AN ABSTRACT OF THE THESIS OF

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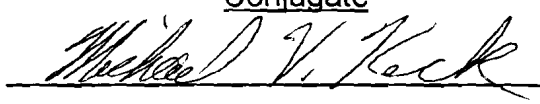
in Physical Science presented on April 7, 2000

Title: Design, Synthesis, and DNA/Avidin Binding Properties of a Platinum-biotin Conjugate

Abstract approved: 

This thesis entails the design, synthesis, and DNA/Avidin binding properties of a platinum-biotin conjugate, which would serve as a "proof-of-concept" model for a new strategy for rational chemotherapy drug design. A compound was designed which would be able to simultaneously bind to DNA and the protein avidin. A two-step synthetic strategy was used to obtain the desired conjugate. First, reaction of N-hydroxysuccinimidobiotin with diethylenetriamine produced the diethylenetriaminobiotin intermediate **1**. After purification, this intermediate was allowed to react with *cis*-bis(acetonitrile)dichloroplatinum (II) to produce the target compound, cis-diethlyenetriaminobiotinylchloro(acetonitrile)-platinum (II) chloride **2**. Compound **2** was bound to DNA, whereupon agarose gel electrophoretic mobility shift assays showed that it did indeed recruit avidin binding to the nucleic acid.

It is thus shown that compounds can be designed to recruit preselected proteins to bind to DNA, even when the normal physiological function of that protein is unrelated to DNA binding. If a protein can be preselected which is specific to tumor cells, this strategy may have implications with regard to rational chemotherapy drug design.

DESIGN, SYNTHESIS, AND DNA/AVIDIN BINDING PROPERTIES OF A PLATINUM-BIOTIN CONJUGATE

A Thesis

Presented to

The Division of Physical Sciences

EMPORIA STATE UNIVERSITY

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Jeffrey Ray Groth
May 2000

Approved by Division Chair

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Dean of Graduate Studies and Research

ACKNOWLEDGEMENTS

I would like to thank my thesis advisor, Dr. Michael Keck, and committee members Dr. Arthur Landis and Dr. David Saunders. Their aid in writing the thesis, along with experimental advice, was greatly appreciated. I would also like to thank Emporia State University for financial assistance through Research and Graduate Teaching Assistantships.

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CHAPTER 1-INTRODUCTION

Cancer and Chemotherapy

Cancer is a condition in which cells proliferate uncontrollably, and can form malignant and metastatic tumors that grow in an invasive manner. It is caused by a series of genetic mutations. Most current treatment methods involve chemotherapy and radiation. Both of these treatment types have little or no selectivity for cancerous or normal cells; hence, both types of cells are killed using these treatments. In cases where there does seem to be some cell type selectivity, the mechanism of selectivity is usually not known.

Since cancer is genetic in origin, a logical route to a possible cure would be through DNA targeting. Indeed, many current chemotherapy drugs are believed to have DNA as the intramolecular target. Drugs that target DNA have a number of different interaction modes available to them. One such mode is DNA cleavage; that is, the drug can bind to DNA and produce single- or double-stranded breaks with great efficiency. An example of a currently used anti-neoplastic agent which works in this manner is the natural product bleomycin. A second DNA modification mode is noncovalent association with the minor groove of the DNA as exemplified by netropsin and distamycin. A third DNA modification mode is intercalation. Intercalating agents possess planar aromatic rings that insert between adjacent base pairs in DNA. Examples of intercalating drugs include actinomycin D, adriamycin, and daunomycin. A fourth DNA modification mode is alkylation. Molecules employing this mechanism bind covalently to DNA,

resulting in an adduct which inhibits nucleic acid synthesis or transcription. One example of an alkylating agent is anthramycin.[1]

Despite the success to date with anti-neoplastic agents that apparently target DNA intracellularly, these drugs are far from perfect. One major drawback, which is manifested via limited success rates and severe side effects, remains the inability to selectively target the cancerous cells without affecting the normal cells. Common side effects originating at least in part from this lack of selectivity include hair loss and nausea, as well as the development of secondary tumors resulting from the chemotherapy.

An additional problem with chemotherapeutic agents is that their effectiveness frequently becomes limited by the emergence of drug-resistant tumor cell populations.[2] This is especially true for re-emergent tumors, which often do not respond to the same drug used to treat the original tumor. One mechanism for resistance to DNA-targeted chemotherapy agents is the ability of cells to repair DNA adducts formed by the drug. DNA repair effectively reverses the therapeutic effects. Indeed, an ideal chemotherapy agent would be one which is selective for cancerous cells and which creates a DNA lesion that cannot be repaired, thereby ultimately ensuring the death of the cancer cell. A strategy for rationally designing and delivering such selective compounds has not yet been realized, but is an active research goal in many laboratories throughout the world. This thesis describes the development of one such strategy, based on the chemistry of cisplatin.

Cisplatin

cis-Diamminedichloroplatinum(II) (cisplatin), Figure 1a, has been demonstrated to be a clinically effective anticancer drug.[3] The compound, known since before the twentieth century, is a prototypical square-planar transition metal complex, whose properties are similar to those of the other group VIIIB transition metals.[3] In fact, cisplatin is a very important compound in the history of inorganic chemistry, having been studied by Alfred Werner, who correctly formulated its structure and used it to elucidate the phenomenon of cis/trans isomerism. Its antitumor activity was discovered serendipitously in 1965 by Barnett Rosenberg and coworkers.[4] They were studying the effects of electric fields on *Escherichia coli* bacteria. In their experiments, these electric fields were generated using platinum electrodes immersed in cell growth medium. It was observed that cell division was inhibited under these conditions. It was subsequently found that this inhibition was due to the presence of Pt(II) in the medium as a result of electrode oxidation. Further investigation showed that concentrations of only 1-10 ppm of group VIIIB transition metal ions could cause this inhibition.[4] Of all the metal complexes tested, *cis*-diamminedichloro-platinum(II) was the metal complex that was found to be the most effective antitumor agent. Clinical use began in the early nineteen seventies. Interestingly, the *trans*-isomer, Figure 1b, does not exhibit useful chemotherapeutic activity.

The mode of chemotherapuetic action of cisplatin is believed to involve the formation of platinum-DNA adducts capable of blocking DNA replication.[5] Cisplatin binds with a high affinity to the purine N7 positions of DNA bases, primarily guanine (G) but also adenine (A), both of which are shown in Figure 2. Cisplatin typically forms bifunctional lesions, primarily between adjacent purines on the same DNA strand. The adducts profile has been shown to be 65% cis-GG, 25% cis-AG, and approximately 10% cis-GXG, and interstand crosslinks, where X can be any DNA base pair.[6] Examples of these adducts are illustrated in Figure 3. The 1,2-intrastrand adduct is known[7] to substantially distort the structure of the double helix, causing a 13° unwinding and a bend of 32-34°. The level of structural alteration is dependent upon the type of adduct formed, which in turn affects the extent to which the adducts are recognized by intracellular proteins, including damage recognition proteins and various nucleotide excision repair (NER) complexes. The unwinding is most likely a major determinant in the recognition of cisplatin-induced DNA damage by the *E. coli* (A)BC excinuclease.[7,8,9] Eukaryotic analogs to this NER system are known, and they have also been shown to repair cisplatin-induced DNA damage in an adduct-specific manner.[8,10] Hence, cisplatin lesions can be repaired by intracellular repair systems, thereby leading to a decrease in drug effectiveness and to the development of resistance. It logically follows that if the repair systems can be impeded, the effectiveness of cisplatin as a chemotherapeutic drug should be increased.

There is evidence that such a repair-shielding process occurs *in vivo*. Cisplatin-DNA adducts have been shown to be substrates for binding of certain classes of proteins. The High Mobility Group Domain proteins represent one such class.[8]

High Mobility Group Domain Proteins (HMG-Domain Proteins)

The HMG group proteins are so named because of their anomolously fast electrophoretic migration rates. These proteins are known to bind specifically to certain "unusual" DNA structures, such as cruciforms, although the true cellular function of this protein group remains unknown.[11] All of the proteins within this group share a common sequence and structural element, which is also shared with some other DNA-binding proteins of known function.[9] In 1994, Huang et. al. found that HMG-domain proteins specifically inhibit the repair of major DNA adducts of the anticancer drug cisplatin by the human excision nuclease repair system in HeLa S3 cell extracts.[8] Two other proteins containing the HMG-domain, HMG1 and the human mitochondrial transcription factor (h-mtTFA)[9], when bound to the cisplatin-DNA adduct, also inhibited the human excinuclease. In a later study, rat HMG1 and a murine testis-specific HMG-domain protein showed similar results.[10]

The HMG-domain proteins have been postulated to mediate the antitumor properties of cisplatin. HMG1 has been shown to bind to the widened minor groove of 16-base-pair DNA duplex containing a site-specific platinum-DNA adduct.[12] HMG1 has two domains, both of which recognize and bind to the 1,2-

d(GpG) and 1,2-d(ApG) DNA intrastrand crosslinks created by cisplatin. The binding occurs in the minor groove of the DNA duplex with the concave surface of the HMG domain. The HMG-domain protein causes the DNA to bend towards the major groove by around 61°.[12] As previously noted, the platinum-DNA lesion causes a 32-34° bend to the DNA duplex. Thus, HMG nearly doubles the bend when it attaches to the platinum-DNA lesion. The combination of both HMG and platinum binding to the DNA is believed to be such a large physical barrier that it impedes recognition or processing of the lesion by the DNA repair system.

