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Synthesis of Novel Temozolomide-Fatty Acid Imide Hybrid Compounds for the Chemotherapeutic Treatment of Glioblastoma Multiforme

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Abstract

Glioblastoma multiforme (GBM) is an aggressive form of brain cancer that originates from glial cells, which make up the supportive tissue surrounding neurons. Temozolomide (TMZ) is the current chemotherapeutic drug administered to treat GBM as it works to inhibit the growth of the cancer cells. This research study focused on developing a method for synthesizing novel hybrid compounds that combines TMZ with various fatty acids known to have anticancer properties, forming a series of imide compounds with potential chemotherapeutic effects. Before synthesizing the compounds, various methods for synthesizing an imide from a primary amide were tested to ensure that TMZ and fatty acids would successfully react with each other to form the novel hybrid compounds. Once the novel hybrid compounds are synthesized, they will be tested for their anticancer properties on glioblastoma cells.

Background

Glioblastoma multiforme (GBM) is a type of brain cancer known as an astrocytoma, as it originates from glial cells supporting neurons. Because of its aggressive and invasive nature, it is classified as a grade IV glioma, the most severe form of brain cancer (Thakkar *et al.*, 2014). While GBM has a relatively low incidence rate of around 10 cases per 100,000 population, it is the most common type of brain cancer as it is found in about 60% of adults with a form of the disease. Patients with GBM are not expected to live long after diagnosis, with the average life expectancy being 15 months (Hanif *et al*., 2017; Rock *et al.*, 2012). The shortened lifespan is partly due to the difficulty of detecting and diagnosing the disease in its early stages since some of the symptoms, such as headaches and seizures, are common and typically attributed to other neurological conditions (Jovčevska *et al.*, 2013). Removing the tumor safely and entirely also presents a challenge as it can grow rapidly and uncontrollably throughout the central nervous system (CNS) (Liu and Mischel, 2017). Although GBM is a rare disease, its severity has made it a global health concern and increases the need to find a cure.

While there is currently no cure for GBM, there are treatment options available to help prolong the lives of individuals with the disease. Surgery is primarily conducted to remove as much of the tumor as possible. However, because the tumor invades large portions of the brain, removing it entirely risks negatively affecting neurologic activity; because of this, surgery is not the only treatment administered to patients with GBM (Yong and Lonser, 2012). Following surgery, radiation and chemotherapy are frequently used to minimize the size and growth of the tumor. In combination with radiation, patients are typically given an oral medication known as Temozolomide (TMZ), which is currently the standard chemotherapeutic drug for treating GBM (Yin *et al.*, 2013; Wesolowski *et al.*, 2010). This particular drug works as an alkylating agent, meaning that it can bind to the DNA of the glioblastoma cells, preventing the strands from

forming the complete double helix structure and ultimately inhibiting cell division and growth. TMZ is also able to pass through the blood-brain barrier (BBB), a selectively permeable membrane that allows certain drugs to enter the brain and attack cancer cells (Agarwala and Kirkwood, 2000; Sarkaria *et al.*, 2018). These characteristics of TMZ have made it the optimal chemotherapeutic drug to treat GBM for the time being.

While TMZ is currently the most successful chemotherapeutic drug against GBM, glioblastoma cells tend to develop a resistance to the compound. About half of the patients who receive TMZ are not receptive to the drug for this reason. The resistance is due to the expression of an enzyme found in glioblastoma cells called O6-methylguanine methyltransferase (MGMT). This protein, when expressed, repairs O6-methylguanine lesions, the areas where TMZ has blocked DNA formation by binding to the guanine bases of the strands (Chen *et al.*, 2018). Currently, there is no other chemotherapeutic drug as potent as TMZ to treat GBM, so overcoming this resistance has become an area of focus to find a more effective treatment or cure for the disease (Jiapaer *et al.*, 2018).

In an attempt to overcome the resistance of glioblastoma cells to TMZ, we worked to synthesize novel hybrid compounds that retain the anticancer properties of TMZ but also increase the efficacy of the drug with the help of another biologically active compound. Previous research done in Dr. Desmond Murray's lab involved synthesizing various types of novel hybrid compounds including arylidene heterocycles and arylidene pyrazolones, some of which successfully decreased the viability of glioblastoma cells and others that had no significant effect (Flores, 2020; Hiramoto, 2020; Deonarine, 2020). A potential new class of novel hybrid compounds containing TMZ is hypothesized to have some anticancer activity considering the effect TMZ has on glioblastoma cells. The compounds specific to this study are known as novel temozolomide-fatty acid imide hybrid compounds. Fatty acids have been studied extensively for

their biological properties such as antibacterial, antifungal, and anticancer activity. In chemotherapy, hybrid compounds containing fatty acids have been known to work well in treating various types of cancer since they increase the cytotoxicity of the compounds, making them more effective for suppressing the growth of the tumor. Previous research concluded that the fatty acids with the strongest anticancer effect are those with saturated, short-carbon chains (<10 carbons) and polyunsaturated, long-carbon chains (Jozwiaka *et al.*, 2020); these types of fatty acids were taken into consideration when choosing the compounds for this study. Because both TMZ and fatty acids are known to have anticancer properties, we believe that combining them will potentially create more effective drugs than TMZ alone.

The combination of TMZ and fatty acids has not been widely studied, but there are a few experiments where cancer cells were exposed to a mixture of the two compounds. An experiment done by Maor *et. al*. (2018) studied the anticancer activity of TMZ and various fatty acids (2-hydroxyoleic acid [2-OHOA], Gamma-Linoleic acid [GLA], and Fish oil [FISH]) on glioblastoma, colon cancer, and endothelial cells. Unfortunately, physically combining the two compounds did produce an antagonistic effect; it was found that fatty acids could inhibit the cytotoxicity of TMZ and vice versa, especially for fatty acids such as GLA and 2-OHOA.

In another experiment done by Ryu *et al*. (2012), TMZ was combined with valproic acid (VPA), a fatty acid used as a drug for certain health conditions such as seizures, bipolar disorder, and migraines. Contrary to Maor *et al.* (2018), the results showed that this combination of TMZ and VPA did have anticancer effects, as it helped inhibit MGMT expression in the glioblastoma cells, both *in vivo* and *in vitro*. Like the experiment mentioned previously, TMZ and the fatty acid were physically combined by simply mixing the two compounds. There are no known studies that chemically combine the two compounds to form a hybrid compound. This concept

may provide a solution to the antagonism described by Maor *et al.* (2018) and hopefully work as well as the experiment by Ryu *et al*. (2012).

When TMZ is combined with fatty acids, we believe that the general structure of the novel hybrid compounds will contain an imide (Figure 1). Imides and imide derivatives are another group of compounds known to have anticancer properties, including cytotoxic effects and the ability to influence DNA binding, like TMZ (Tumiatti *et al.*, 2009). Because imides are known to have anticancer properties, this functional group is thought to also contribute to the efficacy of the novel hybrid compounds. While imide synthesis is a well-studied area of research, there is not much literature on producing an imide from a primary amide, which is a functional group found on the structure of TMZ (Figure 2). In one research study, Schnyder and Indolese (2002) developed a method that involved combining primary amides such as formamide, acetamide, and benzamide with aryl bromides through the process of carbonylation using carbon monoxide; however, such reaction conditions were not possible in our lab. Thus, we needed to develop a new method to synthesize the imide and ensure that the novel hybrid compounds will form.

There were two main aims for this research project: 1) to develop a method for synthesizing an imide from a primary amide and 2) to use the new method to generate a series of novel temozolomide-fatty acid imide hybrid compounds. Unfortunately, because of the time constraint on the project, I was not able to synthesize the novel hybrid compounds, but it will be done in the future as a continuation of this study. Once the compounds have been synthesized, they will be tested on glioblastoma cells to determine their anticancer activity, if any, and be analyzed further for the potential chemotherapeutic treatment of GBM.

Methodology

The first step of this experiment was to develop a method for synthesizing an imide from a primary amide to ensure that the reaction between TMZ and fatty acids would be successful. Because TMZ and fatty acids were both limited in the lab and fairly expensive for small amounts, similar compounds were used for most of the trials to confirm the hypothesis that an imide would form. With the guidance of Dr. Murray, certain variables were modified to determine the best reaction conditions; a total of seven variables were tested for each reaction – a primary amide, an aryl or acyl halide to mimic the fatty acid, a catalyst to promote the reaction, a base to allow the formation of the imide, a solvent, reaction time, and reaction temperature. Each trial was essentially organized into three steps – Reaction, Isolation, and Analysis. While many of the reactions seemed to produce the desired compound, one of the more promising methods for synthesizing an imide was used for Reactions #4 and #7, where 4-methoxybenzamide was the primary amide and valeryl chloride acted as the fatty acid.

Reaction

The first step was to prepare the reaction mixture in a round bottom flask (RBF). The reagents were added in the following order: acetonitrile for the solvent, potassium carbonate for the catalyst, N,N-diisopropylethylamine (Hünig's base), 4-methoxybenzamide, and lastly, valeryl chloride dissolved in acetonitrile, which was added slowly to the reaction mixture using a syringe. The reaction was stirred for three hours in an ice bath. After the three hours, the mixture was poured into a beaker containing ice and 1M HCl to neutralize the mixture and form a precipitate which was expected to be the solid product. This mixture was then left overnight to allow the solid to form and observe any changes in the mixture.

Isolation

The following day, the product was extracted from the rest of the reaction mixture using fresh ethyl acetate. The organic layer from the extraction, which contained the product, was dried using anhydrous sodium sulfate to remove any water that may have mixed with the organic layer. The product was then isolated by filtering out the anhydrous sodium sulfate and using a rotary evaporator to remove the ethyl acetate. In an attempt to fully dry the product, it was placed on a vacuum overnight.

