Synthesis of non-sugar GAG mimetics

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List of abbreviations

DCM- dichloromethane
DMF- dimethylformamide
EWG – electro withdrawing group
GAG - glycosaminoglycans
HGF- hepatocyte growth factor
HS -heparan sulfate
HPLC - high-performance liquid chromatography
IR -infrared spectroscopy
mCPBA- meta-Chloroperoxybenzoic acid
MS - mass spectrometry
MTBE methyl tert-butyl ether
RAF - rapidly accelerated fibrosarcoma
RTK -receptor tyrosine kinase
PI3K - phosphatidylinositol 3-kinase

Abstract

The Met receptor is a well-known motility, metastasis, and apoptosis modulator within human cells. Its activity is known to be accelerated in cancer cells. Its activation has been found to be co-dependent on the attachment of the hepatocyte growth factor ligand. The cofactor site, binding glycosaminoglycan molecules within the ligand, has been found to be crucial for the activity of the Met receptor. The approach to modulate the activity of Met by synthesising the molecules able to competitively bind to the cofactor side is known in the current literature. Two new synthetic routes to develop novel non-sugar GAG mimetics are presented within the project. The first proposed route, with a 5-membered ring system as a core, could not be completed due to synthetic problems and low yields of reactions, however, the second route, using cyclohexenes as a central structure, was completed. Further characterisation of the resulting compounds was advised. Also, after the completion of purification and analytical work, to fully evaluate the pharmacophore within the resulting compounds a biological assay-wound healing assay is advised.

1. Introduction

1.1 Cancer as a worldwide threat

According to the World Health Organisation, cancer is a worldwide health problem and a major cause of death, contributing to one out of six fatalities overall. Cancer is described as a broad term, applied to a large group of diseases affecting all parts of the human body. It is characterized by abnormal growth of the defunctionalized cells which can affect the neighbouring tissues and spread across the human body. The most occurring cancers are breast, lung, and colon cancer (World Health Organisation, 2022).

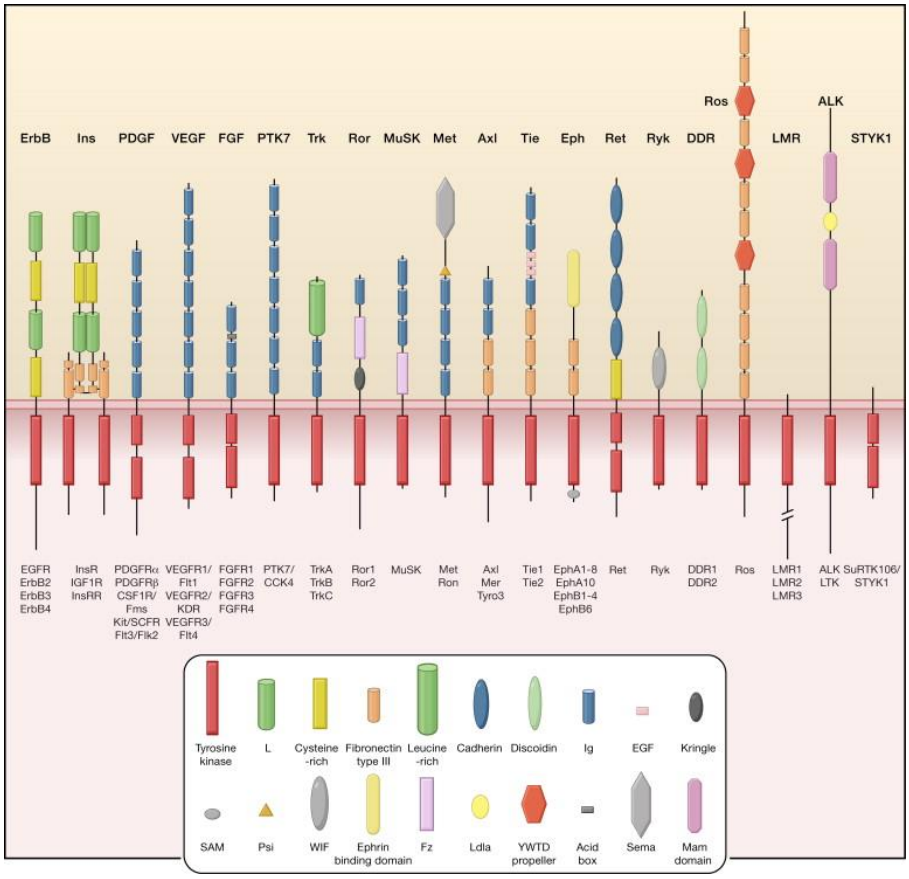
Cancer treatments vary depending on the type of cancer and its severity. The available treatments include chemotherapy, where drugs are used to kill the cancer cells, hormone therapy, used mainly in breast and prostate cancer in which hormones aim to slow down the cancer growth, hyperthermia where heat up to 45 ° C is applied to damage the affected cells, immunotherapy boosting the immune system to fight cancer, photodynamic methods, radiation, surgery, and targeted therapy (National Cancer Institute, 2022).

Targeted therapy is a relatively new treatment method offering an alternative to exhausting chemotherapy. The advancement in the sequencing of the human genome and the development of novel technologies that allow spotting of genomic, epigenetic, or transcriptional changes, contributed to the emergence of precision medicine (Tsimberidou, 2015). This treatment method focuses on targeting specific enzymes, signal transducers or growth receptors that play a significant role in cellular processes that are accelerated in cancerous cells. Currently, few known carcinogenesis drivers involved in the RAS–RAF–MEK and PI3K–AKT–mTOR pathways have attracted researchers' attention. The first pathway plays a significant role in cell life processes such as motility, proliferation, migration, differentiation, and apoptosis of cells. The second one is considered crucial for the growth and progression of cancer (Tsimberidou, 2015).

1.2 Receptor Tyrosine Kinase

Receptor Tyrosine Kinases belong to the tyrosine kinase group within the human body. Considering their structure, receptors can be divided into 20 subgroups (Figure 1). RTKs are key regulators in biological processes such as cell survival, metabolism, and proliferation of cells. Also, their activity regulates the whole cell-life cycle. (Lemmon & Schlessinger, 2010).

Figure 1. The diversity within the family of RTKs.



Note From " Cell Signaling by Receptor Tyrosine Kinases" by Lemmon, M. & Schlessinger, J. 2010. Cell, 141(7), p. 1118. (https://doi.org/10.1016/j.cell.2010.06.011). Copyright 2010 by Lemmon, M. & Schlessinger.

The structures of RTKs are consistent and can be divided into an extracellular region with a ligand binding domain, a transmembrane helix and a cytoplasmic region with the protein tyrosine kinase and carboxy-terminal and juxtamembrane regulatory areas. In most cases, the activation of the RTK takes place by ligand attachment which causes receptor dimerization;

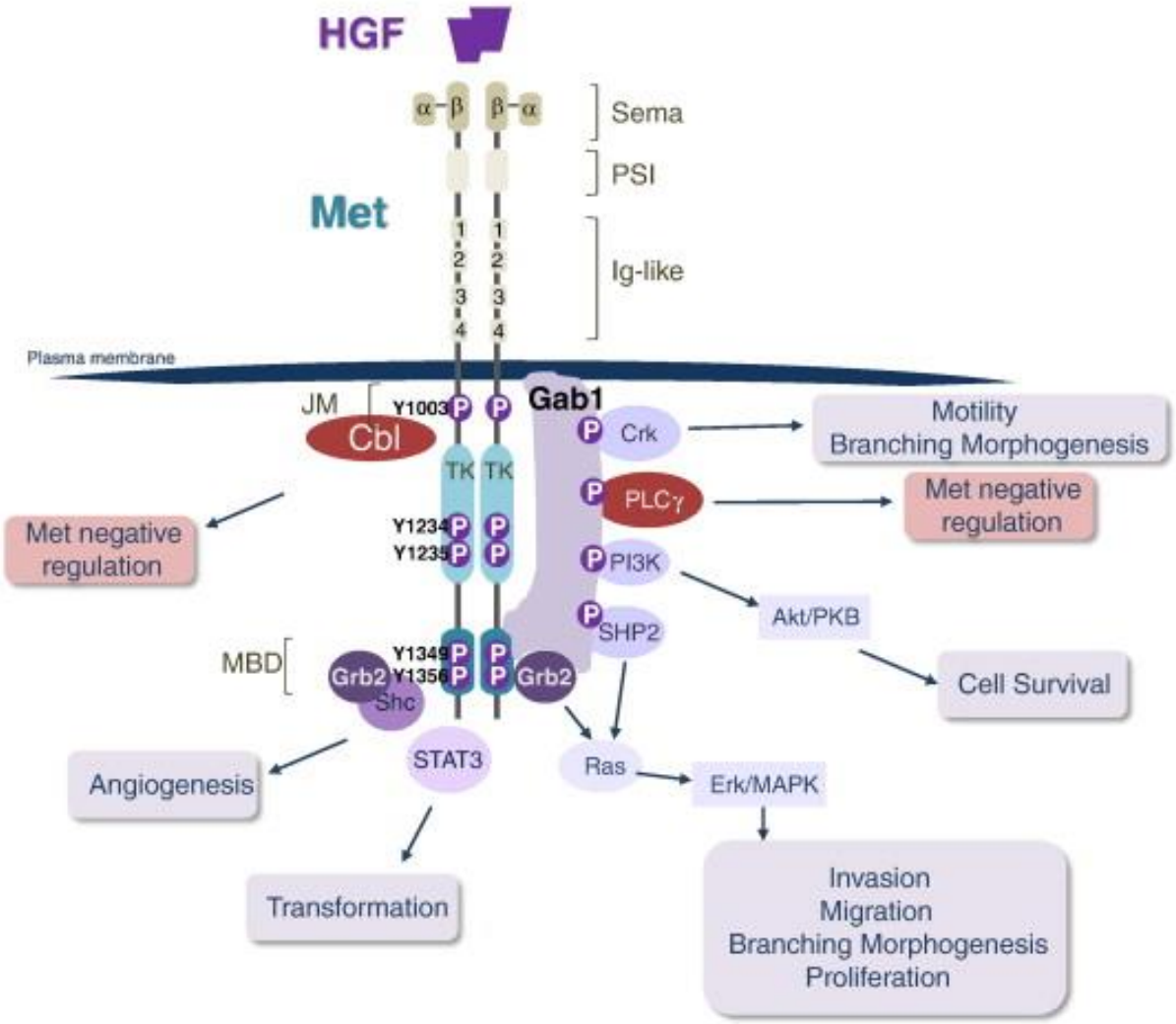
however, dimeric forms of inactive receptors are also known e.g., insulin and IGF1 receptors. (Lemmon & Schlessinger, 2010).

Genetic changes and abnormalities concerning the receptors' regulation, abundance or distribution lead to numerous diseases and are directly linked to cancers, inflammation, bone disorders and diabetes. Therefore, there is considerable interest in the development of a new generation of drugs with the ability to alter the activity of RTKs (Lemmon & Schlessinger, 2010).

1.3 Met Receptor

The Met receptor belongs to the RTK series and follows the structural similarity observed within the family (Figure 2). A ligand specific for the Met receptor is a hepatocyte growth factor. The receptor itself is 190 kDa in size with 45 kDa contributing to the amino-terminal extracellular alpha-chain and a 145kDa beta-chain which follows through the cell membrane. The next intracellular part of the receptor contributes to the juxtamembrane domain with significance for the signalling tyrosine residues and the carboxy-terminal domain which, following the receptor activation, docks the signalling adaptors and receptors (Maroun &Rowlands, 2014).

Figure 2. Structure of the Met receptor and its biological functions.



Note From "The Met receptor tyrosine kinase: A key player in oncogenesis and drug resistance" by C.R. Maroun, & T. Rowlands. 2014. *Pharmacology & Therapeutics,* 142(3), p 317 (https://doi.org/10.1016/j.pharmthera.2013.12.014) Copyright 2014 by M.C. Maroun & T. Rowlands.

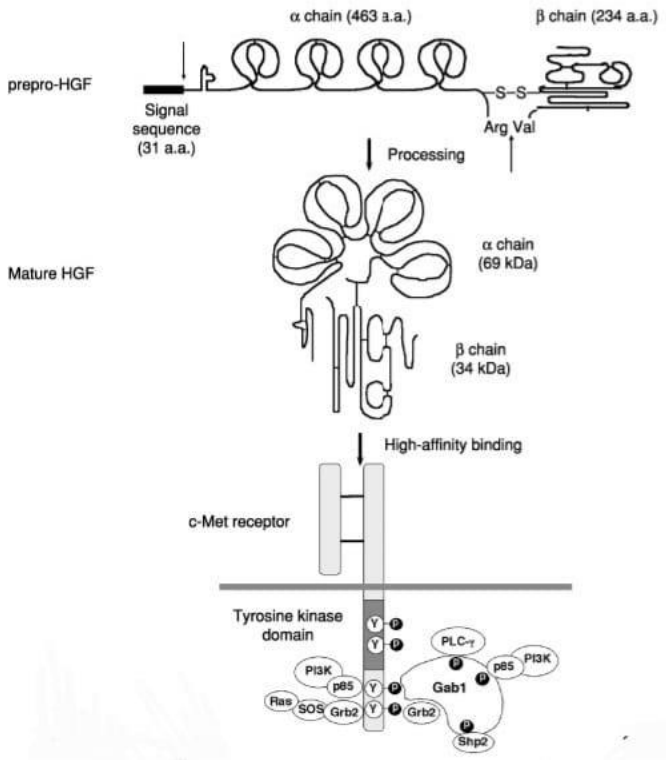
Semaphorin, PSI and immunoglobin-like units are the main blocks of the beta-chain part of the Met receptor, however, only SEMA-PSI seems to be a crucial site for HGF ligand binding (Maroun &Rowlands, 2014).

The receptor is a well-known oncological target as its upregulation and accelerated activity is responsible for the development and growth of various type of cancer. (Fasolo & Sessa, 2013) Also, it is known to be an important factor in tissue regeneration (Zhao et al., 2022).

1.4 HGF and Glycosaminoglycans

The HGF ligand, or scatter factor, is secreted by fibroblasts and acts in a mitogenic manner towards epithelial and endothelial cells. It is produced as an inactive form and processed into the active heterodimer version with alpha and beta chains linked by a disulfide bond (Sherbet, 2011).

Figure 3. Structure of inactive and active forms of HGF and the binding mode with Met.



Note from "Structure of HGF and the C-Met Receptor" by K. Matsumoto, &T. Nakamura. 2004.*Encyclopedia of Endocrine Diseases.* Copyright 2004 by K. Matsumoto, &T. Nakamura.

The alpha chain folded at the amino N-terminal domain followed by 4 Kringle domains are considered a site of binding to the Met receptor. Also, the carboxy parts of the alpha chains; NK1, NK2 and NK4 take part in competitive binding but are not able to activate the receptor (Sherbet, 2011).

The optimal Met receptor activation mediated by attachment of HGF is glycosaminoglycan dependent (Catlow at al. 2008) and the studies carried out by Catlow et al. (2008) provided a closer insight into the structural requirements of competitive binding to the above site. HGF exhibits a high affinity to sugar-structured polysaccharides – glycosaminoglycans like heparan sulfate (HS), heparin, and dermatan sulfate (Catlow et al. 2008). Previous *in vivo*

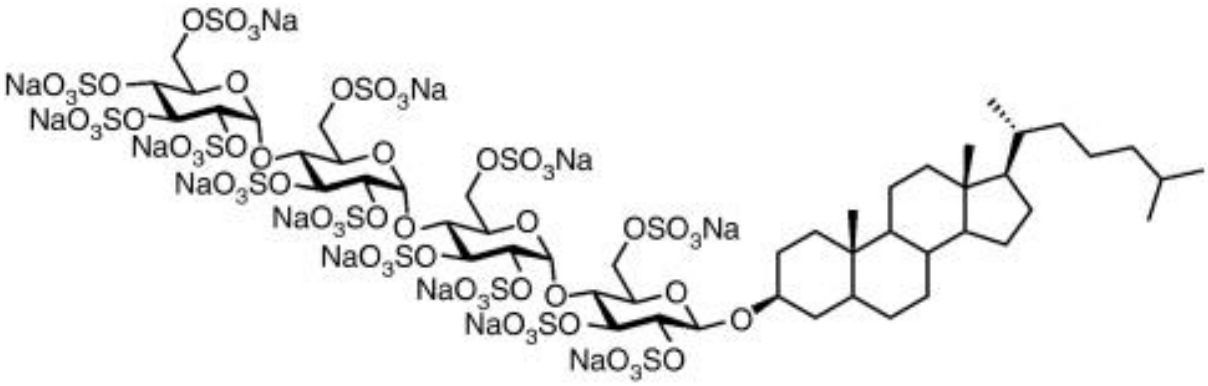
research indicated that in the absence of the GAG co-factor, the HGF still binds to the Met receptor, however, its activity and downstream signalling are poor (Deakin & Lyon, 1999). The research carried out by Catlow et al. (2008), on natural and modified GAG molecules, indicated the structural features significant for the competitive binding to occur and revealed that the interaction between GAG-HGF is flexible and exhibits a low specificity level, which creates the possibility of chemical synthesis of competitors to the binding. During the investigations, in the case of monosulfated compounds, the structure of L-ido-hexopyranuronic acid has been found to increase the affinity to the co-factor HGF site, however, with the increased level of sulfation, the IdoA ring was not crucial in achieving a level of affinity, however, the 6-member core might have a physiological significance (Catlow et al., 2008).

To sum up, the most important factor in achieving the competitive binding to the HGF site is the level of sulfation within the molecule, however, when designing a new generation of GAG competitors, the 6-membered heterocycle/hexene core could be considered to retain the physiological relevance and similarity to naturally occurring GAGs.

1.5 Previous research

The use of GAG mimetics to modulate the antiangiogenic and antimetastatic activity of tumour cells is an emerging idea in current research. An example of this is PG545, a molecule synthetically designed by Progen Pharmaceuticals LTD, Australia (Dredge et al., 2011). The molecule mimics heparan sulfate and consists of 4 fully sulfated sugar residues and an additional group adding to its functionality - cholestanyl aglycon (Figure 4).

Figure 4 The chemical structure of heparan sulfate mimic- PG545.

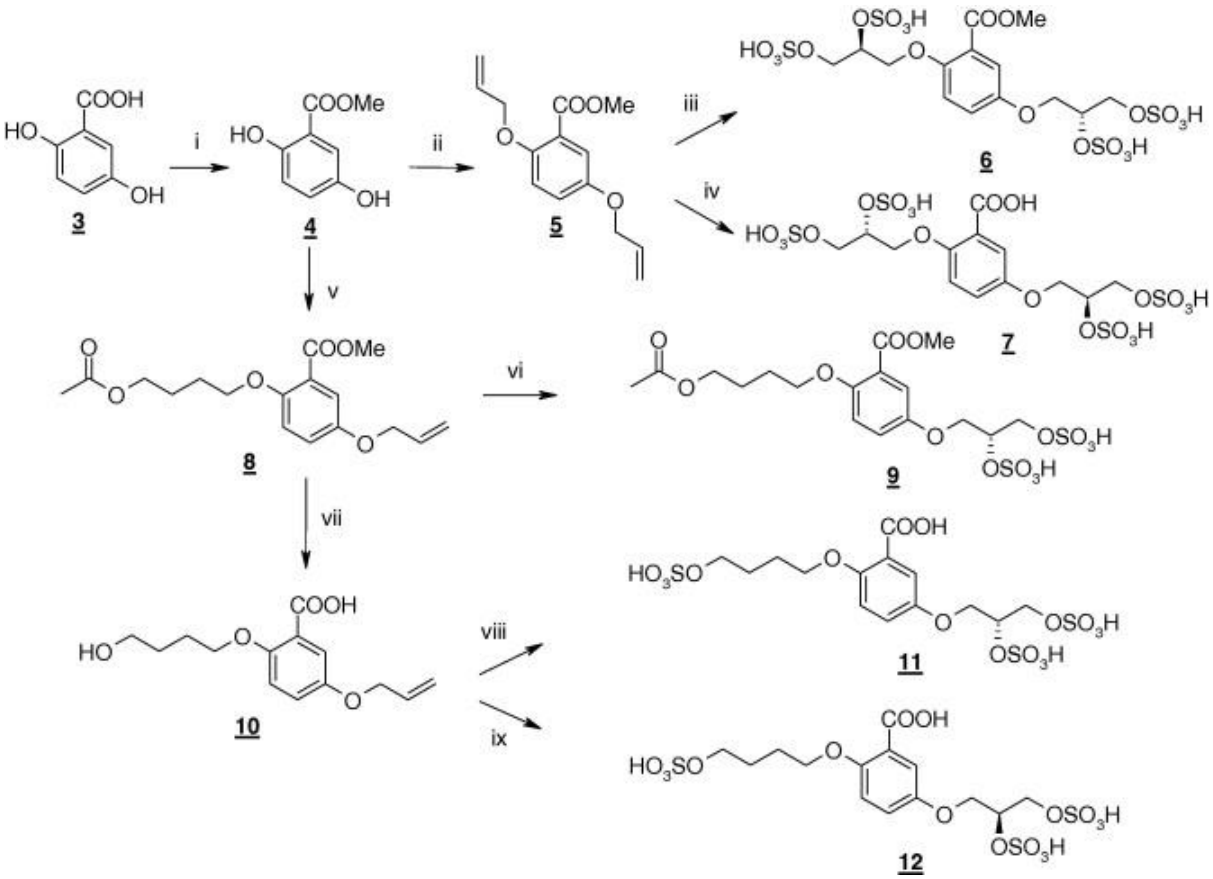


Note from "PG545, a dual heparanase and angiogenesis inhibitor, induces potent anti-tumour and anti-metastatic efficacy in preclinical models" by Dredge et al,. 2011. British Journal of Cancer, 104(4). Copyright Dredge et al.

Its activity involves modulation of the functionality of RTKs and inhibition of heparinase, an enzyme responsible for the degradation of HS. The drug candidate is currently in phase one clinical trials as *in vivo* and *in vitro* investigations indicated its high potency in anti-tumour activity in breast, liver, prostate, head, and neck cancers (Dredge et al. 2011).
Moreover, the research carried out by Winterhoff et al., 2015, showed that PG545, a mimic of HS, shows promising results in epithelial ovarian cancer as an addition to the standard paclitaxel and carboplatin therapies, significantly reducing the proliferation of the cancer cells. Moreover, the addition of PG545 to standard chemotherapy allows for a decrease in the dosage of cytotoxic agents with retention of inhibited proliferation which could result in a reduction of the tumour burden for the patient (Winterhoff et al., 2015).

The GAG mimetic molecule - P545 incorporates the idea of a sulfated drug with central sugar residues within its core. The approach taken by Raiber et al. (2007) was based on the non-sugar structured HS mimetics potent to inhibiting the HGF-regulated activation of ERK in Chinese hamster ovary cells and in a migration assay of Madin-Darby canine kidney cells. Based on previous research, Raiber and colleagues decided to mimic the central sugar residue using a benzene ring as a core and to retain the spatial orientation of the trisaccharide, two aliphatic chains were located opposite each other on the central benzene ring. A series of compounds with different patterns of sulfation (Figure 5) were synthesized and the potency to inhibit the HGF-mediated migration of the cell was tested.

Figure 5. Overview of the synthesis carried out by Raiber and colleagues.



Note from "Novel heparin/heparan sulfate mimics as inhibitors of HGF/SF-induced MET activation" by Raiber et al. 2007. Bioorganic & Medicinal Chemistry Letters, 17, p. 6323. (https://doi.org/10.1016/j.bmcl.2007.08.074). Copyright 2007 by Raiber et al.

Compounds 7 and 12 (Figure 5) turned out to be able to inhibit ERK activity at a concentration of 100 µg/mL. Also, in the migration assay, both compounds inhibited the movement of the kidney cells by 57% and 70% respectively. To further investigate the possible binding sites of molecule 12 to HGF, Raiber and colleagues carried out computational docking studies. The amino acid residues taking part in the hydrogen bond/ionic interactions were identified; Arg35, Arg76, Arg73, Lys78, Lys60, and Lys62 and interestingly, 3 of the identified residues are known to take part in heparin-HGF bonding.

Interestingly, more recent research carried out by Mahmoud et al. (2019) used non-sugar GAG mimetics in preventing vascular calcification, a major clinical problem linked to upregulation in HGF activity, with no current treatment available. *In-vivo* investigations of β-glycerophosphate-induced human vascular smooth muscle cells showed that the small non-sugar GAG mimetics synthesized by Mahmoud and colleagues reduced calcification and

prevented the upregulation of osteogenic markers. Also, the mode of action of the most potent compounds synthesized by the research group, C2 and C4, was investigated and found to be analogous to the known Met inhibitor Crizotinib. The docking studies of C2 and C4 compounds revealed that the lowest energy binding positions to the HGF correlate to the region naturally binding to heparan sulfate (Mahmoud et al. 2019).

1.6 Aims

The evidence supporting the significance of continuing the research involving targeted therapy and the promising results of using compounds able to modulate the activity of the various signalling receptors, providing alternative treatment to various diseases and cancers, illustrate the urgency for further research within this area.

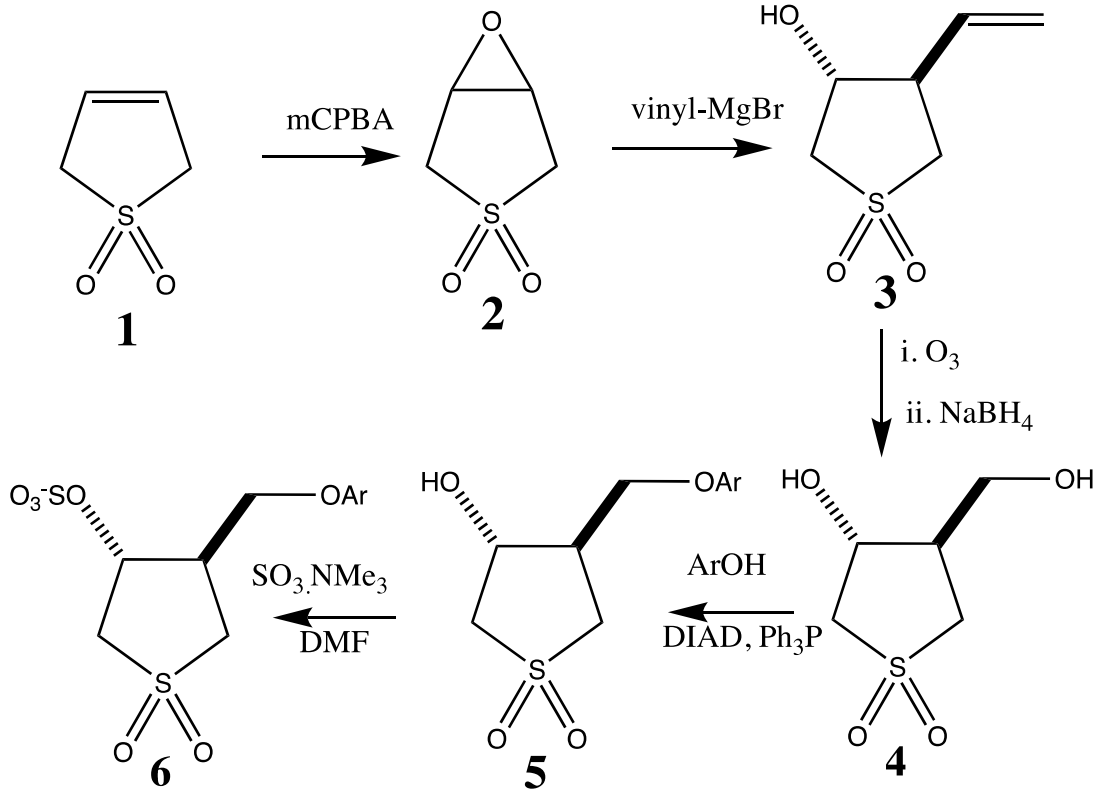
The research described here aims to synthesize a new generation of sulfated molecules, possibly potent in inhibiting the HGF-mediated proliferation of cells. The synthetic approach of Raiber et al. (2007) is beneficial as the chemistry to achieve a new generation of non-sugar GAG mimetics is transferrable to the synthetic lab. Also, a synthesis adapting cheap starting materials/reagents is possible. Previously Raiber et al. (2007) synthesized molecules with an aromatic benzene ring as a centre of the molecule. Research within this project focuses on the synthesis of novel cyclic but not aromatic compounds with a 5-membered-ring molecule-sulfolene and cyclohexenes as a core of the molecule. The new generation of sulfated compounds will supply further additional information regarding the pharmacophore present within the structure, especially the potency of GAG mimetics with non-aromatic core. Also, the use of sulfolene, a very cheap starting material, which has S=O already present in the structure, adds to the level of sulfation needed to achieve competitive binding to HGF.