It is believed that the HMG proteins function as a "molecular shield", covering up the platinum-DNA lesion and protecting the lesion from repair. The molecular shield that is formed also prevents cell replication and transcription. This shielding effect is dependent on the type of adduct present, with the greatest effect seen for intrastrand GG and AG crosslinks, and with little effect on GXG.[9] Results indicate that the HMG-domain proteins can block excision repair of the major cisplatin-DNA adducts and suggest that such an activity could contribute to the unique sensitivity of certain tumors to cisplatin.[9] Interestingly, it may also contribute to therapeutic effectiveness by presenting a more substantial obstacle for replication and transcription enzymes.

The observations discussed above, namely that proteins bound to drug/DNA adducts can shield these adducts from repair and/or enhance replication and transcription disruption, has several potential implications for chemotherapy. One of these is the suggestion of novel chemotherapeutic

strategies. For example, can lesions be shielded specifically in tumor cells? This might be possible if a tumor-specific protein could be recruited to bind to the DNA/drug adduct and shield the lesion from the DNA repair system. To do this, three major questions need to be addressed: (1) Can an appropriate tumor-specific protein or other macromolecule be identified? (2) Can a chemotherapeutic drug be designed or modified so that it can recruit this protein to bind to the DNA? (3) If the first two questions are answered in the affirmative, will the desired biological effects be achieved? The first two questions are discussed below as relevant principles, and the third is addressed experimentally in this thesis.

Telomerase

One potential tumor-specific protein that might serve as a reasonable target in this "shielding" strategy is telomerase. Telomerase is a catalytic ribonucleoprotein, containing both essential proteins and an essential RNA component.[13] It recognizes chromosome ends, called telomeres, and extends the 3' termini by addition of specific repeat sequences.[11] Such activity is necessary because, due to the inherent chemistry of the DNA replication process, the telomeres shorten during every cell division.[14] An unavoidable result of telomere shortening is that after some finite number of replications, important DNA sequence information must be lost and cell death ensues. Thus, telomere shortening has been correlated with the aging process.[15] Telomerase activity is thus necessary to ensure that no important DNA information is lost.

The telomeres from distantly related eukaryotes show conserved features of short sequence motifs that typically contain guanine-rich strands and cytosine-rich complementary strands.[16] One example is the ciliate *Tetrahymena,* whose DNA contains tandem TTGGGG repeats. Another example is the TTAGGG repeat of the DNA of humans and other mammals.[17] A single cell may have 40,000-1,000,000 telomeric repeats, depending on the species.[18] Telomere addition proceeds by using as a template a short region within the RNA component of the telomerase enzyme.

Normal cells appear to lack telomerase activity. Telomerase is typically active only during fetal development, and is inactivated shortly after birth. Thus, during the lifetime of the organism, DNA gets progressively shorter during each round of replication. In most human tumor cells, the telomerase is activated and the telomere length does not decrease. Immortality is thereby conferred to the tumor cell.[15] Telomerase thus represents a tumor-specific protein which might be a target for the chemotherapeutic strategy presented above.

Hypothesis and Experimental Goals

Based on the foregoing discussion, a novel strategy for chemotherapy could be to impair the repair of drug-DNA adducts selectively in tumor cells by recruiting a tumor-specific protein to bind to and shield the lesion. In order to accomplish this, a target drug must be made which can simultaneously bind to the DNA and to a tumor-specific protein, thereby forming a ternary complex which is resistant to repair and which can block DNA replication and transcription.

In this thesis, a well-known ligand-binding interaction of biotin and avidin is utilized in a "proof of concept" set of experiments. Biotin, also known as vitamin H, is a cofactor for several enzymes which catalyze carboxylation reactions. In the 1940s, an unknown protein found in chicken egg whites was discovered to bind very tightly to biotin.[19] This protein, named avidin, is a basic glycoprotein consisting of four identical subunits. These four identical subunits are the binding sites of biotin; hence, one avidin molecule can bind four biotin molecules.[20]

The tight binding of biotin to avidin is utilized in this set of experiments to recruit the protein to bind to DNA. The biotin is attached to DNA through a cisplatin-derived conjugate. The target compound synthesized to mediate these interactions for this purpose is *cis*-diethylenetriaminobiotinylchloro(acetonitrile)-platinum(II) chloride. This compound was found to bind DNA, as demonstrated by the unwinding of supercoiled plasmid DNA. It was subsequently shown using bandshift assays that this compound could in fact recruit the binding of the protein avidin to DNA.

CHAPTER 2-MATERIALS AND METHODS

Diethylenetriamine, N-hydroxysuccinimidobiotin, and avidin were purchased from Sigma Chemical Company. N,N-Dimethylformamide was purchased from Fisher and vacuum distilled from barium oxide. *cis*-Bis(acetonitrile)dichloroplatinum(II) was purchased from Aldrich Chemical Company. pBR322 plasmid DNA was purchased from Boehringer Mannheim. The deuterated solvent d^6-DMSO was purchased from Cambridge Isotope Laboratories.

Fourier transform infrared spectra were obtained using a Bomem MB-100 FTIR. UV-Vis spectra were recorded using a GC McPherson EU-700 Series spectrophotometer. Proton nuclear magnetic resonance spectra (400 MHz) were obtained at the University of Kansas with the assistance of Jeff Botts and Erick Honores. Fast atom bombardment mass spectra [FABMS(+)] were obtained at the University of Kansas with the assistance of Professor Andrew Borovik.

Synthesis of Diethylenetriaminobiotin(1) N-hydroxysuccinimidobiotin (18.0 mg, 5.27×10^{-5} mol) was allowed to react with 11.4 μL (1.06×10^{-4} mol) of diethylenetriamine in 9 mL of N,N-dimethylformamide (DMF). The reaction was allowed to proceed for approximately 24 hours at room temperature. An initial white precipitate was removed by centrifugation. To the decantant was added 100 ml of diethyl ether. After standing for 2 hours, a colorless crystalline precipitate **1** had formed. The solvent was decanted, and the solid was dissolved in 1 mL of deionized water and transferred to a pre-weighed test tube.

Lyophilization yielded 10.2 mg of a white solid, which was analyzed by silica gel thin layer chromatography (TLC). TLC was performed using a 60% methanol/30% chloroform/10% acetic acid mobile phase. The mobility of **1** by TLC was compared to the solvent of the reaction and the reactants. Product visualization was achieved either using ninhydrin (0.3% solution in n-butanol) or iodine vapors. TLC results allowed tentative identification of **1** as diethylene-triaminobiotin (58.7% yield).

Test for Avidin Binding By 1 A mixture of 0.5 mL of 0.56 mM 4-hydroxyazobenzoic acid (HABA) and 0.5 mL of 0.18 mg/mL avidin was titrated with 5 μL increments of 0.4 mM biotin, up to a total of 30 μL, and the absorbance was measured at 500 nm after each addition. A similar experiment was performed using 3 mM diethylenetriaminobiotin. The titration was carried out in increments of 5 μL for the first 30 μL, then increments of 10 μL up to 160 μL, followed by 20 μL increments up to 260 μL, 40 μL increments up to a volume of 420 μL, plus an additional 10 μL for a final volume added of 430 μL. Again, absorbances were measured at 500 nm after each incremental addition.

Synthesis of cis-Diethylenetriaminobiotinylchloro(acetonitrile)platinum(II) Chloride (2) Diethylenetriaminobiotin **1** (7.2 mg, 2.2×10^{-5} mol) was allowed to react with *cis*-bis-(acetonitrile)dichloroplatinum(II) (7.1 mg, 2.0×10^{-5} mol) in 10 mL of DMF overnight at room temperature. Addition of 100 mL of diethyl ether induced the precipitation of a solid yellow product after about two hours. The solvent was decanted, and the solid, which was largely insoluble in water, was

suspended in deionized water, and transferred to a pre-weighed test tube. It was then isolated by centrifugation and washed with water three additional times. The yellow solid was then dried by vacuum centrifugation to yield 9.1 mg (1.5×10^{-5} mol) of *cis*-diethylenetriaminobiotinylchloro(acetonitrile)platinum(II) chloride **2** (68.8% yield). The mobility of **2** by TLC was compared to the solvent of the reaction, reactants, and **1**. Product **2** did not migrate on the TLC and could not be visualized using ninhydrin, but was visualized using iodine vapors. Product identity was confirmed by FABMS(+), M^+ 601.

