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Synthesis of a Precursor for a Carotenoid Cationic Lipid

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Preface

The presented work has been carried out at the Norwegian University of Science and Technology, faculty for Natural Sciences and Technology, Department of Chemistry spanning the period of August 2011 – June 2012. The project has been supervised by Professor Vassilia Partali and phD candidate Muhammad Zeeshan, NTNU.

Acknowledgements

I would like to thank my supervisor, Professor Vassilia Partali, for including me in her research group and for sparking my interest in carotenoids and its many applications. Thank you for all your help and encouragement. I am also thankful for all the guidance I have got from phD candidate Muhammad Zeeshan. The first months in the laboratory would not have gone as smoothly without your help. I also owe a great thank you to the rest of the amazing research group, particularly my fellow students Marius Myreng Haugland and Esther Blijleven, for encouragement and practical assistance in the lab. Thank you also to dr. Richard Sliwka, and phD candidates Asma Zaidi and Eugenia Sandru for all your advice and help along the way.

I would also like to thank Head Engineer Susana Gonzalez and Master of Science Anders Brunsvik at Sintef for obtaining all my MS spectra, as well as Senior engineer Trygve Andreassen for helping me process all my NMR spectra. Thank you also to Staff Engineers Roger Aarvik and Gunnar Svare for providing reactants and solvent, respectively.

Lastly I would like to thank my friends and family for all the support they have given me. Your love encourages me to do my best.

Truc Mong Vo
Trondheim, June 2012

Abstract

The goal of this work was the synthesis of the carotenoid cationic lipid **10** for the research of carriers of nucleic acids into defective cells via transfection therapy. The scheme for the entire reaction can be seen in figure 0.1. In the course of this project compound **5**, a precursor for compound **10**, was successfully obtained, but because of the limited time the synthesis of the carotenoid cationic lipid itself (10) was not performed.

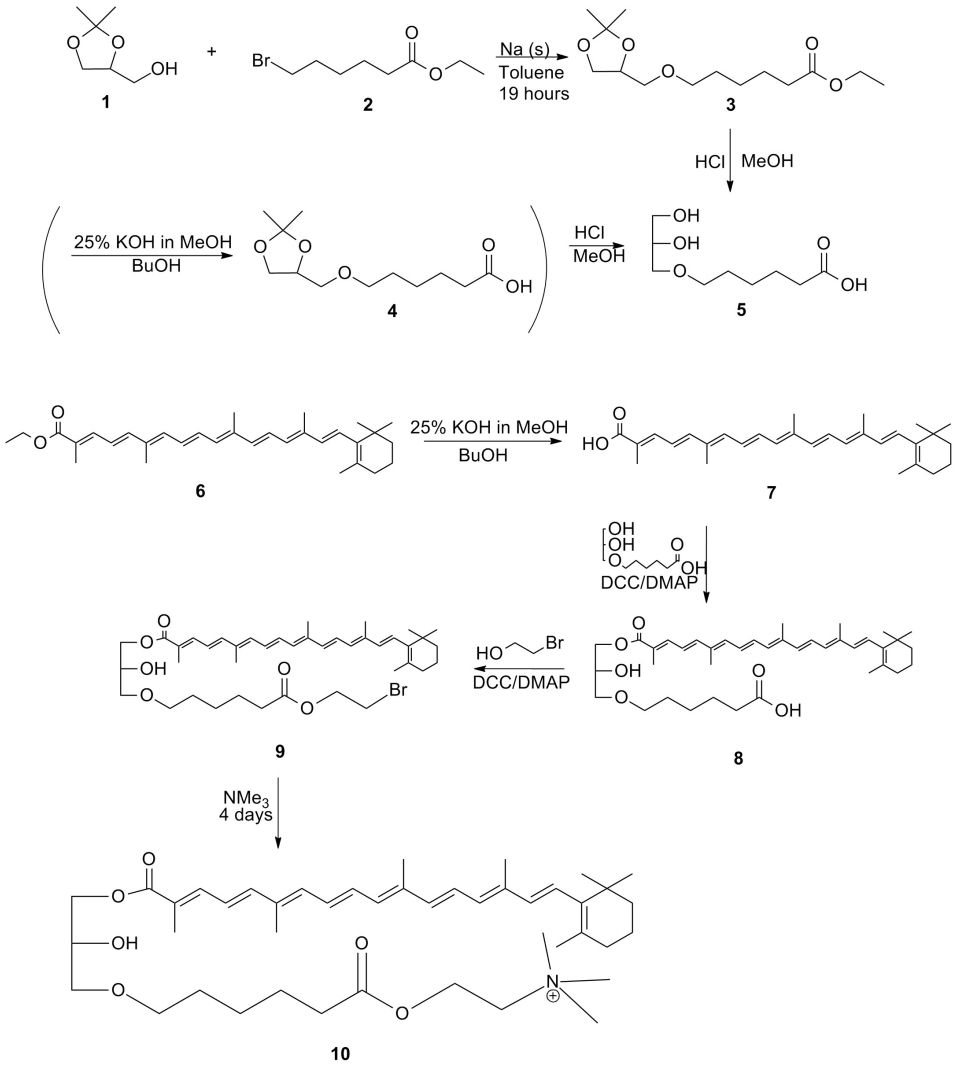


Figure 0.1: Reaction scheme for the carotenoid cationic lipid (10)

Sammendrag

Målet med dette arbeidet var å syntetisere det karotenoide kationlipidet **10** som en del av forskning på bærere av nukleinsyrer til defekte celler via transfeksjonsterapi. Hele reaksjonen kan ses i figur 0.2. I løpet av dette prosjektet har forbindelse **5**, som er en forløper til forbindelse **10**, blitt syntetisert, men grunnet den begrensede tiden har ikke syntesen av det karotenoide kationlipidet (10) blitt gjennomført.

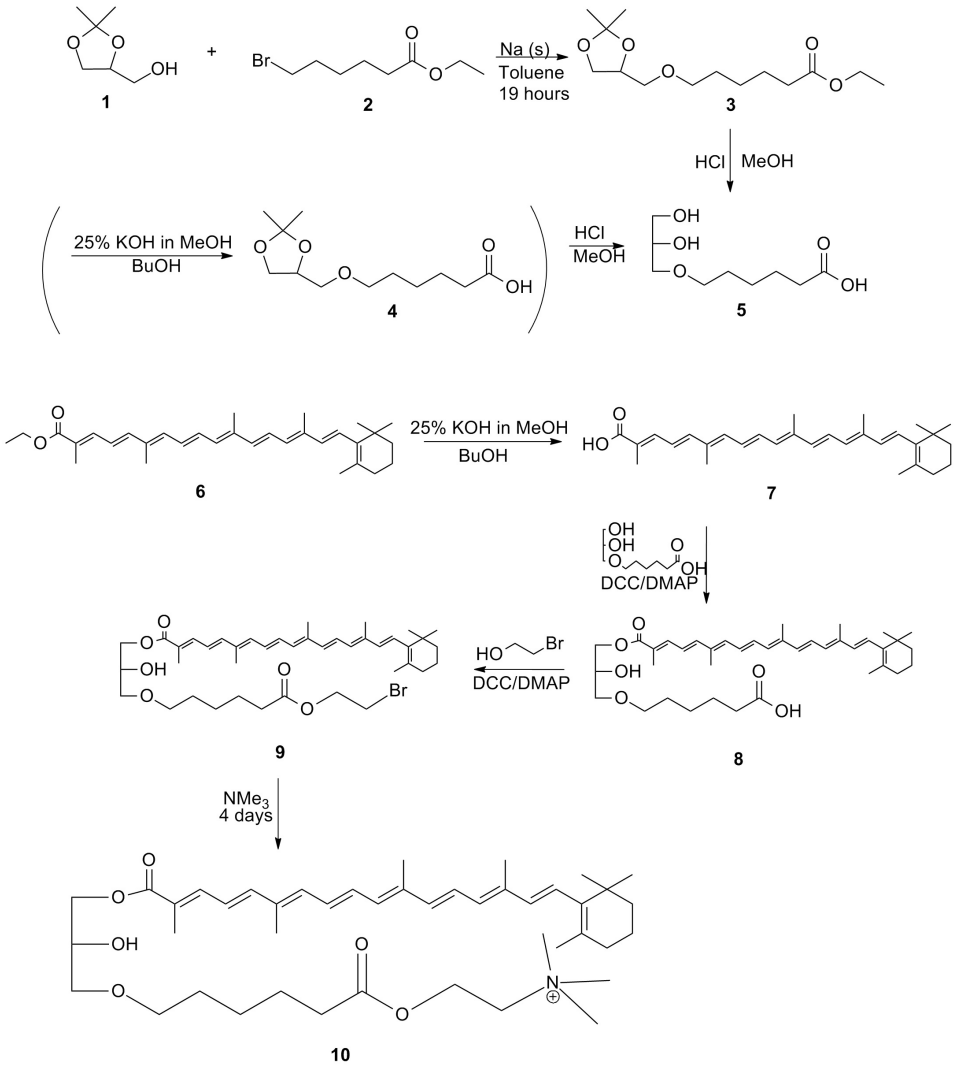


Figure 0.2: Hele reaksjonsforløpet for syntesen av det karotenoide kationlipidet **10**

Symbols and abbreviations

δ	Chemical shift
δ_{as}	Scissoring (bending)
δ_s	Out-of-plane bending or scissoring
ν_{as}	Asymmetrical stretching
ν_s	Symmetrical stretching
^{13}C	Carbon-13
^{1}H	Hydrogen-1
Car	Carotenoid
COSY	Correlated Spectroscopy
DCU	*N,N'*-dicyclohexylurea
dd	Doublet
DCC	*N,N'*-Dicyclohexylcarbodiimide
DMAP	4-Dimethylaminopyridin
DMF	Dimethyl formamide
DNA	Deoxyribonucleic acid
EI	Electron Impact
ESI	Electrospray Ionization
EtOAc	Ethyl acetate
EtOH	Ethanol
HMBC	Heteronuclear Multiple Bond Correlation
HR-MS	High-Resolution Mass Spectroscopy
HSQC	Heteronuclear Single Quantum Coherence
IR	Infrared Spectroscopy
J_t	Coupling constant [Hz]
LR-MS	Low-Resolution Mass Spectroscopy
M	Multiplicity
m	Multiplet
m/z	mass/charge ratio
M+	Molecular ion

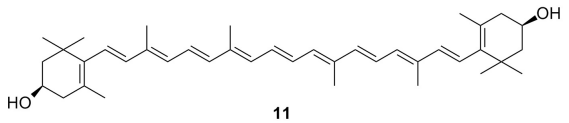
MeOH	Methanol
MS	Mass Spectroscopy
NMR	Nuclear Magnetic Resonance
p	Pentet
q	Quartet
R_f	Retention factor
ROO·	Peroxyl radical
ROO-Car·	Carotenoid peroxyl radical
s	Sextet
t	Triplet
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography

Contents

1 Carotenoids[1]

Carotenoids are organic pigments that are naturally occurring in for example the yellow-orange of flowers, the orange-red colours of fruit and the orange roots from carrots. The greatest production of carotenoids, however, occurs in the photosynthetic tissues of plants and algae. Here, the carotenoids are found universally in the photosynthetic apparatus, though their presence is generally masked by the green of the chlorophyll and revealed only when the chlorophyll is degraded as, for example, in autumn leaves.

There are over 750 known naturally occurring carotenoids,[2] all derived from the same basic C_{40}-isoprenoid skeleton by modifications such as cyclization, substitution, elimination, addition and rearrangement.[3] The carotenoids are divided into two classes: the carotenes are pure hydrocarbons and do not contain oxygen, whereas xanthophylls do. Zeaxanthin (11) and β-carotene (12) are examples of carotenoids and carotenes, respectively. The oxygen in xanthophylls gives them a more polar character, which allows separation from carotenes in many types of chromatography.[4]



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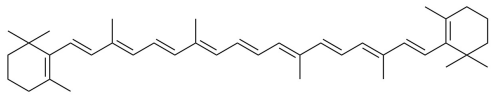

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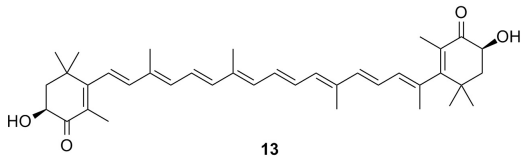
Animals appear to be incapable of biosynthesizing carotenoids, but many animals are coloured by carotenoids that originate from their food. Some examples are the pink-red feathers of flamingos (provided by ketocarotenoids) and the skin and flesh of some fish (like astaxanthin (13) in goldfish and salmon).



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In marine invertebrate animals, pigmentation is often due to carotenoprotein complexes in which the orange-red of the free carotenoid, usually astaxanthin (13), is modified to provide blue, purple or green colours. Thus, the lobster, which is

blue when it is alive, becomes red when it is cooked, as the astaxanthin is liberated from the denatured protein. Colouration by carotenoids is normally not seen in mammals, though humans ingesting large amounts of carotenoid supplements or of carotene-rich food may show yellow or orange colour in the skin.

1.1 The properties of carotenoids

Carotenoids have special properties that reflect their chemical structures. The most characteristic structural feature, which is responsible for the observed colour of the carotenoids, comes from the presence of the long chromophore of conjugated double bonds, called the polyene chain. The number of double bonds in the carotenoid largely determines the spectral properties of the given carotenoid, which typically absorbs light between 400 and 500 nm.[5] Their strong light absorption renders carotenoids in only microgram quantities visible to the human eye. The colour, which can readily be seen in solution and during chromatography, greatly facilitates isolation and makes it easy to monitor purification steps and chemical reactions. The intense light absorption also provides the basis for the quantitative determination of carotenoids. In addition, the polyene system renders the molecule extremely susceptible to oxidative degradation and to geometrical isomerization brought about by light, heat or acids. This is seen as loss or change of colour and gives an immediate warning of decomposition or structural modification.[6]

A very important property of carotenoids is the presence of conjugation. The conjugation in the structure allows the electrons to move freely in the particular areas of the molecule. As the number of double bonds increases, electrons associated with conjugated systems have more room to move, and require less energy to change states. This causes the range of energies of light absorbed by the molecule to decrease. As more frequencies of light are absorbed from the short end of the visible spectrum, the compounds acquire an increasingly red appearance. The increased amount of double bonds also results in a rigid rod-like structure, which seems to play a key role in the stabilization of carotenoids.[2]

1.2 Carotenoids as antioxidants

Studies have shown that β-carotene and other carotenoids, which are present in many food products, have lipid-soluble antioxidant activity.[7] The antioxidant actions of carotenoids are based on their singlet oxygen quenching properties and their ability to trap peroxyl radicals. The best documented antioxidant action of

carotenoids is their ability to quench singlet oxygen *(Eq 1)*.

$$ROO\bullet + Car \rightarrow ROO - Car\bullet \quad (1)$$

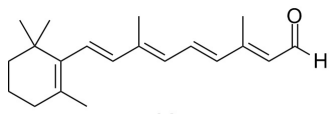
This results in an excited carotenoid, which has the ability to regenerate the original unexcited carotenoid, which can be reused for further cycles of singlet oxygen quenching. The quenching activity of a carotenoid mainly depends on the number of conjugated double bonds of the molecule.

In plants and algae, the most important function of the carotenoid in the reaction centre-core complexes is to protect against photosensitized oxidation, by quenching the excitation energy of the chlorophyll triplet state or singlet oxygen. If carotenoids had not acted in this manner, photosynthesis in an aerobic environment would not have been possible. In addition, the xanthophylls in the light-harvesting chlorophyll-proteins act as accessory light-harvesting pigments, passing on their excitation energy to chlorophyll by singlet energy transfer.

1.3 Carotenoids and health benefits

It has been reported that a diet containing food with carotenoids has a positive effect on the general health. Research seems to support the theory that carotenoids have a protective effect against various diseases, such as stroke, ischemic heart diseases and cancer.[8] Observational studies have also connected high dietary intake of foods providing zeaxanthin with lower incidence of age-related macular degeneration (AMD).[9]

The most important nutritional role of carotenoids is as provitamin A. The involvement of vitamin A aldehyde, retinal (14), as the chromophore of the visual pigments in the eye is central to the process of vision. The consequences of vitamin A deficiency are xerophthalmia, blindness and premature death. Vitamin A also has important systemic functions in maintaining growth and reproductive efficiency and in the maintenance of epithelial tissues and prevention of their keritinization.[10] The importance of the latter effect has led to the synthesis of a wide range of related compounds, the retinoids, and to the evaluation of these substances for therapeutic use to treat skin problems such as acne and also as cancer-prevention agents.



