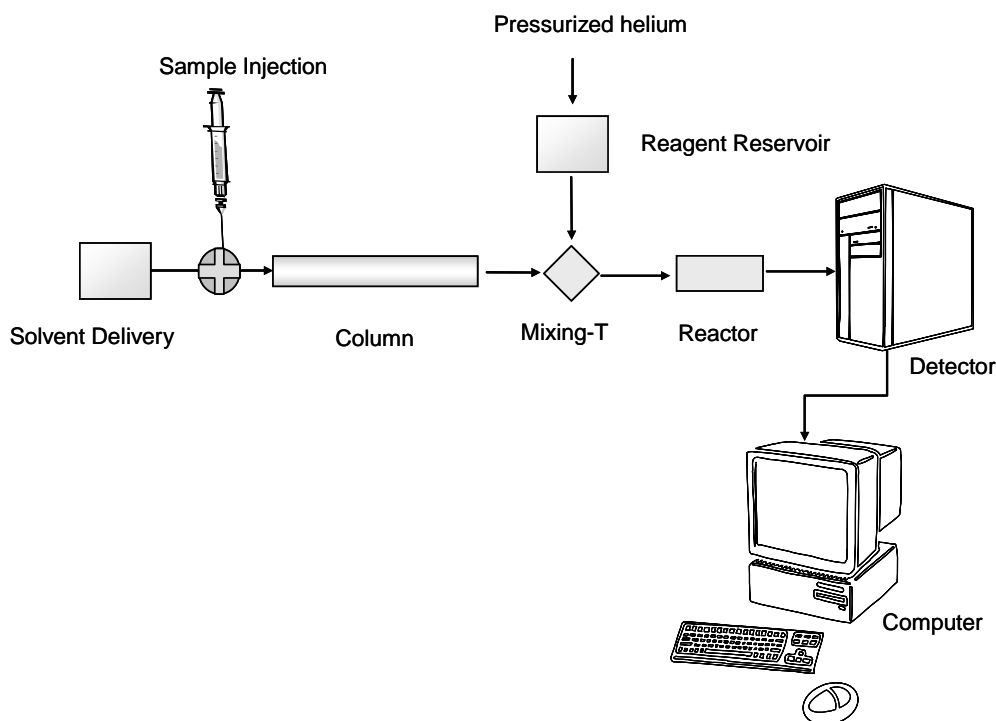
* Reprinted in part with permission from the *Journal of the American Chemical Society*, 2005, Volume 127, pages 15949-15958; Weihua Wang, Oleksandr Rusin, Xiangyang Xu, Kyu Kwang Kim, Jorge O. Escobedo, Sayo O. Fakayode, Kristin A. Fletcher, Mark Lowry, Corin M. Schowalter, Candace M. Lawrence, Frank R. Fronczek, Isiah M. Warner, and Robert M. Strongin; Detection of Homocysteine and Cysteine. Copyright 2005 American Chemical Society.

5.1 Experimental Section

- **Materials**

All materials were purchased from Sigma-Aldrich and used without further purification. HPLC grade methanol and water were obtained from EMD chemicals Inc. Both of the postcolumn reagents were freshly prepared and degassed by helium sparging right before use.

- **Apparatus**



Scheme 5.1. An automatic HPLC postcolumn detection system

The postcolumn detection system includes (i) a helium cylinder; (ii) a CM4000 multiple solvent delivery system (LDC/Milton Roy) (iii) a RDR-1 reagent delivery/reaction module (Timberline), which contains a pressurized reagent reservoir, a mixing tee, and a thermostated reaction block with a Teflon reaction coil; (iv) a Lichrospher reverse-phase column (4.6×250 mm, 5 μ m; Alltech Associates Inc.); (v) a SpectroMonitor

3100 UV/Vis detector (LDC/Milton Roy) (Scheme 5.1). The detection conditions for each detection reagent are described below.

- **Use of MV²⁺ as a Postcolumn Detection Reagent**

Mobile phase: TFA 0.01 M; mobile phase flow rate: 1.5 mL/min; reactor temperature: 80 °C; detector wavelength: 610 nm.

MV²⁺ concentration: 0.01 M in 0.25 M carbonate buffer, pH 9.5.

- **Use of FB as a Postcolumn Detection Reagent**

Mobile phase: 100 % of HPLC grade water; mobile phase flow rate: 1.5 ml/min; reactor temperature: 80 °C; detector wavelength: 505 nm.

FB concentration: 1.25×10^{-5} M in 50/50 (v/v) mixture of MeOH and aqueous carbonate buffer (0.25 M, pH 9.5).

5.2 Results and Discussion

- **Use of MV²⁺ as a Postcolumn Detection Reagent**

Figure 5.1 shows a chromatogram of a mixture of Hcy and Cys (85 nmol each) using MV²⁺ as a post-column detection reagent. Two well-resolved peaks are observed. Both of the peaks are identified by comparing retention times with those of standard Hcy and Cys.

- **Use of FB as a Postcolumn Detection Reagent**

Figure 5.2a shows a chromatogram of a mixture of Hcy and Cys (28.8 nmol each) using FB as a postcolumn chromogenic reagent. Two sharp peaks corresponding to Cys and Hcy are observed. All other amino acids (histidine, methionine, glutamine, lysine, glycine and serine) tested don't exhibit any peaks (Figure 5.2b and Figure 5.3c). This, on

one hand, excludes possible interferences from other common amino acids. On the other hand, it is further evidences that MV^{2+} and FB are chemoselective probes for aminothiols.

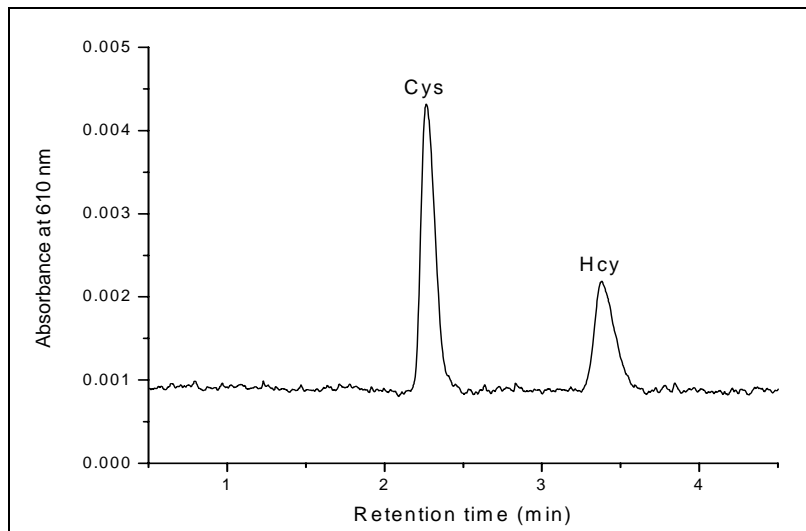


Figure 5.1. Chromatogram of a mixture of Cys and Hcy using MV^{2+} as the post-column reagent at 610nm.

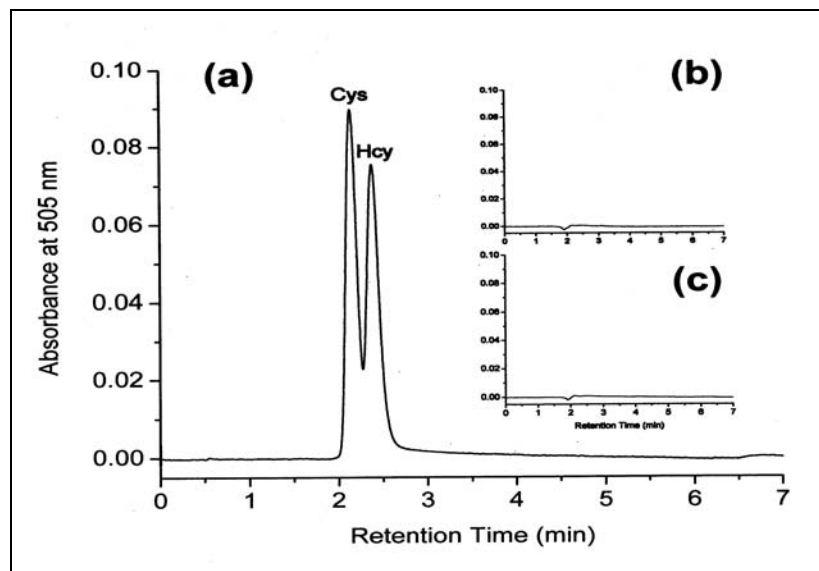


Figure 5.2. Chromatograms of mixtures of: (a) Cys and Hcy, (b) histidine, methionine and glutamine and (c) lysine, glycine and serine using FB as the postcolumn reagent.

5.3 Conclusion

In conclusion, the above HPLC studies indicate that the kinetics of both postcolumn reactions are fast enough for the detection of Hcy. Additionally, no interference from other amino acids is observed. MV^{2+} and FB can thus serve as prospective postcolumn reagents for the determination of Hcy and/or Cys. Currently, optimization, calibration, application in human blood plasma and method validation are still ongoing.

5.4 References

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CHAPTER 6. SYNTHESSES OF LANTHANIDE COMPLEXES AS BIOSENSORS*

6.1 Introduction

My specific contribution to this collaborative work was the optimization of the synthesis of **1** and **2**. This included improvements in yield and product purity as well as the development of an HPLC method to detect and assay **1** and **2**.

The detection of neutral sugars has been of interest for over a century. A challenge remaining in this area is to achieve sensitive and selective detection of mono- and oligosaccharides at physiologically relevant pH. The molecular recognition of sugars by lectins (carbohydrate-binding proteins other than enzymes and antibodies) in nature has inspired work in the field.¹ An important mode in this type of recognition is the coordination of carbohydrates to a metal center, such as Ca^{2+} , Mn^{2+} or other transition metals. The carbohydrate selectivity is often achieved by a network of coordination and hydrogen bonds.² Many innovative metal-based detection methods have been developed.^{3,4} However, since saccharides are weak ligands, elevated solution pH is typically used to attain a useful degree of coordination and signal transduction. The need for the detection of carbohydrate at physiological pH still persists.

Recently, metallomacrocycles have attracted great interest in the field of supramolecular chemistry.^{5,6} Most of the work has focused on the design of selective

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receptors, in which metal cation(s) is/are incorporated into organic ligands. The design of macrocyclic receptors is based on the appropriate organization of the binding sites and the size of analytes. For instance, a class of functionalized UO₂-salophene (or salen) complexes have been developed for the detection of anionic and neutral analytes.^{7,8}

The Shinkai group has reported a salen-Co (II) complex bearing two boronic acid groups for sugar sensing wherein Co (II) provides a well-defined distance between two boronic acid groups.⁹

La³⁺ and Ca²⁺ have been shown to exhibit relatively stronger affinity for sugars compared to most other metal ions.^{10,11} The similar properties of lanthanides allow trivalent lanthanide ions to be employed as useful substitutes for Ca²⁺ in biomedical research. In addition, lanthanides can extend their ligand coordination number via “ligand-sphere” extension, leading to highly coordinated complexes.¹²

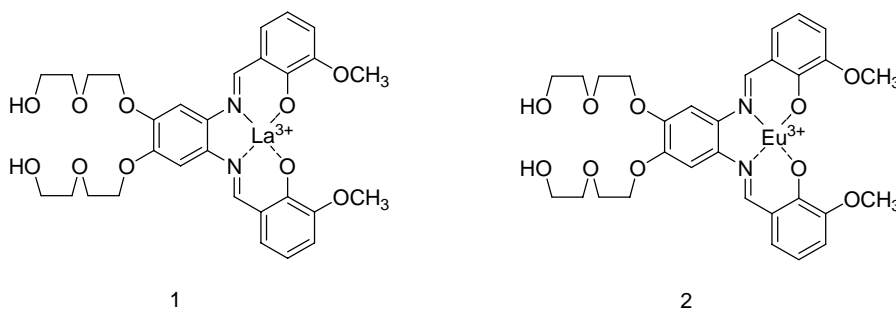


Figure 6.1. Salophene-lanthanide complexes **1** and **2**.

Hence, we propose that La³⁺-salophene complex **1** (Figure 6.1) may be useful for the determination of saccharides at neutral pH. La³⁺, which has good sugar affinity, would be a binding site. Glycolic side chains would help to achieve water solubility.

The determination of anionic sugars, such as sialic acid, at neutral pH is also a challenge of great current interest. An increase or decrease in total sialic acid levels (free circulating and glycoconjugated) in biological fluids or tissues can indicate the

occurrence of certain cancers. A variety of methods, including colorimetric methods, fluorometric methods, GLC and HPLC methods have been developed.¹³⁻¹⁸ In these procedures, a hydrolysis step is typically required to release bound sialic acid residues from the glycoconjugates. The acid-catalyzed liberation of sialic acid often results in the destruction of analytes.^{14, 19-21} In the case of enzymatic hydrolysis, incomplete release of sialic acid residues has been observed.²² New effective sensing agents for the determination of sialic acid are thus needed.

