In Vivo Inhibition of Carbonic Anhydrase using Click Chemistry

In Vivo Inhiberen van Koolzuur Anhydrase met behulp van Click Chemie

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Summary

The main objective of this research is to find conditions for chemical coupling reactions which are compatible with a complex biological environment. To accomplish this two things were needed: 1) a reaction that can be performed under mild conditions compatible with biological conditions and 2) a very sensitive analysis technique for the detection of low substrate and product concentrations. In this study we used the click reaction between an azide and an acetylene in the presence of a copper(I) catalyst since it tolerates many reactions conditions including biomolecules. For analysis, fluorescence spectroscopy was explored since it can detect very low concentrations of fluorescently active molecules in complex media. Since fluorescence spectroscopy has never been used before to monitor catalytic reaction, also HPLC-analysis was used as a control reference.
Using this strategy, we developed new click catalysis conditions using 3-azide-7-hydroxycoumarin (**1**) and 2-azide-*N*-benzyl-3-methylbutanamide (**6**) and we tested three different catalysts. The click product of **1** showed much higher fluorescent emission than **1** and thus we successfully developed a fluorescence-based analysis technique that could be used to monitor this reaction at even very low concentrations (~2 mM). In addition, we showed that this reaction depends greatly on the catalysts used, catalyst **2** being the best at these diluted conditions. Finally, we also performed a click reaction in which a biologically active product is formed at much higher concentrations (~100 mM) using the same catalysts. Here we observed catalyst **1** was the best catalyst, which is probably due to concentration effects.

Samenvatting

Het doel van dit project is het vinden van katalytische chemische reactie die kunnen worden uitgevoerd in biologisch oplossingen. Om dit te bereiken zijn er twee dingen nodig: 1) een milde reactie die kan worden uitgevoerd onder biologische omstandigheden en 2) een gevoelige analyse techniek voor het detecteren van lage substraat en product concentraties. In dit project is er gebruik gemaakt van click chemie tussen een azide en een acetyleen met behulp van een kopper(I) katalysator aangezien deze reactie veel condities tolereert waaronder biomoleculen. Voor analyse is fluorescentie spectroscopie gebruikt omdat deze zeer lage concentraties van fluorescerende moleculen kan detecteren. Omdat fluorescentie spectroscopie nog nooit is gebruikt om katalyse mee te volgen is HPLC als een controle/referentie analyse techniek gebruikt.
Met deze strategie zijn er twee click katalyses gekozen, met 3-azide-7-hydroxycoumarin (**1**) en 2-azide-*N*-benzyl-3-methylbutanamide (**6**) als azide substraten. Verder zijn in deze reactie drie katalysatoren getest. Het click product van **1** geeft een veel hogere fluorescente emissie dan **1** en dus hebben we succesvol een fluorescentie analyse techniek ontwikkeld waarmee we deze reactie kunnen volgen bij lage concentraties (~2 mM). Daarbij hebben we ook aangetoond dat het verloop van de reactie zeer afhankelijk is van de katalysator. Katalysator **2** was de beste onder deze erg verdunde omstandigheden. Uiteindelijk hebben we ook een click reactie gedaan met **6**, die een biologische actieve stof vormt, bij veel hogere concentraties (~100 mM) waarbij gebruik werd gemaakt van dezelfde katalysatoren. Hierbij zagen we dat katalysator **1** de beste was. Dit verschil tussen katalysator snelheden in beide reacties word waarschijnlijk veroorzaakt door concentratie effecten.

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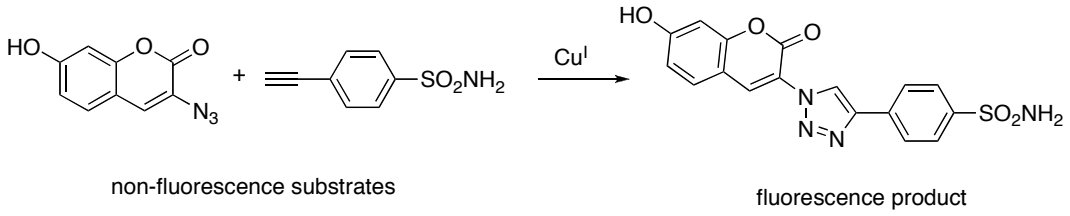
1. Introduction

Chemical biology has received a lot of attention in recent years. It uses chemical tools to study biological processes. The ideal is to study the (often unknown) functions of many proteins in the human body. This is a very important research for finding new methods to treat diseases. Many diseases occur when a protein does not work properly anymore and because more then one protein often trigger functions of organs it is difficult to determine which protein fails. One way to find out how a specific protein works is by tagging or blocking the active side of a protein. So far most chemical biological methods to study proteins are done *in vitro*[a]. Suitable chemical transformations to study proteins *in vivo*[b] are still lacking. However the best information is obtained when a specific protein is studied in its natural environment. But performing reactions in a cell of organisms or in biological media requires certain demands. Biological substances are fragile and cannot handle extreme reaction conditions like heat or organic solvent. The reactions have to occur in biological medium and they should tolerate many functionalities. The reaction should not produce any potentially toxic side-products. One of the few reactions that fulfils these criteria is the so called click reaction. The most famous click reaction is the cycloaddition reaction between an azide and an acetylene and this reaction occurs at very mild (in biological media) conditions only in the presence of a copper(I)-catalyst. This is the type of reaction that will be explored in this study.

The goal is to make a biologically active product using the click reaction that subsequently interacts with a specific protein, in this study carbonic anhydrase. To achieve this we must develop a suitable catalytic protocol for the click reaction to occur under *in vivo* reaction conditions. For this purpose a click reaction is studied that produces a fluorescent product from non-fluorescent substrates. The reactions that will be studied are depicted in *Scheme 1.1*. Since the products are fluorescent it is possible to follow the reaction through time using fluorescence spectroscopy.

a.



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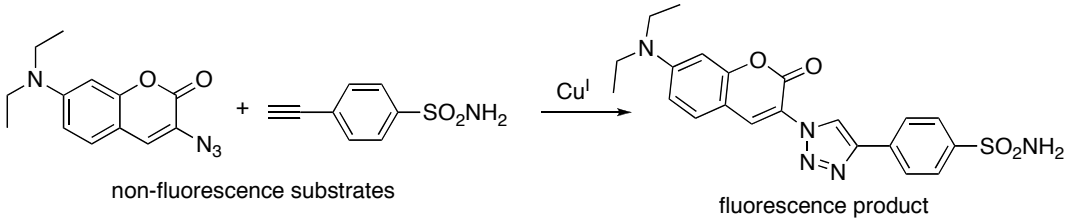
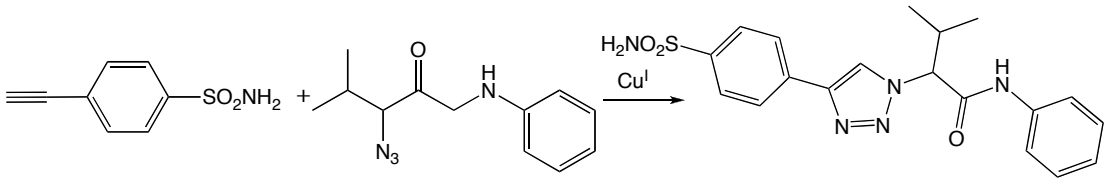


Scheme 1.1. Click reaction non-fluorescence substrates giving a fluorescence product.

[a] *In vitro*: Adjective & adverb biology (of processes or reactions) taking place in a test-tube, culture disk ore elsewhere outside a living organism.
[b] *In vivo*: Adjective & adverb biology (of processes) taking place in a living organism

For *in vivo* conditions, it is clear that the catalyst has to perform in the presence of biomolecules. The click reaction is already used for this purpose, but the reaction conditions are not compatible for in *vivo* reaction condition and thus the reaction has only been done *in vitro*. Therefore the most suitable copper(I)-catalyst must be selected. Once suitable catalytic conditions are developed, the catalysis will be done inside vesicles. Many controls experiments has to be performed to make sure that the reaction really occurs inside the vesicle. For example it have be to tested whether the catalyst can be encapsulated inside the vesicle and whether the substrates and/or the product can diffuse through the lipid bilayer of the vesicle. It is also important to know if the substrates and/or the product can puncture to the membrane of the vesicles, since that can cause indicated leakage of the catalyst from the vesicles



Protein inhibitor of carbonic anhydrase

Scheme 1.2. Click reaction of protein inhibitor of carbonic anhydrase

Eventually we would like to perform the reaction depicted in *Scheme 1.2* inside vesicles and study if the product, an inhibitor of carbonic anhydrase, can inhibit carbonic anhydrase when it is encapsulated in the vesicle. If this is possible it is maybe also possible to do this in a living cell.
In the theoretical section an more in depth introduction will be given about chemical biology, click chemistry, homogenous catalysis, vesicles as models for cells and fluorescence spectroscopy. All these subjects are important for the research that will be conducted.

2. Theory

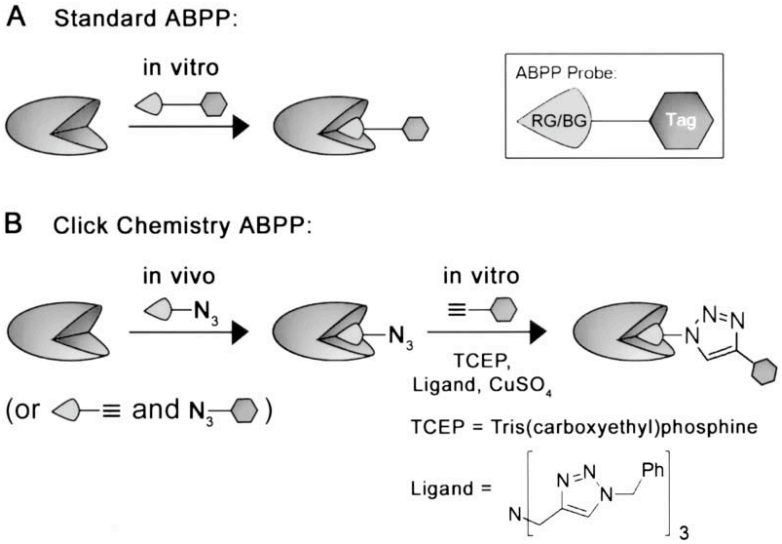
2.1 Chemical Biology

Chemical biology is a growing business and with reason. A lot about the human body is already discovered but there is even more to explore, especially in the area of genome and protein research. In this section the research of activity-based protein profiling (ABPP) will be outlined.[1]

ABPP makes use of the natural activity of the protein in order to study their functions. In ABPP, an inhibitor binds to the active site of a specific protein there by knocking out the natural functions of the protein. Using such an active side approach assures that only the non-natural fragment (the inhibitor) is introduced into the protein. By introducing a diagnostic tag into the inhibitor, it is possible to study/profile the function of proteins utilizing the specific physical properties of the tagged-inhibitor. The tagged proteins can be analyzed in many ways one of the most common ways is with 1D-SDS-PAGE. It is similar to, in the chemistry, TLC only 1D-SDS-PAGE works from up to down. It separates the proteins according to their weight. The heavier the protein the lower it is on the gel and to determine the weight a cocktail of known proteins are run with your tagged proteins. With UV light it is possible to see what protein is tagged and with the side run you know in what weight class it is. And in this way there are more analytical possibilities like micro arrays and LC-MS/MS.

But there is downside in the studies so far. All of the protein tagging has been done (partly) *in vitro*. However, it is known that several proteins often trigger the catalytic reactions in the human body and thus when a protein is studied *in vitro* it does not give accurate information about its behavior inside the body. That is why studies are nowadays are focusing on achieving ABPP studies *in vivo*. A good example is a study of by Cravatt.[2] In this study they study the possibility of labeling aldehyde dehydrogenase 1 (ALDH-1) *in vitro* and *in vivo*. *Scheme 2.1* shows how they compared the two protocols. The second protocol is not completely *in vivo* since they could not do the click reaction *in vivo*.

They studied what worked best by using either an acetylene probe or an azide probe. This is done by labeling, with a rhodamine tag, the ALDH-1 protein out of the liver of mouse *in vitro*. The *in vivo* labeling was done inside the mousses themselves and they were sacrificed after an hour. The proteins who were labeled *in vivo* were tagged with rhodamine acetylene or azide tag. The result was that the acetylene probe had a greater fluorescence signal than the azide probe, so it labels better.
But still the primary goal of ABPP is to identify enzyme activities expressed in human diseases. Therefore they also studied the activities of protein form breast cancer cells.



Scheme 2.1 Comparison of standard and click chemistry ABPP.[2]

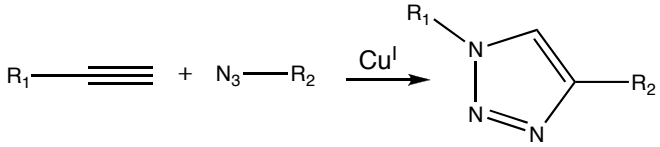
The proteins of breast cancer cells were labeled with the click reaction *in vitro* en *in vivo*. Both of the proteins of these cells were than analyzed. The results are that the *in vivo* prepare probe labeled better then those made *in vitro*. They were labeled with equal intensity probes. And by measure this the intensity of the tagged proteins it showed that the intensity of the *in vivo* proteins was higher and therefore the labeling was better.

These studies are the first step of labeling a protein in vivo. In this case they made a start but there is a believe that the use of click chemistry must be possible completely in vivo because of its properties.

2.2. Click chemistry

2.2.1. Basic of click chemistry between an azide and an acetylene

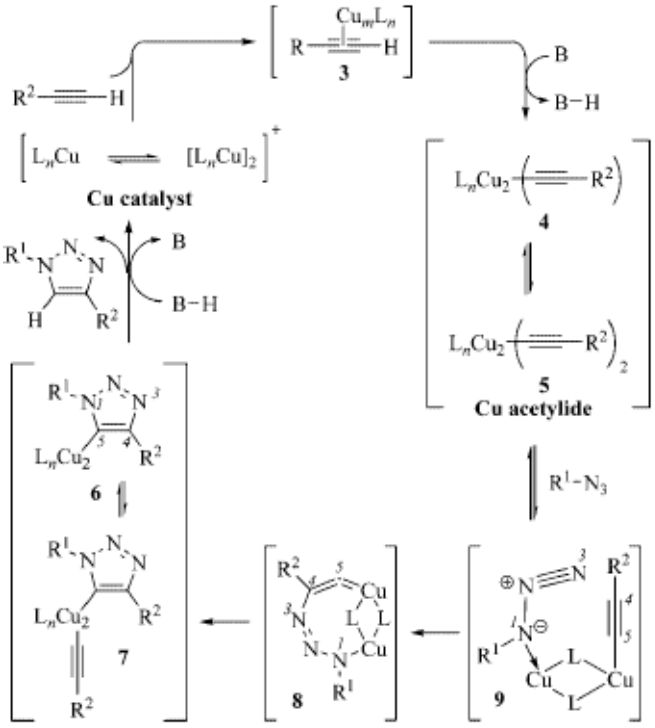
Click chemistry between an azide and an acetylene (*Scheme 2.2*) can be used for ABPP for many reasons.



Scheme 2.2 Click reaction between an azide and an acetylene.

