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Direct detection of homocysteine and syntheses of lanthanide complexes as biosensors

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DIRECT DETECTION OF HOMOCYSTEINE AND SYNTHESES OF LANTHANIDE COMPLEXES AS BIOSENSORS

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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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amu	Atomic Mass Units
ATP	Adenosine tiphosphate
ASFB	4-Aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole
BMF	Bromomethylfluorescein
BSA	Bovine serum albumin
Ca^{2+}	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
e.g.	*exempli gratia* (for example)
Eq.	Equation
EIA	Enzyme immunoassay

ELISA	Enzyme-linked immunosorbent assay
et al.	*et alii* (and others)
EtOAc	Ethyl acetate
Eu^{3+}	Europium ion
$EuCl_3$	Europium chloride
FB	Fluorone black
$FeCl_3$	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
HNO_3	Nitric acid
H_2O	Water
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography

H_2S	Hydrogen sulfide
IAAF	6-Iodoacetamidofluorescein
IR	Infrared
K_2CO_3	Potassium carbonate
KOH	Potassium hydroxide
La^{3+}	Lanthanum ion
$LaCl_3$	Lanthanum chloride
LC	Liquid chromatography
LC-MS	Liquid chromatography – mass spectrometry
MALDI	Matrix-Assisted Laser Desorption/Iionization
mBrB	Monobromobimane
MeOH	Methanol
MHz	Megahertz
min	Minute
Mn^{2+}	Manganese ion
MS	Mass spectrometry
MV^{2+}	Methyl viologen
$MV^{+\cdot}$	Methyl viologen radical cation
m/z	Mass-to-charge ratio in mass spectrometry
NAD^+	Nicotinamide adenine dinucleotide
$NaHCO_3$	Sodium bicarbonate (sodium hydrogen carbonate)
Na_2SO_4	Sodium sulphate
NH_3	Ammonia
nm	Nanometer

NMDA	N-methyl-D-aspartate
NMR	Nuclear Magnetic Resonance
NO	Nitric oxide
OPA	*O*-phthalaldehyde
O=PPh$_3$	Triphenylphosphine oxide
pH	Measure of the acidity of a solution
pKa	Measure of the strength of an acid
PPh$_3$	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
UO$_2$	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 amnino 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, La^{3+} 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.

1.4 References

1.1 Refsum H.; Ueland P. M.; Nygard O.; Vollset S. E. "Homocysteine and cardiovascular disease". *Annu. Rev. Med.*, **1998**, *49*, 31-62.

1.2 Seshadri S.; Beiser A.; Selhub J.; Jacques P. F.; Rosenberg I. H.; D'Agostino R. B.; Wilson P. W. F.; Wolf P. A. "Plasma homocysteine as a risk factor for dementia and Alzheimer's disease". *N. Engl. J. Med.*, **2002**, *346*, 476-483.

1.3 Smithells R. W.; Sheppard S.; Schorah C. J.; Seller M. J.; Nevin N. C.; Harris R.; Read A. P.; Fielding D. W. "Possible prevention of neural-tube defects by periconceptional vitamin supplementation". *Lancet.*, **1980**, *i*, 339-340.

1.4 Eskes T. K. A.B.; Steegers E. A. P.; Merkus H. M. W. M. "Hyperhomocysteinemia in obstetrics and gynecology: A unifying concept". *Neth. J. Med.*, **1998**, *52*, S16-17.

1.5 Wheeler D. C. "Cardiovascular disease in patients with chronic renal failure". *Lancet.*, **1996**, *348*.1673-1674.

1.6 Lentz S. R.; Haynes W. G. *Cleveland Clin. J. Med.*, **2004**, *71*, 730.

1.7 van Veggel F. C. J. M.; Verboom W.; Reinhoudt D. N. "Metallomacrocycles: supramolecular chemistry with hard and soft metal cations in action". *Chem. Rev.*, **1994**, *94*, 279-299.

1.8 Liu G.-D.; Yang X.; Chen Z.-P.; Shen G.-L.; Yu R.-Q. "Interaction of metal complexes of bis(salicylidene)ethylenediamine with DNA". *Anal. Sci.*, **2000**, *16*, 1255-1259.

1.9 Tsukube H.; Shinoda S. "Lanthanide complexes in molecular recognition and chirality sensing of biological substrates". *Chem. Rev.*, **2002**, *102*, 2389-2403.

1.10 Angyal S. J. "Complexes of carbohydrates with metal cations. I. determination of the extent of complexing by NMR spectroscopy". *Aust. J. Chem.*, **1972**, *25*, 1957-1966.

1.11 Sillerud L. O.; Prestegard J. H.; Yu R. K.; Schafer D. E.; Konigsberg W. H. "Assignment of the carbon-13 nuclear magnetic resonance spectrum of aqueous ganglioside GM1 micelles". *Biochemistry*, **1978**, *17*, 2619-2628.

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.

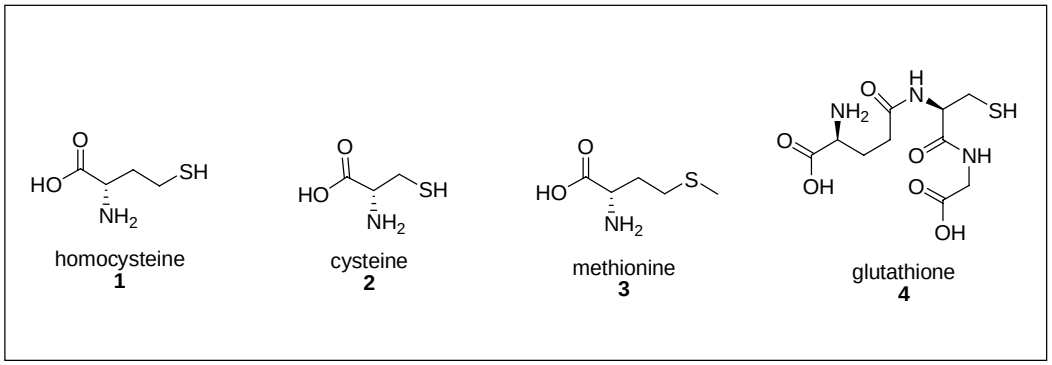


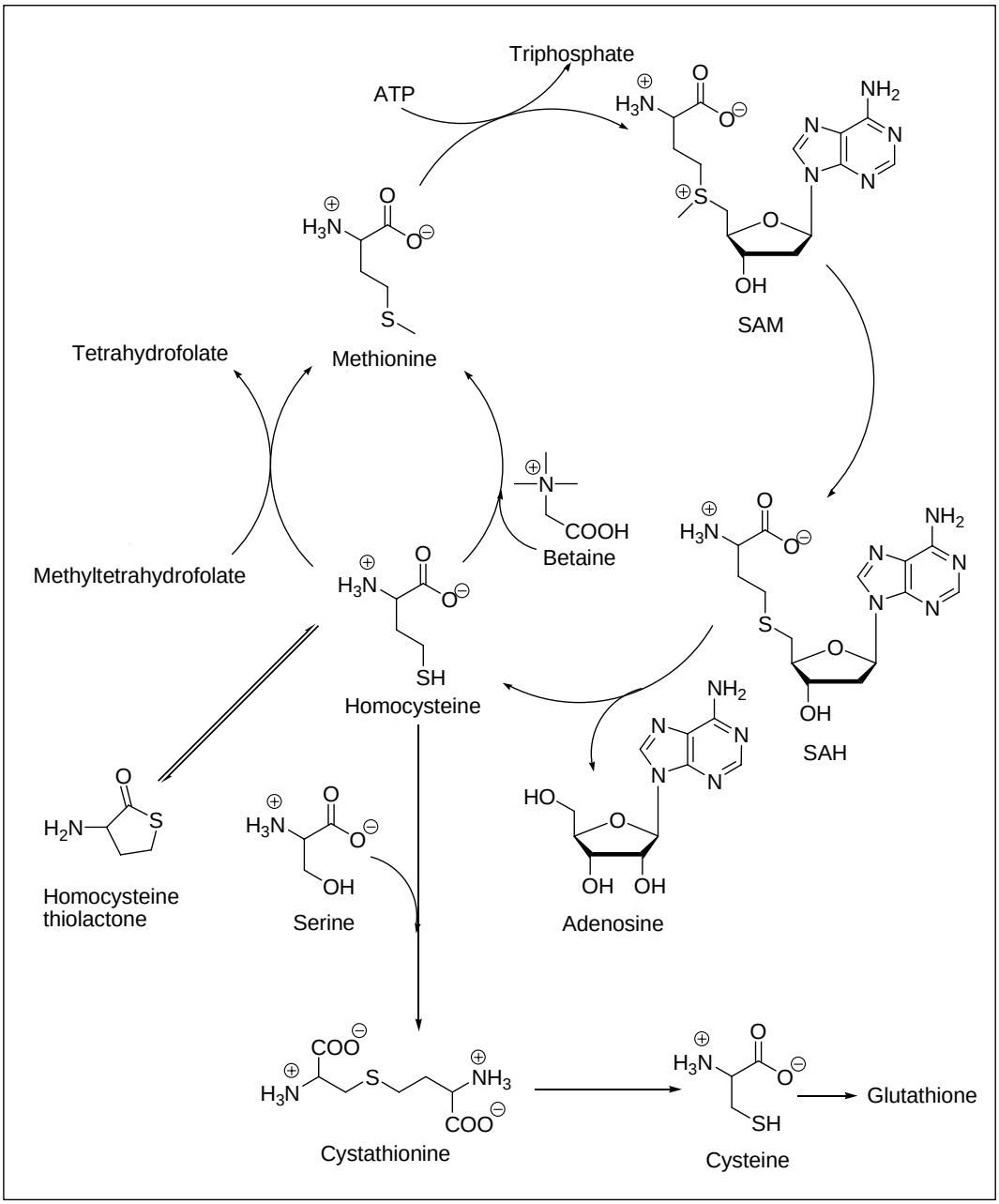
Figure 2.1. Structures of some biological thiols

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 B_6-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 O_2 giving rise to the formation of hydrogen peroxide (H_2O_2), 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. H_2O_2) 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.

2.9 References

2.1 Butz LW; du Vigneaud V. "The formation of a homologue of cystine by the decomposition of methionine with sulfuric acid". *J. Biol. Chem.*, **1932**, *99*, 135-142.

2.2 Carson NAJ; Cusworth DC; Dent CE; Field CMB; Neill DW; Westall RG. "Homocystinuria: A new inborn error of metabolism associated with mental deficiency". *Arch. Dis. Child*, **1963**, *38*, 425-436.

2.3 Cantoni G. L. "S-adenosylmethionine: A new intermediate form enzymatically from L-methionine and adenosine triphosphate". *J. Biol. Chem.*, **1953**, *204*, 403-416.

2.4 Finkelstein, J. D. "The metabolism of homocysteine: pathways and regulation". *Eur. J. Pediatr.*, **1998**, *157*, *Suppl. 2*, S40-44.

2.5 Cantoni, G. L. "Biological methylation: selected aspects". *Annu. Rev. Biochem.*, **1975**, *44*, 435-451.

2.6 Finkelstein, J. D. "Methionine metabolism in mammals". *J. Nutr. Biochem.*, **1990**, *1*, 228-237.

2.7 Mudd S. H.; Finkelstein J. D.; Irreverre F.; Laster L. "Transsulfuration in mammals: Microassays and tissue distribution of three enzymes of the pathway". *J. Biol. Chem.*, **1965**, *240*, 4382-4392.

2.8 Selhub J. "Homocysteine Metabolism". *Annu. Rev. Nutr.*, **1999**, *19*, 217-246.

2.9 Rao A. M.; Drake M. R.; Stipanuk M. H. "Role of the transsulfuration pathway and of γ-cystathionase activity in the formation of cysteine and sulfate from methionine in rat hepatocytes". *J. Nutr.*, **1990**, *120*, 837-845.

2.10 Sturman J. A.; Rassin D. K.; Gaull G. E. "Distribution of transsulphuration enzymes in various organs and species". *Int. J. Biochem.*, **1970**, *1*, 251-253.

2.11 Jakubowski, H. "Metabolism of homocysteine thiolactone in human cell cultures". *J. Biol. Chem.*, **1997**, *272*, 1935-1942.

2.12 Refsum H.; Helland S.; Ueland P. M. "Radioenzymic determination of homocysteine in plasma and urine". *Clin. Chem.*, **1985**, *31*, 624-628.

2.13 Araki A.; Sako Y. "Determination of free and total homocysteine in human plasma by high-performance liquid chromatography with fluorescence detection". *J. Chromatogr. Biomed. Sci. App.*, **1987**, *422*, 43-52.

2.14 Pasas S. A.; Lacher N. A.; Davies M. I.; Lunte S. M. "Detection of homocysteine by conventional and microchip capillary electrophoresis/electrochemistry". *Electrophoresis*, **2002**, *23*, 759-766.

2.15 Sengupta S.; Wehbe C.; Majors A. K.; Ketterer M. E.; DiBello P. M.; Jacobsen D. W. "Relative roles of albumin and ceruloplasmin in the formation of homocystine, homocysteine-cysteine-mixed disulfide, and cystine in circulation". *J. Biol.Chem.*, **2001**, *276*, 46896-46904.

2.16 Jacobsen, D. W. "Homocysteine-to Test and to Treat", DPC Technical Report. **2001**.

2.17 Medina M. A.; Urdiales J. L.; Amores-Sánchez M. I. "Roles of homocysteine in cell metabolism". *Eur. J. Biochem.*, **2001**, *268*, 3871-3874.

2.18 Rasmussen K.; Moller J. "Total homocysteine measurement in clinical practice". *Ann. Clin. Biochem.*, **2000**, *37*, 627-648.

2.19 Refsum H.; Ueland P. M.; Nygard O.; Vollset S. E. "Homocysteine and cardiovascular disease". *Annu. Rev. Med.*, **1998**, *49*, 31-62.

2.20 Seshadri S.; Beiser A.; Selhub J.; Jacques P. F.; Rosenberg I. H.; D'Agostino R. B.; Wilson P. W. F. Wolf P. A. "Plasma homocysteine as a risk factor for dementia and Alzheimer's disease". *N. Engl. J. Med.*, **2002**, *346*, 476-483.

2.21 Smithells R. W.; Sheppard S.; Schorah C. J.; Seller M. J.; Nevin N. C.; Harris R.; Read A. P.; Fielding D. W. "Possible prevention of neural-tube defects by periconceptional vitamin supplementation". *Lancet.*, **1980**, *i*, 339-340.

2.22 Eskes T. K. A.B.; Steegers E. A. P.; Merkus H. M. W. M. "Hyperhomocysteinemia in obstetrics and gynecology: A unifying concept". *Neth. J. Med.*, **1998**, *52*, S16-17.

2.23 Wheeler D. C. "Cardiovascular disease in patients with chronic renal failure". *Lancet.*, **1996**, *348*.1673-1674.

2.24 Clarke R.; Smith A. D.; Jobst K. A.; Refsum H.; Sutton L.; Ueland P. M. "Folate, vitamin B_{12}, and serum total homocysteine levels in confirmed Alzheimer's disease". *Arch. Neurol.*, **1998**, *55*, 1449-1455.

2.25 Lipton S. A.; Kim W. K.; Choi Y. B. Kumar S.; D'Emilia D. M.; Rayudu P. V.; Amelle D. R.; Stamler J. S. "Neurotoxicity associated with dual actions of homocysteine at the N-methyl-D-aspartate receptor". *Proc. Natl. Acad. Sci.*, **1997**, *94*, 5923-5928.

2.26 Boushey C. J.; Beresford S. A. A.; Omenn G. S.; Motulsky A. G. "A quantitative assessment of plasma homocysteine as a risk factor for vascular disease: Probable benefits of increasing folic acid intakes". *JAMA.*, **1995**, *274*, 1049-1057.

2.27 Kilmer S.; McCully M. D. "Homocysteine, vitamins, and prevention of vascular disease". *Military Medicine*, **2004**, *169*, 325-329.

2.28 Refsum H.; Ueland P. M.; Nygard O.; Vollset S. E. "Homocysteine and cardiovascular disease". *Annu. Rev. Med.*, **1998**, *49*, 31-62.

2.29 Wlcken D. E. L.; Gupta V. J.; Reddy S. G. "Accumulation of sulphur-containing amino acids including cysteine-homocysteine in patients on maintenance haemodialysis". *Clin. Sci.*, **1980**, *58*, 427-430.

2.30 Wlcken D. E. L.; Gupta V. J.; Reddy S. G. "Accumulation of sulphur-containing amino acids in chronic renal failure with particular reference to homocystine and cysteine-homocysteine mixed disulphide". *Eur. J. Clin. Invest.*, **1979**, *9*, 301-307.

2.31 Rosenquist T. H.; Ratashak S. A.; Selhub J. "Homocysteine induces congenital defects of the heart and neural tube: effect of folic acid". *Proc. Natl. Acad. Sci.*, **1996**, *93*, 15227-15232.

2.32 Steegers-Theunissen R.; Boers G.; Trijbels F. J.; Eskes T. K. "Neural-tube defects and derangement of homocysteine metabolism". *N. Engl. J. Med.*, **1991**, *324*, 199-200.

2.33 Kang S. S.; Zhou J.; Wong P. W. K.; Kowalisyn J.; Strokosch G. "Intermediate homocysteinemia: a thermolabile variant of methylenetetrahydrofolate reductase". *Am. J. Hum. Genet.*, **1988**, *43*, 414-421.

2.34 Dalery K.; Lussier-Cacan S.; Selhub J.; Davignon J.; Latour Y.; Genest J. "Homocysteine and coronary artery disease in French Canadian subjects: relationship with vitamin B12, B6, pyridoxal phosphate and folate". *Am. J. Cardiol.*, **1995**, *75*, 1107-1111.

2.35 Franken D. G.; Boers G. H. J.; Blom H. J.; Trijbels F. J. M. "Effect of various regimens of vitamins B-6 and folic acid on mild hyperhomocysteinemia in vascular patients". *J. Inherit. Metab. Dis.*, **1994**, *17*, 159-162.

2.36 Naurath H. J.; Joosten E.; Riezler R.; Stabler S. P.; Allen R. H.; Lindenbaum J. "Effects of vitamin B12, folate, and vitamin B6 supplements in elderly people with normal serum vitamin concentrations". *Lancet.*, **1995**, *346*, 858-859.

2.37 Pancharunity N.; Lewis C. A.; Sauberlich H. E.; Perkins L. L.; Go R. C. Alvarez J. O. Macaluso M.; Acton R. T.; Copeland R. B.; Cousins A. L. "+ Plasma homocysteine, folate and vitamin B-12 concentrations and the risk for early-onset coronary artery disease". *Am. J. Clin. Nutr.*, **1994**, *59*, 940-948.

2.38 Ubbink J. B.; Vermaak W. J. H.; van der Merwe A.; Becker P. J. "Vitamin B12, Vitamin B6, and folate nutritional status in men with hyperhomocysteinemia". *Am. J. Clin. Nutr.*, **1993**, *57*, 47-53.

2.39 Selhub J.; Jacques P. F.; Wilson P. W. F.; Rush D.; Rosenberg I. H. "Vitamin status and intake as primary determinants of homocysteinemia in the elderly". *JAMA*, **1993**,*270*, 2693-2698.

2.40 Andus, T.; Mollers, C.; Geissler, A.; Vogl, D.; Gross, V.; Schmitz, G.; Hermann, W.; Scholmerich, J. *Gastroenterology, Suppl. S,* **1996,** *110* A855.

2.41 van Meurs, J. B. J; Dhonukshe-Rutten, R. A. M.; Pluijm, S. M. F.; van der Klift, M.; de Jonge, R.; Lindemans, J.; de Groot, L. C. P. G. M.; Hofman, A.; Witteman, J. C. M.; van Leeuwen, J. P. T. M.; Breteler, M. M. B.; Lips, P.; Pols, H. A. P.; Uitterlinden, A. G. "Homocysteine levels and the risk of osteoporotic fracture". *N. Engl. J. Med*, **2004,** *350,* 2033-2041.

2.42 Bienvenu, T.; Ankri, A.; Chadefaux, B.; Kamoun, P. "Plasma homocysteine assay in the exploration of thromobosis in young subjects". *Presse Medicale*, **1991,** *20*, 985-988.

2.43 Fang Y. Z.; Yang S.; Wu G. "Free radicals, antioxidants and nutrition". *Nutrition*, **2002**, *18*, 872-879.

2.44 Lei X. G. "In vivo antioxidant role of glutathione peroxidase: evidence from knockout mice". *Methods Enzymol.*, **2002**, *347*, 213-225.