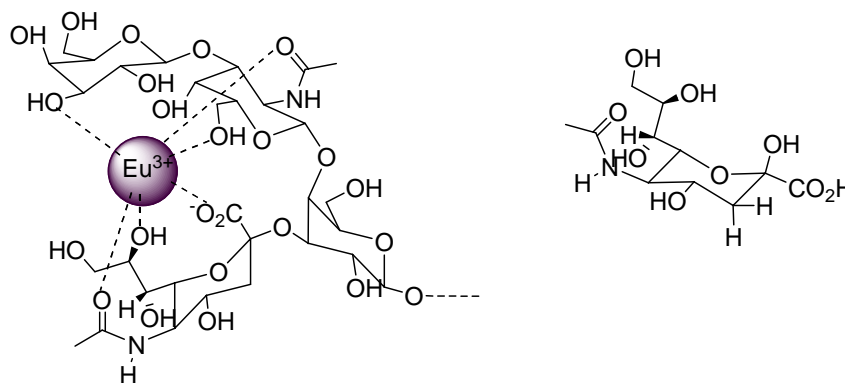
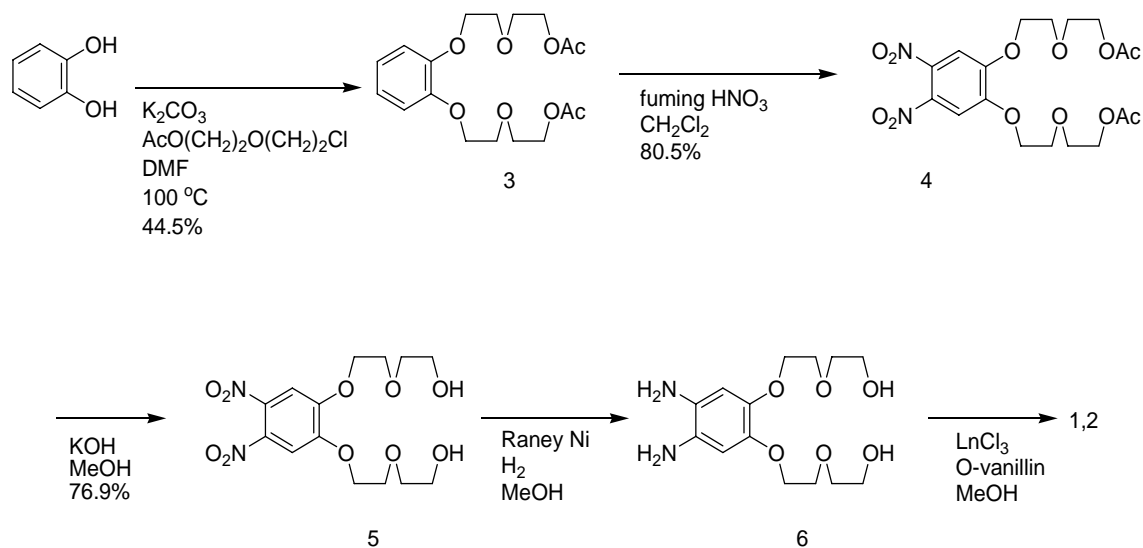


Figure 6.2. Left: Coordination of GM1 to Eu^{3+} . Right: Structure of free sialic acid

Sillerud *et al.* have reported favorable cooperative interactions of the oligosaccharide and sialic acid moieties of gangliosides with Eu^{3+} . As compared with sialic acid, the higher affinity of Eu^{3+} to GM1 was attributed to: i) an electrostatic interaction of Eu^{3+} with GM1 sialic acid carboxylate group; ii) secondary interactions with the proximal hydroxyls of the oligosaccharide (Figure 6.2).²³

Additionally, the smaller the ionic radius of a lanthanide, the larger are the intramolecular interactions among its ligands. Since Eu^{3+} has a smaller ionic radius compared to La^{3+} , we hypothesized that compound **2** (Figure 6.1) may function as a good sensing agent for anionic gangliosides under neutral conditions.

6.2 Results and Discussion



Scheme 6.1. Syntheses of Compounds **1** and **2**. **1**, Ln=LaCl₃, **2**, Ln=EuCl₃.

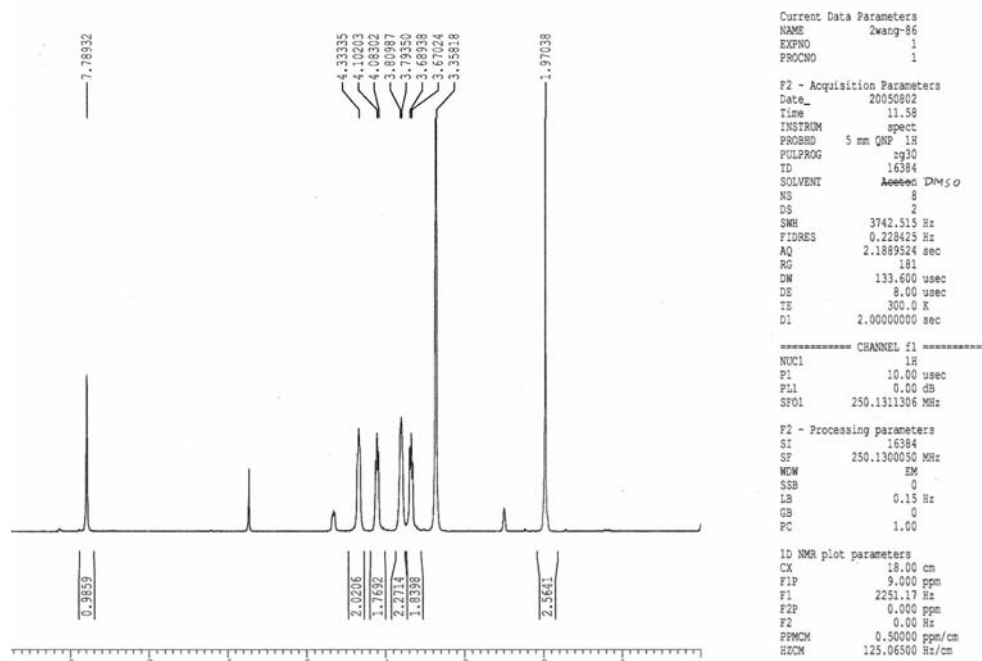


Figure 6.3. ¹H-NMR spectrum of **4** in DMSO-*d*₆.

The synthesis of **1** and **2** starts with the alkylation of catechol with o-acetyl-2-(2-chloro-ethoxy)-ethanol in the presence of K_2CO_3 to give the diacetates **3** (Scheme 6.1). The diacetates are chosen because the unprotected diol would lead to some byproducts under the acidic conditions of the following nitration step. Aromatic nitration of **3** gives the dinitro compound **4** in high yield. The 250 MHz 1H NMR spectrum of **4** is well resolved and clearly shows the symmetrical nature of the molecule. The presence of a two proton singlet at ca. δ 7.79 indicates the formation of the dinitrated product (Figure 6.3). Deacetylation in methanolic potassium hydroxide in the following step affords **5**. Catalytic hydrogenation of **5** gives rise to **6**, which is used immediately for the synthesis of **1** or **2** to prevent any unwanted oxidation.

- **HPLC Purity Screening of Compounds 1 and 2**

To verify the purity of **1** and **2**, high performance liquid chromatography is used. At the detection wavelength of 360 nm, only one peak is observed at 3.96 min for **1**, 4.02 min for **2**, respectively (Figure 6.4, 6.5). This indicates that both compounds are pure enough to be used in the following analytical detection processes.

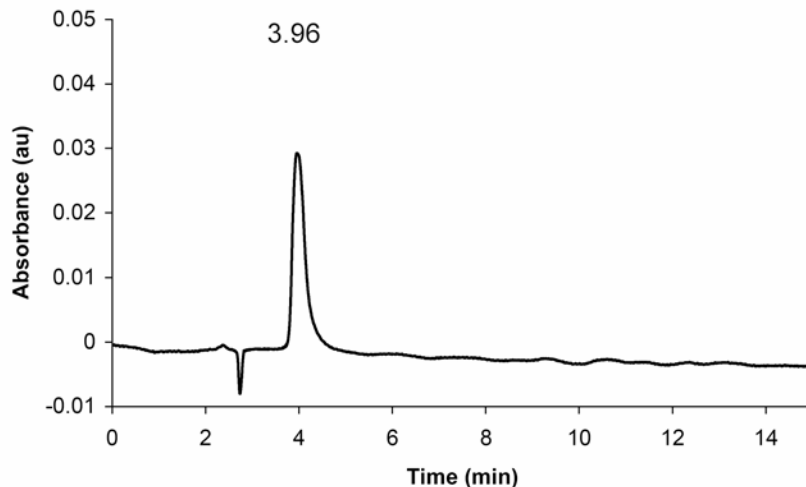


Figure 6.4. Chromatogram of a solution of **1** in MeOH.

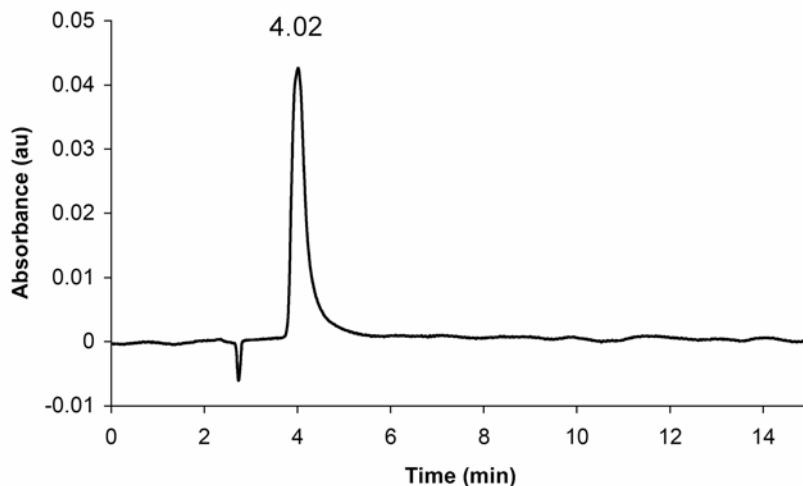


Figure 6.5. Chromatogram of a solution of **2** in MeOH.

- **Detection of Neutral Sugars at Physiological pH with Compound 1**

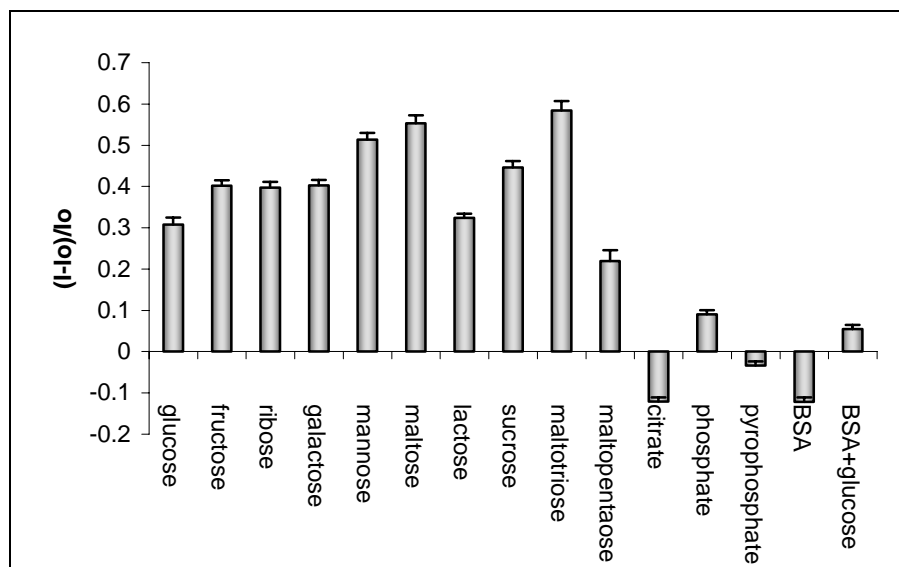


Figure 6.6. Relative fluorescence emission (400 nm) changes observed in solutions of **1** (5.53×10^{-6} M) in the presence of mono-, oligosaccharides, anions (1.1×10^{-3} M), BSA (1 mg/mL) and a mixture of BSA and glucose (1 mg/mL and 1.1×10^{-3} M, respectively) in HEPES buffer solution (pH 7.0). The standard deviation ($n=3$ for each analyte) of the relative fluorescence intensity ranges from 0.01-0.027.

Addition of saccharides (1.1×10^{-3} M) to a solution of **1** (5.53×10^{-6} M, 0.1 M HEPES buffer, pH 7.0) gives rise to fluorescence emission increases (Figure 6.6).

Common anions (citrate, phosphate and pyrophosphate), which exist in biological fluids promote relatively weaker emission responses under these conditions (Figure 6.6). In addition, bovine serum albumin-containing solutions exhibit emission increases only when glucose is present.

- **Detection of Gangliosides at Neutral Condition with Compound 2**

Compared with free sialic acid and non-sialic acid-containing ganglioside asialo GM1 (Figure 6.7), sialic acid-containing ganglioside GM1 is selectively detected by compound 2 (Figure 6.8). Free sialic acid binding to metals has been reported by *Saladini et al.*²⁴ The carboxylate, pyranose ring and oxygens of glycerol side-chain are all involved in the coordination. Sialic acid-containing GM1 binds to Eu^{3+} via multiple coordination sites. Besides the sialic acid moiety, proximal oligosaccharide hydroxyls also play important roles in the coordination (Figure 6.2).