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2 Transfection therapy

Transfection is a method of gene therapy where nucleic acids are deliberately introduced into cells.[11] Transfection therapy treats diseases of genetic origin by providing the missing genetic material, correcting defective genes or enhancing the expression of genes that are already present.[12] In the future gene therapy may provide a way to cure genetic disorders, such as severe combined immunodeficiency, cystic fibrosis or Haemophilia A.

Due to the multianionic character of DNA, its permeation across the negative cellular membrane is limited, and vectors for transporting it are needed.[13] The success of gene therapy chiefly depends on the development of efficient and safe vectors for gene delivery. The two main types of vectors that are used in gene therapy are based on viral and non-viral gene delivery systems.[14]

The use of viral vectors show efficient transfection but it has many disadvantages, such as oncogenic effects and immunogenicity.[15;16] The use on non-viral vectors, however, such as cationic lipids or polymers, does not trigger such immune response. The non-viral vectors also have greater carrier capacity; they are easier to produce in large scale and are more stable.

2.1 Carotenoid cationic lipids as non-viral vectors

Cationic lipids are formed from either a single cationic amphiphile or in combination with a neutral lipid. Because of its permanent cationic charge, the cationic lipids interact electrostatically with the negatively charged DNA and form clusters of aggregated vesicles along the nucleic acid (figure 2.1).[15;17]

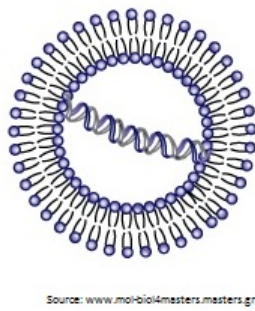


Figure 2.1: The cationic lipids form vesicles along the nucleic acid

In order for the lipoplex to cross the cell membrane, the complex should be

charge-neutral or have an excess positive charge overall. The use of cationic lipids facilitates lipoplex formation by developing a charge-neutral complex with the negatively charged nucleic acid.[18] Most cationic lipid/DNA-based systems are taken up efficiently through an endocytosis pathway (figure 2.2).[19] Once they have entered the cell, the nucleic acids are slowly released either to be expressed in the cell nucleus or to control gene expression.[20]

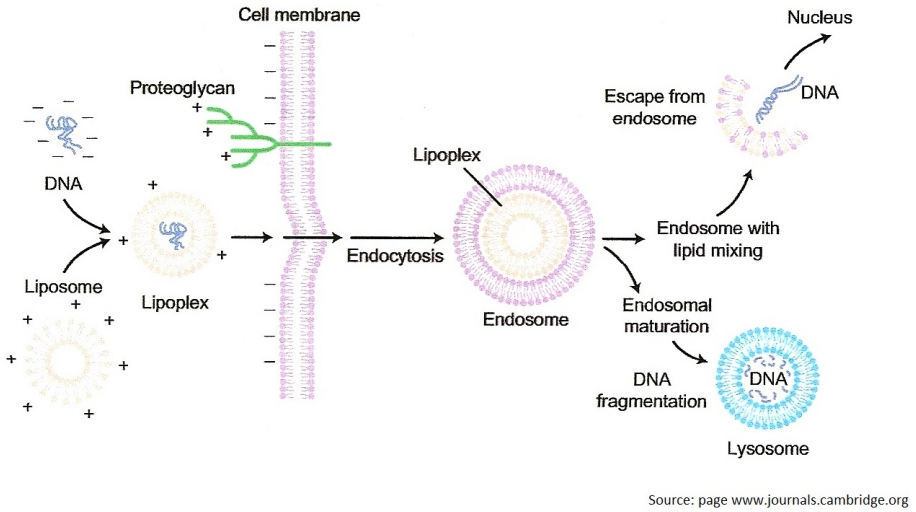
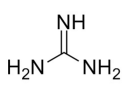


Figure 2.2: Lipoplex-mediated transfection and endocytosis

Nucleic acid binding by the cationic lipid vector requires a head group that can sustain a positive charge at physiological pH (7.4). Some commonly applied head group moieties that achieve this include primary, secondary or tertiary amines, in addition to quaternary ammonium salts. The quaternary amines generally express higher activity, though in cationic cholesterols the tertiary amines are often more active.[20] Guanidine (15) and imidazole (16) groups have also been successfully used.[18] A large number of cationic lipids are functionalized with polyamine head groups, where spermine (17) and spermidine (18) groups are very common.



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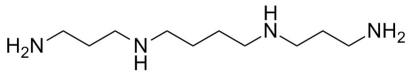

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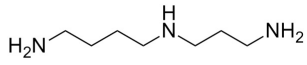

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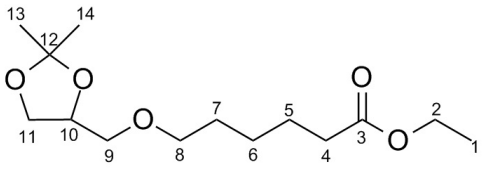
Carbon atoms act as a spacer between the charged and non-polar parts of the molecule. The length of spacer is important and can influence the activity of the overall molecule. The length of the non-polar hydrocarbon moiety is also very important. Chains shorter than 14 carbon atoms can destabilize the lipid layer and express toxicity for cells. Very long and saturated chains can have undesirable high temperature of melting.

3 Results and discussion

Three methods were applied to synthesize ethyl 6-[(2,2-dimethyl-1,3-dioxalan-4-yl)methoxy]hexanoate (3), one of which was deemed unsuccessful. Afterwards, two methods were applied to hydrolyse the ester, with little success. The method applied to open the ring also proved beneficial for hydrolysing the ester. Finally, β-apo-8'-carotenoic acid (7) was reacted with 6-(2,3-dihydroxypropoxy)hexanoic acid (5) in an attempt to synthesize 6-(2-hydroxy-3-(((2E,4E,6E,8E,10E,12E, 14E,16E)-2,6,11,15-tetramethyl-17-(2,6,6-trimethylcyclohex-1-en-1-yl)heptadeca-2, 4,6,8,10,12,14,16-octaenoyl)oxy)propoxy)hexanoic acid (8).

3.1 Synthesis of ethyl 6-[(2,2-dimethyl-1,3-dioxalan-4-yl) methoxy]hexanoate (3)



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3.1.1 Method 1: KOH, with toluene as solvent

In the initial attempt, 1,2-isopropylideneglycerol (1), ethyl-6-bromohexanoate (2) and KOH were refluxed in toluene for 17 hours with a Dean Stark trap to give compound **3** (figure 3.1).

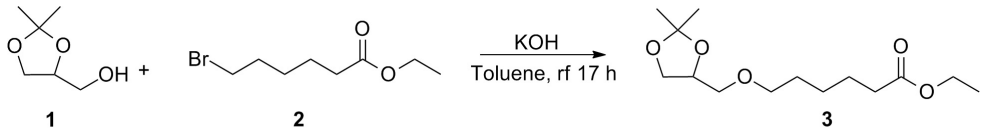


Figure 3.1: Initial attempt to synthesize compound **3**

This reaction was repeated three times. In the literature the yield is reported to be 98 %,[21] whereas in this case spectroscopic analyses imply that the desired compound has been obtained in only very small amounts. From the NMR spectra (figure 3.2) it is difficult to distinguish the shifts that are characteristic for compound **3**. The proton signals for the methyl groups should appear as singlets

between 1.36 ppm and 1.42 ppm, and the carbon signals should appear between 25.5 ppm and 26.8 ppm, though they are not observed.

However, analysis by mass spectroscopy indicates that the reaction was successful. It is possible that small amounts of compound **3** has been produced. Observation of weak signals in the proton spectrum supports this theory. The triplet at 1.20 ppm coincides with the methyl group at C-1, although the signal for the neighbouring CH_2-group is not observed in the expected area of the spectrum. The multiplet at 2.49 ppm - 2.56 ppm coincides with protons belonging to an ether, specifically C-8 and C-9. The signals at 3.57 ppm - 3.62 ppm and 4.05 ppm - 4.10 ppm may belong to the protons at C-11. The carbon signals at 115.54 ppm and 179.90 ppm may belong to the quaternary carbon C-12 and the carbonyl carbon C-3, respectively, but the shifts are somewhat higher than expected. As mentioned above, all of proton signals are very weak compared to the signals that belong to main product obtained from this reaction, thus this method is deemed unsuccessful.

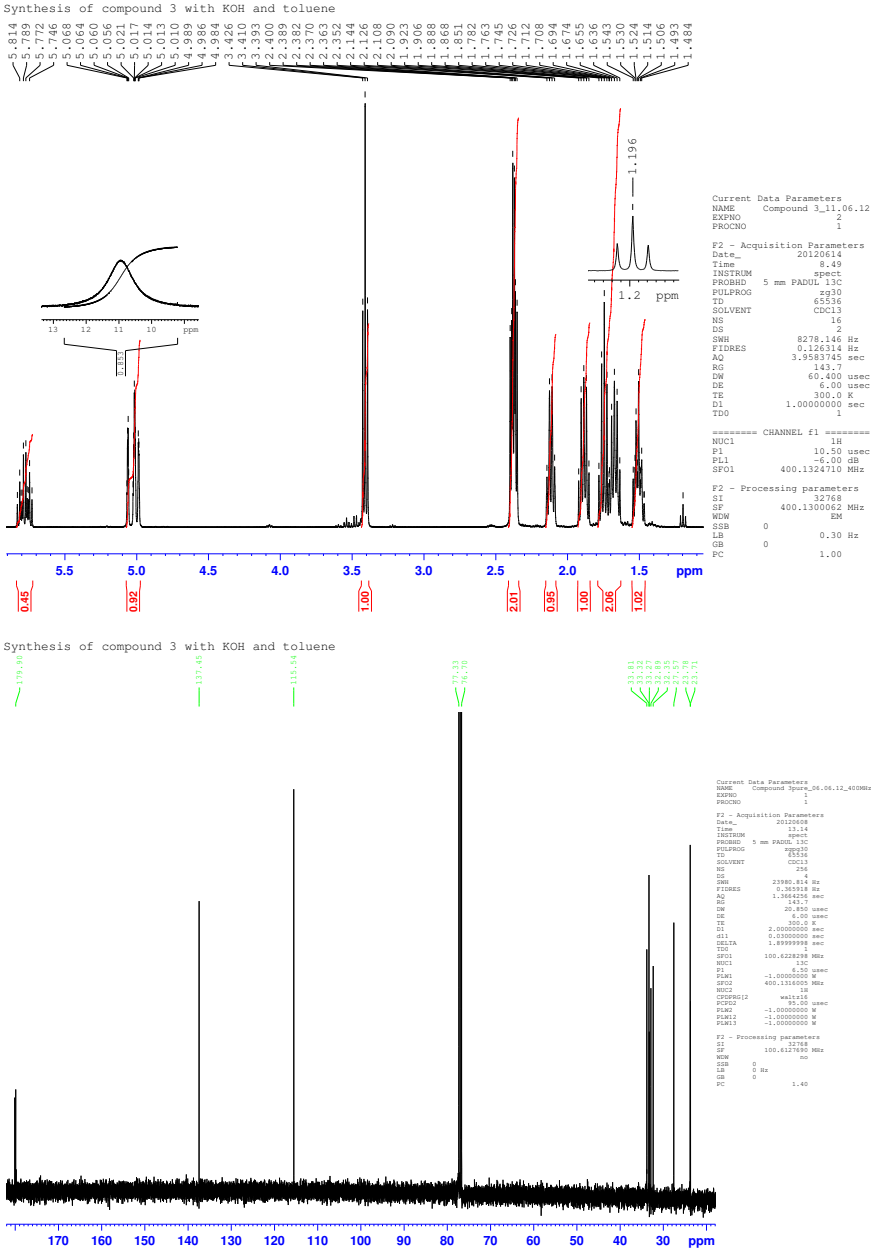


Figure 3.2: NMR spectra of product formed by reaction with KOH in toluene

One possible reason why the reaction was not successful is that not all of the water that was formed during the reaction was not removed. In theory, removing

the water will cause the equilibrium to be shifted towards the product side. However, if the water is not removed, this might cause the reaction to reach equilibrium at an earlier point, which can result in a lower yield.

Analyses by TLC shows several spots, indicating that byproducts has been formed. The hydroxide group may prefer to attack the carbon in the α-position to the bromide in ethyl-6-bromohexanoate over deprotonating the hydroxyl group on 1,2-isopropylideneglycerol (figure 3.3).

Figure 3.3: Substitution of bromide with hydroxide

3.1.2 Method 2: NaH, with DMF as solvent

In the second method 1 equivalence of 1,2-isopropylideneglycerol (1) is deprotonated with 1.53 equivalence of the strong base NaH (figure 3.4).

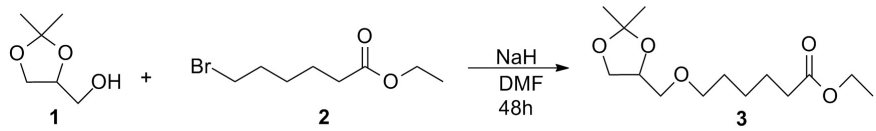


Figure 3.4: Synthesis of compound 3 with NaH

Once the deprotonation of the ketal is complete, 1.19 equivalence of ethyl-6-bromohexanoate (2) is added to the reaction mixture. This is to prevent the base from deprotonating the ester in stead of the alcohol, resulting in undesired products. If too much NaH is used, or if the ester is added before all the ketal has been deprotonated, the ester might also be deprotonated (figure 3.5).

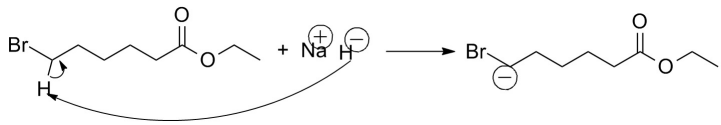


Figure 3.5: Deprotonation of ethyl-6-bromohexanoate as a competing reaction

Another complication that might occur is deactivation of NaH. If the base is exposed to the air for too long, it might be deactivated by the humidity. The result

would be that some of the alcohol (1) may not be deprotonated, leading to a poorer yield. It is therefore important to maintain an inert atmosphere.

This reaction has been repeated 4 times, but after purification by flash column chromatography the desired product has been obtained only twice, with very low yield (5 - 7 %). Compound **1** is the limiting reagent, but TLC reveals that it is still present at the end of the reaction, which indicates that some of the NaH was deactivated and/or that compound **2** was deprotonated as well. The literature confirms that this reaction results in low yield (35 %),[22] though their value is somewhat higher than what was achieved in this project. The TLC also revealed that, as with the previous method (section 3.1.1), byproducts has been formed (figure 3.6).

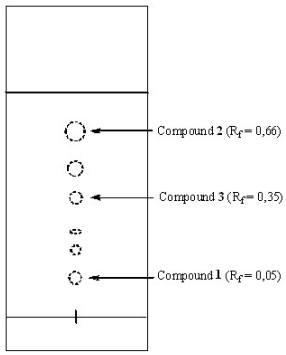


Figure 3.6: TLC of crude after step 1 (toluene-acetone, 24:1)

3.1.3 Method 3: Na, with toluene as solvent

Since the first reaction was deemed unsuccessful (section 3.1.1), and the second reaction gave the desired compound in very low yield (section 3.1.2), a third method was applied, where compounds **1** and **2** were refluxed with Na for 19 hours (figure 3.7).

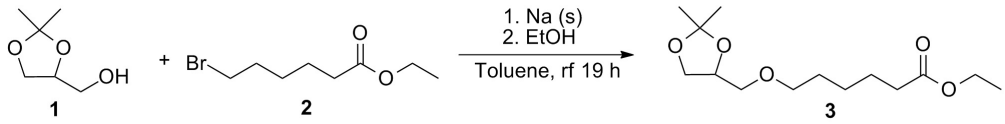
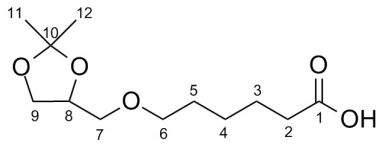


Figure 3.7: Synthesis of compound **3** using Na(s)

Analysis by TLC reveals that the R_f value of the main product coincides with the R_f value of compound **3**, which is a strong indication that the reaction was

successful. The desired product was obtained in 38% yield, which is lower than the value in the literature (63 %)[23], but better than for the previous attempts.