The first reason is that only the 1,4 regioselectivity occurs when a Cu^{I}-catalyst is used versus the 1,5 regioselectivity that normally occurs with this cycloaddition when heated and no catalyst is used. Additionally, the reaction is 100% atom efficient, meaning that all atoms of the substrates are incorporated in the triazole product. This means no side-products formation and no purification needed[3]. Furthermore has the click reaction proven to be very robust. The reaction occurs in a variety of solvents, including aqueous *tert*-butyl alcohol or ethanol and, most importantly, in water without needing an organic co-solvent. The reaction is also suitable in a broad pH-range. The catalysis seems to work well at pH values ranging from approximately 4 to 12.[4] The yields are near quantitative.

Al these properties are ideal when the reaction has to occur in a biological environment. It has to able to proceed even in de presence of biomolecules and proteins should not interphere with the chemical click transformation.



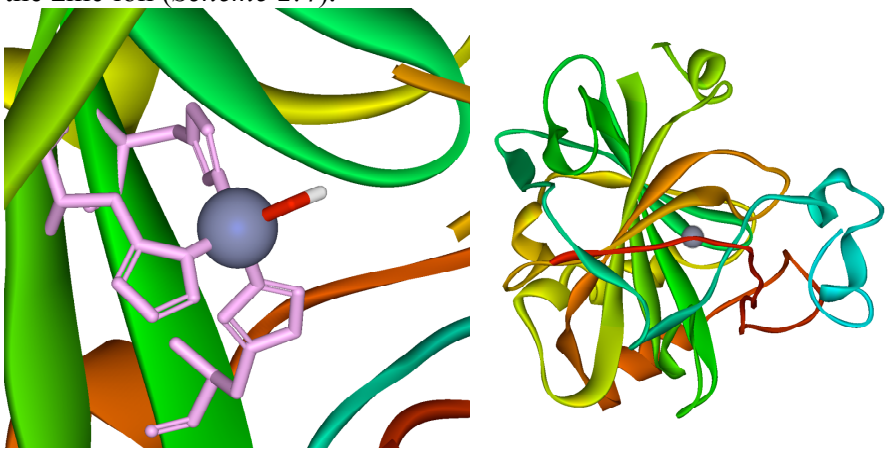
Scheme 2.3. Proposed reaction mechanism of the click reaction of an azide and an acetylene with a copper(I) catalyst.[5]

In *Scheme 2.3* the proposed reaction mechanism is showed. It starts with a formation of the acetylene Cu^{I}, adduct the π complex **3**. For the deprotonation a base is necessary. Because of the coupling of the Cu^{I} to the acetylene the pKa of the acetylene-H is decreased which makes it possible to deprotonate with adding a base. Next a dimeer is formed between Cu^{I} and ligands. The azide subsequently coordinates with the negatively charged nitrogen to the Cu^{I} adduct **9**. By a nucleophilic attack of the acetylene carbon C(4) at N(3) of the azide a metallocycle **8** is formed. This mechanism and experimental results suggest that electron-withdrawing substituents on the alkyne accelerate the Cu^{I}-catalyzed alkyne-azide coupling. Because of the transannular interaction of the N(1) and the C(5) the 1,2,3-triazole **7** can be formed. The transfer of **7** to **6** happens fast. Studies suggest that the protonation in the end occurs through intertaction with an external base. But further studies are needed to conclusively establish the proton source.[3]

As mentioned earlier, the catalytic reaction only occurs in the presence of a Cu^{I} catalyst. However, Cu^{I} is from itself not a stable ion and can oxidize to a Cu^{II}. Therefore in most literature procedures a Cu^{II}-salt is used as the catalyst precursor which is reduced *in situ* to active Cu^{I}. Often copper sulfate is used an sodium ascorbate or tris(carboxyethyl)phosphine as reducing agent. This method can be used for biological environments but not *in vivo*. The advantages, for this method, are; it is cheap and there is a high yield. That is why it is most commonly used as a catalyst.[3]

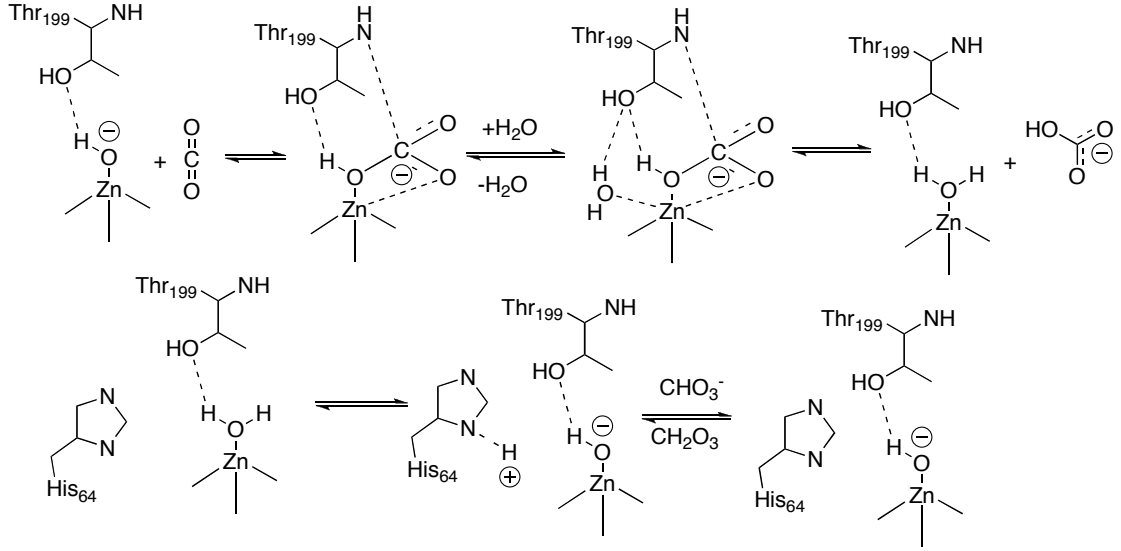
2.2.2 Carbonic anhydrase and its inhibitor

Carbonic anhydrase is a metalloënzyme. Metalloënzymes are enzymes where a metal is responsible for the activity of the enzyme. In the case of carbonic anhydrase this is the zinc ion (*Scheme 2.4*).



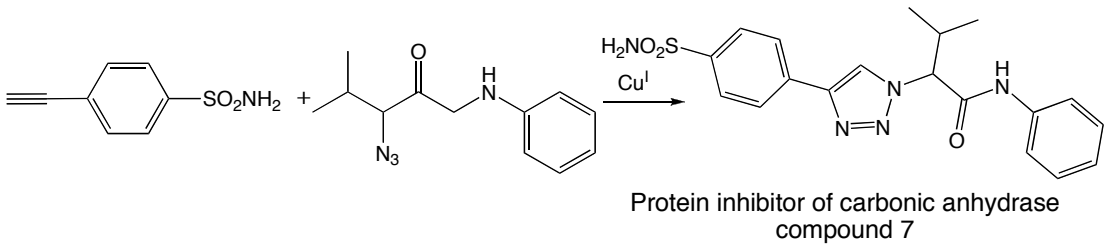
Scheme 2.4. Human carbonic anhydrase II, left side is the active side of the protein. The purple chains are histidine residues. Red/white chain is the hydroxide. The blue/grey sphere is the Sn^{II} ion. Right side is the whole ribbon diagram.[6]

It functions are to catalyze the reaction of carbon dioxide and water into protons and bicarbonate ions. The reaction is in balance. When there is a lot of carbon dioxide, for instance in muscles, bicarbonate is formed with water and when the bicarbonate is transport to for instance the longs the return-reaction into carbon dioxide and water occurs. The mechanism of carbonic anhydrase is depicted in *Scheme 2.5*.



Scheme 2.5. Reaction mechanism of carbonic anhydrase enzyme.[7] Thr is threonine and His is histidine. The number shows on which place in the chain the amino acid stands.

Carbonic anhydrase can be inhibit by any benzenesulfonamide. But the binding strength depends on the other functional groups present in the inhibitor. In former studies the inhibitor in *Scheme 2.6* has proven to have a strong binding capacity for carbonic anhydrase and can be formed by using click chemistry



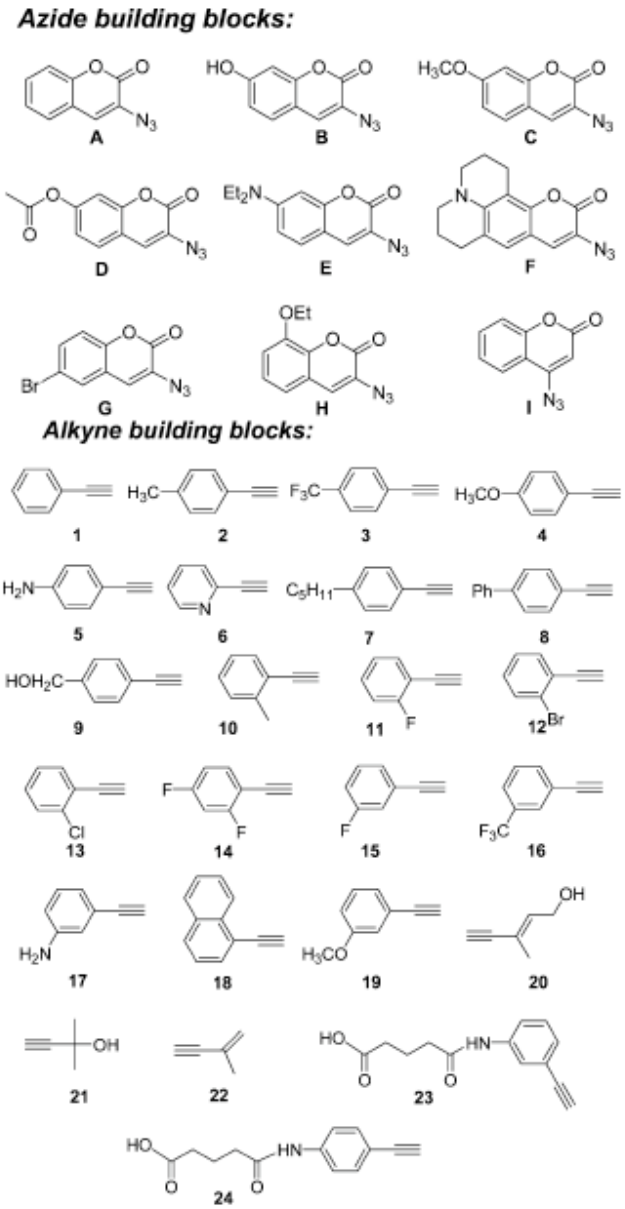
Scheme 2.6. Click reaction of the protein inhibitor of carbonic anhydrase

The sulfonamide does not bind covalent to the zinc ion of the protein. It makes hydrogen bounds with the zinc ion and the histidine of the protein.[8] That is part of the reason why there is a preference of the protein for the best inhibitor; different side groups play a part in the electro negativity of the sulfonamide and therefore influences the binding to the protein. Even though the bonds of the sulfonamide is not covalent, it blocks the possibilities of carbon dioxide ore bicarbonate ion to bind and this prevents the carbonic anhydrase catalysis of the protein.

2.2.3 Fluorescence click product for testing

As mentioned in paragraph 2.1 many studies of protein are done *in vitro* and there is made a start to perform reactions *in vivo*, however this still remains impossible. Fluorescent techniques are potentionally ideal to study proteins *in vivo*. In this part we will describe how fluorescence click products can be used for testing catalytic click reaction *in vivo*.
If you want to do a reaction inside a cell it is necessary to have a powerful technique to monitor the occurrence of the reaction. The fluorescent signal of a compound can be measured through a cell membrane.

Thus a reaction in which a fluorescent product is formed in ideal to develop a chemical transformation inside living cells. Since in cells one works at very low concentrations it is necessary to produce products with high fluorescent intensity. Preferentially. The substrates should not be fluorescently active in order to get the optimum result. For this purpose we will study the reaction depicted in *Scheme 2.7* and *Scheme 2.8* reported by *the department of chemistry & biochemistry, university of South Carolina.*[9]
They investigated the possibility of making a fluorescence product out of non-fluorescence substrates using click chemistry. They used the azides and acetelynes building blocks shown in *Scheme 2.7* The reaction mixtures were diluted to 1 or 10 μM for quantitative fluorescent detection. The excitation and emission maximum of the products were widespread. λmax^{Ex} = 298-445 nm; λmax^{Em} = 388-521 nm.



Scheme 2.7. The different building blocks for the fluorescence click product. The azides have a letter assigned to them and the acetylenes a number.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	w	w	w			w	m	m	m	w		w	w			w				w	w	w		
B	s	s	s	m	w	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s
C	m	s	s			s	m	s	m	m	m	s	m	m	m	m		w	w	w	m	w		
D	m	m	s	w		s	m	m	m	m	m	m	m	m	s	m			m	m	m	w	w	w
E	s	s	s	s	m	s	s	s	s	s	s	s	s	s	s	s	m	s	s	s	s	s	s	s
F	s	s	s	s	m	s	s	s	s	s	s	s	s	s	s	s	m	s	s	s	s	s	s	s
G	w	w	w	w			w	w	w	w	w	w					m							
H	w	w	w	w		w	w	m	w	w	w	w	w	w	w	w			w	w	w	w	w	w

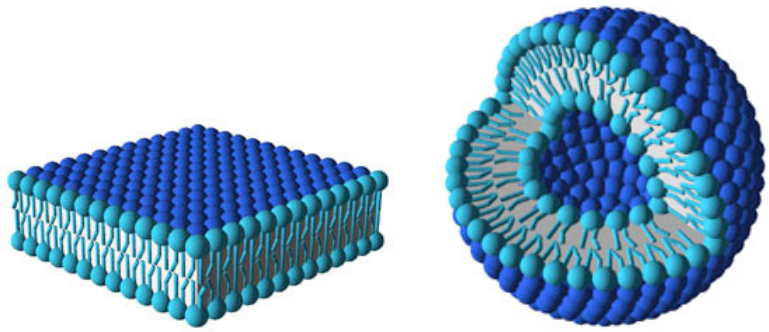
510-525 nm
500-509 nm
490-499 nm
480-489 nm
450-479 nm
420-449 nm
380-419 nm
non fluorescence

Scheme 2.8. Intensities and emission wavelengths of the final triazolocoumarin products. The rows and columns refer to different building blocks as indicated in *Scheme 2.6.* The relative fluorescent intensities are labeled with different letters, in which w=weak; m=medium; and s=strong. The color of each cell represents the emission wavelength.

It appeared that mainly the properties of the azide was important for the strength of the fluorescence of the click product (see *Scheme 2.8*) . From this data it is concluded that products obtained from azides B, E and F have the strongest fluorescence and therefore are most suitable for the purpose of this research. Because of the apolar group of compound F, which is not handy in a biological environment, the main focus will be on compound B and E.

2.3. Vesicles

As mentioned earlier, we want to develop a click catalysis protocol that can be performed inside living cells. Therefore it must be tested with model systems if the reaction cab really be performed in biological medium. Vesicles are very useful for this because the are cell models. Similar to living cells vesicles are made from a lipids that form a bilayer and form a spherical membrane, the vesicle (*Scheme 2.9)*.



Scheme 2.9. Schematic example of a bilayer and a vesicle.

