University of
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Enzymatic Biodegradation of Waste Plastics: Degradation of Ethyl Benzoate from Lipase *Pseudomonas Cepacia*

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I: Introduction

Synthetic polymers, waste plastics, are infamously persistent in the biosphere[1]. Current chemical or mechanical recycling methods of the world's plastic waste often fail to process plastics deemed contaminated, due to stringent purity requirements for recycling[2]. Equally, traditional plastics recycling often lowers the quality of the plastic, thus limiting the number of times a plastic can be recycled.[3],[4],[5] Enzymatic degradation offers a biocatalytic pathway to polymer recycling that requires minimal inputs[6], can produce recycling products of equal quality to virgin plastics[7] (plastics derived directly from fossil fuels or other feedstocks), and which contribute to circular bioeconomic models of materials sourcing and disposal[8]. This report offers an introductory route to understanding enzyme-catalysed depolymerization of two common plastic wastes: an ether (PE plastic waste similar) and a urethane (PU plastic waste similar).

II: Plastic Waste Contexts

Plastic wastes are now ubiquitous, from the tonnage of plastics accumulating in the Great Pacific Garbage Patch[9], to the micro- and nano-plastics accumulating across the food chain in the water we drink and the air we breathe[10],[11]. By 2050, one study estimated that by mass there will be more plastic waste in the ocean than there are fish[12]. Equally, another averaged the polymer fragments bioaccumulating in soils and across agriculture[13]; in an average apple eaten, there are upwards of 225,000 plastic fragments[14].

The persistence and bioaccumulation of plastics can be attributed to several factors; some being insufficient recycling and disposal methods[15], the growing predominance of plastics production and usage in commodities[16], and the chemical and biophysical properties of plastics which render them inherently incompatible with wider ecosystem life.[17] Associated toxicology of microplastic contamination remains an emerging sector of research: the impacts of plastics incineration (one solution to processing plastic waste, branded as waste-to-energy)[18] is long known to have carcinogenic and developmental impacts associated with human inhalation of the incineration plumes[19],[20]. Interactions between microplastics and human biology is largely considered to be harmful,[21],[22],[23] but persistence in human biology is also ubiquitous now even from birth; plastic fragments have been found in placenta of new-born children[24].

Current disposal and recycling infrastructure is largely insufficient, as established by the endemic environmental and biological contamination of plastic wastes[15],[16]. Only 9% of all plastics have ever been recycled[16]; amongst these, there is often a limit to the continued recyclability of each polymer[25]. PET, for example, a plastic bottle, can typically only be recycled three times due to decrease in material quality after each recycling cycle.[26] This is largely because recycling infrastructure largely relies on mechanical (shredding), or crude chemical recycling processes, resulting in impurities throughout the polymeric structure. Some chemical approaches can retain polymer quality, thus theoretically expanding recyclability to near-infinity, by cleaving individual polymeric bonds and reducing a polymer to its substituent monomers, removing any impurities, and undergoing repolymerization to a chemically-identical polymer to the feedstock plastic.[25] This ensures retention of desirable polymeric structural and mechanical properties, retaining near-identical plastic quality.[27] However, these synthetic chemical methods often involve disproportionately large input requirements (energy / reagents) due to the chemical strength of polymeric bond linkages. These large inputs necessarily attach additional carbon and economic

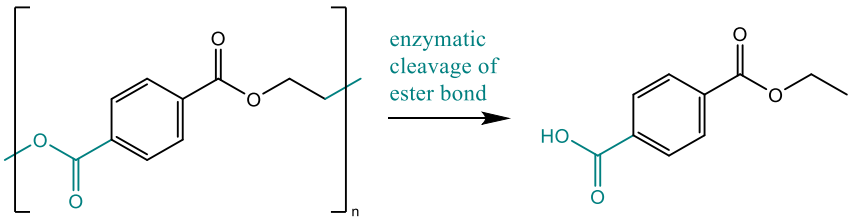
footprints to the recycling process, such that overall environmental and social benefit is often reduced.[28]

