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The Impact of sequencing Human Genome on A Novel Spectroscopic Method of Detecting Aminoglycoside Antibiotic Residues in part per billion (PPB) Range in the Tissue Extracts of Food Producing Animals such as Chickens, cows, pigs, fish, crabs and lobster. This novel analytical method of detecting PPB residue will help FDA food inspectors in the field to identify low level residues in meat eating population and they could not only prevent drug resistance in humans, but also will prevent the transmission of animal diseases to humans.

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Abstract

The purpose of this article is to describe how to spectroscopically identify invisible aminoglycoside antibiotic residues in tissue extract of food producing animals (meat extracts from the tissues of chickens, cows, pigs, fish, crabs or lobsters) by attaching powerful chromophores such as Di-nitrophenyl (DNP) or Tri-nitrophenyl (TNP) groups. The idea of using DNP (Dinitrophenyl) and TNP (Trinitro phenyl) derivatives came from my doctoral work where I was designing drugs to bind to DNA shutting off genes that causes cancers. The DNA binding agents such as Aziridines and Carbamate are attached to DNP and TNP moiety so that they become visible to spectroscopic detection. Farmers use Aminoglycoside Antibiotics not only to prevent infections in food producing animals, but also to promote growth and increase body mass of these animals. The presence of too much antibiotics residues in the tissues could be unsafe for human consumption. The confirmation of these aminoglycoside antibiotics by conventional Electron Impact (EI) mass spectrometry or Chemical Ionization (CI) mass spectrometry did not produce a molecular ion peak for underivatized antibiotics because the antibiotics do not carry chromophore moiety, difficult to volatize and are thermally unstable. By attaching powerful chromophores such as DNP and TNP analogs, and by using a new time-of flight mass spectrometer attached to a radioactive 252-Californium Plasma desorption mass Spectrometry (CPDM) source, we successfully obtained mass spectra with the molecular ion peak of all nine derivatized antibiotics as their sodium positive ion peaks. In most cases, there are very few fragmentation peaks and the molecular ion was primary peak in the spectrum. These spectra could be used as Reference Spectra when comparing with the unknown samples obtained during inspection. These spectra will help the food inspectors in the field not only to identify the misuse of any of antibiotics in the tissue extracts, but also will identify the amount of residue present.

Keywords: Epigenetic disorder, Methylation, Acetylation, Phosphorylation, Glioblastoma. BBB, Aziridine, Carbamate, AZQ

Introduction

Historical Background:

How a CSO (Consumer Safety Officer) becomes a researcher in FDA?

I answer this question about 40 years after the fact. I waited for years hoping that some of those people in power are retired, dead, gone and buried. They were powerful enough to hurt me when they were alive. I conducted an experiment without informing my colleagues and the Big Boss to develop a novel method to

detect drug residue level in part per billion range useful in the field for the food inspectors looking for misuse of drugs in the tissues extract of food producing animals. This was my idea, I conducted and I completed and I plan to publish the paper without their names. If I were to share my project with the Big Boss, the idea will be his and I will be kick out of this project. First, I wanted to complete this project in my own time then I want to share with them. The research idea came from a review committee member, who shared with us during a coffee break.

Price of Disobedience

I served FDA with great honor. FDA offered me the first Government job. Over the years, FDA provided me with all the training to become a good Consumer Safety Officer (CSO). Over the years, I reviewed hundreds of research proposals, wrote summary statements, and dozens of Federal Notices. and submitted to My superior, Dr. Luther, who made some minor changes and returned to me to finalize the document. Once he approved, the final document goes to his Superior, a truly Yes Man, who signed the document and send to the Big Boss. When the Big Boss signed, the documents are published with his name and all other names are removed. The Big Boss was really Big absolute arrogant and nasty. He frequently used Potty language. Everyone is afraid of him. Whenever he comes across, he expects you to say Good Morning Sir, but he never responds. He acts as if he owns the FDA and rest of us are slaves working for him. Everyone obeyed him. FDA is good; the job is secured and good; the salary is good. I obeyed as everyone did. I never dream of disobeying. I never thought that I will confront the Big Boss one day.

In one of those Review meetings during a coffee break, I heard an astonishing story. The reviewer shared the story about an incident in Porto Rico. From a hilltop a tourist saw a bunch of teenagers riding bikes toward the seashore. Soon they took off their shirts and jumped into the water. He was shocked when he saw big breasted girls playing in the water. Public indecency, he thought and he called the police. Soon a Polic lorry arrived and they were all arrested and huddled up into the Lorry. Upon arrival at the Police Station, Police discovered that they were not girls; they were all boys. When the news appeared, in the local papers, people thought that these boys were exposed to some toxic chemicals. In the contaminated water, they were swinging and fishing at a specific area of a lake. FDA and EPA were alerted. The inspectors analyzed the water and found nothing; they analyzed the soil and found it safe. It was a farming area where livestock are kept. In the olden days, the solution to pollution was dilution. It was suspected that someone dumped large quantities of chemical waste in the lake water. The toxic particles were picked up by the microbial plankton of lake which were eaten by fish. The toxic residues were concentrated in fish. The fish was eaten by those boys. Since the contaminated fish concentrate the toxic chemicals, some fish were caught and tissue extracts were sent to Govt. Lab for analysis.

I immediately realized that farmers use all sorts of antibiotics to promote animal growth and to prevent infection in livestock. Large scale waste generated in the farms and factories is quietly dumped in the lake. The most frequently used chemicals are aminoglycoside antibiotics. Unfortunately, at the Part Per Billion (PPB) level, they are undetectable because they lack any chromophore which make them visible to spectroscopic analysis. For almost five years, (as doctoral candidate and 2 years,as a postdoctoral student), I had made over 120 analogs of Di-nitrophenyl (DNP) and Trinitro phenyl (TNP) as chromophores carrying Aziridines for treating experimental animal tumors [1-3].

When the Coffee Break was over and the conference resumed, the reviewer returned to the conference, but I kept on thinking how easy it is to attach the chromophores to all eleven aminoglycoside antibiotics and they can be detected at PPB level. and I can publish a nice paper.

After completing my Doctorate and post-doctorate work at the London University, England, I came to America to serve as the Fogarty International Awardee to work at the National Cancer Institute (NCI) of the National Institutes of Health (NIH). My work involved the synthesis of Dinitrophenyl Aziridine a powerful chromophore alkylating agent to treat animal cancers.

I can use this knowledge to develop method to detect antibiotic residues in farm waste. My doctoral and postdoctoral work involved making powerful chromophores derivative such as DNP and TNP analogs of Aziridine. These compounds were developed to bind to the information molecule DNA (Deoxyribose Nucleic Acid). DNA is made of a long string of four nucleotide namely Adenine (A), Thymine (T), Guanine (G) and Cytosine (C). As a part of my doctoral work, I synthesized Aziridine derivatives DNP and TNP to shut off genes that causes cancers in experimental animals. We took advantage of the genetic revolution.

The true information revolution begins to appear when we broke the genetic code and unlocked the secrets of life. During the last one hundred year, we discovered several sub revolutions. The first is the New World Order based on Crick Watson's structure of the double stranded DNA world [4]. According to the New World Order, life did not come to Earth from Heaven. All evidence indicate, Life is evolved on the surface of Earth from the ingredients already present on Earth. The second sub-revolution was the Gregor Mandel's work. When Mandel cross-bred red flower pea plant, with White flower pea plant. The next generation of flowers were all Red (Dominant allele) White flower disappeared (Recessive allele). When he cross bred muted first-generation pea plants, two third red flower plants and one third of White flower plant returned. He called the red flower plant as Dominant allele and the White as Recessive allele. The White flower did not blend or mixed, the traits traveled in its entirety. What is true with plants is also true with humans. Intermarriage among close relations., bring the defected member back in the third generation.

Sequencing Human Genome:

Genetic revolution began when using the restriction enzymes, scientists around the world, started cutting, pasting and isolating genes from various parts of species patenting and claiming as their own. The US government rejected their claim. Instead, the US Government decided to provide funds to decipher the entire book of life of a human being called the Human Genome and release all the genes free of charge to anyone who wants it. Sequencing Human Genome, reading the number and the order in which these nucleotides are arranged is called sequencing. Sequencing will answer the most fundamental questions, we have asked ourselves since the dawn of human civilization. Questions like, what does

it means to be human? What is the nature of our memory and our conscientiousness? Our development from a single cell to a complete human being? The Biochemical nature of our senses, the process of our aging? Scientific biases of our similarity and dissimilarity. Dissimilarity that all living creatures from a tiny blade of grass to mighty elephant, including man mouse and monkey are all made of the similar building blocks the nucleotides and yet we are so diverse that no two individuals are alike even identical twin are no exactly identical, they grow up to become two separate individuals.

In 1990, US Congress authorized three billion dollars to our Institute (NIH) to decipher the entire human Genome under the title, "The Human Genome Project" We found that our Genome contains six billion four hundred million nucleotides bases half comes from our father and another half comes from our mother. Less than two percent of our Genome contains genes which code for proteins. The other 98 percent of our genome contains non-coding nucleotides forming switches, promoters, terminators etc. The 46 Chromosomes present in each cell of our body are the greatest library of the Human Book of Life on planet Earth. The Chromosomes carry genes which are written in nucleotides. Before sequencing (determining the number and the order of the four nucleotides arranged on a Chromosomes), it is essential to know how many genes are present on each Chromosome in our Genome. The Human Genome Project has identified not only the number of nucleotides on each Chromosome, but also the number of genes on each chromosome.

A single cell is so small that we cannot even see with our naked eyes. We must use a powerful microscope to enlarge its internal structure. Under an electron microscope, we can enlarge that one cell up to nearly a million times of its original size. Under the electron microscope, a single cell looks as big as our house. There is a good metaphor with our house. For example, our house has a kitchen, the cell has a nucleus. Imagine for a moment, that our kitchen has 23 volumes of cookbooks which contain 24,000 recipes to make different dishes for our breakfast, lunch, and dinner. The nucleus has 23 pairs of chromosomes which contain 24,000 genes which carry instructions to make proteins. Proteins interact to make cells; cells interact to make tissues; tissues interact to make an organ and several organs interact to make a man, a mouse, or a monkey. To confirm the composition of nucleotides, we must sequence the entire human genome. As you know, every cell of our body carries sixteen thousand good genes, six thousand mutated (bad) genes responsible for six thousand diseases and two thousand Pseudo-genes that have lost their functions.

The Human Genome: The greatest Catalog of Human Genes on planet Earth

We deciphered all 46 chromosomes, 23 from each parent. The 46 chromosomes present in each cell of our body are the greatest library of the Human Book of Life on planet Earth. The Human Genome Project has identified the following genes on each chromosome:

We found that the chromosome-1 is the largest chromosome carrying 263 million A, T, G and C nucleotide bases and it has only 2,610 genes. The chromosome-2 contains 255 million nucleotides bases and has only 1,748 genes. The chromosome-3 contains 214 million nucleotide bases and carries 1,381 genes. The chromosome-4 contains 203 million nucleotide bases and carries 1,024 genes. The chromosome-5 contains 194 million nucleotide bases and carries 1,190 genes. The chromosome-6 contains 183 million nucleotide bases and carries 1,394 genes. The chromosome-7 contains 171 million nucleotide bases and carries 1,378 genes. The chromosome-8 contains 155 million nucleotide bases and carries 927 genes. The chromosome-9 contains 145 million nucleotide bases and carries 1,076 genes. The chromosome-10 contains 144 million nucleotide bases and carries 983 genes. The chromosome-11 contains 144 million nucleotide bases and carries 1,692 genes. The chromosome-12 contains 143 million nucleotide bases and carries 1,268 genes. The chromosome-13 contains 114 million nucleotide bases and carries 496 genes. The chromosome-14 contains 109 million nucleotide bases and carries 1,173 genes. The chromosome-15 contains 106 million nucleotide bases and carries 906 genes. The chromosome- 16 contains 98 million nucleotide bases and carries 1,032 genes. The chromosome-17 contains 92 million nucleotide bases and carries 1,394 genes. The chromosome-18 contains 85 million nucleotide bases and carries 400 genes. The chromosome-19 contains 67 million nucleotide bases and carries 1,592 genes. The chromosome-20 contains 72 million nucleotide bases and carries 710 genes. The chromosome-21 contains 50 million nucleotide bases and carries 337 genes. The chromosome-22 contains 56 million nucleotide bases and carries 701 genes. Finally, the sex chromosome of all females called the chromosome-X contains 164 million nucleotide bases and carries 1,141 genes. The male sperm called chromosome-Y contains 59 million nucleotide bases and carries 255 genes.

If you add up all genes in the 23 pairs of chromosomes, they come up to 26,808 genes and yet we keep on mentioning 24,000 genes needed to keep us function normally. There are 16,000 good genes, 6,000 defected or mutated genes and 2,000 Pseudogenes. A gene codes for a protein, not all 24,000 genes code for proteins at the same time. (Alternative splicing could generate thousands of new combinations of proteins. (The cells use new combination to generate new proteins as needed). It is estimated that less than 19,000 genes code for protein. Because of the alternative splicing, each gene codes for more than one protein. As needed by our body, a small number of genes interact to make specific proteins. All the genes in our body make less than 50,000 protein which interact in millions of different ways to give a single cell. Millions of cells interact to give a tissue and hundreds of tissues interact to give an organ and several organs interact to make a human [5,6].

Identification, Prevention and Treatment of a mutated or diseased gene

No two individuals look alike because when you compare their genome in every thousand nucleotide bases, one nucleotide base is located at a different place. This variation is called the Single Nucleotide Polymorphism (SNP). In a six billion four hundred million nucleotide base pairs of a human genome, there are 4-5 million SNPs. A single nucleotide polymorphism (abbreviated SNP, pronounced snip) is a genomic variant at a single base position (either A, T, G or C) in the DNA. Scientists study if and how SNPs in a genome influence health, disease, drug response and other traits.

Are there any mutation in the SNP related to human diseases. By genotyping or sequencing the a single cell in the tissue extract of a diseased person's genome and comparing with the Reference Sequence, we can easily identify the SNP carrying the mutation responsible for causing the disease. Single

nucleotide polymorphisms (SNPs) may act as biological markers, as they can relate to the genes that are associated with various complex diseases such as heart diseases, diabetes, cancer, schizophrenia, blood pressure, migraine, and Alzheimer.

Rational Drug Design

The supreme intellect for Drug Design is Ross, an Englishman, who is a Professor of Chemistry at the London University. Professor WCJ Ross is also the Head of Chemistry Department at the Royal Cancer Hospital, a post-graduate medical center of the London University. Ross was the first person who designed drugs for treating Cancers. He designed drugs to cross-link both strands of DNA that we inherit one strand from each parent. Cross-linking agents such as Nitrogen mustard are extremely toxic and were used as chemical weapon during the First World War. More toxic derivatives were developed during the Second World War. Using the Data for the toxic effect of Nitrogen Mustard used during the First World War, Ross observed that Soldiers exposed to Nitrogen Mustard showed a sharp decline of White Blood Cells (WBC) that is from 5,000 cell/CC to 500 cells/CC. Children suffering from Childhood Leukemia have a very high WBC count over 90,000 cells/CC. In sick children, most of the WBCs are premature, defected and unable to defend the body from microbial infections. Ross rationale was that cancer cells divide faster than the normal cell, by using Nitrogen Mustard to cross linking both strands of DNA, one can control and stop the abnormal WBC cell division in Leukemia patients. It was indeed found to be true. Professor Ross was the first person to synthesize a large number of derivatives of Nitrogen Mustard. By using an analog of Nitrogen Mustard, called Chlorambucil, 9., he was successful in treating Childhood Leukemia. In America, two Physicians named Goodman and Gilman from the Yale University were the first to use Nitrogen Mustard to treat cancer in humans. Nitrogen Mustards and its analogs are highly toxic. Ross was a Chemist, over the years, he synthesized several hundred derivatives of Nitrogen Mustard molecules to modify toxicity of Nitrogen Mustard [7-13].

Although analogs of Nitrogen Mustard are highly toxic. They cross-link both strands of DNA. They are more toxic to cancer cells and more cancer cells are destroyed than the normal cells. Toxicity is measured as the Chemotherapeutic Index (CI) which is a ratio between toxicity to Cancer cells versus the toxicity to Normal cells. Higher CI means that the drugs are more toxic to cancer cell. Most cross-linking Nitrogen Mustard have a CI of 10 that is they are ten times more toxic to cancer cells. Some of the Nitrogen Mustard analogs Ross made over the years are useful for treating cancers such as Chlorambucil for treating childhood leukemia (which brought down the WBC level down to 5,000/CC). Children with Childhood Leukemia treated with Professor Ross Chlorambucil showed no sign of Leukemia even after 20 to 25 years. Chlorambucil made Ross one of the leaders of the scientific world. He also made Melphalan and Myrophine for treating Pharyngeal Carcinomas.

At the London University, I was trained as an Organic Chemist in the Laboratory of Professor WCJ Ross of the Royal Cancer Hospital, a post-graduate medical center of the London University. After working for about ten years at the London University, I moved to America when I was honored by the Fogarty International Fellowship Award by the National Institutes of Health, NIH, and the National Cancer Institute, NCI, of the USA. NIH has been my home for over a quarter of a century, I designed drugs to shut off mutated genes. All three Common Allele diseases have genetic origin. The rationale I used to synthesize anti-cancer drugs could be used to treat the other two old age diseases like Alzheimer or cardiovascular diseases. In the following sections, I will describe in detail how anti-cancer drug like AZQ was designed to shut off Glioblastoma genes which cause Brain Cancer in humans. Using the same rational, we will consider how each of the other two diseases namely cardiovascular disease and Alzheimer could be treated by shutting off their genes to save human life: The order of these diseases are arranged based on the level of funding provided by NIH specifically by the NCI (National Cancer Institute).

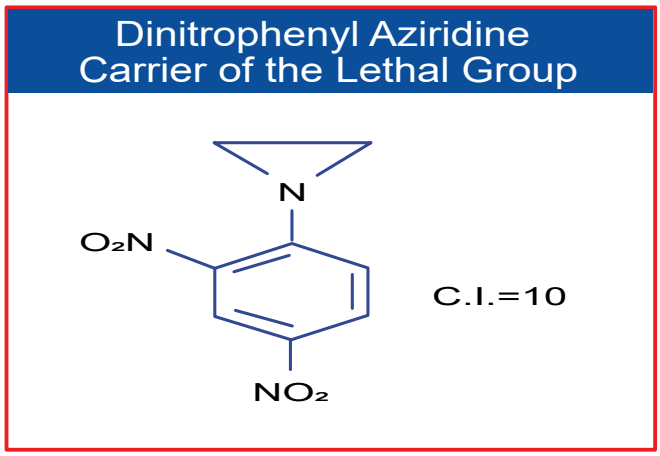
As I said above, Professor Ross was designing drugs to attack both strands of DNA simultaneously by cross-linking using Nitrogen Mustard analogs, which are extremely toxic. As a part of my doctoral thesis, I was assigned a different path. Instead of cross-linking DNA, I am to design drugs to attack only one strand of DNA. This class of drugs is called Aziridines. Over the years, I made over 100 Dinitrophenyl Aziridines derivatives. One of them is Dinitro benzamide (CB1954) which gives a CI of 70 highest ever recorded. CB1954 wipes out a solid tumor by attacking the DNA of Walker Carcinoma 256, a solid aggressive tumor in Rat.

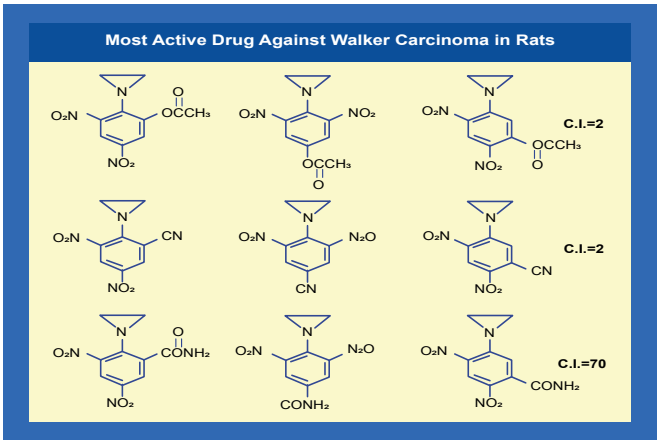
Designing drugs to bind to a Single Stranded DNA to Treat Animal Cancers

As a part of my doctoral thesis, [1.,2., 3] I was assigned a different path instead of cross-linking both strands of DNA by Nitrogen Mustard, I am to design drugs to attack only one strand of DNA by making Aziridine analogues. We decided to use Aziridine moiety (as an intermediate of Nitrogen Mustard) that would be an excellent active component to shut off a gene by binding to a single strand of DNA. To deliver Aziridine to the target site which is the N-7 Guanine of DNA, we decided to use Dinitrophenyl (DNP) moiety as a drug delivery agent. DNP is a dye which colors the tumor tissues of the experimental animal tumor such as Walker Carcinoma 256 in Rats. It is well known that analogs of DNP such as Dinitrophenol disrupts the Oxidative Phosphorylation (OXPHOS) of the ATP (Adenosine Triphosphate) which provides energy to perform all our body functions. To provide energy to our body function, the high energy phosphate bond in ATP is broken down to ADP (Adenosine Diphosphate) which is further broken down to AMP (Adenosine Mono Phosphate), the enzyme Phosphokinase put the inorganic phosphate group back on the AMP giving back the ATP. This cyclic process of Oxidative Phosphorylation is prevented by Dinitrophenol.

The Binding of aminoglycosides to DNP and TNP as a Chromophores is my idea not the FDA Big Boss.

As a part of my two-year post-doctoral work, I used powerful chromophores such as Dinitrophenol (DNP) and Trinitrophenol (TNP) derivatives as drug delivery method for the active ingredient aziridine. The analog of DNP such as Aziridine Dinitrophenol could also serves as a dye which stains Walker Carcinoma 256, a solid and most aggressive tumor in Rat. The first compound I made by attaching the C-14 radiolabeled Aziridine to the DNP dye. The Dinitrophenyl Aziridine was synthesized using Dinitrochlorobenzene with C-14 radiolabeled Aziridine in the presence of Triethyl amine





which removes the Hydrochloric Acid produced during the reaction. When the compound Dinitrophenyl Aziridine was tested against the implanted experimental animal tumor, the Walker Carcinoma 256 in Rats, it showed a TI (Therapeutic Index) of ten. The TI of ten was like most of the analogs of Nitrogen Mustard. Since this Aziridine analog was not superior to Nitrogen Mustard, it was dismissed as unimportant.

On further reexamination of the X-ray photographs of Dinitrophenyl Aziridine, it appeared that most of the radioactivity was concentrated at the injection site. Very little radioactivity was observed at the tumor site. It was obvious that we need to make derivatives of Dinitrophenyl Aziridine to move the drug from the injection site to the tumor site. Because of the lack of fat/water solubility to be effective drug delivery method, Dinitrophenyl Aziridine stays at the injection site, a very small amount of radioactivity was observed on the tumor site.

Structure-Activity Relationship:

I immediately realized that by altering structure, I could enhance biological activity by making water and fat-soluble analogs of Dinitrophenyl Aziridine. By attaching water soluble groups, I should be able to move the drug from the injection site to the tumor site. To deliver 2,4-Dinitrophenylaziridine form the injection site to tumor site, I could alter the structure of 2,4-Dinitrophenylaziridine by introducing the most water-soluble group such as ethyl ester to the least water-soluble group such as Cyano- group or to introduce an intermediate fat/water soluble such as Amido group.

An additional substituent in the Dinitrophenyl Aziridine could give three isomers, Ortho, Meta, and Para substituent. Here confirmational chemistry plays an important role in drug delivery method. Ortho substituent always give inactive drug. Model building showed that because of the steric hinderance, Aziridine could not bind to DNA shutting off the genes. On the other hand, Meta and Para substituents offer no steric hindrance and drug could be delivered to DNA. When injected in Rat, because of the high solubility, most of the drugs was pass down through urine and extracted the drug from Rat urine by chloroform, The following chart showed that I synthesized all nine C-14 radiolabeled analogs of 2,4-Dinitrophenyl aziridines and tested them against implanted Walker Carcinoma 256 in Rats.

Derivatization of Dinitro phenyl Benzamide based on Partition Coefficient

The most water-soluble substituent

The first three compounds on top line of the above chart carry all three isomer of the most water-soluble **Ethyl Ester group** attached to 2,4-Dinitropehny aziridine. The compound in vivo is hydrolyzed Ethyl Ester to produce most water-soluble carboxylic group. Since it is the most water-soluble substituent, within 24 hours of injection in Rats, the entire radioactive compound was passed down from in the Rat urine and it can be extracted by Chloroform. Since the Ortho position was not available for DNA binding, it showed no biological activity, but the third compound in which Ortho position was free to bind to DNA showed some anti-tumor activity in Rats.

The least water-soluble substituent

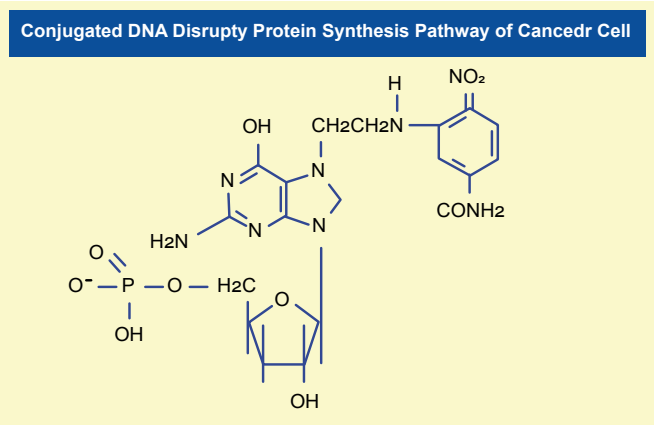
On the other hand, when the least water-soluble **Cyano-group** was attached to all three isomers of the 2,4-Dinitrophenyl aziridine compound as shown in the second line of the above chart, most of the compound stayed at the injection site. Only the last Cyano-derivative attached to DNA showed some anti-tumor activity.

The moderately soluble Amido-substituent

The last line of the above chart showed that the first two **Amido groups** were sterically hindered and did not bind to DNA and showed no biological activity, but the last compound presents the perfect drug delivery method. The entire drug was delivered from the injection site to the tumor site. The drug 1-Aziridine, 2,4-dinitro, 5-benzamide (CB1954) showed the highest anti-tumor activity. It has a CI of seventy; it is seventy times more toxic to cancer cells, highest toxicity ever recorded against Walker Carcinoma 256 in Rats [14-16].

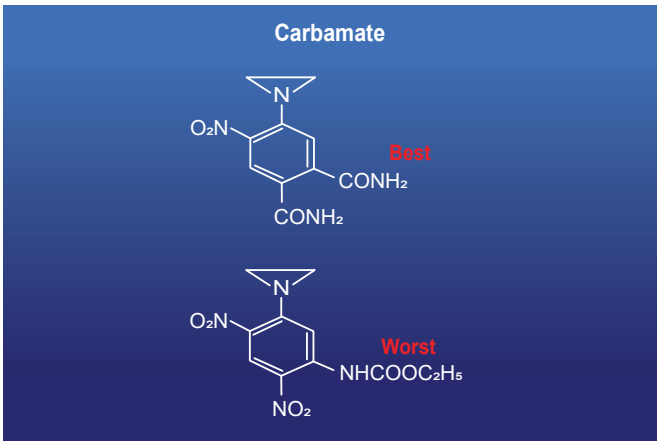
As I said above, Nitrogen Mustards are highly toxic because they have neither specificity nor selectivity. They attack all dividing cells whether they are normal or abnormal. On the other hand, the analogs of Aziridines and Carbamates serve as prodrug and remain inactive in the basic and neutral media. They become activated only in the presence of acid produced by growing cancer cells. Aziridine attacks DNA in acidic medium, particularly the N-7 Guanine. The dye Dinitro benzamide has great affinity for Walker Tumor. The Aziridine Dinitro benzamide (CB1954) has the highest toxicity to Walker Tumor cells ever recorded. As the tumor grows, it uses

Glucose as a source of energy. Glucose is broken down to Lactic Acid. It is the acid which activates the Aziridine ring. The ring opens to generate a carbonium ion which attacks the most negatively charged N-7 Guanine of DNA (as shown below) shutting off the Walker Carcinoma gene in Rat. The following conjugate structure show how CB1954 binds to a single stranded of DNA shutting off the gene.



Conjugated DNA Disrupting Protein Synthesis Pathway of Cancer Cell

For the discovery of CB1954, The University of London, honored with the Institute of Cancer Research (ICR) post-doctoral fellowship award to synthesize more analogs of CB1954. To improve drug delivery method, over the years, I made over a hundred additional analogs of Dinitro phenyl aziridines. To increase the toxicity of CB1954 to Walker Carcinoma, I made additional 20 analogs as a postdoctoral fellow. When I attached one more Carbonium ion generating moiety, the Carbamate moiety to the Aziridine Dinitrobenzene, the compound Aziridine Dinitro benzamide Carbamate was deadliest. It was so toxic that its Therapeutic Index could not be measured. We stop the work. Further work in London University was discontinued for safety reason.



The Best and the Worst Dinitro phenyl Aziridine Analogs

Although Aziridine Carbamate is extremely toxic, it is also very useful in testing the sensitivity of tumors in Tumor Bank. Over the years, some tumors in the tumor bank could become resistant. If a tumor culture survives in a petri dish by adding a solution of Aziridine Dinitrobenzene Carbamate, it means that this tumor has become resistant over the years and must be replaced by new sensitive tumor cells.

As a part of the inter-government agreement between UK and USA, all novel drugs developed in England were sent to the National Cancer Institute (NCI) in America for further screening. To translate animal work to human, I was invited to continue my work on the highly toxic Aziridine/Carbamate combination in America when I was offered the Fogarty International Fellowship Award to continue my work at the National Cancer Institute (NCI) of the National Institutes of Health (NIH), USA. For making more Aziridine/Carbamates, I brought the idea from London University of attacking one strand of DNA using not only Aziridine, but also Carbamate without using the same dye Dinitro benzamide. My greatest challenge at NCI is to translate the animal work to humans.

In developing drugs for treatments, we poison bad DNA selectively. All poisons are a class of chemicals that attacks all DNA good and bad alike. Chemicals that cause cancer, at a safe level, can also cure cancer. Science teaches us to selectively attack bad sets of DNAs without harming the good sets of DNAs. Poisons are injurious to living creatures. There is a small class of chemical, when exposed to humans, disrupt the function of DNAs, and make normal cells abnormal and they are called cancer causing chemicals or carcinogens. I must confess, we still use surgery to cut off a cancerous breast; we still burn cancer cells by radiations; and we still poison cancer cells by chemicals. The largest killer of women is breast cancer. After all the treatment, the traces of remaining cancer cells return in three years as metastatic cells and kill breast cancer patients. A decade from now, these methods could be considered as brutal and savage, but today that is all we have. We hope to develop new treatment for Breast Cancer. Hopes means never ever to give up.

Nitrogen Mustards are highly toxic because they have neither specificity nor selectivity. They attack all dividing cells whether they are normal or abnormal. On the other hand, the DNP and TNP analogs of Aziridines and Carbamates serve as pro-drug and they remain inactive in the basic and neutral media. They become activated only in the presence of Acidic media. The growing tumor uses Glucose as a source of energy, but the Glucose break down to Lactic Acid.

I used a simple rationale, the Aziridine attacks DNA in acidic medium, particularly the N-7 Guanine. The dye Dinitro benzamide has great affinity for Walker Tumor [14-16]. The Aziridine dinitro benzamide (CB1954) stain the tumor. As the tumor grows, it uses Glucose as a source of energy. Glucose is broken down to Pyruvic Acid. It is the acid which attacks the Aziridine ring. The ring opens to generate a Carbonium ion which attacks the most negatively charged N-7 Guanine of DNA shutting off the Walker Carcinoma gene in Rat. To continue my work, I was honored with the Institute of Cancer Research Post-Doctoral Fellowship Award of the Royal Cancer Hospital of London University. To increase the toxicity of CB1954 to Walker Carcinoma, I made additional 20 analogs as a postdoctoral fellow. When I attached one more Carbonium generating moiety, the Carbamate moiety to the Aziridine Dinitrobenzene, the compound Aziridine Dinitro benzamide Carbamate was so toxic that its Therapeutic Index could not be measured. We stop the work at the London University for the safety concern.

I continued my work on the highly toxic Aziridine/Carbamate combination in America when I was offered the Fogarty International Fellowship Award to continue my work at the National Cancer Institute (NCI) of the National Institutes of

Health (NIH). I brought the idea from London University of attacking one strand of DNA using not only Aziridine, but also Carbamate without using the same dye Dinitro benzamide.

My greatest challenge at NCI is to translate the animal work which I did in London University to humans. One day, I came across a paper which described that radio labeled Methylated Quinone cross the Blood Brain Barrier in mice. When injected in mice, the X-ray photograph showed that the entire radioactivity was concentrated in the Mice's Brain within 24 hours. I immediately realized that Glioblastoma multiforme, the brain tumor in humans, is a solid aggressive tumor like Walker Carcinoma in Rats. I decided to use Quinone moiety as a carrier for Aziridine rings to attack Glioblastomas. By introducing an additional Carbamate moiety, I could increase its toxicity several folds. I planned to use this rational to translate animal work to human by introducing multiple Aziridine and Carbamate moieties to the Quinone to test against Glioblastomas in humans. Attaching two Aziridines and two Carbamate moieties to Quinone, I named this novel compound AZQ. By treating brain cancer with AZQ, we observed that Glioblastoma tumor not only stop growing, but also start shrinking. I could take care of at least one form of deadliest old age cancers that is Glioblastomas. Literature search showed that AZQ is extensively studied.

As I said above, Glioblastomas, the brain cancers, is a solid and aggressive tumor and is caused by mutations on several Chromosomal DNA. Mutations on DNA is the result of damaging DNA nucleotides by exposure to radiations, chemical and environmental pollution, viral infections or genetic inheritance. The other factors responsible for causing DNA mutations are due to the fast rate of replication of DNA. For example, the bacteria E-coli grows so rapidly that within 24 hours, a single cell on a petri dish forms an entire colony of millions when incubated on the Agar Gel. Mistakes occur in DNA during rapidly replication such as Insertion of a piece of DNA, Deletion, Inversion, Multiple Copying, Homologous Recombination etc.

When an additional piece of nucleotide is attached to a DNA string, it is called Insertion or a piece of DNA is removed from the DNA string; it is called Deletion or structural Inversion of DNA is responsible for mutations. Since the gene in a DNA code for Proteins, Insertion and Deletion on DNA have catastrophic effects on protein synthesis. Glioblastomas represent such an example. In Glioblastomas, three major changes occur on Chromosomes (C-7, C-9 & C-10) and two minor changes occur on Chromosomes (C-1 & C-19). These mutations are responsible for causing brain cancers in humans. In a normal human cell, Chromosome-7 which is made of 171 million nucleotide base pairs and it carries 1,378 genes. When Insertion occurs on Chromosome-7. Ninety-seven percent of Glioblastoma patients are affected by this mutation. On the other hand, a different mutation occurs on Chromosome-9 which is made of 145 million nucleotide base pairs and it carries 1,076 genes. A major Deletion of a piece of DNA occurs on Chromosome-9 which results in eighty- three percent patients who are affected by this mutation. A minor Deletion of DNA also occurs on Chromosome-10 which is made of 144 million base pairs and it carries 923 genes. Although it is a minor deletion of a piece of DNA and yet it contributes to ninety-one percent patients with Glioblastoma. To a lesser extent, small mutation occurs on Chromosome-1 (the largest Chromosome in our Genome). It is made of 263 million nucleotide base pairs and carries 2,610 genes) and Chromosome-19 (it is made of 67 million base pairs and carries 1,592 genes) is also implicated in some forms of Glioblastomas.

All known Glioblastomas causing genes are located on five different Chromosomes and carries a total of 9,579 genes. It appears impossible to design drugs to treat Glioblastomas since we don't know which nucleotide on which gene and on which Chromosome is responsible for causing the disease. With the completion of 1,000 Human Genome Project, it becomes easier. By simply comparing the patient's Chromosomes with the one thousand genomes, letter by letter, word by word and sentence by sentence, we could identify the difference called the variants with precision and accuracy, the exact variants or mutations responsible for causing the disease. Once the diagnosis is confirmed, the next step is how to treat the disease.

With the Quinone ring, I could introduce different combinations of Aziridine rings and Carbamate moieties and could create havoc for Glioblastomas. My major concern was how toxic this compound would be to the human brain cells. Fortunately, brain cells do not divide, only cancer cells divide.

Our Rational Drug Design work began in the University of London, England, and completed in the Laboratory of the National Cancer Institute (NCI), of the National Institutes of Health (NIH), in Bethesda, Maryland, USA. Over this period, we conducted over 500 experiments which resulted in 200 novel drugs. They were all tested against the experimental animal tumors. Forty-five of them were considered valuable enough to be patented by the US Government (US Patent 4,146,622). One of them is AZQ. Radiolabeled studies showed that AZQ has the ability to cross organ after organ, cross the Blood Brain Barrier, cross the nuclear membrane and attack the nuclear DNA shutting off the gene. X-ray studies showed that the radioactivity is concentrated in the tumor region. Glioblastoma stop growing and start shrinking. The toxicology studies on AZQ have taken many years to complete [17,18].

My Fogarty Internation Fellowship ended after three years; I could apply for US citizen after five- year stay in US. I worked for two Years in Southwest Research Foundation in San Antonio, Texas. later when I became US citizen, I was offered the first US Government job by FDA where I worked for many years. When I returned to NIH, for. The discovery of AZQ, I was honored with the "2004 NIH Scientific Achievement Award" one of America's highest awards in medicine and I was also honored with the India's National Medal of Honor, "Vaidya Ratna" a Gold Medal. (see Exhibits 1,2,3,4).

Exhibit # 1

2004 NIH Scientific Achievement Award Presented to **Dr. Hameed Khan** By **Dr. Elias Zerhouni,** The Director of NIH During the NIH/APAO Award Ceremony held on December 3, 2004.

Dr. Khan is the Discoverer of AZQ (US Patent 4,146,622), a Novel Experimental Drug Specifically Designed to shut off a Gene that causes Brain Cancer for which he receives a 17-year Royalty for his invention (License Number L-019-01/0). To this date, more than 300 research papers have been published on AZQ. The award ceremony was broadcast live worldwide by the Voice of America (VOA). Dr. Khan is the first Indian to receive one of America's highest awards in Medicine.

Exhibit # 2

His Excellency,
Dr. A.P.J. Abdul Kalam,
The President of India Greeting
Dr. A. Hameed Khan

Discoverer of anti-cancer AZQ, after receiving 2004, Vaidya Ratna, The Gold Medal, One of India's Highest Awards in Medicine At The Rashtrapathi Bhavan (Presidential Palace), in Delhi, India, During a Reception held on April 2, 2004.

2004 NIH Scientific Achievement Award

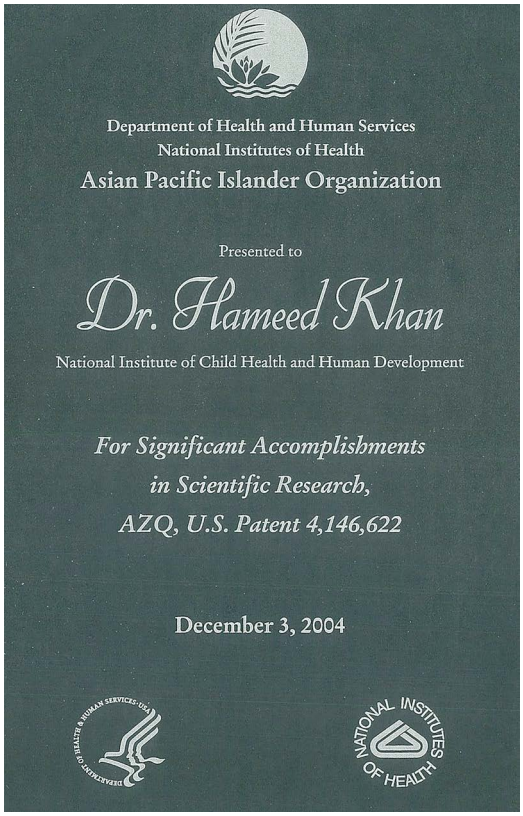
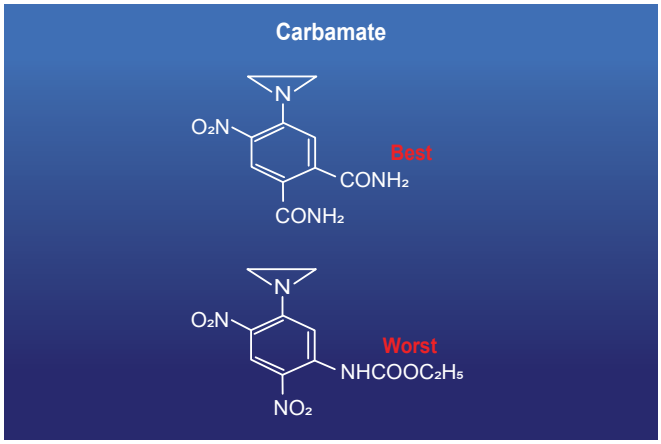


Exhibit # 3

Single Strand DNA Binding Aziridine and Carbamate



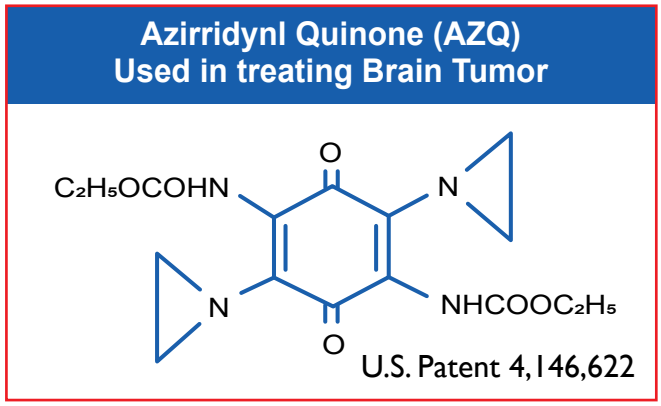


Exhibit # 4

Gold Medal for Dr. Khan

Dr. A. Hameed Khan, a Scientist at the National Institutes of Health (NIH) USA, an American Scientist of Indian Origin was awarded on April 2, 2004. Vaidya Ratna; The gold Medal, one of India's Highest Awards in Medicine for his Discovery of AZQ (US Patent 4,146,622) which is now undergoing Clinical Trials for Treating Bran Cancer.

After staying for five years in America working in private institutions, I became a US citizen. I was offered my first US Government job in the Food & Drug Administration (FDA) as the Consumer Safety officer (CSO). FDA is the best place to receive training to go into science administration. My job involved reading enormous industry application for the approval of novel food and drugs. I set up Study Sections; I invited best and the brightest scientists, expert in the field to identify strengths, weaknesses in the proposals and make recommendation and what additional studies are needed to make the product safe and effective and summarize their comments and recommendation in a Summary Statement. Over the years, I conducted dozens of study sections for FDA, wrote hundreds of summary statements making recommendations.

In spite of all precautions, small amount of drug residues passed on in the tissues of food-producing animals. Constant exposure to small amount of drug could either make human resistant to the drugs or cause future health problems. Detecting tiny number of drugs in tissues extracts is our greatest challenge. NIH has annual budget of $50 billion each year, it can afford to buy the latest and most expensive equipment for various research project. One of the latest instruments is a new time-of flight mass spectrometer attached to a radioactive 252-Californium Plasma desorption mass Spectrometry (CPDM) source, in my other project, I was successful in obtained mass spectra with the molecular ion peak of all nine derivatized antibiotics as their sodium positive ion peaks. In most cases, there are very few fragmentation peaks and the molecular ion was primary peak in the spectrum. I immediately realized that tiny amount of drug residue of aminoglycosides could be detected in the extracts of food-producing animal by making their DNP and TNP analogs of aminoglycoside antibiotics. These spectra could be used as Reference Spectra when comparing with the unknown samples obtained during inspection. These spectra will help the food inspectors in the field not only to identify the misuse of any of antibiotics in the tissue extracts, but also will identify the amount of residue present.

Synthesis of DNP and TNP Analogs of Aminoglycoside Antibiotics

Over the weakened, I wrote a 250 words concept research proposal describing how I plan to synthesize DNP and TNP analogs of all eleven aminoglycoside antibiotics and use a novel mass spectral method of detection in the (part per billion) PPB range. These spectra will help the food inspectors in the field not only to identify the misuse of any of antibiotics in the tissue extracts, but also will identify the amount of residue present. I submitted the concept review proposal to my immediate supervisor, Dr. Lonnie Luther, a true gentleman, with a request to work on this proposal if he gave me a day off in a week. After reviewing the proposal, Dr. Luther told me that our current workload does not allow him to give me a day off to work on the proposal. I asked him what if I were to work ten hours a day and four days a week, could he give me Friday off. Since he also come to work very early in the morning, he approved and I promised him that I will work on my own time on Saturdays and Sundays working 12 hours a day and will try to finish the work as quickly as possible. To avoid any interference, we informed no one in the office about my new working arrangement.