Vesicles can be used in a variety of ways. [10] One of them is for drug delivery because the vesicle can encapsulate molecules in an aqueous solvent for transport purposes. Because of the hydrophobic membrane (the tails of the lipid) the aqueous solvent cannot get out of the vesicle. The transport can be completed by fusion with another bilayer such as that of a cell membrane. Vesicles can also be designed to deliver drugs in other ways. Vesicles that contain low (or high) pH substance can be constructed such that dissolved aqueous drugs will be charged in solution. As the pH naturally neutralizes within the liposome (protons can pass through a membrane), the drug will also be neutralized, allowing it to freely pass through a membrane. These vesicles work to deliver drug by diffusion rather than by direct cell fusion. Another strategy is to trigger the immune system so that the macrophages attack the vesicles and destroy it and in that way the drug is released. This shows already how flexible vesicles are and it also demonstrates the possibility to encapsulate molecules, e.g. catalysts.

An example of such a study is the enzymatic reaction of α-chymotrysin with 2 different substrates, the long chained succinyl-L-Ala-L-Ala-L-Pro-Phe-p-nitroanilide and the sort chain benzoyl-L-Tyr-p-nitroanilide. [11] α-Chymotrysin was entrapped in vesicles, the substrates were dissolved in the aqueous solvent surrounding the vesicles. The enzyme could normally react with both substrates, however following the kinetic constants and determining the mixture inside the vesicles it became clear that the bulky long chain nitroanilide could not diffuse through the membrane of the vesicle and reach the enzyme. The short chained nitroanilide on the other hand was able to react to the enzyme, leading to hydrolysis of the substrate. This clearly shows

how vesicles, like real cells, can discriminate between substrates and how it can separate the inside from the outside. This is ideal for the research described in this report.

In conclusion it is without a doubt that the chemical biology will play a major role in elucidating the function of proteins in living organisms. One step that we still need to make is the ability to perform chemical transformations *in vivo*, e.g. living cells. Although click chemistry fulfils many of the requirements, new conditions required for *in vivo* applications vesicles seem ideal model systems for living cells to develop such conditions. In the research described in this report are initial attempts to reach that goal.

3. Experimental

General section:
The following compounds were purchased

- 2,4-dihydroxybenzaldehyde 98%, Aldrich
- 4-diethylaminosalicylaldehyde 98%, Aldrich
- Acetic acid p.a., 96%, Acros
- Acetic anhydride p.a., Acros
- Acetonitrile 99%, Acros
- Anhydrous sodium acetate, Acros
- Copper(II)sulfate pentahydrate, Acros
- Dicyclopentadienyl iron, Baker
- Ethylnitroacetate 98%, Acros
- Methanesulfonyl chloride 99.5%, Acros
- *N*-acetylglycine 99%, Aldrich
- Piperidine 99%, Acros
- Sodium acetate, Acros
- Sodium ascorbate, Acros
- Sodium azide, Acros
- Sodium nitrite 97+%, Acros
- Tetrabutylammoniumhexafluorophosphat 99%, Fluka
- Tin(II)chloride dihydrated 97%, Acros
- Triethylamine 99%, Acros

Analysis where performed on an Oxford NMR 300 Varian for ^{1}H-NMR, Perkin Elmer spectrum one FT-IR spectrometer for IR, Perkin Elmer series 200 diode array detector and pump with the column GraceSmart RP 18 5u, Lot NO. 39/138, 250 mm x 4.6 mm SN: 0170100055, PN 5138810, Hardware type: female (B) for HPLC and for fluorescence Fluorolog Spex 1680 0.22m double spectrometer, with a split of 1 mm.

3.1. Synthesis of 3-azide-7-hydroxycoumarin (compound 1).[9]

A mixture of 2,4-dihydroxybenzaldehyde (2.76 g 20 mmol), *N*-acetylglycine (2.34 g 20 mmol) and anhydrous sodium acetate 4.92 g (60 mmol) in acetic anhydride (100 ml) was refluxed for 4 hours. The mixture was poured over ice resulting in a yellow precipitate subsequently. The yellow solid was filtrated and washed with ice-cold water. The solid was refluxed in HCl(conc.):EtOH (20 ml:10 ml) mixture for an hour. The reaction mixture was cooled to room temperture and ice water was added to the mixture to dilute it. The mixture was placed in an ice bath and sodium nitrite (2.76 g 40 mmol) was added to the mixture and the resultingg mixture was stirred for 15 minute. Next sodium azide (3.90 g 60 mmol) was added in small portions to the mixture and after complete addition the mixture was stirred for another 15 minute in the ice bath. The resulting precipitate was filtered and washed with water. The 3-azide-7-hydroxycoumarin was dried *in vacuo*. The brown solid was collected and stored cooled. ^{1}H-NMR (DMSO: 300 MHz) δ 6.74 ppm (d,$^{4}J_{H,H}$ 3) ArH, 6.78 ppm (dd, $^{3}J_{H,H}$ 9; 3.6) ArH, 7.45 ppm (d, $^{3}J_{H,H}$ 9) ArH, 7.56 ppm (s) ArH, 10.5 ppm (s) H from OH. IR 2100 cm $^{-1}$ N_3. Attachment 1: ^{1}H-NMR spectrum, IR spectrum and reaction mechanism

3.2. Synthesis of 3-azide-7-diethylaminocoumarin (compound 2).[9]

A mixture containing n-butanol (20 ml), 4-diethylaminosalicylaldehyde (1.4 g 7.2 mmol), ethylnitroacetate (0.8 ml 7.2 mmol), piperidine (0.1 ml 1.0 mmol), acetic acid (0.2 ml 3.5 mmol) and molsieves 4 Å was refluxed for 24 hours. Upon cooling the mixture to room temperature, a yellow solid was formed. The solid was colleted and dissolved in DMF (15 ml)at 80 °C. This mixture was filtrated fast to remove the molsieves 4 Å. The filtrate was poured over 100 ml ice-cold water. Resulting in the precipitation of an orange solid. Over a period of one hour ,the orange solid was added in small portions to a mixture of HCl conc. (40 ml) and tin(II)chloride dihydrated (12.2 g 54 mmol). When everything was added stirring was continued for an other 4 hours at room temperature. The mixture was poured over 20 g ice and made alkaline with sodium hydroxide (4M) at 15 °C using an ice bath. The suspension was extracted with 2x100 ml diethyl ether, the organic layer was washed with alkaline water (100 ml) and dried with anhydrous sodium sulfate. The solvent was evaporated *in vacuo* leaving a pasty residue. The pasty residue was triturated with hexane (30 ml). The yellow solid was collected. The solid was slowly dissolved in 17.2% HCl (30 ml) at room tempeture. This mixture was cooled to 0-5 °C and sodium nitrite (223.9 mg 3.2 mmol) was added stirred for an hour at 0 °C. Next sodium acetate (37.5 ml of 4.9 μmol/ml) was added to make the pH of the solution 4. Sodium azide (425.3 mg 6.4 mmol) was added in portions at a temperature of 0-5 °C. After complete addition the mixture was stirred for another 5 hours. The precipitated product was filtrated rapidly and washed with ice-cold water. The 3-azide-7diethylaminocoumarin was dried *in vacuo* and the compound stored stored at -20 °C. H^1-NMR ($CDCl_3$: 300 MHz) δ 1.2 ppm (t, $^3J_{H,H}$ 30) 6 H 2xCH_3, 3.4 ppm (q, $^3J_{H,H}$ 30) 4 H 2xCH_2, (DMSO: 300 MHz) δ 6.6 ppm (s) ArH, 6.7 (dd, $^3J_{H,H}$ 12) ArH, 7.4 ppm (d, $^3J_{H,H}$ 9) ArH 7.5 ppm (s) H. IR 2100 cm^{-1} N_3. Attachment 2: ^{1}H-NMR spectrum, IR spectrum and reaction mechanism

3.3. Click reaction of 3-azide-7-hydroxycoumarin and p-ethynylbenzenesulfonamine (compound 4).[9]

Compound **1** (34.5 mg 0.17 mmol) and the p-ethynylbenzenesulfonamide, compound **3**, (30.8 mg 0.17 mmol) was dissolved in a mixture of ethanol and water (1:1, 5 ml). Freshly prepared 1 M sodium ascorbate (34 μl 0.034 mmol) was added, followed by the addition of copper(II)sulfate pentahydrate (28 μl 0.0085 mmol, 7.5 mol%). This mixture was stirred overnight at room temperature in the dark. The ethanol was removed under reduced pressure and the residue was diluted with 5 ml in ice cooled water. The precipitate was collected by filtration. It was washed with ice cold water and dried *in vacuo*. H^1-NMR (DMSO: 300 MHz) δ 6.86 ppm (s) ArH, 6.92 ppm (d, $^3J_{H,H}$ 6) ArH, 7.7 ppm (m) 2 H amine ARH, 7.91 ppm (d,$^3J_{H,H}$ 6) 2 ArH, 8.14 ppm (d, $^3J_{H,H}$ 6) 2 ArH, 8.68 ppm (s) ArH, 9.14 ppm (s) H6. Attachment 3: ^{1}H-NMR spectrum

3.4. Click reaction of 3-azide-7-diethylaminocoumarin and p-ethynylbenzenesulfonamide (compound 5).

Compound **2** (43.9 mg 0.17 mmol), compound **3** (30.8 mg 0.17 mmol) and cat. **2** (5.4 mg 0.025 mmol) were placed in a schlenk tube and put under nitrogen atmosphere. Under a nitrogen flow, 5 ml dry and degassed acetonitrile was added. Stir the mixture overnight under room temperature. The precipitate is filtrated and carefully washed with acetonitrile leaving en light brown grey solid. This was dried *in vacuo*. H^1-NMR (DMSO: 300 MHz) δ 1.1 ppm (t) 2x CH_3, 3.5 (m) 2x CH_2, 6.7 ppm (s) ArH, 6.8 ppm

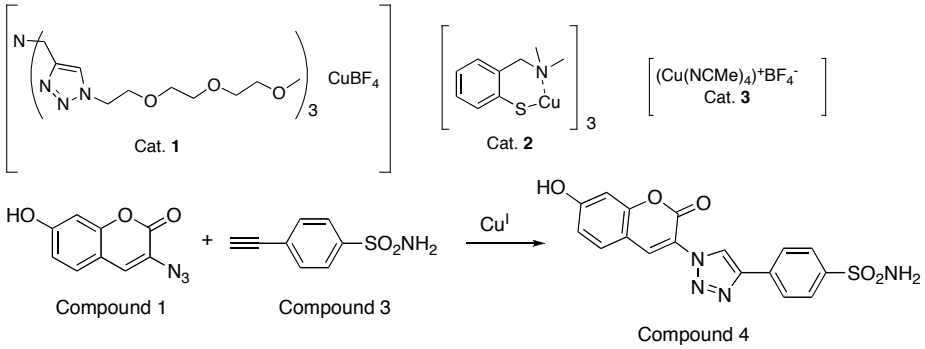
(d,$^{4}J_{H,H}$ 6) ArH, 7.3 ppm (s) 2 H amine, 7.6 ppm (d, $^{3}J_{H,H}$ 6) ArH, 7.9 ppm (d, $^{3}J_{H,H}$ 6) 2x ArH, 8.1 ppm (d, $^{3}J_{H,H}$ 6) 2x ArH, 8.5 ppm (s) ArH, 9.1 ppm (s) H. IR the azide peek at 2100 nm has disappeared. Attachment 4: ^{1}H-NMR spectrum and IR spectrum

3.5. Preparation of Cat. 1

The tris triazoleligand (390 mg 0.56 mmol) and [Cu(NCMe)$_4$](BF$_4$) (160 mg 0.56 mmol) were dissolved in 5 ml methanol and this solution was stirred for an hour. The solvent was evaporated giving a sticky oil. The oil was dissolved in 1 ml methanol and on addition of diethyl ether a sticky oil came out of the solution. The oil was collected and dried *in vacao*. This reaction is carried out under nitrogen atmosphere. ^{1}HNMR (Acetone: 300 MHz) δ 3.3 ppm (s) CH3, 3.5 ppm (m) 4x CH_2, 3.9 (s) CH_2, 4.1 ppm (s) CH_2, 4.7 ppm (s) CH_2, 8.1 ppm (s) H triazole. In attachment 5a cat. **1** is showed in 5b the ligand. Because of the ligand coupling the protons in the complex near the copper have limited rotation freedom, which cause the peak broadening. Attachment 5: ^{1}H-NMR spectra.

3.6. Catalysis of reaction 1

Catalysts



30 mol% catalyst

Cat. 1	12.7 mg
Cat. 2	3.4 mg
Cat. 3	4.7 mg
Cat. 3 + base 2,6-lutidine	4.7 mg + 5.8 µl

All catalytic experiments were performed under a nitrogen atmosphere. Compound **1** (10.2 mg 0.05 mmol) and compound **3** (9.1 mg 0.05 mmol)were placed in a schlenk tube with a stirrer under nitrogen. This mixture was dissolved in degassed acetonitrile (25 ml) and veratrole (25 µl), as internal standard, was added and the first sample* was taken. Next the appropriate catalyst mixture was added. The reaction was followed in time with HPLC and fluorescence.

* Sample for HPLC: 5 µl from the reaction mixture was directly injected. All measurements on the HPLC were done with the same column with 25% acetonitrile, 75% ammonium acetate buffer as eluens. for fluorescence 15 µl of the reaction mixture was diluted with 2985 µl acetonitrile to obtain a 10 µM solution. All measurements on the fluorescence spectrometer were done with an excitation of 386 nm and in the emission area from 400-550 nm.

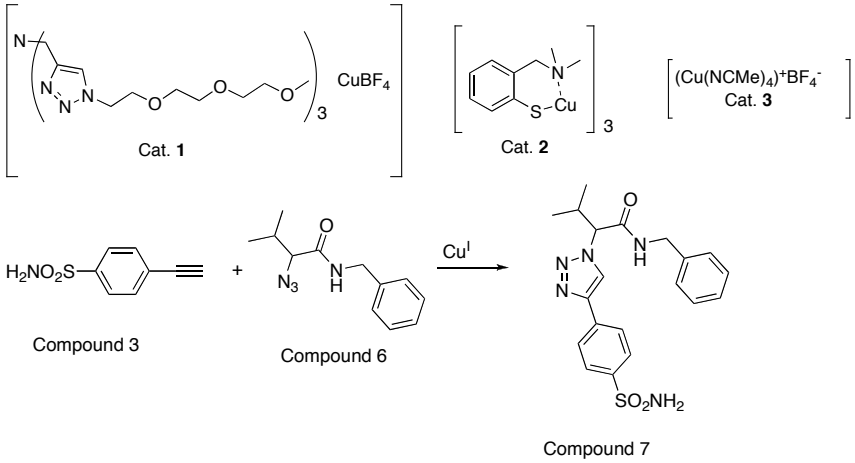
3.7. Synthesis of 2-azide-*N*-benzyl-3-methylbutanamide (compound 6)

N-benzyl-2-hydroxy-3-methylbutanamide (1.31 g 6.32 mmol) and triethylamine (1.8 ml 12.63 mmol) were mixed in dry THF (35 ml) and cooled with an ice-bath. Next,

methanesulfonyl chloride (0.54 ml 6.95 mmol) was added drop wise resulting in the immediate appearance of a white precipitate. This mixture was continued to stir overnight at room temperature. Next water (40 ml) was added and the product was extracted with dichloromethane (3 x 30 ml). The combined organic abstract was washed with water (40 ml) and dried with $MgSO_4$ and concentrated, leaving a yellowish oil that solidified upon standing.
The yellowish oil and sodium azide (500 mg 7.69 mmol) were mixed in DMF (75 ml) and this mixture was stirred for 3 days at 60 °C. Next, DMF was removed under vacuum and the sticky oil was participated between water (30 ml) and dichloromethane (30 ml). The organic layer was collected and the water layer was extracted with dichloromethane (2 x 20 ml). The combine organic layer was washed with water (20 ml), dried with $MgSO_4$ and concentrated leaving a slightly yellow oil. ^{1}HNMR ($CDCl_3$: 300 MHz) 1.0 ppm (dd, $^3J_{H,H}$ 60;6) 2x CH_3, 1.3 ppm (m) H, 2.4 ppm (m) H, 3.9 ppm (d, $^3J_{H,H}$ 3) H from NH, 4.5 ppm (m) CH_2, 7.3 (m) 5 ArH.
Attachement 6: ^{1}H-NMR spectrum and reaction mechanism.

