UiT
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Faculty of Health Sciences – Department of Pharmacy

Evaluation and Late-Stage Fluorination of Small Molecule GnRH-R Antagonists

Synthesis and human serum stability of potential PET radiotracers for the early diagnosis of Alzheimer's disease

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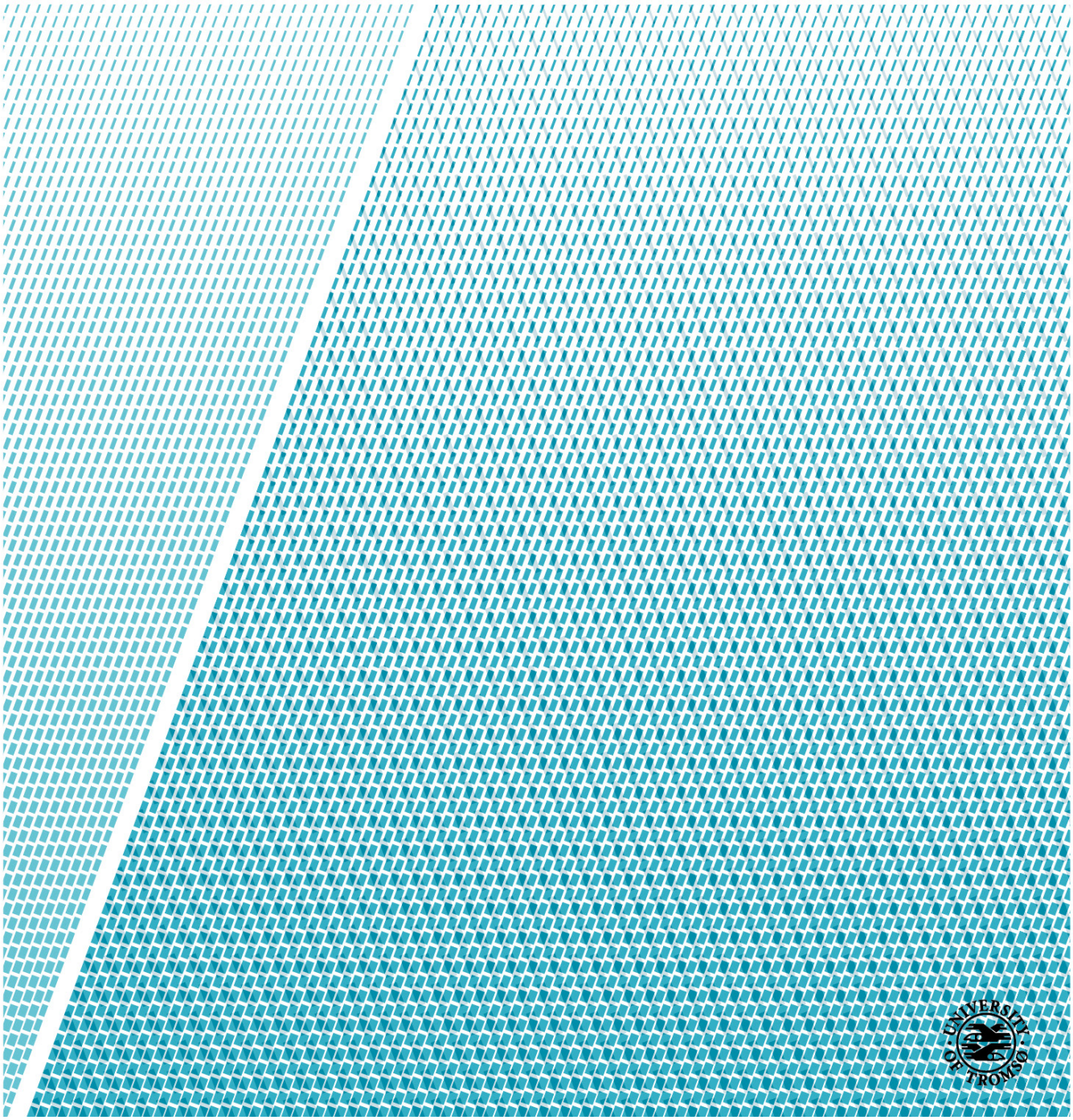
Master's thesis in Pharmacy, May 2019



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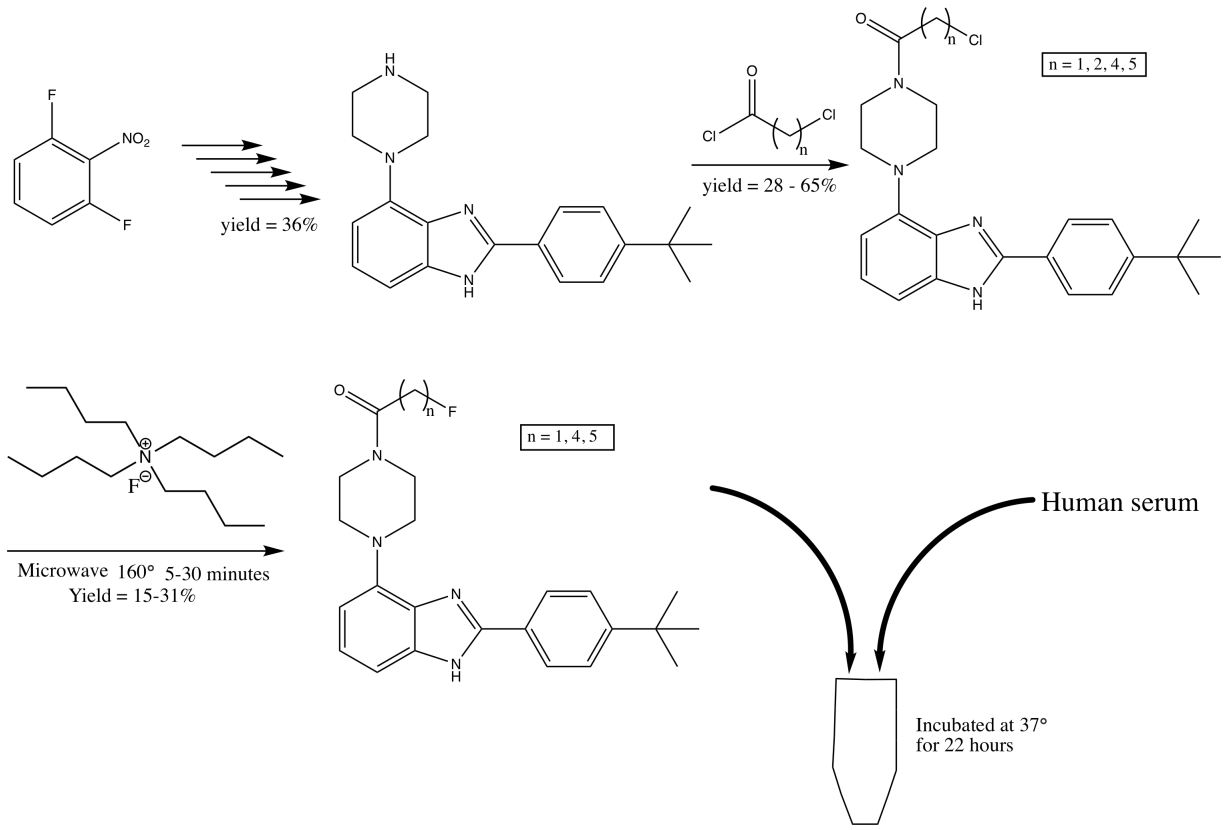
Abstract

Alzheimer's disease is the most common type of dementia; causing problems with memory, thinking and behaviour. The seemingly protective effect of hormone replacement therapy against Alzheimer's disease sparked an interest in the role of the sex hormone system. Sex steroid production is dependent on gonadotropins released in the pituitary. The gonadotropin level is controlled by gonadotropin releasing hormone and its receptor (GnRH-R) is dispersed throughout the body, but is interestingly expressed on neurons in the same brain regions affected by Alzheimer's disease. Positron emission tomography (PET) is a non-invasive imaging technique which utilises radioactively labeled molecules. A radioactively labelled GnRH-R antagonist allows for the observation and quantification of GnRH-R density which may reflect early pathological changes in Alzheimer's disease.

This thesis includes the synthesis, fluorination and biological *in vitro* evaluation by human serum stability of four piperazine tert-butyl benzimidazole GnRH-R antagonist analogues via direct halide exchange from chloroalkyl precursors.

Three compounds were successfully fluorinated in the last reaction step. The reactions all had reaction times less than 30 minutes, suitable for radioactive labelling where a short reaction time is essential. In the last part of this thesis, the three fluorinated compounds were tested for stability in human serum and incubated for 22 hours. All of the fluorinated compounds showed good stability and were found to be suitable for future experiments with radiolabellnig *in vitro* and *in vivo*.



Abbreviations

- **Aβ:** Amyloid β
- **AβPP:** Amyloid β Protein Precursor
- **ACN:** Acetonitrile
- **AD:** Alzheimer's Disease
- **ApoE:** Apolipoprotein E
- **CT:** Computed Tomography
- **DCM:** Dichloromethane
- **DIPEA** N,N-diisopropylamine
- **DMF:** Dimethylformamide
- **DMSO:** Dimethyl Sulfoxide
- **EtOAc:** Ethyl Acetate
- **EtOH:** Ethanol
- **FDG:** Fluordeoxyglucose
- **FSH:** Follicle-stimulating Hormone
- **GnRH:** Gonadotropin Releasing Hormone
- **GnRH-R:** Gonadotropin Releasing Hormone Receptor
- **HPG-axis:** Hypothalamus Pituitary Gonadal-axis
- **HPLC:** High Performance Liquid Chromatography
- **prep-HPLC:** Preparative High Performance Liquid Chromatography
- **HRT:** Hormone Replacement Therapy
- **LDL:** Low Density Lipoprotein
- **LH:** Luteinizing Hormone
- **MCI:** Mild Cognitive Impairment
- **MRI:** Magnetic Resonance Imaging
- **NIA:** National Institute on Aging
- **PET:** Positron Emission Tomography
- **SPECT:** Single-photon emission computed tomography
- **TBAF:** Tetra-butylammonium fluoride
- **THF:** Tetrahydrofuran
- **TLC:** Thin-layer chromatography
- **UPLC:** Ultra Performance Liquid Chromatography

Contents

List of schemes

List of Figures

1 Introduction

There were an estimated 46,8 million people living with dementia in 2015 and it is estimated that this number will almost double every 20 years. The increased longevity leads to a population where people are getting older than before and the prevalence of dementia increases. Dementia usually affects elderly, even though there is a growing awareness of early onset dementia. The costs of dementia is immense, estimated to be a trillion US$. To put it into perspective: if dementia was a country, it would be the 18th largest country in the world. A large portion of the people living with dementia does not have the formal diagnosis, not getting treatment and creating a treatment gap.[1]

The World Alzheimer report from 2011 underlined the importance of early diagnosis in closing the treatment gap. An early diagnosis can improve disease progression through drug and non-drug interventions and prepare the patient leading to delayed institutionalisation and greater quality of life. The patient can make important life decisions before becoming too cognitively impaired and the surrounding family can get information and support on how to handle this disease.[2]

1.0.1 Alzheimer's Disease

Alzheimer's Disease (AD) is the most common type of dementia, being the cause of approximately 60% of the dementia patients. AD is categorised as early or late onset based on if the onset was before or after the age of 60.[3]

AD is a chronic progressive neurodegenerative disease. The symptoms can be divided into three primary groups: (1) Cognitive dysfunction which manifests as memory loss, language difficulties and lack of planning abilities. (2) Psychiatric symptoms such as depression, hallucinations and agitation. (3) Daily living difficulties like eating, shopping and dressing.[4]

AD is categorised into three stages by NIA (National Institute on Aging). The preclinical stage is when the brain changes, but clinical symptoms are not prominent. This can then progress to mild cognitive impairment (MCI) where symptoms start to emerge. The last stage is the Alzheimer's dementia diagnosis with all or some of the symptoms stated above.[4] [5]

The disease is characterised by neurofibrillary tangles, β-amyloid plaque and cell loss. The neurofibrillary tangles are caused by the hyperphosphorylation of tau proteins, and the low solubility of β-amyloid leads to aggregation which is toxic to the neurons and ultimately leading to cell loss.[3]

The research into β-amyloid plaques is vast, but pharmacotherapy targeting these has shown little effect.[6] This indicates that these physiological changes are secondary to the AD and is caused by the underlying mechanisms and not themselves a cause of AD.[7]

1.0.2 Diagnosis

The physician makes the diagnosis based on the patient's ability to function normally and the presence of cognitive and behavioural symptoms and through thorough investigation of the patient's history and neuropsychological testing.[5][8] We divide the dementia diseases based on the physiological cause: (1) The degenerative brain diseases which include AD, Huntingtons and Parkinson, (2) Vascular dementia and (3) Secondary dementia caused by other non-degenerative diseases in the brain.[8] Vascular dementia usually has a more sudden onset than AD and is an important factor in the differential diagnosis.

Brain imaging has traditionally been performed using magnetic resonance imaging (MRI) and computed tomography (CT) to observe atrophy (reduction in size) in certain areas of the brain. Positron Emission Tomography (PET) (discussed later in this chapter) has gained more and more interest as resolution and radiotracer availability has increased. PET uses different radioactive tracers to observe binding in the brain. Trials has been done using fluordeoxyglucose

(FDG), tau protein binders and β-amyloid binders to stage AD.[9][10] Most PET-tracers can be useful in the differential diagnosis of dementia, but there is still missing good radiotracers for the early diagnosis.

1.0.3 Risk factors

Risk factors include age, genetic predispositions for example diseases affecting the chromosomes such as Down's disease, ApoE (Apolipoprotein E) genotypes and head injuries.[4] Lifestyle diseases like Diabetes Mellitus, hypertension[11][12], obesity, physical inactivity and smoking also gives an increased risk of AD. Barnes et al.[13] estimates that a reduction in these lifestyle diseases by 25% will reduce the prevalence of AD cases with 3 million worldwide.

1.0.4 Pharmacotherapy

There is no cure or effective treatment of AD today, but there are drugs that help with some of the symptoms.[8] Pharmacotherapy mainly focuses on cholinesterase inhibitors such as donepezil, rivastigmine and galantamine. Their mechanism of action is the blocking of cholinesterase enzymes that breaks down acetylcholine, thereby increasing the concentration of naurotransmitter in the synaptic gaps. Their efficacy is well documented to be beneficial for people with AD, although the effects are small and the clinical importance discussed.[14][15][16] There are also another class of drugs that block NMDA-receptors (N-methyl-D-aspartate) in glutamatergic neurons. Drugs include memantine and the class has showed good results in several studies.[17][18] Patients with AD also has an increased drug burden using in average more drugs than other controls without cognitive diseases.[19]

1.1 Gonadotropins

Gonadotropins are a part of the endocrine system. The endocrine system consists of a range of hormones controlling bodily functions and activities together with the nervous system. The bodily functions include metabolism and electrolyte balance, regulating temperature, stress response, blood cell production and growth and reproduction. The gonadotropins are tropic hormones, meaning that their primary function is the regulation of hormone secretion by other glands. Gonadotropins act on the gonads, which is the reproductive glands that stimulates the production of gametes (sex cells) and sex hormones.

Gonadotropins are a group of polypeptide hormones including Follicle-stimulating hormone (FSH) and Leuteinizing Hormone (LH). The gonadotropins are essential to the production of sex hormones and disturbances in the release of gonadotropins leads to unnormal growth, lack of sexual development and infertility.

The anterior pituitary gland is responsible for the production of gonadotropins and gets its signals from the hypothalamus. The hypothalamus secretes Gonadotropin Releasing Hormone (GnRH), a decapeptide ($pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-$) which binds to its receptors (GnRH-Rs) in the anterior pituitary gland leading to the production and secretion of LH and FSH.[20] The GnRH-Receptors are G-protein coupled receptors mainly located in the anterior pituitary gland, but can also be found on neoplastic cells. GnRH is used clinically in two ways: to mimic the normal GnRH cycle in patients with infertility or to block the receptor in the treatment of sex-hormone dependent cancers such as prostate, ovary or mammary.[21] The main focus in this thesis is the gonadotropins' connection to Alzheimer's Disease, but radiolabelled GnRH-R binders can also prove important in the diagnosis of certain cancers.[22]

1.1.1 HPG-axis

LH, GnRH and activins have receptors throughout the body, including neurons and these receptors are regulated by hormonal feedback. The hormones and their receptors regulate different processes in the the brain and the dysregulation during meno- and andropause can cause changes in the brain and may be a driver for degenerative diseases such as AD.[23]

The hypothalamus-pituitary-gonadal-axis (HPG-axis) consists of the hypothalamus, pituitary gland and the gonads. Testosterone and oestrogen are released in the gonads from stimuli by gonadotropins produced in the pituitary gland. Testosterone and oestrogen will then have a negative feedback on the pituitary gland. During meno- and andropause oestrogen and testostoreone, respectively, decreases. The testosterone and oestrogen levels decrease and the negative feedback on the pituitary gland leads to increased gonadotropin hormones. The levels of LH and FSH are significantly elevated in women with AD compared with normal controls.[24]

The sex hormones are steroids made from cholesterol. Cholesterol is stored in lipoproteins which transports lipids around the body. Apolipoproteins are proteins on the outside of lipoproteins and bind to the Low-density lipoprotein receptor. ApoE has three phenotypes, E2, E3 and E4. Having the E4 is a known risk factor for developing AD.[25]

1.1.2 Gonadotropins and Alzheimer

Each gonadotropin plays a role in the aging and changing brain. Estrogens and androgens shows a trophic and neuroprotective ability and decreasing concentrations has been connected to disease progression through epidemiological studies. Early studies on the meno-/andropauses influence on AD showed HRT to reduce the risk of AD through reduction of tau phosphorylation and β-amyloid plaques [25][26][27][28][29], but these believes are now being questioned.[30][31] Studies now show that Hormone replacement therapy (HRT) has a critical period in which it exerts its effect [29] and this critical period corresponds with the peak time of GnRH levels. The hormone levels are directly related to the gonadotropin levels due to the negative feedback loop. It was then hypothesized that it may not be the levels of oestrogen and testosterone, but the levels of gonadotropins that played a crucial role.

Gonadotropin levels has shown to be elevated in AD patients compared to age-matched controls and the increased risk of AD in women can be explained by the earlier and more sudden rise in gonadotropin levels. The gonadal hormone receptors are located throughout the brain, but is concentrated in the limbic system and are known for regulating brain development and brain structure. The same neurons that is a part of the AD pathology.[27] Several studies has shown that LH levels modulate Amyloid-$_\beta$ Protein Precursor (A$_\beta$PP) and the generation of β-amyloid. This is supported by mouse studies eliminating the effect of sex hormone alterations.[32][33][34][35]

During meno- and andropause the GnRH-levels are close to that of an infant, and as this is a phase of growth and GnRH activins and sex steroids are promoters of cell proliferation it's likely that this promotes the signals for development in the brain.[23] Studies have shown that the AD brain undergoes cell signalling indicative of cell cycle activity and the reintroduction of neuronal cells in this cycle. Adult neuronal cells normally stop at the G_0-phase and remain in this state through adult life. The AD brain surprisingly shows that neuronal cells shows activated pathways ultimately leading to cell death and atrophy.[36][23] This hypothesis is further explained by the biochemical changes like Aβ-plaques, tangles and hyperphosphorylation of tau and presenilin expression is evident in the infants developing brain. These changes are present in the developing brain (where neuronal cells die during the shaping of the brain) and support that Aβ-proteins are a part of cell death and tau-phosphorylation in the division of cells, destabilizing the microtubule.