Reaction of cis-Diethylenetriaminobiotinylchloro(acetonitrile)platinum(II) Chloride With pBR322 Plasmid DNA A 3 mg/mL sample of cis-diethylene-triaminobiotinychloro(acetonitrile)platinum(II) chloride (**2**) was prepared with deionized water. One reaction consisted of 5 μL of the 3 mg/mL **2** and 2.5 μL of 0.25 μg/μL pBR322 plasmid DNA, producing a 0.007 ratio of drug to nucleotide. A second reaction consisted of 10 μL of 3 mg/mL **2** with 2.5 μL of 0.25 μg/μL pBR322 plasmid DNA producing a 0.014 ratio of drug to nucleotide. Each of the reactions were allowed to proceed at approximately 35 °C for 24 hours in triplicate. After 24hours, a 1 μg portion of avidin was added to one tube of each reaction and a 5 μg portion to another.

Avidin Interactions with pBR322 To each of five microfuge tubes was added 2.5 μL of 0.25 μg/μL pBR322 plasmid DNA. Different amounts of avidin were added to four of the five microfuge tubes; the amounts are shown in Table 1. All samples were then combined with Type IV loading buffer (0.1M Tris, 40% sucrose and

bromophenol-blue) and analyzed by 1% agarose gel electrophoresis using 0.1 M Tris running buffer (pH 9). Electrophoresis was carried out for 3-4 hours at 105 V. After staining with ethidium bromide, the bands were visualized by UV trans-illumination. Gels were photographed using Polaroid 667 film. Images were scanned using a Musktec scanner and imported into Photo Plus4.

Mobility Shift Assay of *cis*-Diethylenetriaminmobiotinylchloro(acetonitrile)-platinum(II) Chloride with pBR322 Plasmid DNA Platinated pBR322 DNA samples were prepared as described above using the amounts indicated in Table 2. A 1 μg portion or a 5 μg portion of avidin was then added to two tubes in each set (Table 2). All samples were analyzed on a 1% agarose gel using 0.1 M Tris running buffer (pH 9). Electrophoresis was carried out for 3-4 hours at 105 V, stained with ethidium bromide (3 μg/mL), and the bands were visualized by UV trans-illumination. Gels were photographed using Polaroid 667 film. Images were scanned using a Musktec scanner and imported into Photo Plus4.

CHAPTER 3-RESULTS AND DISCUSSION

Synthesis of Diethylenetriaminobiotin The progress of diethylenetriamino-biotin synthesis was followed by TLC. The reaction scheme is shown in Figure 4. The Fourier transform infrared (FTIR) spectrum of the diethylenetriamino-biotin product is shown in Figure 5. The carbonyl stretches for the ring and chain are evident at approximately 1651 cm^{-1} and 1562 cm^{-1}. The amine (N-H) stretch appears at approximately 2938 cm^{-1}. The IR spectrum is consistent with the predicted product. The product was also characterized by proton nuclear magnetic resonance (NMR). Peak assignments are shown in Table 3 for the NMR spectrum (Figure 6). Peak assignments were aided by the COSY NMR (Figure 7). COSY NMR identifies the interactions of adjacent hydrogens on adjacent carbons. The 2-D NMR (COSY) confirms the results of the one dimensional NMR.

Binding of Avidin to Diethylenetriaminobiotin Diethylenetriaminobiotin was tested at this point to ensure that the introduced chemical modification did not disrupt avidin binding. This test was done by following the biotin (diethylenetriaminobiotin)-mediated displacement of HABA from the protein. This assay takes advantage of the differences in the visible spectra of HABA in the bound and unbound forms (ε_{500} = 34,500 and 600 $L \cdot mol^{-1} \cdot cm^{-1}$) for the bound and free HABA, respectively). The experiment was first carried out using unmodified biotin as a control. The mixture of HABA, Tris buffer and avidin yielded a yellow mixture, caused by the color of the HABA bound to avidin (λ_{max}= 500 nm). As biotin was added, it displaced HABA from the protein, and as a result the yellow

color lightened as the absorbance at 500 nm decreased. The absorbance, measured as a function of the volume of biotin added, after 10 μL of biotin had been added to the mixture, as shown in Figure 8. The data are presented in Table 4. The calculated saturation point works out to a ratio of 4 moles of biotin to 1.36 mole of avidin, or about 3:1. This value differs from the expected ratio of 4:1. This discrepancy may have been caused by some denatured avidin in the sample.

A similar experiment performed using the diethylenetriaminobiotin yielded comparable results. The 3 mM diethylenetriaminobiotin solution, determined by weight, caused the absorbance to decrease gradually when added to the HABA/avidin mixture. The data are shown in Table 5, and the plot of absorbance versus added compound is shown in Figure 9.

These results indicate that the intermediate, diethylenetriaminobiotin, does bind to avidin, although apparently not as strongly as does pure biotin. Thus, rather than the plateau observed for saturation due to complete stoichiometric displacement, the HABA displacement observed for our ligand is better represented as an equilibrium binding superimposed on some effect due to dilution. It is simple to rationalize why the binding affinity of avidin for biotin would decrease upon diethylenetriamine modification. Avidin is a basic glycoprotein containing four essentially identical subunits.[19] One avidin can therefore normally bind 4 molecules of biotin. These results suggest that diethylenetriaminobiotin may not bind to avidin at four sites as does biotin. The diethylenetriamine group

may cause some steric hindrance, which does not allow the intermediate to bind in the four possible binding sites. Alternatively, since avidin is a basic protein, and therefore positively charged at neutral pH, the added cationic tail of diethylenetriaminobiotin may also lead to a decreased binding affinity relative to unmodified biotin due to repulsive electrostatic interactions. Either explanation would qualitatively account for the differences in the two binding curves.

Synthesis of *cis*-Diethylenetriaminobiotinylchloro(acetonitrile)platinum(II) Chloride (2) The reaction of *cis*-bis(acetonitrile)dichloroplatinum(II) with diethylenetriaminobiotin produced 9.1 mg of the yellow product *cis*-diethylene-triaminobiotinylchloro(acetonitrile)-platinum(II) chloride **2** (68.8% yield assuming chloride counterion). The FTIR spectrum of the solid product is shown in Figure 10. The infrared spectrum is similar to the diethylenetriaminobiotin spectrum shown in Figure 5, except for a shift in peaks before the carbonyl stretches. The peaks at the wavenumbers less 1562 cm^{-1} appear to have shifted to a lower wavenumber. These peaks are tentatively assigned to be N-H stretches, and a shift in these to lower wavenumbers upon platinum binding is expected. The carbonyl stretches for the ring and chain are evident at approximately 1651 cm^{-1} (ring) and 1562 cm^{-1} (chain).

The solid product was also analyzed by fast atom bombardment mass spectrometry in the positive ion mode (FABMS(+)). The mass spectrum is shown in Figure 11. The parent peak appears at a mass/charge ratio of 601, which corresponds to the calculated molecular weight of *cis*-diethylenetriamino-

biotinylchloro(acetonitrile)-platinum(II). Peak distribution is consistent with isotope ratios expected for a product containing one chlorine and one platinum. The primary fragment peak appears at a mass/charge ratio of 329, which corresponds to the calculated molecular weight of diethylenetriaminobiotin. This evidence positively identifies this product as *cis*-diethylenetriaminobiotinylchloro-(acetonitrile)platinum(II).

A gel mobility shift assay was used to investigate changes in migration of DNA that is modified due to binding or other phenomena compared to the unmodified DNA. The DNA mobility is determined by size, shape, and charge. When binding of either an antitumor drug or protein occurs, one or more of these parameters can change, thus affecting mobility. The binding of proteins can change the shape and add bulkiness to the DNA strand. The bulkier strand will then migrate slower. This rationale is the basis for gel mobility shift assays.

Avidin Reaction with pBR322 Prior to testing whether platinum compound **2** could recruit avidin binding to DNA, the interaction between avidin and pBR322 was examined as a control. Four different avidin:DNA ratios were tested using an agarose gel DNA mobility shift assay. The results are shown in Figure 12. As the concentration of avidin was increased, the mobility of the DNA decreased as evidenced by the amount of DNA being retained in the well at the higher amounts of avidin. The intensity of this band seemed to increase proportionally with avidin concentration. These results indicate that avidin has an inherent binding affinity for the DNA. In retrospect, this is not surprising, considering the basic nature of

the protein and anionic nature of the DNA backbone. Notably, supercoiled DNA with normal mobility (avidin free) was still present at 7.58 x 10^{-11} moles of avidin, the highest level tested.