During a meeting in FDA at the Parklawn Building, I shared my concept proposal with Dr. Edward Allen, Chief of Pharmacology and Toxicology at the USDA (United States Department of Agriculture).at the Beltsville Labs, He loved it and thought it was a brilliant idea. With a big laugh he said that my work would challenge the existing scientists in the FDA Lab, If I publish this paper, I will thumb their noses particularly of Dead Woods sitting in the Lab in Downtown. He said that he will provide every assistance I need., Lab space, chemicals and equipment. I offered him the co-authorship. Dr. Allen was the most powerful and his support was essential. On my way back to the office, I stopped at NIH and met with my old friend Ed. Sokolowski with whom I published some work, he is an expert in running Mass Spectrometer. I had worked with him before. When I shared the proposal with him, he was more than happy to collaborate with me. It will be a joint effort between FDA/USDA and NIH. All these buildings are about 20 miles apart from each other. The synthetic work will be done on Saturdays and Sundays at the USDA Labs, I will clean up and purify the analogs on Sundays and the Mass Spectral analysis will be done at NIH, on Fridays when Ed was working, I will write the paper at my office at FDA, Parklawn Building.

The stage was set, there was no time to waste. I rushed through my work collecting chemicals and equipment. One thing is certain that I will share the progress of my work only with my supervisor, but not a word with the FDA hierarchy. If I seek their permission of the Big Boss, he will remove all other names and place his name as he had been doing with all official documents. This is not an official document. This is my idea; I conceived it; I will complete it. When I complete the work, I will publish it without their name. I fear what would happen when he sees the publication without his name. Let us proceed if I succeed. I will confront the Big Boss when times come.

My biggest challenge was to work with no information on the boys exposed to antibiotics showing big breast for example their blood analysis data showing the presence of drug residue; no health affecting residue was available on the analysis of the air, land or water analysis. I cannot even ask for such data from the inspectors in the field. It will upset a lot of people. In the absence of this information, I chose to derivatize all available aminoglycosides antibiotics and measure the lowest level of detection by Mass Spectral analysis without sharing the story of their exposure to teenage boys. I will simply say that the method of detection will help the food inspectors in the field to detect misuse of these residues.

The following two years, by working three days a week and almost twelve hours a day, I completed the work and wrote over a hundred-page long report entitled, "A Confirmatory Mass Spectrometry Method for Aminoglycoside Antibiotics." Before circulating throughout FDA, I sent the first copy of the bound volume to Dr. Ed Allen. He was delighted and wrote the following letter of commendation to my Supervisor, Dr. Lonnie Luther: To avoid any confrontation among basses, I did not release this letter for almost quarter of a century, hoping some of them will retire, die and disappear from the face of the earth.

Date October 15, 1985

From Edward H. Allen, HFV-520

Subject Research conducted by Dr. A. Khan aL

Confirmatory Mass Spectrometry Method

To Lonnie Luther, Ph.D.

Chief, Swine and Poultry Drugs Branch

Division of Biometrics and Production

Memorandum

DVMR and NIH. Report: A

for Aminoglycoside Antibiotics".Drugs

Dr. Khan is to be commended for recommending the use of 25zc^Litornium plasma desorption mass spectrometry for identifying the aminoglycoside antibiotics. I believe that this work is a pioneering effort to use a new type of mass spectrometry (MS) to identify and distinguish among the aminoglycosides. Other types of MS do not give the necessary data to adequately identify these drugs. Dr. Khan has put in long hours to prepare the best possible derivatives of nine aminoglycosides. This work is absolutely essential to apply the new MS technique to a multiresidue method for aminoglycosides isolated from edible animal tissues and milk. The latter work is not complete at this time; however, the preliminary work on the nine aminoglycosides is necessary to show that it is possible to identify aminoglycosides with several different structures. Not only is Dr. Khan a very persistent and diligent scientist, but he has, the diplomatic talent to work with the research scientists at several locations in this area to complete his research and other assignments related to his overall work assignment.

This report gives the details for synthesis of three derivatives and the corresponding MS spectra for nine aminoglycoside antibiotics. The spectra have few lines, a very strong molecular ion peak, and clearly show the application to residue identification. That is, the molecular ion peak is always very intense which will a11ow the detection of small qualities of each drug. The completed draft, I gave it to Dr. Allen. He wrote the following: It has been a pleasure working with Dr. Khan and I expect continued significant performance from him as a researcher exploring problems important to CVM.

Edward H. Allen, Ph.D.

Acting Chief, Veterinary

Pharmacology/Toxicology Branch

Division of Veterinary Medical Research

Once I completed the project, I had the work nicely typed and bound in a 100page volume. I wanted to release the volume to see the effect of my secret work on my colleagues and bosses. Next, I presented a copy of the report to the Big Boss. Dr. Luther and I went to his office with the copy of the report. The Big Boss had a Ph.D. degree in Statistics and did not know any Chemistry. This report contains Synthetic Organic Chemistry and Mass Spectrometry. He turned page after page looking at Organic Molecular Structure searching for his name. He found his name in the acknowledgement section. Not finding his name as the co-author, he was furious and almost screamed and showered us with questions. Who Authorized this work? Who prepared this report? Who worked on it? Who approved it? Where was this work done? He said he disapprove the report and launch an investigation. He did not allow us to say a word. He threw the report in front of me and got up said that he would like to see who will publish it; who will approve it and who will fund it acceptable and now get out of here.

Upon returning to my office, I called Dr. Ed Allen and gave him the detail discussion with the Big Boss. Dr. Ed Allen is equally powerful. He immediately called the Big Boss. There was clash of Titans, a shouting match between the two giants. Ed Allen told him that he is recommending to the director launching an investigation about the Big Boss, what has he done during the last five weeks in his office, during the last five months and during the last five years in the office except signing the documents prepared by his staff removing everyone's name except his.

Dr. Allen told the Big Boss that the project Dr. Khan completed has nothing to do with his office work. He worked in his private time making contribution to FDA effort to detect misuse of antibiotics in food producing animals. You cannot be a co-author. The work involved is beyond your expertise. It described the synthesis of Organic molecules and Mass Spectrometry.

Soon after our conversation with the Big Boss, Dr. Allen sent his letter he had written to Dr. Luther about the significance of my work to the main office which was circulated throughout the agency. Soon after the meeting with the Big Boss, my Fridays off authorization was cancelled and my working four days a week 10 hours day authorization was also cancelled.

In spite of the crisis in the office, I prepared an abstracted paper for publication, and circulated among the co-authors. They all approved it. I put the draft away in a locker hoping to bring it out after the crisis is over. An Angle appeared in the mid of crisis. Dr. Catherine Fenselau, was a professor at the Johns Hopkins University specializing in Mass Spectrometry. She was serving as a reviewer in a meeting in FDA at the Parklawn Building. I showed her the draft paper. She loved it because we were used Californium-252 probe a new method of detection in Mass Spectrometry. She rushed through the

publication without accepting any payment. I submitted the paper on February 1988, the paper was reviewed, accepted and published in the Journal of Biomedical and Environmental Mass Spectrometry on April 1988. The following abstracted paper was published in, Biomedical and Environmental Mass Spectrometry, Vo. 17, 329-355 (1988) [19].

Californium-252 Plasma Desorption Mass Spectrometry of Aminoglycoside Antibiotics

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Edward A. Sokoloski

Laboratory of Chemistry, NHLBI, National Institutes of Health, Bethesda, Maryland 20205, USA

The paper was published without the name of Big Boss name as a co-author. The paper was well received, but it created the biggest crisis. The Big Boss was furious. I know my chances of staying in FDA is zero. During the ten-year period while serving in FDA, from 1978 to 1988, I must have written hundreds of documents and not one document mentions my name except the research paper. The paper describes a quick method of detection of misuse of any antibiotics. The field inspector collects a suspect tissue extract in which he adds an acetone solution of coloring agents like picryl chloride or Dinitrochlorobenzene. He heats the solution for five minutes. The coloring agents bind to antibiotics. The solution is cooled and extracted the compound with chloroform. The chloroform solution is injected into the GC/MS (Gas Chromatograph/ Mass Spectrometry) . The GC separate each derivatized antibiotic and MS shatter the molecule by a beam of neutrons to get the Molecular ion peak corresponding to the molecular weight of the derivatized antibiotics. By comparing the spectrum with the Reference Spectra published in the paper, we can identify the kind of antibiotic used and GC identify the level of antibiotic used.

What is the reward for my two years effort? I have a choice either to get kicked out dishonorably or quit honorably. I chose to quit honorably.

NIH is full of Angles; they recommended my transfer from FDA to NIH.

The Angle who rescued me from the wrath of the Big Boss was Dr. Laurance Johnson, affectionately called Larry, a former colleague of mine. We worked together in FDA years earlier. Larry was brilliant, he had a Ph.D. degree in Biochemistry and also completed MBA (Master in Business Administration), he was invited by NICHD to assembled a group to clear a massive backlog of research proposals build up over the years. Larry personally traveled from USDA to FDA to NIH, picking up the best and the brightest scientists to join his group to clear the backlog Larry assembled about 20 scientists. He created and headed a new branch called (DSR) Division of Scientific Review in the NICHD. Larry was most admired and most respected. He treated us all as friends. To reduce the backlog, he set a race among us to conduct as many study sections as fast as possible. When Larry asked us to work over the weakened, we love to work for him. One Sunday afternoon, we finished the work earlier, he organized a picnic in the Needwood Park. He ordered food and drink and we all played volley ball and some of us took boat rides. We loved to work for him. Unlike most supervisor, Larry ran a study section himself.

Larry was a perfect Boss, but he had one major flaw. This flaw is considered an asset in women, but in men it is a major curse. Larry was very handsome. Women from the lowest to the highest rank fall over him. His office was always crowded with women. Although we work next door, if we seek his advice, we had to call him on the phone. He went to lunch with them and played Tennis with them. There were also old and wiser women who suffered from jealousy, anger and hatred. They hated him and they brought his down fall. They spread stories about him, some true, some false and some exaggerated. He fell from the grace into the oblivion. No one knows the fine detail except the front office. He accepted the responsibilities. He was divorced, resigned and vanished from the face of the Earth. No one knew where he went. Someone claimed to have spotted him in Vales, Colorado grown ugly beard to hide his face, teaching skiing to school kids. You were the best boss and best friend. God Bless you Laurance Johnson whereever you are.

Although Larry was gone, I continued to participate in the race to conduct as many Study Sections as possible, within a few years, I won the race I conduced more study sections than any of my colleagues. For my effort, NIH honored me with Merit Award for Supporting Research.

Presented To Dr. A Hameed Khan

By Dr. Duane Alexander, M.D. Director, NICHD

Dr. Robert Stretch, Director DSR and

Dr. Yvonne Maddox, Deputy Director, NICHD

In recognition of his superior commitment, dedication and accomplishment in the planning and executing of over 250 Peer Review Meetings for both Grants and Contracts. Dr. Khan was honored during the Director's Award Ceremony held on October 11, 2006.

What is the future of the CSO?

What is the future of young CSO scientists who conceived novel brilliant ideas? How can they translate theories into practice. I have good news and I have bad news. The good news for young bright minds is if you have a novel idea, NIH is here to help you. You write a Concept Review Proposal of 250 words and send it to NIH Concept Review Committee If the review committee of expert approves the proposal, NIH provides $100.000 per year for two years to conduct the study and generate data. You have to attach yourself with local research institution. They will love to provide you the space and facility. Your work will promote their institutions. (Google NIH Home Page to get more detail). If you succeed in generating the data by proving the idea, you can use the data to apply for an R01 grants for three-to-five-year research support. The bad news is how to handle the Big Boss the dead wood. Since you work in his office, he claims every piece of your work belongs to him. The only solution is to look for another job, Years later, I heard my Big Boss fell from the grace into the gutter. He was sent on a permanent vacation.

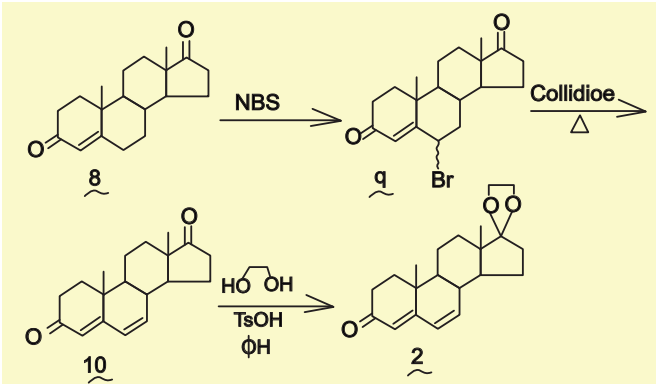
Here is an example, how to write a Concept proposal:

Concept proposed rationale for treating Breast tumor:

Although the breast cancer causing BRCA1 gene is located on Chromosome-17 (which is made of 92 million nucleotide bases carrying 1,394 genes) has been identified years ago, we wonder why it has been so difficult to treat Breast Cancer. By the time the Breast Cancer diagnosis is confirmed in a patient, the BRCA1 gene has accumulated more than three thousand mutations. Genotyping of the blood would also show that composition of many cells carrying mutated cell for creating secondary deposits. It is also believed that by the time Breast Cancer is confirmed, metastatic cancer cells have already been spread from liver lung on its way to brain.

Radiolabeled studies showed that male hormone Testosterone has great affinity for female Breast, Ovary, and Fallopian tube cells. On the other hand, Estrogen, the female hormone, has great affinity for male prostate gland. By attaching multiple Aziridine rings and Carbamate moieties to both Hormones, I could attack the Breast and the Prostate cancer.

In a Breast tumor, within the start (AUG) and stop codon (UGG or UGA, or UAG), BRCA1 gene has captured over two hundred thousand nucleotide bases. The BRCA1 genes carries about three thousand mutations. These mutations are caused by radiations, chemical or environmental pollutants, viral infection or genetic inheritance. To attack the mutated nucleotides among the three thousand cells in BRCA1 gene, I could use male hormone, Testosterone, and bind multiple radio-labeled Aziridine and Carbamate moieties to attack BRCA1 mutations. By using MRI, I could show how many



radio-labeled nucleotides were bound to which mutations. Out of seventeen positions available for substitutions on Testosterone. There are only three positions that is 1,3 and 17 positions are available on Testosterone ring system. I could activate position 9 and 10 by reacting with Bromo-acetamide which introduce a Bromo ion on position 10 which could be dibrominated by Collidine to introduce a 9,10 double bond which I could further brominate to produce 9,10 dibromo compound. These bromo ion could be replaced by additional Aziridines or Carbamate moieties. I could increase or decrease the number of Aziridine and Carbamate ions to get the maximum benefit by further brominating position 15 and 16 to introduce additional Aziridine and Carbamate moieties.

[14] Carl Djerassi C. Djerassi et al. J. Amer. Chem. Soc. 72. 4534 (1950)] had demonstrated that we could activate additional positions for substitutions on hormone ring system such as the position 9 and 10 by reacting with Bromo-acetamide which introduce a Bromo ion on position 10 which could be de-brominated by Collidine to introduce a 9,10 double bond which we could further brominate to produce 9,10 dibromo compound. These bromo ion could be replaced by additional Aziridines or Carbamate ions. We could increase or decrease the number of Aziridine and Carbamate ions to get maximum benefit by further brominating position 15 and 16 to introduce additional Aziridine and Carbamate moieties.

Similarly, I could use the female hormone Estrogen and attach multiple Aziridine and Carbamate ions to attack Prostate tumor. Since there are seventeen positions available on Estrogen ring as well; again, I could increase or decrease the number of Aziridine and Carbamate ions to get the maximum benefit. Future generation of scientists (my students) will use this method to develop drugs to treat all cancers.

Summary

This report describes successful method to obtain the mass spectra for all nine aminoglycoside antibiotics, namely: neomycin, kanamycin, paromomycin, tobramycin, apramycin, amikacin, gentamicin, netilmicin, and sisomicin separated from an aqueous solution as their dinitrophenyl {DNP) and/or trinitrophenyl {TNP) derivatives. Derivatization of the antibiotics was useful for the following three reasons: First, the pure antibiotics in biological fluids are too hydrophilic and could not be extracted in organic solvents for mass spectral analysis. However, after derivatization, the antibiotic derivatives were soluble in.Organic solvents and mass spectra.were obtained in methanol/THF solution. A second advantage of the derivatization of the antibiotics is to increase their molecular weights by several factors.· This increase in molecular weight faci'l itates the measurement of small quantities of the antibiotics extracted from the edible tissues of food-producing animals. -For xample, when neomycin with a.molecular weight of 614 is derivatized to its TNP derivative, its·molecular weig·ht increases to 1882.,- a 3-fold increase.

Thus, the mass spectrum obtained from 10.·mcg quantity of the TNP-neomycin derivative, in fact, corresponds to about 3 mcg·quantity of the pure neomycin. Finally, the reaction of DNP and TNP derivatizing·agents produces a unique DNP and TNP derivative of antibiotic in the presen e of impurities in biological fluids. For example, the neomycin molecule contains 5 reacting amino groups on each molecule and produces 5 DNP or 5 TNP derivatives.

Thus, derivatized antibiotics can easily be distinguished from impurities by mass spectral analysis, giving this process an even higher degree of specificity than is possible by any other available method. These spectral data are used to confirm the presence of residues of antibiotics in the multicomponent aqueous tissue extracts and may be used for FDA or USDA compliance and regulatory activities concerning the abuse of illegal aminoglycoside antibiotic residues in the tissues of food-producing animals.

The confirmation of these aminoglycoside antibiotics by conventional Electron Impact (EI) or Chemical Ionization (CI) mass spectrometry did not produce a molecular ion peak for underivatized antibiotics because the antibiotics are difficult to volatilize and are thermally unstable. By contrast, with the confirmation of the aminoglycoside antibiotics by a new time-of-flight mass spectrometer with a radioactive ^{252}californium plasma desorption source, was obtained by producing mass spectra with the molecular ion peak of all nine antibiotics as their sodium positive ion peaks...In most cases, there were very few fragmentation peaks and the molecular ion was the primary peak in.,the spectrum.

This is a pioneering and innovative concept in that the ^{252}californium plasma desorption mass spectrometry has never been used as a tool in the development of analytical methodology for detecting residues of antibiotics in the tissue extracts of food-producing animals.

Furthermore, since this is a relatively new technique, published reports could not be found for the application of this technique to aminoglycoside antibiotics. This report contains the first known mass spectra of nine aminoglycoside antibiotics using ^{252}californium plasma desorption mass spectrometry.

Nine DNP and nine TNP derivatives of these antibiotics were synthesized. Each DNP or TNP antibiotic derivative produces two different kinds of mass spectra: 1) a negative ion mass spectrum which corresponds to the molecular weight of the DNP or TNP derivative and 2) a positive ion mass spectrum which corresponds to the molecular weight of its sodium or potassium salts. When a negative ion mass spectrum of a derivative gives a weak molecular ion peak, the same derivative may be identified by a stronger positive ion spectrum. For all 18 derivatives, we obtained 36 mass spectra, 18 negative ion mass spectra and 18 positive ion mass spectra. A total of 45 mass spectra are reported. The remaining nine represent the positive ion spectra of the underivatized antibiotic.

Thus, four different methods have been developed to monitor the residues of the same antibiotic in n aqueous solution. These mass spectral data may be used for FDA or USDA compliance and reg latory activities concerning the abuse of illegal antibiotic residues in the tissues of food-producing animals.

This work was·completed in Bel.tsville {FDA, CVM, DVMR) and NIH Laboratories by working one day per week from August 1984 to October 1985.

Acknowledgments

I wish to express my sincere thanks to the following scientists of the Center for Veterinary Medicine without whose encouragement and authorization this work could never have been completed:

Drs. L. W. Luther, R. Lehmann, R. Carnevale, M. Norcross, R. Benson,

R. E. Osterberg, J. A. Settepani, N.E. Alderson and R. E. Teske.

Mass spectrometry work was conducted at the NIH Laboratories and I am deeply indebted to Dr. Henry Fales, Dr. Helen Lloyd and Mr. E. Sokoloski.

My thanks to Ms. Iris Hyman for reading the entire manuscript.

Background

Aminoglycoside aminocyclitol antibiotics are valuable therapeutic agents. They are considered broad spectrum antibiotics because they inhibit the growth of both gram-positive and gram-negative bacteria, as well as mycobacteria. All aminoglycoside antibiotics are relatively small, (molecular weights range from 440 to 590) basic water soluble molecules that form stable salts. Most aminoglycosides are products of the fermentation of filamentous actinomyc tes of the genus Streptomyces (for example, neomycin, kanamycin, paromomycin, and apramycin) although other groups, including the gentamicins, are derived from species of the genus Micromonospora. In Micromonospora-derived antibiotics (for example, gentamicin, and sisomicin), the terminal portion of their generic names is spelled micin to distinguish them from mycins that are derived from the Streptomyces species.

The newer semisynthetic aminoglycoside antibiotics are derived from antibiotics that are produced by natural fermentation, such as kanamycin, and that have been subsequently altered structurally to produce semi synthetic antibiotics such as tobramycin or amikacin. Another example is netilmicin which is similar structurally to gentamicin and sisomicin. Netilmicin is synthesized from naturally occurring sisomicin. A critical difference between netilmicin and sisomicin is in the! position of the 2-deoxystreptamine moeity in which sisomicin has an amino group and ne.tilmicin has an N-ethyl group. The presence of this N-ethyl group protects netilmicin sterically from inactivation by many common aminoglycoside-inactivating enzymes. Otherwise, netilmicin1s spectrum of activity is similar to that of other aminoglycosides.

The agency has approved the use of a number of aminoglycoside antibiotics in combination with a variety of drugs to prevent diseases in food producing animals. The sponsors rely mostly on the microbiological assay to detect residues of these antibiotics in the edible tissues. Although the microbiological method is rapid, it is neither specific nor sensitive. In the absence of any specific mass spectrometry method, we developed a confirmatory mass spectrometric method for all nine antibiotics and their nine DNP and nine

TNP derivatives. The confirmatory mass spectro metric method is both specific and sensitive. It is specific as the molecular ion will identify a specific antibiotic in _the tissue extracts in the· presence of multi-component residue mixture, and it is sensitive as it measures antibiotic residues in sub-part per million quantities.

Objective

The purpose of this study was to develop a mass spectrometric confirmatory procedure for aminoglycoside antibiotics in tissue extracts of food-producing animals. The confirmation of the aminoglycoside antibiotics by conventional Electron Impact (EI) or Chemical Ionization (CI) mass spectrometry procedure did not produce a molecular ion peak for underivatized antibiotics because the antibiotics are difficult to volatilize and are thermally unstable. If the parent ion intensity is weak or non-existent, identification must be made primarily on·the basis of the fragmentation pattern which may give an ambiguous result. By contrast, with the confirmation of the presence of aminoglycoside antibiotics by a new time-of-flight mass spectrometer with a radioactive 252californium plasma desorption source, we were able to obtain mass spectra with the molecular ion peak of all nine commercially available antibiotics as their sodium salt positive ion. In most cases, there were very few peaks and the molecular ion was the primary peak in the spectrum. These are the first spectra of these antibiotics in which 252cf-Plasma desorption source has been used and molecular ions are produced with and without derivatization. In developing this method, we were faced with two problems: The first problem was that the pure antibiotics are difficult to volatilize and are thermally unstable. The Electron Impact (EI) or Chemical Ionization (CI) mass spectrometer was unable to produce a parent molecular ion peak spectrum for pure antibiotics. The second problem was that the pure antibiotics in biological fluids are too hydrophilic and could not be extracted in organic solvents for mass spectral analysis.

The first problem was overcome by the use of a time-of-flight mass spectrometer with a 252californium plasma desorption (252cf-POMS) source for ionizing non-volatile and thermally unstable molecules. The 252cf-PDMS instrument produces a mass spectrum of non-volatile compounds from solid state at room temperature.

The second problem was overcome by derivatizing the antibiotics with chromophoric reagents such as picryl chloride and 2,4-dinitroflurobenzene in an alkaline aqueous solution. This produced the nitrophenyl derivatives which were less soluble in water and could then be separated and analyzed on the mass spectrometer.

This report describes the first segment of activity which was to develop methods of derivatization on the pure antibiotics in an alkaline aqueous solution. Derivatizations of all nine aminoglycoside antibiotics in an aqueous alkaline solution have been completed. Nine dinitrophenyl (DNP) derivatives and nine trinitrophenyl (TNP) derivatives have been synthesized. Mass spectral analyses of all 18 derivatives are presented. The second segment of the activity will be to test the same derivatization ethods on antibiotics extracted from biological tissues and to determine the applicability of the mass spectral method for these derivatized antibiotics. Part of the second segment which was to develop the mass spectral method on the tissue extracted antibiotics has been completed. So far, different concentrations of an incurred sample of neomycin extracted from the kidney tissues of cattle have been derivatized with picryl chloride and 2,4-dinitroflurobenzene.

The following two approaches are being considered to obtain the mass spectra of the derivatized neomycin from tissue extracts. One is to purify the derivatized neomycin from tissue extracts by chromatography (TLC) for mass spectral analysis (although neomycin obtained from the kidney tissues extracts has been purified by HPLC, derivatization introduces new impurities which requires further purification).

The other is to increase the sensitivity of the 252cf-PDMS instrument to obtain the mass spectra of the derivatized neomycin without purification. The mass spectral analyses of the derivatized neomycin for this portion of the study will be reported later.

Materials and Methods

All aminoglycoside antibiotics are basic water soluble molecules and are commercially available as their sulfate salts. The following antibiotics were purchased from the Sigma Chemical Company: neomycin, kanamycin, paromomycin, tobramycin, amikacin, and gentamicin. Apramycin was donated by Elanco Products, Inc. and netilmicin and sisomicin were donated by Schering Corporation. Picryl chloride was purchased from Polyscience Inc. and 2,4-dinitroflurobenzene was purchased from the Aldrich Chemical Company.

The aliphatic amino groups in the antibiotics molecule are quite basic and readily react with the picryl chloride or 2,4-dinitroflurobenzene in aqueous potassium carbonate solution at 80°C to form the corresponding nitrophenyl derivatives in high yields. After cooling, the product separates out of the solution and is collected by filtration.

Trituration of the product in methanol leaches out unreacted impurities, 1 eavi ng behind a fai rly pure product. Thin 1 ayer chromatography (TLC) of the product on a silica gel plate in THF:Hexane (4:1) gives a single spot. Further purification by either recrystallization from THF:Hexane or purification on a silica gel column from THF:Hexane causes the decomposition of the product as the mass spectral analysis of the purified sample from chromatrography column shows the loss of a nitrophenyl group from the derivatized neomycin. No further purification was attempted.

This report contains 27 structure sheets; nine describe the structures of underivatized antibiotics and the remaining 18 describe the structures of nine dinitrophenyl derivatives and nine trinitrophenyl derivatives. Also included are 18 experimental description sheets. Nine sheets each, respectively, describe the syntheses of dinitrophenyl and trinitrophenyl derivatives. A brief description of the derivatization of a gentamicin (C_1, c_2 & c_1a) mixture is reported. The above information is summarized in Table I & II. The two tables (pages 120 & 121) describe the characteristics of the dinitrophenyl and trinitrophenyl derivatives of the following aminoglycoside antibiotics: neomycin, kanamycin, paromomycin, tobramycin, apramycin, amikacin, gentamicin, netilmicin and sisomicin.

The tables also include the melting point (mp), percentage yield, and molecular weight of all 18 derivatives. The ^{252}cf mass spectral data describe the molecular ions of each of the derivatives. For all derivatives, both negative and positive ion peaks are reported. The negative molecular ion peaks correspond to the molecular weights of the derivatives. The positive ion peaks correspond to the molecular weights of their sodium or potassium salts. With underivatized aminoglycoside antibiotics, only positive ion peaks were stable enough to be recorded. Because the aminoglycoside antibiotics are difficult

to volatilize and are thermally unstable, the mass spectrum obtained from the electron impact or chemical ionization instrument is devoid of parent ion peak. For this reason, we have chosen for detecting molecular ions (M+H+) of the nitrophenyl derivatives of aminoglycoside antibiotics, a time-of-flight mass spectrometer with ^{252}californium plasma desorption source for ionizing non-volatile and thermally unstable antibiotics.

^{252}cf plasma desorption mass spectrometry (^{252}cf-PDMS) has been developed to obtain mass spectra of non-volatile compounds from solid state at room temperature. This method involves fast heavy-ion bombardment of a solid sample using radioactive ^{252}cal1•forn1•um. Volatilization and ionization of the sample are thus affected. Mass spectra are measured using the time-of-flight mass spectrometer featuring single-ion counting and fast electronics. The sample is deposited on a thin sheet of aluminized Mylar that is placed close to the ^{252}cf fission fragment source. When the fission fragment from the radionuclide passes through the thin aluminum foil, a thermal spike is created in the vicinity generating a localized temperature of 20,000° to 30,000°C for about 10-11 second. This high temperature mobilizes quantities of mobile impurity ions in the sample, for example, hydrogen, sodium and hydride ions (H+, Na+, H-) etc.

These secondary ions are found in combination with sample molecules to produce quasimolecular ions that are accelerated by a high voltage grid. At this constant potential, the time-of-flight of the ion to detector is proportional to the square root of the mass of the ion. This technique provides an effective way to ionize antibiotic molecules with little sample degradation.

Mass spectra were obtained with a ^{252}cf-PDMS system constructed for NIH by

R.D. Macfarlane (Texas A&M, College·station, TX). ·californium-252 (10 nCi) was used as the primary ion source and a 45-cm flight tube was used to separate the ions. The optics of the instrument allow about 60% of the hemisphere of fission particles to penetrate the sample foil. 0rtec Model 473A constant fraction discriminators were used to shape the start and stop pulses whose differences were digitized as flight times and transmitted to a Perkin-Elmer Model 3220 computer for analysis. Intensities are presented in terms of actual number of counts in the channel of maximum intensity (channel widths were 1.25 ns).

Samples of antibiotics dissolved in methanol/methanol-water/water-THF or/acetone were electrosprayed on a 1.7 cm2 zone of a 1 nm thick aluminized Maylar film for analysis. For convenience, the typical sample size was about 10 mcg although satisfactory spectra may be obtained on less than 1 mcg. Ions were collected for 20 minutes to four hours depending on the sample and spectral quality desired. 10 Kv accelerating voltage was used at room temperature and at a pressure of 3.10-7mm.

An important feature of this method is that samples may be recovered virtually intact (10-6 of the sample is used in the typical measurement), although losses may be incurred through irreversible absorption on the film, by volatilization in the spectrometer, or during electrospraying.

Results and Discussion

NEOMYCIN

The antibiotic neomycin carries 5 primary amino groups. These groups are quite basic and under alkaline aqueous conditions react with nitrophenyl halides (such as 2,4-dinitrofluorobenzene or picryl chloride) to produce either the penta-N-dinitrophenyl (DNP) derivative or the pent -N-trinitro phenyl (TNP) derivative in which five DNP or TNP units are attached to each molecule of neomycin. Neomycin has a molecular weight of 556; when 5 units of DNP are attached, its molecular weight increases to 1610. The 252Cf-PDMS negative ion mass spectrum corresponds to its molecular weight of DNP-neomycin derivative, although the mass spectrum shows a parent ion peak at m/z = 1612. The additional two atomic mass units (amu) represent two hydrogen atoms that the parent molecular ion has picked up during its transition state. The positive ion spectrum of the DNP derivative corresponds to its sodium salt giving the molecular ion peak at m/z = 1634. With the TNP derivative, 5 TNP units are attached to each molecule of neomycin which increases its molecular weight from 556 to 1863. The negative ion mass spectrum of the TNP derivative has a molecular ion peak at m/z = 1863 (with a water molecule). The positive ion mass spectrum of the TNP derivative corresponds to its sodium salt giving the molecular ion peak at m/z = 1905.

KANAMYCIN

Kanamycin consists of two isomers, Kanamycin A and Kanamycin B. Commercially available kanamycin predominantly consists of kanamycin A. The small quantity of kanamycin Bis lost during derivatization and purification. Therefore, dinitrophenyl and trinitrophenyl derivatives of only kanamycin A are reported.

The antibiotic kanamycin carries 4 primary amino groups. These groups are quite basic and under alkaline aqueous conditions react with nitrophenyl halides (such as picryl chloride or 2,4-dinitrofluorobenzene) to produce either the tetra-N-dinitrophenyl (DNP) derivative of kanamycin (mp= 210°C; yield= 72%) or the tetra-N-trinitrophenyl (TNP) derivative of kanamycin (mp= 218°C; yield= 72%) in which 4 DNP or TNP units are attached to each molecule of kanamycin. Kanamycin has a molecular weight of 484; when 4 units of DNP are attached, its molecular weight increases to 1147. The 252cf-PDMS negative ion mass spectrum corresponds to its molecular weight of DNP-kanamycin derivative, although the mass spectrum shows a parent ion peak at m/z = 1148. The additional one atomic mass unit (amu) represents one hydrogen atom that the parent molecular ion has picked up during its transition state. The positive ion spectrum of the DNP derivative corresponds to its sodium salt giving the molecular ion peak at m/z = 1171. With the TNP derivative, 4 TNP units are attached to each molecule of kanamycin which increases its molecular weight from 484 to 1328. The negative ion mass spectrum of the TNP derivative has a molecular ion peak at m/z = 1329 (with a hydrogen atoms). The positive ion mass spectrum of the TNP derivative corresponds to its potassium salt giving the molecular ion peak at m/z = 1367.

PAR0M0MYCIN

The antibiotic paromomycin carries 5 primary amino groups. These groups are quite basic and under alkaline aqueous conditions react with nitrophenyl halides (such as picryl chloride or 2,4-dinitrofluorobenzene) to produce either the penta-N-dinitrophenyl (DNP) derivative of paromomycin (mp= 237°C; yield= 72%) or the penta-N-trinitrophenyl (TNP) derivative of paromomycin (mp= 210°C; yield= 66%) in which 5 DNP or TNP units are attached to each molecule of paromomycin. Paromomycin has a molecular weight of 585; when 5 units of DNP are attached, its molecular

weight increases to 1446.9 The 252Cf-PDMS negative ion mass spectrum corresponds to its molecular weight of DNP-paromomycin derivative, although the mass spectrum shows a parent ion peak at m/z = 1447.2 The positive ion spectrum of the DNP derivative corresponds to its sodium salt giving the molecular ion peak at m/z = 1470.

With the TNP derivative, 5 TNP units are attached to each molecule of paromomycin which increases its molecular weight from 585 to 1670. The negative ion mass spectrum of the TNP derivative has a molecular ion peak at m/z = 1672. The additional 2 atomic mean units (amu) represent 2 hydrogen atoms that the parent molecular ion has picked up during its transition state. The positive ion mass spectrum of the TNP derivative corresponds to its sodium salt giving the molecular ion peak at m/z = 1995.

TOBRAMYCIN

The antibiotic tobramycin carries 5 primary amino groups. These groups are quite basic and under alkaline aqueous conditions react with nitrophenyl halides (such as picryl chloride or 2,4-dinitrofluorobenzene) to produce either the penta-N-dinitrophenyl (DNP) derivative of tobramycin (mp= 94°C; yield= 82%) or the penta-N-trinitrophenyl (TNP) derivative of tobramycin (mp= 225°C; yield= 64%) in which 5 DNP or TNP units are attached to each molecule of tobramycin. Tobramycin has a molecular weight of 476; when 5 units of DNP are attached, its molecular weight increases to 1297.9. The ^{252}cf-PDMS negative ion mass spectrum corresponds to its molecular weight of DNP-tobramycin derivative, although the mass spectrum shows a parent ion peak at m/z = 1298.1. The positive ion spectrum of the DNP derivative corresponds to its potassium salt giving the molecular ion peak at m/z = 1338. With the TNP derivative, 5 TNP units are attached to each molecule of tobramycin which increases its molecular weight from 476 to 1523.9. The negative io mass spectrum of the TNP derivative has a molecular ion peak at m/z = 1523. The positive ion mass spectrum of the TNP derivative corresponds to its potassium salt giving the molecular ion peak at m/z = 1562.

AMIKACIN

The antibiotic amikacin carries 4 primary amino groups. These groups are quite basic and under alkaline aqueous conditions react with nitrophenyl halides (such as picryl chloride or 2,4-dinitrofluorobenzene) to produce either the tetra-N-dinitrophenyl (DNP) derivative of amikacin (mp= 102°C; yield= 94%) or the tetra-N-trinitrophenyl (TNP) derivative of amikacin (mp= 192°C; yield= 90%) in which 4 DNP or TNP units are attached to each molecule of amikacin. Amikacin has a molecular weight of 585; when 4 units of DNP are attached, its molecular weight increases to 1233.9. The 252cf-PDMS negative ion mass spectrum corresponds to its molecular weight of DNP-amikacin derivative, although the mass spectrum shows a parent ion peak at m/z = 1250. The additional 17 atomic mass unit (amu) represent a hydroxyl group that the parent molecular ion has picked up during its transition state. The positive ion spectrum of the DNP derivative corresponds to its potassium salt giving the molecular ion peak at m/z = 1273.

With the TNP derivative, 4 TNP units are attached to each molecule of amikacin which increases its molecular weight from 585 to 1433. The negative ion mass spectrum of the TNP derivative has a molecular ion peak at m/z = 1429 (with a loss of 4 hydrogen atoms). The positive ion mass spectrum of the TNP derivative corresponds to its sodium salt giving the molecular ion peak at m/z = 1452.

NETILMICIN

The antibiotic netilmicin carries 3 primary amino groups. These groups are quite basic and under alkaline aqueous conditions react with nitrophenyl halides (such as picryl chloride or 2,4-dinitrofluorobenzene) to produce either the tri-N-dinitrophenyl (DNP) derivative of netilmicin (mp= 234°C; yield= 87%) or the tri-N-trinitrophenyl (TNP) derivative of netilmicin (mp= 194°C; yield= 96%) in which 3 DNP or TNP units are attached to each molecule of netilmicin. Netilmicin has a molecular weight of 475; when 3 units of DNP are attached, its molecular weight increases to 976. The ^{252}cf-PDMS negative ion mass spectrum corresponds to its molecular weight of DNP-netilmicin derivat1ve, although the mass spectrum shows a parent ion peak at m/z = 975.4. The positive ion spectrum of the DNP derivative corresponds to its potassium salt iving the molecular ion peak at m/z = 1011.

With the TNP derivative, 3 TNP units are attached to each molecule of netilmicin which increases its molecular weight from 475 to 1110. The negative ion mass spectrum of the TNP derivative has a molecular ion peak at m/z = 1109.4. The positive ion mass spectrum of the TNP derivative corresponds to its potassium salt giving the molecular ion peak at m/z = 1149.8.

SISOMICIN

The antibiotic sisomicin carries 4 primary amino groups. These groups are quite basic and under alkaline aqueous conditions react with nitrophenyl halides (such as picryl chloride or 2,4-dinitrofluorobenzene) to produce either the tetra-N-dinitrophenyl (DNP) derivative of sisomicin (mp= 218°C; yield= 80%) or the tetra-N-trinitrophenyl (TNP) derivative of sisomicin (mp= 184°C; yield= 81%) in which 4 DNP or TNP units are attached to each molecule of sisomicin. Sisomicin has a molecular weight of 447; when 4 units of DNP are attached, its molecular weight increases to 1111. The ^{252}Cf-PDMS negative ion mass spectrum corresponds to its molecular weight of DNP-sisomicin derivative, although the mass spectrum shows a parent ion peak at m/z = 1111.9. The additional one atomic mass unit (amu) represents one hydrogen atom that the parent molecular ion has picked up during its transition state. The positive ion spectrum of the DNP derivative corresponds to its sodium salt giving the molecular ion peak at m/z = 1134.

With the TNP derivative, 4 TNP units are attached to each molecule of sisomicin which increases its molecular weight from 447 to 1291. The negative ion mass spectrum of the TNP derivative has a molecular ion peak at m/z = 1292 (with a hydrogen atom). The positive ion mass spectrum of the TNP derivative corresponds to its potassium salt giving the molecular ion peak at m/z = 1332.

APRAMYCIN

The antibiotic apramycin carries 4 primary amino groups. These groups are quite basic and under alkaline aqueous conditions react with nitrophenyl halides (such as picryl chloride or 2,4-dinitrofluorobenzene) to produce either the tetra-N-dinitrophenyl (DNP) derivative of apramycin (mp= 78°C; yield= 95%) or the tetra-N-trinitrophenyl (TNP) derivative of apramycin (mp= 210°C; yield= 96%) in which 4 DNP or TNP units are attached to each molecule of apramycin.

Apramycin has a molecular weight of 539.8; when 4 units of DNP are attached, its molecular weight increases to 1207. The 252Cf-PDMS negative ion mass spectrum corresponds to its molecular weight of DNP-apramycin derivative, although the mass spectrum shows a parent ion peak at m/z = 1206.7. The positive ion spectrum of the DNP derivative corresponds to its sodium salt giving the molecular ion peak at m/z = 1338.

With the TNP derivative, 4 TNP units are attached to each molecule of apramycin which increases its molecular weight from 539.8 to .1387. The negative ion mass spectrum of the TNP derivative is too weak to be recorded, but it shows a strong positive ion peak. The positive ion mass spectrum of the TNP derivative corresponds to its potassium salt giving the molecular ion peak at m/z = 1414.

GENTMICINS

Commercially available gentamicin consists of three isomer , namely: gentamicin c_1, gentamicin c_2 and gentamicin c_1a. The presence of any of these isomers could not be detected using the Electron Impact (EI) or Chemical Ionization (CI) mass spectrometer which caused the total degradation of the molecule. With the ^{252}cf plasma desorption mass spectrometer, however, all three isomers were identified as their sodium positive ion peaks in the same spectrum.

The underivatized Gentamicins mixture showed the following three molecular ion peaks as their sodium salt:

Gentamicin c_1 m/z = 500.8

Gentamicin c_2 m/z = 486.7, and

Gentamicin c_1a m/z = 472.9.

The isomer c_1a appears to be the least in the Gentamicin mixture and is lost during derivatization and purification. The remaining two major derivatized isomers of gentamicin, isomer c_1 and c_2, run very closely on a silical gel plate {TLC} either in THF:Hexane (4:1) or in THF:DMF (4:1) and could not be separated by chromatography. Table II (page 121) describes the melting points and yields of the c_1 and c_2 mixture of both the dinitrophenyl and triniropenyl derivates. The remaining two major isomers of Gentamicins, isomers c_1 and c_2, carries 3 and 4 primary amino groups, respectively. These groups are quite basic and under alkaline aqueous conditions react with nitrophenyl halides (such as picryl chloride or 2,4-dinitrofluorobenzene) to produce either the tri or tetra-N-dinitrophenyl (DNP) derivatives of gentamicin mixture of c_1 and c_2 (mp= 182°C; yield= 73%) or the tri or tetra-N-tri itrophenyl {TNP) of gentamicin mixture of c_1 and c_2 (mp= 100°C; yield= 79%) in which 3/4 DNP or TNP units are attached to each molecule of gentamicin.

Gentamicin c1 has a molecular weight of 447.59; when 3 units of DNP are attached, its molecular weight increases to 976.8. The ^{252}Cf-PDMS negative ion mass spectrum corresponds to its molecular weight of DNP derivative of gentamicin c_1 although the mass spectrum shows a parent ion peak at m/z = 977.

Gentamicin c_2 has a molecular weight of 463.57; when 4 units of DNP are attached, its molecular weight increases to 1127.9. The ^{252}Cf-PDMS negative ion mass spectrum corresponds to its molecular weight of DNP-gentamicin c_2 derivative, although the mass spectrum shows a parent ion peak at m/z = 1129. The additional one atomic mass unit (amu) represents one hydrogen atom that the parent molecular ion has picked up during its transition state. The DNP positive ion spectra of c_1 & c_2 isomers correspond to their potassium salt giving the molecular ion peaks at m/z = 1119 and 1167, respectively. With the TNP derivative, 3 TNP units are attached to each molecule of gentamicin c1 which increases its molecular weight from 447.5 to 1118. The negative ion mass spectrum of the TNP derivative has a molecular ion peak at m/z = 1111.8. The positive ion mass spectrum of the TNP derivative corresponds to its potassium salt giving the molecular ion peak at m/z = 1151.7.

With the TNP derivative, 4 TNP units are attached to each molecule of gentamicin c_2 which increases its molecular weight from 463.57 to 1307.9. The negative ion mass spectrum of the TNP derivative has a molecular ion peak at m/z = 1307.4. The positive ion mass spectrum of the TNP derivative corresponds to its potassium salt giving the molecular ion peak at m/z = 1346.9.

Conclusion

It has been successfully demonstrated that ^{252}californium Plasma desorption Mass Spectrometry (252cf-PDMS) can provide information about molecular weight and structure for non-volatile and thermolabile aminoglycoside antibiotics which cannot be obtained with the conventional electron Impact {EI) or Chemical Ionization {CI) mass spectrometer. This is a pioneering and innovative concept in that the ^{252}cf-PDMS has never been used as a tool in the development of analytical methodology for detecting residues of antibiotics in the tissue extracts of food-producing animals. We have developed four different methods to monitor the residues of the same antibiotic in an aqueous solution. To produce a stable molecular ion peak, two different derivatives of the same antibiotic have been prepared. If the dinitrophenyl (DNP) derivative did not produce an intense peak, its trinitrophenyl (TNP) derivative was used. For each derivative, two separate mass spectra was obtained: 1) A negative ion mass spectrum which corresponds to the molecular weight of the compound and 2) A positive ion mass spectrum which corresponds to the molecular weight of the sodium or potassium salt of the derivatized antibiotic. Thus, four different methods have been developed to monitor the residues of the same antibiotic in an aqueous solution. These are the first spectra of these antibiotics in which ^{252}californium plasma desorption source has been used and molecular ion spectra are produced with and without derivatization. To determine the applicability of this work, we have derivatized trace residues of antibiotic in biological fluid. Our preliminary work on an incurred sample of neomycin extracted from the kidney tissues of cattle indicates that derivatization in a biological sample is successful. Purification of this sample for ^{252}Cf-PDMS analysis is in progress. Preliminary work on the measurement of the sensitivity of the ^{252}cf-PDMS instrument indicates that the instrument is capable of measuring a 10 microgram sample without difficulty. Using a pure sample of 10 micrograms of TNP-neomycin derivative in methanol, we were able to obtain a mass spectrum (see page 119) at m/z = 1881.8 formw = 1882.