3.2 Synthesis of 6-[2,2-dimethyl-1,3-dioxalan-4-yl)methyoxy] hexanoic acid (4)



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Two methods were applied to hydrolyse the ester (figure 3.8).

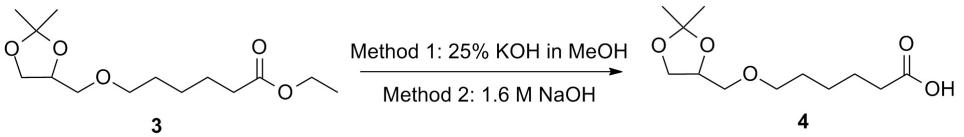


Figure 3.8: Hydrolysis of compound **3**

3.2.1 With KOH (25 % in MeOH)

In the first approach compound **3** is reacted with KOH under heating for 2 hours. The ^{1}H NMR spectrum (figure 3.9) indicates that compound **4** was not obtained. The missing signal at 1.3 ppm - 1.4 ppm, which denotes methyl groups, may be an indication that a ring-opening occurred, but there are no signals at 3.6 ppm - 3.9 ppm to indicate formation of the glycerol moiety. There is a weak signal at 1.17 ppm - 1,22 ppm which may be the methyl group on the ethyl ether moiety. If this is the case, it would mean that the hydrolysis reaction was unsuccessful.

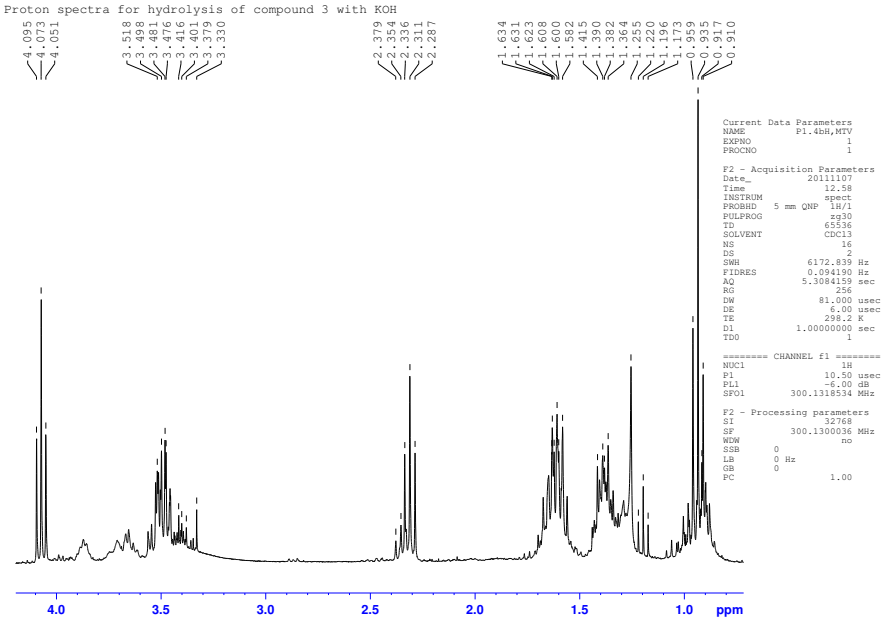


Figure 3.9: Proton spectra for hydrolysis of compound 3 with KOH

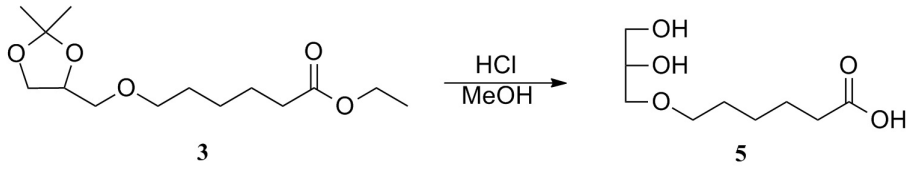
Based on the results from HR-MS, however, it seems like the synthesis of compound **4** was a success. The molecular mass of compound **4** is 246.3013 g/mol, and the found m/z was 202.1566, which is consistent with the loss of CO_2. It is possible that the obtained amount was too small to be detected on the ^{1}H NMR spectra.

3.2.2 With 1.6 M NaOH

In the second approach compound **3** is reacted with 1.6 M NaOH for 4 days. Analyses by TLC revealed that none of the products had the expected R_f value, thus this method was also deemed unsuccessful.

3.3 Synthesis of 6-(2,3-dihydroxypropoxy)hexanoic acid (5)

Compound **5** is obtained by refluxing compound **3** under acidic conditions (figure 3.10). After purification the reported yield was 69 %.



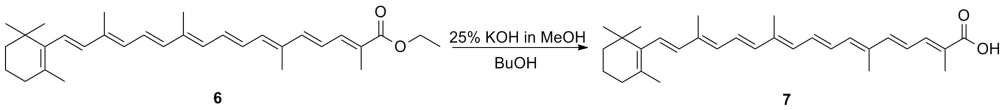
3 5

Figure 3.10: Ring-opening and hydrolysis of compound **3**

^{1}H and ^{13}C NMR spectra confirm that compound **5** was obtained through hydrolysis and ring-opening of compound **3** (appendix C). The signal at 3.85 ppm - 3.87 ppm in the ^{1}H spectrum is typical for the single proton of the middle carbon in the glycerol moiety; the lack of singlets in the area 1.3 ppm - 1.4 ppm confirms that the ring was opened, and the lack of signals at 1.25 ppm and 4.15 ppm is a clear indication that the hydrolysis was successful.

3.4 Hydrolysis of ethyl β-apo-8'-carotenoate (6) to give β-apo-8'-carotenoic acid (7)

Ethyl β-apo-8'-carotenoate (6) was refluxed with KOH (25% in MeOH) in n-BuOH for up to two hours to give β-apo-8'-carotenoic acid (7) (figure 3.11).



6 7

Figure 3.11: Hydrolysis under basic conditions

Analysis on TLC showed that the conversion of ester to acid was completed, and no purification was required. The reported yield was higher than theoretically possible, due to inadequate drying on vacuum pump overnight.

3.5 Synthesis of 6-(2-hydroxy-3-(((2E,4E,6E,8E,10E,12E,14E, 16E)-2,6,11,15-tetramethyl-17-(2,6,6-trimethylcyclohex-1-en-1-yl)heptadeca-2,4,6,8,10,12,14,16-octaenoyl)oxy)propoxy) hexanoic acid (8)

Compounds **5** and **7** were stirred with DCC and DMAP in dry dichloromethane under inert atmosphere to give compound **8** (figure 3.12).

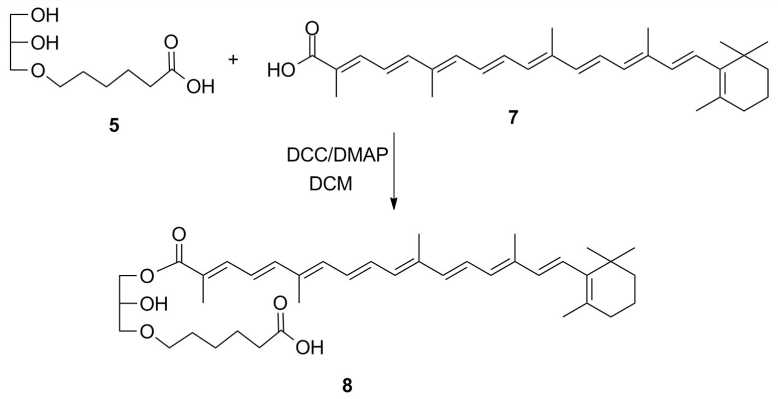


Figure 3.12: Esterification of compound **7**

Compound **5** was used in excess in order to minimize the possibility of reaction on both of the hydroxyl groups and the formation of carotenoid anhydride. The reaction was monitored by TLC, and stopped when most of the alcohol had been consumed. There was also precipitate, which indicates the formation of the byproduct DCU, meaning that the DCC was successful in reacting with the deprotonated carotenoic acid. The TLC showed that several other byproducts had also been formed. The reaction requires completely dry conditions, though the reactants and/or catalyst may have contained some solvent or humidity. This may have resulted in the many byproducts, but spectroscopic analyses of the different fractions were inconclusive, so it is not possible to confirm their structure. The crude was run through the flash column and the fraction with the expected R_f value was spectroscopically analysed. The ^{1}H NMR spectrum (figure 3.13) reveals signals at 6.1 ppm - 6.8 ppm, which is characteristic for the polyenic protons of the carotenoic part of compound **8**. In addition, the signal at 4.2 ppm - 4.3 ppm coincides with the value of the shift of the single proton on the middle carbon in the glycerol moiety.[24] Due to the lack of further spectroscopic analyses, however, it is not possible to characterize the obtained compound.

The reaction was repeated twice, with the same result.

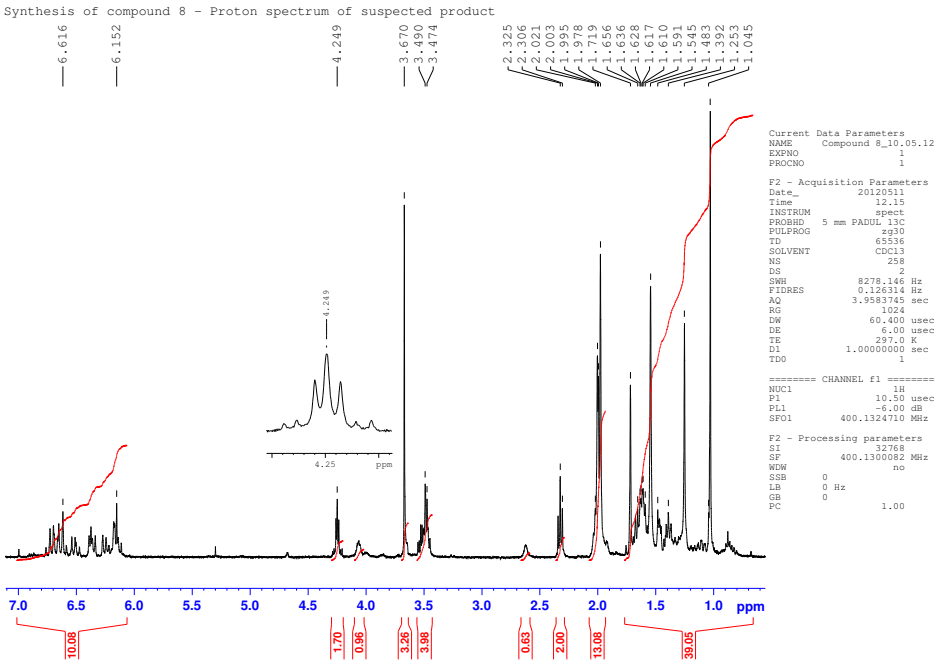


Figure 3.13: Synthesis of compound **8** - ^{1}H NMR spectrum of suspected product

3.6 Other comments on yield

The yield of the products in each step was relatively low. In addition to the reasons listed above, the low yield could be the consequence of inadequate work-up. If insufficient amounts of organic phase were used during extraction of the product from the aqueous phase, some of the desired product could have remained in the aqueous phase, and the resulting yield would have been somewhat lower than the potential yield.

4 Structural analyses

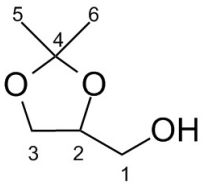
The structure of the synthesized compounds was analyzed by using different spectroscopic methods. ^{1}H NMR experiments were performed on all compounds, while the ^{13}C NMR, HMBC, HSQC and COSY experiments were performed on compounds **1**, **3** and **5**. Mass spectroscopy was performed to confirm the molecular mass and formula, while IR was performed to confirm the functional group(s).

4.1 Structural characterization of 1,2-isopropylideneglycerol (1)

The structure of compound **1** was determined by running ^{1}H NMR, ^{13}C NMR, HSQC, HMBC and COSY experiments (appendix A). The spectra were used as reference for the compounds that were synthesized in this project.

4.1.1 NMR spectroscopy

The assignment of ^{1}H NMR shifts, integral, number of protons, multiplicity, coupling constants, ^{13}C NMR shifts and coupling in COSY for compound **1** is shown in table 4.1.



1

Table 4.1: Assigned shifts for compound **1**

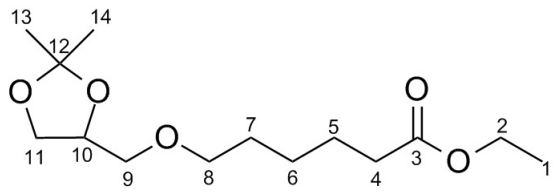
Position	^{1}H (ppm)	Integral	Number of protons	M	J_t (Hz)	^{13}C (ppm)	COSY
1	3.60	1.03	1	m	1.6	62.90	2
	3.74	1.06	1	m			2
2	4.23	1.00	1	m	1.6	76.08	1, 3
3	3.80	1.02	1	dd	1.6; 6.8	65.58	2
	4.04	1.04	1	dd	1.6; 6.4; 6.8;		2
4	-	-	-	-	-	109.40	-
5	1.38	3.05	3	s	-	25.26	-
6	1.44	3.17	3	s	-	26.70	-
OH	1.97	0.98	1	t	6.4	-	1

4.2 Structural characterization of ethyl 6-[(2,2-dimethyl-1,3-dioxalan-4-yl)methoxy]hexanoate (3)

The structure of compound **3** was determined by running ^{1}H NMR, ^{13}C NMR, HSQC, HMBC, COSY, LR-MS and IR experiments (appendix B).

4.2.1 NMR spectroscopy

The assignment of ^{1}H NMR shifts, integral, number of protons, multiplicity, coupling constants, ^{13}C NMR shifts and coupling in COSY for compound **3** is shown in table 4.2. Proton and carbon shifts are assigned in accordance with ^{1}H (figure B.1), ^{13}C (figure B.2), HSQC (figure B.3), COSY (figure B.4) and HMBC (figure B.5) spectra (appendix B).



3

Table 4.2: Assigned shifts for compound **3**

Position	^{1}H (ppm)	Integral	Number of protons	M	J_t (Hz)	^{13}C (ppm)	COSY
1	1.25	3.01	3	t	7.2	14.27	2
2	4.10 - 4.15	2.02	2	q	7.2	60.22	1
3	-	-	-	-	-	173.7	-
4	2.30	2.00	2	t	7.6	34.3	3, 5
5	1.6		2	m		29.25	4, 6
6	1.36		2	m		25.45	5, 7
7	1.7		2	m		24.79	6, 8
8	3.5		2	m		71.53	7
9	3.42 3.50	4.06	1 1	m m		71.89	10
10	4.24	0.98	1	p	5.8; 6.2	74.77	9, 11
11	3.70 - 3.74	1.01	1	dd	6.4; 1.8	66.93	10
	4.03 - 4.07	1.00	1	m	6.4; 1.8		10
12	-	-	-	-	-	109.39	13, 14
13	1.42		3	-	-	26.79	12
14	1.36		3	-	-	25.68	12

Note 1: The integrals for the protons on carbons 6, 13 and 14 are indistinguishable from each other, as are the integrals for the protons at carbons 5 and 7, as well as the integrals for the protons at carbons 8 and 9
Note 2: The high value for the integral at 1.56 ppm - 1.66 ppm might be because of impurities

4.2.2 IR spectroscopy

The IR spectrum of compound **3** (figure B.6, appendix B) shows C-H stretching at 2937 cm^{-1} and 2867 cm^{-1}, which is characteristic for the methyl group. The spectrum also shows C=O stretching at 1736 cm^{-1}, as well as C-O-C stretching at 1118 cm^{-1}.

4.2.3 MS fragmentation

The main fragmentation of compound **3** from LR-MS by EI ionization (appendix B) are shown in table 4.3.