DNA Mobility Shift Assay Using 2-Modified DNA Results of the electrophoretic analysis of avidin binding to DNA and to the platinum DNA complex are shown in Figure 13. The pBR322 plasmid DNA control shows two bands, as expected. The upper band represents nicked circular DNA, and the lower band is the supercoiled plasmid DNA. Addition of 1.52 x 10^{-11} moles of avidin does not affect the mobility of the DNA (lane 2); a reduction in mobility of both the nicked and the supercoiled DNA occurred with the platinum compound **2** bound (lane 3). Addition of 1.52 x 10^{-11} moles of avidin to this platinum-modified DNA caused no change in mobility (lane 4). Addition of 7.58 x 10^{-11} moles of avidin to the Pt-DNA complex generated a ternary complex, which did not migrate from the wells of the gel (lane 5). These results demonstrated that the avidin was binding to the platinated DNA, but did not indicate unequivocally that avidin was binding to the platinum-adduct and not simply interacting non-specifically with the DNA. At higher levels of bound platinum, the DNA revealed an additional small decrease in mobility, and only one thick band appeared in the gel (lane 6). This band had approximately the same mobility as the nicked DNA in the lower ratio, an observation consistent with a complete removal of supercoils as is known to occur with other platinum compounds.[9] Addition of 1.52 x 10^{-11} moles of avidin yielded no significant change in the mobility (lane 7). However, when 7.58 x 10^{-11} moles of avidin was

added to the **2**-modified DNA, all DNA was retained in the well (lane 8). This provides evidence of avidin recruitment to the **2**-modified DNA.

Comparison of lanes 5 and 8 of Figure 13 with the control experiment lanes 1 through 5 shown in Figure 12 provides evidence that the platinum/biotin conjugate effectively recruits avidin to bind to the DNA. As the amount of bound avidin increases, the migration decreases due to the size of the complex that is formed upon binding. Lane 5 of Figure 12 and lane 8 of Figure 13 both contained the same amount of DNA and avidin. The difference is that in the latter case, the DNA had been modified by **2**. The platinated DNA bound avidin quantitatively under these conditions, whereas unmodified DNA did not, as evidenced by the presence of supercoiled DNA in Figure 12, lane 5. Also, lanes 2 and 4 of Figure 13 have a different intensity in the upper band near the well. Again, these lanes contain the same amount of DNA and avidin. Thus, the increased intensity of the upper band in lane 4 is attributed to the increased binding of avidin to the DNA caused by conjugate **2**.

CHAPTER 4-CONCLUSIONS

The spectra from both the intermediate and final products demonstrate that *cis*-diethylenetriaminobiotinylchloro(acetonitrile)platinum(II), **2**, was successfully synthesized. The mass spectrum showed a parent peak at 601 mass-to-charge ratio, which does not coincide with the molecular weight of the originally-predicted dichloride product. Rather, this parent peak agrees with the mono-chloro, mono-acetonitrile complex. The fragment at m/e = 329 agrees with the predicted molecular weight for diethylenetriaminobiotin, **1**, a reasonable fragmentation product. The avidin binding assay performed on the intermediate demonstrated protein binding to the biotin derivative, albeit with reduced affinity. This binding is critical if the final product is to recruit avidin binding to the DNA.

The mobility shift assay performed with unmodified pBR322 plasmid DNA and avidin showed some binding of avidin to DNA. This non-specific avidin-DNA interaction, however, seems to be relatively weak, as evidenced by the fact that avidin-free supercoiled DNA is present, even in the presence of 5 μg of avidin (a drug to nucleotide ratio of 0.014). In retrospect, this interaction is reasonable on the basis of electrostatic considerations, even though it was originally unanticipated. The second mobility shift assay performed with **2**, avidin, and pBR322 plasmid DNA showed binding of both **2** and avidin to the DNA. Not surprisingly, **2** binding to pBR322 seems to cause superhelical unwinding similar to *cis*-diamminedichloroplatinum(II). The unwinding is important because it allows avidin-product **2** binding to occur. The presence of avidin caused gel mobility

retardation of product **2**-modified DNA to a significantly greater extent than for unmodified DNA, an effect which was especially evident at higher avidin concentrations.

The fact that one can design and create a system in which a specific protein can be recruited to bind to DNA is of critical importance with respect to rational anti-neoplastic drug design utilizing the shielding effect. These experiments demonstrate the ability to synthesize a compound that will bind a protein while the compound is bound to DNA. This binding seems to be in many respects analogous to the binding of HMG-domain proteins. The HMG1 protein has been shown to recognize platinum-DNA adducts, most notably the 1,2-d(GpG) and 1,2-d(ApG) DNA intrastrand crosslinks created when cisplatin reacts with DNA. The 1,2-d(GpG) and 1,2-d(ApG) DNA intrastrand crosslinks are the major adducts formed by cisplatin. The structural difference caused by the platinum-DNA adduct of the DNA allows HMG1[12] to bind and cause a bend of 61°. This large distortion of the helix of DNA inhibits the repair system from fixing the lesion and does not allow the DNA to be transcribed or replicated. The product **2** appears to produce the same type of platinum-DNA adduct as cisplatin. The addition of avidin to this system appears to act in a similar manner as the HMG-domain protein does with cisplatin. DNA migration was reduced at higher concentrations of avidin. This lack of migration suggests that avidin is binding to DNA which has been modified by product **2**. Thus, at least in some ways, this system mimics the interaction of HMG1 with platinated DNA.[12]

The work described in this thesis has demonstrated that rational design of "drugs" which can directly recruit proteins to bind to DNA is possible. Ideally, a protein with no inherent DNA-binding affinity would be recruited to bind tightly to DNA. However, it was found that avidin does have an inherent DNA binding affinity, presumably due to electrostatic interactions between polyanionic DNA and the basic protein. Additionally, the strength of the avidin-biotin interaction was shown to be diminished by the chemical modification required to attach the platinum, thereby limiting its ability to induce strong ternary complex formation. Despite these unanticipated complications, rationally-designed drug-mediated protein-DNA complex formation was clearly demonstrated.

The effect of this ternary Pt-DNA-avidin complex on the function of intracellular DNA-processing proteins has not yet been determined. Remaining questions include: (1) Does the DNA repair system recognize these avidin-shielded lesions? (2) Can the DNA repair system fix these lesions? and (3) Does the avidin-shielded lesion block or hinder DNA replication and transcription? These questions are important, because the tight protein-DNA binding interaction demonstrated here is a necessary but insufficient condition for an effective drug candidate. The ternary adduct, when formed, must have the desired biological consequences on DNA-processing enzymes, thereby ultimately resulting in the death of malignant cells. Although the system described here is simply "proof-of-concept" and will not be clinically useful, it will still be important to show that the desired *in vitro* effects on DNA processing proteins will occur. In order for this

shielding strategy to be a general one, it must have general applicability, even in systems which are not clinically relevant.

Successful implementation of this strategy may ultimately increase the selectivity of chemotherapuetic drugs. Some proteins are known to be present in higher concentrations in cancerous cells. This strategy may exploit such proteins. A tantalizing target is telomerase, which is found in dramatically higher concentrations in cancerous cells. If this strategy can be utilized by attaching a compound to the DNA which binds tightly and specifically to telomerase, then selectivity for cancerous cells should be improved. A compound containing a platinum oligonucleotide conjugate that binds with telomerase could be the next step in preventing the spread of cancer.

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TABLES

Table 1: Mobility Shift Assay Performed With pBR322 DNA and Avidin

Lane	pBR322 (μg)	Avidin (μg)
1	0.625	0
2	0.625	2
3	0.625	3
4	0.625	4
5	0.625	5

Table 2: Mobility Shift Assay Performed With pBR322, Product **2**, and Avidin

Lane	pBR322 (μg)	Product **2** (mol)	Avidin (μg)	Drug : DNA Ratio
1	0.625	0	0	0
2	0.625	0	1	0
3	0.625	2.52×10^{-8}	0	140 μmol: 1mol
4	0.625	2.52×10^{-8}	1	140 μmol: 1mol
5	0.625	2.52×10^{-8}	5	140 μmol: 1mol
6	0.625	5.05×10^{-8}	0	70 μmol: 1mol
7	0.625	5.05×10^{-8}	1	70 μmol: 1mol
8	0.625	5.05×10^{-8}	5	70 μmol: 1mol

Table 3: NMR Peak Assignments for Diethylenetriaminobiotin

δ (Downfield Shift)	Carbon(s)
1.25-1.70	H6,H7,H8
2.15-2.25	H9
2.35-2.38	H1
2.65-2.92	H11,H12,H13,H14
2.92-3.00	H2
4.3-4.5	H3,H4