2. Results and Discussion

2.1. Synthetic overview

The proposed route of synthesis involved 5 steps (Figure 6). 3-Sulfolene, an unsaturated 5-membered cyclic compound, was chosen as a starting material. The reason for this was to take the advantage of the S=O bond, adding an additional level of sulfation, already present within the starting material.

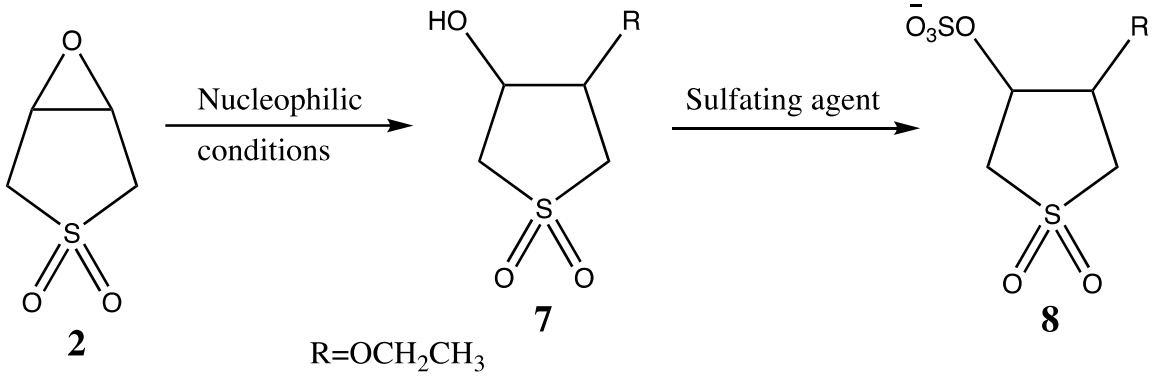
Figure 6. The proposed route of synthesis of sulfated compounds



The first step was an epoxidation of the starting material (**1**) with mCPBA to obtain the epoxide (**2**), which next was opened with the Grignard reagent – vinyl magnesium bromide, to build up the side chains to the central 5-membered ring core (**3**) following ozonolysis and reduction reactions. The last two proposed steps were supposed to be a Mitsonobu reaction and sulfation with a sulfur trioxide trimethylamine complex.

Unfortunately, due to the relatively low yield of steps I and II and technical issues with the university's ozone generator, compounds **4**, **5** and **6** were not synthesized. An alternative route to synthesising sulfated molecules based on compound **2** was therefore proposed (Figure 7).

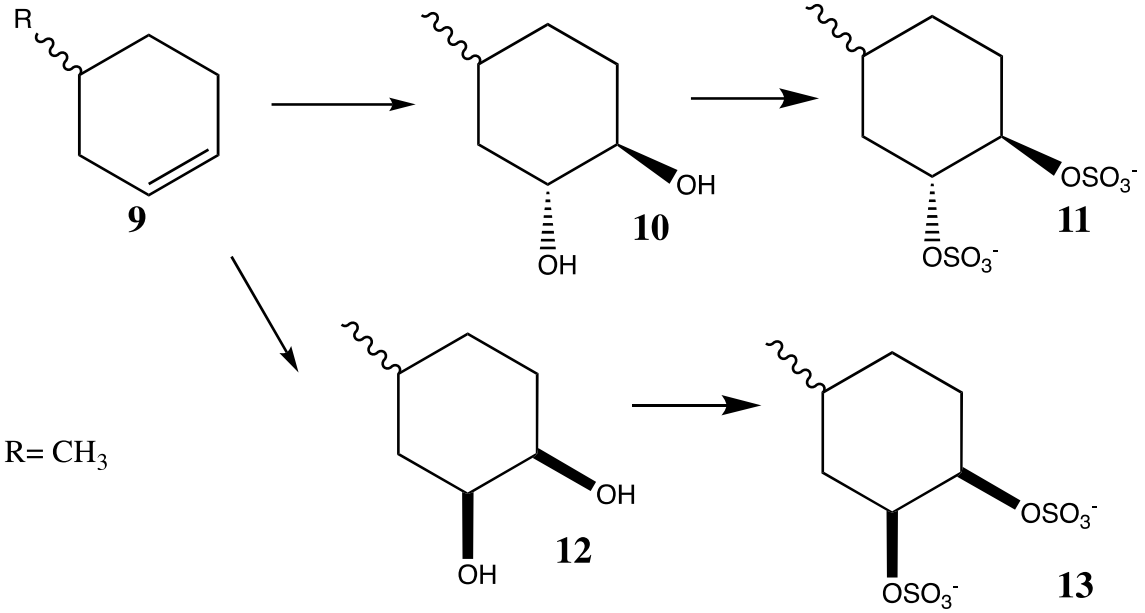
Figure 7. The alternative route to the synthesis of sulfated compounds based on sulfolene



Compound **2**, obtained after the first step proposed in Figure 5, can be opened with a hydroxy compound to add aliphatic chains to the molecule (7), followed by sulfation to achieve compound **8**.

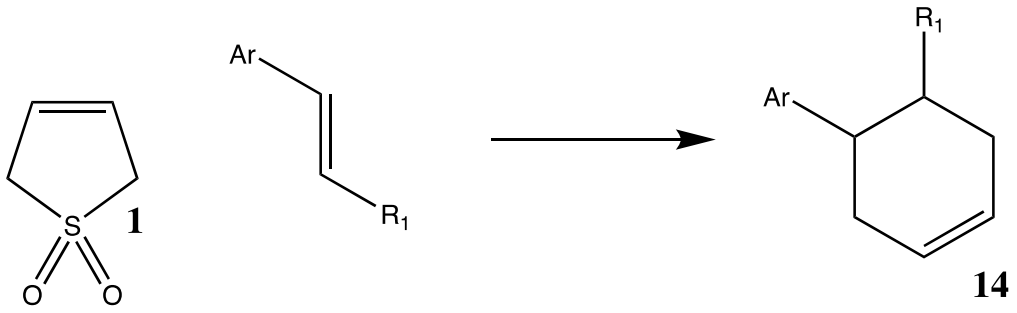
Also, a further series of sulfated molecules can be synthesized with the use of commercially available cyclohexene starting materials (Figure 8). The different ways of creating diol compounds could supply information about the preferred stereochemistry for competitive binding – either *cis* or *trans* orientation of the sulfated chains.

Figure 8. The synthesis of sulfated compounds derived from commercially available cyclohexenes.



Due to the high price of commercially available cyclohexenes, the idea to use sulfolene as a precursor to butadiene and hence create starting materials for the scheme presented in Figure 8 was proposed. (Figure 9) Sulfolene can act as masked butadiene, reacting with various dienophiles in Diels- Alder pericyclic reaction resulting in new cyclohexenes (Lopez et al. 2003, Bouhadir et al. 2016).

Figure 9. Possible use of sulfolene as a starting material to synthesise cyclohexenes

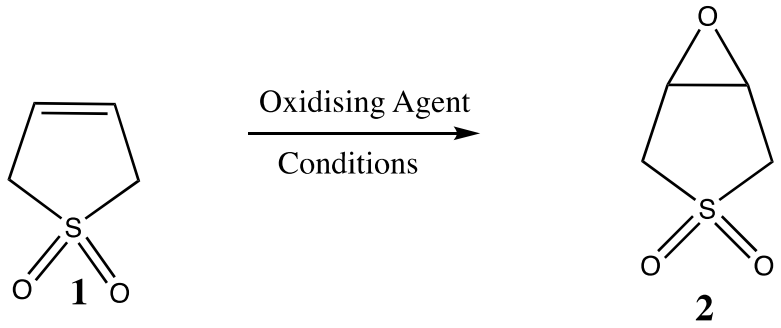


Diels-Alder cycloaddition is an extremely useful reaction in organic chemistry as it leads to the formation of two carbon-carbon bonds in a single step. The mechanism of the reaction is neither polar nor radical, but it is a pericyclic process in which a cyclic redistribution of bonding electrons takes place. The diels-Alder reaction is a general method for supplying unsaturated cyclic compounds. It occurs between a species containing two double bonds

within a conjugated structure-‘dienes’ and ‘dienophiles’ and an alkene species preferably with an electron-withdrawing substituent group (McMurry, 2008).

2.2 Synthesis of sulfolene oxide

Figure 10. Epoxidation of sulfolene to achieve sulfolene oxide.

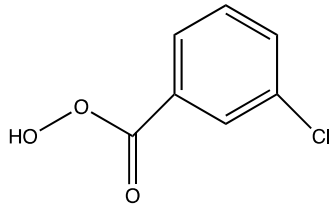
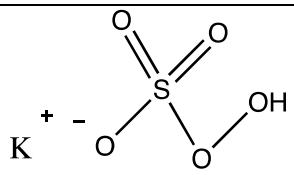
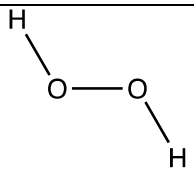
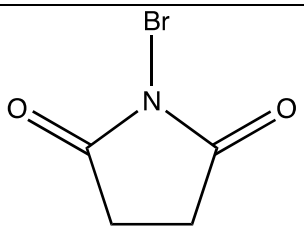
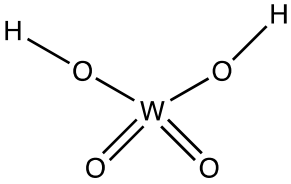


3-Sulfolene, the starting material for the first proposed route of synthesis, was chosen due to the presence of the S=O bonds within the compound. That could contribute to the overall level of sulfation required for competitive binding to HGF to occur. In the current literature, only 3 methods of epoxidations of 3-sulfolene has been reported so far and these were performed with peroxyformic acid (Cadogan et al. 1991), mCPBA (Xue &Seto, 2006) and peracetic acid (Aitken et al. 1984).

The method using mCPBA reported by Xue & Seto (2006) was chosen for the synthesis of compound **2**. Surprisingly, this step was found to be challenging. Initially, the first proposed method of epoxidation- with mCPBA, gave the product in low yield-30%.

The investigations described for epoxidizing compound **1** in cited earlier papers were carried out almost 30-40 years ago, and the mCPBA one- 16 years ago. The alternative methods to achieve compound **2** were proposed and tested within this project including ones using NBS, Oxone, tungstic acid, and hydrogen peroxide (Table 1).

Table 1. Reagents used to epoxidize sulfolene and corresponding yields of reactions.

	Epoxidizing agent	Chemical structure	Yield of the reaction (%)
1	mCPBA, 3-chloroperbenzoic acid		46%
2	Oxone, potassium peroxymono sulfate		17%
3	H_2O_2, hydrogen peroxide		0%
4	NBS, N-Bromo succinimide/hydroxide		23%
5	Tungstic acid		25%

For the epoxidation of compound **1** with Oxone, the reaction had a low yield of 17%, moreover, the conversion of the starting material to the product was also very low at only 25%. The ^{1}H NMR of the obtained product, beside the desired product- **2**, revealed the presence of impurities from ethyl acetate, water, and a significant amount of starting material (Fulmer et al. 2010).

For the method using hydrogen peroxide to prepare **2** the yield of the reaction was 0% The hydrogen peroxide did not affect the sulfolene and only the starting material was visible in the ^{1}H NMR spectrum.

The experiment using NBS/hydroxide as an epoxidizing agent for **1** was carried out 3 times to ensure no error in following the protocol had occurred. In the best run, the yield of the reaction was 23%. Conversion of starting material to the product was constantly in the range of 10%. In the ^{1}H NMR spectrum, signals from compounds **1**, **2** and impurities from used reagents NBS and acetone were visible (Fulmer et al. 2010).
The method using tungstic acid as an epoxidizing agent provided the yield of the reaction at 25%. The ^{1}H NMR spectrum indicated that the conversion of starting material to products after the time required was less than 10%. Impurities from dichloromethane and sodium acetate were present (Fulmer et al. 2010).

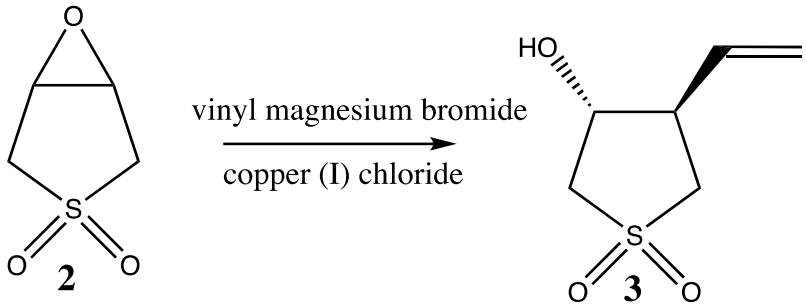
After testing the above methods, the mCPBA one turned out to be the best one for sulfolene.
The reaction was repeated multiple times to establish the optimal reaction time for the maximal conversion of the starting material to the product. Each trial provided a yield of around 40-50%, however, a longer time of reaction provided a better conversion. The reaction kept under reflux for 7 days gave no compound **1** visible in the NMR spectrum while the reaction kept at reflux for 48 hours provided the conversion of starting material to the product of only 50%. During the process of work-up, the reaction solution requires multiple filtrations as after single filtration using filter paper, the remaining mCPBA is present in the NMR spectrum.
The reason for the low yield and conversion of sulfolene to the epoxide might be the nature of the compound itself. The sulfonyl group is strongly electron-withdrawing and has charge distribution, molecular size and properties matching the carbonyl, carboxyl, or phosphate groups (Fei et al. 2016). Therefore, the double bond within the structure can be considered as electron-deficient and a different, more reactive method of epoxidation could be beneficial. A more current approach to the epoxidation of unsaturated sulfones was investigated by Lewis & Grayson (2012), where compounds structurally different than sulfolene-vinyl sulfones were exposed to nucleophilic epoxidation under the Meth-Cohn conditions. The main focus of the above studies was the achievement of enantiopure compounds, which is not the concern in the case of compound **1**, however, besides enantiopurity, Lewis & Grayson (2012) managed to achieve the sulfone epoxides in yields of up to 100% for some of the investigated compounds. The nucleophilic oxidating agent was butyllithium in a solution of tert-butyl hydroperoxide under nitrogen.

If the epoxidation of 3-sulfolene was to be repeated different epoxidation method would be proposed.

2.3 Opening of sulfolene oxide to 4-ethenyl-1,1-dioxothiolan-3-ol

The second step of the proposed route was to open compound 2 with a Grignard reagent to achieve compound 3 as shown in the scheme in Figure 6.

Figure 11. Scheme of the attempted opening of sulfolene oxide with vinyl magnesium bromide.

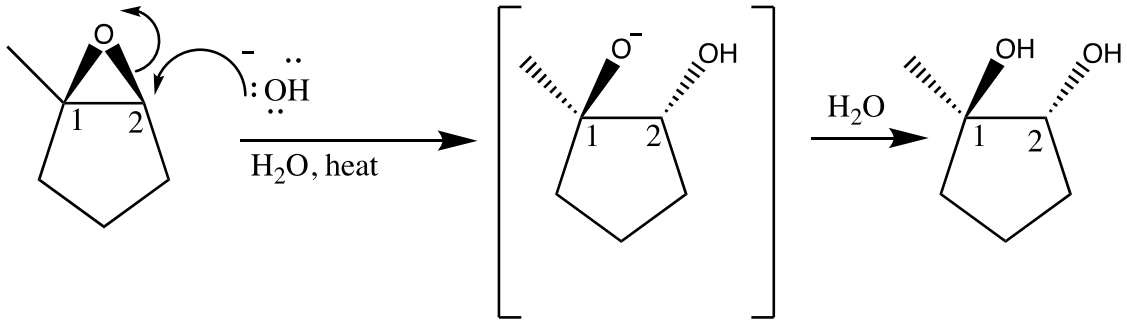


The reaction of nucleophilic addition of vinyl magnesium bromide to 3-sulfolene epoxide was unsuccessful. The ^{1}H NMR spectrum of the resulting product showed signals belonging to allyl bromide (Nayak et al. 2020), some impurities and water. Grignard reagents are very strong bases, so they react with mild acids like water to give conjugated acid and magnesium bromide hydroxide ions (McMurry, 2008). The reaction was repeated twice using solvent (THF), coming from the same bottle. The outcome for both reactions was identical, no desired product was obtained. Therefore, there is a possibility that the bottle of THF was contaminated with traces of water which destroyed the Grignard reagent (McMurry, 2008). If the reaction is to be repeated the new solvent would be bought to eliminate the risk of destroying the Grignard reagent.
The initially proposed 3rd step could not be continued, also, the newly bought equipment to produce ozone required for the next step of reaction was not ready to use yet. An alternative route of producing non-sugar GAG mimetics involving sulfolene was therefore proposed.

2.4. Synthesis of 4-ethoxy-1,1-dioxo-tetrahydro-1-thiophen-3-ol

Epoxide species can be cleaved by acid or base. Even though the ether oxygen is usually a poor leaving group in S_N2 reactions, the extra strain of the three-membered ring causes epoxides to react with hydroxide ion in presence of heat. (Figure 12)

Figure 12. General mechanism of nucleophilic attack on an epoxide

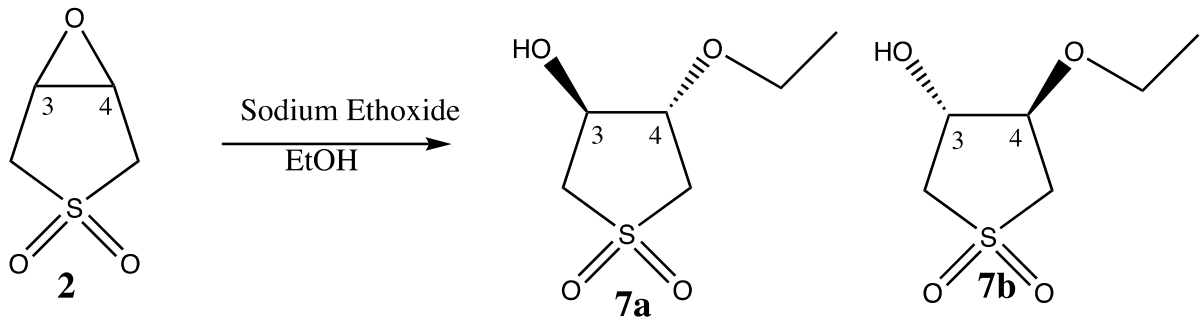


The base-catalysed reaction occurs through an S_N2 mechanism in which hydroxide ion attacks the less hindered epoxide carbon causing inversion of the stereochemistry. The resulting intermediate species undergoes a protonation to give a neutral alcohol product. The same mechanism applies to other nucleophilic species too like alkoxides in an alcohol solvent. The protonating species in this case is the alcohol solvent (McMurry, 2008).

As the first proposed scheme was not continued due to low reaction yield and an unsuccessful reaction with the Grignard reagent, compound **2** was opened with another nucleophile-sodium ethoxide in ethanol as a solvent to achieve **7a** and **7b**.

Sodium ethoxide was chosen as a nucleophile because it does not undergo irreversible protonation (as is in the case with vinyl magnesium bromide) and should easily react with compound **2**.

Figure 13. The mixture of 1:1 enantiomers resulting after opening compound 2 with sodium ethoxide



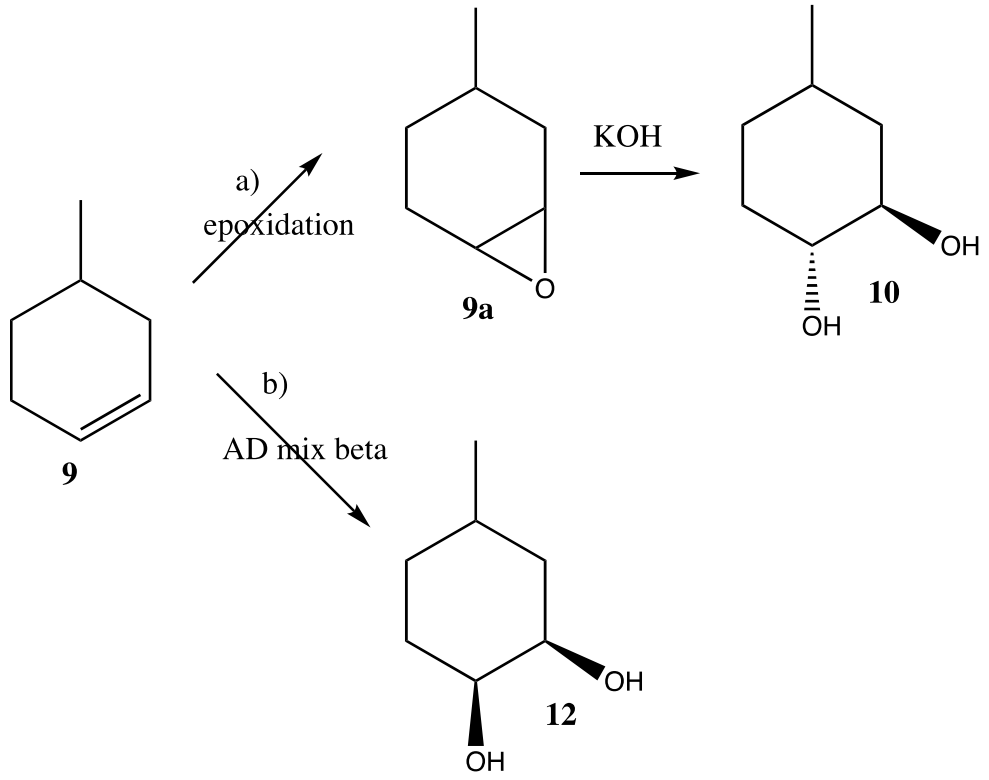
The method of using $NaOCH_2CH_3$ in EtOH has been reported as being applied to open compound **2** previously by Mukhamedova et al. (1975), however, only a melting point of the resulting compound was recorded by the author. However, if the ^1H NMR spectra of compound, and the resulting 4-ethoxy-1,1-dioxo-tetrahydro-1-thiophen-3-ol (**7**) are compared, the hydrogens from the epoxide carbons in **2** are gone and new identical multiplets in the ratio 1:1 at δ 4.5 and 4.2 ppm are present. From a mechanistic point of view, it is logical as compound **2** is symmetrical, moreover, the epoxide carbons are equal then the chance of an attack on carbons 3 and 4 in compound **2** are identical.

Also, in the literature, the racemic mixture of isomers after the opening of a similar compound-cyclopentene oxide, with nucleophile has been widely reported (Chakraborti & Kondaskar, 2003, and Chandrasekhar et al. 2002)

2.5 Reactions of 4-methylcyclohexene

The commercially available 4-methylcyclohexene (**9**) was bought from Sigma-Aldrich to explore the potency of GAG mimics with a 6-membered ring in the centre of the molecule. The first proposed scheme within this project supplies the GAG mimetics with a 5-membered ring in the centre of the molecule. The approach with the use of cyclohexene derivatives within the core of synthesized GAGs should provide additional information about the pharmacophore of molecules potentially able to competitively bind to HGF. The route to the synthesis of these mimetics included two pathways supplying different molecules with hydroxy groups in the *cis* or *trans* relationship (Figure 14).

Figure 14. Two different pathways to dehydroxylated cyclohexane molecules with different relative stereochemistry of the OH groups.

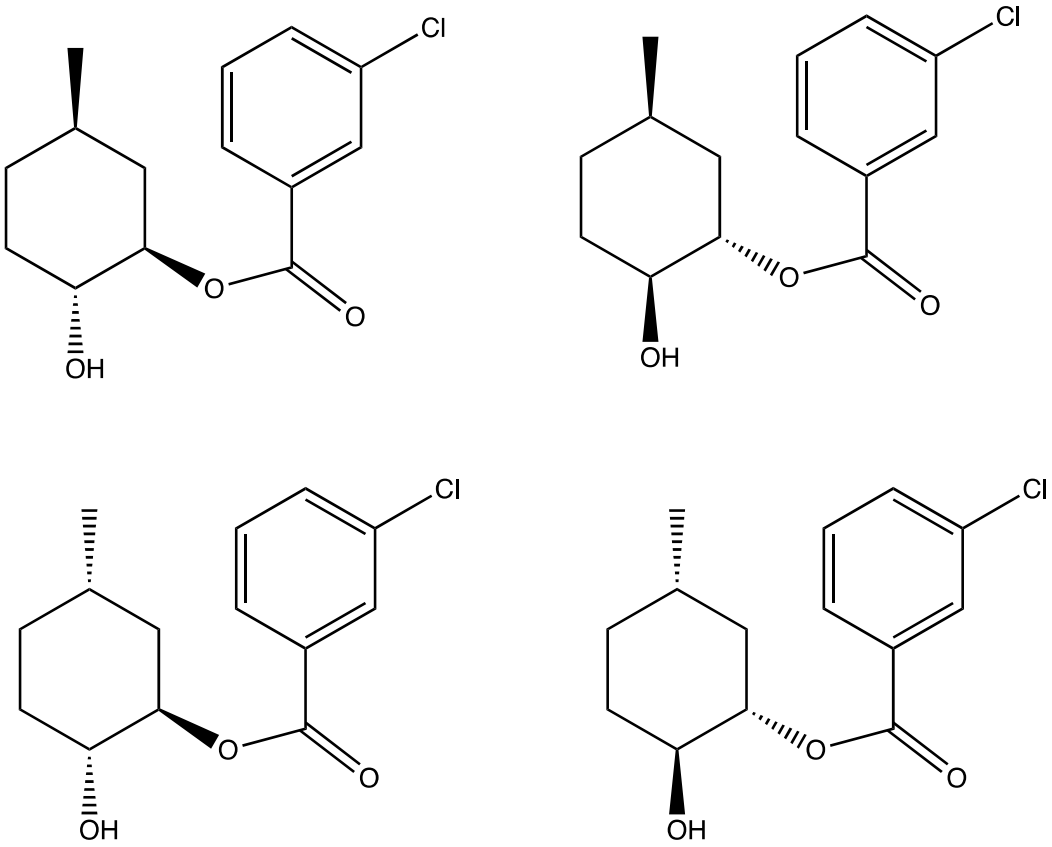


a) The route to the *trans* diol product **10**.

The first step to achieve the *trans* diol product **10** was to epoxidize compound **9**. As the mCPBA previously was the best method to achieve that with compound **1**, it was decided to use it for compound **9a.**

Based on previous experience with the use of mCPBA, the reaction was left refluxing for 6 days, however, the ^{1}H NMR spectrum information did not provide evidence of the formation of **9a** and was not consistent with the spectrum of 4-methyl-1,2-cyclohexene oxide (compound **9a**) found in the literature (Raban et al. 1994). There was a presence of aromatic protons, a set of 4 multiplets in the δ 3.7-5.1 region, and peaks from the cyclohexene ring in the CH_2 and methyl group CH_3 areas. The ^{1}H NMR spectrum suggested that the long reaction time may have caused the opening of the desired epoxide with a by-product of mCPBA- 3-chlorobenzoic acid (Figure 18). The reagent can be used as a nucleophile which was previously reported in the use of metal-catalysed ring-opening reactions (Jacobsen et al. 1997).

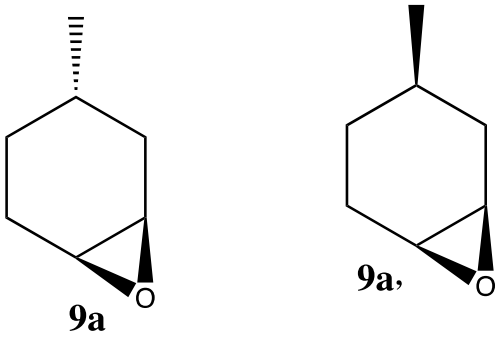
Figure 15. The possible stereoisomers resulting after opening racemic 4-methylcyclohexene oxide with 3-chlorobenzoic acid



In the case of opening of 4-methylcyclohexene oxide with the 3-chlorobenzoic acid, 4 stereoisomers are possible to be formed (Figure 15). That could explain the 4 identical multiplets in the δ 3.7-5.1 region. Also, the ^{1}H NMR and ^{13}C NMR data are consistent with 2-hydroxycyclohexyl benzoate (Reddi et al. 2014), which only differs from the proposed 2-hydroxy-5-methylcyclohexyl benzoate in the absence of the CH_3 group.