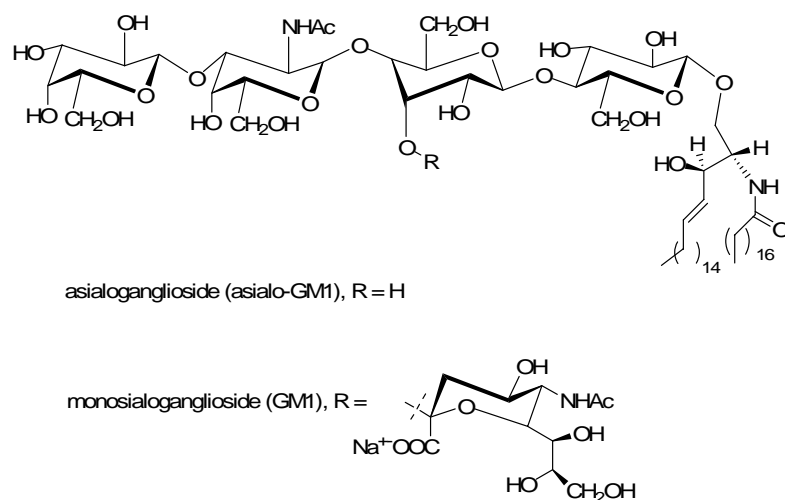


Figure 6.7. The structures of asialo-GM1 and GM1.

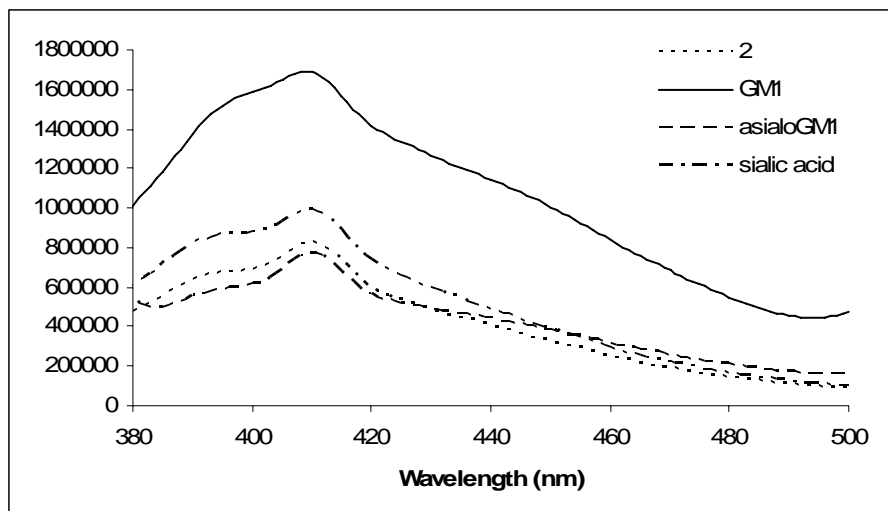


Figure 6.8. Fluorescence intensity change of solutions of **2** (5.53×10^{-6} M) in response to added gangliosides (0.5 mg/mL, *ca.* 10^{-4} M each) and sialic acid (1.0×10^{-3} M) in 0.1 M HEPES buffer solution (pH 7.0). Excitation 360 nm, emission 400 nm.

6.3 Conclusion

Two salophene-lanthanide complexes, **1** and **2**, have been synthesized and characterized. The design of compound **1** is inspired by calcium-dependent recognition of saccharides by C-type lectins. It can be used in the detection of neutral sugars at physiological pH. Compound **2** has shown potential for the selective detection of sialic acid-containing gangliosides under neutral conditions. Electrostatic interactions of Eu^{3+} with the carboxylate group of the sialic acid moiety as well as the secondary interactions of Eu^{3+} with proximal oligosaccharide hydroxyls plays important roles in the signal transduction.

6.4 Experimental Section

• General Methods

All chemicals were purchased from Sigma-Aldrich or Cambridge Isotope Labs and used without further purification. Gangliosides were purchased from Calbiochem. Fluorescence spectra were recorded on a spectrofluorimeter SPEX Fluorolog-3 equipped

with double excitation and emission monochromators and a 400W Xe lamp. MS were acquired on a Bruker ProfFLEX III MALDI-TOF mass spectrometer. ^1H and ^{13}C NMR spectra were acquired on a Bruker DPX-250 or DPX-300 spectrometer. All δ values were reported in ppm. FT-IR spectra were recorded on a Bruker Tensor 27 IR spectrophotometer.

HPLC purity screening was performed on a CM4000 multiple solvent delivery system connected to a LiChrospher 100 RP-18 (5 μm) endcapped column (250 \times 4.6 mm) and a SpectroMonitor 3100 UV/Vis detector (LDC/Milton Roy). The mobile phase is 30/70 (v/v) water/MeOH, the flow rate 1.0 mL/min and the detector wavelength was set to 360 nm.

- **Synthesis of 1,2-bis(2-(2(2-acetoxy(ethoxyethoxy))))benzene 3.**²⁵ To 90 mL of DMF under N_2 , 3.76 g of K_2CO_3 (27.24 mmol), 1.0 g of catechol (9.80 mmol) and 2.1 g of *O*-acetyl-2-(2-chloro-ethoxy)-ethanol (18.16 mmol) are added. The final mixture is heated overnight at 100 $^\circ\text{C}$. The reaction is monitored by thin layer chromatography (EtOAc / Hexane 1:3). Upon completion, the reaction mixture is cooled to room temperature. K_2CO_3 is filtered. The filtrate is diluted with EtOAc (60 mL) and washed with H_2O (30 mL \times 4). The organic phase is dried over Na_2SO_4 and concentrated under reduced pressure. The product is purified by column chromatography and obtained as a yellow oil (1.5 g, 44.5%). ^1H NMR (250 MHz, $\text{DMSO-}d_6$) δ (ppm): 1.99 (6H, s, CH_3) 3.69 (8H, m, CH_2) 4.09 (8H, m, CH_2) 6.92 (4H, m, ArH). ^{13}C NMR (62.5 MHz, $\text{DMSO-}d_6$) δ (ppm): 21.6, 64.0, 69.1, 69.3, 69.8, 115.2, 122.1, 149.2, 171.2.

- **Synthesis of compound 4.**²⁵ 1.6 g of **3** (4.32 mmol) is dissolved in 50 mL of DCM. The solution is cooled on an ice bath. To the solution, 12 mL fuming HNO_3 is added. The

solution is stirred overnight at room temperature. The reaction mixture is poured onto a mixture of ice and water (80 mL). The organic phase is collected, neutralized with 10% NaHCO₃ and washed with H₂O. The organic layer is dried over anhydrous Na₂SO₄. The solvent is removed under reduced pressure. The product is chromatographed on silica gel to give compound **4** (1.6 g, 80.5%). ¹H NMR (250MHz, DMSO-*d*₆) δ (ppm): 1.97 (6H, s, CH₃) 3.68 (2H, t, CH₂) 3.80 (2H, t, CH₂) 4.10 (2H, t, CH₂) 4.33 (2H, t, CH₂) 7.79 (2H, s, ArH). ¹³C NMR (62.5 MHz, DMSO-*d*₆) δ (ppm): 21.4, 55.7, 64.0, 69.3, 70.4, 110.1, 136.7, 152.0, 171.2.

- **Synthesis of compound 5.** The deprotection of **4** is carried out with 2 equiv KOH in a MeOH. 0.52 g compound **4** (1.13 mmol) is dissolved in 30 mL MeOH. 0.13 g KOH (2.26 mmol) is added. The mixture is stirred at room temperature for 4 hours. The reaction mixture is neutralized with 2N HCl. MeOH is removed under reduced pressure. The product is extracted with DCM. After washing with H₂O, the organic layer is dried over anhydrous Na₂SO₄. After removing DCM under reduced pressure, compound **5** is obtained (0.33 g, 76.9%). Compound **5** is used without further purification.

- **Synthesis of compound 1.** Compound **5** (0.2 g, 0.53 mmol) is dissolved in MeOH (15 mL). Raney Ni is added to the solution. Hydrogenation is carried out at 50 psi and monitored via hydrogen consumption. The reaction mixture is filtered through celite. The filtrate solution containing **6** is immediately used in the next step to prevent any unwanted oxidation. To a refluxing solution of LaCl₃ (0.13 g, 0.53 mmol) in 10 mL MeOH, *O*-vanillin (0.16 g, 1.1 mmol) in 10 mL MeOH and the solution containing **6** are simultaneously added over 20 min. The solution is heated at reflux for 2 h. The reaction mixture is concentrated under reduced pressure and washed with EtOAc (5 mL × 3). The

product is obtained as a dark-red solid (0.37 g). ^{13}C NMR (62.5 MHz, DMSO- d_6) δ (ppm): 49.4, 56.5, 56.9, 61.1, 69.7, 69.8, 73.1, 73.3, 113.8, 114.0, 118.4, 120.0, 120.9, 123.4, 149.2, 151.4, 192.8. MALDI-TOF (m/z): calcd. $\text{C}_{30}\text{H}_{34}\text{LaN}_2\text{O}_{10}$, 721.13; obsd, 721.48. IR 3206.20, 1614.33, 1439.22, 1209.10, 1036.87.

• **Synthesis of 2.** Compound **2** is synthesized according to the procedure described above for **1** except EuCl_3 is used instead of LaCl_3 . The product is obtained as a dark-red solid (0.35 g). ^{13}C NMR (62.5 MHz, DMSO- d_6) δ (ppm): 49.4, 56.6, 57.0, 61.1, 69.7, 69.8, 73.1, 73.4, 118.4, 119.3, 120.1, 120.9, 123.4, 147.3, 149.0, 149.3, 151.6, 192.8. MALDI-TOF (m/z): calcd. $\text{C}_{30}\text{H}_{34}\text{EuN}_2\text{O}_{10}$, 735.14; obsd, 735.34. IR 3104.00, 1638.44, 1444.54, 1214.76, 1018.07.

6.5 References

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APPENDIX A. COLORIMETRIC DETECTION OF HCY

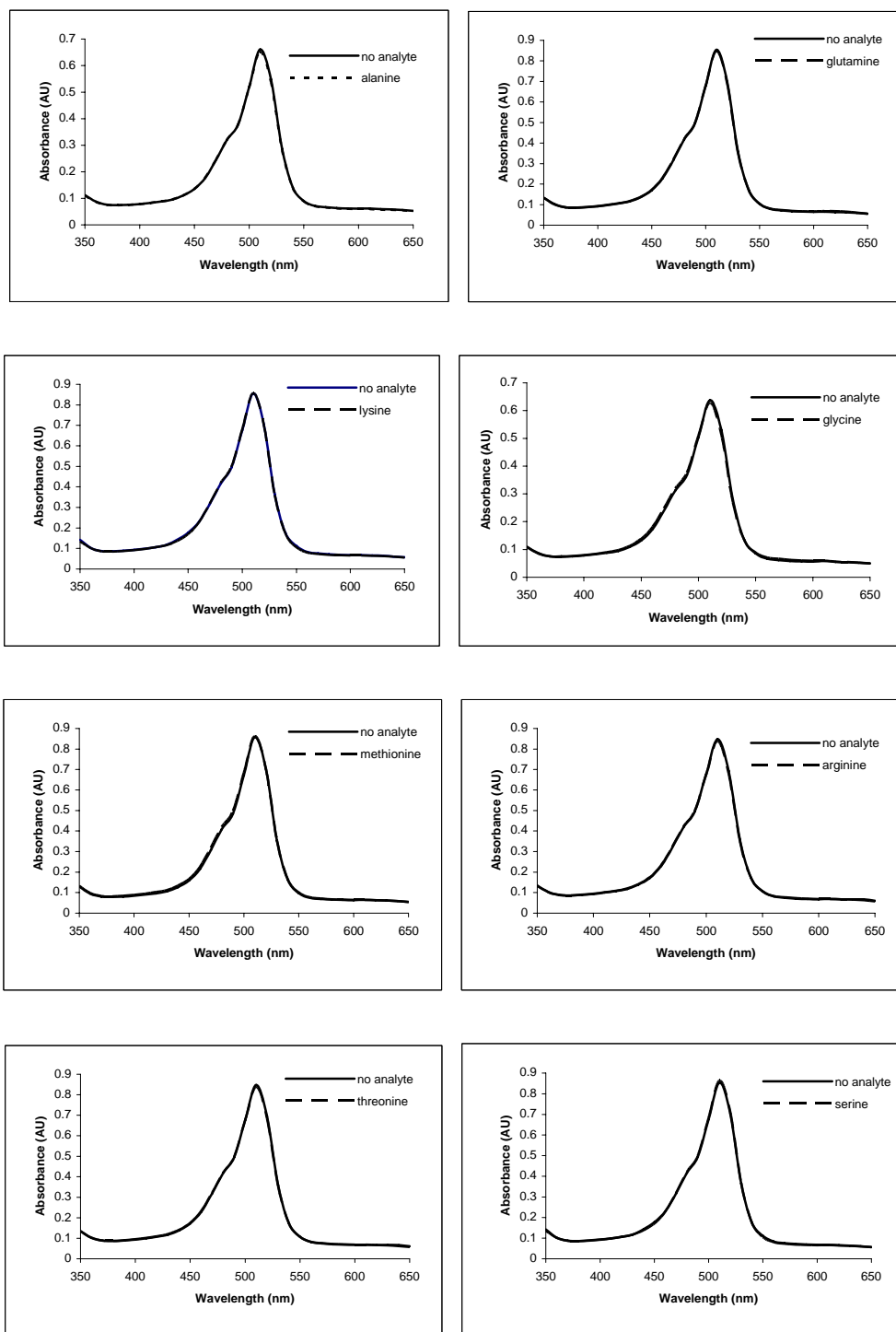


Figure A1. UV-Vis spectra of FB (1.0×10^{-5} M) in 30 % phosphate buffer (0.015 M, pH 7.3), 70 % MeOH in the presence of various amino acids (1.0×10^{-4} M). No absorbance changes are observed due to the presence of the amino acids.