2.45 Lang D.; Kredan M. B.; Moat S. J.; Hussain S. A.; Powell C. A.; Bellamy M. F.; Powers H. J.; Lewis M. J. "Homocysteine induced inhibition of endothelium dependent relaxation in rabbit aorta: role of superoxide anions". *Arterioscler. Thromb. Vasc. Biol.*, **2000**, *20*, 422-427.

2.46 Zhang X.; Li H.; Jin H.; Ebin Z.; Brodsky S.; Goligorsky M. S. "Effects of homocysteine on endothelial nitric oxide production". *Am. J. Physiol., Renal. Physiol.*, **2000**, *279*, F671-678.

2.47 Heydrick S. J.; Weiss N.; Thomas S. R.; Cap A. P.; Pimentel D. R.; Loscalzo J.; Keaney J. F. Jr. "L-homocysteine and L-homocystine stereospecifically induce endothelial nitric oxide synthase-dependent lipid peroxidation in endothelial cells". *Free Radic. Biol. Med.*, **2004**, *36*, 632-640.

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-[^{14}C] adenosylhomocysteine. Quantification of S-[^{14}C] 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_2O; (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.
o-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

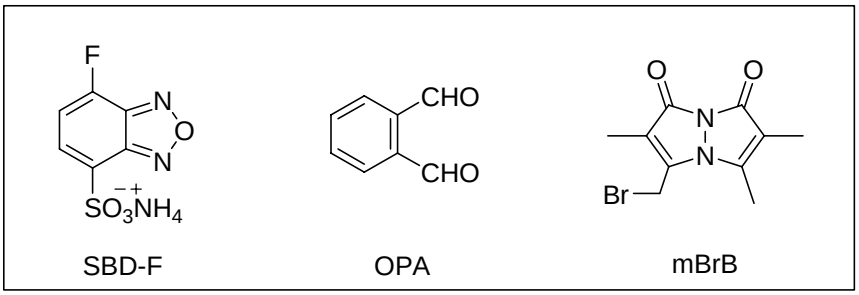
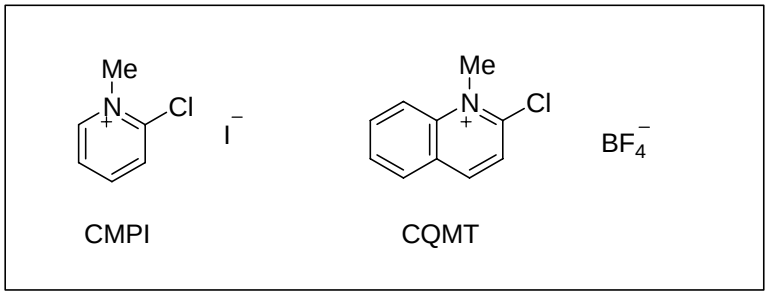


Figure 3.1. Examples of fluorescent derivatizing agents

- **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]



Fgure 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 silver[28] have been exploited. To improve poor voltammetric responses with these common substrates, the use of bismuth-doped lead dioxide[29] and boron doped diamond electrodes[30] 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 pyrroloquinoline quinone shows potential.[45]

- **CE with Fluorescence Detection**

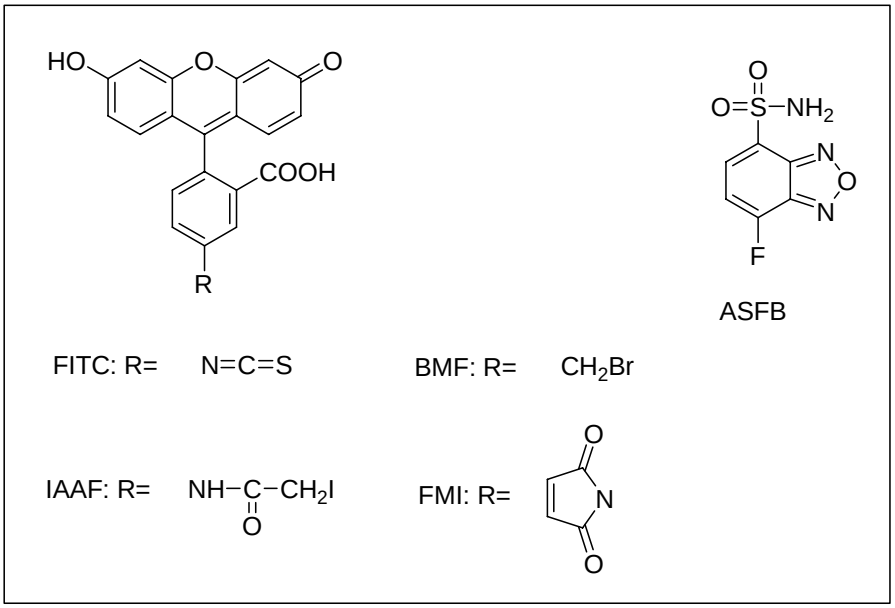


Figure 3.3. Commonly used fluorophores for CE

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 (ASFB),[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 intrapatient 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.

3.8 References

3.1 Ueland P.; Refsum H.; Stabler S.; Malinow M.; Andersson A.; Allen R. "Total homocysteine in plasma or serum: methods and clinical applications". *Clin. Chem.*, **1993**, *39*, 1764-1779.

3.2 Krijt J.; Vackova M.; Kozich V. "Measurement of homocysteine and other aminothiols in plasma: advantages of using tris(2-carboxyethyl)phosphine as reductant compared with tri-n-butylphosphine". *Clin. Chem.*, **2001**, *47*, 1821-1828.

3.3 Gilfix B.; Blank D.; Rosenblatt D. "Novel reductant for determination of total plasma homocysteine". *Clin. Chem.*, **1997**, *43*, 687-688.

3.4 Refsum H.; Helland S.; Ueland P. “Radioenzymic determination of homocysteine in plasma and urine”. *Clin. Chem.*, **1985**, *31*, 624-628.

3.5 Frantzen F.; Faaren A.; Alfheim I.; Nordhei A. “Enzyme conversion immunoassay for determining total homocysteine in plasma or serum”. *Clin.Chem.*, **1998**, *44*, 311-316.

3.6 Shipchandler M.; Moore E. “Rapid, fully automated measurement of plasma homocyst(e)ine with the Abbott IMx analyzer”. *Clin. Chem.*, **1995**, *41*, 991-994.

3.7 Nexo E.; Engbaek F.; Ueland P.;Westby C.; O’Gorman P.; Johnston C. *et al*. “Evaluation of novel assays in clinical chemistry: quantification of plasma total homocysteine”. *Clin. Chem.*, **2000**, *46*, 1150-1156.

3.8 Magera M.; Lacey J.; Casetta B.; Rinaldo P. “Method for the determination of total homocysteine in plasma and urine by stable isotope dilution and electrospray tandem mass spectrometry”. *Clin. Chem.*, **1999**, *45*, 1517-1522.

3.9 Gempel K.; Gerbitz K.; Casetta B.; Bauer M. “Rapid determination of total homocysteine in blood spots by liquid chromatography-electrospray ionization-tandem mass spectrometry”. *Clin. Chem.*, **2000**, *46*, 122-123.

3.10 Araki A.; Sako Y. “Determination of free and total homocysteine in human plasma by high-performance liquid chromatography with fluorescence detection”. *J. Chromatogr.*, **1987**, *422*, 43-52.

3.11 Tcherkas Y.; Denisenko A. “Simultaneous determination of several amino acids, including homocysteine, cysteine and glutamic acid, in human plasma by isocratic reversed-phase high-performance liquid chromatography with fluorimetric detection”. *J. Chromatogr. A.*, **2001**, *913*, 309-314.

3.12 Fiskerstrand T.; Refsum H.; Kvalheim G.; Ueland P. “Homocysteine and other thiols in plasma and urine: automated determination and sample stability”. *Clin. Chem.*, **1993**, *39*, 263-271.

3.13 Jacobsen D.; Gatautis V.; Green R.; Robinson K.; Savon S.; Secic M. *et al*. “Rapid HPLC determination of total homocysteine and other thiols in serum and plasma: sex differences and correlation with cobalamin and folate concentrations in healthy subjects”. *Clin. Chem.*, **1994**, *40*, 873-881.

3.14 Ivanov A.; Nazimov I.; Baratova L. “Determination of biologically active low molecular-mass thiols in human blood. I. Fast qualitative and quantitative, gradient and isocratic reversed-phase high-performance liquid chromatography with photometric and fluorescence detection”. *J. Chromatogr. A.*, **2000**, *895*, 157-166.

3.15 Kaniowska E.; Chwatko G.; Glowacki R.; Kubalczyk P.; Bald E. "Urinary excretion measurement of cysteine and homocysteine in the form of their S-pyridinium derivatives by high-performance liquid chromatography with ultraviolet detection". *J. Chromatogr. A.*, **1998**, *798*, 27-35.

3.16 Bald E.; Kaniowska E.; Chwatko G.; Glowacki R. "Liquid chromatographic assessment of total and protein-bound homocysteine in human plasma". *Talanta*, **2000**, *50*, 1233-1243.

3.17 Chwatko G.; Bald E. "Determination of different species of homocysteine in human plasma by high-performance liquid chromatography with ultraviolet detection". *J. Chromatogr. A.*, **2002**, *949*, 141-151.

3.18 Krien P.; Margou V.; Kermici M. "Electrochemical determination of femtomole amounts of free reduced and oxidized glutathione. Application to human hair follicles". *J. Chromatogr.*, **1992**, *576*, 255-261.

3.19 Wang A.; Zhang L.; Zhang S.; Fang Y. "Determination of thiols following their separation by CZE with amperometric detection at a carbon electrode". *J. Pharm. Biomed. Anal.*, **2000**, *23*, 429-436.

3.20 Carvalho F.; Remiao F.; Vale P.; Timbrell J.; Bastos M.; Ferreira M. "Glutathione and cysteine measurement in biological samples by HPLC with a glassy carbon working detector". *Biomed. Chromatogr.*, **1994**, *8*, 134-136.

3.21 D'Eramo J.; Finkelstein A.; Boccazzi F.; Fridman O. "Total homocysteine levels in plasma: high-performance liquid chromatographic determination with electrochemical detection and glassy carbon electrode". *J. Chromatogr. B*, **1998**, *720*, 205-210.

3.22 Gogia R.; Richer S.; Rose R. "Tear fluid content of electrochemically active components including water soluble antioxidants". *Curr. Eye Res.*, **1998**, *17*, 257-263.

3.23 Solomon B.; Duda C. "Homocysteine determination in plasma". *Curr. Separ.*, **1998**, *17*, 3-7.

3.24 Vandeberg P.; Johnson D. "Comparison of pulsed amperometric detection and integrated voltammetric detection for organic sulfur compounds in liquid chromatography". *Anal. Chim. Acta*, **1994**, *290*, 317-327.

3.25 Owens G.; LaCourse W. "Pulsed electrochemical detection of thiols and disulfides following capillary electrophoresis". *J. Chromatogr. B*, **1997**, *695*, 15-25.

3.26 Vandeberg P.; Johnson D. “Pulsed electrochemical detection of cysteine, cystine, methionine, and glutathione at gold electrodes following their separation by liquid chromatography”. *Anal. Chem.*, **1993**, *65*, 2713-2718.

3.27 Wu L.; Wu J.; Hunt S.; James B.; Vincent G.; Williams R.; Hopkins P. “Plasma homocyst(e)ine as a risk factor for early familial coronary artery disease”. *Clin. Chem.*, **1994**, *40*, 552-561.

3.28 Jovanovic T.; Stanovic B. “Silver and platinum electrodes in the direct potentiometric determination of N-acetyl-L-cysteine with copper (II)”. *Acta Pharm. Jugosl.*, **1989**, *39*, 117-121.

3.29 Popovic N.; Cox J.; Johnson D. “Electrocatalytic function of Bi (V) sites in heavily-doped PbO_2-film electrodes applied for anodic detection of selected sulfur compounds”. *J. Electroanal. Chem.*, **1998**, *455*, 153-160.

3.30 Chailapakul O.; Aksharanandana P.; Frelink T.; Einaga Y.; Fujishima A. “The electrooxidation of sulfur-containing compounds at boron-doped diamond electrode”. *Sens. Acts. B: Chem.*, **2001**, *80*, 193-201.

3.31 Hou W.; Wang E. “Liquid chromatography with electrocatalytic detection of cysteine. N-acetylcysteine and glutathione by a Prussian blue film-modified electrode”. *J. Electroanal. Chem.*, **1991**, *316*, 155-163.

3.32 Li H.; Wang E. “Determination of reduced glutathione in human plasma using liquid chromatography with electrochemical detection with a glassy carbon electrode chemically modified with indium ferricyanide”. *Microchem. J.*, **1994**, *49*, 91-98.

3.33 Zhang S.; Sun W.; Zhang W.; Qi W.; Jin L.; Yamamoto K.; Tao S.; Jin J. “Determination of thiocompounds by liquid chromatography with amperometric detection at a Nafion/indium hexacyanoferrate film modified electrode”. *Anal. Chim. Acta*, **1999**, *386*, 21-30.

3.34 Cataldi T.; De Benedetto G.; Bianchini A. “Electrocatalysis and amperometric detection at a ruthenium-modified indium-hexacyanoferrate film electrode”. *Electroanalysis*, **1998**, *10*, 1163-1167.

3.35 Shi G.; Lu J.; Xu F.; Sun W.; Jin L.; Yamamoto K.; Tao S.; Jin J. “Determination of glutathione in vivo by microdialysis using liquid chromatography with a cobalt hexacyanoferrate chemically modified electrode”. *Anal. Chim. Acta*, **1999**, *391*, 307-313.

3.36 Zhou J.; Wang E. “Electrocatalytic oxidation and amperometric determination of sulfhydryl compounds at a copper hexacyanoferrate film glassy carbon electrode in liquid chromatography”. *Electroanalysis*, **1994**, *6*, 29-35.

3.37 Wring S.; Hart J.; Birch B. “Development of an amperometric assay for the determination of reduced glutathione, using glutathione peroxidase and screen-printed carbon electrodes chemically modified with cobalt phthalocyanine”. *Electroanalysis*, **1992**, *4*, 299-309.

3.38 Huang X.; Kok W. “Conductive carbon cement as electrode matrix for cobalt phthalocyanine-modified electrodes for detection in flowing solutions”. *Anal. Chim. Acta*, **1993**, *273*, 245-253.

3.39 Park J.; Shaw B. “Improved performance of unmodified and cobalt phthalocyanine-modified carbon-Kel-F composite electrodes”. *J. Electrochem. Soc.*, **1994**, *141*, 323-330.

3.40 Wang J.; Pamidi P.; Parrado C.; Park D.; Pingarron J. “Sol-gel-derived cobalt phthalocyanine-dispersed carbon composite electrodes for electrocatalysis and amperometric flow detection”. *Electroanalysis*, **1997**, *9*, 908-911.

3.41 O’Shea T.; Lunte S. “Chemically modified microelectrodes for capillary electrophoresis/electrochemistry”. *Anal. Chem.*, **1994**, *66*, 307-311.

3.42 Zhang S.; Sun W.; Xian Y.; Zhang W.; Jin L.; Yamamoto K.; Tao S.; Jin J. “Multichannel amperometric detection system for liquid chromatography to assay the thiols in human whole blood using the platinum microelectrodes chemically modified by copper tetraaminophthalocyanine”. *Anal. Chim. Acta*, **1999**, *399*, 213-221.

3.43 O’Shea T.; Lunte S. “Selective detection of free thiols by capillary electrophoresis-electrochemistry using a gold/mercury amalgam microelectrode”. *Anal. Chem.*, **1993**, *65*, 247-250.

3.44 Pasas S.; Lacher N.; Davies M.; Lunte S. “Detection of homocysteine by conventional and microchip capillary electrophoresis/electrochemistry”. *Electrophoresis*, **2002**, *23*, 759-766.

3.45 Inoue T.; Kirchhoff J. “Determination of thiols by capillary electrophoresis with amperometric detection at a coenzyme pyrroloquinoline quinone modified electrode”. *Anal. Chem.*, **2002**, *74*, 1349-1354.

3.46 Causse E.; Terrier R.; Champagne S.; Nertz M.; Valdiguie P.; Salvayre R.; Couderc F. “Quantitation of homocysteine in human plasma by capillary electrophoresis and laser-induced fluorescence detection”. *J. Chromatogr. A*, **1998**, *817*, 181-185.

3.47 Vecchione G.; Margaglione M.; Grandone E.; Colaizzo D.; Cappucci G.; Fermo I.; D’Angelo A.; Di Minno G. “Determining sulfur-containing amino acids by

capillary electrophoresis: a fast novel method for total homocyst(e)ine human plasma". *Electrophoresis*, **1999**, *20*, 569-574.

3.48 Causse E.; Issac C.; Malatray P.; Bayle C.; Valdiguie P.; Salvayre R.; Couderc F. "Assays for total homocysteine for total homocysteine and other thiols by capillary electrophoresis-laser-induced fluorescence detection. I. Preanalytical condition studies". *J. Chromatogr. A*, **2000**, *895*, 173-178.

3.49 Kang S.; Kim J.; Chung D. "Determination of homocysteine and other thiols in human plasma by capillary electropgoresis". *J. Pharm. Biomed. Anal.*, **1997**, *15*, 1435-1441.

3.50 Chassaing C.; Gonin J.; Wilcox C.; Wainer I. "Determination of reduced and oxidized homocysteine and related thiols in plasma by thiol-specific pre-column derivatization and capillary electrophoresis with laser-induced fluorescence detection". *J. Chromatogr. B*, **1999**, *735*, 219-227.

3.51 Ling B.; Baeyens W.; Dewaele C. "Capillary zone electrophoresis with ultraviolet and fluorescence detection for the analysis of thiol. Application to mixtures and blood". *Anal. Chim. Acta*, **1991**, *255*, 283-288.

3.52 Myung S.; Kim M.; Min H.; Yoo E.; Kim K. "Determination of homocysteine and its related compounds by solid-phase microextraction-gas chromatography-mass spectrometry". *J. Chromatogr. B*, **1999**, *727*, 1-8.

3.53 Pietzsch J.; Julius U. "Hanefeld M. Rapid determination of total homocysteine in human plasma by using N (O, S)-ethoxycarbonyl ethyl ester derivatives and gas chromatography –mass spectrometry". *Clin. Chem.*, **1997**, *43*, 2001-4.

3.54 Stabler S.; Lindenbaum J.; Savage D.; Allen R. "Elevation of serum cystathionine levels in patients with cobalamin and folate deficiency". *Blood*, **1993**, *81*, 3404-3413.

3.55 Ducros V.; Schmitt D.; Pernod G.; Faure H.; Polack B.; Favier A. "Gas chromatographic-mass spectrometric determination of total homocysteine in human plasma by stable isotope dilution: method and clinical applications". *J. Chromatogr. B*, **1999**, *729*, 333-339.

3.56 Kataoka H.; Takagi K.; Makita M. "Determination of total plasma homocysteine and related aminothiols by gas chromatography with flame photometric detection". *J. Chromatogr. B*, **1995**, *664*, 421-425.

3.57 Myung S.; Chang Y.; Yoo E.; Park J.; Min H.; Kim M. "Determination of homocysteine, cysteine and methionine in human plasma by gas chromatography with electron capture detector". *Analytical Science & Technology*, **1999**, *12*, 408-414.

3.58 Refsum, H.; Smith, A. D.; Ueland, P. M.; Nexo, E.; Clarke, R.; McPartlin, J.; Johnston, C.; Engbaek, F.; Schneede, J.; McPartlin, C.; Scott, J. M. “Facts and recommendations about total homocysteine determinations: an expert opinion”. *Clin. Chem*. **2004**, *50*, 3-32.

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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* Reprinted in part with permission from *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.

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% CD_3OD, 30% D_2O on a Bruker DPX-250 or DPX-300 spectrometer, unless otherwise indicated.

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

Equimolar amounts of Hcy, Cys and GSH are dissolved in three 1mL solutions of MV^{2+} (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 MV^{2+} 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 MV^{2+}**

Commercial lyophilized human blood plasma is reconstituted with distilled H_2O. 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 MV^{2+} (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 (PPh_3)**

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. PPh_3 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 PPh_3 solution and 0.4mL of FB solution. 0.3mL of 0.005 M, pH 7.3 phosphate buffer mixed with 0.3mL of PPh_3 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 H_2O. 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 PPh_3 (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 PPh_3 from the absorbance of a reduced plasma sample containing FB and PPh_3.

- **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 Fe^{3+} 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 $FeCl_3$ (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 $FeCl_3$, the UV/Vis spectrum is acquired. A solution containing same amounts of Hcy and FB except for $FeCl_3$ 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 (·OH), nitrogen dioxide radical (NO_2·), and peroxyl radical (ROO·) can often react with thiols to generate thiyl radicals.