Analysis

The product was analyzed in two ways: by calculating the percent yield (how much product formed) and using Nuclear Magnetic Resonance (NMR) Spectroscopy, which helps identify the compound based on the presence and location of hydrogens in the structure.

Results

We completed a total of ten trials with several variations made to determine the best reaction conditions for synthesizing the imide (Figure 3). Table 1 provides a summary of the percent yield and NMR results for each product. Among the 10 trials, we tested four sets of starting materials with three primary amides and two halide compounds; the reactions are illustrated in Figures 4-7. Figures 8-12 provide the NMR spectra for the starting materials and Figures 13-22 show the NMR spectra for the products.

Reaction #1

The starting materials for this reaction were 4-methoxybenzamide (primary amide) and methyl 4-chlorobenzoate (fatty acid); the proposed reaction is illustrated in Figure 4. In addition to the starting materials, the following reagents were added to a round bottom flask: dimethylformamide (DMF) for the solvent, lithium hydride for the catalyst, and t-butyl alcohol for the base. The reaction mixture was then refluxed, which involved heating the mixture for three hours. To form the solid, I poured the reaction mixture into a beaker containing ice and 1M

HCl and left it overnight. The next day, to isolate the product, the mixture was filtered using a vacuum filter. Once the product was completely dry, it was ready for analysis with NMR and calculating the percent yield.

The product of this reaction was a white and chalky powder. After analyzing the product, we determined that it was not the compound that we expected from the reaction. The percent yield was 55.4%. In the NMR spectrum, only a few of the necessary peaks were present, indicating that the desired compound did not form.

Reaction #2

For this trial, we repeated the same procedure that was used for Product #1, with the only change being the reaction time – instead of a 3-hour reflux, the reaction ran for 24 hours to see if more time would allow the reaction to take place. However, we determined that increasing the reaction time from 3 hours did not make a significant difference. Like the previous trial, the obtained product was white and chalky. Upon analysis, the percent yield was 43.0% and the NMR spectrum again did not show all the peaks for the product we expected to obtain.

Reaction #3

The primary amide for this trial remained the same, but we used valeryl chloride instead of methyl 4-chlorobenzoate to act as the fatty acid (Figure 5). The following reagents were also added to a round bottom flask: acetonitrile (solvent), potassium carbonate (catalyst), and diisopropylethylamine (base). The valeryl chloride was first dissolved in acetonitrile before slowly being added to the reaction mixture. The mixture was then stirred for 90 minutes on an ice bath to observe the effect of colder temperatures on the reaction. Like the previous trials, the mixture was added to a beaker with ice and 1M HCl to form a precipitate and left overnight. The product, however, was not solid and had to be extracted using ethyl acetate. To remove the ethyl

acetate and obtain the isolated product, the reaction was placed on a rotary evaporator then left overnight on the vacuum to remove any excess liquid.

The product from this reaction was orange and had some crystallized solid, which was different from previous trials. Although the product was left overnight on the vacuum, it did not completely dry. The percent yield was 84.7% and the NMR spectrum showed that the desired product may be present among several extra peaks.

Reaction #4

The reaction for this trial was the same as it was for Reaction #3, with the only change being an increase in reaction time for 90 minutes to 3 hours. Like the previous trial, the product was orange and contained both solid and liquid. The percent yield was slightly higher at 91.2%. The NMR spectrum looked very similar to the one for Product #3, as the product was present, but there was still a number of extra peaks.

Reaction #5

The purpose of this trial was to test the effect of using different bases. This procedure repeated Reaction #4, but diisopropylethylamine was replaced with triethylamine. The product was a white, crystallized solid, which was a new observation. The percent yield was 20.7%. The NMR spectrum was cleaner than the spectra for Products #3 and #4, with fewer extra peaks, although the peaks we expected to see were much weaker, specifically, those representing the hydrogen atoms on the carbon chain from valeryl chloride, found between 0 and 3.0 ppm.

Reaction #6

Like the previous trial, we were working to determine the best base for the reaction. Instead of using triethylamine, we used 1,8-Diazabicyclo [5.4.0]undec-7-ene, commonly known as DBU. Like Product #5, this product was white and crystallized. The percent yield was 28.3%.

The NMR spectrum did indicate that the peaks for the desired product were present along with many extra peaks.

Reaction #7

Based on the results from Reactions #4 and #5, we determined that diisopropylethylamine was the best base for the reaction as we had higher percent yields and more promising NMR spectra for both. For consistency purposes, this trial was a repeat of the fourth trial. Like Product #4, the product was orange and crystallized with some liquid remaining. The percent yield for this product was 78.0%. The NMR spectrum again showed multiple extra peaks, however, the desired compound was present in the product. We did notice at this time that some of the extra peaks may suggest that some starting materials (4-methoxybenzamide and valeryl chloride) remained after the reaction, as there were duplicate peaks in the regions that indicated the presence of the product.

Reaction #8

This trial continued to test the effect of using stronger bases. In addition to diisopropylethylamine, 3 drops of DMF were added to the reaction mixture. This new step was tested based on a graduate student's current work in Dr. Murray's lab, which also involves finding an effective method for synthesizing an imide from a primary amide. In addition to incorporating DMF, there were some other variations – the solvent was changed from acetonitrile to dichloromethane and, due to availability, 4-methoxybenzamide was replaced with 4-fluorobenzamide, which has a similar structure (Figure 6). The product had the same physical appearance as Products #3, #4, and #7. The percent yield was 31.9% and NMR showed that the desired compound was present, but there was still the presence of extra peaks.

Reaction #9

Although the previous reaction had a low percent yield, we decided to continue with the same conditions using DMF since the imide still formed. Because we were getting the desired product and there was not much time left to conduct this research, we replaced the benzamide with TMZ to see if we could still obtain the imide compound using the reaction from the previous trial (Figure 7). The product was yellow and had a larger amount of liquid than the crystallized solid. The percent yield was 25.8%. NMR, unfortunately, showed that the reaction was not successful as there were some peaks missing from the spectrum.

Reaction #10

To increase the amount of product, the moles of the limiting reagents (starting materials) were multiplied by three. The product looked very different from the others as it was yellow with a paste-like consistency. The percent yield was 39.1%. Like Product #9, the NMR spectrum did not show all the desired peaks, indicating that the reaction was not successful.

Discussion

Based on the results, there is still quite a bit of work that needs to be done before synthesizing the novel temozolomide-fatty acid imide hybrid compounds. Out of the ten trials, six of the products contained the predicted imide compound. However, there was an issue with low percent yields for many of the products. The true reason for the low percent yields is unknown, although one contributing factor could be the loss of product as the reaction mixture was transferred to several vessels such as round bottom flasks, funnels, and beakers within one trial. In an attempt to increase the percent yield, we tried testing the effect of various bases on the reaction. Increasing the strength of the base was believed to allow more of the starting materials to react and form more of the product. However, the results show that increasing the strength of the base yielded less product. The reactions with the highest percent yields used

diisopropylethylamine alone as the base, which is not as strong as the other bases that were tested. This correlation is currently not well understood and will need to be analyzed further to increase product yield.

Another problem that we encountered in this study was the presence of extra peaks in the NMR spectra for all the products that contained the compound. As previously mentioned for Products #7 and #8, we noticed duplicate peaks in the areas of the spectra that represented the hydrogens in the benzene ring of the primary amide and those in the hydrocarbon chain of the valeryl chloride. As indicated by Dr. Murray, these peaks give reason to believe that the product also contained unreacted starting materials. While Product #7 had a much higher percent yield than #8, it is possible that even small amounts of the starting materials did not undergo the reaction and were detected by NMR Spectroscopy. The additional peaks throughout the spectra may also indicate the presence of impurities, which may have come from the environment or simply remaining reagents from the reaction, similar to the starting materials. To resolve the issue with extra NMR peaks, one of the next steps in this study would be to develop a method for purifying the products.

It was also observed after performing the ten experiments that all the products synthesized using Hünig's base had some liquid remaining, whereas the others were completely dry. It is possible that the liquid is not a part of the compound and is contributing to higher percent yields and extra NMR peaks. While the reason for the difference in the appearance of the products is not yet understood, we have decided that another step for this study is to try to remove the liquid. In the first two reactions, lithium hydride was used but was later replaced with potassium carbonate to see test the effect of a different catalyst. While potassium carbonate seemed to work well, using lithium hydride again may help remove the liquid as it works to absorb excess water, indicated by the first two products that were synthesized.

Although we determined that the best reaction conditions out of the 10 trials were for Products #4 and #7 and many of the products contained the imide compound, more time needs to be spent improving the methodology. We believe that both solvents tested, acetonitrile and dichloromethane, worked well in the formation of the imide. Regarding the reaction time and temperature, we found that stirring the mixture in an ice bath for three hours worked the best compared to heating the mixture and letting the reaction run for 90 minutes or 24 hours. However, as previously mentioned, the base and catalyst need to be further studied to optimize the methodology. Another variable that may need to be tested in the future is the primary amide. When using 4-methoxybenzamide and 4-fluorobenzamide as the primary amides, the formation of the imide was successful. However, when TMZ was used, the imide was not present in the product. The reason for this change is unclear and will need to be further analyzed before continuing the attempt to form the imide using TMZ.

Although we are still in the beginning stages of this study, we still have a contribution to the development of a method for imide synthesis from a primary amide. There are elements in the tested methods that have shown promise and others that have not. Scientists can use the information in future studies to consider what may or may not work well in their experiments. Once a method has been developed and works well to create a series of novel hybrid compounds with TMZ and fatty acids, the compounds can then be tested on glioblastoma cells. Based on previous research in collaboration with Dr. Denise Smith in the Biology Department, the method for testing the anticancer properties of the novel hybrid compounds will involve exposing the glioblastoma cells to various concentrations of the compounds in a 12-well cell viability assay to determine the lethal concentration at which 50% or more of the cells die as a result of the exposure, a value known as the LC_{50} (Hiramoto, 2020). This will determine if the compound is effective and can be developed into a potential chemotherapeutic drug to treat GBM.

