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UV Irradiation of Thymine Molecules and Gas Chromatography – Mass Spectrometry

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UV irradiation of thymine molecules and gas chromatography – mass spectrometry

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Abstract

Cyclobutane pyrimidine dimers (CPDs) are products of a photochemical reaction caused by ultraviolet (UV) irradiation of adjacent pyrimidine molecules. Thymine dimers, the most common form of CPDs in the body, are of particular interest because they are the cause of the majority of UV-induced mutations in mammals, often resulting in the development of skin cancers. Because of the photochemical properties of the reactions of thymine dimers, and the relation of these dimers to cancer pathogenesis, the formation and analysis of thymine dimers in a laboratory setting could be a useful educational tool for pre-medicine students learning about organic and photochemistry. Previous studies have shown that thymine monomers, which are normally not volatile enough for gas chromatography, are able to undergo gas chromatography after being derivatized with isobutyl chloroformate (IBCF). In this study, using IBCF as a derivatizing agent for the analysis of UV-irradiated thymine dimers, the use of gas chromatography – mass spectrometry for analysis is attempted.

Introduction

Skin Cancer

Cancer is a term which covers a variety of medical conditions involving rapid, unneeded, and uncontrolled cell division. Cancerous cell division can occur in almost any tissue or organ in mammals, and may result from a variety of different causes, including (but not limited to), "genetic predisposition, environmental influences, infectious agents, and ageing" (Pulverer, Anson, Surridge, & Allen, 2001). Whatever the cause, cancer typically involves malfunctions within the systems of cell signaling, and a failure of the organism to successfully regulate the cell cycle and cell division. Since the tumors – the groups of rapidly dividing cells which accumulate into problematic masses of tissue – are made of the body's own cells, it is difficult not only for the immune system to differentiate between normal and cancerous tissue, but it is difficult for oncologists to find targets which are specific enough to target and eradicate cancerous cells without damaging the normally-functioning cells of the body in the process. Because of this, cancer remains a serious and often fatal health problem, and research regarding the prevention of cancer attracts the attention of scientists in many different areas of the health sciences.

The most common types of cancer in the United States are the skin cancers, which come in a variety of forms. Although it is not the most common, the deadliest form of skin cancer is melanoma. Melanoma skin cancer originates from skin cells called melanocytes, which are, "responsible for giving skin its color and that protects the body from damage caused by the sun's ultraviolet rays" (Pluta, Burke, & Golub, 2011). These melanocytes protect the individual from UV irradiation by producing melanin, which

absorbs UV light. Melanoma skin cancer occurs when melanocytes are altered through genetic mutation and are transformed into cancerous cells. Multiple factors can contribute to the risk of developing melanoma skin cancer, however the factor which results in the highest risk is exposure to UV light (Environmental Protection Agency and Centers for Disease Control and Prevention, 2010). Although any individual can develop melanoma, fairer-skinned individuals are more likely to develop the condition, since their melanocytes produce less melanin to protect the skin from damage by UV exposure.

Skin cancers – both melanoma and non-melanoma – are extremely prevalent health problems in the United States, with non-melanoma skin cancer alone being, "more common than all other forms of skin cancer combined" (Perera & Sinclair, 2013). This high occurrence of skin cancer is especially noticeable in Oregon, which reports 28.4 cases of melanoma skin cancer per 100,000 residents each year; the fourth highest rate of melanoma skin cancer in the United States (Environmental Protection Agency and Centers for Disease Control and Prevention, 2010). This is most likely due to the large white population in Oregon. Since the lack of protective melanin in fair-skinned individuals puts them at a higher risk for skin cancer, a higher rate of skin cancer is seen in these populations, as "More than 9 out of 10 cases of melanoma are diagnosed in non-Hispanic whites" (Centers for Disease Control and Prevention, 2016). Although we have acquired a better understanding of cancer over the last few decades, cancer still remains a very serious public health issue. Because of this, the understanding of the pathogenesis and treatment of cancers are necessary for the improvement of the health of individuals in the United States and all over the world, and require the attention of researchers of many different fields to fully understand these conditions.

Cyclobutane Thymine Dimers

Thymine is a type of pyrimidine molecule, one of the two classes of nucleobases which make up the structure of DNA. Studies have shown that UV exposure of DNA strands can cause adjacent pyrimidine nucleobases to react with each other and dimerize, forming cyclobutane pyrimidine dimers (CPDs) within the strands of DNA (Durbeej & Eriksson, 2002). Although these dimers can form between other pyrimidines as well, cyclobutane thymine dimers (CTDs) are cited as the most common occurrence of CPDs in the body.

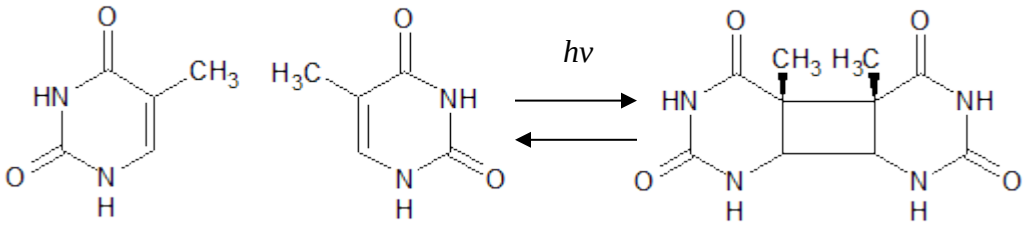


Figure 1. A schematic overview of the reversible photochemical reaction between two adjacent thymine molecules to form a CTD, show in the *cis-syn* conformation. The symbol *hv* is used to denote the presence of light as a photochemical catalyst for the reaction.

The reaction which causes the formation of CPDs – shown schematically in Figure 1 – is a photoreaction, meaning that it can only take place when the molecules in question are exposed to energy in the form of light. This is different from a thermal process, since thermally-catalyzed reactions are caused by energy derived from a heat source. In the case of CPDs, the reaction is catalyzed by UV exposure in the range of

200-320 nm, also called “far UV light” (Podmore, Cooke, Herbert, & Lunec, 1996). This specific photoreaction can be best described as a [2 + 2] cycloaddition, also known as a pyrimidine radical mediated process (Durbeej & Eriksson, 2002). This reaction involves breaking two π bonds – one from the internal alkene group of each thymine molecule – and the formation of two σ bonds between the molecules, resulting in the formation of a cyclobutane ring. This process can take place either as a concerted pericyclic reaction – meaning that the formations of both bonds in the cyclobutane ring take place at the same time, and the reaction does not involve any intermediates – or via a radical intermediate, usually taking the form of an enone (an alkene conjugated to a ketone functional group) or enamine radical (Junkers, 2005). Often, both reaction mechanisms take place simultaneously within a solution.