(iii) Enzymatic oxidation reaction

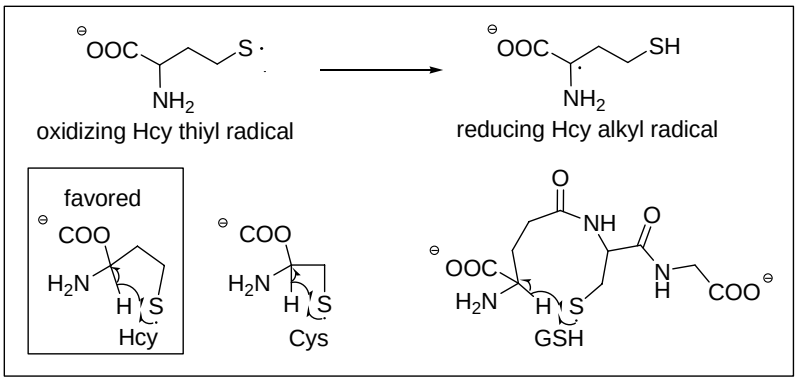
Enzymes that can catalyze the oxidation of thiols are mainly peroxidase, such as horseradish peroxidase, lactoperoxidase, and myeloperoxidase.

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.

$$RS^{\bullet} + RS^{-} \rightleftharpoons RSSR^{\bullet -} \qquad (4.1)$$

$$RS^{\bullet} \rightleftharpoons {}^{\bullet}CR \qquad (4.2)$$

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 intra-molecularly 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).

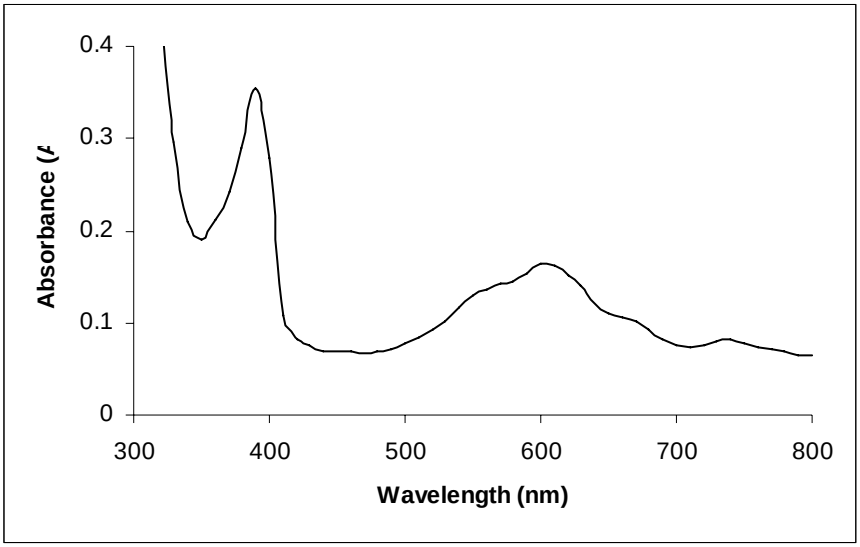


Figure 4.2. UV/Vis spectrum of a MV^{2+} solution (4.0 mM) in the presence of Hcy (17 mM) in tris buffer (0.1 M, pH 7.5) after gentle reflux for 5 min.

- **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+}.

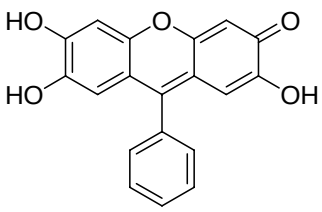


Figure 4.4. The structure of FB

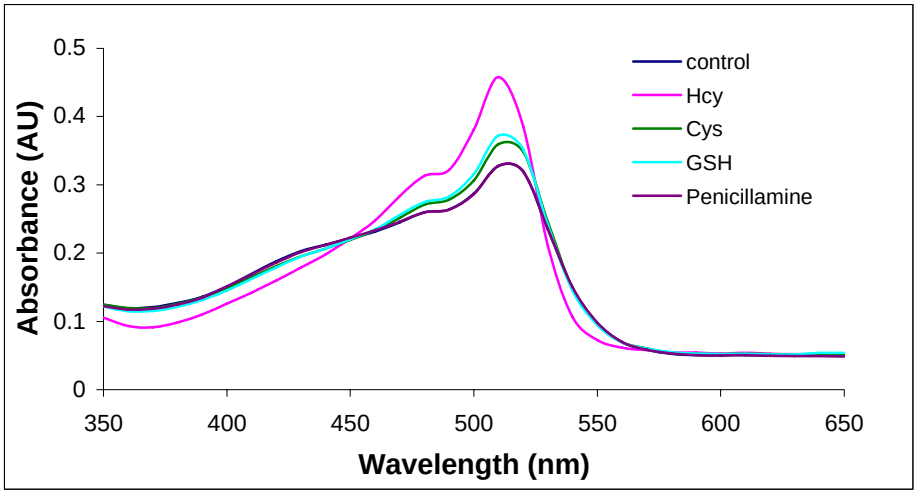


Figure 4.5. UV/Vis spectra of solutions containing FB (1.0×10^{-5} M) and different biological thiols (1.0×10^{-4} M).

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 PPh_3**

The disulfide reduction in biological samples is often accomplished with a reducing agent such as a phosphine. We investigate the effect of PPh_3 on this process, initially in order to mimic the conditions used to prepare biological samples for analysis via disulfide reduction. When PPh_3 (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).

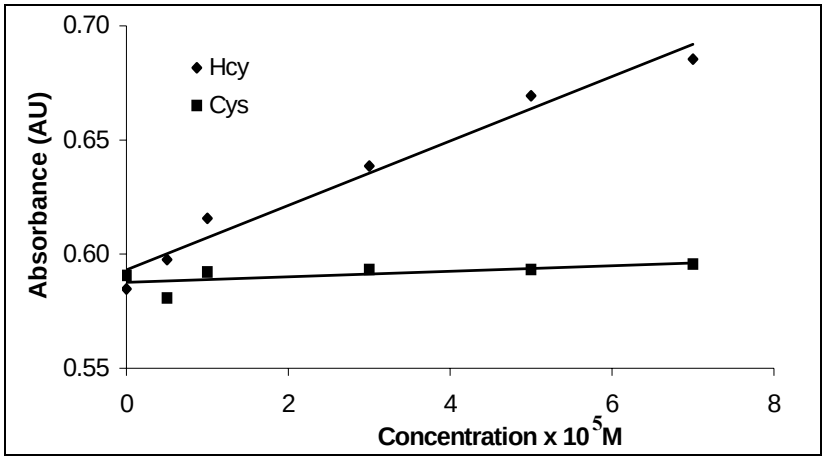


Figure 4.6. Absorbance at 510 nm vs. concentrations of Cys or Hcy in the presence of FB (1.0×10^{-5} M) and PPh_3 (4.5×10^{-4} M).

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 ($O=PPh_3$) in a solution of FB and PPh_3 (Figure A4). After 19 h, the formation of $O=PPh_3$ 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 $O=PPh_3$ is clearly observed. The formation of $O=PPh_3$ 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).

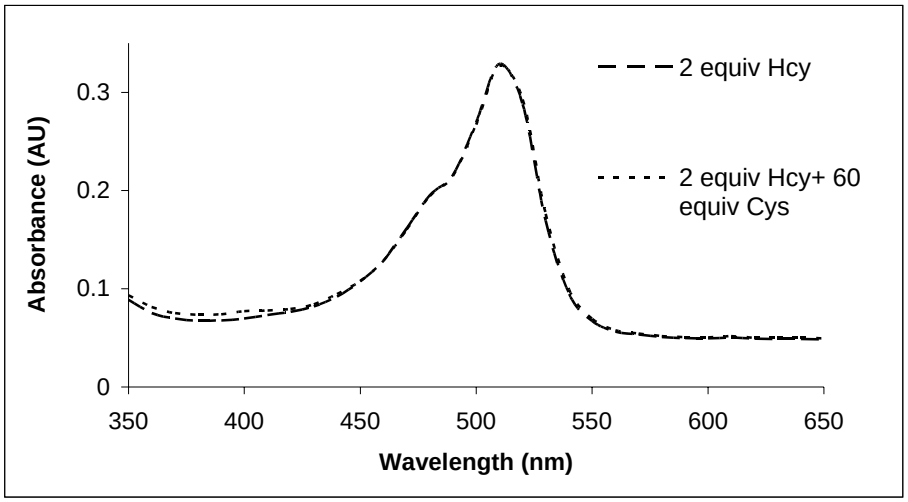


Figure 4.7. UV/Vis spectra of solutions containing FB (0.5×10^{-5} M), Hcy (1.0×10^{-5} M) and PPh_3 (5.0×10^{-4} M) in 30 % phosphate buffer (0.015 M, pH 7.3) and 70 % MeOH in the absence/presence of 30 equivalent Cys (to Hcy). No absorbance change is observed due to the added excess Cys.

- **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).

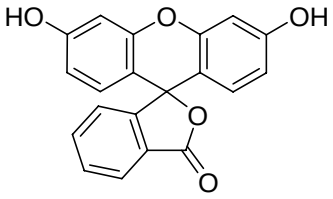


Figure 4.8. The structure of fluorescein

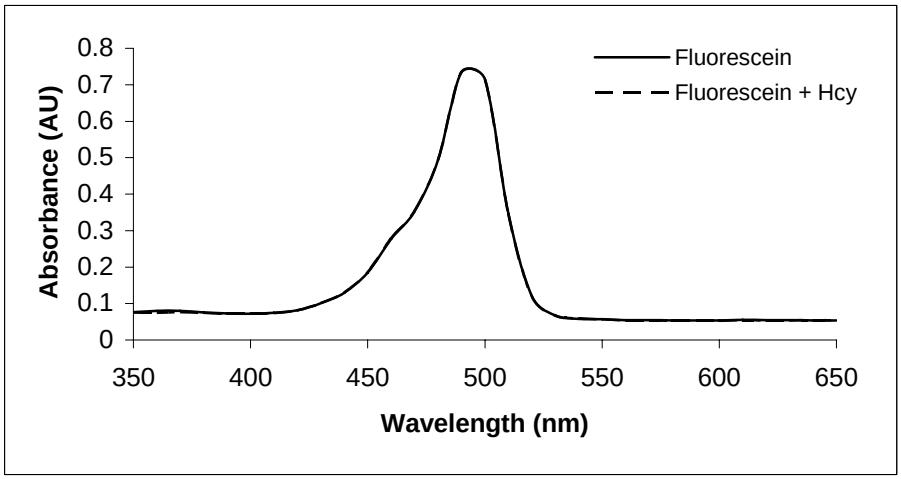


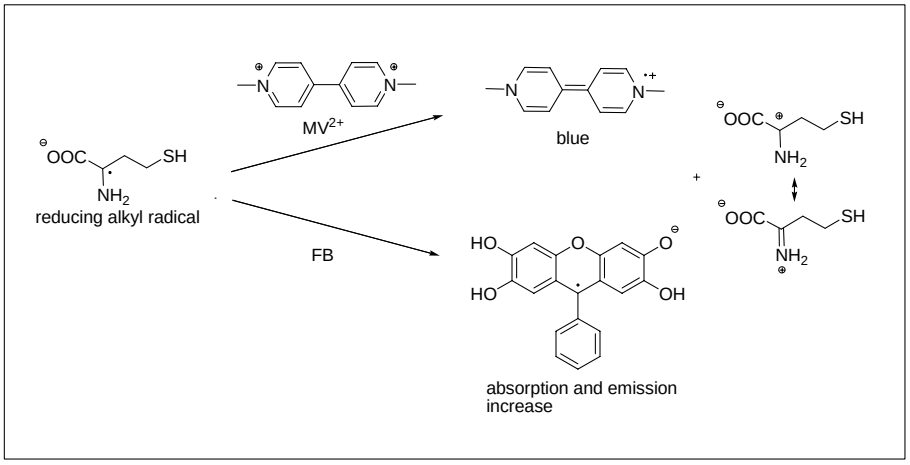
Figure 4.9. Detection of Hcy (1.0×10^{-4} M) with fluorescein (1.0×10^{-5} M) in 0.015 M phosphate buffer pH 7.3.

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 $MV^{+\cdot}$, 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 μM, which is inclusive of the upper limit of healthy Hcy

concentration. The percent recovery of Hcy is 102.9 ± 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.

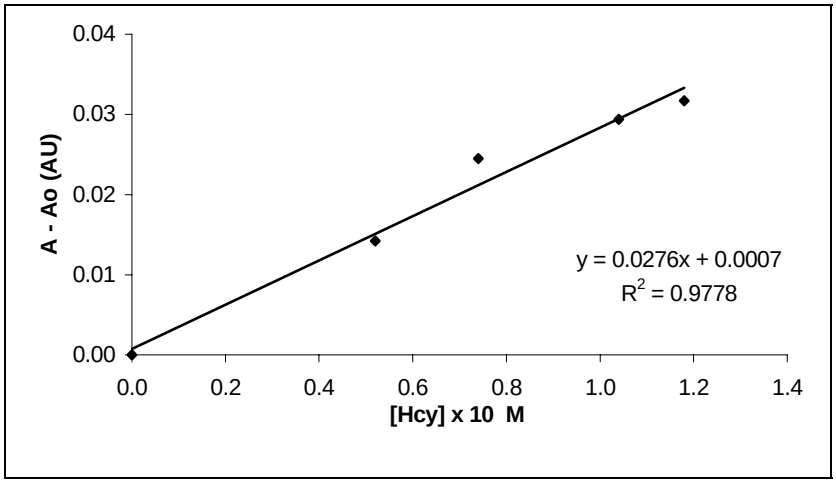


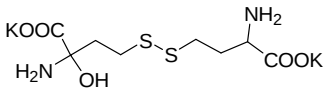
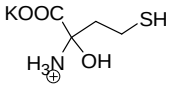
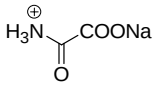
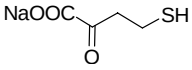
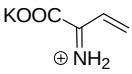
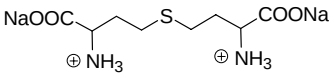
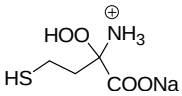
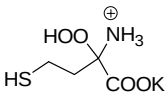
Figure 4.10. Calibration curve for the determination of Hcy in human plasma in the presence of PPh_3 and FB. (**A** is the absorbance of samples with added Hcy at 510nm; **Ao** is the absorbance of samples without added standards at 510nm).

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

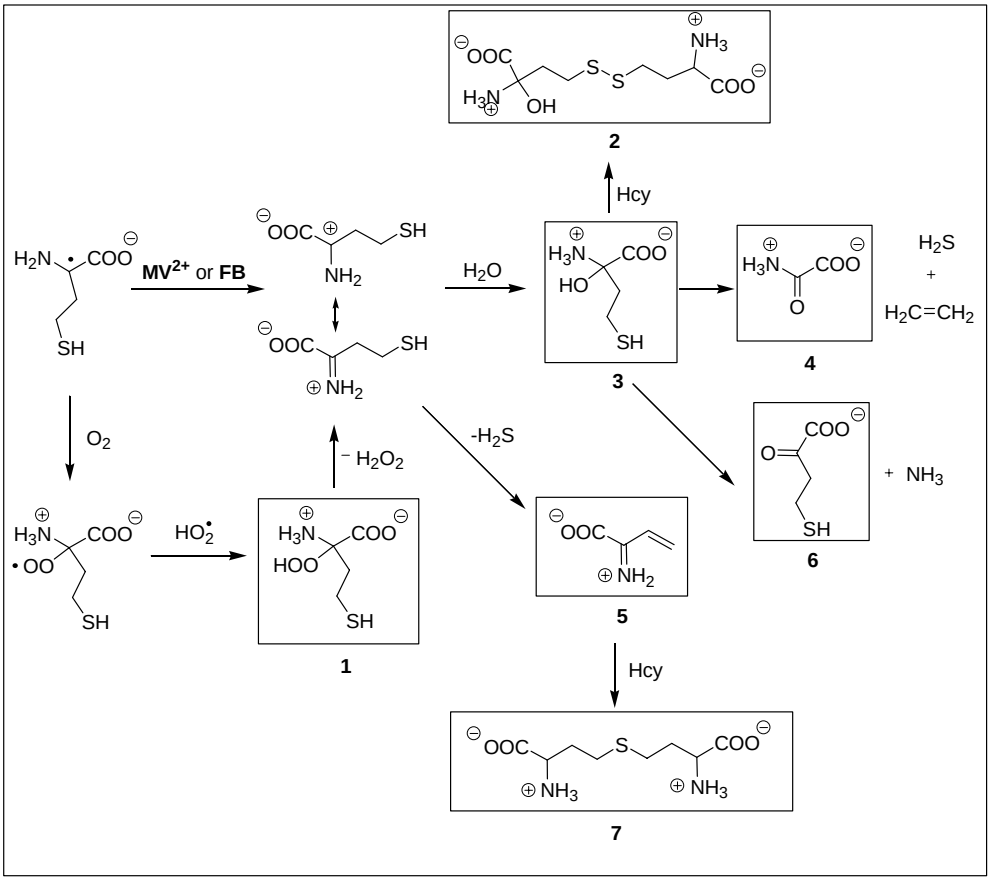
There is growing interest in the study of the metabolites of hyperhomocysteinemia.[7] Amino acid-derived free radicals are often implicated in DNA

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 H_2S. Should Hcy produce H_2S more readily than GSH and Cys? It may have significant implications for Hcy-based toxicity compared to GSH and Cys.

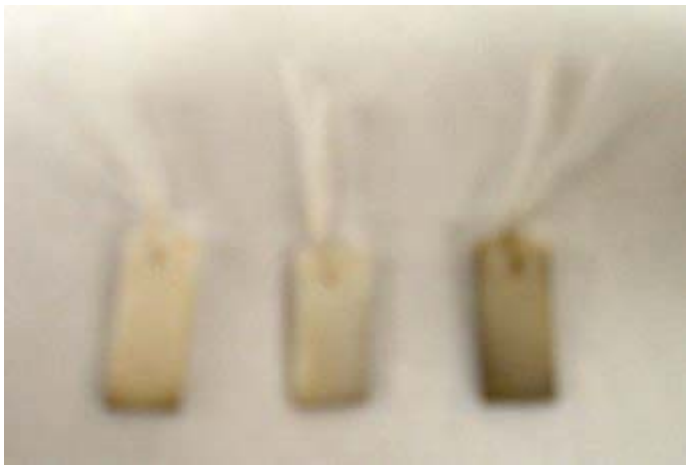


Figure 4.11. H_2S test strips after contact with vapors produced by heated solutions containing equimolar amounts of GSH (left), Cys (center) and Hcy (right) in the presence of MV^{2+}.

- **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 (Fe^{3+}) 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).

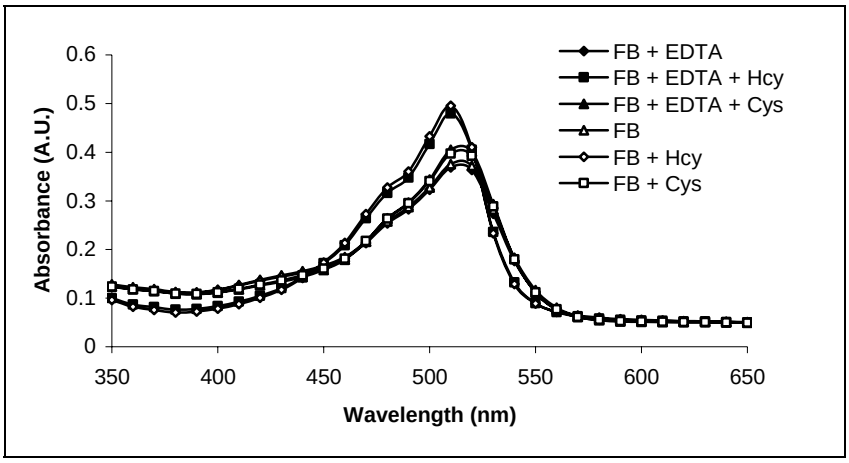


Figure 4.12. UV/Vis spectra of solutions containing FB (1.0×10^{-5} M) and Hcy or Cys (1.0×10^{-4} M) in the presence/ absence of EDTA (2.0×10^{-4} M).