Enzymatic degradation has comparatively far lower input requirements, due to their catalytic nature and their favourable kinetics for reaction.[29], As such, enzymatic pathways for recycling and mitigating plastic waste contamination are an interesting area of research that merits its own sect of academia in effectuating sustainable transitions.[30]

III: Wider Research Contexts

Enzymatic degradation of synthetics polymers has been a topic of research for several decades. In recent years, academic discourse and publications of enzyme-polymer interactions have grown exponentially, as shifts towards both a circular economy and advances in biochemical applications have grown.[31,[32] Enzymes provide kinetically favourable degradative mechanisms: they provide a catalytically lower enthalpy (lower activation energy pathway, E_A) required for polymeric bond cleavage than synthetic chemical -lysis comparisons.[29]

Frontline research includes bespoke synthesis of PET-ase enzymes[33} and AI-screened enzyme complexes, optimised for degradation of PET (polyethylene terephthalate) waste polymers.[34] PET is one of the most commonly wasted plastics, found in single-use plastic bottles and containers, in textiles (themselves a major source of marine microplastic contamination), and many other commodities with a short usage-disposal lifespan.[35] PET enzymatic degradation occurs via hydrolysis of the ester polymeric linkage:



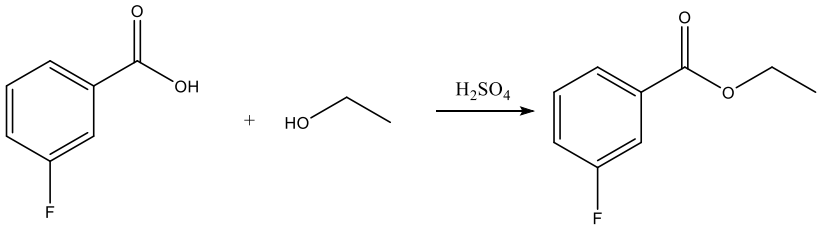
[Fig 1.]

Each active site on an enzyme is uniquely adapted to catalyse a particular polymeric bond structure; a PETase enzyme often cannot cleave a PU (polyurethane) polymeric linkage bond and vice versa. Due to this active site specificity, enzymatic degradation for other common waste plastics like PU (polyurethane), PVC (polyvinyl carbamate), and PS (polystyrene) remain in their nascence: a PETase enzyme can only degrade a small variety of PET polymers, with different synthetic plastic chemical structures requiring specific enzymatic active sites to biodegrade.[36] Further research is needed to both identify a wide spectrum of specific polymer-enzyme interactions and to optimize these for useful polymeric linkage bond degradation for polymers outside of the PET synthetic polymer family.[37]

Key challenges for enzymatic biodegradation of waste plastics include rate of reactions and catalytic efficiency: due to the scale of plastic wastes already present (legacy plastics), rate of catalysis for near-complete or complete polymer degradation is an essential factor.[38]

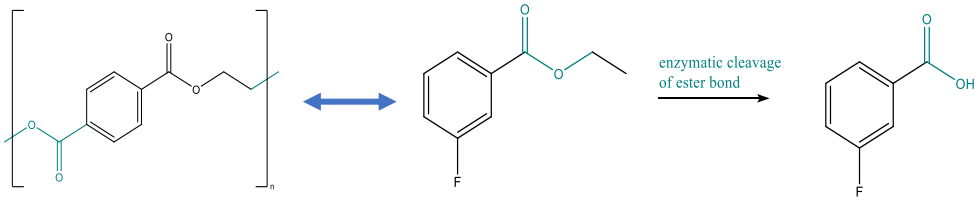
IV: Experimental Overview

Ethyl benzoate (0.200 g, 72.5%) was synthesised from phenyl carboxylic acid and ethanol, wherein the aromatic ring was fluorine tagged (3-fluoro) for TLC analysis under UV radiation, and for [^{19}F] NMR analysis.