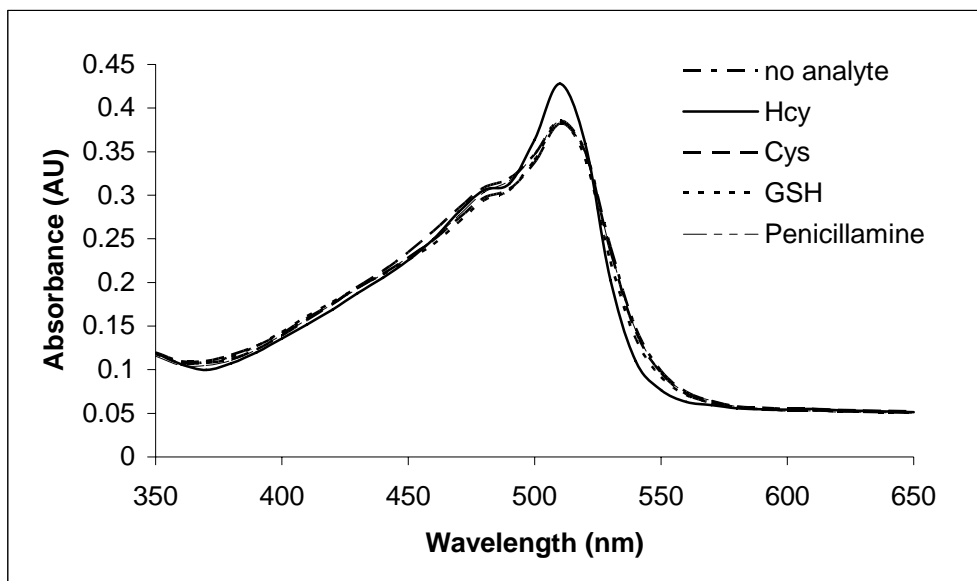


Figure A2. UV-Vis spectra of FB (1.0×10^{-5} M) in 30 % phosphate buffer (0.015 M, pH 7.3) and 70 % MeOH in the presence of various biothiols (1.0×10^{-4} M) and PPh₃ (4.5×10^{-4} M).

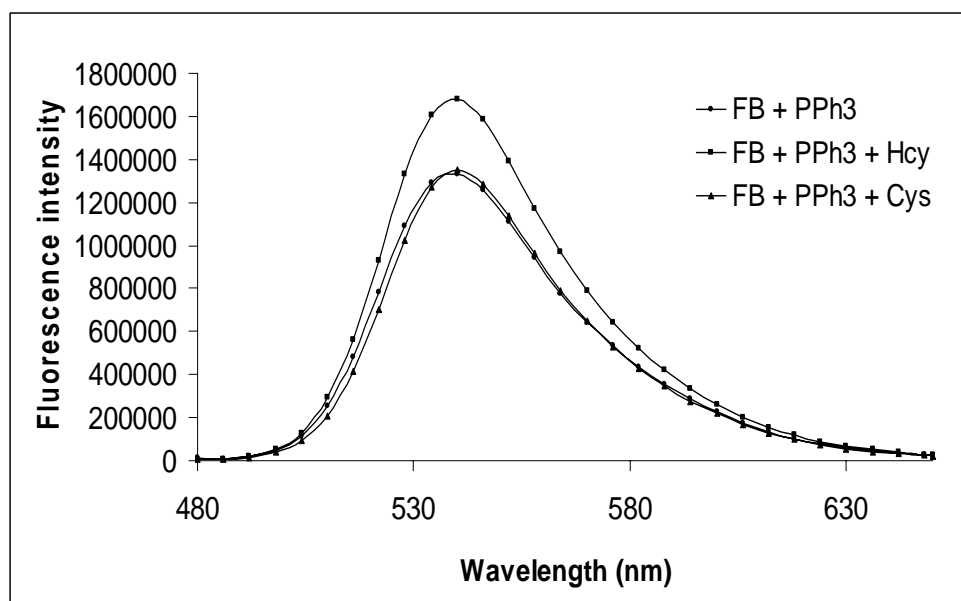


Figure A3. Fluorescence emission spectrum of FB (1.0×10^{-6} M) in the presence of PPh₃ (4.5×10^{-5} M) and Hcy or Cys (1.0×10^{-5} M). This demonstrates selective fluorescence emission responses to the presence of Hcy.

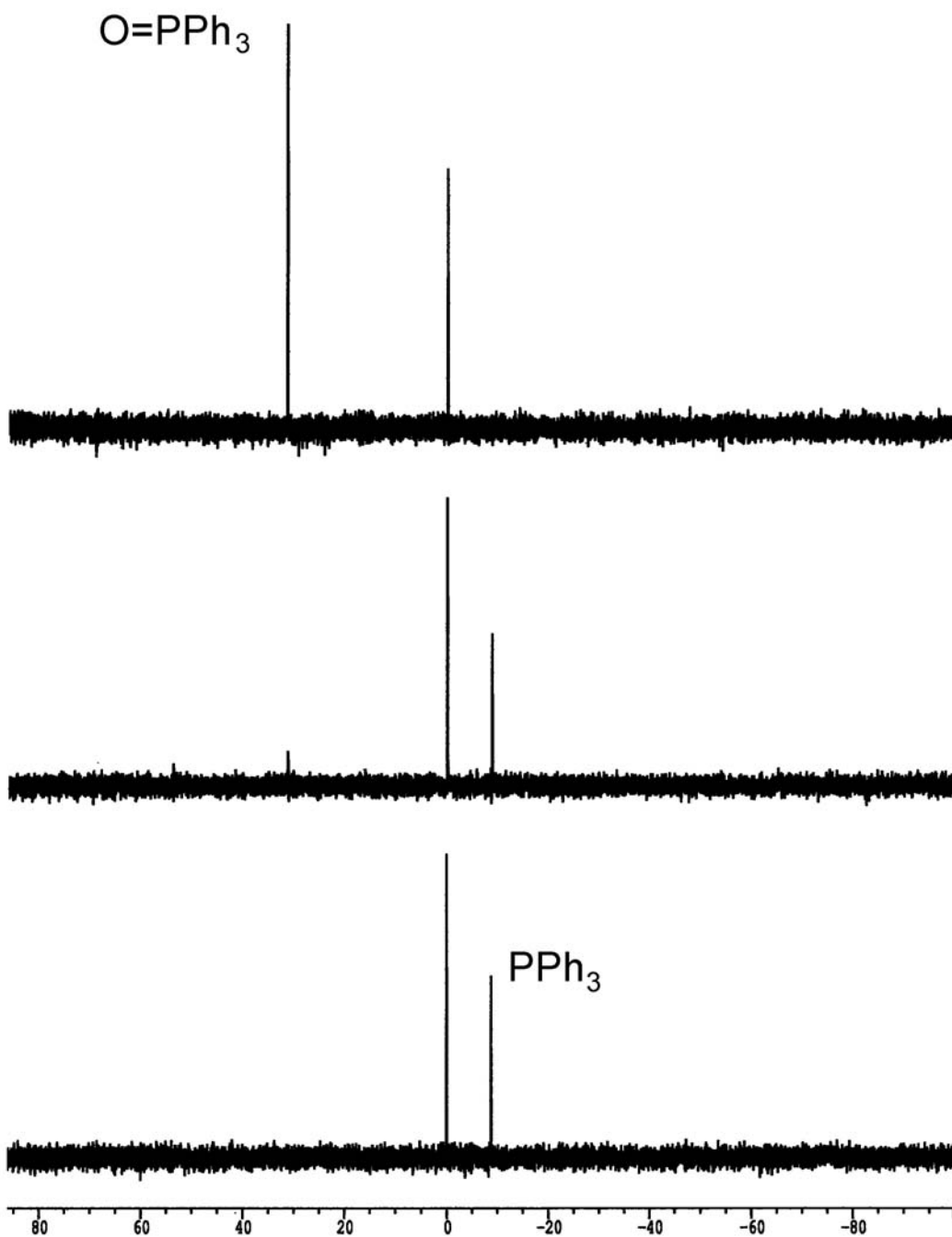


Figure A4. ^{31}P -NMR spectra of PPh_3 in $\text{CD}_3\text{OD}:\text{D}_2\text{O}$ 7:3 in the absence of FB (bottom) and in the presence of FB (middle) after 19 hours. The resonance at 8.80 ppm corresponds to PPh_3 . The resonance at 31.25 ppm corresponds to O=PPh_3 (top). It shows that the formation of O=PPh_3 is promoted in the presence of FB.

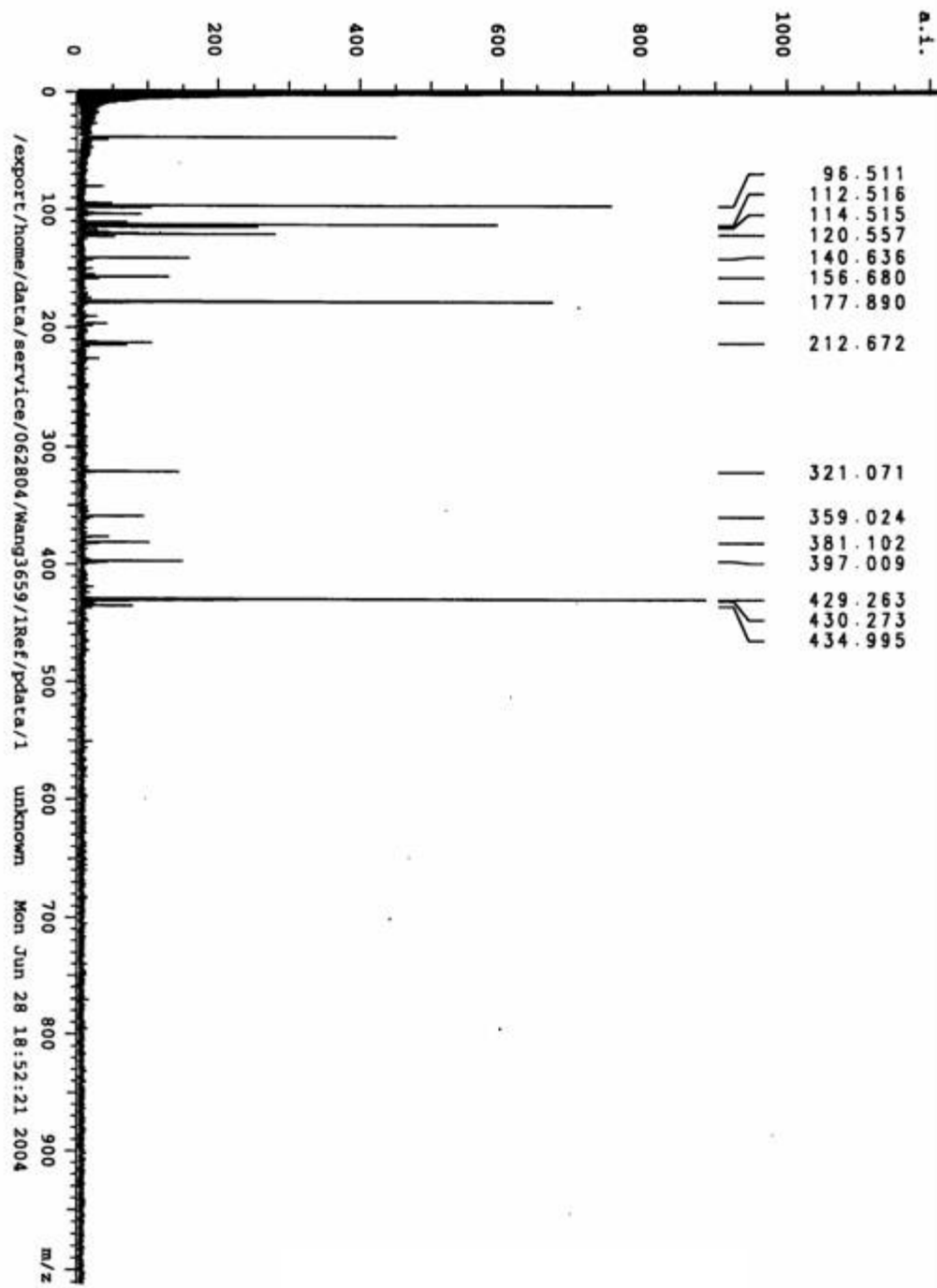


Figure A5. MALDI-TOF of the reaction mixture of FB and Hcy (no matrix)