2+2 Cycloaddition and HOMO-LUMO Interactions

In each alkene group within the ring of the thymine molecules, there are two π orbitals; a bonding orbital (consisting of a lower energy state) and an anti-bonding orbital (in a much higher energy state). In the non-excited state, both of the π electrons in each alkene group are present in the bonding orbital, creating the alkene double bond between the cyclic carbons atoms, and causing the carbon atoms to be sp^2 hybridized. This makes the bonding orbital containing these electrons the highest occupied molecular orbital (HOMO) as well, while the anti-bonding orbital – which holds no electrons – is the

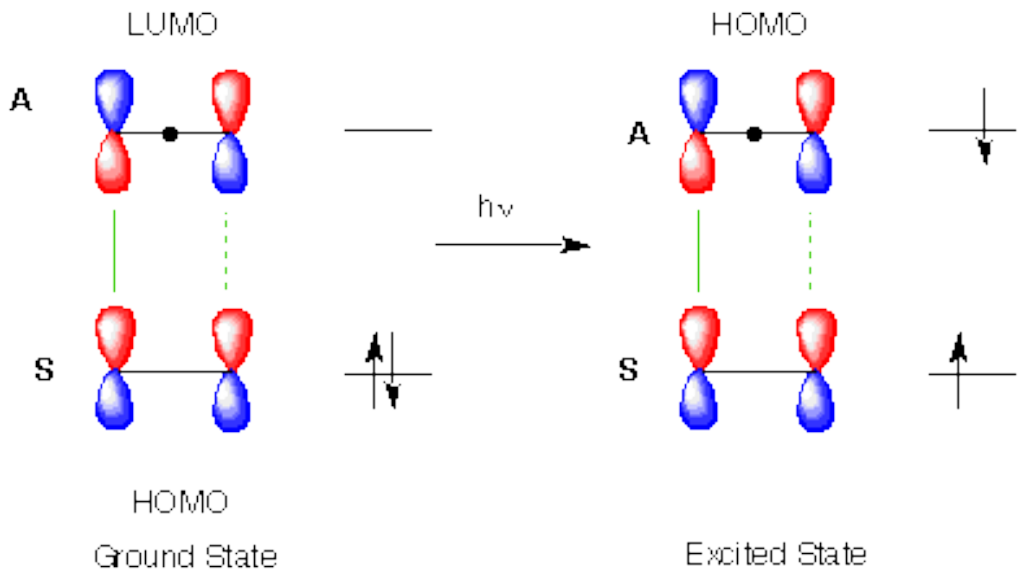


Figure 2: A representation of the excitement of electrons from the ground state to the excited state. In this figure, the symbol *hv* is used to denote the presence of UV light as a catalyst for the HOMO/LUMO interactions. This displays how the HOMO is changed from the bonding orbital to the anti-bonding orbital once the electrons have been irradiated with UV light (Figure citations: Ziegler, 2002).

lowest unoccupied molecular orbital (LUMO). Since the formation of the sigma bonds requires the HOMO of one molecule to overlap with the LUMO of the other molecule, these molecules are not able to form σ bonds under normal conditions, and remain as monomers. However, when the molecules are irradiated with UV light, one of the electrons becomes excited, and travels to a higher energy state, breaking the π bond which formed the internal alkene. In the radical-mediated pathway, an enone radical is formed during this part of the reaction (Junkers, 2005). This means that this electron now occupies the anti-bonding orbital. Since this anti-bonding orbital is in a higher energy state than the bonding orbital, and it is now occupied by an excited electron, this orbital now becomes the HOMO (Arceo and Melchiorre, 2012). This process of electron

excitation and HOMO-LUMO transfer is displayed in Figure 2. When the HOMO and the LUMO of the two adjacent thymine molecules are able to rotate and correctly overlap, the excited electron (or the enone radical) reacts with the adjacent molecule, and σ bonds form between the two molecules. These electrons now occupy newly created bonding orbitals. This reaction causes the formation of the cyclobutane ring, which connects the two molecules.

Orientation and Stereochemistry of CPDs

When the thymine molecules are contained within a strand of DNA, they are able to form these dimers fairly easily, since they are bound to the DNA backbone in a way which keeps them adjacent to each other. However, in a purely aqueous solution, thymine molecules are fairly resistant to dimerization, even with exposure to UV light. This poses a problem when trying to synthesize thymine dimers in a lab for research purposes. However, CTDs have been found to form more easily when a frozen thymine solution is used instead of an aqueous one (Yein, 1987). When the solution is frozen, water is excluded from the space between the molecules, and the thymine molecules are able to compact together, forming a more organized crystal structure. This allows the thymine molecules to be properly oriented to one another in an adjacent fashion which is similar to that of a typical DNA strand. Since the molecules are forced to compact into this favorable orientation, CTDs are more readily formed (Yein, 1987).

Cyclobutane thymine dimers exist in a number of isomeric forms, displayed in Figure 3. The most predominant form of the cyclobutane dimer found within DNA is the *cis-syn* conformation, however the *trans-syn, cis-anti,* and *trans-anti* conformations are

also possible to create through UV irradiation. (Podmore, Cooke, Herbert, & Lunec, 1996,).

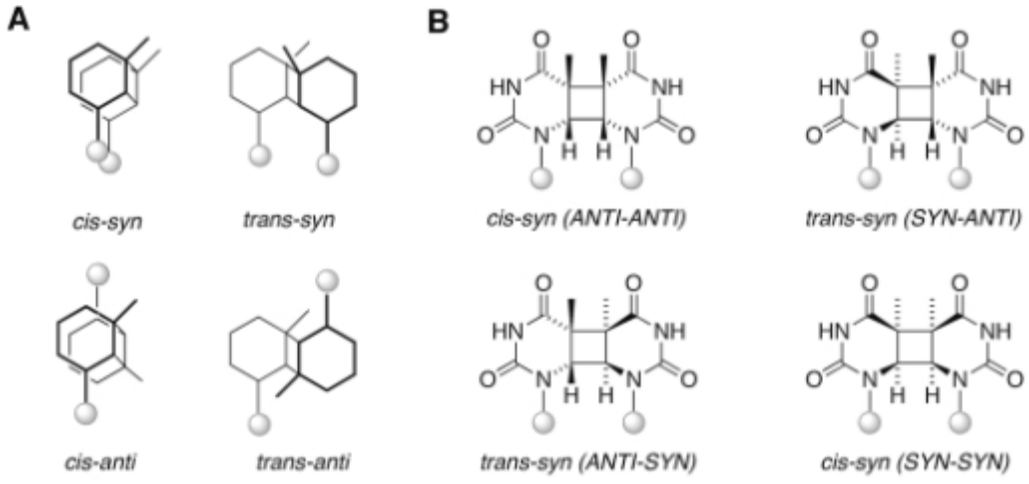


Figure 3: (A) The four possible structures of CPDs resulting from UV irradtion, of which the *cis-syn* is the most common in DNA. (B) The four possible structures of CPDs as viewed from the sugar-phosphate backbone of a DNA strand (Figure citation: Yamamoto, et al, 2011).

Thymine molecules which are adjacent to each other on a DNA strand typically form in the *cis-syn* conformation because of the geometric constraints which the DNA backbone enforces on the molecules (Yamamoto et al., 2011). Dimers which are formed in alternative conformations are generally between non-adjacent thymine molecules on the DNA strand. Similar to adjacent DNA bases, thymine dimers which are synthesized by irradiating a frozen aqueous solution are only found in the *cis-syn* conformation (Varghese, 1970). This is because the crystal structure that forms when the thymine

molecules are frozen causes them to be in a stacked conformation. This allows the molecules not only to be close to each other, which mimics the conformation of DNA, but also places them in the orientation though which the *cis-syn* conformation is formed.

CPD Repair and Mutations

DNA repair of CPDs can happen through a number of different pathways (displayed in Figure 4).

