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CHARLES UNIVERSITY IN PRAGUE

TRABAJO FIN DE GRADO

Grado en Química

Phosphate based self-immolative linkers aimed for delivery of drugs

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Resumen

Los profármacos de nucleótidos (ProTide) basados en compuestos de fosfato o fosfonato son fármacos antivirales potentes y comercializados con éxito. La estrategia ProTide consiste en enmascarar temporalmente el grupo fosfónico libre polar para que facilite la administración del fármaco en el lugar deseado. A pesar de que sus propiedades biológicas son bien conocidas, hay una falta de información en el mecanismo de activación.

En este TFG hemos preparado nuevos profármacos basados en fosfato para la administración de fármacos que contienen un grupo amino, como por ejemplo anilinas, aminas alifáticas y heterocíclicas, etc, y hemos estudiado el mecanismo de activación con radiación UV para la liberación del fármaco.

Abstract

Nucleotide prodrugs (ProTides) based on phosphate or phosphonate compounds are potent and successfully marketed antiviral drugs. The ProTide strategy temporary masks the polar free phosphonic group facilitating the drug delivery inside the target site. Although their biological properties are well known there is a lack of data on the mechanism of their activation pathway.

In this thesis I present new phosphate-based prodrugs for delivery of drugs containing an amino group, such as anilines, aliphatic and heterocyclic amines, suitable for the mechanistic study of drug release pathway mediated by UV light irradiation.

Abbreviations and acronyms:

AZT	3´-azidothymidine
Bn	Benzyl
Boc	*Tert*-butoxycarbonyl
ºC	Centigrade (Celsius scale)
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCM	Dichloromethane
dd	Doublet of doublets
DMF	*N*,*N*-dimethylformamide
DMNB	4,5-dimethoxy-2-nitrobenzyl
DMSO	Dimethyl sulfoxide
dt	Doublet of triplets
eq	Equivalent(s)
Et_3N	Triethylamine
Et	Ethyl
EtOAc	Ethyl acetate
h	Hour(s)
Hz	Hertz(s)
J	Coupling constant
m	Multiplet
Me	Methyl
MeOH	Methanol
mg	Milligram
ml	Milliliter
MMPA	Methoxymethylphosphonic acid
Mmol	Millimol
MS	Mass Spectrometry
NAs	Nucleoside Analogues
NMR	Nuclear Magnetic Resonance
pKa	Acid disociation constant
q	Quartet
R	Substituent
Rt	Room temperature

s	Singlet
SI	Self-immolative
SN_2	Bimolecular nucleophilic substitution
TAF	Tenofovir alafenamide
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
UV	Ultraviolet

1. Introduction

1. Introduction

1.1. Nucleosides and nucleotides analogues

The study of nucleic acids has been carried out for more than one hundred years and, finally, in the 1950s, the discovery of DNA structure made possible to understand its fundamental importance for cell viability in vivo. Almost immediately the idea appeared that the nucleoside components of DNA or their analogues can affect the biochemical processes in bacteria and viruses. In the early 1960s the first nucleoside analogues (NAs) were synthesized.[1] A nucleoside is composed of a sugar moiety and nucleobase, whereas a nucleotide is composed of a sugar, nucleobase and, at least, one phosphate. Nucleosides and nucleotides play a key role in the replication and transcription of genetic information. Ideally, a nucleoside or nucleotide analogue would have similar structure to a natural nucleoside to be recognized by cellular or viral enzymes and to be incorporated into the DNA or RNA replication cycle. However, these analogues would possess one or more modifications that would lead to the disruption and/or termination of replication. During numerous studies various modifications to the nucleoside structure have been performed, including alteration to the sugar, nucleobase, glycosidic bond and phosphate group, as it is shown in the figure 1.

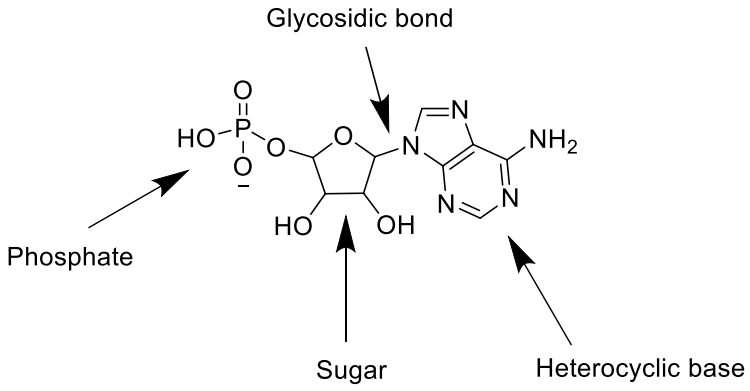


Figure 1. Sites for potential modification of nucleosides analogues

There is a number of possible modifications, e.g. adding a substituent to the heterocyclic base or sugar, replacing an atom in either moiety, moving an atom to a different position, or a combination of above mentioned approaches. This led to a wide array of potent nucleoside therapeutics, with complex structures.[2] Azidothymidine and didanosine, which suppressed HIV DNA replication in monocytes and macrophages *in vivo*, were the first nucleoside analogues

approved for the treatment of the previous diseases. Over the next years many nucleoside analogues have been created and used to fight viruses and cancer.[3]

However, the delivery of nucleoside analogues into the cells has two main limitations. In order to be recognized by DNA/RNA polymerases or reverse transcriptase and incorporated into the growing nucleic acid chain, during DNA/RNA replication, the nucleosides (or nucleotides) analogues first have to be phosphorylated by host cells or viral kinases into their triphosphate form. The triphosphate is highly charged which means the nucleotide cannot be directly delivered into the cell. Also, the first phosphorylation step is often highly specific and rate-limiting,[4] thus the nucleoside analogue is often not recognized and appears inactive.[2] NAs, as hydrophilic molecules, cannot rapidly enter cell membranes by passive transport. Instead they penetrate the cell by carrier-mediated endocytosis,[5] an active transport mechanism that requires energy and specific proteins in the cell surface. In addition they are not really suitable for oral administration, which is a desirable requisite for the treatment of most diseases.[6] To overcome these obstacles the ProTide approach was developed.[7,8]

1.2. ProTide prodrugs and ProTide activation pathway

Several prodrugs of biologically active phosphate and phosphonate analogues have been developed. The aim is to achieve temporary masking of the polar free phosphonic functional group until its systemic absorption and delivery, allowing the *in vivo* release of the active drug at the target site. Phosphate prodrugs have increased lipophilicity, and they are able to alter distribution and/or elimination patterns of the parent drug in the cells and tissues.[6]

The ProTide approach was developed by Chris McGuigan[9–11] (Cardiff University) in 1992 and it has recently been proven to be a very successful method for the intracellular delivery of nucleoside monophosphate into the cell, improving the activity of the parent drug.

ProTides[9] (PROdrug + nucleoTIDE), as it is shown in figure 2, consist of a 5´-nucleoside monophosphate in which the two hydroxyl groups are masked with an amino acid ester, promoiety linked via P–N bond, and an aryloxy component which once in the cell is enzymatically metabolized to deliver free 5´-monophosphate, which is further transformed to the active 5´-triphosphate form of the nucleoside analogue.[6] The aryl group and the amino acid ester mask the negative charges on the monophosphate, allowing the ProTide to efficiently cross the cell membrane.[2]

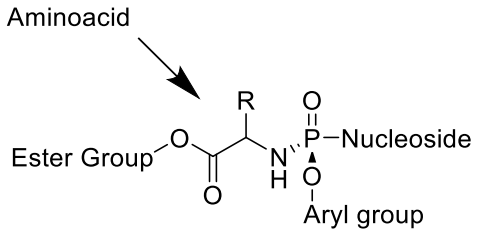


Figure 2. General structure of a McGuigan ProTide

ProTide compounds are enzymatically activated inside the target cells via metabolic pathways[12], following the mechanism shown in figure 3.

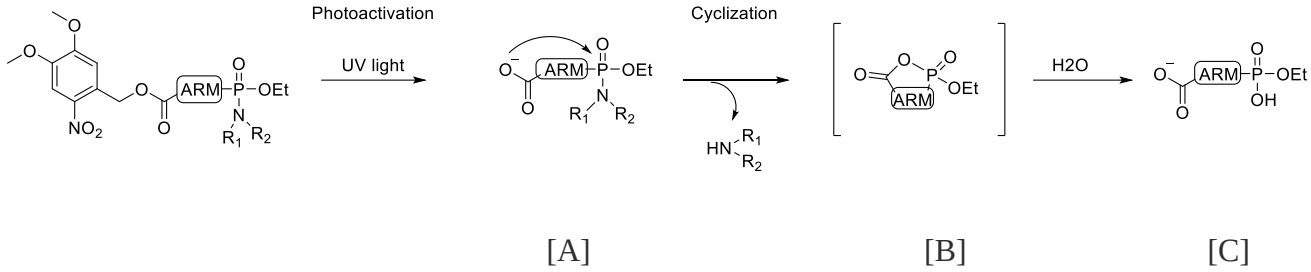


Figure 3. General metabolic pathway for phosphor(n)amidate ProTides

The mechanism consists of hydrolysis of the carboxylic ester of amino acid leading to intermediate [A]. The ester cleavage is followed by an internal nucleophilic attack of the acid residue on the phosphorous centre, displacing the amino group and forming a five-membered cyclic[13] intermediate [B]. The cyclic mixed anhydride is quickly hydrolized to the phosphoramidate [C].