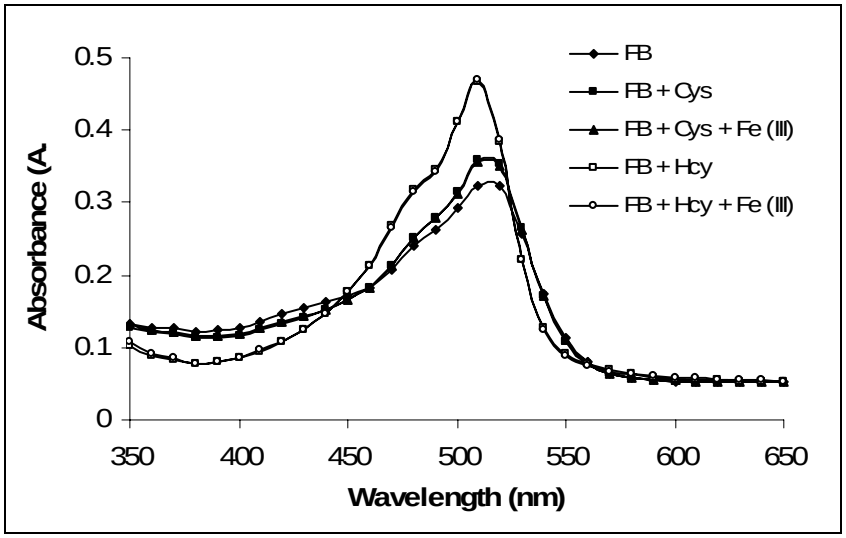


Figure 4.13. UV/Vis spectra of solutions containing FB (1.0×10^{-5} M) and Hcy or Cys (1.0×10^{-4} M) in the presence/ absence of $FeCl_3$ (1.0×10^{-5} M).

In addition, no spectral changes are observed upon addition of $FeCl_3$ (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, MV^{2+} and FB. By using these two compounds, we have developed colorimetric methods for the selective detection of Hcy at neutral pH. MV^{2+} solutions turn color selectively corresponding to the presence of Hcy upon heating. We also selectively detect Hcy by utilizing FB and PPh_3 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

4.1 (a) Davis, C. J.; Lewis, P. T.; McCarroll, M. E.; Read, M. W.; Cueto, R.; Strongin, R. M. "Simple and rapid visual sensing of saccharides". *Org. Lett*. **1999**, *1*, 331-334. (b) Lewis, P. T.; Davis, C. J.; Cabell, L. A.; He, M.; Read, M. W.; McCarroll, M. E.; Strongin, R. M. "Visual sensing of saccharides promoted by

resorcinol condensation products". *Org. Lett*. **2000**, *2*, 589-592. (c) He, M; Johnson, R.; Escobedo, J. O.; Beck, J. A.; Kim, K. K.; St. Luce N. N.; Davis C. J.; Lewis, P. T.; Fronczek F. R.; Melancon, B. J.; Mrse, A. A.; Treleaven, W. D.; Strongin, R. M. "Chromophore formation in resorcinarene solutions and the visual detection of mono- and oligosaccharides". *J. Am. Chem. Soc.* **2002**, *124*, 5000-5009. (d) Rusin, O.; St. Luce, N. N.; Agbaria, R. A.; Escobedo, J. O.; Jiang, S.; Warner, I. M.; Dawan, F. B.; Lian, K.; Strongin, R. M. "Visual detection of cysteine and homocysteine". *J. Am. Chem. Soc.* **2004**, *126*, 438-439.

4.2 Abedinzadeh, Z. "Sulfur-centered reactive intermediates derived from the oxidation of sulfur compounds of biological interest". *Ca. J. Physiol. Pharmacol.*, **2001**, *79*, 166-170.

4.3 Fessenden, R. W.; Neta, P. "Electron spin resonance study of radicals produced in irradiated aqueous solutions of thiols". *J. Phys. Chem.* **1971**, *75*, 2277-2283.

4.4 (a) Sjöberg, L.; Eriksen, T. E.; Revesz, L. "The reaction of the hydroxyl radical with glutathione in neutral and alkaline-aqueous solution". *Radiat. Res.* **1982**, *89*, 255-263. (b) Grierson, L.; Hildenbrand, K.; Bothe, E. "Intramolecular transformation reaction of the glutathione thiyl radical into a non-sulfur-centered radical: a pulse-radiolysis and EPR study". *Int. J. Radiat. Biol.* **1992**, *62*, 265-277.

4.5 (a) Zhao, R.; Lind, J.; Merényi, G.; Eriksen, T. E. "Kinetics of one-electron oxidation of thiols and hydrogen abstraction by thiyl radicals from α-amino C-H bonds". *J. Am. Chem. Soc*. **1994**, *116*, 12010-12015. (b) Zhao, R.; Lind, J.; Merényi, G.; Eriksen, T. E. "Significance of the intramolecular transformation of glutathione thiyl radicals to α-aminoalkyl radicals. Thermochemical and biological implications". *J. Chem. Soc., Perkins Trans.*, **1997**, *2*, 569-574, and references cited therein.

4.6 For example, Rauk, A.; Armstrong, D. A.; Fairlie, D. P. "Is oxidative damage by β-amyloid and prion peptides mediated by hydrogen atom transfer from glycine α-carbon to methionine sulfur within β-sheets?" *J. Am. Chem. Soc*. **2000**, *122*, 9761-9767, and references cited therein.

4.7 Böger, R.H.; Bode-Böger, S. M.; Sydow, K.; Heistad, D. D.; Lentz, S. R. "Plasma Concentration of Asymmetric Dimethylarginine, an Endogenous Inhibitor of Nitric Oxide Synthase, is Elevated in Monkeys with Hyperhomocyst(e)inemia or Hypercholesterolemia". *Arterioscler. Thromb Vasc. Biol.*, **2000**, *20*, 1557-1564.

4.8 Stadtman, E. R. "Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions". *Ann. Rev. Biochem*. **1993**, *62*, 797-821.

4.9 Cooper, A. J. L. “Biochemistry of sulfur-containing amino acids”. *Ann. Rev. Biochem*. **1983**, *52*, 187-222.

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 MV^{2+} 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.

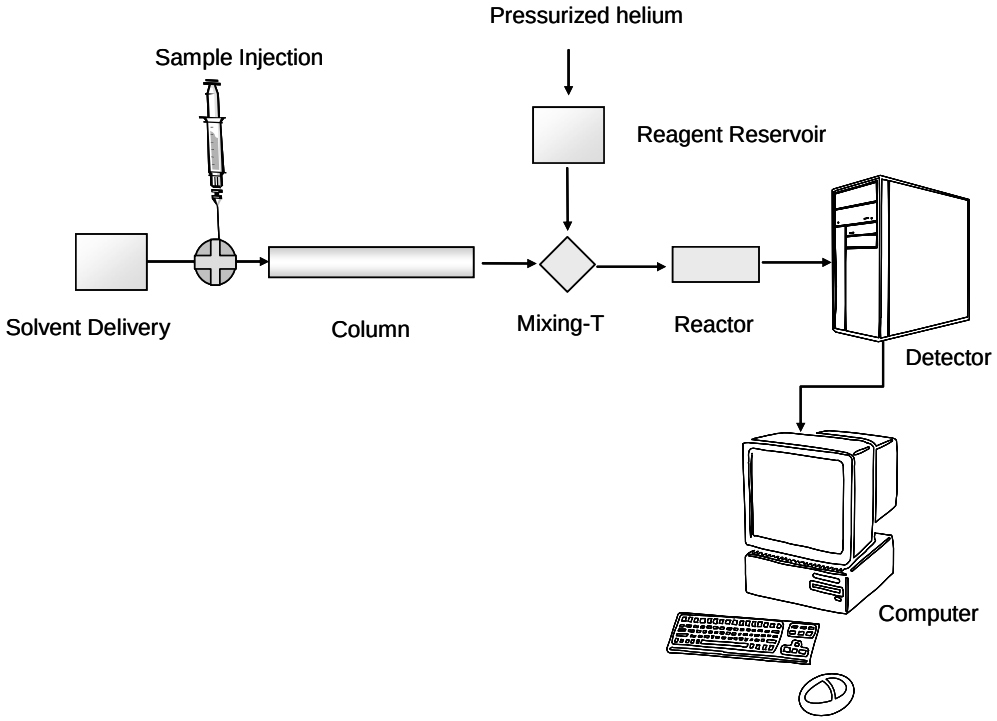
* Reprinted in part with permission from the *Journal of the American Chemical Society*, 2005, Volume 127, pages 15949-15958; Weihua Wang, Oleksandr Rusin, Xiangyang Xu, Kyu Kwang Kim, Jorge O. Escobedo, Sayo O. Fakayode, Kristin A. Fletcher, Mark Lowry, Corin M. Schowalter, Candace M. Lawrence, Frank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

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, 5µm; 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 MV^{2+} 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.

MV^{2+} 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 MV^{2+} as a Postcolumn Detection Reagent**

Figure 5.1 shows a chromatogram of a mixture of Hcy and Cys (85 nmol each) using MV^{2+} 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.

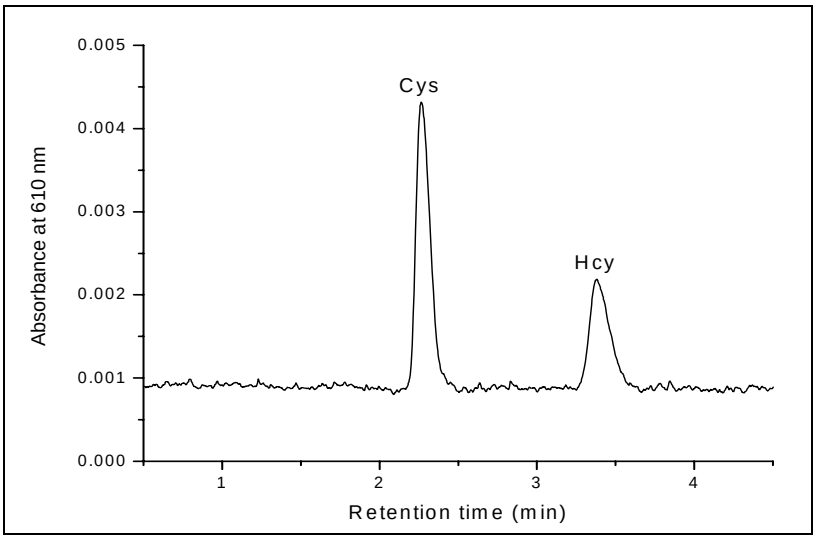


Figure 5.1. Chromatogram of a mixture of Cys and Hcy using MV^{2+} as the post-column reagent at 610nm.

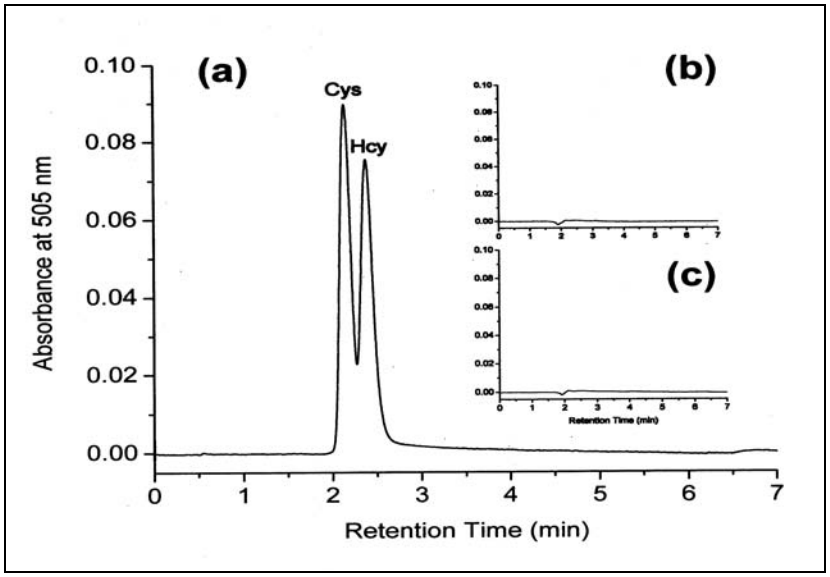


Figure 5.2. Chromatograms of mixtures of: (a) Cys and Hcy, (b) histidine, methionine and glutamine and (c) lysine, glycine and serine using FB as the postcolumn reagent.

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

4.1 (a) Nekrassova, O.; Lawrence, N. S.; Compton, R. G. "Analytical determination of homocysteine: a review". *Talanta*, **2003**, *60*, 1085-1095. (b) Refsum, H.; Smith, A. D.; Ueland, P. M.; Nexo, E.; Clarke, R.; McPartlin, J.; Johnston, C.; Engbaek, F.; Schneede, J.; McPartlin, C.; Scott, J. M. "Facts and recommendations about homocysteine determinations: an expert opinion". *Clin. Chem*. **2004**, *50*, 3-32.

4.1 (a) Ubbink, J. B. "Assay methods for the measurement of total homocyst(e)ine in plasma". *Seminars in Thrombosis and Hemeostasis* **2000,** *26*, 233-242. (b) Haugland, R. P. *Molecular Probes – Handbook of Fluorescent Probes and Research Products*, Molecular Probes, Inc., Eugene, O.R., Ninth Ed., **2002**, p. 79-98.

5.3 Kim, K. K.; Escobedo, J. O.; St. Luce, N. N.; Rusin, O.; Wong, D. and Strongin, R. M. "Postcolumn HPLC detection of mono- and oligosaccharides with a chemosensor". *Org. Lett.* **2003**, *5*, 5007-5010.

CHAPTER 6. SYNTHESES 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.

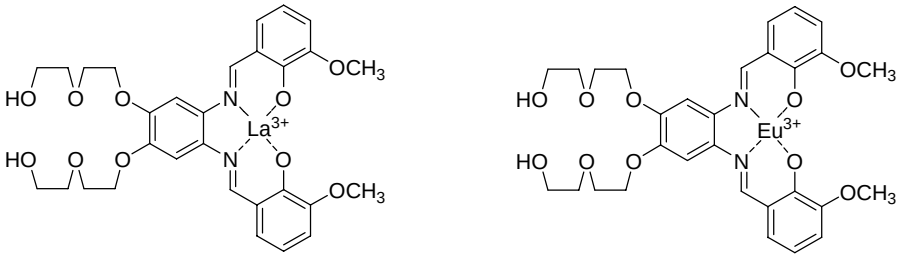
Recently, metallomacrocycles have attracted great interest in the field of supramolecular chemistry.[5,6] Most of the work has focused on the design of selective

*Reprinted in part with permission from *Proceedings of the National Academy of Sciences of the United States of America*, 2006, Volume 103, pages 9756-9760; Onur Alpturk, Oleksandr Rusin, Sayo O. Fakayode, Weihua Wang, Joege O. Escobedo, Isiah M. Warner, Williams E. Crowe, Vladimir Kral, Jeff M. Pruet, and Robert M. Strongin, Lanthanide Complexes as Fluorescent Indicators for Neutral Sugars and Cancer Biomarkers. Copyright 2006 National Academy of Sciences, U.S.A.

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 UO_2-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]

La^{3+} and Ca^{2+} 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 Ca^{2+} in biomedical research. In addition, lanthanides can extend their ligand coordination number via "ligand-sphere" extension, leading to highly coordinated complexes.[12]



1 2

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

Hence, we propose that La^{3+}-salophene complex **1** (Figure 6.1) may be useful for the determination of saccharides at neutral pH. La^{3+}, 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.

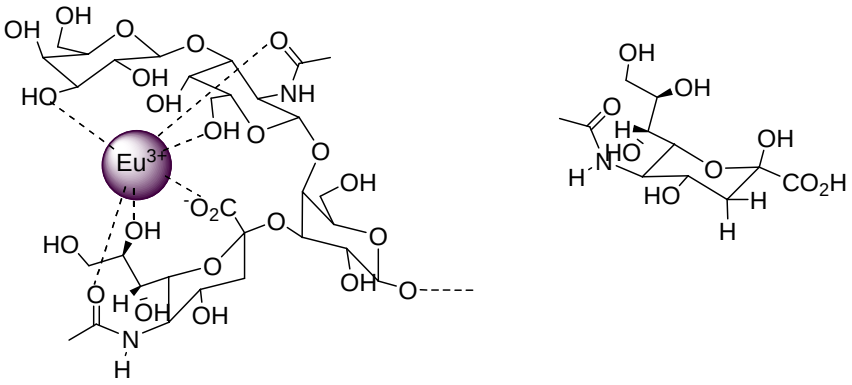
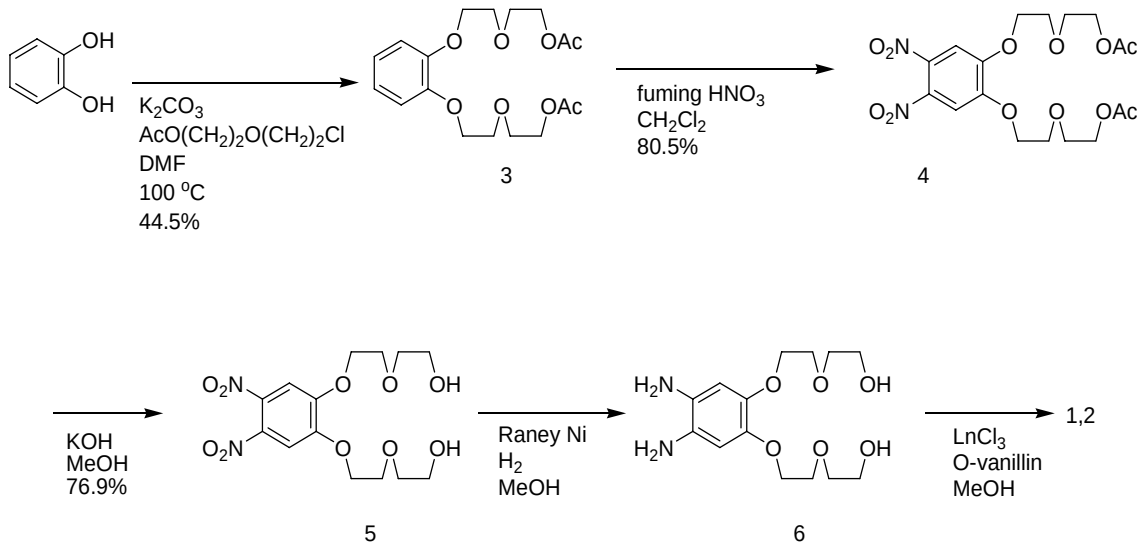


Figure 6.2. Left: Coordination of GM1 to Eu^{3+}. Right: Structure of free sialic acid

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=$LaCl_3$, **2**, Ln=$EuCl_3$.

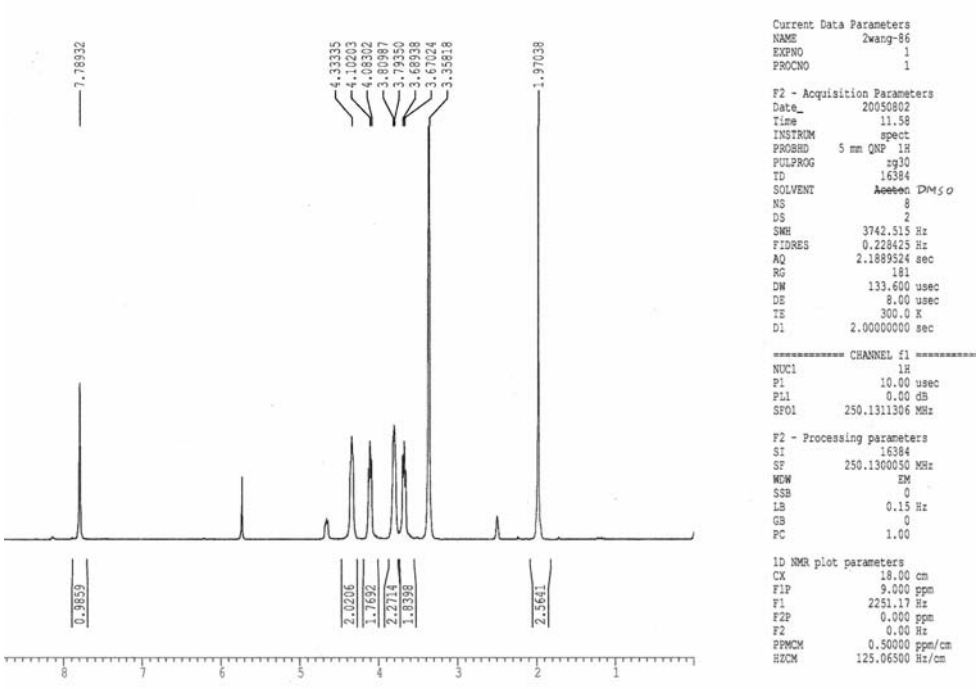


Figure 6.3. ^{1}H-NMR spectrum of **4** in DMSO-d_6.