As the mCPBA method did not supply the desired compound, the Oxone method was used to prepare 4-methylcyclohexene oxide. The reaction was successful, and the obtained data were consistent with those reported in the literature (Raban et al.1994 and Chan et al. 1996). The stereoisomeric mixture (Figure 16) of *trans*-4-methylcyclohexene oxide - compound **9a** and *cis*-4-methylcyclohexene oxide-compound **9a'** was formed.

Figure 16. The mixture of cis and trans-4-methylcyclohexene oxide



In the case of work done by Raban et al. (1994) and Chan et al. (1996), different methods of epoxidation were used; hydrogen peroxide in trichloroacetonitrile, methylene chloride and hydrogen peroxide in acetonitrile, potassium bicarbonate and methanol respectively. Both papers report the resulting isomeric mixture of compounds in a ratio of 1:1. However, the integration of signals for the diastereoisomeric mixture obtained from compound **9** suggests the ratio of isomers of 1:2.7. The applied method using Oxone generates an oxidising agent derived from acetone dimethyl dioxirane, which is the actual oxidising species (Hashimoto & Kanda, 2002). The *trans* product of epoxidation has been found favoured in cyclic unsaturated compounds using methyltrioxorhenium, a similar oxidising species, with the *trans* selectivity increasing over *cis* selectivity in a linear correlation with the size of the substituent within the ring (Adam et al. 1999). Therefore the formation of diastereoisomers in the ratio of 73% *trans*-4-methylcyclohexene and 27% *cis*-4-methylcyclohexene is suggested.
The mixture moved as a single spot on a TLC plate, therefore, it is not possible to seperate the mixture by column chromatography.

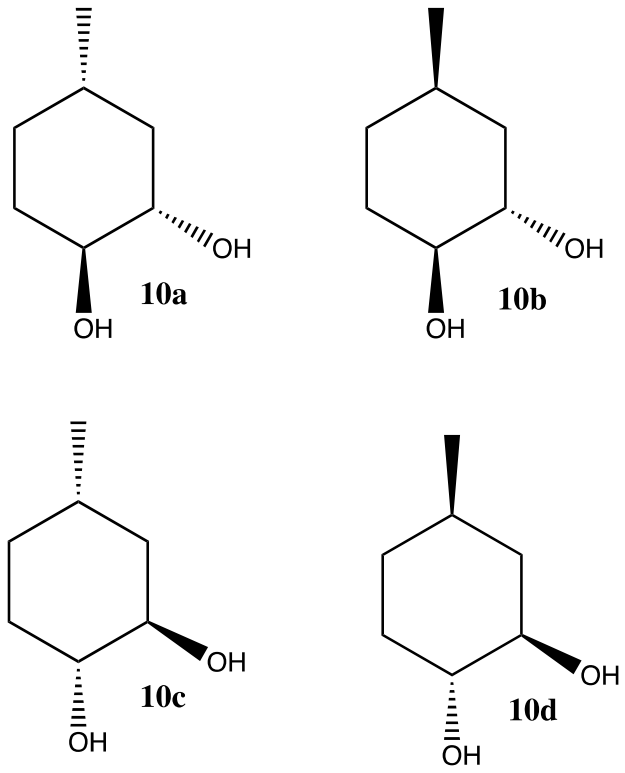
To obtain the trans-diol product (**10**) the resulting mixture of diastereomeric epoxides **9a** and **9a'** was opened with KOH in EtOH.
The method was previously used by Seifert et al. (2012) as a second step to achieve a dialdehyde product - Methyl-adipaldehyde from compound **9**, however, the first step to achieve it was an epoxidation of **9** with the use of mCPBA, not Oxone as in case of this project. Hence, the resulting isomeric mixture differed in ratio from the one obtained within this project. Seifert et al. (2012) did not include any NMR data or information about the

yield obtained specifically after the epoxide-opening step of their compound, but the overall yield of dialdehyde synthesis was 43% so the method used by Seifert et al. (2012) was sufficient to form dihydroxylated compound **10**.

The reaction was successful. ^{1}H NMR data were consistent with (*1R, 2R, 4R*)-4-methylcyclohexane-1,2-diol (**11b**) synthesized by Plummer et al. (2015). However, the integration between the hydrogens from the CH_3 groups and the hydrogens from the OH groups suggested that the diastereomeric mixture of products was produced in a ratio of 1:1.5. The mixture appears as one spot during the TLC, therefore column chromatography would not provide the separation of isomers.

However, if considering possible diastereoisomeric configurations, from the mechanistic view, 2 different diastereoisomeric pairs hence 4 different stereoisomers (Figure 17) could be formed after opening **9a** and **9a'**.

Figure 17. Possible diastereoisomers resulting after the base-catalysed opening of compounds **9a**ı and **9a'**.



To establish which exactly isomers were formed further investigations should be carried out. To help with the identification of the stereoisomers the technique HPLC/ESI-MS would be beneficial as the identification of diastereoisomeric mixtures by this method was recently reported (Chuchracka et al. 2020).

An alternative synthetic method to obtain compound **10** from compound **9** was reported by Habala et al in 2008. Compound **10** was achieved from **9** in one pot and one step with an overall high yield of 88%. Readily available reagents were used in the reaction (hydrogen peroxide, formic acid, sodium hydroxide) and the resulting product was a mixture of 2 stereoisomers. If the synthesis was repeated the method used by Habala et al. 2008 would be used, as a one-pot synthesis provides less waste and potentially an overall higher yield for the reaction.

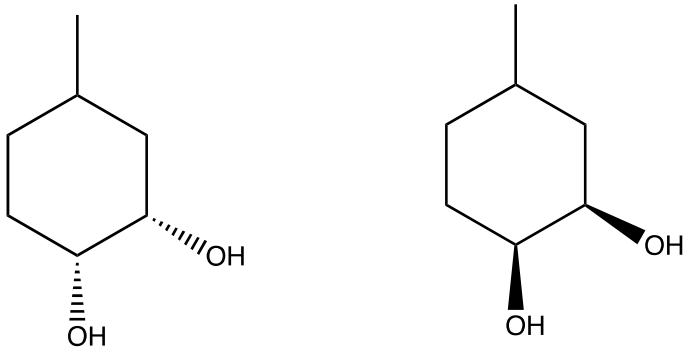
b) Route to obtain *cis*- diol product (**12**)

To synthesise compound **12** where hydroxyl groups are in the '*cis*' position relative to each other, the method with the use of AD mix *beta* was selected.

In the current literature compound, **9** was previously dihydroxylated to achieve **12** by Chanteau & Tour (2003) with the use of osmium (VIII) oxide, *N*-methyl-2-indolinone in water, acetone, and *tert*-butyl alcohol. However, the reported yield of the reaction was low(23%) , therefore within this project, the method with the use of commercially available osmium tetroxide containing AD mix beta was proposed. The osmium tetroxide was previously found efficient in asymmetric dihydroxylation of cyclohexene compounds resulting in a decent yield for the reaction up to 89% (Matt et al. 2022)

The dihydroxylation of 4-methylcyclohexene worked with the AD mix β; however, the reaction yield was 77%, the ^{1}H NMR and ^{13}C NMR data are consistent with the literature (Chanteau & Tour, 2003). The resulting mixture appeared as one spot by the TLC, however, the ^{1}H NMR data indicate a mixture of diastereomers in a ratio of 1:1 (Figure 18), which is also consistent with the literature (Chanteau & Tour, 2003).

Figure 18. The resulting mixture of 1:1 diastereoisomers of *cis*-4-methylcyclohexene after treatment with AD mix.



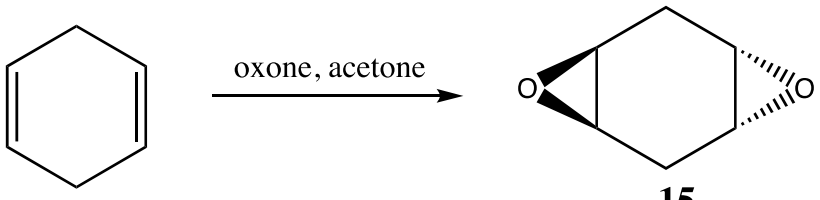
12a **12b**

To improve the reaction yield, an alternative method using an Os/Cu-Al-hydrotalcite catalyst could be applied. Friedrich et al. (2003) introduced an easy preparation of the multimetallic hydrotalcite catalyst which, with *N*-methylmorpholine oxide as co-oxidant dihydroxylated cyclohexene, which differs from compound **9** only in the absence of the CH_3 group, in a yield of 100%.

2.6. Epoxidation of 1,4-cyclohexadiene

Commercially available 1,4-cyclohexadiene was suggested to be explored in the scheme using cyclohexene molecules as a starting material for the scheme supplying GAG mimics with 6-membered ring as a central core in a molecule.

Figure 19. The synthesis of *trans*-1,4-cyclohexene diepoxide



14 **15**

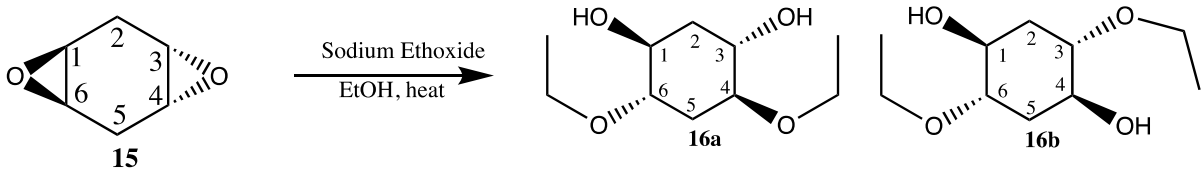
The synthesis succeeded, and the 1,4-cyclohexene diepoxide was achieved with yield – 32%. The NMR data were consistent with the literature. (D'Accolti et al. 2012). Interestingly, only the *trans* diepoxide isomer was synthesized (Figure 19).

In the studies carried out by Cavdar & Saracoglu (2008), where 1,4-cyclohexadiene was treated with mCPBA, the method used supplied both isomers, *cis* and *trans*. The separation of the isomers was achieved by column chromatography and the ^{1}H NMR data for both isomers

were obtained. The *cis* isomer's presence is associated with a signal at δ 2.74 ppm corresponding to CHO hydrogen within the ^{1}H NMR spectrum. In the case of the experiment carried out within this project, the resulting product does not exhibit any signal in this region, therefore, specifically *trans*-1,4-cyclohexadiene epoxide (**15**) was synthesized. Also, in the studies carried out by D'Accolti et al. (2012), where the epoxidation of 1,4-cyclohexadiene was carried out with the use of dimethyldioxirane only the *trans* diepoxide isomer was produced as well. Thos suggests, that the mechanism of epoxidation of 1,4-cyclohexadiene with mCPBA and dimethyloxirane differs and the second method is found to be diasterospecific supplying only a *trans* epoxide product.

2.7 Opening of *trans*-1,4-cyclohexenediepoxide with sodium ethoxide

Figure 20. The mechanistically possible diastereomeric configurations of compound 16.



The reaction is another example of a nucleophilic attack on epoxide carbon of compound **15**. From the mechanistic point of view, a nucleophile, sodium ethoxide, attacks a back side of less substituted carbon causing an inversion of stereochemistry. Compound **15** has 2 epoxides within its structure and could form diastereoisomers with a hydroxyl group in relative positions 1,3 (**16a**) or 1,4 (**16b**) illustrated in Figure 20.

The ring opening of **15**with alkoxides like sodium ethoxide has not been reported so far. However, many reactions with nitrogen-containing nucleophiles like 1°, 2°amines or pyrazole give predominantly 1-3-diols due to the Furst-Platter control of second ring opening (Monceaux & Carlier, 2009).

Further investigations of **15** with various aniline derivatives as a nucleophile and different reaction conditions influenced the mechanism of the reaction to produce the 1:1 isomeric mixture of 1,3-diols and 1,4-diols. It was reported that the electronic nature, electron- poor' or 'electron-rich', of a nucleophile and the solvent factor (neat reaction or reaction in water as a solvent) has a crucial significance for the resulting isomeric ratio. (Monceaux & Carlier, 2009).

The outcome of the reaction of **15** with sodium ethoxide was evaluated by ^{13}C NMR and ^{1}H NMR. The reaction has not been reported earlier in the literature hence further analytical techniques should be used to confirm the formation of **16**, however, if comparing the ^{1}H NMR spectra of **15** and **16**, the spectrum of **16** has not changed much; shifts of two peaks associated with epoxide carbons in **15** have moved up field and now appear at δ 2.95 ppm and δ 2.19 ppm and the aliphatic region at integrates to 10 hydrogens from the 2x -OCH_2CH_3 in **16**,

Also, within the C NMR, only 7 different carbon environments appear within the spectrum which would be the case if only isomer **16b**, the symmetrical compound, was synthesized. Therefore, the formation of a 1,3-diol isomer is suggested. To further evaluate the resulting sample of **16,** it is advised to use HPLC analysis.

2.8. Microwave-assisted reactions with the use of 3-sulfolene as 'masked' precursor to butadiene

3-Sulfolene has been broadly used as a precursor supplying, 3-butadiene into a reaction upon heating, and the resulting product can act as a diene in Diels-Alder cycloaddition reactions (Lopez et al. 2003, Andrade et al. 2003).

The idea to use sulfolene as a reagent in microwave-assisted Diels-Alder reactions seemed like a good way to 'accelerate' the cycloaddition of dienophiles without a powerful EWG conjugated to the double bond.

2.8.1 Styrene as a dienophile

As a first dienophile, commercially available styrene was selected. The reason for that was the fact that the resulting product of the reaction would be a good starting material for the scheme using cyclohexenes in the first step to achieving GAG mimetics.

According to the rules of cycloaddition reactions (McMurry, 2008), the theoretical resulting product from buta-1,3-diene and styrene would be compound **17** (Figure 21).

A literature search for any previously recorded microwave reactions between **1** and styrene did not provide any hints regarding the possible conditions. However, Quiroga et al. (2017) worked on the synthesis of Pyrazolo[3,4-*b*]pyridines by using β-nitrostyrenes as dienophiles in Diels-Alder cycloadditions in a microwave reactor, therefore the initial time of the reactions and choice of solvent was based on their work

Figure 21. The failed synthesis of 3-phenylcyclohexene from styrene and sulfolene

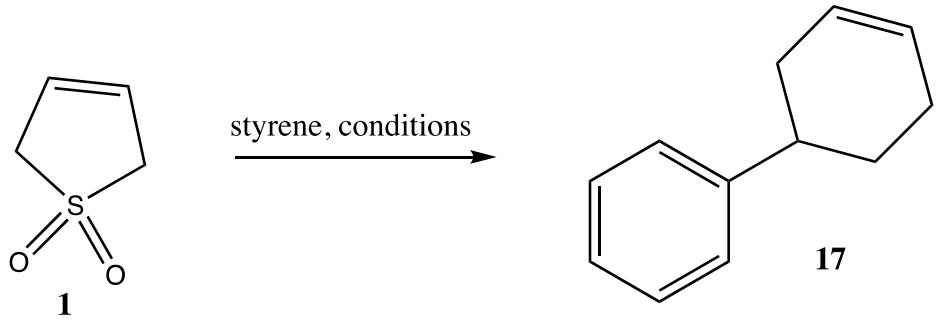


Table 2. Conditions for the microwave-assisted Diels-Alder reactions

Entry No.	Quantity of sulfolene	Quantity of styrene	Solvent	Time [mins]	Temperature [Celsius degrees]	Power [Watts]	Yield of reaction
1.	0.118g/ 1mmol	0.104g/ 1mmol	toluene	4	230	300	0%
2.	0.118g/ 1mmol	0.104g/ 1mmol	neat	4	230	300	0%
3.	0.118g/ 1mmol	0.104g/ 1mmol	neat	60	150	300	0%
4	0.118g	0.104g	neat	10	150	300	0%

Various reaction conditions were tested for sulfolene and styrene (Table 2), however, in every run, starting materials: styrene and sulfolene were present in the ^{1}H NMR spectrum. The presence of a new double bond would be a sign that the reaction had worked. Only in the case of entry no. 3, in the ^{1}H NMR spectrum, two multiplets in δ 3.7-4.2 ppm were visible, however, the chemical shift value does not correlate to the literature value for 3-phenylcyclohexene (**17**) (Imboden et al.1999). and therefore, the approach, using styrene as a dienophile for a microwave-assisted Diels-Alder reaction was stopped at this time.

2.8.2 3-methylcyclohexene-1-one as a dienophile in Diels-Alder microwave-assisted reaction

As the previous idea to the use sulfolene with styrene to synthesize a cyclohexene under microwave-assisted conditions failed, a new dienophile was chosen to react with **1**.

The alkene species was 3-methylcyclohexene-1-one, and compound which was available in the University's inventory and the structure of the alkene with the carbonyl group conjugated with the double bond was supposed to help the cycloaddition reaction to occur (McMurry, 2008).

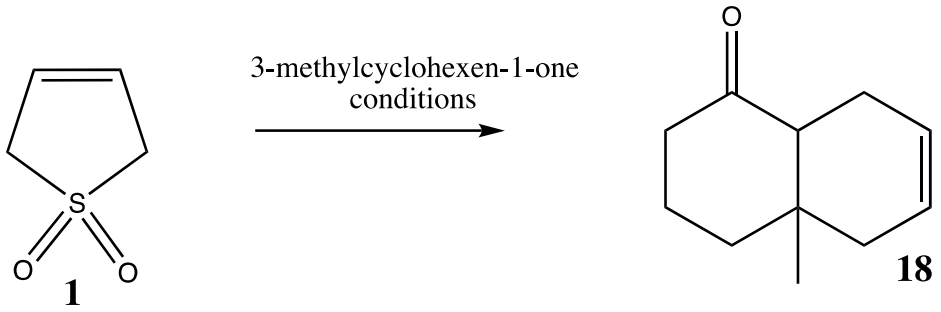
Various conditions for the microwave-assisted Diels-Alder reaction of **1** and 3-methylcyclohexene-1-one were tested (Table 3).

Table 3. Conditions and the outcome for the microwave-assisted Diels-Alder reactions of **1** and 3-methylcyclohexene-1-one

Entry No.	Quantity of sulfolene	Quantity of 3-methylcyclohexene-1-one	Solvent	Time [Mins]	Temperature [Celsius degrees]	Power [W]	Yield of reaction
1	0.118g 1mmol/	0.110g /1mmol	neat	30	180	300	0%
2	0.118g 1 mmol	0.110g /1mmol	neat	60	180	300	0%
2	0.118g 1 mmol	0.110g /1mmol	toluene	15	150	300	0%
3	0.118g 1 mmol	0.110g /1mmol	neat	25	110	300	0%
4	0.118g 1 mmol	0.110g /1mmol	toluene	25	110	300	0%

Considering the rules for stereoelectronic and conformational factors for Diels-Alder cycloaddition of **1** and 3-methylcyclohexene-1-one, the resulting product would be 4-methyl-3,4,4a,5,8,8a-hexahydro-1(2H)naphthalenone – compound **18** (Figure 22) which was confirmed by Angell et al. (1989).

Figure 22. The theoretical product resulting from Diels-Alder reaction of butadiene and 3-methylcyclohexen-1-one



According to the literature (Angell et al. 1989), the CH_3 group in the 4a position within the compound **18** exhibits a hydrogen signal in the NMR spectrum at the δ 1.1 ppm, however, none of the products after microwave-assisted runs showed absorption in this area of the ^1H NMR spectrum. The peaks belonging to 3-methylcyclohexene-1-one, sulfolene and 1,3-butadiene were all still present. Therefore, none of the runs provided the desired compound **18** in a microwave-assisted Diels-Alder reaction.

Compound **1** did not work in microwave-assisted Diels-Alder reactions with either of the provided dienophiles. The problem could be the behaviour of **1** in the microwave reactor. Any previous use of sulfolene acting as masked butadiene under microwave conditions has not been reported so far. In the case of standard conditions, sulfolene was used as a part of a ring system (Govaerts et al. 2002, Govaerts et al. 2003), Diels-Alder reactions had intramolecular character (Leonard et al. 1994) or the used dienophile had strong withdrawing groups, for example, maleic anhydride (Roshchin et al. 2007). In the two first cases, the configuration of the double bonds in the diene relative to each other is predetermined to be *cis (*E*)*. In the last case, the 'strong' dienophile will react with either configuration of diene. It suggested that **1**, under the microwave-assisted conditions tested within this project, decomposes to SO_2 and butadiene in *trans* (Z) configuration, which is less favoured in Diels-Alder reactions. Further investigations to establish the configuration of the diene derived from **1** are encouraged to support this hypothesis.

2.9 Sulfation reactions

The last step to synthesize non-sugar GAG mimetics within this project was adding the level of sulfation needed for competitive binding to HGF to occur. In the current literature, the sulfation of carbohydrates providing a level of sulfation specific to the target of its biomolecular partner has been carried out widely for different targets (Ueki et al. 2001, Mand et al. 2015).

The reported sulfation reactions were mostly carried out for carbohydrates, where the sulfated species is the oxygen from the OH groups within the sugar structure. The yields of these reactions are generally high in the region of 80-100% (Nishimura et al. 2013, Takeda &Tamura, 2014). The reagent used to achieve this is the commercially available sulfur trioxide−trimethylamine complex, one of the most stable SO_3 complexes available on the market. Its stability comes from the base strength of trimethylamine when compared with for examples the complex with another basic compound, pyridine. This stability allows the sulfur trioxide−trimethylamine complex to be used even in the presence of water; in a solution containing 25% of water, only 6.4% of the SO_3 complex is hydrolyzed during 24 hours. The complex is known to be best dissolved in dimethylformamide (Gilbert, 1962).

The use of sulfur trioxide−trimethylamine complex to treat non-sugar alcohol compounds was previously reported with good yields and it is present in plenty of patent literature. (Gilbert, 1962). Alcohols are treated with $SO_3.NMe_3$ are converted into alkyl acid sulfates. In the case of this project synthesized compounds, **10** and **12** are good candidates to be sulfated using the sulfur trioxide−trimethylamine complex in DMF as a solvent.

Recently, interesting use of diarylborinic acid-catalysed method of sulfation with use of $SO_3.NMe_3$ has been reported. (Gorelik et al. 2019). The method could be used for future research in the synthesis of sulfated GAG mimetics with more than 2 hydroxy groups within the structure. The diarylboronic acid coordinates to two neighbouring oxygens from OH groups within the polyalcohol's structure and the following addition of SO_3 complex causes the interaction between diarylboronic acid and sulfur complex, resulting in attaching the SO_3- to only one of the oxygens. This approach would be useful if future research is aimed towards testing the potency of a specific location of sulfation within the non-sugar GAG mimetics.

2.9.1 Attempted sulfation of *trans* and *cis* isomers of 4-methylcyclohexene diol

Compounds **10** and **12** were treated with earlier proposed $SO_3.NMe_3$ in DMF as a solvent. However, the outcome of both reactions was difficult to establish. The ^1H NMR spectra of resulting products did not provide enough information about the successful synthesis of either **11 or 13.**

Comparing the ^1H NMR spectrum of **12** and supposed compound **13,** the signals correlating to two hydrogens from OH in **12** were no longer present which suggests that there was no starting material left.

In the case of supposed compound **11,** the signals correlating to two hydrogens from OH in **10** were no longer present as well, however, it does not give the certainty that the product was a desired sulfated compound.

In both cases, further identification of remaining signals was difficult because of the presence of impurities from solvents; DMF and toluene. Due to the fire accident in the university's organic chemistry lab, access to the lab was restricted within the last month of the project. The work planned for the purification of the resulting compounds could not be carried out. Also the planned analytical work on the characterization of sulfated compounds by other techniques like IR, MS and HPLC could not be completed due to the absence of staff responsible for the supervision of the analytical equipment (person affected by the fire) and time restrictions for the above project.

If there would be more time to continue work on this project, the first task would be to remove DMF from both samples obtained after treatment of **10** and **12** with $SO_3.NMe_3$ complex**.** The method to remove DMF applied within this project where toluene was added to the resulting sample to lower the overall boiling point of the mixture and allow it to evaporate together with DMF failed. The probable reason for that is the previous rotary evaporator with a strong vacuum generated by the pump was damaged during the accident with the fire. The rotary evaporator from the other lab did not have a strong vacuum supplied and it was not powerful enough to evaporate DMF/Toluene mixture from the reaction flask. The alternative work-up could be done by dissolving the resulting mixture in DCM and extracting it a few times with water or brine. DMF is soluble in organic solvents and in water as well, so multiple washes would need to be applied to completely get rid of the solvent.

After the removal of the solvent, the resulting mixture should be checked by TLC to see if it is possible to separate diastereoisomers (as the starting materials for the sulfation reactions-compounds **10** and **12** were mixtures of isomers, to begin with).

If the stereoisomeric mixtures could be separated, then the further characterization of each isomer would take place.

The next logical step would be to test the synthesized compounds in a biological assay.

2.10 Wound healing assay as a biological assay to establish the potency of GAGs synthesized in future research.

If the last step of synthesis of GAG mimetics could be completed, the resulting compounds would need biological evaluation to prove their potency. Especially if the synthesis of compounds **11** and **13** would be a success, the biological assay could provide information regarding the pharmacophore within the structure, especially the preferred positions *cis* or *trans* of disulfated molecules.
A good technique to test the synthesized GAGs mimetics would be a wound healing assay with the use of live-cell microscopy. The above *in vitro* technique investigates collective cell migration in 2D. The assay is widely used for cancer metastasis or tissue injury (Jonkman et al. 2014). The fact that sulfated non-sugar GAG mimetics have been synthesized to competitively bind to HGF and, therefore, affect the activity of Met receptor responsible for motility of cells and known to be accelerated in cancer metastasis, this would make wound healing assay a good measure of the potency of the synthesized compounds.

2.11 Conclusions

Two routes to synthesize GAG mimetics proposed within this project were found challenging. In particular, the first scheme with the use of sulfolene as a starting material for GAG mimetic molecules with a 5-member ring as a core, could not be completed due to low yield, and the unsuccessful 2nd step of the synthesis. The reasons for this and solutions to improve future synthesis based on compound **1** were proposed.
The route supplying cyclohexene derivatives was more successful, however, the sulfation of compounds **10** and **12** needs further purification and evaluation by analytical techniques. The biological evaluation of resulting sulfated compounds would supply valuable information about the pharmacophore for further research on sulfated non-sugar GAG mimetics.

3. Methods

All ^{1}H and ^{13}C NMR spectroscopy was performed at Salford University on a Bruker Ultrashield 600 MHz spectrometer for ^{1}H and 150 MHz for ^{13}C at ambient temperature and edited using Bruker Topspin software. The chloroform-d was used as a solvent unless stated otherwise.

3.1 Epoxidation of sulfolene

1. mCPBA

mCPBA (75.6 mmol, 13 g) was added to a solution of sulfolene (28.6 mmol, 3.47 g) in dichloromethane (60 mL) and heated under reflux for 48 h. The solution was cooled down to room temperature and filtered. The filtrate was diluted with dichloromethane (250 mL) and then washed with saturated sodium sulfite (300 mL), saturated sodium bicarbonate (300 mL) and saturated sodium chloride (300 mL). The organic layer was dried with magnesium sulfate and the solvent evaporated *in vacuo* provided 1.63 g of crude product. Yield 46%.

The product had been formed, as the H NMR values for sulfolene oxide were consistent with the literature. (Xue & Seto, 2006)

^{1}H NMR (600 MHz, $CDCl_3$) δ 11. 6 (s,1H), 8.1 (s, 2H), 8.0 (t, J=6.4, 2H) 7.7 (t, J=3.2, 2H), 7,5 (m, 2H), 7,3 (s, 1H), 6,10 (s, 2H), 3,9 (s, 2H), 3.8 (s, 4H), 3.5 (d, J= 7.3, 2H), 3.4 (d, J=3.5, 2H).