3.8. Catalysis of reaction 3

Catalysts



5 mol% cat.

cat. 1	4.24 mg
cat. 2	1.07 mg
cat. 3	1.57 mg
cat. 3 + base 2,6-lutidiene	1.57 mg + 2 µl

All catalytic experiments were performed under a nitrogen atmosphere. Compound **6** (23.2 mg 0.1 mmol), compound **3** (18.1 mg 0.1 mmol) and veratrole, (50 µl), as internal standard were placed in a schlenk tude with a stirrer. The mixture was dissolved in degassed acetonitrile (1 ml). After taking the first sample* the appropriate catalyst mixture was added. The reaction was follow in time with HPLC.

*dilute 50 times: 10 µl reaction mixture in 490 µl acetonitrile. All measurements on the HPLC were done with an eluens of 60% acetonitrile and 40% ammonium acetate buffer.

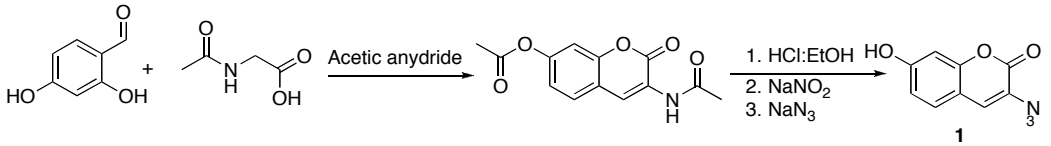
^{1}HNMR (DMSO) 0.85 ppm (dd, $^3J_{H,H}$ 75;6) 2x CH_3, 4.3 ppm (m) CH_2, 5.1 ppm (d, $^3J_{H,H}$ 9) H from NH, 7.2-7.4 ppm (m) 2 H from NH_2 and 5 ArH, 7.9 ppm (d, $^3J_{H,H}$ 4.5) 2 ArH, 8.1 (d,$^3J_{H,H}$ 4.5) 2 ArH, 8.9 ppm (s) H triazole, 9.1 (t, $^3J_{H,H}$ 3) H. Attachment 7: ^{1}H-NMR spectrum

4. Results and Discussion

In this research the aim is to perform a reaction in a biological environment and be able to follow this reaction, even at very low concentrations. To accomplice this, a new method had to be set up. The plan was to follow the reaction with fluorescence spectroscopy because this can be measured at very low concentrations. In this research we looked at three reactions shown in *Scheme 4.4.* Reaction **1** and **2** give a fluorescent product from non-fluorescent substrates. Reaction **3** is chosen because the product is a biological inhibitor of carbonic anhydrase, an protein involved in cancer development.

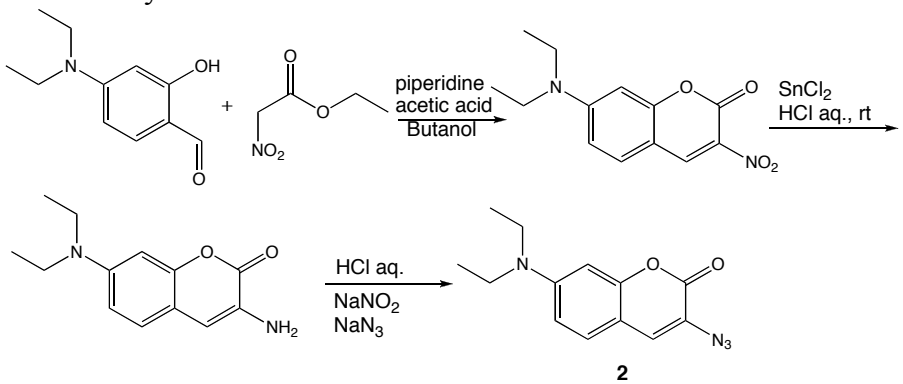
4.1. Synthesis

Based on the literature the choice was made to explore two possibilities for the fluorescence study: reactions **1** and **2**. The synthesis of compound **1** started by making an ester via an intramolecular aldol addition followed by water elimination (*Scheme 4.1*). Next amine and ester deprotection with HCl is followed by making a diazonium-salt with sodiumnitrite. Next the azide **1** is formed by a nucleophilic substitution with sodium azide. The yield was 12.5%. This low yield is caused by the fact that **1** is made in four steps including many washing steps.



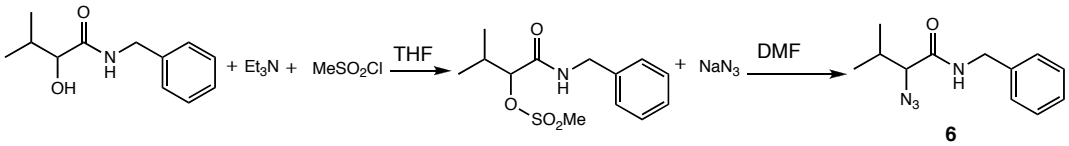
Scheme 4.1. Reaction of compound **1**

The synthesis of compound **2** started by making an ester via an intramolucular aldol addition and water elimination (*Scheme 4.2*). Next a reduction of the nitro group with HCl and tin(II)chloride was conducted followed by formation of a diazonium with sodiumnitrite. Finally, **2** was obtained by a nucleophilic substitution with sodium azide. The yield was 10.3%.

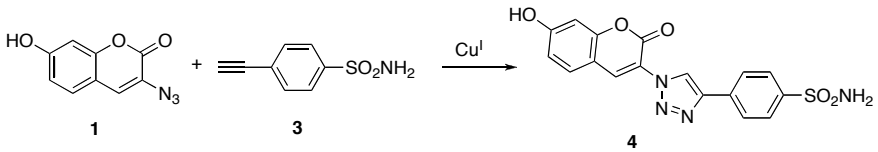


Scheme 4.2. Reaction of compound **2**

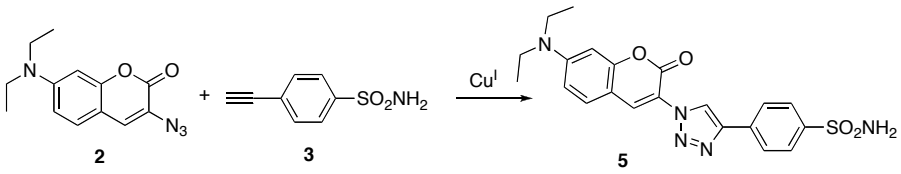
Compound **6** was made by making a mesylate ester using methanesulfonyl chloride, followed by a nucleophilic substitution with sodium azide (*Scheme 4.3*). The yield was 22%. This low yield was caused by the by the last step, which needed to be performed three times in order to get full conversion.



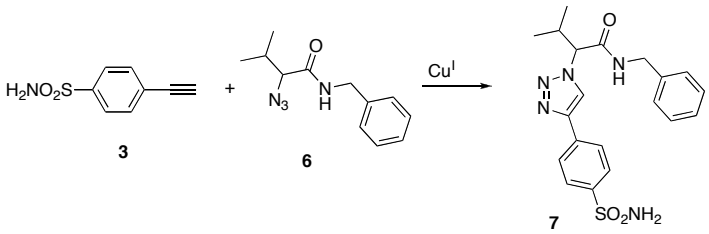
Scheme 4.3. Reaction of compound **6**



Reaction **1**



Reaction **2**



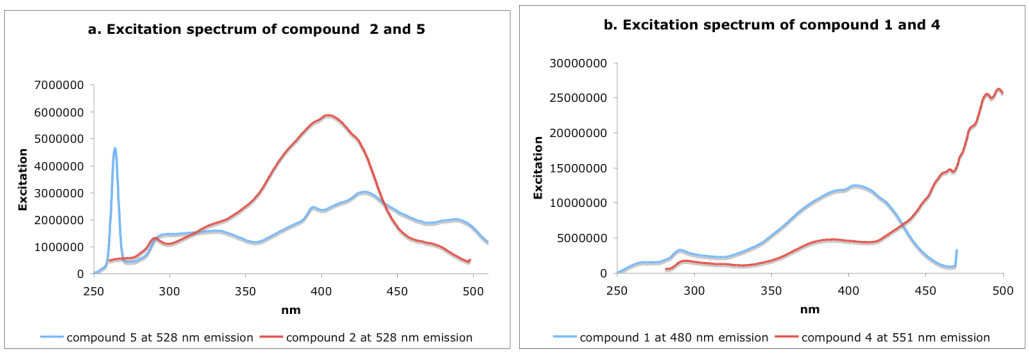
Reaction **3**

Scheme 4.4. Click reaction of compound **4**, **5** and **7**

With compounds **1**, **2** and **6** in hands, reaction **1**, **2** and **3** (*Scheme 4.4*) were conducted in MeCN with a copper(I) catalyst. Products **4**, **5** and **7** were obtained in good yields and were used to set up suitable fluorescent and HPLC analysis techniques for the click catalysis experiments.

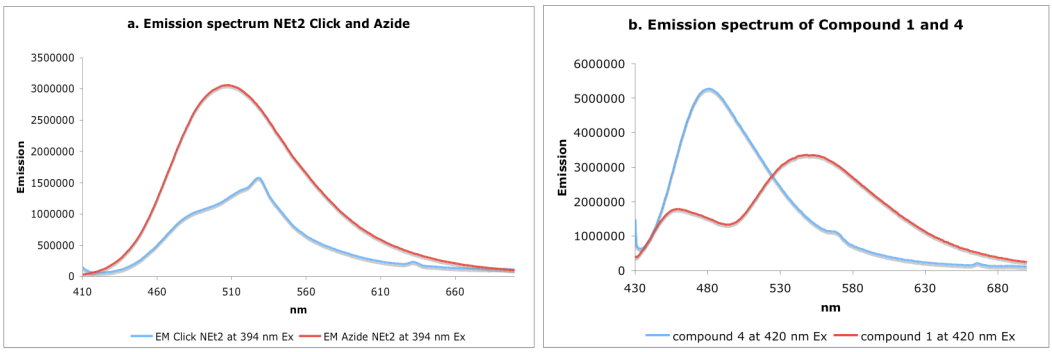
4.2. Analysis

Firstly, for the set up of the fluorescence, the excitation spectra of compounds **1**, **2**, **4** and **5** were measured to determine the maximum absorption wavelengths of these molecules (*Graph 4.1*). This was subsequently used to determine at what wavelength the emission of compounds **4** and **5** are high and those of compounds **1** and **2** are low. The wavelength with the largest difference in fluorescent emission between substrate and product will be used monitor the click reaction.



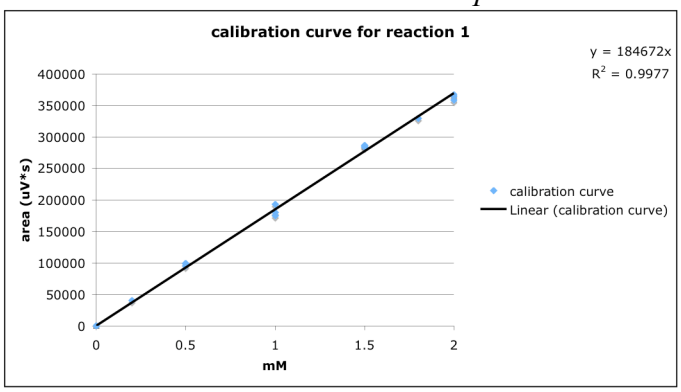
Graph 4.1. Excitation spectra of compound **2**, compound **5**, compound **1** and compound **4**.

The measurements were done in tris/triton buffer pH 8, because the final aim is to perform the click reactions in a biological environment. The excitation spectra of compounds **2** and **5** are shown in *Graph 4.1.a* . The difference in absorption was the highest at a wavelength of 394 nm with the highest absorption for compound **5**. Therefore the emission spectra of compound **2** and **5** were measured by exciting the solutions at 394 nm. *Graph 4.2.a* shows that the emission of substrate **2** is higher than of product **5**. Both compounds have a maximum at about 510 nm. The literature mentioned that compound **2** is non-fluorescence, but this was measured only in THF and because the strength of the fluorescence is solvent dependent, compound **2** apparently has a strong emission in tris/triton buffer. For compound **1** and **4** the emission spectra were measured by exciting the solution at 420 nm. At that point for these compounds the difference in absorption the was highest (see *Graph 4.1.b*). In *Graph 4.2.b* the emission spectra are shown. Also in this case the compound **1** is fluorescence but at the maximum of compound **4** (480 nm) compound **1** has a local minimum.



Graph 4.2. Emission spectra of compound **2**, compound **5**, compound **1** and compound **4**.

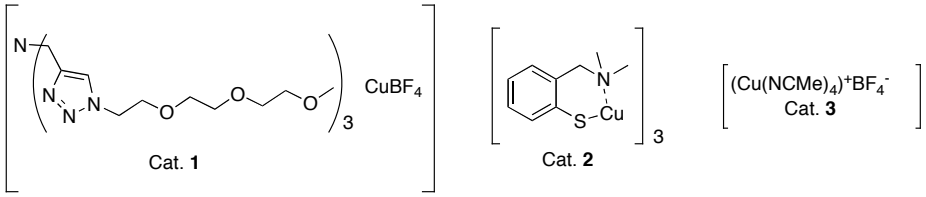
Based on these results we choose not to continue with reaction **2**. The fluorescence differences between substrate **2** and product **5** were to small and the product was less fluorescent than the substrate. Another advantage reaction **1** has is that the fluorescence of compound **4** is very strong and thus we decided to test this reaction using fluorescence spectroscopy as the analysis technique. Since fluorescence has never been used before for monitoring catalytic reaction, we decided to also use High Performance Liquid Chromatography (HPLC) as a control reference analysis technique. Therefore, we first made a HPLC calibration curve of compound **4** that can be used to monitor reaction **1**. In *Graph 4.3.* the calibration curve is shown.



Graph 4.3. HPLC calibration curve of **4** that was used to determine the conversion of catalysis of reaction **1**.