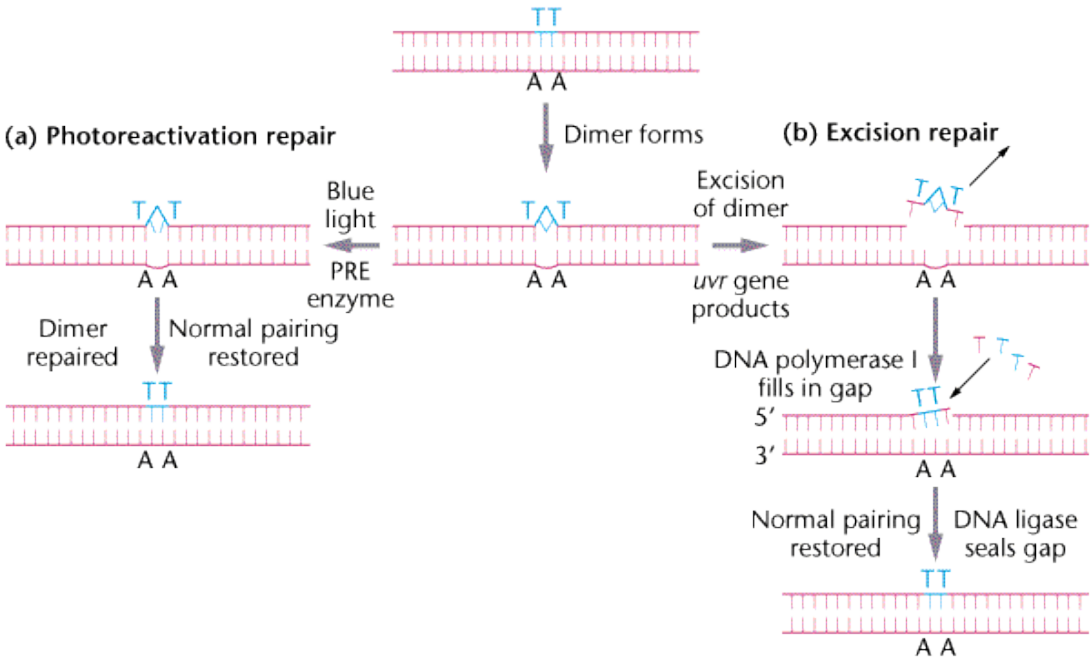


Figure 4: Schematic representation of CTD repair through (A) photoreactivation repair and (B) excision repair (Figure citation: Carr, 2014).

The first of these pathways is photoreactivation repair. In this method, cells which have been irradiated with far UV light – which forms the CPDs – are able to use high energy visible light (also known as blue light) to repair the dimers (Oguma et al, 2001). DNA photolyase, a repair enzyme, uses this visible light to break bonds between the CPDs, causing them to go back to their original conformations, and allowing the DNA to be transcribed as normal. Although this method is still used in a large population of bacteria and other microorganisms, as well as some reptiles and amphibians, it is not used within humans, which must rely on alternative methods of repair.

The second type of DNA repair used – which is present in mammals – is nucleotide excision repair (NER). In this pathway, lesions in DNA strands which are caused by specific mutagenic changes are recognized as sequences which need to be removed from the strand. A collection of different proteins – which are often highly specific to a certain lesion or mutation – then cleave the phosphodiester bonds between the nucleobases within the DNA backbone, removing the segment from the DNA strand (Reguly, 2012). DNA polymerase the refills the gap where the mutagenic molecule once was with the original, unaffected nucleobase sequence. This stops the mutagenic sequences from being transcribed, and will halt the progression of any negative effects of the mutation.

Although the human body is usually able to excise CPDs within the DNA strand before transcription occurs, sometimes dimers can go undetected by repair mechanisms (Durbeej & Eriksson, 2002). These dimers can cause kinks in DNA stands to be formed (as displayed in Figure 5). This causes the cell to have issues with transcription of the DNA, since enzymes are not able to properly read and transcribe the dimerized

nucleobases. These issues often lead to mutations, some of which are involved in the pathogenesis of cancers. CPDs have been found to be fairly frequent sources of mutation within the DNA strand, with CPDs being responsible for, “at least 80% of the UVB-induced mutations” in mammalian cells (You, Lee, Yoon, Nakajima, Yasui, & Pfeifer, 2001). Because of their high rate of occurrence and high mutagenicity, CPDs are considered one of the major sources of mutations involved in skin cancer, especially since melanocytes are so frequently exposed to UV light.

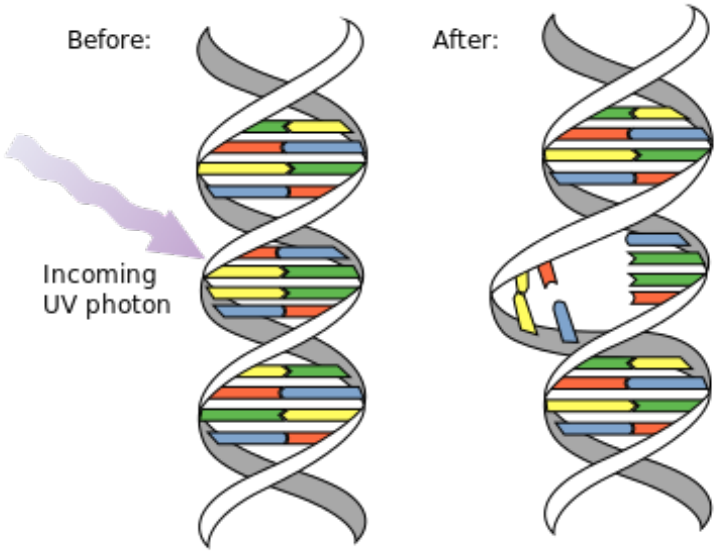


Figure 5: Diagram of kinks in DNA strand which are caused by the formation of CTDs between adjacent thymine molecules (Figure citation: Pyrimidine dimer, 2016).

Gas Chromatography-Mass Spectrometry

Gas Chromatography-Mass Spectrometry (GCMS) is an analytical technique that is used to separate and analyze samples of chemical compounds using specialized

instruments in order to determine some of their physical characteristics. The first portion – the gas chromatography – separates the compounds based on characteristics, while the second portion – the mass spectrometry – analyzes the mass of compound fragments once it has been ionized. GCMS is frequently used as an analytical technique because it provides clear and detailed results in a relatively short amount of time (sometimes even within minutes) without the use of expensive or dangerous organic solvents, resulting in little to no waste treatment (McNair & Miller, 2009). Because of this, GCMS is a very attractive method of analysis for research an educational use within an undergraduate institution.

Like any other type of chromatography, gas chromatography (GC) is a method of separating components of a mixture by using physical means, called phases. One of these phases is stationary, while the other is mobile, passing through the stationary phase during the chromatography session. As the different components of the compound are introduced to the chromatography environment, they are separated based on the ways in which they interact with the stationary phase, which can keep them from moving along with the mobile phase. Molecules which have more interactions with the stationary phase will adhere more strongly to it, while less interactive ones will move through the phase more quickly. In GCMS, the stationary phase consists of a long, narrow column with a liquid coating on the inside. The mobile phase consists of a carrier gas – usually nitrogen, hydrogen, or helium – which flows through the column. Using GCMS requires the analysis of a compound which is sufficiently volatile, since the compound must become a gas when entering the mobile phase (McNair & Miller, 2009). As the sample is introduced into the system (which is displayed in Figure 6) through the sample injector,

compounds within the sample are separated based on how strongly they interact with the column coating, as well as their volatility. As compounds come out of the column (since compounds with the same physical characteristics and volatilities should all come off the column around the same time), they are detected by the instrument. The compounds are then processed by some other type of analytical method, since, although GC separates compounds based on their characteristics, it does not determine their identity (McNair & Miller, 2009).

