

Synthetic Lipids from Natural Alcohol Building Blocks

A thesis submitted by

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in partial fulfillment of the requirements for the degree of Master of Science

in

Chemical and Biological Engineering

Tufts University

February 2022

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## Abstract

Lipid nanoparticles (LNPs) have found their way to the forefront of modern drug delivery systems by utilizing the many advantages of their predecessors while avoiding the shortcomings. The amphipathic properties of lipids allow for membrane permeability and therefore more effective delivery.

We study two classes of lipids— ester O-series and amide N-series. Previously studies have found that O-series lipids tend to target the liver, and N-series lipids tend to target the lungs. To expand the potential target organs, it is critical to expand the lipid library. This was accomplished by attempting to incorporate natural alcohols— geraniol, 10-undecen-1-ol, 2-butyl-1-octanol, farnesol, citronellol, phytol, 9-heptadecanol, and 2-hexyl-1-decanol— via Michael Addition reactions between amine head groups and acrylate tails.

Electrospray Ionization-Mass Spectrometry (ESI-MS) confirmed the successful incorporation of the natural alcohols into the lipid structure, and *in vivo* studies have highlighted the alcohols with greatest delivery efficacy and the potential for even greater expansion of the lipid library.

## Acknowledgements

First, I would like to thank my adviser, Dr Qiaobing Xu, for his patience as we navigated COVID-19 and found a project that could be successful despite the many unforeseen obstacles that the pandemic created that impacted how research was conducted and labs were operated. I would also like to thank Dr Hyunmin Yi, as well as Dr Mingdi Yan from University of Massachusetts Lowell for serving on my thesis committee.

I would also like to thank all of lab members and Changfeng Huang in particular. Thanks for mentoring me into an organic chemist and always being willing to further explain the reaction mechanisms and how everything fits into the bigger picture as questions arose. I would also like to thank Min Qiu, who is responsible for formulating the synthesized lipids into LNPs for drug delivery.

Finally, I'd like to thank my friends and family who have continued to support me and have kept me sane and afloat as I have been on this journey.

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## Introduction

Lipid nanoparticles (LNPs) have been a major focus in drug delivery since the early 1990's.<sup>[12-39]</sup> LNPs have great potential as drug delivery vehicles because they are both biocompatible and physiochemically diverse.<sup>[40,41]</sup> Some benefits include the ability to transport across semipermeable membranes and remain functional in harsh microenvironments such as the acidic gastrointestinal tract (GI). Lipids are also advantageous for delivering poorly water-soluble drugs because of their amphipathic properties—hydrophilic head groups and hydrophobic tails, combined through Michael Addition reactions. LNPs have various potential therapeutic applications. These include oral delivery<sup>[42]</sup>, targeted delivery<sup>[43]</sup>, vaccines<sup>[44]</sup>, and mRNA therapies<sup>[45]</sup>.

LNPs have been extensively studied as potential vehicles for protein and genetic delivery.<sup>[1-5]</sup> The Xu lab studies two main groups of tails: esters (O-series) and amides (N-series). One common O-series tail studied is O12B and N12B the equivalent N-series tail. The naming convention is as follows: O indicates an ester, 12 refers to the number of atoms following the ester group, and B indicates the presence of a disulfide bond in the tail. The same head group, 3,3'-diamino-N-methyldipropyl-amine (306), was used for each lipid studied. 306 was chosen because it has recently been demonstrated to have a higher delivery efficacy than a current FDA approved lipid formulation.<sup>46</sup> Dependent on the molar ratios of 306 and the corresponding tails, a maximum of four tails can be combined. 306-4O12B (4 indicates four tails) has been studied extensively in the group.

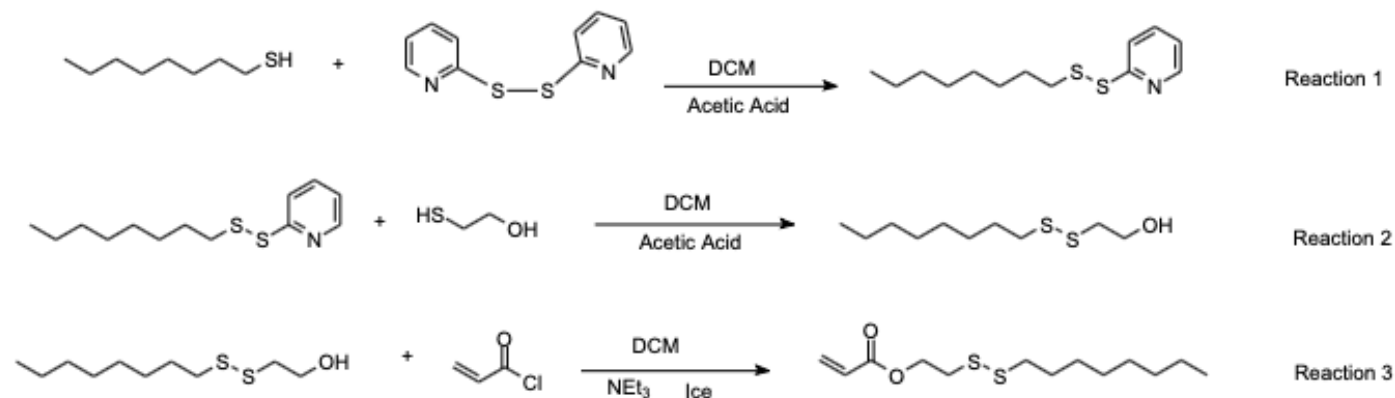
Recently it was discovered that three tails could provide comparable efficacy of drug delivery. This discovery allowed for further exploration in additional tails that could be incorporated into this newly created vacancy. Incorporation of natural alcohols is one such

possibility. This paper will discuss the potential for natural alcohols in LNP drug delivery. A few key properties of the natural alcohol tails are length, saturated versus unsaturated, and branching versus linear alcohols.

## Materials and Methods

### Synthesis of O12B

O12B is synthesized through a series of three reactions as shown in **Figure 1**.



**Figure 1.** O12B Synthesis

First, 2,2'-dipyridyl disulfide (26.4 g, 120 mmol, Oakwood Chemical) is dissolved in dichloromethane (200 mL, DCM, Sigma Aldrich). Acetic acid (1.5 mL, Sigma Aldrich) is then added. Octan-1-thiol (20.24 g, 100 mmol, Millipore) is added dropwise. The reaction progresses overnight.

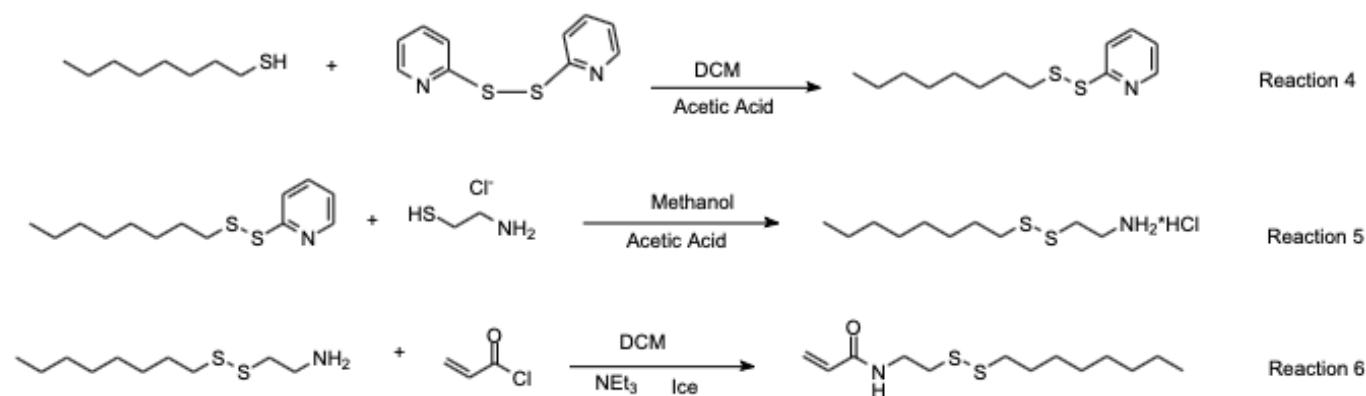
Reaction 1 product is then concentrated using a Heidolph rotary evaporator. The product is then extracted using a 10:1 ratio of hexane to ethyl acetate and concentrated again before purification by silica flash chromatography (Teledyne) with hexane and ethyl acetate (0.05 ethyl acetate by volume) as the mobile phase. Reaction progression and product identification is confirmed using thin layer chromatography (TLC,  $R_f=0.6$ ).

The purified Reaction 1 product (21.3 g, 68.4 mmol, 68.4% yield) is then added to DCM (140 mL) followed by acetic acid (1.02 mL). 2-mercaptoethanol (5.09 g, 65.1 mmol, Sigma Aldrich) is added dropwise and Reaction 2 proceeds overnight. The workup for the Reaction 2 product is like that of Reaction 1 apart from a 2:1 ratio of hexane to ethyl acetate used instead. The product is then purified using silica flash chromatography (0.2 ethyl acetate by volume).