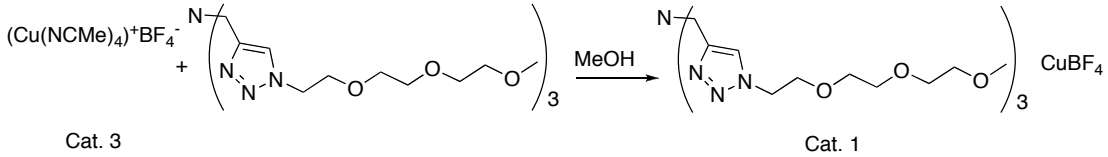
4.3. Catalysis

Thus, for the click catalysis we decided to only explore reaction **1** and **3** in *Scheme 4.4.* Furthermore we also wanted to investigate different catalysts for these reactions since so far no reports have been published yet in which the role of the catalysts in click reactions has been investigated. From homogeneous catalysis it is known that coordination of different ligands to the catalytic metal centre can have big advantages for the catalysis. In this research we looked at three possibilities: catalysts **1** – **3** (*Scheme 4.5*). The difference between the catalysts is the ligands that are bound to the metal centre. Cat.**3** was used as a control for cat. **1** because cat. **1** is prepared from of cat. **3** and cat. **3** is also often used in literature as a catalyst in click reactions.



Scheme 4.5. The catalyst that were tested in this research.

Cat. 1 is made by ligand exchange by mixing cat **3** with a tristriazole amine ligand (*Scheme 4.6*).



Scheme 4.6. Reaction of cat. **1**

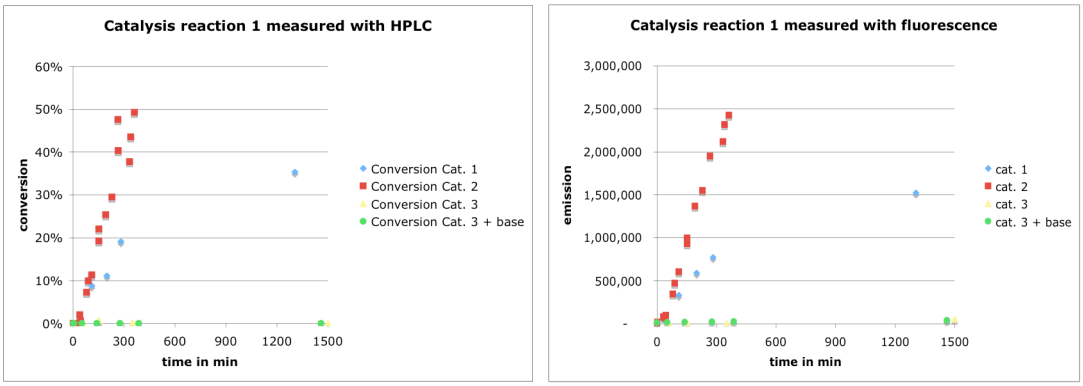
The catalysts were tested in reactions **1** and **3** and the reaction progress was monitored with HPLC and in case of reaction 1 also with fluorescence spectroscopy. The results and conditions are shown in *Table 4.1.*

catalyst	time in min at 20% reaction 1	time in min at 50% reaction 3
cat. 1	325	35
cat. 2	150	203
cat. 3	>>[a]	225
cat. 3 + base	>>[a]	265

Table 4.1. Results of catalysis of reaction **1** and **3**. a. below detection limit of analysis methode, thus conversion never exceeded 1%. Conditions reaction **1**: Compound **1** (2 mM), compound **3** (2 mM) in degassed acetonitrile with 30 mol% catalyst. Conditions reaction **3**: Compound **3** (100 mM), compound **6** (100 mM) in degassed acetonitrile with 5 mol% catalyst.

Reaction 1

Reaction **1** was follow in time with HPLC and fluorescence. In case of fluorescence analysis the samples taken were diluted to reach concentrations of ~ 10 μM (200 x dilution) In *Scheme 4.7.* the graphs are given.

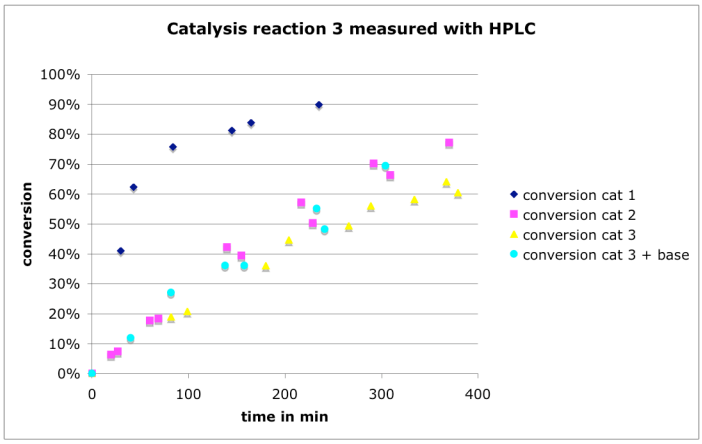


Scheme 4.7. Comparison of HPLC measurements and fluorescence measurements of the catalysis reaction **1**.

Cat. **2** was the best catalyst in this reaction. Cat. **3** could not perform this reaction, not even by addition of a base which is commonly done in literature. Because the behaviour of both curves are equal it is possible to calibrate the fluorescent results with the HPLC curve and convert emission intensities to conversion rates for this reaction at these conditions. These results clearly show that fluorescence spectroscopy can be used to monitor catalytic conversion and holds great promise for following reactions under very dilute conditions, *e.g.* in biological media.

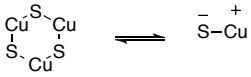
Reaction 3

Reaction **3** was followed in time with HPLC. In *Scheme 4.8.* the results are given. The calibration curve of reaction **3** is given in attachment 8.



Scheme 4.8. Catalysis results of reaction **3**.

Surprisingly, in reaction **3** cat.**1** is the fastest while reaction **1** cat. **2** is the fastest. This might come from solvability issues of cat. **2** in acetonitrile. Cat. **2** forms a trimer which adopts a six-member ring between the Cu atom and the S atom (*Scheme 4.9*). In this form the catalyst in thought to be inactive and therefore these bonds have to be broken before the copper(I) can function as a catalyst. Thus it can be because of the high catalyst concentration in reaction **3** as compared to reaction **1** that there are less active catalyst particles in solution and therefore the real catalyst concentration is lower and therefore the reaction is slower.



Scheme 4.9. Schematic representation of the six-membered ring formation of cat. **2** and its equilibrium with its monomer.

These results clearly show that the click catalysis is dependent on the catalyst used and also on the reaction conditions. Further study is necessary to fully understand the reactivity of the different catalysts in these particular reactions under the mentioned conditions.

5. Conclusion

The synthesis of compound **1**, **2** and **6** was accomplished successfully and they were applied in the click reaction to synthesise their corresponding click products **4**, **5** and **7**. From the fluorescence experiments performed with these compounds it appeared that compound **2** was not useful because the click product **(5)** has poor fluorescence in tris/triton buffer.

We have shown that reaction **1** can be conducted at low concentrations of 2 mM and we showed for the first time that the conversion of such a reaction can be determined with fluorescence spectroscopy. This was accomplished by following reaction **1** with both HPLC and fluorescence spectroscopy and by using the HPLC results to reference the fluorescent data. This creates the possibility to perform the reaction at even lower concentrations since the detection limit of fluorescence (1 – 20 μM) has not been reached yet.

Furthermore cat. **1**, cat. **2** and cat. **3** were tested in reactions **1** and **3**. From these results it can be concluded that cat. **2** and cat. **1** are the best catalyst and thus that ligand design is very useful to obtain better catalysts. In reaction **1** cat. **2** is the fasted catalyst followed by cat. **1** whereas with cat. **3** reaction **1** does not occur not even with addition of a base.
In reaction **3** cat. **1** was by far the best catalyst. The reason that cat. **2** is slower in reaction **3** might come from solvability issues with cat. **2** in acetonitrile.

6. Future work

In future research the catalytic behaviour of catalysts **1** and **2** in water at the same condition of reaction **1** has to be studied. Also lowering the concentration more and more until the point that concentration compatible with biological conditions are reached (<20 μM) have to be explored . When this goes well the reaction will be studied in the presence of biomolecules, inside vesicles as models for cells and in complex biological media.

7. Reference:

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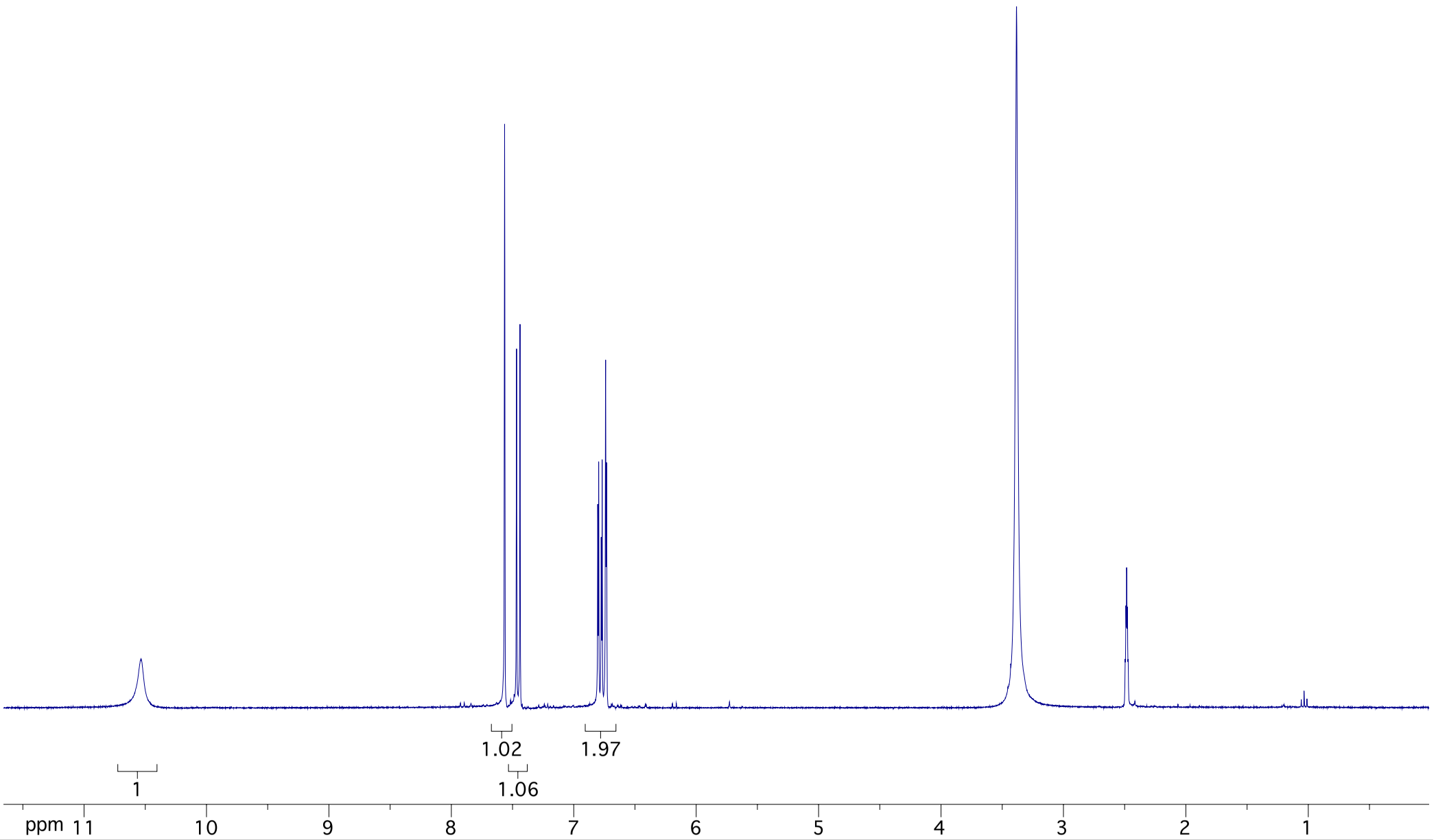
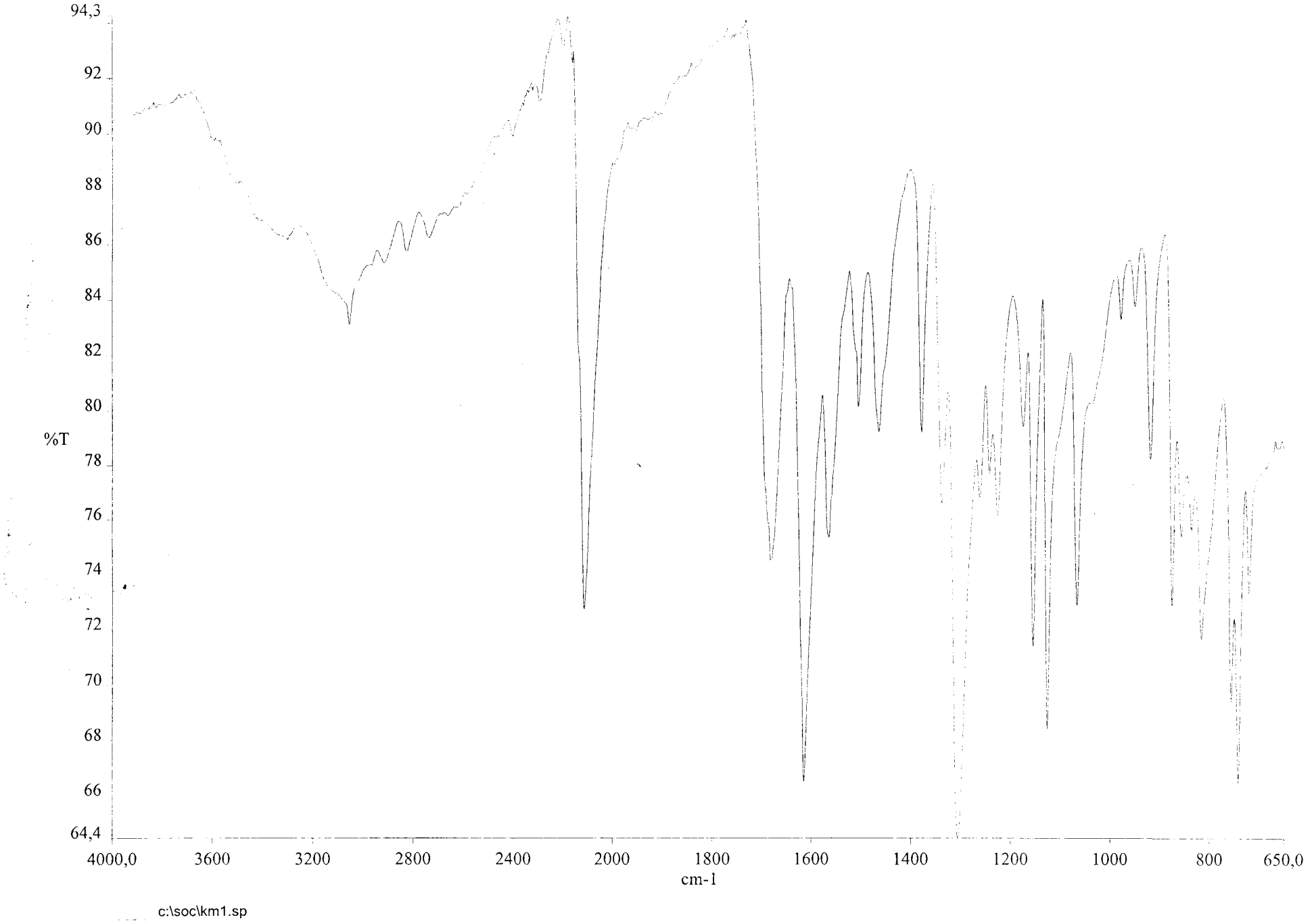
8. Attachments