As it is explained above, the activation of the prodrugs inside the target cells is carry out *via* metabolic pathways, in a cascade of decomposition reactions,[14] which could be considered a self-immolative (SI) processes.[15]

Structure-activity studies suggested[6] that the carboxylester group linked to the amino acid moiety has a significant influence on the pharmacokinetic of the prodrugs and their

stability. It was demonstrated that some simple esters such as methyl, ethyl or benzyl are quickly activated in contrast to the branched esters, such as *tert*-butyl ester. Also, the modification of the amino acid moiety has a big impact on the metabolism of the prodrug and α-amino acids were demonstrated to be necessary for biological activation. Although compounds bearing β-amino acids (longer amino acid) also showed efficient ester cleavage, they showed to be biologically inert and displayed no phenyl loss. L-Alanine was then identified and selected as the preferred amino acid. [6,16]

1.3. Examples of ProTide strategies, ProTide technology

As mentioned above, the phosphoramidate prodrug technology, commonly referred to as the ProTide technology, is one of the prodrugs technologies that mask one or two of the oxygens of the monophosphate group making the molecule more lipophilic so that the transport into the cells is improved. This prodrug technology has proved to be a powerful tool in the research of effective nucleoside monophosphate and monophosphonate prodrug therapeutics. This has so far translated into numerous ProTide clinical candidates, some of them are shown in figure 4, that act as antiviral agents against HIV, HBV, HCV and Ebola[17] virus. The most recent ones are being pursued as anticancer agents.[3,18] Currently, there are several ongoing clinical trials of Remdesivir, also called GS-5734, as potential drug against Covid-19 infection.[19,20]

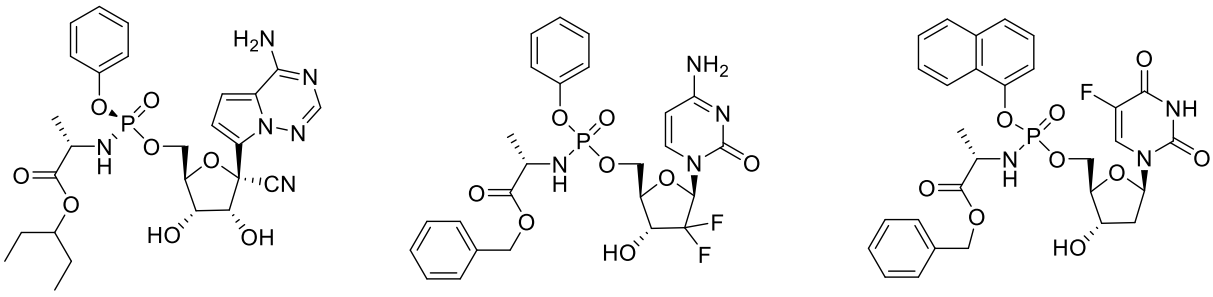


Figure 4. Examples of ProTide clinical candidates along with diseases they are being pursued to treat.

Moreover, the ProTide prodrug technology has so far led to the discovery of two FDA-approved antivirals, shown in figure 5: sofosbuvir[21–24], for the treatment of Hepatitis C Virus

(HCV) and tenofovir alafenamide (TAF)[25–28], for the treatment of Human Immunodeficiency Virus (HIV) and Hepatitis B Virus (HBV).

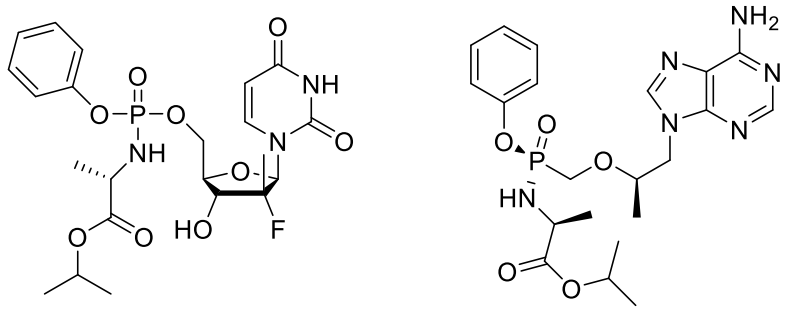


Figure 5. At the left, structure of Sofosbuvir, approved antiviral ProTide for the treatment of Hepatitis C virus. At the right, structure of Tenofovir Alafenamide, approved antiviral ProTide for the treatment of Hepatitis B Virus and Human Immunodeficiency Virus.

1.4. Methoxymethylphosphonic acid (MMPA) delivery vehicle for oral delivery of phenolic drugs

Propofol, figure 6, is one of the most extensively used intravenous anaesthetic drug.[29] It was reported[30] that HSK3486 (figure 6), a close analogue of propofol, shows equivalent therapeutic potentials in insomnia, migraine, anxiety, analgesia, etc.

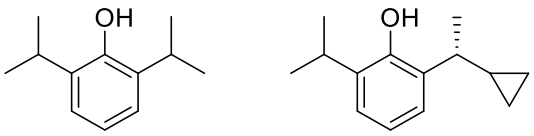


Figure 6. At the left, structure of Propofol. At the right, structure of HSK3486, a close analogue of Propofol.

Given the above-mentioned properties of both compounds, a research in their oral delivery through prodrug strategy was carried out by Y. Wei´s team[30]. Based on the previous work of Gilead Sciences[31,32] with the oral delivery of tenofovir prodrug, and using the ProTide strategy, Wei and his team proposed the same mechanism but replacing the phenol with propofol or HSK3486 and with methoxymethylphosphonic acid as the delivery vehicle.

Such work demonstrated that MMPA could be used as delivery vehicle for oral administration of phenolic drugs such as propofol or HSK3486, as it is shown in figure 7. Here,

the ProTide technology is altered and the phosphate moiety is used as a masking group releasing the active drug (Propofol).

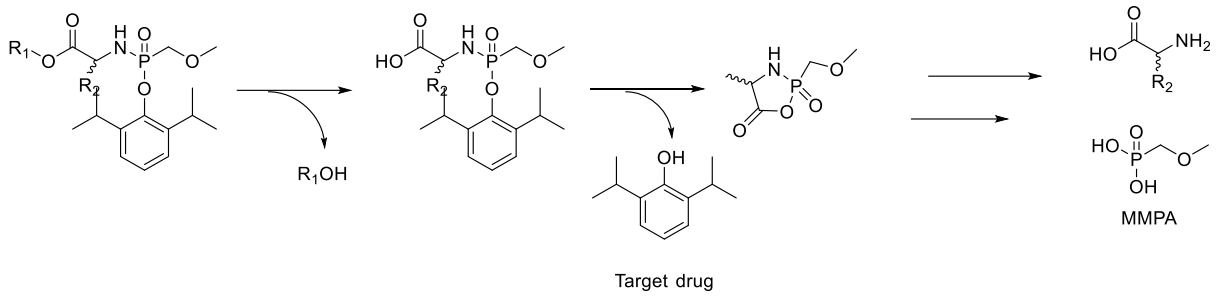


Figure 7. MMPA based oral delivery of propofol.

1.5. Self-immolation and photoactivated ProTides

Phosphoramidate self-immolation, first described by Imbach´s group[16], consists of an activation reaction[33] (enzymatic, chemical or physical) that cleaves the photoremovable[34–36] group and triggers a series of reactions that leads to the release of the cargo molecule.[37] These released chemical cargoes could be biomolecules, drugs or reporters such as fluorescent dyes.

It was observed that phosphoramidate self-immolation is strongly sensitive to the structure of phosphoramidate linker and to conditions used, such as solvent, temperature and concentration.[12] Regarding the influence of solvent, it was discovered that water presence is crucial for the cleavage of the photoremovable group and the whole activation pathway.[12,38]

Photoactivated ProTides carry a photoremovable group which is cleaved by UV light irradiation, initiating a self-immolation reaction that leads to the release of the cargo molecule inside the target cell.

Photoactivated prodrugs are involved in light activated cancer therapies[39–41], as antitumoral agents. The approach of using photoactivated compounds represents an alternative to surgery, chemotherapies and radiotherapies. These three techniques have to face several challenges such as lack of tumor selectivity and systemic toxicity during chemotherapy and radiotherapy and complete removal of cancer tissues during surgery. Hence, several studies were focused on the design of "smart" tumor environment responsive systems that can improve the capability of therapeutic molecules to reach the target cells.

To continue with the research on novel phosphate-based prodrugs, where the drug is released *via* cyclization (self-immolative) process from the phosphate core, in this work we will focus on various aliphatic and heterocyclic amines as possible leaving groups. The subsequent synthesis of photoactivated compounds will allow us the study of the self-immolation of phosphoramidates *in situ* inside the NMR.

2. Objectives and synthetic plan

2. Objectives and synthetic plan

The aim of this thesis is to study and prepare new phosphate-based prodrugs for delivery of drugs containing an aminogroup, e.g. aliphatic amines, anilines, heterocyclic amines, etc. Two strategies will be examined during this research.

At first, we will study a group of the phosphorbisamidate prodrugs where the phosphate core binds an alanine arm, responsible for self-immolation, NH_2, containing model substituent (cargo, drug) and a control group (ethyloxy group). At second, we will continue further with the study of phosphormonoamidate prodrugs with the phosphate core decorated with the lactate arm, responsible for self-immolation, NH_2, containing model substituent (cargo, drug) and a control group (ethyloxy group).

Our objectives are following:

1) Synthesis of the L-alanine and L-lactate photoarms protected with the 4,5-dimethoxy-2-nitrobenzyl (DMNB) photocaging group.

2) Preparation of model bisamidate prodrugs to screen the feasibility of the bisamidate synthesis and stability of prepared compounds.

3) Synthesis of model bisamidate prodrugs equipped with L-alanine photoarm (photoactivation study).

4) Synthesis of model monoamidate prodrugs to screen the feasibility of the monoamidate synthesis and stability of prepared compounds.

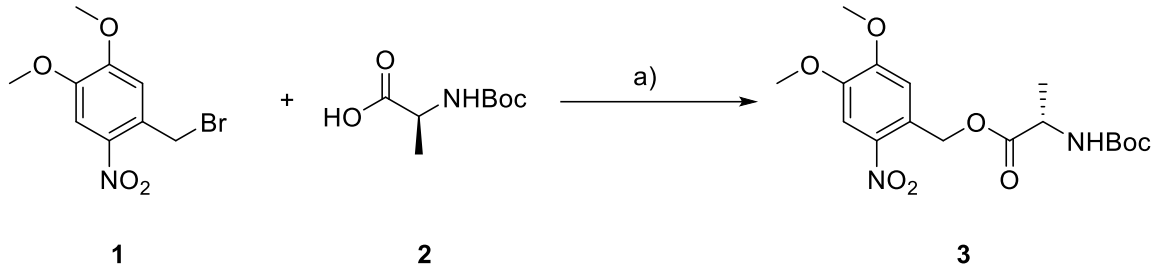
5) Synthesis of model monoamidate prodrugs equipped with L-lactate photoarm (photoactivation study).

6) Conclusion: Evaluation and impact of the study.