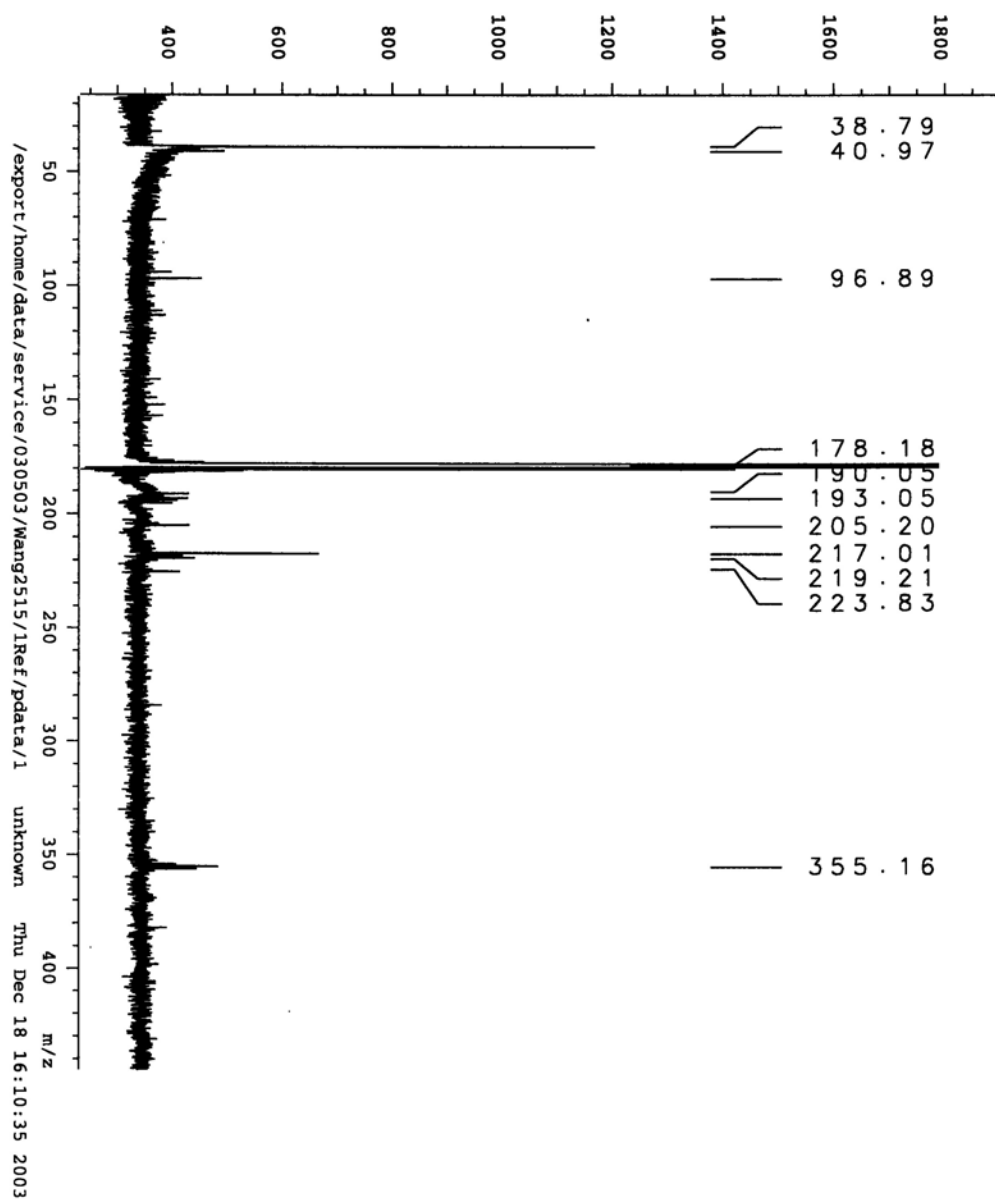


Figure A6. MALDI-TOF of the reaction mixture of FB and Hcy (anthracene matrix)

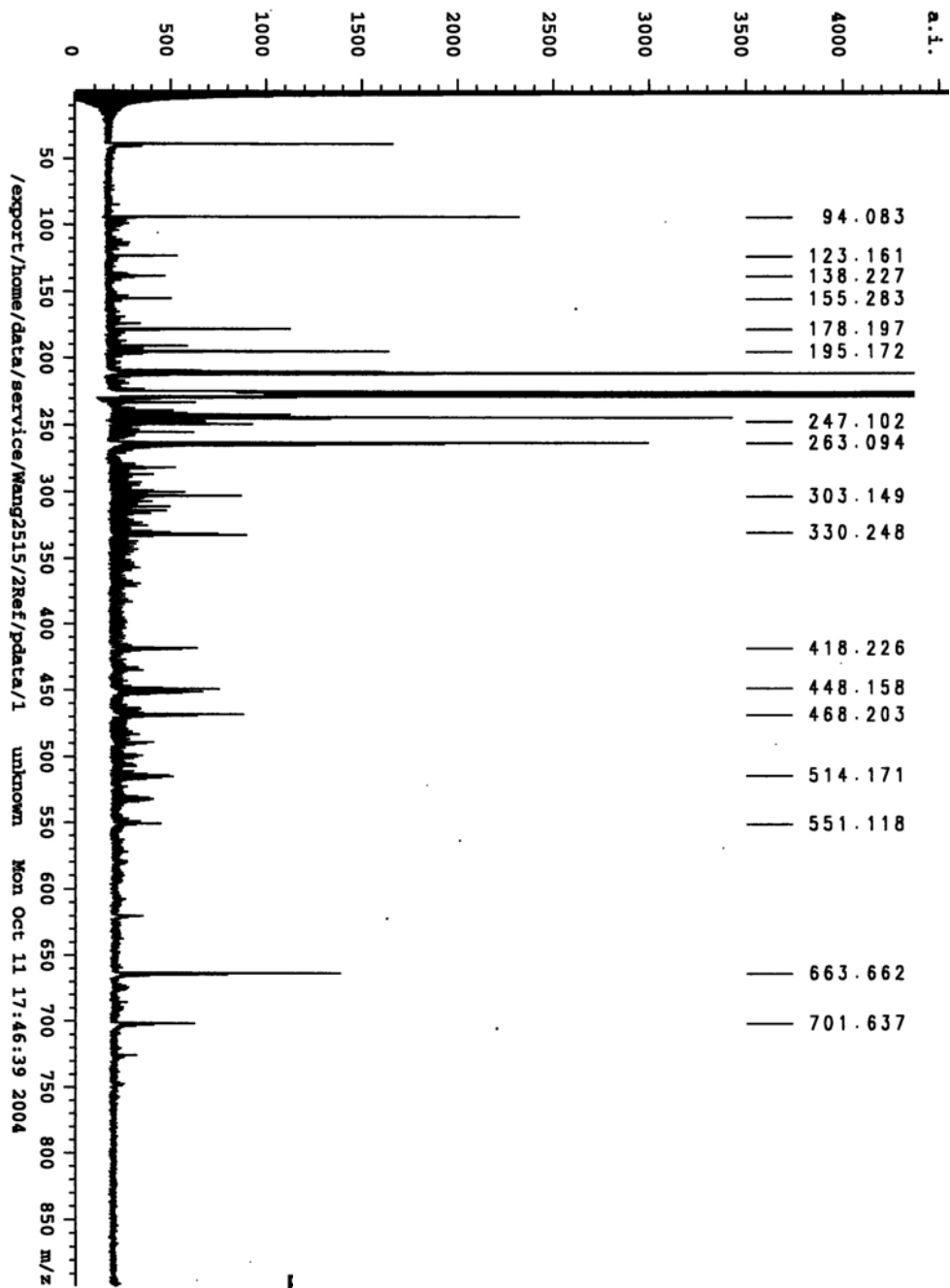


Figure A7. MALDI-TOF of the reaction mixture of FB and Hcy (dithranol matrix)

APPENDIX B. SYNTHESIS OF LANTHANIDE COMPLEXES

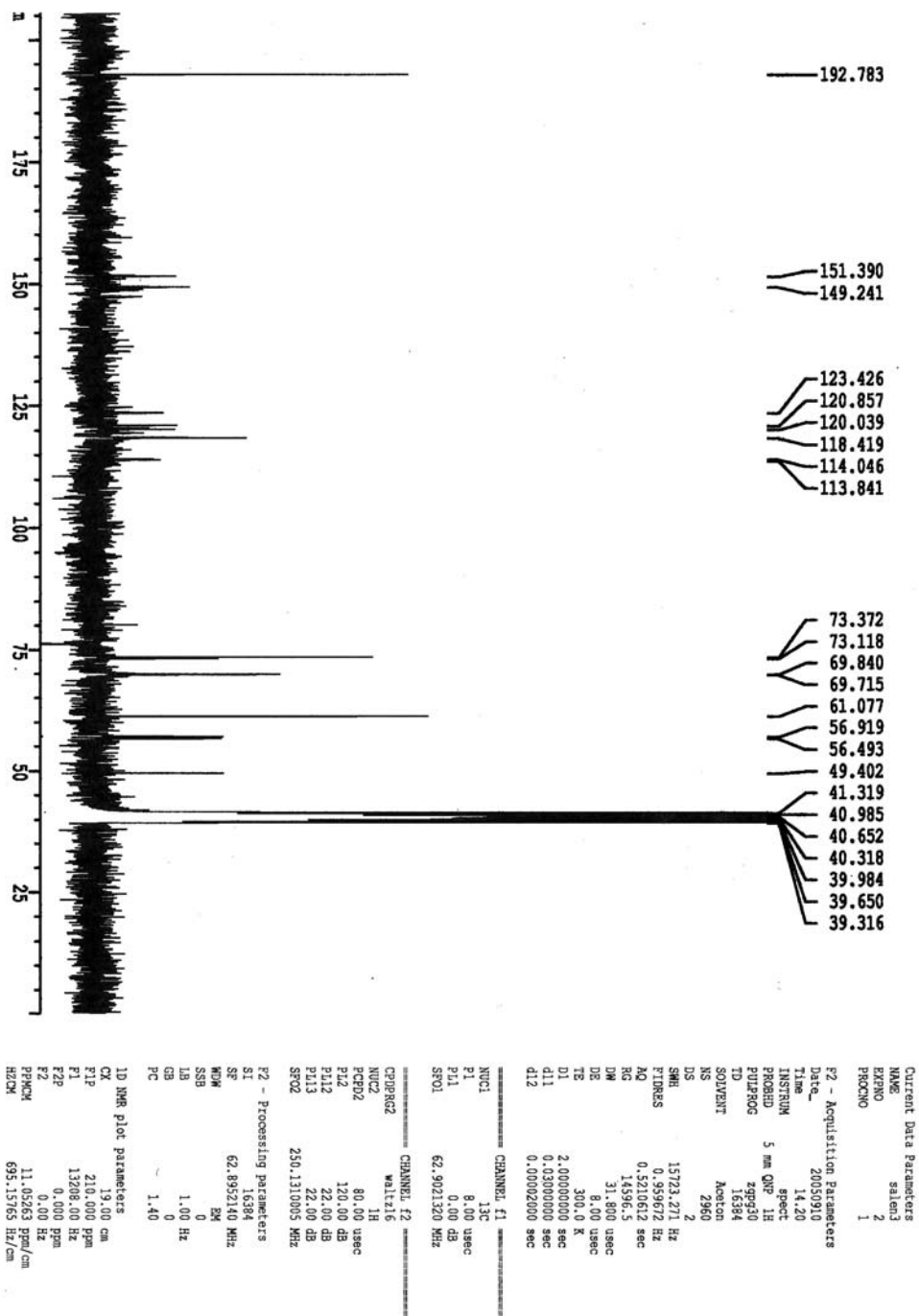


Figure B1. ¹³C-NMR spectrum of **1** in DMSO-*d*₆.

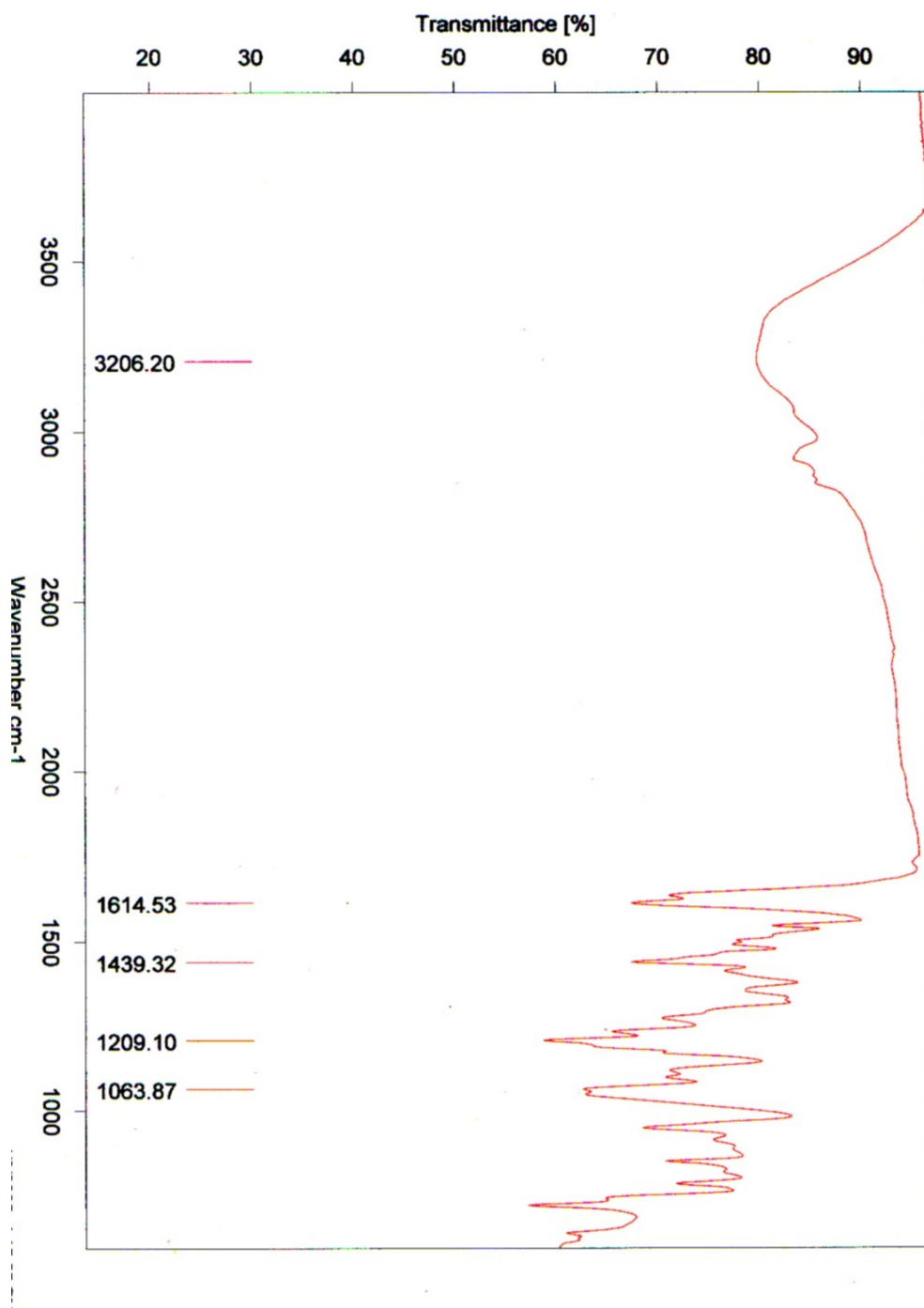


Figure B2. FTIR spectrum of **1**.