The purified Reaction 2 product (18 g, 64.7 mmol) is then added to DCM (325 mL). Triethylamine (12 mL, Sigma Aldrich) is then added, and the reaction is cooled with ice before acryloyl chloride (7.03 g, 77.7 mmol, 6.2 mL, Sigma Aldrich) is added dropwise because Reaction 3 is exothermic, and reactants may vaporize at elevated temperatures which may limit reaction progression and final yield. Reaction 3 continues to mix at room temperature overnight. O12B is concentrated, filtered (10:1 hexane: ethyl acetate), and purified (0.1 ethyl acetate by volume). O16B is synthesized in a similar method with dodecanthiol used instead of octanthiol.

### Synthesis of N12B

N12B is synthesized in a series of reactions illustrated by Reactions 4-6 in **Figure 2**.



**Figure 2.** N12B Synthesis

First, 2,2'-dipyridyl disulfide (45.6 g, 207 mmol) is dissolved in DCM (350 mL). Acetic acid (2.6 mL) is added, and then octanthiol (25.23 g, 172.5 mmol) is added dropwise. The reaction mixture is stirred overnight. Reaction 4 product is concentrated using a rotary evaporator before being filtered over silica gel with a 10:1 hexane/ ethyl acetate mixture. The product is then concentrated again before being purified using silica flash chromatography with hexane and ethyl acetate as the mobile phase. Product is eluted at 5% ethyl acetate. Reaction progression and product identification is confirmed using TLC.

The purified Reaction 4 product (17.6 g, 68.9 mmol, 39.9% yield) is then dissolved in methanol (140 mL). Acetic acid (1.0 mL) is added then 2-mercaptoethylamine hydrochloride (7.45 g, 65.6 mmol) is added gradually. The reaction mixture is mixed overnight. The reaction 5 product is concentrated. Next, the concentrated product is dissolved in methanol. The dissolved mixture is added dropwise to cold diethyl ether (dry ice). The reaction mixture is stirred for 15-20 minutes before being filtered using VWR 417 filters and is flushed with cold ether and hexane. The process is repeated several times until a white crystalline product remains. The crystallized Reaction 5 product is then neutralized using sodium hydroxide (2M) and extracted with DCM three times. The product is concentrated before proceeding to Reaction 6, which has a similar procedure to Reaction 3, which has been discussed in *Synthesis of O12B*. N16B synthesis follows the same procedure except for dodecanthiol instead of octanthiol.

#### *Synthesis of 306-O12B*

306-O12B is synthesized in a combinative Michael Addition reaction.<sup>[6-8]</sup> A 3.2:1 molar ratio of O12B to 306 is used to obtain a mixture of 306-3O12B and 306-4O12B after mixing for 48 hours at 70°C. The reaction product is purified using silica flash chromatography. 306-4O12B elutes at 5 v% ethyl acetate, and 306-3O12B elutes at 10 v% ethyl acetate. Products were confirmed using Electrospray Ionization-Mass Spectrometry (ESI-MS). 306-O12B samples were stored at -20°C.

#### *Incorporation of Natural Alcohols*

Incorporation into the lipid structure requires the alcohols to be converted to acrylates under the same conditions as Reaction 3 described previously. An example reaction is shown by Reaction 7 in **Figure 3**.

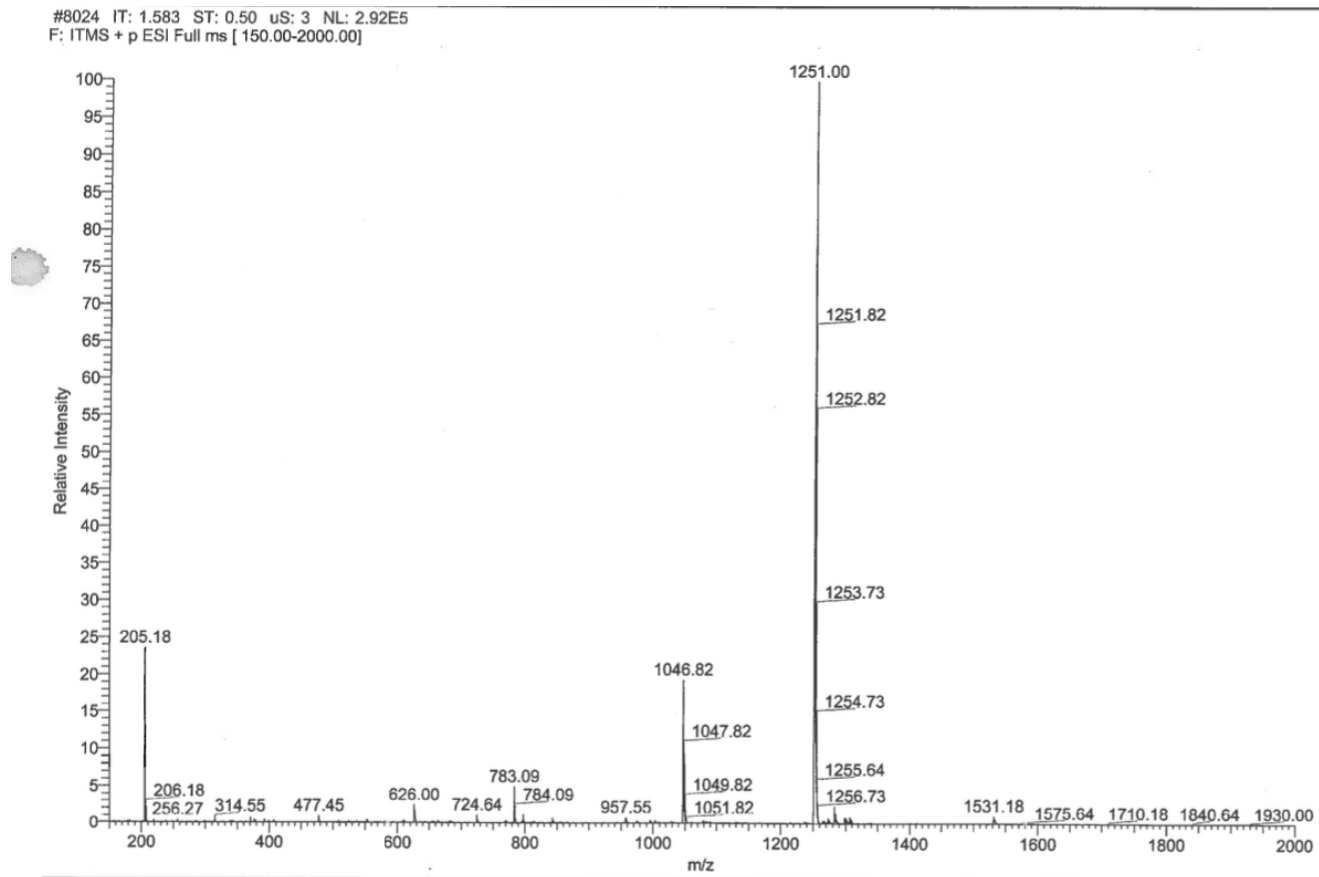


and DMG-PEG at 50:38.5:10:1.5 molar ratios and dissolved in enough ethanol for a final lipidoid concentration of 10 mg/mL. The solution is then mixed with an acidic sodium acetate buffer containing mRNA using a NanoAssembler microfluidic system. The resulting LNPs are dialyzed against phosphate buffer saline (PBS) overnight. Next, mRNA is mixed with sodium acetate buffer. The mRNA and LNP solutions are injected into the microfluidic device at a 3:1 ratio. The microfluidic device promotes rapid mixing that allows for the self-assembly of LNPs. LNPs are dialyzed again overnight against PBS before they can be further studied and analyzed.