This mechanism can also be explained by the observed increase in AD risk for people with Down's syndrome.[25] [37] The Down's syndrome patient has sex-hormone levels comparable

to the normal population, but their gonadotropin levels are significantly higher. Especially the gonadotropin FSH is increased in these patients and contrary to the normal population, this occurs more frequently in male patients.

Studies has shown that gonadotropins enhanced the number of LDL-receptors (Low Density Lipoprotein) and their transport into neurons. Lipoproteins are globular complexes made of proteins and lipids (fat) in the body and are responsible for the transport of fat through the body. On the surface of these lipoproteins there are ligands known as Apolipoprotein E and its receptor is β-amyloid. Individuals carrying a specific allele of the gene coding for this protein, ApoE4 are known to have an increased risk of AD. The elevated circulating gonadotropin levels could increase plaques formed from ApoE and its ligand β-amyloid if we assume that these mechanisms are also present in neural cells.[25][38] This mechanism can also help to explain the protective effect of HRT as increased testosterone and oestrogen levels would decrease the gonadotropin levels and thereby reduce plaques.

Targeting gonadotropin release can affect the incidence and progression of AD, as showed by Casadesus et. al when they treated late stage AD mice with leuprolide acetate showing improved cognition and β-amyloyd depositions.[32] Leuprolide acetate is a GnRH analogue acting as an agonist on the GnRHR initially inducing stimulation of gonadotropins, but since a steady administration does not mimic the natural endogenic cycle the receptors soon become desensitized and LH and FSH levels decrease. GnRH antagonists does not have this initial increase in gonadotropins. As well as having the potential in therapy of AD, the GnRH antagonists has the potential as PET tracers. This non-invasive tool can help us understand the expression of GnRHR in AD and possibly prove to be a valuable diagnostic tool.

1.2 Positron Emission Tomography

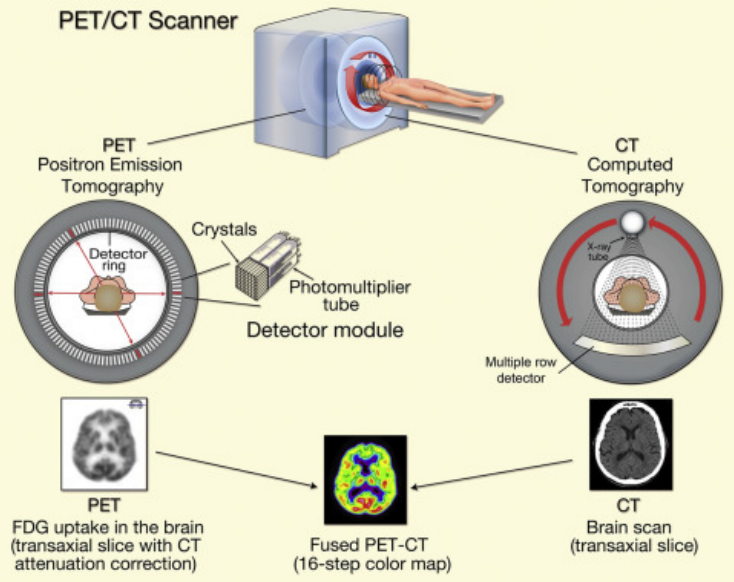


Figure 1: A PET/CT-scan can be combined to give both structural and functional images [39].

Positron Emission Tomography (PET) is a nuclear imaging technique using small amounts of a radionuclide to give functional images of uptake in the body. The most common radionuclide is [^{18}F]-FDG (2-Fluoro-Deoxy-D-glucose), a glucose analogue which accumulate in energy demanding cells such as cancerous, brain and heart.

The ^{18}F is made in a cyclotron which accelerates and shoots protons at water enriched with ^{18}O. When a proton "crashes" into the nucleus of the ^{18}O it kicks out a neutron and you are left with ^{18}F.[40] The reaction can be written like this: $^{18}\mathrm{O}\,(\mathrm{p},\mathrm{n})\,^{18}\mathrm{F}$. $^{18}\mathrm{F}^{-}$ is a good nucleophile,

but it makes strong hydrogen bonds with water creating a solvation effect making it almost chemically inert. The $^{18}F^-$ is therefore extracted from the water using Kryptofix 222 ®, a cryptand that complexes with K^+ which creates a salt with the fluorine. The salt will be more hydrophobic, allowing for the use of organic solvents. The rest of the water is usually removed using acetonitrile (ACN) which makes an azeotropic mixture with water and makes it possible to evaporate them together.

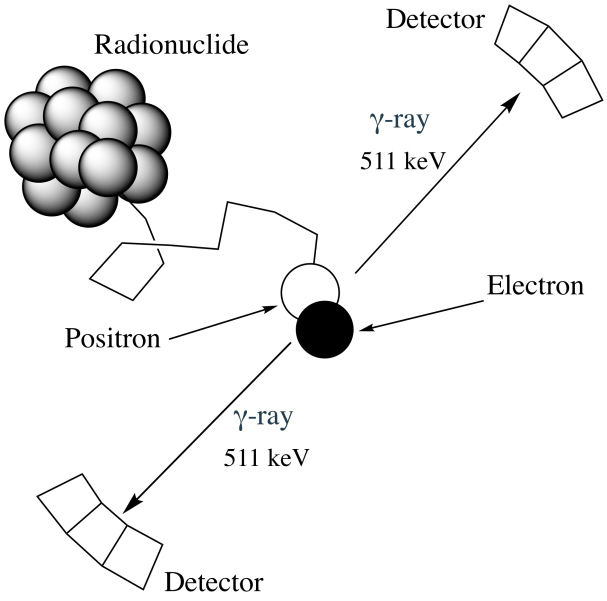


Figure 2: This shows the principle of PET on an atomic level. The readionuclide emit a β^+-particle which annihilates with an electron resulting in two γ-rays which is then detected.

As illustrated in Figure 2, radionuclides used in PET emit β^+-particles, also known as positrons. Positrons are similar to electrons, having the same mass but with a positive charge. This makes the positron the antiparticle of an electron. The positrons are short-lived and only travel a couple of millimetres in tissue until it meets an electron and annihilates into two γ-rays of 511 keV almost 180° from each other. This angle is to maintain the momentum each of the particles had when they were annihilating. 511 keV is the resting potential of an electron, and the positron has to come close to this to be able to annihilate and is the reason for its travel. It does not need to reach exactly 511 keV, the consequence being that the angle of which the two γ-photons is not exactly 180°. The different radionuclides used in PET-scanning (^{11}C, ^{13}N, ^{15}O, ^{18}F and ^{68}Ga) emit β^+-particles with different energy levels. ^{18}F, the most common radionuclide has a decay energy of 634 keV, close to that of an electron's resting potential. The practical difference between the energy levels is the distance from where the radionuclide decays to where the β^+-particles annihilates with an electron. A simulation of positron travel between ^{68}Ga and ^{18}F shows that the ^{68}Ga positron travels about three times further in lung tissue than ^{18}F resulting in poorer resolution.[41]

PET gives good functional images as opposed to CT which gives structural information. To interpret PET images alone can be difficult as it is difficult to localise the uptake in the body since there is little structural information. The combined image obtained from a PET/CT gives both radiotracer activity and its distribution.

The advantage of PET-scans is that it allows us to get structural and functional images of the body, including the brain. This would be beneficial in understanding the development of AD in patients as the disease progresses and the importance of GnRH-R expression.

Theranostics is an interesting field which is comprised of two words: therapy and diagnostics. Within nuclear medicine the main focus of theranostics is cancer, where a PET-radiotracers can

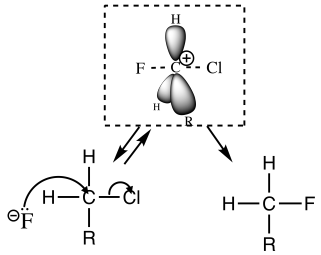


Figure 3: A S_N2-reaction mechanism, most commonly done during nucleophilic substitution of incorporating fluorine in PET tracers. This shows the nuclophilic fluoride attacking the carbon and through a transition state kicking out chloride as the leaving group.

be used for diagnosis and stage-determination of the cancer and then radiolabelled with a nuclide emitting more tissue damaging radiation. When changing the diagnostic radionuclide i.e. ^{18}F with a β^--emitter such as ^{177}Lu, the radiation can be specific and the total radiation dose to the patient minimised compared to conventional radiation therapy. One of the earlier theranostic applications were the "magic bullet" for thyroid cancer: ^{131}I which emits both β^- and γ-rays. Since iodine is mainly absorbed in the thyroid gland to produce thyroid hormones, ^{131}I can be administered and will effectively irradiate the thyroid gland with β^-. Since approximately 10% of the ^{131}I decay is γ-rays, the patient can also be examined using a single photon emission computed tomography (SPECT), another nuclear medical modality.

1.3 Radiochemistry

1.3.1 PET radiochemistry

The synthesis of radioactive PET-tracers needs to be quick to increase radiochemical yields due to the short half-life of most PET radionuclides.[42] The most common PET radionuclide ^{18}F has a half-life of 109,7 minutes.[43] Since ^{18}F usually is extracted using a class of crown ethers called cryptands (Kryptofix), the most common way of incorporating the radionuclide is nucleophilic substitution via S_N2 shown in Figure 3. Direct nucleophilic substitution calls for a good nucleophile and a good leaving group and most common in radiolabelling are tosylates and mesylates.

1.3.2 The piperazine tert-butyl benzimidazole class

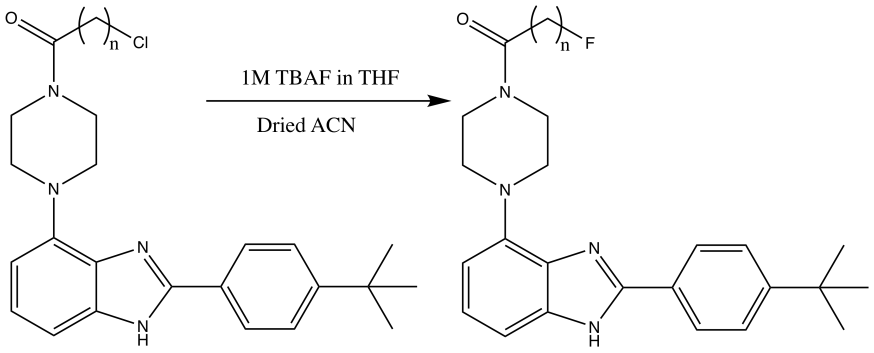
The synthesis of small molecule non-peptide GnRH-R antagonists has gained increasing scientific interest. There are 10 different classes of non-peptide GnRH receptor antagonists including the piperazine-benzimidazole derivatives presented in this thesis.[44]

The piperazine tert-butyl benzimidazole class has shown nanomolar binding affinities to the human GnRH-R receptor and good oral bioavailability [45] making this a class of interest for future optimisation.

Previous studies on similar compounds prepared to be SPECT-tracers, using radioactive iodine showed certain instability issues in serum [46] In serum, the issue is thought to be the hydroxylation, cleaving the C-I-bond.

1.3.3 Fluorination

As a further optimisation of these compounds we wanted to incorporate fluorine and prepare them as potential PET-radiotracers The C-F-bond is a short bond ($\sim$1.47 Å) and one of the strongest bonds in organic chemistry[47] and should improve serum stability over the previously synthesised iodides. The fluorination method is a direct primary alkyl halide substitution with tetrabutyl ammonium fluoride (TBAF) as the fluoride carrier shown in Scheme 1.



Scheme 1: The scheme shows the fluorination method used in this thesis and is adapted from [48].

^{18}F is, as described earlier made in a cyclotron from ^{18}O-enriched water and traditionally the preparation of radiolabelled pharmaceuticals were made with water as solvent. Even though $^{18}F^-$ is a strong nucleophile, the solvation effect of water reduces its reactivity substantially and requires harsh reaction conditions.

Despite several methods of incorporating fluorine in organic molecules [49] relatively few methods are used for radiolabelling because the introduction of ^{18}F requires the method to be selective, efficient and convenient.[50] To facilitate the [^{18}F]-radiolabelling and improve the reaction conditions it is now common to use a *"naked"* fluoride ion attached to a cation such as tetrabutylammonium or a crown ether complex.[51][50]

The ammonium salt creates a salt with fluorine making the fluorine less affected by the solvation effect and fascilitating the nucleophilic fluorination. Because the fluorine ion is less covered in solvents it is often referred to as *"naked"*. TBAF is thought to mimic the Kryptofix222, a cryptand wich binds a K^+ creating a salt with F^-.

As described earlier, the $^{18}F^-$ is bound to a carrier and the reaction usually performed in an aprotic solvent such as ACN. Recent studies have shown that adding some amounts of a sterically hindered protic solvents such as tert-butanol can maintain nucleophilicity while reducing by-product formation and reduce reaction time substantially with.[50][48] The thought behind this is that the sterically hindered protic solvent will help stabilise the fluorine and solubilise it. This can help bring the fluorine to the molecule and initiate the S_N2-reaction. Since the molecules are prepared to become PET radiotracers it is an advantage to use late-stage radiolabelling where the fluorine is incorporated in the molecule as late as possible.

Direct fluorination from chlorides is attempted according to [48]. This study uses a molecule where the C−Cl carbon is more electron dense than in our short-chained molecules and thus our molecules should have a faster reaction time than the reported 48 hours. The long reaction time is not suitable for PET due to the short $t_{1/2}$ of ^{18}F and methods to reduce reaction time is essential.

1.3.4 Microwave assisted synthesis

The method of using microwave to enhance reaction speed and selectivity is known within organic synthesis and radiolabelling.[52] The matter undergoing microwave irradiation will heat up as long as it has a dipole moment. The oscillation of the molecules corresponds to the frequency, wavelength and energy of the irradiation and the fast changing in the electric field leads to an even heating of the reaction mixture.[53] This heating and activation of molecules help decrease reaction times substantially, which is sought after in a radiolabelling situation.

1.4 Serum stability

Serum stability analysis is an important first step in the *in vitro* evaluation of potential new drugs. Previously reported studies on an α-iodinated piperazine tert-butyl benzimidazole [46] showed sufficient serum stability for rat brain SPECT analysis and biodistribution, but with room for improvement. The previous studies were conducted with the aim of creating SPECT radiotracers with radioactive iodine. SPECT as a nuclear medicine modality is still important, but lack the possibility of quantification as obtainable in PET. To overcome these potential problems, we want to synthesise fluorinated compounds which should be more stable than iodinated molecules as well as develop a fluorination method indicating suitability for ^{18}F-radiolabelling.

A problem emerging from alkyl iodides, especially α-iodides such as presented in [46] is the potential alkylation effect. In medicinal chemistry it is rarely a good thing to have α-halides as these molecules has the potential of reacting with DNA and other nucleophilic aminogroups and thiols in proteins. DNA consists of base-pairs, all of which contain nucleophilic nitrogen's that is able to attack the electron poor α-carbon leading to an irreversible alkylation of the DNA-pair leading to cell damage and possible cancer. The same reaction is shown in Figure 3 where DNA in this instance would act as F^- does in the figure.

2 Aims

It is known that gonadotropins can play an important role in Alzheimer's Disease. Small molecule GnRH antagonists can help understand the mechanism behind gonadotropin involvement in AD and may be used as a method for early diagnosis of the disease.

The first aim was to synthesise the intermediate **Compound 5** as shown in Scheme 2 as this forms the basis for the four chlorinated analogues which is the starting material for the fluorination.

The second aim was to develop a fluorination method which was effective and provided good yields as the last step in the synthesis. The fluorination in radioactive labelling preferably happens in the last synthesis step to minimise time from radioactive labelling to finished product. A good fluorination method would indicate suitability for ^{18}F-fluorination and the possibility of radioactive labelling and subsequent preclinical *in vivo* experiments. The fluorides are presented in Figure 1.

The third aim was to determine the human serum stability of the fluorinated analogues and compare them to previously reported iodide compounds. The serum stability has been an issue with alkyl halides, making them less suitable as drugs. The serum stability would also give an indication of the alkylating effect, giving rise to potential cytotoxic sideeffects.

3 Results and discussion

The results and discussion chapter are divided into three sections: Firstly the synthesis of intermediate and the chloroalkyl ketones which forms the basis of starting material for the fluorination is presented. The second part presents the results of the fluorination method, its effectiveness and the chloroalkyl moieties' suitability as starting material. Finally the human serum stability performed on the fluorinated products and compare them to a previously synthesised iodoacetyl ketone with known human serum stability performance.

3.1 Synthesis of intermediate and analogues

The intermediate (Compound 5) presented in Scheme 2 is the starting material for the chloroalkyl ketone analogues. The chloroalkyl ketones are produced by reacting the intermediate with chloroacyl chlorides with different chain lengths to produce Compound 62, 63, 65 and 66. The chlorinated is presented as starting material in Scheme 1.

The synthesis of intermediate (Compound 5) was done according to Pelletier et al.[54] with the modifications done by Fjellaksel and colleagues [55].

Compound 5 was synthesised successfully with a total yield of 36% over five which is comparable to Fjellaksel [55] with synthetic route shown in 2.

Table 1: Key reaction data from the synthesis of intermediate Compound 5 as shown in Scheme 2 and the four chloroalkane ketones.