Synthetic plan:

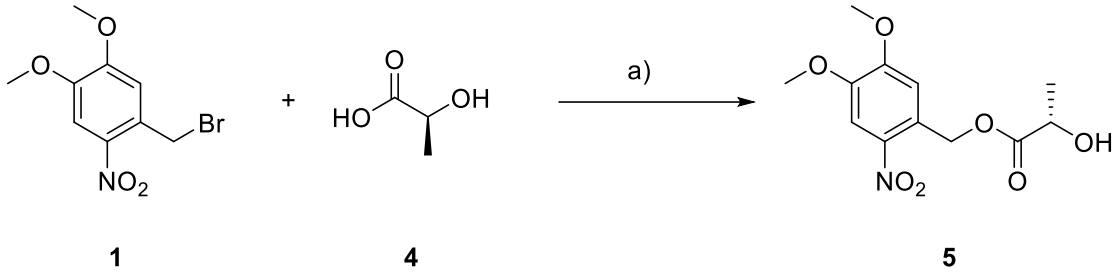
2.1. Synthesis of L-alanine and L-lactate photoarm protected with the 4,5-dimethoxy-2-nitrobenzyl (DMNB) photocaging group

The synthesis of the alanine photoarm[35,36] will be performed by using the substitution reaction shown in scheme 1.



Scheme 1: Synthesis of L-alanine photoarm. *Reagents and conditions*: (a) Cesium carbonate, dimethylformamide.

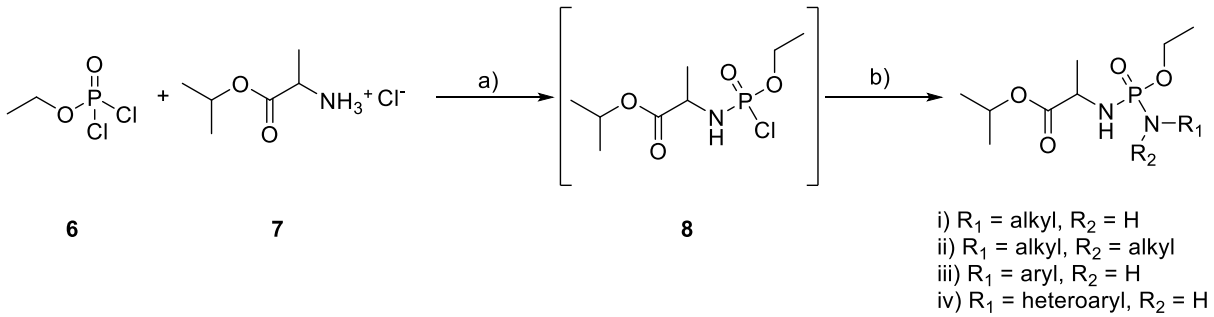
The synthesis of the lactate photoarm will be carried out using several methods, which will be discuss later. General synthetic procedure is shown in scheme 2.



Scheme 2: Synthesis of L-alanine photoarm. *Reagents and conditions***:** (a) Potassium carbonate, dimethylformamide.

2.2. Preparation of model bisamidate prodrugs to screen the feasibility of the bisamidate synthesis and stability of prepared compounds.

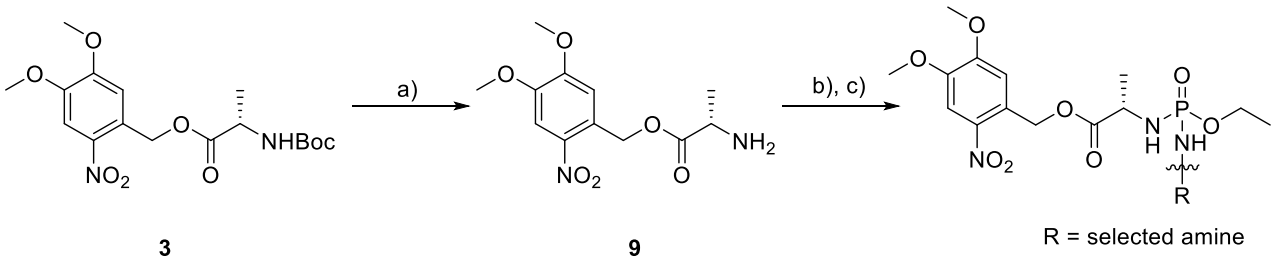
Due to the relatively high price of the L-alanine photoarm, we are going to screen the feasibility of the bisamidate synthesis and stability of prepared compounds first, by using simple L-alanine isopropyl ester (scheme 3).



Scheme 3: Synthesis of model bisamidate prodrugs. *Reagents and conditions*: (a) Triethylamine, dichloromethane; (b) Amine, triethylamine.

2.3. Synthesis of model bisamidate prodrugs equipped with L-alanine photoarm (photoactivation study)

Once we confirm the synthesis of bisamidate is successful and the prepared compounds are stable, we will continue with the synthesis of photoactivated prodrug (scheme 4) for the self-immolation study using the L-alanine photoarm **3**.

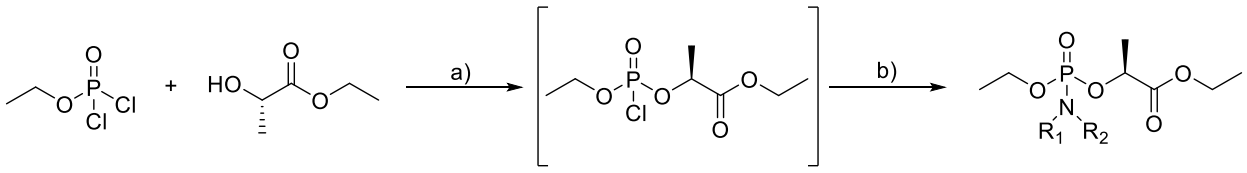


3 **9**

Scheme 4: Synthesis of the model bisamidate prodrugs equipped with L-alanine photoarm. *Reagents and conditions*: (a) Trifluoroacetic acid, dichloromethane; (b) Ethyldichlorophosphate, triethylamine, dichloromethane; (c) Amine, triethylamine.

2.4. Synthesis of model monoamidate prodrugs to screen the feasibility of the monoamidate synthesis and stability of prepared compounds

Similar strategy will be used for model phosphate prodrugs having L-lactate photoarm. At first, we are going to screen the feasibility of the monoamidate synthesis and stability of prepared compounds, by using simple (-) ethyl-L-lactate (scheme 5).



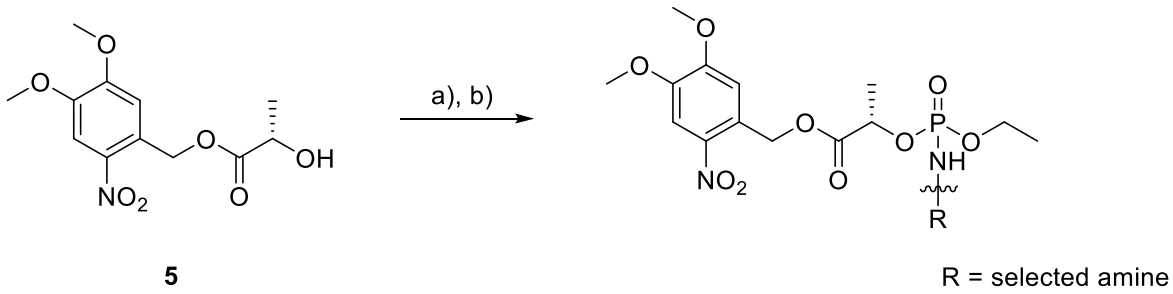
6 **10** **11**

i) R_1 = alkyl, R_2 = H
ii) R_1 = alkyl, R_2 = alkyl
iii) R_1 = aryl, R_2 = H
iv) R_1 = heteroaryl, R_2 = H

Scheme 5: Synthesis of model monoamidate prodrugs. *Reagents and conditions*: (a) Triethylamine, dry toluene; (b) Amine, triethylamine.

2.5. Synthesis of model monoamidate prodrugs equipped with L-lactate photoarm (photoactivation study)

Once we confirm the synthesis of monoamidate is successful and the prepared compounds are stable, we will continue with the synthesis of photoactivated prodrugs (scheme 6) for the self-immolation study using the L-lactate photoarm **5**.



5

Scheme 6: Synthesis of the model monoamidate prodrugs equipped with L-lactate photoarm. *Reagents and conditions*: (a) Ethyldichlorophosphate, triethylamine, dry toluene; (b) Amine, triethylamine.

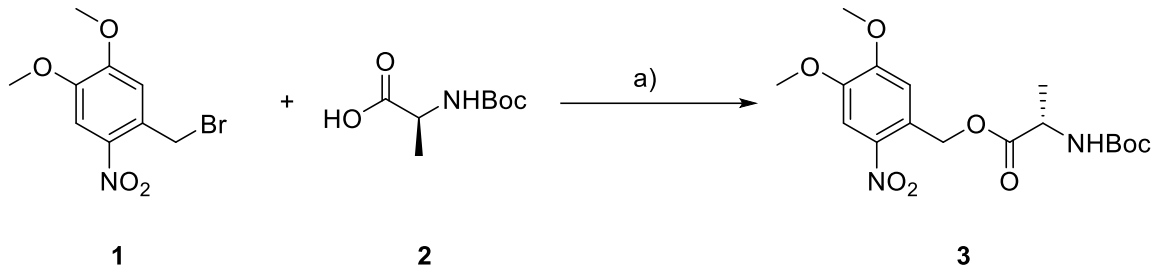
3. Results and discussion

3. Results and discussion

3.1. Synthesis of the L-alanine photoarm protected with the 4,5-dimethoxy-2-nitrobenzyl (DMNB) photocaging group

The synthesis of the L-alanine photoarm **9** consisted of two steps. The first synthetic step was the synthesis of the Boc protected alanine photoarm using DMNB[42] as photocaging group. Second synthetic step was the reaction of deprotection of the Boc group to obtain the (L)-alanine photoarm **9**.

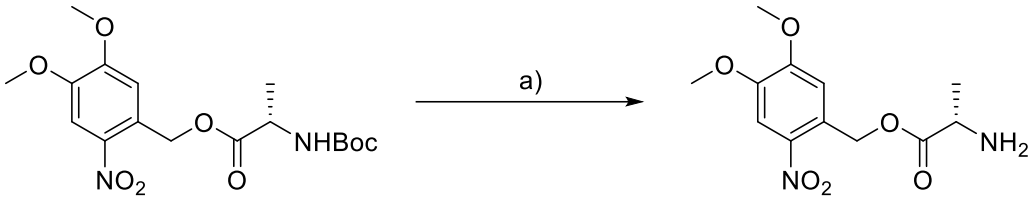
3.1.1. Synthesis of Boc protected alanine photoarm



Scheme 1: Synthesis of L-alanine photoarm. *Reagents and conditions*: (a) Cesium carbonate, dimethylformamide, rt, overnight.