[Fig. 2]

The ester -COO bond formed in the product aims to simulate the ester bond linkage in PE and PET polymers, for subsequent enzymatic bond cleavage. Confirmation of enzymatic degradative activity by for [^{19}F] NMR and subsequent kinetic analysis, was undertaken for this report.



PET repeating unit

Enzymatic degradation of ester mimetic dimer

[Fig. 3]

Synthesis of ethyl 3-fluorobenzoate:

3-fluorobenzoic acid (209 mg) and ethanol (5 mL) were refluxed with H_2SO_4 (0.3 mL, 4M) until judged complete by TLC analysis. The reaction mixture was partitioned between ethyl acetate (20 mL) and water (20 mL). The aqueous layer was washed with ethyl acetate (2 x 10 mL). The organic layers were combined and washed with $NaHCO_3$ (aq. sat., 20 mL), brine (20 mL), and dried over magnesium sulphate. The product was analysed by [^{1}H] NMR and [^{19}F] NMR and found to be a successful synthesis of ethyl 3-fluorobenzoate (200 mg, 72.5%). No further purification was required.

Synthesis of ethyl (4-fluorophenyl)carbamate:

4-fluoroaniline (0.426 mL) and ethyl chloroformate (0.430 mL) was added to potassium carbonate (1.241 g) and dissolved in dichloromethane (DCM) (10 mL). The reaction mixture was refluxed overnight, cooled, and quenched with water (15 mL) once judged to be complete by TLC analysis. The aqueous fraction was extracted with DCM (3 x 10 mL). The organic fractions were combined and washed with HCl (2 x 10 mL), water (10 mL), and brine (5 mL), then dried over sodium sulphate, filtered, and concentrated under reduced pressure. The resulting product proved too soluble for recrystallization with ethanol, so a trituration with hexane was conducted and judged to result in sufficient product purity by [^{1}H] NMR and [^{19}F] NMR. Ethyl (4-fluorophenyl)carbamate was produced (321 mg, 93.1%).

Buffer Solution Preparation:

Two buffer solutions, pH7 and pH8, were created from KH_2PO_4 / K_2HPO_4 solution in 500 mL of sterile water. The pH7 buffer solution comprised of monobasic KH_2PO_4 (1.574 g) and dibasic K_2HPO_4 (2.332 g); the pH8 buffer solution comprised of monobasic KH_2PO_4 (222 mg) and dibasic K_2HPO_4 (4.071 g). Both solutions were filtered under reduced pressure and the pH was verified by calorimetry.

Assay of Lipase, *Pseudomonas sp.*:

Lipase from *Pseudomonas sp.* (20.2 mg, solubility 5 mg / mL) was dissolved in distilled water (3.8 mL) and acetonitrile (20 µL).

V: Assessing Enzymatic Degradative Activity

For test of enzymatic degradative activity, four assay sets were conducted; positive control (enzymatic activity on olive oil), negative control (buffer solution only), as well as the two experimental substrates (ethyl 3-fluorobenzoate and ethyl (4-fluorophenyl)carbamate). Three repeats of each substrate and the positive control included addition of the enzyme assay; the 4th repeat of each excluded the enzyme assay to measure any baseline degradative activity.

For those with added enzyme, the enzyme assay (400 µL) and substrate (mass denominated in Table 1) were made up to 2.5 mL with pH7 buffer solution (KH_2PO_4 / K_2HPO_4). For those without enzyme, each substrate (mass denominated) was made up to 2.5 mL with buffer solution. Buffer-only negative controls contained only 2.5 mL of the buffer solution.