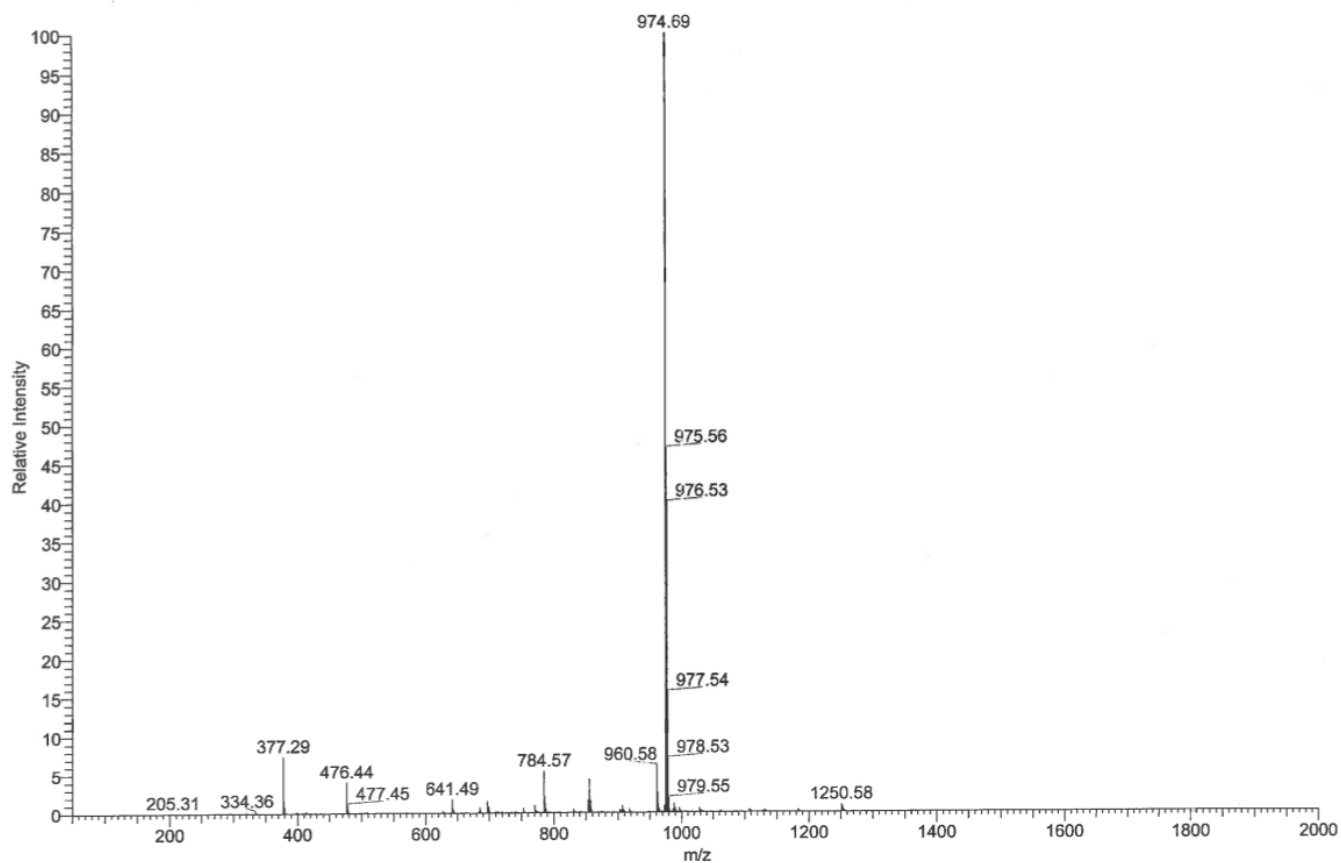
## Results

### ESI-MS

Final lipid composition is confirmed using ESI-MS. The first two figures (**Figures 5 and 6**) demonstrate the ability to isolate both 306-4O12B and 306-3O12B.



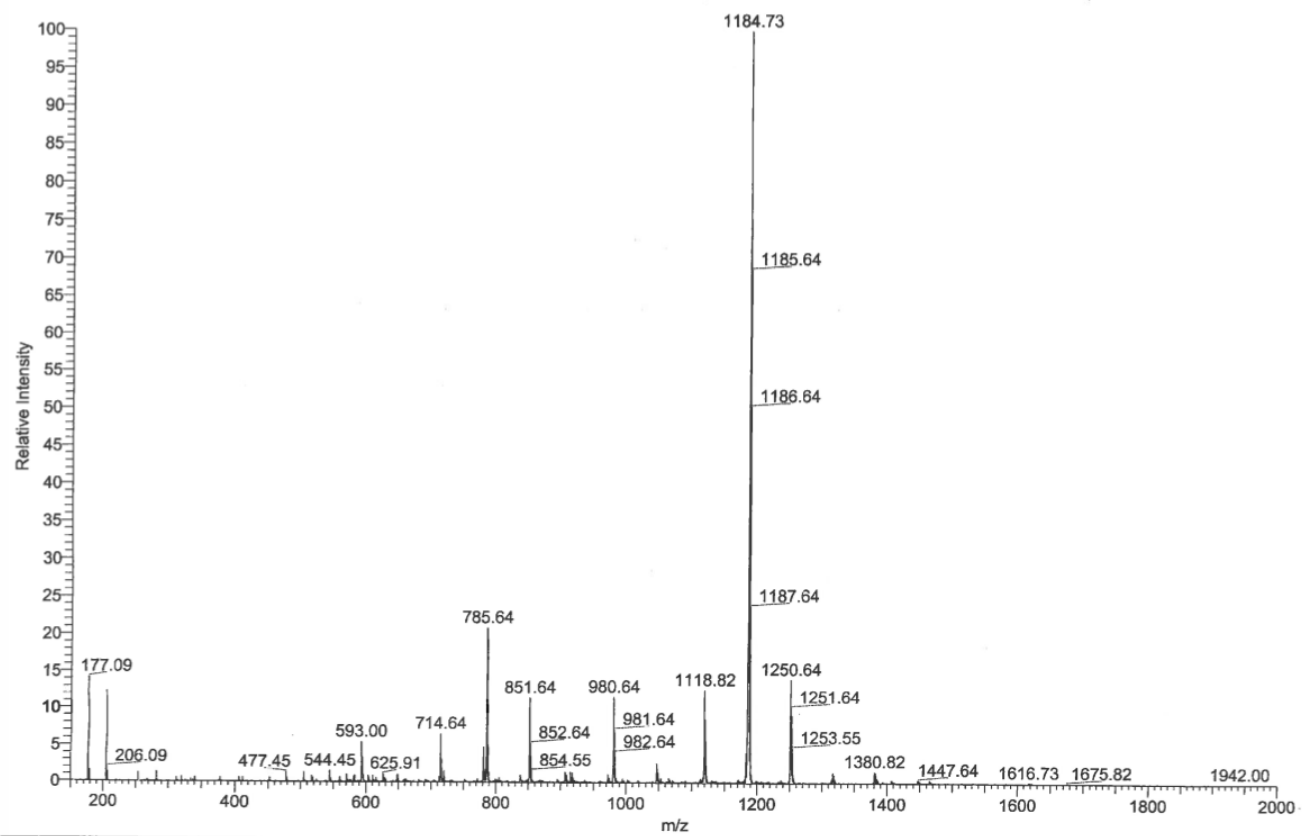
**Figure 5.** ESI-MS for 306-4O12B



**Figure 6.** ESI-MS for 306-3O12B

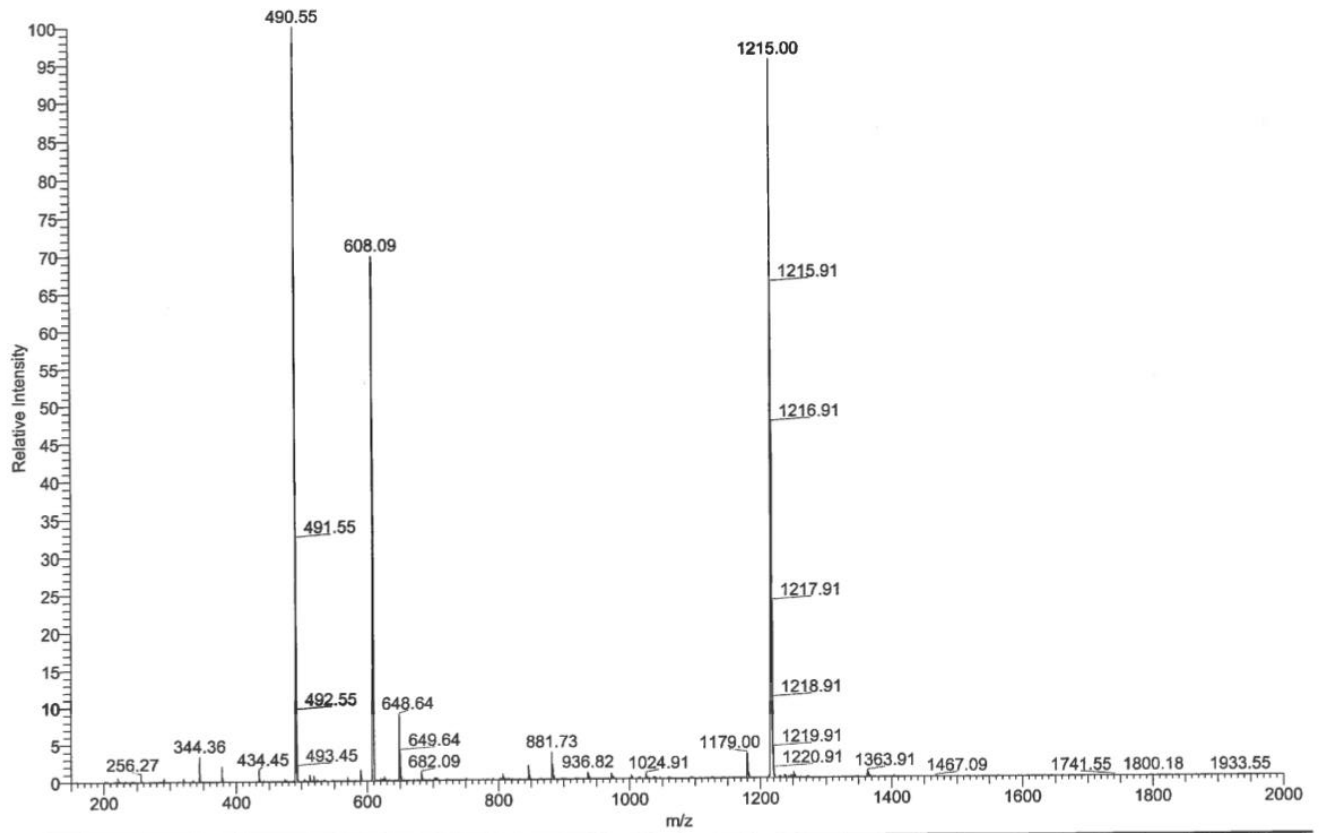
Confirming the ability to isolate 306-3O12B allowed for the exploration of the potential expansion of the lipid database. Successful incorporation of the acrylated alcohols is shown in the succeeding **Figures 7-13**. **Table 1** lists the molecular weights of the different alcohols and their corresponding lipids for reference.