The synthesis of Boc protected alanine photoarm was performed by a reaction (SN_2) between Boc protected L-alanine **2** and 1-(bromomethyl)-4,5-dimethoxy-2-nitrobenzene **1** in dry DMF in presence of cesium carbonate as a base. After stirring the reaction overnight at room temperature, a silica TLC (Hexane/Acetate 1:1) was performed to verify the reaction course. Starting compound disappeared and the reaction was extracted with water/EtOAc (3 x 20mL). Organic phase was dried by $MgSO_4$ and solvent was removed under vacuum. Crude product was purified by using silica chromatography (hexane/EtOAc, 1/1, v/v) to give a title compound **3** with 80% yield.

3.1.2. Deprotection of the Boc group



3 **9**

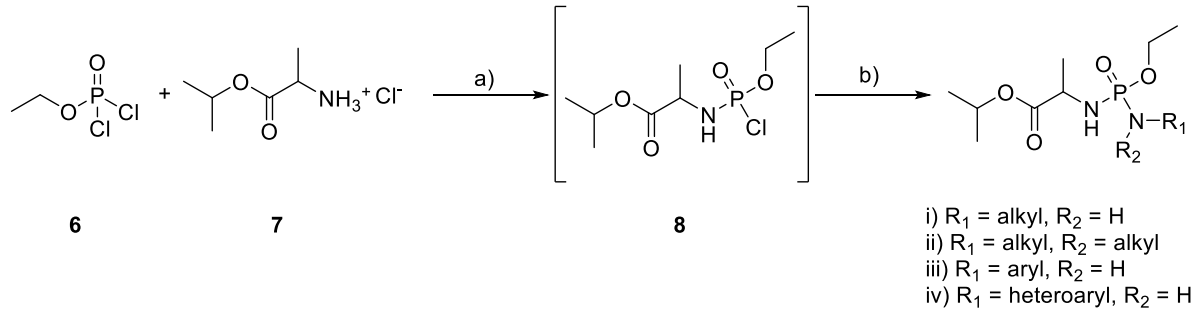
Scheme 7: Deprotection of the Boc group. *Reagents and conditions*: (a) Trifluoroacetic acid, dichloromethane, rt, 2h.

The last synthetic step consisted of deprotection of the alanine Boc group of the compound **3** by using trifluoroacetic acid[43]. After 2 hours of stirring, a silica TLC (Hexane/Acetate 1:1) was performed to verify the reaction course. Starting compound disappeared hence solvent was removed under vacuum. Desired photoarm **9** was obtained with a 90% yield as yellow solid. Photoarm **9** was later used for the synthesis of model bisamidate prodrugs.

3.2. Preparation of model bisamidate prodrugs to screen the feasibility of the bisamidate synthesis and stability of prepared compounds

As it was mentioned above, due to the relatively high price of the (L)-alanine photoarm **9**, we studied the scope of bisamidate synthesis and stability of prepared compound first, by using simple L-alanine isopropyl ester. Once we verified the synthetic feasibility and stability of target bisamidates, we repeated the synthesis again using the (L)-alanine photoarm to have the photoactivated prodrug for the self-immolation study.

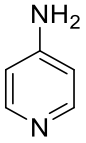
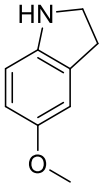
As it was mentioned in the chapter Objectives, the synthesis of model bisamidates consisted of two steps. The first synthetic step (a) is always identical for all the experiments performed in this screening study. Firstly, we performed substitution of the first chlorine on ethyldichlorophosphate by the reaction with L-alanine isopropyl ester in presence of Et_3N. Then, we monitored the formation of intermediate **8** by using a ^{31}P NMR. Two signals belonging to the two corresponding diastereomers of the intermediate **8** were observed.

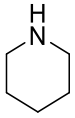
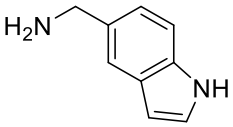
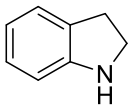
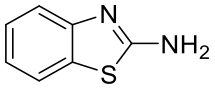
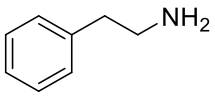


Scheme 3: Synthesis of model bisamidate prodrugs. *Reagents and conditions*: (a) Triethylamine, dichloromethane, rt, 1h; (b) Amine, trimethylamine, rt, 1h.

Screening the bisamidate synthetic scope: In order to screen the reactivity and stability of target bisamidate, the second step of the synthesis (b) consisted of the reaction of the phosphorchloridate intermediate **8** with an amine (a cargo), in the presence of trimethylamine (base). ^{31}P NMR was used to monitor the reaction course. A change in the ^{31}P NMR chemical shift was observed, resulting with a formation of the two new peaks belonging to the diastereomeric mixture of the final products.

As mentioned above, the synthesis was carried out with several different amines to perform an extensive study on the reactivity and stability on target compounds. The next table shows the amines used for the synthetic screen, their acidity (pKa), and, moreover, whether they worked or not in the above mentioned synthesis.

Experiment	Amine	pKa	Successful in the synthesis?	Isolated	Other observations
A		9,11	NO	NO	Various approaches were tried.
B		6,07	NO	NO	Did not react.

C		11	YES	YES	Multiple chromatography.
D		17,07	NO	NO	Multiple approaches were tried.
E		4,9	YES	NO	Detected in the ^{31}P NMR. Isolation not successful.
F		4,48	YES	NO	Detected in the ^{31}P NMR. Isolation not successful.
G		9,8	YES	YES	

In the experiments A, B and D we did not observe formation of any product in the ^{31}P NMR. Here we mostly observed the decomposition of the starting phosphorchloridate resulting in complex phosphorus NMR spectra. In case of experiments E and F we could see the formation of the diastereoisomeric product in the ^{31}P NMR but the stability of those compounds prevented product isolation. Finally, experiments C and G afforded clean spectra and the isolation of final products was possible.

Above mentioned reactivity between the tested amines and phosphochloridate **8**, and the stability of final bisamidates led us to following theory: Amines with lower pKa have more acidic nature, therefore the potencial product is less stable since they are better leaving groups. If the amines are ordered in increasing order of the acid dissociation constant (pka) we obtain following trend: 2-aminobenzothiazole < indoline < 5-methoxy-2,3-dihydroindoline < 4-aminopyridine < **phenetylamine** < **piperidine** < 5-(aminomethyl)indole. From the screen of amines presented above we could determine that performing the synthesis with piperidine and phenethylamine led to stable compounds, which is in correlation with their higher pKa.

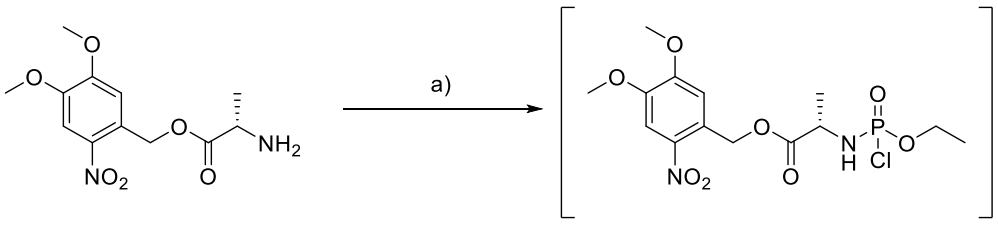
One experiment was carried out to test the stability of the phosphorbisamidate with the 2-aminobenzothiazole. The synthesis of this product was performed as described above and a NMR reaction monitoring was carried out, showing a clean ^{31}PNMR spectra where we observed the two new signals belonging to the product. Similarly, to previous attempts, the isolation and purification of this final product was not successful. The quenching experiment consisting of addition of phenethylamine to the reaction mixture resulted in the substitution of the 2-aminobenzothiazole by phenethylamine since the phenethylamine is a better nucleophile and 2-aminobenzothiazole is a good leaving group. The corresponding substitution product **19** was isolated.

This experiment confirmed that most final products can be synthesized following the synthesis described above but they are not stable enough to be isolated and purified.

3.3. Synthesis of model bisamidate prodrugs equipped with L-alanine photoarm (photoactivation study)

Based on the extensive study on the reactivity and stability on bisamidate compounds described above, I was able to demonstrate that piperidine (C) and phenethylamine (G) are suitable for the proposed synthesis of target compounds and therefore, the next step was to prepare target bisamidates **13** and **14** equipped with L-alanine photoarm for the photoactivation studies.

The L-alanine photoarm synthesis was already described above; thus, the next synthetic step was the reaction of **9** with ethyldichlorophosphate in dry DCM in presence of triethylamine. After stirring the reaction for one hour at room temperature a ^{31}P NMR monitoring was performed to follow the reaction course.

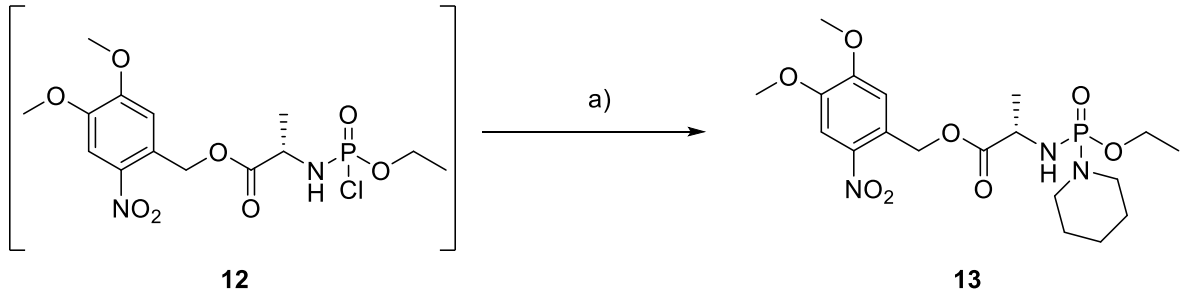


9 **12**

Scheme 8: Synthesis of phosphorchloridate intermediate 12. *Reagents and conditions*: Ethyldichlorophosphate, triethylamine, dichloromethane, rt, 1h.