Compound	**Yield (%)**
1	98*
2	50
3	100
4	81
5	90
62	39
63	37
65	28
66	65

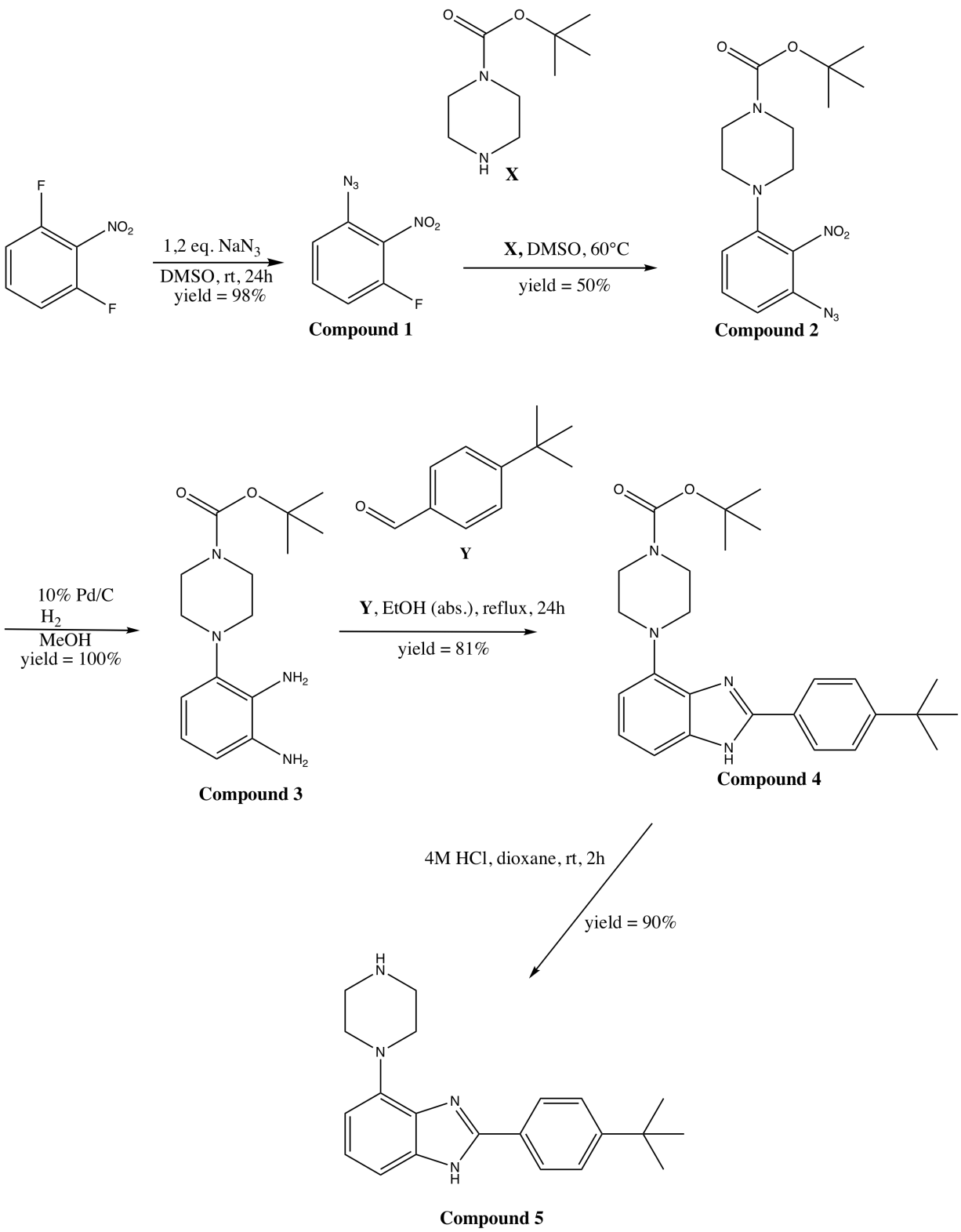
**increased NaN_3 from 1 to 1,2 eq to increase yield from 83%*

3.1.1 1-azido-3-fluoro-2-nitrobenzene Compound 1

2,6-difluoronitrobenzene was dissolved in dimethyl sulfoxide (DMSO) and 1,2 molar equivalents (eq) NaN_3 was added. The amount of NaN_3 was increased from 1,0 to 1,2 equivalents to obtain a yield of 98% compared to previously reported 83%[55] This could increase the disubstituted by-product, but this would easily be removed in the next synthesis step as it can not react with piperazine. The reaction mixture was extracted using ethyl acetate (EtOAc) and washed with distilled water. If the water phase had a strong yellow colour, the water phase was washed with EtOAc to minimise loss of product. The reaction is a nucleophilic aromatic substitution. The product was confirmed using ^{1}H-NMR, and the yield found to be 98%.

3.1.2 tert-butyl 4-(3-azido-2-nitrophenyl) piperazine-1-carboxylate Compound 2

Compound 1 was dissolved in DMSO and DIPEA and 1-boc-piperazine for a nucleophilic aromatic substitution. The N,N-Diisopropylethylamine (DIPEA) was added to increase pH and ensure nucleophilicity of the 1-boc-piperazine. The reaction was stirred for 22 hours. The reaction mixture was extracted using EtOAc and washed with distilled water. The organic phase



Scheme 2: The scheme shows the synthetic route to reach the intermediate Compound 5

was then dried on a rotavapor and the product dry mounted on celite and chromatographed using flash chromatography. A 15:85 EtOAc : pentane mobile phase was first tried, but did not give satisfactory separation and too many fractions making work-up more time consuming. 40:60 EtOAc : pentane gave a good separation and the best yields = 50%. The product was confirmed using ^{1}H-NMR.

3.1.3 tert-butyl 4-(2,3-diaminophenyl) piperazine-1-carboxylate Compound 3

Compound 2 was dissolved in methanol (MeOH) and transferred to a hydrogenation reaction vessel. 10% Palladium on carbon (Pd/C) was carefully transferred to a test tube under constant N_2 stream and carefully added MeOH. Once the Pd/C was wetted with MeOH it was transferred to the bomb which was mounted in the Parr apparatus. The reaction vessel was flushed with H_2 three times to ensure a saturated atmosphere and maintained a pressure of 150 psi for 3 hours. The reaction vessel was dismounted carefully and the reaction mixture filtered through celite and the solvents removed on the rotavapor to provide a brown powder. The yield was found to be 101% which indicates some solvent residues. The product was confirmed using ^{1}H-NMR.

3.1.4 tert-butyl 4-(2-(4-(tert-butyl)phenyl) -1H-benzo[d]imidazol-4-yl) piperazine-1-carboxylate Compound 4

Previous studies showed that the best yields could be obtained in the 1 mmol scale. Compound 3 was dissolved in ethanol (EtOH) and tert-butyl benzaldehyde was added to the solution and heated to 80°C and the reaction refluxed for 24 hours. The aldehyde forms a cyclic structure with the two amine groups obtaining the benzimidazole. The reaction was monitored using Thin-layer chromatography (TLC) and stopped when the TLC started to show spots for the unwanted disubstituted product (Figure 4) or there were no starting material left. The solvents were removed using rotavapor and chromatographed using flash chromatography. The mobile phase was a 40:60 EtOAc : pentane mixture. The fractions were collected and the solvents removed on the rotavapor to obtain the desired product with 81% yield. The product was confirmed using ^{1}H-NMR.

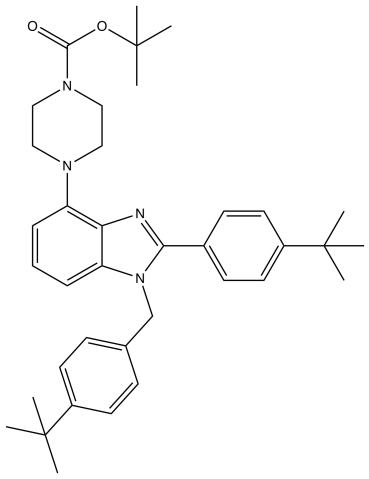


Figure 4: The disubstituted by-product in the synthesis of Compound 4

3.1.5 2-(4-(tert-butyl)phenyl)-4-(piperazin-1-yl)-1H-benzo[d]imidazole Compound 5

Compound 4 was dissolved in 4M hydrochloric acid (HCl) in dioxane and stirred for 2 hours to remove the Boc protection group. The solvents were removed using a water ejector vacuum

pump with a trap between. When the solvents were removed, the product was extracted with water and washed with EtOAc. The waterphase was lyophilized to obtain a white powder. The product is the hydrochloride salt of Compound 5 and this was taken into account on the following reactions. The yield were found to be 90%. The product was confirmed using ^{1}H-NMR.

3.1.6 1-(4-(2-(4-(tert-butyl)phenyl)-1H-benzo[d]imidazol-4-yl)piperazin-1-yl)-2-chloroethan-1-one Compound 62

Compound 5 was dissolved in dry DMF under inert conditions and DIPEA was added to the solution. The round bottom flask (RBF) was lowered into an ice bath. When the solution was ice cold, chloroacetyl chloride was added to the solution dropwise. The solution was stirred for 1 hour under N_2. The reaction was quenched using distilled water and transferred to a separatory funnel and washed with dichloromethane (DCM). Brine was used if the phases did not separate. The organic phase was collected and evaporated on the rotavapor. The product was chromatographed on Biotage C18 column and the fractions lyophilized to obtain a white powder with a yield of 39% The product was confirmed using ^{1}H-NMR.

3.1.7 1-(4-(2-(4-(tert-butyl)phenyl)-1H-benzo[d]imidazol-4-yl)piperazin-1-yl)-3-chloropropan-1-one Compound 63

The method for synthesising Compound 63 resembles the synthesis of Compound 62. Compound five dissolved in dry dimethylformamide (DMF), added DIPEA and put in an ice bath. 3-chloropropionyl chloride added to the solution dropwise. Main difference is the reaction time, this reaction taking 3 hours. Work-up was done using a Biotage C18 column and the fractions lyophilized to obtain a white powder. The yield was found to be 37%. The product was confirmed using ^{1}H-NMR.

3.1.8 1-(4-(2-(4-(tert-butyl)phenyl)-1H-benzo[d]imidazol-4-yl)piperazin-1-yl)-5-chloropentan-1-one Compound 65

The method of synthesising Compound 65 resembles the synthesis of Compound 62. 5-chlorovaleroyl chloride was added to ice cold reaction mixture of Compound 5, DIPEA and dry DMF. Purification was done using a Biotage C18 column and the fractions lyophilized to obtain a white powder yield = 28% The product was confirmed using ^{1}H-NMR.

3.1.9 1-(4-(2-(4-(tert-butyl)phenyl)-1H-benzo[d]imidazol-4-yl)piperazin-1-yl)-6-chlorohexan-1-one Compound 66

The method of synthesising Compound 66 resembles the synthesis of Compound 62. 6-chlorohexanoyl chloride was added to ice cold reaction mixture of Compound 5, DIPEA and dry DMF. Work-up was done using a Biotage C18 column and the fractions lyophilized to obtain a white powder yield = 28% The product was confirmed using ^{1}H-NMR.

3.1.10 Improvements

Ultra Performance Liquid Chromatography (UPLC) analysis of the reaction showed the formation of 60% compound 72. The yield was found to be 30% and indicates that there is substantial loss of product in the work-up. Sometimes the issue could be explained by overlapping peaks at the Biotage as the difference in polarity between the chloride- and fluoride compounds is minimal. Better separation was seen on the preparative high performance liquid chromatography (prep-HPLC) as can be expected from a bigger column with smaller silica particles. The use of prep-HPLC was limited by technical instrument issues.

3.2 Fluorination method

The fluorination method for radioactive labelling with ^{18}F needs to be effective and selective to maximise radiochemical yields.[42] The fluorination method was developed using non-radioactive ^{19}F and conditions similar to that obtainable in a PET-center. Since ^{18}F and ^{19}F has the same basic chemical properties there should not be a difference in reactivity between the two isotopes, but some stoichiometric and condition differences must be expected and optimisation and adjustments are anticipated in a potential radioactive labelling experiment.

The fluorination method uses TBAF shown in Figure 5 as the fluorine source because it was available in the laboratory and mimics the cryptand. Few studies compare the two fluorination methods head-to-head, but som future development must be expected if a crptand is used. Due to the relatively long reaction time reported by Kim and colleagues [48] the method presented in this thesis was a microwave-assisted reaction. The TBAF available in the laboratory was a 1M solution in tetrahydrofuran (THF), a polar aprotic solvent. The reactions were done at 160°C with 5 minute holds for a total of 30 minutes. The samples were analysed on high resolution Mass spectrometry (HRMS) to follow the reaction and check for product formation. The samples were later analysed on UPLC to determine reaction time showed in Figure 7.

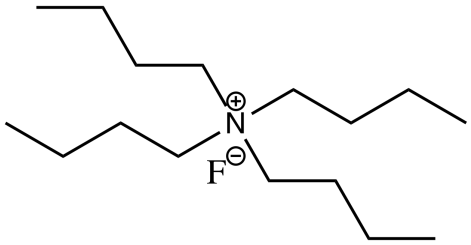


Figure 5: Tetrabutyl ammonium fluoride

3.2.1 1-(4-(2-(4-(tert-butyl)phenyl)-1H-benzo[d]imidazol-4-yl)piperazin-1-yl)-2-fluoroethan-1-one Compound 72

The chloride analogue was dissolved in dry ACN in a microwave vial and 2 eq 1M TBAF (Figure 5) in THF was added to the solution. The reaction is a halide substitution. Since chlorine is not the best leaving group and halide substitutions previously has been reported to have reaction times up to 48 hours[48] the reaction was attempted in the microwave. The microwave was set for 160°C for a total of 30 minutes with samples taken every 5 minutes. The samples were analysed on HRMS during the reaction and later on UPLC to determine reaction time and efficiency. The UPLC data concluded that the reaction was done after 5 minutes as there was no starting material left. The yield was found to be 30%. The product was confirmed using ^{1}H-NMR and HRMS.

3.2.2 1-(4-(2-(4-(tert-butyl)phenyl)-1H-benzo[d]imidazol-4-yl)piperazin-1-yl)-3-fluoropropan-1-one Compound 73

This reaction did not lead to the desired product. The chloride analogue was dissolved in dry ACN in a microwave vial and 2 eq 1M TBAF in THF was added to the solution. The reaction was done in the microwave at 160°C for a total of 30 minutes. The reaction was chromatographed on Biotage C18 column. During work-up of this product, HRMS was utilised to detect which fractions contained the desired product. The results did not show the mass of the desired product, but instead found the mass corresponding to the elimination product shown in Figure 6.

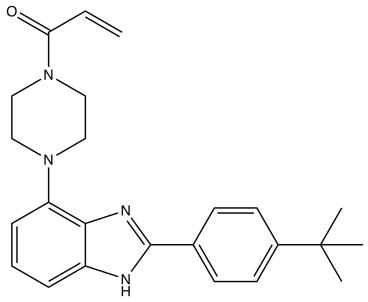


Figure 6: The elimination by-product formed in the attempted synthesis of Compound 73

3.2.3 1-(4-(2-(4-(tert-butyl)phenyl)-1H-benzo[d]imidazol-4-yl)piperazin-1-yl)-5-fluoropentan-1-one Compound 75

This reaction was done in the same way as the other fluorinations, but with substantial formation of by-products compared to Compound 72. The product was worked up using Biotage and the fractions containing product was confirmed using HRMS. The yield was found to be 15%.

3.2.4 1-(4-(2-(4-(tert-butyl)phenyl)-1H-benzo[d]imidazol-4-yl)piperazin-1-yl)-6-fluorohexan-1-one Compound 76

Due to adequate amounts of starting material it was decided to test the difference in selectivity and reaction time when the reaction was dissolved in either tert-butanol or dry ACN. The reaction mixtures were prepared with the same amount of starting material, solvent and TBAF. The reaction was monitored using HRMS and even though MS is not quantifiable without the use of internal standard calibration, the peak heights could give an indication of relative abundance. The HRMS-results indicated that the reaction with tert-butanol were marginally slower than the one performed in ACN, but the tert-butanol seemed to provide less of the elimination product. They were both worked up using Biotage and the fractions containing product was confirmed using HRMS. Yield from tert-butanol experiment: 31% Yield from ACN experiment: 18%.

3.3 Reaction time experiment

Figure 7 shows the reaction time of direct fluorination with chlorine as a leaving group in dried ACN. The graph shows the results from a single experiment, but similar results were observed for every reaction. The peak height was determined by the UPLC-peak height and showed that the reaction peaked at 60% product after 5 minutes and stayed consistently at that level. After 5 minutes, there was no more starting material left, indicating 40% other reaction products. In addition to the product peak there were four other peaks: One larger peak found to be 18% of the total area and the other three around ∼5%. The fractions were not worked up and analysed, but it is known that TBAF is a weak base and it is speculated that the acidic α-protons leaves, leading to an elimination of the chloride resulting in an olefin as the one shown in Figure 6.

It was estimated that the shorter chained molecules would have a faster reaction time than the longer carbon chained. The reactions were monitored using HRMS, even though not quantifiable the peak height can give an indication on relative abundance. The MS results confirmed that reaction time was somewhat slower for the longer chained compounds, but all reactions were completed within 30 minutes at 160°C in the microwave. As can be seen in Figure 7 the graph shows that the longer chained Compound 75 had 10% starting material left after 5 minutes, but never went down to 0%. The UPLC-spectra was difficult to analyse due to the presence of other peaks with similar retention times which could contribute to the peak height leading to an overestimation of starting material left.

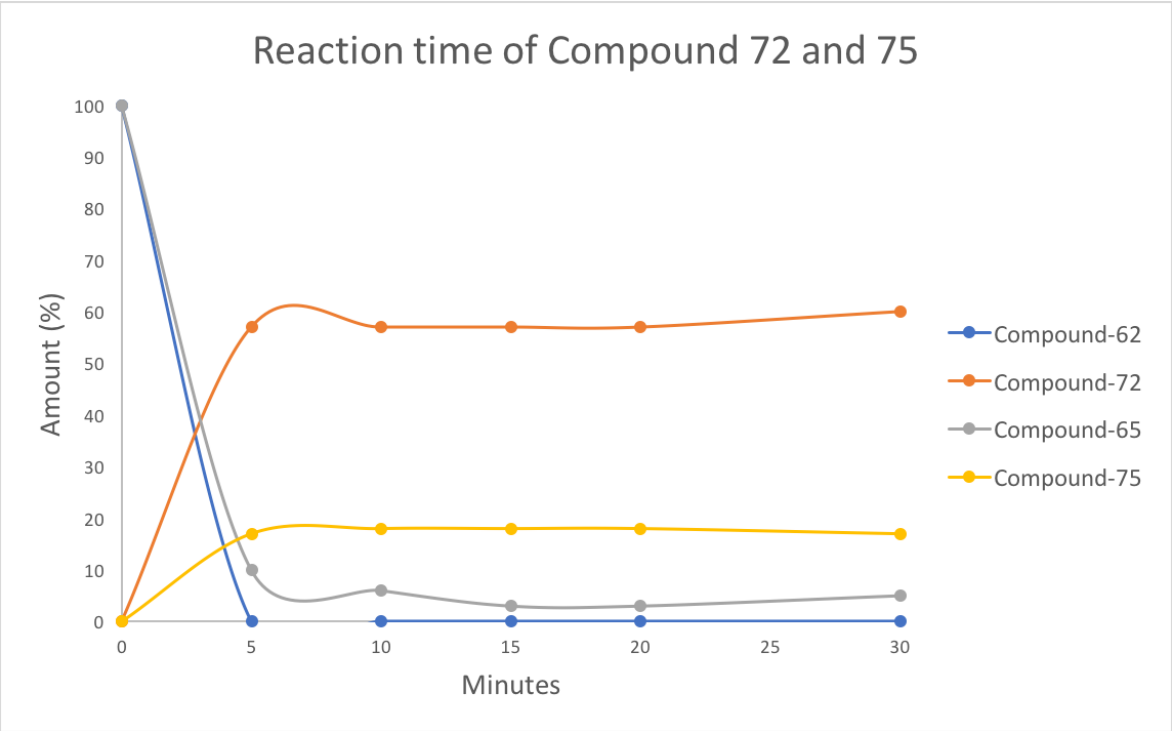


Figure 7: The reaction time of the two compounds, 72 and 75. Direct fluorination from chloride monitored by UPLC at 0, 5, 10, 15, 20 and 30 minutes at 160°C in the microwave

The fluorinated compounds were purified by first extracting the reaction mixture with an organic solvent and washed with distilled water. The organic phase were dried and the product was further purified using C18 Biotage flashchromatography columns. The fractions were collected based on HRMS results and lyophilized to obtain the pure product used in the serum analysis.

The reaction time using microwave-assisted reaction are satisfactory with reaction times not exceeding 30 minutes. The main issue found in this experiment is the by-product formation and is considerably higher than Kim et al. [48] which reported 60% yield from a compound comparable to that of Compound 75 which yielded 20% when using ACN.

The by-product formation can arise from a multitude of factors such as leaving group, solvents used and fluorine source It would be interesting to compare the fluorination efficacy with other leaving groups such as tosylates or mesylates which could increase yields as shown by Pilcher and colleagues [56]. Pilcher also compared two different fluorine sources: TBAF and TBAT (tetrabutylammonium (triphenyl- silyl)difluorosilicate). TBAF is considered having a greater nucleophilicity than TBAT, but TBAT is less basic. This means that the reaction time is expected to be longer, but the by-product formation should be less. In this experiment, where the reaction times are low, a slightly longer reaction time with less by-product formation might be beneficial.