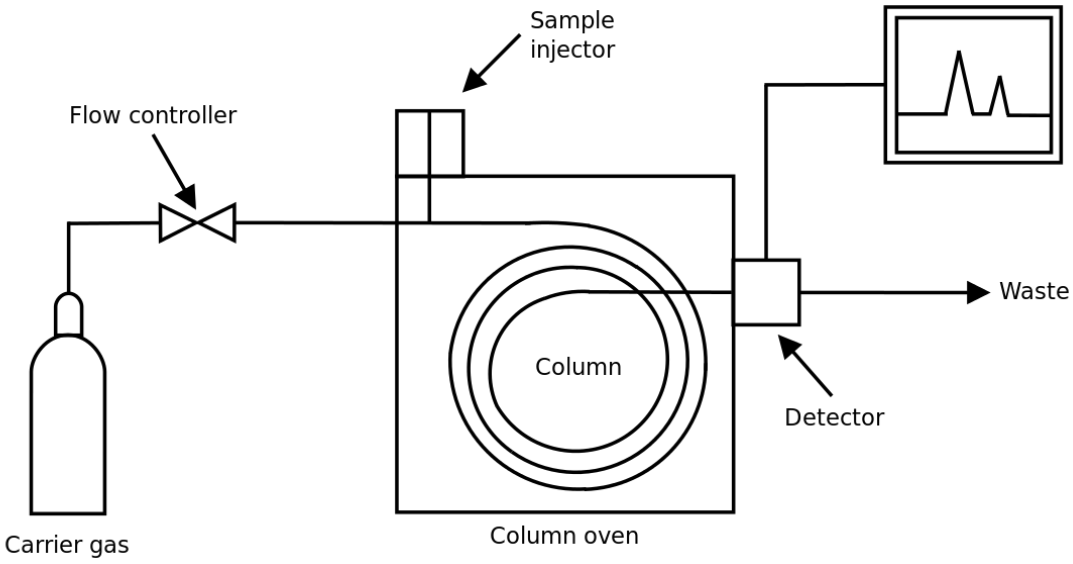


Figure 6: Basic overview of a gas chromatograph (GC). As the carrier gas enters the system through the flow controller, it carries the sample through the column. There, the components of the compound are separated by their characteristics such as size and ability to adhere to the column coating. (Figure citation: Gas chromatography, 2016).

Electron ionization mass spectrometry is an analytical method which can elucidate the molecular mass and structure of a particular molecule using mass fragmentations. Compounds which are analyzed through mass spectrometry are exposed to a high-energy electron beam, which causes the molecules to break into fragmented

ions (Silverstein, Webster, & Kiemle, 2005). The fragments are then accelerated to a high velocity and separated using electromagnetic deflection. The deflection patterns of the ions are then recoded based on their mass-charge ratio (*m/z*). These values are then used to generate a mass spectrum, which is a visual representation of this data from which the fragment masses can be determined (Silverstein, Webster, and Kiemle, 2009). This allows not only the total mass of the molecule to be identified, but also the masses of functional groups which have broken off of the molecule during ionization. Even though it does not explicitly identify the bonding pattern, mass spectrometry can help to determine the functional groups which are present in an analyte.

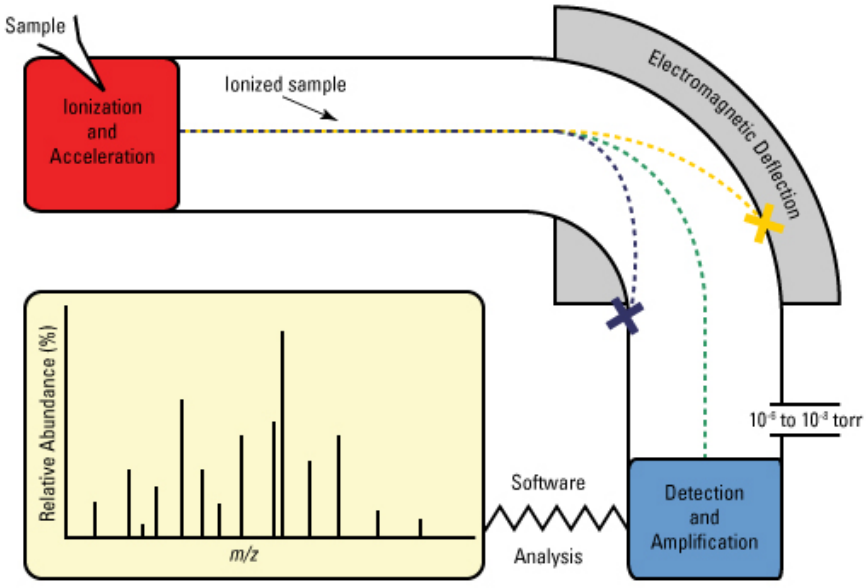


Figure 7: Diagram of a mass spectrometer. As a sample is injected into the spectrometer, the molecules of the compounds form ionized fragments, which are accelerated to high speeds. As the ions go through the system, electromagnetic deflection separates them by their mass and charge. These signals are then detected and amplified, allowing for a mass spectrum to be generated for the compound of interest. The pressure within the system is kept fairly low in order to avoid unexpected fragmentation. (Figure citation: Overview of mass spectrometry for protein analysis, 2016)

Derivatization of CPDs with Isobutyl Chloroformate

Although GCMS can be used as a very simple and efficient way to separate and analyze compounds, it does have its limitations, one of these being the limitation of only being able to use sufficiently volatile compounds for analysis. Compounds which are not volatile enough will not be able to enter the gaseous phase, and therefore will not be able to travel through the chromatography environment. Nucleobases in their original state are not able to be used in GCMS, because they are not sufficiently volatile or thermally stable to be eluted from the GC column (Brohi, Khuhawar, & Khuhawar, 2016). In order to analyze these nucleobases using GCMS, they need to go through a derivatization process in order to become volatile enough for the procedure. This can be done using a variety of silyl agents, such as trimethylsilyl (TMS) compounds, which are highly effective. However, these procedures require the use of a non-aqueous medium, are time-intensive, and are often difficult to conduct (Brohi, Khuhawar, Channa, Laghari, & Abbasi, 2016). An alternative method has been tested and verified, which involves the use of isobutyl chloroformate (IBCF) to derivatize the nucleobases. This process is relatively simple and quick, and is carried out in an aqueous medium (Brohi, Khuhawar, & Khuhawar, 2016). This process also does not require the use of many expensive organic solvents like many of the silyl reagent processes, making it much safer and cost-effective, while limiting the amount of waste to be processed. Although IBCF has been proven to be an effective derivatization reagent for single nucleotide molecules, it has not been used on nucleotide dimers, such as CPDs.

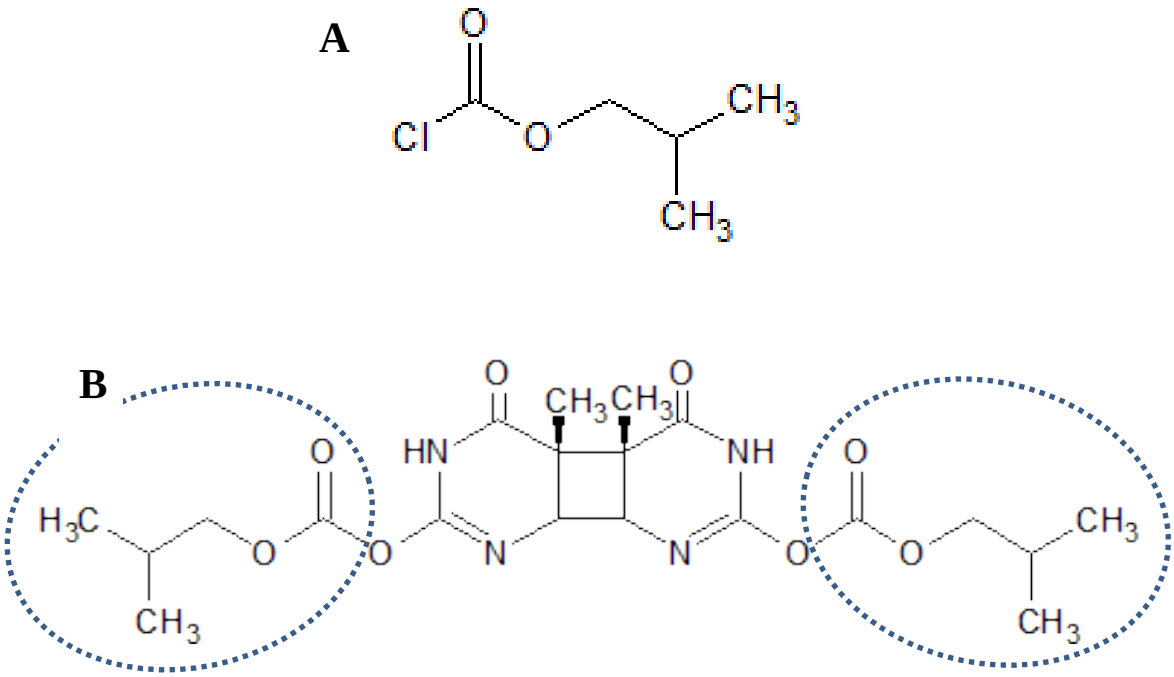


Figure 8: (A) Structure of IBCF and (B) Proposed structure of a derivatized *cis-syn* CTD using IBCF. The circled sections show molecules of IBCF which have been added to the thymine dimer through derivatization.