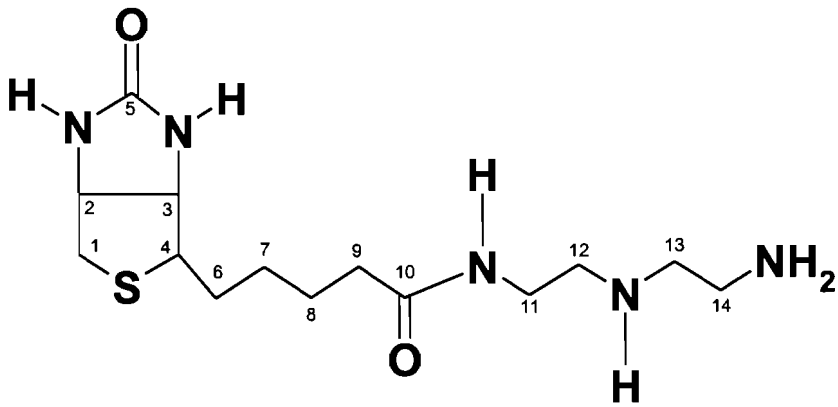


Table 4: Binding of Pure Avidin to Biotin

The initial solution contained 0.5mL 0.56mM HABA and 0.5mL 0.18mg/mL Avidin

Volume of 0.4 mM Biotin Added (μL)	Absorbance
0	0.246
5	0.215
10	0.156
15	0.164
20	0.157
25	0.157
30	0.157

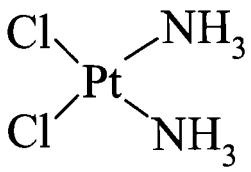
Table 5: Binding of Diethylenetriaminobiotin to Avidin

The initial solution contained 0.5mL 0.56mM HABA and 0.5mL 0.18mg/mL Avidin

Volume of 3 mM **1** Added (μL)	Absorbance
0	0.266
5	0.264
10	0.260
15	0.257
20	0.258
25	0.257
30	0.256
40	0.255
50	0.250
60	0.250
70	0.245
80	0.244
90	0.238
100	0.231
110	0.233
120	0.231
130	0.227
140	0.225
150	0.219
160	0.219
180	0.208
200	0.212
220	0.202
240	0.194
260	0.188
300	0.183
340	0.169
380	0.159
420	0.148
430	0.155

FIGURES

a)



b)

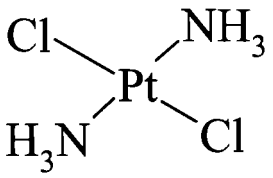
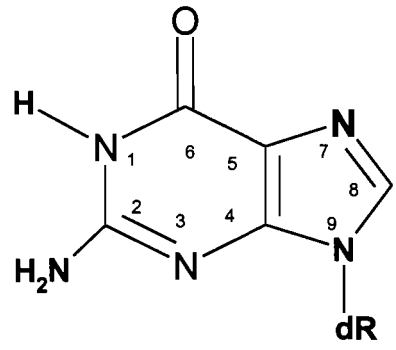


Figure 1. *cis*-Diamminedichloroplatinum(II) (a) and *trans*-Diamminedichloroplatinum(II) (b)

a)



b)

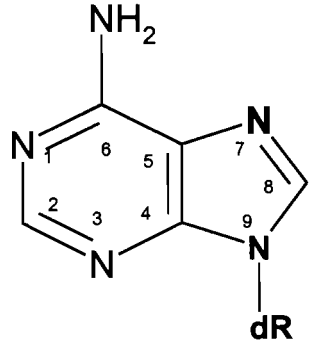


Figure 2. Structures of Guanine (a) and Adenine (b). Attachment to the Deoxyribose ring (dR) is through the N-9 Position. Platinum binds to N7.

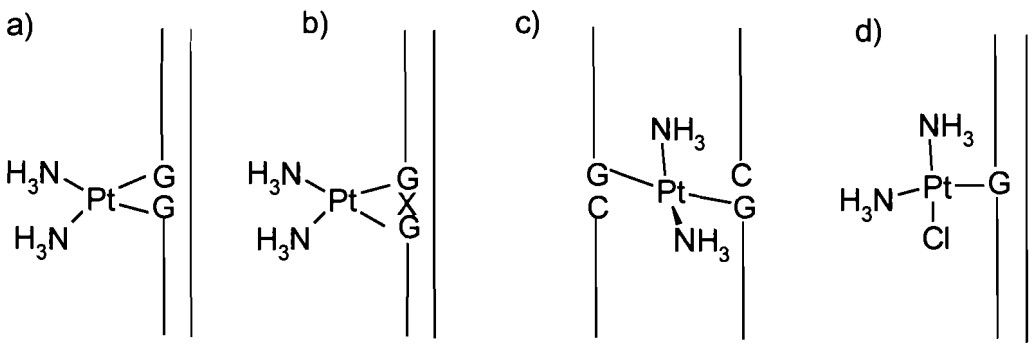


Figure 3. Schematic Representations of the Various Platinum-DNA Adducts. a) 1,2-Intrastrand; b) 1,3-Intrastrand; c) Interstrand; d) Monoadduct