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Figures and Tables

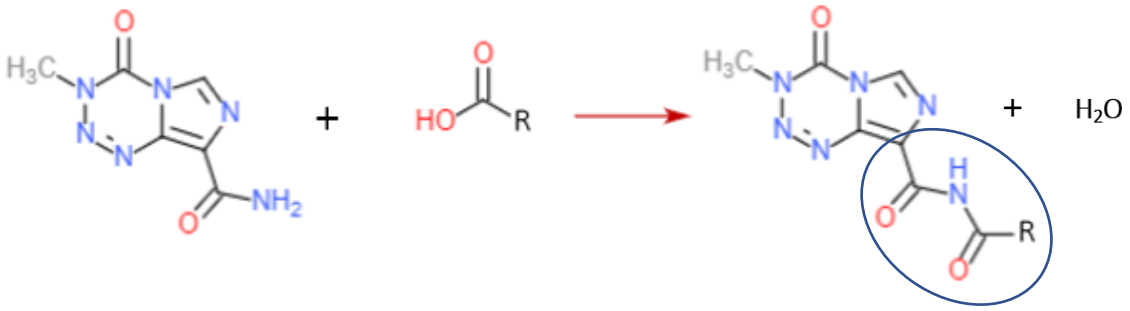

Figure 1. Proposed Reaction Between Temozolomide (left) and Fatty Acids (general structure in the middle). The reaction is believed to form an imide (circled on the right), a functional group characterized by the two carbonyl groups and NH group in the middle. The "R" represents the various hydrocarbon chains of fatty acids. When TMZ and the fatty acid combine to form the novel hybrid compound, excess water is also produced.

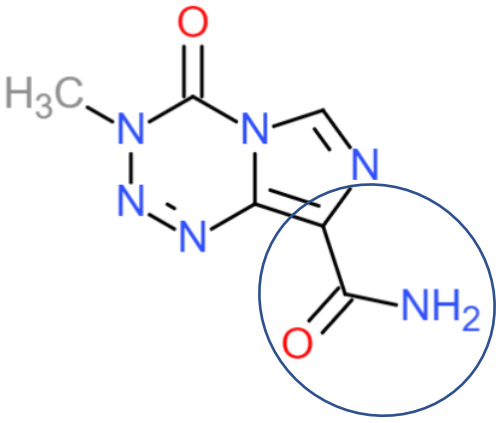

Figure 2. Primary Amide in Temozolomide. The primary amide functional group is characterized by the amine (NH2) group attached to a carbonyl group.

Figure 3. Products Obtained from the Ten Trials (labeled #1-10 from left to right). Products #5, #7, #8, and #9 have been dissolved in ethanol to easily transfer the product from the round bottom flask after using the rotary evaporator to the vial to store the product.

Reactions

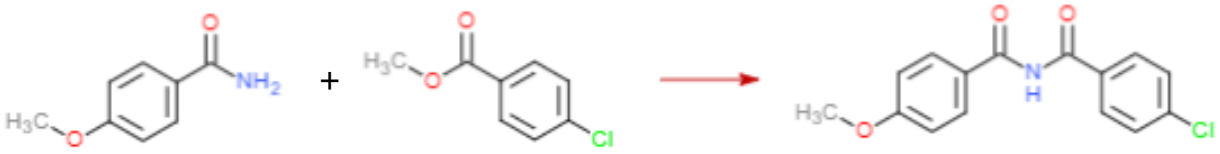

Figure 4. Reaction between 4-methoxybenzamide (left) and Methyl 4-chlorobenzoate (middle). This reaction was used for Products #1 and #2. Not pictured – excess CH_3OH is also produced when forming the imide.

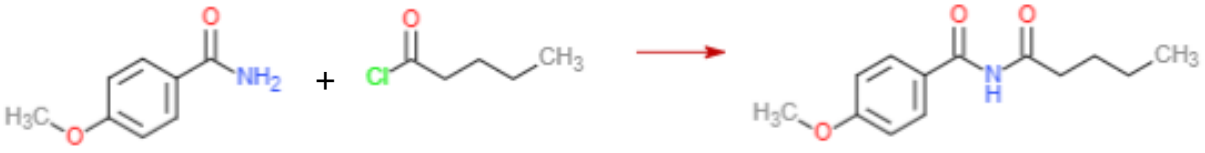

Figure 5. Reaction between 4-methoxybenzamide (left) and valeryl chloride (middle). This reaction was used for Products #3 through #7. Not pictured – excess HCl is also produced when forming the imide.

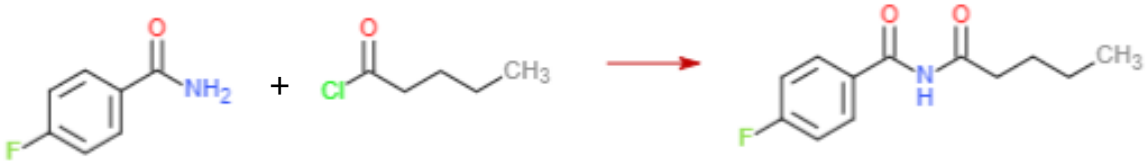

Figure 6. Reaction between 4-fluorobenzamide (left) and valeryl (middle). This reaction was used for Product #8. Not pictured – excess HCl is also produced when forming the imide.

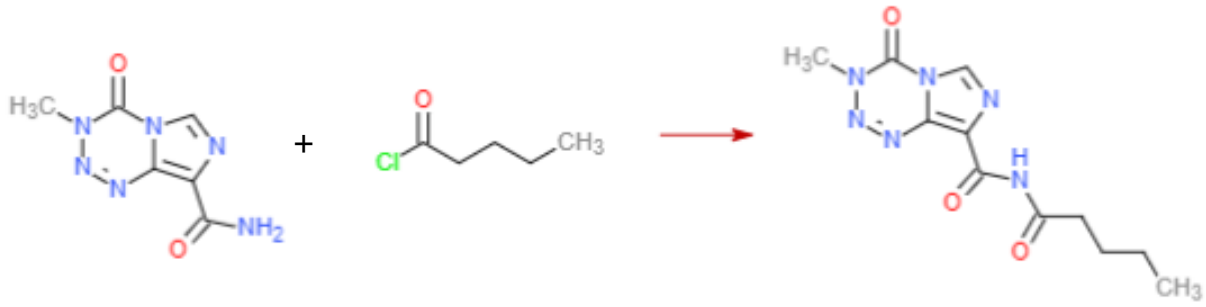

Figure 7. Reaction between Temozolomide (TMZ) (left) and valeryl chloride (middle). This reaction was used to produce Products #9 and #10. Not pictured – excess HCl is also produced when forming the imide.

Table 1. Percent Yield and NMR Results

Product #	Primary Amide/Fatty Acid	Percent Yield (%)	NMR Results (Product Absent/Present)
1	4-methoxybenzamide/ Methyl 4-chlorobenzoate	55.4	Absent
2	4-methoxybenzamide/ Methyl 4-chlorobenzoate	43	Absent
3	4-methoxybenzamide/ Valeryl chloride	84.7	Present
4	4-methoxybenzamide/ Valeryl chloride	91.2	Present
5	4-methoxybenzamide/ Valeryl chloride	20.7	Present
6	4-methoxybenzamide/ Valeryl chloride	28.3	Present
7	4-methoxybenzamide/ Valeryl chloride	78.0	Present
8	4-fluorobenzamide/ Valeryl chloride	31.9	Present
9	TMZ/Valeryl chloride	25.8	Absent
10	TMZ/Valeryl chloride	39.1	Absent

NMR Results

Starting Materials

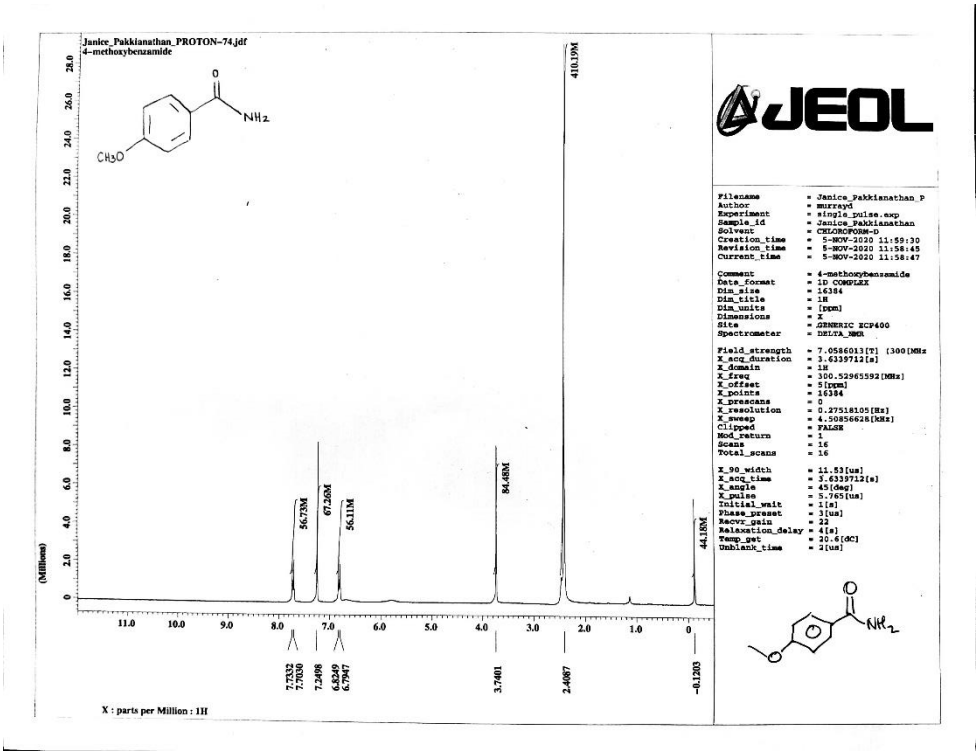

Figure 8. NMR Spectrum for 4-methoxybenzamide.