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 ^{1}H 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.

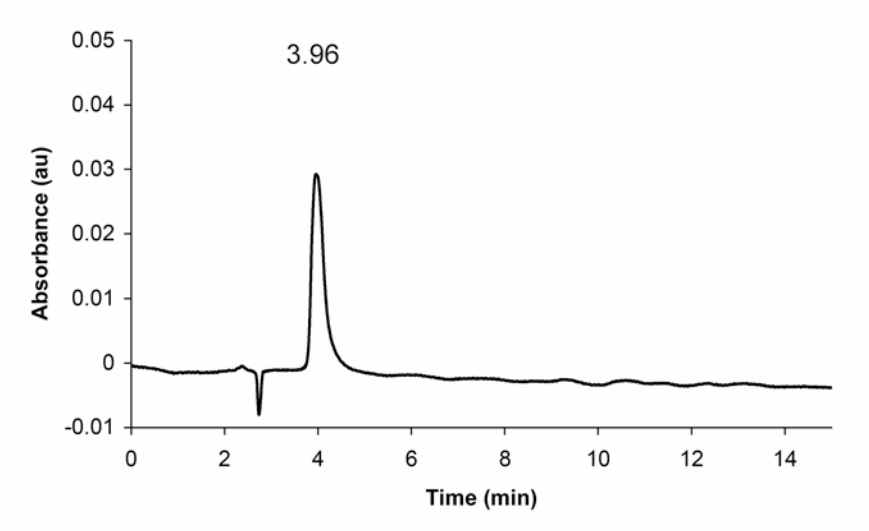


Figure 6.4. Chromatogram of a solution of **1** in MeOH.

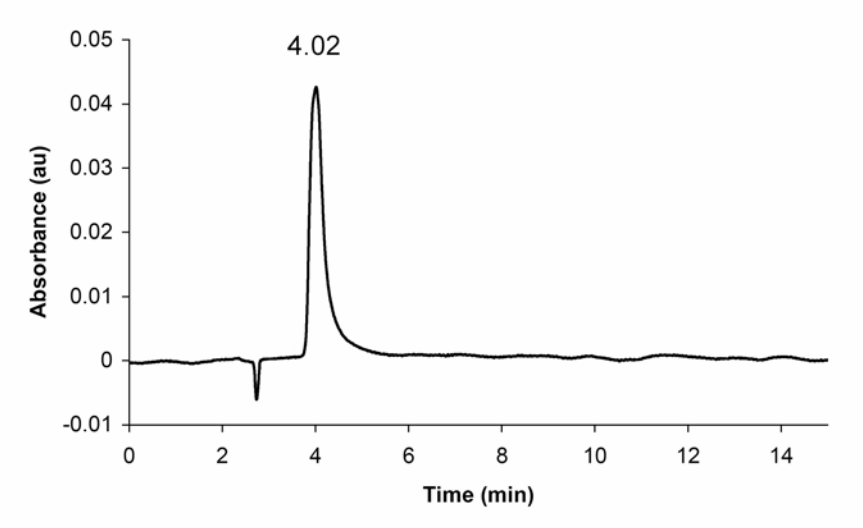


Figure 6.5. Chromatogram of a solution of **2** in MeOH.

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

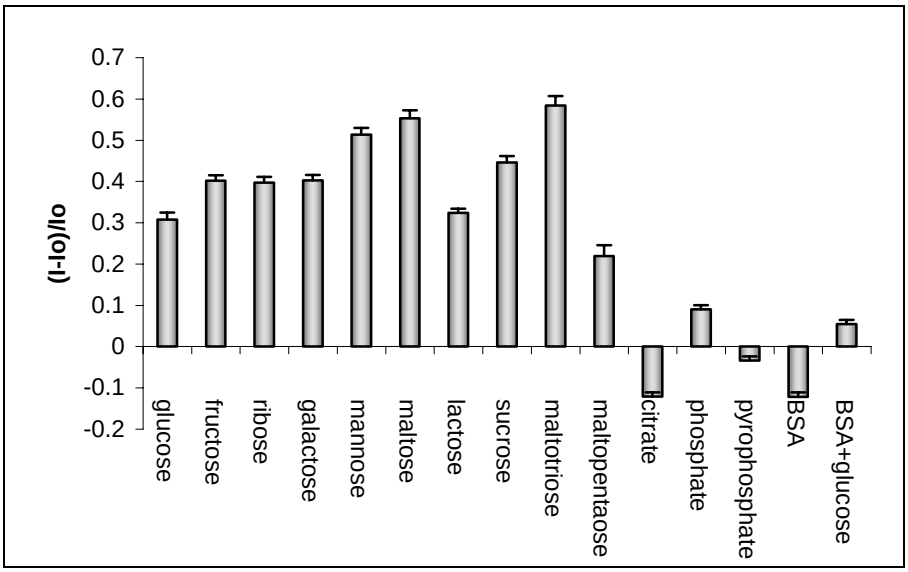


Figure 6.6. Relative fluorescence emission (400 nm) changes observed in solutions of **1**(5.53×10^{-6} M) in the presence of mono-, oligosaccharides, anions (1.1×10^{-3} M), BSA (1 mg/mL) and a mixture of BSA and glucose (1 mg/mL and 1.1×10^{-3} M, respectively) in HEPES buffer solution (pH 7.0). The 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).

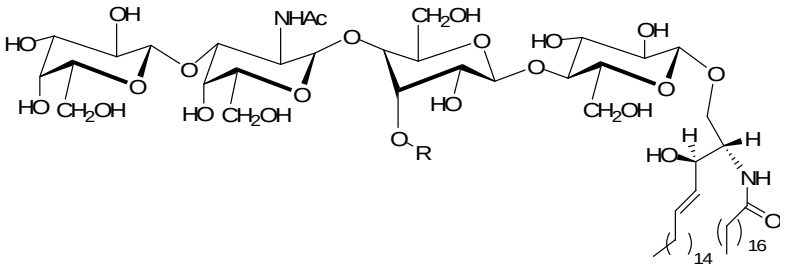


Figure 6.7. The structures of asialo-GM1 and GM1.

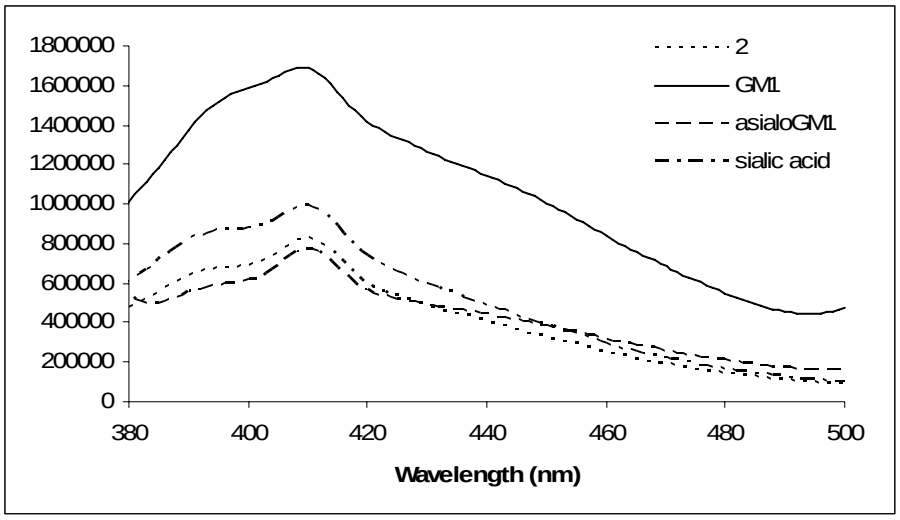


Figure 6.8. Fluorescence intensity change of solutions of **2** (5.53×10^{-6} M) in response to added gangliosides (0.5 mg/mL, *ca*. 10^{-4} M each) and sialic acid (1.0×10^{-3} M) in 0.1 M HEPES buffer solution (pH 7.0). Excitation 360 nm, emission 400 nm.

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 ProFLEX III MALDI-TOF mass spectrometer. ^{1}H 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 × 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 ˚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 × 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%). ^{1}H NMR (250 MHz, 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, 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% $NaHCO_3$ and washed with H_2O. The organic layer is dried over anhydrous Na_2SO_4. The solvent is removed under reduced pressure. The product is chromatographed on silica gel to give compound **4** (1.6 g, 80.5%). ^{1}H NMR (250MHz, DMSO-d_6) δ (ppm): 1.97 (6H, s, CH_3) 3.68 (2H, t, CH_2) 3.80 (2H, t, CH_2) 4.10 (2H, t, CH_2) 4.33 (2H, t, CH_2) 7.79 (2H, s, ArH). ^{13}C 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 H_2O, the organic layer is dried over anhydrous Na_2SO_4. 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 $LaCl_3$ (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. $C_{30}H_{34}LaN_2O_{10}$, 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. $C_{30}H_{34}EuN_2O_{10}$, 735.14; obsd, 735.34. IR 3104.00, 1638.44, 1444.54, 1214.76, 1018.07.

6.5 References

6.1 Ambrosi M.; Cameron N. R.; Davis B. G. "Lectins: tools for the molecular understanding of the glycocode". *Org. Biomol. Chem.*, **2005**, *3*, 1593-1608.

6.2 Weis W. I.; Drickamer K.; Hendrickson W. A. "Structure of a C-type mannose-binding protein complexed with an oligosaccharide". *Nature*, **1992**, *360*, 127-134.

6.3 Striegler S.; Dittel M. "A sugar discriminating binuclear copper (II) complex". *J. Am. Chem. Soc.*, **2003**, *125*, 11518-11524.

6.4 Davis A. P.; Wareham R. S. "Carbohydrate recognition through noncovalent interactions: a challenge for biomimetic and supramolecular chemistry". *Angew. Chem. Int. Ed.*, **1999**, *38*, 2979-2996.

6.5 van Veggel F. C. J. M.; Verboom W.; Reinhoudt D. N. "Metallomacrocycles: supramolecular chemistry with hard and soft metal cations in action". *Chem. Rev.*, **1994**, *94*, 279-299.

6.6 Liu G.-D.; Yang X.; Chen Z.-P.; Shen G.-L.; Yu R.-Q. "Interaction of metal complexes of bis(salicylidene)ethylenediamine with DNA". *Anal. Sci.*, **2000**, *16*, 1255-1259.

6.7 Rudkevich D. M.; Verboom W.; Brzozka Z.; Palys M. J.; Stauthamer W. P. R. V.; van Hummel G. J.; Franken S. M.; Harkema S.; Engbersen J. F. J.; Reinhoudt D.

N. "Functionalized UO_2 salenes: neutral receptors for anions". *J. Am. Chem. Soc.*, **1994**, *116*, 4341-4351.

6.8 van Doorn A. R.; Bos M.; Harkema S.; van Eerden J.; Verboom W.; Reinhoudt D. N. "Molecular recognition of neutral molecules by metalloclefts: synthesis, x-ray structure, ^{1}H NMR spectroscopy, electrochemistry, and molecular modeling". *J. Org. Chem.*, **1991**, *56*, 2371-2380.

6.9 Mizuno T.; Takeuchi M.; Shinkai S. "Sugar sensing using chiral salen-Co(II) complexes". *Tetrahedron*, **1999**, *55*, 9455-9468.

6.10 Angyal S. J. "Complexes of carbohydrates with metal cations. I. determination of the extent of complexing by NMR spectroscopy". *Aust. J. Chem.*, **1972**, *25*, 1957-1966.

6.11 Angyal S. J.; Mills J. A. "Complexes of carbohydrates with metal cations. XI. paper electrophoresis of polyols in solutions of calcium ions". *Aust. J. Chem.*, **1979**, *32*, 1993-2001.

6.12 Tsukube H.; Shinoda S. "Lanthanide complexes in molecular recognition and chirality sensing of biological substrates". *Chem. Rev.*, **2002**, *102*, 2389-2403.

6.13 Reuter G.; Schauer R. "Determination of sialic acids". *Methods Enzymol.*, **1994**, *230*, 168-199.

6.14 Svennerholm L. "Quantitative estimation of sialic acids. a colorimetric resorcinol-hydrochloric acid method". *Biochim. Biophys. Acta*, **1957**, *24*, 604-611.

6.15 Kobayashi K.; Akiyama Y.; Kawaguchi K.; Tanabe S.; Imanari T. "Fluorometric determination of N-acetyl and N-glycolyl neuraminic acids by HPLC as their 4'-hydrazino-2-stilbazole derivatives". *Anal. Sci.*, **1985**, *1*, 81-84.

6.16 Yu R.; Ledeen R. W. "Gas-liquid chromatographic assay of lipid-bound sialic acids: Measurement of gangliosides in brain of several species". *J. Lipid Res.*, **1970**, *11*, 506-516.

6.17 Honda S.; Suzuki S. "Common conditions for high performance liquid chromatographic microdetermination of aldoses, hexosamines, and sialic acids in glycoproteins". *Anal. Biochem.*, **1984**, *142*, 167-174.

6.18 Anumula K. R. "Rapid quantitative determination of sialic acids in glycoproteins by high performance liquid chromatography with a sensitive fluorescence detection". *Anal. Biochem.*, **1995**, *230*, 24-30.

6.19 Schauer R.; Kelm S.; Rerter G.; Roggentin P. Shaw L. "Biology of the sialic acids". **1995**, Plenum, New York, p. 7.

6.20 Mattoo R. L.; Roseman S. "Quantitative determination of sialic acid in the monosialoganglioside, GM1, by the thiobarbituric acid method". *Anal. Biochem.*, **1997**, *246*, 30-33.

6.21 Varki A.; Diaz S. "The release and purification of sialic acids from glycoconjugates: methods to minimize the loss and migration of o-acetyl groups". *Anal. Biochem.*, **1984**, *137*, 236-247.

6.22 Hikita T.; Tadano-Aritomi K.; Iida-Tanaka N.; Toyota H.; Suzuki A.; Toida T.; Imanari T.; Abe T.; Yanagawa Y. Ishizuka I. "Determination of N-acetyl and N-glycolylneuraminic acids in gangliosides by combination of neuraminidase hydrolysis and fluorometric high performance liquid chromatography using a GM3 derivative as an internal standard". *Anal. Biochem.*, **2000**, *281*, 193-201.

6.23 Sillerud L. O.; Prestegard J. H.; Yu R. K.; Schafer D. E.; Konigsberg W. H. "Assignment of the carbon-13 nuclear magnetic resonance spectrum of aqueous ganglioside GM1 micelles". *Biochemistry*, **1978**, *17*, 2619-2628.

6.24 Saladini M.; Menabue L.; Ferrari E. "Binding ability of sialic acid towards biological and toxic metal ions. NMR, potentiometric and spectroscopic study". *J. Inorg. Biochem.*, **2002**, *88*, 61-68.

6.25 Duggan S. A.; Fallon G.; Langford S. J.; Lau V. L.; Satchell J. F.; Paddon-Row M. N. "Crown-linked porphyrin systems". *J. Org. Chem.*, **2001**, *66*, 4419-4426.

APPENDIX A. COLORIMETRIC DETECTION OF HCY

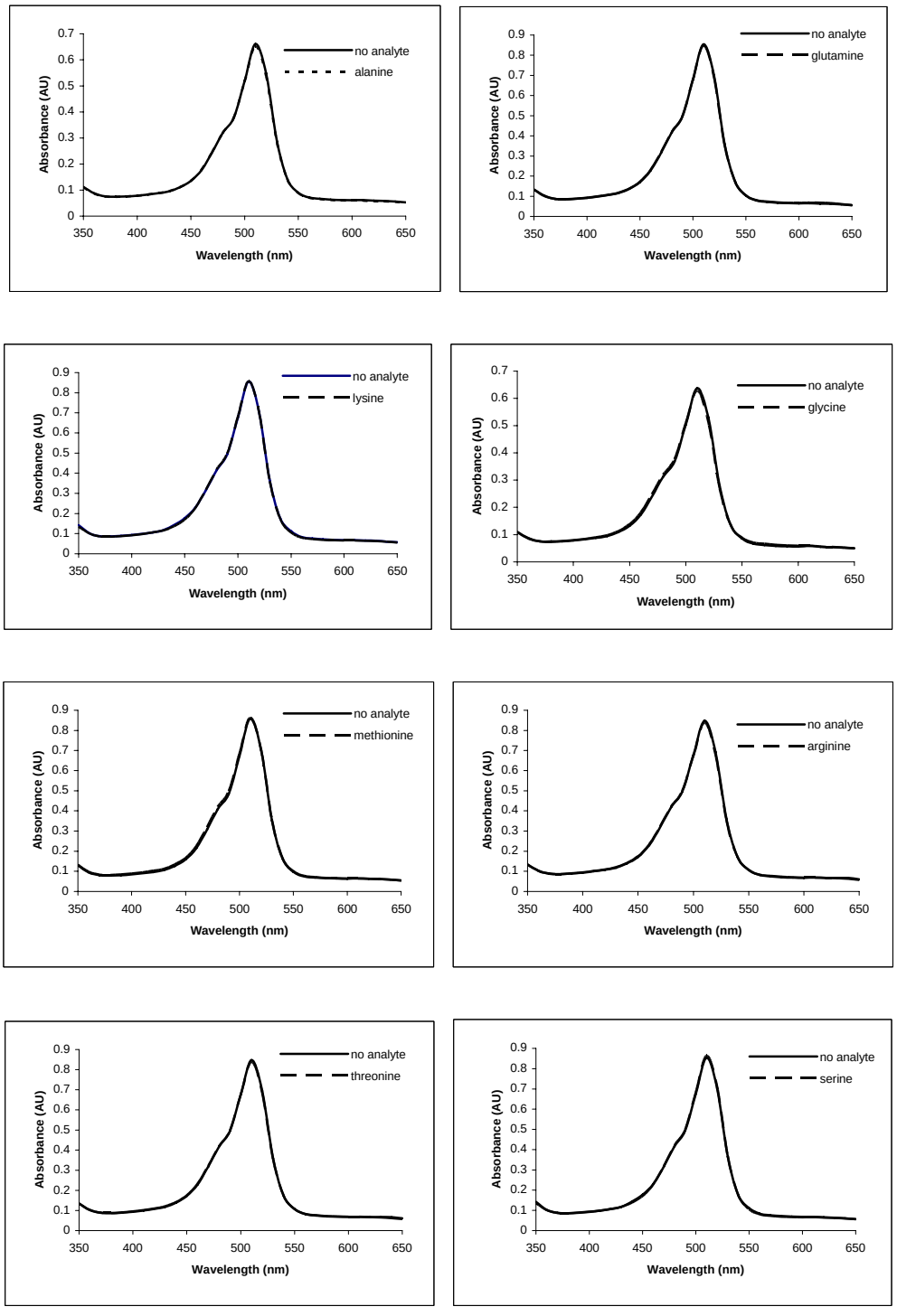


Figure A1. UV-Vis spectra of FB (1.0×10^{-5} M) in 30 % phosphate buffer (0.015 M, pH 7.3), 70 % MeOH in the presence of various amino acids (1.0×10^{-4} M). No absorbance changes are observed due to the presence of the amino acids.

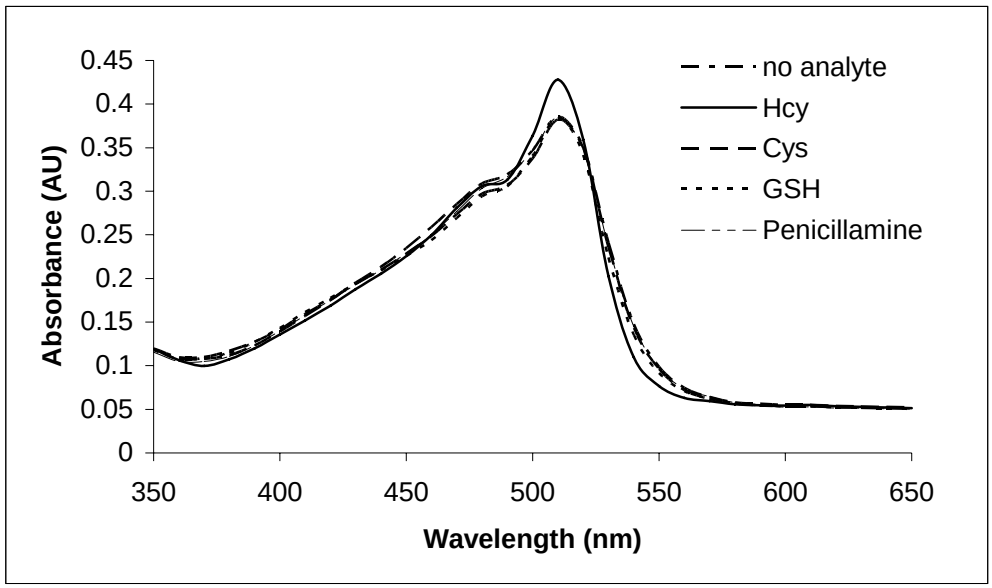


Figure A2. UV-Vis spectra of FB (1.0 x 10^{-5} M) in 30 % phosphate buffer (0.015 M, pH 7.3) and 70 % MeOH in the presence of various biothiols (1.0 x 10^{-4} M) and PPh_3 (4.5 x 10^{-4} M).