1. Potassium peroxymonosulfate

Sulfolene (2.5 mmol, 0.303 g) was dissolved in a mixture of ethyl acetate and acetone (100 mL:100 mL). At the same time, a solution of sodium bicarbonate (10 g) and benzyltriethylammonium chloride (0.12 g) in water (70 mL) was prepared and added to the sulfolene. The mixture was cooled in the ice bath and stirred. A solution of potassium peroxymonosulfate (12 g) in water (150 mL) was added dropwise with the use of the syringe pump at the rate of 75 mL per hour. The reaction was left stirring for 3 days. After that time, the layers were separated and the organic layer was washed with water (100mL), dried with magnesium sulfate and solvent has been removed in *vacuo* giving 0.05g of crude product. The reaction provided a yield of 17%.

^{1}H NMR (600 MHz, $CDCl_3$) δ 7.32 (s, 1H), 6.1 (s, 2H), 4,12 (m, 2H), 3,9 (s, 2H), 3.8(s, 4H), 3.5 (d, J= 7.3, 2H), 3.4 (d, J=7.4, 2H), 2.05 (s,3H), 1.55 (s, 2H) 1.26, (t, J=3,2, 3H)

2. Hydrogen peroxide

Sulfolene (24 mmol, 2.91 g) was added to a mixture of methanol (300 mL), potassium bicarbonate (0.2g) and acetonitrile (12.5 mL) and stirred. 30% Hydrogen peroxide solution (5.8 mL) was added dropwise over 10 mins and the mixture was left to stir at room temperature overnight. The following day, the solvents were evaporated in vacuo and the residue was dissolved in ethyl acetate (200 mL) and washed with water (150 mL), the resolving organic layer was dried with magnesium sulfate and again solvent was removed using a rotary evaporator.

The hydrogen peroxide did not affect the sulfolene; only the starting material was visible in the ^{1}H NMR spectrum. Yield: 0%.

^{1}H NMR (600 MHz, $CDCl_3$) δ 7.3 (s, 1H), 6.1 (s, 2H), 3.8 (s, 4H).

3. *N*-Bromosuccinimide/base

   A mixture of sulfolene (10.4 mmol, 1,23 g) and NBS (1.6g, 9.0 mmol) was added to a mixture of acetone (10 mL) and water (4 mL), and the solution was stirred further for 30 min in an ice bath. After that time, the ice bath was removed, and the reaction was left stirring at room temperature for another 60 min. A second portion of NBS (0.2g, 1.1 mmol) was added and left to react for another 90 mins. The reaction was cooled down with the ice bath and a 5 M solution of sodium hydroxide (20mL) was added. The mixture was left to reach room temperature, and stirred for another 60min, after which time it was diluted with 20% citric acid (20 mL) and extracted with dichloromethane (50 mL). The separated organic layer was dried with magnesium sulfate and evaporated *in vacuo* providing 0.28 g of crude product.

   The experiment was carried out 3 times to ensure no error in following the protocol occurred. In the best run, the yield of the reaction was 23%. Conversion of starting material to the product was constant, in the range of 10%. In the ^{1}H NMR spectrum, starting material, product, and impurities from used reagents; NBS and acetone were visible.

   ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.10 (s, 1H), 3.9 (s, 2H), 3.8 (s, 2H), 3.5 (d, J=7.3, 2H), 3.4 (d, J=7.6, 2H), 2.7 (s, 4H), 2.17 (s, 4H).

4. Tungstic acid

   Sulfolene (2.72 mmol, 0.33 g), and tungstic acid (2.72 mmol, 0.68 g) were added to a solution of 30% hydrogen peroxide (10 mL), methanol (5 mL) and sodium acetate (0.68 g). The reaction was left stirring at room temperature overnight. The following day, a solid was filtered off from the reaction mixture and washed with dichloromethane (30

mL) The filtrate was extracted with dichloromethane (3x 30 mL), dried over magnesium sulfate and the solvent was evaporated in *vacuo* giving 0.08 g of crude product.
The yield of the reaction was 25%. The ^{1}H NMR spectrum indicated that the conversion of starting material to products after the time required was less than 10%. Impurities from dichloromethane and sodium acetate were present.
^{1}H NMR (600 MHz, $CDCl_3$) δ 6.10 (s, 1H), 5.3 (s, 2H), 3.9 (s, 2H), 3.8 (s, 2H), 3.5 (d, J=8.2, 2H), 3.4 (d, J=6.2, 2H), 2.1 (s)

3.2 Opening of sulfolene oxide to 4-ethenyl-1,1-dioxothiolan-3-ol

Under argon, a solution of vinylmagnesium bromide in THF (1 M, 0.2 mL, 1,5 mmol), and copper(I) chloride (5 mol %, 0.005 g) was made and cooled down to a temperature of -10 °C (cooling bath with ice and acetone in ratio 1:1). A solution of sulfolene oxide (1 mmol, 0.12 g) in THF (4 mL) was added dropwise over 60 min. During this time the temperature of the solution was kept constantly around -10 °C . After 1 hr the solution was allowed to warm to 0 °C and methanol (2.5 mL) was added to dilute the reaction mixture and aqueous HCl (2 M, 3 mL) was added to quench the reaction. The solution was stirred for another 60 mins and methyl tert-butyl ether (5 mL) was added. The organic layer was separated followed by washing with aqueous HCl (2 M, 3 mL), water (2 mL), saturated sodium thiosulfate solution (2 mL) and water (2 mL). The resulting organic layers were combined, and the solvent was evaporated *in vacuo*.
The reaction was not successful; in the ^{1}H NMR spectrum, signals corresponding to allyl bromide were present (Nayak et al. 2020).
The yield of the reaction was 0.
H NMR (600 MHz, $CDCl_3$) δ 6.28 (m, 1H), 5.5 (m, 1H), 5.3 (m, 1H), 4.00 (d, J=8.4, 2H), 1.5 (s, 2H).

3.3 Opening of 3-Sulfolene epoxide with sodium ethoxide.

A solution of sodium ethoxide (1.5 mmol, 0.102 g) in ethanol (10 mL) was prepared and 3-sulfolene oxide (0.84 mmol, 0.112 g) was added. The reaction was heated at reflux for 18 h. After that time, the solvent was removed *in vacuo* and a mixture of ethyl acetate and water (20 mL:20 mL) was added and transferred to a separatory funnel. The solution was

extracted with ethyl acetate (2 x 20 mL) and dried over magnesium sulfate. The crude product was obtained by removing the solvent *in vacuo* giving 0.05 g (0.37 mmol) Yield: 45% .

^{1}H NMR for a mixture (600 MHz, $CDCl_3$) δ 4.5 (m, 1H), 4.2 (m, 1H), 3.2-3.7 (m, 2x6H), 1.2 (m, 2x3H)

^{13}C 135 dept NMR for isomers mixture (150 MHz, $CDCl_3$) δ 15.15 (CH_2), 15.16 (CH_2), 26.52 (CH_3), 29.39 (CH_3), 47.45 (CH), 49.85 (CH), 69.40 (CH_2), 71.76 (CH_2)

3.4 Reactions of 4-methylcyclohexene.

3.4.1. Epoxidation of 4-methylcyclohexene

4-Methylcyclohexene was epoxidized by two methods, using mCPBA and dimethyldioxirane

a. mCPBA

   mCPBA (0.45 g, 2.64 mmol) was added to a solution of 4-methylcyclohexene (1 mmol, 1 mL) and dichloromethane (20 mL) and heated under reflux for 6 days. The solution was cooled down to room temperature and filtered. The filtrate was diluted with dichloromethane (100 mL) and then washed with saturated sodium sulfite (100 mL), saturated sodium bicarbonate (100 mL) and saturated sodium chloride (100 mL). The organic layer was dried with magnesium sulfate and the solvent evaporated in *vacuo.*

   Yield 0%.

   Data not consistent with the spectrum of 4-methyl-1,2- cyclohexene oxide found in the literature. (Raban et al. 1994). There was a presence of an aromatic compound (impurities from mCPBA) and a set of 4 triplets in the δ 3.7-5.1 region and peaks from the cyclohexene ring in CH_2 and CH_3 areas.

   ^{1}H NMR (600 MHz, $CDCl_3$) 8.1 (s, 1H), 8.0 (t, J=6.2, 1H) 7.7 (t, J=6.4, 1H), 7.5 (m, 1H), 5.1 (t, J=7.2, 1H), 4.8 (t, J=7.3, 1H), 3.8 (t, J= 6.3,1H), 3.7 (t, J=6.4, 1H), 1.2-2 (m, CH_2), 0.8 (m, CH_3).

b. Conversion of 4-methylcyclohexene to 4-methyl-1,2- cyclohexene oxide by Oxone.

   4-methylcyclohexene (1cmmol, 1cmL) was dissolved in a mixture of ethyl acetate and acetone (50 mL:50 mL). At the same time, a solution of sodium bicarbonate (5g) and benzyltriethylammonium chloride (0.06 g) in water (35 mL) was prepared and added to the 4-methylcyclohexene. The mixture was cooled in an ice bath and stirred. A

solution of potassium peroxymonosulfate (6g) in water (75mL) was added dropwise with the use of the syringe pump at the rate of 75mL per hour. The reaction was left stirring for 3 days. After that time, the layers were separated and the organic layer was washed with water (50 mL), dried with magnesium sulfate and solvent removed *in vacuo* gave 0.80 g of crude product.

The yield of the reaction was 80%, and the ^{1}H NMR and ^{13}C NMR data are consistent with the literature (Raban et al. 1994). Also the integration of the peaks corresponding to the epoxides and methyl group supplied the information that the reaction results in a mixture of diastereoisomers in a ratio 1:2.7.

H NMR (600 MHz, $CDCl_3$) δ 3.12, 3.15 (s, 2H, CHO), 1.2-2.0 (m, 14H, cyclohexene ring), 0.85, 0.86 (s, CH_3)

3.4.2 Opening the 4-methyl-1,2- cyclohexene oxide with base

The diastereomeric mixture of 4-methyl-1,2-cyclohexene oxide (1 mmol, 0.112 g) was added to a solution of KOH (5 mmol, 0.337 g) in EtOH (20 mL). The reaction was heated at reflux for 48 hours.

After completion of the reaction, the solvent was removed *in vacuo*. A mixture of ethyl acetate and water (20 mL;20 mL) was added to the reaction flask and then transferred to a separatory funnel where the organic layer was separated. The aqueous layer was washed further with ethyl acetate (3 x20 mL). The combined organic layers were dried with magnesium sulfate and solvent dried in *vacuo* resulting in 0.110g of crude product.

Yield: 98%

^{1}H NMR (600 MHz, $CDCl_3$) δ 3.46-3.30 (m, 2H), 2.15 (d, J=7.2 1H), 2.10 (d, J=7.6 1H), 2.00-1.91(m, 2H), 1.73-1.46 (m, 2H), 1.40-1.24 (m, 1H), 1.09 – 0.92 (m, 2H), 0.94 (d, J=6.8, 3H)

^{13}C NMR (150 MHz, $CDCl_3$) δ 78.7, 76.8, 37.

3.4.3 Dihydroxylation of 4-methylcyclohexene using AD mix.

AD mix beta (1.4 g) was added to a mixture of 5 ml tert-butyl alcohol and 5 ml of water. Methanesulfonamide (0.10 g) was added to the mixture and stirred at room temperature to the point where two clear phases were obtained. The reaction mixture was cooled in an ice bath and 4-methylcyclohexene (1 mmol, 0,12 g) was added and stirred for 18 hours. The temperature of 0 °C was maintained during the reaction. Upon completion, the sodium sulfite (1.5 g) was added and the reaction continued stirring for 30 mins at 0 °C, after which it was left stirring for another 30 mins at room temperature. The reaction mixture was extracted with ethyl acetate (3 x 30 ml) and brine (50 ml) and dried over magnesium sulfate. The final product was obtained by removing the solvent *in vacuo* resulting in 0.09g of **12**.

The yield of the reaction was 77%. The spectroscopic information was consistent with the literature (Chanteau & Tour, 2003). Also consistent with the literature, the reaction results in two isomers in a ratio of 1:1, which appear as one spot in the TLC;

^{1}H NMR (600 MHz, $CDCl_3$) δ 3.95 (m, 2 H), 3.61 (m, 2 H), 2.20 (br s, 4 H), 1.90 (m, 2 H), 1.82-1.63 (m, 6 H), 1.45-1.14 (m, 6 H), 0.93 (d, J=6.5, 3 H), 0.88 (d, J=6.3, 3 H).

^{13}C NMR dept135 (150 MHz, $CDCl_3$) δ 71.78, 71.75, 69.60, 68.71, 39.35, 37.33, 32.44, 30.40, 28.51, 27.35, 22.09, 21.52.

3.5. Epoxidation of 1,4- Cyclohexadiene.

Due to previous experience in the epoxidation of cyclohexenes, the dimethyloxirane method was chosen to synthesise the cyclohexa-1,4-diene epoxide.

1,4-cyclohexadiene (8.5 mmol, 0.7 g) was dissolved in a mixture of ethyl acetate and acetone (80 mL:80 mL). At the same time, a solution of sodium bicarbonate (2g) in water (40mL) was prepared and added to the reaction. The mixture was cooled in an ice bath and stirred. A solution of potassium peroxymonosulfate (10.4 g) in water (80 mL) was added dropwise with the use of the syringe pump at the rate of 75 mL per hour. The reaction was left stirring for 3 days. After that time, the layers were separated and the organic layer was washed with water (100 mL), dried with magnesium sulfate and the solvent was removed *in vacuo* to give 0.22g of crude.

The yield of the reaction was 32%. The synthesis of the reaction was successful, and the data were consistent with the literature (Clique et al. 2005, Cavdar & Saracoglu, 2008)
^{1}H NMR (600 MHz, $CDCl_3$) δ 3.09 (d, J= 6.3, 4H), 2.28 (d, J=7.0, 4H).

3.6 Opening of *trans*-1,4-cyclohexadiene dioxide with sodium ethoxide.

The solution of sodium ethoxide (5 mmol, 0.34 g) in ethanol (20 mL) was prepared and *cis*-1,4-cyclohexadiene (2 mmol, 0.23 g) was added. The reaction was heated at reflux for 18 h. After that time, the solvent was removed *in vacuo* and a mixture of ethyl acetate and water (20 mL:20 mL) was added and transferred to a separatory funnel. The solution was extracted with ethyl acetate (2x 20 mL) and dried over magnesium sulfate. The crude product was obtained by removing the solvent *in vacuo* giving 0.10g
The yield of the reaction was 32%.
^{1}H NMR 600 MHz, $CDCl_3$) δ 2.95 (s,2H), 2.19 (s,2H), 1.12-0.90 (m, 10H).
^{13}C NMR (150 MHz, $CDCl_3$) δ 77.2, 60.4, 48.9, 29.4, 23.8, 21.0, 14.2.

3.7 Synthesis of cyclohexenes by Diels-Alder reaction carried out in microwave reactor.

3.7.1 Styrene.

Sulfolene (1 mmol, 0.118 g) and styrene (1 mmol, 0.104 g) were added to a 10 mL microwave-safe flask. Toluene (1 mL) was added as a solvent, or the reaction was run as neat in a microwave reactor. The conditions for each run are specified in Table 2. In the case of the reaction where toluene was added, after the run, the solvent was removed *in vacuo*.
After the reaction, the analytical sample was obtained, and the reaction was evaluated according to the H NMR data. Yield 0%.
^{1}H NMR (600 MHz, $CDCl_3$) δ 7.9-7.0 (m, 6H aromatic area), 6.5 (dd, J=5.7 1H styrene), 6.1 (s, 2H, sulfolene), 5.8 (dd, J=8.1, 1H, styrene), 5.2 (dd, J=8,2, 1H styrene), 4.2 (m, unidentified), 3.9 (s, 2H sulfolene), 4.6 (m, unidentified), 2.2-1.2 (b, m, unidentified), 0.8 (m, unidentified).

3.7.2 3-methylcyclohexene-1-one

The sulfolene (1 mmol, 0.118g) and 3-methylcyclohexene-1-one (1mmol, 0.110 g) were added to a 10 mL microwave-safe flask. Toluene (1mL) was added as a solvent, or the reaction was running as neat in a microwave reactor. The conditions for each run were specified in Table 3. In the case of the reaction where toluene was added, after the run, the solvent was removed in vacuo.

Yield 0%

^{1}H NMR (600 MHz, $CDCl_3$) δ 6.3 (m, 1H, from 1,3-butadiene), 6.1 (s, 2H, sulfolene), 5.8 (s, 1H from double bond within 3-methylcyclohexene-1-one), 5.2 (d, J=6.3, 1H, 1,3-butadiene), 5.1 (d, J=6.2, 1H, 1,3-butadiene), 3.6 (s, 4H, sulfolene), 2.3-2.4 (b, m, 6H), 2.0-1.8 (m, 3H).

3.8 Sulfation reactions.

3.8.1

cis-diol compound

Compound **11** (0.21g, 1.7 mmol) was dissolved in anhydrous DMF (1 mL). The Sulfur-trioxide-trimethylamine complex (0.49 g, 3.5 mmol) was added and the reaction was stirred for 16h at 40 degrees. After that time, toluene (3 mL) was added and the solvent was removed in *vacuo* giving 1.83g of colourless oil.

Yield: not able to calculate due to the presence of the solvent.

^{1}H NMR (600 MHz, $CDCl_3$) δ 8.02 (s, 1H), 2.96 (s,3H), 2.88 (s, 3H),

3.8.2

trans-diol compound

Compound **10** (0.11 g, 1.01 mmol) was dissolved in anhydrous DMF (1 mL). The Sulfur-trioxide-trimethylamine complex (0.417 g, 3 mmol) was added and the reaction was stirred for 16 h at 40 °C. After that time, toluene (3 mL) was added and the solvent was removed in *vacuo* giving 2.63 g of colourless oil.

Yield: not able to calculate due to the presence of the solvent.

^{1}H NMR (600 MHz, $CDCl_3$) δ 8.02 (s, 1H), 2.96 (s,3H), 2.88 (s, 3H)

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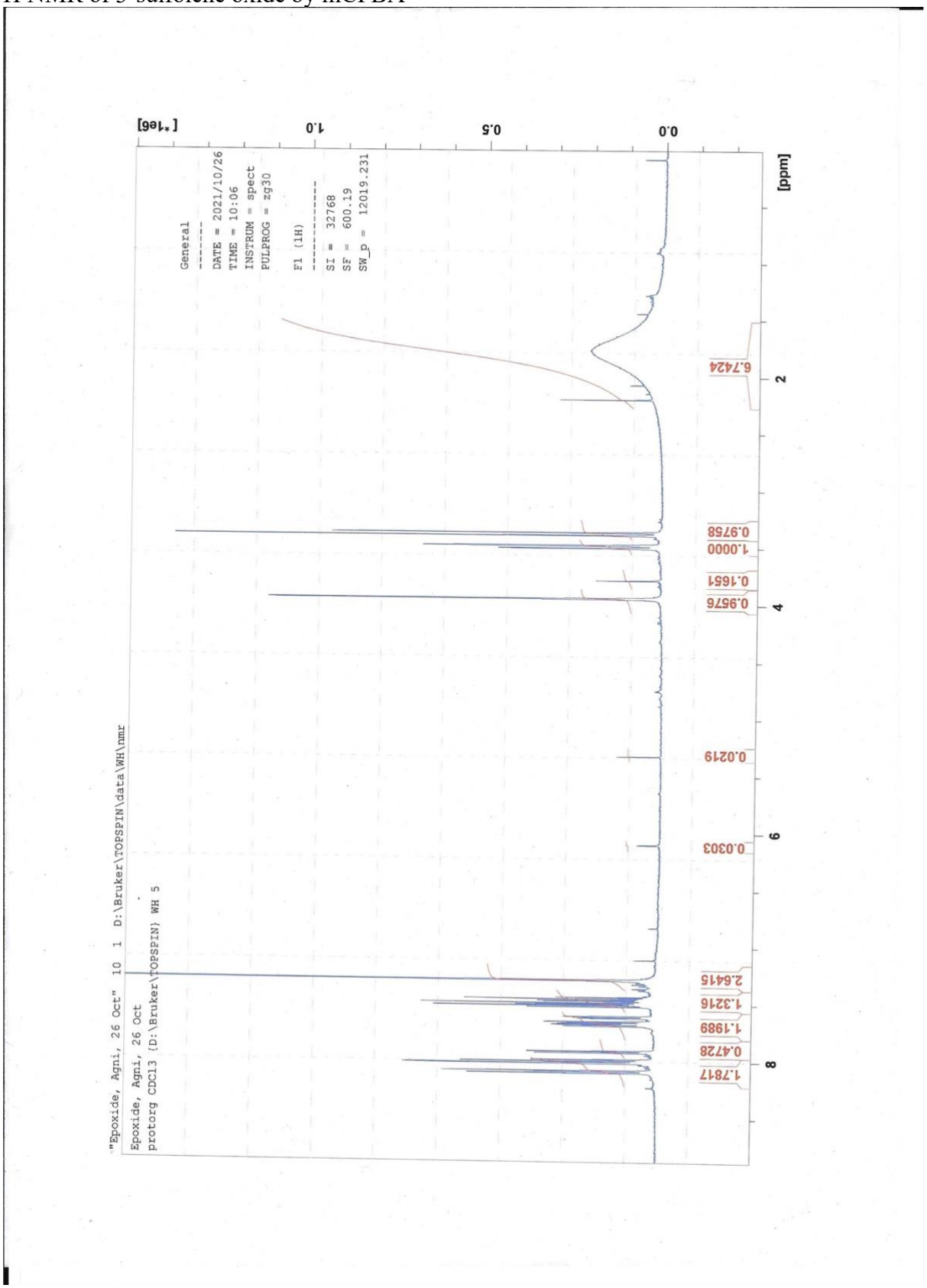
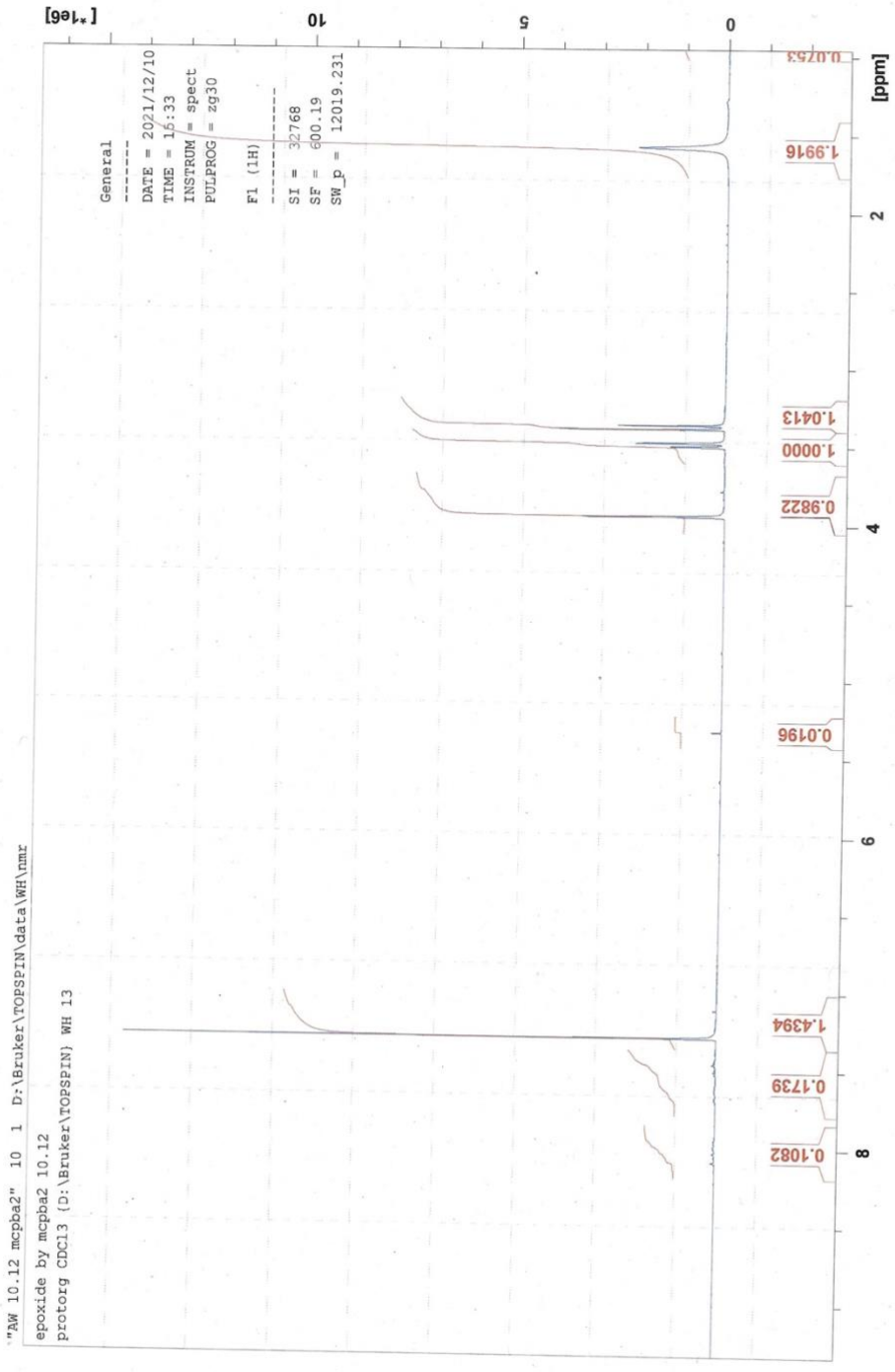
Supporting Information:

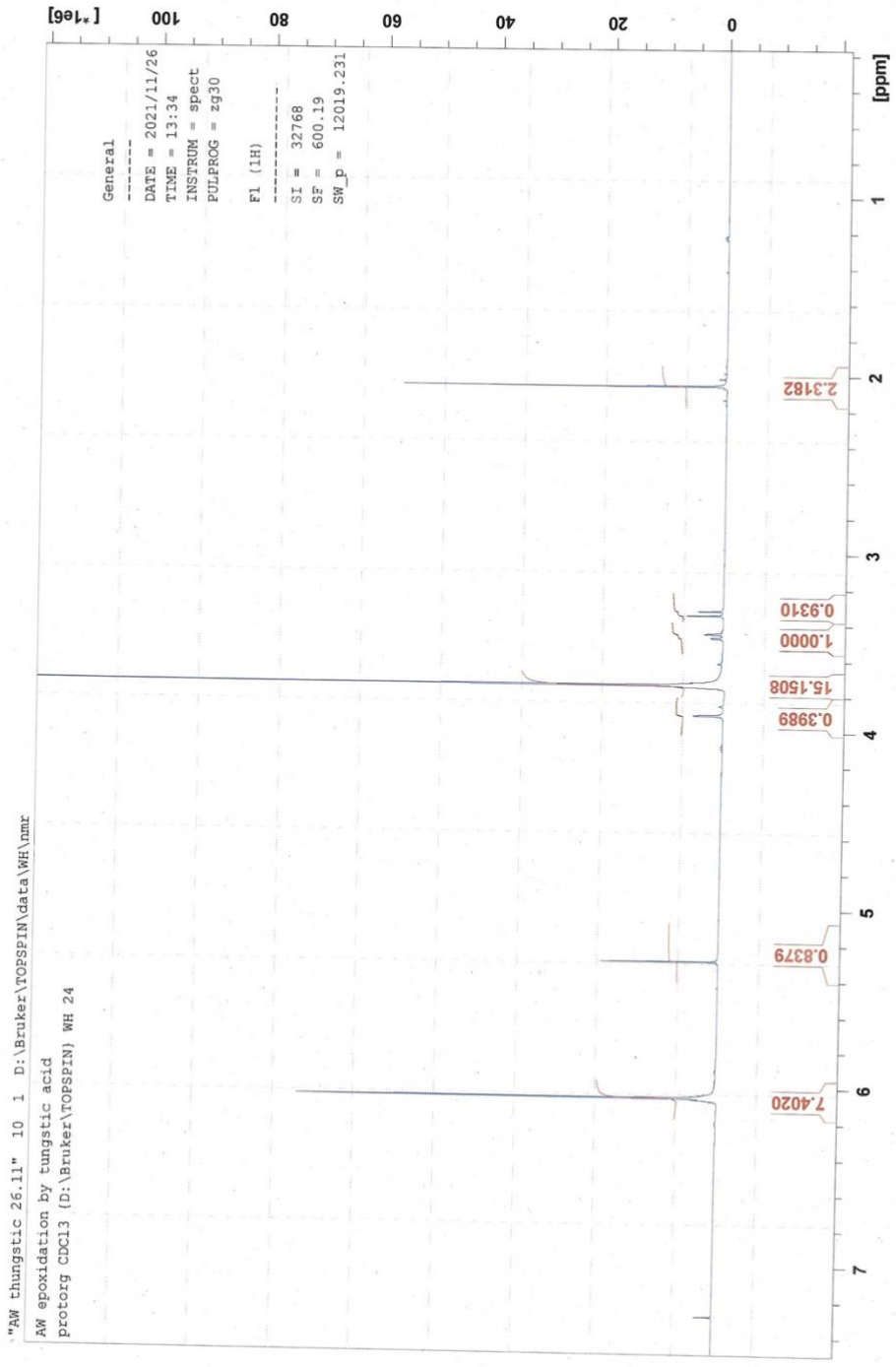
H NMR of 3-sulfolene oxide by mCPBA



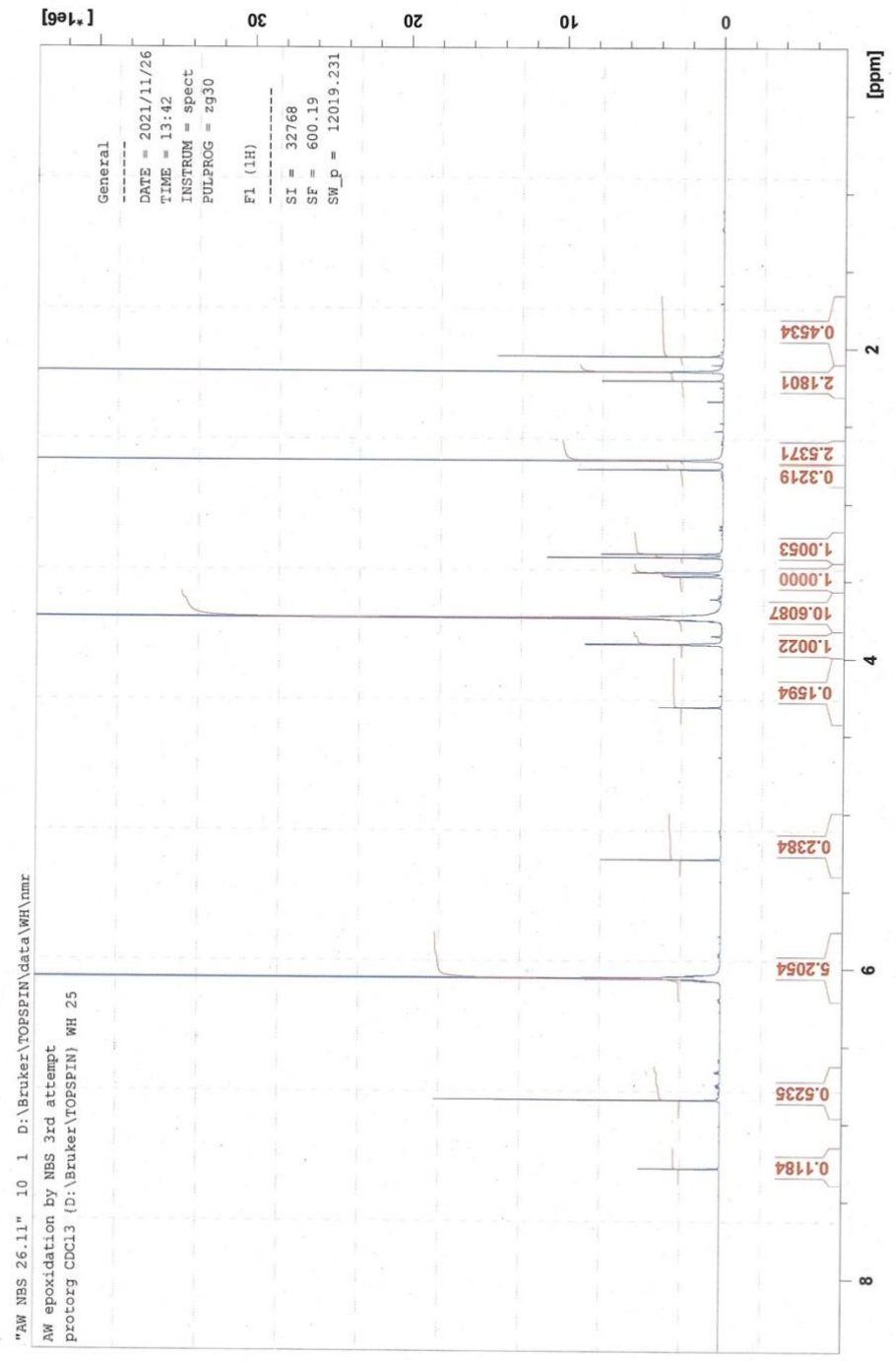
H NMR of 3-Sulfolene by mCPBA/ after method development



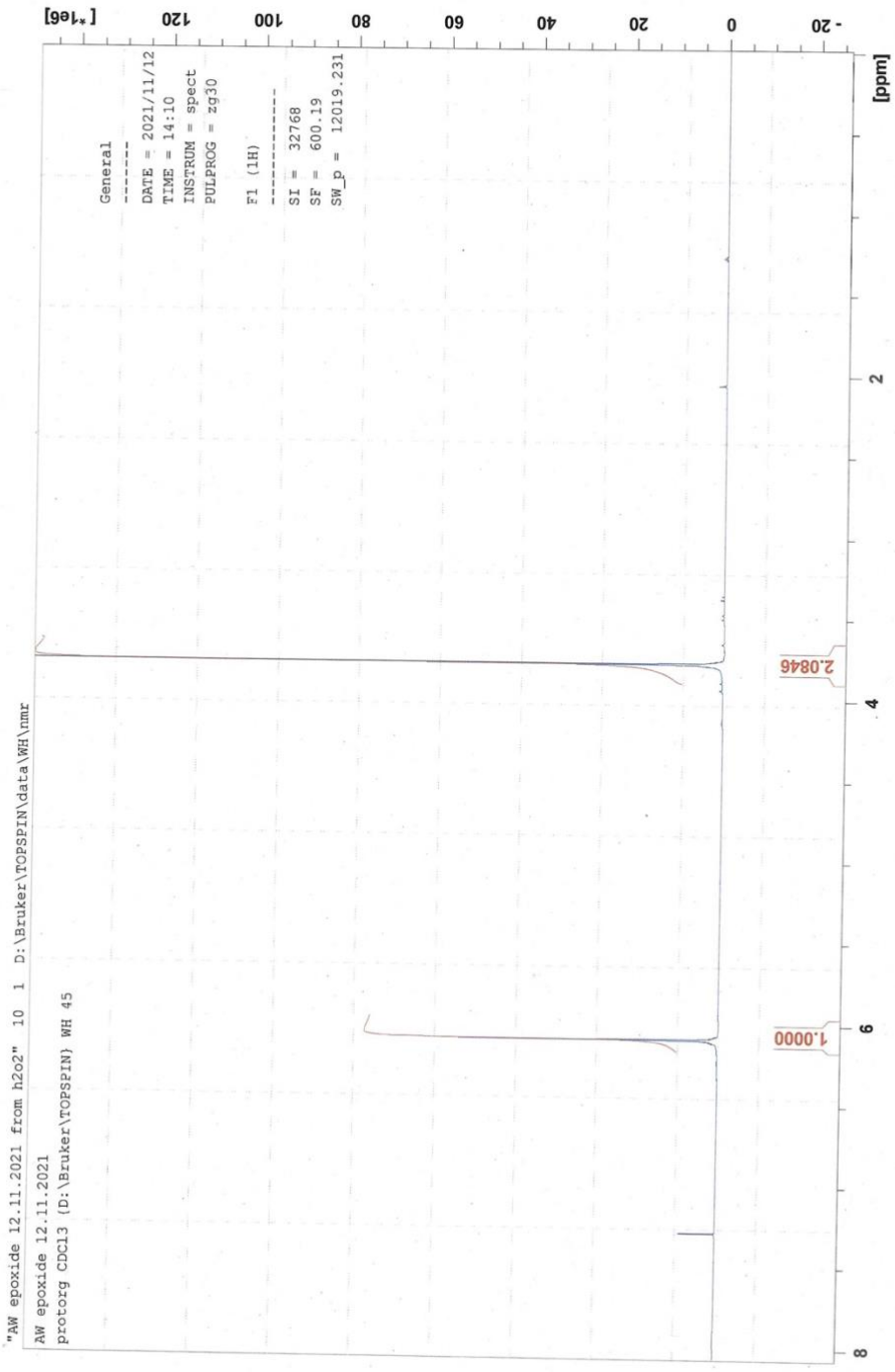
H NMR of sulfolene oxide / tungstic acid method



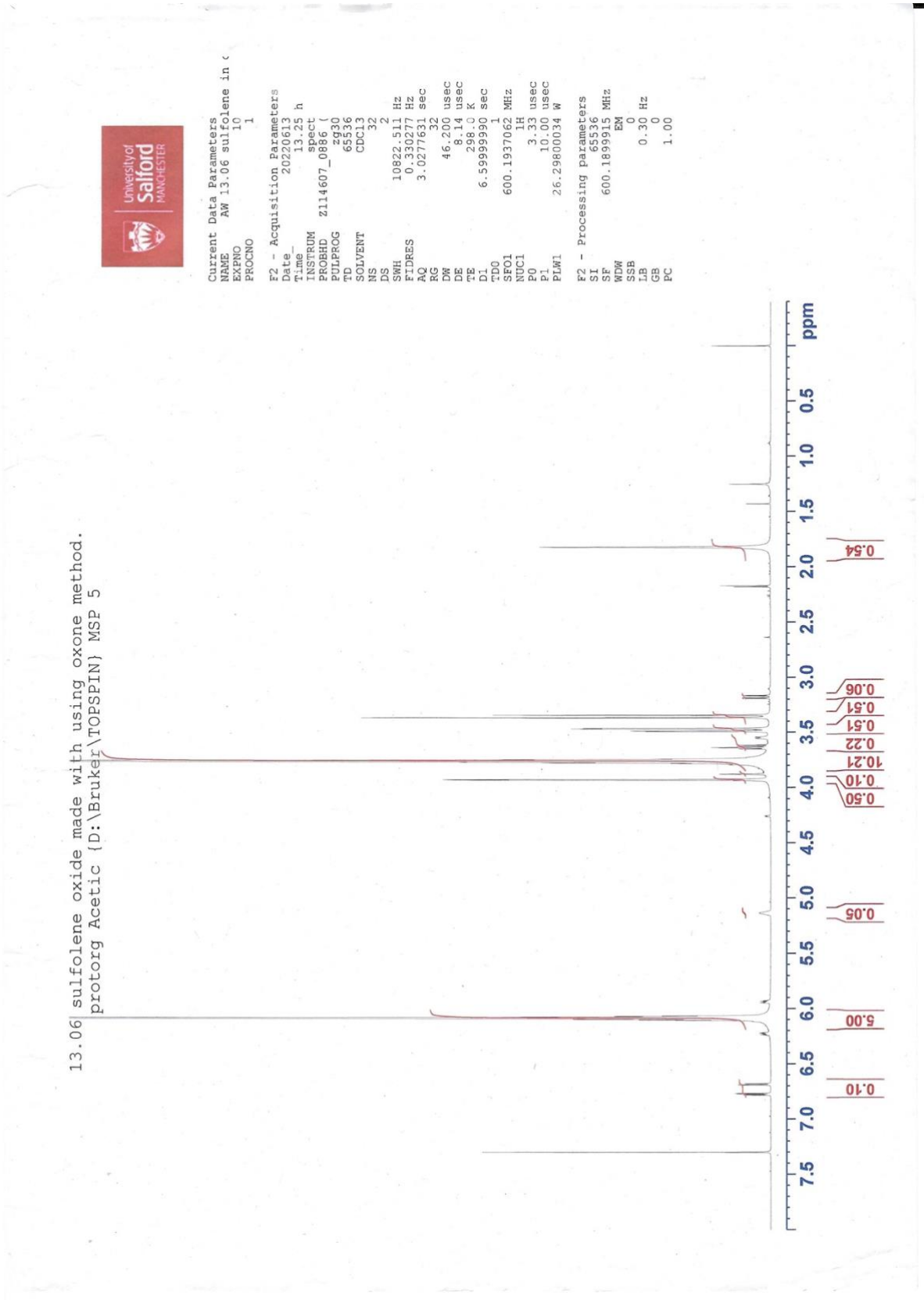
H NMR of 3-Sulfolene oxide/ NBS method



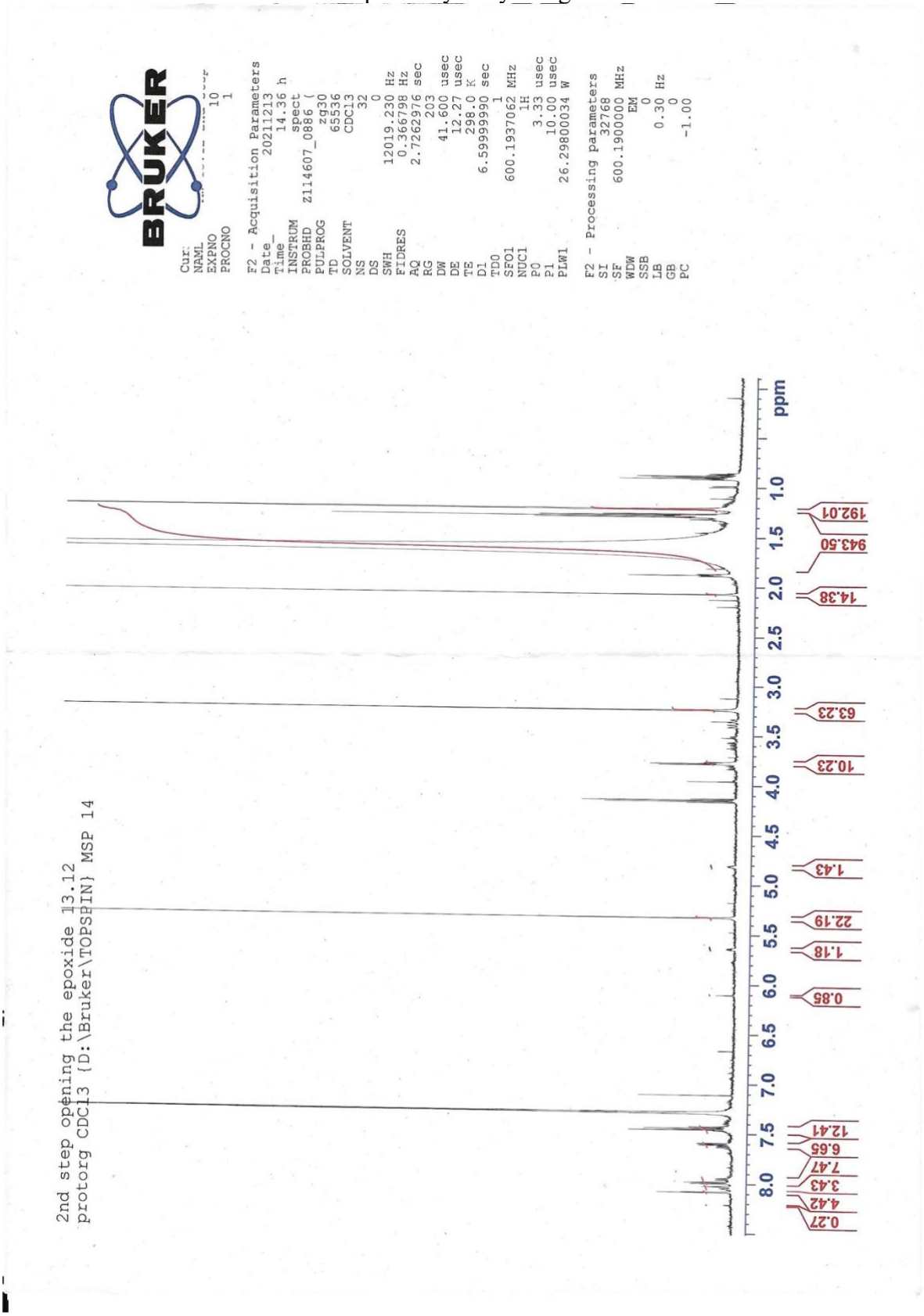
H NMR 3-Sulfolene oxide/ hydrogen peroxide method



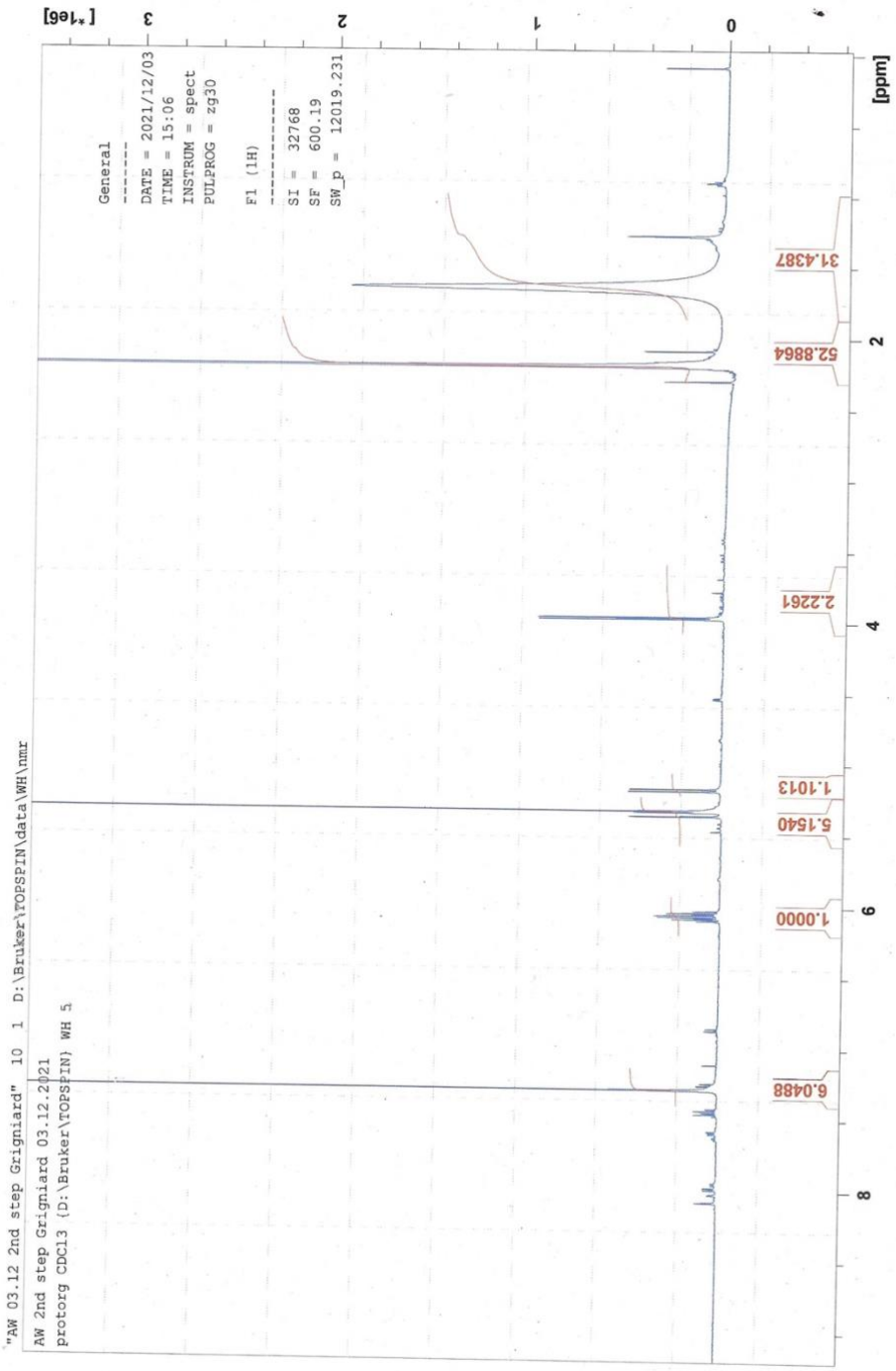
H NMR Sulfolene epoxide by Oxone method



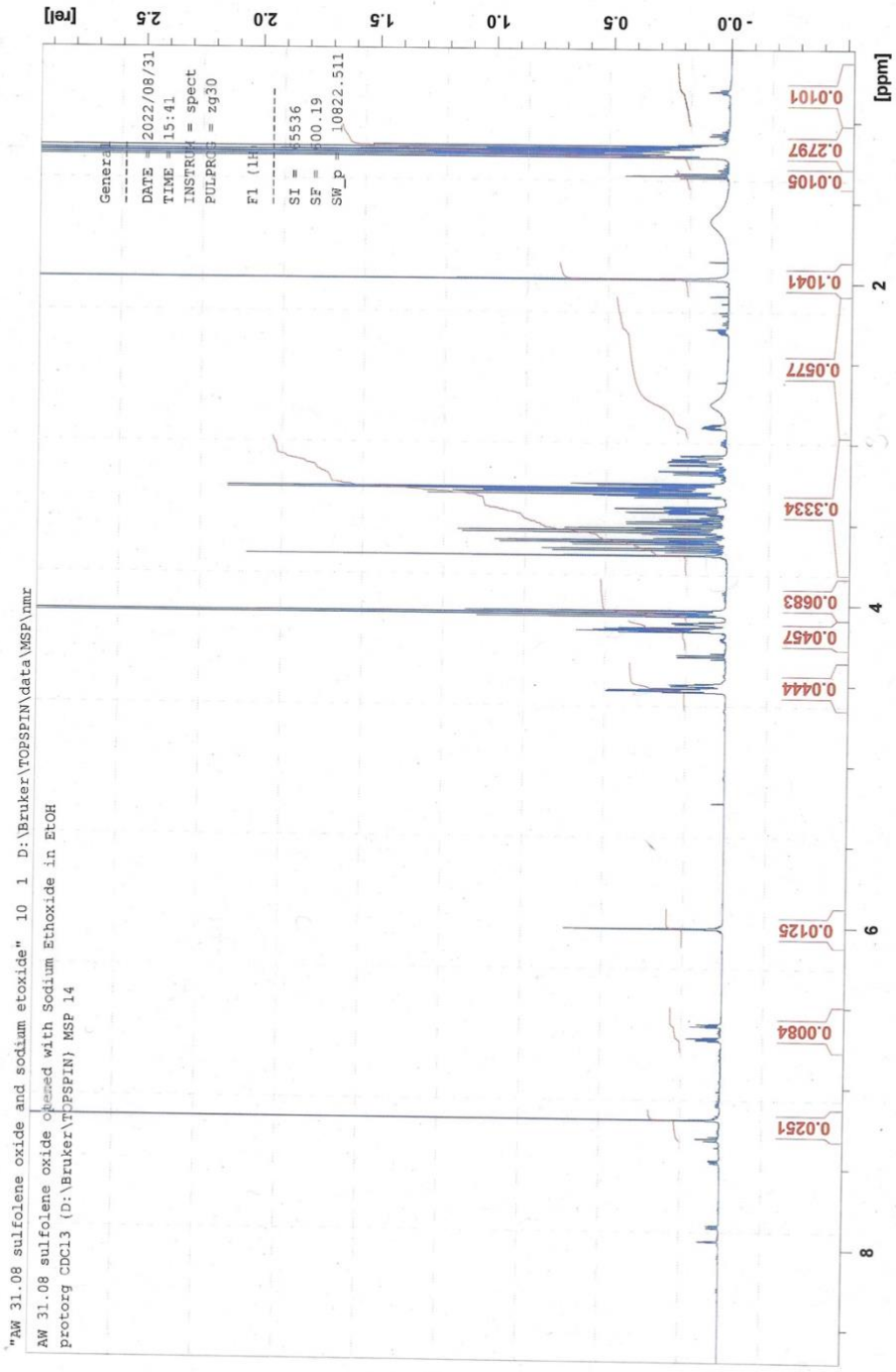
H NMR of sulfolene oxide opened by Vinyl Magnesium Bromide



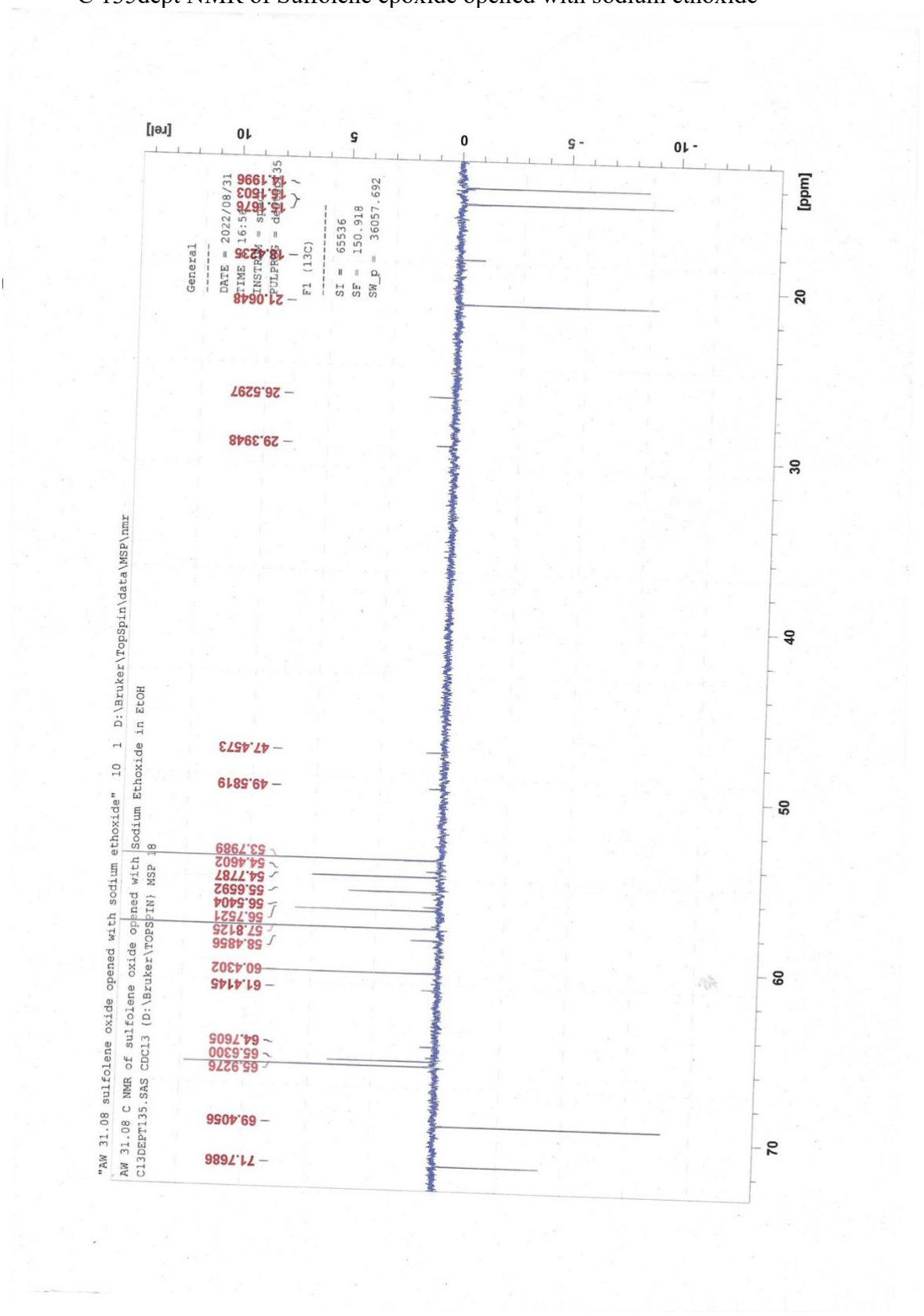
H NMR of sulfolene epoxide with vinyl magnesium bromide/ another attempt, failed