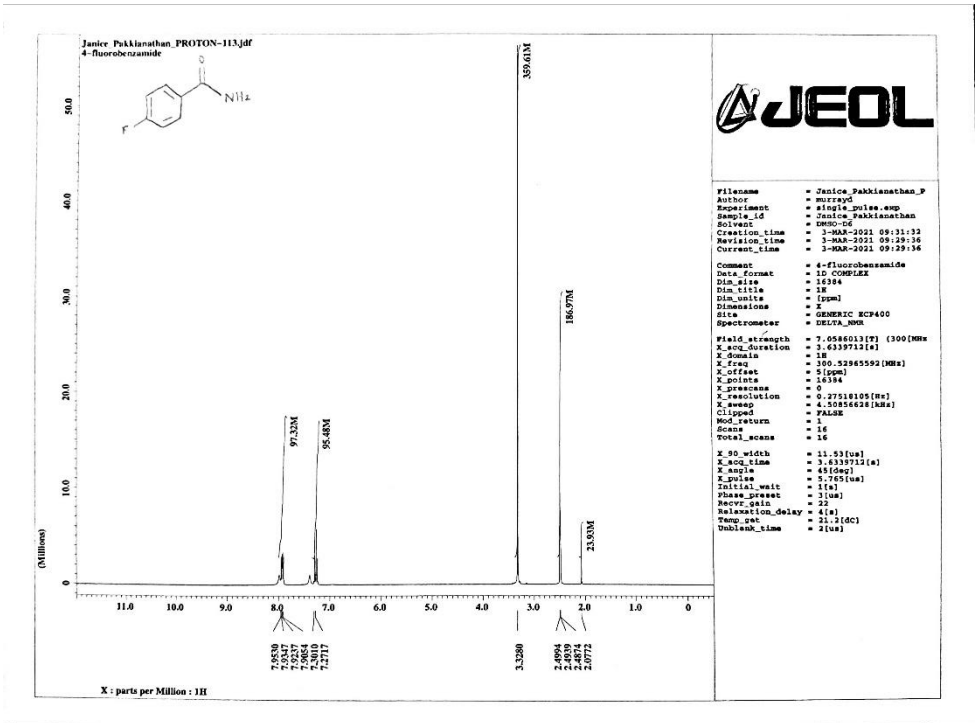

Figure 9. NMR Spectrum for 4-fluorobenzamide.

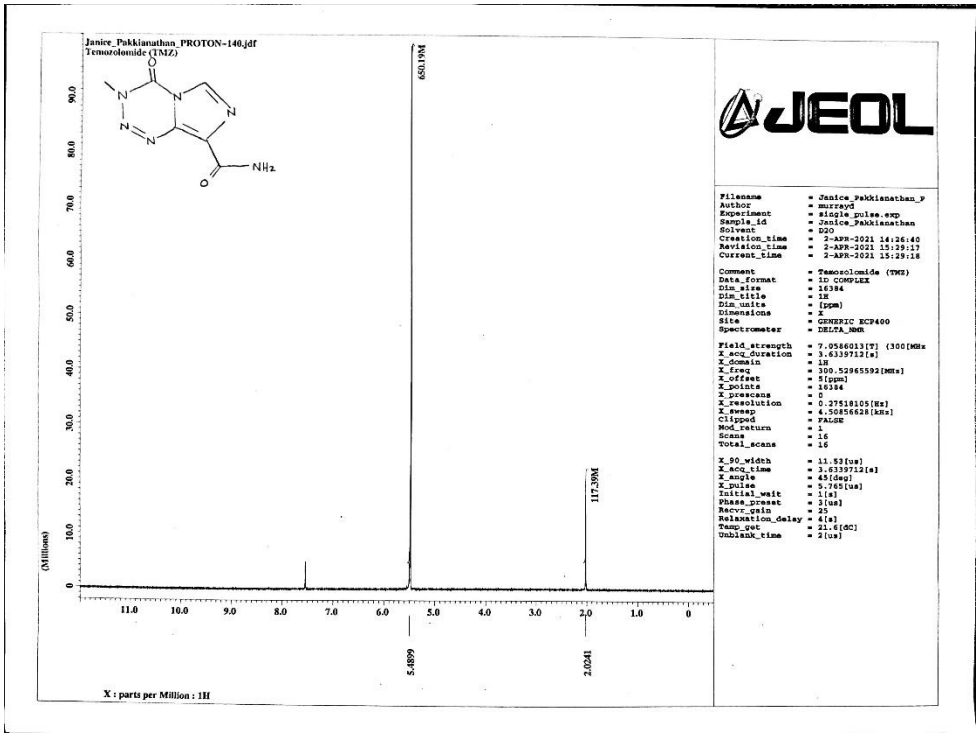

Figure 10. NMR Spectrum for Temozolomide (TMZ).

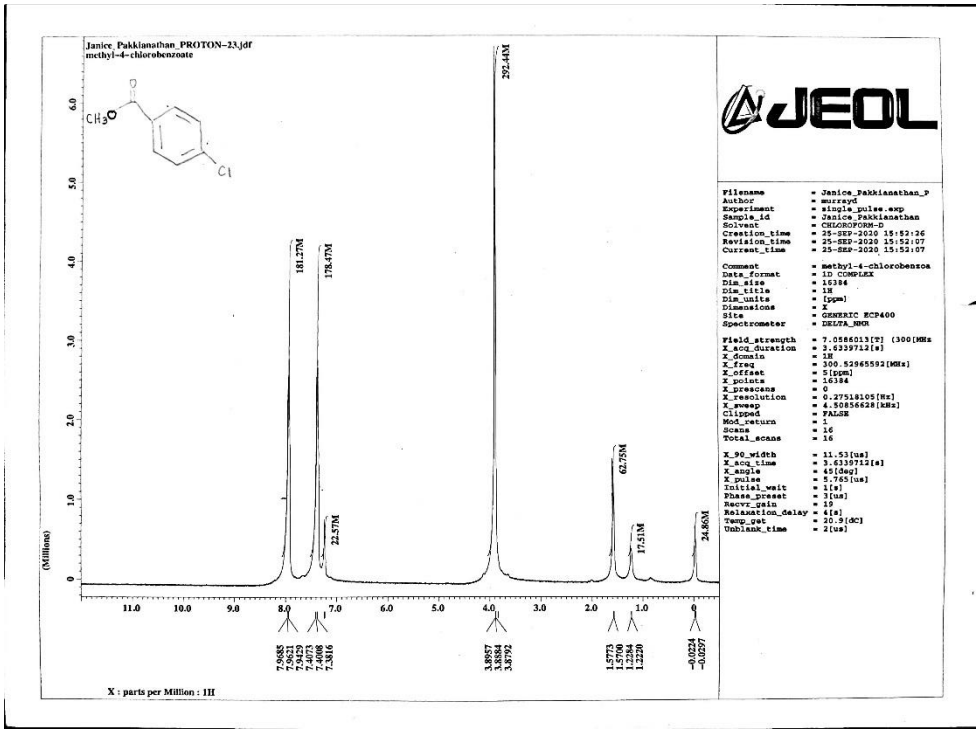

Figure 11. NMR Spectrum for Methyl 4-chlorobenzoate.

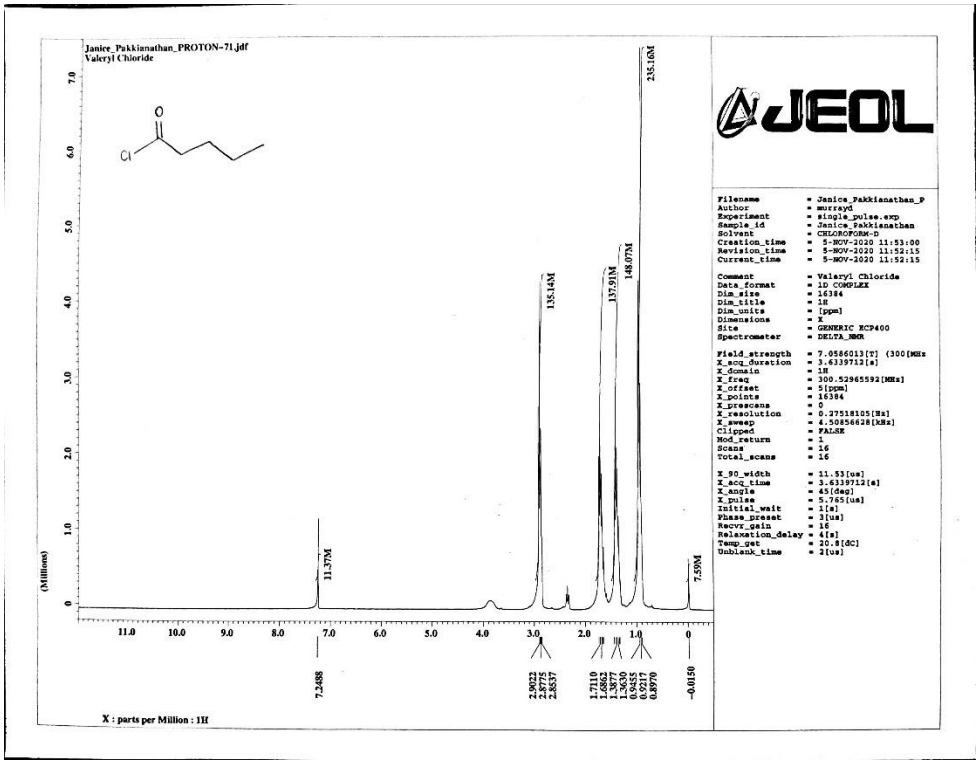

Figure 12. NMR Spectrum for Valeryl Chloride.