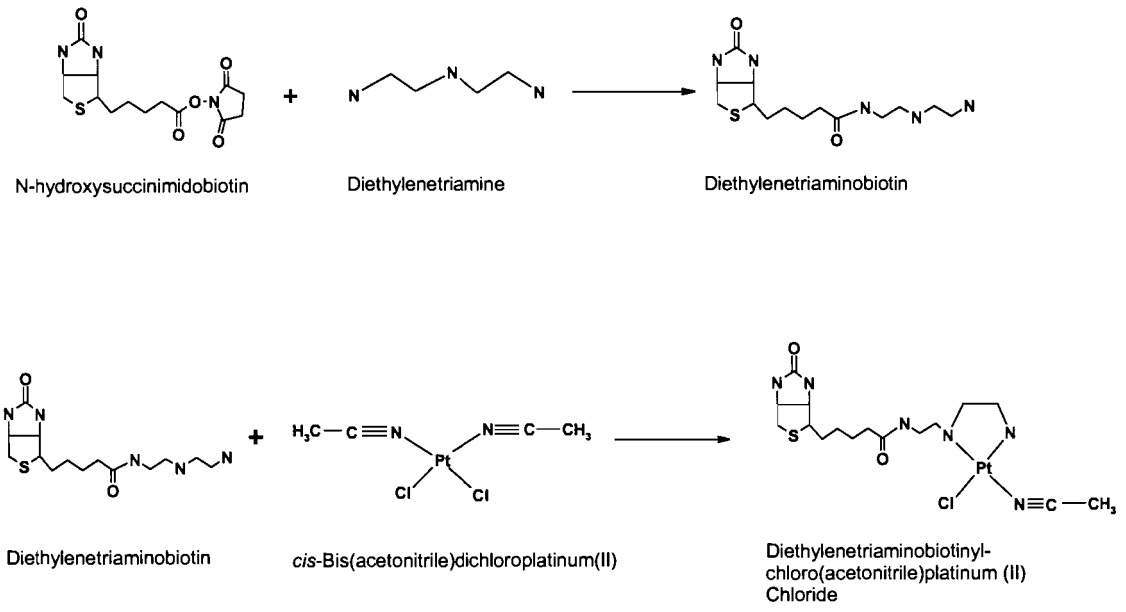


Figure 4. Synthetic Scheme

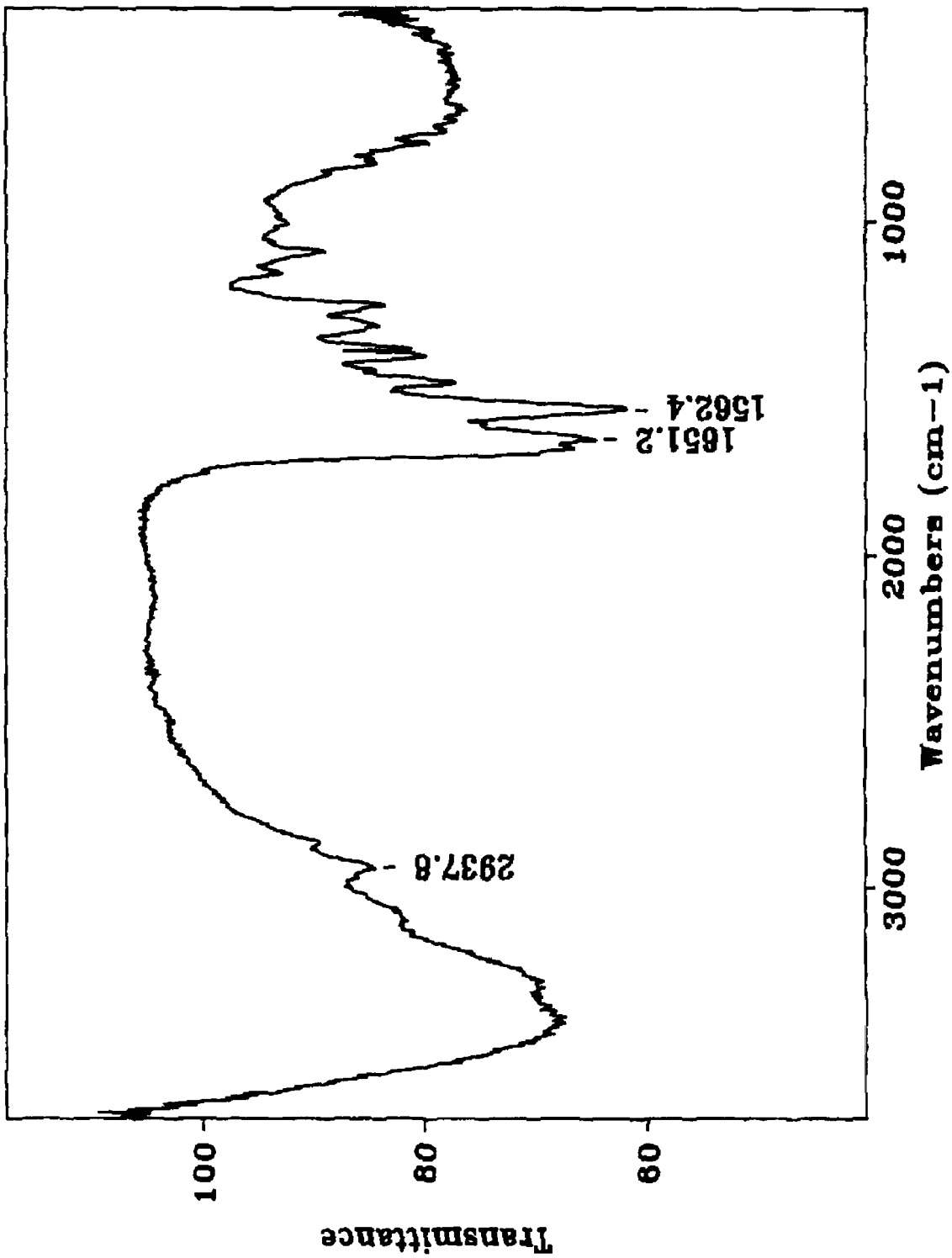


Figure 5. Infrared Spectrum of Diethylenetriaminobiotin (**1**)