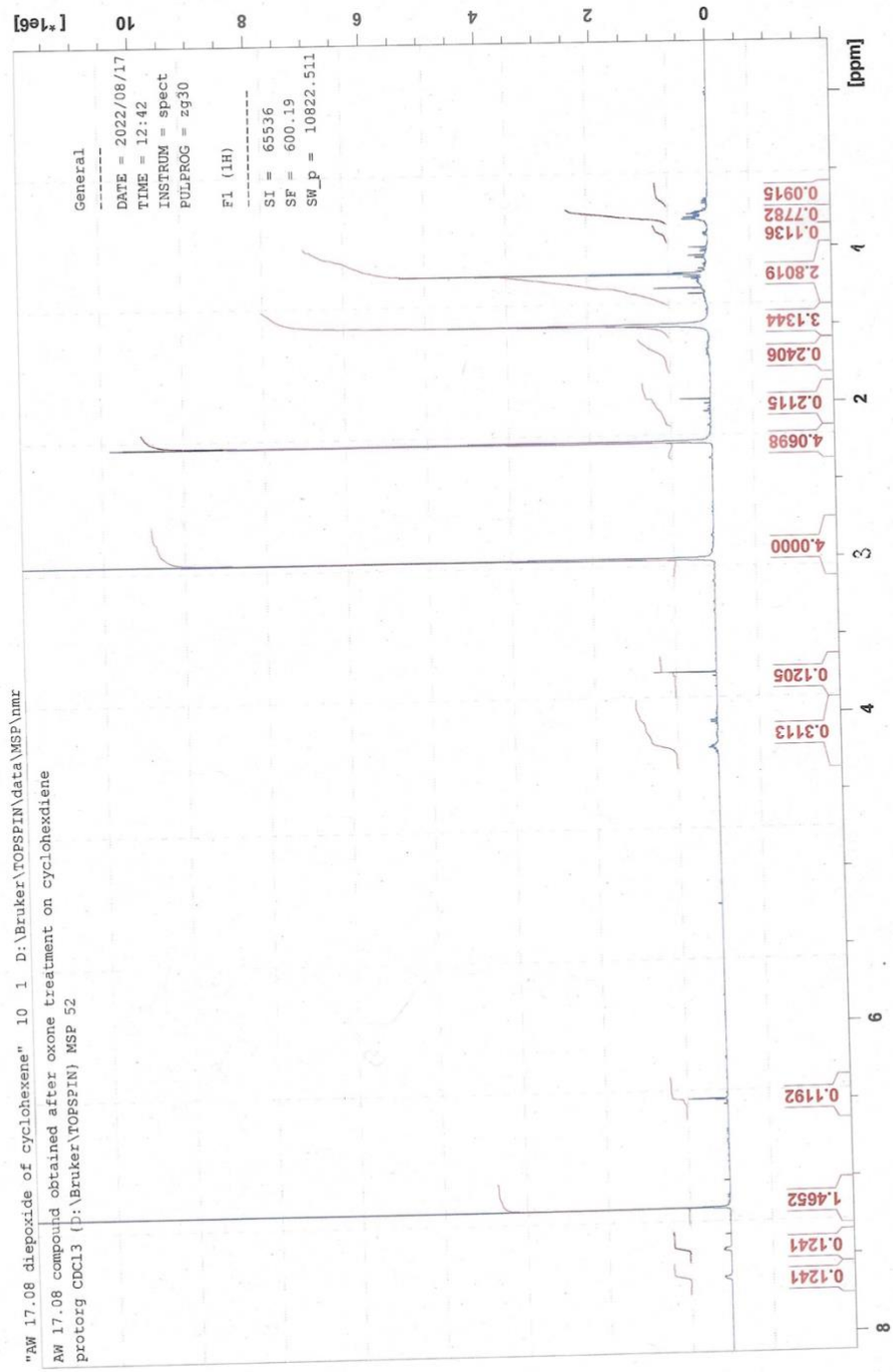
H NMR of sulfolene epoxide opened with sodium ethoxide



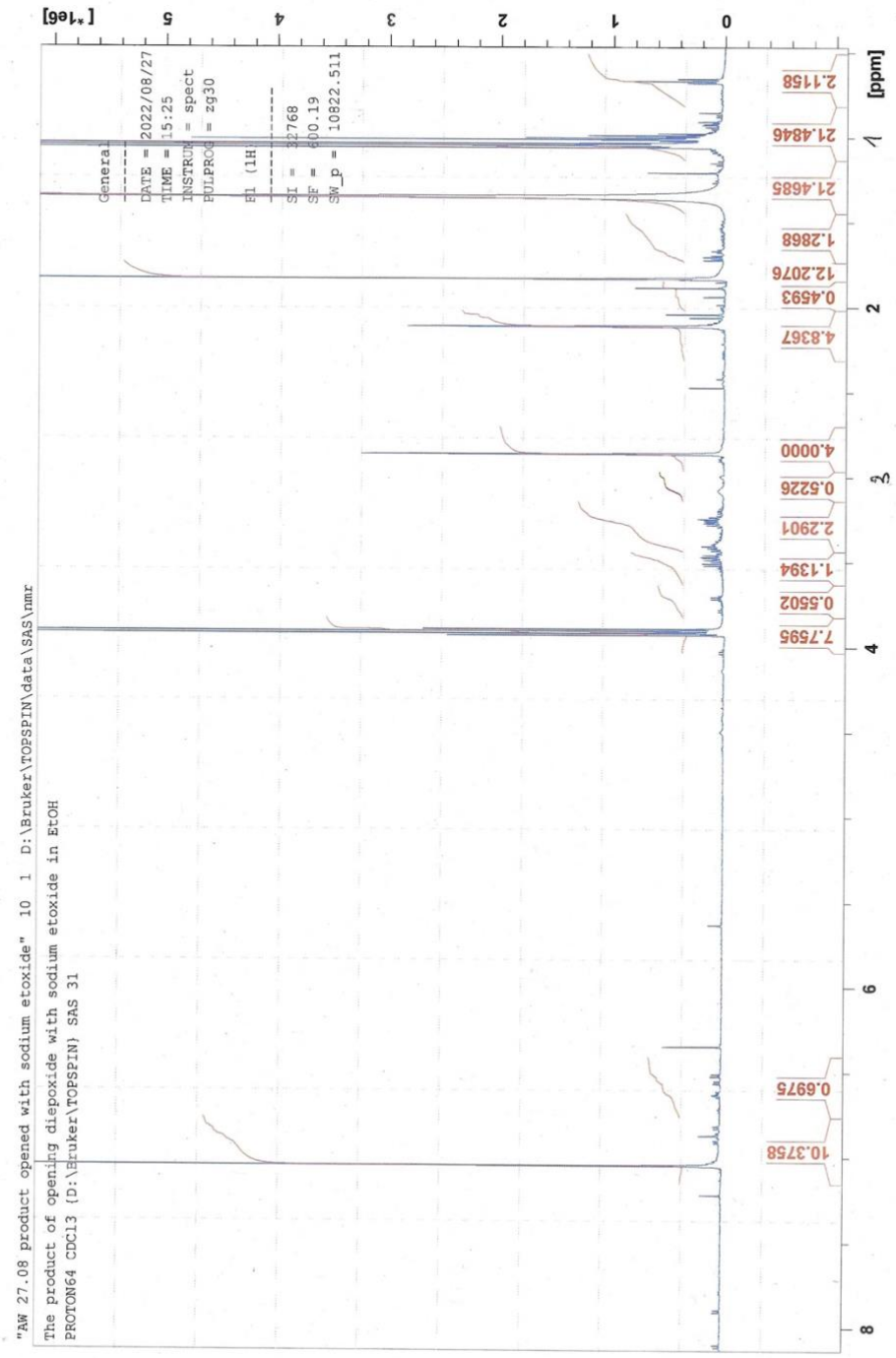
C 135dept NMR of Sulfolene epoxide opened with sodium ethoxide



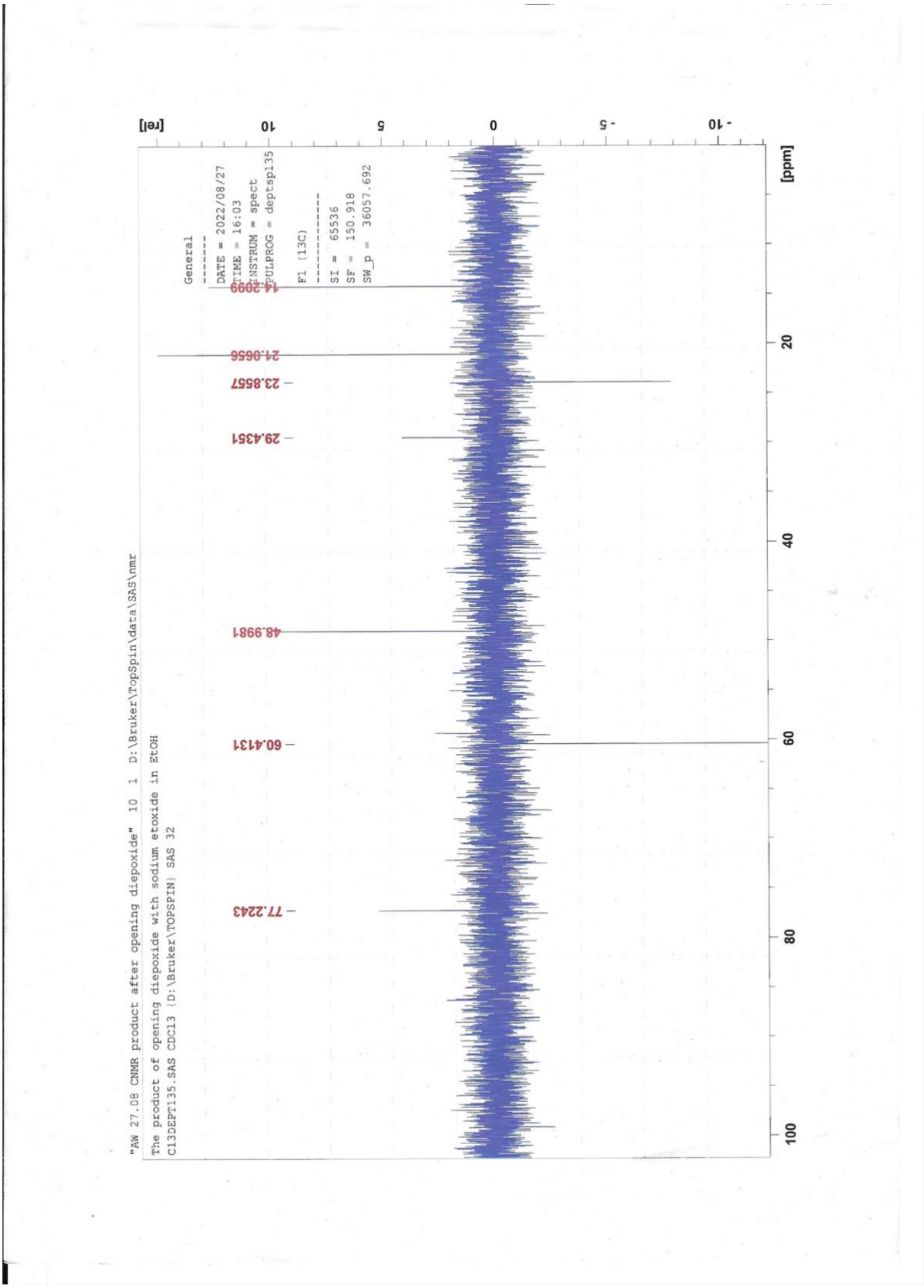
H NMR of trans 1,4-cyclohexene dioxide



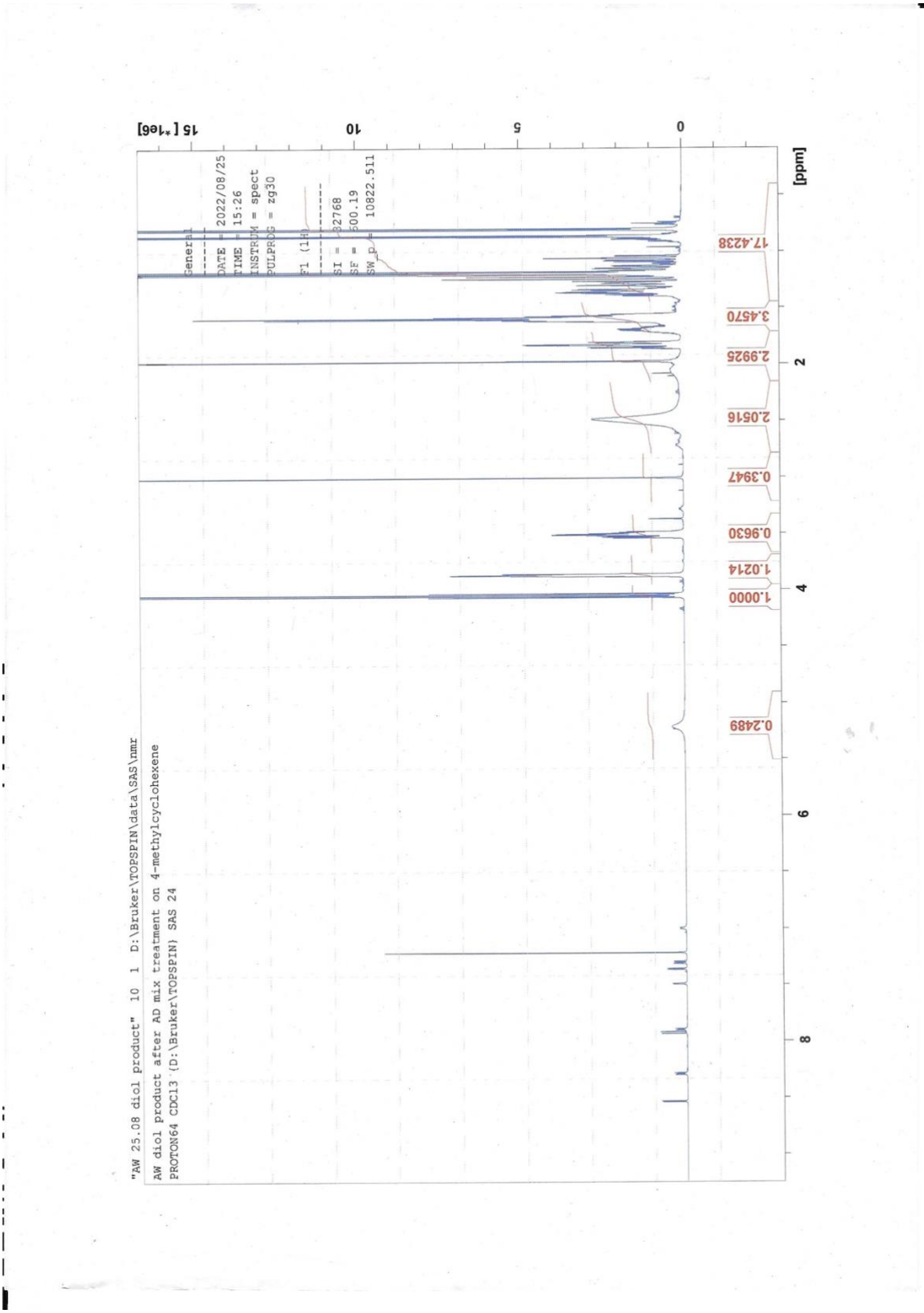
H NMR of trans 1,4-cyclohexene oxide opened with sodium ethoxide



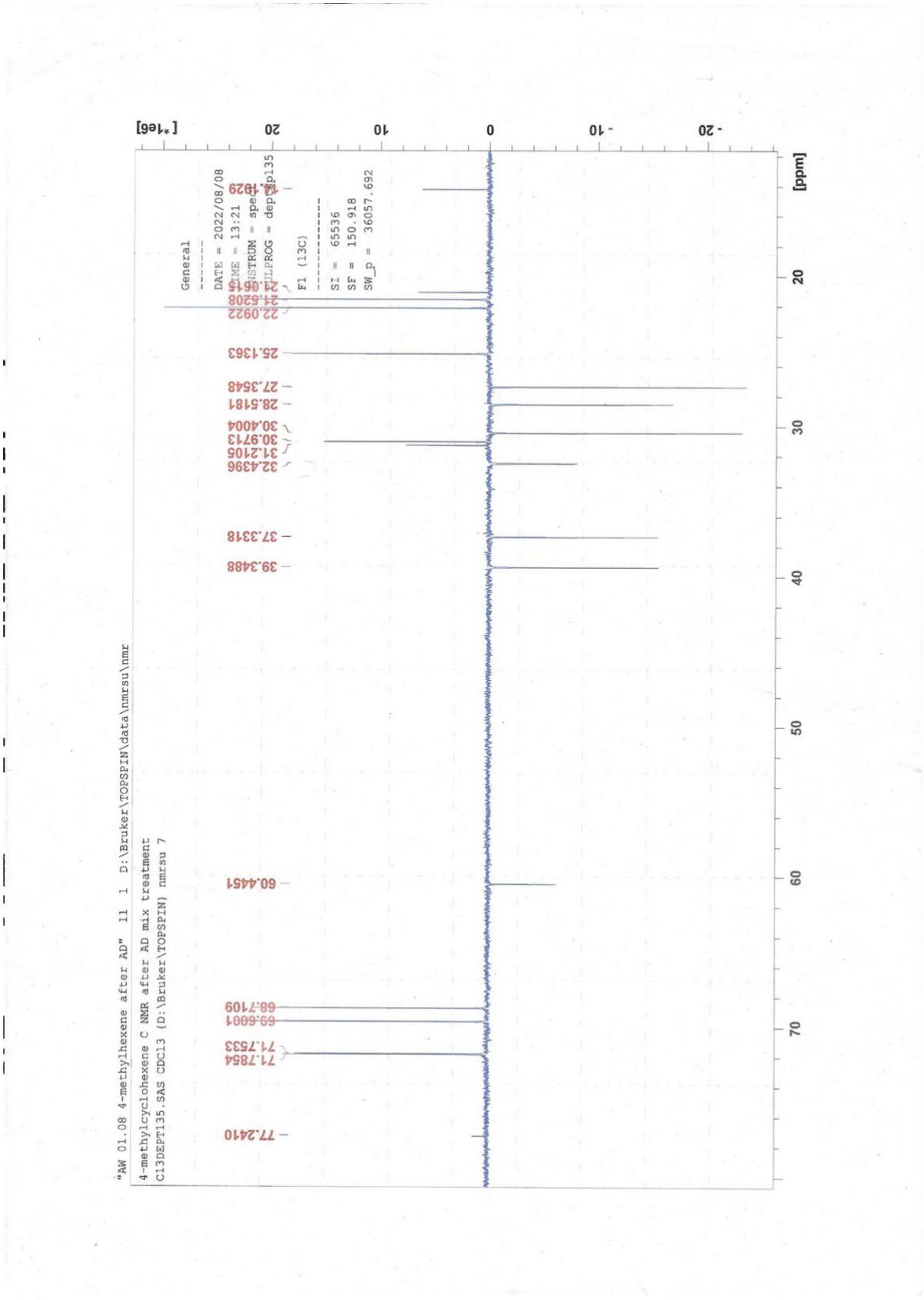
C 135dept NMR of trans 1,4-cyclohexene oxide opened with sodium ethoxide



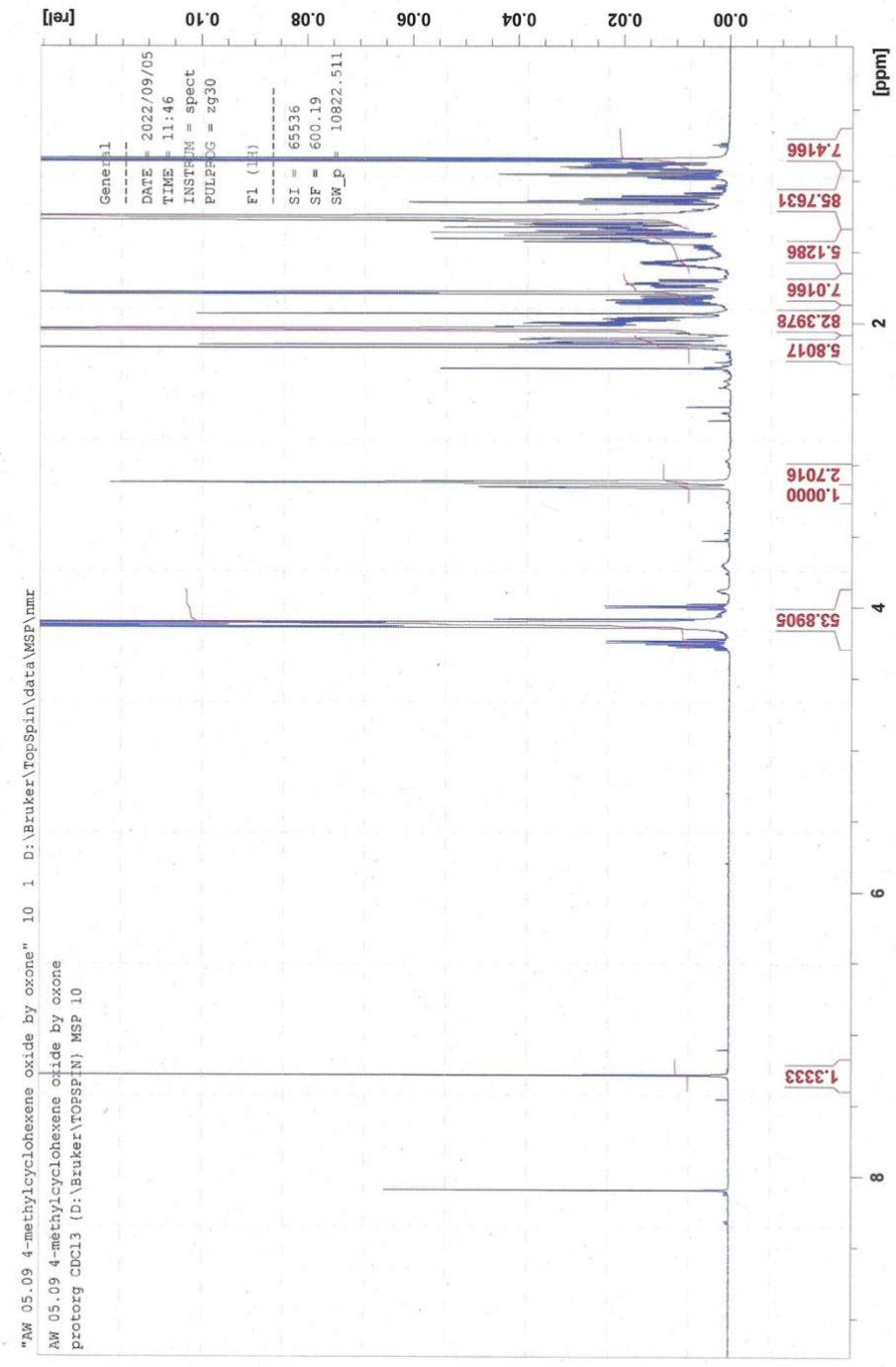
H NMR AD mix treatment on 4-methylcyclohexene- *cis* diol product.



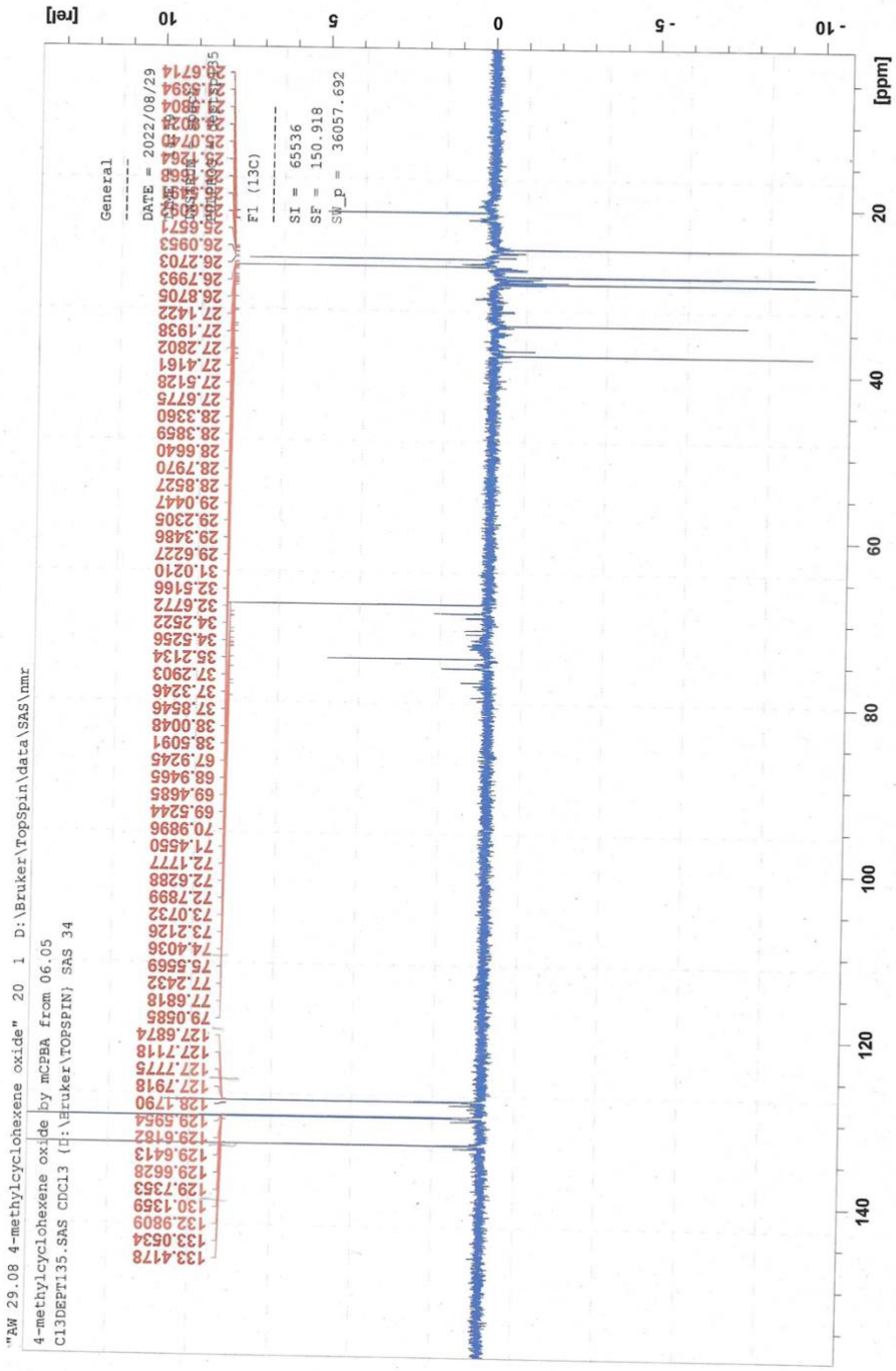
C 135dept NMR of cis diol product/ 4-methylcyclohexene after AD mix treatment