Currently, we are attempting to obtain a mass spectrum at the established tolerance of neomycin 0.25 ppm (0.25 microgram).

For nine underivatized aminoglycoside antibiotics, nine positive ion mass spectra are presented. Two derivatives of

each aminoglycoside antibiotic were synthesized. For all 18 derivatives, 36 mass spectra were obtained (18 negative and 18 positive ion mass spectra). A total of 45 mass spectra are reported. These mass spectral data may be used for FDA or USDA compliance and regulatory activities concerning the abuse of illegal aminoglycoside antibiotic residues in the tissues of food-producing animals.

Experimental Section

This section contains the following information on each antibiotic:

Structure of the pure antibiotic.

Positive-ions mass spectrum of the pure antibiotic.

Structure of the Dinitrophenyl (DNP) derivative of antibiotic.

Synthesis of the DNP derivative.

Negative-ions mass spectrum of the DNP derivative.

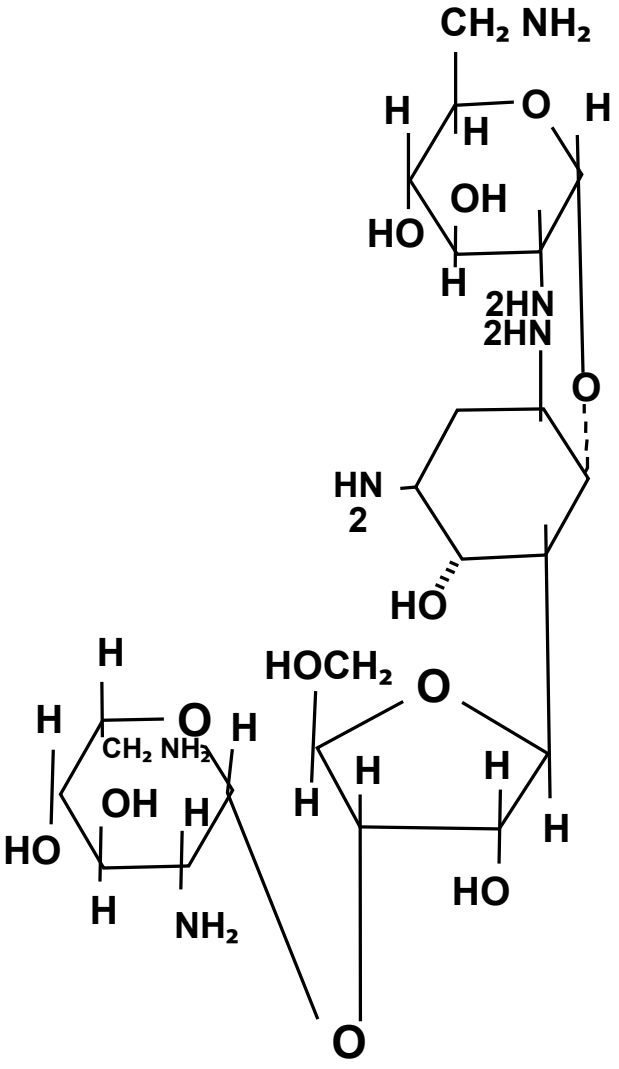
Positive-ions mass spectrum of the DNP derivative.

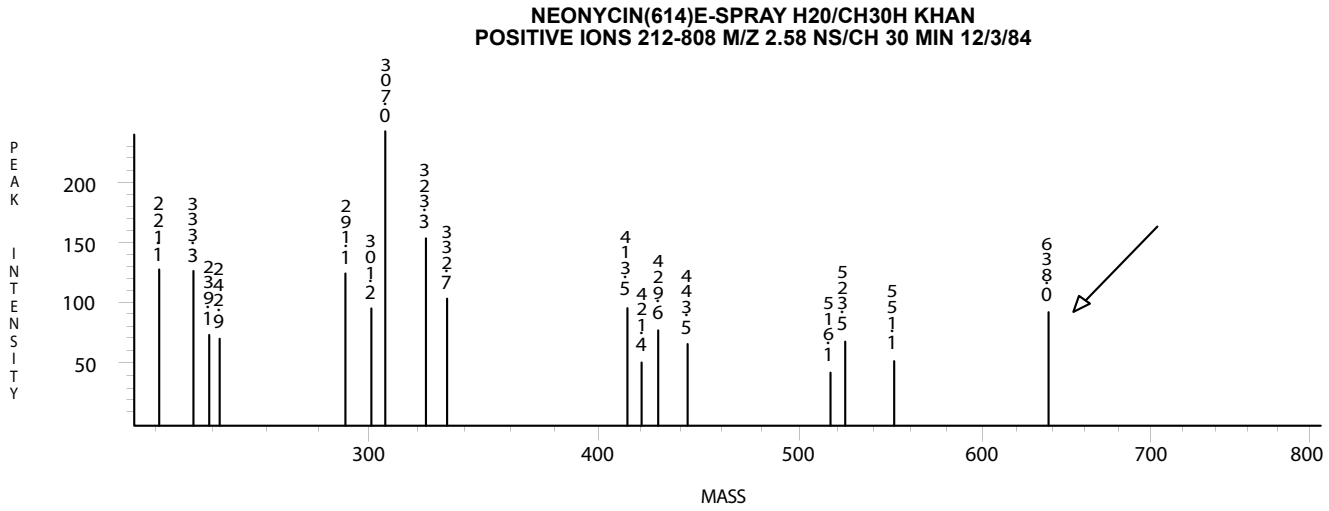
Structure of the Trinitrophenyl (TNP) derivative of antibiotic.

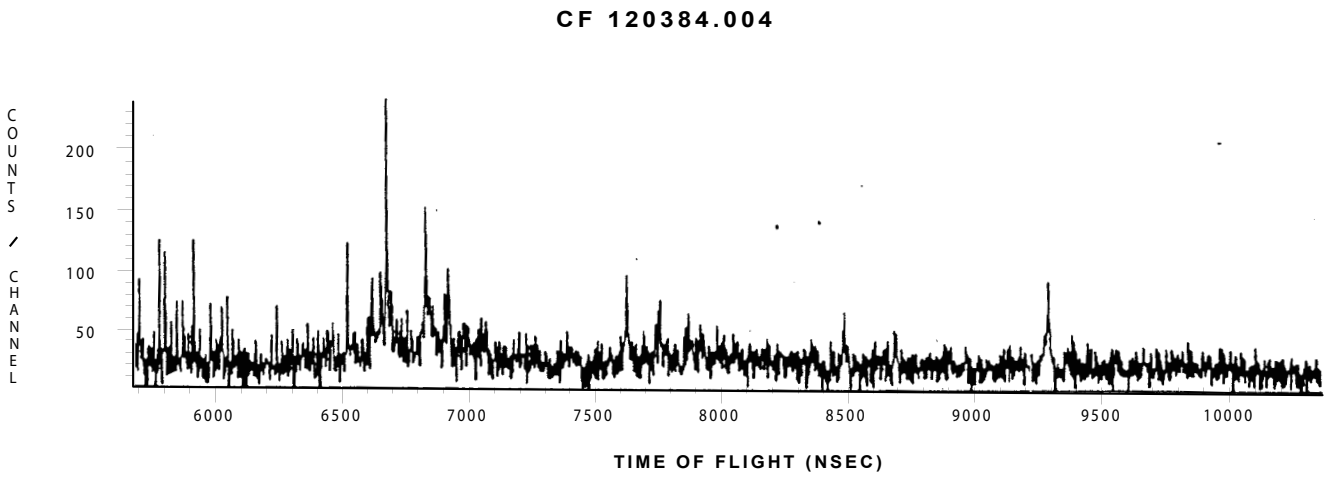
Synthesis of the TNP-derivative.

Negative-ions mass spectrum of the TNP derivative.

Positive-ions mass spectrum of the TNP derivative.



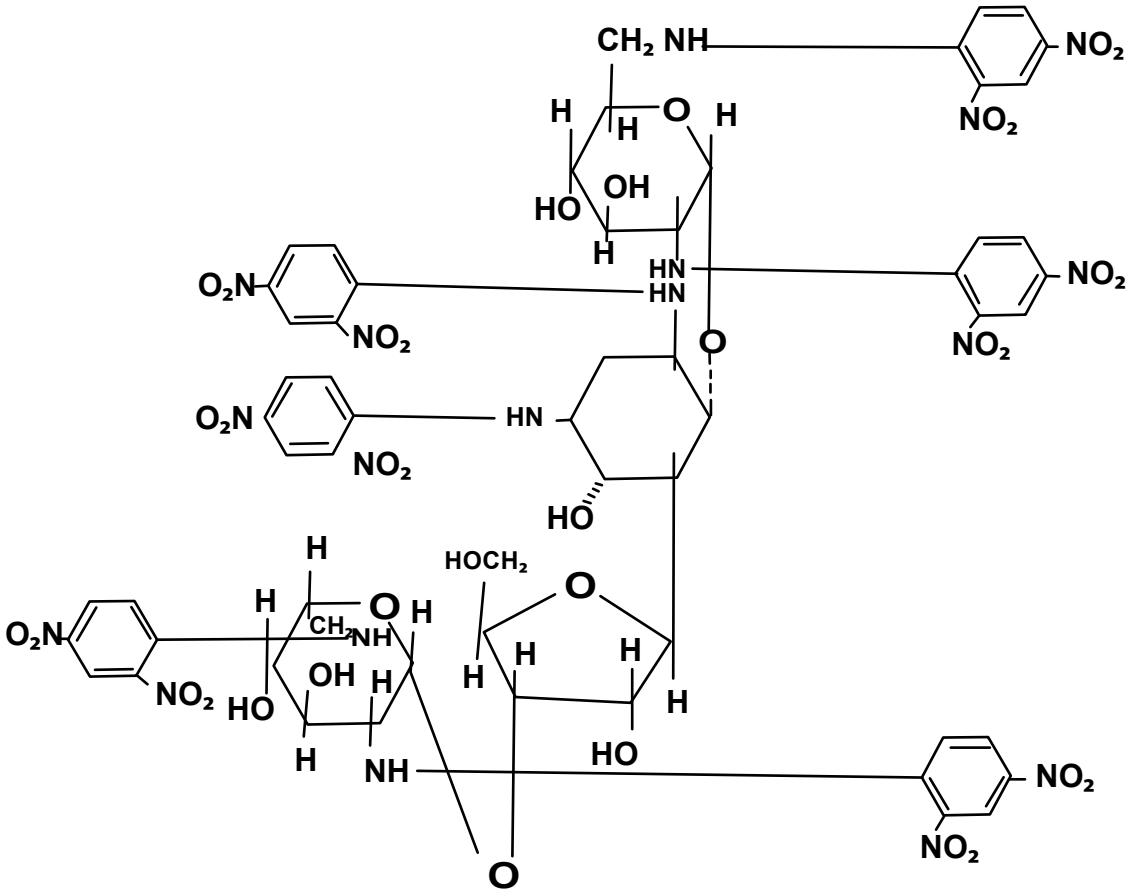




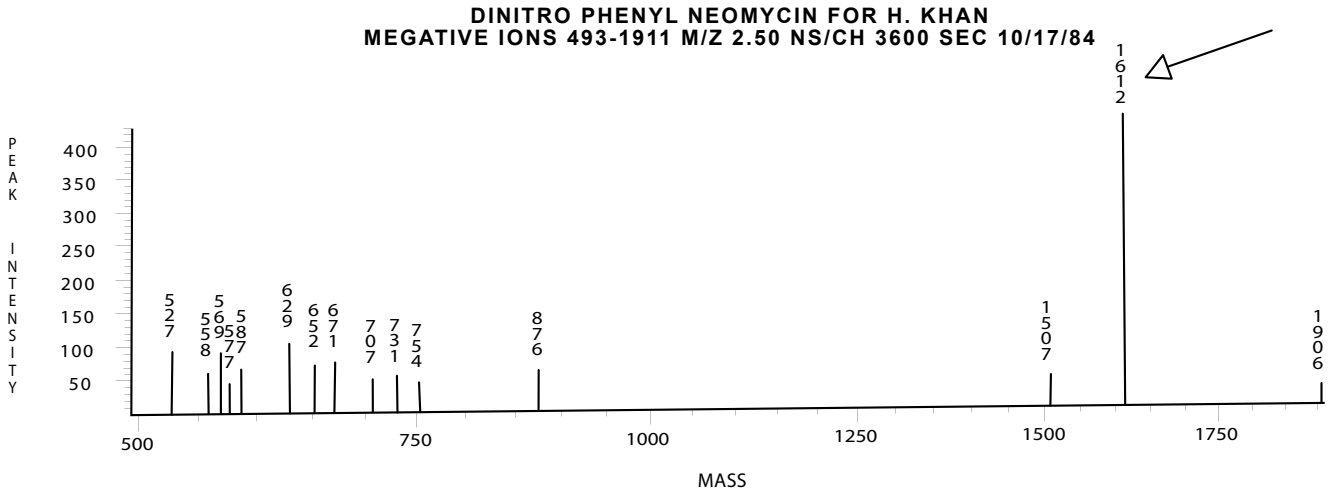
Synthesis of NEOMYCIN, N-hexa(2,4-dinitrophenyl)

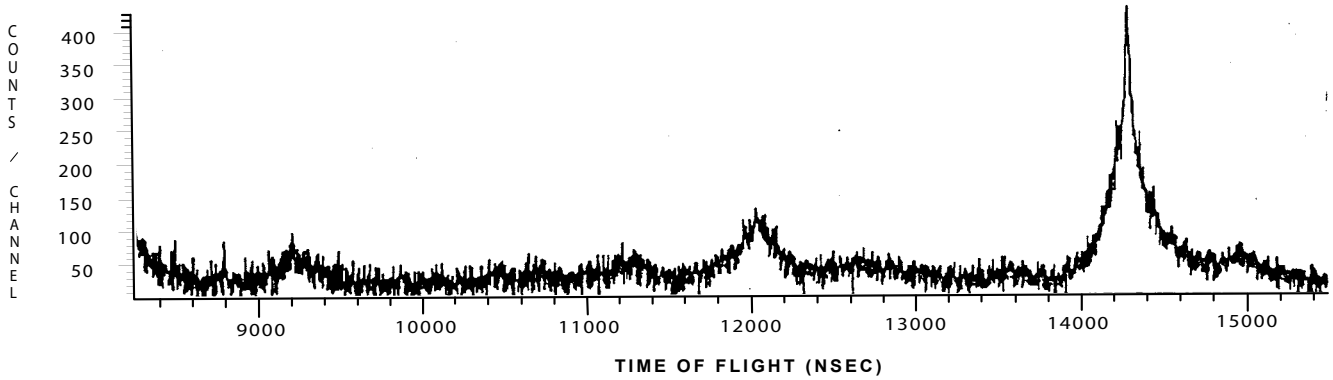
Neomycin Sulfate (2.0 g free base) was dissolved in an aqueous solution of 0.1 M potassium carbonate (50 ml containing an additional 0.23 g of potassium carbonate). The above solution was heated in an oil bath at 80°C. While stirring mechanically, a solution of 2,4-dinitrofluorobenzene (3.7 g) in methanol (50 ml) was added dropwise over 1/4 hour. After the addition was complete, the reaction mixture was heated for one hour. The product, Neomycin, N-hexa(2,4-dinitrophenyl), which was an orange colored semisolid oil separated from the reaction mixture. The hot solution was decanted to remove the solvent; the orange oily product was washed with cold potassium carbonate solution and then with cold water. The oily product solidified when kept in an ice-water bath for 1/2 hour. The solid was ground to fine powder, collected by filtration, washed with cold water, and dried on a suction pump. The structure of the Neomycin, N-hexa(2,4-dinitrophenyl) was confirmed by The Californium (252 Cf) Fast Atom Bombardment (FAB) mass spectrometry. The 252 Cf mass spectrum showed the following ions: m/z = 1612 (negative ion) and m/z = 1634 (positive ion).

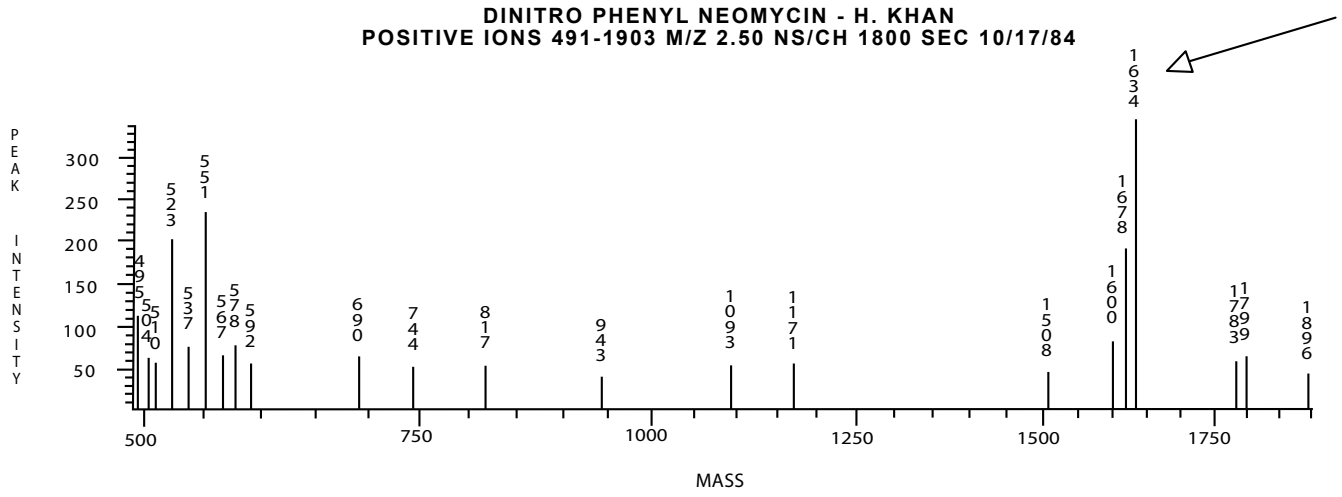
The following data on the above product were also collected: mp= 225°C; yield= 3.1 g (70%).



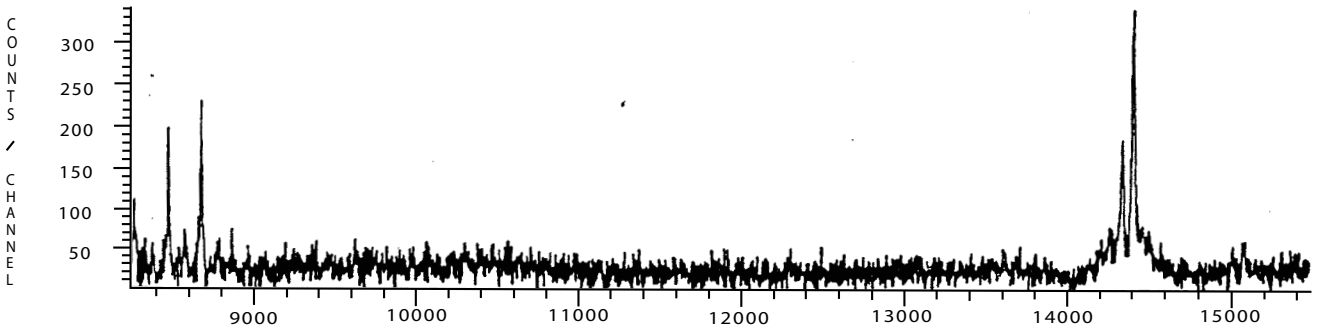
NEOMYCIN, 6-N (2,4 - dinitropheny 1)







DK 2: FILE 1017.007

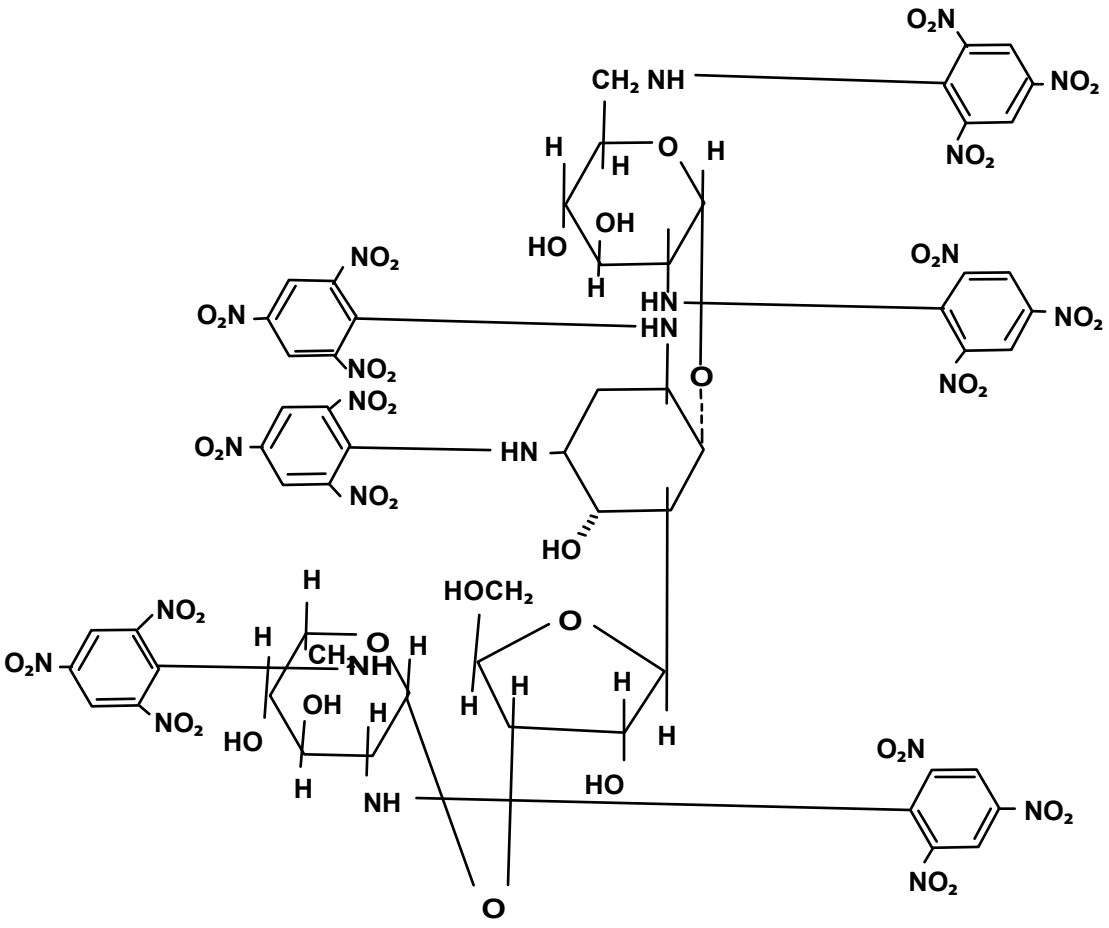


Synthesis of NEOMYCIN, N-hexa(2,4,6-trinitrophenyl)

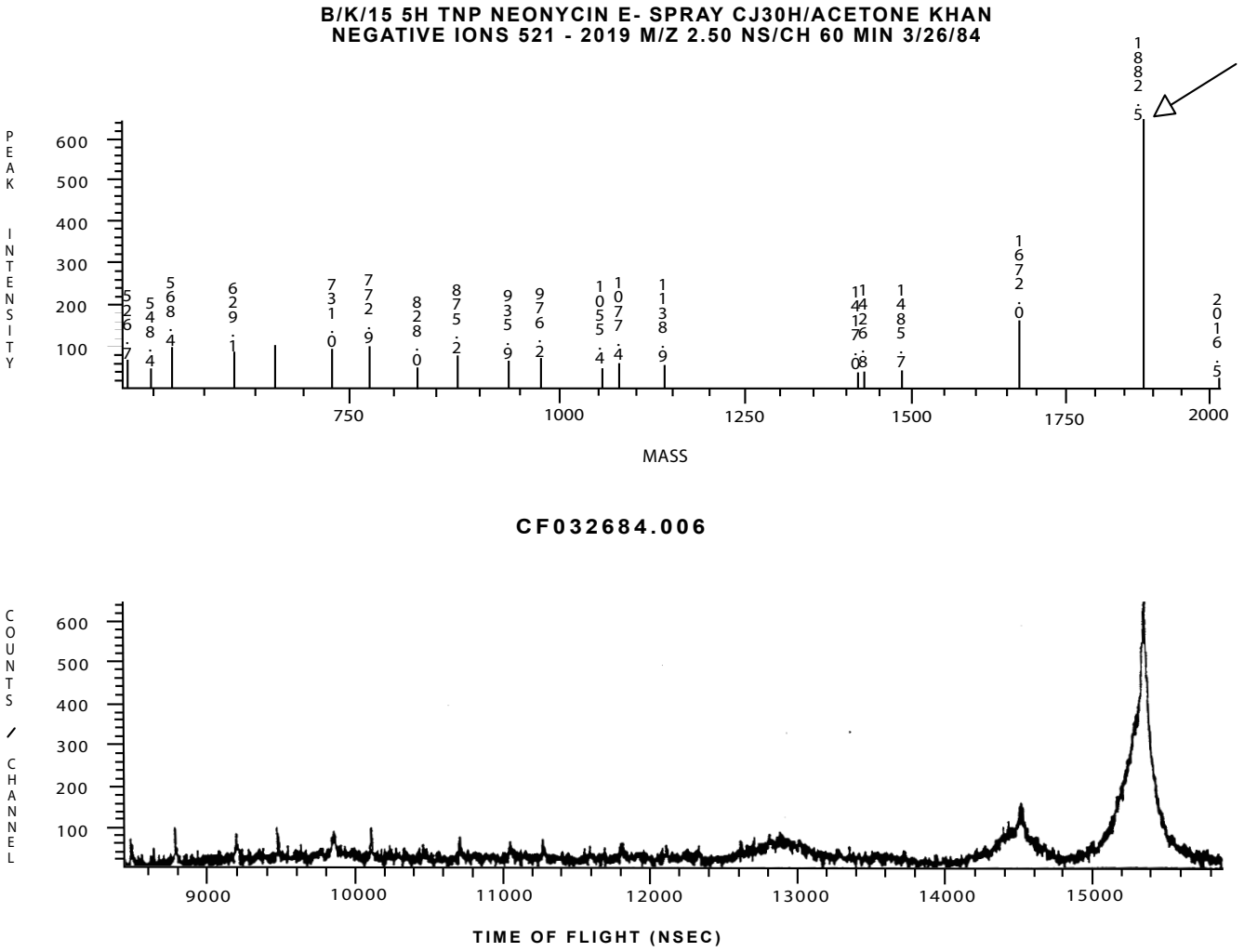
Neomycin Sulfate (0.5 g free base) was dissolved in an aqueous solution of 0.1 M potassium carbonate (25 ml containing an additional 0.23 g of potassium carbonate). The above solution was heated in an oil bath at 80°C. While stirring mechanically, a solution of Picryl Chloride (2.0 g) in methanol (25 ml) was added dropwise over 1/4 hour. After the addition was complete, the reaction mixture was heated for one hour. The product, Neomycin, N-hexa{2,4,6•trinitrophenyl), which was an orange colored semisolid oil separated from the reaction mixture. The hot solution was decanted to remove the solvent; the orange oily product was washed with cold potassium carbonate solution and then with cold water. The oily product solidified when kept in an ice-water bath for 1/2 hour. The solid was ground to fine powder, collected by filtration, washed with cold water, and dried on a suction pump. The structure of the Neomycin, N-hexa(2,4,6-trinitrophenyl) was confirmed by The Californium (252 Cf) Fast Atom Bombardment (FAB)

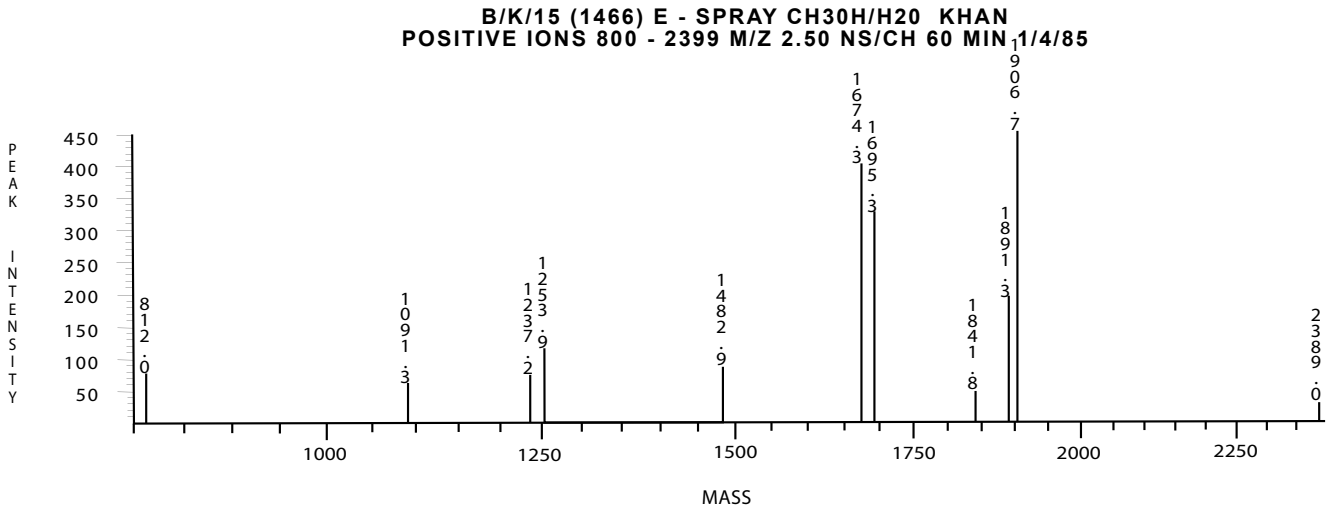
mass spectrometry. The 252 Cf mass spectrum showed the following ions: m/z = 1882 (negative ion) and m/z = 1905 (positive ion).

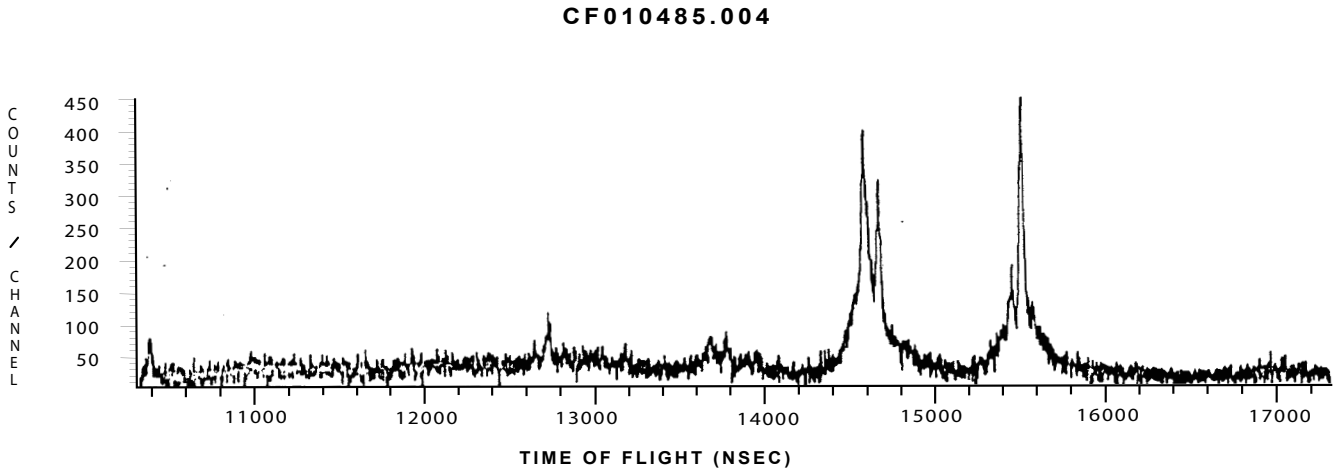
The following data on the above product were also collected: mp= 130°C; yield= 2.4 g (71%).

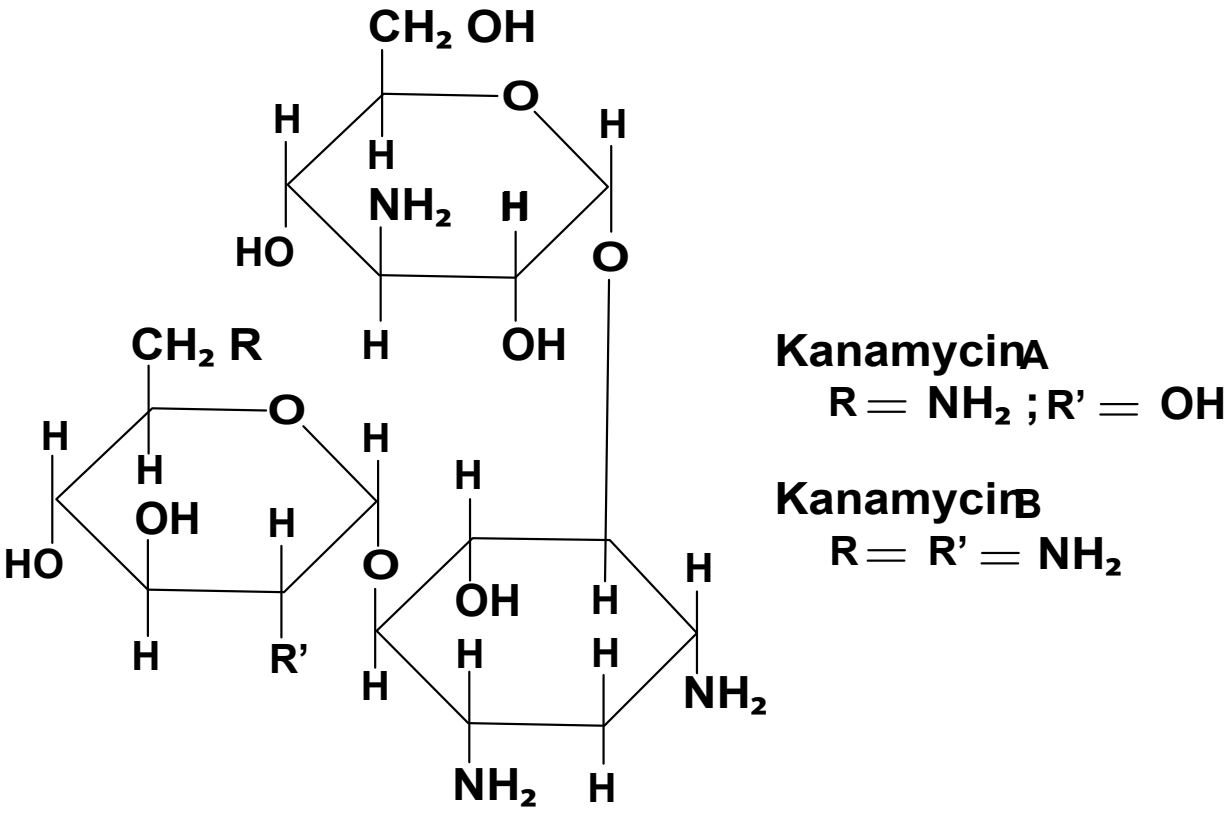


NEOMYCIN, 6-N (2,4,6 - trinitropheny 1)

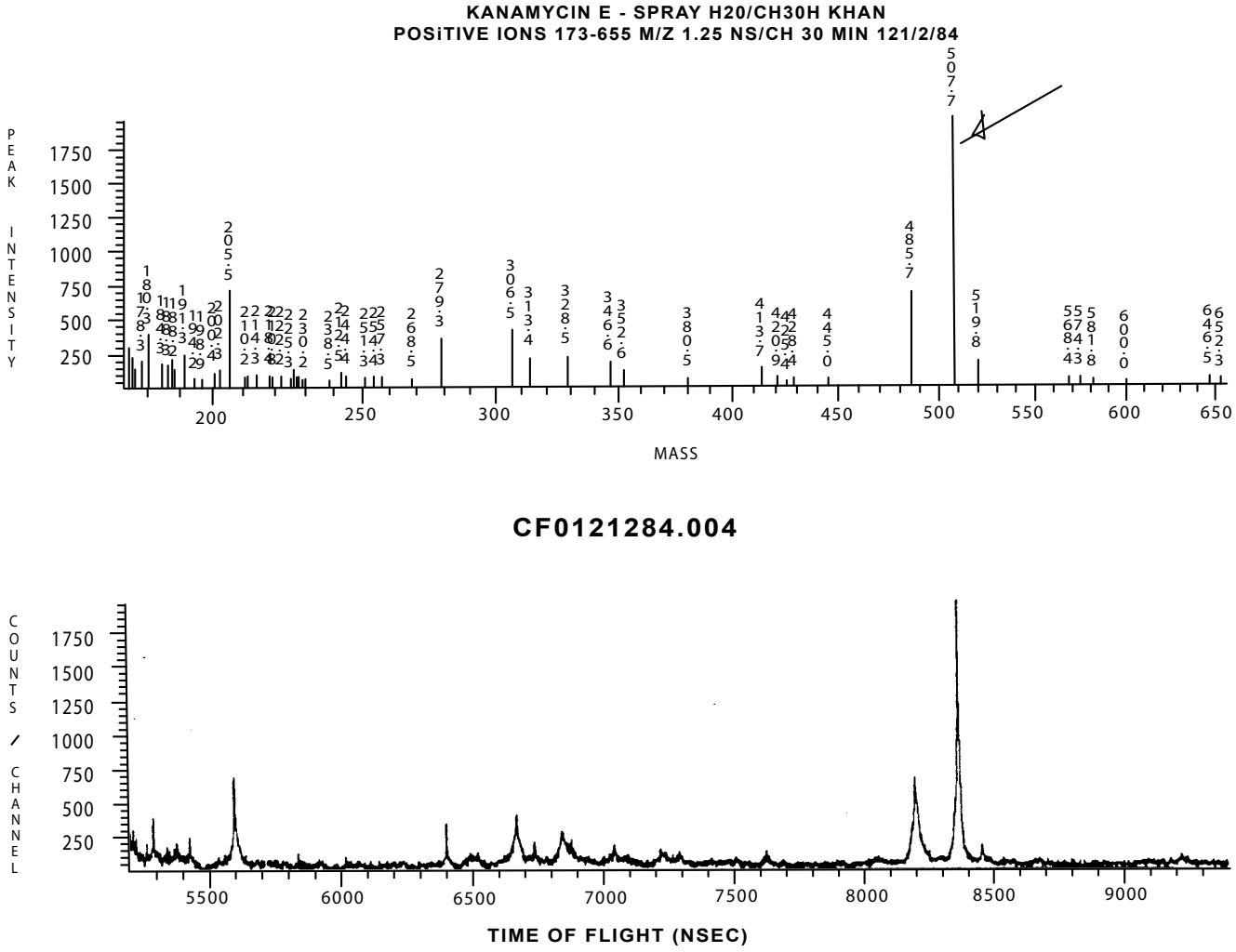








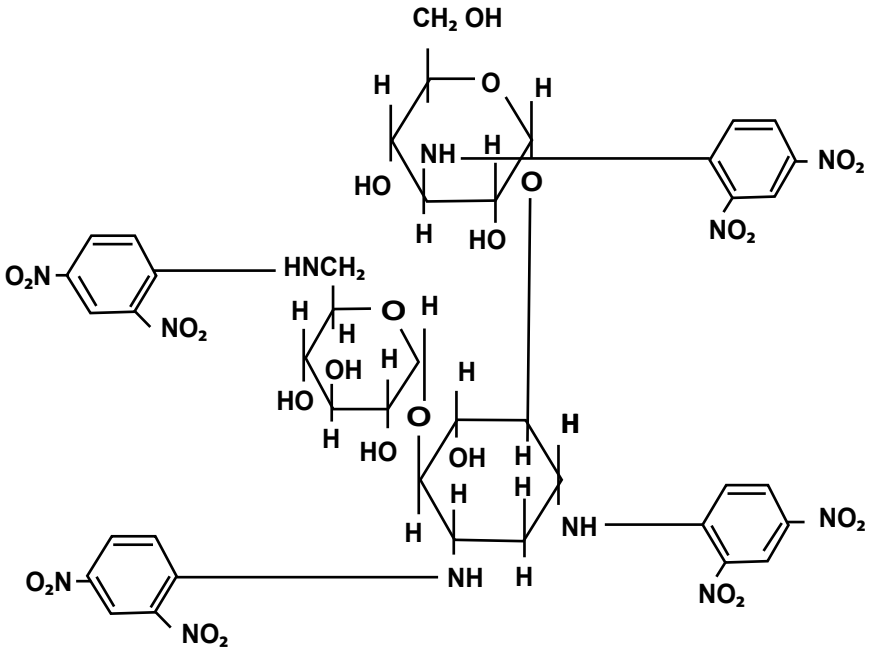
KANAMYCIN



Synthesis of KANAMYCIN, N-tetra(2,4-dinitrophenyl)

Kanamycin Sulfate (0.5 g free base) was dissolved in an aqueous solution of 0.1 M potassium carbonate (15 ml containing an additional 0.1 g of potassium carbonate). The above solution was heated in an oil bath at 80°C. While stirring mechanically, a solution of 2,4-dinitrofluorobenzene (1.92 g) in methanol (15 ml) was added dropwise over 1/4 hour. After the addition was complete, the reaction mixture was heated for one hour. The product, Kanamycin, N-tetra(2,4-dinitrophenyl), which was an orange colored semisolid oil separated from the reaction mixture. The hot solution was decanted to remove the solvent; the orange oily product was washed with cold potassium carbonate solution and then with cold water. The oily product solidified when kept in an ice-water bath for 1/2 hour. The solid was ground to fine powder, collected by filtration, washed with cold water, and dried on a suction pump. The structure of the Kanamycin, N-tetra(2,4-dinitrophenyl) was confirmed by The Californium (252 Cf) Fast Atom Bombardment (FAB) mass spectrometry. The 252 Cf mass spectrum showed the following ions: m/z = 1148.5 (negative ion) and m/z = 1171.5 (positive ion).

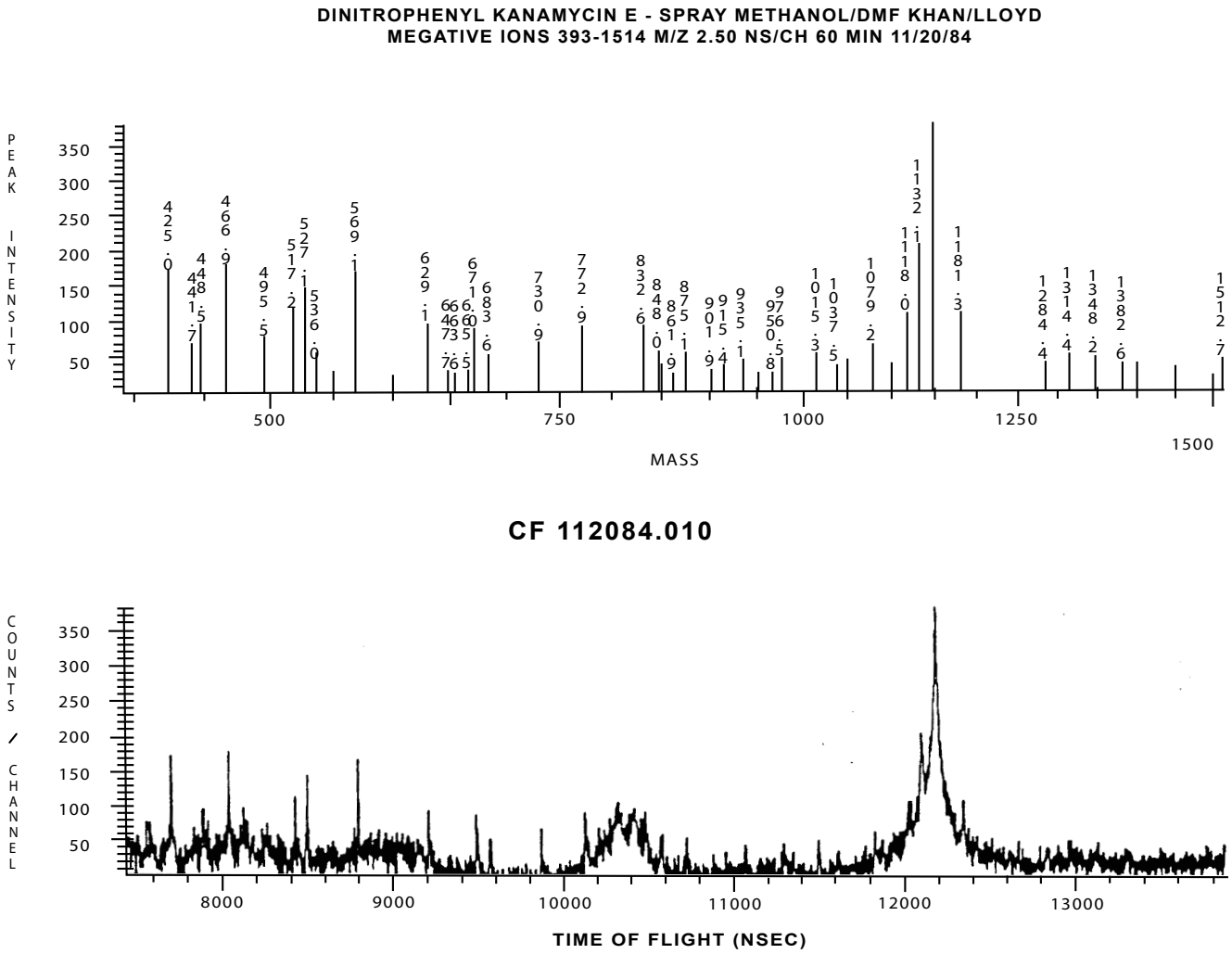
The following data on the above product were also collected: mp= 210°C; yield= 0.98 g (72%).