Even though polar aprotic solvents generally are considered good solvents for nucleophilic halide exchange reactions, there has been an increased interest of using sterically hindered protic solvents. Kim et. al[48] compared the yields and reaction time in a nucleophilic fluorination using a sterically hindered protic solvents such as tert-amyl- or tert-butyl alcohol and a polar aprotic solvent such as ACN. In this comparison experiment in the synthesis of Compound 76, the yields obtained were 31% using tert-butanol and 18% using ACN as solvent. Even though the results presented in this thesis does not reach the same yields as Kim et. al. reported (95% in tert-butanol and 59% in ACN), the trends are similar. Using HRMS during this reaction it was apparent that the reaction time seemed longer in tert-butanol than ACN, but both reactions

were completed at 30 minutes. This indicates that higher yields than presented in the other fluorination could be obtained using tert-butanol. The other fluorination reactions were not performed in tert-butanol because ACN is the solvent traditionally used in a PET-centre and the method presented should resemble these conditions.

The fluorination method presented above showed adequate yield for three of the four attempted fluorination. Compound 72, 75 and 76 were successfully synthesised and purified.

3.4 Human serum stability

The identification and optimisation of characteristics and properties of a compound are important in drug development. A good lead candidate must possess good binding, potency, low toxicity and stability. The stability analysis compared the three fluorinated compounds synthesised in this thesis with a iodoacetyl ketone analogue previously synthesised by Fjellaksel et. al. [57] (Compound 82) shown in Figure 8.

Fluorinated products are generally thought of as more stable than the other halides (Cl, Br, I as the C-F-bond is a short bond ($\sim$1.47 Å) and one of the strongest bonds in organic chemistry. [47]

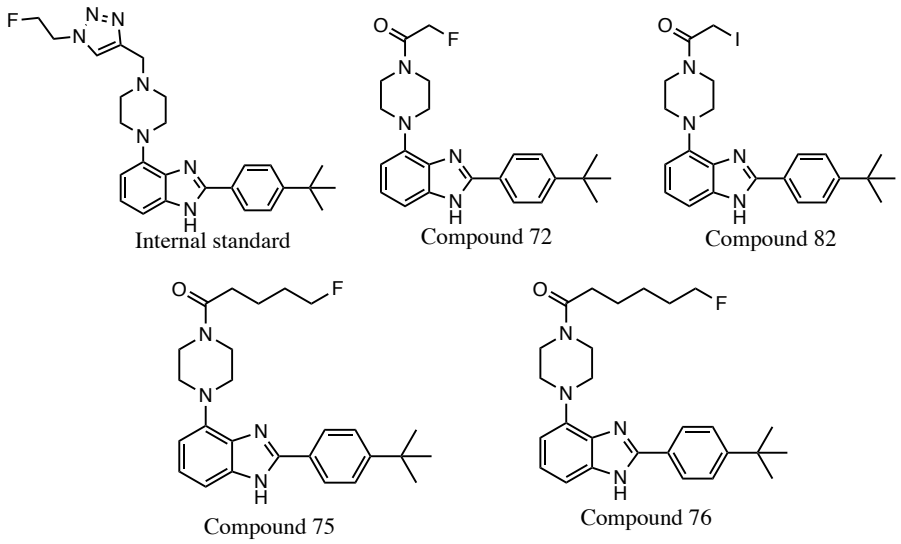


Figure 8: The compounds used in the serum stability study and the internal standard.

The stability testing was done by dissolving the compound in 5% ethanol, 5% polysorbate 80 and 90% water to make a 4μM solution. 50μl solution was added to 400μl serum and aliquots of 50μl were taken at timepoints 0, 10, 30, 60, 120, 240 and 1320 (22h) minutes. The aliquots were quenched with ice cold ACN and centrifuged at 13000 rpm for five minutes. 50μl of the supernatant was transferred to HPLC-vial and 950μl internal standard (IS) solution was added. The samples where then frozen until it were analysed on HPLC-MS. The serum experiments were performed in triplicates.

The IS was added after the work-up and primarily served the purpose of reducing instrument variations and not variations in the method. A new batch of IS was made before each stability

test. The IS chosen was a compound previously synthesised in [55] and was used because it had a mass similar to our compounds. The IS's stability in water was tested to ensure that it would not influence the measured stability. This was tested by freezing two HPLC-vials with a $85\mu M$ solution and keeping two HPLC-vials with the same solution at room temperature for 24 hours. Analysis showed that 95% of the IS was left after 24 hours.

The results presented in Figure 9 are ratios of the compound peak compared to the IS. To ease the comparison, the results are presented as relative ratios where all the samples are compared to $t = 0\ min$. This ensures that all the lines start at the same point.

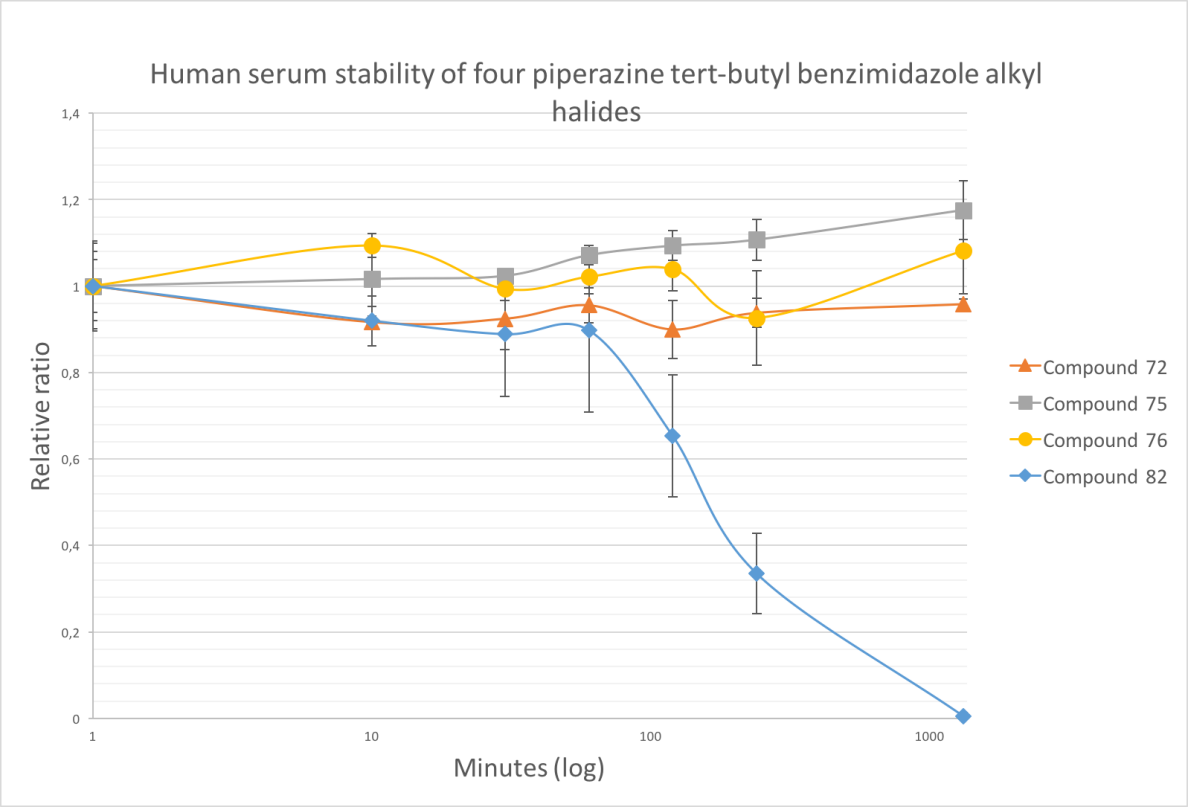


Figure 9: The human serum stability of compounds 72, 75, 76 and 82. The graph show that the fluorinated compounds 72, 75 and 76 shows good stability in human serum compared to the iodinated short-chained compound 82.

Figure 9 shows the stability of **Compound 72, 75, 76** and **82** in human serum. The figure shows that all the fluorinated compounds 72, 75 and 76 are more stable than the iodinated compound 82. The fluorinated compounds all show that more than 90% of the product remains after 22 hours. There are some fluctuation in the graphs, but common for the fluorinated compounds is a increase towards the end. The IS stability experiment showed that 95% of the IS was left after 24 hours. Since the graph is presented as a ratio between compound peak height and IS peak height, this could overestimate the amount. To minimise the effect the IS stability would have, a fresh batch was made before each experiment so that each compound could be compared.

The figure confirms the assumptions that the C-F bond is more stable than C-I. It has previously been known that α-halides next the electron withdrawing groups such as amides and other carbonyls can exhibit alkylating mechanisms. These are therefore not considered good drug candidates as alkylating agents can be cancerous through the alkylation and modification of DNA. Since the main stability issue is thought to be the elimination of the halide, the serum stability can give an indication of alkylation effect. Based on these stability data, the fluorides should not possess this alkylation effect.

The iodinated compound 82 showed greater stability in this study compared to previously reported data [46], but the trends are similar. Previously reported stability for compound 82 showed that 50% remained after 1 hour and 23% after 2 hours [46].

In the development of the MS method there were some carry-over of the previous samples. After changing the needle wash to a stronger solution containing more ACN this effect was minimal and should not affect the results.

The IS had a retention time similar to a lot of signals from the serum and some of the variation in results can be explained by this. A suitable IS added at the same time as the compound could eliminate method variations such as pipette volume variations and condensation on the eppendorf test tubes and subsequently reducing the amount of variation.

The detector used was an orbitrap MS and ion suppression due to several other ions can make the results vary. MS results can be quantifiable if it's calibrated with a standard solution. Before the samples were analysed, standards with known concentrations were made so that we could quantitatively compare the concentration of compound in each sample. When performing the analysis of the samples, substantial disturbances from the serum were observed in the same retention time as the IS. The disturbances can the suppress the signal from the IS making it lower than expected. Because the signal suppression would lead to a reduction in IS peak height, the compound concentration would be overestimated in the serum samples if it was compared to the standard without serum. Under the presumption that the serum LC-MS peaks did not vary substantially between each sample, the IS peak could still be used to correct for instrument variation. None of the compounds peaks had the same retention time as disturbances from the serum and should not be affected by the ion suppression effect.

In the analysis of Compound 82, one of the three parallels was excluded as an outlier as the concentration measured was only 15% of the other two parallels. This was due to too little solution prepared making it difficult to measure out the correct amount before adding it to the serum. The other two parallels were consistent in the concentration declined, but only having two parallels explains the wider standard deviation.

4 Conclusions

In this thesis, we presented the synthesis of three fluorinated compounds, adding to the existing library of small-molecule piperazine tert-butyl benzimidazoles.

We synthesised the intermediate Compound 5 in 36% yield, which is comparable to previous studies. The four chloroalkyl analogues were successfully synthesised with yields ranging from 28 to 65%.

One of the most important contributions of this thesis was to develop a fluorination method for ^{19}F fluorination with potential for ^{18}F radiolabelling. The direct halide exchange method proved useful and gave adequate yields in the fluoro-ethanone (Compound 72), -pentanone (Compound 75) and -hexanone (Compound 76) using microwave. No desired product was formed in the attempted fluorination of the chloro-propanone analogue (Compound 63). The fluorination method had good reaction times, all reaction done within 30 minutes. The formation of by-products should be further improved.

In the last part of this thesis we presented the human serum stability of the three fluorinated compounds (Compound 72, 75 and 76). The study showed good serum stability after 22 hours proving that these alkyl fluorides are suitable for further *in vivo* and *in vitro* experiments.

5 Future work

This thesis presented the ^{19}F fluorination with low reaction times and usable yields. The synthesis could be improved to increase yields and some methods has been discussed. Even though ^{18}F and ^{19}F has mostly the same chemical properties, reaction conditions is expected to be different in a PET-environment. A cryptand such as Kryptofix and TBAF are expected to exert different fluorination selectivity and future experiments with radiolabelling should expect some method development.

None of the analogues presented has been tested for binding affinities against the GnRH-R, which is essential before future development. Should the compounds show satisfactory binding, future *in vitro* and *in vivo* studies such as toxicological testing, biodistribution and preclinical PET can be performed.

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6 Experimental

General:
All chemicals were bought from Sigma-Aldrich. Silica gel 60 TLC plates with fluroscence indicator (F254) from Merck were used.

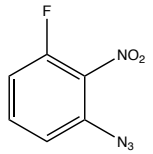
Instrumentation:
Microwave: Anton Parr Monowave 300®. Biotage SP1 systems®with 12g Snap-Ultra C18 columns.
HPLC system: Thermo scientific Accela with Accela pump, sample manager and autosampler with Ascentis ®Express C18 2,7 μm, 2,1 mm x 5 cm column.
Prep-HPLC system: Waters 2545 HPLC pump. Waters 2998 PDA detector, 200-500nm. Waters 2767 sample manager. XBridge®prep C18 5μm OBDtm 19x250mm column.
HRMS: Thermo scientific LTQ Orbitrap XL + Electrospray ion source (ION-MAX).
NMR: 400 MHz Bruker Avance III HD equipped with a 5 mm SmartProbe BB/1H (BB=19F, 31P-15N).
UPLC system: Acquity UPLC H class. Acquity column manager. Acquity PDA detector. Acquity UPLC ®BEH C18 1,7μ m. 2,1 mm x 5 cm column.
Flash chromatography: Davisil 20 - 45 μm silica.

Determination of purity by UPLC:
Column at 50°C, the same method used for all analysis. Method: 0,6ml/min. Initial 95% H_2O 0,1% TFA(Trifluoroacetic acid), 5% ACN(Acetonitrile) 0,1% TFA then a linear change during 10 minutes to 5% H_2O 0,1% TFA, 95% Acetonitrile 0,1% TFA.

Method for Bitoage purification
Method A: Initial 95% H_2O and 5% ACN then a linear change during 20 minutes to 5% H_2O and 95% ACN

Method for prep-HPLC purification
Method A: 25 ml/min. Initial 95% H2O 0,1% TFA, 5% ACN 0,1% TFA then linear change during 15 minutes to 5% H2O 0,1% TFA, 95% ACN 0,1% TFA.

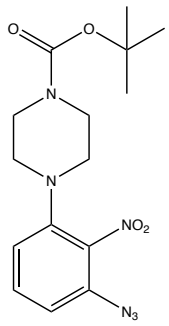
6.1 1-azido-3-fluoro-2-nitrobenzene Compound 1



21,9 mmol NaN_3 was added to a solution containing 18 mmol 2,6-difluoronitrobenzene and 12 ml DMSO. The solution was stirred for 22 hours. The reaction was monitored using TLC. After 22 hours the reaction mixture was extracted with 250 ml EtOAc and washed with 3 × 250 ml distilled water. The organic phase was dried using Na_2SO_4 and the solvents removed by rotavapor. The product is a yellow powder. (3,2 g, 17,6 mmol, yield = 98%)

^{1}**H-NMR**: (400 MHz $CDCl_3$) δ 7,53-7,47 (m, 1H), 7,11-7,08 (m, 1H), 7,05-7,01 (m, 1H)

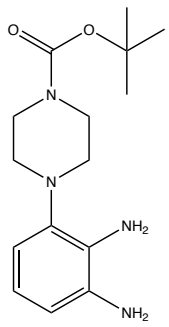
6.2 tert-butyl 4-(3-azido-2-nitrophenyl) piperazine-1-carboxylate Compound 2



18,7 mmol 1-azide-3-fluoro-2-nitrobenzene was dissolved in 25 ml DMSO and 3,6 ml DIPEA was added to the solution. The solution was heated to 60°C and 3,7g 1-boc-piperazine was added to the solution and stirred for 22 hours. The reaction was monitored using TLC. The reaction was extracted using EtOAc and washed with distilled water two times. The water phase was washed with EtOAc. The organic phase was dried using Na_2SO_4 and the solvents removed using rotavapor. The product was dissolved in DCM and dry mounted on silica and purified on flashchromatography column. Mobile phase 15:85 EtOAc : pentane The fractions were collected and the solvents removed using rotavapor to obtain a yellow powder. (3238mg, yield = 50%)

^{1}**H-NMR**: (400 MHz $CDCl_3$) δ 7,43 (t, 1H), 7,0 (d, 1H), 6,95 (d, 1H), 3,5 (m, 4H), 2,92 (m, 4H), 1,46 (s, 9H)

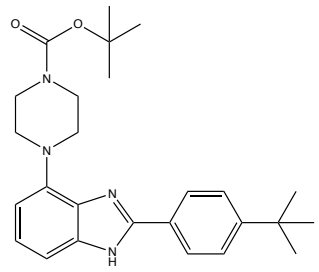
6.3 tert-butyl 4-(2,3-diaminophenyl) piperazine-1-carboxylate Compound 3



2,92 mmol dissolved in 10 ml MeOH and added to bomb containing Pd/C and 10 ml MeOH. Total MeOH amount: 20 ml. Hydrogenation was performed at 150 psi for 3 hours. The reaction mixture was filtered through celite and the solvents removed using rotavapor to provide a brown powder. (865,9 mg, yield = 101%)

^{1}**H-NMR**: (400 MHz $CDCl_3$) δ 6,67 (t, 1H), 6,58 (dd, 1H), 6,54 (dd, 1H), 3,9-3,2 (broad, 8H), 2,83 (s, 4H), 1,47 (s, 9H)

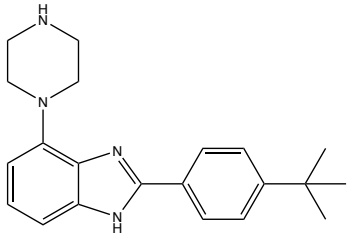
6.4 tert-butyl 4-(2-(4-(tert-butyl)phenyl) -1H-benzo[d]imidazol-4-yl) piperazine-1-carboxylate Compound 4



Two parallels were done simultaneously. 1,0 mmol tert-butyl 4-(2,3-diaminophenyl)piperazine-1-carboxylate was added to a solution containing 10 ml abs EtOH and 1 mmol tert-butylbenzaldehyde. The reaction was stirred and refluxed at 80°C for 24 hours. The reaction was monitored using TLC. The solvents were removed using rotavapor. The product was dry mounted on celite and chromatographed using flashchromatography. Mobile phase 40:60 EtOAc : pentane. The fractions were collected and the solvents evaporated using rotavapor to provide a white powder (699,7 mg, yield 81%)

HRMS-ESI+: m/z found: 435,2754 calculated: 435,2755 ^{1}**H-NMR**: (400 MHz $CDCl_3$) δ 7,98 (d, 2H), 7,49 (m, 2H), 7,16 (bs, 2H), 6,68 (bs, 1H), 3,71 (m, 4H), 3,6-3,4 (bs, 4H), 1,5 (s, 9H), 1,35 (s, 9H)

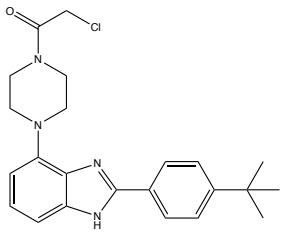
6.5 2-(4-(tert-butyl)phenyl)-4-(piperazin-1-yl)-1H-benzo[d]imidazole Compund 5



1,81 mmol tert-butyl 4-(2-(4-(tert-butyl)phenyl) -1H-benzo[d]imidazol-4-yl) piperazine-1-carboxylate was dissolved in 30 ml 4 M HCl/Dioxane and stirred for 2 hours. The reaction mixture was extracted using distilled water and washed with 150 ml EtOAc three times. The waterphase was lyophilised to provide an orange powder (602,9 mg, yield = 90%)