The second step of the reaction consisted of adding 1 equivalent of the amine followed by 1 equivalent of triethylamine dropwise to substitute the second chlorine of the phosphochloridate.

3.3.1. Reaction with piperidine



Scheme 9: Synthesis of model bisamidate prodrug with piperidine. *Reagents and conditions*: (a) Piperidine, triethylamine, rt, 1h.

To the reaction mixture of intermediate **12** was added piperidine followed by triethylamine as a base and hydrogen chloride scavenger. The reaction was monitored by ^{31}P NMR to verify that the final product **13** was formed. Solvent was removed under vacuum and the crude product was purified by preparative TLC with a 30% yield.

Successful synthesis of compound **13** allowed us to evaluate the efficiency of model piperidine drug release. Photoremovable dimethoxynitrobenzyl (DMNB) group releases free carboxylate during the irradiation by 365 nm UV light, which triggers the whole process of prodrug activation.

Irradiation experiment was performed for the piperidine prodrug **13** using 365 nm LED lamp connected to an optical fiber fixed inside an NMR tube.

The sample (0.5 mM) was dissolved in mixture of 50% cacodylate buffer (0.1 M, pH 7.4) in DMSO-d6 at 25 °C. The utilization of cacodylate buffer allowed to adjust the physiological pH and avoided blurring the ^{31}P NMR by one broad peak belonging to the phosphate (from PBS buffer). Though, one major disadvantage of our approach could be the increased toxicity of cacodylate buffer.

In fact, after 30 minutes the piperidine was completely released from the phosphoramidate. Final phosphoramidate product was detected at 6.37 ppm (figure 8).

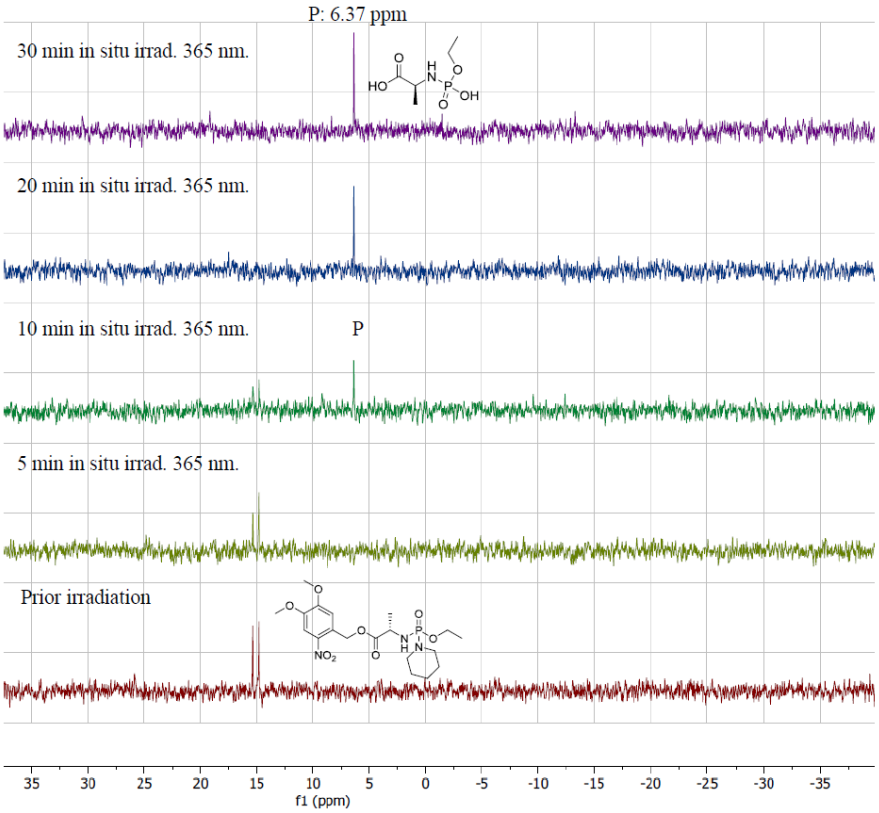
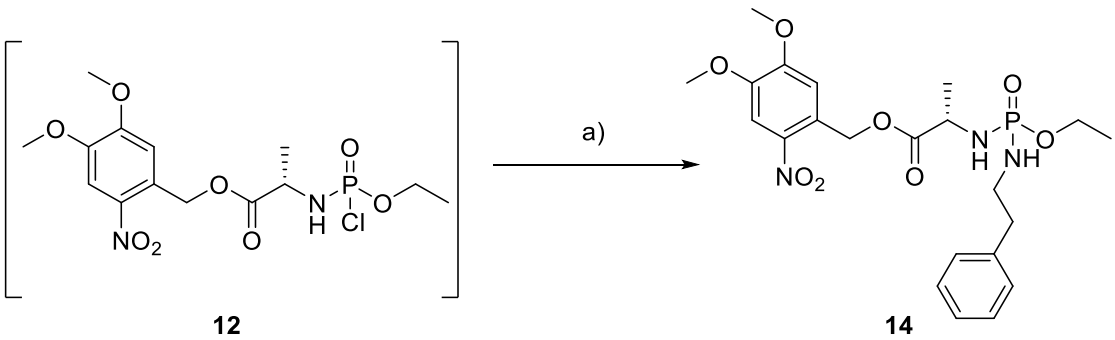


Figure 8. ^{31}P NMR spectra obtained from irradiation experiments

3.3.2. Reaction with phenethylamine



Scheme 10: Synthesis of model bisamidate prodrug with phenethylamine. *Reagents and conditions*: (a) Phenethylamine, triethylamine, rt, 1h.

Phenethylamine was added into the reaction mixture containing the intermediate **12** followed by addition of triethylamine. The reaction was stirred for one hour at room temperature. Then, a ^{31}P NMR was performed to monitor the reaction course and to verify that

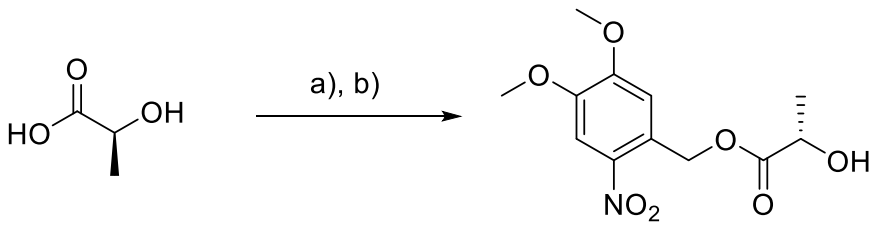
the final product **14** was formed. Solvent was removed under vacuum and the crude product was purified by using preparative TLC with a 25% yield.

This compound will undergo, in the future, the same irradiation experiment used in the case of the piperidine to evaluate the efficiency of model phenethylamine drug release.

3.4. Synthesis of the (L)-lactate photoarm protected with the 4,5-dimethoxy-2-nitrobenzyl (DMNB) photocaging group

The synthesis of lactate photoarm **5** was performed by three different methods to find the optimal procedure regarding the yield and the purity.

3.4.1. First approach



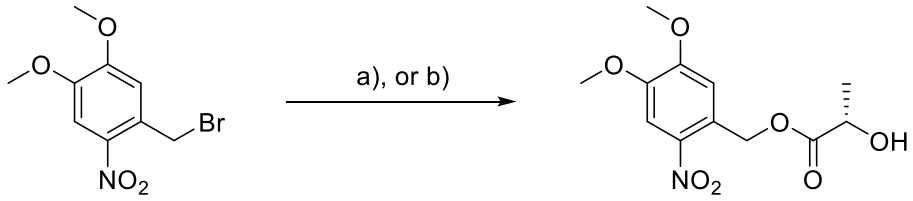
4 **5**

Scheme 11: Synthesis of L-lactate photoarm. *Reagents and conditions*: (a) DBU, methanol, rt, 30 min; (b) **1**, dimethylformamide, rt, overnight.

In this approach, the synthesis of lactate photoarm was performed by the addition of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) to a solution of anhydrous L-lactic acid (98%) in methanol. After formation of the corresponding lactate salt the solution of 1-(bromomethyl)-4,5-dimethoxy-2-nitrobenzene in dry DMF was added to the reaction. Crude product was purified by using silica chromatography to give a title compound **5** in 61%. The ^{31}P NMR spectrum showed that the product was not completely pure hence it was purified again with another silica chromatography affording compound **5** in 51% yield.

Due to the fact that two chromatography columns were needed to purify the product and that the yield obtained was not high we decided to look for another approach.

3.4.2. Second and third approaches



1 **5**

Scheme 12: Synthesis of (L)-lactate photoarm. *Reagents and conditions*: (a) Silver lactate, dimethylformamide, rt, overnight; or b) Anhydrous L-lactic acid (98%), dimethylformamide, potassium carbonate, rt, overnight.

Conditions (a): The synthesis of lactate photoarm was performed by a reaction (SN_2) between silver lactate and 1-(bromomethyl)-4,5-dimethoxy-2-nitrobenzene in dry DMF. Silica TLC analysis was performed to verify the reaction course. Starting compound disappeared and solvent was removed under vacuum. Crude product was purified by using silica chromatography to give a title compound **5** in 46%.

Purity of the product **5** was examined by using ^{31}P NMR which showed that obtained compound **5** is clean, however, the yield was low regarding the expenses of the synthesis, therefore it was necessary to look for another approach.

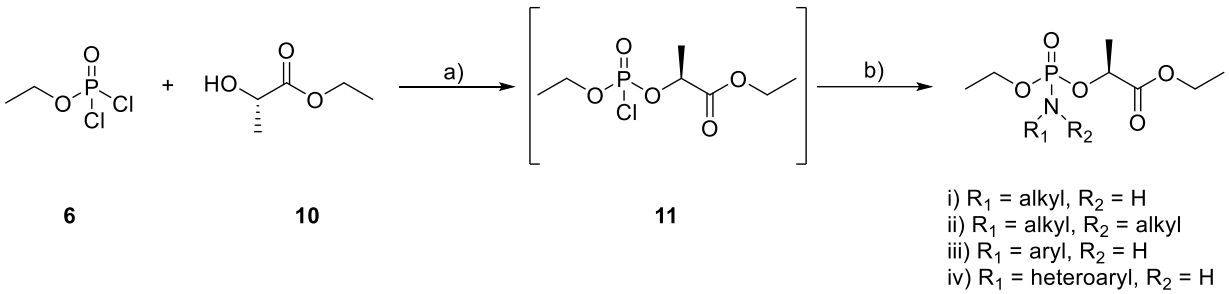
Conditions (b): The synthesis of lactate photoarm was performed by a reaction (SN_2) between anhydrous L-lactic acid (98%) and 1-(bromomethyl)-4,5-dimethoxy-2-nitrobenzene in dry DMF in presence of base K_2CO_3. Silica TLC was performed to verify the reaction course. Solvent was removed under vacuum and the crude product was purified by using silica chromatography to give a title compound **5** in 70%.