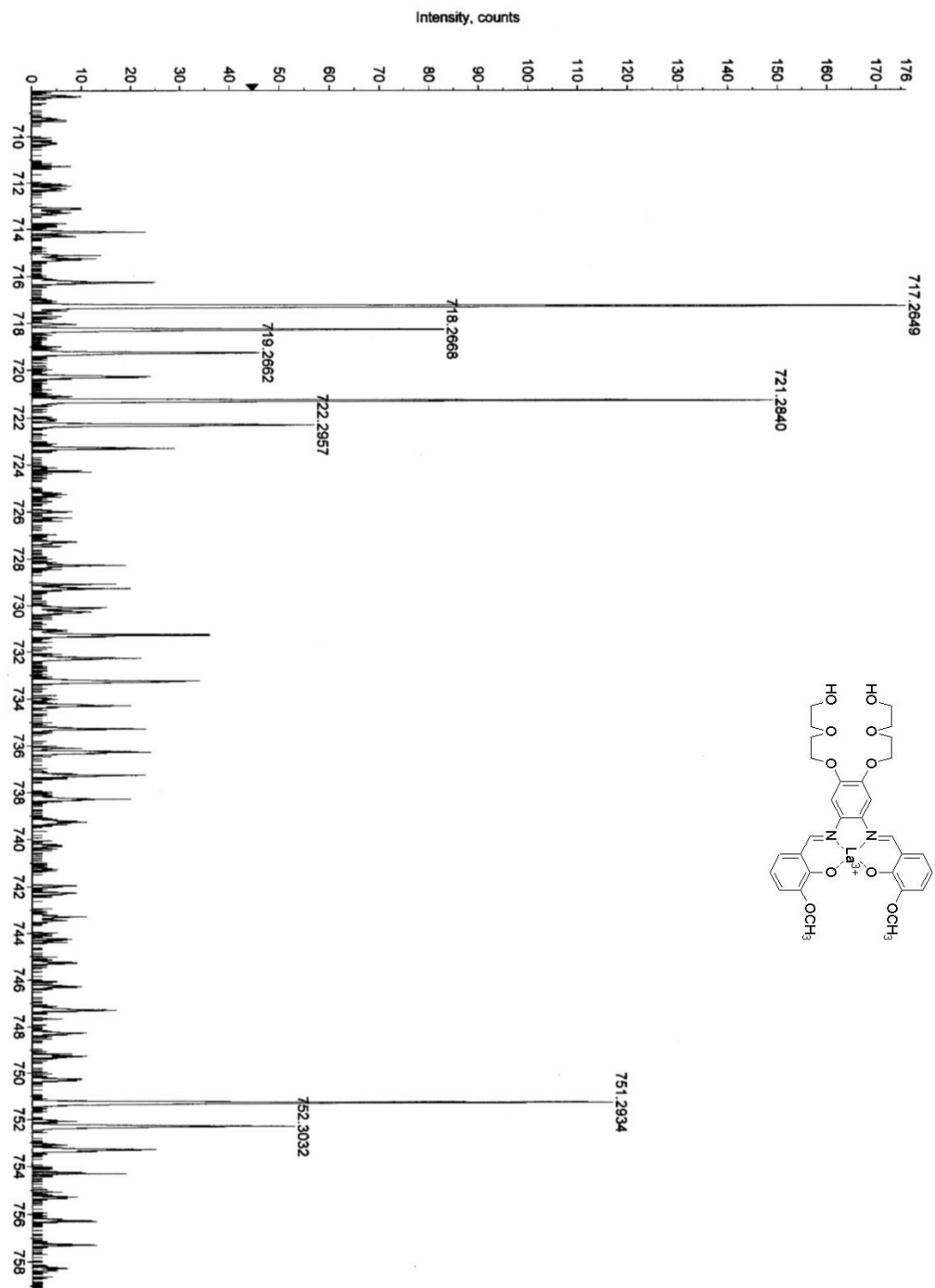


Figure B3. MALDI TOF MS spectrum of 1.

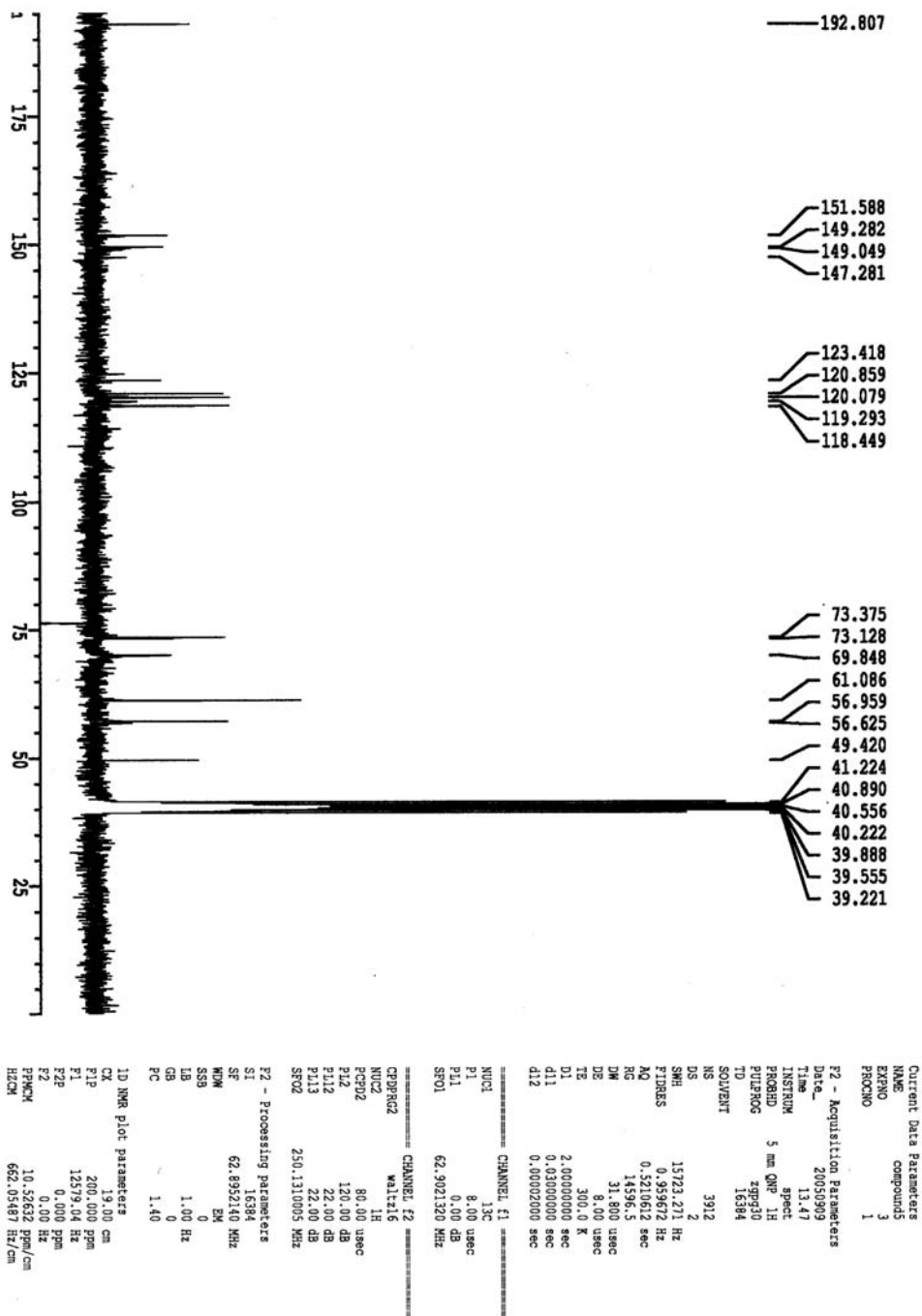


Figure B4. ¹³C-NMR spectrum of **2** in DMSO-*d*₆.

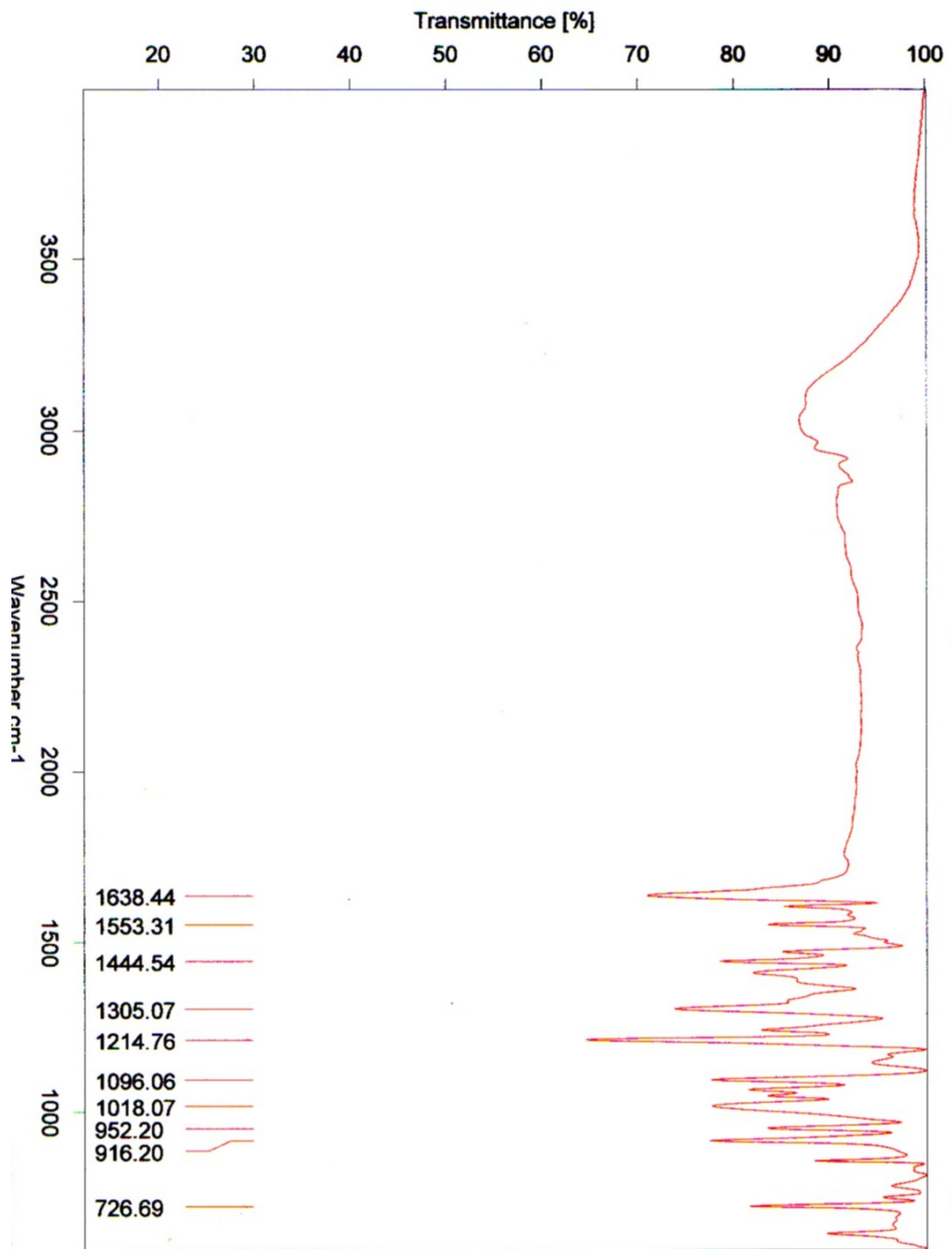


Figure B5. FTIR spectrum of 2.