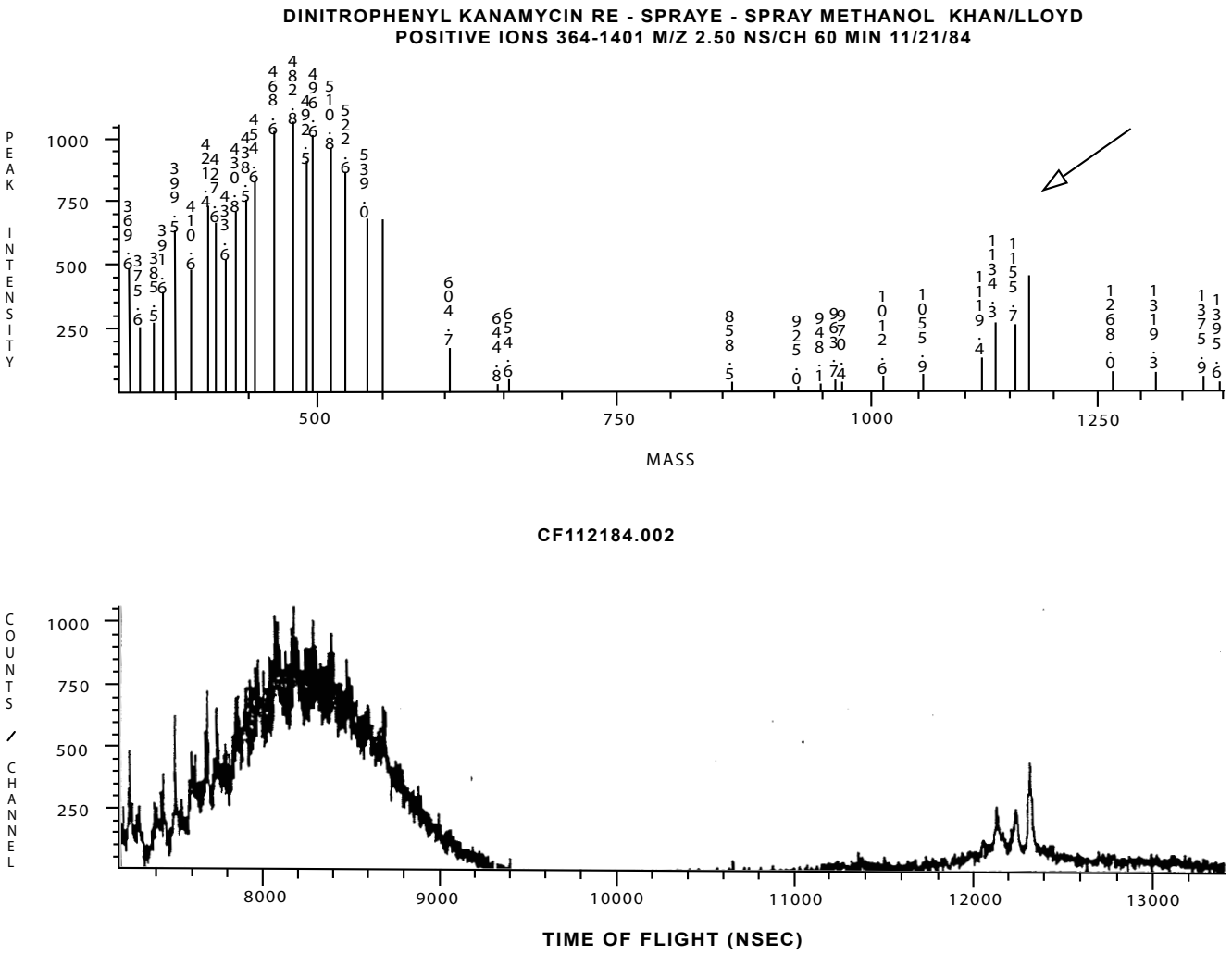


$C_{42}\ H_{43}\ N_{12}\ O_{27}$

mw = 1147.965

KANAMYCIN A, 4-N (2,4 - dinitropheny 1)

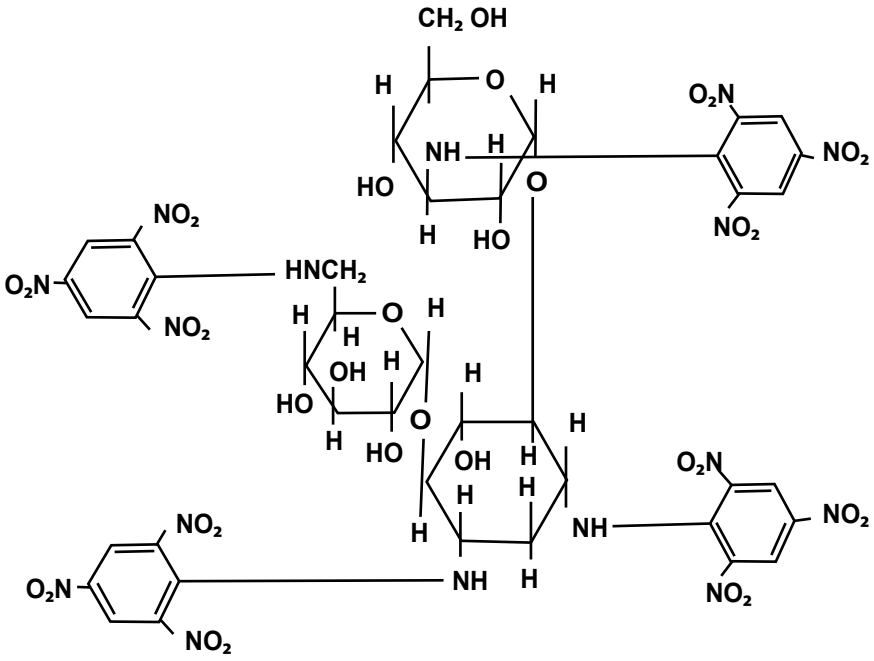




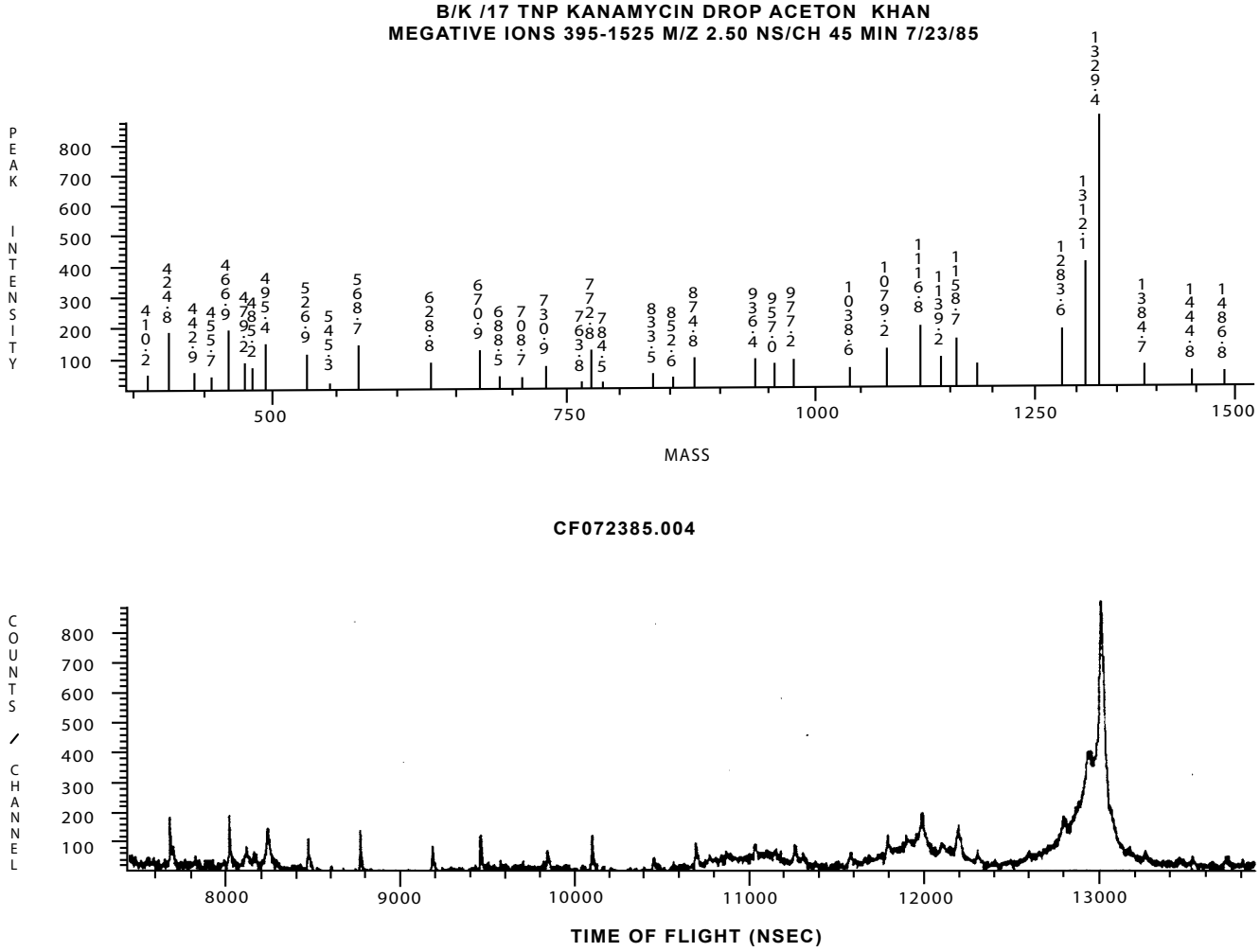
Synthesis of KANAMYCIN, N-tetra(2,4,6-trinitrophenyl)

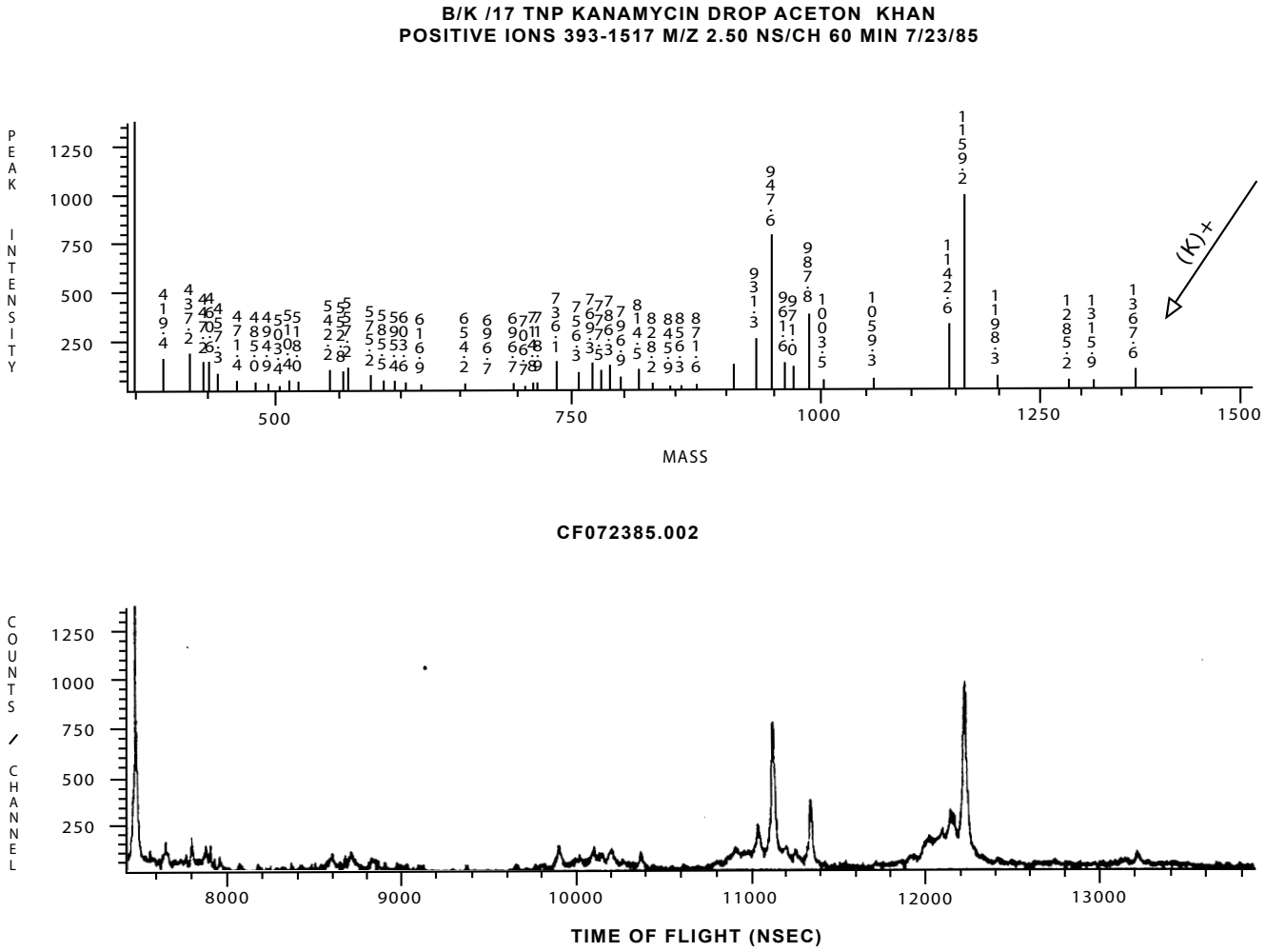
Kanamycin Sulfate (0.5 g free base) was dissolved in an aqueous solution of 0.1 M potassium carbonate (25 ml containing an additional 0.2 g of potassium carbonate). The above solution was heated in an oil bath at 80°C. While stirring mechanically, a solution of Picryl Chloride (1.2 g) in methanol (25 ml) was added dropwise over 1/4 hour. After the addition was complete, the reaction mixture was heated for one hour. The product, Kanamycin, N-tetra(2,4,6 trinitrophenyl), which was an orange colored semisolid oil separated from the reaction mixture. The hot solution was decanted to remove the solvent; the orange oily product was washed with cold potassium carbonate solution and then with cold water. The oily product solidified when kept in an ice-water bath for 1/2 hour. The solid was ground to fine powder, collected by filtration, washed with cold water, and dried on a suction pump. The structure of the Kanamycin, N-tetra(2,4-dinitrophenyl) was confirmed by The Californium (252 Cf) Fast Atom Bombardment (FAB) mass spectrometry. The 252 Cf mass spectrum showed the following ions: m/z = (negative ion) and m/z = (positive ion).

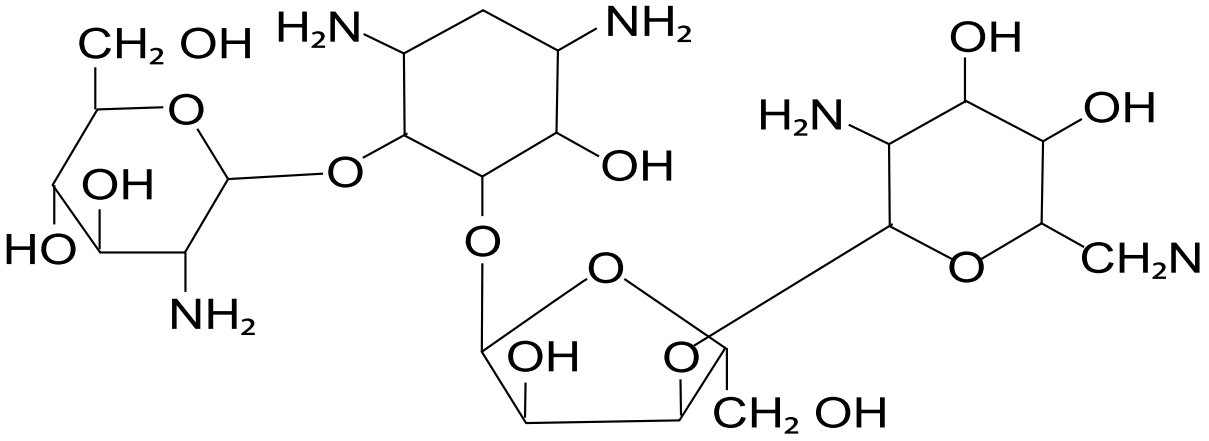
The following data on the above product were also collected: mp= 218°C; yield= 1.0 g (72%).



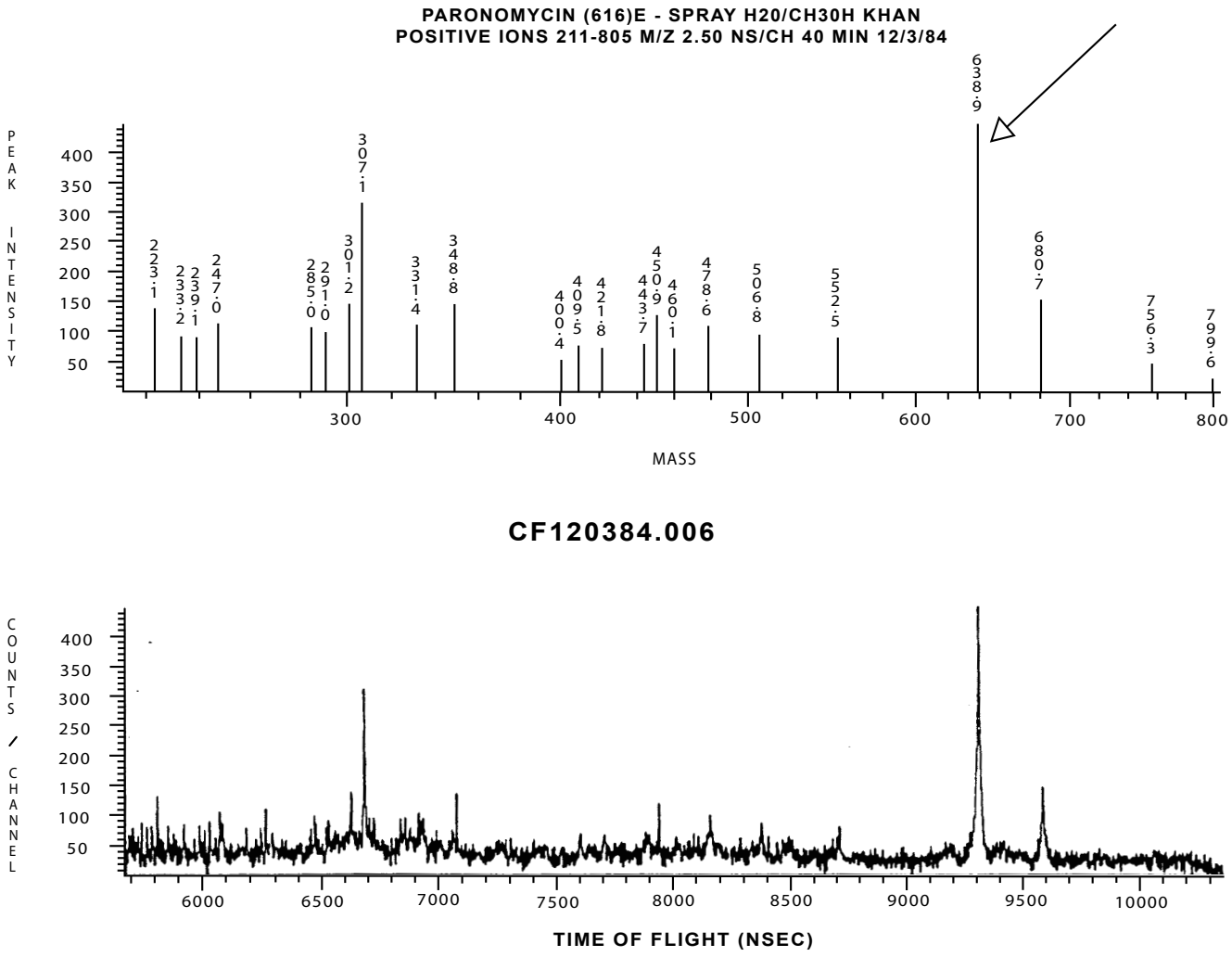
KANAMYCIN A, 4-N (2,4,6-trinitrophenyl)







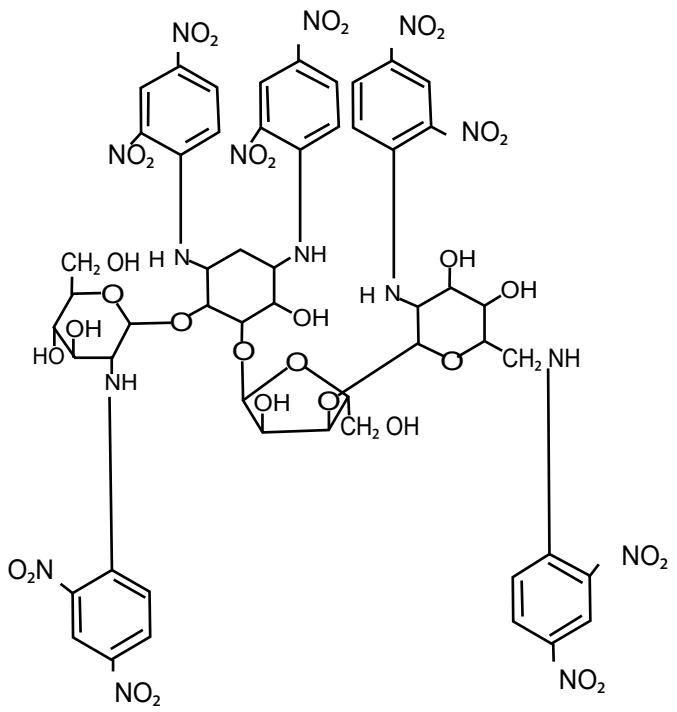
PAROMOMYCIN



Synthesis of PAR0M0MYCIN, N-penta(2,4-dinitrophenyl)

Paromomycin (0.5 g free base) was dissolved in an aqueous solution of 0.1 M potassium carbonate (15 ml containing an additional 0.23 g of potassium carbonate). The above solution was heated in an oil bath at 80°C. While stirring mechanically, a solution of 2,4-dinitrofluorobenzene (1.5 g) in methanol (20 ml) was added dropwise over 1/4 hour. After the addition was complete, the reaction mixture was heated for one hour. The product, Paromomycin, N-penta(2,4-dinitrophenyl), which was an orange colored semisolid oil separated from the reaction mixture. The hot solution was decanted to remove the solvent; the orange oily product was washed with cold potassium carbonate solution and then with cold water. The oily product solidified when kept in an ice-water bath for 1/2 hour. The solid was ground to fine powder, collected by filtration, washed with cold water, and dried on a suction pump. The structure of the Paromomycin, N-penta(2,4-dinitrophenyl) was confirmed by The Californium (252 Cf) Fast Atom Bombardment (FAB) mass spectrometry. The 252 Cf mass spectrum showed the following ions: m/z = 1447.2 (negative ion) and m/z = 1470.5 (positive ion).

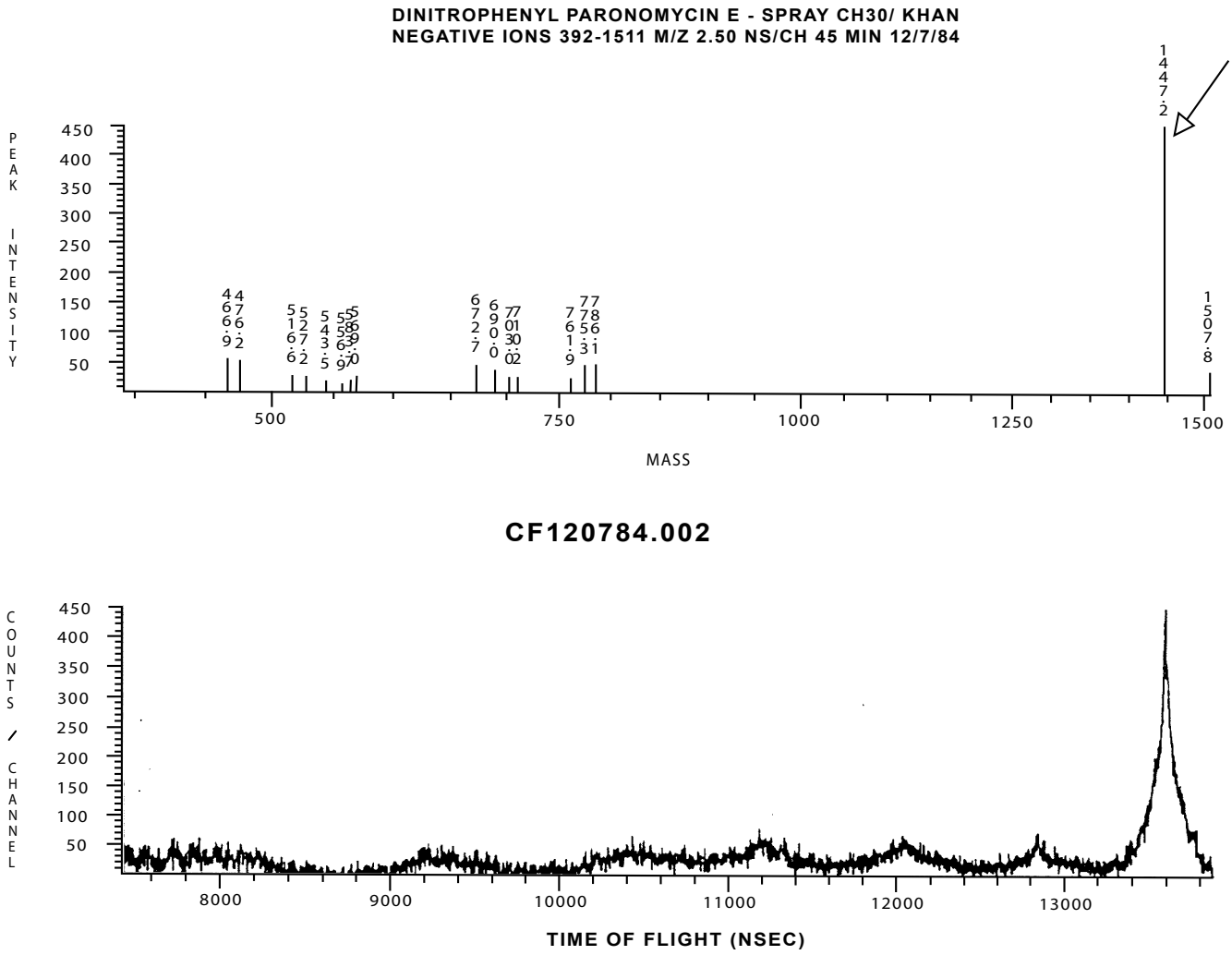
The following data on the above product were also collected: mp= 237°C; yield= 0.71 g (70%).

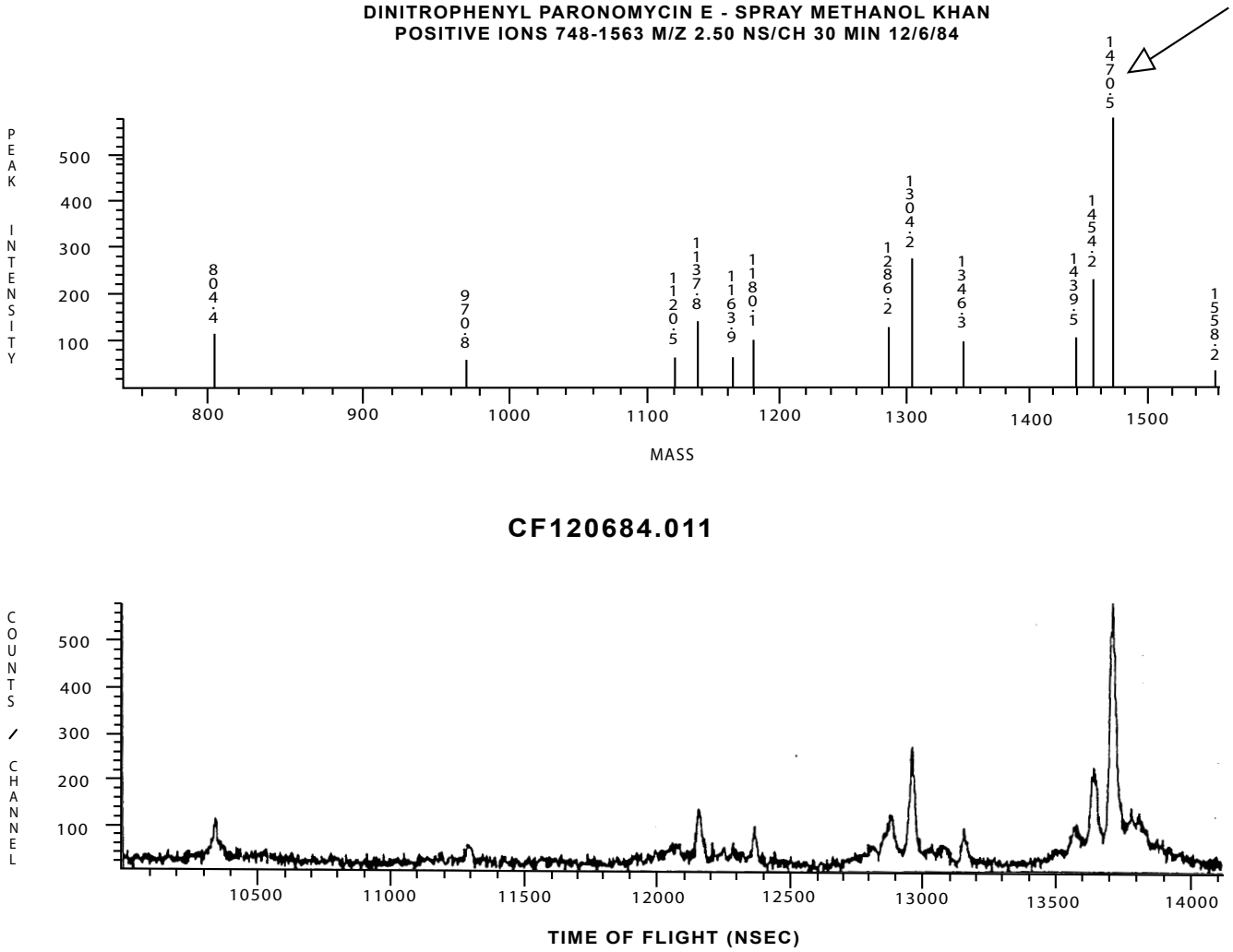


C_{53} H_{55} N_{15} O_{34}

mw = 1446.089

PAROMOMYCIN, 5-N (2,4 - dinitropheny 1)

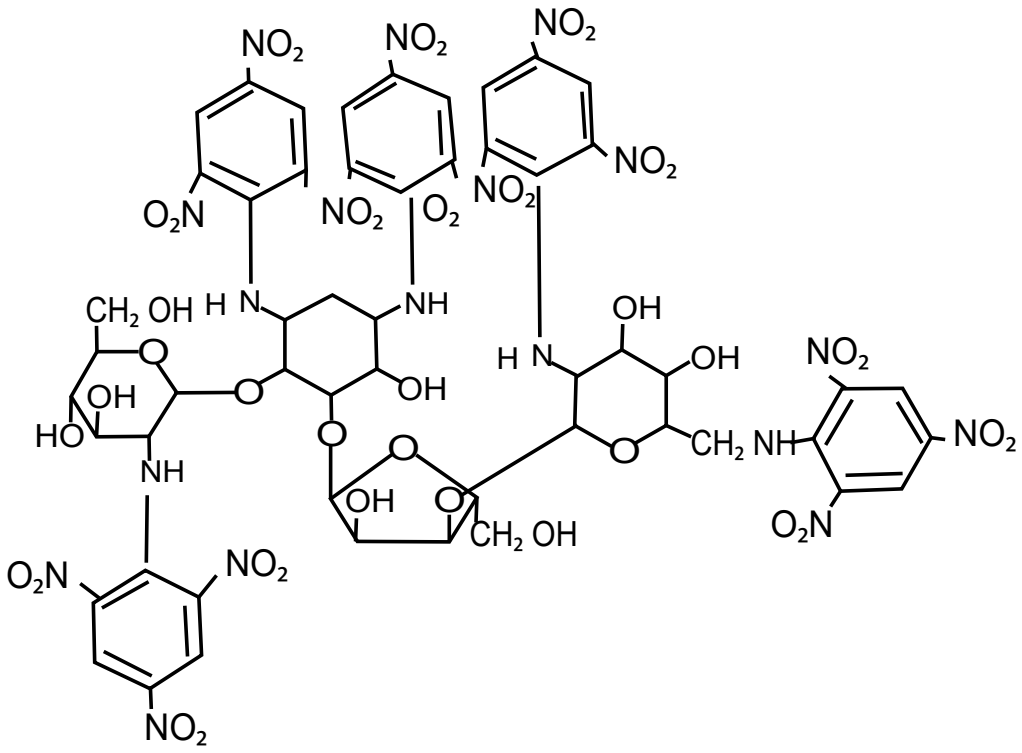




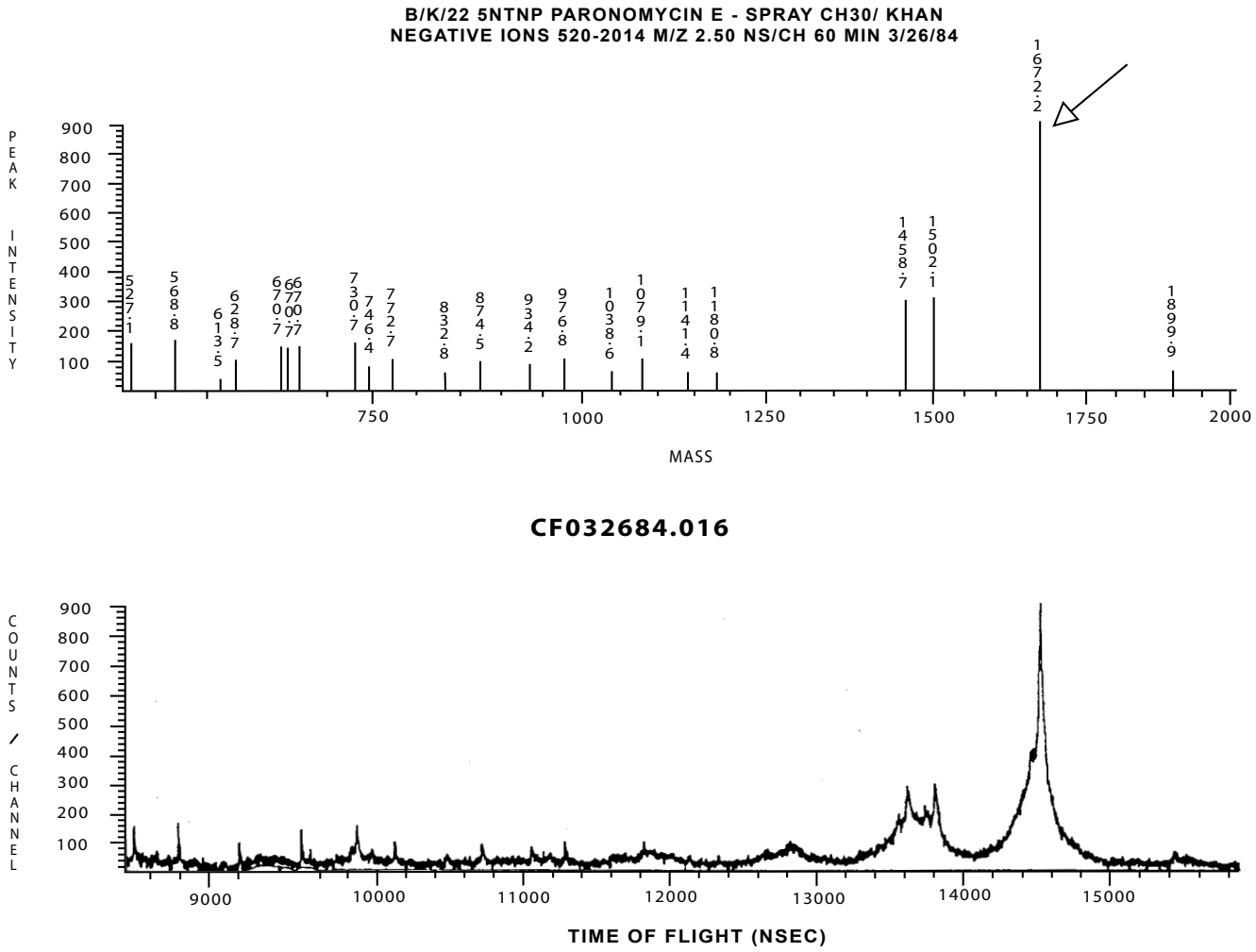
Synthesis of PAR0M0MYCIN, N-penta(2,4,6-trinitrophenyl)

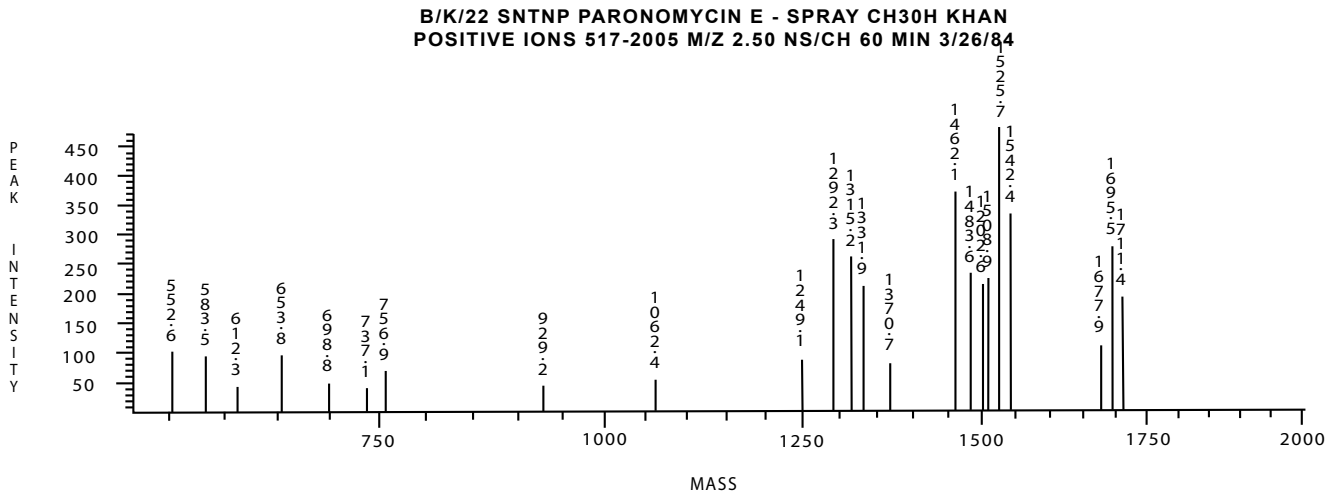
Paromomycin (0.5 g free base) was dissolved in an aqueous solution of 0.1 M potassium carbonate (15 ml containing an additional 0.23 g of potassium carbonate). The above solution was heated in an oil bath at 80°C. While stirring mechanically, a solution of Picryl Chloride (1.5 g) in methanol (25 ml) was added dropwise over 1/4 hour. After the addition was complete, the reaction mixture was heated for one hour. The product, Paromomycin, N-penta(2,4,6-trinitrophenyl), which was an orange colored semisolid oil separated from the reaction mixture. The hot solution was decanted to remove the solvent; the orange oily product was washed with cold potassium carbonate solution and then with cold water. The oily product solidified when kept in an ice-water bath for 1/2 hour. The solid was ground to fine powder, collected by filtration, washed with cold water, and dried on a suction pump. The structure of the Paromomycin, N-penta(2,4,6-trinitrophenyl) was confirmed by The Californium (252 Cf) Fast Atom Bombardment (FAB) mass spectrometry. The 252 Cf mass spectrum showed the following ions: m/z = 1672 (negative ion) and m/z = 1995 (positive ion).

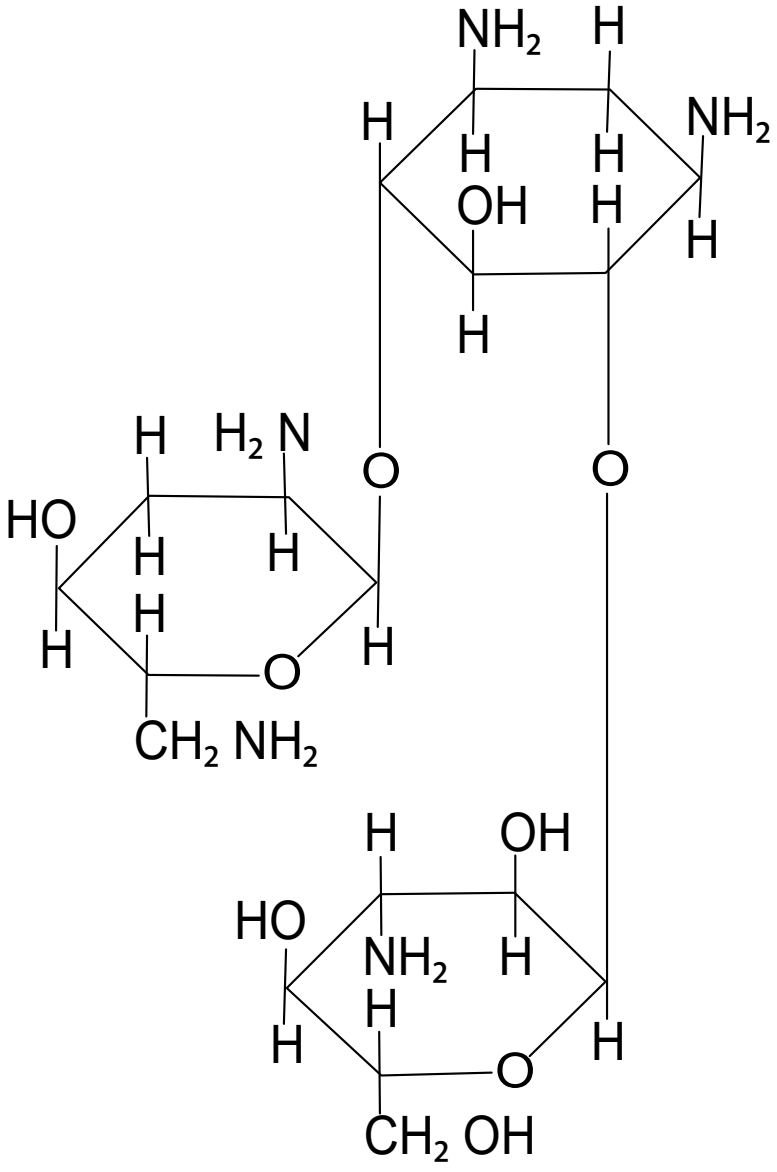
The following data on the above product were also collected: mp= 210°c; yield= 0.9 g (66%).