Products

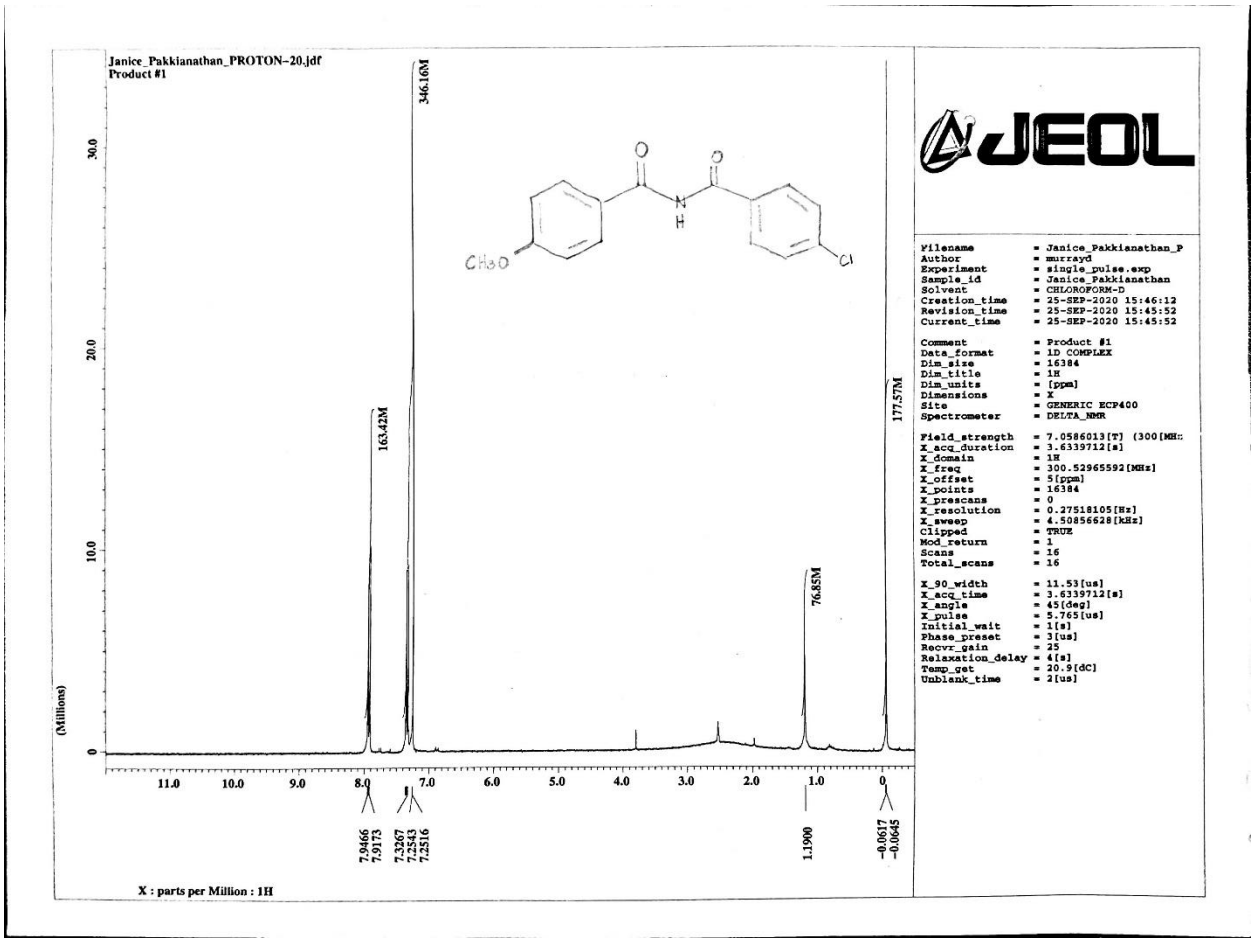

Figure 13. NMR Spectrum for Product #1.

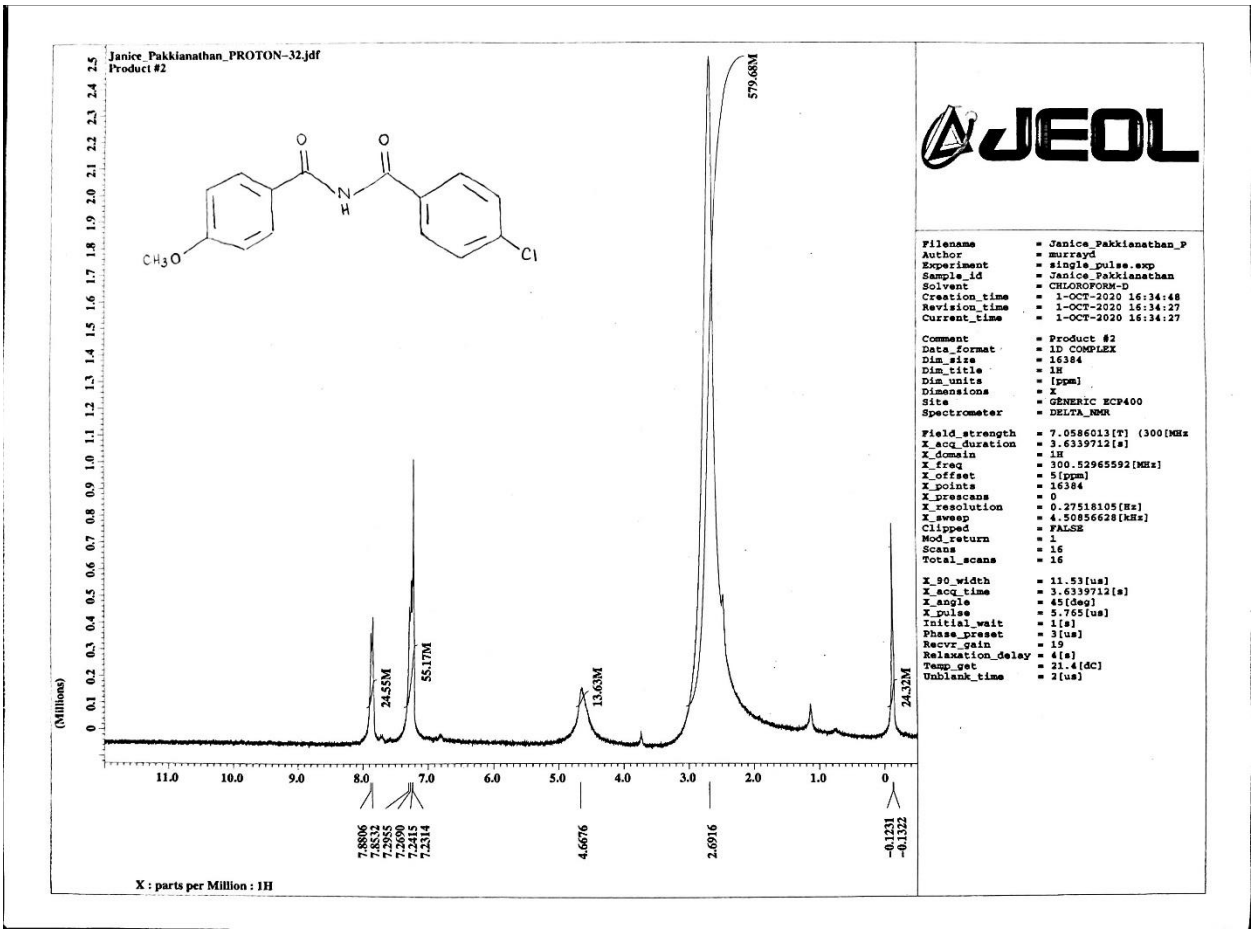

Figure 14. NMR Spectrum for Product #2.