Table 4.3: Selected MS fragments of compound **3**

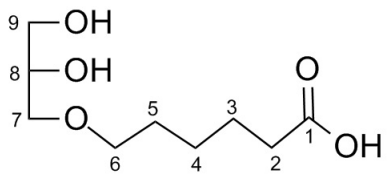
m/z	Rel. intensity (%)	Fragment
293.1	4	$[M+H_3O]^+$
274.2	2	$[M]^+$
259.2	30	$[M\text{-}CH_3]^+$
234.3	5	$[M+2H\text{-}C_3H_6]^+$
189.1	8	$[M+H\text{-}C_5H_{12}O]^+$
171.2	25	$[M+H\text{-}C_5H_{14}O_2]^+$
143.2	32	$[M+H\text{-}C_7H_{18}O_2]^+$
115.1	25	$[M\text{-}C_8H_{18}O_3]^+$
101.1	100	$[M\text{-}C_9H_{20}O_3]^+$
97.1	40	$[M\text{-}C_9H_{22}O_4]^+$
73.0	20	$[M\text{-}C_{10}H_{20}O_4]^+$
69.0	36	$[M\text{-}C_{11}H_{24}O_4]^+$

4.3 Structural characterization of (S)-6(2,3-dihydroxy propoxy)hexanoic acid (5)

The structure of compound **5** was determined by running ^{1}H NMR, ^{13}C NMR, HSQC, HMBC, COSY and MS experiments (appendix C).

4.3.1 NMR spectroscopy

The assignment of ^{1}H NMR shifts, integral, number of protons, multiplicity, coupling constants, ^{13}C NMR shifts and coupling in COSY for compound **5** is shown in table 4.4. Proton and carbon shifts are assigned in accordance with ^{1}H (figure C.1), ^{13}C (figure C.2), HSQC (figure C.3), COSY (figure C.4) and HMBC (figure C.5) spectra (appendix C).



5

Table 4.4: Assigned shifts for compound **5**

Position	^{1}H (ppm)	Integral	Number of protons	M	J_t (Hz)	^{13}C (ppm)	COSY
1	-	-	-	-	-	174.22	-
2	2.32	2.10	2	t	7.2; 7.6	33.92	3/5
3	1.65	0.5∗4.03	2	m	7.6; 8.0	24.61	2, 4, 6/7
4	1.38	2.00	2	m	7.2; 7.6; 8	25.63	3/5
5	1.60	0.5∗4.03	2	m	7.6	29.14	2, 4, 6/7
6	3.42 - 3.53	3.97	4	m	4.0;	71.33	3/5, 8
7					5.2	72.42	3/5, 8
8	3.86	0.97	1	m		70.54	7/6, 9
9	3.61 - 3.70	4.86	4	m	4.0	64.15	8

Note 1: The integrals for the protons at carbons 3 and 5 are indistinguishable from each other, as are the signals for the protons at carbons 6 and 7
Note 2: It is assumed that the hydroxyl groups contribute to the value of the integral at 3.61 ppm - 3.70 ppm
Note 3: The peak for the proton on the carboxylic carbon is observed at 10.03 ppm

4.3.2 MS fragmentation

The main fragmentation of compound **3** from HR-MS by ESI ionization (appendix C) are shown in table 4.5.

Table 4.5: Selected MS fragments of compound **5**

m/z	Rel. intensity (%)	Fragment
453.2324	15	(2M+CH_3COO)-[H_2O]
393.2121	36	(2M-H)-[H_2O]
241.0851	7	$(M+Cl)^-$
187.0978	100	(M-H)-[H_2O]

5 Experimental

5.1 General methods

5.1.1 Solvents and reagents

Anhydrous solvents were obtained from a MB SPS-800 Solvent Purification System (MBraun) and stored over molecular sieves (4 Å). The starting compounds 1,2-isopropylideneglycerol and ethyl-6-bromohexanoate were bought from Sigma-Aldrich.

5.1.2 Chromatographic methods

Silica gel TLC plates (Al-sheets, 60, F254, Merck) were used to monitor the reactions, developed with 20 wt.% phosphomobdilic acid solution in ethanol. MN silica gel 60 (70-230 mesh ASTM) was used for flash chromatography. The eluent systems are specified for each compound.

5.1.3 Spectroscopic analyses

Nuclear Magnetic Resonance All samples were dissolved in $CDCl_3$ containing tetramethylsilane (TMS, 0.05% V/V) as internal reference.^{1}H NMR experiments were recorded on a Bruker Avance DPX 400 and Bruker Avance DPX 300 instruments. ^{13}C NMR and all 2D experiments were recorded on the Bruker Avance DPX 400. All spectra were analyzed using the TopSpin NMR Software.

Mass Spectroscopy Accurate mass determination was performed on a MAT95XL ThermoFinnigan (EI ionization mode) and Agilent G1969 TOF IS (ESI ionization mode) instruments. For ESI analyses, samples were injected into the instrument using an Agilent 1100 series HPLC instrument. A direct injection analysis without any chromatography was performed for the EI analyses.

Infrared Spectroscopy The IR experiments were performed on an Avatar FT-IR (Thermo Nicolet) instrument. The spectra were analyzed using the EZ OMNIC software.

5.2 Synthesis of ethyl 6-[(2.2-dimethyl-1,3-dioxalan-4-yl) methoxy]hexanoic acid (3)

5.2.1 Method 1: KOH, with toluene as solvent

A suspension of KOH (3.51 g, 62.6 mmol) in toluene (50 mL) was added to a solution of 1,2-isopropylideneglycerol (1) (4.40 g, 25.7 mmol) in toluene (50 mL). The mixture was heated to 70 ^{o}C, then added a solution of ethyl-6-bromohexanoate (2) (9.76 g, 43.7 mmol) in toluene (20 mL). The mixture was then heated to 150 ^{o}C and left to stir for 5 hours. The reaction was monitored by TLC. Spectroscopic analyses deem this method as unsuccessful to synthesize compound **3**.

5.2.2 Method 2: NaH, with DMF as solvent

A solution of 1,2-isopropylideneglycerol (76.01 mmol, 10.05 g) in dry DMF (20 mL) was added dropwise to a suspension of NaH (116.11 mmol, 2.79 g of 60% dispersion in mineral oil, washed with petroleum ether under N_2) in dry DMF (250 mL). The mixture was stirred at room temperature for 3 hours before a solution of ethyl-6-bromohexanoate (90.19 mmol, 20.12 g) in dry DMF (10 mL) was added dropwise. The resulting mixture was stirred for 4.5 days at room temperature, diluted with water (100 mL) and extracted with diethyl ether (3×200 mL). The combined organic phases were washed with water (3×50 mL) and dried (Na_2SO_4), and the solvents were removed under reduced pressure. The removal of solvents afforded an oil, which was purified by flash column chromatography (silica gel, toluene-acetone, 24:1) to give ethyl 6-[(2,2-dimethyl-1,3-dioxalan-4-yl)methoxy]hexanoate (1.39 g, 5.07 mmol, 7 % yield).

This procedure was repeated, with modifications; NaH was washed with dry diethyl ether instead of petroleum ether. The crude product was purified by flash column chromatography (silica gel, n-hexane-ethyl acetate, 7:3) to give ethyl 6-[(2,2-dimethyl-1,3-dioxalan-4-yl)methoxy]hexanoate (5 % yield).

TLC (toluene-acetone, 24:1): R_f = 0.50; (n-hexane:EtOAc, 7:3): R_f = 0.64

IR (Neat NaCl tablet): C–H stretch: 2937 cm^{-1} ν_{as} CH_3, 2867 cm^{-1} ν_s CH_3, C–O stretch: 1736 cm^{-1} δ_{as}, C–O–C stretch: 1118 cm^{-1} δ_s

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.25 (t, J_t = 7.2 Hz, 3H, C-1), 4.10 - 4.15 (q, J_t = 7.2 Hz, 2H, C-2), 2.3 (t, J_t = 7.6 Hz, 2H, C-4), 1.60 (m, 2H, C-5), 1.36 (m, 2H, C-6), 1.70 (m, 2H, C-7), 3.50 (m, 2H, C-8), 3.42 (m, 1H, C-9), 3.50 (m, 1H, C-9),

4.24 (p, J_t = 5.8 Hz, 6.2 Hz, 1H, C-10), 3.70 - 3.74 (dd, J_t = 1,8 Hz, 6,4 Hz, 1H, C-11), 4.03 - 4.07 (m, 1H, C-11), 1.42 (3H, C-13), 1.36 (3H, C-14)

^{13}C NMR (400 MHz, $CDCl_3$): δ 14.27 (CH_3, C-1), 60.22 (CH_2, C-2), 173.70 (quaternary C, C-3), 34.30 (CH_2, C-4), 29.25 (CH_2, C-5), 25.45 (CH_2, C-6), 24.79 (CH_2, C-7), 71.53 (CH_2, C-8), 71.89 (CH_2, C-9), 74.77 (CH, C-10), 66.93 (CH_2, C-11), 109.39 (quaternary C, C-12), 26.79 (CH_3, C-13), 25.68 (CH_3, C-14)

MS (EI, LR): 293.1 (4) $[M+H_3O]^+$, 274.2 (2) $[M]^+$, 259.2 (30) $[M\text{-}CH_3]^+$, 234.3 (5) $[M+2H\text{-}C_3H_6]^+$, 189.1 (8) $[M+H\text{-}C_5H_{12}O]^+$, 171.2 (25) $[M+H\text{-}C_5H_{14}O_2]^+$, 143.2 (32) $[M+H\text{-}C_7H_{18}O_2]^+$, 115.1 (25) $[M\text{-}C_8H_{18}O_3]^+$, 101.1 (100) $[M\text{-}C_9H_{20}O_3]^+$, 97.1 (40) $[M\text{-}C_9H_{22}O_4]^+$, 73.0 (20) $[M\text{-}C_{10}H_{20}O_4]^+$, 69.0 (36) $[M\text{-}C_{11}H_{24}O_4]^+$

5.2.3 Method 3: Na, with toluene as solvent

Pieces of Na (0.58 g, 25.23 mmol) and dry toluene (6 mL) were heated to weak refluxing followed by vigorously stirring to form a grey suspension. 1,2-isopropylideneglycerol (1) (2.8 mL, 22.53 mmol) was added under stirring to the cooled mixture, which was then heated to 110 ^{o}C and kept at this temperature for 2 hours, and then cooled. Ethyl-6-bromohexanoate (2) (4.4 mL, 24.73 mmol) was added dropwise at 80 ^{o}C, and the mixture was stirred at this temperature for 19 hours. The reaction was monitored by TLC. The reaction mixture was cooled to room temperature, then added EtOH (6 mL) and stirred for 45 minutes. The solvents were removed under reduced pressure. Water (20 mL) and DCM (30 mL) were added, and the phases were separated. The compound was extracted with DCM (3×20 mL); the combined organic phases were dried (Na_2SO_4) and the solvents were removed under reduced pressure. The crude was purified by flash column chromatography (silica gel, n-hexane-ethyl acetate, 7:3) to give ethyl 6-[(2,2-dimethyl-1,3-dioxalan-4-yl)methoxy]hexanoate (3) (38 % yield).

TLC (n-hexane:EtOAc, 7:3): R_f = 0.68

5.3 Synthesis of 6-[2,2-dimethyl-1,3-dioxalan-4-yl) methoxy]hexanoic acid (4)

5.3.1 With KOH (25 % in MeOH)

A solution of KOH (25 % in MeOH, 8.6 mL) was added to a solution of ethyl 6-[(2,2-dimethyl-1,3-dioxalan-4-yl)methoxy]hexanoate (3) (1.39 g, 5.07 mmol) in n-BuOH (150 mL) and the reaction mixture was heated to 145 ^{o}C for 2 hours, after which

the solvent was removed under reduced pressure. Aqueous H_2SO_4 (25 %, 17 mL) was added, and the reaction mixture was stirred for 5 minutes. Distilled water was added and the solution was stirred vigorously for 30 min. The product was extracted with DCM (3×50 mL), and the combined organic phases were washed with water (4×40 mL) and dried (Na_2SO_4), and the solvents were removed under reduced pressure to give 6-[2,2-dimethyl-1,3-dioxalan-4-yl)methyoxy]hexanoic acid (4). This compound was not purified further.

MS (EI, HR): 202.1566 [M-CO_2]

5.3.2 With 1.6 M NaOH

A solution of 1.6 M NaOH (1 mL) was added to a solution of ethyl 6-[(2,2-dimethyl-1,3-dioxalan-4-yl)methoxy]hexanoate (3) (0.12 g, 0.45 mmol) in dioxane (35 mL). The mixture was left to stir for 4 days at room temperature, before it was neutralized with 1 M HCl. The mixture was extracted with DCM (3×15 mL), and the combined organic phases were dried (Na_2SO_4) and removed under reduced pressure which gave an oil. Analyses by TLC revealed that none of the products had the expected R_f value.

5.4 Synthesis of 6(2,3-dihydroxypropoxy)hexanoic acid (5)

Ethyl 6-[(2,2-dimethyl-1,3-dioxalan-4-yl)methoxy]hexanoate (3) (0.50 g, 1.83 mmol) was mixed with concentrated HCl (0.3 mL) and MeOH (15 mL) and refluxed for 4 hours. The cooled mixture was added to water (40 mL) and extracted with chloroform (2×20 mL). The aqueous layer was then saturated with $NaHCO_3$ (observed pH = 9), then extracted with chloroform (3×20 mL). The combined organic phases were dried ($MgSO_4$) and removed under reduced pressure. The removal of solvents afforded and oil which was purified by flash column chromatography (silica gel, toluene, then MeOH) to give 6-(2,3-dihydroxypropoxy)hexanoic acid (5) (0.26 g, 1.26 mmol, 69 % yield).

TLC (toluene-acetone, 4:1): R_f = 0.68

^{1}H NMR (400 MHz, $CDCl_3$): δ 2.32 (t, J_t = 7.2 Hz, 7.6 Hz, 2H, C-2), 1.57 - 167 (m, J_t = 7.6 Hz, 8.0 Hz, 4H, C-3, C-5), 1.38 (m, J_t = 7.2 Hz, 7.6 Hz, 8.0 Hz, 2H, C-4), 3.42 - 3.53 (m, J_t = 4.0 Hz, 5.2 Hz, 4 H, C-6, C-7), 3.86 (m, 4H, C-8), 3.16 - 3.70 (m, J_t = 4.0 Hz, 4H, C-9)

^{13}C NMR (400 MHz, $CDCl_3$): δ 174.22 (CO, C-1), 33.92 (CH_2, C-2), 24.61

(CH_2, C-3), 25.63 (CH_2, C-4), 29.14 (CH_2, C-5), 71.33 (CH_2, C-6), 72.42 (CH_2, C-7), 70.54 (CH, C-8), 64.15 (CH_2, C-9)

MS (EI, HR): 187.0976 (M-H)-[-H2O], 241.0848 (M+Cl)-, 393.213 (2M-H)-[H2O], 453.2324 (2M+CH3COOO)-[H2O]

5.5 Hydrolysis of ethyl-β-apo-8'-carotenoate (6)

Ethyl-β-apo-8'-carotenoate (6) (0.50 g, 1.09 mmol) was dissolved in n-BuOH (100 mL). KOH (25 % in MeOH, 3.6 mL) was added, and the solution was heated to 135 ^{o}C for 2 hours. The solution was cooled until room temperature and the solvent was removed under reduced pressure. Aqueous H_2SO_4 (25 %, 6 mL) was added to the solution which was stirred for 5 min. Water (50 mL) was added to the mixture which was then stirred vigorously for 30 min. The product was extracted with DCM (6×30 mL) and the combined organic phases were washed with water (4×100 mL), dried (Na_2SO_4) and the solvents were removed under reduced pressure to give β-apo-8'-carotenoic acid (7).

TLC (toluene-acetic acid-methanol, 6:1:1): R_f = 0.52, which is in accordance with the literature.[24]

5.6 Synthesis of 6-(2-hydroxy-3-(((2E,4E,6E,8E,10E,12E,14E, 16E)-2,6,11,15-tetramethyl-17-(2,6,6-trimethylcyclohex-1-en-1-yl)heptadeca-2,4,6,8,10,12,14,16-octaenoyl)oxy)propoxy) hexanoic acid (8)

6-(2,3-dihydroxypropoxy)hexanoic acid (5) (0.11 g, 0.55 mmol) was dissolved in dry DCM (25 mL). β-apo-8'-carotenoic acid (7) (0.15 g, 0.39 mmol) and DMAP (0.07 g, 0,56 mmol) was dissolved in dry DCM (5 mL) and added to the solution under stirring. DCC (0.14 g, 0.68 mmol) was dissolved in dry DCM (2 mL) and added dropwise to the reaction mixture, which was then left to stir under N_2 at room temperature for 1 day. The reaction was monitored by TLC. The reaction mixture was then filtered to remove byproduct of DCC and the solvents were removed under reduced pressure to give a powdery crude (0.4 g) which was run through the flash column (silica gel, n-pentane - acetone, 8:2) to give five fractions.