Attachment 1

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3600
3200
2800
2400
2000
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1600
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1200
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800
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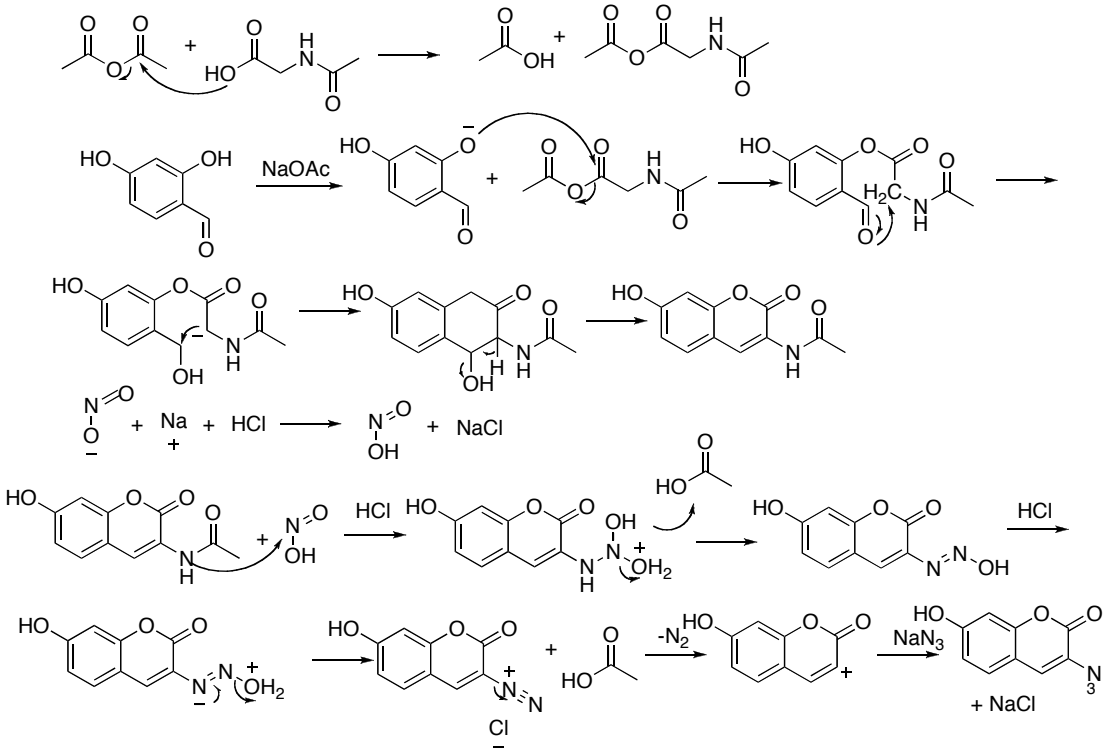
Reaction mechanism of compound 1



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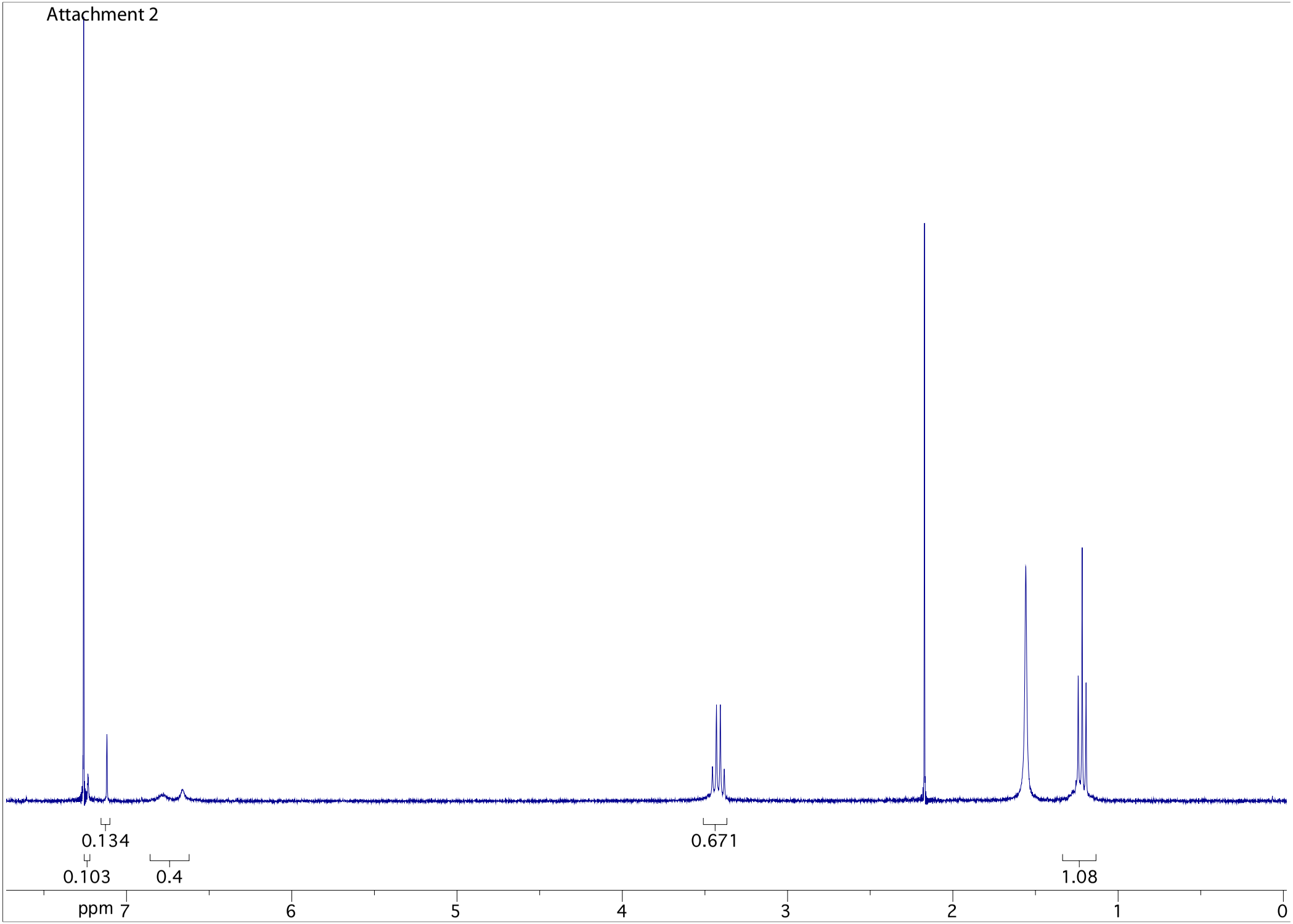

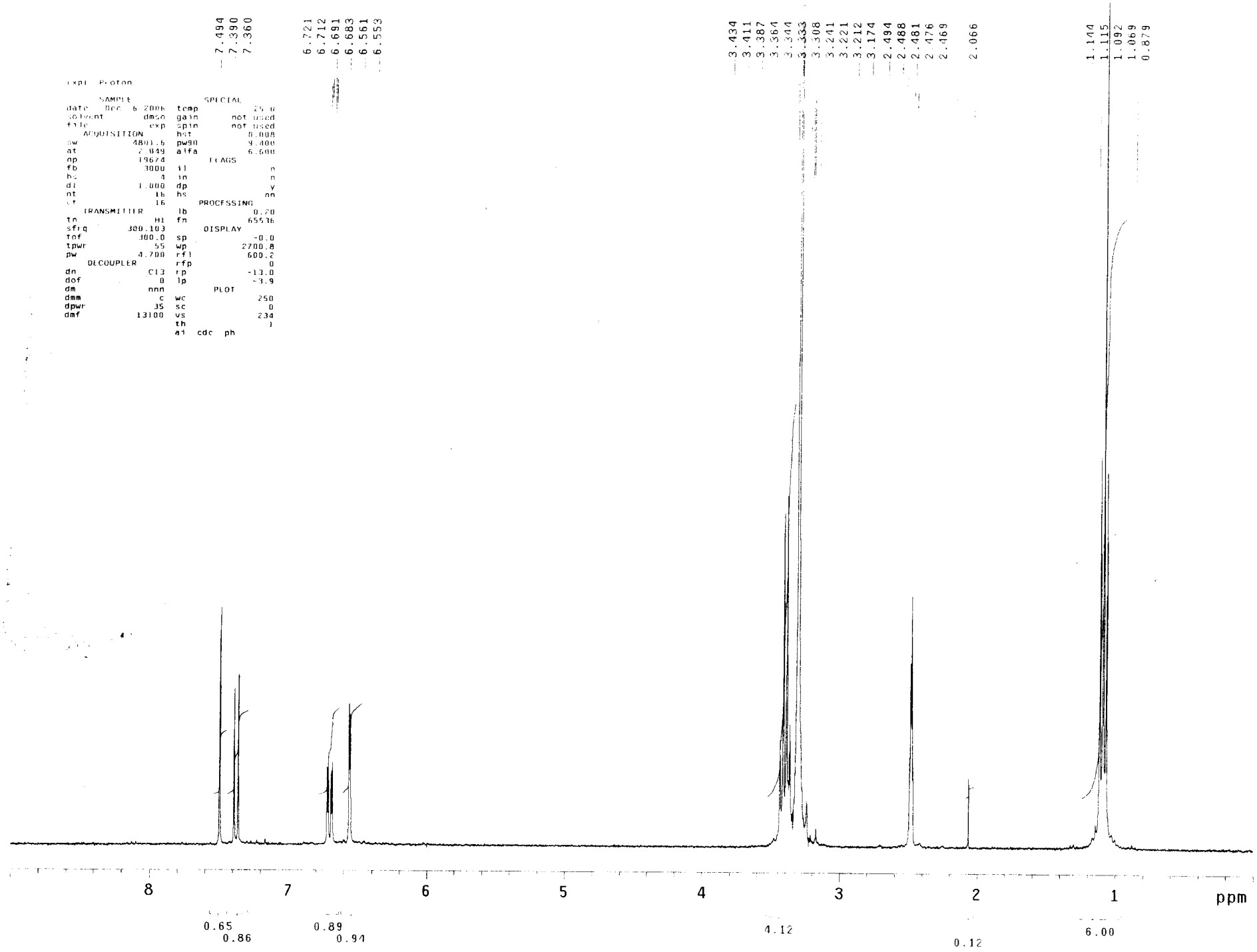

expt Proton

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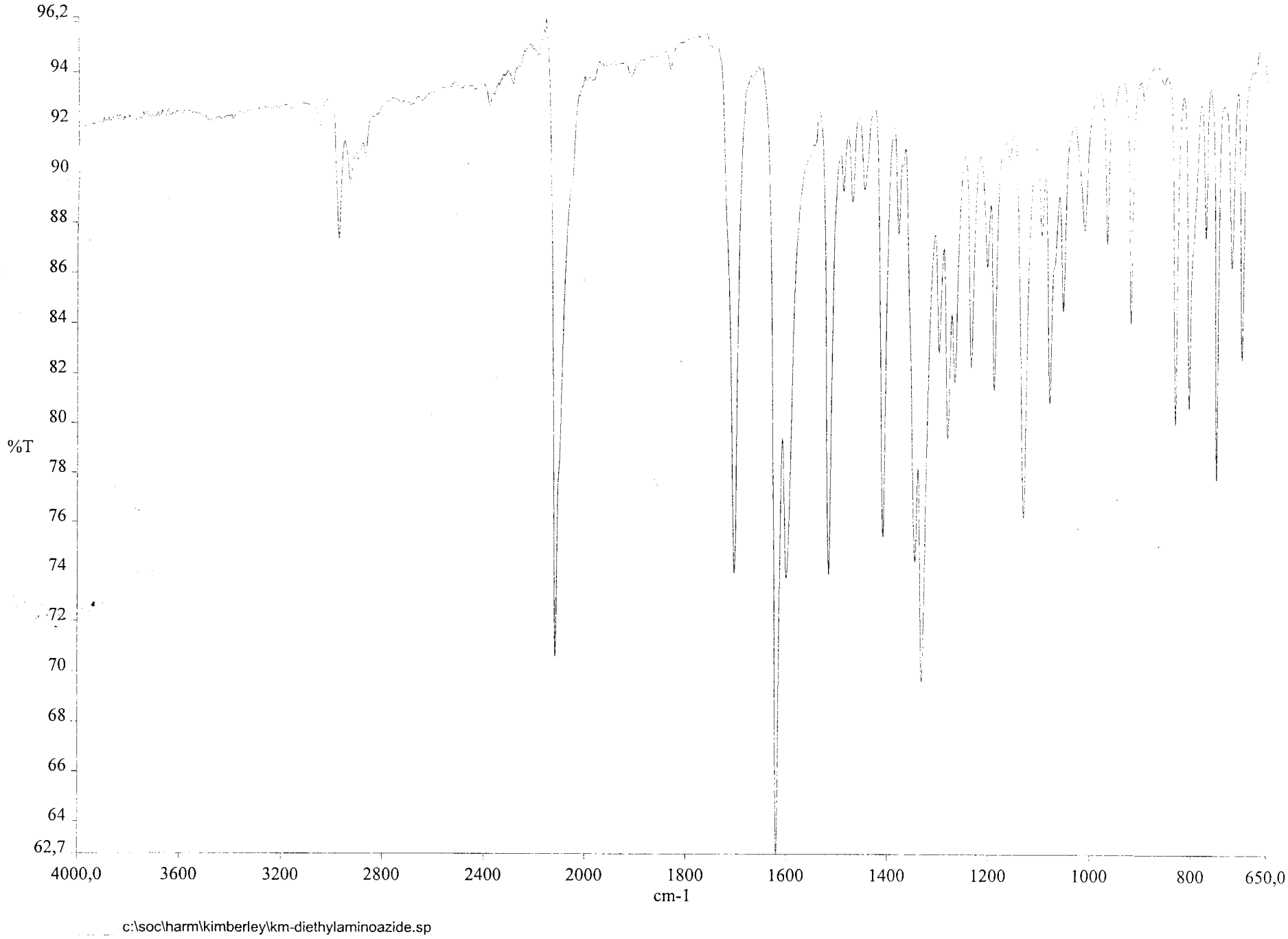
solvent	dmso	gain	not used
file	exp	spin	not used
ACQUISITION		hst	0.008
sw	4801.6	pw90	9.400
at	2.049	alfa	6.600
np	19674	FLAGS	
fb	3000	il	n
bs	4	in	n
dl	1.000	dp	y
nt	16	hs	nn
ct	16	PROCESSING	
TRANSMITTER		lb	0.20
tn	H1	fn	65536
sfrq	300.103	DISPLAY	
tof	300.0	sp	-0.0
tpwr	55	wp	2700.8
pw	4.700	rfl	600.2
DECOUPLER		rfp	0
dn	C13	rp	-13.0
dof	0	lp	-3.9
dm	nnn	PLOT	
dmm	c	wc	250
dpwr	35	sc	0
dmf	13100	vs	234
		th	3
		ai	cdc ph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2800
2400
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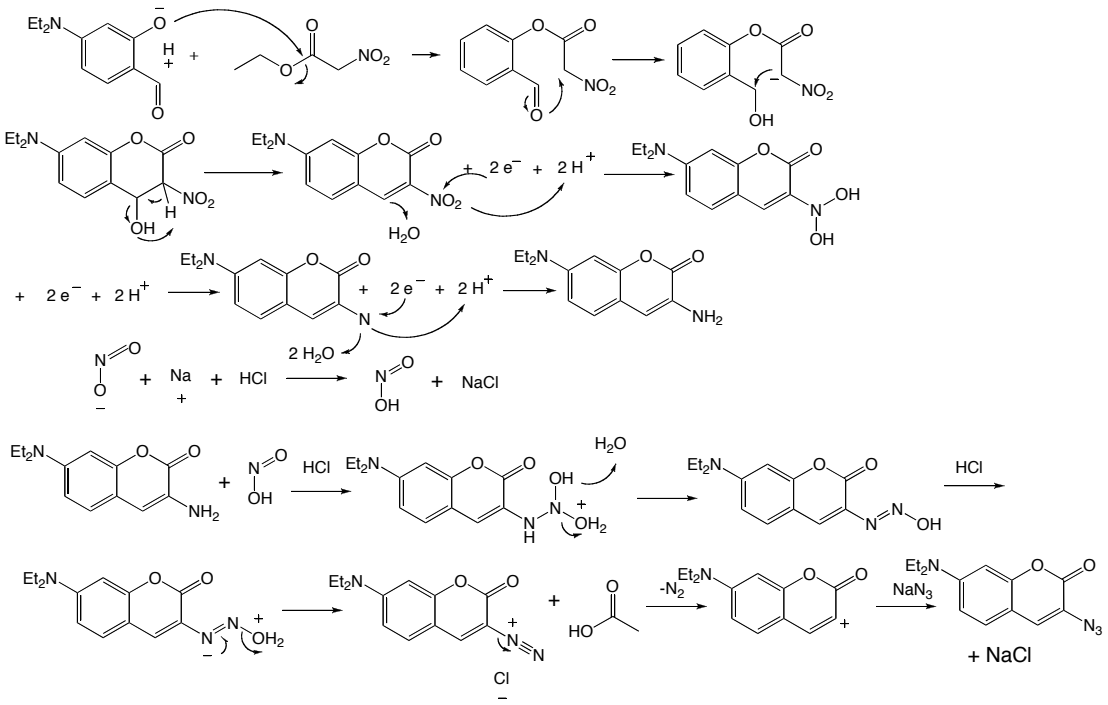
Reaction mechanism of compound 2