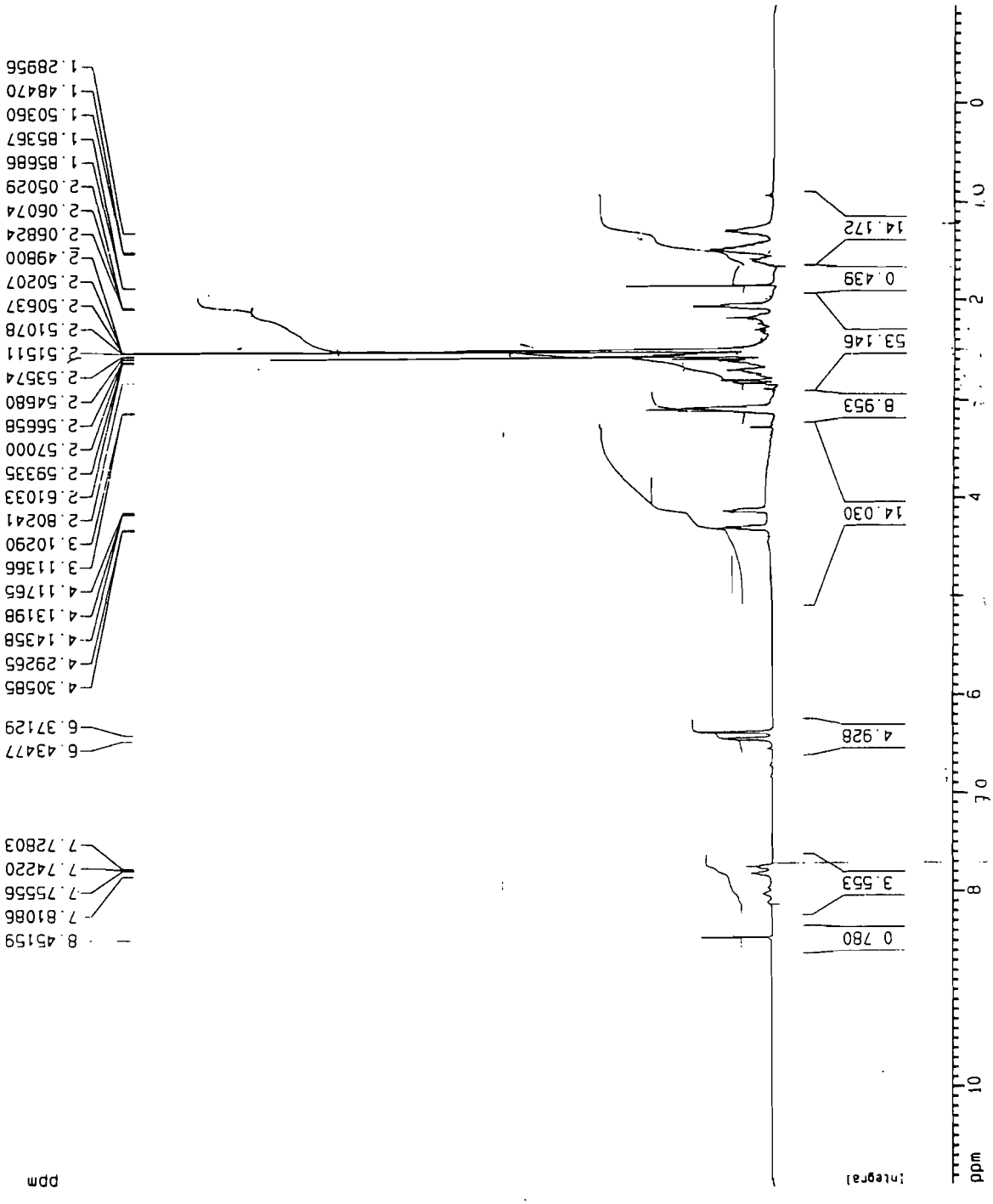


Figure 6. Proton NMR of Diethylenetriaminobiotin

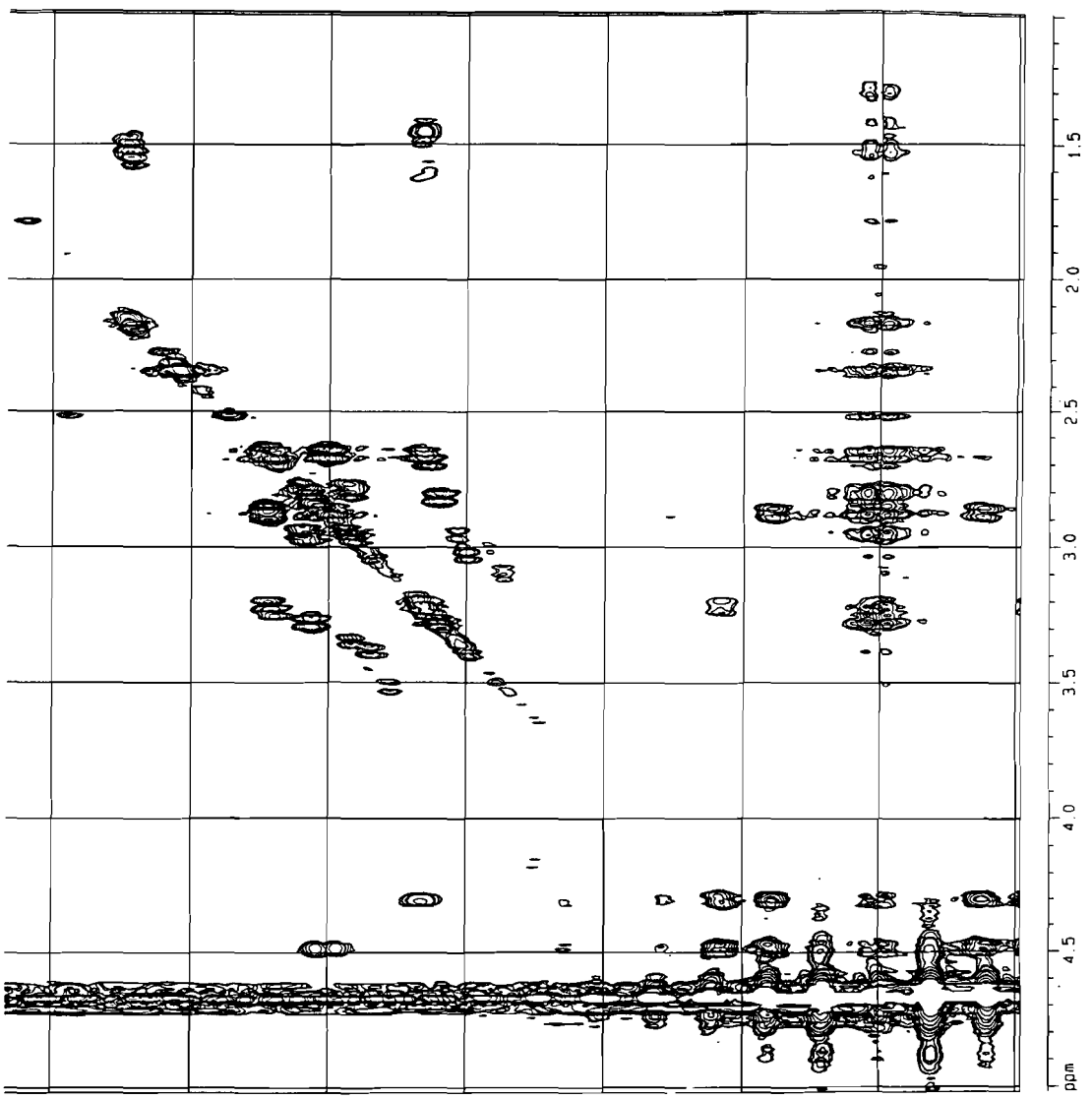


Figure 7. COSY 2D-NMR of Diethylenetriaminobiotin

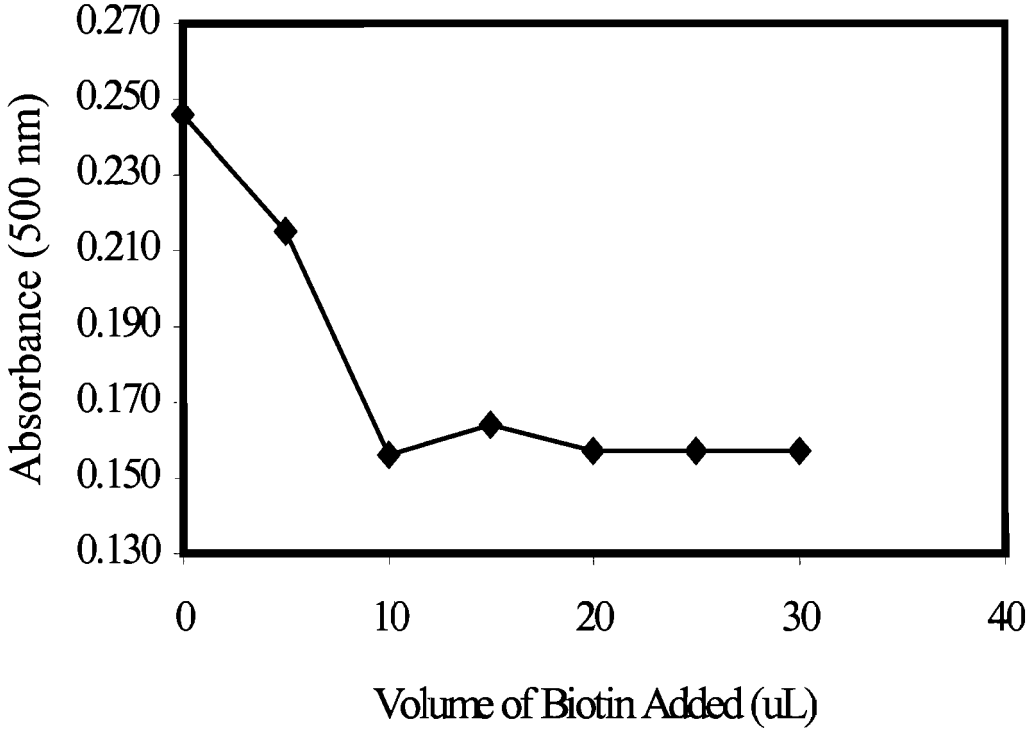


Figure 8. Change in the absorbance at 500 nm of the HABA/Avidin mixture as a function of added biotin.

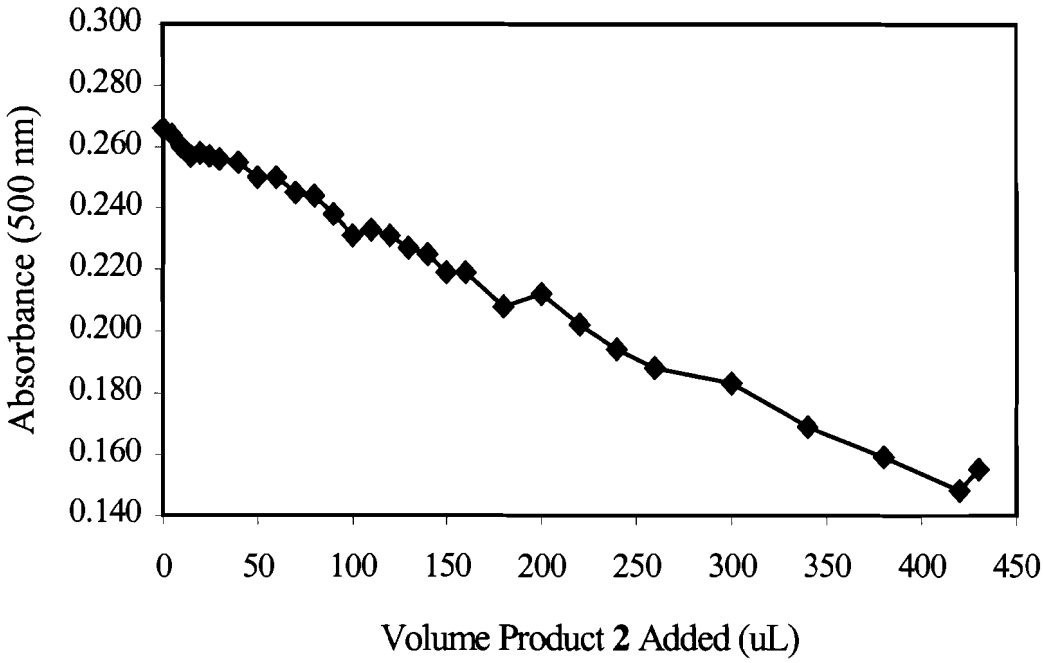


Figure 9. Change in the absorbance at 500 nm of the HABA/Avidin mixture as a function of added product **2**.