5.6.1 Reaction mechanism

The Steglich esterification is a variation of an esterfication with dicyclohexylcarbodiimide (DCC) as a coupling reagent and 4-dimethylaminopyridine (DMAP) as a catalyst. The carboxylic acid reacts with DCC to form O-acyl-isourea, which is more reactive than the free acid. The alcohol then attacks this intermediate, forming DCU, which is observed as white precipitate, and the corresponding ester. The detailed mechanism of the Steglich is shown in figure 5.1.

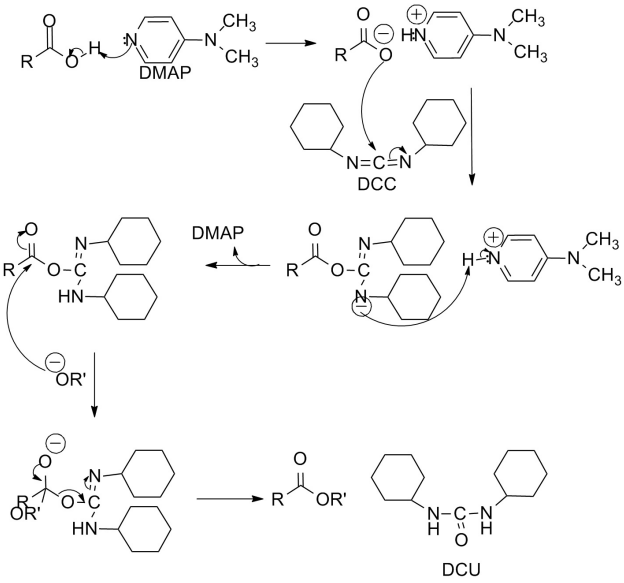


Figure 5.1: The Steglich esterification

6 Conclusion

The synthesis of compound **3** was performed 7 times; the desired compound was obtained successfully three times, although the yield was only 38 % at the most. Compound **4** was also successfully synthesized, but the exact yield could not be calculated due to inadequate purification of the product. The synthesis of compound **5** was performed successfully twice from compound **3**, with the yield being 69 %. Synthesis of compound **8** was attempted three times, but spectroscopic analyses were inconclusive about the success.

Further works that must be done in order to obtain the carotenoid cationic lipid **10** include attachment of bromoethanol and trimethylamine.