HRMS-ESI+: m/z found: 335,2231 calculated: 335,2230 ^{1}**H-NMR**: (400 MHz CD_3OD) δ 8,06, (d, 2H), 7,65 (d, 2H), 7,40 (m, 2H), 7,09, (d, 1H), 3,49 (m, 4H), 3,41 (m, 4H), 1,35 (s, 9H)

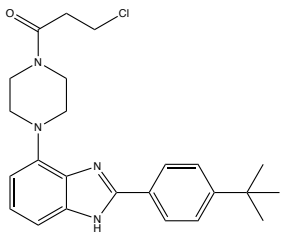
6.6 1-(4-(2-(4-(tert-butyl)phenyl)-1H-benzo[d]imidazol-4-yl)piperazin-1-yl)-2-chloroethan-1-one Compound 62



0,27 mmol 2-(4-(tert-butyl)phenyl)-4-(piperazin-1-yl)-1H-benzo[d]imidazole was dissolved in 10 ml DMF. 0,16 mmol DIPEA was added to the solution and the RBF put in an icebath (0°C). 0,07 ml chloroacetyl chloride was added to the solution and stirred for 1 hour. The reaction was monitored using TLC. The reaction mixture was extracted using distilled water and washed two times with 25 ml DCM. The organic phase was dried using NA_2SO_4 and the solvents evaporated using rotavapor. The product was dissolved in DCM and applied to Biotage precolumn and chromatographed using Method A. The fraction were collected and lyophilised to obtain a white powder (43,7 mg, yield = 39%)

HRMS-ESI+: m/z found:411,1958 calculated: 411,1973 ^{1}**H-NMR**: (400 MHz CD_3OD) δ 8,05 (d, 2H), 7,59 (d, 2H), 7,20 (m, 2H), 6,76 (d, 1H), 4,34 (s, 2H), 3,92-3,86 (m, 4H), 3,44 (m, 2H), 3,36 (m, 2H), 1,38 (s, 9H)

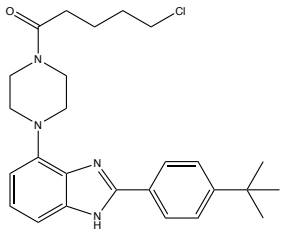
6.7 1-(4-(2-(4-(tert-butyl)phenyl)-1H-benzo[d]imidazol-4-yl)piperazin-1-yl)-3-chloropropan-1-one Compound 63



0,198 mmmol 2-(4-(tert-butyl)phenyl)-4-(piperazin-1-yl)-1H-benzo[d]imidazole was dissolved in 2,5 ml DMF. 0,11 ml DIPEA was added to the solution and the RBF put in to an icebath (0 °C). 0,06 ml 3-chloropropinoyl chloride was added to the solution and the solution was stirred for 3 hours. The reaction mixture was extracted using 25 ml distilled water and washed with 30 ml DCM three times. The organic phase was washed using 20 ml brine. The solvents were removed using rotavapor. The product was dissolved in DCM and added to a Biotage precolumn and chromatographed using Method A. The fractions were collected and lyophilised to provide a white powder. (30,9 mg, yield = 37%)

HRMS-ESI+: m/z found: 425,2100 calculated: 425,2103 ^{1}**H-NMR**: (400 MHz $CDCl_3$) δ 8,0 (d, 2H), 7,56 (d, 2H), 7,30 (bs, 2H), 6,65 (bs, 1H), 3,93 (m, 2H), 3,78 (m, 2H), 3,60-3-52 (bm, 6H), 2,43 (m, 2H), 1,9-1,78 (bs, 4H), 1,35 (s, 9H)

6.8 1-(4-(2-(4-(tert-butyl)phenyl)-1H-benzo[d]imidazol-4-yl)piperazin-1-yl)-5-chloropentan-1-one Compound 65

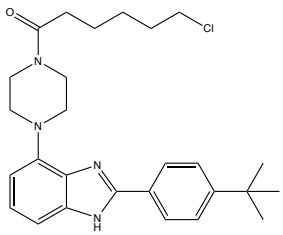


0,19 mmol 2-(4-(tert-butyl)phenyl)-4-(piperazin-1-yl)-1H-benzo[d]imidazole was dissolved in 3 ml DMF. 0,11 ml DIPEA was added to the solution and the RBF put in to an icebath (0 °C). 3 eq chlorovaleroyl chloride added to the solution and stirred for 23 hours. The reaction was monitored using TLC. The reaction mixture was extracted using 50 ml distilled water and washed three times with 50 ml DCM. The organic phase was dried using Na_2SO_4 and the solvents evaporated using rotavapor.

The product was dissolved in DCM and applied to Biotage precolumn and chromatographed using Method A. The fractions were collected and lyophilised to obtain a white powder. (24,3 mg, yield = 28%)

HRMS-ESI+: m/z found: 453,2416 calculated: 453,2416 ^{1}**H-NMR**: (400 MHz $CDCl_3$) δ

6.9 1-(4-(2-(4-(tert-butyl)phenyl)-1H-benzo[d]imidazol-4-yl)piperazin-1-yl)-6-chlorohexan-1-one Compound 66

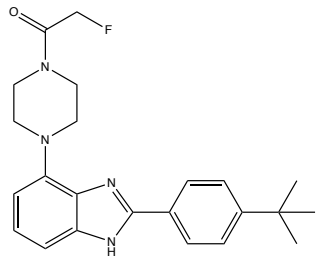


0,274 mmol 2-(4-(tert-butyl)phenyl)-4-(piperazin-1-yl)-1H-benzo[d]imidazole was dissolved in 5 ml DMF. 0,16 ml DIPEA was added to the solution and the RBF put in to an icebath (0 °C). 0,11 ml 6-chlorohexanoyl chloride was added to the solutino and the reaction mixture stirred for 24 hours. The reaction was monitored using TLC. The reaction mixture was extracted using 50 ml distilled water and washed three times with 25 ml DCM. The organic phase was dried using Na_2SO_4 and the solvents were evaporated using rotavapor.

The product was dissolved in DCM and applied to Biotage precolumn and chromatographed using Method A. The fractions were collected and lyophilised to obtain a white powder (83,7 mg, yield = 65%)

HRMS-ESI+: m/z found: 467,2472 calculated: 467,2572 ^{1}**H-NMR**: (400 MHz CD_3OD) δ 8,04 (d, 2H), 7,58 (d, 2H), 7,20-7,14 (m, 2H), 6,73 (bs, 1H), 3,87 (bd, 4H), 3,59 (m, 2H), 3,31 (s, 4H), 2,53-2,48 (bm, 4H), 1,85 (m, 2H), 1,68 (m, 2H), 1,54 (m, 2H), 1,38 (s, 9H)

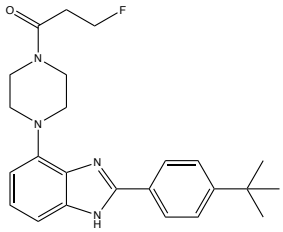
6.10 1-(4-(2-(4-(tert-butyl)phenyl)-1H-benzo[d]imidazol-4-yl)piperazin-1-yl)-2-fluoroethan-1-one (Compound 72



20,7 mg 1-(4-(2-(4-(tert-butyl)phenyl)-1H-benzo[d]imidazol-4-yl)piperazin-1-yl)-2-chloroethan-1-one was dissolved in 2 ml ACN in a microwave vial. 0,1 ml 1M TBAF in THF added to the solution. The reaction was performed in a microwave at 160 °C for 10 minutes. The reaction mixture was extracted using distilled water and washed with DCM two times. The organic phase was dried using Na_2SO_4 and the solvents removed using rotavapor. The product was dissolved in DCM and applied to Biotage precolumn on chromatographed using method A The fractions were collected and lyophilised to obtain a white powder. (6,0 mg, 30 %)

Purity: 95% **HRMS-ESI+:** m/z found: 395,2242 calculated: 395,2242 ^{1}**H-NMR**: (400 MHz $CDCl_3$) δ 7,97 (d, 2H), 7,51 (d, 2H), 7,15 (m, 2H), 6,64 (bs, 1H), 5,12 (s, 1H), 5,01 (s, 1H), 3,93 (bs, 2H), 3,72 (m, 2H), 3,67 (m, 2H), 3,54 (m, 2H), 1,36 (s, 9H)

6.11 1-(4-(2-(4-(tert-butyl)phenyl)-1H-benzo[d]imidazol-4-yl)piperazin-1-yl)-3-fluoropropan-1-one Compound 73

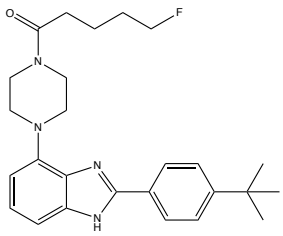


No desired product

0,047 mmol 1-(4-(2-(4-(tert-butyl)phenyl)-1H-benzo[d]imidazol-4-yl)piperazin-1-yl)-3-chloropropan-1-one dissolved in 2 ml ACN in a microwave vial. 0,1 ml 1M TBAF in THF added to the solution. The reaction was performed in a microwave at 160 °C for a total of 30 minutes. Samples were taken at 0, 5, 10, 15, 20 and 30 minutes. The reaction mixture was extracted using distilled water and washed with DCM two times. The organic phase was dried using Na_2SO_4 and the solvents removed using rotavapor. The product was dissolved in DCM and applied to a Biotage precolumn and chromatographed using Method A. No fraction contained the mass of desired product.

The fractions were collected and lyophilised to obtain a white powder 13,7 mg *This is of the elimination product*

6.12 1-(4-(2-(4-(tert-butyl)phenyl)-1H-benzo[d]imidazol-4-yl)piperazin-1-yl)-5-fluoropentan-1-one Compound 75

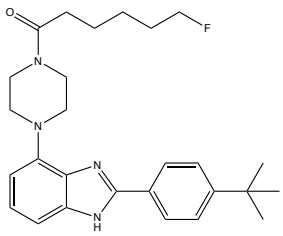


6.12.1 Experiment No. 1

0,045 mmol 1-(4-(2-(4-(tert-butyl)phenyl)-1H-benzo[d]imidazol-4-yl)piperazin-1-yl)-5-chloropentan-1-one dissolved in 2 ml ACN in a microwave vial. 0,09 ml 1M TBAF in THF added to the solution. The reaction was performed in a microwave at 160 °C for a total of 30 minutes. Samples were taken at 0, 5, 10, 15, 20 and 30 minutes. The reaction mixture was extracted using distilled water and washed with DCM 2 times. The organic phase was dried using Na_2SO_4 and the solvents removed using rotavapor. The product was dissolved in DCM and applied to a Biotage precolumn and chromatographed using Method A. The fractions were collected and lyphillized to obtain a white powder. (3,0 mg, 15%)

Purity: 96% **HRMS-ESI+:** m/z found: 437,2714 calculated: 437,2711 ^{1}**H-NMR**: (400 MHz $CDCl_3$) δ 8,15 (d, 2H), 7,36 (d, 2H), 7,28 (d, 1H), 7,17 (m, 1H), 6,73 (d, 1H), 4,11 (s, 2H), 3,91 (bs, 2H), 3,84 (bs, 2H), 3,38 (bs, 2H), 3,25 (bs, 2H), 1,23 (s, 9H)

6.13 1-(4-(2-(4-(tert-butyl)phenyl)-1H-benzo[d]imidazol-4-yl)piperazin-1-yl)-6-fluorohexan-1-oneCompound 76



6.13.1 Experiment No. 1

0,043 mmol 1-(4-(2-(4-(tert-butyl)phenyl)-1H-benzo[d]imidazol-4-yl)piperazin-1-yl)-6-chlorohexan-1-one dissolved in 2 ml tert-butanol. The reaction was performed in a microwave at 160 °C for 30 minutes. The reaction mixture was extracted using distilled water and washed with 20 ml diethyl ether two times. The organic phase was dried using Na_2SO_4 and the solvents evaporated using rotavapor. The product was run on prep-HPLC using Method A. The fractions were collected and lyophilised to obtain a white powder (6 mg, yield = 31%)

6.13.2 Experiment No. 2

0,042 mmol Rx66 dissolved in 2 ml ACN. The reaction was performed in a microwave at 160 °C for 30 minutes. The reaction mixture was extracted using distilled water and washed with 20 ml diethyl ether two times. The organic phase was dried using Na_2SO_4 and the solvents evaporated using rotavapor. The product was run on prep-HPLC using Method A. The fractions were collected and lyophilised to obtain a white powder (3,5 mg, yield = 18%)

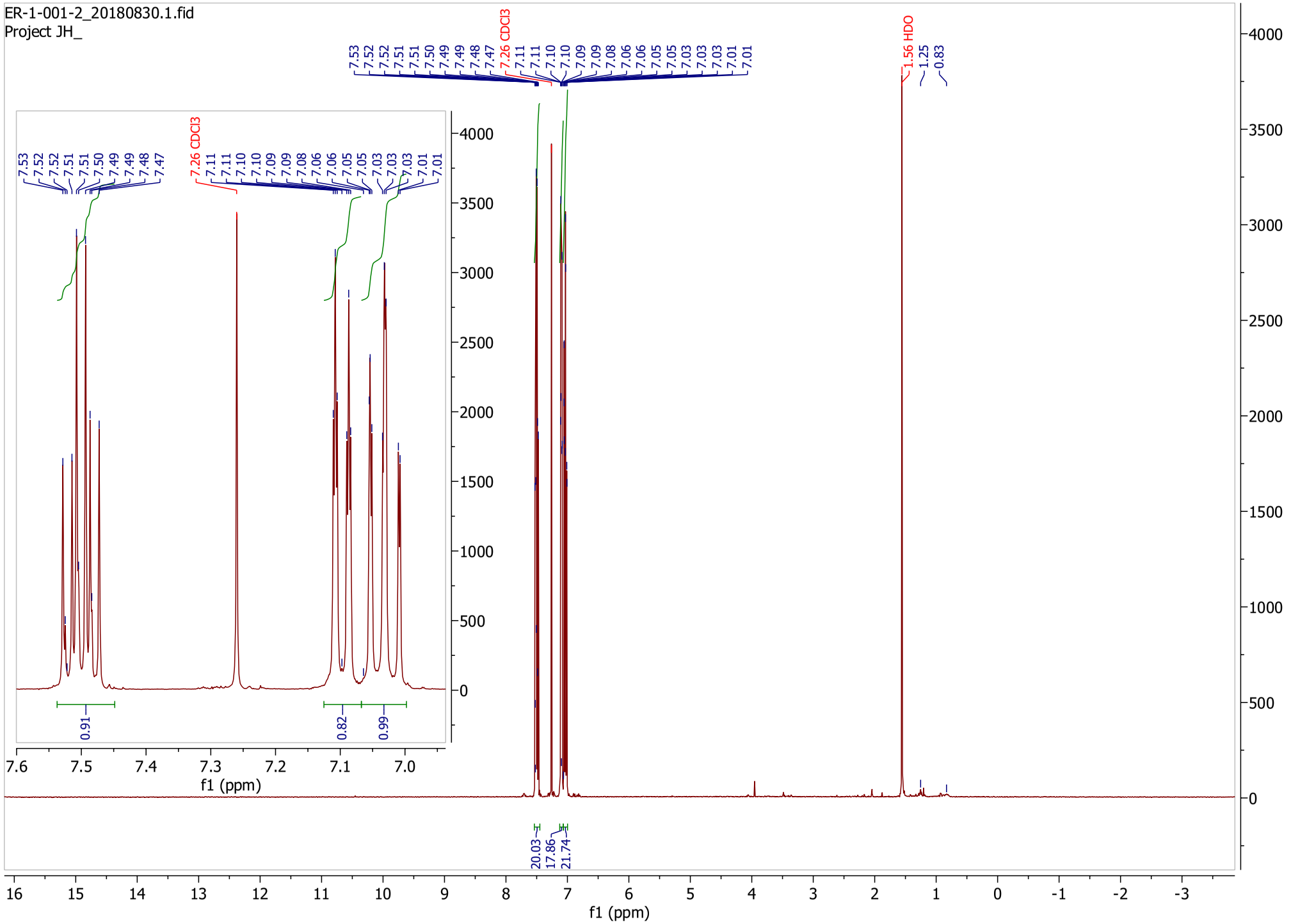
Purity: 95% **HRMS-ESI+:** m/z found: 451,2864 calculated: 451,2868 ^{1}**H-NMR**: (400 MHz $CDCl_3$) δ 8,07 (d, 2H), 7,43 (d, 2H), 7,19 (m, 2H), 6,70 (d, 1H), 5,10 (s, 1H), 4,98 (s, 1H), 3,93 (bs, 2H), 3,74 (bs, 2H), 3,51 (bs, 2H), 3,42 (bs, 2H), 1,29 (s, 9H)

6.14 Serum stability

The four compounds selected for serum stability was dissolved in a solutiion of 5% ethanol, 5% polysorbate 80 and 90% sterile water. The solutions were made to a concentration of 4 mM. The internal standard was diluted to a concentration of 85μl

50μl compound solution was added to an eppindorf vial containing 400μl human serum from a AB-male. The tubes were incubated at 37°C on a shaker. Aliquots of 50μl were taken at timepoints: 0, 10, 30, 60, 120, 240 and 1320 minutes. The samples were quenched using 50μl ice cold acetonitrile. The samples were sentrifuged at 13000 rpm for 5 minutes and 50μl of the supernatant was transferred to a HPLC-vial and diluted with the solution containing the internal standard.