Hypothesis

Previous studies have shown the effectiveness of silyl reagents on the derivatization of CPDs for GCMS analysis, however these methods are not simple enough to be used within the context of an undergraduate laboratory. Although IBCF has shown to be a simple and effective method of derivatizing mononucleotides, the effectiveness of IBCF on the derivatization of CTDs has not been researched (Brohi, Khuhawar, & Khuhawar, 2016). In this study, we aim to determine a simple and effective method of synthesizing, separating, and analyzing CTDs using UV irradiation as our synthesis method, GCMS as our method of analysis, and IBCF as our method of derivatization. We hypothesize that it is possible to use IBCF as a derivatizing agent to allow CPDs to become volatile enough for GCMS analysis. Also, we hypothesize that the formation of the dimer through UV irradiation will be possible to determine and observe using mass spectra from GCMS analysis, since the mass of the dimer should twice as large as the mass of the original thymine molecule. Ultimately, we hope to create a procedure that is effective enough to display clear results, while still being simple enough for undergraduate students to use, understand, and learn from.

Materials and Methods

Thymine Control Trial Using NMR

Before attempts were made to use GCMS as the analysis method for the experiment, trials were conducted to determine whether NMR could be used as a sufficient method to detect thymine dimerization. 3 mL of 16 mM thymine stock solution (0.0210g thymine/10 mL water) was transferred to a plastic petri dish and frozen at -80 ˚C. The solution was then irradiated with a UV germicidal lamp (about 4 cm away) for 4 hours over dry ice, wrapped in foil, and transferred to a freezer. The sample was then melted into a beaker, extracted with 10 mL of chloroform, and dried for one hour. Residue from drying was dissolved into 0.75 mL deuterated chloroform and analyzed using NMR (128 scans). Results were very poor (most likely due to the low concentration of thymine in the sample, since thymine has a very low solubility), so NMR was deemed as an insufficient method of analysis (Figure 9).

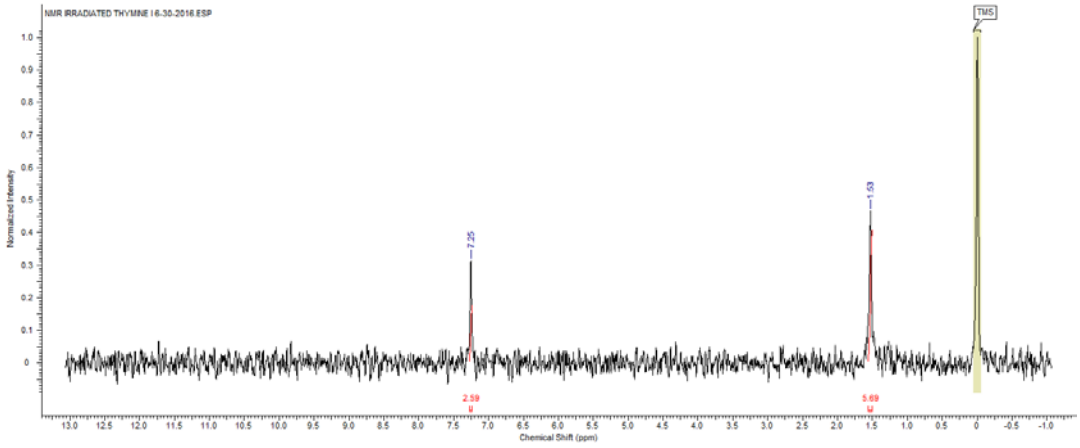


Figure 9: NMR spectrum produced for irradiated thymine sample. Because of the high amount of noise and the large solvent peak (most likely due to the low solubility of thymine in water), NMR was deemed as an insufficient method of analyzation for analysis.

Thymine Control and Preliminary Irradiation Trials Using GCMS

Stock solutions were made for the preparation and derivatization of thymine molecules in order to conduct GCMS trials. The stock solvent consisted of 4.4 mL pyridine, 4.4 mL methanol, 17.6 mL acetonitrile, and 17.6 mL water, while the carbonate buffer solution (acidified with HCl to reach pH 9.00) consisted of 0.1062 g sodium carbonate and 0.3366 g of sodium bicarbonate (Brohi, Khuhawar, & Khuhawar, 2016). Solution of 10% IBCF in methanol was also prepared for this part of the experiment (Brohi, Khuhawar, & Khuhawar, 2016). All GCMS trials were conducted on an HP-5890 GC (with an HP-5972 MS) with an HP-5 capillary column (30 m x 0.32 mm).

In the first control trial, 1 mL of 25 μM thymine solution (diluted from stock solution) was mixed with 0.5 mL pyridine solvent, 0.5 mL carbonate buffer, and 0.25 mL 10% IBCF in methanol. The sample was then sonicated for 15 minutes at 30 ˚C to thoroughly mix the solution, and sample was extracted with 0.5 mL of chloroform. The sample was then run through the GCMS using Setting 1 (Figure 10) (Brohi, Khuhawar, & Khuhawar, 2016). No detection of the capped thymine molecule was found on the mass spectrum produced. In the second control trial, 1 mL of 50 μM thymine in chloroform was mixed with two drops of IBCF, and ran through the GCMS using Setting 1. The capped thymine molecule was still not detected on the mass spectrum.

GCMS Setting	**Injection Port Temperature**	**Transfer Line Temperature**	**Helium Flow Rate**	**Split Ratio**	**Column Temperature Settings**
Setting 1	290 ˚C	290 ˚C	1.5 mL/minute	20:1	100˚C for 1 minute, with a heating rate of 30 ˚C per minute until the sample reached 280 ˚C, which was then held for 2 minutes
Setting 2	250 ˚C	280 ˚C	1.0 mL/minute	20:1	Increased from 120 ˚C to 250 ˚C at a rate of 10 ˚C/minute after an initial 2 minutes at 120 ˚C, and then increased from 250 ˚C to 280 ˚C at 30 ˚C/minute, finally being kept at 280 ˚C for 2 minutes

Figure 10: Parameters of settings ran on GCMS for thymine analysis.

In the third trial (the first irradiation attempt), 5.0 mL of 25 μM thymine solution was frozen in a petri dish at -80 ˚C and irradiated with germicidal lamp over a frozen block of salt water for four hours. The solution was then melted and mixed with 0.5 mL solvent, 0.5 mL buffer, and 0.25 mL IBCF in methanol. After 15 minutes of sonication at 30 ˚C and extraction of 1.0 mL of thymine solution with 1.0 mL of chloroform, the sample was then run through the GCMS using Setting 1. Non-conclusive results were obtained. After analyzing the peaks on the mass spectrum, and matching them with the values of other known chemicals, it was found that most of the peaks on resulting spectra

were coming from outside gasses and chemical leakage from the column. A new HP-5 column was then installed.