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Appendices

A Spectroscopic analyses of compound 1

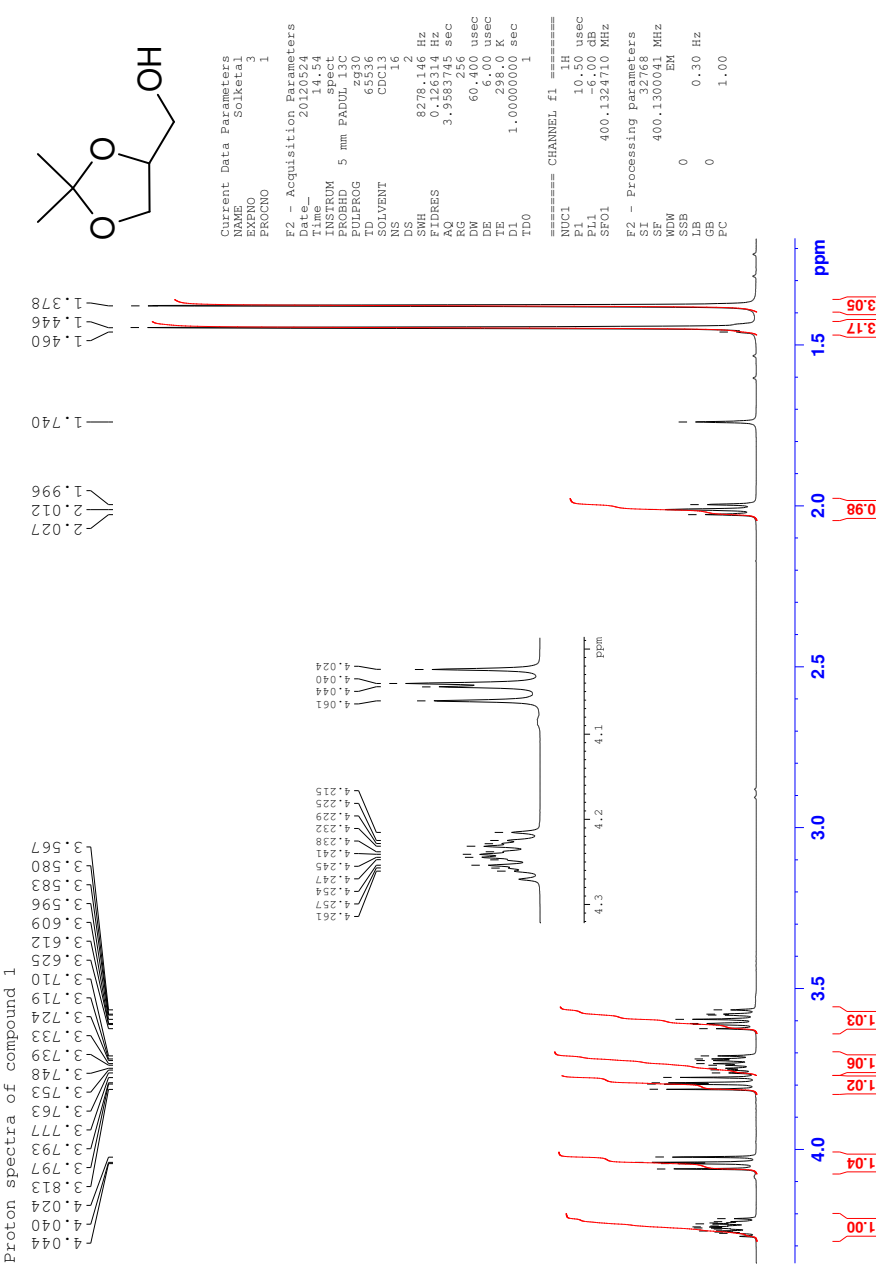

Figure A.1: ^{1}H NMR spectrum of compound **1**

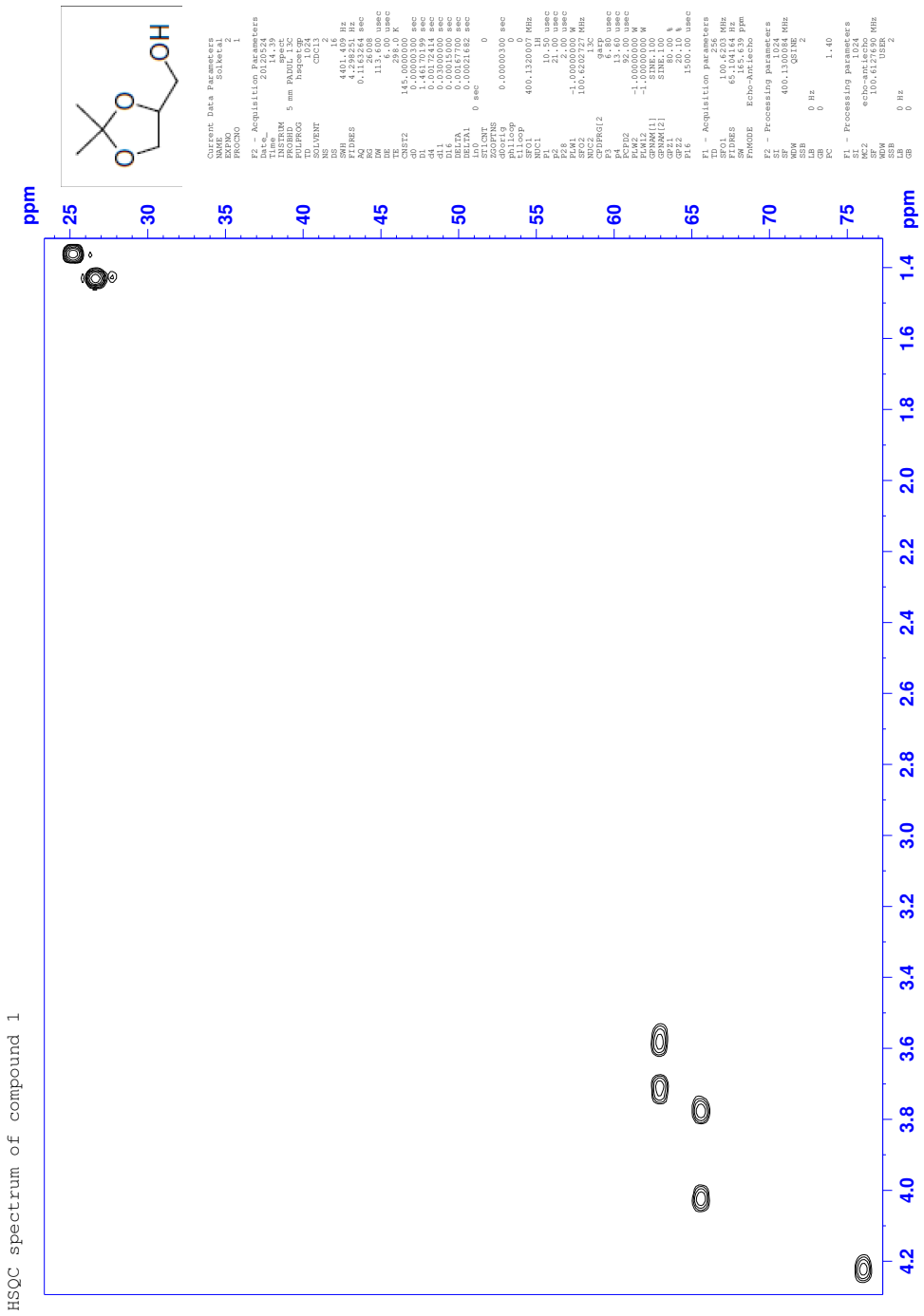

Figure A.2: HSQC spectrum of compound **1**

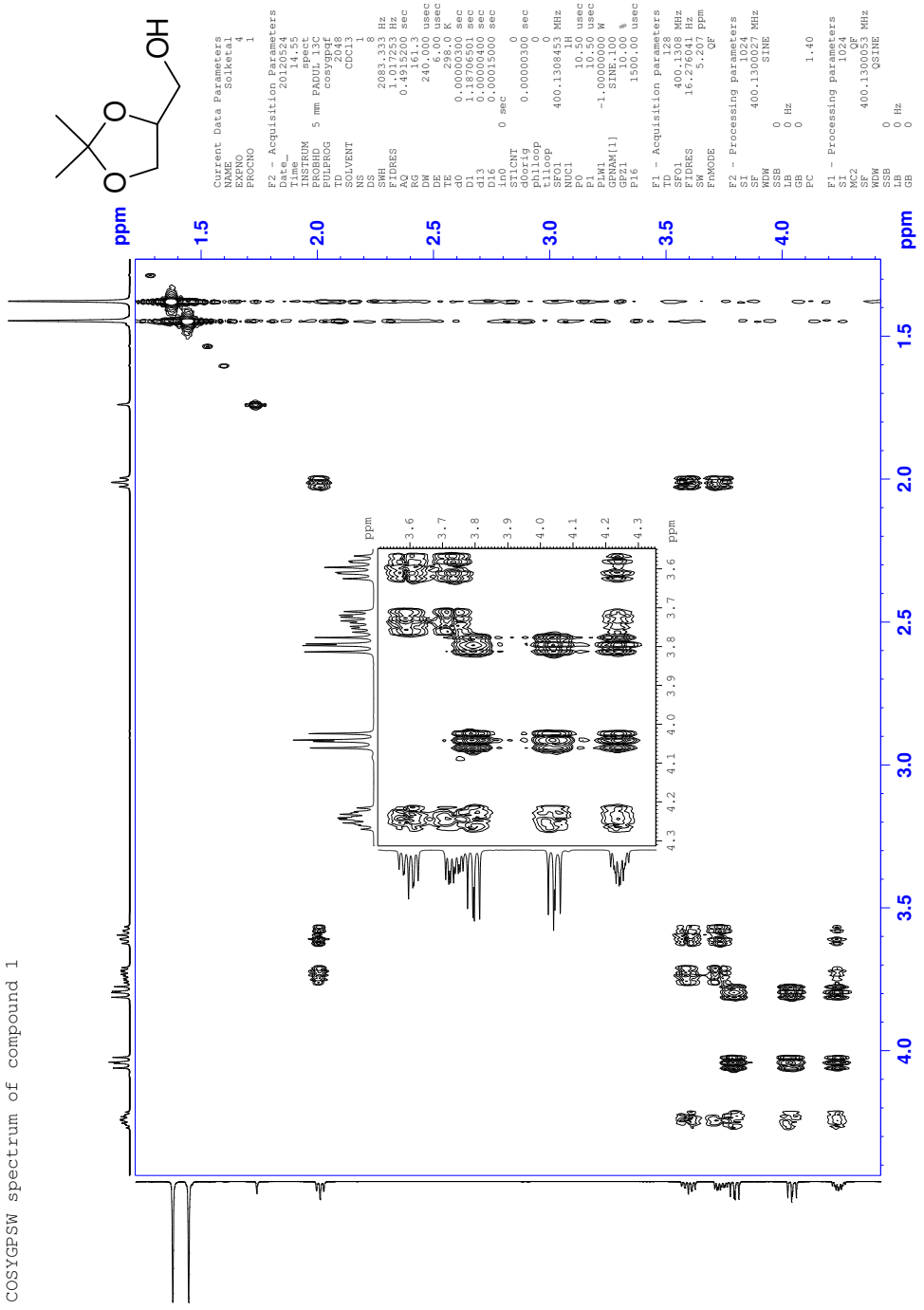

Figure A.3: COSY spectrum of compound **1**

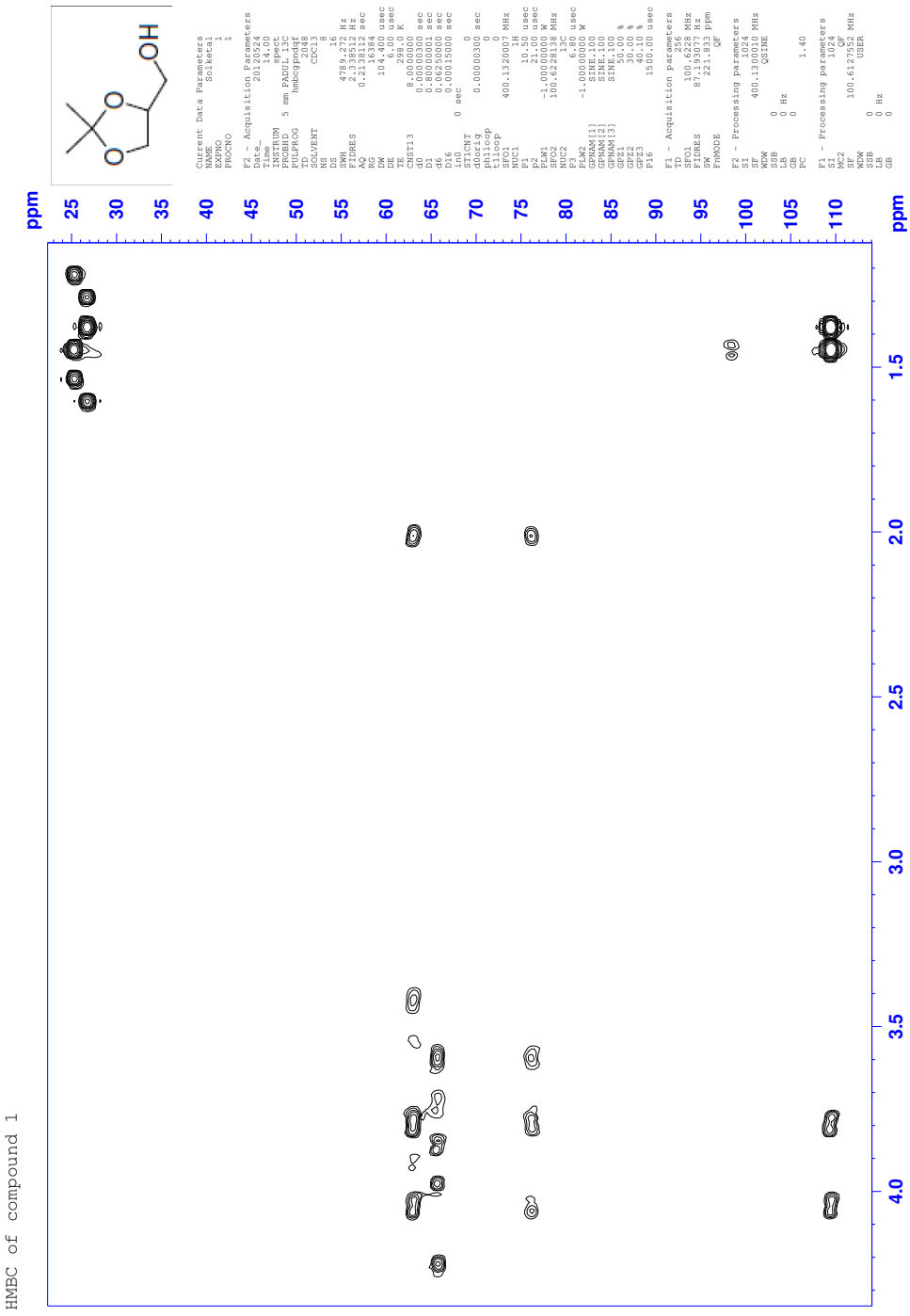

Figure A.4: HMBC spectrum of compound **1**

B Spectroscopic analyses of compound 3

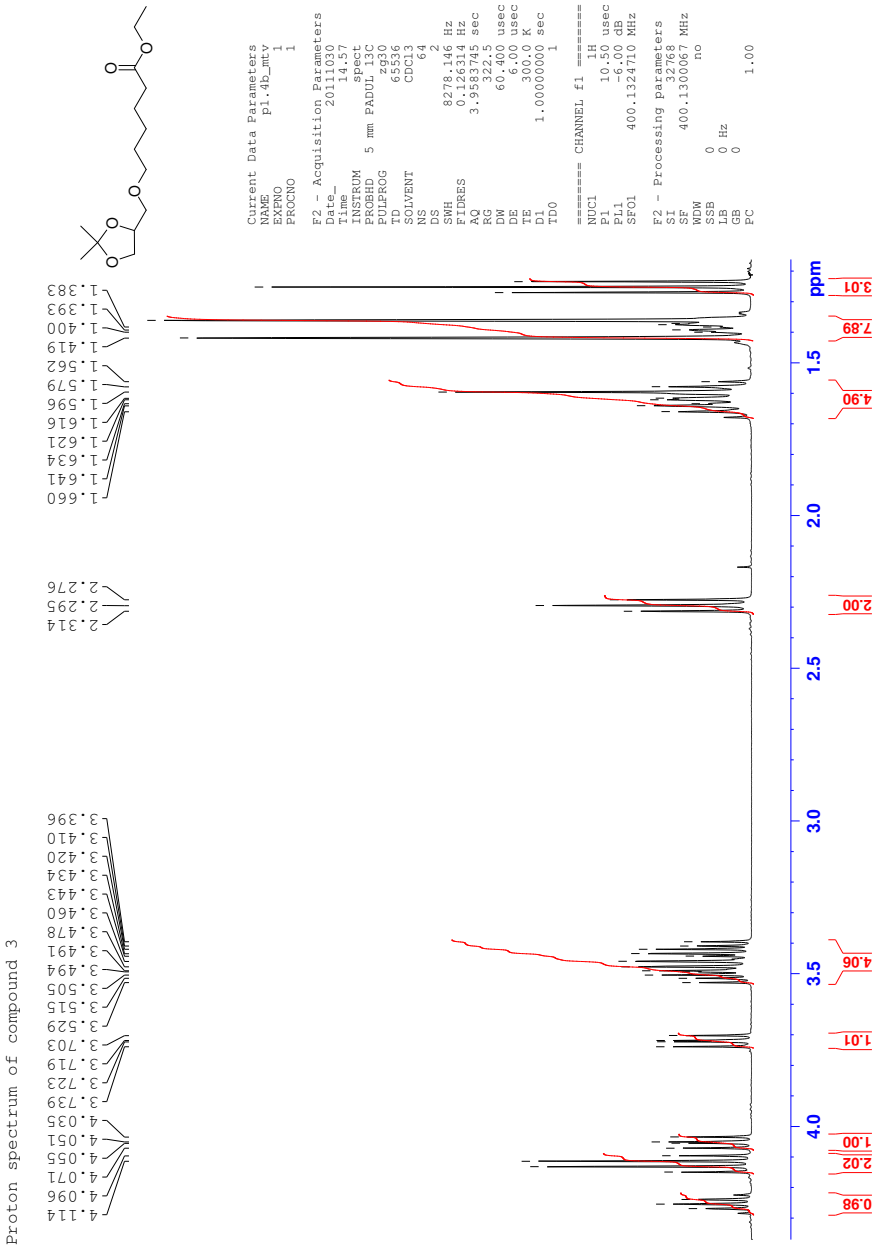