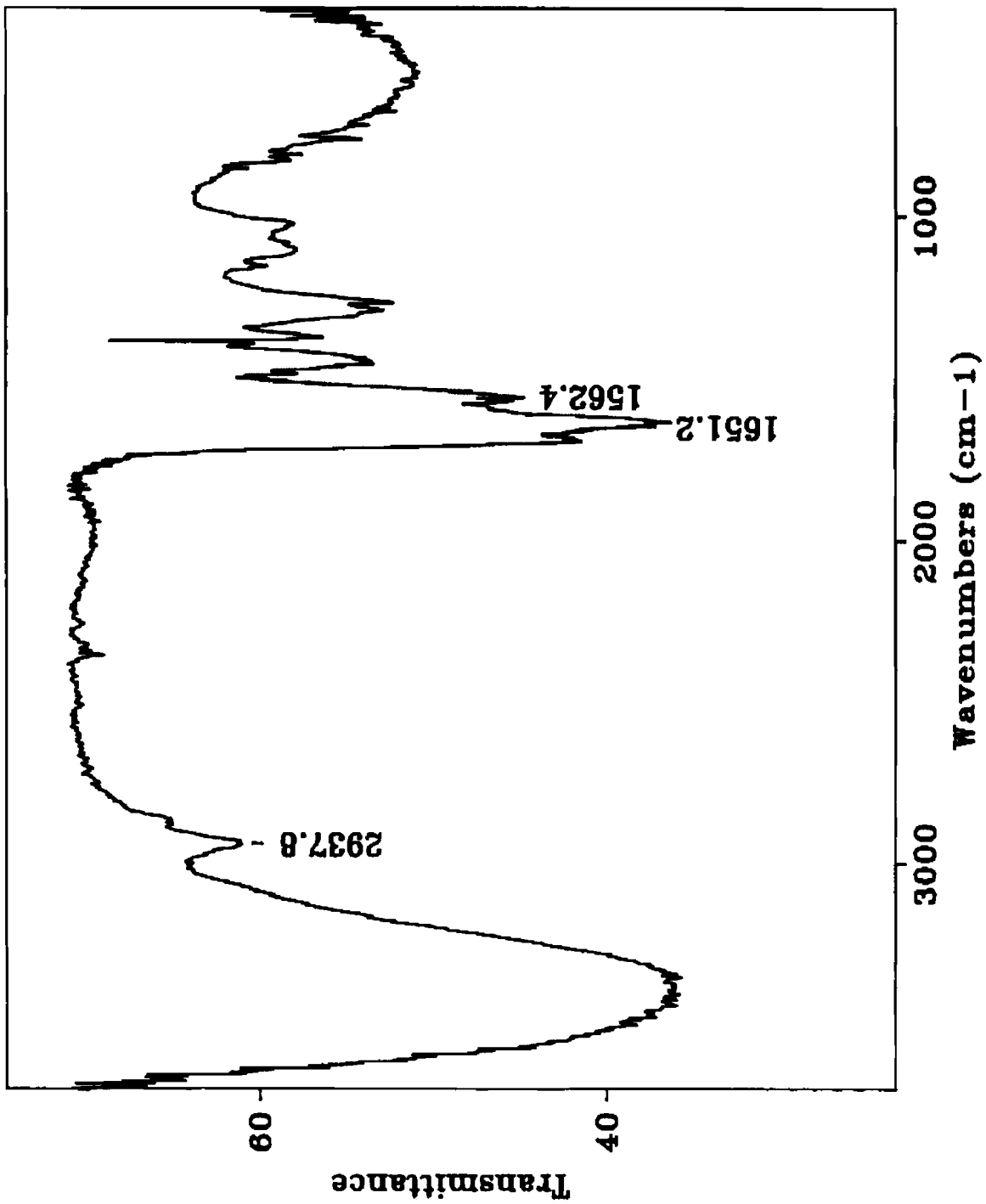


Figure 10. Infrared Spectrum of *cis*-Diethylene-triaminobiotinylchloro(acetonitrile)platinum(II)

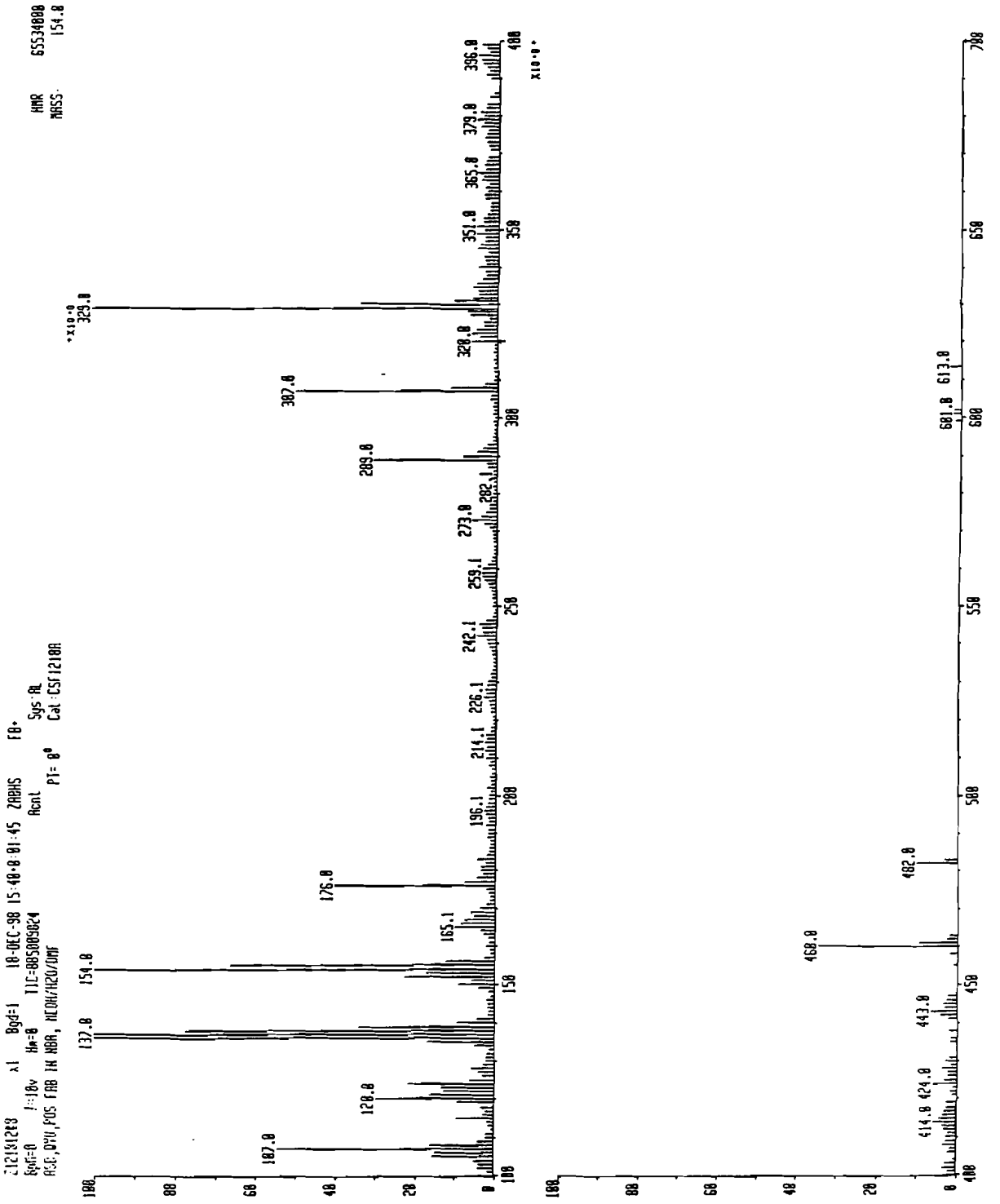
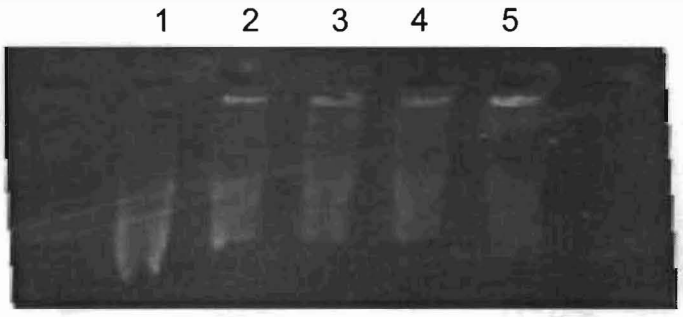
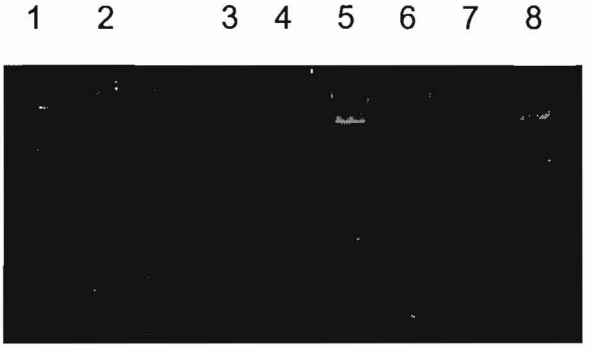


Figure 11. FAB(+) Mass Spectrum of cis-Diethylenetriaminobiotinylchloro(acetonitrile)platinum(II)



Lane 1 – pBR322 DNA
Lane 2 – pBR322 DNA + 2 μg Avidin (30.3 pmol)
Lane 3 – pBR322 DNA + 3 μg Avidin (45.5 pmol)
Lane 4 – pBR322 DNA + 4 μg Avidin (60.6 pmol)
Lane 5 – pBR322 DNA + 5 μg Avidin (75.8 pmol)

Figure 12. Mobility Shift Assay Performed with Avidin and pBR322 Plasmid DNA. All lanes contained 0.625 μg of pBR322 Plasmid DNA.



Lane 1 – pBR322 DNA
Lane 2 – pBR322 DNA + 1 μg Avidin
Lane 3 – pBR322 DNA + 2.52 x 10^{-8} mol Product **2**
Lane 4 – 140 μmol Product **2** : 1 mol pBR322 + 1 μg Avidin
Lane 5 – 140 μmol Product **2** : 1 mol pBR322 + 5 μg Avidin
Lane 6 – pBR322 DNA + 5.05 x 10^{-8} mol Product **2**
Lane 7 – 70 μmol Product **2** : 1 mol pBR322 + 1 μg Avidin
Lane 8 – 70 μmol Product **2** : 1 mol pBR322 + 5 μg Avidin

Figure 13. Mobility Shift Assay Performed with pBR322 DNA, Product **2**, and Avidin. All lanes contained 0.625 μg of pBR322 Plasmid DNA. 1 μg of Avidin corresponds to 15.2 pmol. 5 μg of Avidin corresponds to 75.8 pmol.

Jeffrey Ray Groth
Signature of Graduate Student

Michael V. Keck
Signature of Major Advisor

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4-24-2000
Date

Design, synthesis, and DNA-avidin binding properties of a platinum-biotin conjugate.

Title of the Thesis Report

Doug Cooper
Signature of Graduate Office Staff Member

5-16-00
Date Received