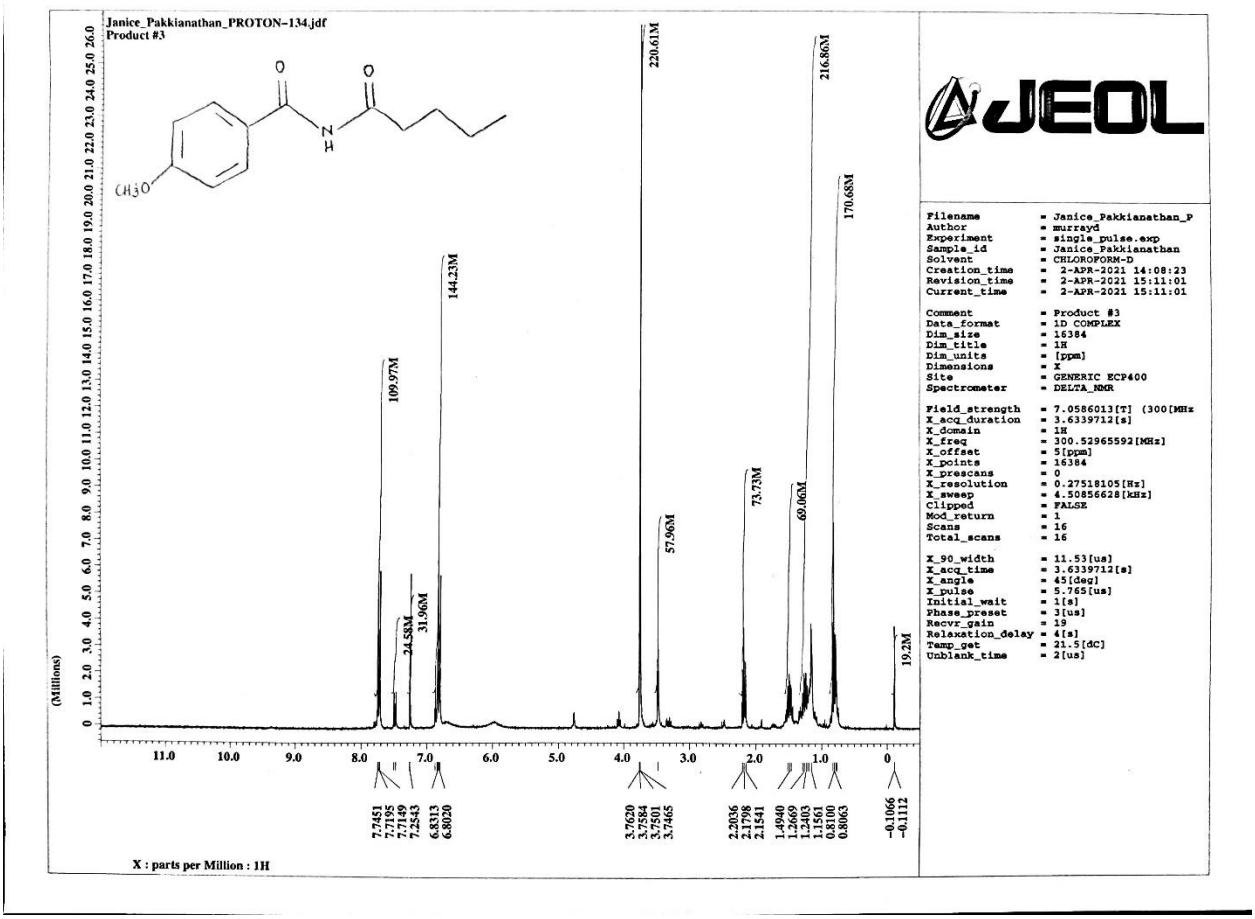

Figure 15. NMR Spectrum for Product #3.

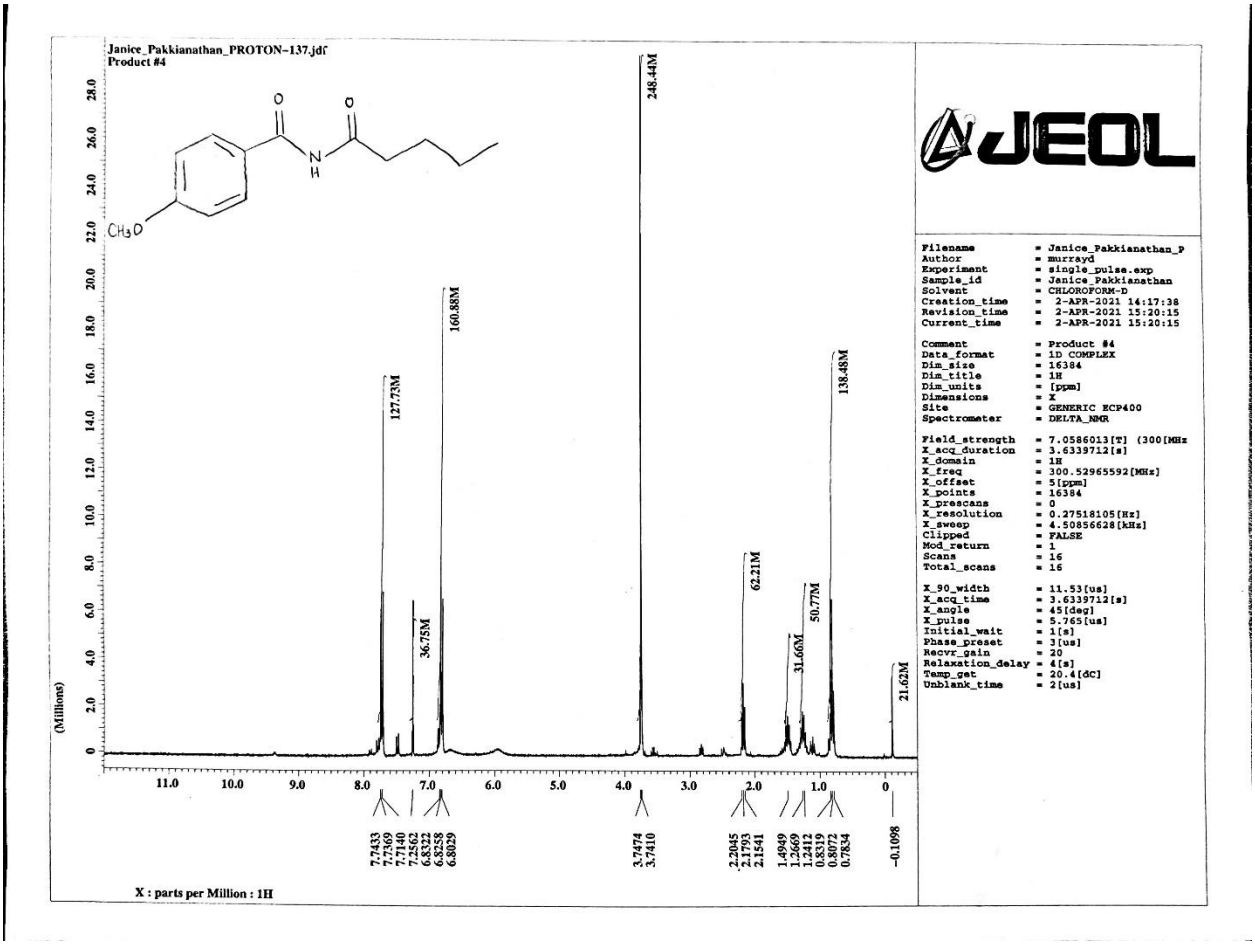

Figure 16. NMR Spectrum for Product #4.

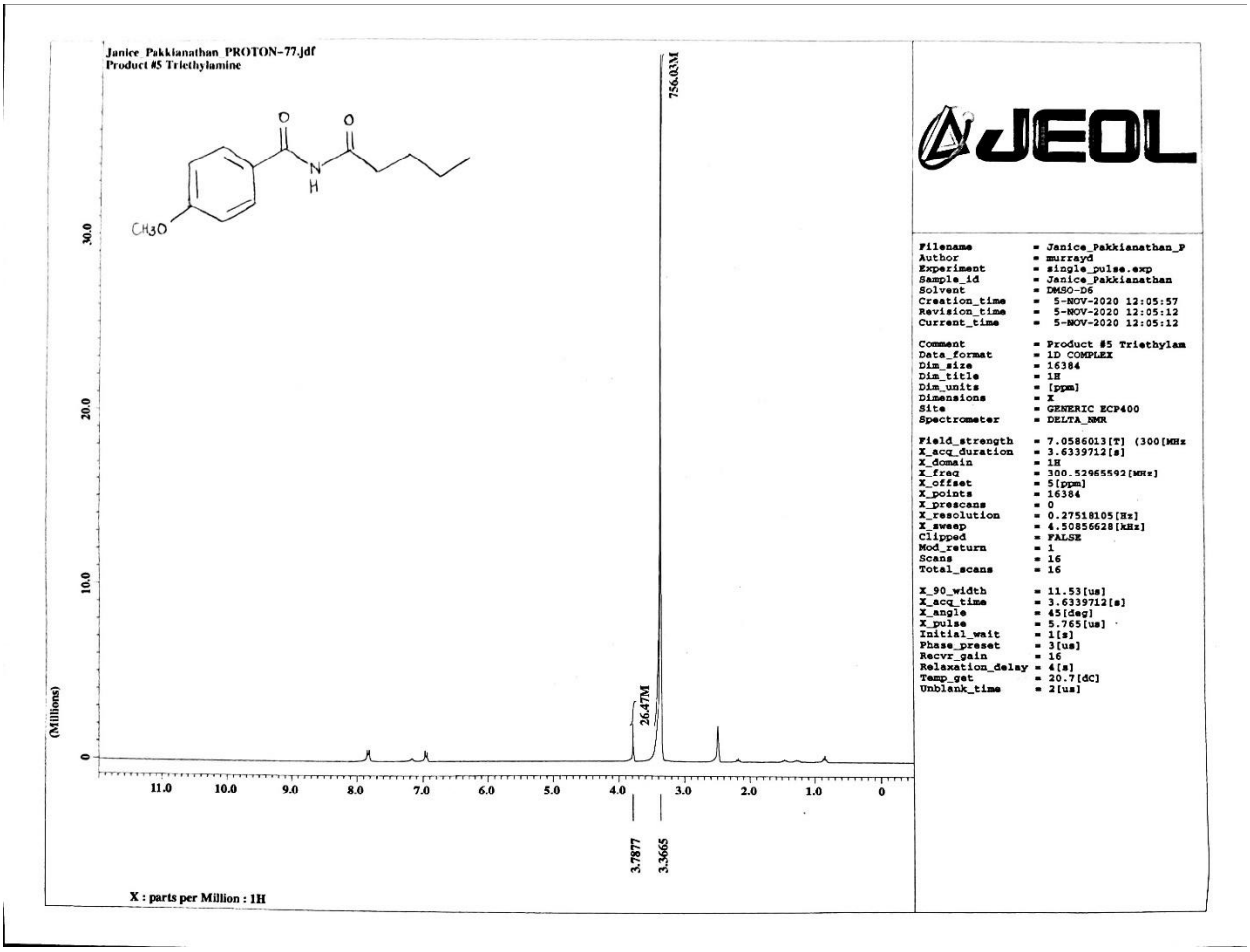

Figure 17. NMR Spectrum for Product #5.