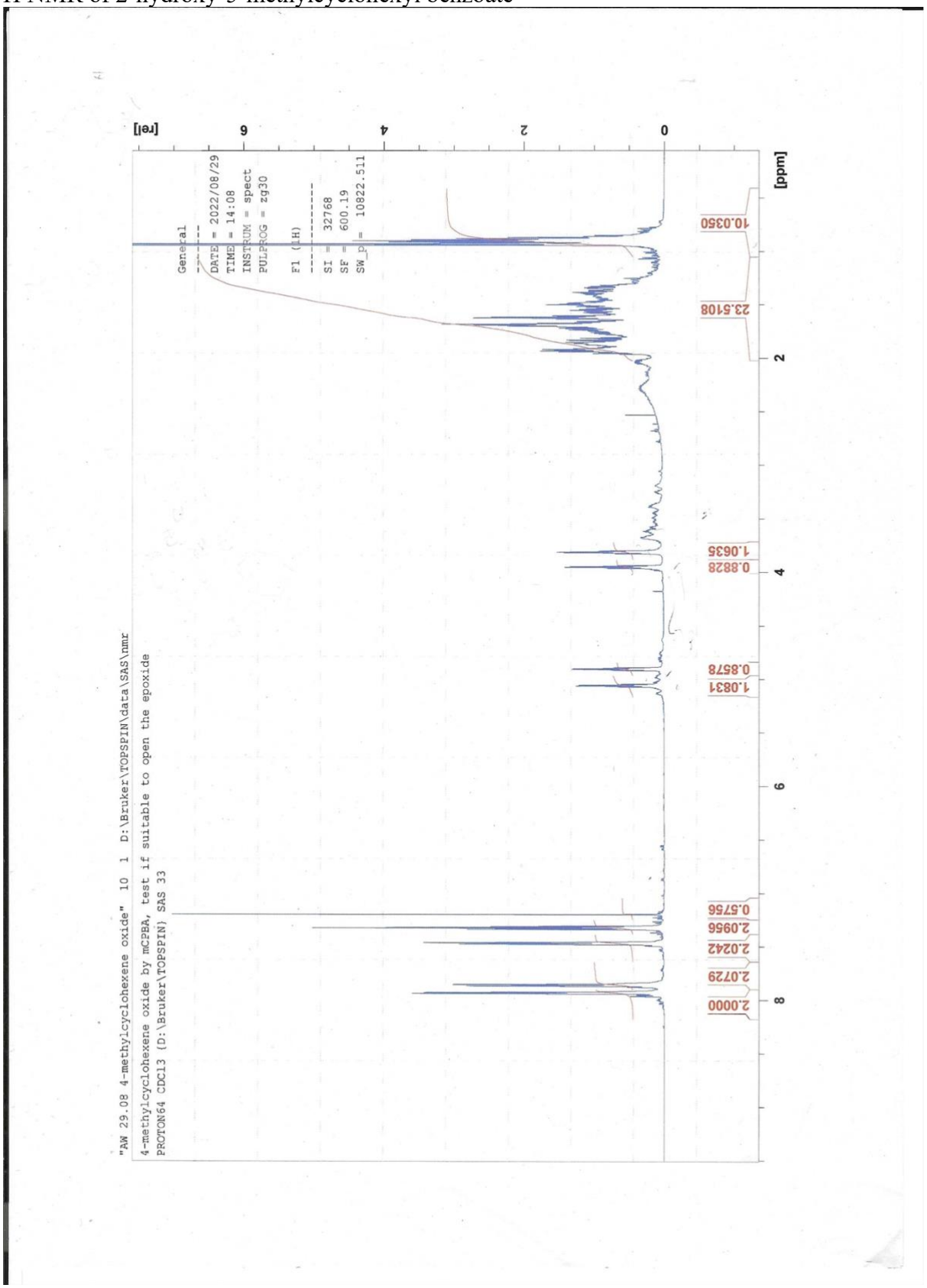
H NMR of 4-methylcyclohexene oxide by Oxone method



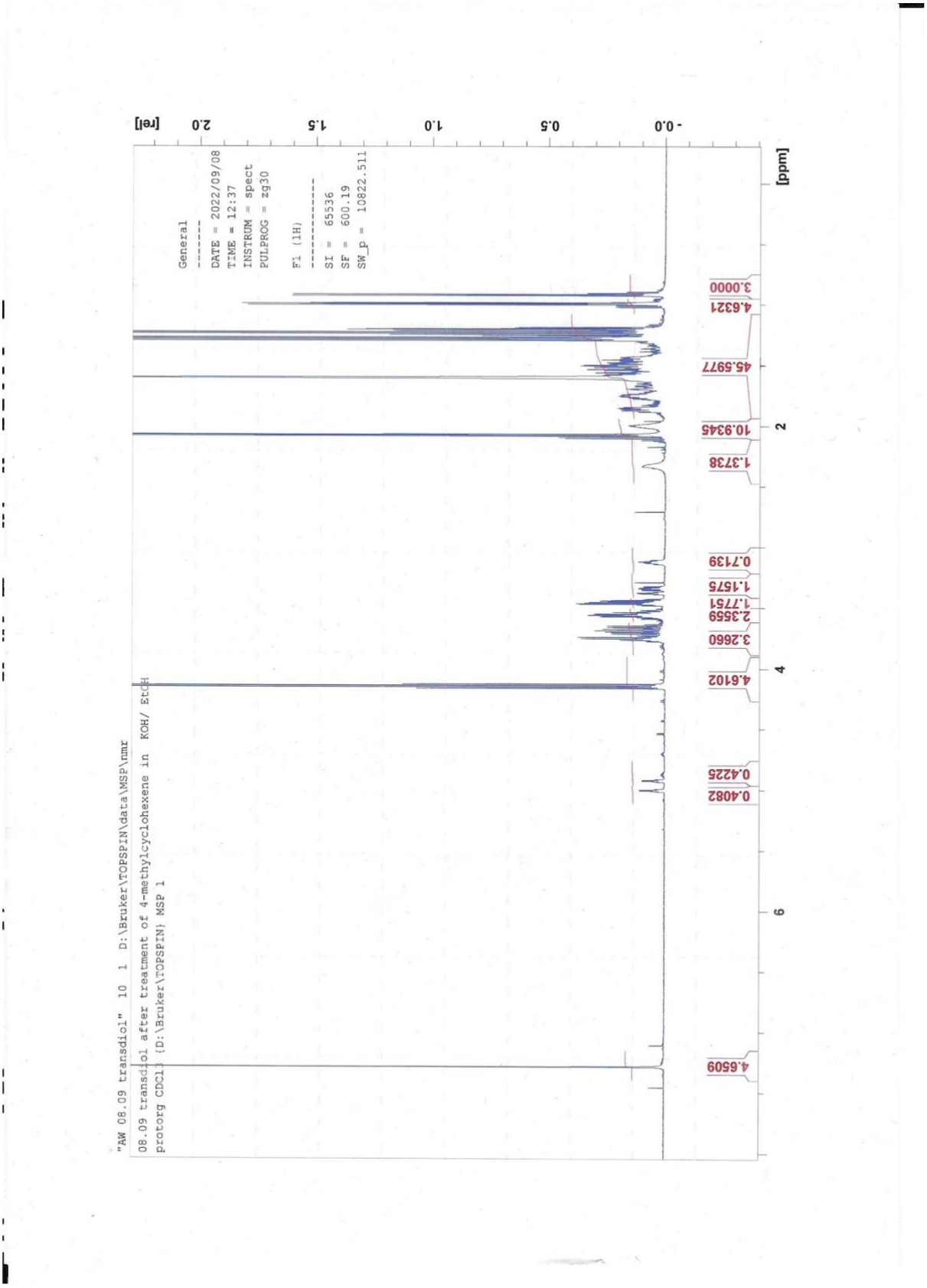
C dept 135 NMR of 2-hydroxy-5-methyl cyclohexybenzoate/ product obtained after the treatment of mCPBA on 4-methylcyclohexene



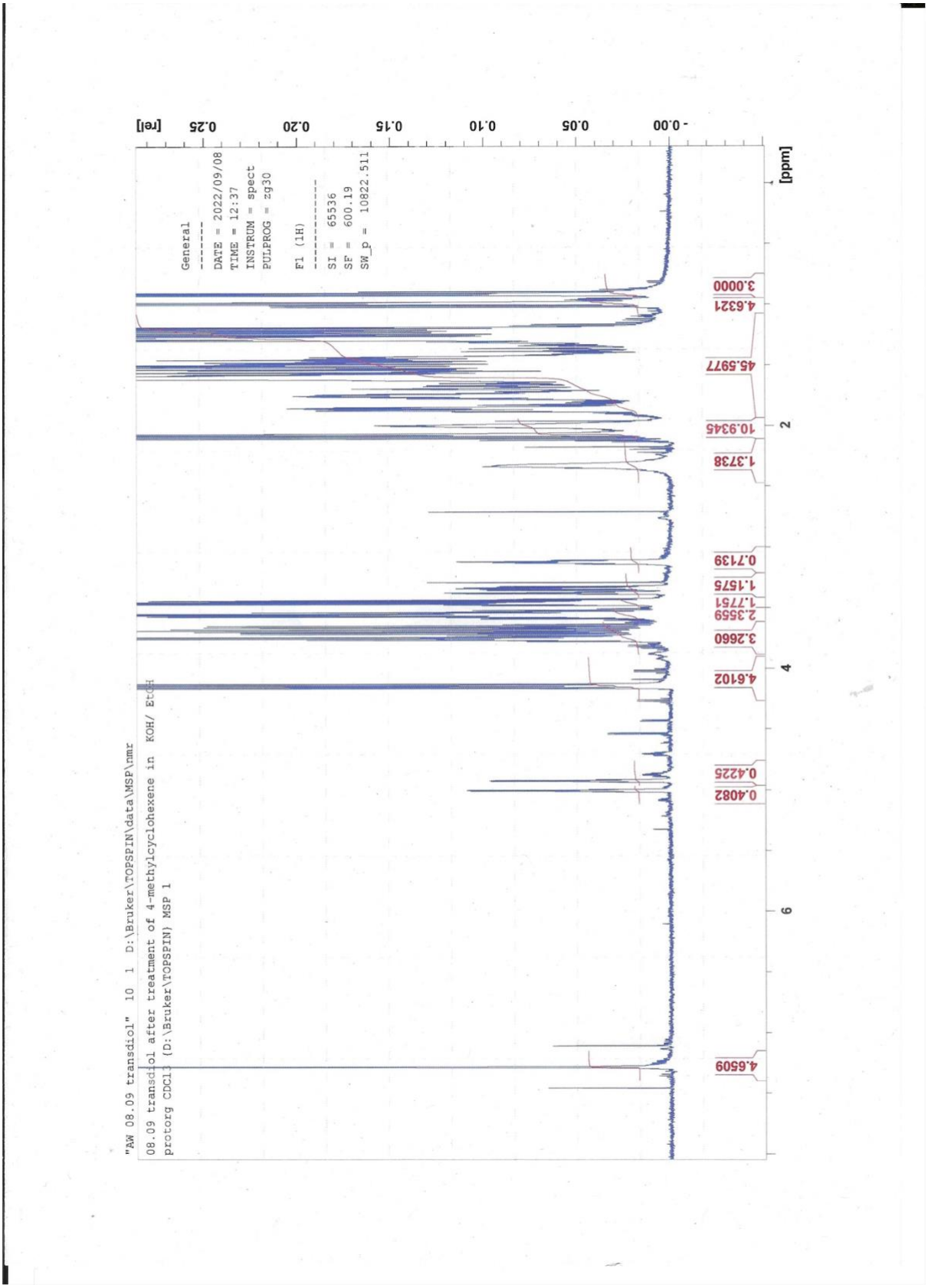
H NMR of 2-hydroxy-5-methylcyclohexyl benzoate



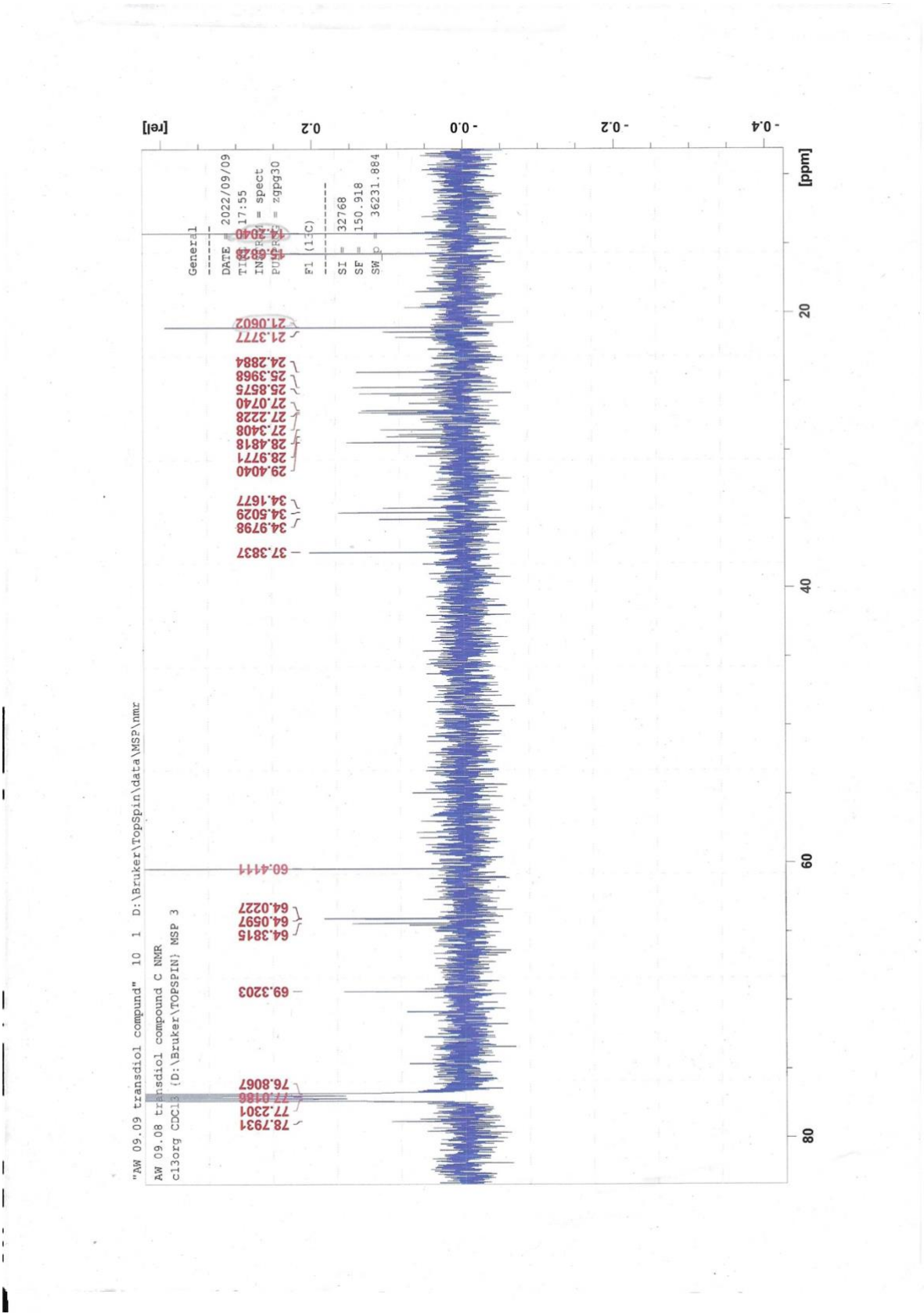
H NMR of the compound resulting after opening 4-methylcyclohexene with KOH (transdiol)



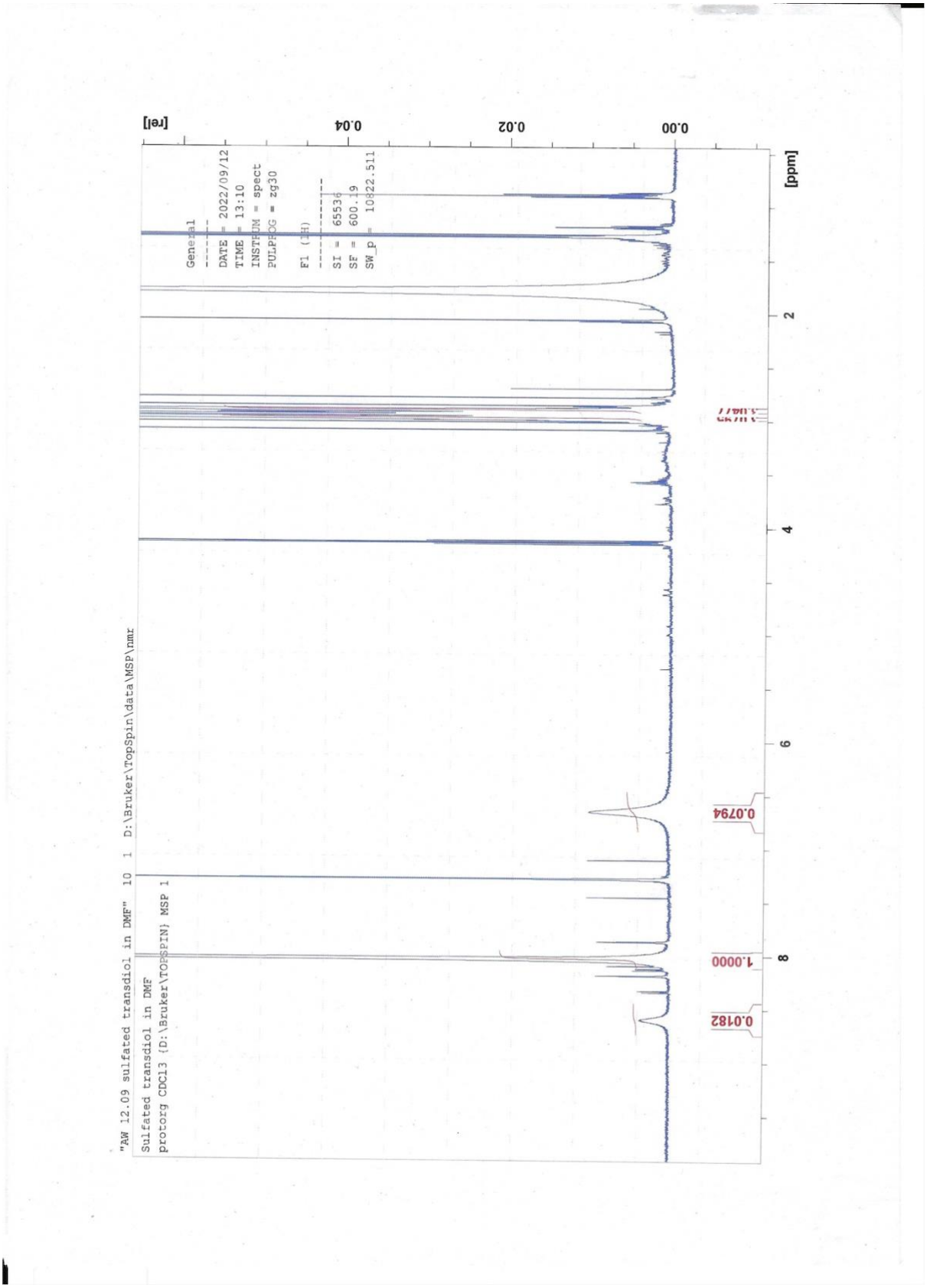
H NMR of the compound resulting after opening 4-methylcyclohexene with KOH (transdiol) /closer view.



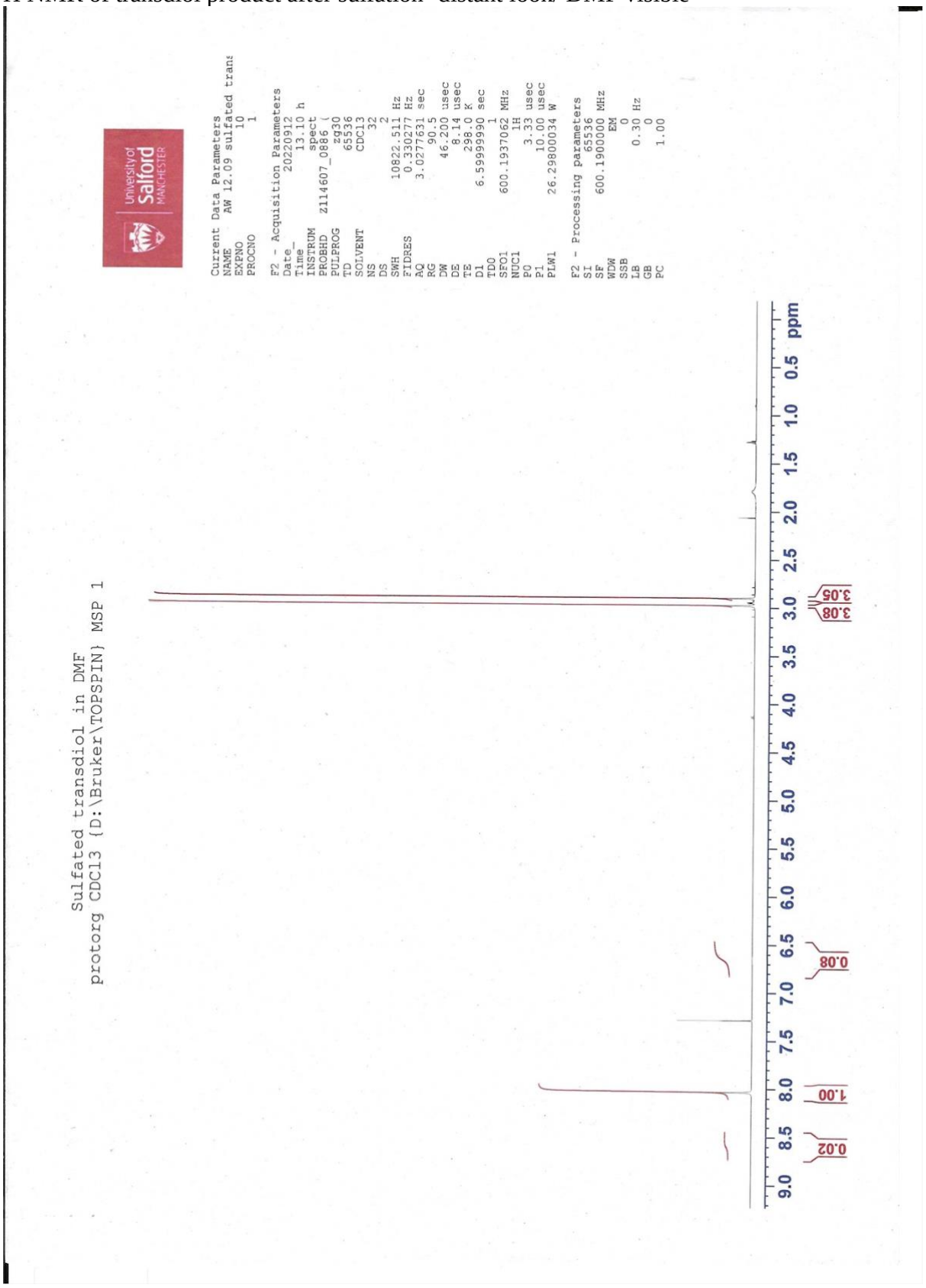
C NMR of 4-methylcyclohexene trans diol



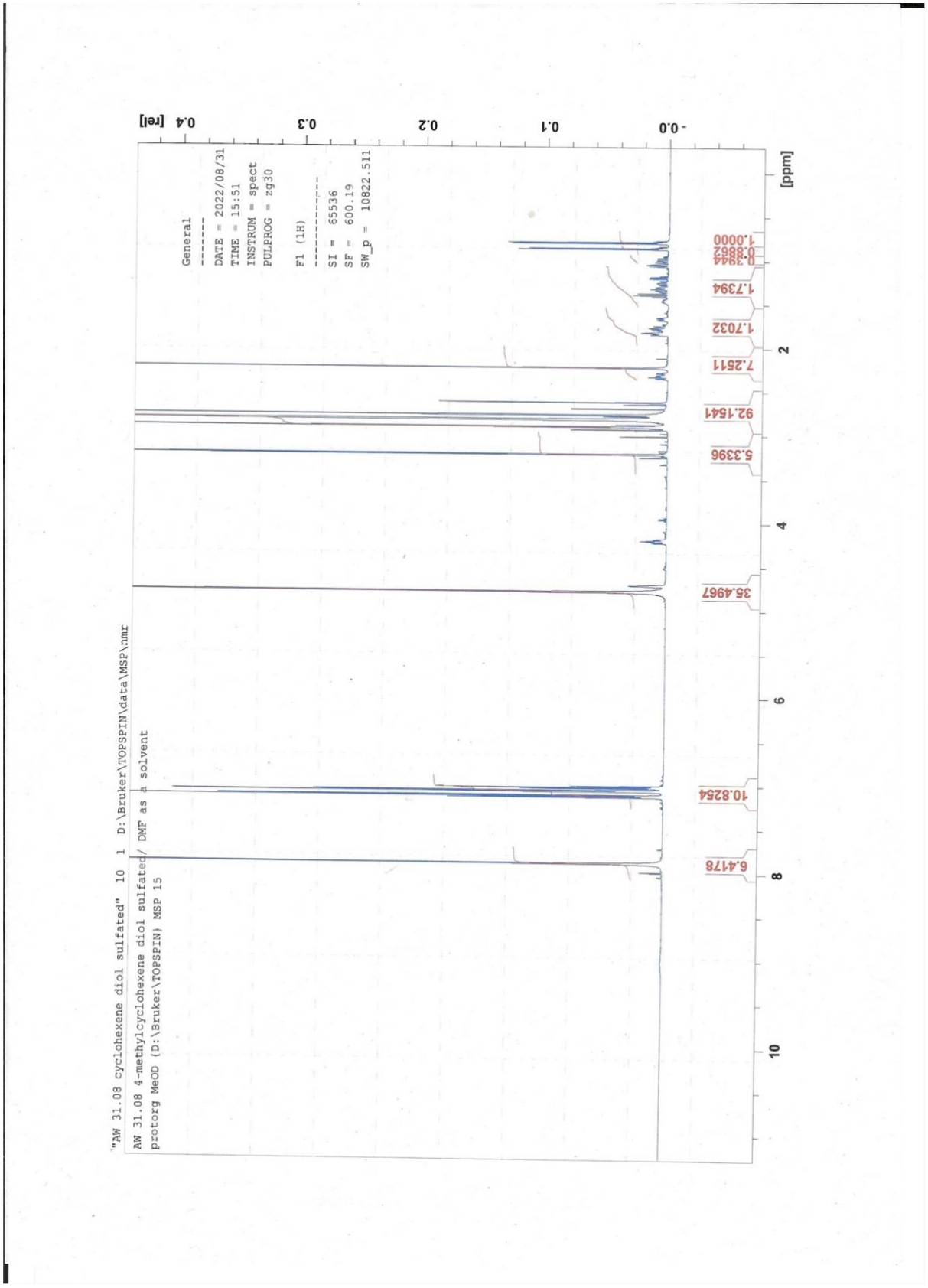
H NMR of 4-methylcyclohexene trans diol after the sulfation



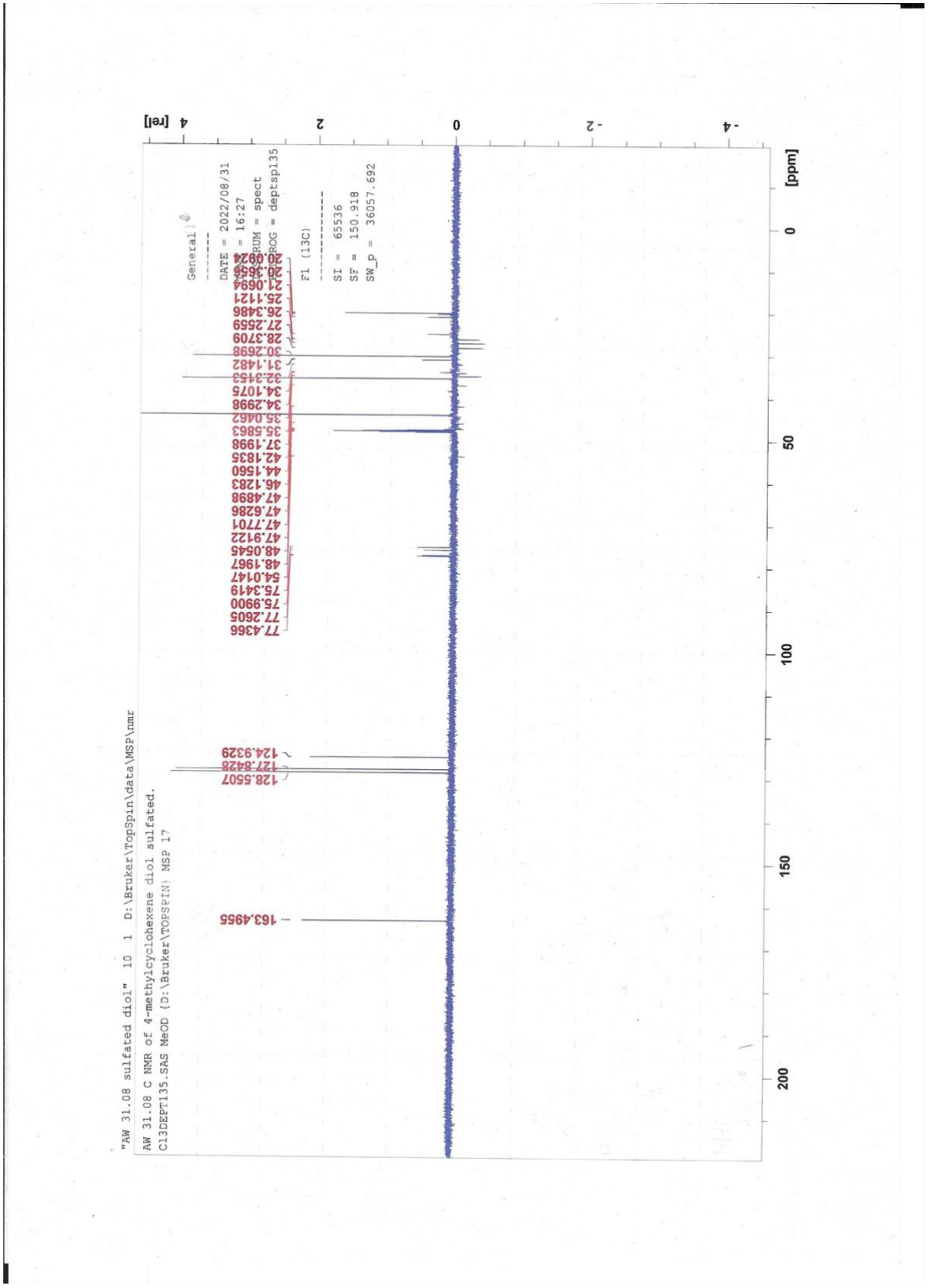
H NMR of transdiol product after sulfation- distant look/ DMF visible