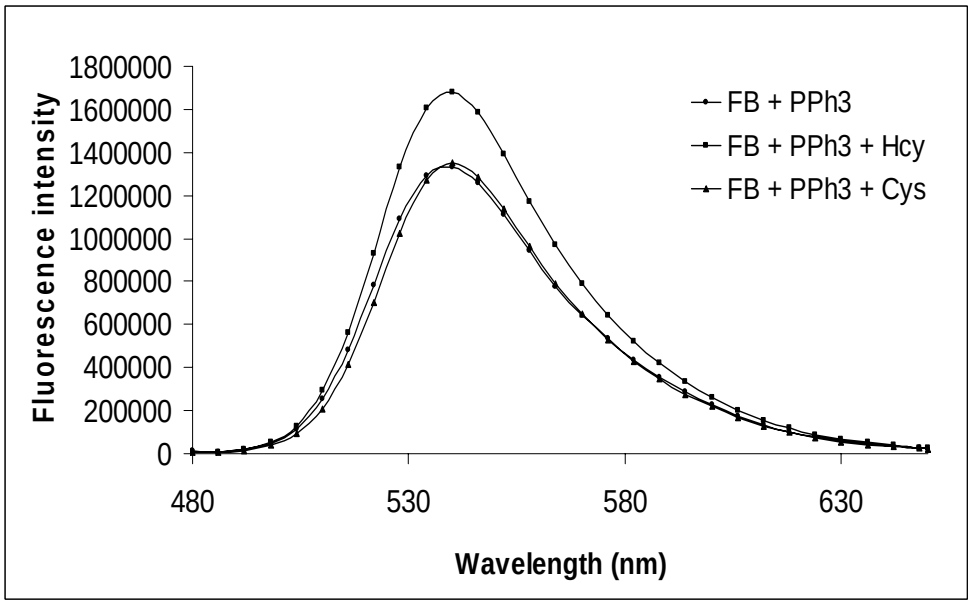


Figure A3. Fluorescence emission spectrum of FB (1.0 × 10^{-6} M) in the presence of PPh_3 (4.5 × 10^{-5} M) and Hcy or Cys (1.0 × 10^{-5} M). This demonstrates selective fluorescence emission responses to the presence of Hcy.

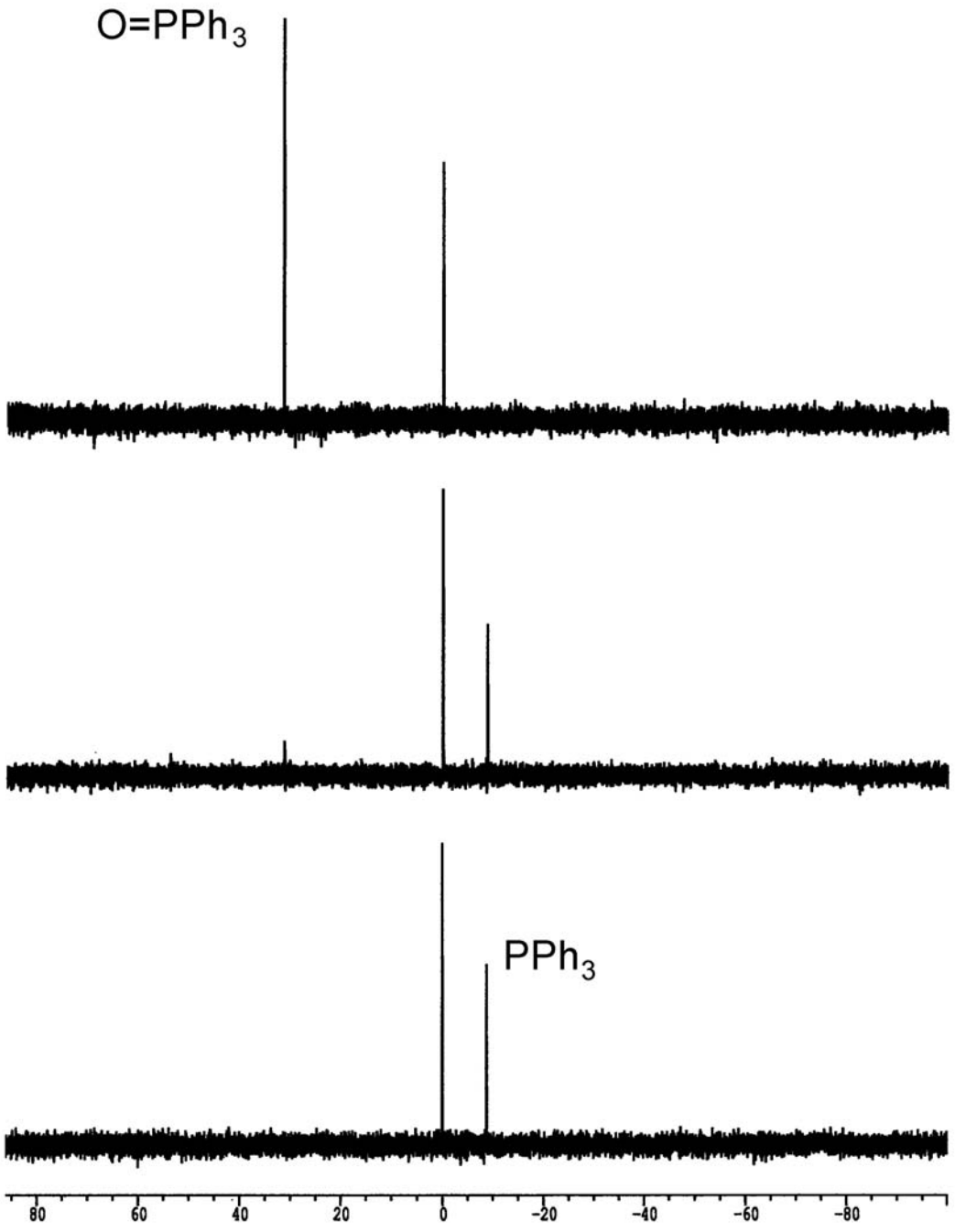


Figure A4. ^{31}P-NMR spectra of PPh_3 in CD_3OD:D_2O 7:3 in the absence of FB (bottom) and in the presence of FB (middle) after 19 hours. The resonance at 8.80 ppm corresponds to PPh_3. The resonance at 31.25 ppm corresponds to $O{=}PPh_3$ (top). It shows that the formation of $O{=}PPh_3$ is promoted in the presence of FB.

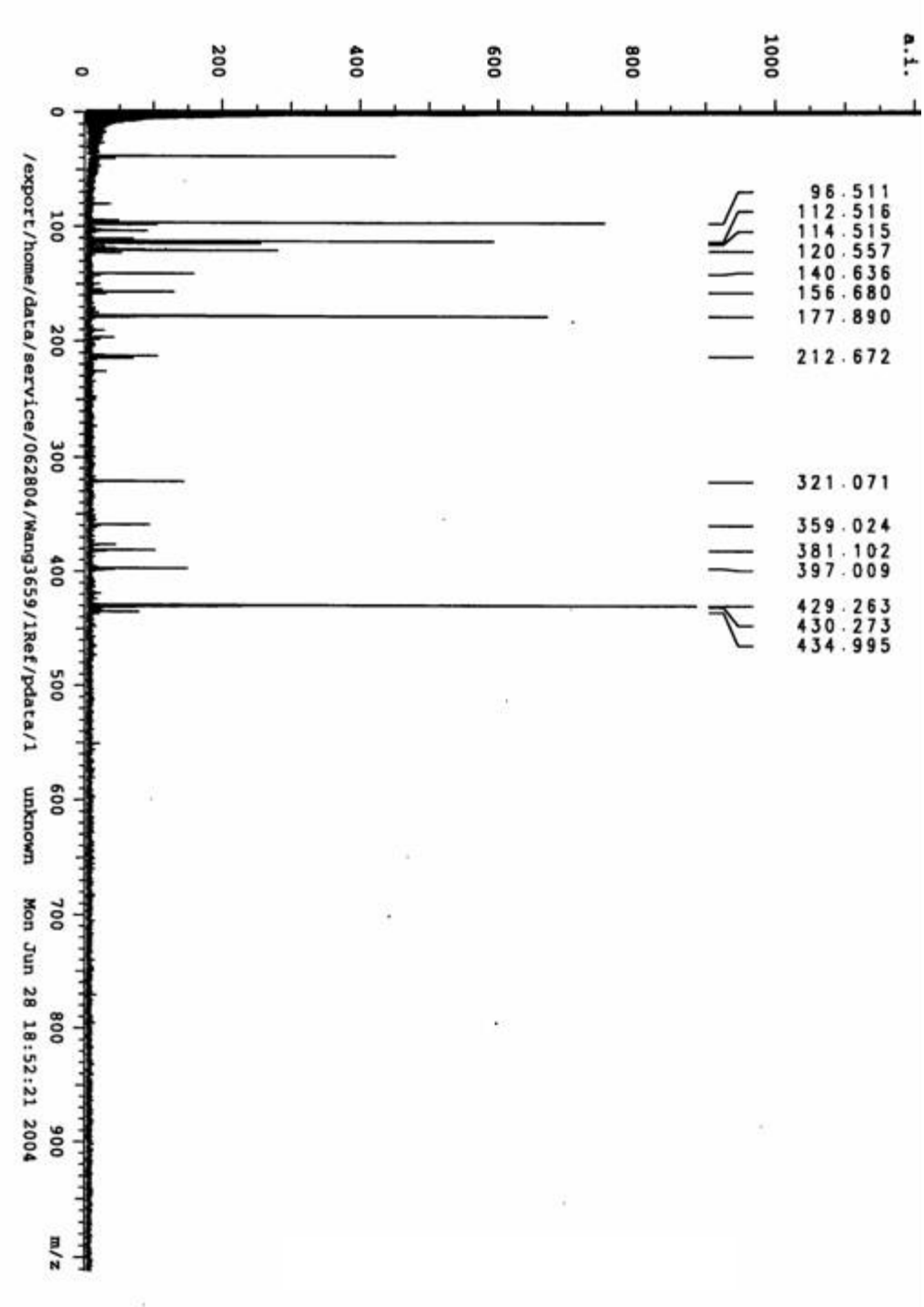


Figure A5. MALDI-TOF of the reaction mixture of FB and Hcy (no matrix)

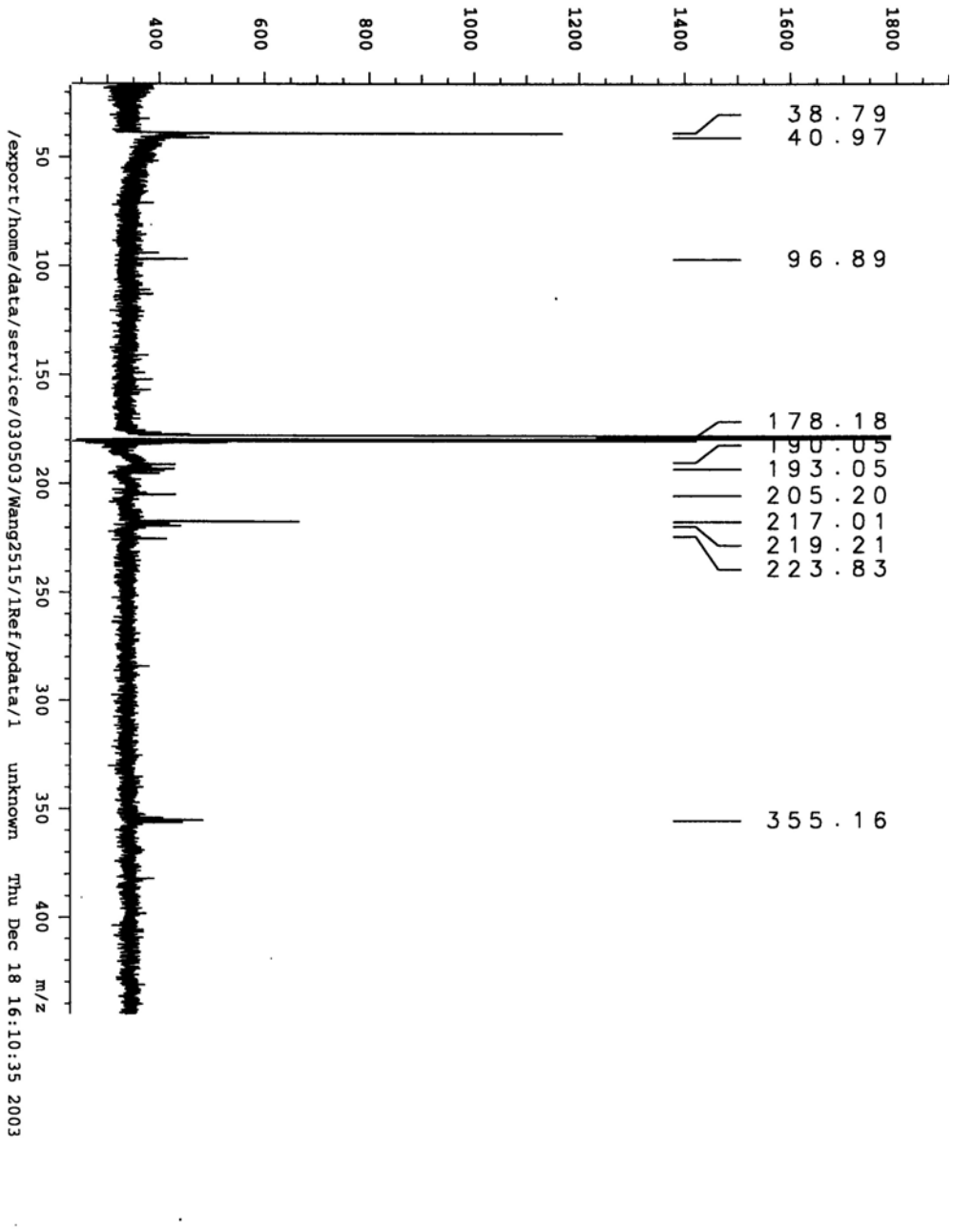


Figure A6. MALDI-TOF of the reaction mixture of FB and Hcy (anthracene matrix)

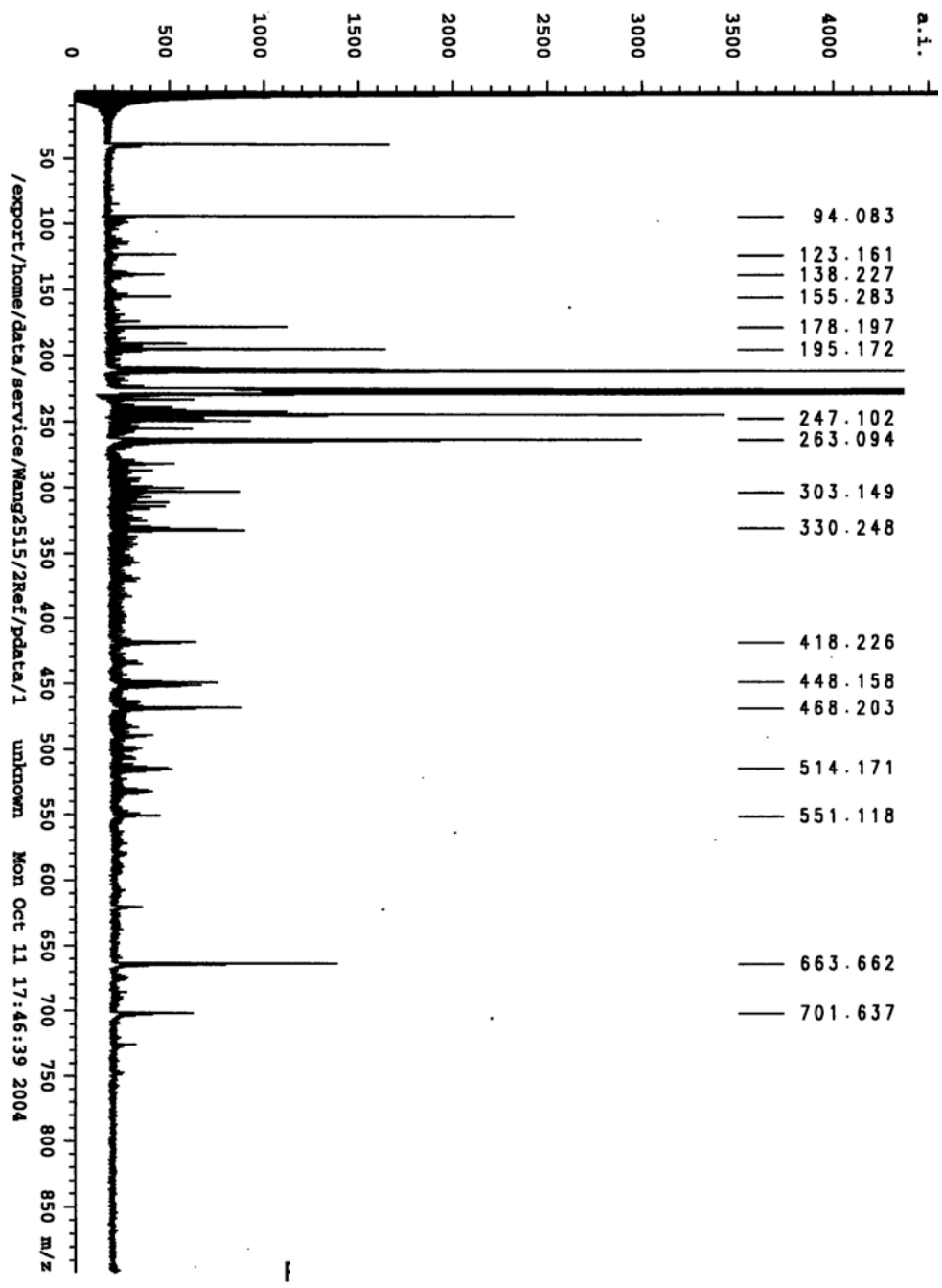


Figure A7. MALDI-TOF of the reaction mixture of FB and Hcy (dithranol matrix)

APPENDIX B. SYNTHESIS OF LANTHANIDE COMPLEXES

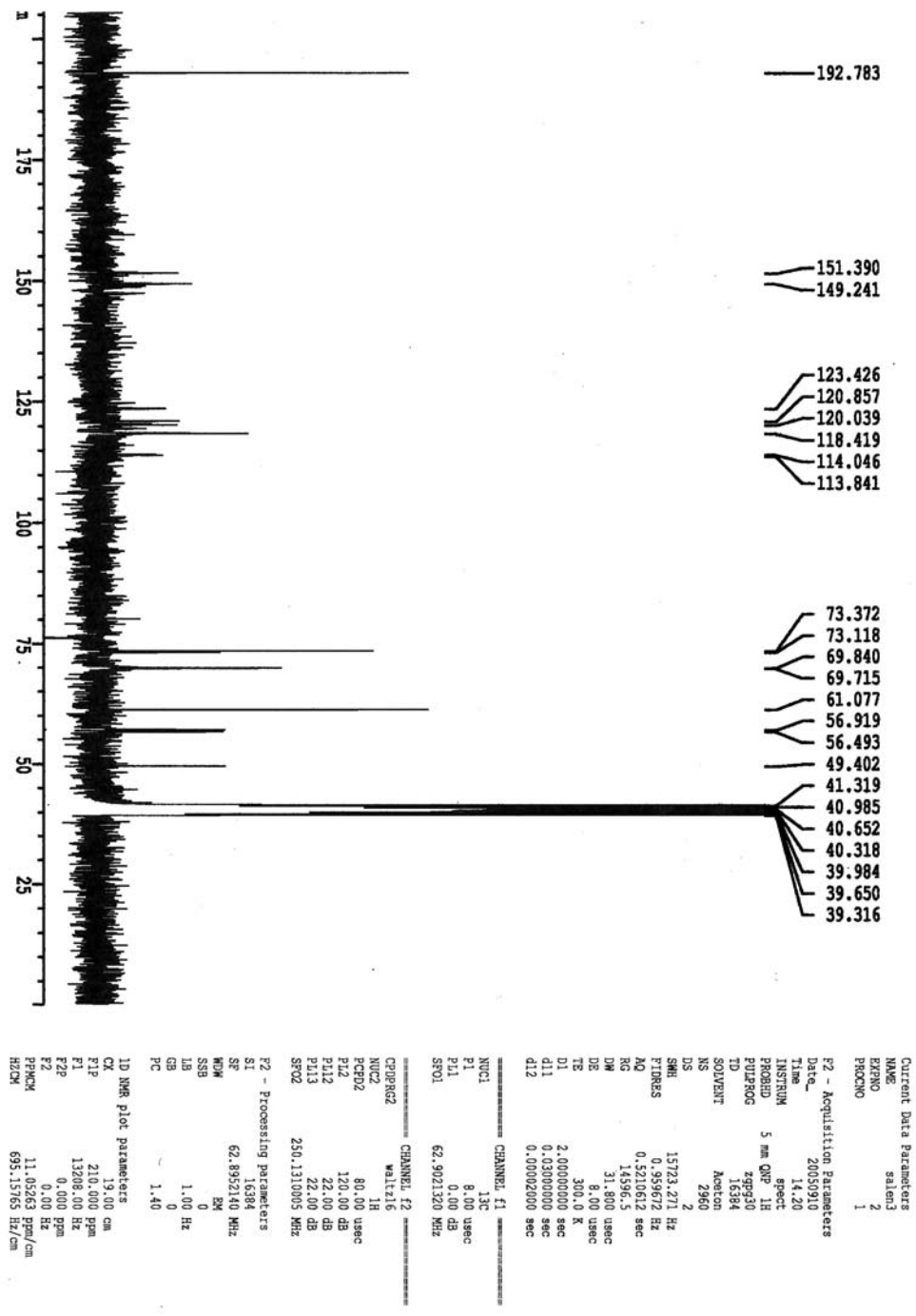


Figure B1. ^{13}C-NMR spectrum of **1** in DMSO-d_6.