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**Figure 7.** ESI-MS for Geraniol

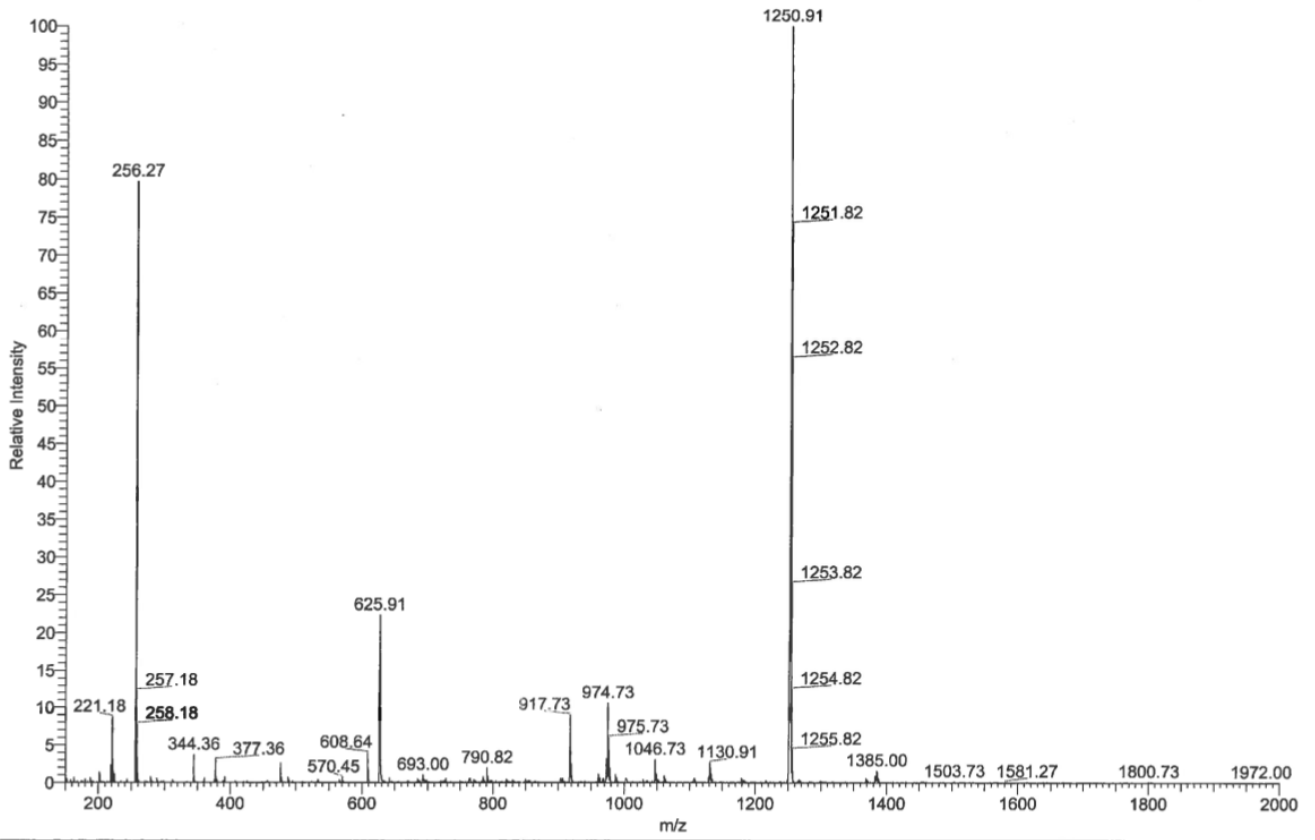
#1844 IT: 0.181 ST: 0.50 uS: 3 NL: 1.69E6  
F: ITMS + p ESI Full ms [ 150.00-2000.00]



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**Figure 8.** ESI-MS for 2-Butyl-1-Octanol

#1371 IT: 0.475 ST: 0.50 uS: 3 NL: 7.33E5  
F: ITMS + p ESI Full ms [ 150.00-2000.00]



**Figure 9.** ESI-MS for Farnesol

#2046 IT: 0.418 ST: 0.50 uS: 3 NL: 7.72E5  
F: ITMS + p ESI Full ms [ 150.00-2000.00]