A ^{31}P NMR spectrum showed that the product was pure. Hence, with this approach we obtained the highest yield of **5** and the purest compound, it was not necessary to look for other approaches.

3.5. Preparation of model monoamidate prodrugs to screen the feasibility of the monoamidate synthesis and stability of prepared compounds

As it was mentioned above, due to the relatively high price of L-lactate photoarm **5** we studied the feasibility of the monoamidate synthesis and stability of prepared compounds first, by using simple (-) ethyl-L-lactate. Once we confirmed the synthesis of monoamidate was successful and the prepared compounds were stable we continued with the synthesis using the (L)-lactate photoarm to obtain the photoactivated prodrugs for the self-immolation study.

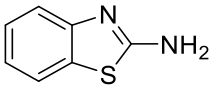
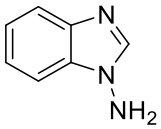
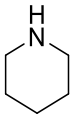
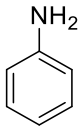
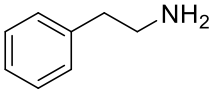
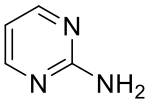
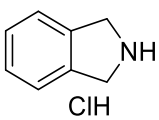
As it was mentioned in the aims, the synthesis of model monoamidates consisted of two steps. The first synthetic step (a) was always identical for all the experiments performed and consisted of the substitution of one ethyldichlorophosphate chlorine by the (-) ethyl-L-lactate in dry toluene in presence of Et_3N as a base (hydrogen chloride scavenger). ^{31}P NMR monitoring was performed and two new peaks belonging to the diastereomeric mixture of the intermediate **11** were observed.

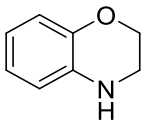
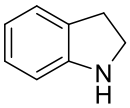
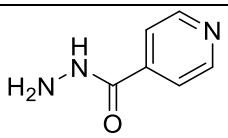


Scheme 5: Synthesis of model monoamidate prodrugs. *Reagents and conditions*: (a) Triethylamine, dry toluene, rt, overnight; (b) Amine, trimethylamine, rt, overnight.

Screening the synthetic monoamidates scope: In order to screen the reaction feasibility and the monoamidate synthetic scope, the second step (b) of the synthesis consisted of the reaction of the phosphochloridate **11** with an amine (a cargo), in presence of trimethylamine. ^{31}P NMR was used to monitor the reaction course. A change in the ^{31}P NMR chemical shift was observed, resulting with a formation of the two new peaks belonging to the diastereomeric mixture of the final product.

As mentioned above, the synthesis was carried out with several different amines to perform an extensive study on the reactivity and stability on target compounds. The next table shows the amines used for the synthetic screen, their acidity (pKa), and, moreover, whether they worked or not in the synthesis previously mentioned.

Experiment	Amine	pKa	Successful in the synthesis?	Isolated	Other observations
F		4,48	NO	NO	According to the NMR spectrum it did not react. According to MS it reacted.
H		28,4[44]	NO	NO	Did not react.
C		11	YES	YES (confirmed by NMR and MS)	MS shows that the biggest peak is the product. Conversion of 50% after 1 h of stirring.
I		4,58	YES	YES (confirmed by NMR and MS)	MS shows that the biggest peak is the product. Low conversion after 1 h of stirring.
G		9,8	YES	YES (confirmed by NMR and MS)	MS shows that the biggest peak is the product. High conversion after 1 h of stirring.
J		3,45	YES	NO	Detected in the ^{31}P NMR. Isolation not successful.
K		9,26	NO	NO	Did not react.
L		4,37	YES	YES	Low conversion after 1 h of stirring.

					
E		4,9	YES	YES	
M		2,00	NO	NO	Did not react.

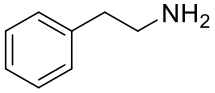
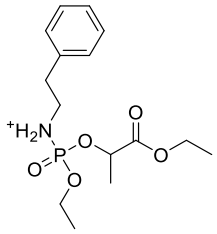
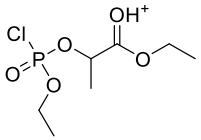
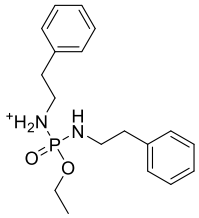
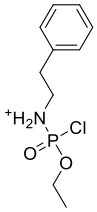
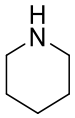
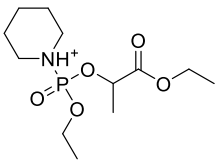
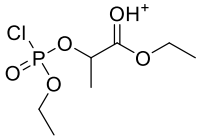
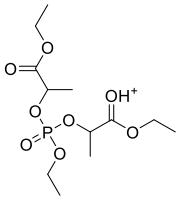
In experiments F, H, K and M we did not observe formation of any product in the ^{31}P NMR, just decomposition of the starting phosphorchloridate leading to complex mixtures. In case of J we could see the formation of the diastereoisomeric product in the ^{31}P NMR spectra but the product isolation using flash chromatography was not successful, most probably because of the limited stability of final product. Finally, the experiments C, E, G, I, and L afforded clean spectra and isolation of final products (**20**, **25**, **22** and **21** respectively) was possible.

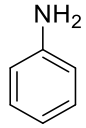
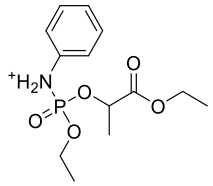
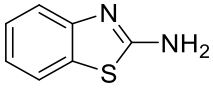
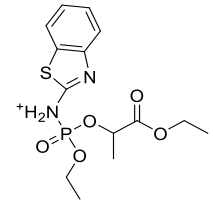
Similarly to the screening study mentioned above, amines with lower pKa have more acidic character, therefore the product is less stable since they are better leaving groups. If the amines are ordered in increasing order of the acid dissociation constant (pka) we obtain following trend: isoniazid < 2-aminopyrimidine < **3,4-Dihydro-2H-benzo[1,4]oxazine** < 2-aminobenzothiazole < **aniline** < **indoline** < Isoindoline hydrochloride < **phenetylamine** < **piperidine** < 1-aminobenzimidazole.

From the screen of amines presented above we could determine that performing the synthesis with 3,4-dihydro-2*H*-benzo[1,4]oxazine, aniline, indoline, piperidine and phenethylamine led to stable compounds, hence we could see that in this case the stability of final products is not really correlated with their pKa values.

A mass spectrometry analysis was performed with 4 amines: piperidine, aniline, 2-aminobenzothiazole and phenethylamine to monitor the structure of the compounds in the reaction mixture. The MS samples were prepared from the reaction mixture of the second step

of the synthesis (scheme 5) once it was stirred overnight. This study had the aim to compare the reactivity and the speed of conversion of four different types of amines: primary amine (phenethylamine), secondary amine (piperidine), aromatic amine (aniline) and heterocyclic amine (2-aminobenzothiazole). The next table contents the biggest signals showed in the mass spectra for the four samples.

Amine	Type of amine	Most intensive signal	Other MS signals
 Phenethylamine	Primary amine	 Chemical Formula: $C_{15}H_{25}NO_5P^+$ Exact Mass: 330,14649 Molecular Weight: 330,34021	 Chemical Formula: $C_7H_{15}ClO_5P^+$ Exact Mass: 245,03401 Molecular Weight: 245,61521  Chemical Formula: $C_{18}H_{26}N_2O_2P^+$ Exact Mass: 333,17264 Molecular Weight: 333,39121  Chemical Formula: $C_{10}H_{16}ClNO_2P^+$ Exact Mass: 248,06017 Molecular Weight: 248,66621
 Piperidine	Secondary amine	 Chemical Formula: $C_{12}H_{25}NO_5P^+$ Exact Mass: 294,14649 Molecular Weight: 294,30721	 Chemical Formula: $C_7H_{15}ClO_5P^+$ Exact Mass: 245,03401 Molecular Weight: 245,61521  Chemical Formula: $C_{12}H_{24}O_8P^+$ Exact Mass: 327,12033 Molecular Weight: 327,28921

 Aniline	Aromatic amine	 Chemical Formula: $C_{13}H_{21}NO_5P^+$ Exact Mass: 302,11519 Molecular Weight: 302,28621	Unknown peak at retention time 5,98 min andm/z: 220,0282
 2-aminobenzothiazole	Heterocyclic amine	 Chemical Formula: $C_{14}H_{20}N_2O_5PS^+$ Exact Mass: 359,08251 Molecular Weight: 359,35621	Some unknown peaks: m/z: 567,1286 m/z: 491,0939 m/z: 499,0386

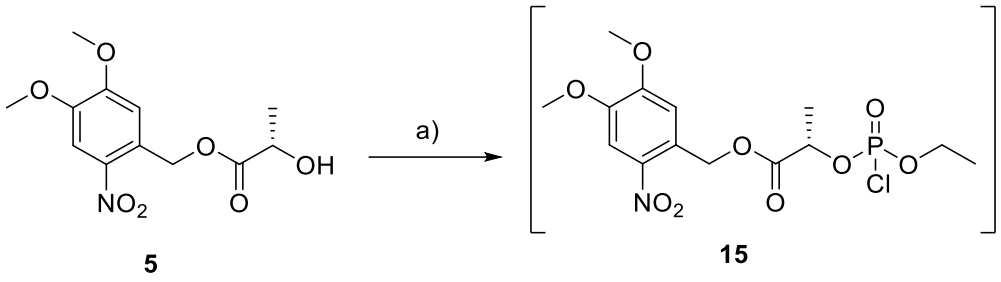
These four measurements showed that in all the cases the final product was in the reaction mixture as the main compound. It was possible to establish an order according to the reactivity, conversion and speed of these four amines due to the appearance of the mass spectra: phenethylamine > piperidine > aniline > 2-aminobenzothiazole.