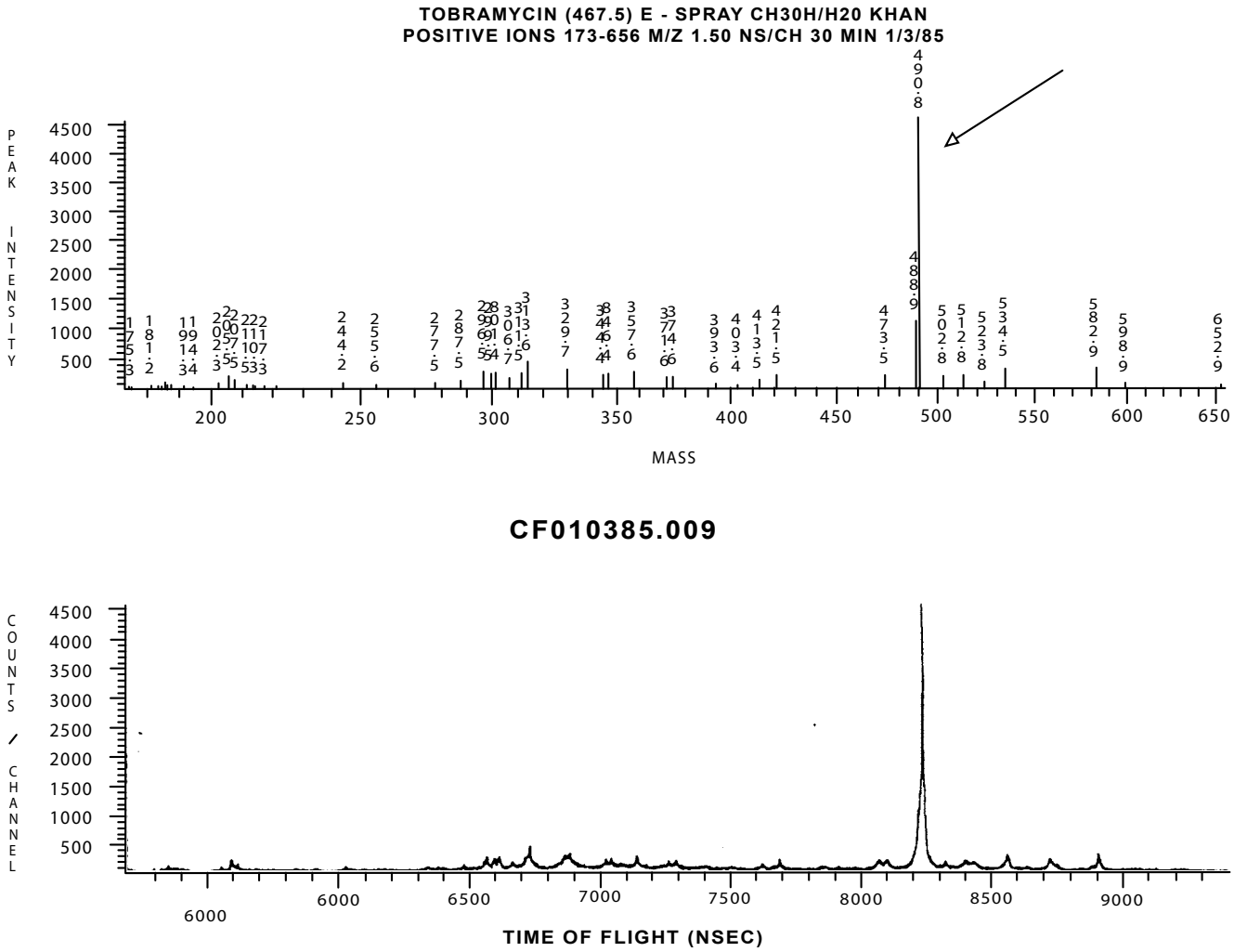
5-N (2,4,6 - Trinitropheny 1) - paromomycin







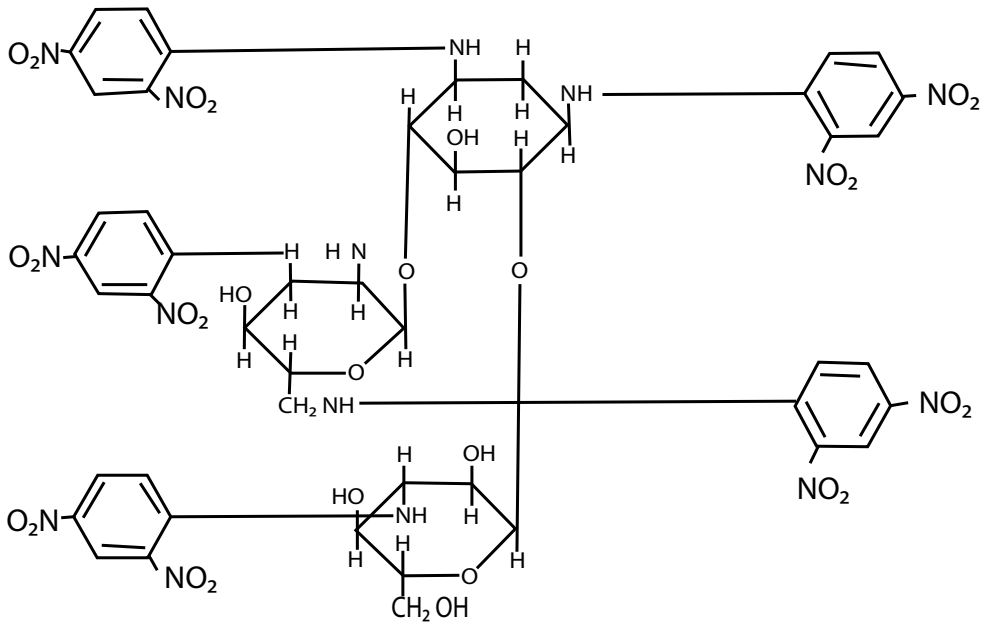
Tobramycin



Synthesis of TOBRAMYCIN, N-penta(2,4-dinitrophenyl)

Tobramycin (0.2 g free base) was dissolved in an aqueous solution of 0.1 M potassium carbonate (15 ml containing an additional 0.23 g of potassium carbonate). The above solution was heated in an oil bath at 80°C. While stirring mechanically, a solution of 2,4-dinitrofluorobenzene (0.4 g) in methanol (15 ml) was added dropwise over 1/4 hour. After the addition was complete, the reaction mixture was heated for one hour. The product, Tobramycin, N-penta(2,4-dinitrophenyl), which was an orange colored semisolid oil separated from the reaction mixture. The hot solution was decanted.to remove the solvent; the orange oily product was washed with cold potassium carbonate solution and then with cold water. The oily product solidified when kept in an ice-water bath for 1/2 hour. The solid was ground to fine powder, collected by filtration, washed with cold water, and dried on a suction pump. The structure of the Tobramycin, N-penta(2,4-dinitrophenyl) was confirmed by The Californium (252 Cf) Fast Atom Bombardment (FAB) mass spectrometry. The 252 Cf mass spectrum showed the following ions: m/z = 1298 (negative ion) and m/z = (positive ion).

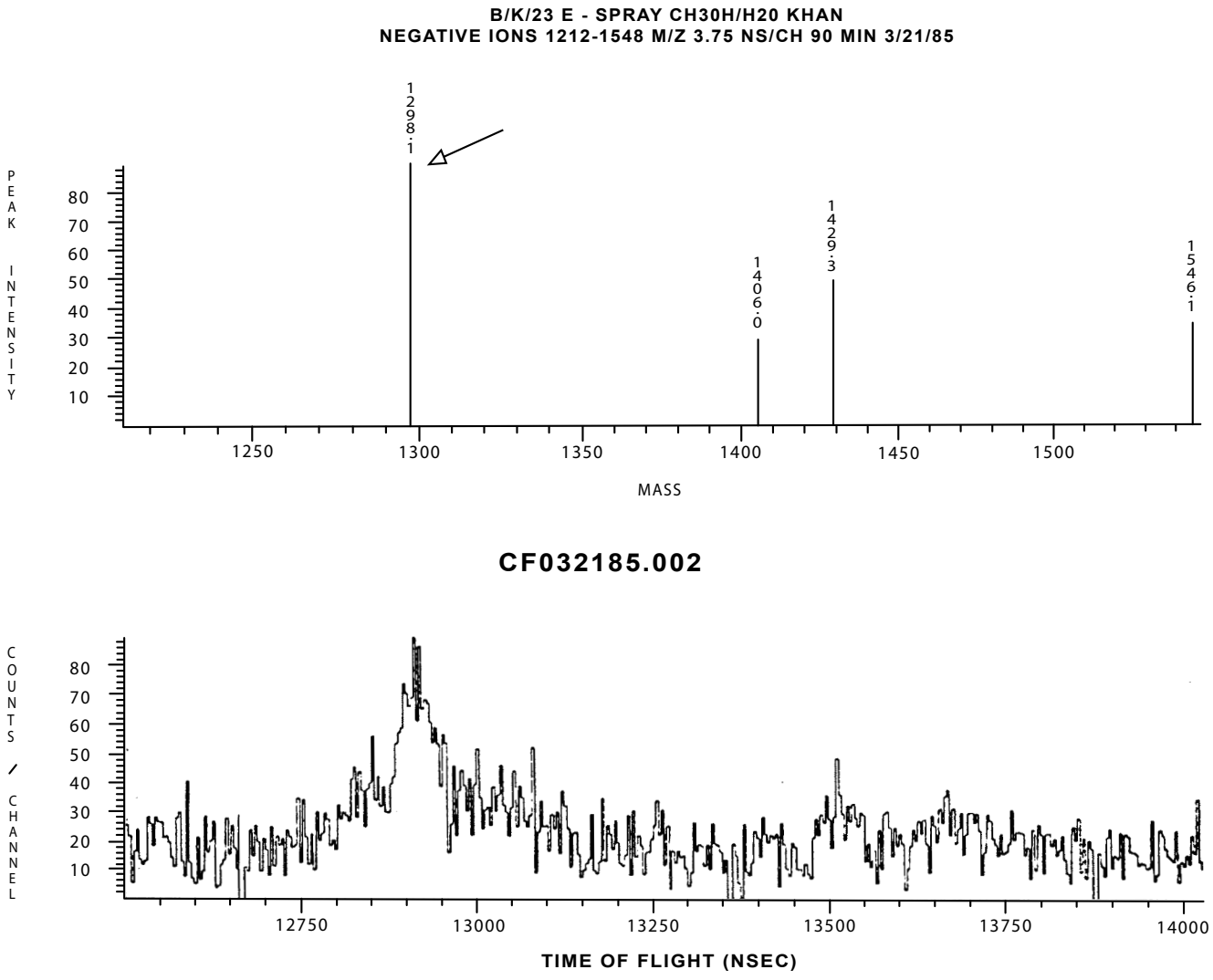
The following data on the above product were also collected: mp= 94°C; yield= 0.28 g (82%).

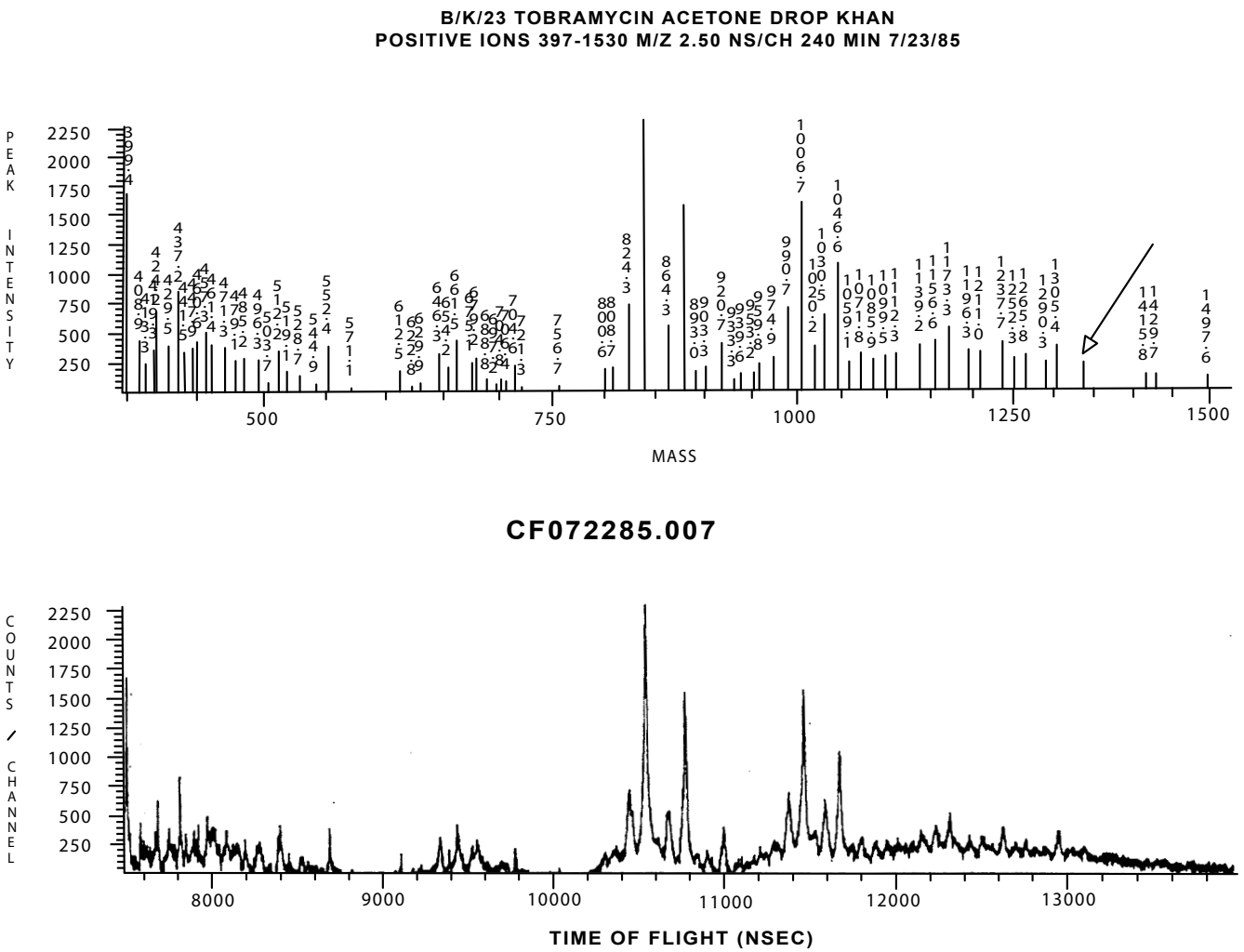


$C_{48}\ H_{47}7\ N_{15}5\ O_{29}$

mv = 1297.982

5 - N (2,4 - Dinitropheny) - Tobramycin

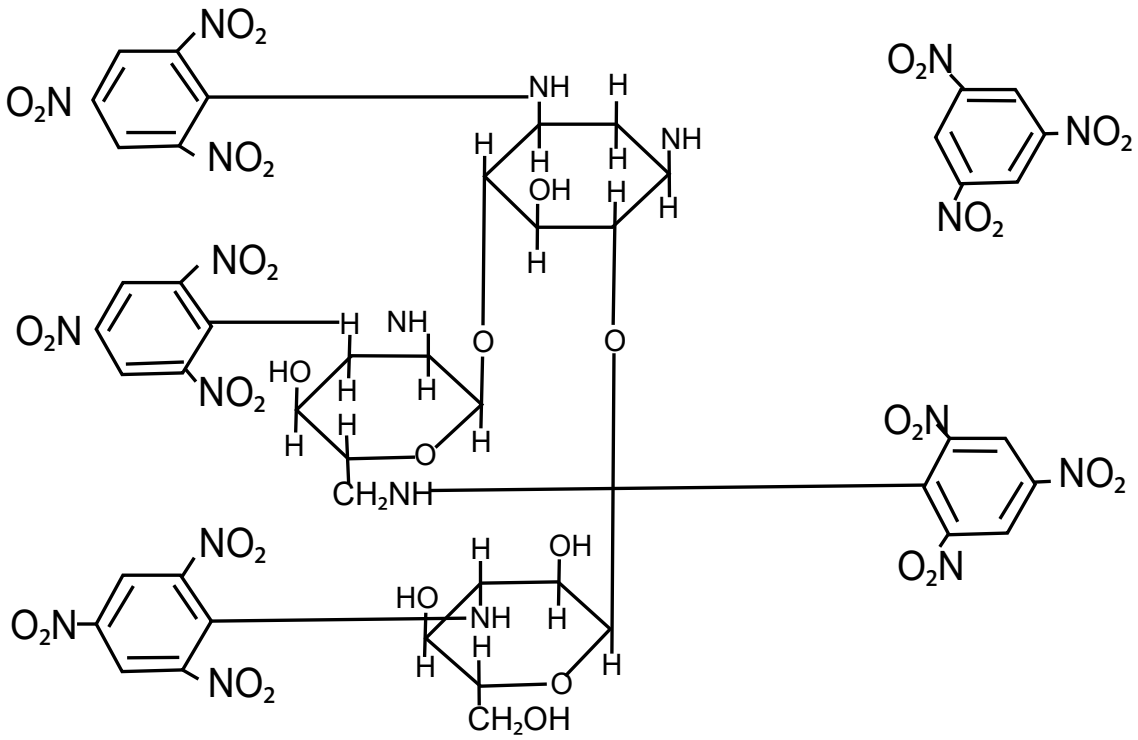




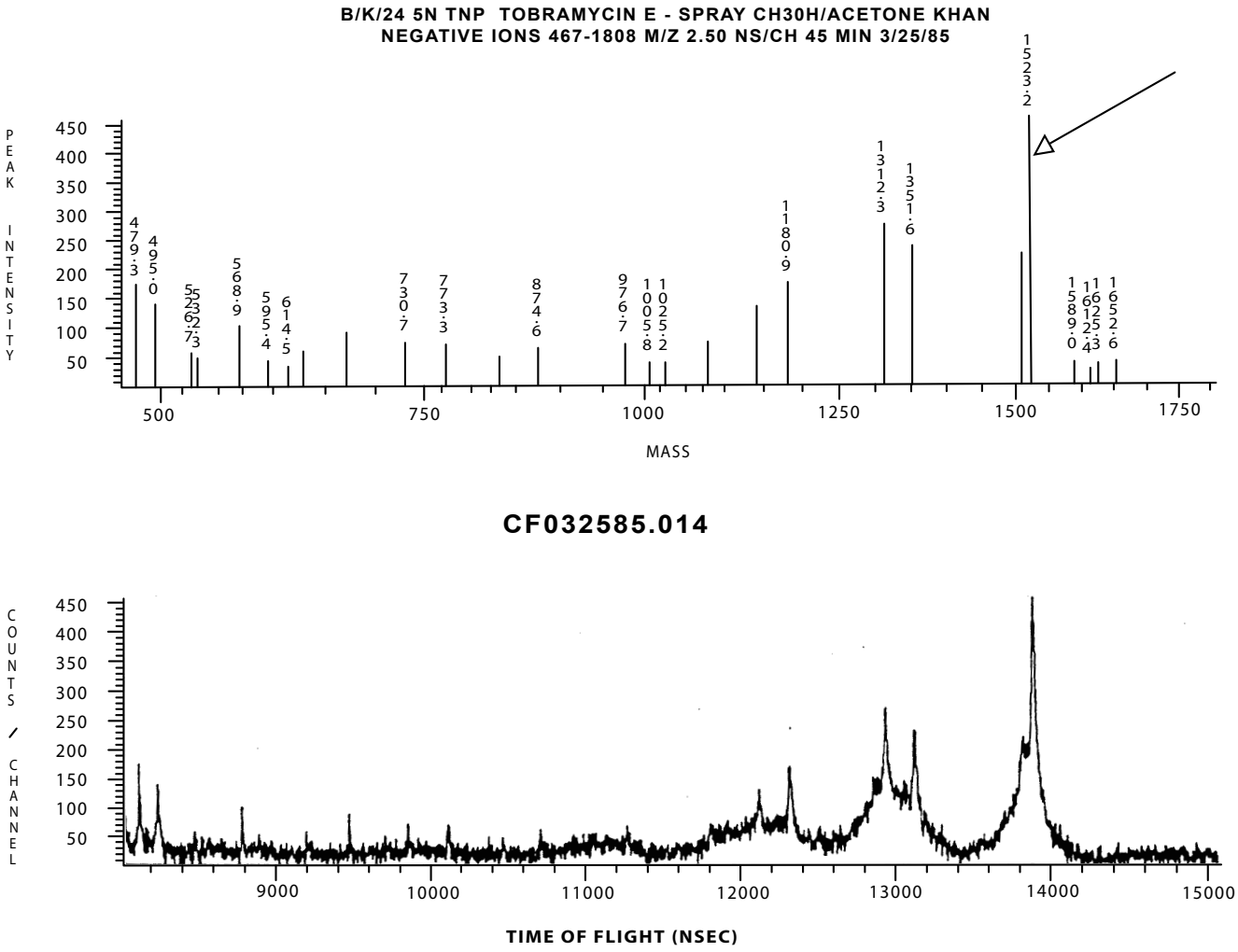
Synthesis of T0BRAMYCIN, N-penta(2,4,6-trinitrophenyl)

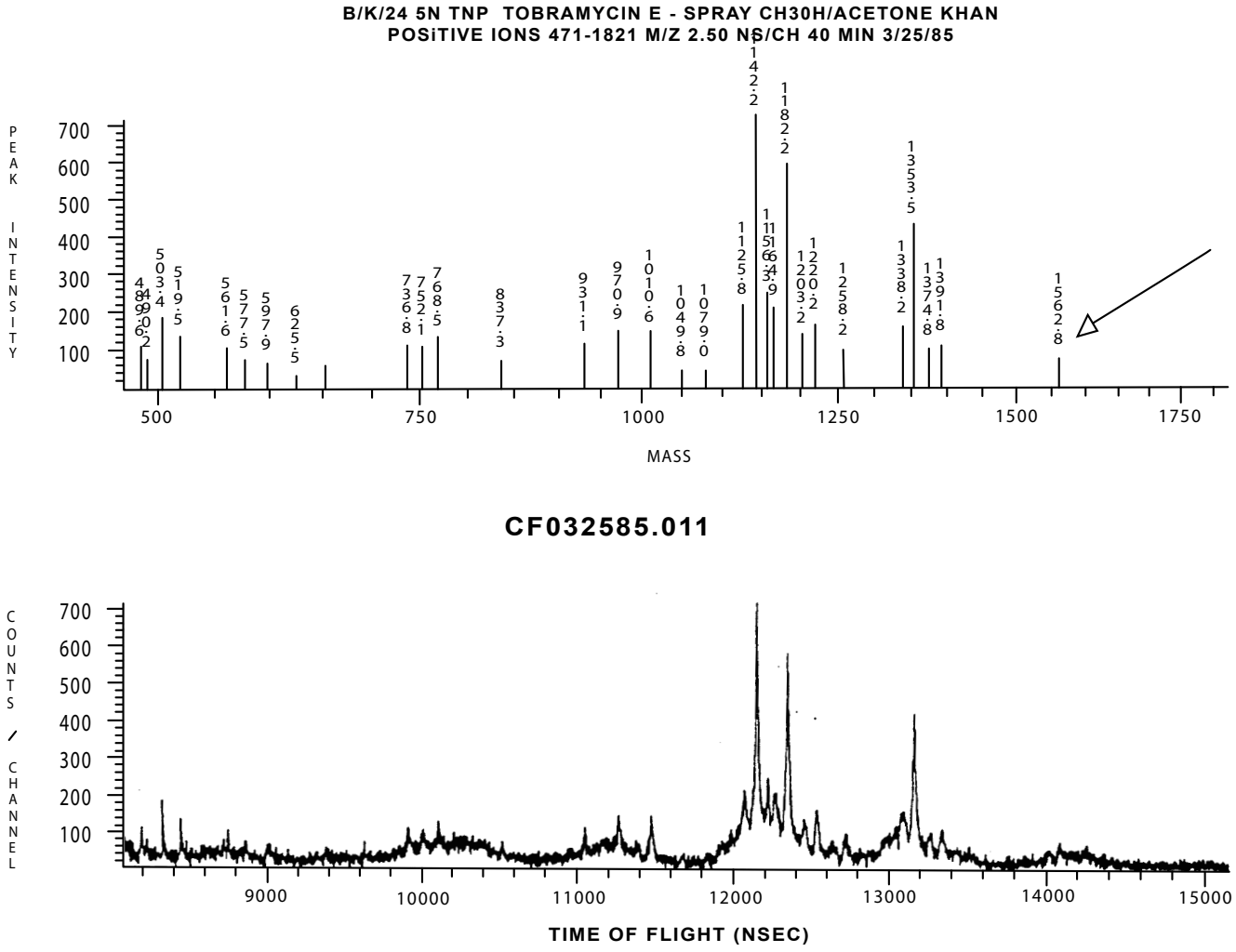
Tobramycin (0.2 g free base) was dissolved in an aqueous solution of 0.1 M potassium carbonate (15 ml containing an additional 0.23 g of potassium carbonate). The above solution was heated in an oil bath at 80°C. While stirring mechanically, a solution of Picryl Chloride (0.53 g) in methanol (15 ml) was added dropwise over 1/4 hour. After the addition was complete, the reaction mixture was heated for one hour. The product, Tobramycin, N-penta(2,4,6-trinitrophenyl), which was an orange colored semisolid oil separated from the reaction mixture. The hot solution was decanted to remove the solvent; the orange oily product was washed with cold potassium carbonate solution and then with cold water. The oily product solidified when kept in an ice-water bath for 1/2 hour. The solid was ground to fine powder, collected by filtration, washed with cold water, and dried on a suction pump. The structure of the Tobramycin, N-penta(2,4,6-trinitro phenyl) was confirmed by The Californium (252 Cf) Fast Atom Bombardment (FAB) mass spectrometry. The 252 Cf mass spectrum showed the following ions: m/z = 1523 (negative ion) and m/z = 1562 (K-positive ion).

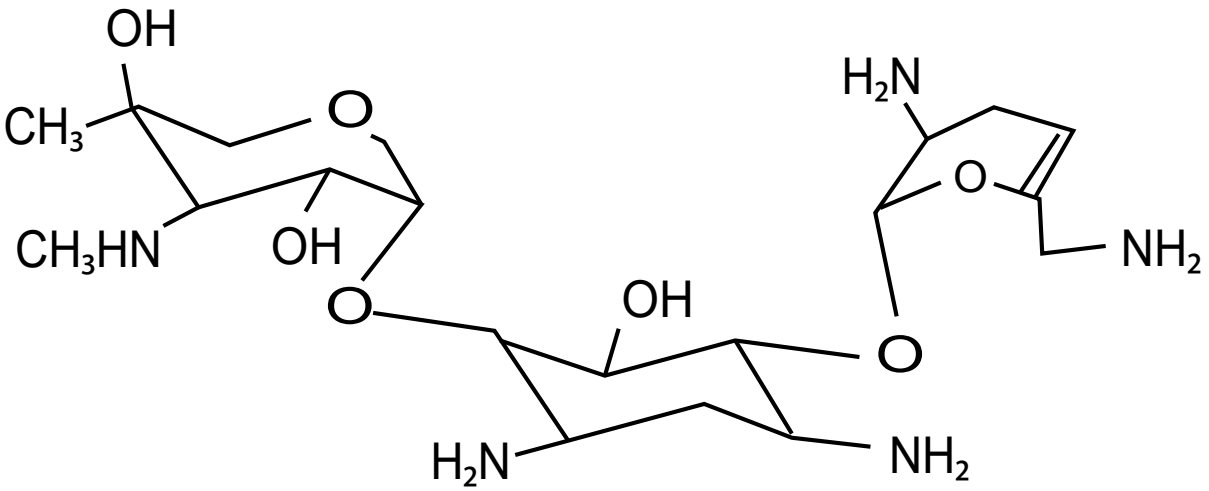
The following data on the above product were also collected: mp= 225°C; yield= 0.5 g (64%).



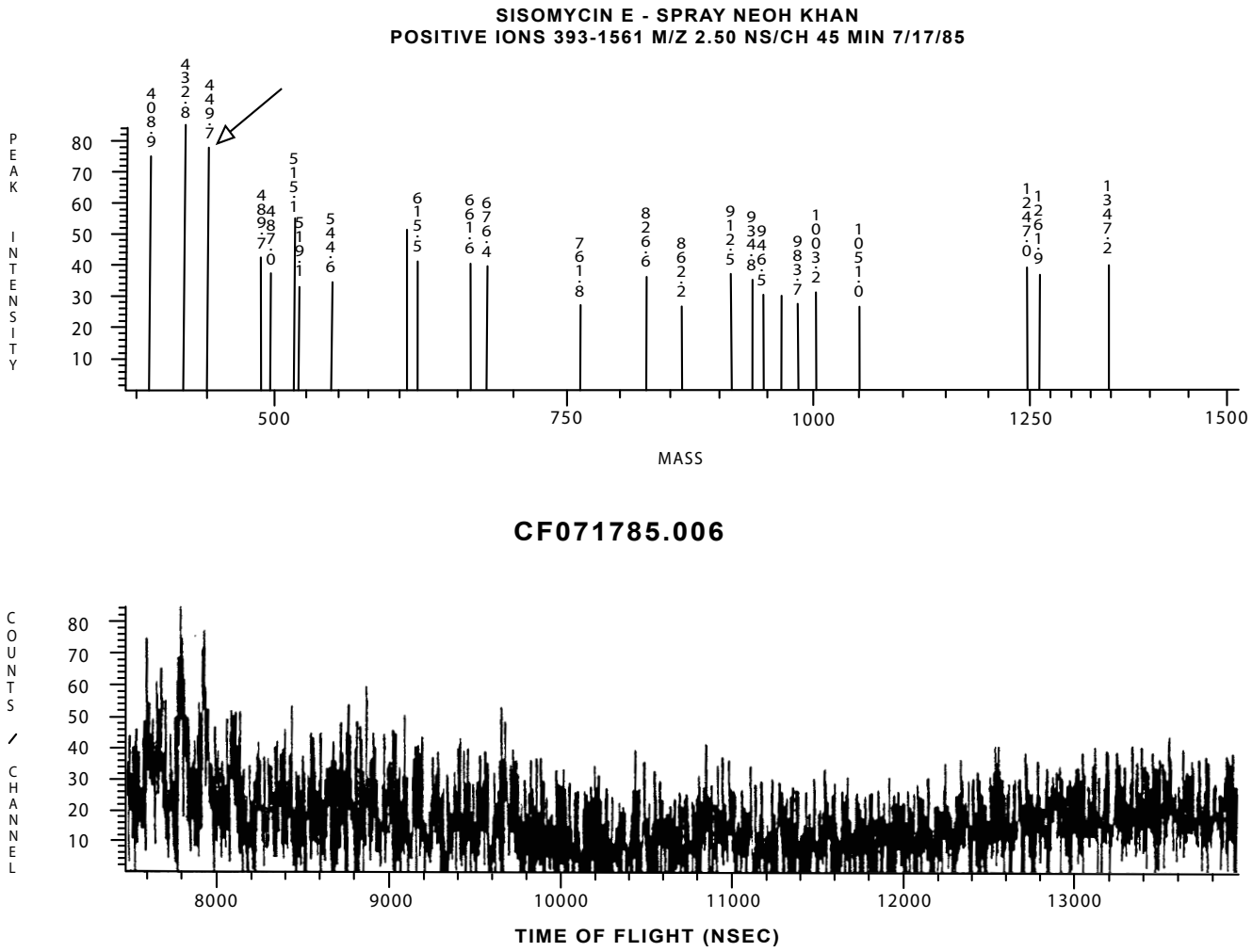
5 - N (2,4,6 - Trinitropheny 1) - Tobramycin

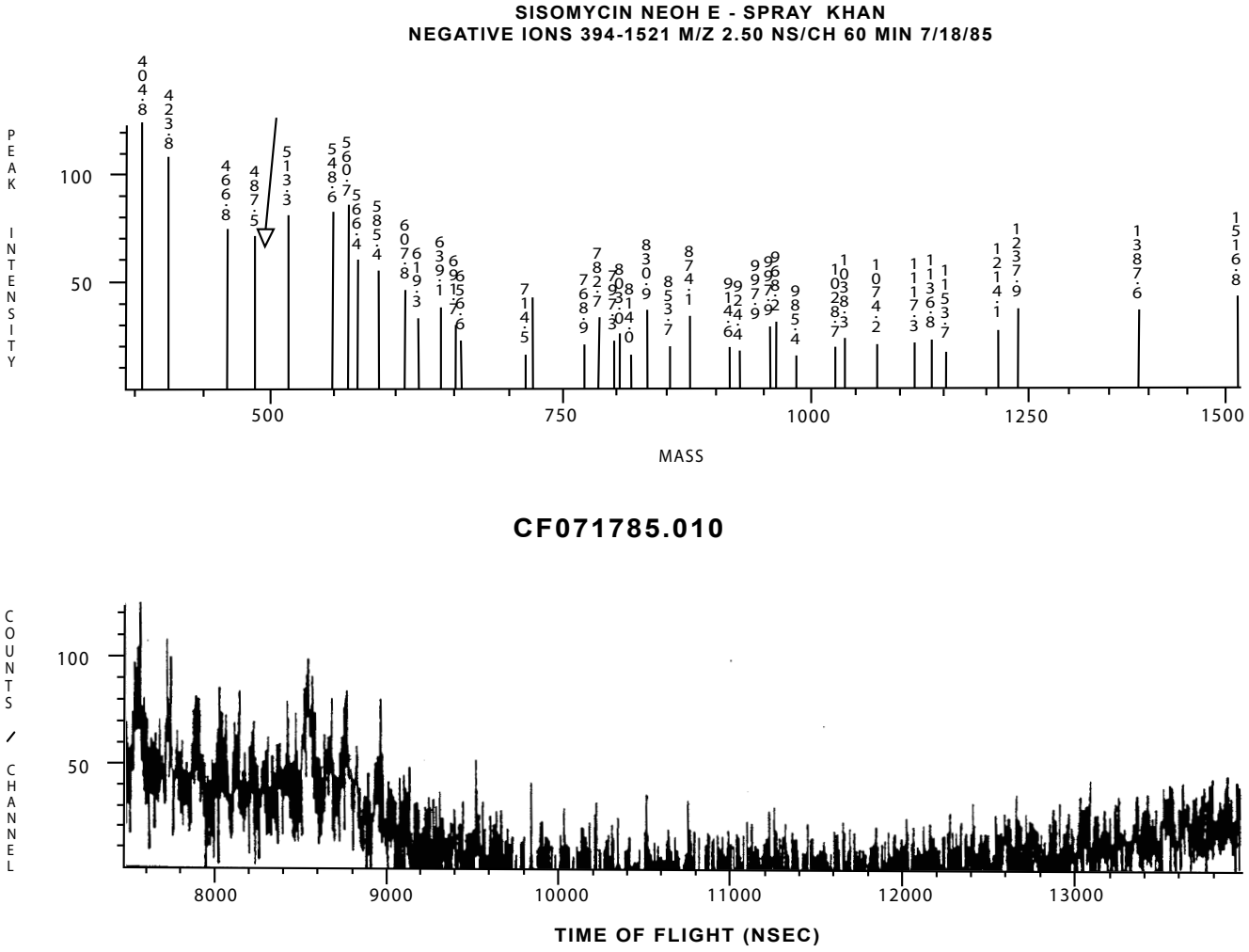






SISOMICIN

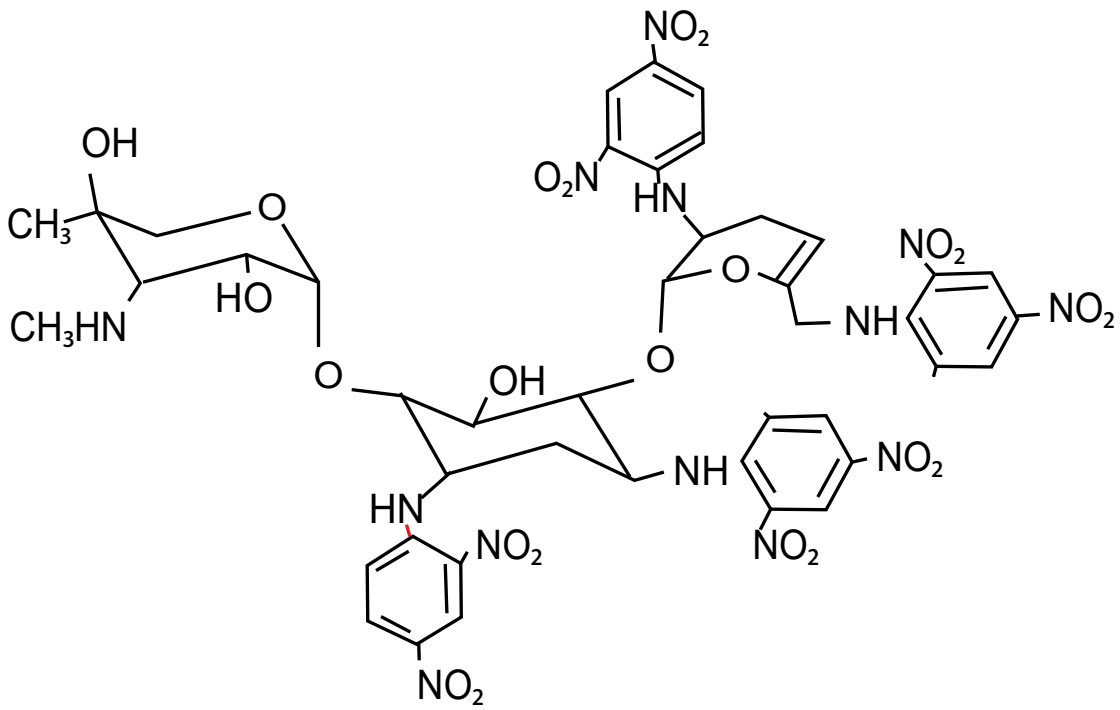




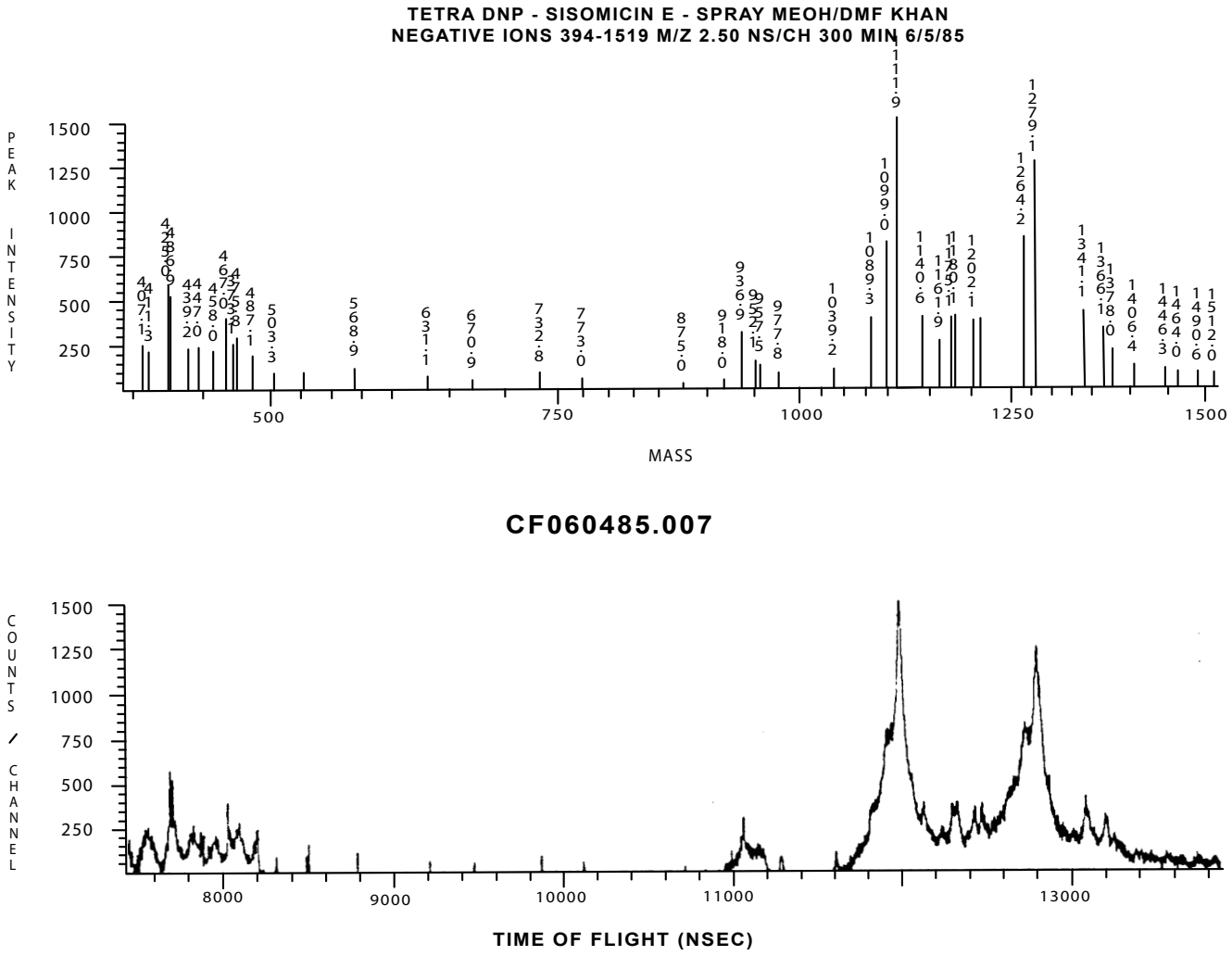
Synthesis of SIS0MICIN, N-tetra(2,4-dinitrophenyl)

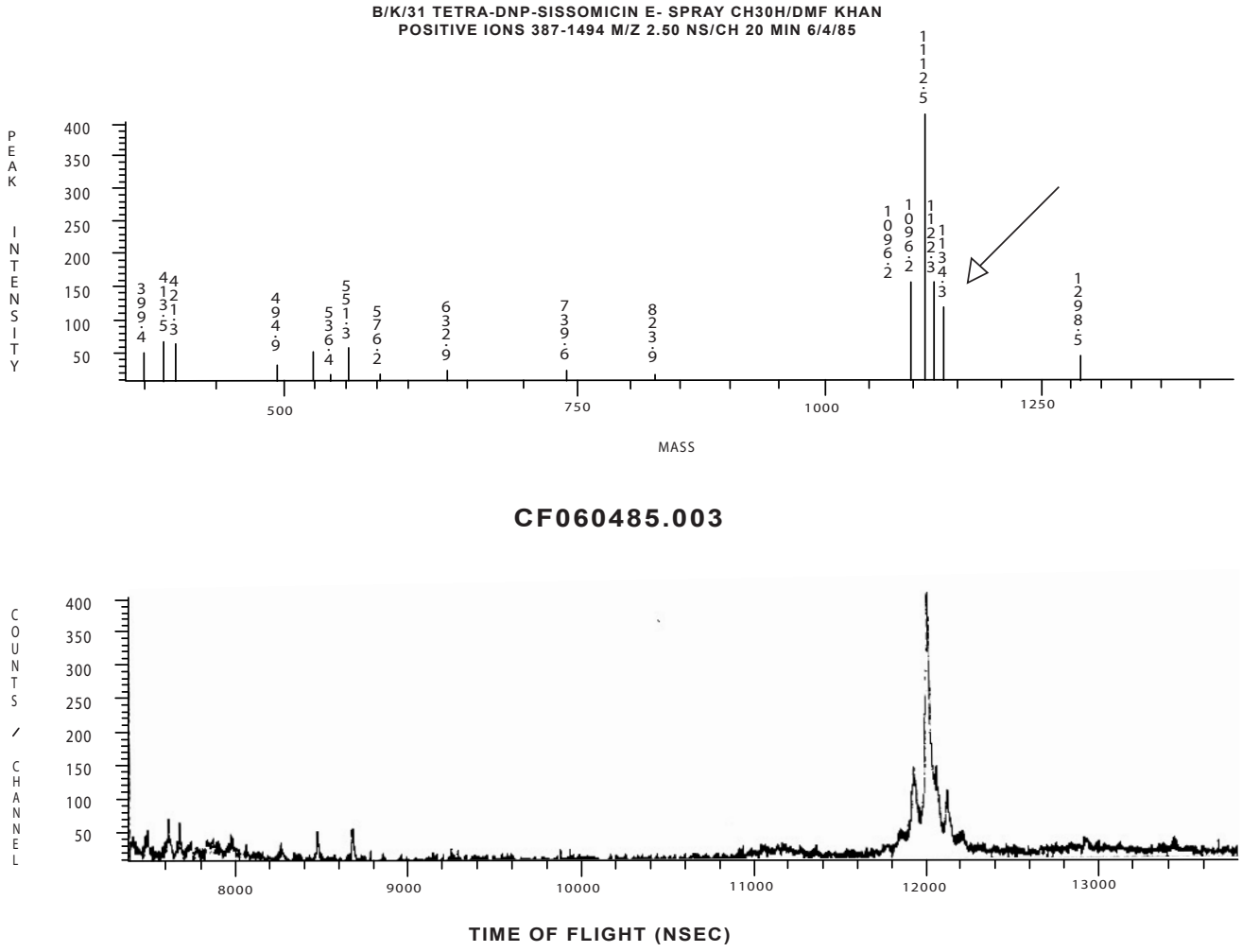
Sisomicin (0.25 g) was dissolved in an aqueous solution of 0.1 M potassium carbonate (15 ml containing an additional 0.3 g of potassium carbonate). The above solution was heated in an oil bath at 80°C. While stirring mechanically, a solution of 2,4-dinitrofluorobenzene (0.5 g) in methanol (15 ml) was added dropwise over 1/4 hour. After the addition was complete, the reaction mixture was heated for one hour. The product, Sisomicin, N-tetra(2,4-dinitrophenyl), which was an orange colored semisolid oil separated from the reaction mixture. The hot solution was decanted to remove the solvent; the orange oily product was washed with cold potassium carbonate solution and then with cold water. The oily product solidified when kept in an ice-water bath for 1/2 hour. The solid was ground to fine powder, collected by filtration, washed with cold water, and dried on a suction pump. The structure of the Sisomicin, N-tetra(2,4-dinitrophenyl) was confirmed by The Californium (252 Cf) Fast Atom Bombardment (FAB) mass spectrometry. The 252 Cf mass spectrum showed the following ions: m/z = 1111.9 (negative ion) and m/z = 1134.3 (positive ion).

The following data on the above product were also collected: mp= 218°C; yield= 0.5 g (80%).