For the fourth and fifth control trials, two solutions of thymine were made with 1.0 mL of 16 mM thymine in water, 0.5 mL solvent, and 0.5 mL buffer. One of the solutions contained 0.25 mL of IBCF solution in methanol, however the other contained 0.5 mL, in order to have IBCF present in excess. Both solutions were sonicated for 15 minutes and extracted with 1.0 mL of chloroform and injected into the GCMS using Setting 2 (Figure 10) (Podmore, Cooke, Herbert, & Lunec, 1996). In both samples, there were base peaks at 126 (the mass of thymine) and the rest of the spectrum resembled the expected mass spectrum of the capped thymine molecule.

Irradiation Trials

For the first irradiation trial, 5.0 mL of 16 mM thymine solution was frozen in an open petri dish and irradiated with a 36W UVC germicidal lamp (254nm) for 4 hours over dry ice. 1.0 mL of irradiated thymine solution was then mixed with 0.5 mL solvent, 0.5 mL buffer, and 0.5 mL IBCF in methanol. Sample was then sonicated for 15 minutes, extracted with 0.5 mL chloroform, and injected into the GCMS using Setting 2. The mass spectrum of this sample displayed no sign of the capped thymine molecule or the thymine dimer. Two more samples were also run, being prepared in the same manner (using 1.0 mL of 16 mM thymine stock solution), except one of the samples contained 0.05 mL of undiluted IBCF instead of the IBCF in methanol. When these samples were run through the GCMS using Setting 2, it was found that the sample which used the IBCF in methanol did not display results of the capped thymine molecule, while the sample

with undiluted IBCF did. It was concluded that the IBCF solution had become less potent over time, and it was decided to continue using undiluted IBCF for the rest of the experiment. However, after irradiating a thymine sample and preparing using the modified method (1.0 mL thymine, 0.5 mL solvent, 0.5 mL buffer, and 0.05 mL IBCF, followed by sonication and extraction with 0.5 mL chloroform), results showed only the normal capped thymine molecule.

In the third irradiation trial, 5.0 mL of the 16 mM thymine solution was frozen in a petri dish and irradiated for 1 minute using a different UV lamp. The sample was then prepared using the modified method and injected into the GCMS using Setting 2. The mass spectrum only displayed the normal capped thymine molecule. For the fourth irradiation, three samples of thymine solution were frozen and irradiated over dry ice for different lengths of time (1 hour, 2 hours, and 4 hours). Each sample was prepared using the modified method and injected into the GCMS using Setting 2. Although each of the samples showed the capped thymine fragment peaks on their mass spectrum, none showed peaks which would be significant of the capped thymine dimer. In the final trial, a frozen thymine sample was irradiated over dry ice overnight (about 17 hours). Although the sample was found to have melted overnight, the sample was still prepared using the modified method and injected into the GCMS using Setting 2.

Results and Discussion

The data obtained from the experimental control trials reflects that of previous experiments, and supports the fact that IBCF can be used as an effective derivatizing agent for the analysis of thymine monomers using GCMS. However, from the data, the formation of the thymine dimer or the reactivity of the dimer to the derivatizing agent,

while using the same derivatization and analyzation methods, was not able to be confirmed, suggesting that this derivatization method is not suitable for CTDs.

Thymine Derivatization Control Trials

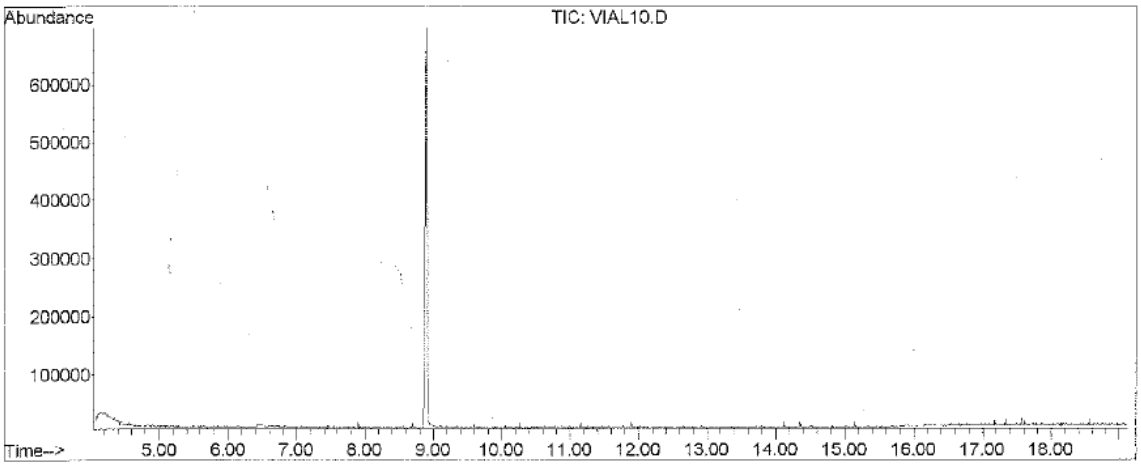


Figure 11: GC column spectrum for the non-irradiated thymine control sample. As is shown here, the derivatized thymine gas was eluted from the GC column around 9 minutes into the elution cycle.

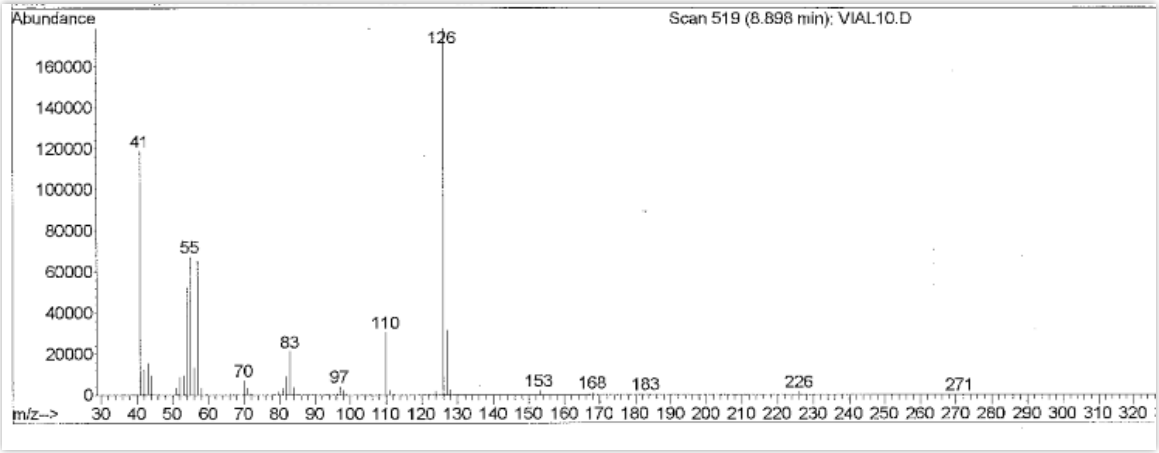


Figure 12: Mass spectrum for the non-irradiated thymine control sample. As is shown here, the base peak for the sample is at 126 m/z, and the parent peak for the sample is at 271 m/z (although the parent peak of the derivatized thymine dimer, 226 m/z, is also present).