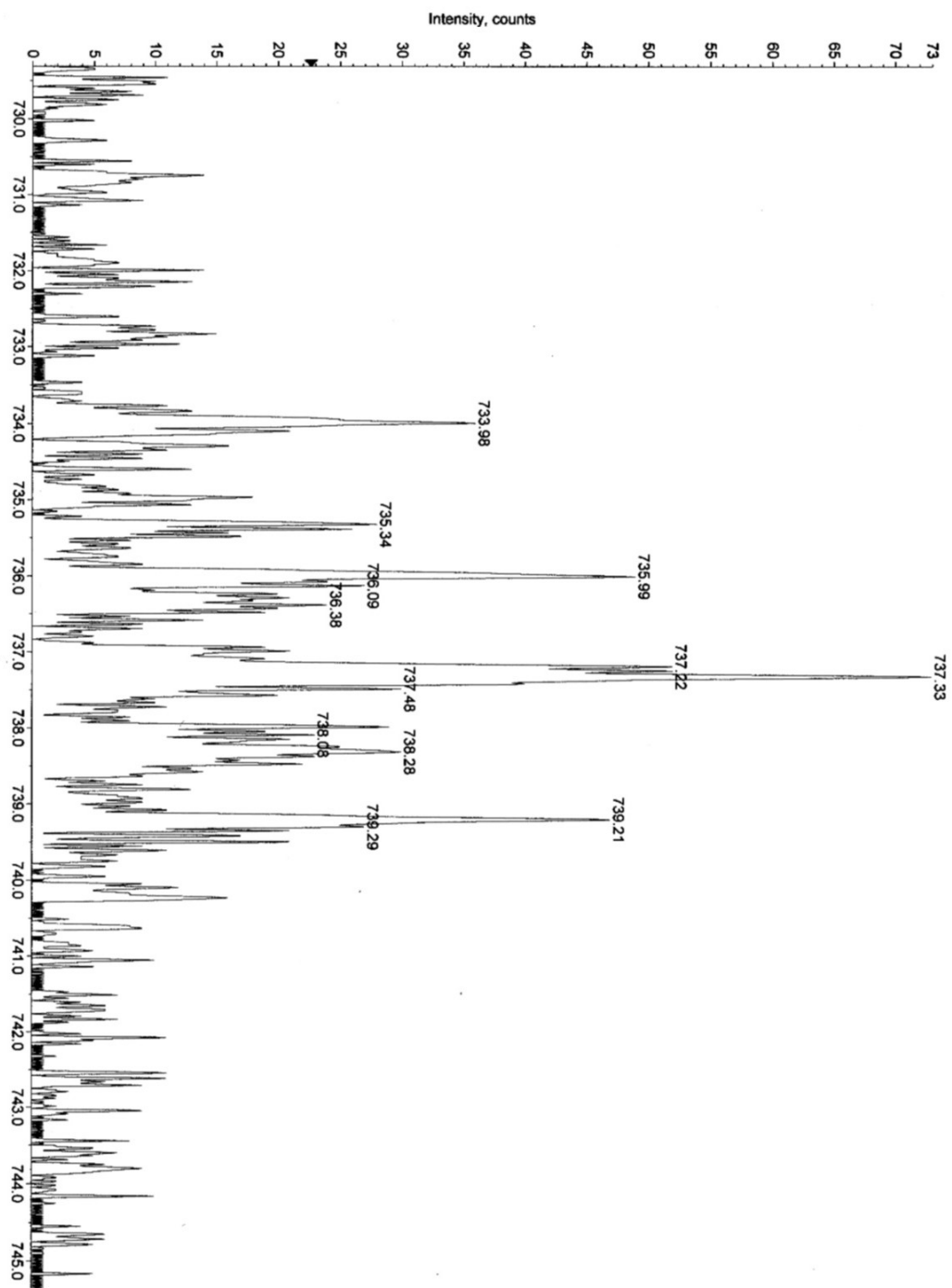
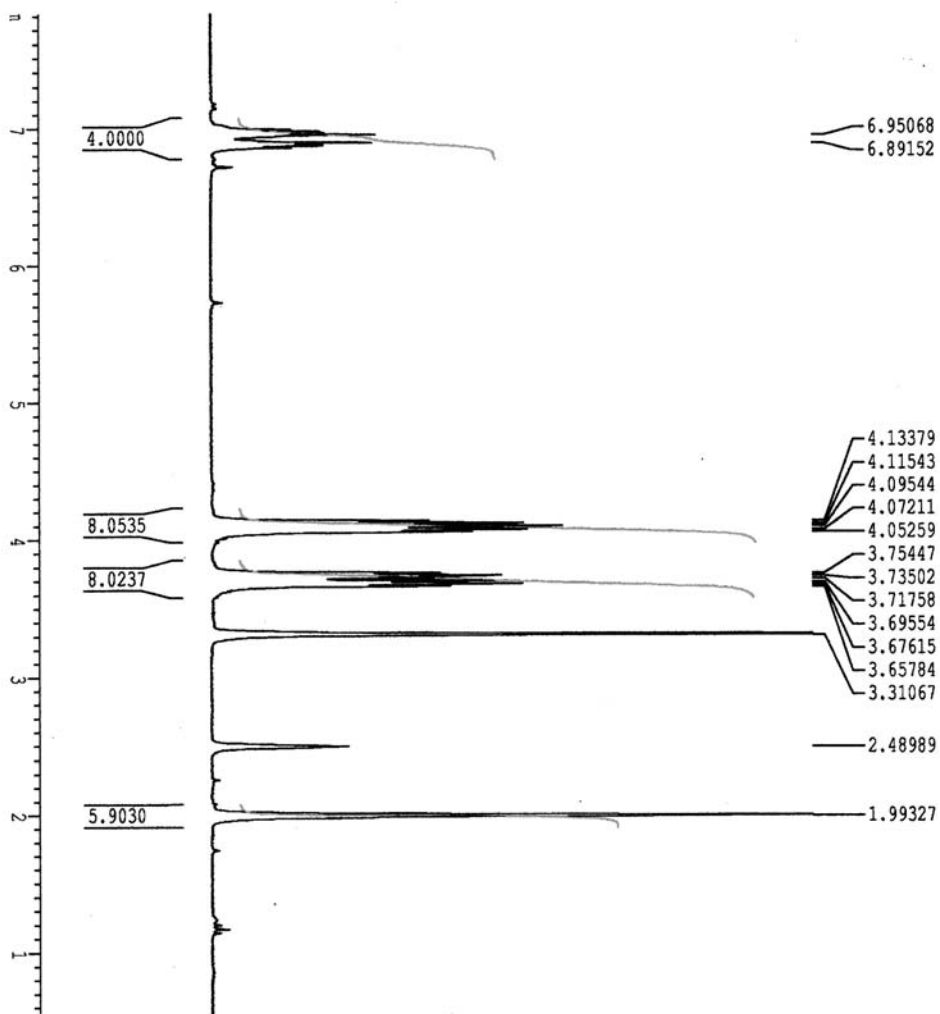


Figure B6. MALDI TOF MS spectrum of **2**.



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PROCNO       1

F2 - Acquisition Parameters
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Time         18.07
INSTRUM      spect
PROBHD       5 mm QNP 1H
PULPROG      zg30
TD           16384
SOLVENT      DMSO
NS           8
DS           2
SMH          3742.515 Hz
FIDRES       0.228425 Hz
AQ           2.1889524 sec
RG           362
DM           133.600 usec
DE           8.00 usec
TE           300.0 K
D1           2.00000000 sec

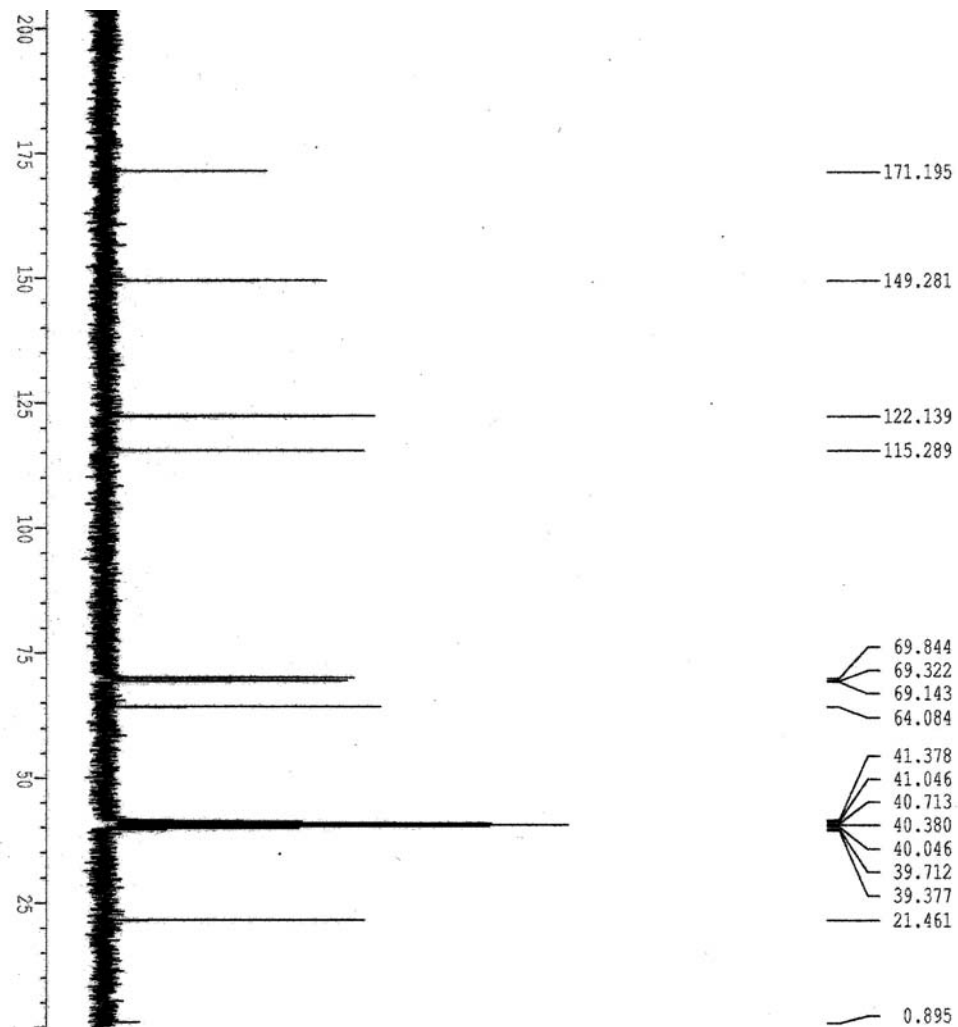
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PLI         0.00 dB
SFO1        250.131306 MHz

F2 - Processing parameters
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SF          250.130050 MHz
WDW         EM
SSB         0
LB          0.15 Hz
GB          0
PC          1.00

1D NMR plot parameters
CX          18.00 cm
F1P         8.000 ppm
F1          2001.04 Hz
F2P         0.500 ppm
F2          125.07 Hz
PPMCM       0.41667 ppm/cm
HZCM       104.22083 Hz/cm

```

Figure B7. ¹H-NMR spectrum of **3** in DMSO-*d*₆.



```

Current Data Parameters
NAME          2mg01-95
EXPNO         4
PROCNO       1

F2 - Acquisition Parameters
Date_         20050801
Time         14.15
INSTRUM      spect
PROBHD       5 mm QNP 1H
PULPROG      zgpg30
TD            65536
SOLVENT      Acetone DMSO
NS           40
DS           2
SMH          15723.271 Hz
FIDRES       0.959672 Hz
AQ           0.5210612 sec
RG           3649.1
DM           31.800 usec
DE           8.00 usec
TE           300.0 K
D1           2.00000000 sec
d11          0.03000000 sec
d12          0.00002000 sec

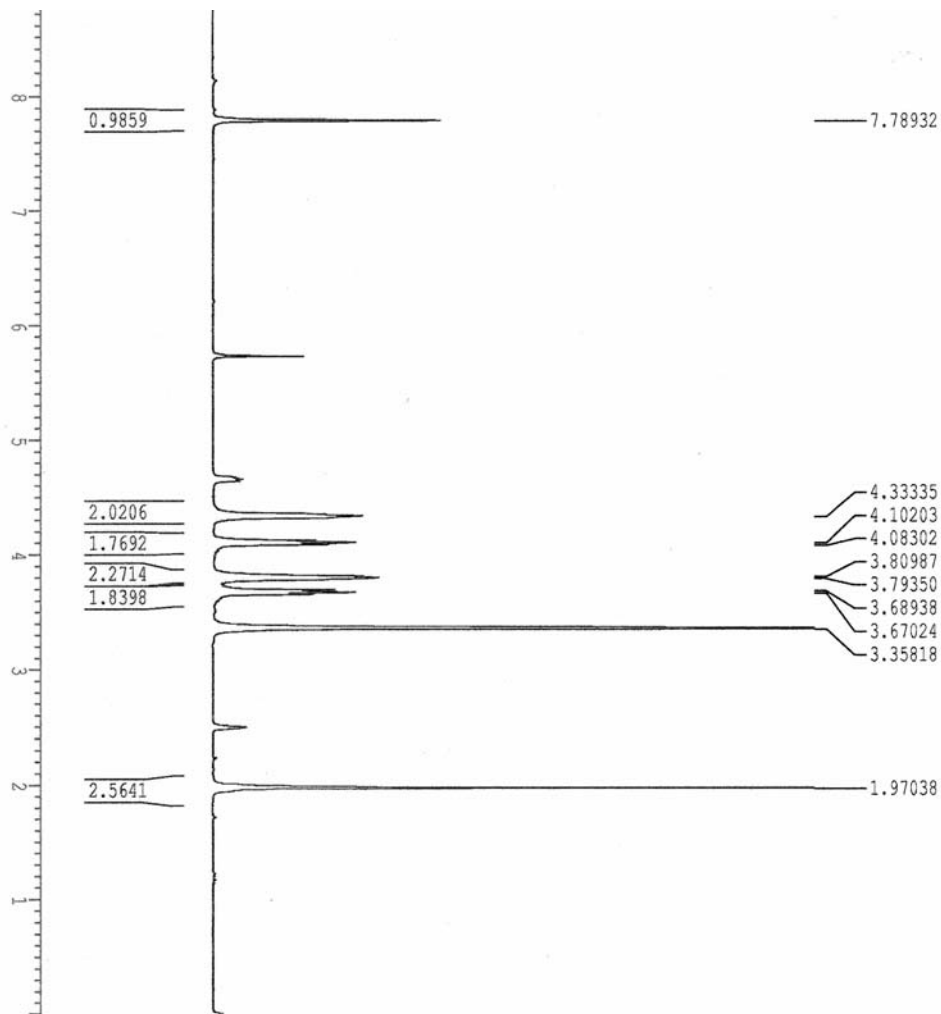
===== CHANNEL f1 =====
NUC1          13C
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PL1          0.00 dB
SFO1         62.9021320 MHz

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CPDPRG2      waltz16
NUC2          1H
PCPD2        80.00 usec
PL2          120.00 dB
PL12         22.00 dB
PL13         22.00 dB
SFO2         250.1310065 MHz

F2 - Processing parameters
SI           16384
SF           62.9392140 MHz
WDW          EM
SSB          0
GB          1.00 Hz
CB           0
PC           1.40

1D NMR plot parameters
CX           19.90 cm
F1P          220.000 ppm
F1           13836.35 Hz
F2P          0.600 ppm
F2           0.80 Hz
PCKM        11.57885 ppm/cm
HRCM        728.26038 Hz/cm
  
```

Figure B8. ^{13}C -NMR spectrum of **3** in $\text{DMSO-}d_6$.