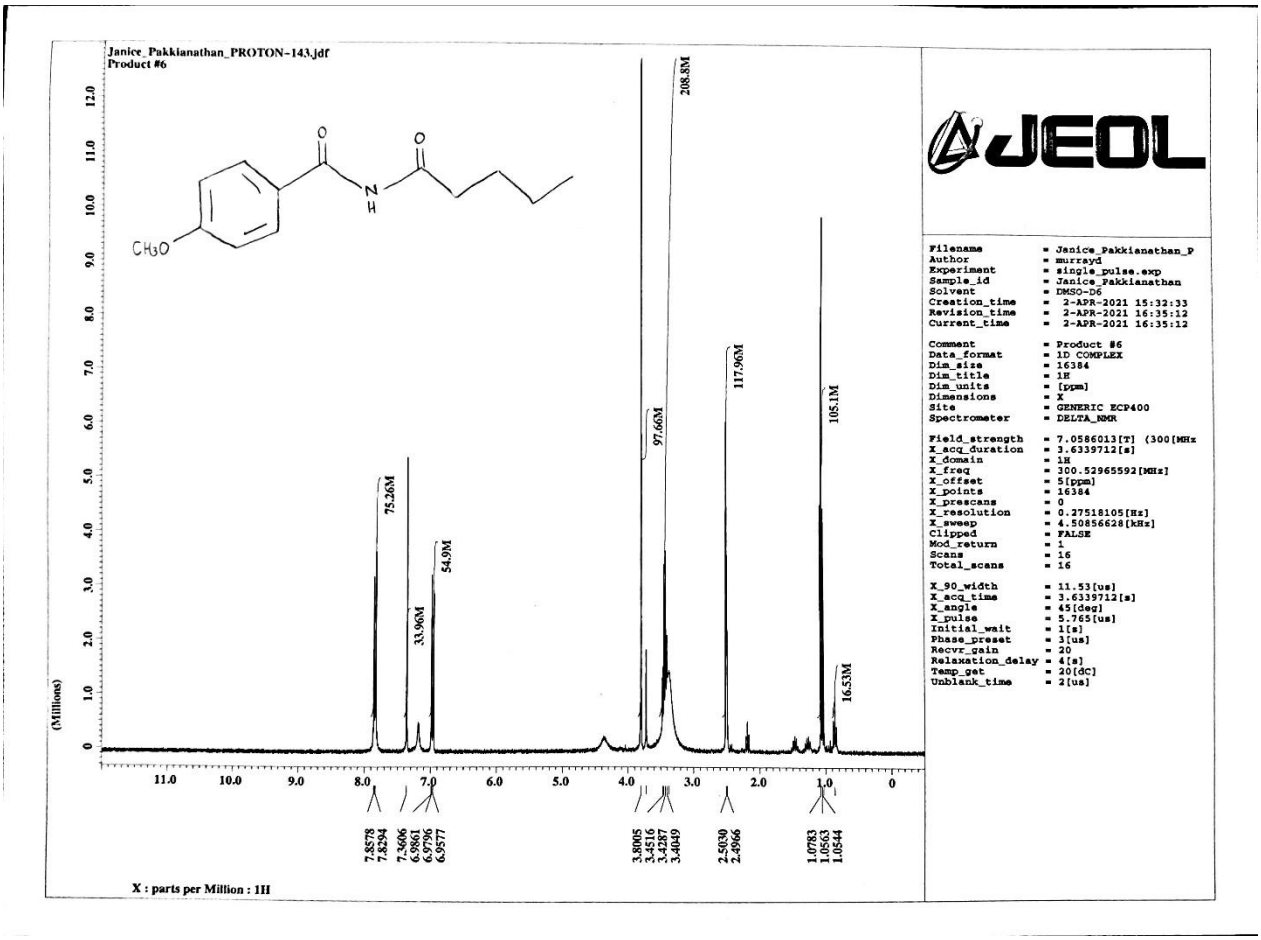

Figure 18. NMR Spectrum for Product #6.

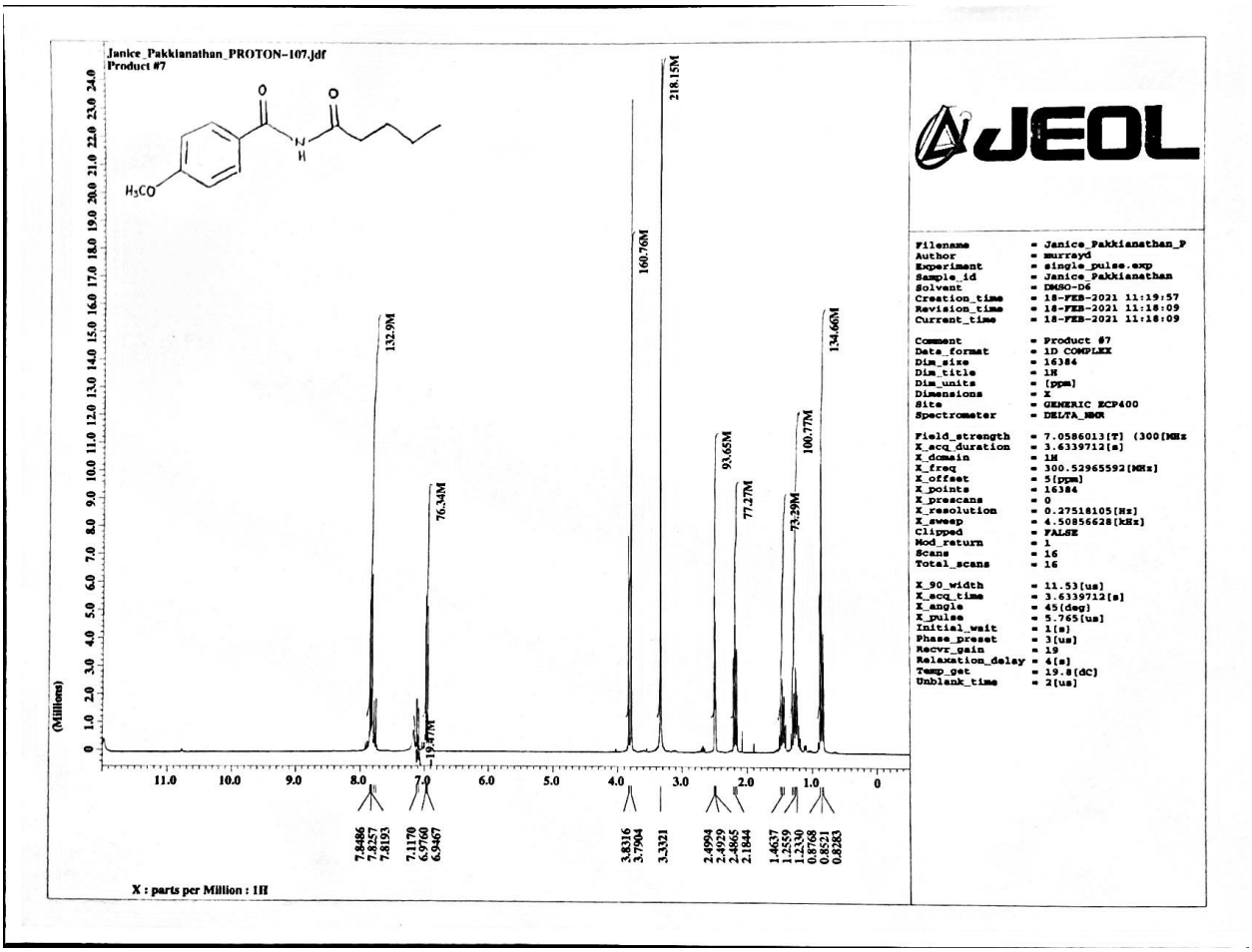

Figure 19. NMR Spectrum for Product #7.