As seen in Figure 11, the derivatized thymine sample was eluted from the GC column 9 minutes into the GCMS cycle. Although this elution time is slightly different than that of previous experiments, which showed derivatized thymine being eluted from the column at a little less than 6 minutes, this is most likely due to differences in the conditions of the GCMS trials (Brohi, Khuhawar, & Khuhawar, 2016). The mass spectrum of this sample (Figure 12) displays peaks which would be expected in the fragmentation pattern of a thymine molecule derivatized with one branch of IBCF. The parent peak is displayed at 226 m/z, which is the molecular weight of the thymine molecule with the addition of one branch of derivatization (Figure 13). The peak at 183 m/z corresponds with the mass fragment resulting from the loss of an isopropyl group on the end of the derivatized branch (Figure 14a), while the peak at 153 m/z corresponds with the mass fragment formed by the loss of the entire ester group (Figure 14b). The peak at 126 m/z is representative of the molecular weight of thymine, and therefore

corresponds with the mass fragment formed from the loss of the entire IBCF branch (Figure 14c). This peak (126 m/z) is also the base peak for the mass spectrum, most likely because the stability of the thymine molecule on its own allows it to form as a fragment more readily than other less stable fragments. The peaks on the lower side of the base peak are characteristic of the expected fragmentation patterns of the thymine molecule. The peak at 110 m/z corresponds with the fragment formed from the loss of an oxygen, the peak at 83 m/z corresponds with the mass fragment formed from the loss of a carbonyl group and a nitrogen group (with hydrogen) from the ring, and the peak at 55 m/z corresponds with the mass fragment formed from loss of both carbonyl groups and one of the ring nitrogen groups (Figure 15).

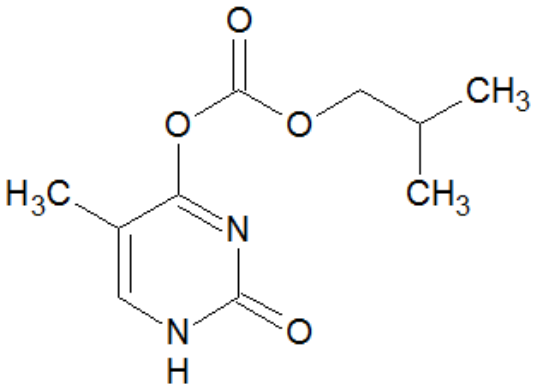
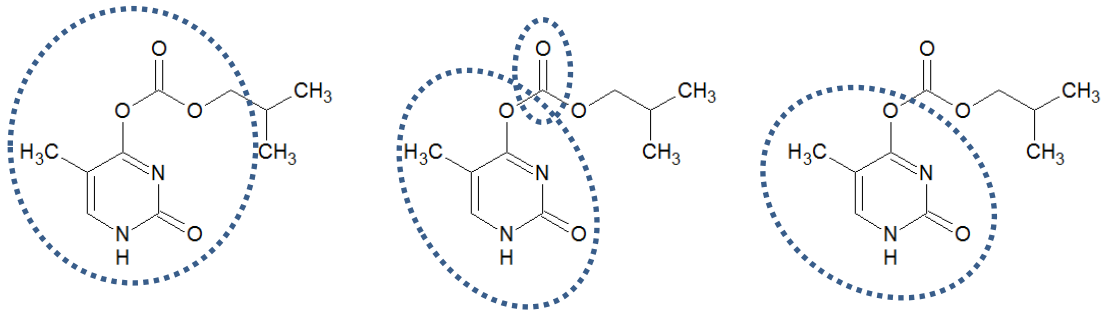
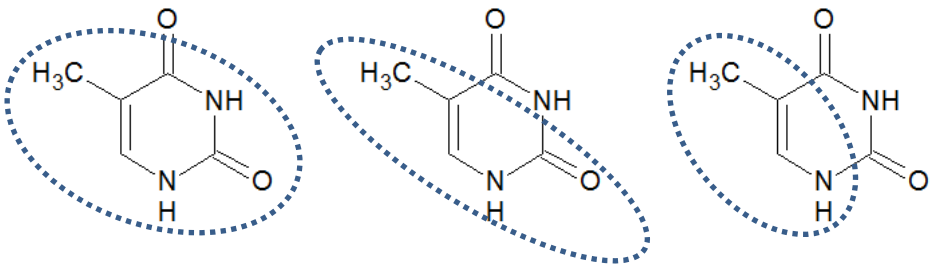


Figure 13: Derivatized thymine molecule capped with one branch of IBCF. This is the form of thymine that has been proposed to have been detected by the GCMS trials after derivatization.



a. b. c.

Figure 14: Possible fragments of capped thymine molecule from the derivatized thymine molecule mass spectrum. The circled areas shown the possible structures of the observed ion fragments: (a) 183 m/z, (b) 153 m/z, and (c) 126 m/z.



a. b. c.

Figure 15: Possible mass fragments of thymine molecule from the derivatized thymine molecule mass spectrum. The circled areas show the possible structures of the observed ion fragments: (a) 110 m/z, (b) 83 m/z, and (c) 55 m/z.

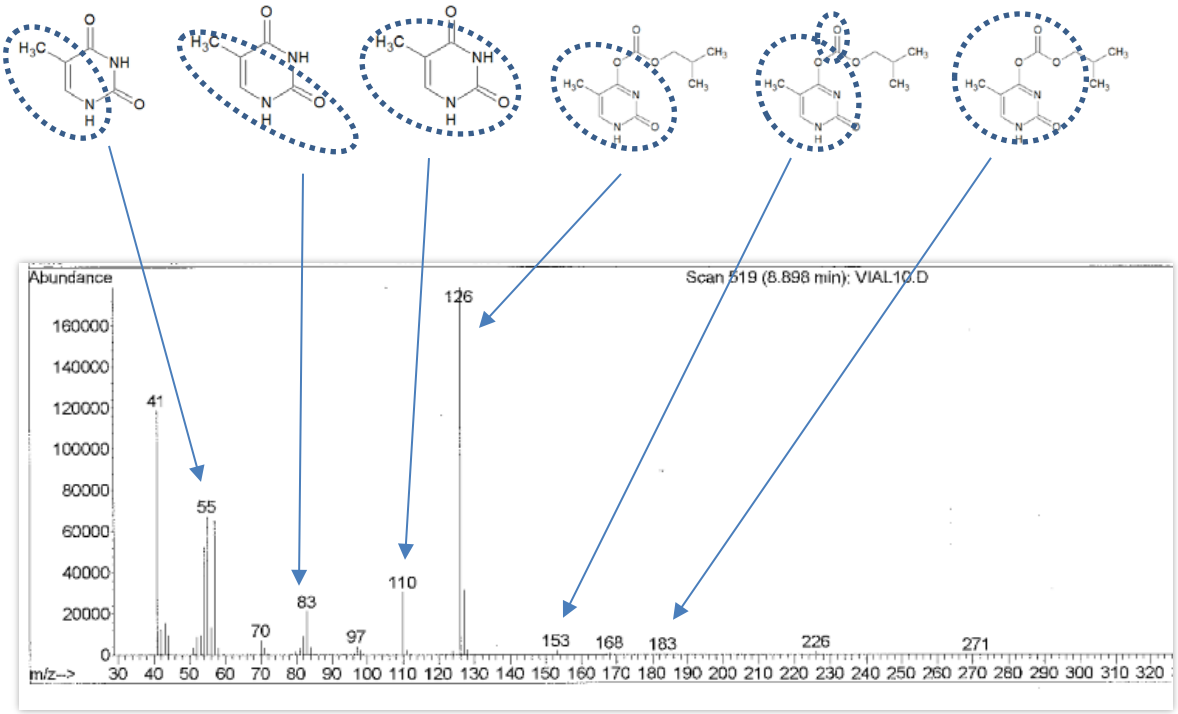


Figure 16: Mass spectrum with corresponding mass fragments assigned to their peaks.

The peak at 271 m/z seems out of place in this fragmentation pattern, since the whole, unfragmented molecule itself only adds up to 226 mu. This means that fragments are being detected which are not expected fragments from the derivatized thymine molecule. However, this may be due to leakage from the column or from outside gasses (which were problematic in some of the earlier trials). This peak at 271 m/z did not appear in many of the other trials, which would suggest that it was not a part of the fragmentation pattern of the derivatized thymine molecule, and instead some sort of contamination from another type of volatile substance.