Moreover, these four reaction mixtures were stirred for five days, after which a ^{31}P NMR measurement was performed to observe the stability of the final products. The results showed that the final products with phenethylamine, piperidine and aniline were stable. However, in case of 2-aminobenzothiazole it was not possible to observe any signal belonging to the product in the ^{31}P NMR spectra, which led to the conclusion that this product was not stable.

3.6. Synthesis of model monoamidate prodrugs equipped with L-lactate photoarm (photoactivation study)

Based on the screening study on reactivity and stability on monoamidate compounds described above, I was able to demonstrate that piperidine (C), indoline (E), phenethylamine (G), aniline (I) and 3,4-dihydro-2*H*-benzo[1,4]oxazine (L) are suitable for the proposed synthesis of target compounds. Therefore, the next step was to prepare target monoamidates equipped with the L-lactate photoarm for photoactivation studies.

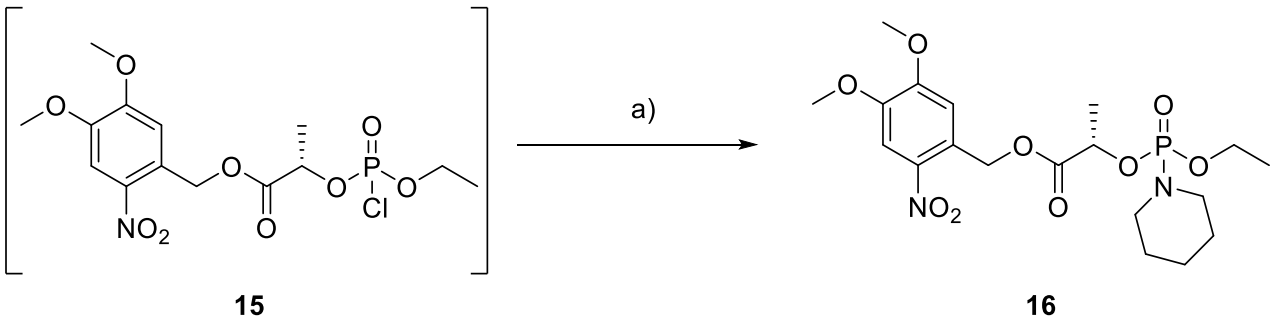
The reaction of the lactate photoarm **5** with ethyldichlorophosphate in dry toluene and in presence of triethylamine afforded the intermediate **15**. ^{31}P NMR monitoring was performed to follow the reaction course.



Scheme 13: Synthesis of phosphorchloridate intermediate 15. *Reagents and conditions*: Ethyldichlorophosphate, triethylamine, toluene, rt, overnight.

The second step of the reaction consisted of adding 1 equivalent of the amine followed by 1 equivalent of triethylamine as a base (hydrogen chloride scavenger).

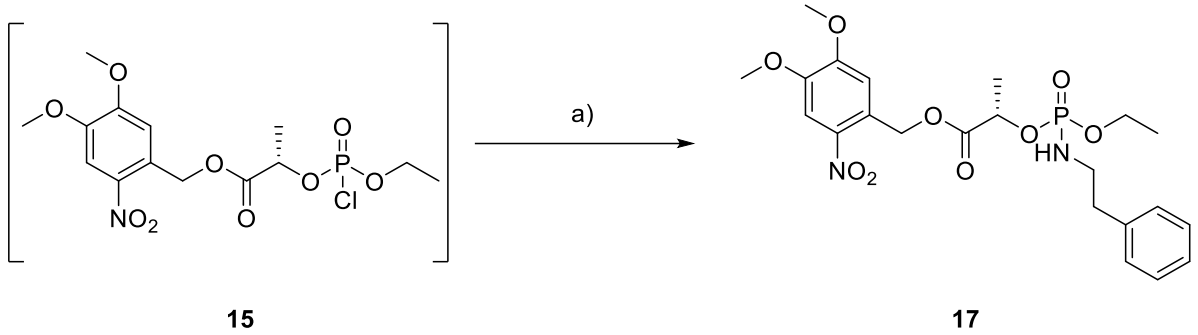
3.6.1. Reaction with piperidine



Scheme 14: Synthesis of model monoamidate prodrug with piperidine. *Reagents and conditions*: (a) Piperidine, trimethylamine, rt, overnight.

To the reaction mixture phosphochloridate **15** was added piperidine followed by triethylamine dropwise. ^{31}P NMR was performed to monitor the reaction course and to verify that the final product was formed. The solvent was removed under vacuum.

3.6.2. Reaction with phenethylamine



15 **17**

Scheme 15: Synthesis of model monoamidate prodrug with phenethylamine. *Reagents and conditions:* (a) Phenethylamine, trimethylamine, rt, overnight.

To the reaction mixture containing intermediate **15** was added phenethylamine followed by triethylamine dropwise. ^{31}P NMR was performed to monitor the reaction course and to verify that the final product was formed. The solvent was removed under vacuum.

These two compounds will be used in the future for other studies of irradiation to evaluate the efficiency of model piperidine and phenethylamine drug release. In addition, it will be possible to compare between the L-alanine photoarm compounds and the L-lactate photoarm compounds in terms of efficiency and duration of drug release.

4. Conclusions

4. Conclusions

1. The first screen for possible bisamidate model prodrugs was performed with L-alanine isopropyl ester as a substitute for the corresponding L-alanine photoarm. Our study shows that only primary and secondary amines were suitable for the synthesis of final products. In our case, piperidine and phenethylamine gave the products which were stable enough for the isolation and purification.

2. Model bisamidate prodrugs equipped with L-alanine photoarm were synthesised by using piperidine and phenethylamine.

3. Photoactivation study was performed with the model piperidine bisamidate and it showed the piperidine drug release after 30 minutes of irradiation.

4. The second screen for suitable monoamidates model prodrugs bearing L-lactate ethyl ester was performed. Most of the tested amines were able to form a final product in the ^{31}PNMR, but only in certain cases it was possible to isolate and purify them. Monoamidate compounds with L-lactate ethyl ester demonstrated higher stability than bisamidates with L-alanine isopropyl ester.

5. Model monoamidate prodrugs equipped with L-lactate photoarm were synthesised by using piperidine and phenethylamine.

5. Experimental

5. Experimental

General

All the reactions were carried under inert atmosphere (argon, Schlenk line).

NMR spectra were recorded with 400 Hz NMR machine (Brüker AsendTM 400 device equipped with an autosampler). Spectra were recorded in $CDCl_3$ or DMSO-d_6 as solvent (^{1}H: 400MHz, ^{13}C: 100.6MHz and ^{31}P: 162MHz). Chemical shifts for protons are reported in ppm from TMS as standard with the residual $CHCl_3$ resonance as internal reference. Chemical shifts for carbons are reported in ppm from TMS as standard and are referenced to the carbon resonance of the solvent. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, dt = doublet of triplet, br = broad signal), coupling constants in Hertz, and integration.

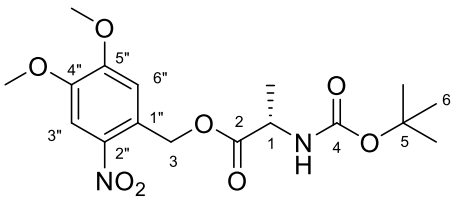
Mass spectra were measured on a LCQ Fleet spectrometer (Thermo Fisher Scientific) using ESI ionisation.

TLC analysis was performed on glass-backed plates coated with silica gel 60 F254 Merck and visualized by either UV irradiation (254 nm UV lamp) or by staining with $KMnO_4$ agent.

Commercially available organic and inorganic compounds were used without further purification. Anhydrous solvents were bought from Acros Organics: 99.8%, extra dry, stored over molecular sieves, AcroSeal®.

5.1. Synthesis of alanine photoarm

5.1.1. Synthesis of Boc protected alanine photoarm (3)

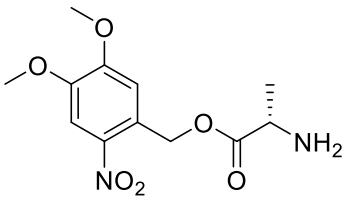


3

To a solution of Boc protected (L)-alanine (189 mg, 1 mmol, 1 eq) in dry DMF (4 ml) was added cesium carbonate (195 mg, 0.6 mmol, 0.6 eq). The reaction was stirred for 25 minutes at 25 ºC. Then, the suspension was cooled down to 0ºC and 1-(bromomethyl)-4,5-dimethoxy-2-nitrobenzene (290 mg, 1.05 mmol, 1.05 eq) was added in one portion. The reaction was stirred at 25°C overnight. Then, the reaction was

partitioned between water/EtOAc and aqueous phase was extracted with EtOAc (3x). The organic phase was dried with magnesium sulfate anhydrous and the solvent was removed under vacuum. The product was immediately purified by chromatography column (cyclohexane/EtOAc 5:1 to cyclohexane/EtOAc 3:1 and to cyclohexane/EtOAc 1:1). A white-yellow solid (309 mg, 0,80 mmol, 80,47%) was obtained. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (s, 1H, 3”), 7.07 (s, 1H, 6”), 5.60 (m, 2H, 3), 5.02 (d, *J* = 8.0 Hz, 1H, N**H**), 4.44 (m, 1H, 1), 4.04 (s, 3H, 4”-OMe or 5”-OMe), 3.98 (s, 3H, 4”-OMe or 5”-OMe), 1.47 (d, *J* = 8.0 Hz, 3H, 1-Me), 1.45 (s, 9H, 6). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.11 (s, HMBC, 2), 153.92 (s, HMBC, 4´´), 148.24 (s, HMBC, 5´´), 139.46 (s, HMBC, 2´´), 127.24 (s, HMBC, 1´´), 111.20 (s, 6´´), 107.44 (s, 3´´), 80.2 (s, HMBC, 5), 64.47 (s, 3), 56.65 (s, 4”-OMe or 5”-OMe), 56.43 (s, 4”-OMe or 5”-OMe), 49.43 (s, 1), 28.31 (s, 6), 18.29 (s, 1-Me).