Sisomicin - N-Tetra - (2,4 - dinitrophenyl)

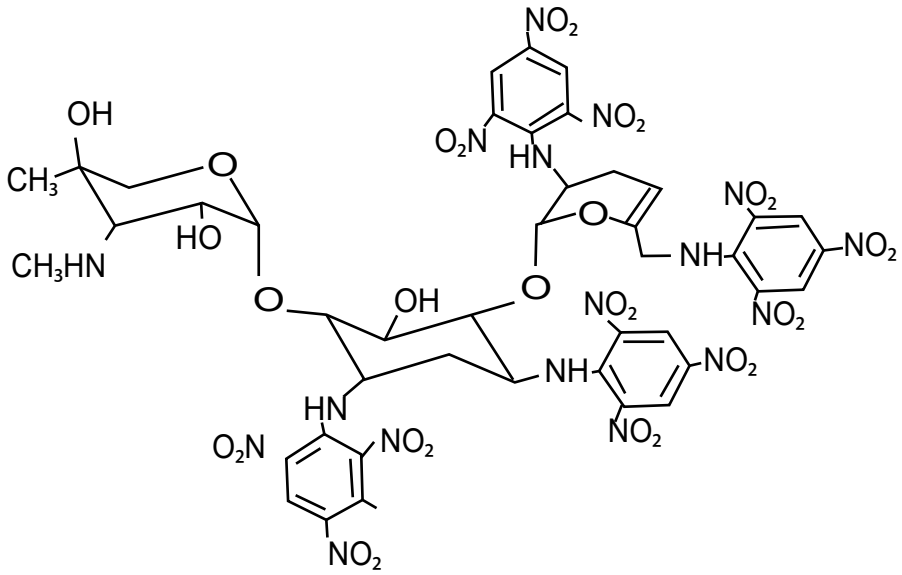




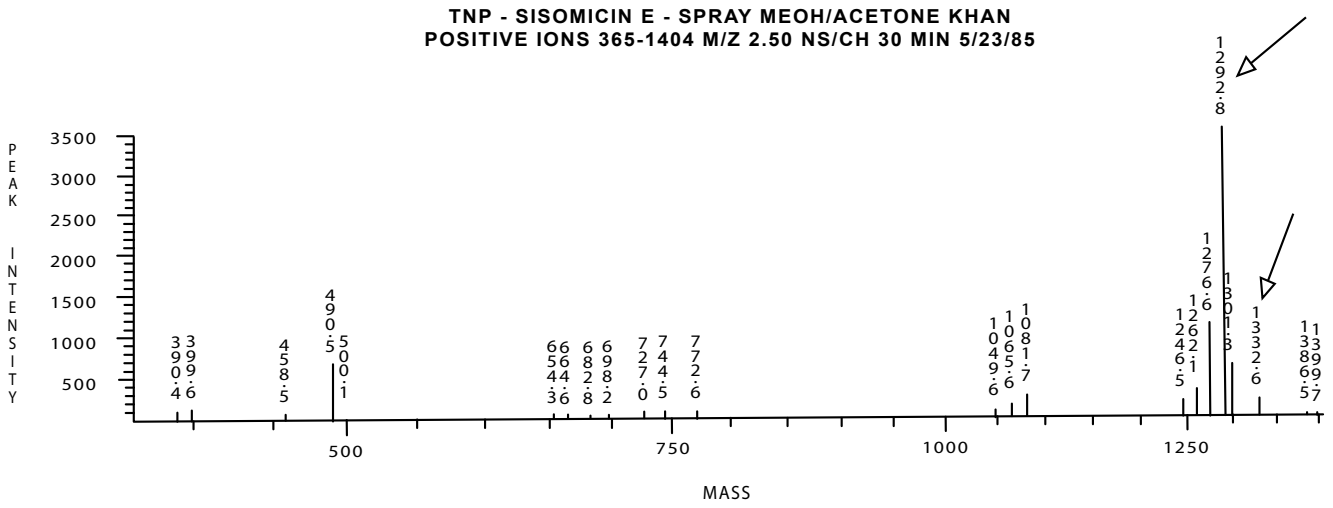
Synthesis of SIS0MICIN, N-tetra(2,4,6-trinitrophenyl)

Sisomicin (0.2 g) was dissolved in an aqueous solution of 0.1 M potassium carbonate (15 ml containing an additional 0.3 g of potassium carbonate). The above solution was heated in an oil bath at 80°C. While stirring mechanically, a solution of Picryl Chloride (0.55 g) in methanol (15 ml) was added dropwise over 1/4 hour. After the addition was complete, the reaction mixture was heated for one hour. The product, Sisomicin, N-tetra(2,4,6-trinitrophenyl), which was an orange colored semisolid oil separated from the reaction mixture. The hot solution was decanted to remove the solvent; the orange oily product was washed with cold potassium carbonate solution and then with cold water. The oily product solidified when kept in an ice-water bath for 1/2 hour. The solid was ground to fine powder, collected by filtration, washed with cold water, and dried on a suction pump. The structure of the Sisomicin, N-tetra(2,4,6- trinitrophenyl) was confirmed by The Californium (252 Cf) Fast Atom Bombardment (FAB) mass spectrometry. The 252 Cf mass spectrum showed the following ions: m/z = 1292 (negative ion) and m/z = 1332(K+) (positive ion).

The following data on the above product were also collected: mp= 184°C; yield= 0.47 g (81%).



Sisomicin - N-Tetra - (2,4,6 - trinitrophenyl)



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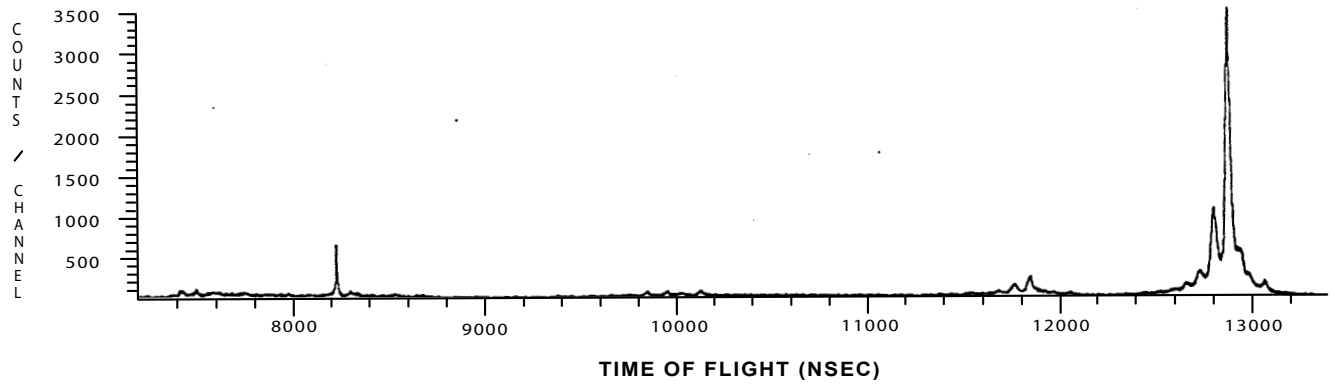
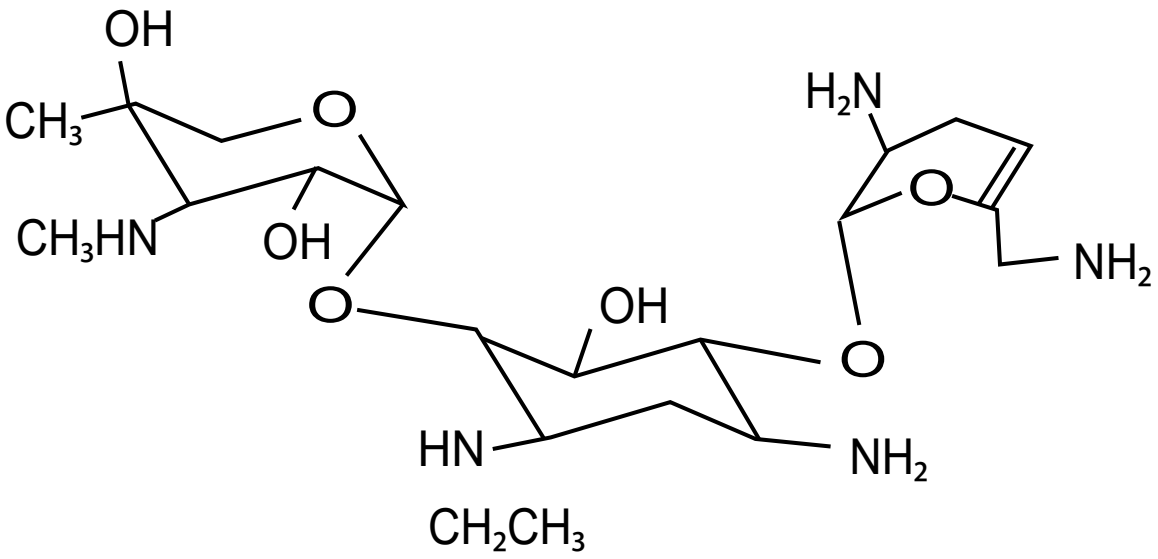
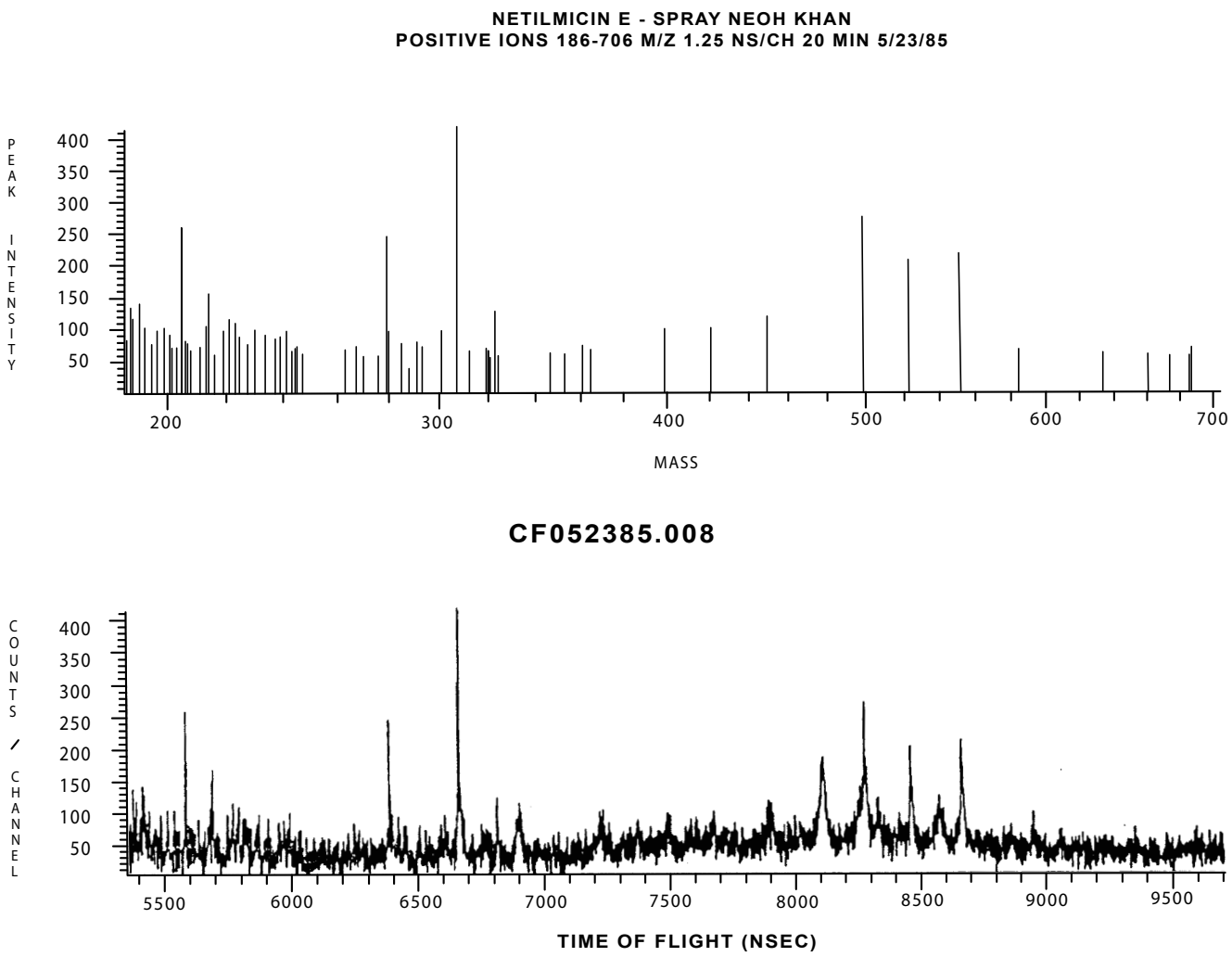




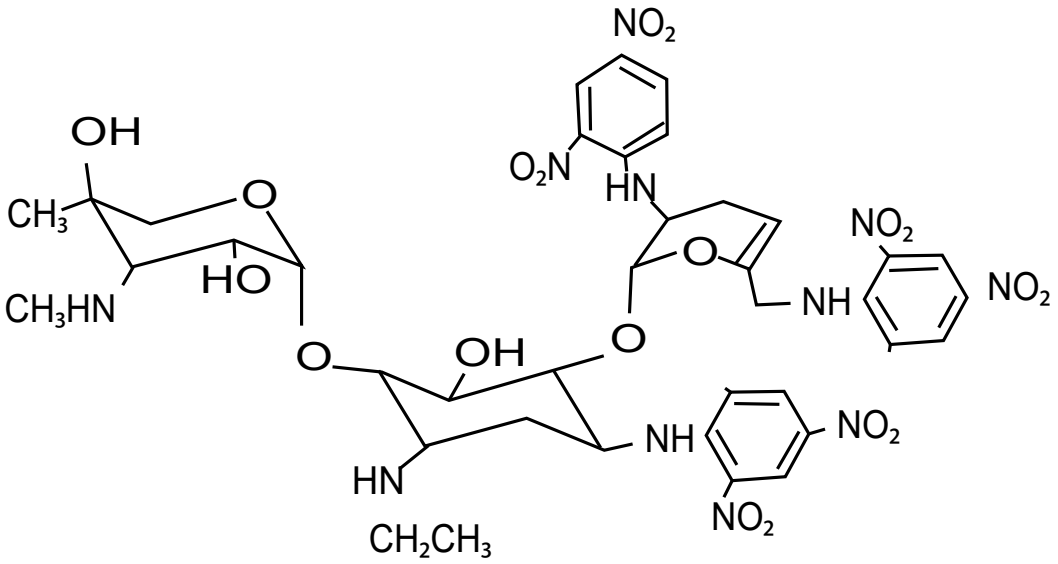
NETLIMICIN



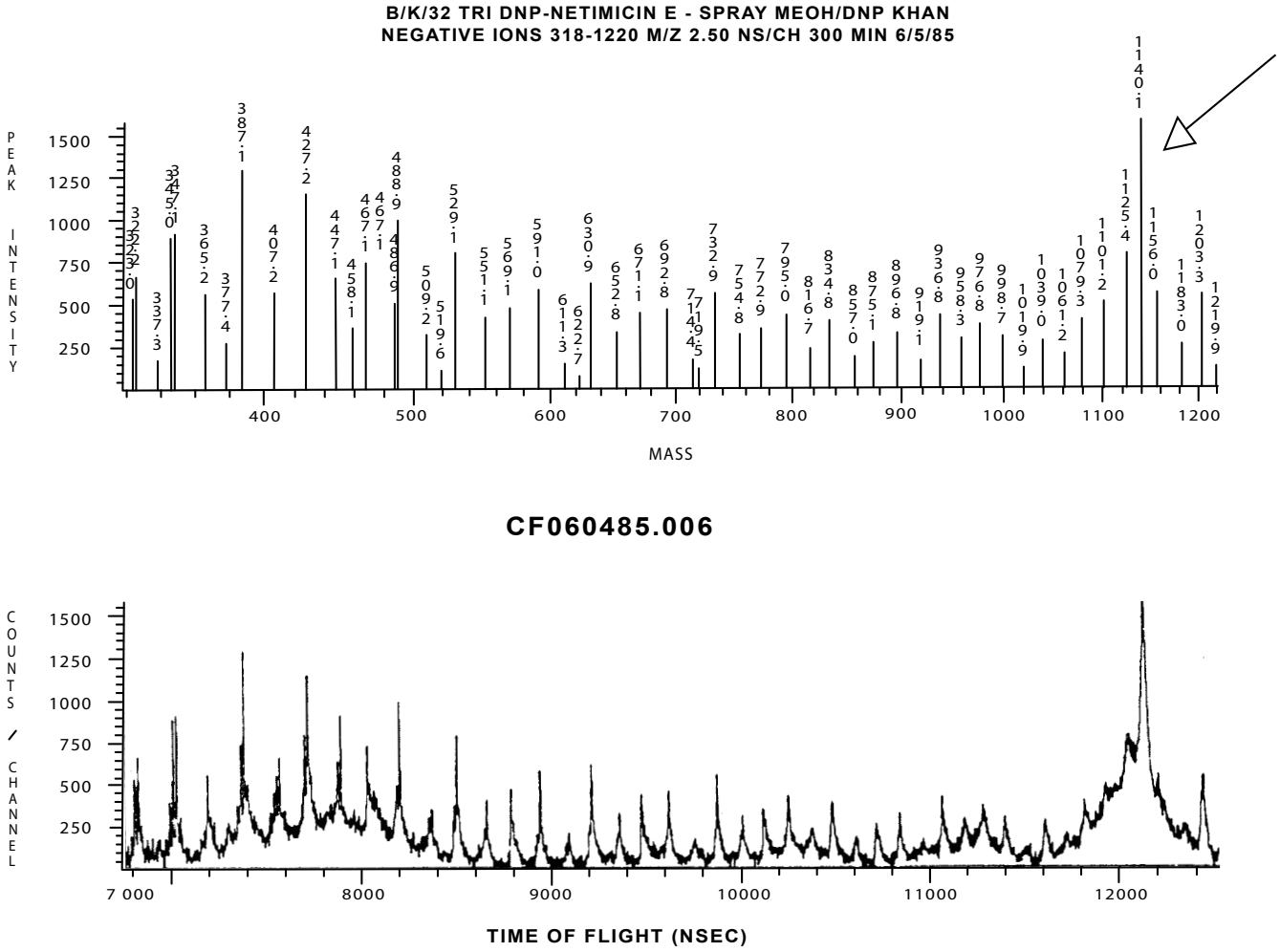
Synthesis of NETILMICIN, N-tri(2,4-dinitrophenyl)

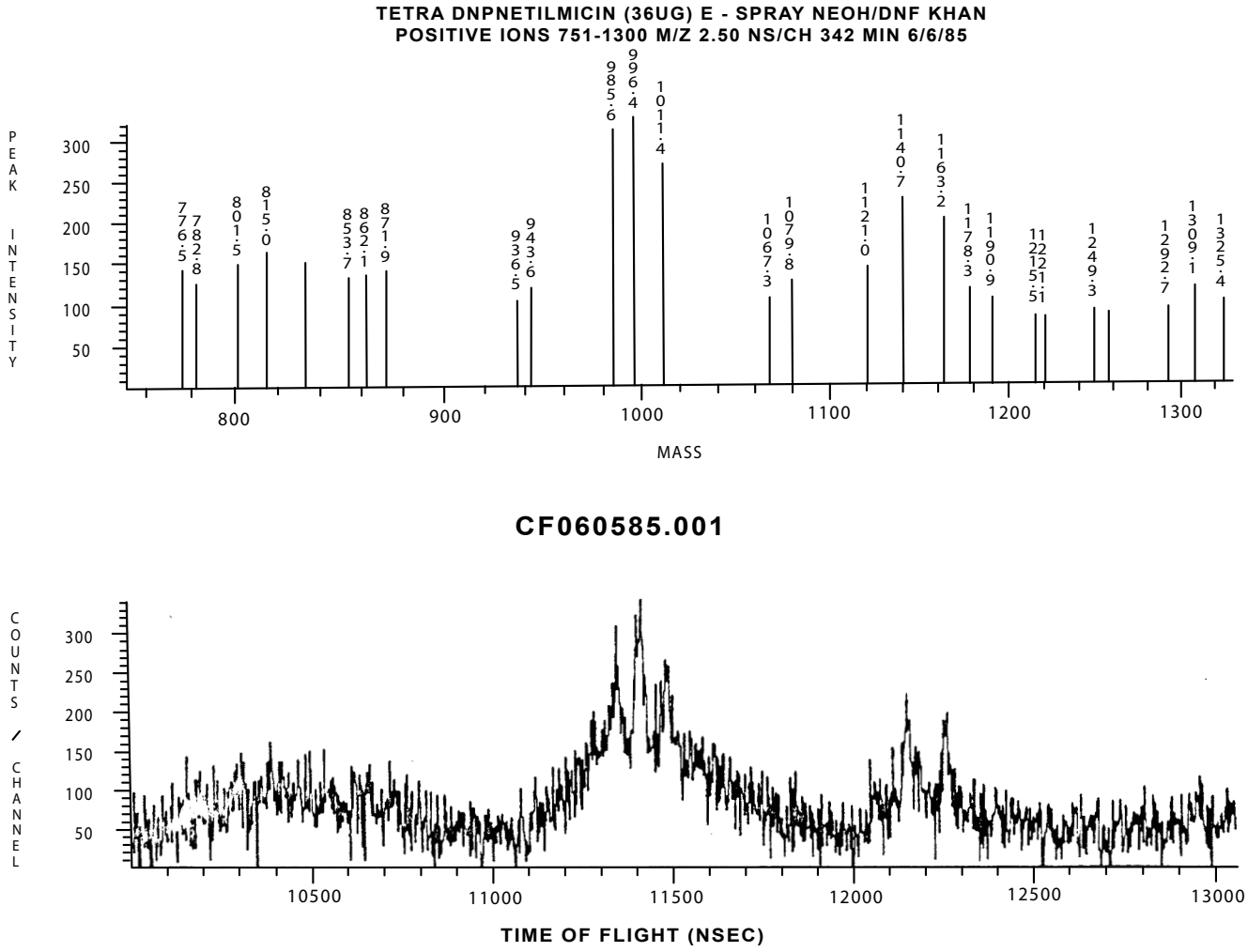
Netilmicin (0.25) was dissolved in an aqueous solution of 0.1 M potassium carbonate (15 ml containing an additional 0.3 g of potassium carbonate). The above solution was heated in an oil bath at 80°C. While stirring mechanically, a solution of 2,4-dinitrofluorobenzene (0.5 g) in methanol (15 ml) was added dropwise over 1/4 hour. After the addition was complete, the reaction mixture was heated for one hour. The product, Netilmicin, N-tri(2,4-dinitrophenyl), which was an orange colored semisolid oil separated from the reaction mixture. The hot solution was decanted to remove the solvent; the orange oily product was washed with cold potassium carbonate solution and then with cold water. The oily product solidified when kept in an ice-water bath for 1/2 hour. The solid was ground to fine powder, collected by filtration, washed with cold water, and dried on a suction pump. The structure of the Netilmicin, N-tri(2,4-dinitrophenyl) was confirmed by The Californium (252 Cf) Fast Atom Bombardment (FAB) mass spectrometry. The 252 Cf mass spectrum showed the following ions: m/z; 975.4 (negative ion) and m/z; 1163.2 (positive ion).

The following data on the above product were also collected: mp; 234°C; yield; 0.45 g (87%).



Netilmicin - N - Tri - (2,4 - dinitrophenyl)

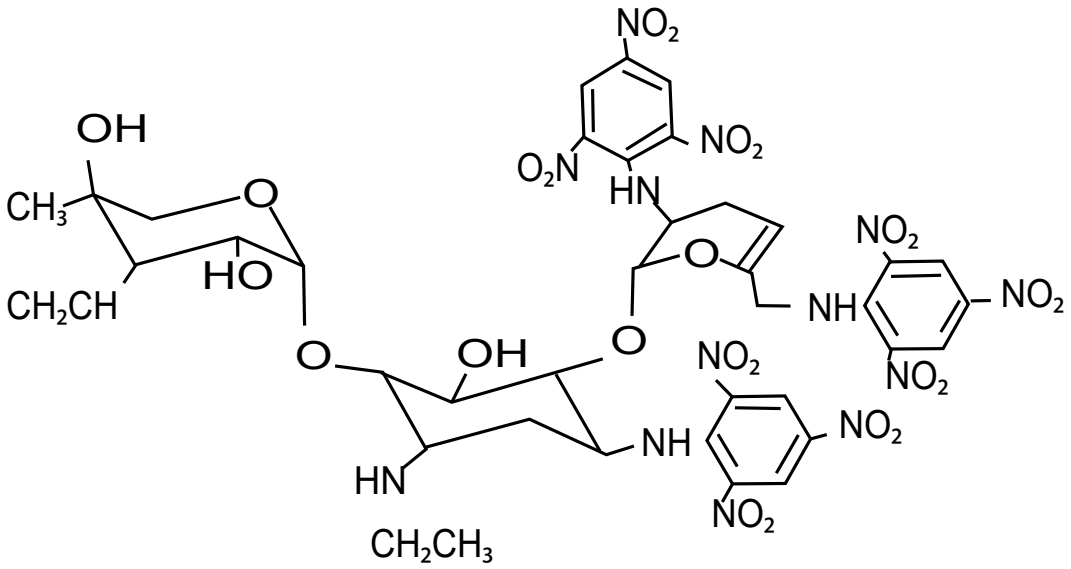




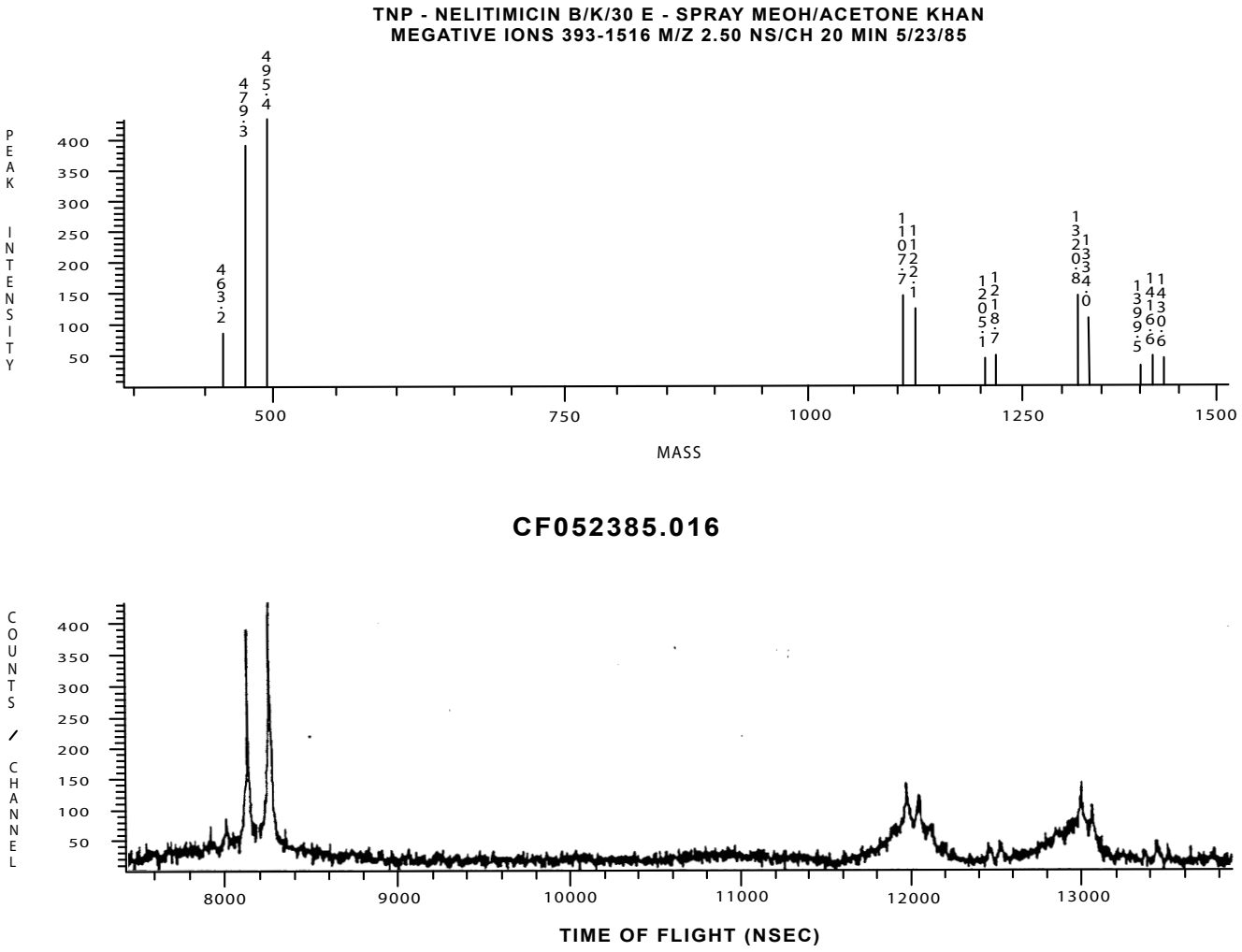
Synthesis of NETILMICIN, N-tri(2,4,6-trinitrophenyl)

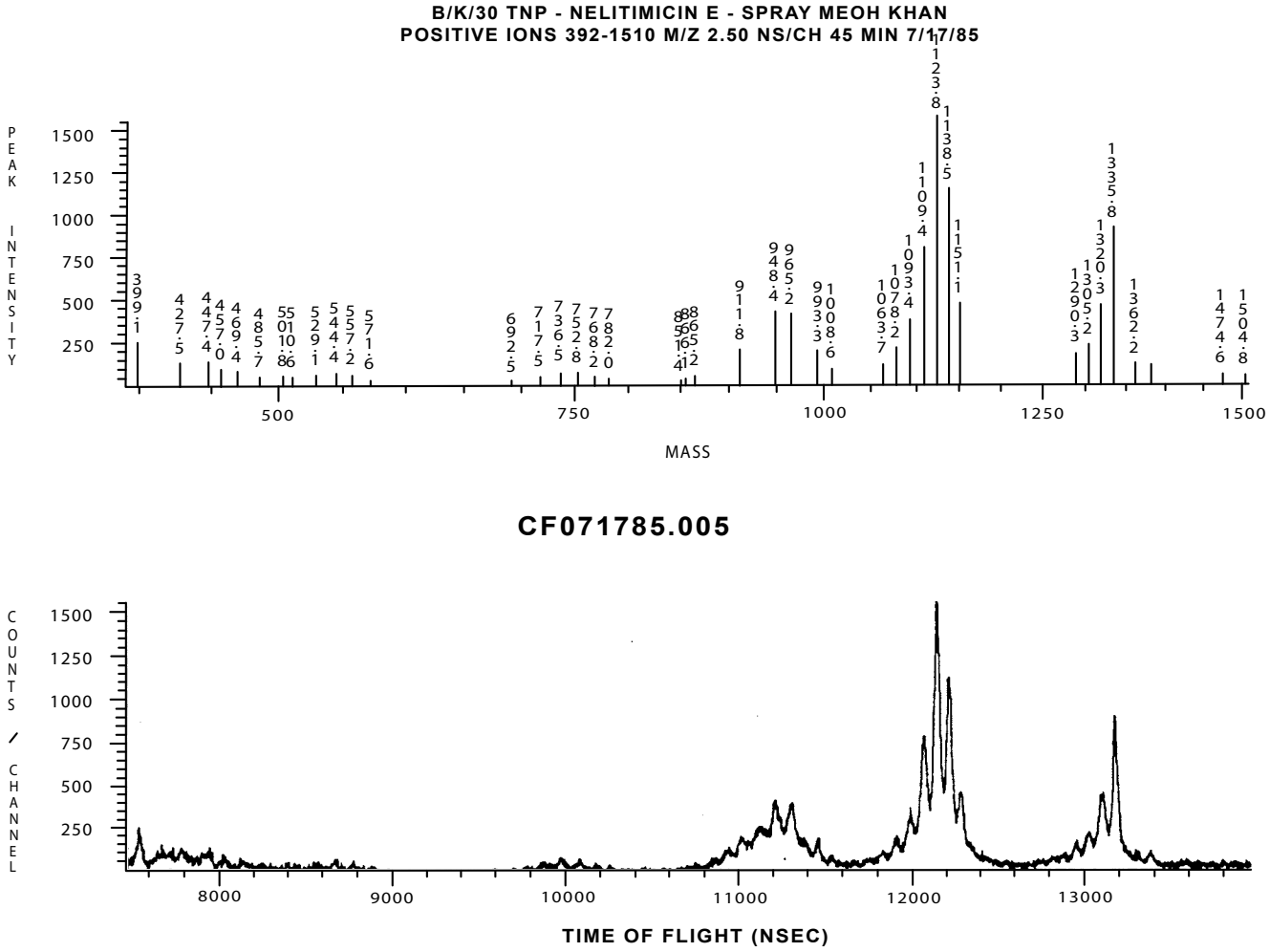
Netilmicin {0.2 g) was dissolved in an aqueous solution of 0.1 M potassium carbonate {15 ml containing an additional 0.3 g of potassium carbonate). The above solution was heated in an oil bath at 80°C. While stirring mechanically, a solution of Picryl Chloride {0.39 g) in methanol {15 ml) was added dropwise over 1/4 hour. After the addition was complete, the reaction mixture was heated for one hour. The product, Netilmicin, N-tri{2,4,6-trinitrophenyl), which was an orange colored semisolid oil separated from the reaction mixture. The hot solution was decanted to remove the solvent; the orange oily product was washed with cold potassium carbonate solution and then with cold water. The oily product solidified when kept in an ice-water bath for 1/2 hour. The solid was ground to fine powder, collected by filtration, washed with cold water, and dried on a suction pump. The structure of the Netilmicin, N-tri(2,4,6-trinitrophenyl) was confirmed by The Californium (252 Cf) Fast Atom Bombardment (FAB) mass spectrometry. The 252 Cf mass spectrum showed the following ions: m/z = (negative ion) and m/z = 1149.8 (K+) (positive ion).

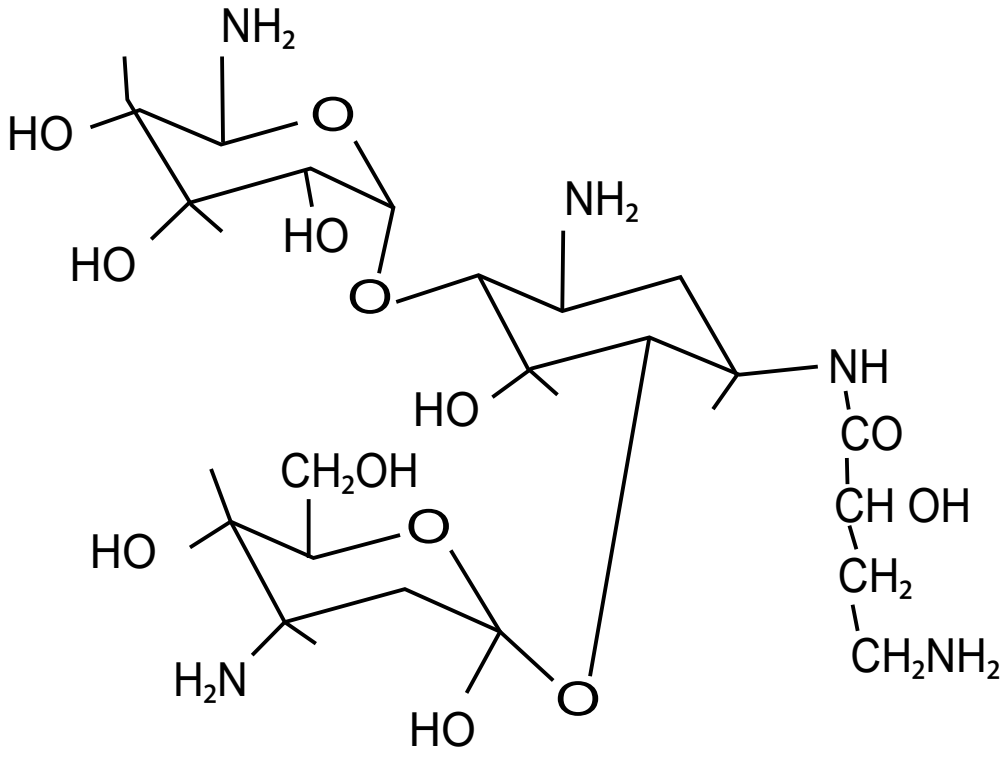
The following data on the above product were also collected: mp= 194°C; yield= 0.45 g {96%).



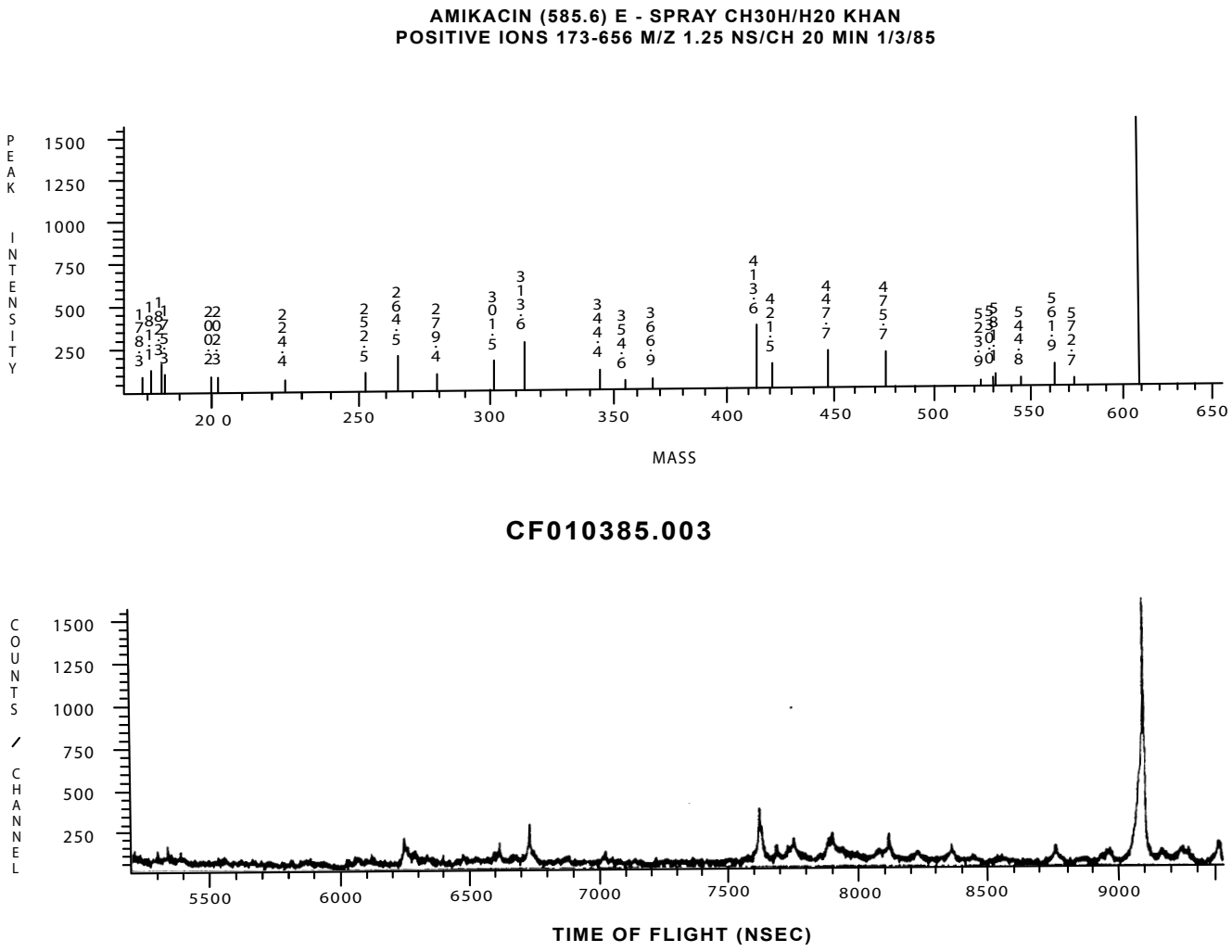
Netilmicin - N - Tri - (2,4,6 - trinitrophenyl)







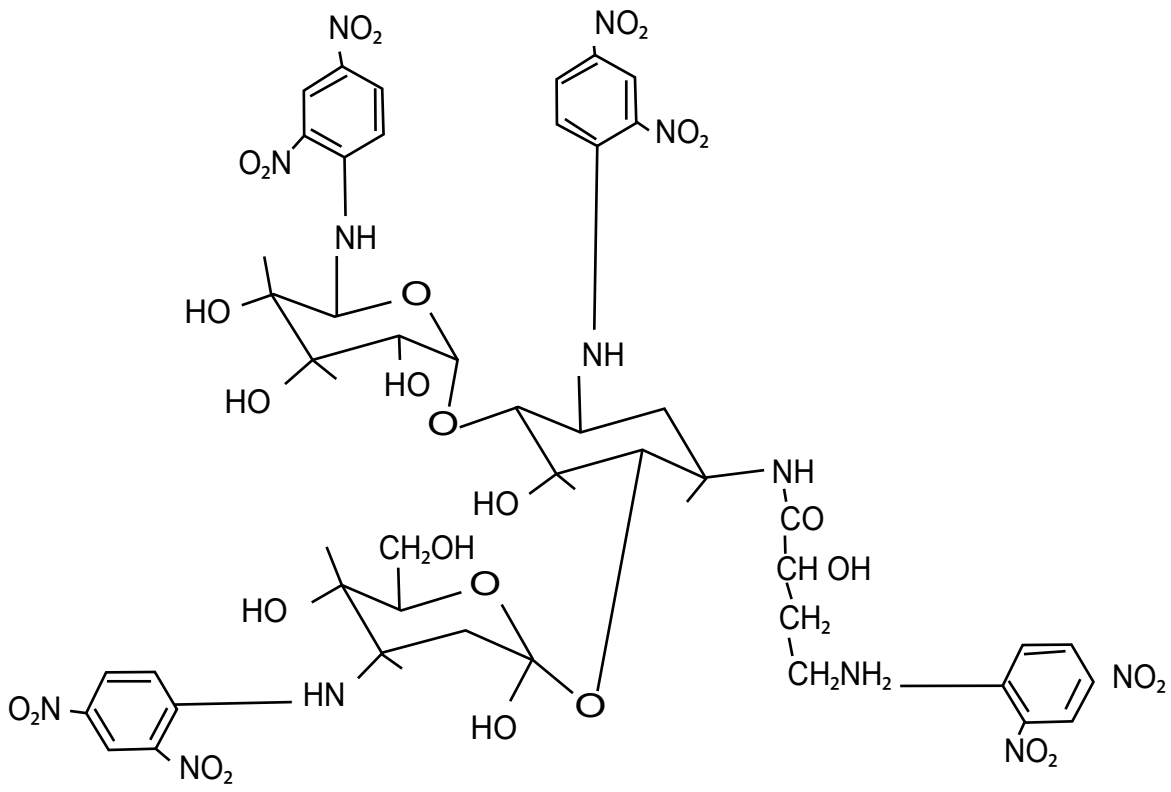
AMIKACIN



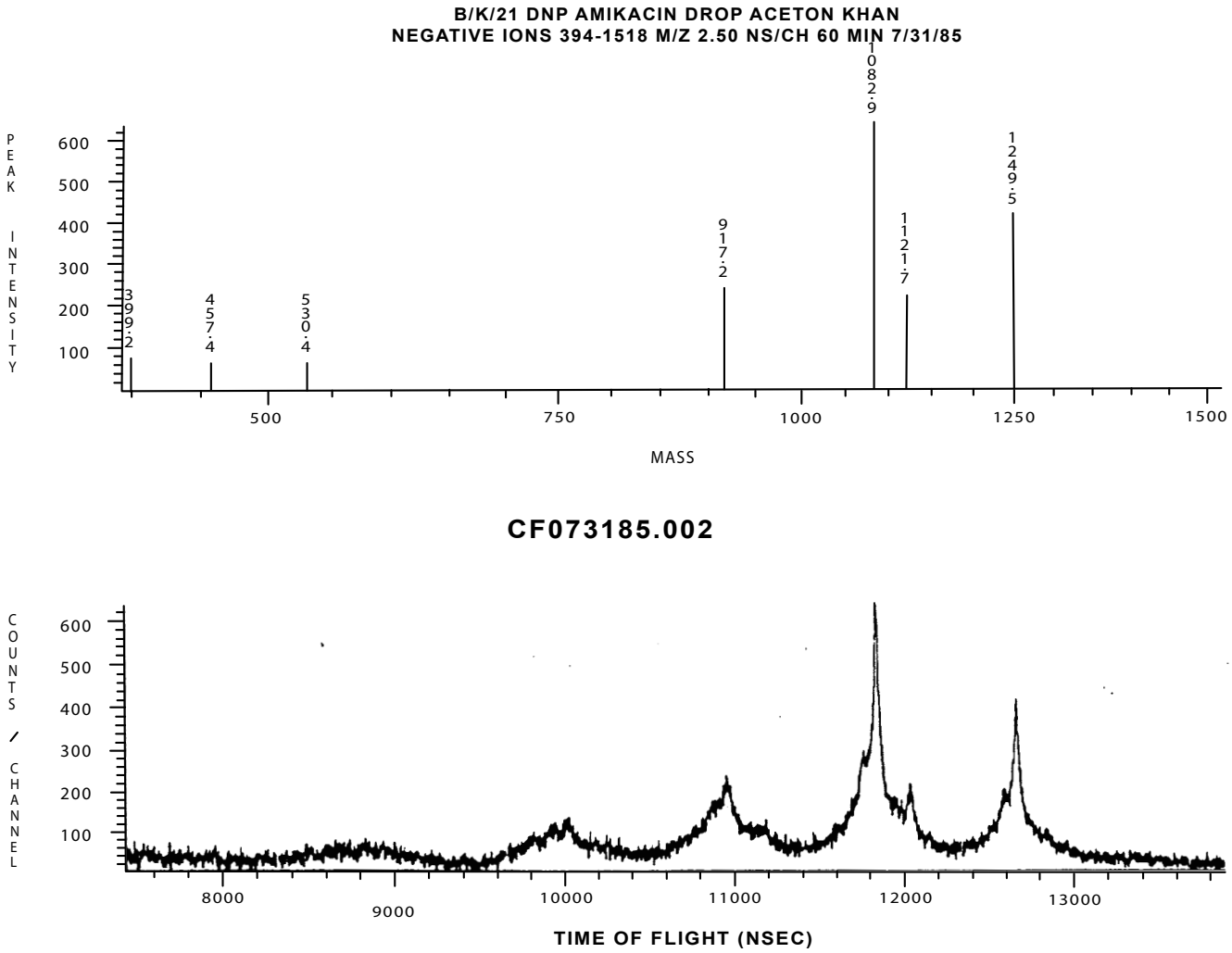
Synthesis of AMIKACIN, N-tetra(2,4-dinitrophenyl)

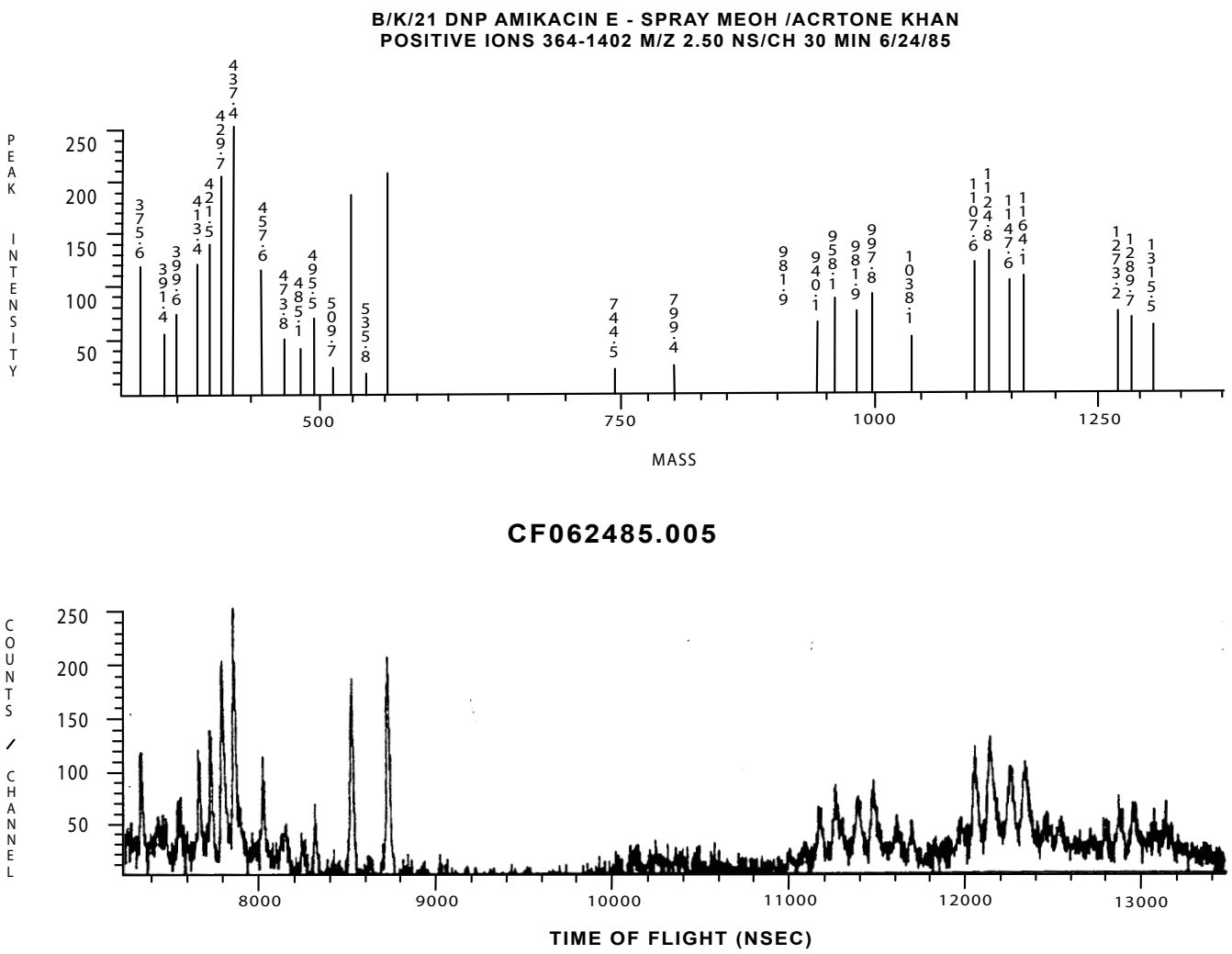
Amikacin (1.0 g free base) was dissolved in an aqueous solution of 0.1M potassium carbonate (25 ml containing an additional 0.23 g of potassium carbonate). The above solution was heated in an oil bath at 80°C. While stirring mechanically, a solution of 2,4-dinitrofluorobenzene (2.0 g) in methanol (30 ml) was added dropwise over 1/4 hour. After the addition was complete, the reaction mixture was heated for one hour. The product, Amikacin, N-tetra(2,4-dinitrophenyl), which was an orange colored semisolid oil separated from the reaction mixture. The hot solution was decanted to remove the solvent; the orange oily product was washed with cold potassium carbonate solution and then with cold water. The oily product solidified when kept in an ice-water bath for 1/2 hour. The solid was ground to fine powder, collected by filtration, washed with cold water, and dried on a suction pump. The structure of the Amikacin, N-tetra(2,4-dinitrophenyl) was confirmed by the Californium (252 Cf) Fast Atom Bombardment (FAB) mass spectrometry. The 252 Cf mass spectrum showed the following ions: m/z = 1250.5 (negative ion) and m/z = 1273.2 (positive ion).