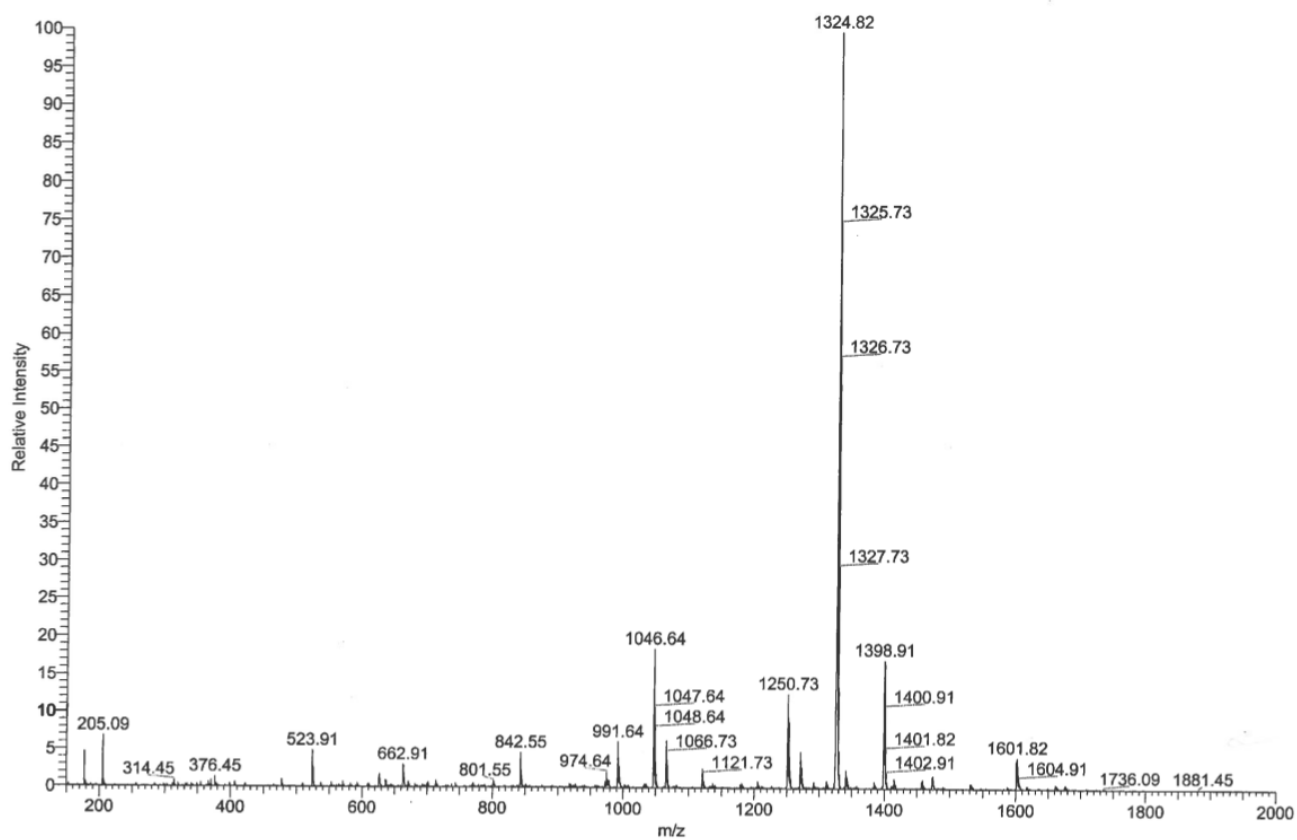


Figure 10. ESI-MS for Phytol

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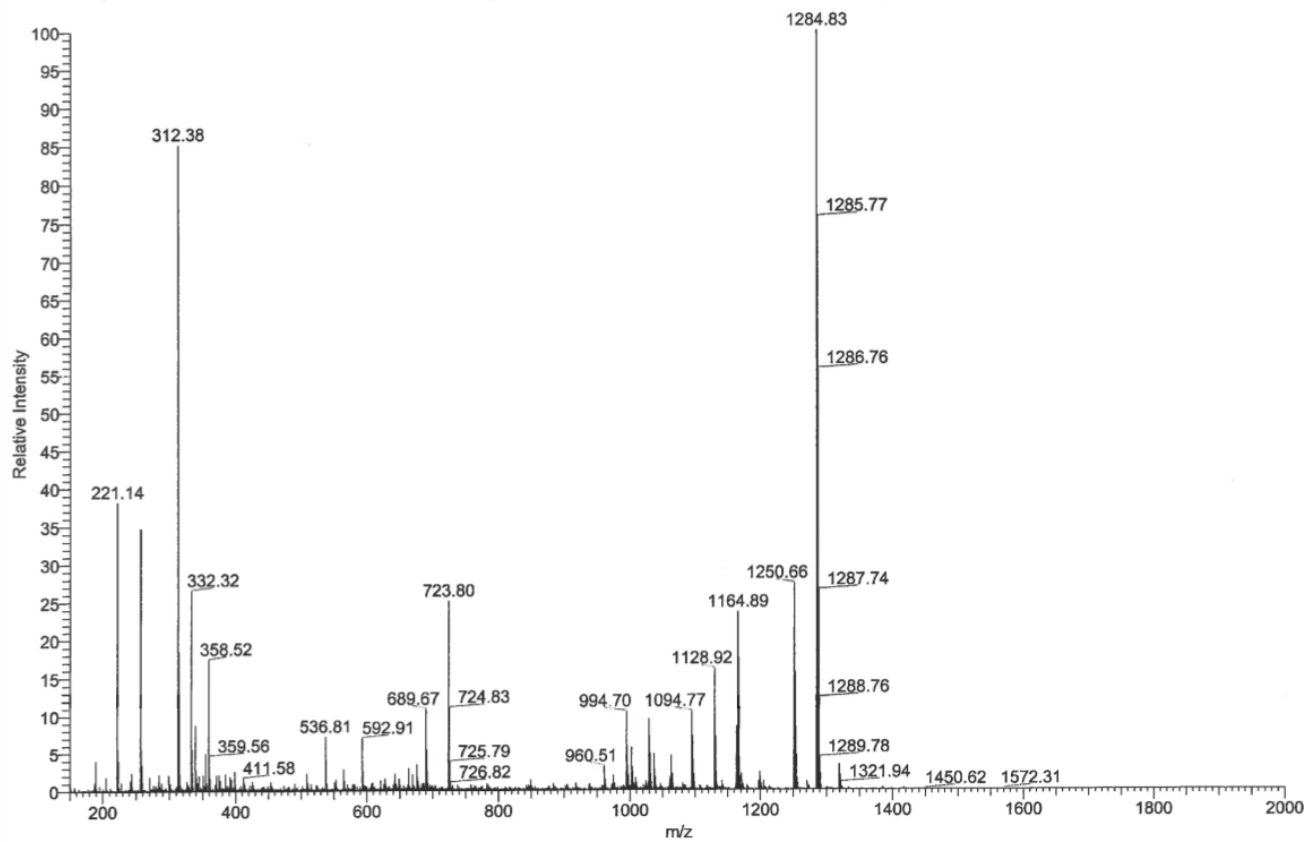


Figure 11. ESI-MS for Citronellol

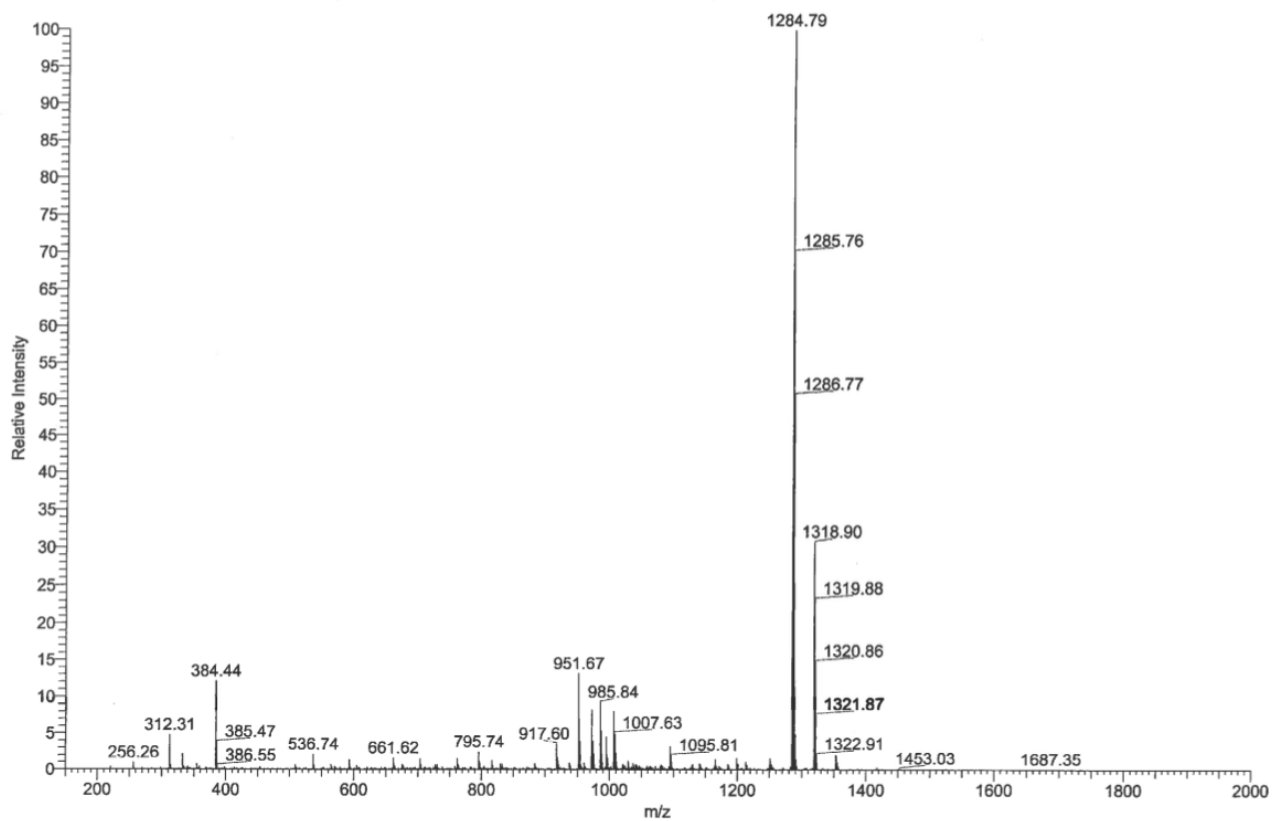
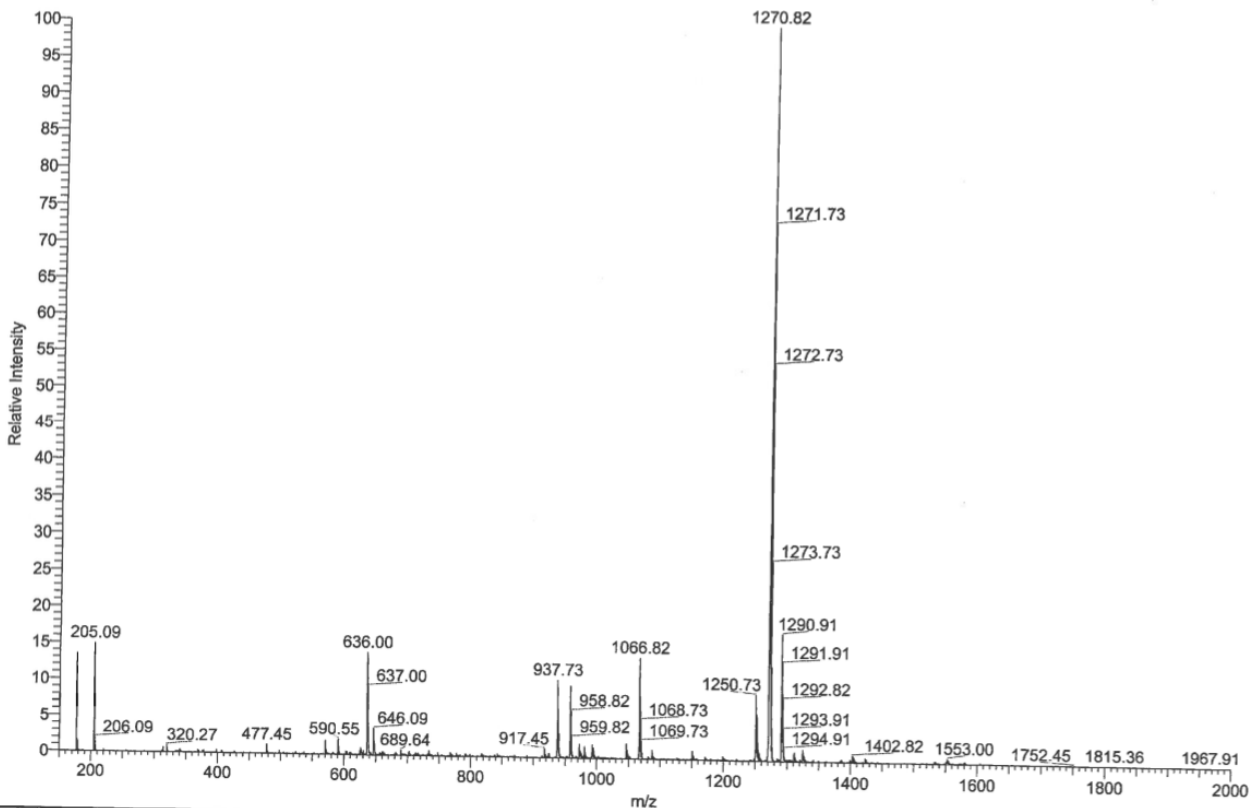