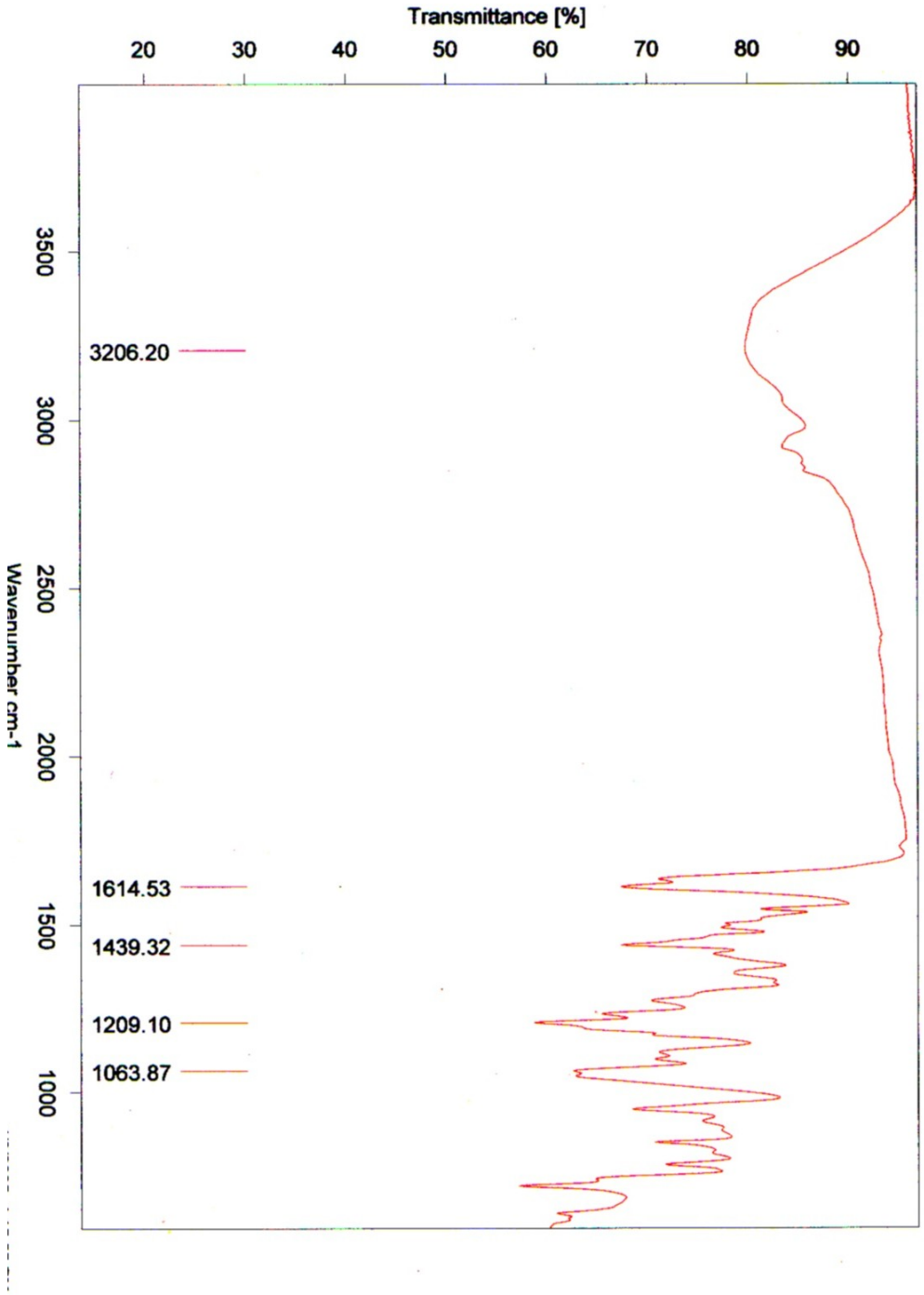


Figure B2. FTIR spectrum of **1**.

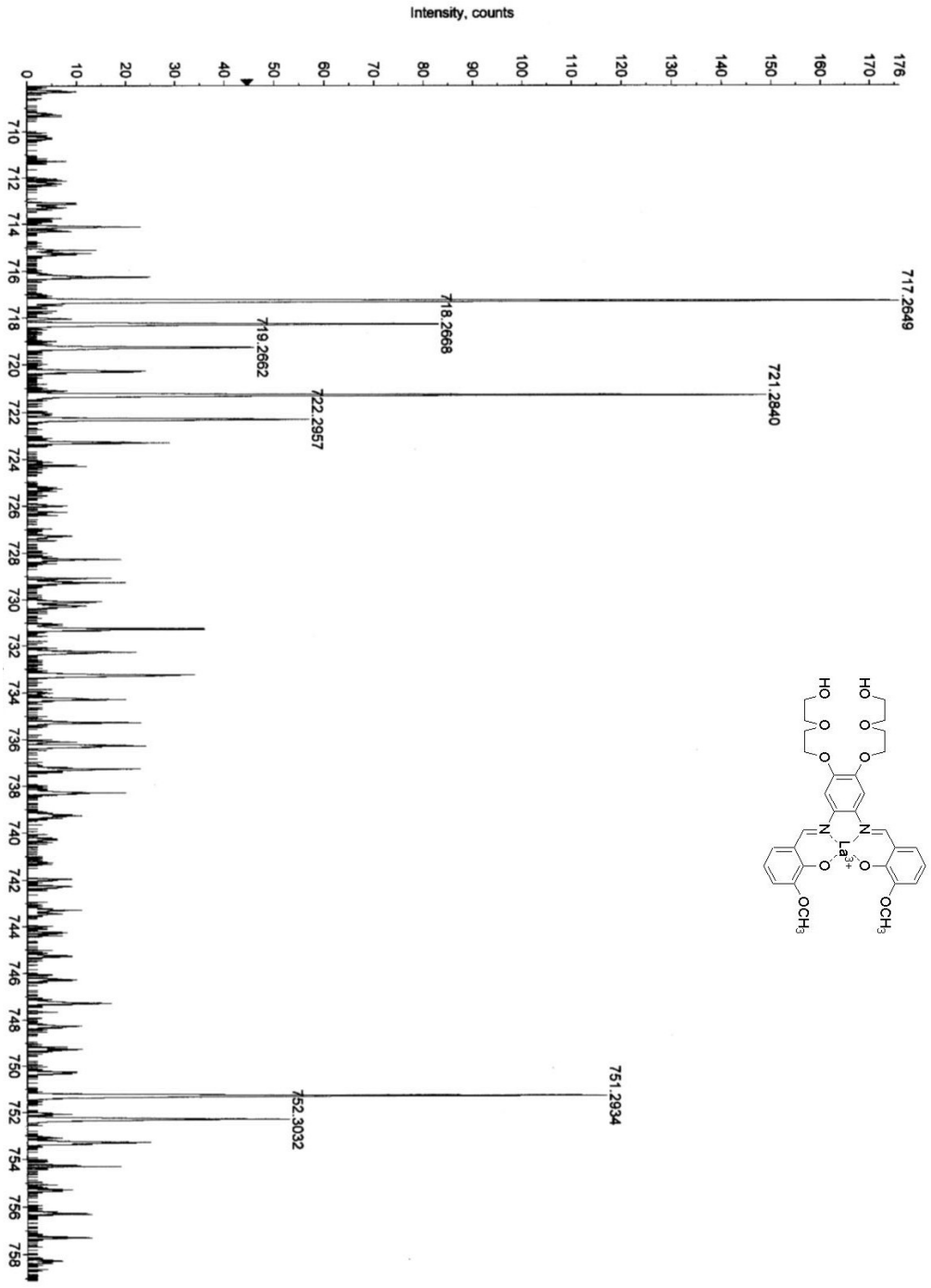


Figure B3. MALDI TOF MS spectrum of **1**.

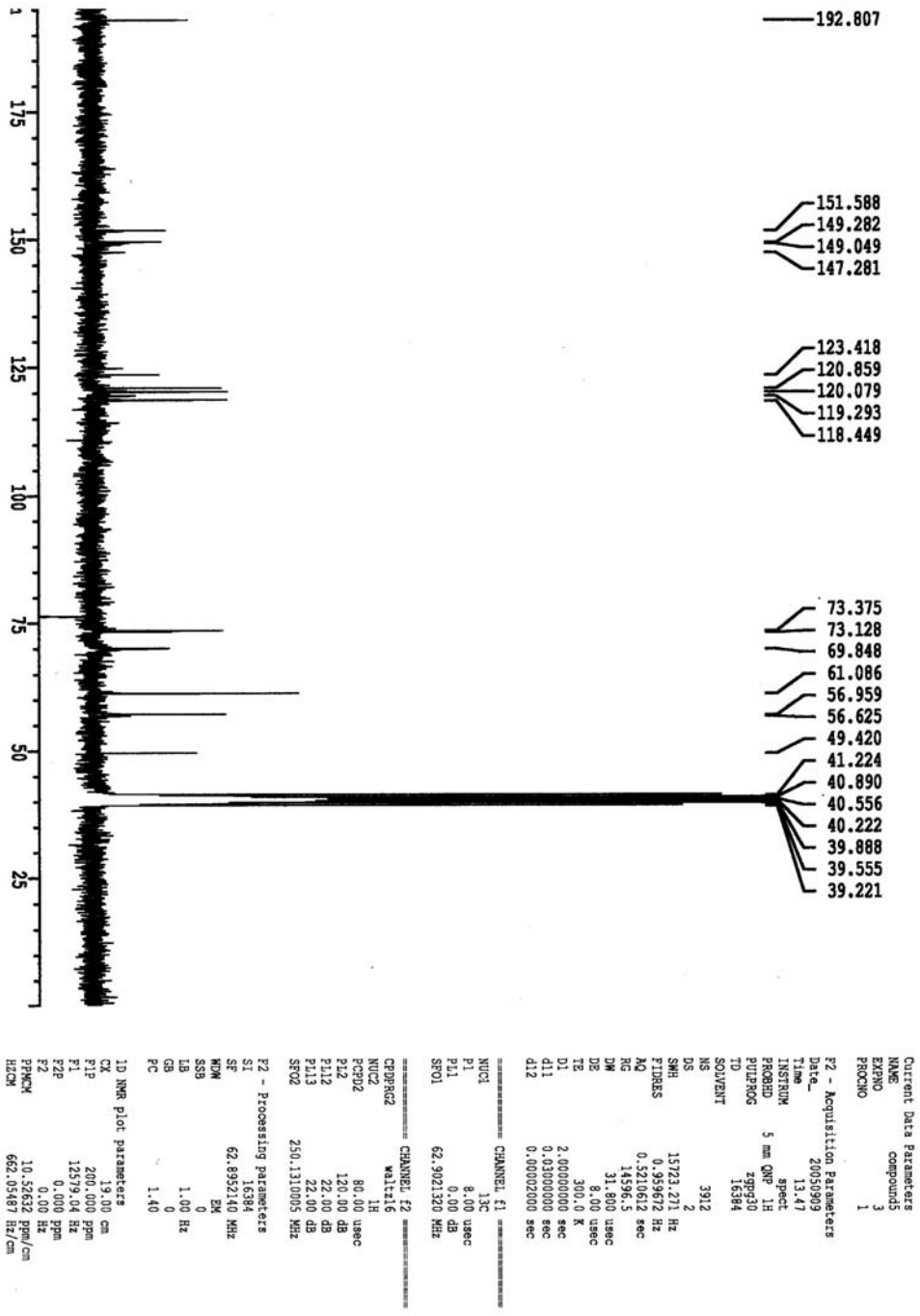


Figure B4. ^{13}C-NMR spectrum of **2** in DMSO-d_6.

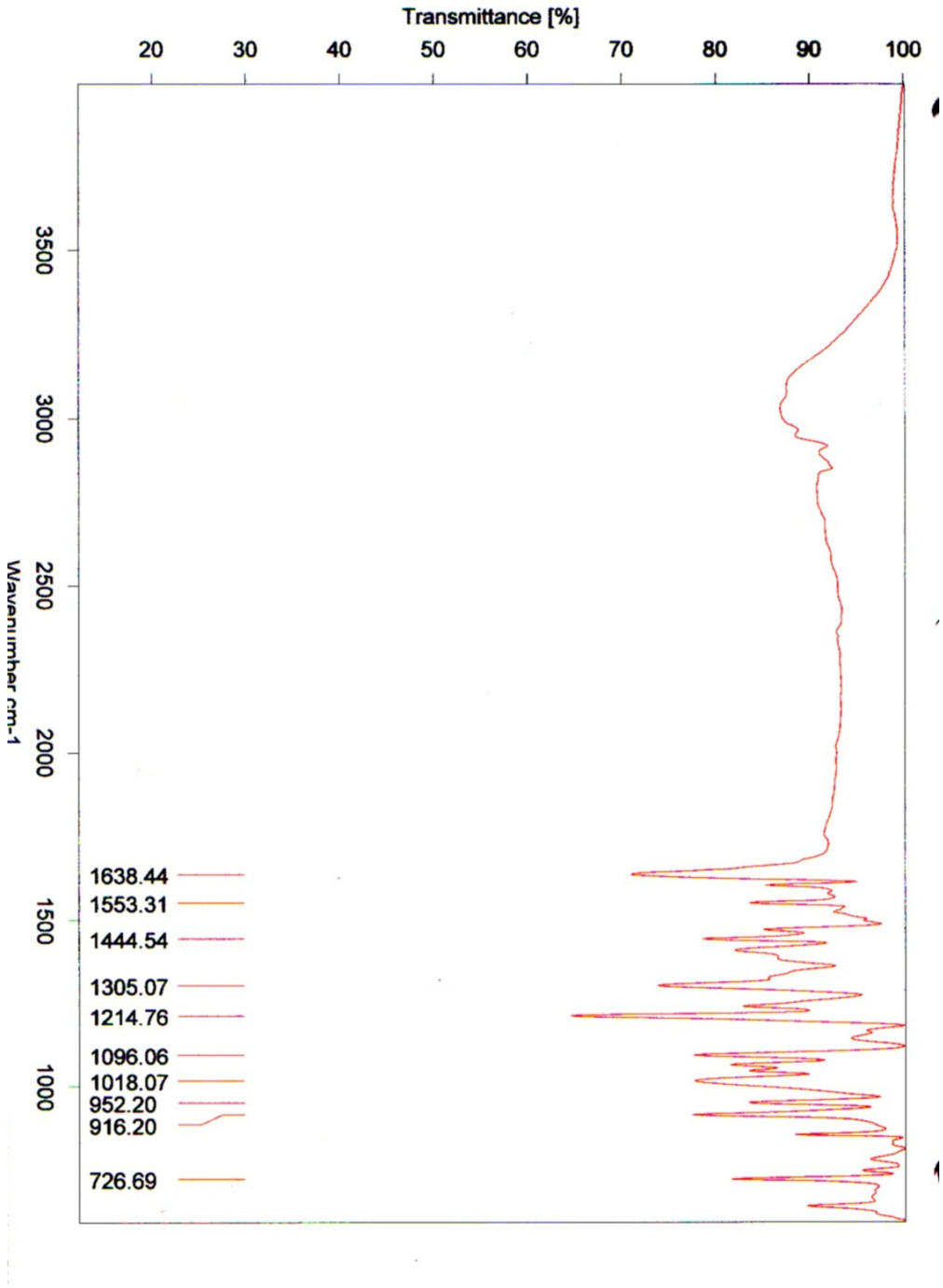


Figure B5. FTIR spectrum of **2**.