The following data on the above product were also collected: mp= 102°C; yield= 94% (wt. = 2.1 g).



AMIKACIN, 4-N (2,4 -dinitrophenyl)



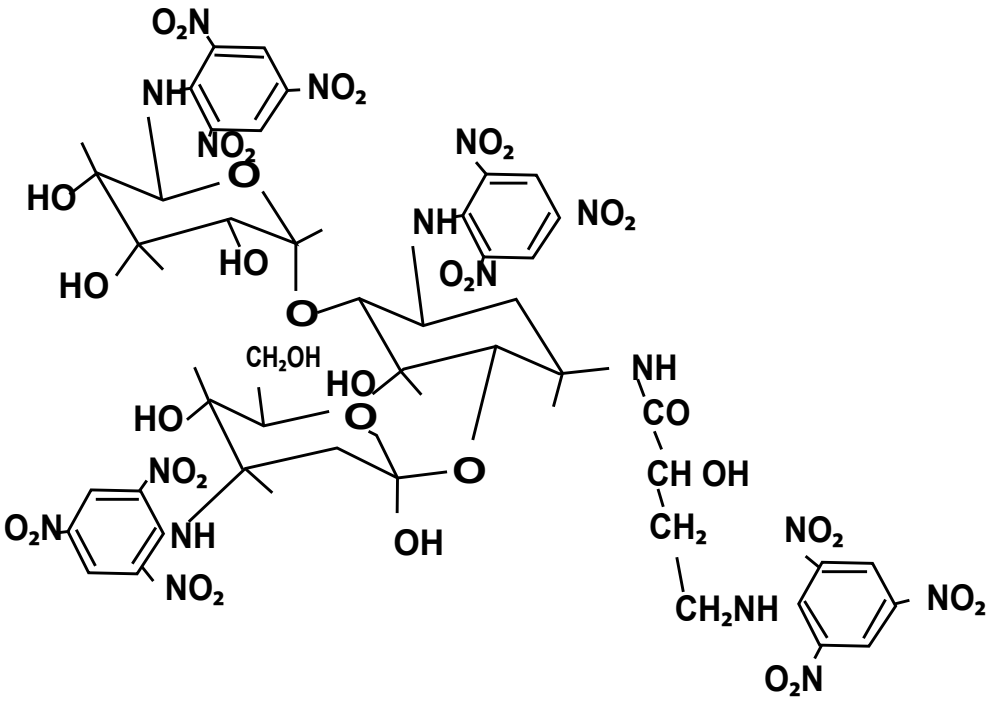


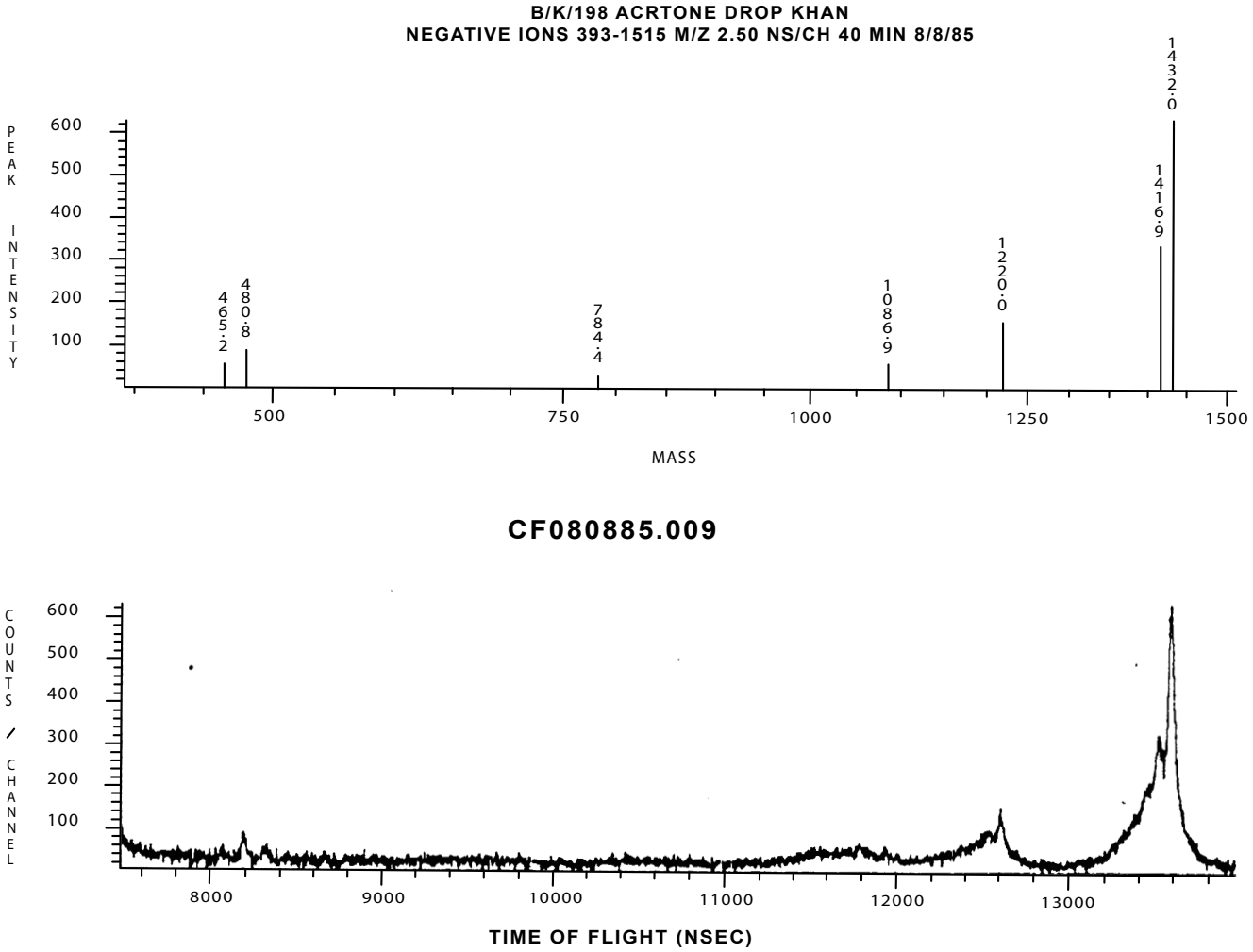
Synthesis of AMIKACIN, N-tetra(2,4,6-trinitrophenyl)

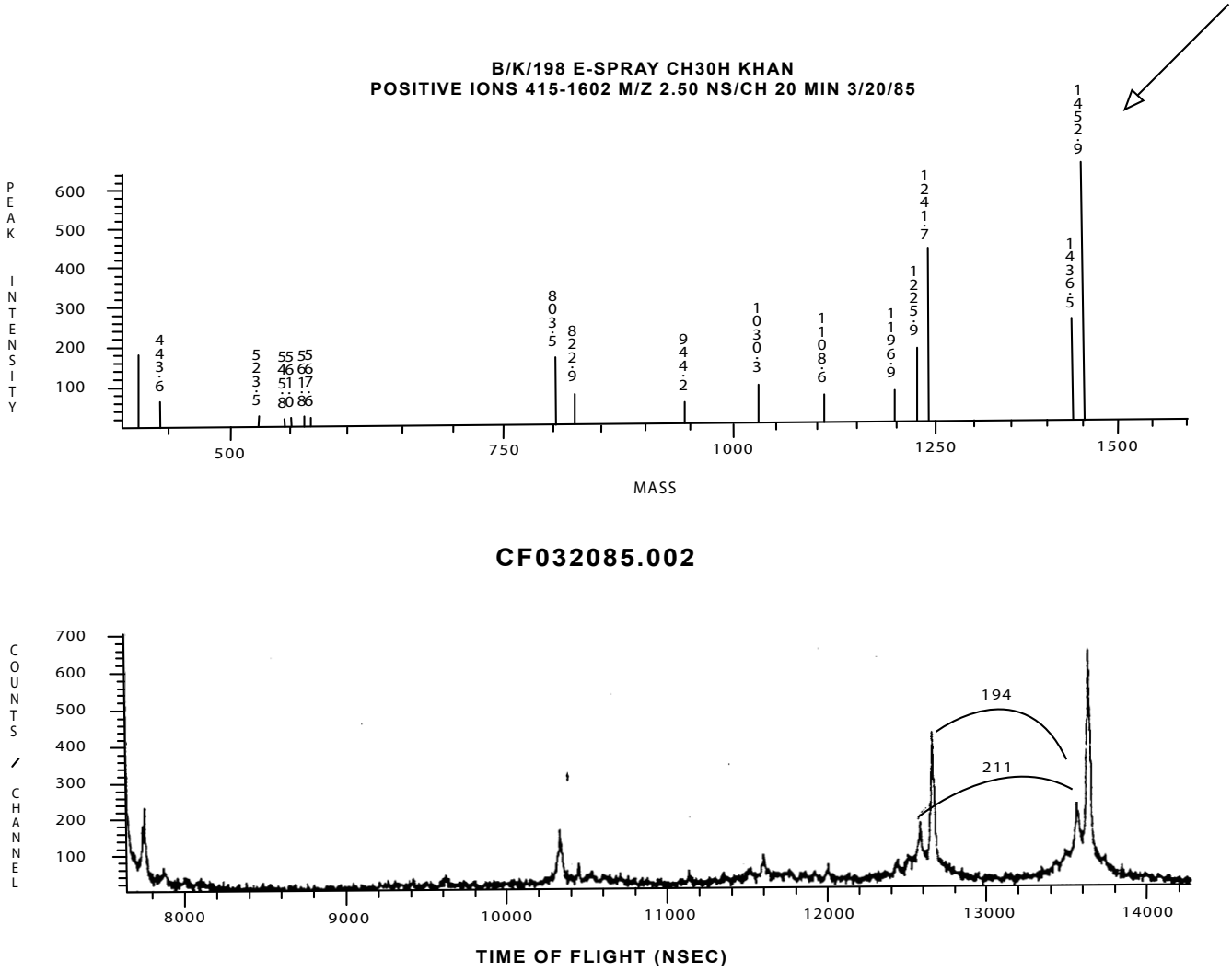
Prnikacin (1.0 g free base) was dissolved in an aqueous solution of 0.1M potassium carbonate (25 ml containing an additional 0.23 g of potassium carbonate). The above solution was heated in an oil bath at 80°C. While stirring mechanically, a solution of Picryl Chloride (2.0 g) in methanol (25 ml) was added dropwise over 1/4 hour. After the addition was complete, the reaction mixture was heated for one hour. The product, Amikacin, N-tetra(2,4,6-trinitrophenyl), which was an orange colored semisolid oil separated from the reaction mixture. The hot solution was decanted to remove the solvent; the orange oily product was washed with cold potassium carbonate solution and then with cold water. The oily product solidified when kept in an ice-water bath for 1/2 hour. The solid was ground to fine powder, collected by filtration, washed with cold water, and dried on a suction pump. The structure of the Amikacin, N-tetra(2,4,6-trinitrophenyl) was confirmed by the Californium (252 Cf) Plasma desorption mass spectrometry. The 252 Cf mass spectrum showed the following ions: m/z = 1429 (negative ion) and m/z = 1452 (positive ion).

The following data on the above product were also collected: mp= 192°C; yield= 90% (wt. = 2.2 g).

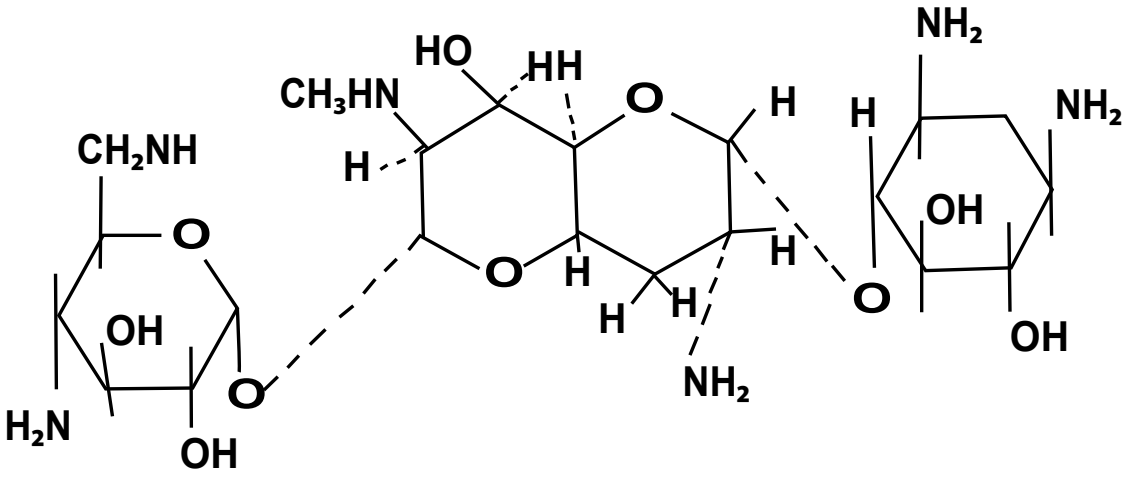
AMIKACIN, 4 - (2,4,6 - Trinitrophenyl)

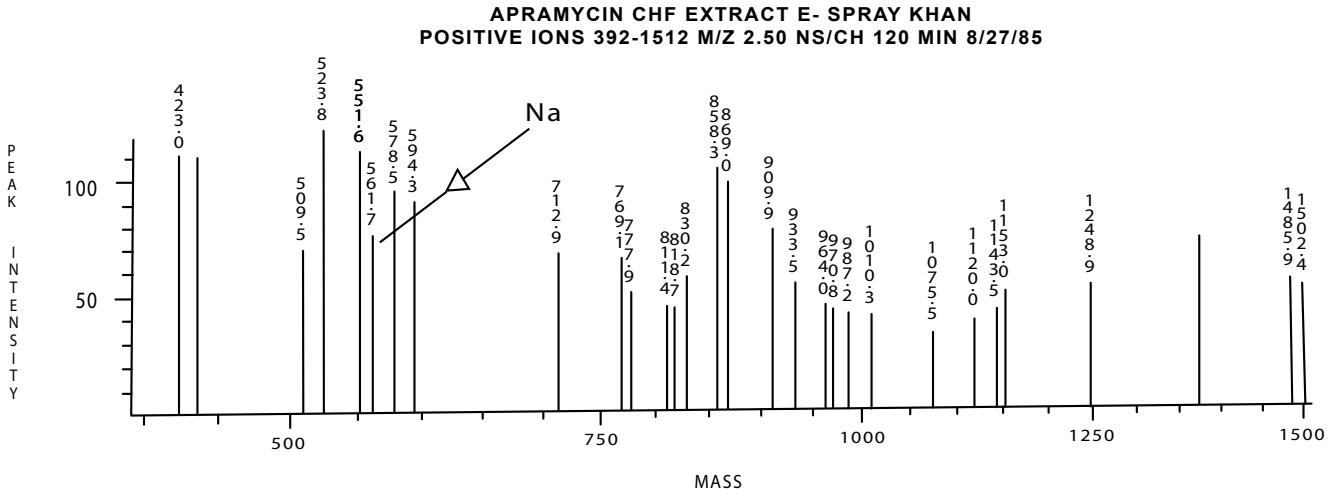






APRAMYCIN





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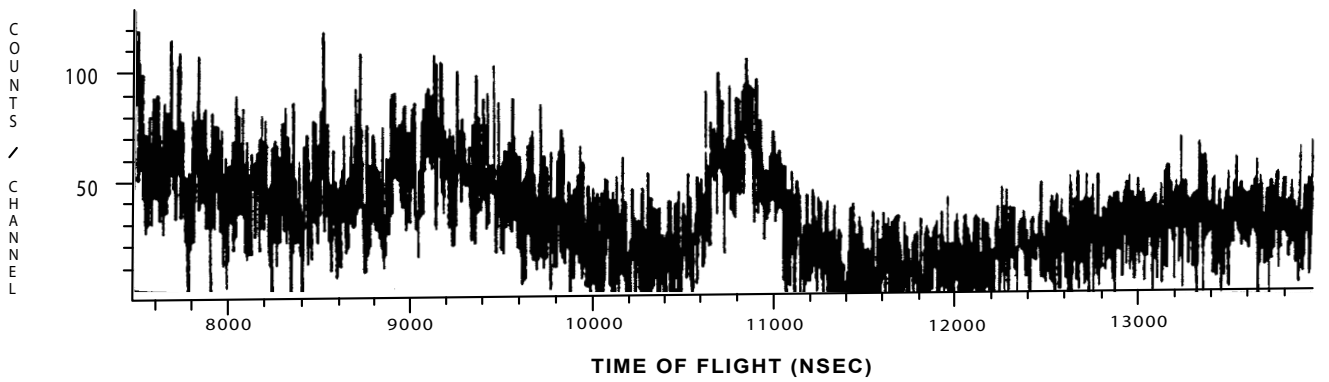
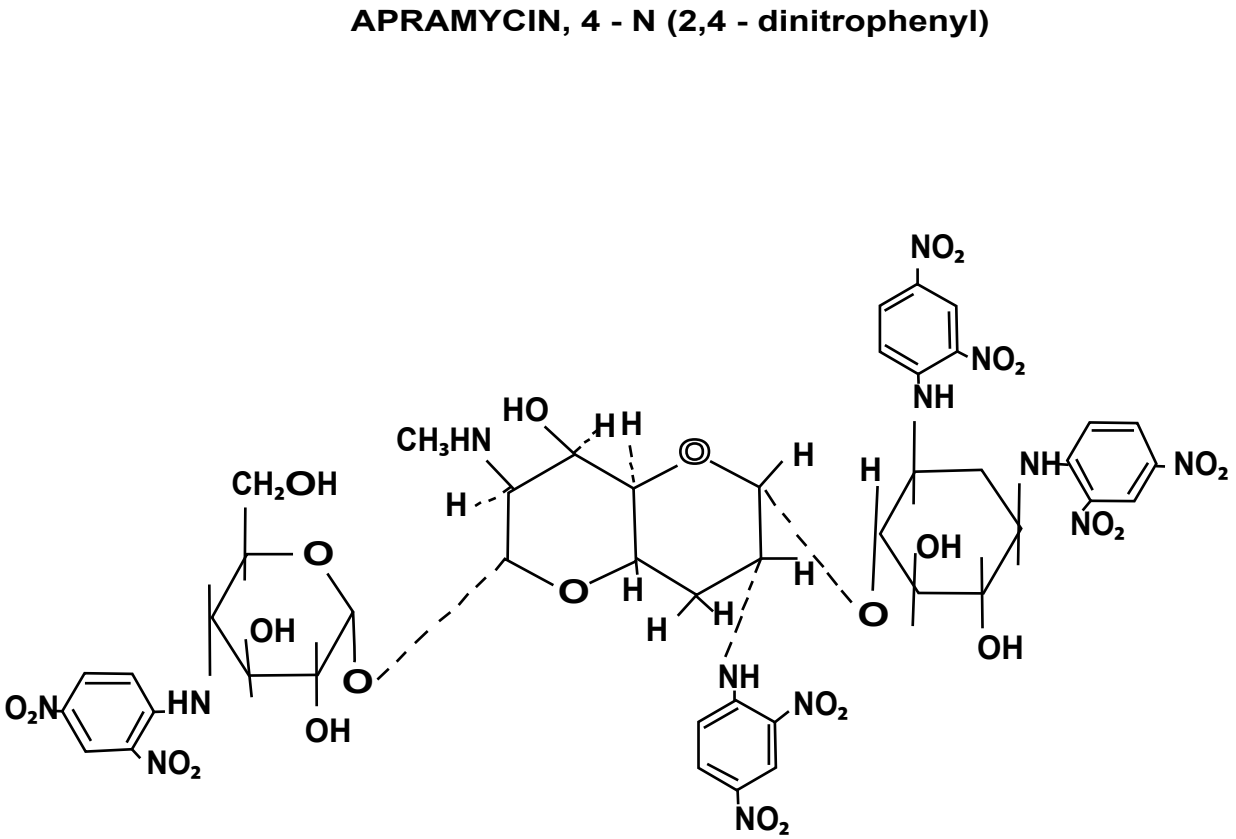
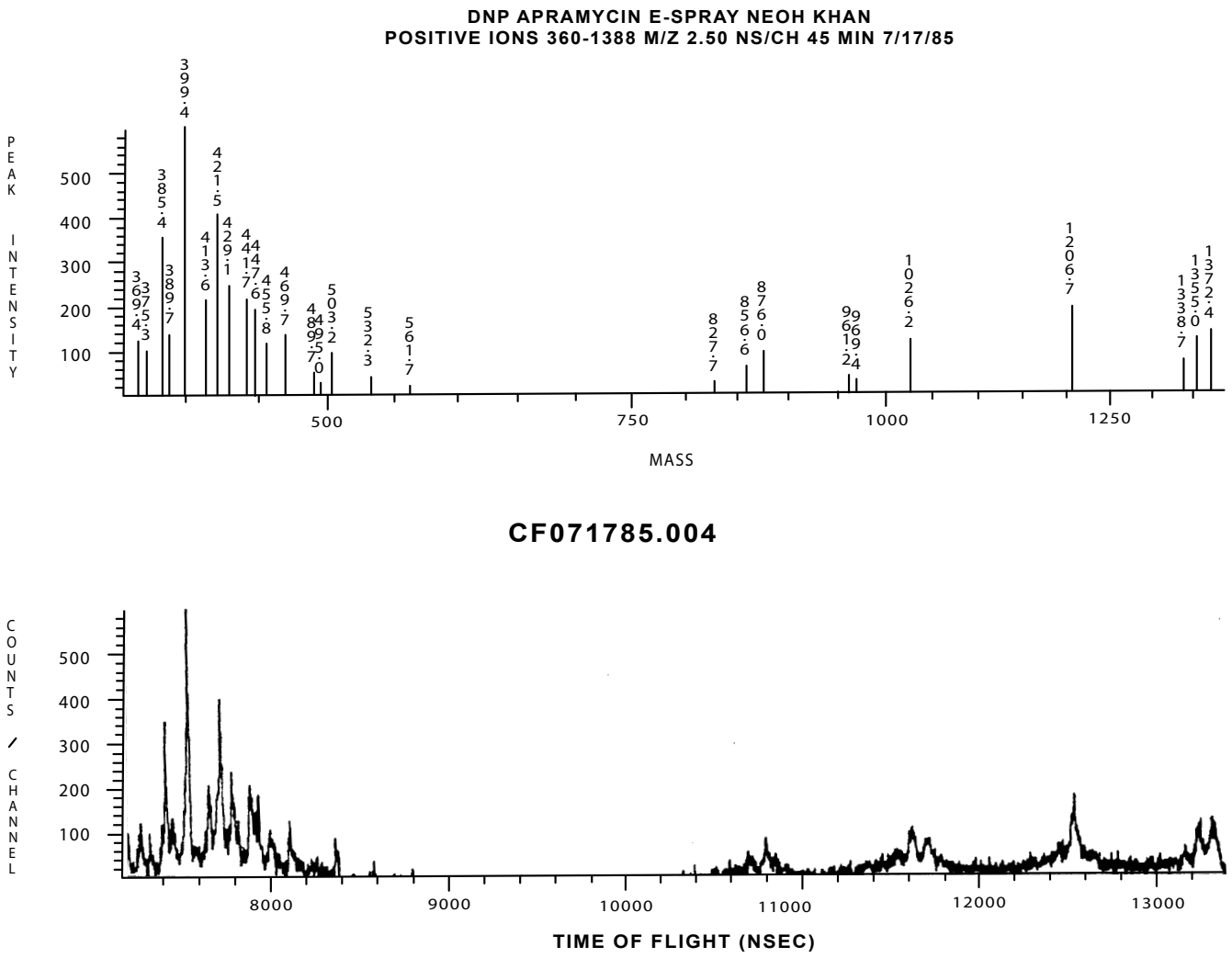


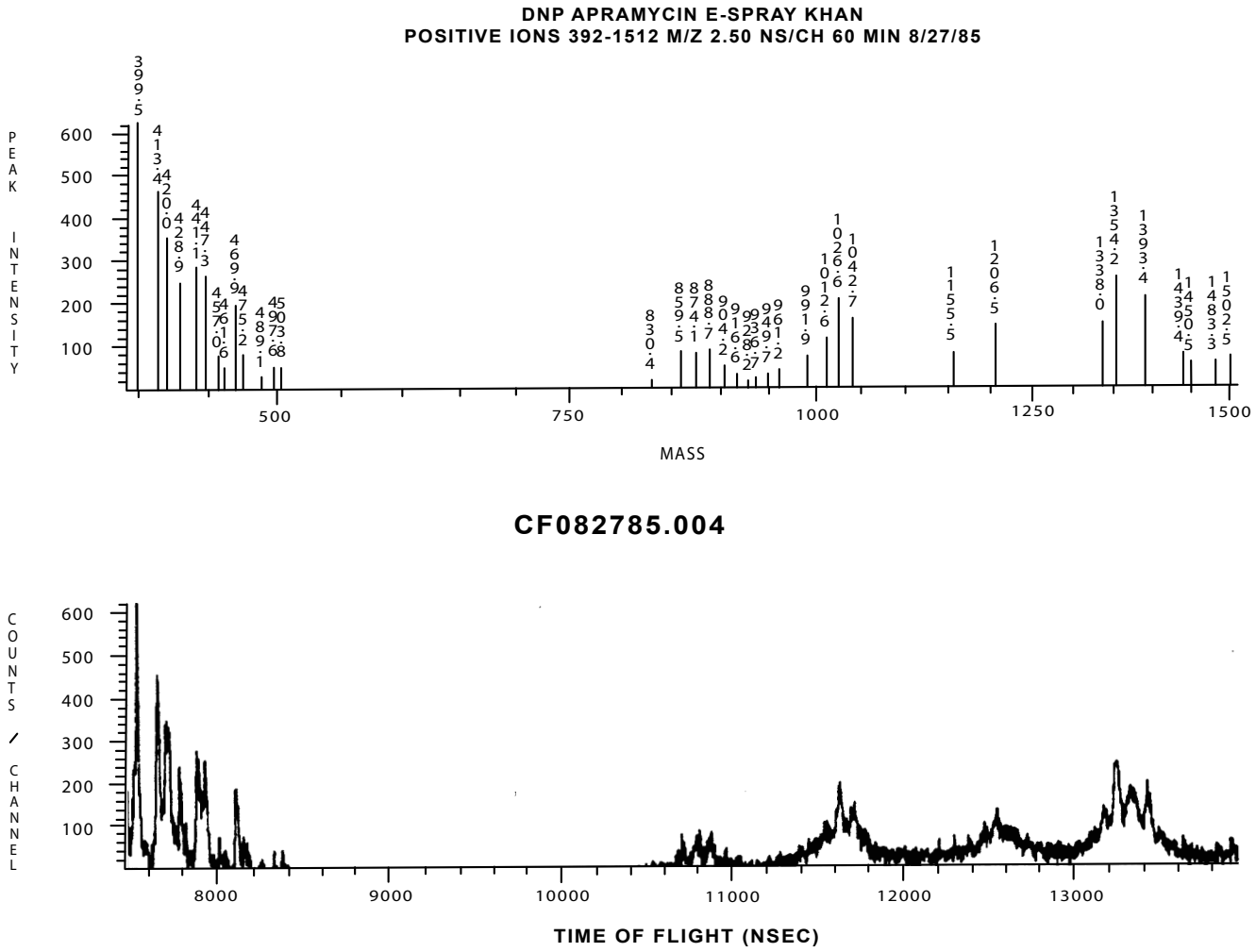
Synthesis of APRAMYCIN, N-tetra(2,4-dinitrophenyl)

Apramycin (0.25 g) was dissolved in an aqueous solution of 0.1 M potassium carbonate (15 ml containing an additional 0.23 g of potassium carbonate). The above solution was heated in an oil bath at 80°C. While stirring mechanically, a solution of 2,4-dinitrofluorobenzene (0.43 g) in methanol (15 ml) was added dropwise over 1/4 hour. After the addition was complete, the reaction mixture was heated for one hour. The product, Apramycin, N-tetra(2,4-dinitrophenyl), which was an orange colored semisolid oil separated from the reaction mixture. The hot solution was decanted to remove the solvent; the orange oily product was washed with cold potassium carbonate solution and then with cold water. The oily product solidified when kept in an ice-water bath for 1/2 hour. The solid was ground to fine powder, collected by filtration, washed with cold water, and dried on a suction pump. The structure of the Apramycin, N-tetra(2,4-dinitrophenyl) was confirmed by The Californium (252 Cf) Fast Atom Bombardment (FAB) mass spectrometry. The 252 Cf mass spectrum showed the following ions: m/z = 1206 (negative ion) and m/z = 133apositive ion).

The following data on the above product were also collected: mp= 78°C; yield= 0.44 g (95%).





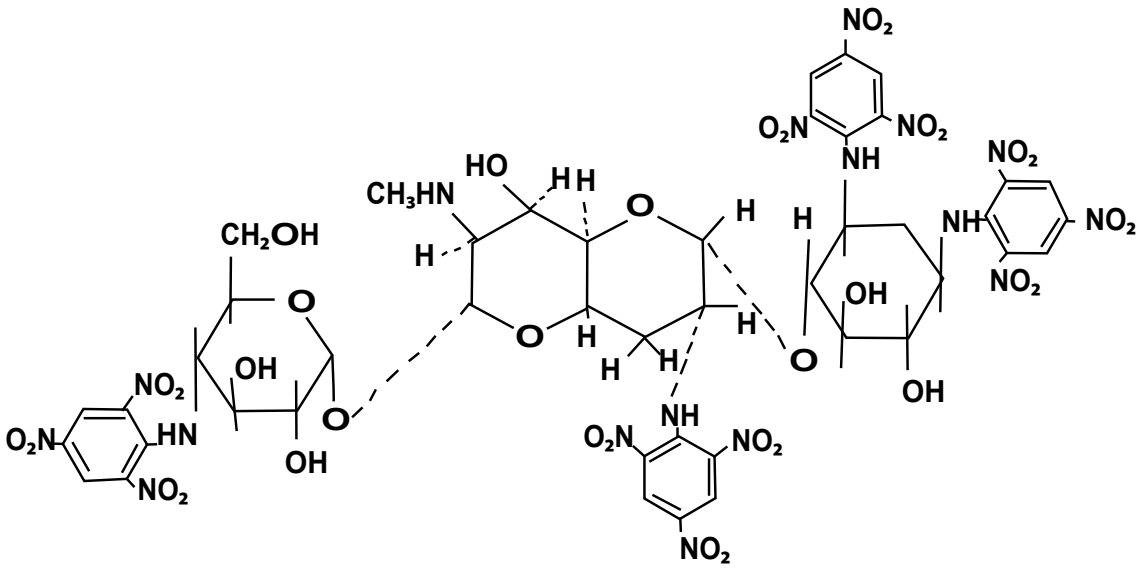


Synthesis of APRAMYCIN, N-tetra(2,4,6-trinitrophenyl)

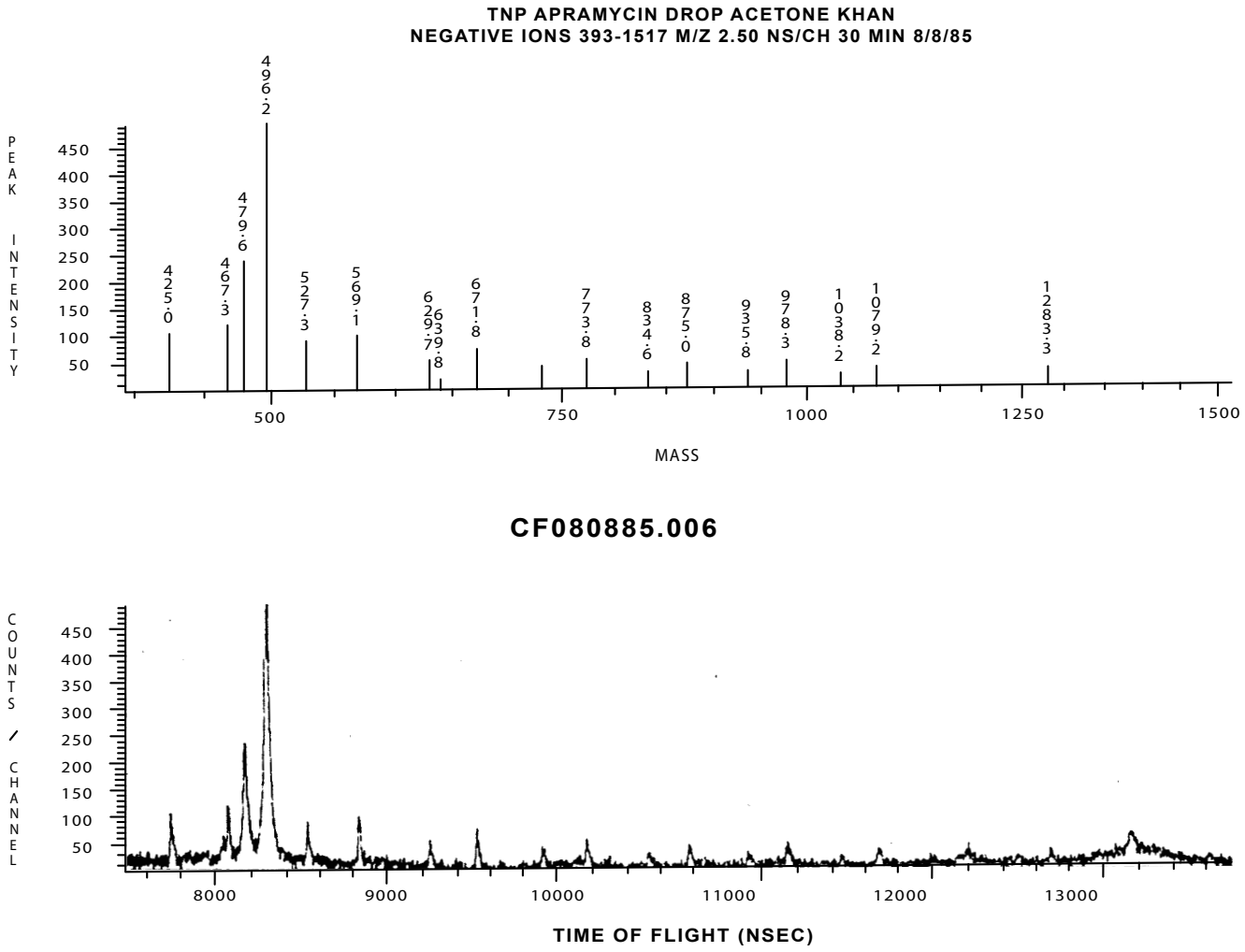
Apramycin (0.25 g) was dissolved in an aqueous solution of 0.1 M potassium carbonate (15 ml containing an additional 0.23 g of potassium carbonate). The above solution was heated in an oil bath at 80°C. While stirring mechanically, a solution of Picryl Chloride (0.57 g) in methanol (15 ml) was added dropwise over 1/4 hour. After the addition was complete, the reaction mixture was heated for one hour. The product, Apramycin, N-tetra (2,4,6-trinitrophenyl) which was an orange colored semisolid oil separated from the reaction mixture. The hot solution was decanted to remove the solvent; the orange oily product was washed with cold potassium carbonate solution and then with cold water. The oily product solidified when kept in an ice-water bath for 1/2 hour. The solid was ground to fine powder, collected by filtration, washed with cold water, and dried on a suction pump. The structure of the Apramycin, N-tetra(2,4,6-trinitrophenyl) was confirmed by The Californium (252 Cf) Fast Atom Bombardment (FAB) mass spectrometry. The 252 Cf mass spectrum showed the following ions: m/z = (negative ion) and m/z =1414(positive ion).

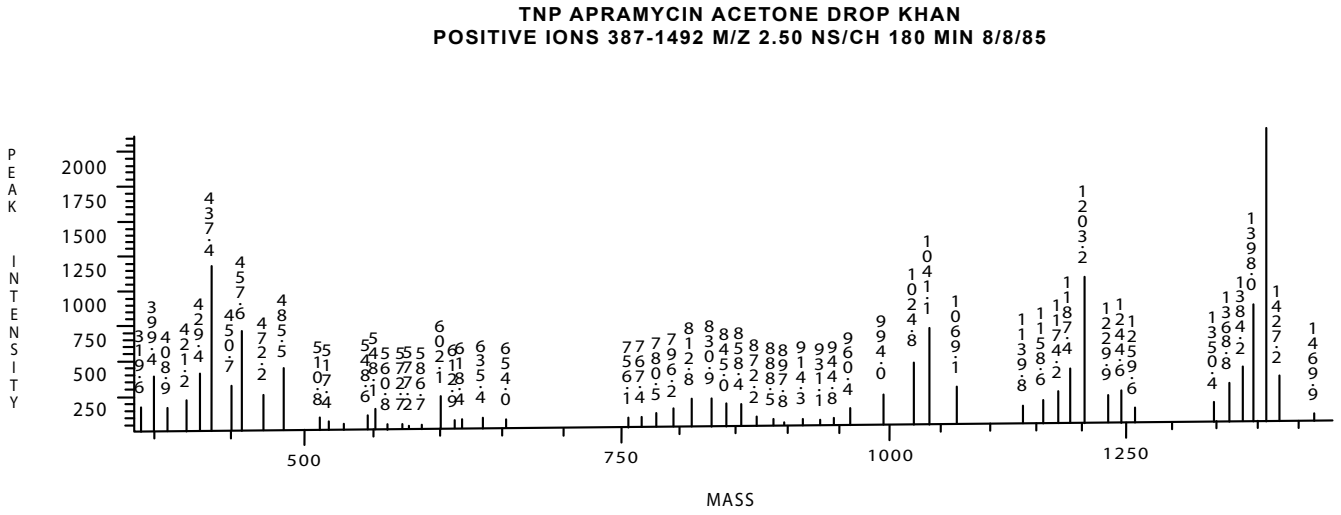
The following data on the above product were also collected: mp= 210°C; yield= 0.5 g (96%).