The samples were frozen until analysed on HPLC-MS. The HPLC-MS results were analysed using SPSS, see syntax in Supplement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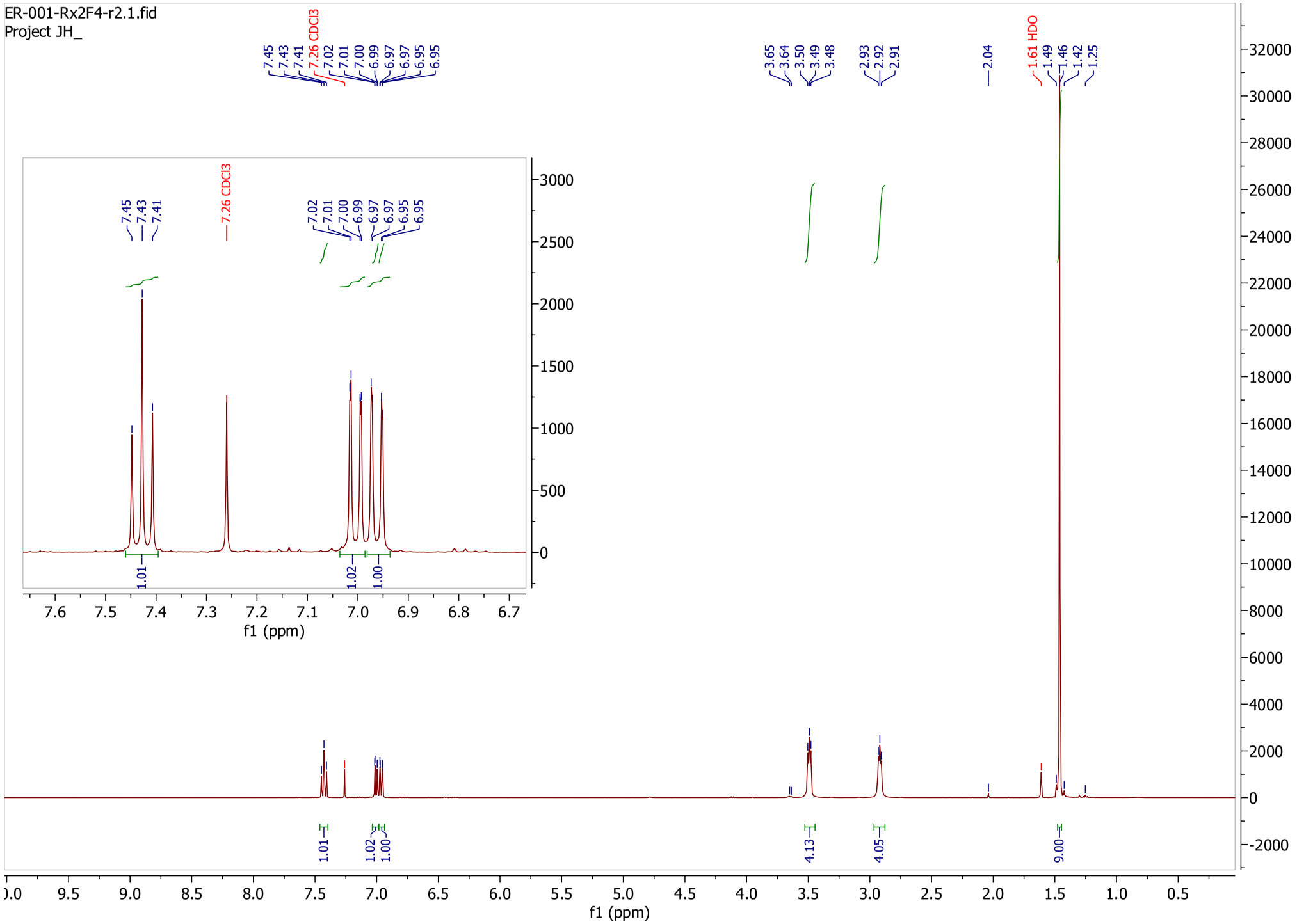
7.52
7.52
7.51
7.51
7.50
7.49
7.49
7.48
7.47
7.26 CDCl3
7.11
7.11
7.10
7.10
7.09
7.09
7.08
7.06
7.06
7.05
7.05
7.03
7.03
7.03
7.01
7.01
1.56 HDO
1.25
0.83
0.91
0.82
0.99
20.03
17.86
21.74
f1 (ppm)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7.41
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7.01
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6.97
6.97
6.95
6.95
3.65
3.64
3.50
3.49
3.48
2.93
2.92
2.91
2.04
1.61 HDO
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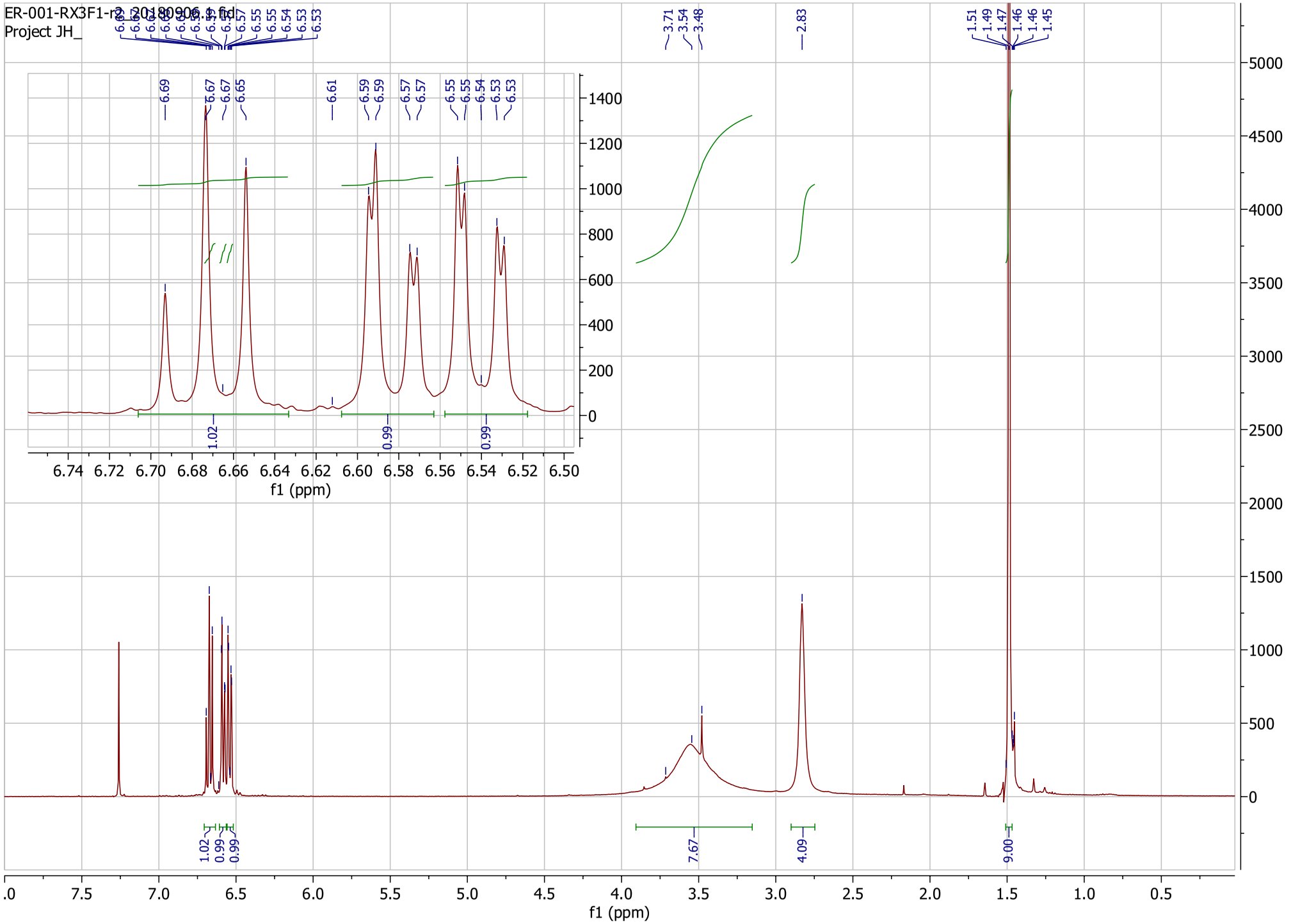
1.46
1.42
1.25
1.01
1.02
1.00
4.13
4.05
9.00
f1 (ppm)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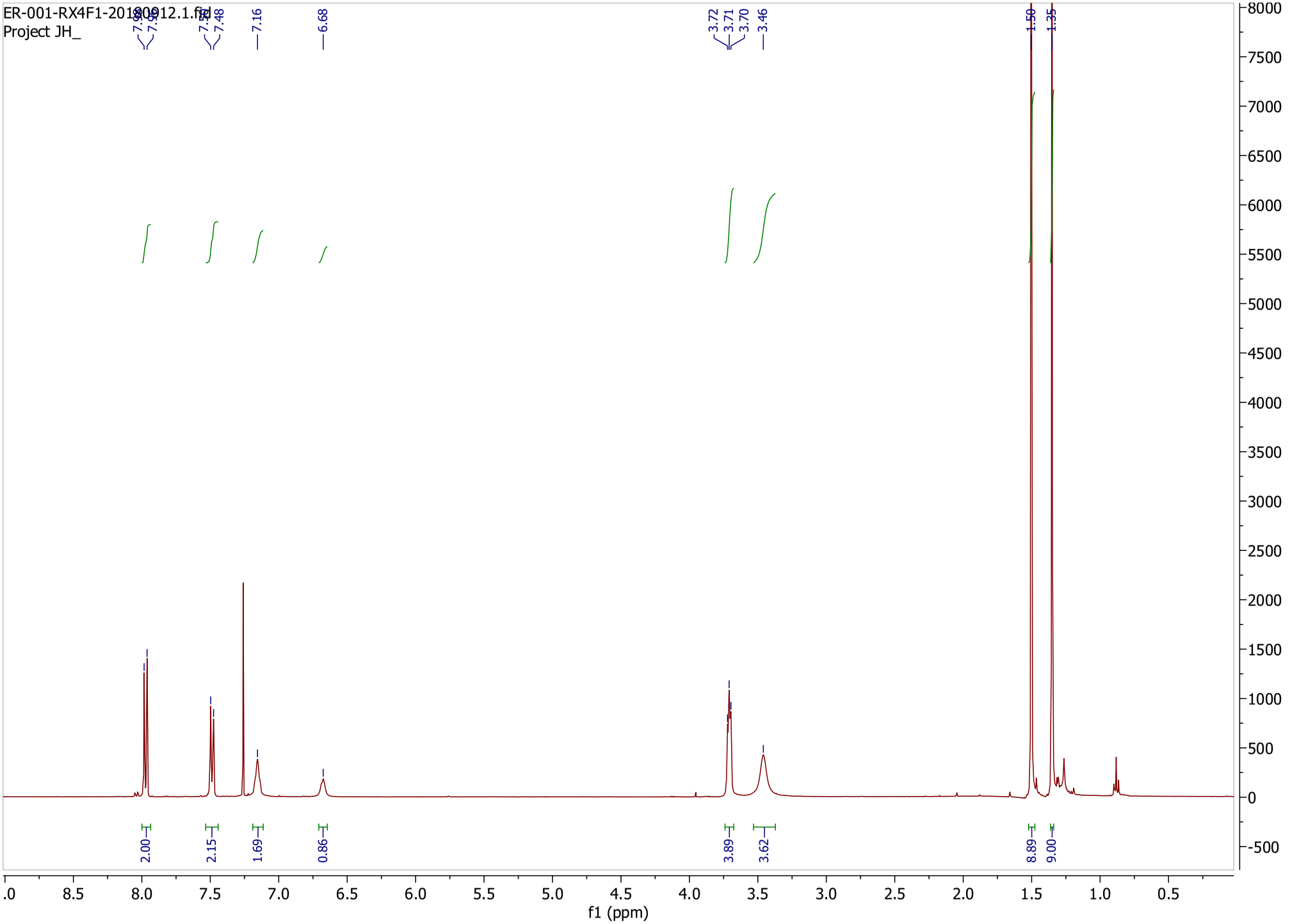
6.67
6.65
6.61
6.59
6.59
6.57
6.57
6.55
6.55
6.54
6.53
6.53
1.02
0.99
0.99
6.74 6.72 6.70 6.68 6.66 6.64 6.62 6.60 6.58 6.56 6.54 6.52 6.50
f1 (ppm)
3.71
3.54
3.48
2.83
1.51
1.49
1.47
1.46
1.46
1.45
1.02
0.99
0.99
7.67
4.09
9.00
7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f1 (ppm)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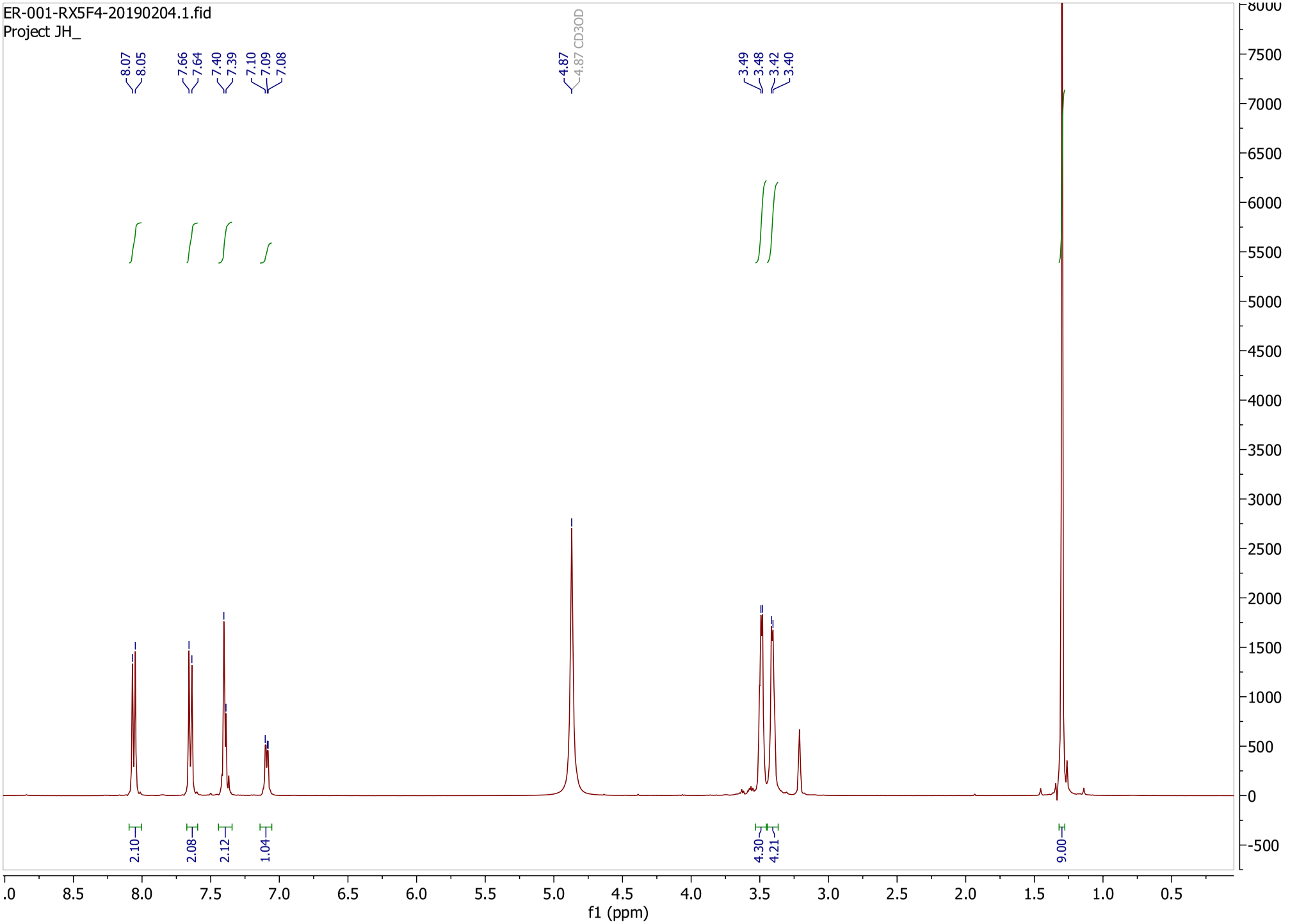
7.98
7.95
7.52
7.48
7.16
6.68
3.72
3.71
3.70
3.46
1.50
1.35
2.00
2.15
1.69
0.86
3.89
3.62
8.89
9.00
f1 (ppm)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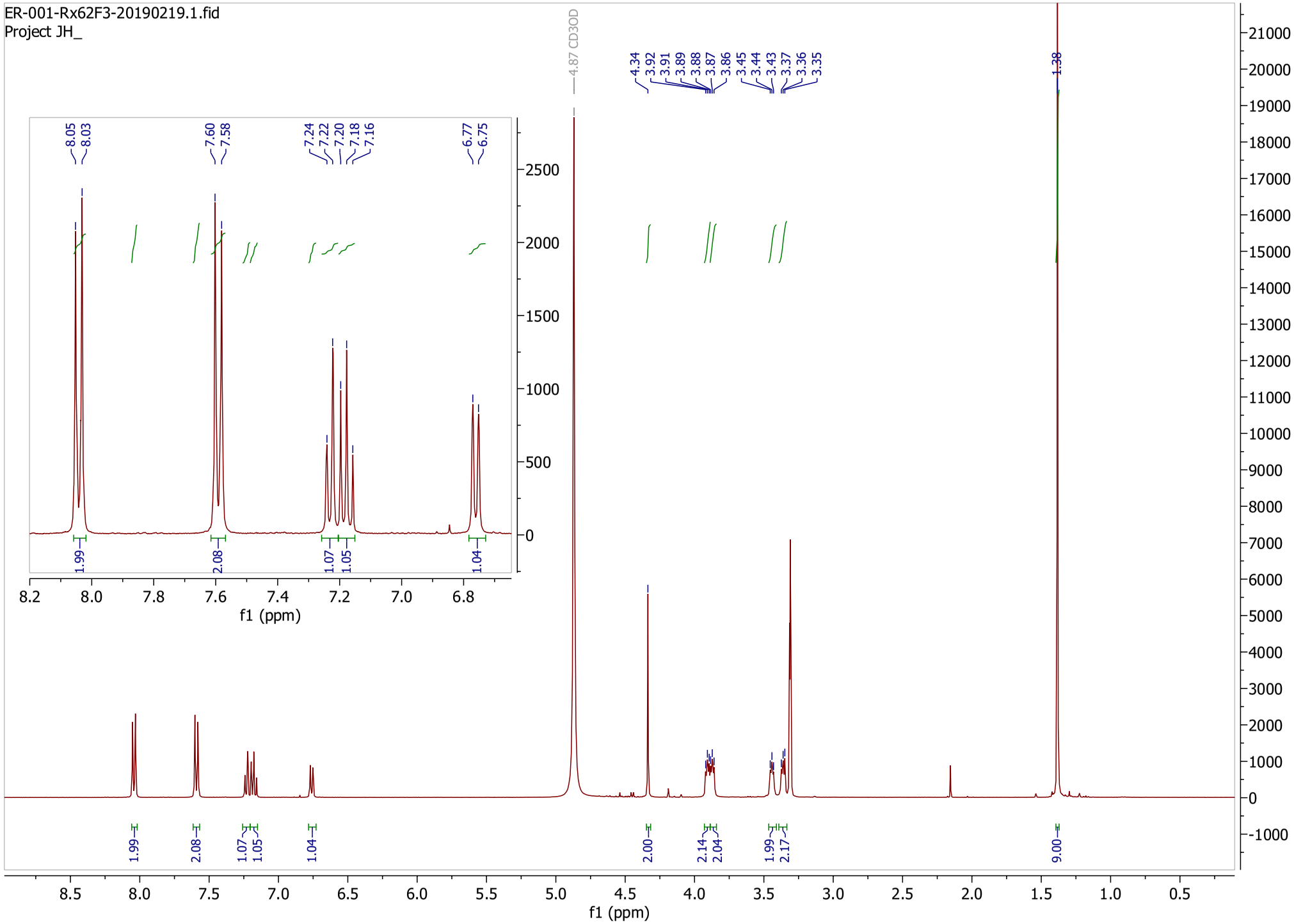
8.05
7.66
7.64
7.40
7.39
7.10
7.09
7.08
4.87
4.87 CD3OD
3.49
3.48
3.42
3.40
2.10
2.08
2.12
1.04
4.30
4.21