Figure B.1: ^{1}H NMR spectrum of compound **3**

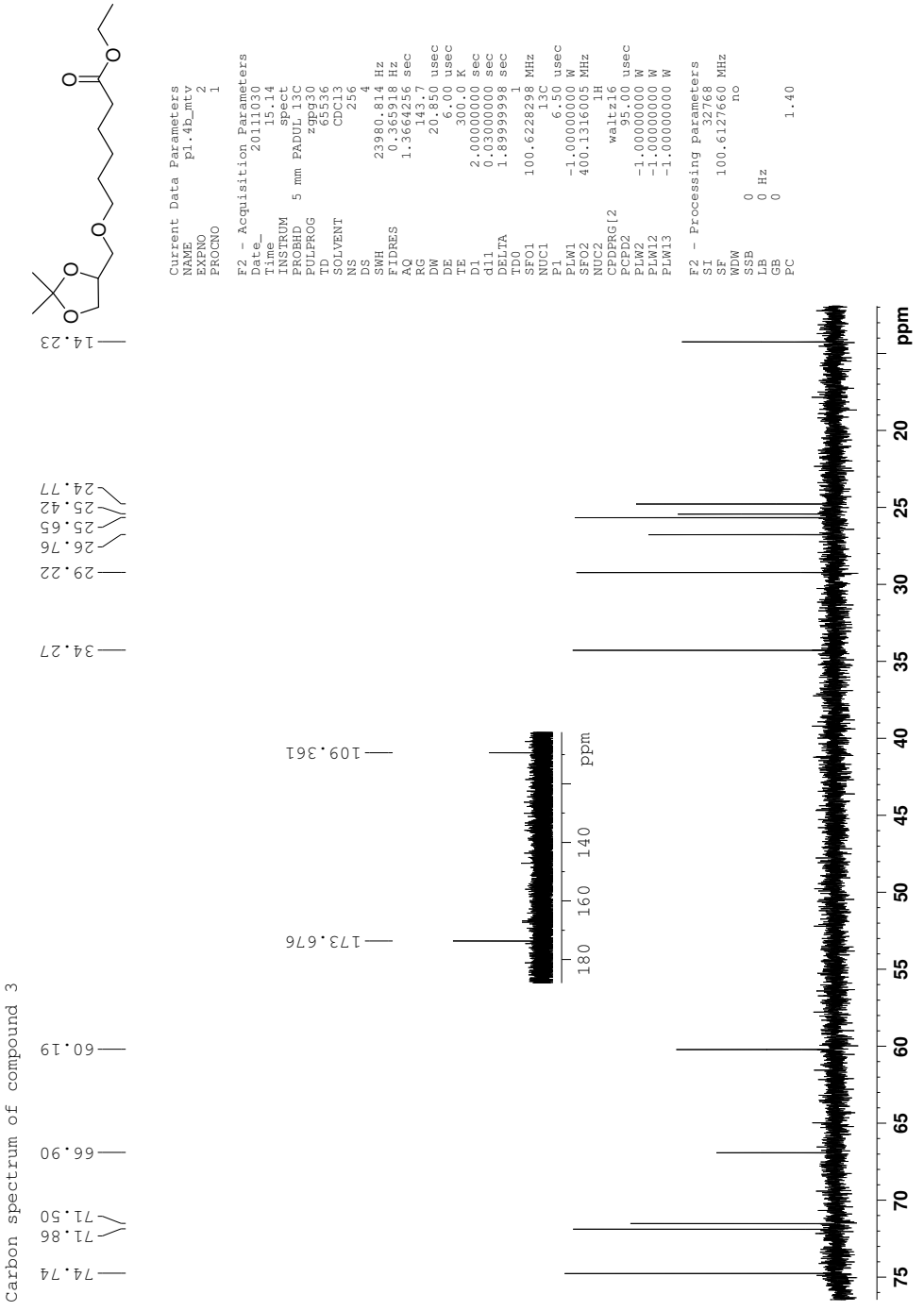

Figure B.2: ^{13}C NMR spectrum of compound **3**

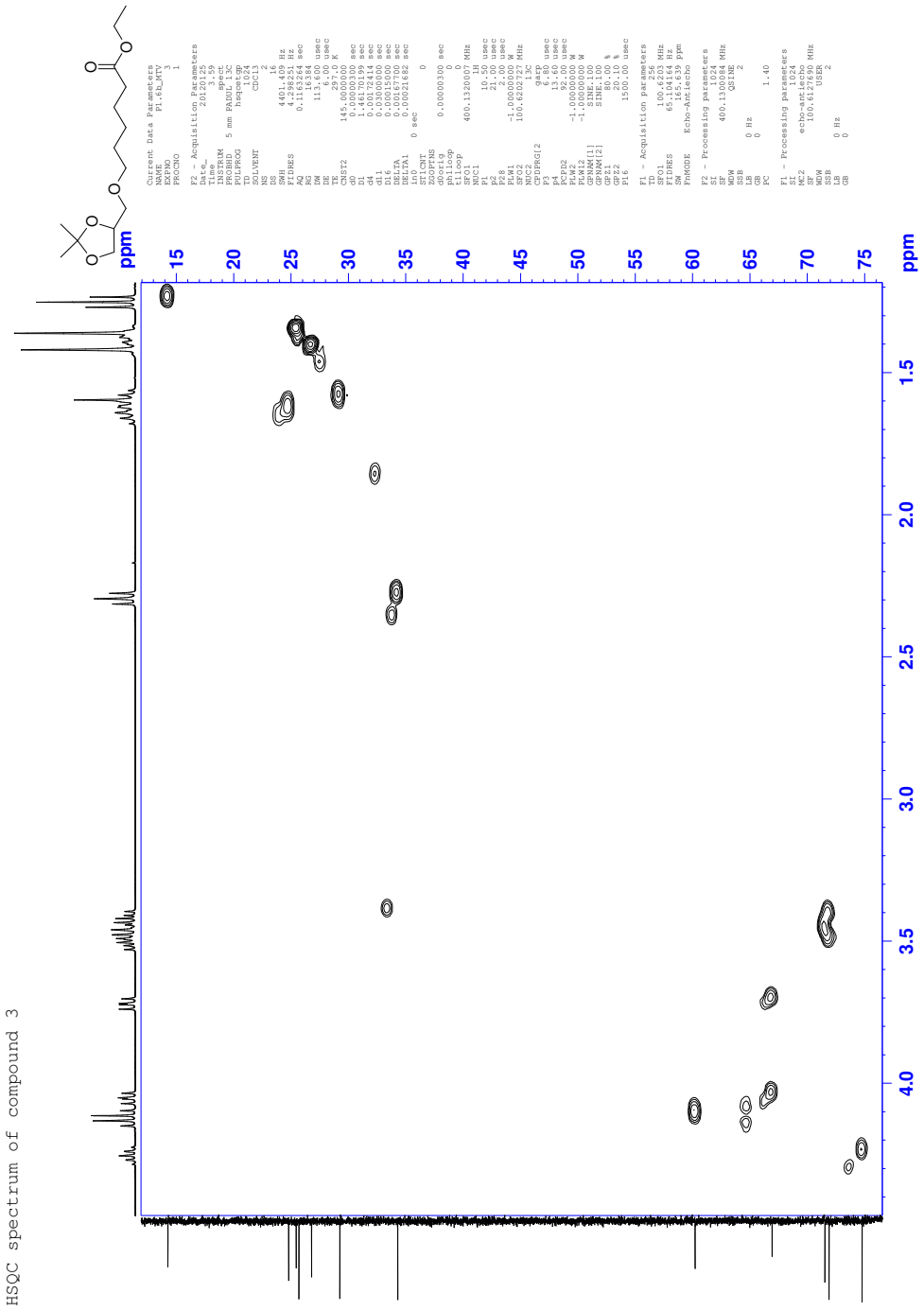

Figure B.3: HSQC spectrum of compound **3**

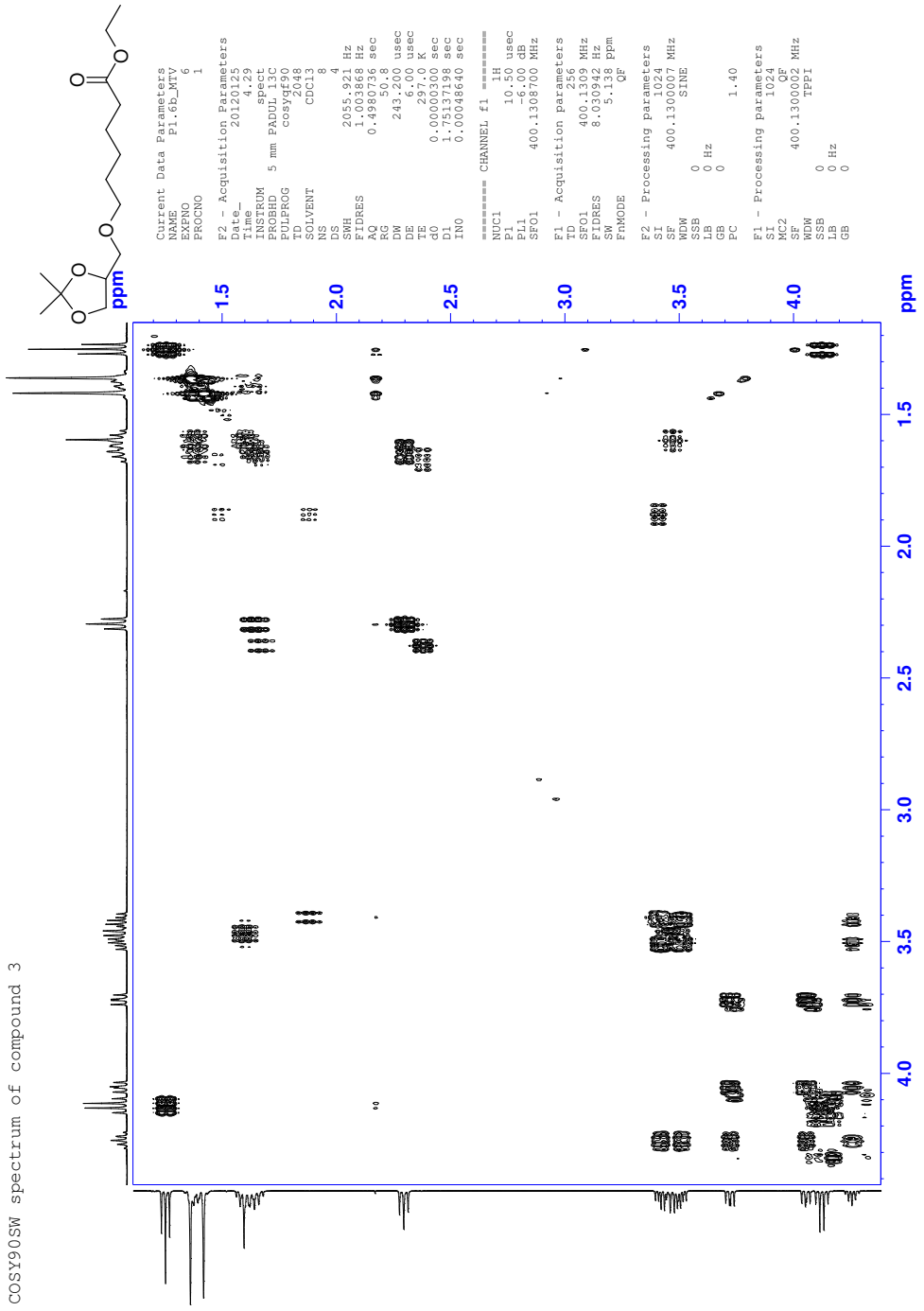

Figure B.4: COSY spectrum of compound **3**

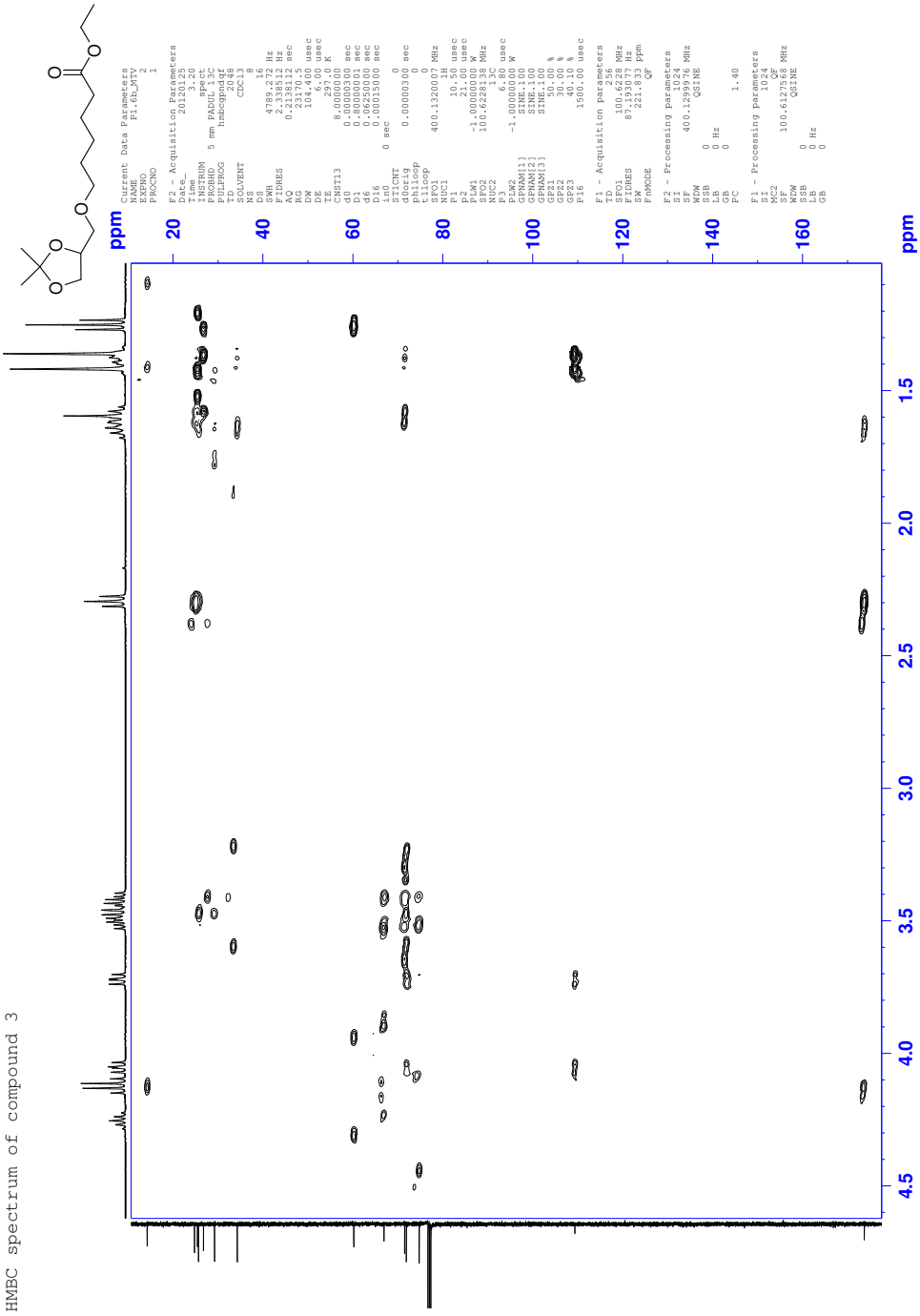

Figure B.5: HMBC spectrum of compound **3**

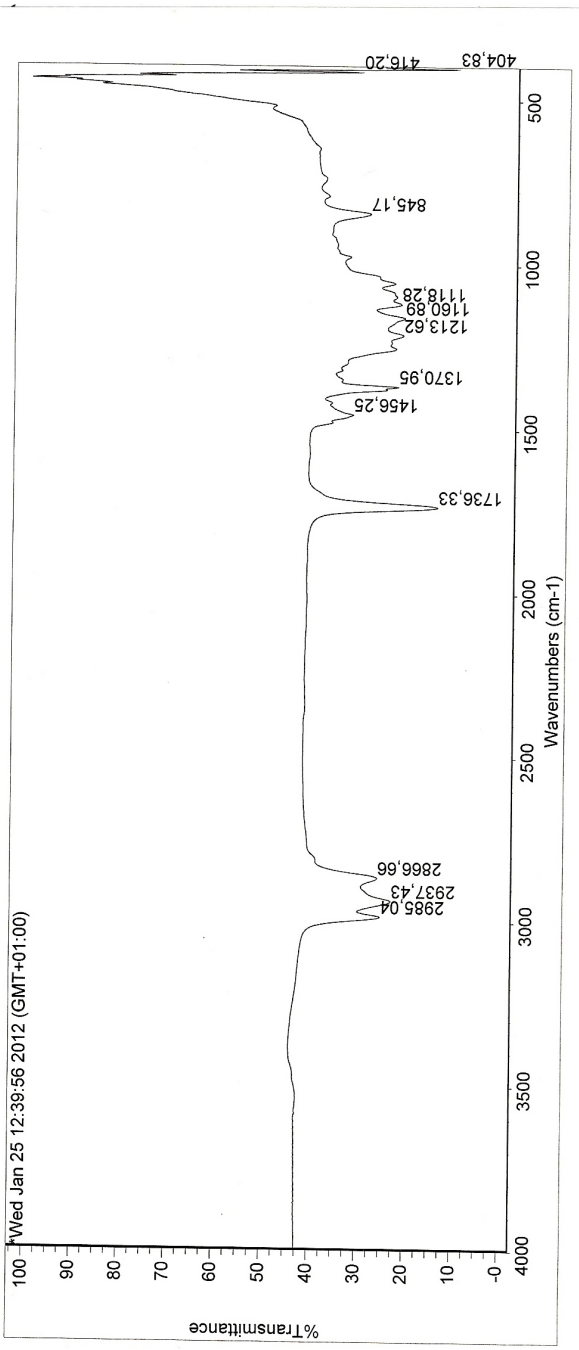

Figure B.6: IR spectrum of compound **3**

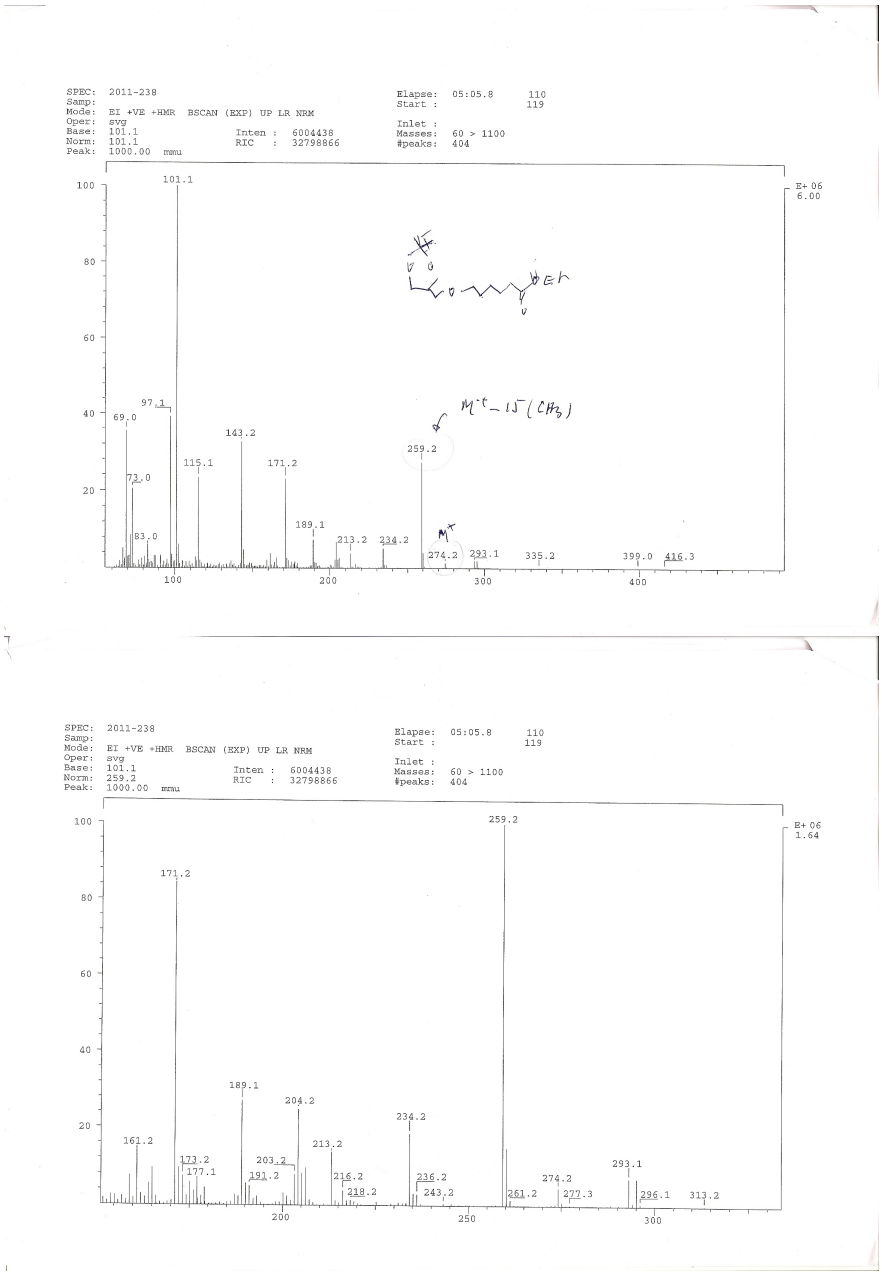

Figure B.7: MS spectrum of compound **3**