APRAMYCIN, 4 - N (2,4,6 - trinitrophenyl)



C_{45} H_{45} N_{17} O_{35} = mw = **1383.943**





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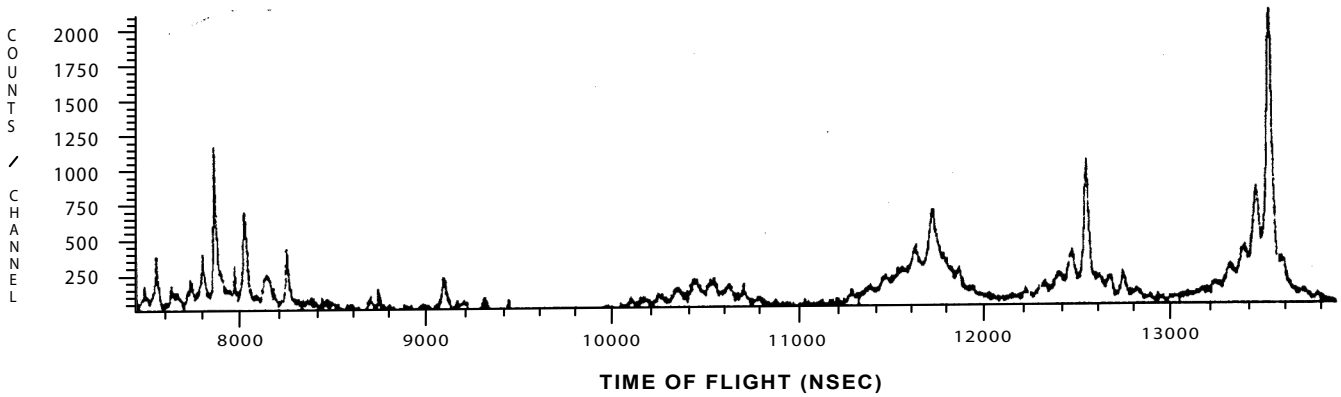
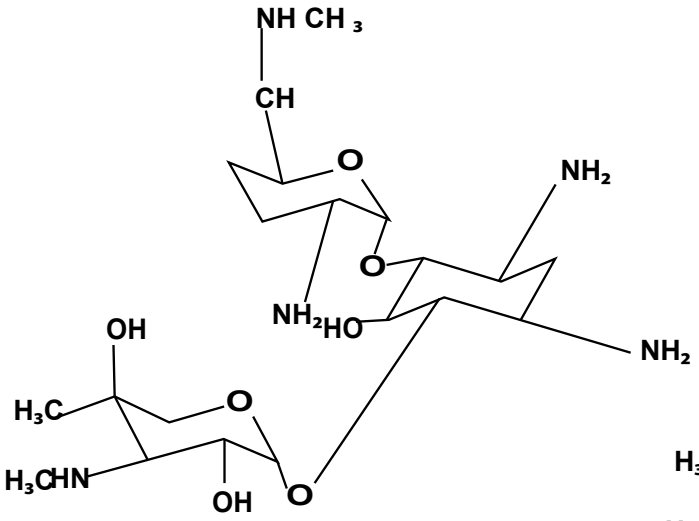
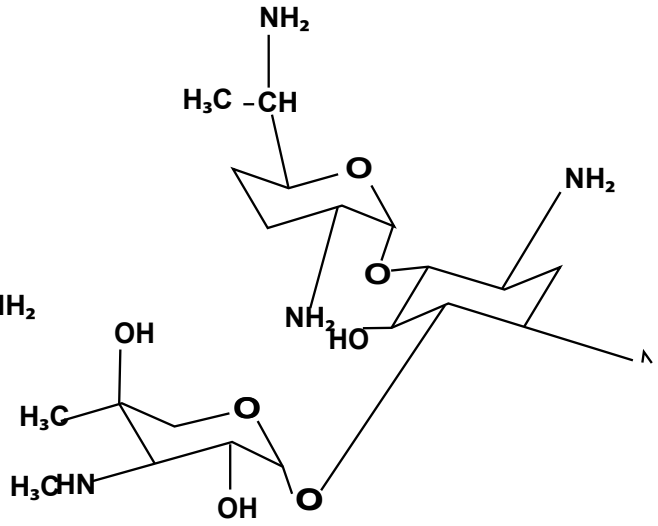
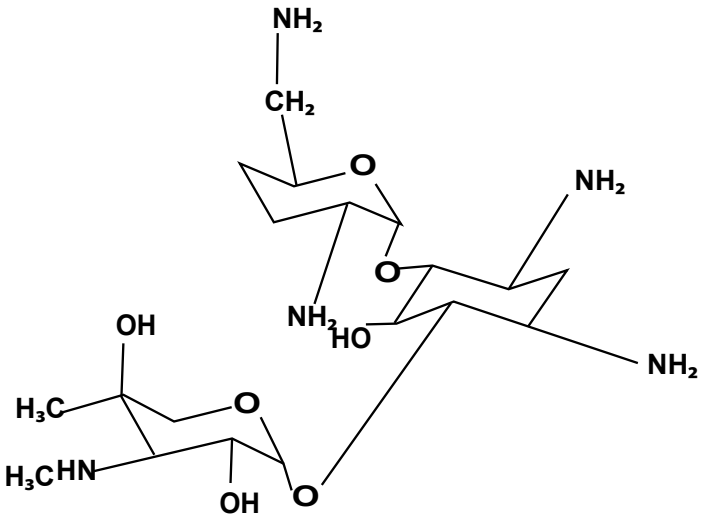




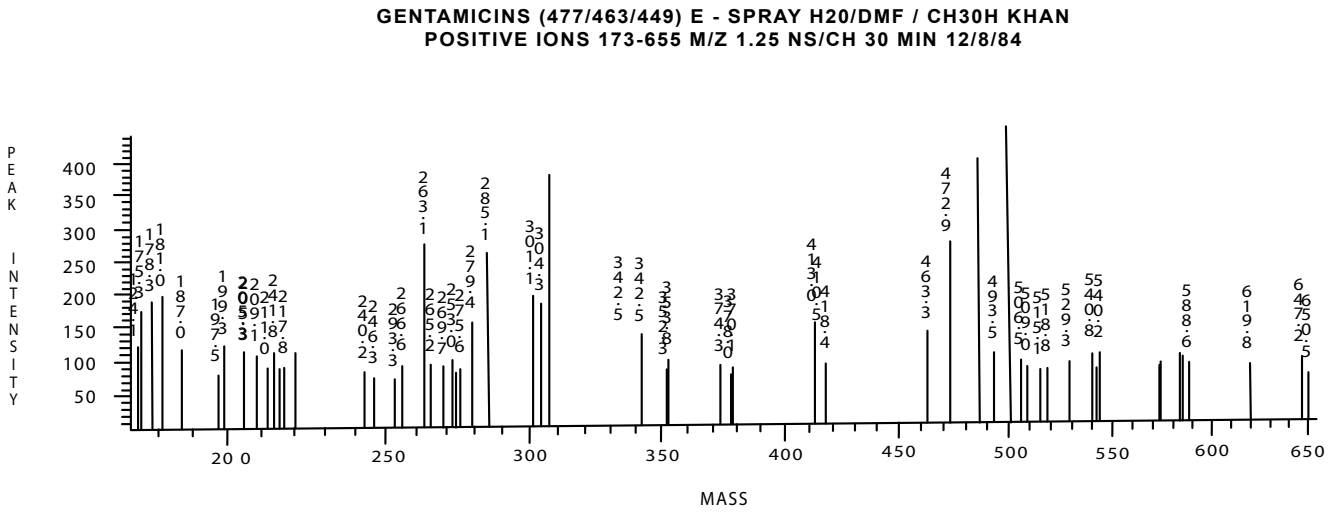
GENTAMICIN C_1 mw = 477.59

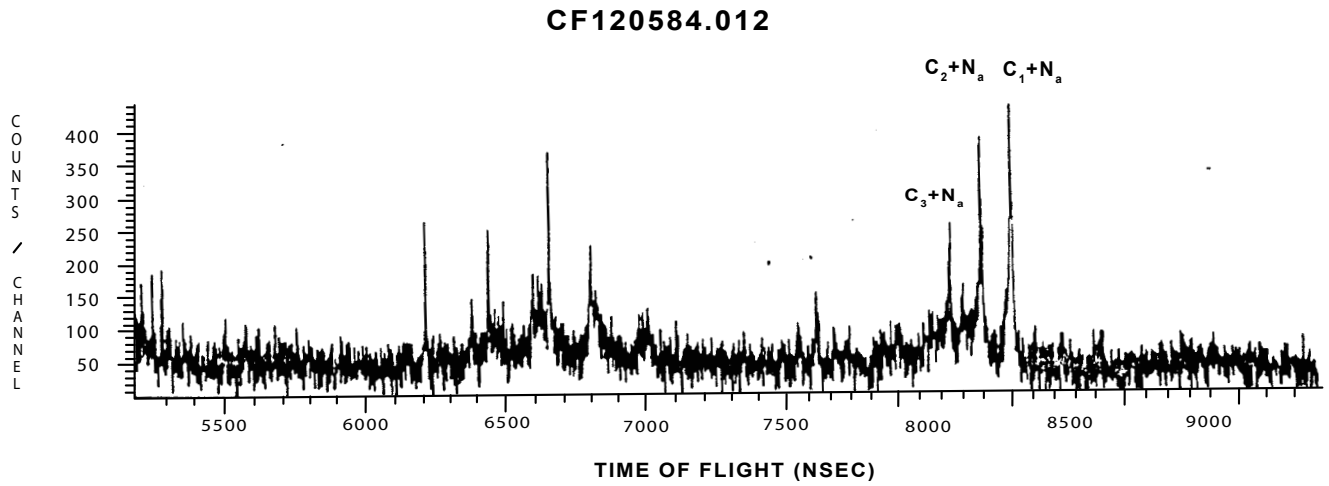


GENTAMICIN C_2 mw = 463.57



GENTAMICIN C_{1a} mw = 449.55

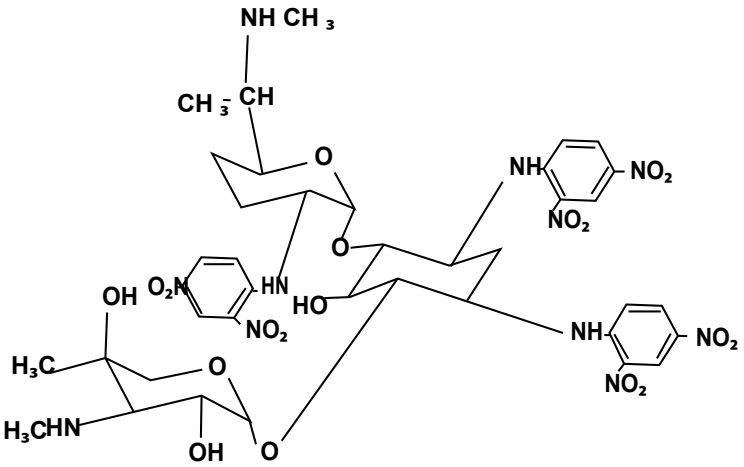




Synthesis of GENTAMICIN C - -dinitrophe GENTAMICIN c2-, -d1n1trop

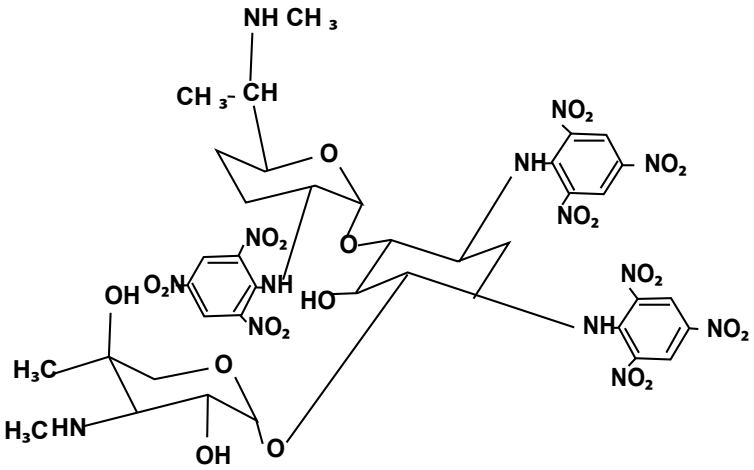
Gentamicin sulfate (1.0 g) was dissolved in an aqueous solution of 0.1M potassium carbonate (25 ml containing an additional 0.23 g of potassium carbonate). The above solution was heated in an oil bath at 80°C. While stirring mechanically, a solution of 2,4-dinitrofluorobenzene (2.0 g) in methanol (25 ml) was added dropwise over 1/4 hour. After the addition was complete, the reaction mixture was heated for one hour. The product, Gentamicin, c1-N-tri(2,4-dinitrophenyl) and c2-N-tetra(2,4-dinitrophenyl) which was an orange colored semisolid oil separated from the reaction mixture. The hot solution was decanted to remove the solvent; the orange oily product was washed with cold potassium carbonate solution and then with cold water. The oily product solidified when kept in an ice-water bath for 1/2 hour. The solid was ground to fine powder, collected by filtration, washed with cold water, and dried on a suction pump. The structure of the Gentamicin, c1-N-tri(2,4-dinitrophenyl) and c2-N-tetra(2,4-dinitrophenyl) mixture was confirmed by the Californium (252 Cf) Plasma desorption mass spectrometry. The 252 Cf mass spectrum showed the following ions: Gentamicin c1 isomer m/z = 977 (negative ion) Gentamicin c2 isomer m/z = 1129.2 (positive ion).

The following data on the above product were also collected: c1 & c2 mp 182°C; yield= 1.83 g (73%)



C_{39} H_{50} N_{11} O_{19}

GENTMICIN C_1 - Tri - N (2,4, - dinitrophenyl) mw = 976.882



C_{39} H_{47} N_{14} O_{25}

GENTMICIN C_1 - Tri - N (2,4,6 - trinitrophenyl) mw = 1111.88

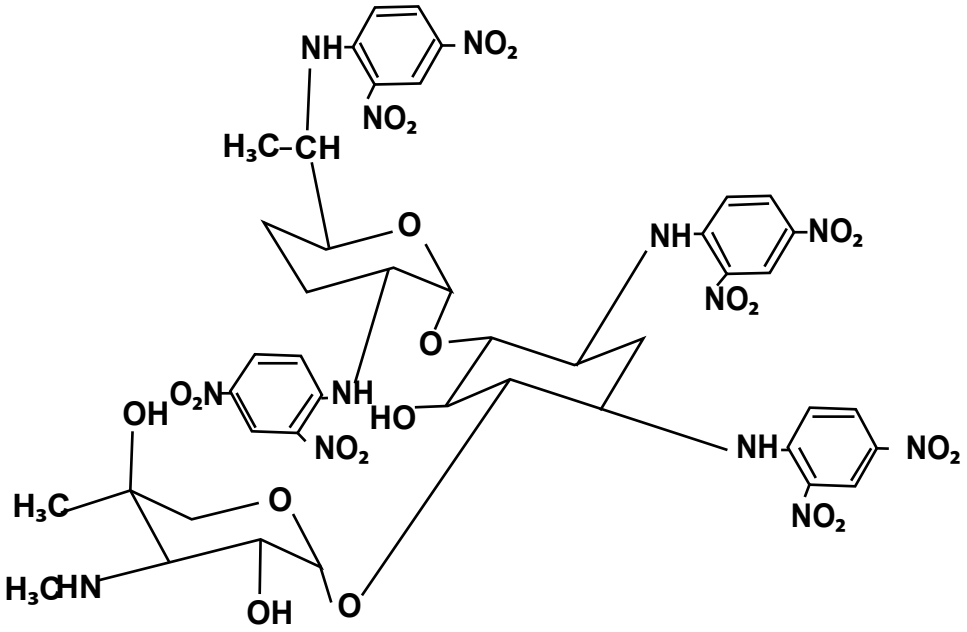
Synthesis of GENTAMICIN c1-N-tri(2,4,6-trinitrophenyl) and GENTAMICIN c -N-tetra(2,4,6-trinitrophenyl) Mixture

Gentamicin sulfate (1.0 g) was dissolved in an aqueous solution of 0.1M potassium carbonate (25 ml containing an additional 0.23 g of potassium carbonate). The above solution was heated in an oil bath at 80°C. While stirring mechanically, a solution of Picryl Chloride (2.0 g) in methanol (50 ml) was added dropwise over 1/4 hour. After the addition was complete, the reaction mixture was heated for one hour. The product Gentamicin, c1-N-tri(2,4,6-trinitrophenyl) and c2-N-tetra(2,4,6-trinitro phenyl) which was an orange colored semisolid oil separated from the reaction mixture. The hot solution was decanted to remove the solvent; the orange oily product was washed with cold potassium carbonate solution and then with cold water. The oily product solidified when kept in an ice-water bath for 1/2 hour. The solid was ground to fine powder, collected by filtration, washed with cold water, and dried on a suction pump. The structure of the Gentamicin, c1-N-tri(2,4,6-trinitrophenyl) and Gentamicin c2-N-tetra(2,4,6-trinitrophenyl) mixture was confirmed by the Californium (252 Cf) Plasma desorption mass spectrometry. The 252 Cf mass spectrum showed the following ions: Gentamicin c1 isomer m/z = 1111.8 (negative ion) Gentamicin c2 isomer m/z = 1307.8 (positive ion).

The following data on the above product were also collected: c1 & c2 mp= 100°c; yield= 3.0 g (79%)

Commercially available Gentamicins consist of three isomers, namely: Gentamicin c1, Gentamicin c2 and Gentamicin c1a. The presence of any of these isomers could not be detected using the Electron Impact (I.C.) mass spectrometer which caused the total destruction of the molecule. But, by using the 252cf Plasma desorption instrument, all three isomers were identified as their sodium positive ions in the same spectrum. Thus, the underivatized Gentamicins mixure showed the following three peaks as their sodium salt: 1) Gentamicin c1 m/z = 500.8 Gentamicin c2 m/z = 486.7, and 3) Gentamicin c1a m/z = 472.9.

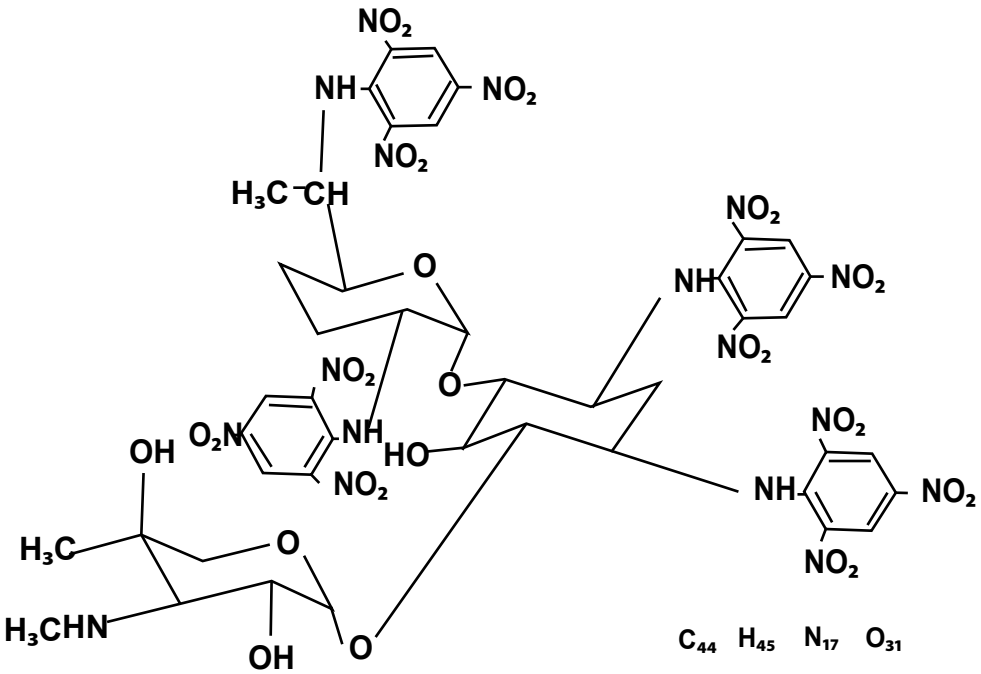
The isomer c1a appears to be the least in the Gentamicin mixture and is lost during derivatization and purification. The remaining two major isomers of Gentamicins, isomers c1 and c2, run very closely on a silica gel plate (TLC) either in THF:HEXANE (4:1) or in THF:DMF (4:1) and could not be separated by chromatography. Table II gives the melting points and yields of the c1 and c2 mixture of both the dinitrophenyl and trinitrophenyl derivatives. The mass spectrum of dinitrophenyl derivatives showed peaks corresponding to both the c1 and c2 isomers and similarly the mass spectrum of the trinitrophenyl derivatives also showed peaks corresponding to c1 and c2 isomers. Purification of either of the derivatives on a silica gel column caused the decomposition of the molecule. Therefore, the crude yield of the mixture is reported.



C_{44} H_{49} N_{13} O_{23}

mw = 1127.944

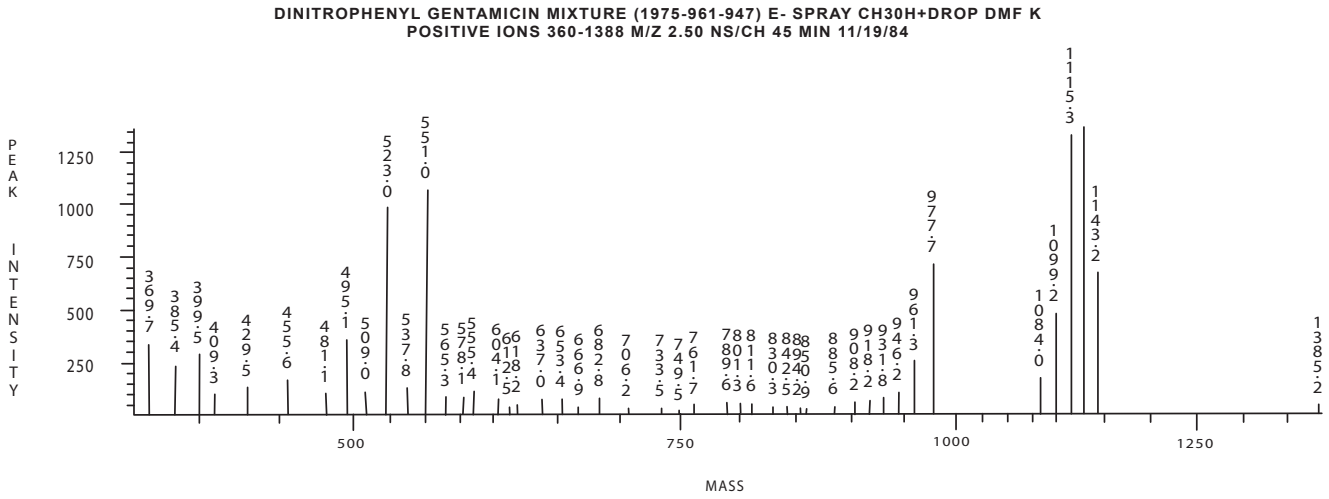
GENTMICIN C_2 - Tetra - N (2,4, - dinitrophenyl)



C_{44} H_{45} N_{17} O_{31}

mw = 1307.93

GENTMICIN C_2 - Tetra - N (2,4,6 - trinitrophenyl)



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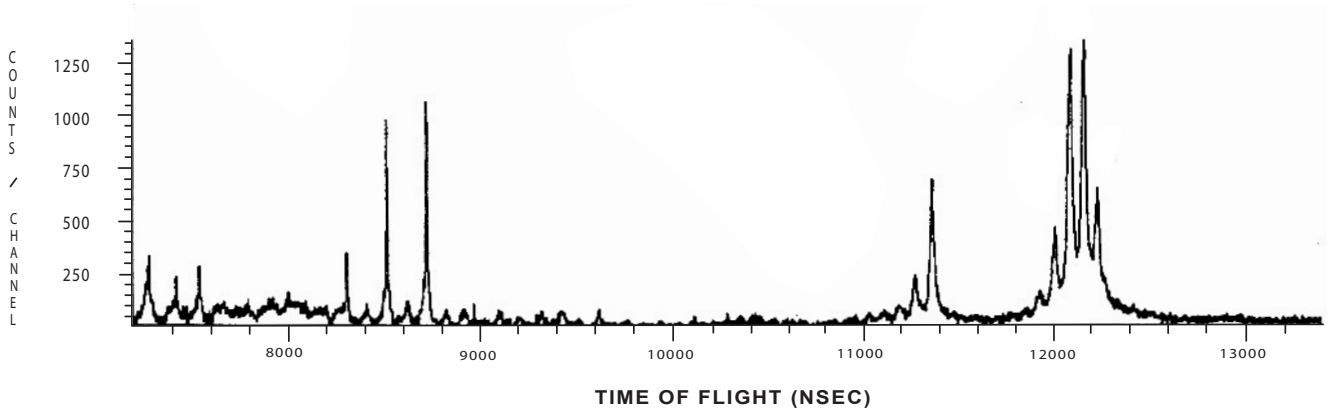

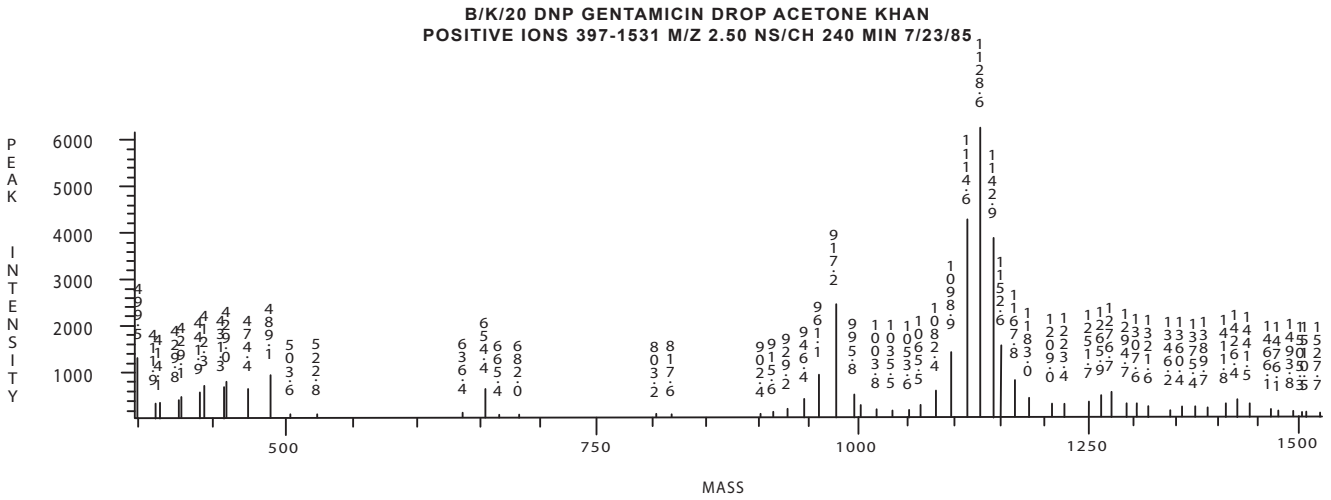

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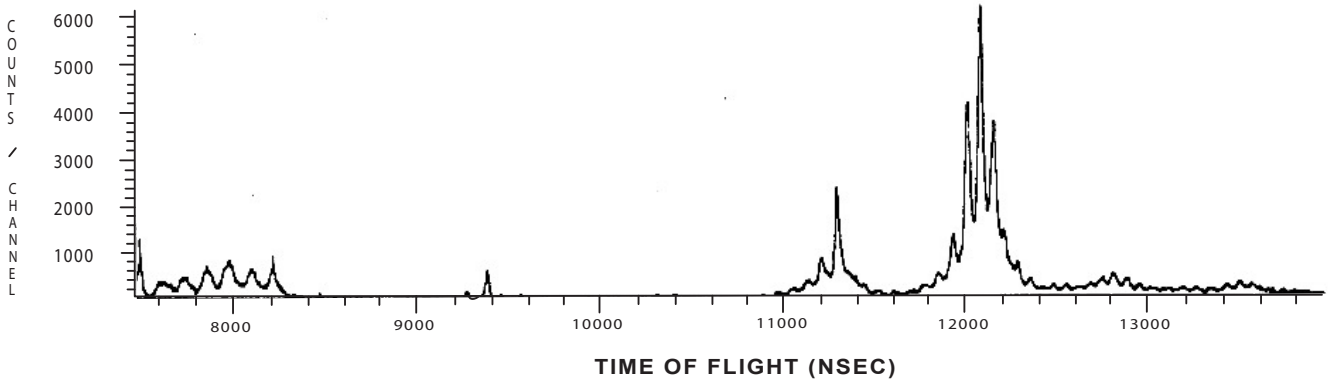
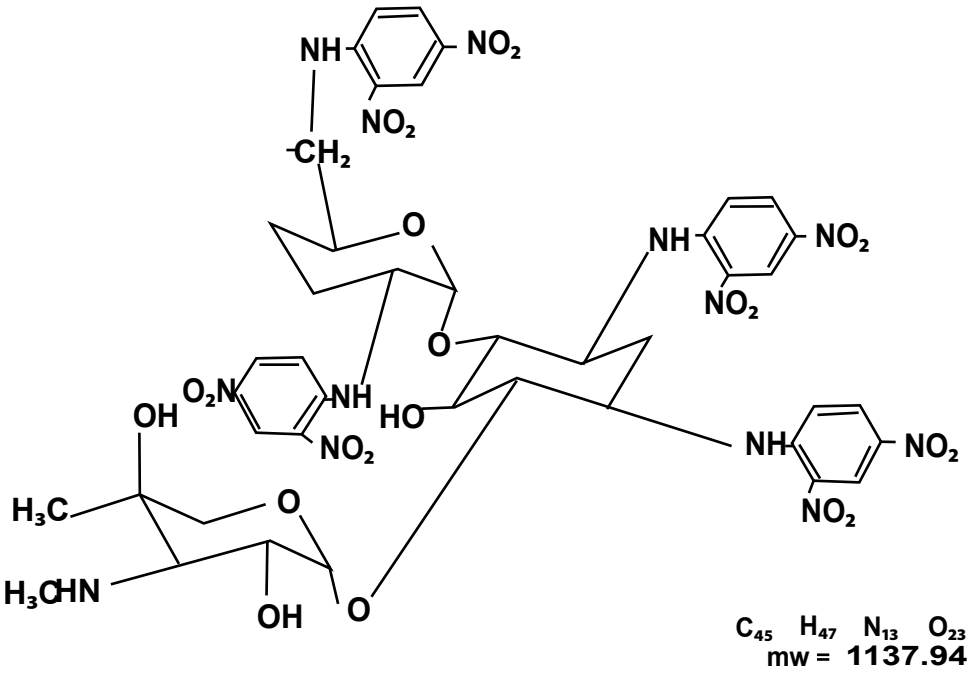
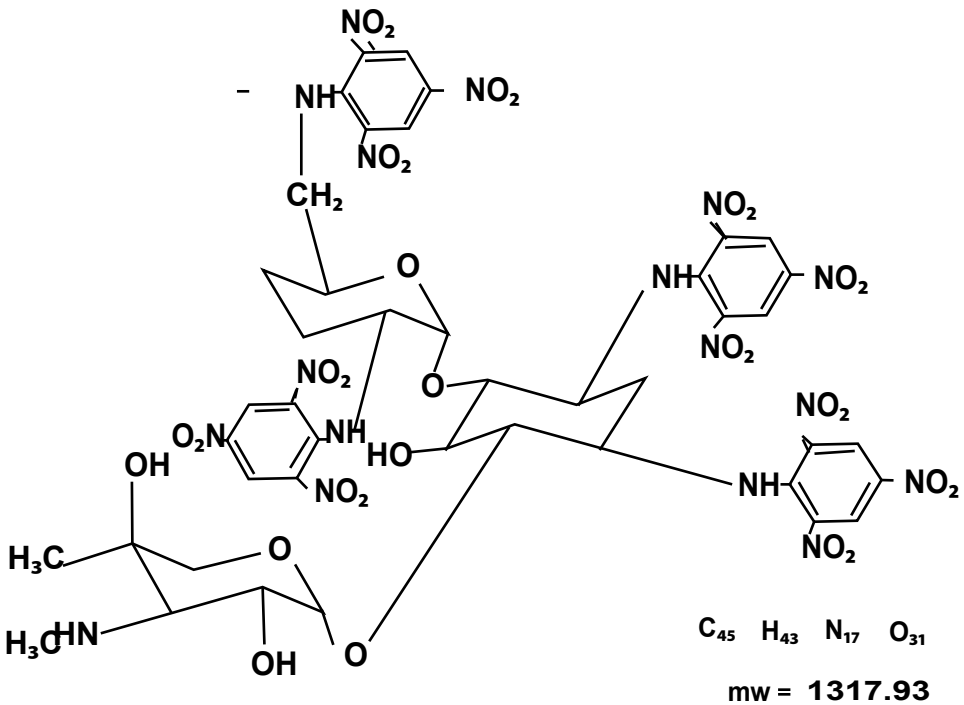
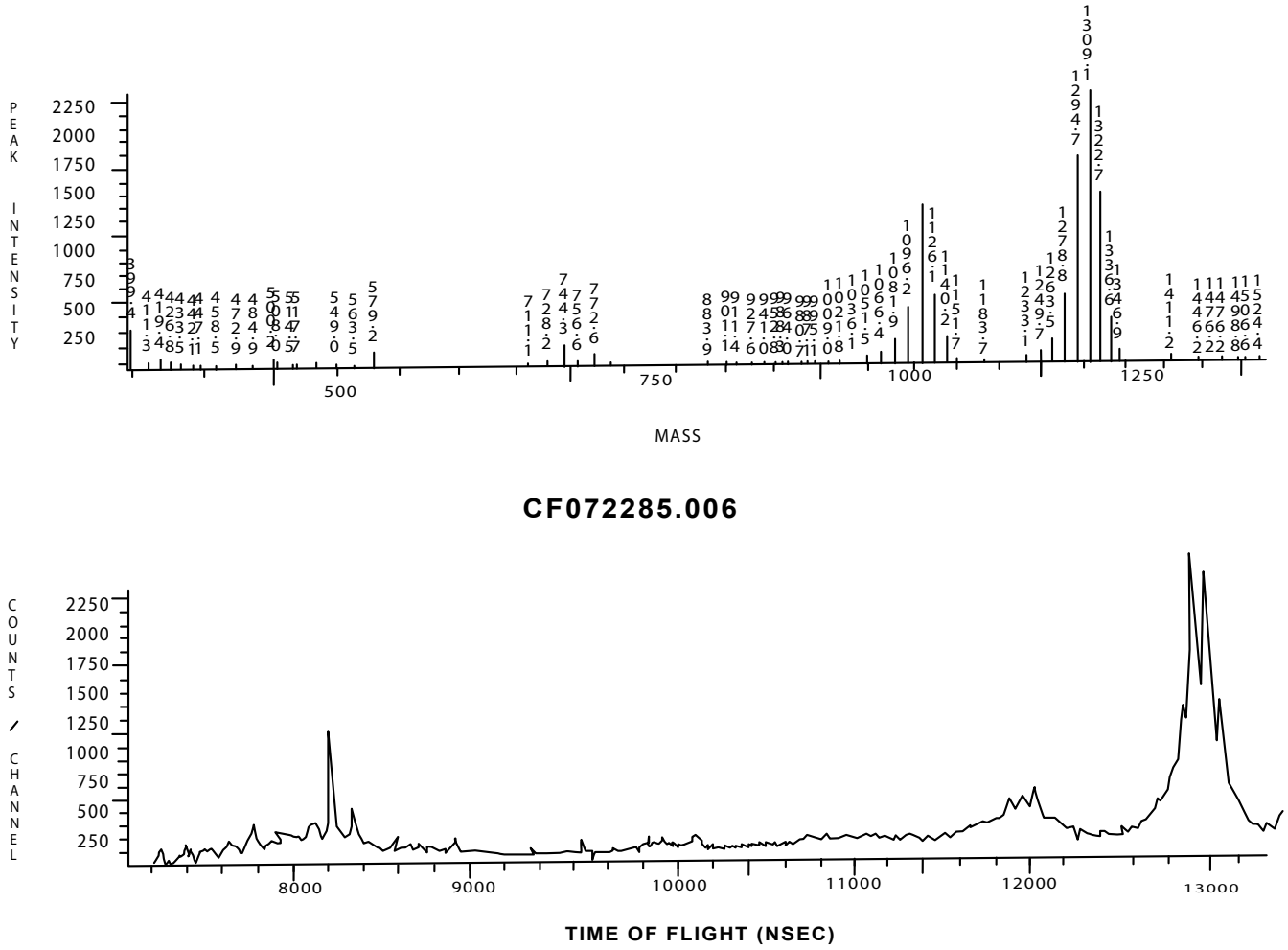


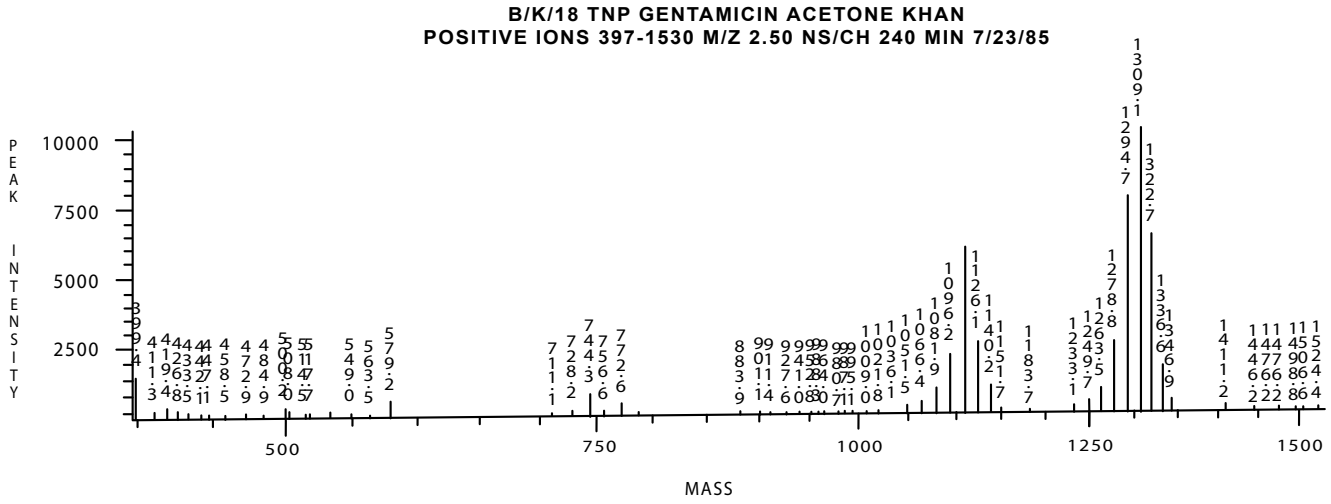


GENTMICIN C_2a - Tetra - N (2,4, - dinitrophenyl)



GENTMICIN C_2a - Tetra - N (2,4,6 - trinitrophenyl)





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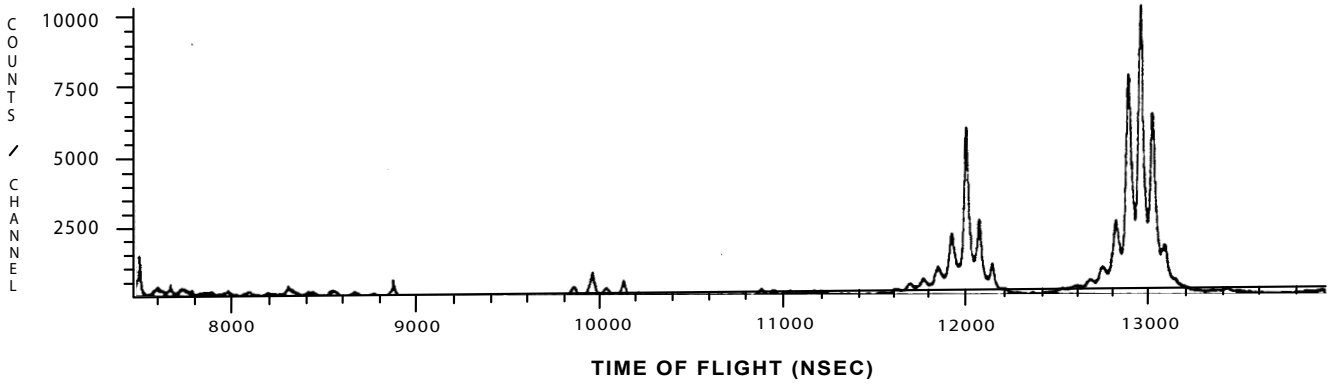

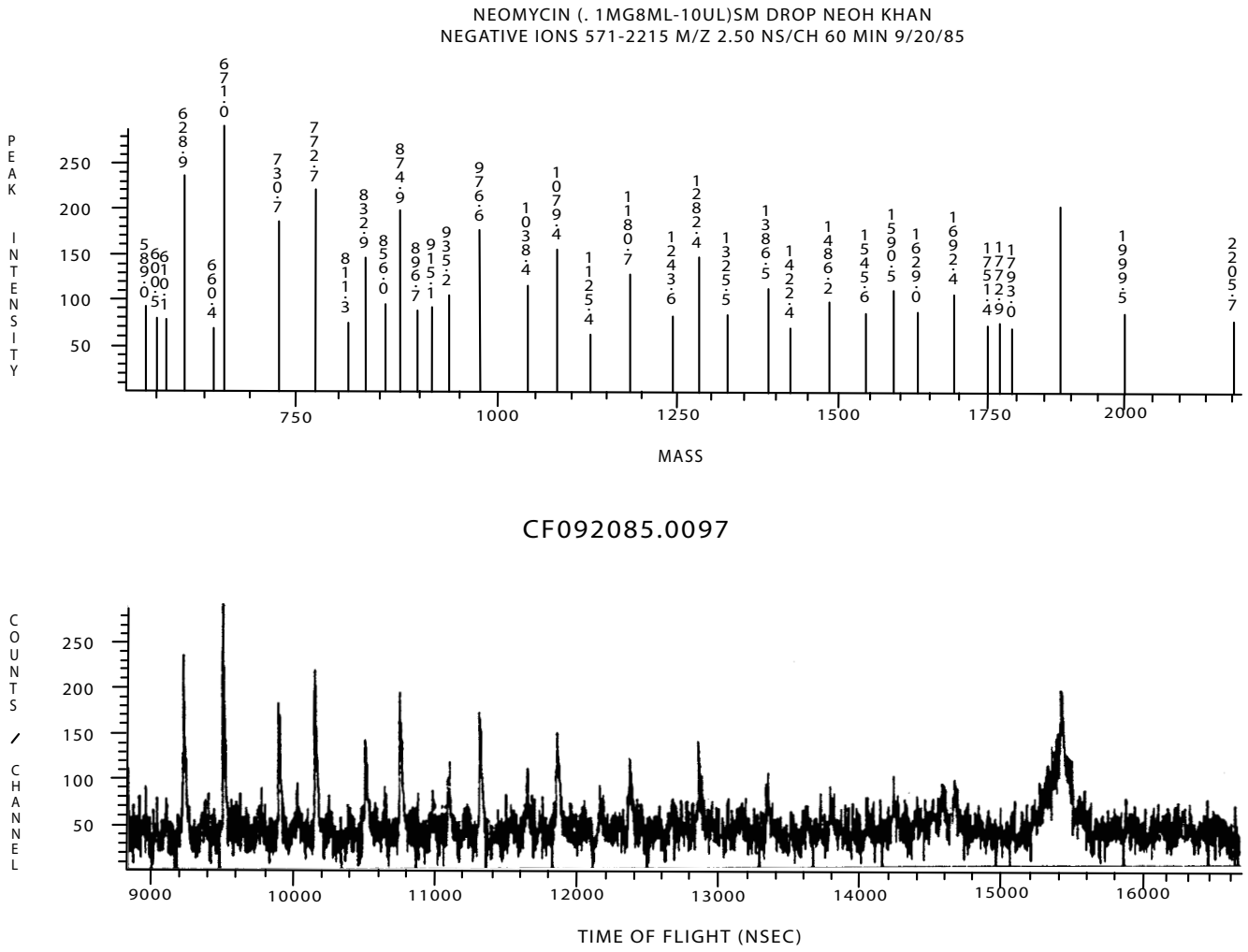

TABLE I

^{252}CALIFORNIUM MASS SPECTRA OF THE DERIVATIZED AMINOGLYCOSIDE ANTIBOTICS

ANTIBIOTICS & DERIVATIVES	MOLECULAR WEIGHTS	MELTING POINTS	PERCENTAGE YIELDS	^{252}Cf MS OF NEGATIVE-IONS	^{252}Cf MS OF POSITIVE-IONS
NEOMYCIN	556.43	-	-	-	m/z = 638
a) 6N-Dinitrophenyl	1610.19	225° C	70	m/z = 1612	m/z = 1634
b) 6N-Trinitrophenyl	1863.176	130° C	71	m/z = 1882	m/z = 1905
KANAMYCIN	484	-	-	-	m/z = 507.7
a) 4N-Dinitrophenyl	1147.865	210° C	72	m/z = 1148.5	m/z = 1171.5
b) 4N-Trinitrophenyl	1328	218° C	72	m/z = 1329.4	m/z = 1367.6(K)
PAROMOMYCIN		-	-	-	m/z = 638.9
a) 5N-Dinitrophenyl		237° C	72	m/z = 1447.2	m/z = 1470.5
b) 5N-Trinitrophenyl		210° C	66	m/z = 1672	m/z = 1995
TOBRAMYCIN	476	-	-	-	m/z = 490.8
a) 5N-Dinitrophenyl	1297.982	94° C	82	m/z = 1298.1	m/z = 1338.2(K)
b) 5N-Trinitrophenyl	1523.979	225° C	64	m/z = 1523	m/z = 1562(K)
AMIKACIN	585.6	-	-	-	m/z = 608.9
a) 4N-Dinitrophenyl	1233.9	102° C	94	m/z = 1250.5	m/z = 1273.2(K)
b) 4N-Trinitrophenyl	1433.6	192° C	90	m/z = 1429	m/z = 1452.9(K)

TABLE II

^{252}CALIFORNIUM MASS SPECTRA OF THE DERIVATIZED AMINOGLYCOSIDE ANTIBOTICS

ANTIBIOTICS & DERIVATIVES	MOLECULAR WEIGHTS	MELTING POINTS	PERCENTAGE YIELDS	^{252}Cf MS OF NEGATIVE-IONS	^{252}Cf MS OF POSITIVE-IONS
NETILMYCIN	475	-	-	-	m/z = 498
a) 3N-Dinitropheny ll	976	234° C	87	m/z = 975.4	m/z = 1111.4(K)
b) 3N-Trinitropheny ll	1110	194° C	96	m/z = 1109.4	m/z = 1149.8(K)
SISOMICIN	447	-	-	-	
a) 4N-Dinitropheny ll	1111	218° C	80	m/z = 1111.9	
b) 4N-Trinitropheny ll	1291	184° C	81	m/z = 1292	
GENTAMICIN (isomers)		-	-	-	
i) Gentamicin C_1	447.59	–	–	–	m/z = 500.8
ii) Gentamicin C_2	463.57	–	–	–	m/z = 486.7
iii) Gentamicin C_1a	449.55	–	–	–	m/z = 472.9
a) 3N-Dinitropheny l C_1	976.88	182° C	73	m/z = 977	m/z = 1119(K)
4N-Trinitropheny l C_2	1127.9	-	-	m/z = +129.2	m/z = 1167.8(K)
a) 3N-Trinitrophenyll C_1	1111.88	100° C	79	m/z = 1111.8	m/z = 1151.7(K)
b) 4N-Trinitropheny ll C_2	1307.93			m/z = 1307.4	m/z = 1346.9(K)
APRAMYCIN	539.8	-	-	-	m/z = 561.7
a) 4N-Dinitropheny ll	1207.58	78° C	95	m/z = 1206.1	m/z = 1338(Cs)
b) 4N-Trinitropheny l	1387.58	210° C	96	m/z =	m/z = 1414(K)

m/z = mass over charqe ratio.

Conclusion:

Sir Winston Churchill, the Prime minister of England, the hero of the WWII, once said, Success comes to those who dare and act and it seldom goes to coward who are ever afraid of consequences. I was lucky; I was saved by NIH and got away. I succeeded in publishing the paper without the Big Boss name and paid the price. I have no regret. After long years of services in FDA, at least I have one publication. I am very grateful to FDA for preparing me to publish numerous papers and earn numerous awards from NIH for my years of services.

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