9.00
f1 (ppm)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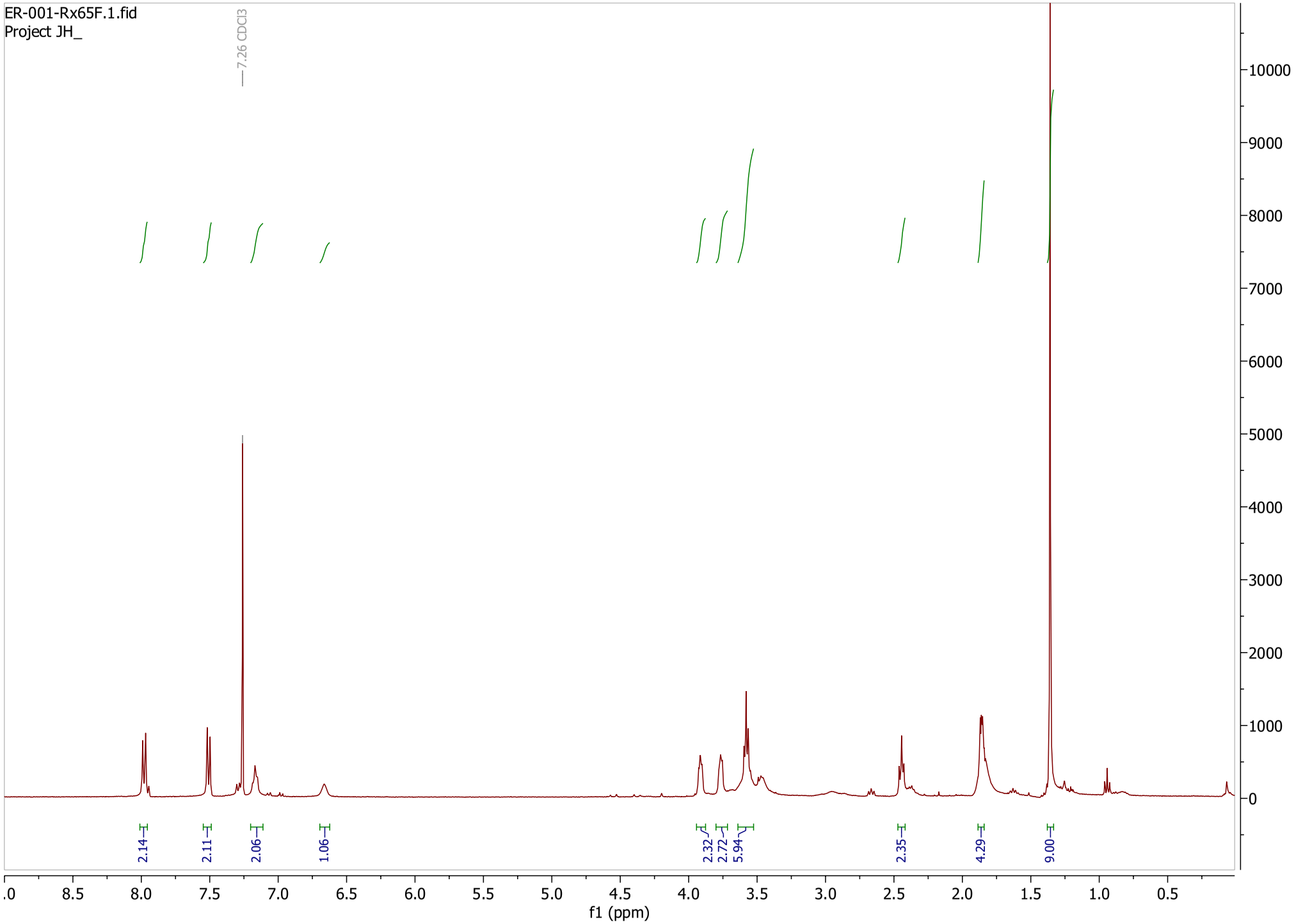
4.87 CD3OD
4.34
3.92
3.91
3.89
3.88
3.87
3.86
3.45
3.44
3.43
3.37
3.36
3.35
1.38
8.05
8.03
7.60
7.58
7.24
7.22
7.20
7.18
7.16
6.77
6.75
1.99
2.08
1.07
1.05
1.04
2.00
2.14
2.04
1.99
2.17
9.00
f1 (ppm)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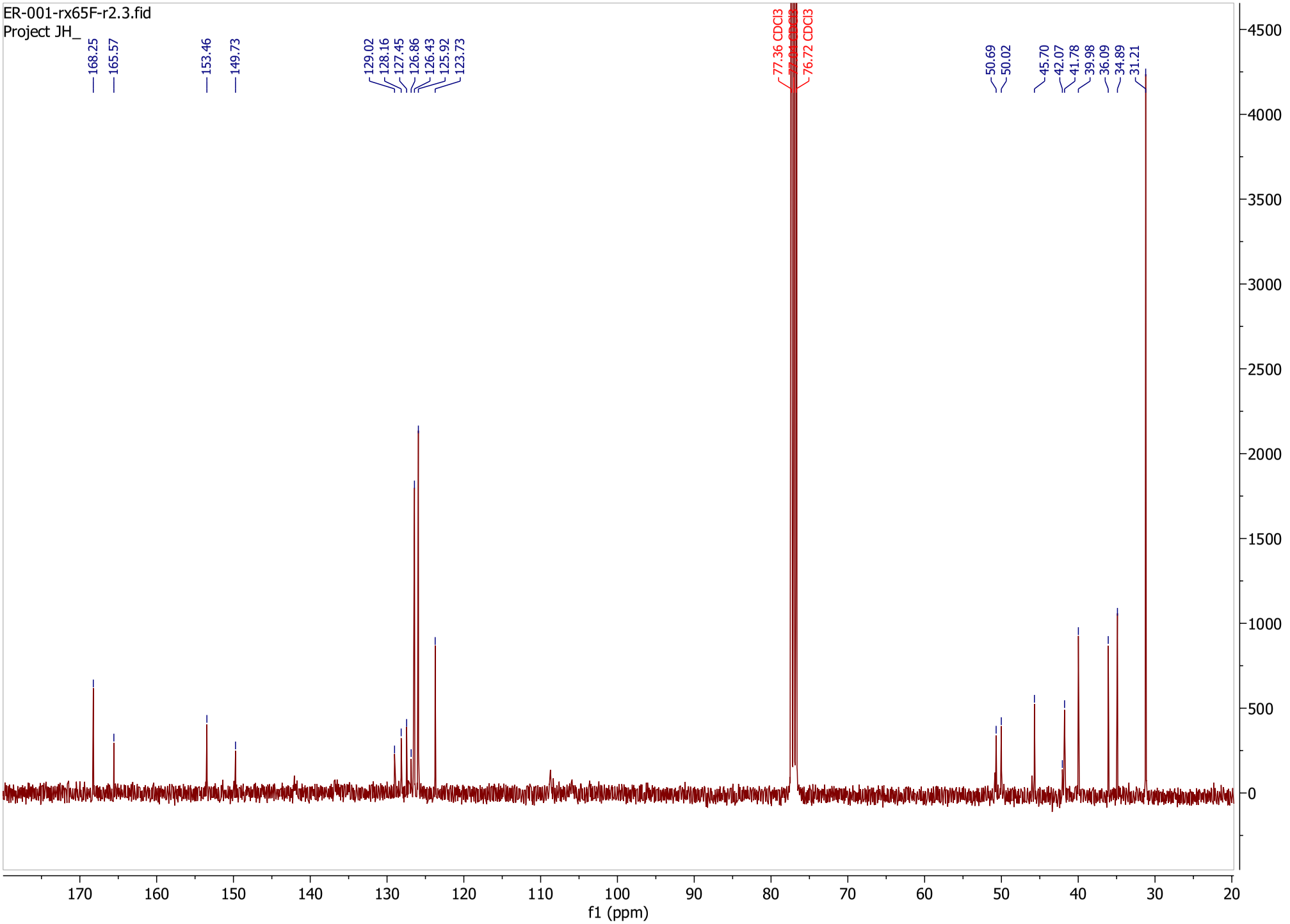
7.26 CDCl3
2.14
2.11
2.06
1.06
2.32
2.72
5.94
2.35
4.29
9.00
f1 (ppm)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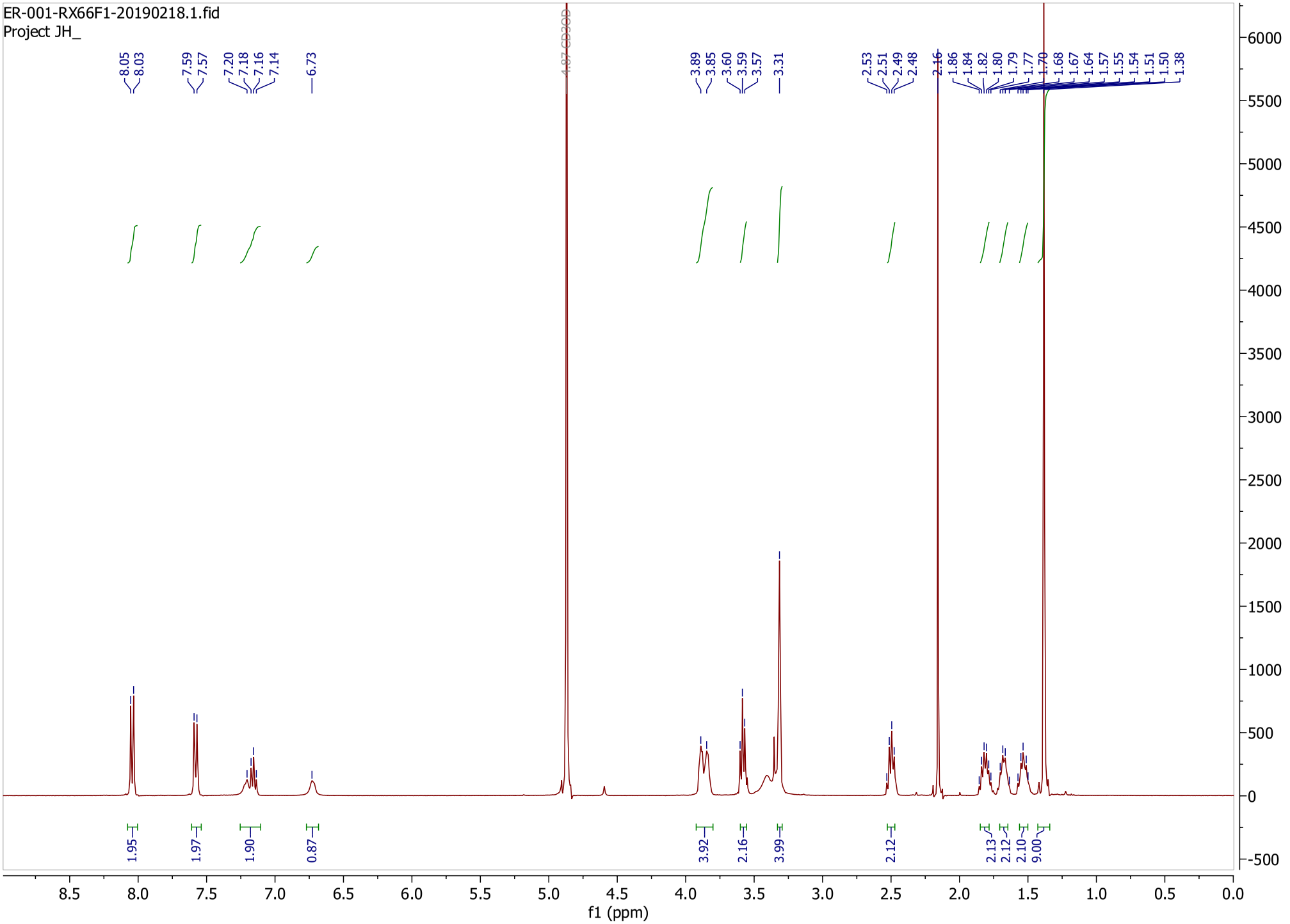
165.57
153.46
149.73
129.02
128.16
127.45
126.86
126.43
125.92
123.73
77.36 CDCl3
77.04 CDCl3
76.72 CDCl3
50.69
50.02
45.70
42.07
41.78
39.98
36.09
34.89
31.21
4500
4000
3500
3000
2500
2000
1500
1000
500
0
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
f1 (ppm)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Project JH_
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8.03
7.59
7.57
7.20
7.18
7.16
7.14
6.73
4.87 CD3OD
3.89
3.85
3.60
3.59
3.57
3.31
2.53
2.51
2.49
2.48
2.16
1.86
1.84
1.82
1.80
1.79
1.77
1.70
1.68
1.67
1.64
1.57
1.55
1.54
1.51
1.50
1.38
1.95
1.97
1.90
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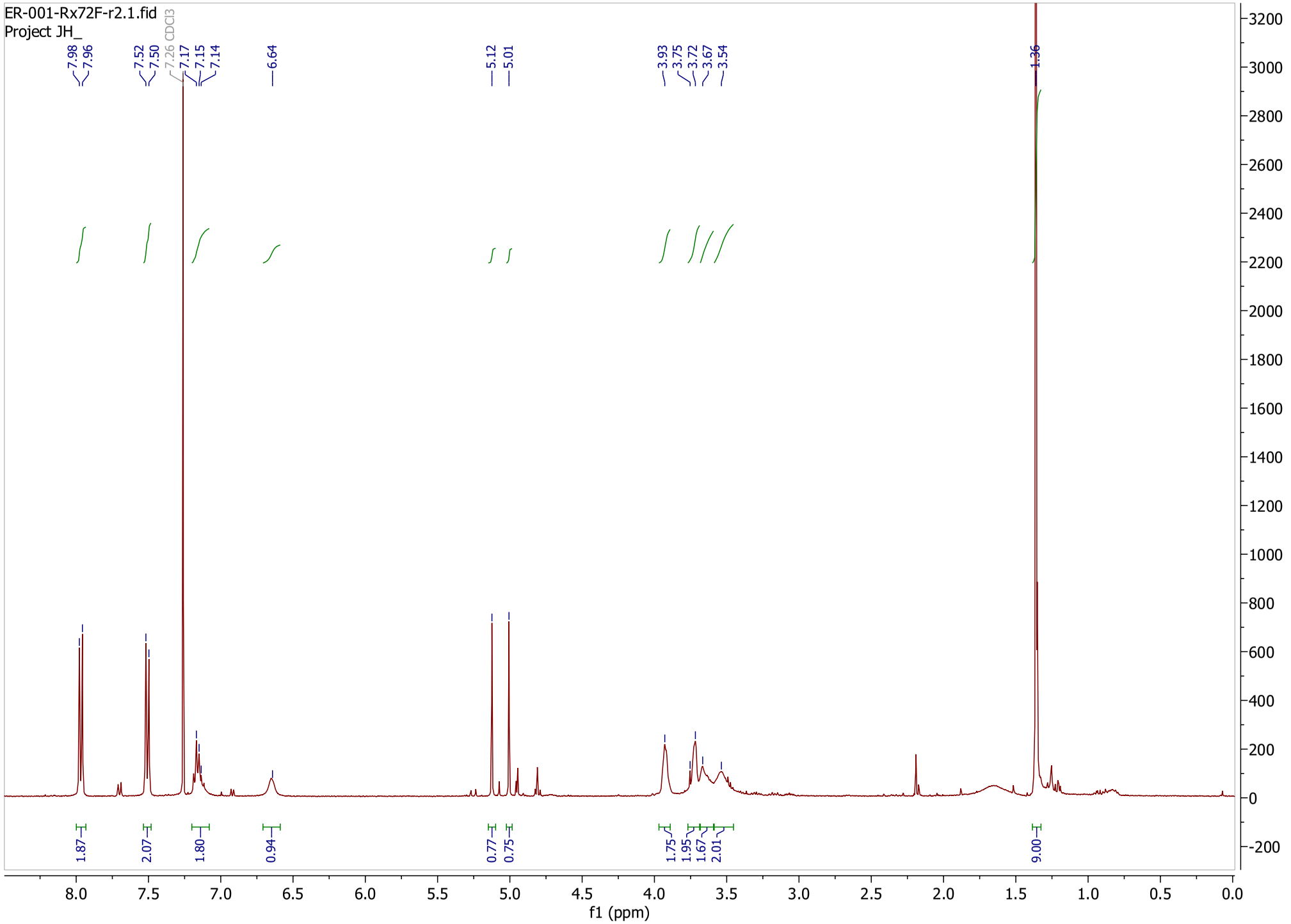
2.16
3.99
2.12
2.13
2.12
2.10
9.00
f1 (ppm)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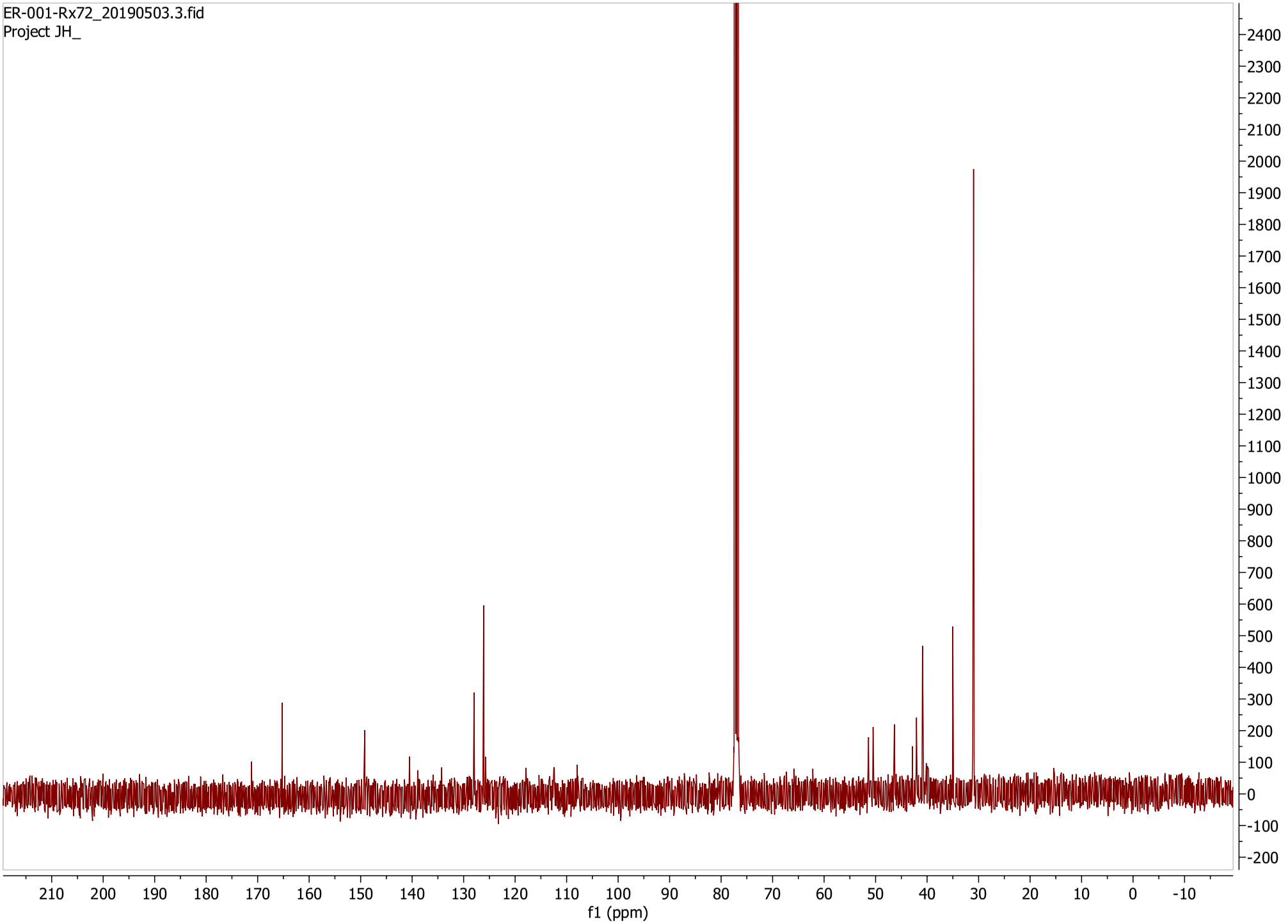
7.98
7.96
7.52
7.50
7.26 CDCl3
7.17
7.15
7.14
6.64
5.12
5.01
3.93
3.75
3.72
3.67
3.54
1.36
1.87
2.07
1.80
0.94
0.77
0.75
1.75
1.95
1.67
2.01
9.00
f1 (ppm)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2300