Irradiations and Thymine Dimer Trials

Although a mass spectrum for the derivatized thymine monomer was able to be produced, a spectrum for the derivatized thymine dimer was not able to be produced in

the same way. After the thymine samples were irradiated and run through the GCMS, most still showed only the non-irradiated capped thymine molecules on their mass spectra, with no other major elutions from the GC column (since the derivatized thymine dimer would be twice the size of the derivatized thymine molecule, it was suspected that it would come off of the column later than the thymine molecule). Although a mass spectrum of a sample which suggested that another significant product was present was able to be produced, fragmentation patterns for the molecule which was eluted were non-conclusive.

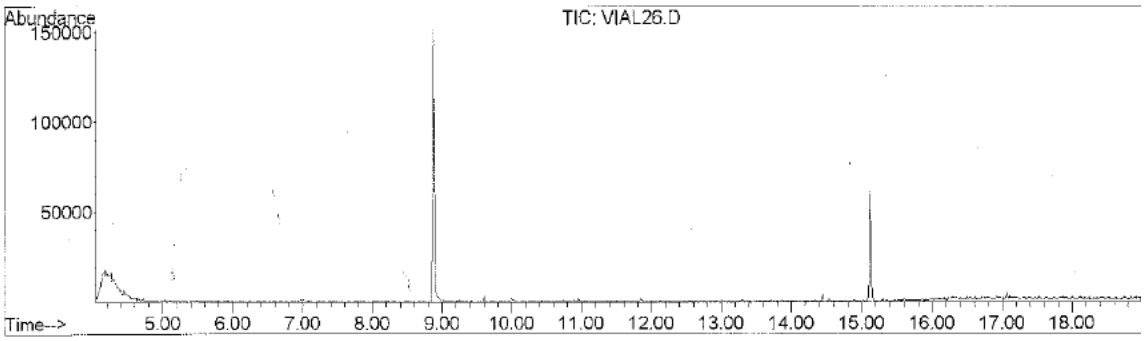


Figure 17: GC column spectrum for the irradiated thymine sample. As is shown here, the derivatized thymine gas was eluted from the GC column around 9 minutes into the elution cycle, while the suspected derivatized thymine dimer was eluted from the GC column around 15 minutes into the elution cycle.

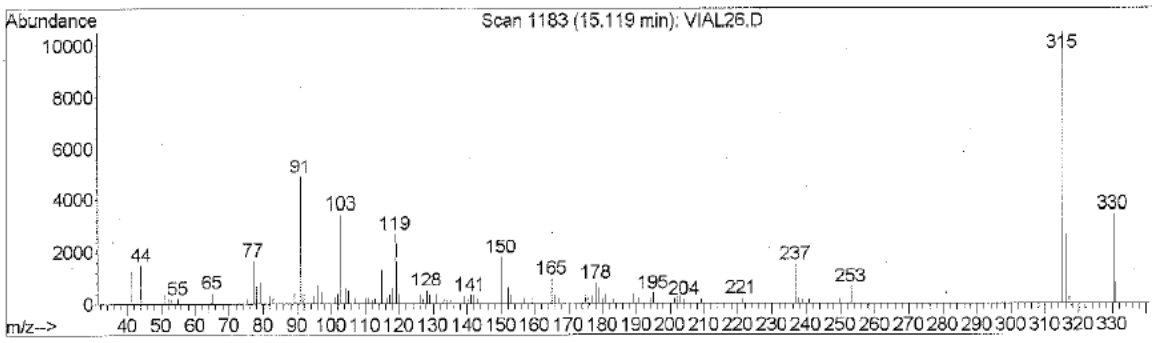


Figure 18: Mass spectrum for the irradiated thymine control sample. As is shown here, the base peak for the sample is at 315 m/z, and the parent peak for the sample is at 330 m/z.

In addition to the typical elution of the derivatized thymine molecule at 9 minutes, an additional, significant elution peak is also observed at 15 minutes (Figure 17). Although the higher fragmentation peaks seem to indicate the formation and derivatization of the thymine dimer, the mass fragments which correspond with the peaks are difficult to identify (Figure 18). The mass of the thymine dimer itself (252 m/z), which one would expect to see present as a fragment, is not found on the mass spectrum (although 253 m/z is). In addition, the base peak of the mass spectrum, found at 315 m/z, does not correspond with any major fragments expected from the derivatized thymine dimer. The mass of the thymine dimer with one branch of derivatization is 352 mu, from which this peak would form from a fragment after a loss of 37 m/z. Since there are not any easily detachable groups which add up to 37 mu on the derivatized thymine dimer, the mass fragment represented by this peak on the mass spectrum has not been able to be identified. Because of the difficulties in identifying the peaks on the mass spectrum,

whether this elution was the derivatized thymine dimer has not yet been able to be determined from the information provided by the mass spectrum.

The problems that were experienced in analyzing the thymine dimer with the GC may be due to a few different factors. First, IBCF may not be a useful capping reagent for the derivatization of the thymine dimer. When the two thymine molecules come together to react, although the carbonyl groups which are eventually derivatized by the IBCF are left unreacted by the dimerization, the thymine molecules lose their inter-ring alkene bonds. This could affect the reactivity of the thymine dimers towards IBCF, making them less reactive towards IBCF than the unreacted thymine molecules. Because of this, the thymine dimers may not be able to be derivatized with IBCF like the thymine monomers are.

Second, the UV lamp may not have been strong enough to create an abundance of dimers which was significant enough to be detected by the GCMS. Since the thymine dimers form in such a low abundance, even with a very powerful source of UV light, the irradiation may have yielded too few thymine dimers to be detected by the GCMS. To test this hypothesis, the irradiated samples could be analyzed using derivatizing agents which are known to work with thymine dimers (such as TMS) to act as a control, and to confirm the formation of the dimer during irradiation with the lamp. If any evidence of the dimers was not seen while using these control methods, it could be decided that the thymine dimers were not being formed by the UV lamp.

Third, the dimers may not have lasted long enough to make it through the derivatization process, which would cause the unreacted thymine to be the only product seen during analysis using GCMS. Although the UV lamp may have been powerful

enough to create the dimers, the dimers are still very unstable, and can revert back to their unreacted thymine state very quickly if they come in contact with UV irradiation. Although most of the derivatization process was conducted in a way which would limit exposure of the samples to UV irradiation, it is still possible that the samples could have been exposed to UV irradiation somewhere along the way, and could have undergone a conversion back to their original confirmation before they were able to be analyzed with the GCMS. This could also be tested using TMS, since the formation and stability of the dimers could be confirmed by using this method. Although the second elution from the irradiated thymine sample still remains identified, there is still possibility that the thymine dimer can be derivatized by IBCF. By using these experimental controls to narrow the possibilities of error, the difficulties faced in the derivatization and analysis of the thymine dimer can be better understood.

Conclusions

The aim of this study was to determine if IBCF could be used as an effective derivatizing agent for CTDs created through UV irradiation, allowing them to be easily analyzed using GCMS in an undergraduate laboratory setting. Although the data displays that IBCF is effective in derivatizing thymine monomers, as seen in Figure 12, we were not able to obtain data which verify the creation or derivatization of CTDs. Because of this, IBCF was not able to be proven effective in derivatizing thymine dimers when using the same method of derivatization as used on thymine monomers.

Although our results are not conclusive, UV irradiation and GCMS analysis of thymine dimers shows promise as an educational tool in the study of organic and photochemistry. Future research in this field will most likely focus on alternative methods of derivatization which are as simple and cost-effective as the derivatization method using IBCF. Most currently known derivatization agents are difficult to use and can be very expensive, and are therefore unsuitable for use in an undergraduate laboratory. However, if a method for the derivatization of thymine dimers using agents such as IBCF can be proven effective, this experiment could be very useful for education of undergraduate chemistry students.

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