Figure 12. ESI-MS for 9-Heptadecanol

F: [TMS + p ESI Full ms [ 150.00-2000.00]]



**Figure 13.** ESI-MS 2-Hexyl-1-Decanol

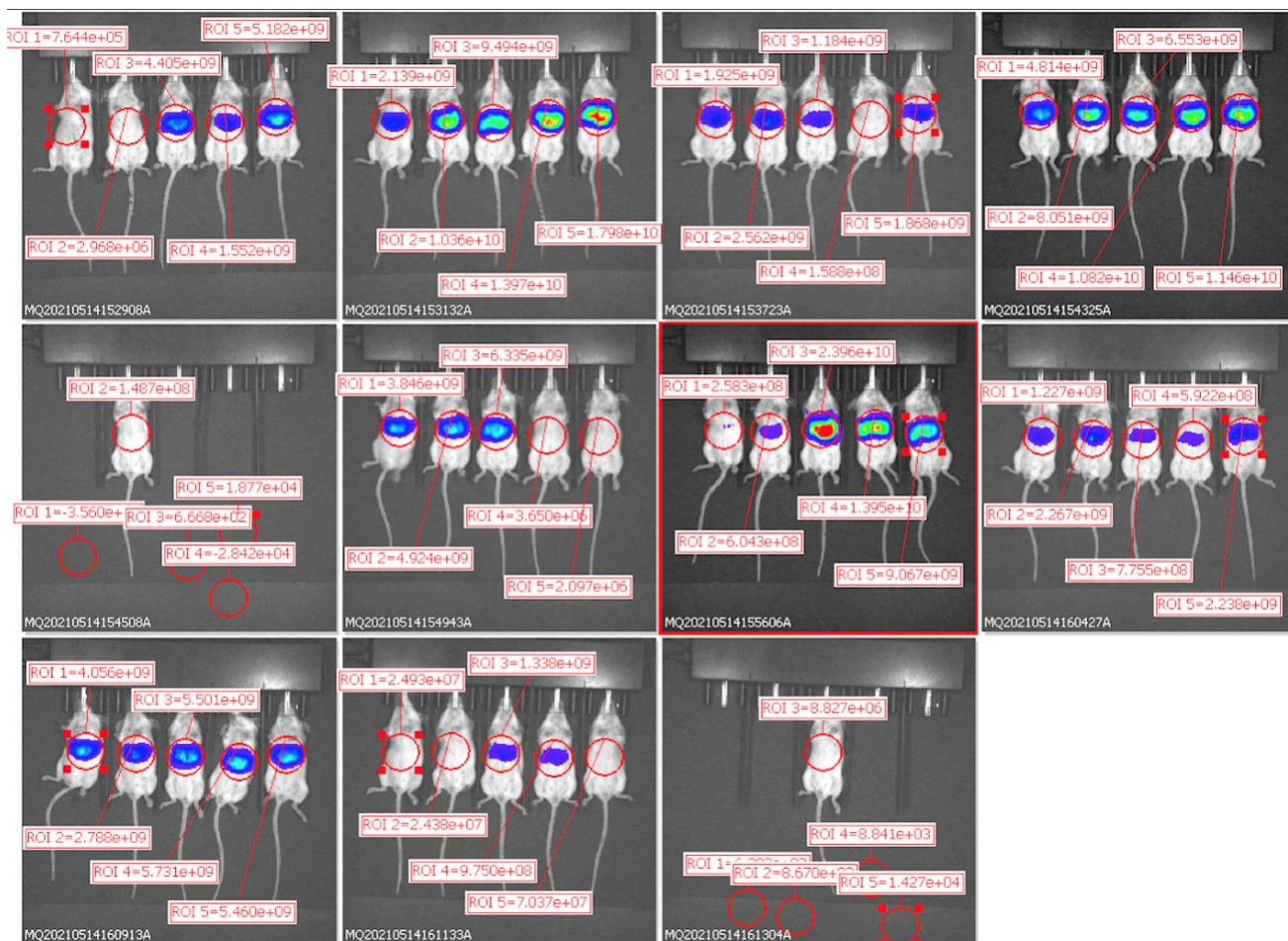
	Compound	MW	MW (after acrylation)	MW (306-3O12B-acrylated alcohol)
1	geraniol	154.25	209.26	1183.8
2	2-butyl-1-octanol	186.33	241.34	1215.88
3	undecenol	170.29	225.3	1199.84
4	farnesol	222.37	277.38	1251.92
5	phytol	296.53	351.54	1326.08
6	citronellol	156.27	211.28	1185.82
7	9-heptadecanol	256.47	311.48	1286.02
8	2-hexyl-1-decanol	242.44	297.45	1271.99
	306	145.25		
	O12B	276.43		
	306-3O12B	974.54		
	Acryloyl chloride	90.51		

**Table 1.** Molecular Weights for Alcohols and Relevant Lipid Components

### *In vivo Results*

All O-series LNPs were tested in mice to determine *in vivo* delivery efficacy. Each LNP was tested, and the results can be seen in **Figure 14**. The results read from top left as 1,1,2,2,3, 3,4,4,5,5, etc. where each number corresponds to the numbers in **Figure 15**. From the *in vivo* results geraniol, 2-butyl 1-octanol, and citronellol incorporated LNPs had the greatest efficacy in mRNA delivery amongst the natural alcohols tested. This is made clear by the ROI values that are an order of magnitude greater than those of the other alcohols. Only boxes 2-5 pertain to lipids synthesized from the natural alcohol building blocks. All other boxes include other lipids that were tested that day. It should also be noted that a cage of mice administered the 9-heptadecanol, 2-hexyl-decanol, and one 306-3O12B-78 thioester was lost, which explains the missing results in the fifth box.

When comparing the three most successful alcohols, it appears that length may be an important property to consider when formulating LNPs. All three have an 8-carbon backbone as opposed to the longer backbones of the other alcohols. Interesting to note, all three lipids also have some degree of branching in their structures. Geraniol and citronellol incorporated LNPs are also unsaturated due to the presence of double bonds.



**Figure 14.** *In vivo* Results.

## 5/12/21 ## 4.738 4.7828 4.782  
 Top left → 4.7858

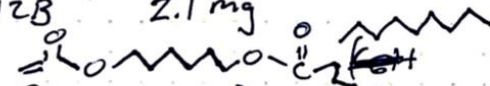

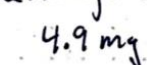
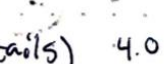
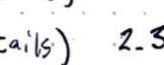
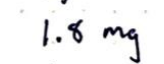
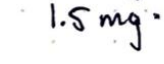
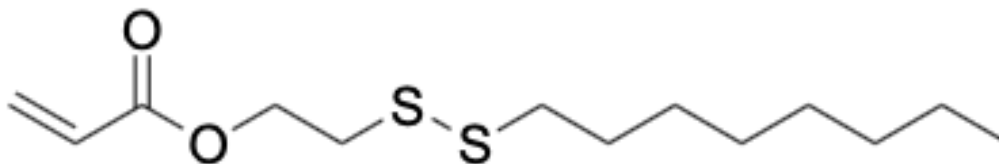
1	306-ethyl 012B	2.1 mg	
2	306-3012B - 	1.8 mg	
3	306-3012B - 	1.1 mg	
4	306-3012B-geraniol	2.5 mg	
5	306-3012B-2butyl octanol	2.0 mg	
6	306-3012B-undecanol	2.9 mg	
7	306-3012B-farnesol	2.1 mg	
8	306-3012B-phytol	2.2 mg	
9	306-3012B-3isodecyl acrylate	2.2 mg	2.3 mg
10	306-3012B-citronellol	2.0 mg	
11	306-3012B-9heptadecanol	1.8 mg	
12	306-3012B-2hexyl 1-decanol	3.1 mg	
13	306-3012B-79 thioester	2.1 mg	
14	306-3 lauryl <sup>acrylate</sup> <del>acetate</del> -1012B	3.1 mg	
15	306-3 2-ethyl hexyl <sup>acrylate</sup> <del>acetate</del> -1012B	2.8 mg	
16	306-4 2-ethyl hexyl acrylate	2.4 mg	
17	306-4 lauryl acrylate	2.2 mg	
18	306-4 isodecyl acrylate	2.9 mg	
19	306-4 C <sub>8</sub> H <sub>17</sub> S-S 	4.9 mg	
20	306-4012B (6.4)	2.5 mg	
21	306-C <sub>6</sub> S-S 	(4 tails) 4.0 mg	
22	306-C <sub>6</sub> S-S 	(4 tails) 2.3 mg	
23	306-C <sub>7</sub> H <sub>15</sub> S-S 	1.8 mg	
24	306-Oleic-S-S 	1.5 mg	
25	306-012B (3.7)	1.7 mg	