Substrate	Code	Type	Enzyme Added?	Mass of Substrate Added (mg)
Olive Oil	PC1	Positive Control	Yes	203
Olive Oil	PC2	Positive Control	Yes	208
Olive Oil	PC3	Positive Control	Yes	201
Olive Oil	PC4	[Control]	No	202
Buffer Only	B1	Negative Control	No	/
Buffer Only	B2	Negative Control	No	/
Ethyl 3-fluorobenzoate	E1	Repeat 1	Yes	21
Ethyl 3-fluorobenzoate	E2	Repeat 2	Yes	21
Ethyl 3-fluorobenzoate	E3	Repeat 3	Yes	20
Ethyl 3-fluorobenzoate	E4	Baseline	No	21
Ethyl (4-fluorophenyl)carbamate	U1	Repeat 1	Yes	20
Ethyl (4-fluorophenyl)carbamate	U2	Repeat 2	Yes	21
Ethyl (4-fluorophenyl)carbamate	U3	Repeat 3	Yes	21
Ethyl (4-fluorophenyl)carbamate	U4	Baseline	No	21

[Table 1]

All assays were incubated simultaneously at 37 °C and 15,000 RPM, and various time points were extracted over the course of the reaction.

Confirmation of Enzymatic Degradative Activity:

At each time point, a fraction of each assay (400 µL) was extracted. Each assay was quenched with methanol (400 µL, 1M) to precipitate out any remaining enzyme with centrifugation (10 min., 15,000 RPM). For substrates which had been fluoro-tagged (ethyl 3-fluorobenzoate and ethyl (4-

fluorophenyl)carbamate), [^{19}F] NMR analysis was run on each repeat of each time point to measure any degradative activity.

Initial time, T_0, was taken prior to any incubation. Subsequent time points were taken and the enzymatic activity quenched as described above.

Time Point	Hours Elapsed (hr)
T_0	0.0
T_1	2.25
T_2	21.5
T_3	76.75

[Table 2]

Enzymatic activity was confirmed by comparing T_0 to T_3 on the positive control (olive oil) via visible increased miscibility of the olive oil and the aqueous assay solute for PC1-PC3. Miscibility on the PC4 (no enzyme added) additionally showed no increase in the olive oil miscibility, confirming enzymatic activity.

Enzymatic activity was confirmed on ethyl 3-fluorobenzoate, analysed by [^{19}F] NMR to be 0% conversion at T_0 (Fig. 1), 15% at T_2 (Fig. 2), and 66% at T_3 (Fig. 3). [^{19}F] NMR analysis on E4 (no enzyme added) additionally showed a 0% conversion at T_3 (Fig. 4), confirming enzymatic activity. The lipase was thus confirmed to cleave polymeric mimetic ester bonds and successfully produce a degradation product.

No enzymatic degradative activity was confirmed on ethyl (4-fluorophenyl)carbamate, with a 0% conversion both at T_0 (Fig. 5) and at T_3 (Fig. 6). This may be due to solubility issues of the organic component restricting enzyme interactions. In future work, this would be mediated with addition of DMSO (1 mL) to improve solubility.

VI: Kinetic Analysis

Kinetic analysis was conducted on ethyl 3-fluorobenzoate following confirmation of enzymatic degradative activity. The enzyme assay (400 µL) and substrate (mass denominated in Table 3) were made up to 2.5 mL with pH8 buffer solution (KH_2PO_4 / K_2HPO_4), in accordance to their respective varying concentrations. pH8 buffer was used in kinetic analysis in correspondence with the particular favourable assay conditions of the enzyme ordered.

Substrate	Code	Type	Enzyme Added?	Mass of Substrate Added (mg)
Ethyl 3-fluorobenzoate	E0	Kinetic Analysis	Yes	0
Ethyl 3-fluorobenzoate	E10	Kinetic Analysis	Yes	12
Ethyl 3-fluorobenzoate	E20	Kinetic Analysis	Yes	20
Ethyl 3-fluorobenzoate	E50	Kinetic Analysis	Yes	50
Ethyl 3-fluorobenzoate	E100	Kinetic Analysis	Yes	99
Ethyl 3-fluorobenzoate	E200	Kinetic Analysis	Yes	203

[Table 3]

All assays were incubated simultaneously at 37 °C and 15,000 RPM, and various time points were extracted over the course of the reaction and analysed via ^{19}[F] NMR as above.