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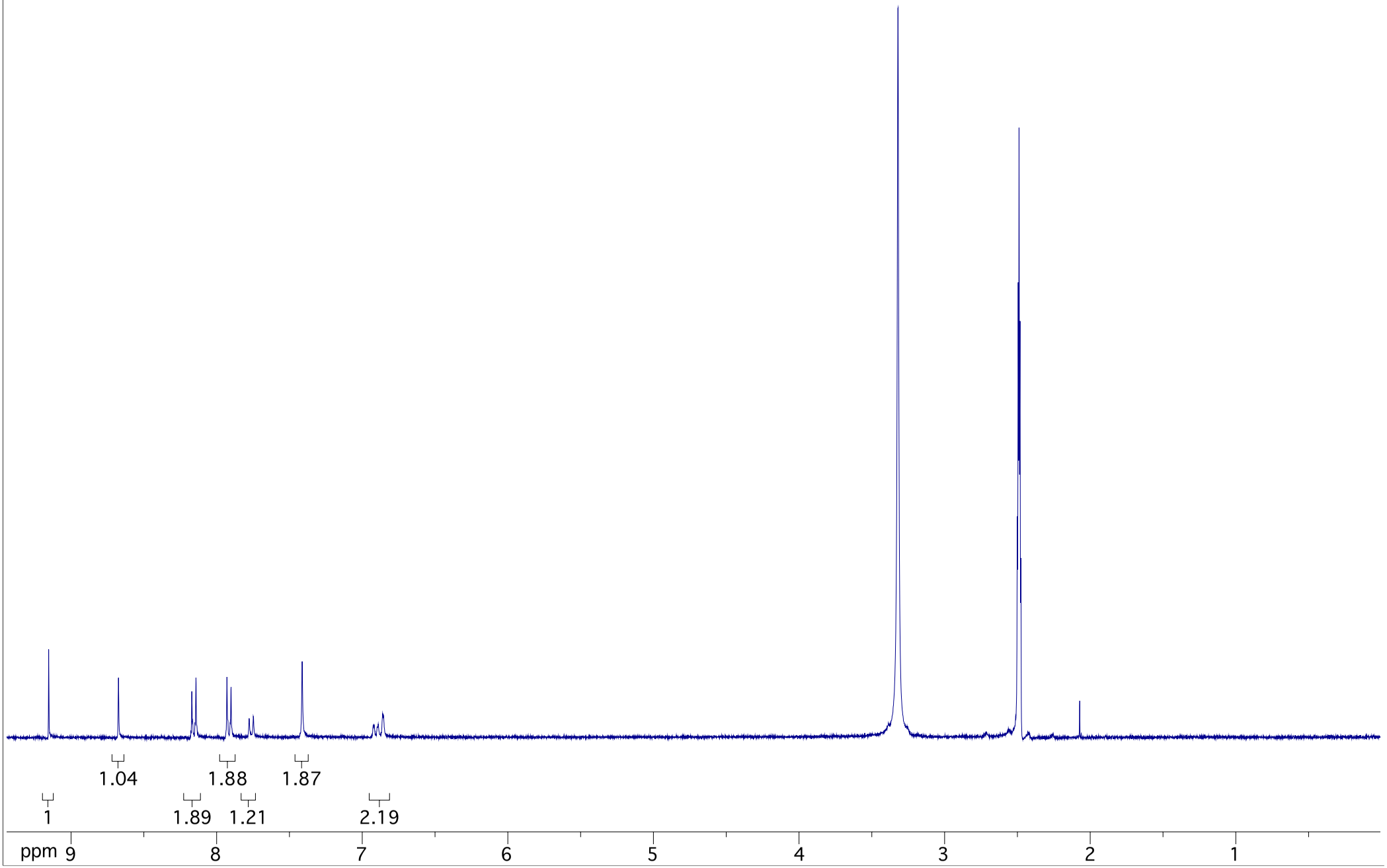

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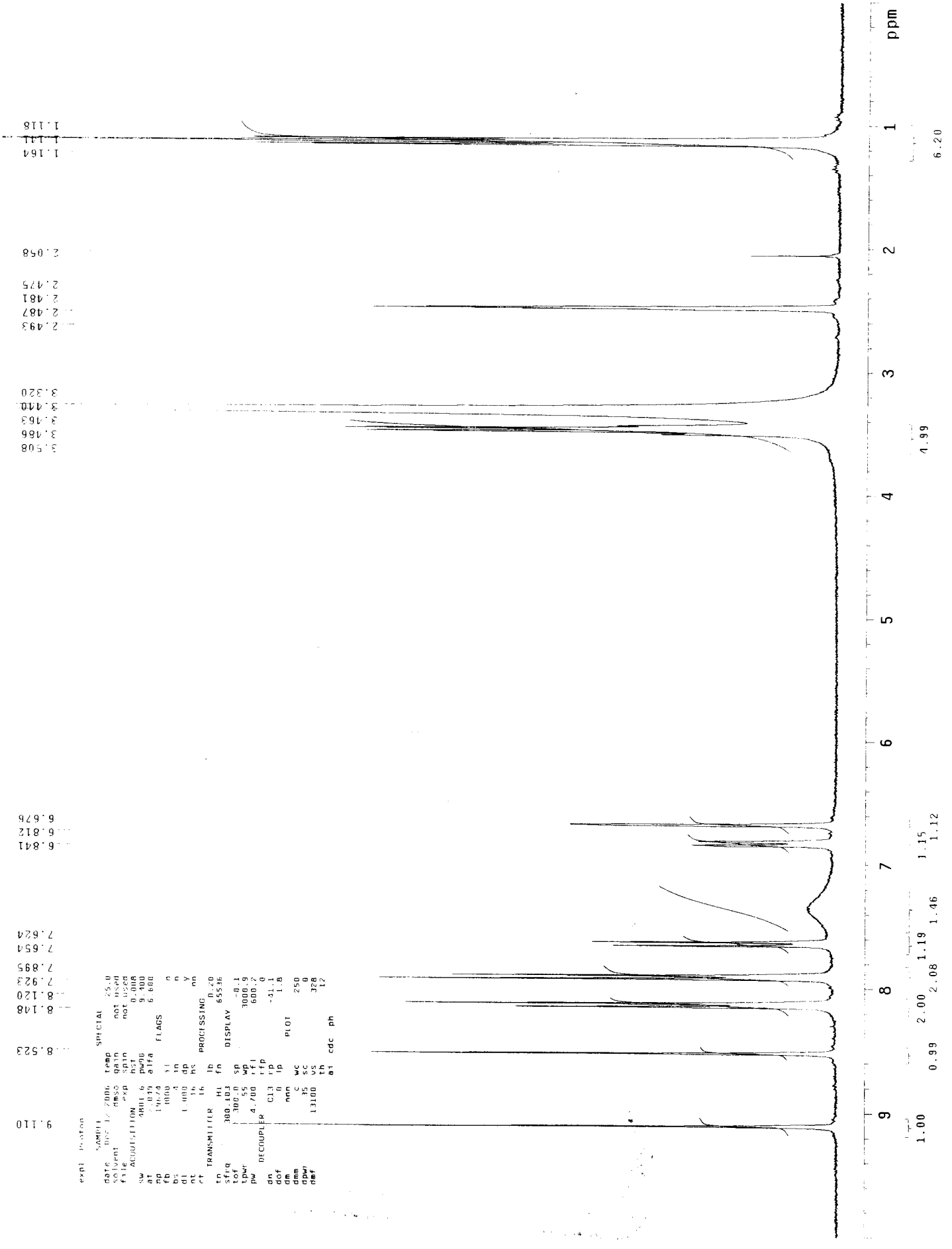