```

Current Data Parameters
NAME      Zhang-96
EXPNO     1
PROCNO    1

F2 - Acquisition Parameters
Date_     20050802
Time      11.58
INSTRUM   spect
PROBHD    5 mm QNP 1H
PULPROG   zg30
TD         16384
SOLVENT   Acetone DMSO
NS         8
DS         2
SWH        3142.515 Hz
FIDRES     0.228425 Hz
AQ         2.1899524 sec
RG         181
DM         133.600 usec
DE         8.00 usec
TE         300.0 K
D1         2.00000000 sec

===== CHANNEL f1 =====
NUC1       1H
P1         10.00 usec
PL1        0.00 dB
SFO1       250.1311306 MHz

F2 - Processing parameters
SI         16384
SF         250.1300050 MHz
WDW        EM
SSB        0
LB         0.15 Hz
GB         0
PC         1.00

ID NMR plot parameters
CX         18.00 cm
FIP        9.000 ppm
F1         2251.17 Hz
F2P        0.000 ppm
F2         0.00 Hz
ZPWCM      0.50000 ppm/cm
HZCM       125.06500 Hz/cm

```

Figure B9. ^1H -NMR spectrum of **4** in $\text{DMSO-}d_6$.

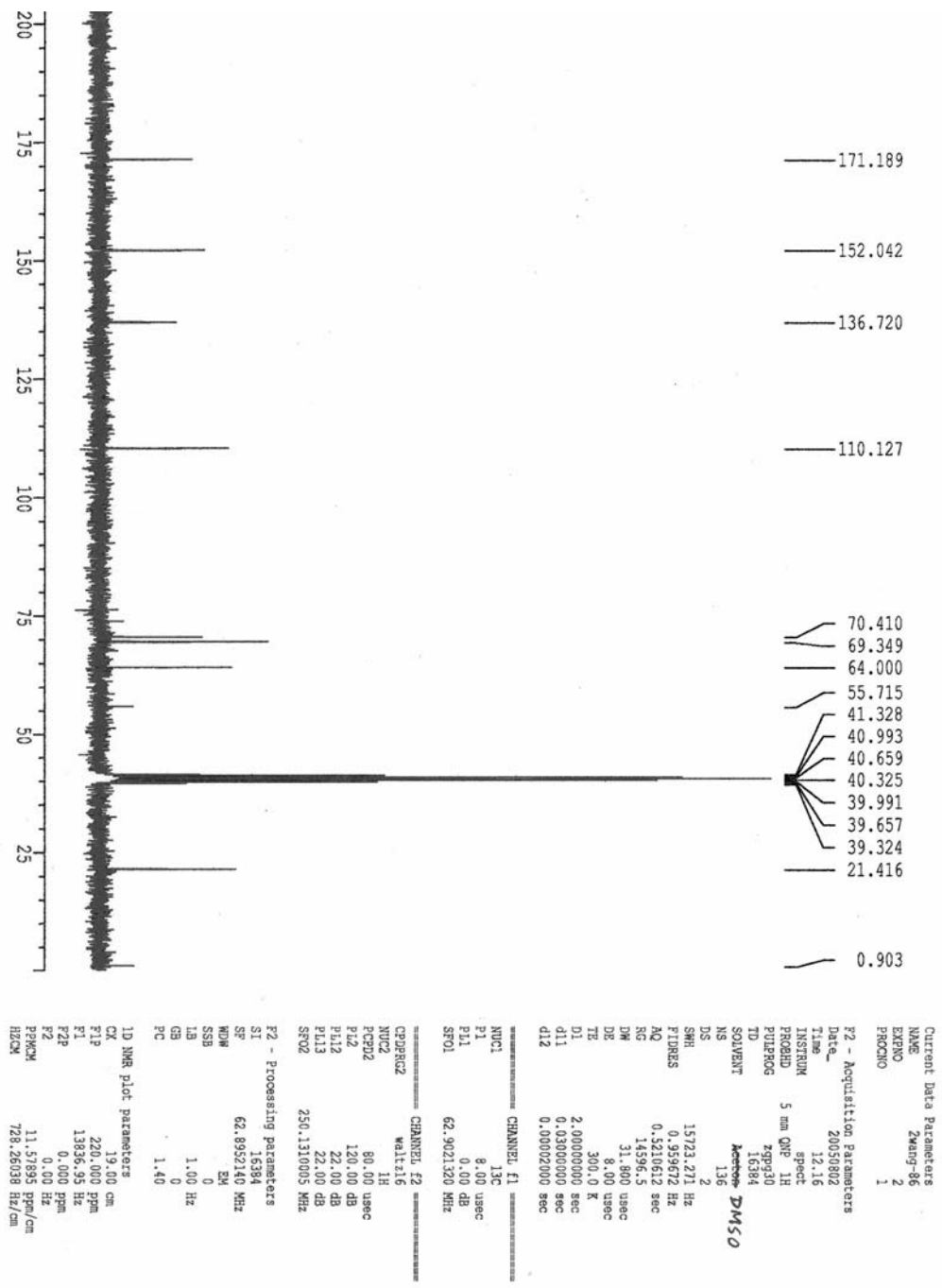
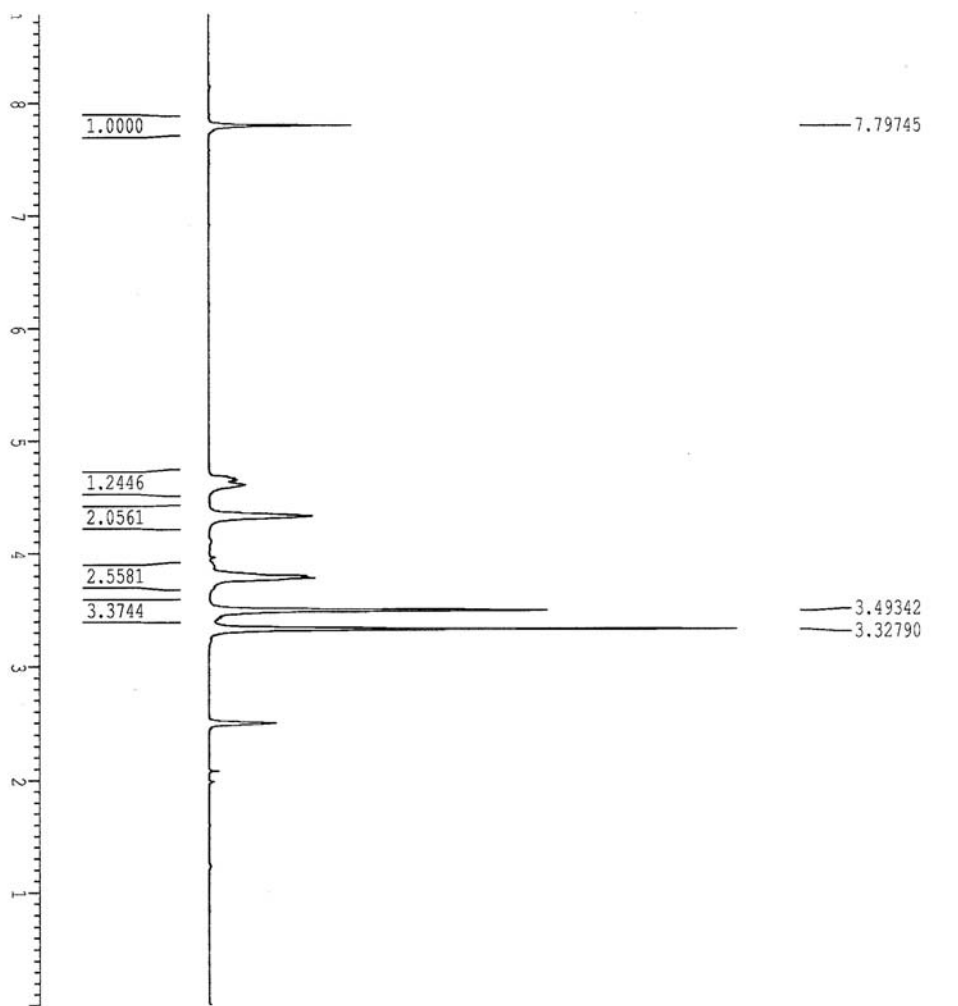


Figure B10. ¹³C-NMR spectrum of **4** in DMSO-*d*₆.



```

Current Data Parameters
NAME      Zhang-89
EXPNO    1
PROCNO   1

F2 - Acquisition Parameters
Date_    20050824
Time     13.06
INSTRUM  spect
PROBHD   5 mm QNP 1H
PULPROG  zg30
TD       65536
FIDRES   0.16384
SOLVENT  Aceton
NS       8
DS       2
SWH      3742.515 Hz
FIDRES   0.228425 Hz
AQ       2.1889524 sec
RG       362
DM       133.600 usec
DE       8.00 usec
TE       300.0 K
D1       2.00000000 sec

===== CHANNEL f1 =====
NUC1     1H
P1       10.00 usec
PL1      0.00 dB
SFO1     250.1311306 MHz

F2 - Processing parameters
SI       16384
SF       250.1300050 MHz
WCOM     EM
SGB      0
LB       0.15 Hz
GB       0
PC       1.00

1D NMR plot parameters
CX       18.00 cm
FIP      9.000 ppm
FI       2251.17 Hz
F2P      0.000 ppm
F2       0.00 Hz
PFGMCM   0.50000 ppm/cm
HZCM     125.06500 Hz/cm

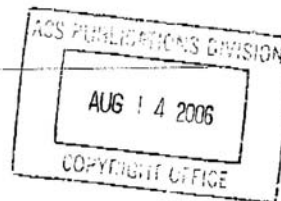
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Figure B11. ¹H-NMR spectrum of **5** in DMSO-*d*₆.

APPENDIX C. LETTERS OF PERMISSION

Arleen Courtney

From: Weihua Wang [wwang4@lsu.edu]
Sent: Friday, August 11, 2006 3:44 PM
To: copyright@acs.org
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- "Direct Detection of Homocysteine," *J. Am. Chem. Soc.*, **2004**, *126*, 3400-3401.
- "Detection of Homocysteine and Cysteine," *J. Am. Chem. Soc.*, **2005**, *127*, 15949-15958.

Thank you very much for your consideration of this request.

Sincerely,

Weihua Wang

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Fax: (225)-578-3458
E-mail: wwang4@lsu.edu



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 Louisiana State University, Baton Rouge, LA

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VITA

Weihua Wang was born in Laizhou, Shandong, the People's Republic of China, on November 29, 1969. In fall 1988, she entered Jiangsu Institute of Chemical Technology, where she received her bachelor's degree in macromolecular materials science and engineering. As an undergraduate student, she received the Scholarship for Outstanding Student consecutively for four years. Between 1992 and 1996, she worked in Yantai Plastics Corporation as an assistant engineer. In fall 1996, she went to Nanjing University of Chemical Technology to pursue her master's degree, majoring in macromolecular materials science and engineering. She was one of a few BASF Outstanding Graduate Student Award winners. After graduation in 1999, she worked in Jiangsu Polytechnic University as a lecturer and undergraduate research advisor. In September 2001, she enrolled in the doctoral program in the Department of Chemistry at Louisiana State University. Since then, she worked as a Graduate Teaching Assistant and Research Assistant under the supervision of Professor Robert M. Strongin. She was awarded Colgate-Palmolive and Departmental Research Scholar in 2004. She has publications involved in the fields of polymer, organic chemistry, analytical chemistry and biochemistry. She is a member of the American Chemical Society and currently a candidate for the Doctor of Philosophy in chemistry.