Time Point	Hours Elapsed (hr)

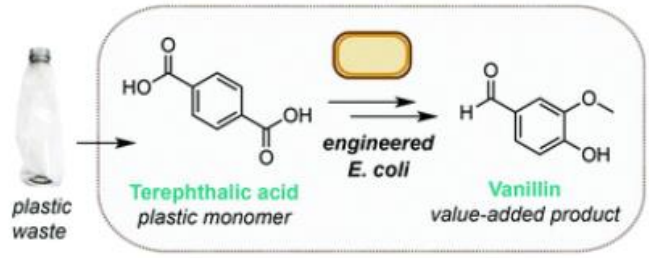
T_0	0.0
T_1	3.5
T_2	23

[Table 4]

With addition of pH8 buffer, the reaction rate was increased two-fold, with 66% conversion at E50 concentration occurring at the 23-hour mark. Further work would be to plot the concentrations over time against absorbance, to provide the kinetics for the rate of reaction for this enzyme-substrate complex.

VII: Further Considerations

Depolymerization, enzymatic or otherwise, returns a polymer to its substituent monomer. Most conventionally, this is then re-polymerized to create a commercially viable product in the form of a high-quality plastic. However, enzymatic degradation pathways do not always have to result in regeneration of a synthetic plastic. Whilst there is expansive opportunity to upcycle high quality plastics (virgin plastic production itself is forecasted to increase three-fold, with circular-economic recycling routes likely a more sustainable option than polymers derived from fossil fuels), note should also be taken on opportunities to biochemically transform plastic waste. One paper for alternate uses of the PET monomers produced from enzymatic PET depolymerization involves the synthesis of vanillin (vanilla extract).[39] Enzymatic transformation from bio-incompatible (plastic) monomer to a biocompatible molecule (vanillin) could be one route to addressing the associated toxicology of microplastics and nano-plastics.



Systems thinking on the biochemical potential of monomeric substituents, finding ways to convert and thus reduce planetary feedstock of waste plastics (rather than just to regenerate a polymer efficiently) is worth considering. Policy incentives on recycling, disincentivizing virgin plastic production, and regulations around plastic contamination all work to define the contexts in which biochemical polymer research can be carried out. As further research expands understanding on the extent of polymer fragment toxicology, and as biochemical transformation research progresses, there will perhaps be increasing impetus to tackle the world's plastic waste problem holistically. Circular alternatives to virgin plastic production, such as enzymatic repolymerization or even polymer synthesis from CO_2 gas captured from the atmosphere, should also consider whole-lifecycle assessments of each plastic, including degradative pathways, if we are to achieve a truly circular material economy.

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Appendix:

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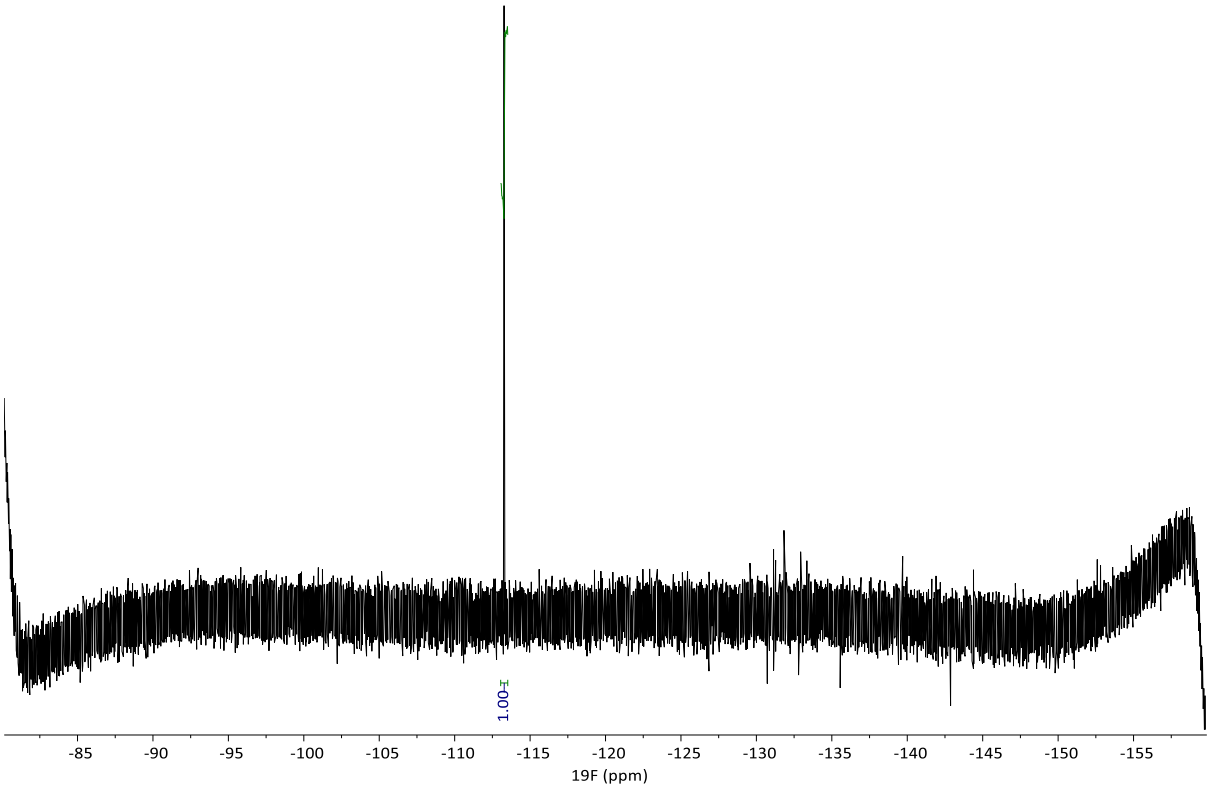

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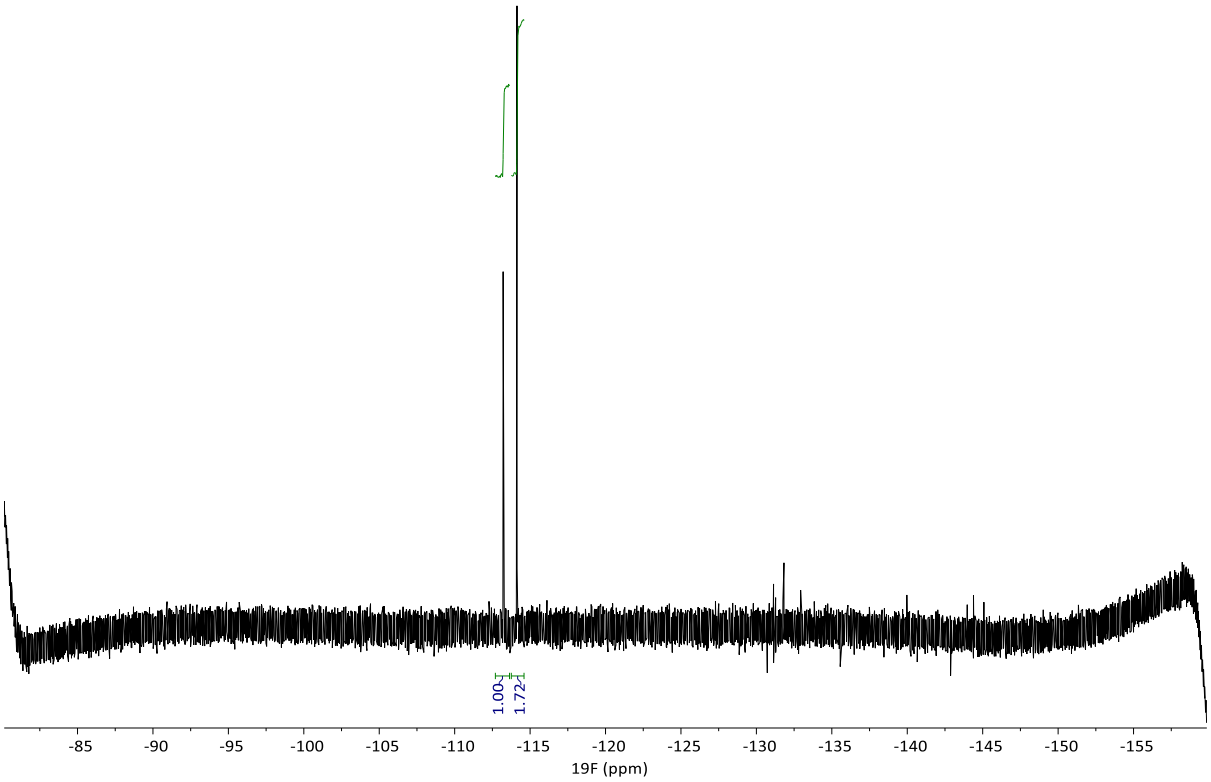