C Spectroscopic analyses of compound 5

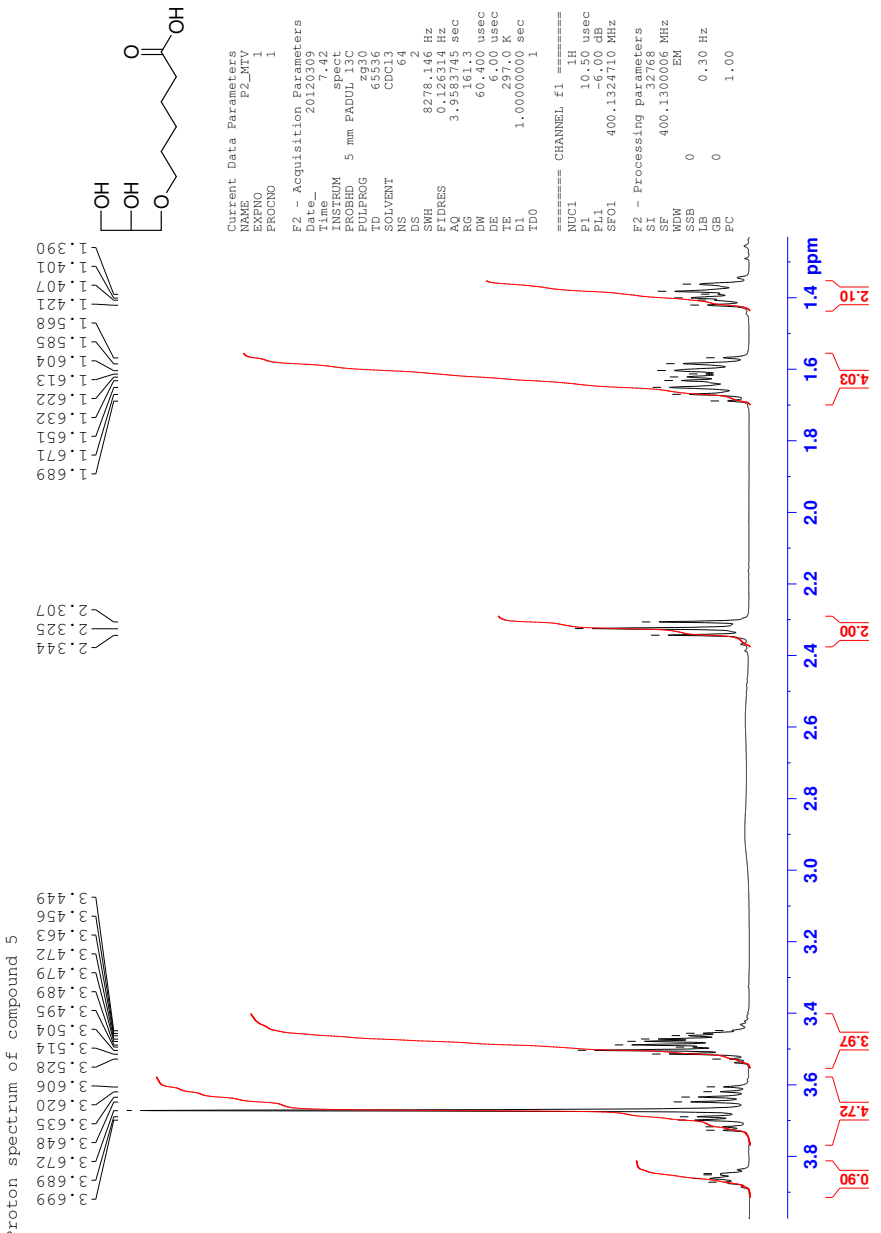

Figure C.1: ^{1}H NMR spectrum of compound **5**

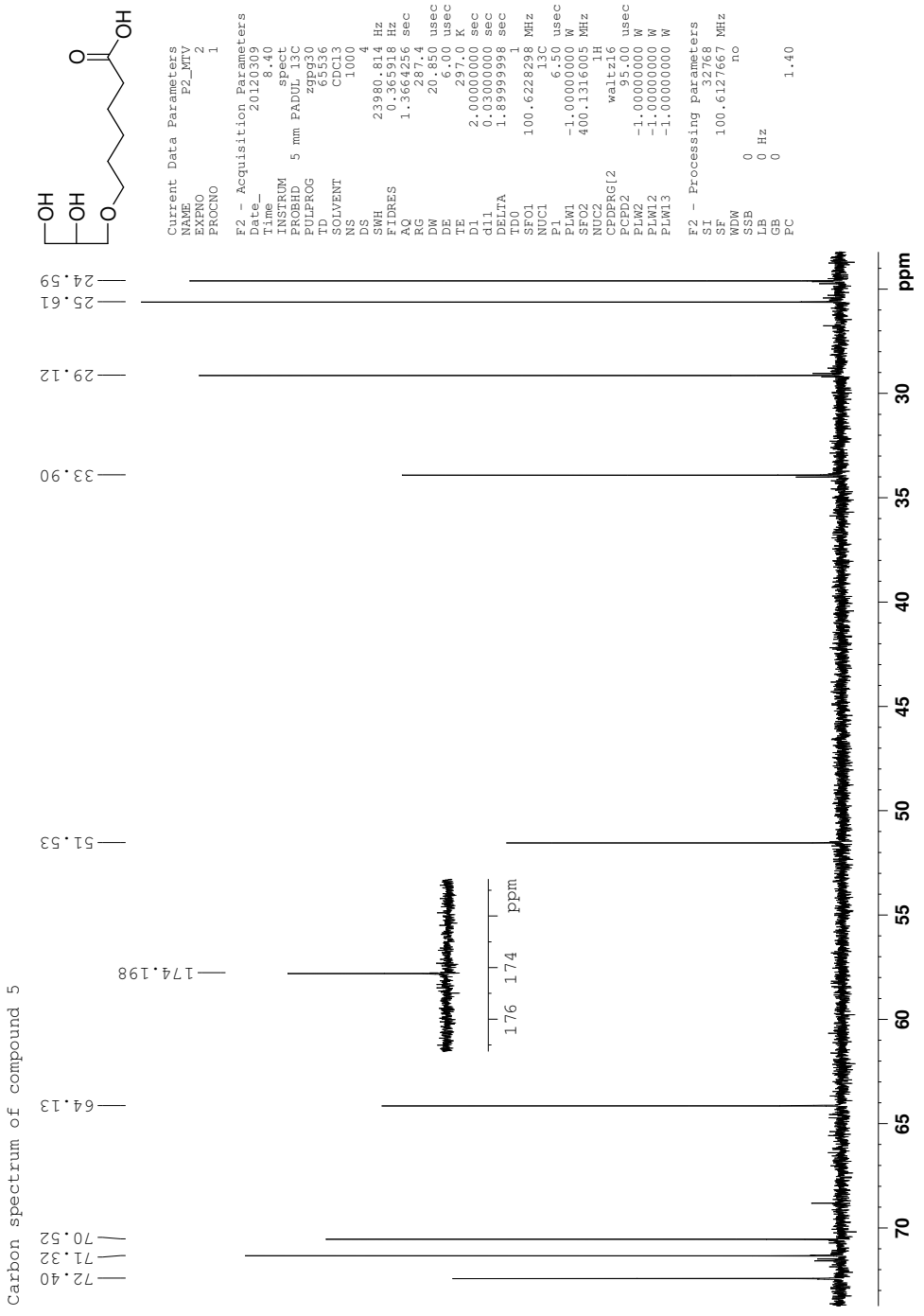

Figure C.2: ^{13}C NMR spectrum of compound **5**

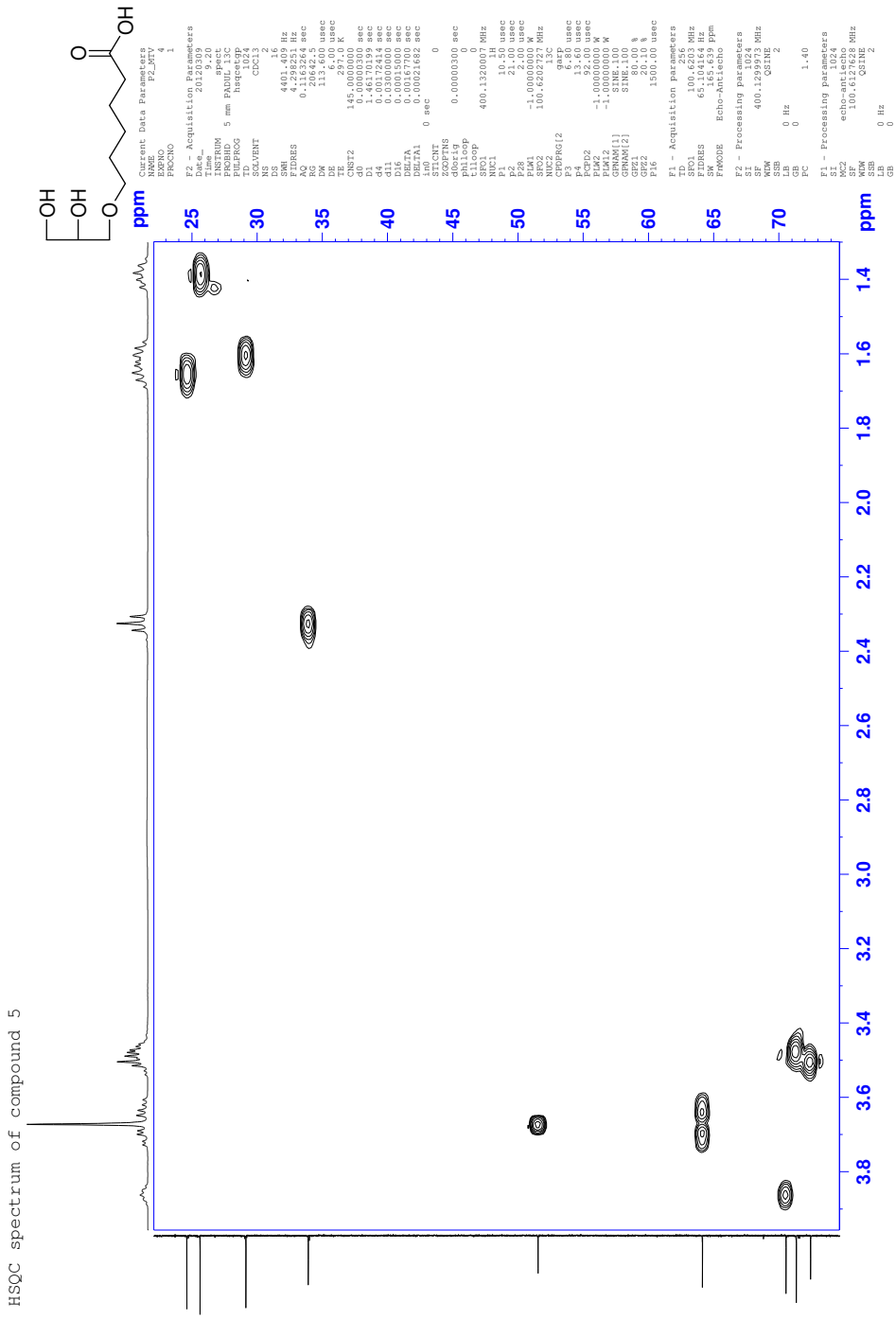

Figure C.3: HSQC spectrum of compound **5**

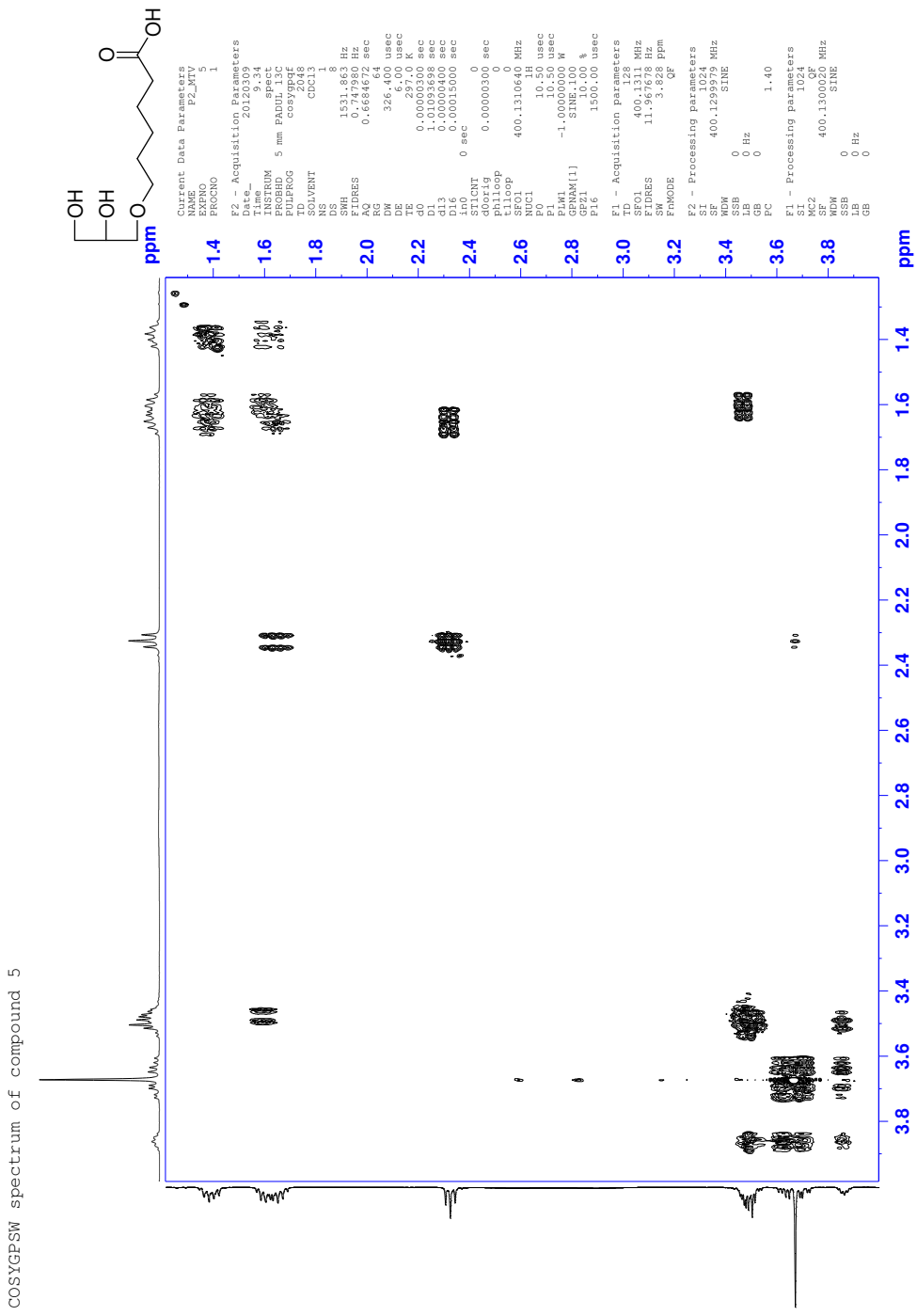

Figure C.4: COSY spectrum of compound **5**

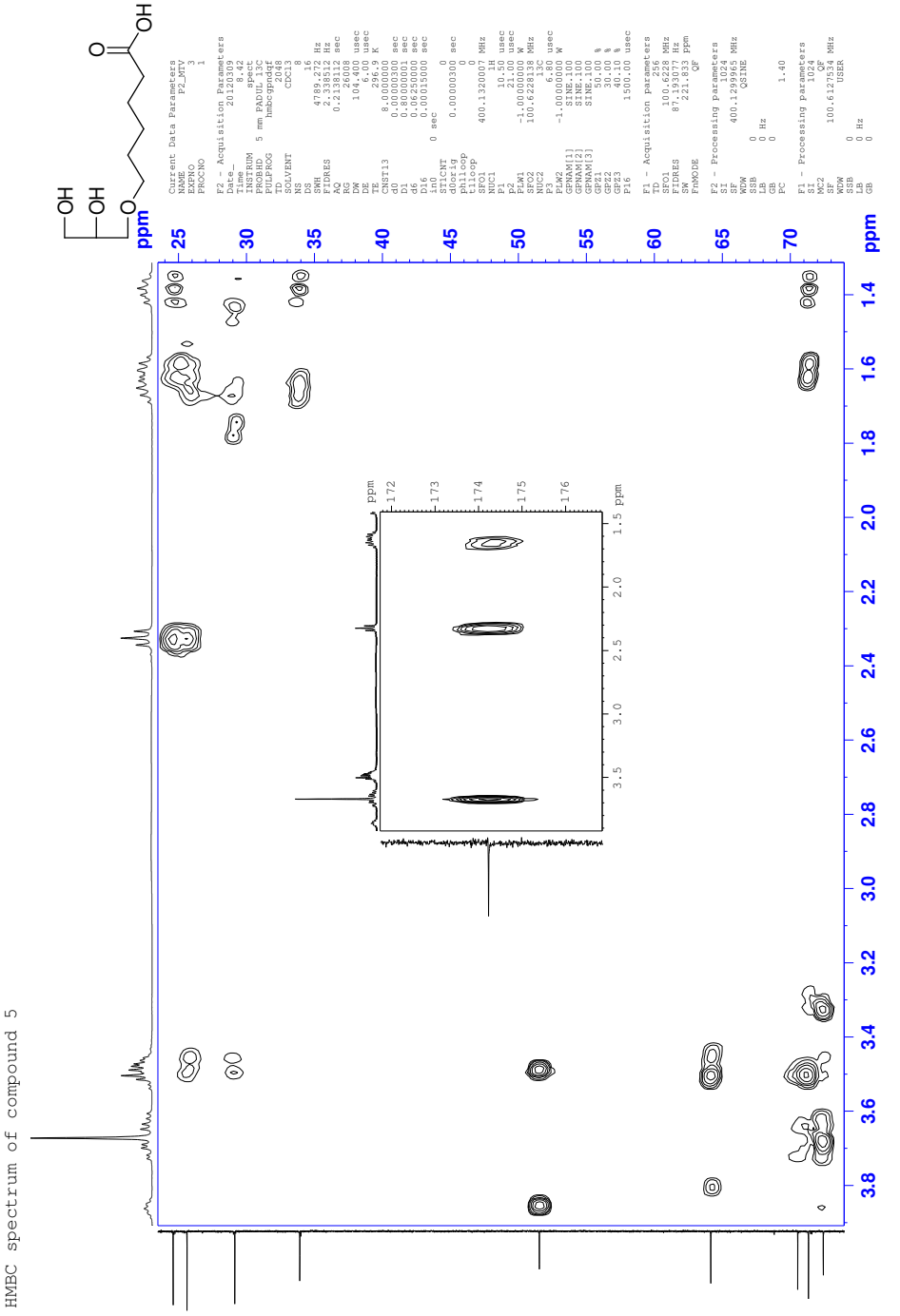

Figure C.5: HMBC spectrum of compound **5**

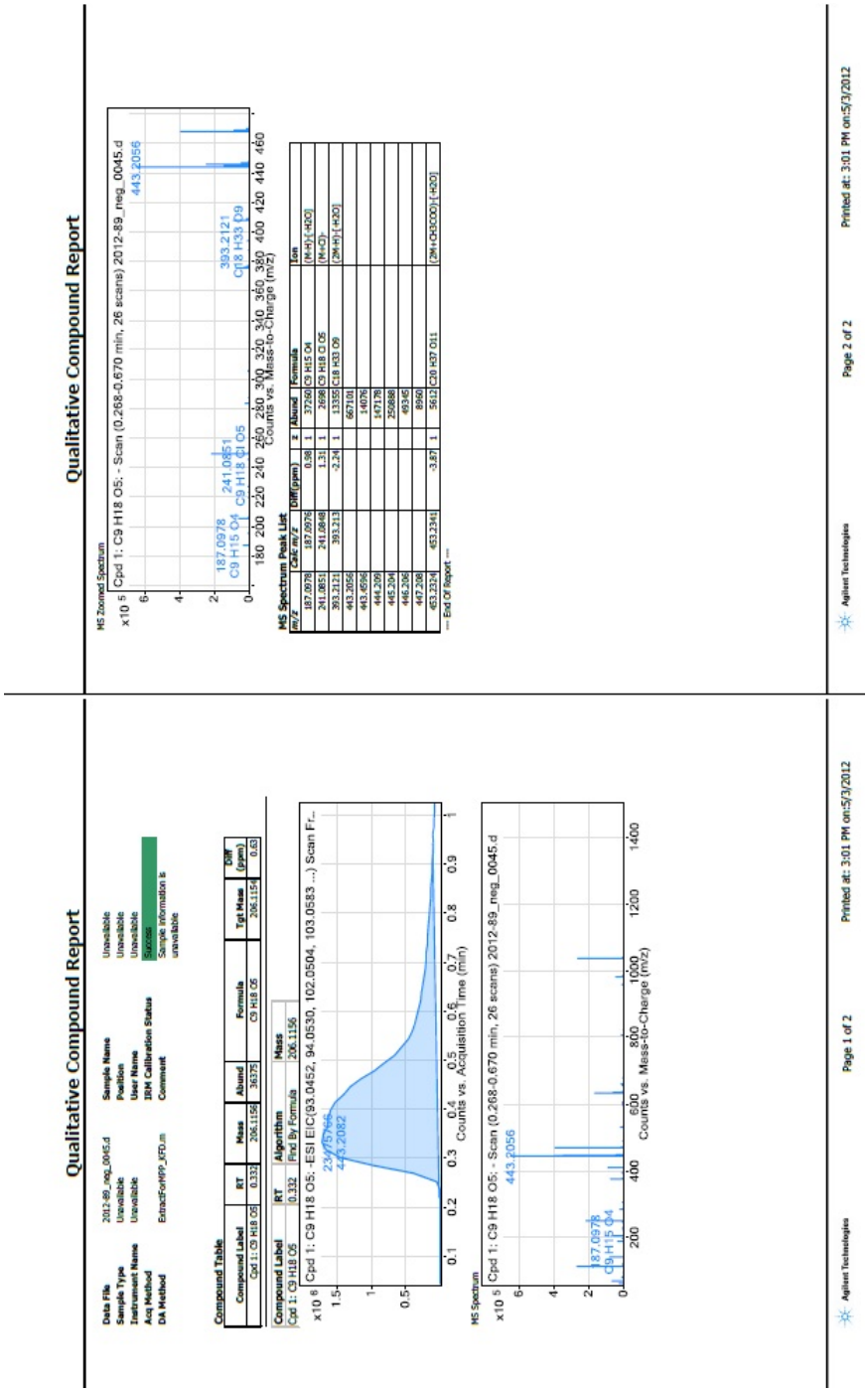

Figure C.6: MS analysis of compound **5**