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2100
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1900
1800
1700
1600
1500
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1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
-100
-200
210
200
190
180
170
160
150
140
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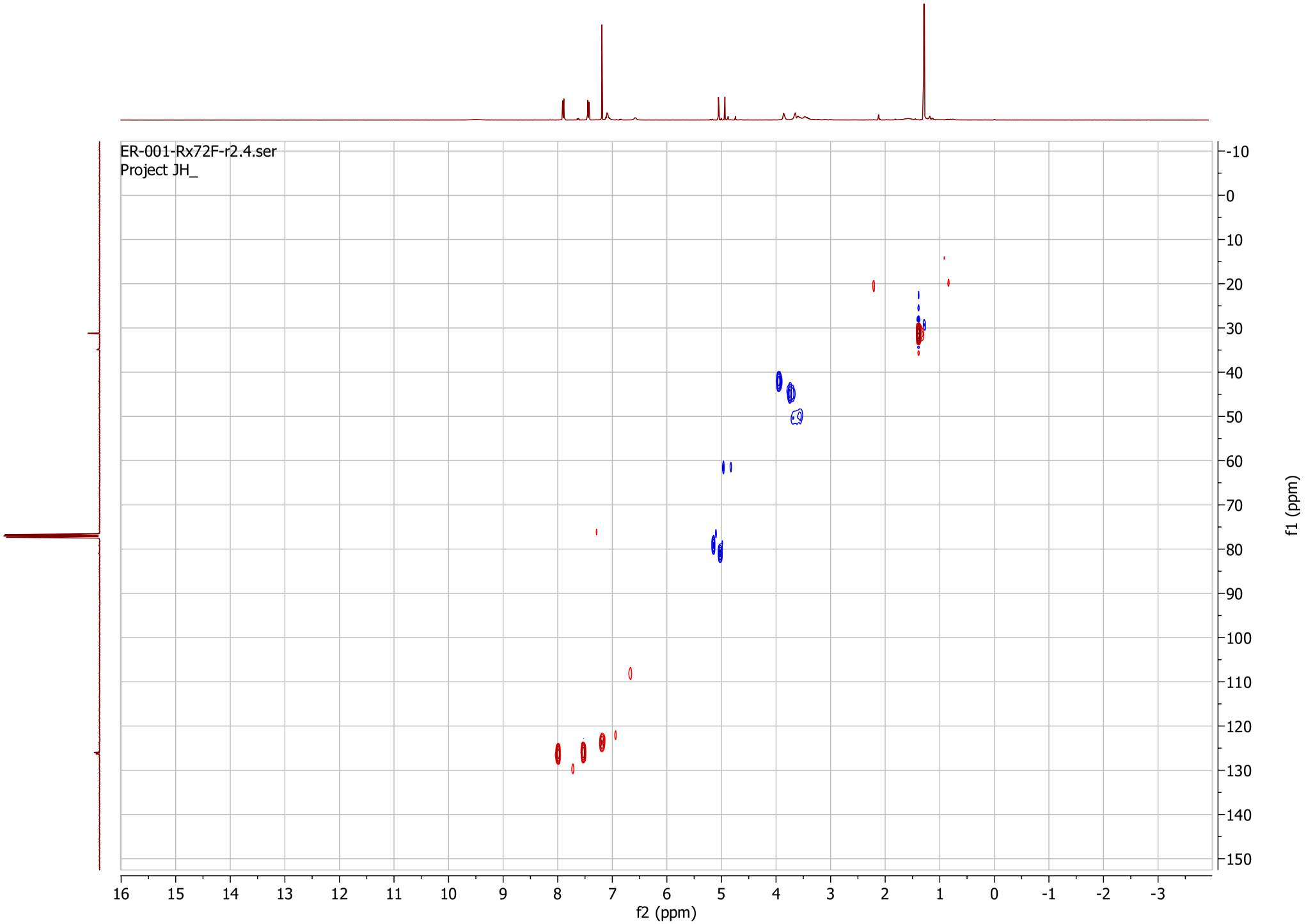
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)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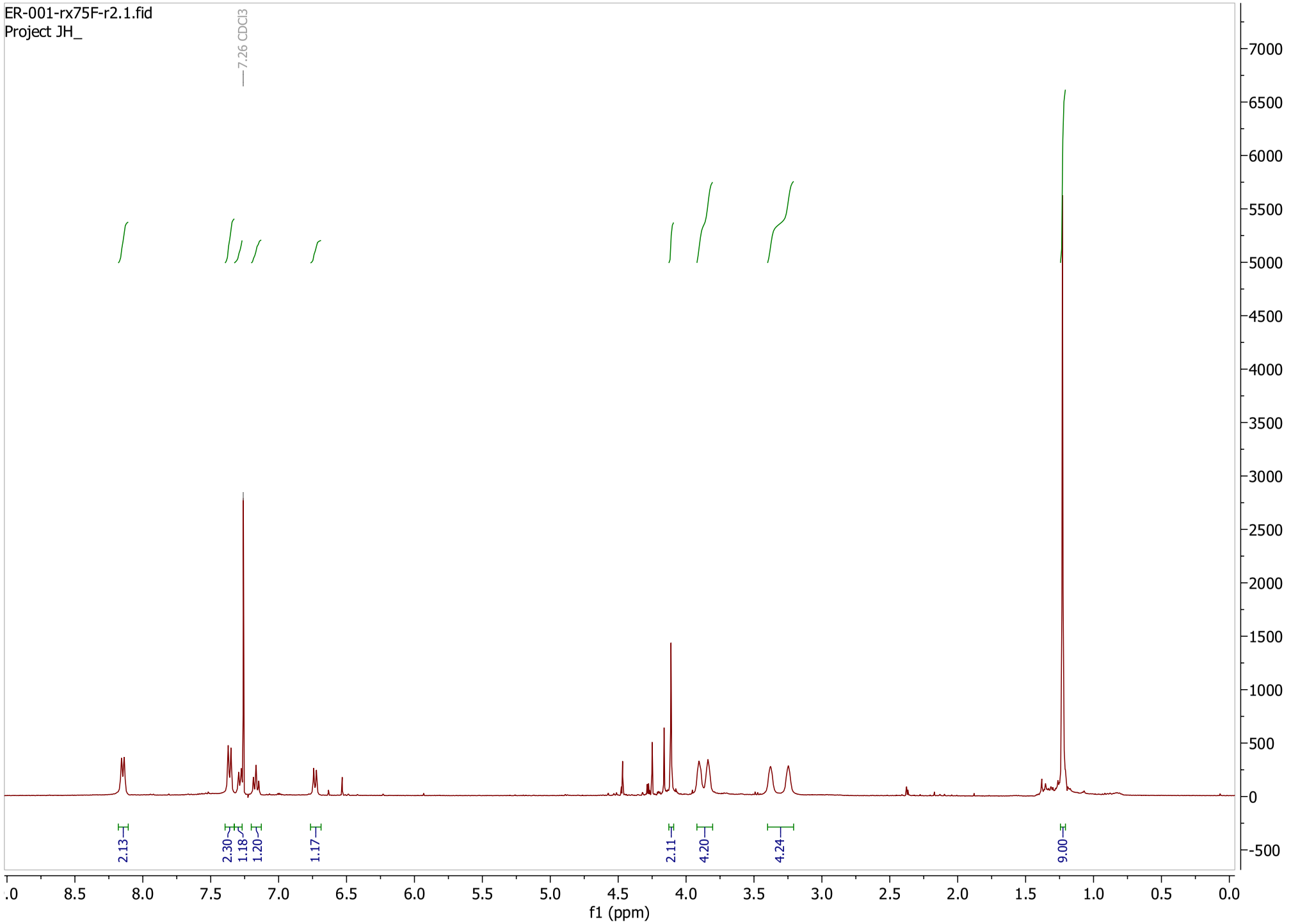
Project JH_
f2 (ppm)
f1 (ppm)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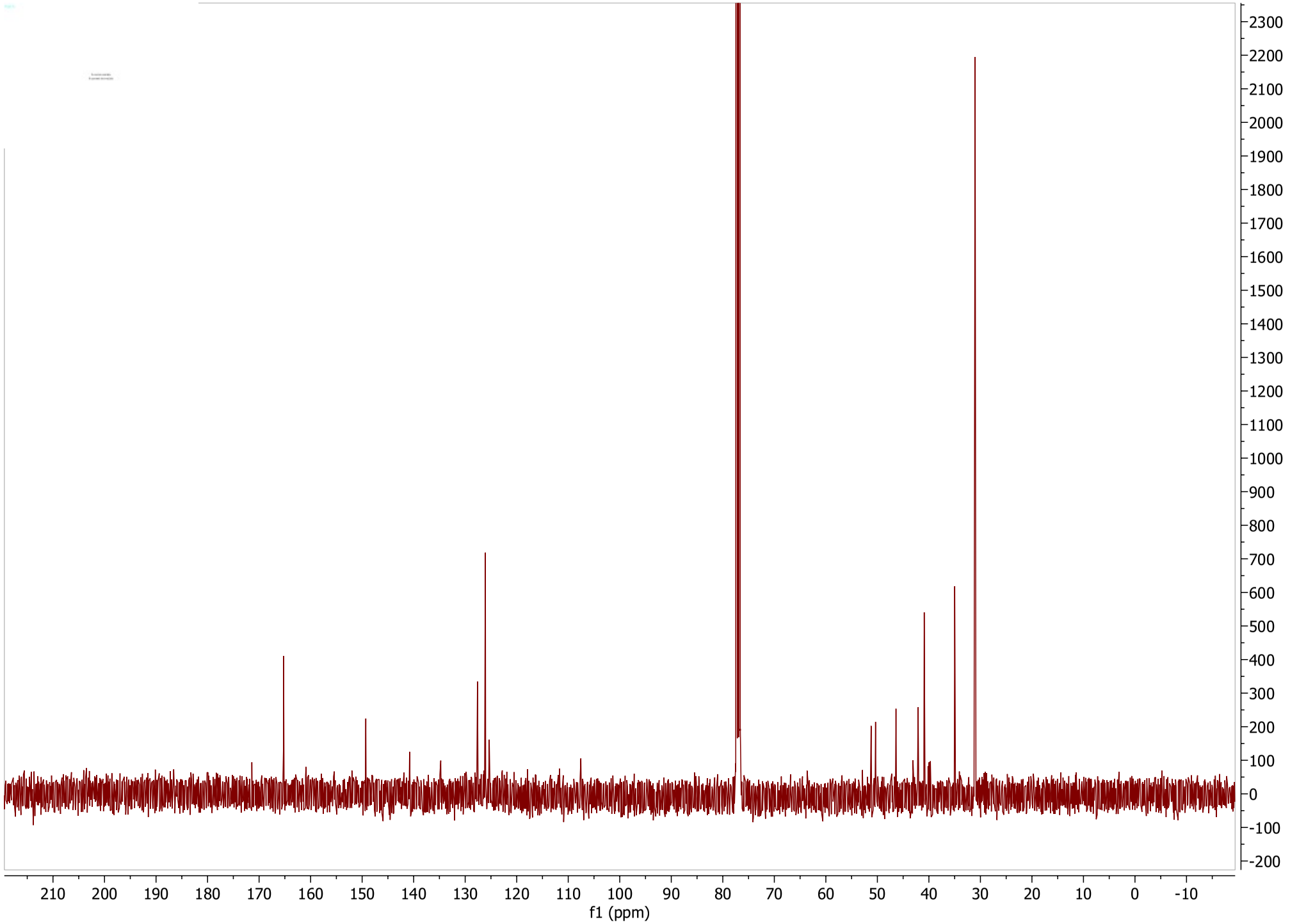
Project JH_
7.26 CDCl3
2.13
2.30
1.18
1.20
1.17
2.11
4.20
4.24
9.00
f1 (ppm)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2200
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1900
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200
100
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200
190
180
170
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80
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40
30
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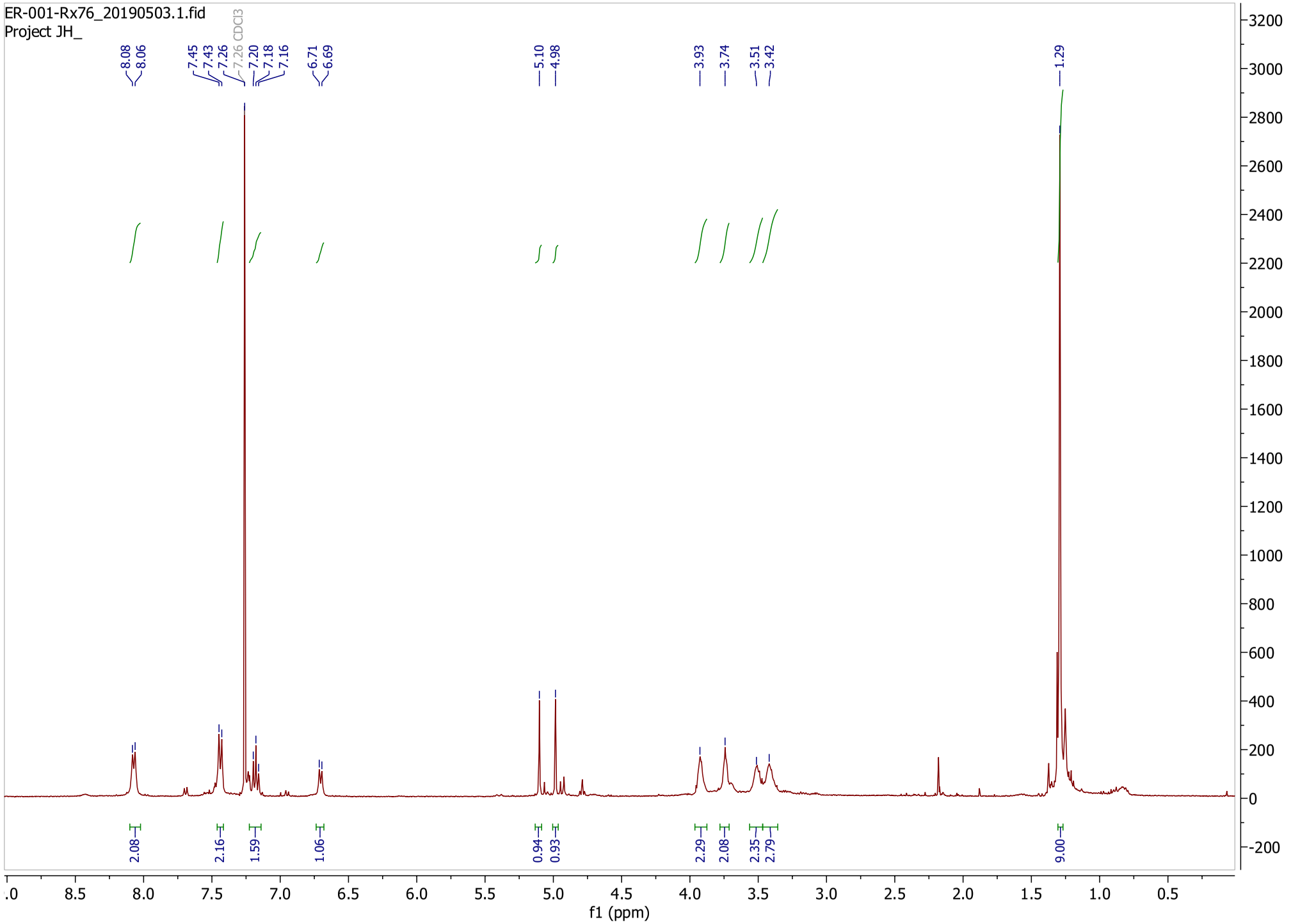
0
-10
f1 (ppm)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8.08
8.06
7.45
7.43
7.26
7.26 CDCl3
7.20
7.18
7.16
6.71
6.69
5.10
4.98
3.93
3.74
3.51
3.42
1.29
2.08
2.16
1.59
1.06
0.94
0.93
2.29
2.08
2.35
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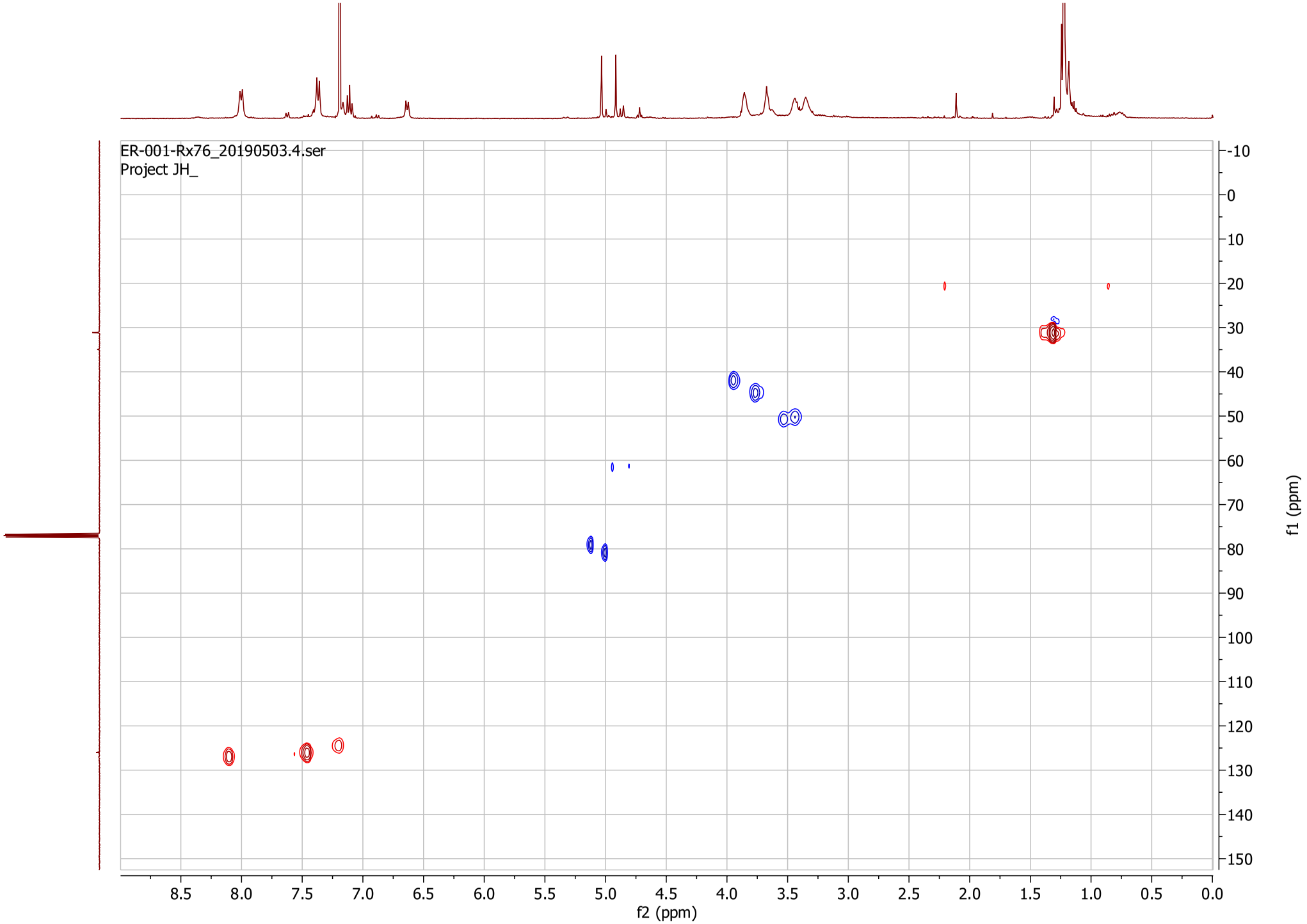
9.00
f1 (ppm)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Project JH_
f2 (ppm)
f1 (ppm)