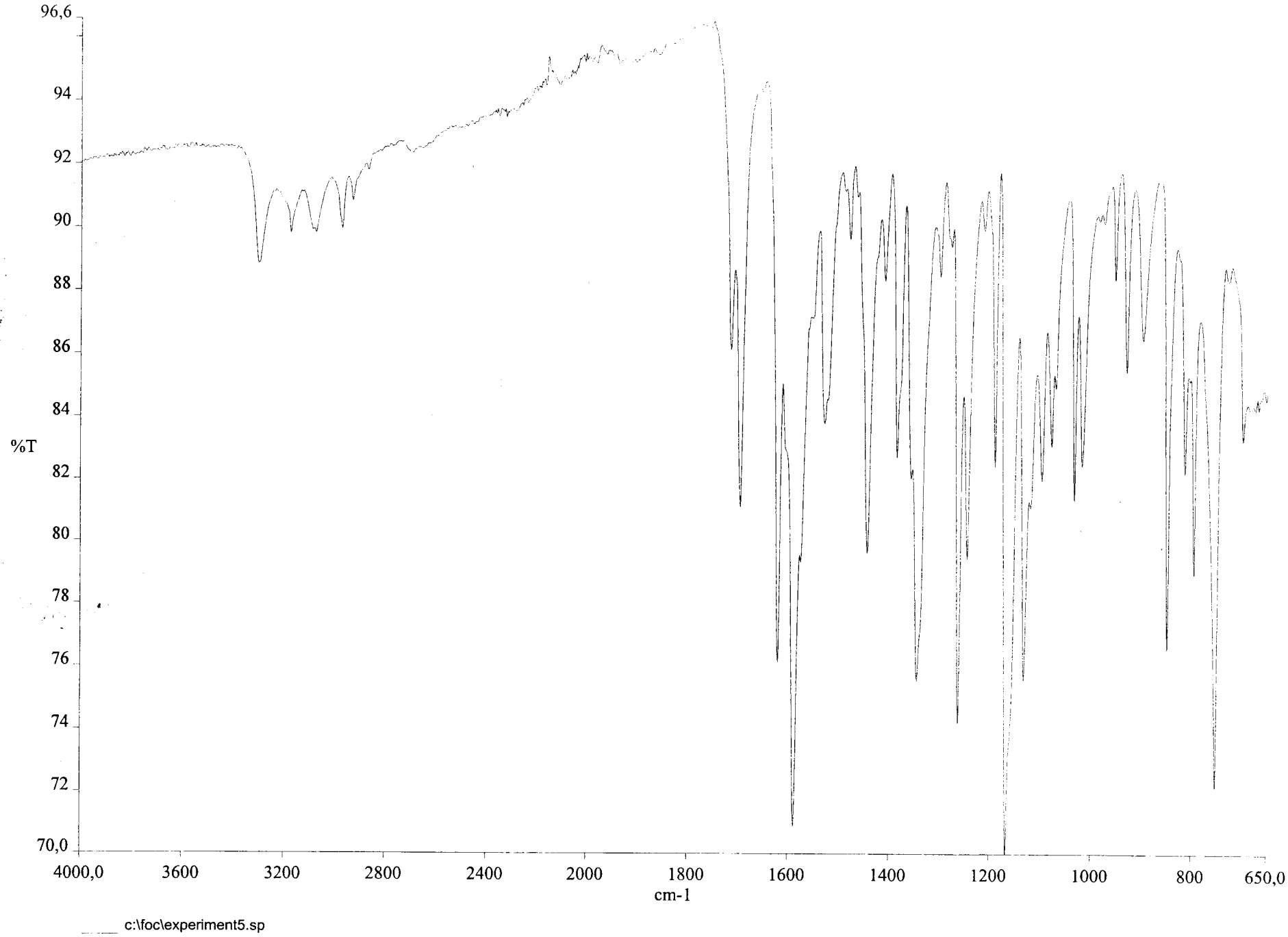
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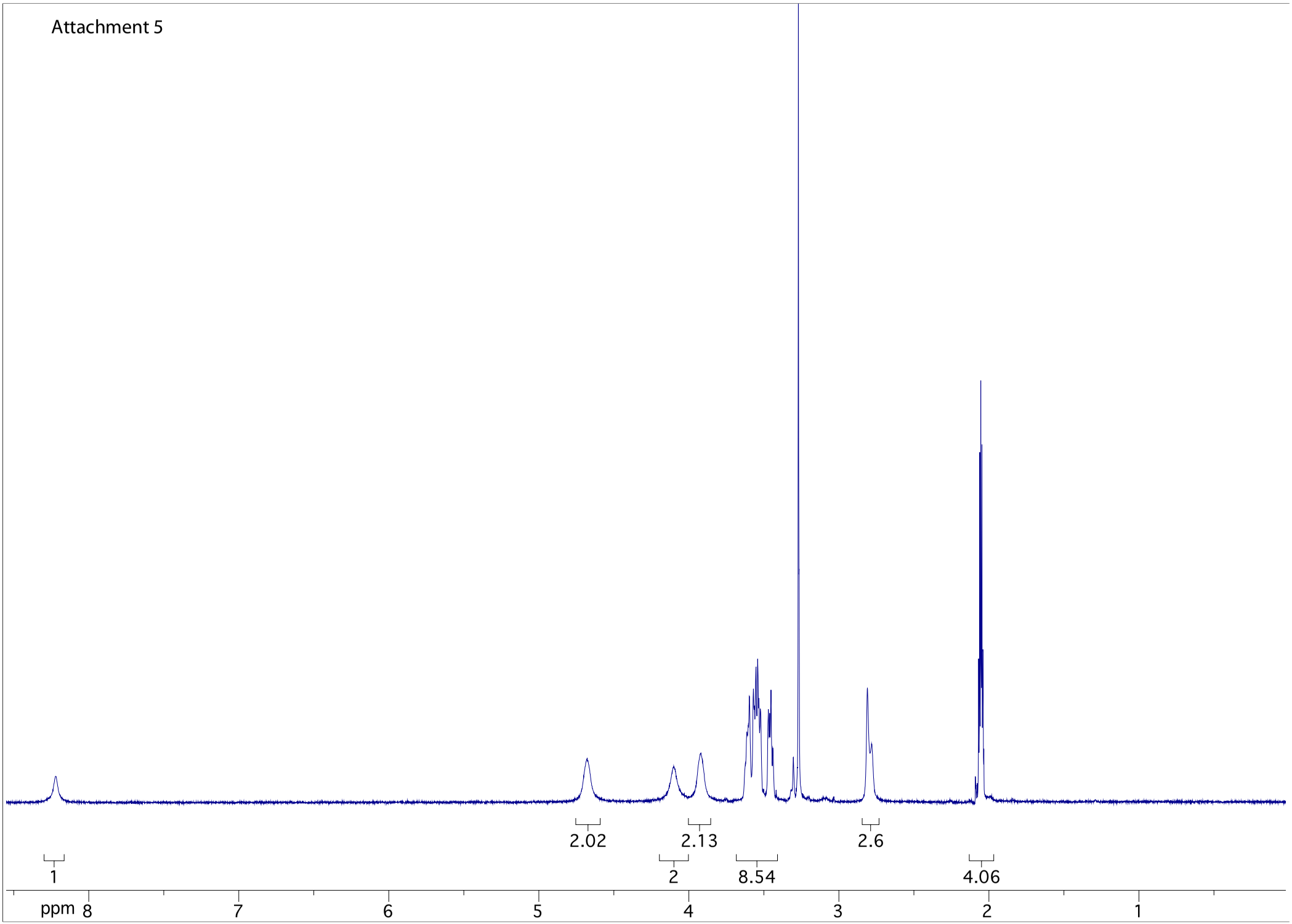
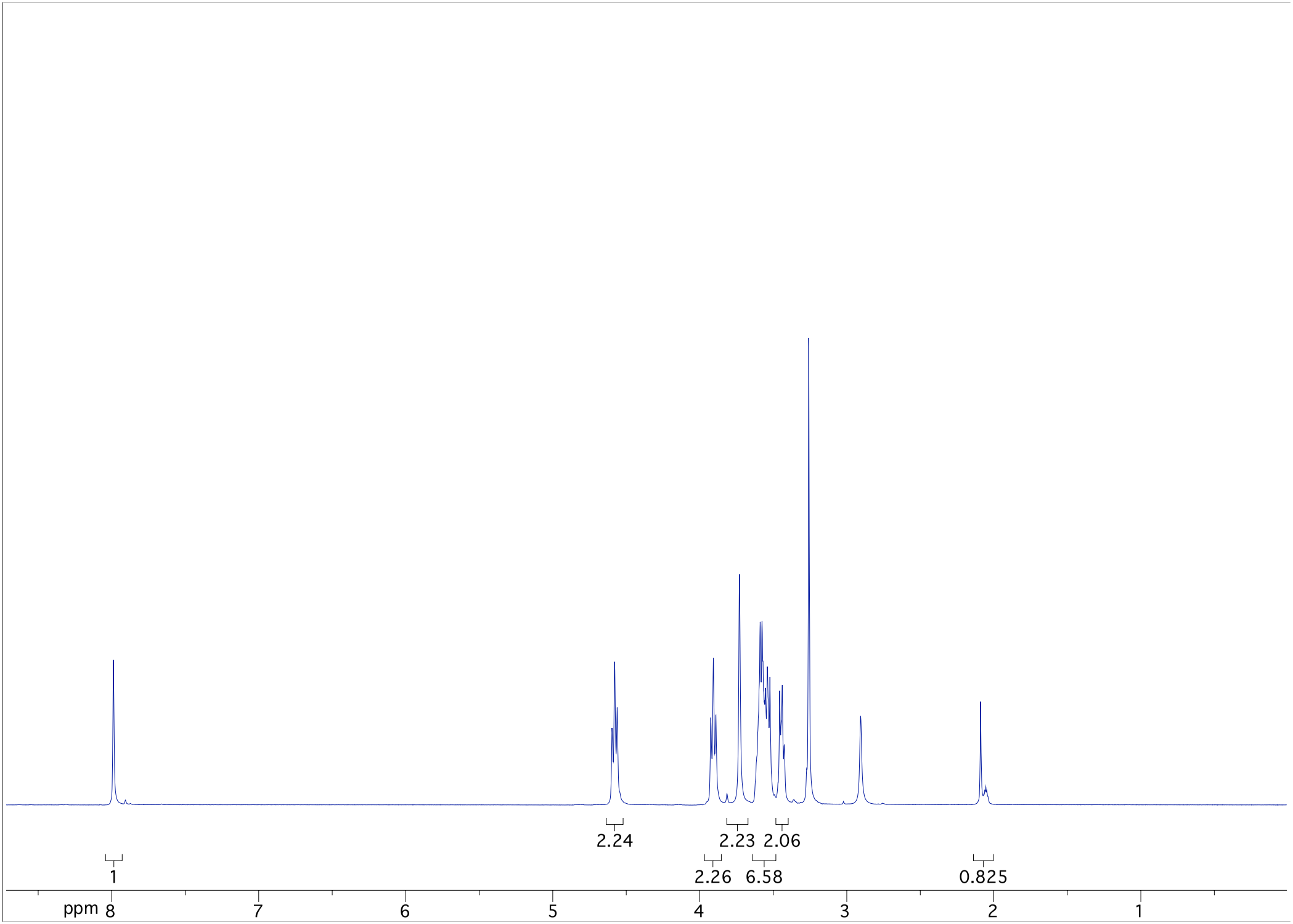
Attachment 5

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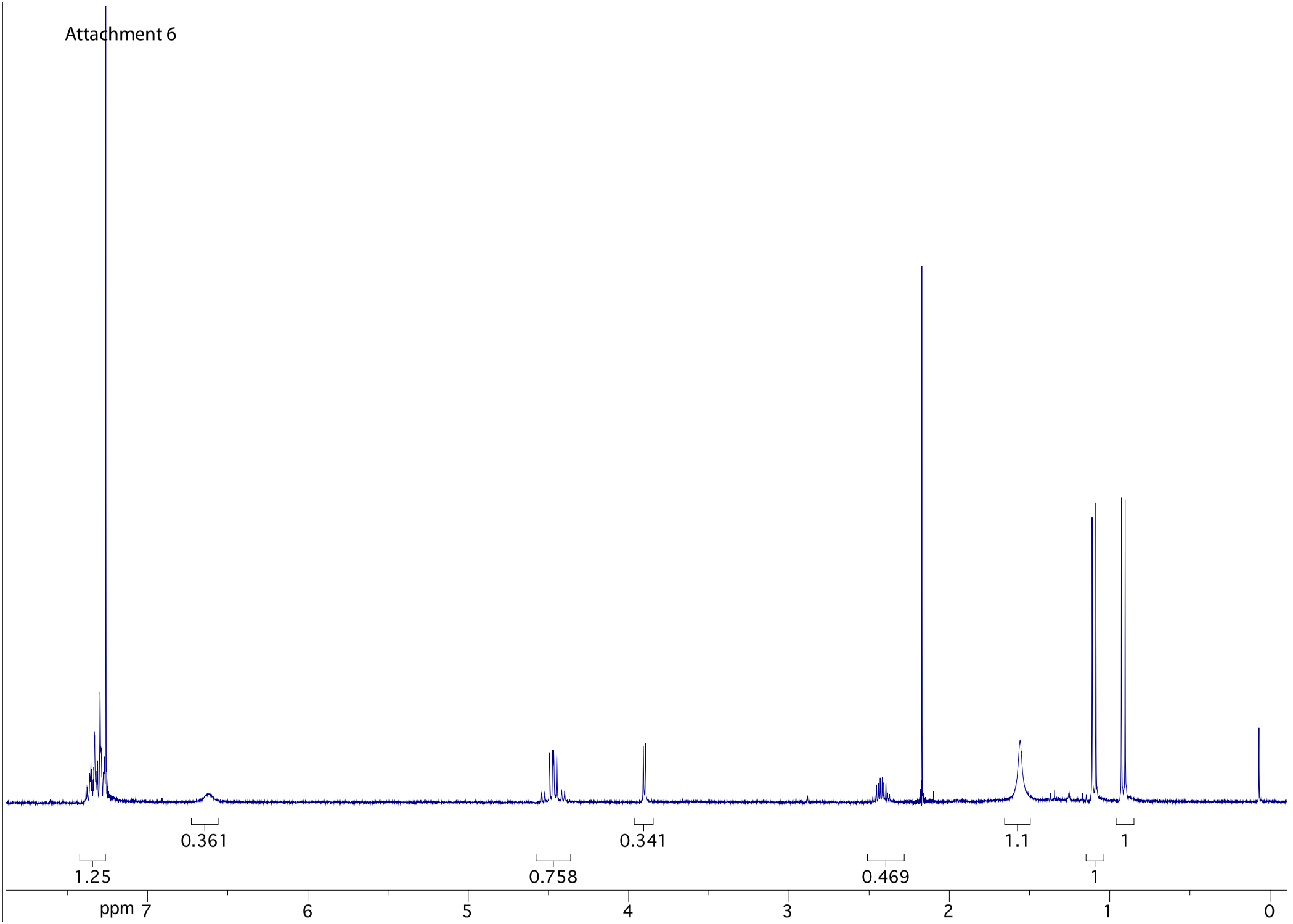
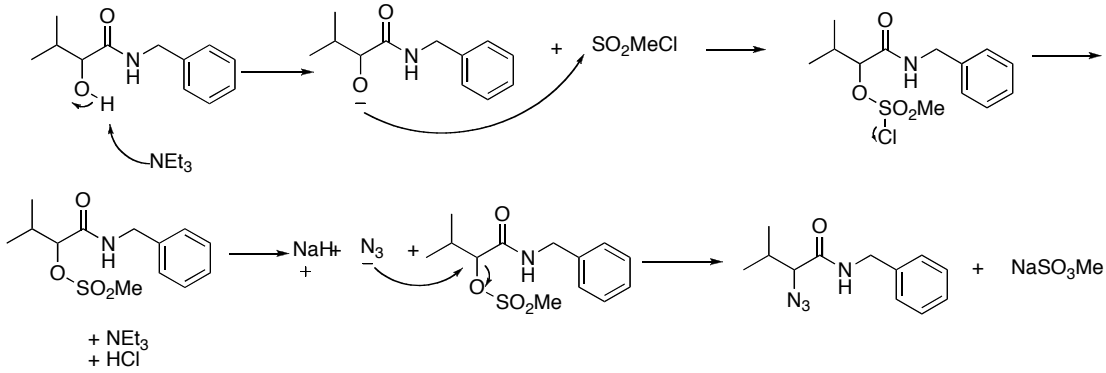
3
2
1

Attachment 6



Reaction mechanism of compound 6



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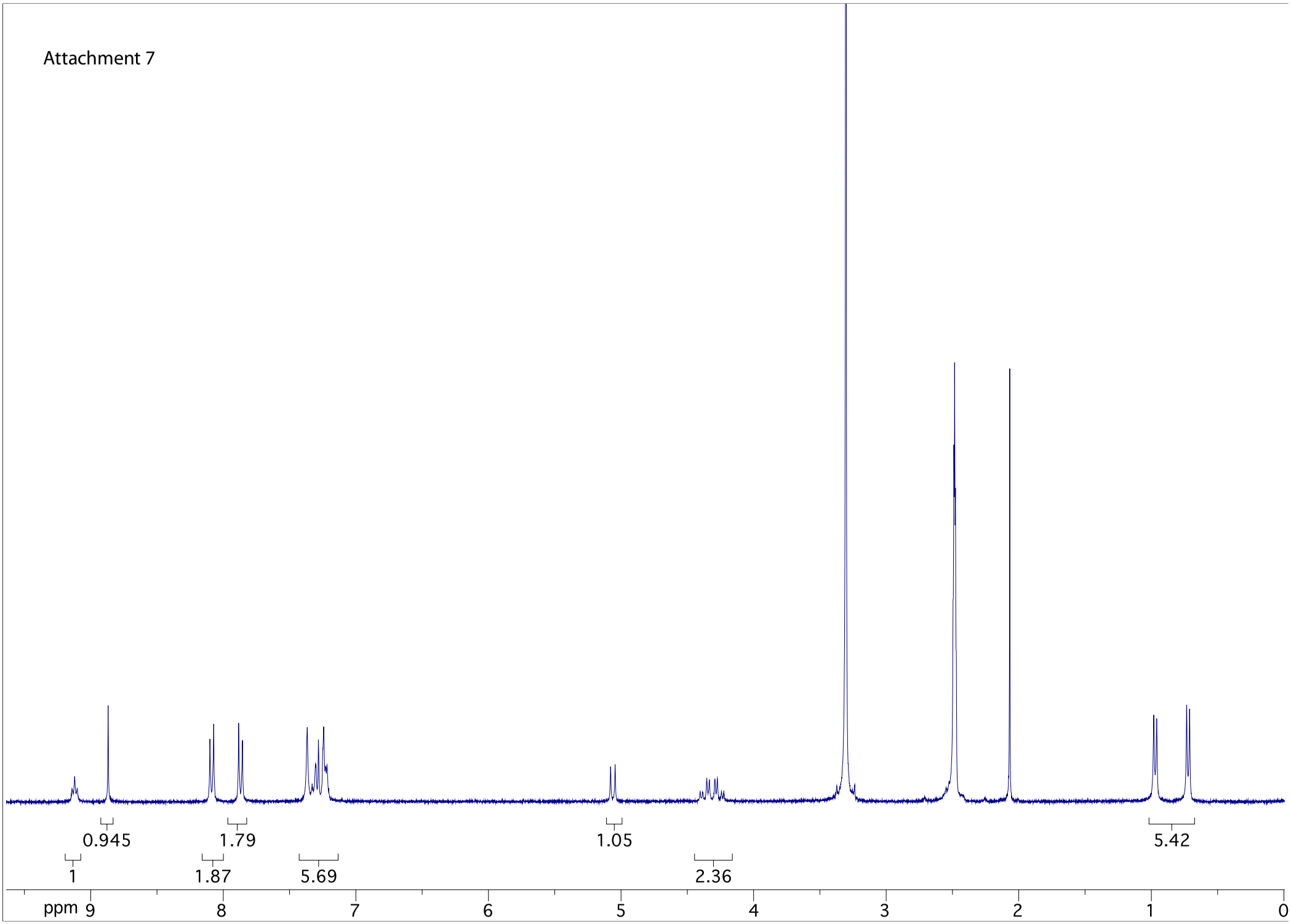

Attachment 8

Calibration curve reaction 3