Figure 15. Lab Notes indicating what LNPs were administered to which mice *in vivo* Results of Figure 14

## Ongoing Work

Ongoing work with the demonstration of the effectiveness of incorporating natural alcohols, in particular geraniol, citronellol, and 2-butyl-1-octanol, will focus on the continued expansion of our lipid library. One potential expansion of the library is to further incorporate the alcohols into the lipid structure. In order to incorporate the natural alcohols into all four tails, the alcohols need to be converted into thiols to create the disulfide bonds present in O12B, which help make the LNPs biodegradable.<sup>[4]</sup> This process will be discussed in more detail later. Other future work may include the incorporation of the natural alcohols into the N-series lipids, as well as changing the positioning of the disulfide bond in lipid structure. As shown in the structure of O12B in **Figure 16**, the disulfide bond is present after the second carbon following the ester group.

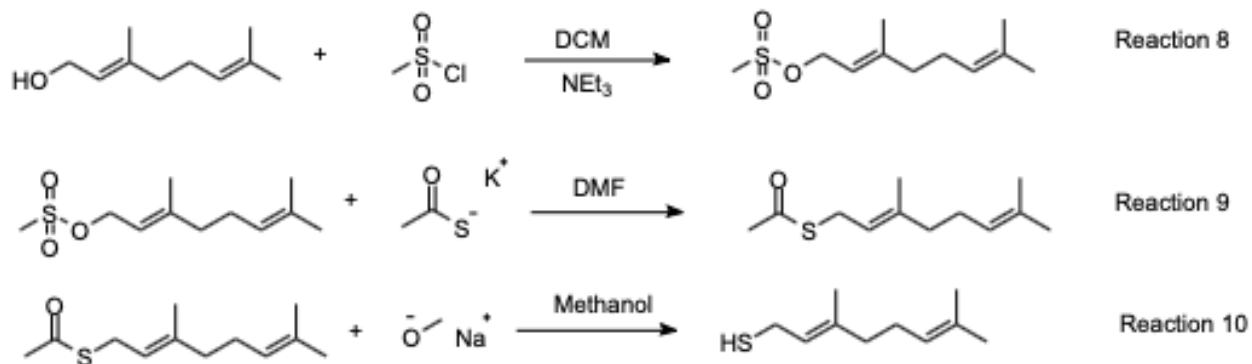


**Figure 16.** O12B Structure

The placement of the disulfide bond farther down the lipid tail may be an interesting characteristic to study. Finally, all of the tails need to be studied with various different head groups to more thoroughly explore their potential efficacy.

### *Synthesis of Natural Alcohol Tails*

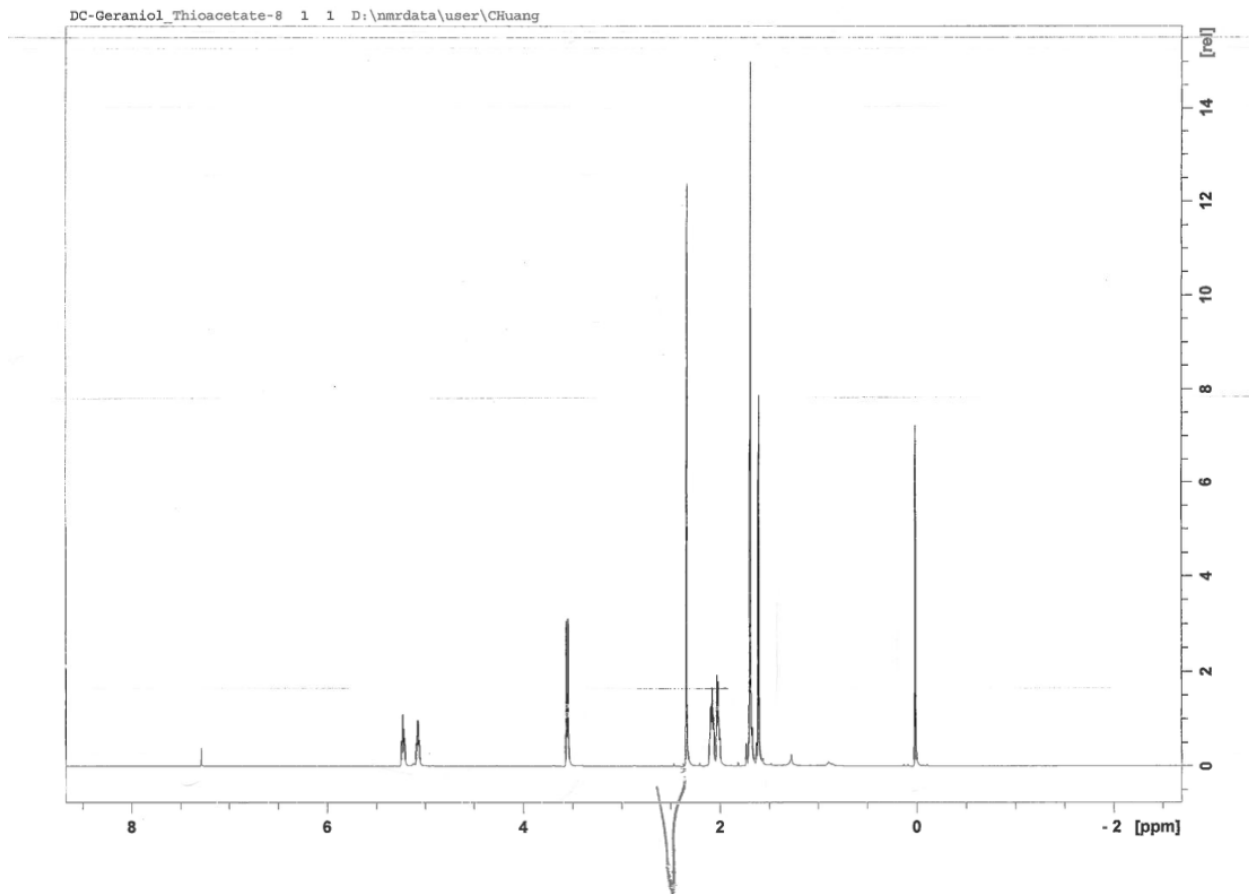
In order to form the disulfide bonds and to incorporate the natural alcohols—geraniol, citronellol, and 2-butyl-1-octanol— into the lipid tail structure, the alcohols must be converted to thiols. This is accomplished through a series of three reactions illustrated by Reactions 8-10 in **Figure 17**.



**Figure 17.** Thiol Formation

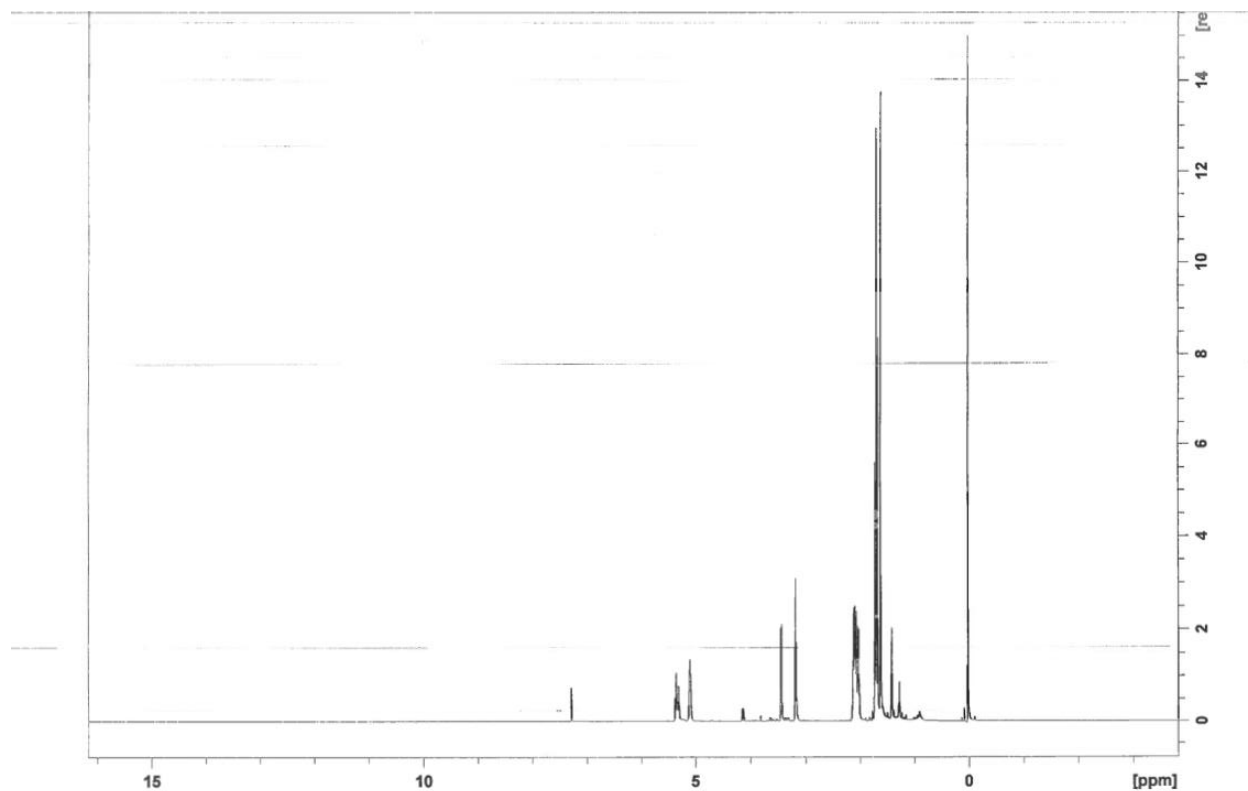
First, geraniol (4.40 g, 28.5 mmol) is added to DCM (90 mL) followed by triethylamine (4.33 g, 42.7 mmol). Methanesulfonyl chloride (4.24 g, 37 mmol, Sigma Aldrich) is then added dropwise. Reaction progress is checked using TLC with hexane and ethyl acetate (10:1) as the mobile phase. The Reaction 8 product is concentrated and purified using flash silica chromatography.