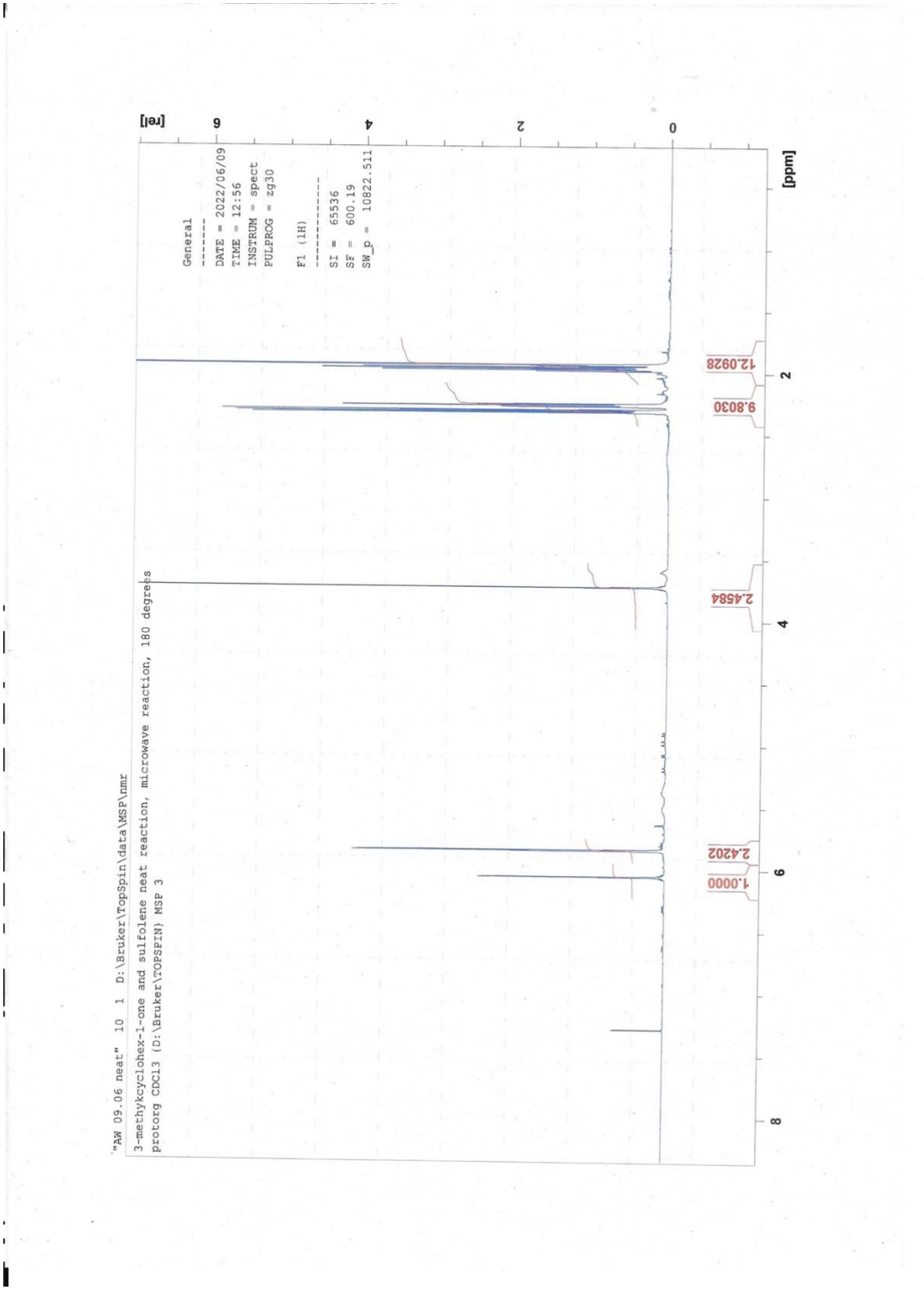
H NMR of 4-methylcyclohexene cis diol sulfated



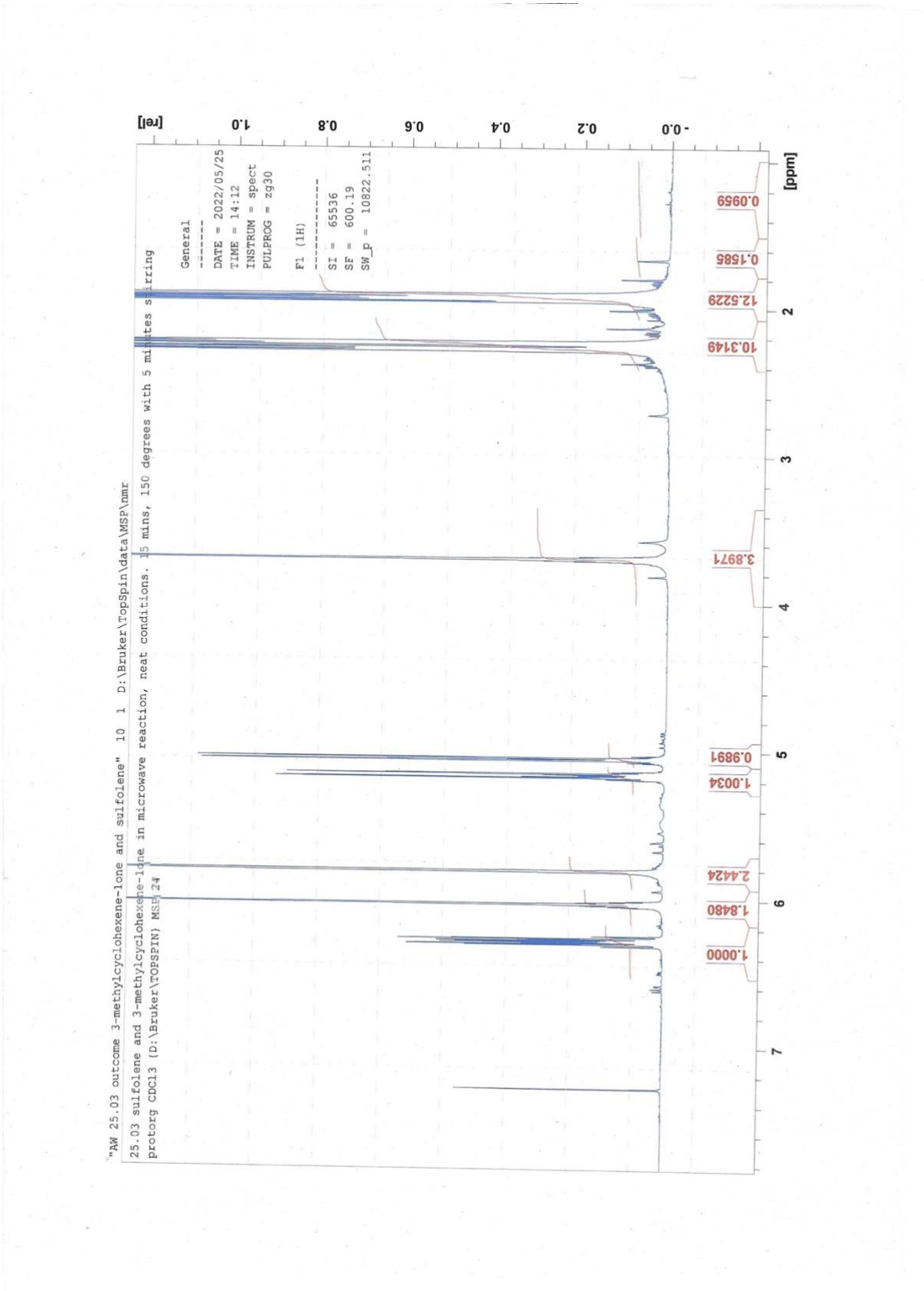
C NMR of 4-methylcyclohexene cis diol sulfated



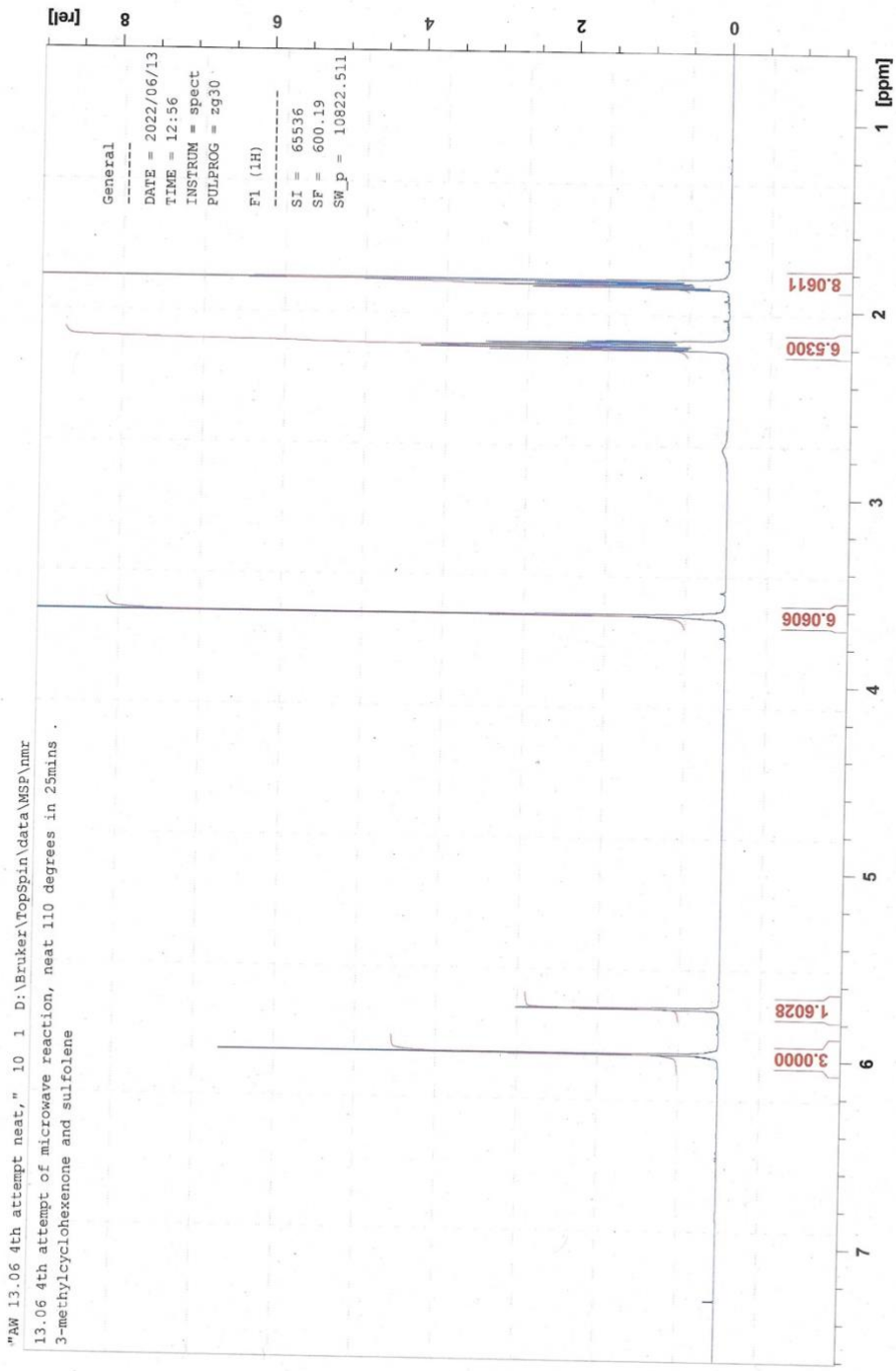
H NMR 3-methylcyclohexene-1one and sulfolene, neat, microwave reaction in 180 degrees



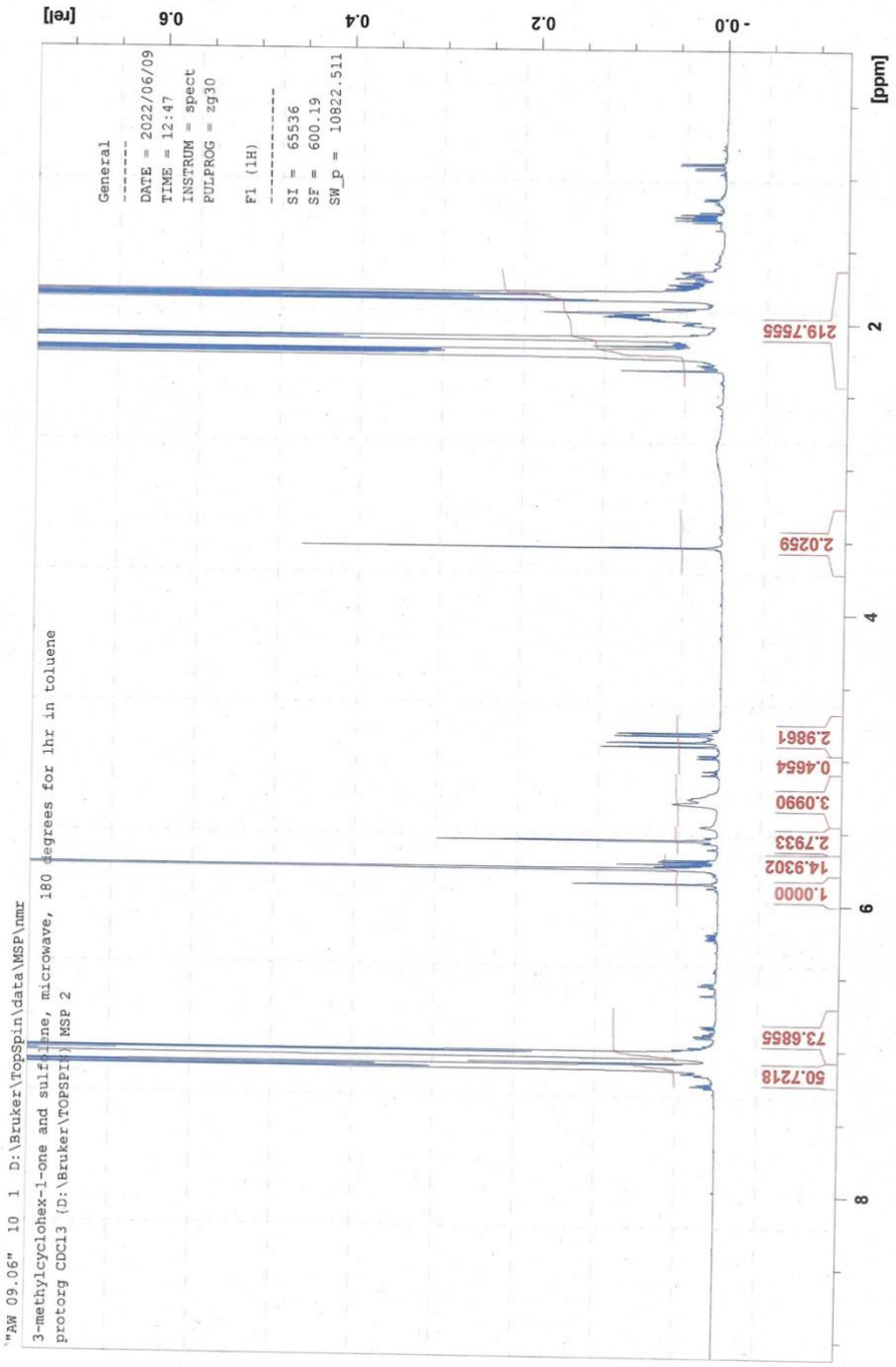
H NMR 3-methylcyclohexene-1one and sulfolene, neat, microwave reaction in 150 degrees



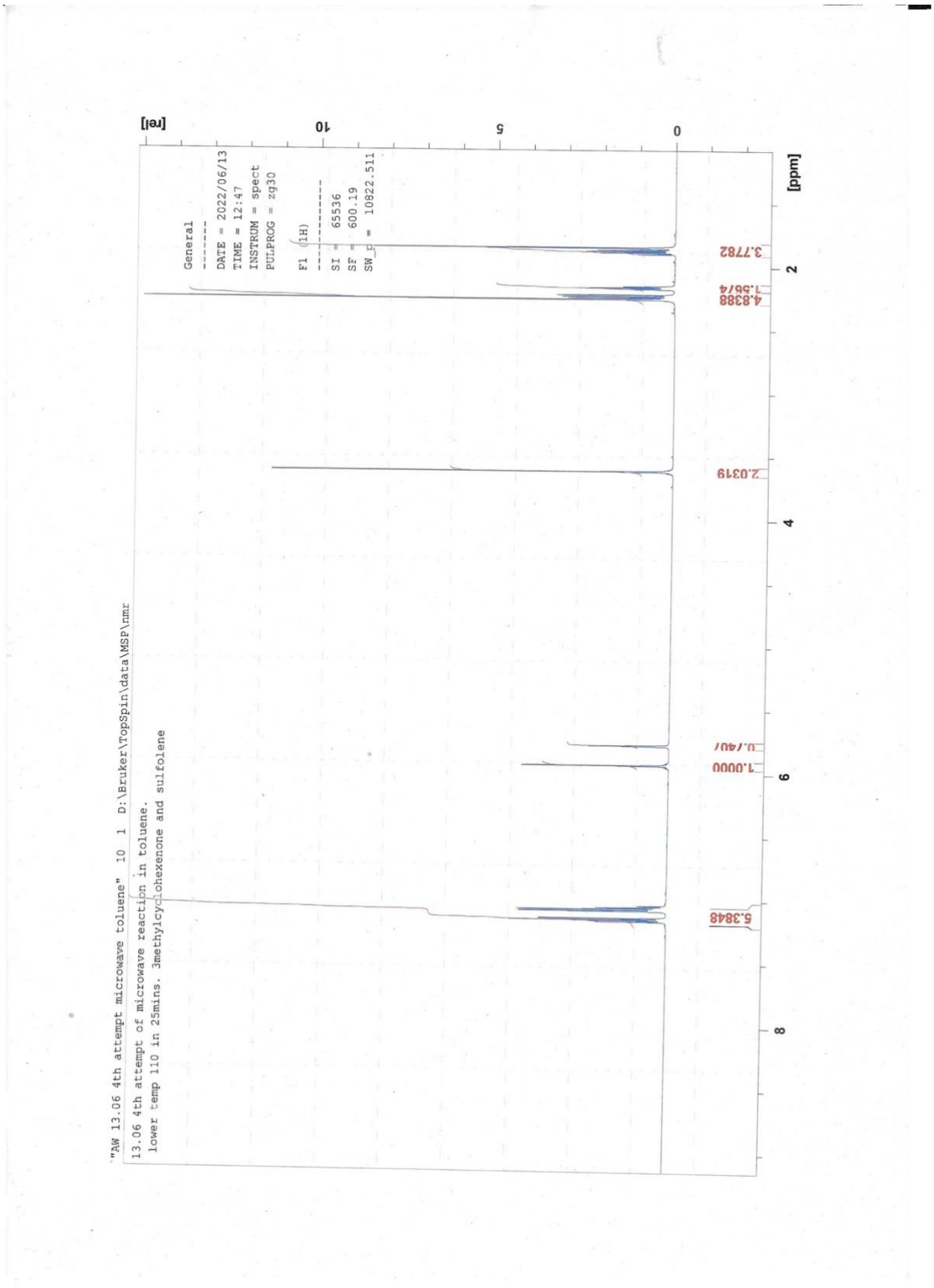
H NMR 3-methylcyclohexene-1one and sulfolene, neat, microwave reaction in 110 degrees



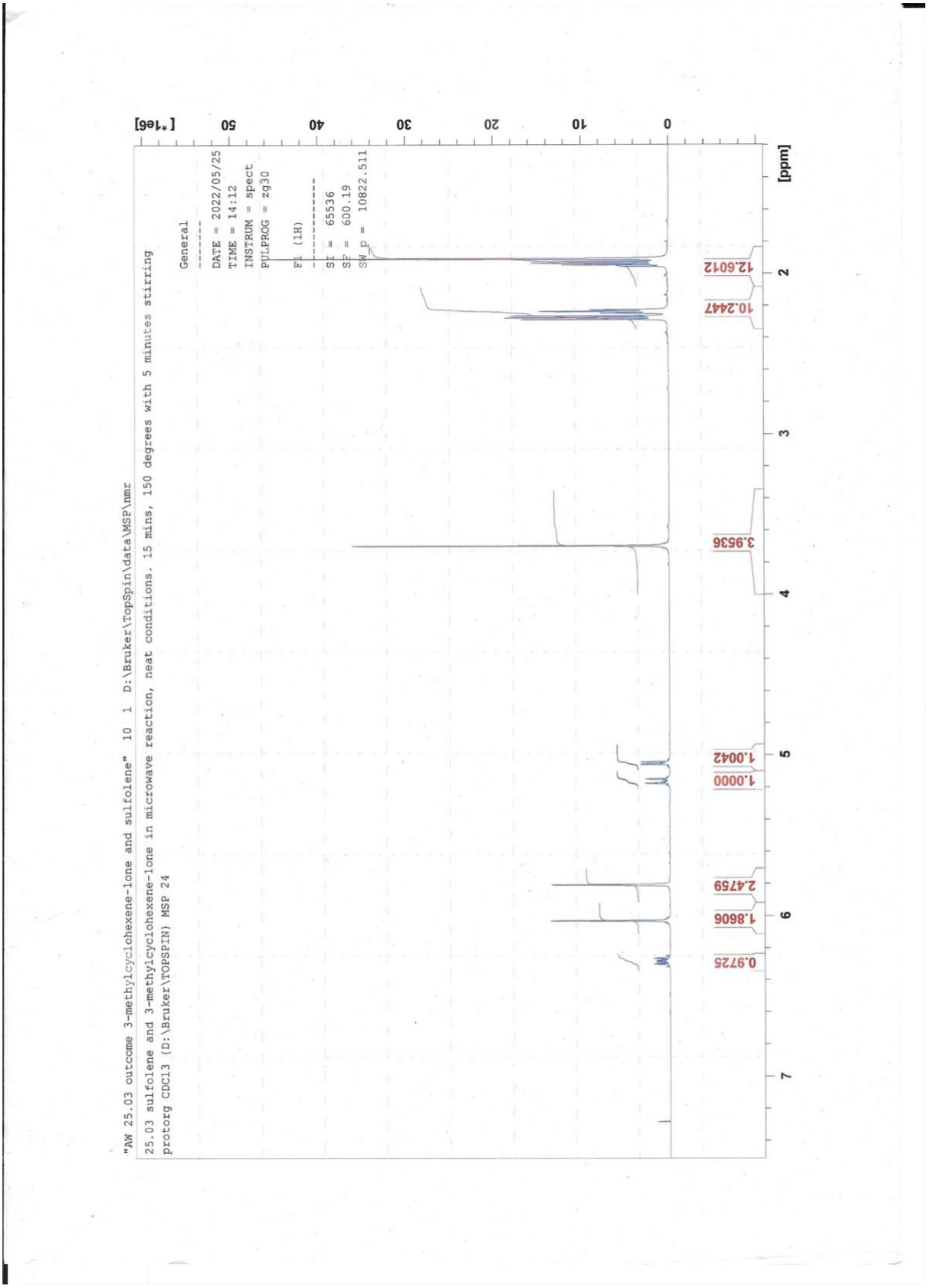
H NMR 3-methylcyclohexene-1one and sulfolene, toluene, microwave reaction in 180 degrees



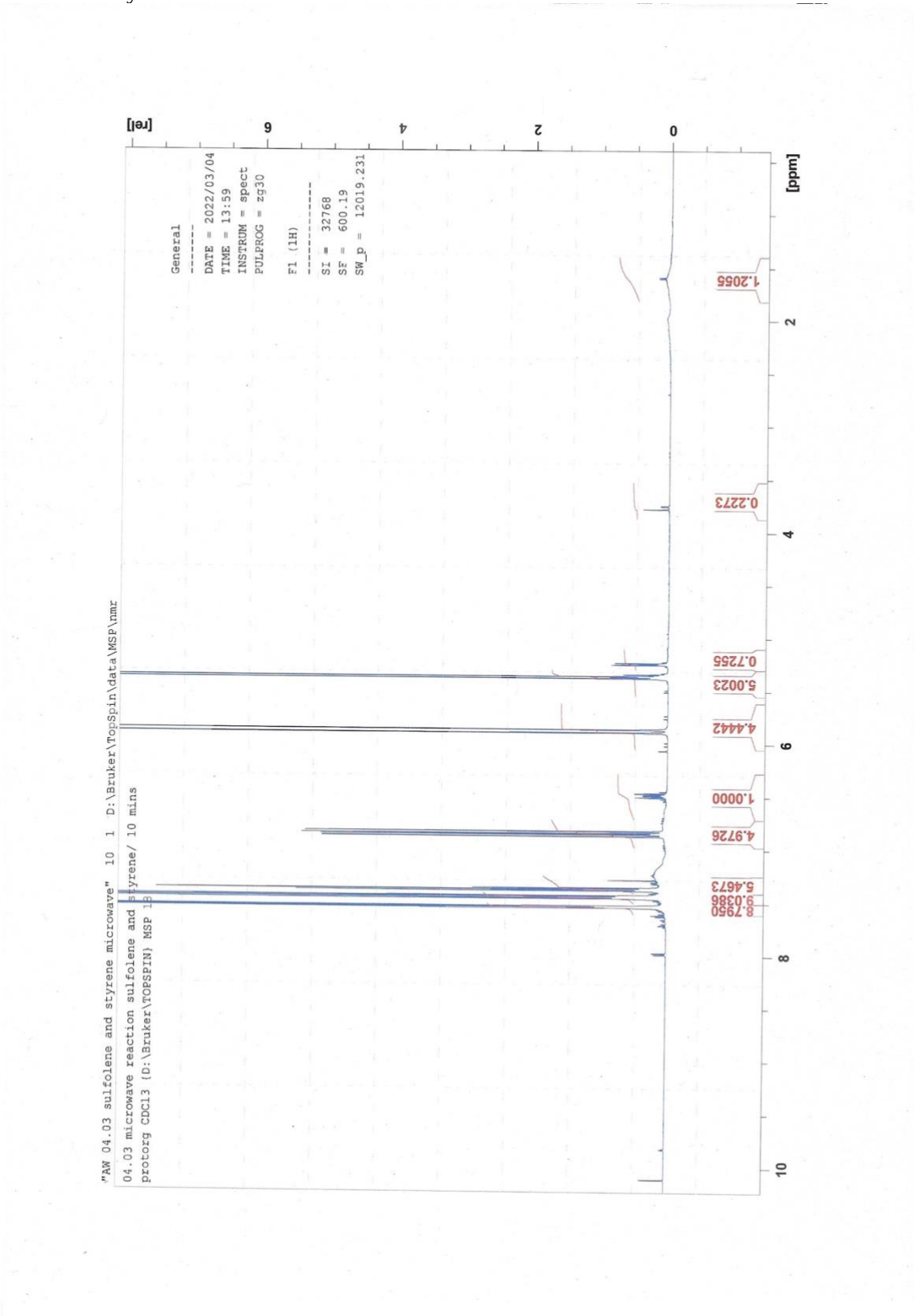
H NMR 3-methylcyclohexene-1one and sulfolene, toluene, microwave reaction in 110 degrees



H NMR 3-methylcyclohexene-1one and sulfolene, neat, 15mins, microwave reaction in 150 degrees



H NMR of styrene and sulfolene in a microwave reactor.



H NMR of H NMR of styrene and sulfolene in a microwave reactor. / different conditions