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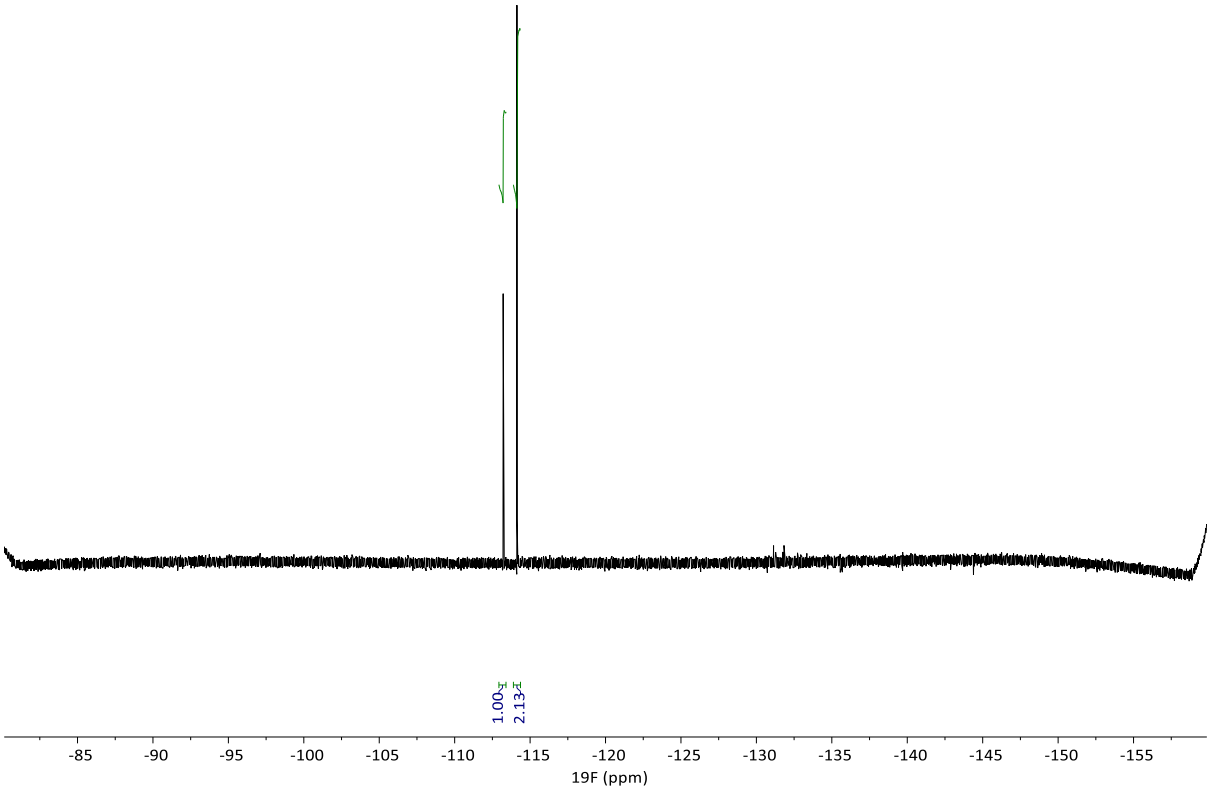

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