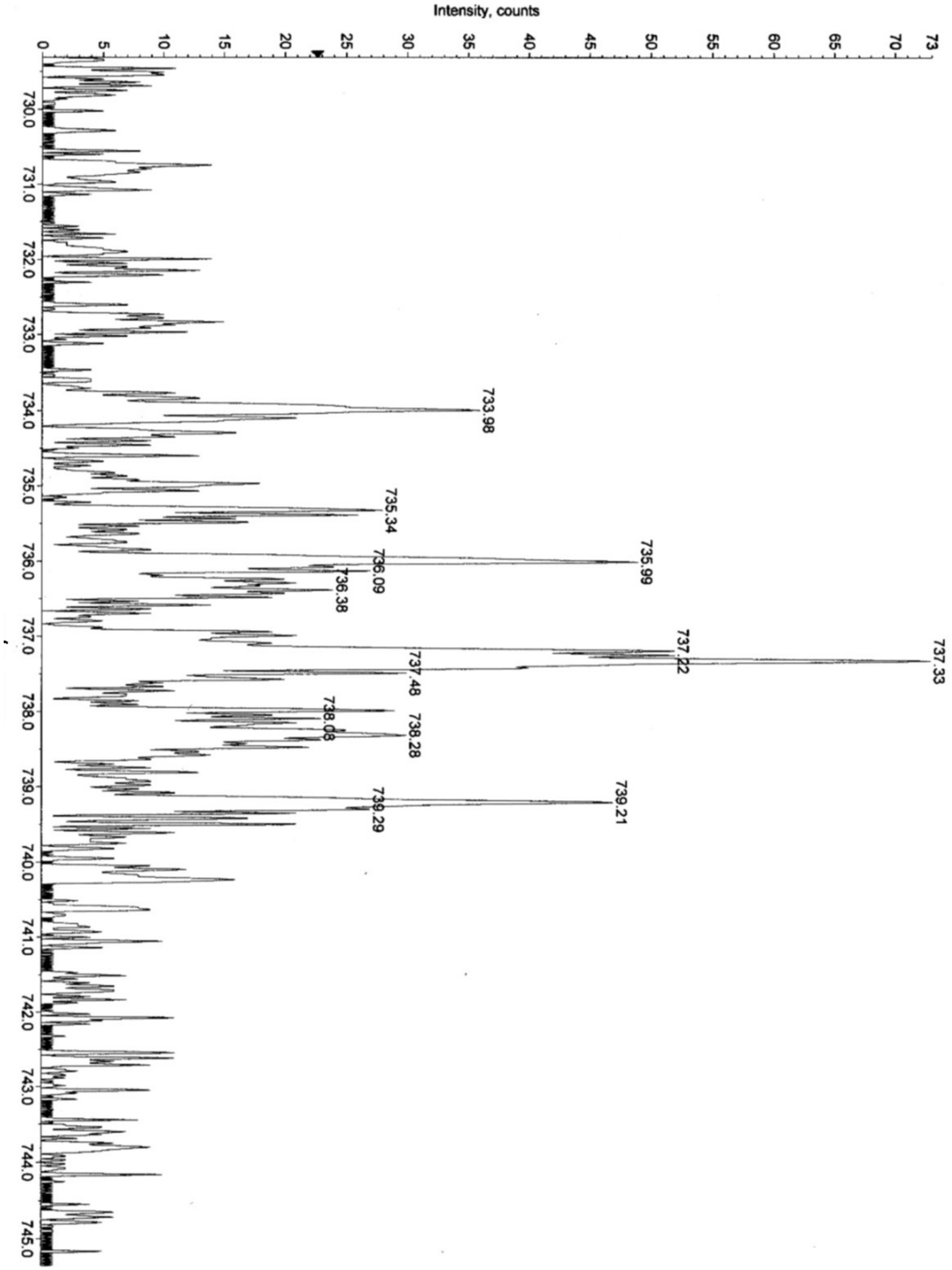
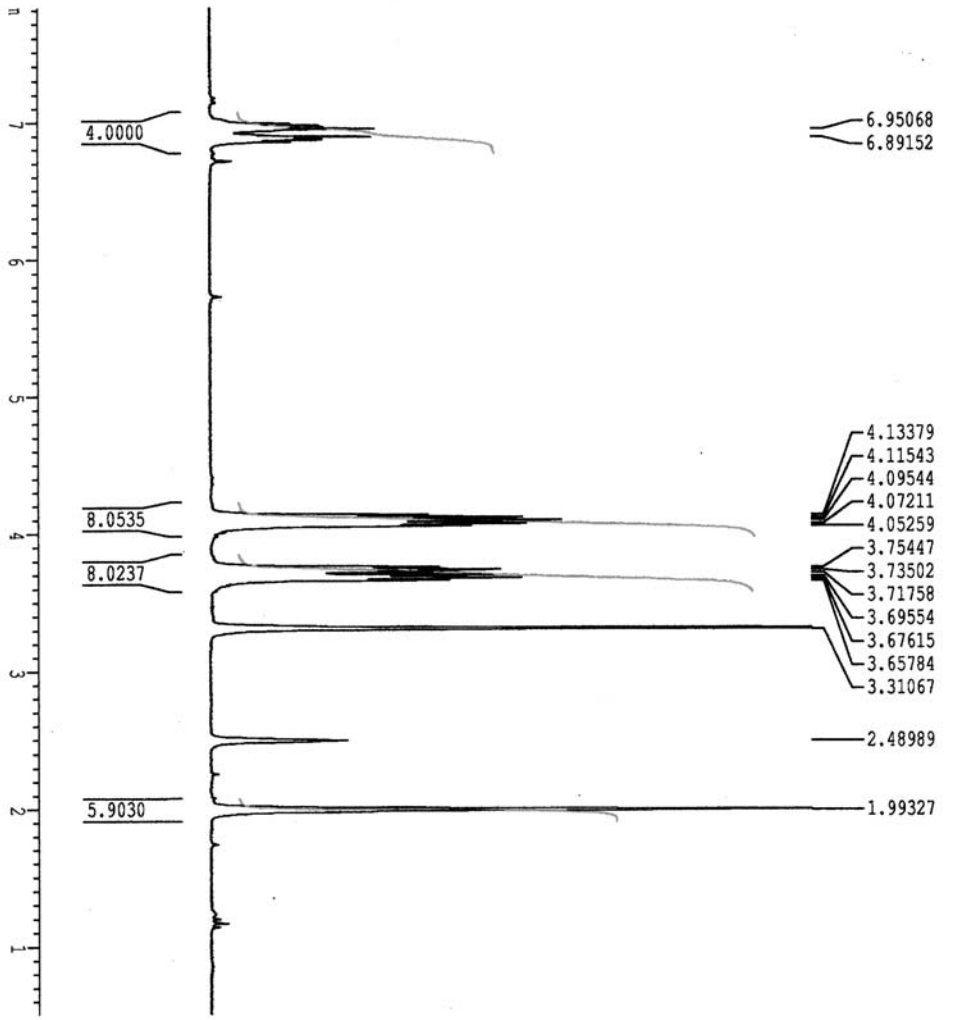


Figure B6. MALDI TOF MS spectrum of **2**.

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F2P                   0.500 ppm
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Figure B7. ^{1}H-NMR spectrum of **3** in DMSO-d_6.

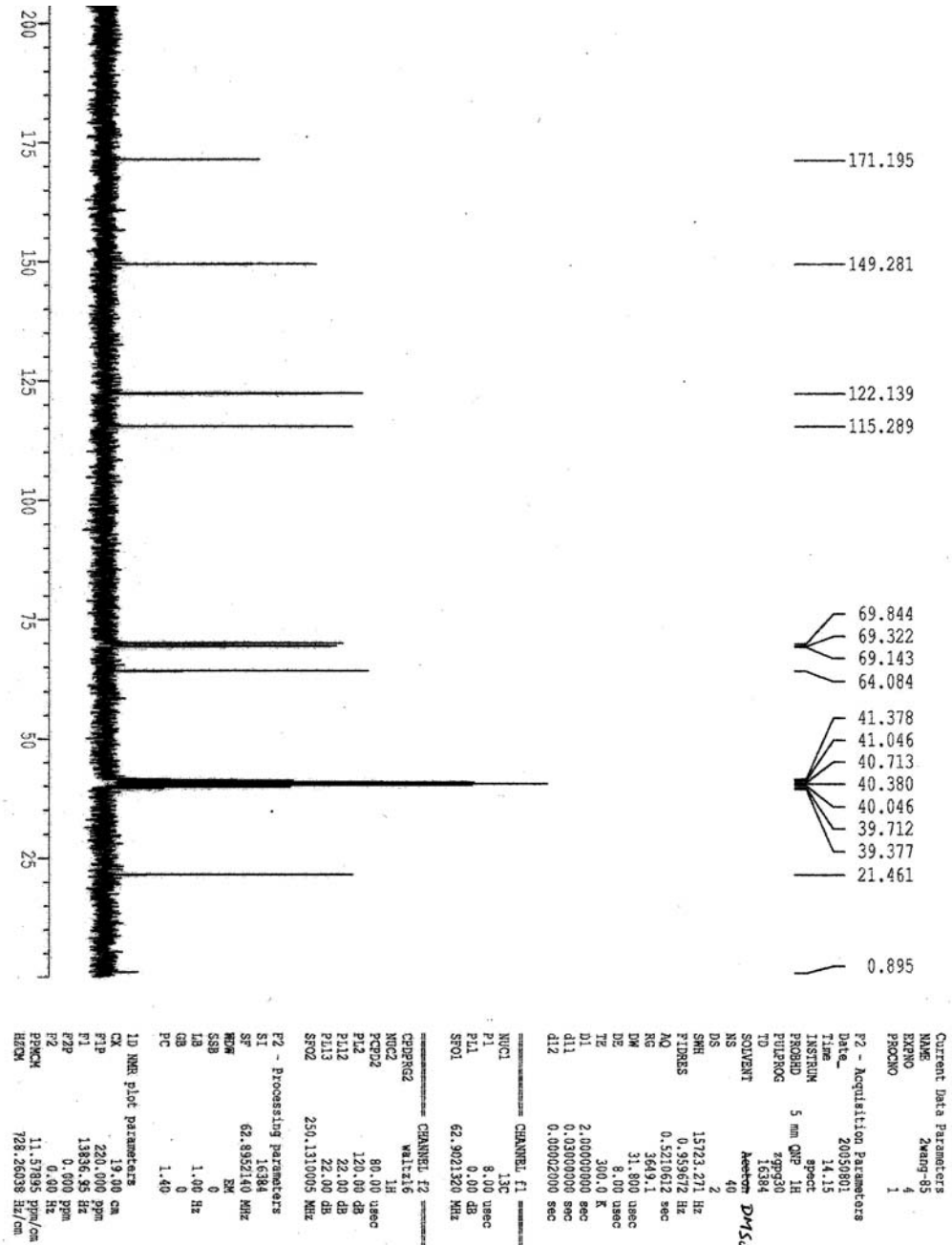


Figure B8. ^{13}C-NMR spectrum of **3** in DMSO-d_6.

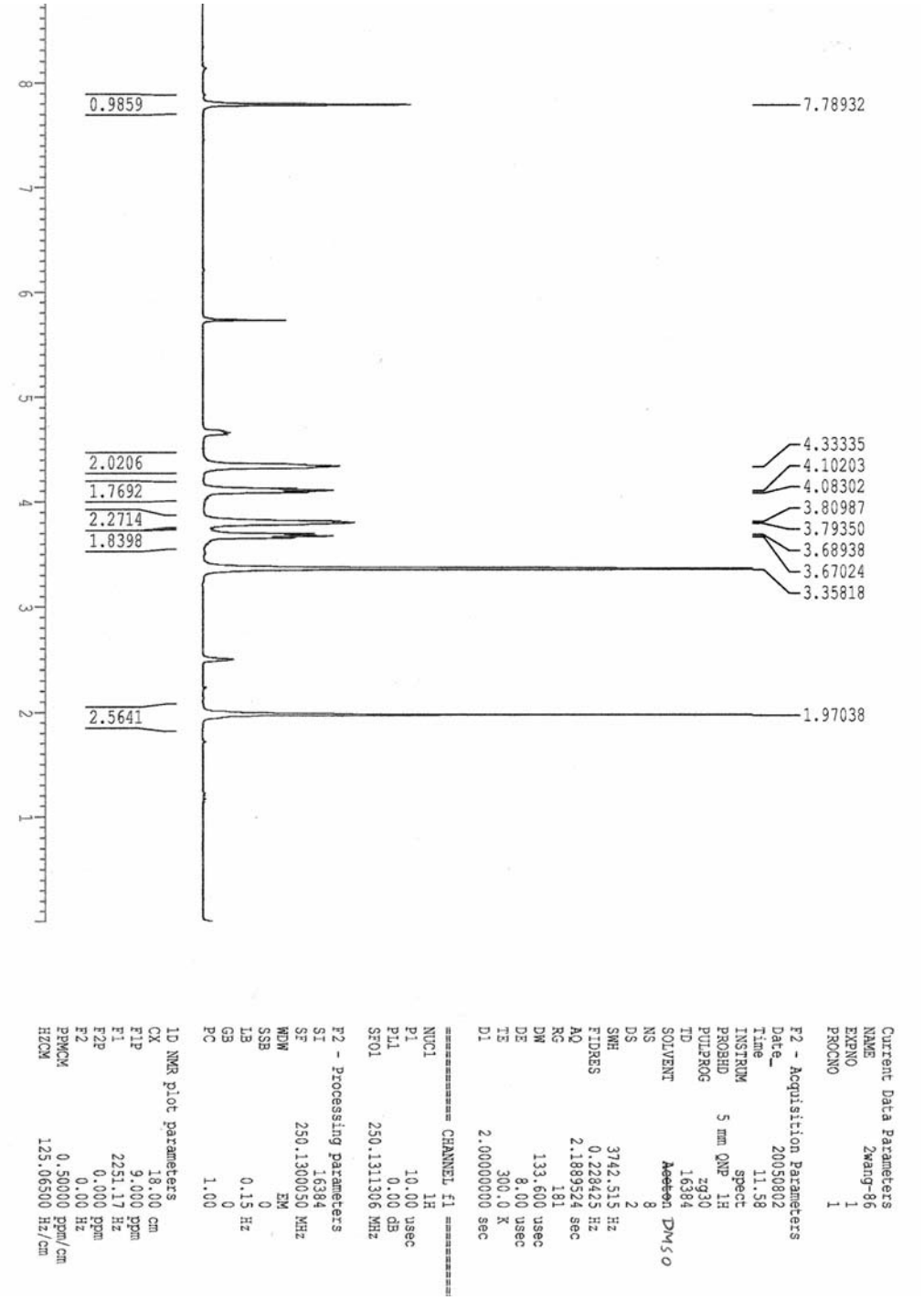


Figure B9. ^{1}H-NMR spectrum of **4** in DMSO-d_6.

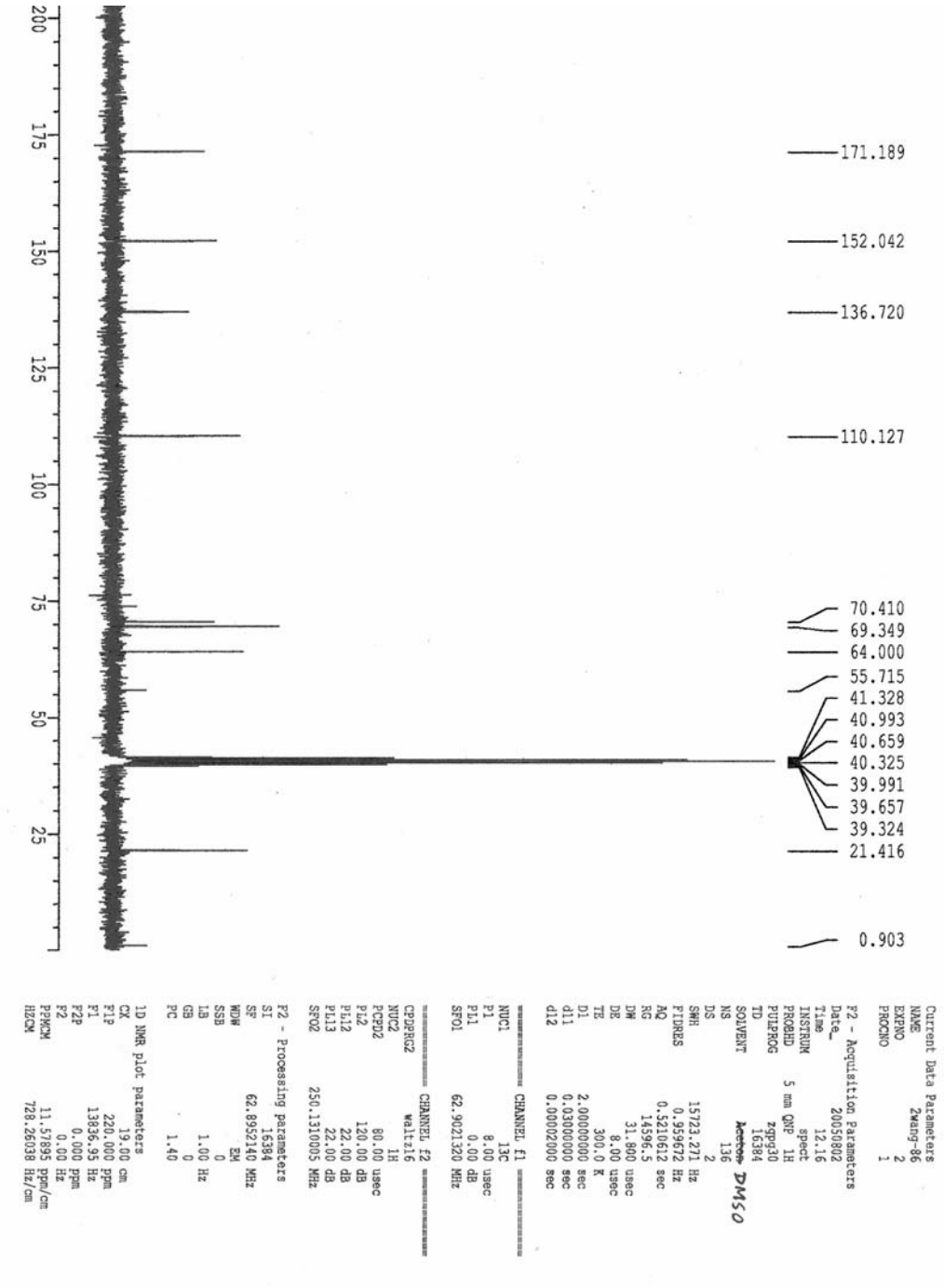


Figure B10. ^{13}C-NMR spectrum of **4** in DMSO-d_6.

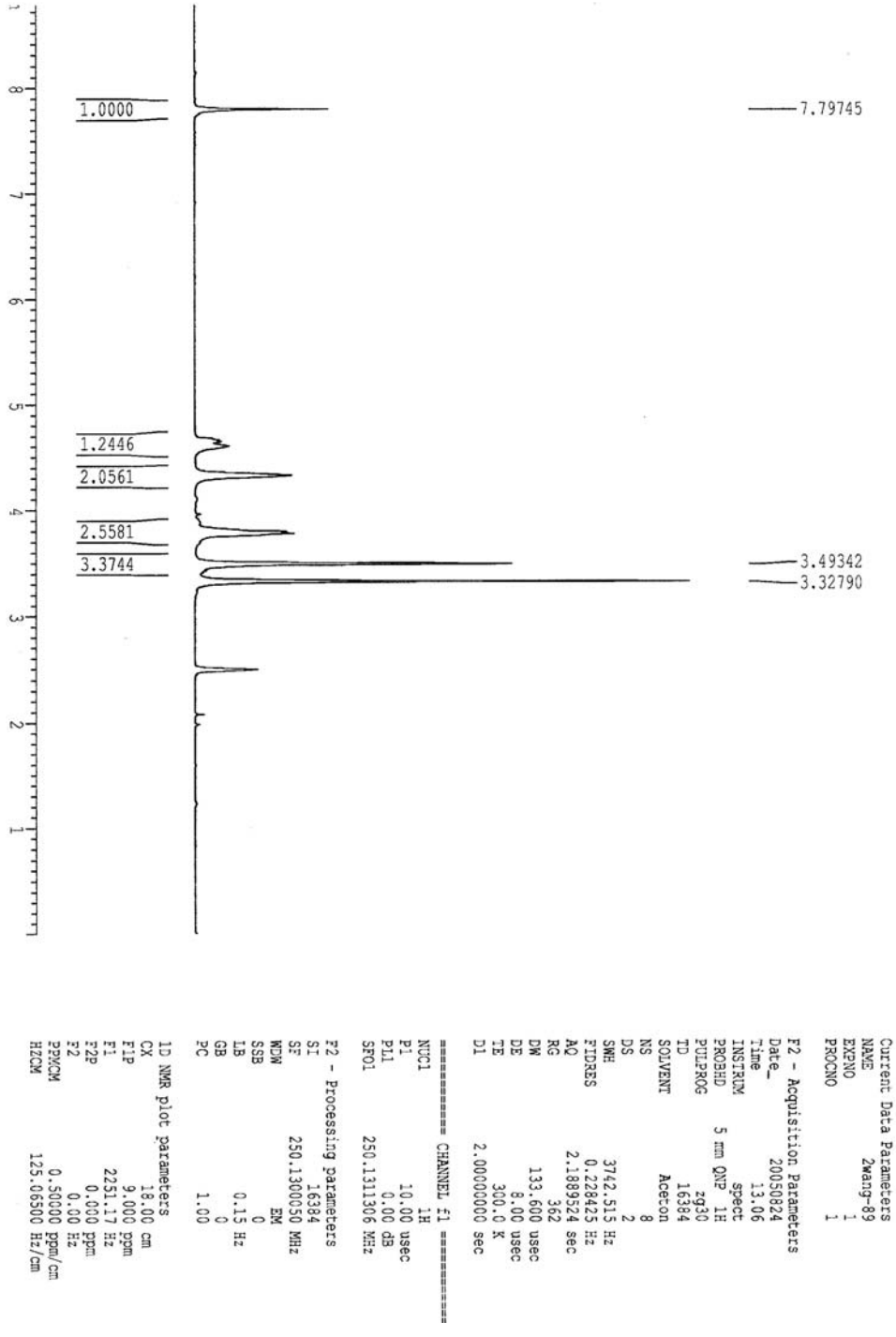
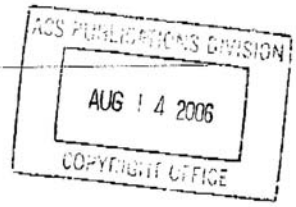


Figure B11. ^{1}H-NMR spectrum of **5** in DMSO-d_6.

APPENDIX C. LETTERS OF PERMISSION

Arleen Courtney



From: Weihua Wang [wwang4@lsu.edu]
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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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My name is Weihua WANG. I'm a graduate student in the Department of Chemistry at Louisiana State University. I'm writing to ask the permission for the use of my contributions to the article published in the "*Proceedings of the National Academy of Sciences of the United States of America*" in my doctoral dissertation. I'm one of the authors of this article. The article is:

"Lanthanide Complexes as Fluorescent Indicators for Neutral Sugars and Cancer Biomarkers", *PNAS*, **2006**, *103*, 9756-9760.

Thank you very much for your consideration of this request.

Sincerely,

Weihua Wang

From: "Millerd, Tiffany" <TMillerd@nas.edu>

To: "Weihua Wang" <wwang4@lsu.edu>

CC:

Subject: RE: permission

Date: Mon, 21 Aug 2006 11:39:37 -0400

Dear Ms. Wang,

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Please feel free to contact us with any additional questions you might have.

Best regards,

Tiffany Millerd for

Diane Sullenberger

Executive Editor

PNAS

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.