Purified Reaction 8 product (is then reacted with potassium thioacetate (Sigma Aldrich) in dimethylformamide (DMF, Sigma Aldrich). The Reaction 9 product is concentrated, purified, and then confirmed using nuclear magnetic resonance (NMR, Bruker AVIII 500 MHz) spectrometer as seen in **Figure 18**.  $\text{H}^+$  NMR analysis was performed using samples in chloroform solvent.



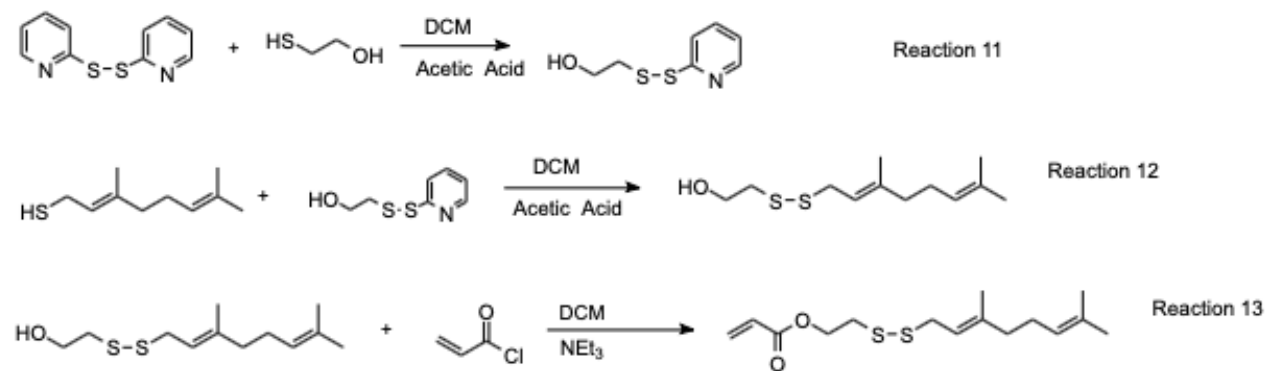
**Figure 18.** Geraniol thioacetate NMR

Reaction 9 product (1.16 g, 5.49 mmol) is next reacted with sodium methoxide (0.44 g, 11 mmol, Millipore) in methanol (30 mL). The reaction product is concentrated and purified. Thiol formation is confirmed using NMR (**Figure 19**).



**Figure 19.** Geraniol thiol NMR

Concurrent to Reactions 8-10, 2,2'-dipyridyl disulfide is reacted with 2-mercaptoethanol (Reaction 11). Reactions 12 and 13 are like Reactions 2 and 3 respectively of the O12B reaction pathway. Reactions 11-13 are shown in **Figure 20**.



**Figure 20.** Disulfide Formation from Natural Alcohols

It is important to have a converging reaction pathway as opposed to a more linear pathway to maximize yield. The reaction pathway would be similar for both citronellol and 2-butyl 1-octanol.

## Conclusion

From vaccines to cancer therapeutics,<sup>[43-45]</sup> LNPs will play a pivotal role in the development of new treatments for previously untreatable diseases. One of the outcomes of the COVID-19 pandemic is that the world has become more aware of the importance lipid nanoparticles will have in modern medicine. It is crucial today to expand our lipid databases in order to find the best possible formulations. The successful incorporation of these natural alcohols— geraniol, 2-butyl 1-octanol, undecenol, farnesol, phytol, citronellol, 9 heptadecanol, and 2-hexyl 1-decanol— into the lipid structure has conclusively expanded our lipid library. The presented success of geraniol, 2-butyl 1-octanol, and citronellol *in vivo* studies allow for the further diversification of the lipid library.

# Appendices

## Appendix I. Additional Figures

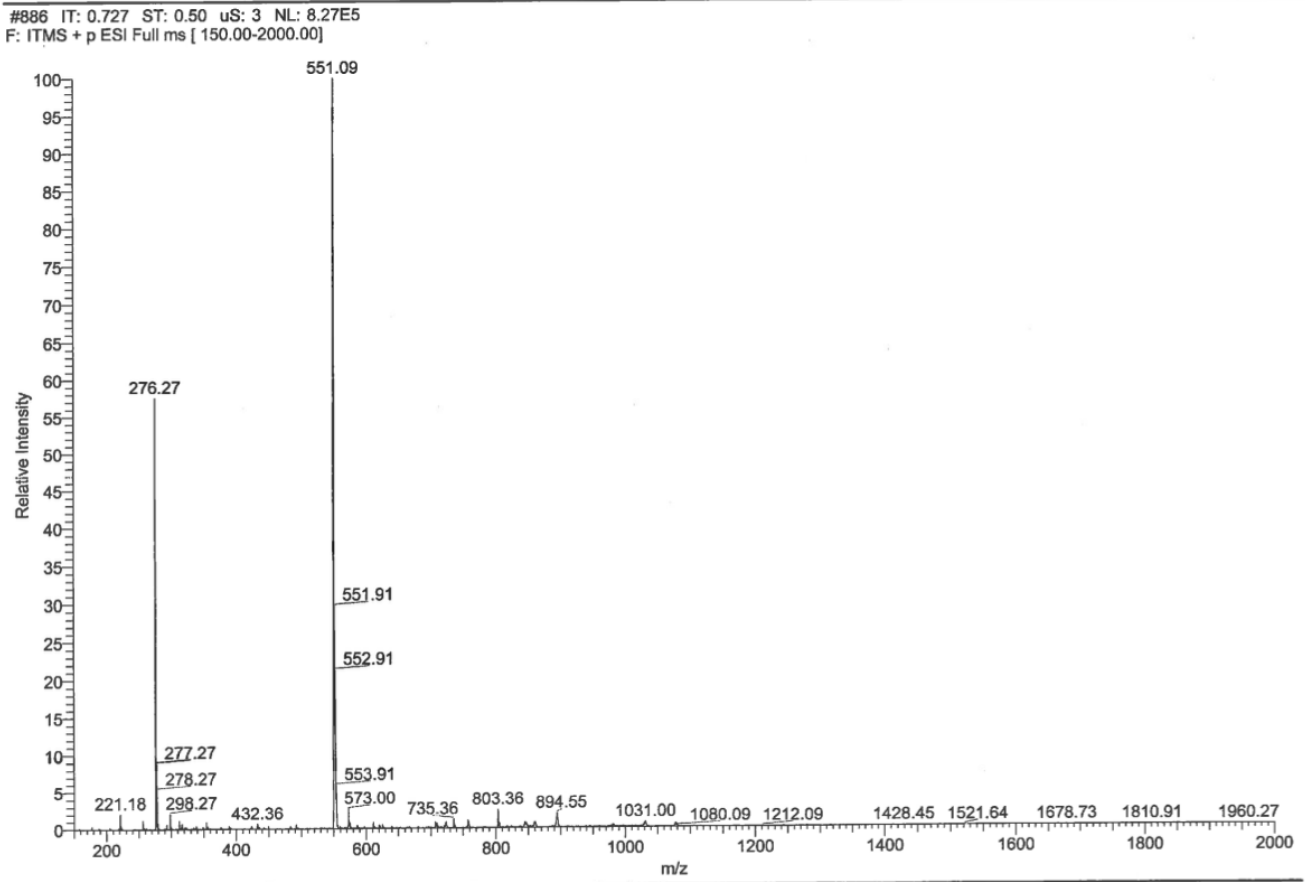


Figure A 1.1 ESI-MS for N12B

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