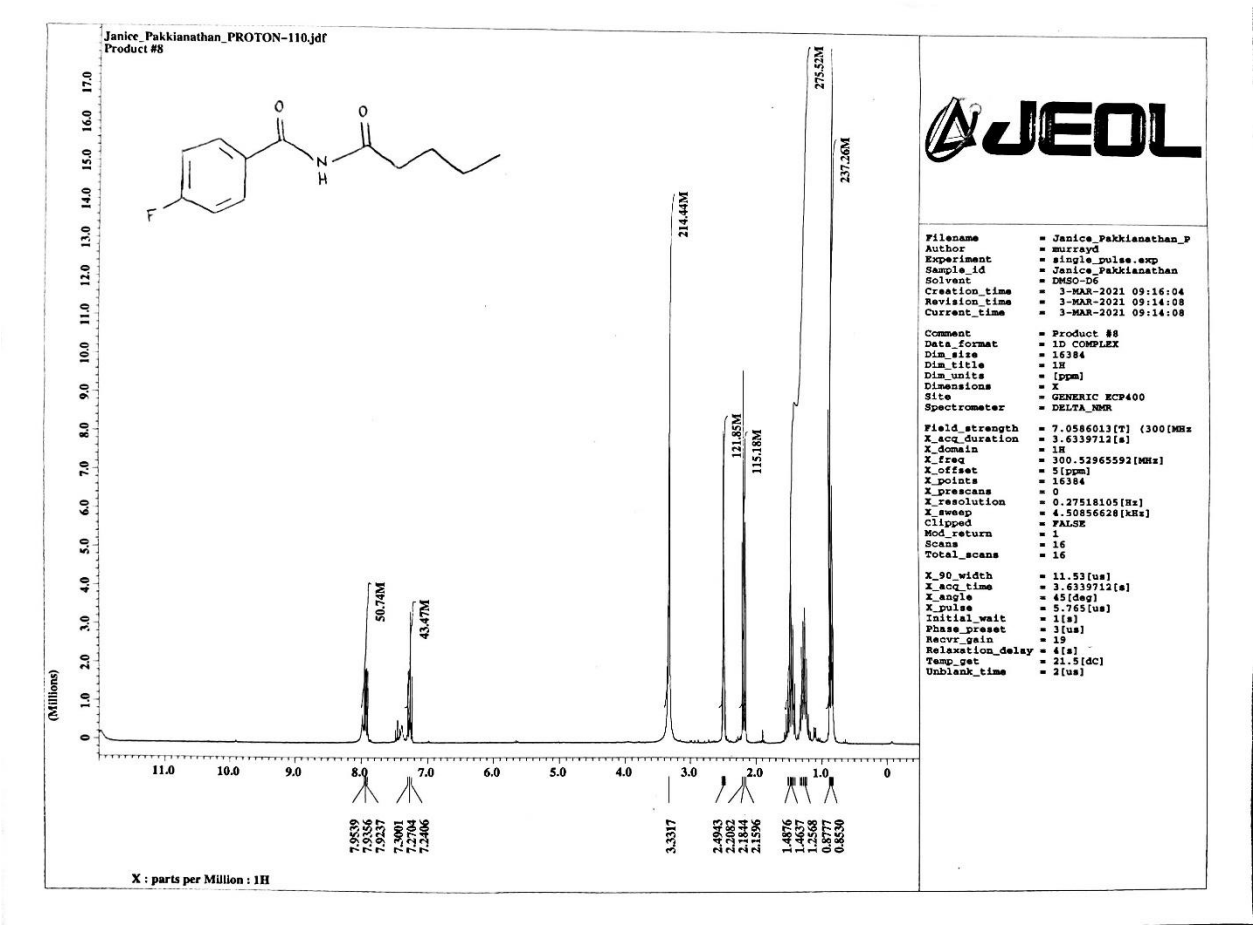

Figure 20. NMR Spectrum for Product #8.

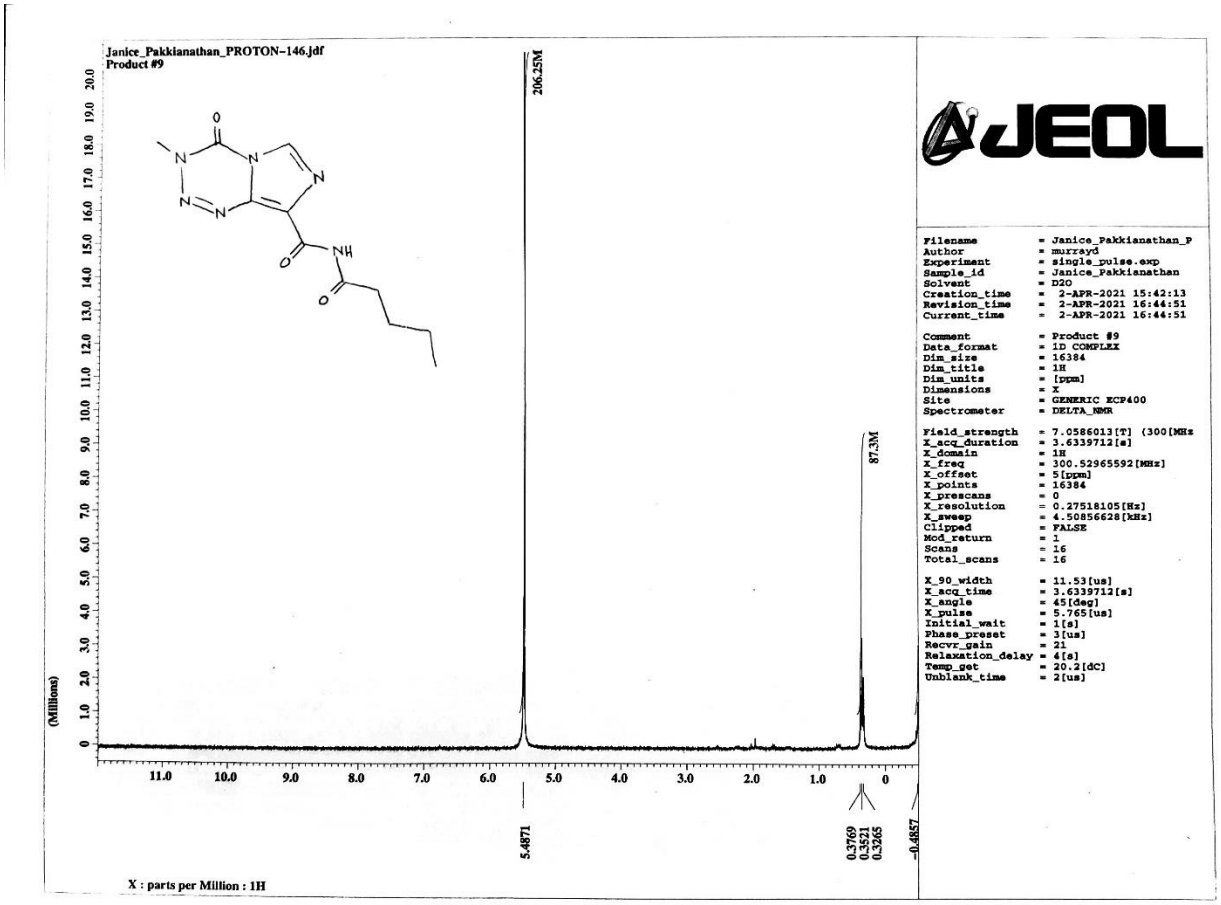

Figure 21. NMR Spectrum for Product #9.

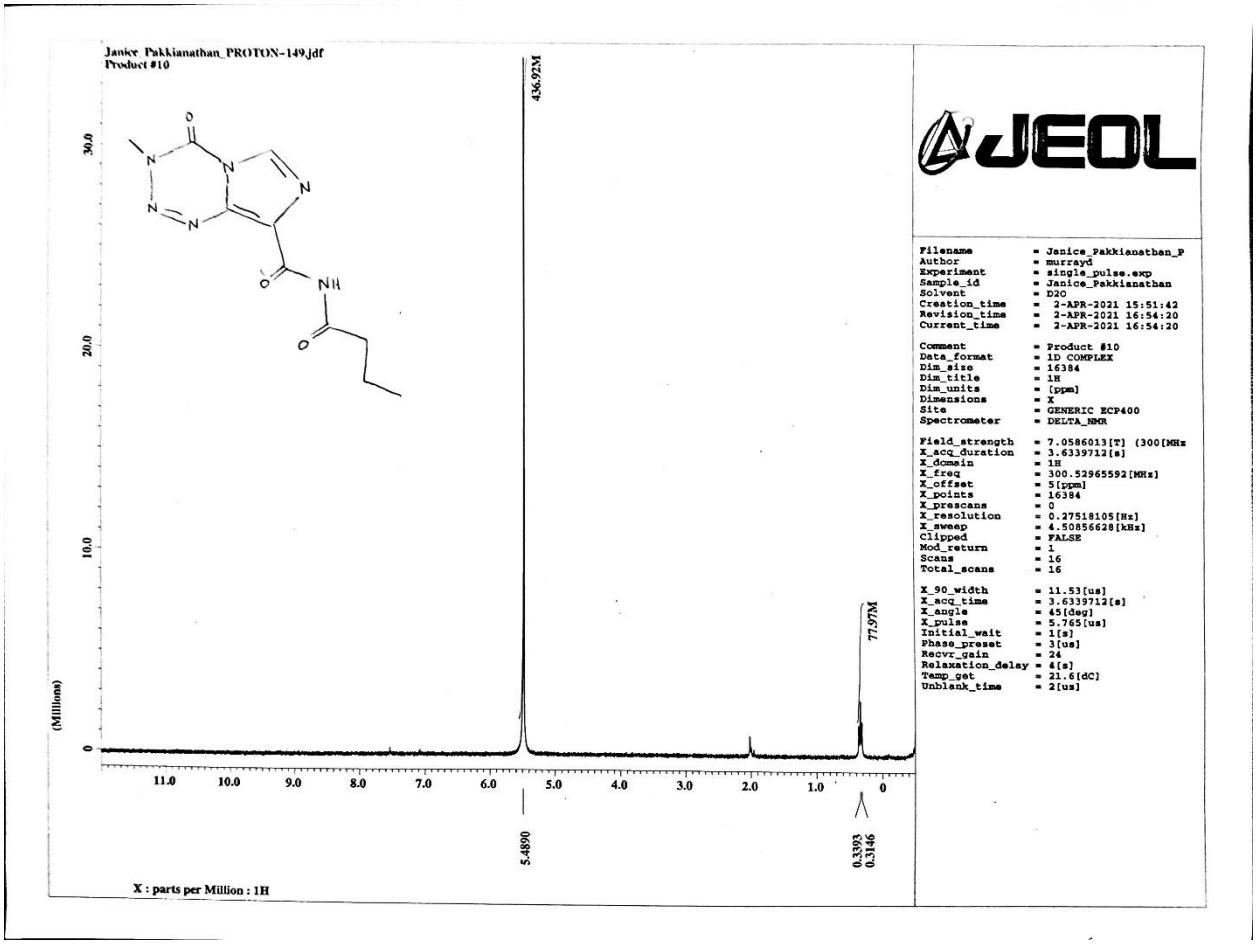

Figure 22. NMR Spectrum for Product #10.