5.1.2. Deprotection of Boc group of the L-alanine photoarm (9)

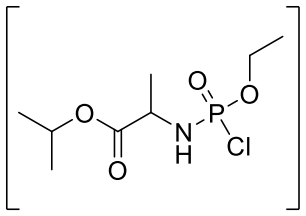


9

To a solution of Boc protected alanine photoarm (**3**) (150 mg, 0.39 mmol, 1 eq) in dry DCM (3 ml) was added trifluoroacetic acid (3 ml). The reaction was stirred for 2 hours at room temperature. Then, the solvent was removed under vacuum. A yellow solid (100 mg, 0.35 eq, 90.2%) was obtained. Crude product was directly used in the synthesis of model photoactivated prodrugs.

5.2. Synthesis of model bisamidate prodrugs having simple L-alanine isopropyl ester

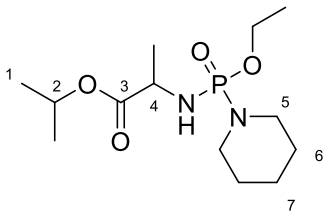
5.2.1. Isopropyl (chloro(ethoxy)phosphoryl)alaninate (8)



8

To a solution of L-alanine isopropyl ester hydrochloride (167.63 mg, 1 mmol, 1 eq) in dry DCM (5 ml), cooled down with dry ice and acetone, was added ethyldichlorophosphate (0.119 ml, 1 mmol, 1 eq) followed by Et_3N (0.279 ml, 2 mmol, 2 eq) dropwise. The solution was stirred for an hour. ^{31}P NMR (162 MHz, $CDCl_3$) δ 12.68, 12.33. Crude product was directly used in the next reaction step (phosphorylation of various amines).

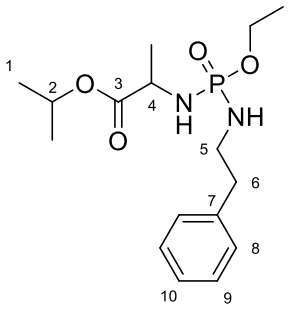
5.2.2. Isopropyl (ethoxy(piperidin-1-yl)phosphoryl)alaninate (18)



18

To a solution of the intermediate **8** was added piperidine (0.207 ml, 2.1 mmol, 2.1 eq). The reaction was stirred for 2 hours at room temperature. Then, the solvent was removed under vacuum. The product was immediately purified by chromatography column (pure DCM to DCM/MeOH 9:1). An orange oil was obtained. It was not completely pure hence the product was repurified by preparative TLC (DCM/MeOH 9:1) with a 60% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.05 (m, 1H, 2), 4.05 – 3.95 (m, 2H, O-**CH_2**-CH_3), 3.92 – 3.79 (m, 1H, 4), 3.15 – 3.02 (m, 4H, 5), 1.68 – 1.45 (m, 6H, 6 and 7), 1.43 – 1.36 (m, 3H, 4-Me), 1.32 – 1.21 (m, 9H, 1 and O-CH_2-**CH_3**). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.17 (s, HMBC, 3), 69.30 (s, 2), 62.18 (s, O-**CH_2**-CH_3), 49.76 (d, *J* = 11.0 Hz, 4), 45.42 (s, 5), 25.88 (m, 6 and 7), 21.56 (m, 1 and 4-Me), 17.56 (s, O-CH_2-**CH_3**). ^{31}P NMR (162 MHz, $CDCl_3$) δ 12.66, 12.29.

5.2.3. Isopropyl (ethoxy(phenethylamino)phosphoryl)alaninate (19)

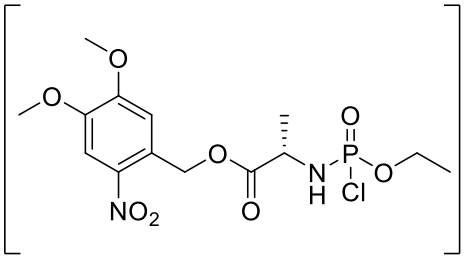


19

To a solution of the intermediate **8** was added phenethylamine (0.126 ml, 1 mmol, 1 eq) followed by Et_3N (0.140 ml, 1 mmol, 1 eq) dropwise. The reaction was stirred for 2 hours at room temperature. Then, the solvent was removed under vacuum. The product was immediately purified by chromatography column (pure DCM to DCM/MeOH 9:1) with 50% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37 – 7.11 (m, 5H, 8, 9 and 10), 5.03 (m, 1H, 2), 4.00 (m, 2H, O-**CH_2**-CH_3), 3.90 – 3.79 (m, 1H, 4), 3.20 (m, 2H, 5), 2.60 – 2.48 (m, 2H, 6), 1.45 – 1.30 (m, 3H, 4-Me), 1.30 – 1.20 (m, 9H, 1 and O-CH_2-**CH_3**). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.71 (s, 3), 139.54 (s, 7), 130.13 (s, 9), 128.50 (s, 8), 126.48 (s, 10), 67.02 (s, 2), 61.27 (s, O-**CH_2**-CH_3), 50.61 (d, *J* = 13.0 Hz, 4), 43.84 (s, 5), 37.59 (s, 6), 21.75 (s, 1), 21.40 (s, 4-Me), 17.31 (s, O-CH_2-**CH_3**). ^{31}P NMR (162 MHz, $CDCl_3$) δ 13.53, 13.10.

5.3. Synthesis of model bisamidate prodrugs equipped with L-alanine photoarm

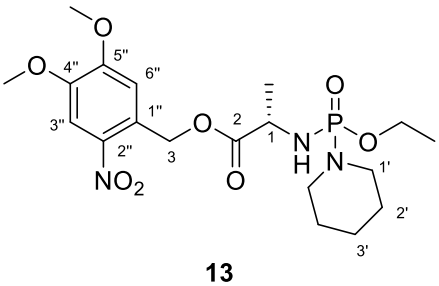
5.3.1. 4,5-dimethoxy-2-nitrobenzyl (chloro(ethoxy)phosphoryl)-L-alaninate (12)



12

To a solution of L-alanine photoarm **9** (150 mg, 0.39 mmol, 1 eq) in DCM (5 ml), cooled down in dry ice and acetone, was added ethyldichlorophosphate (0.046 ml, 0.39 mmol, 1 eq) followed by Et_3N (0.108 ml, 0.78 mmol, 2 eq) dropwise. The solution was stirred for one hour, and directly used in the next reaction step.

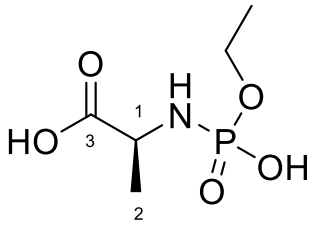
5.3.2. 4,5-dimethoxy-2-nitrobenzyl (ethoxy(piperidin-1-yl)phosphoryl)-L-alaninate (13)



13

To a solution of the intermediate **12** was added piperidine (0.081 ml, 0.819 mmol, 2.1 eq). The reaction was stirred for an hour. The solvent was removed under vacuum and the product was immediately purified by preparative TLC (DCM/MeOH 98:2) with 30% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (s, 1H, 3’’), 7.05 and 7.03 (2 x s, 1H, 6’’), 5.68 – 5.52 (m, 2H, 3), 4.13 – 4.04 (m, 1H, 1), 4.03 – 3.96 (m, 8H, 4’’-OMe, 5”-OMe, O-**CH$_2$**-CH_3), 3.15 – 3.00 (m, 4H, 1’), 1.67 – 1.43 (m, 5H, 1-Me, 3’), 1.36 – 1.23 (m, 7H, 2’, O-CH_2-**CH$_3$**). ^{13}C NMR (101 MHz, $CDCl_3$) δ 149.64 (s, HMBC, 5”), 144.07 (s, HMBC, 4”), 135.73 (s, HMBC, 1”), 122.77 (s, HMBC, 2”), 110.37 (d, *J* = 10.0 Hz, 6”), 108.26 (d, *J* = 2.0, 3”), 63.99 and 63.94 (2 x s, 3), 61.26 (d, *J* = 3.2 Hz, -O-**CH$_2$**-CH_3), 56.63 and 56.58 (2 x s, 5”-OMe), 56.45 (s, 4”-OMe), 49.95 (d, *J* = 2.5 Hz, 1), 45.44 – 45.34 (m, 1’), 29.86 (s, 2’), 26.30 – 26.25 (m, 3’), 21.58 (s, 1-Me), 16.70 (s, O-CH_2-**CH$_3$**). ^{31}P NMR (162 MHz, $CDCl_3$) δ 13.52, 13.15.

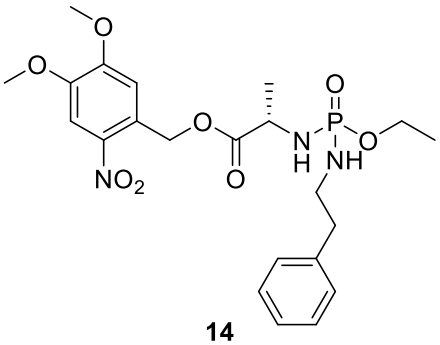
Final product after irradiation



Compound **13** (0.5 mM) was dissolved in mixture of 50% cacodylate buffer (0.1 M, pH 7.4) in DMSO-d6 at 25 °C in NMR tube. Then the sample was irradiated under the optimal conditions investigated previously by 365nm LED lamp using an optical fiber fixed inside an NMR tube. ^{1}H NMR (500 MHz, 50% cacodylate buffer (0.1M, pH 7,4)/DMSO-d6) δ 3.60 (m, 2H, O-**CH$_2$**-CH_3), 3.32 (m, 1H, 1), 1.08 (dd, *J* =

7.0, 2.2 Hz, 3H, 2), 1.03 (dt, *J* = 7.0, 2.1 Hz, 3H, O-CH_2-**CH_3**). ^{13}C NMR (125 MHz, 50% cacodylate buffer (0.1M, pH 7,4)/DMSO-d) δ 182.57 (d, *J* = 8.1 Hz, 3), 61.40 (d, *J* = 5.1 Hz, O-**CH_2**-CH_3), 53.51 (1), 23.44 (d, *J* = 3.3 Hz, 2), 17.60 (d, *J* = 7.5 Hz, O-CH_2-**CH_3**). ^{31}P NMR (202 MHz, 50% cacodylate buffer (0.1M, pH 7,4)/DMSO-d6) δ 6.37.

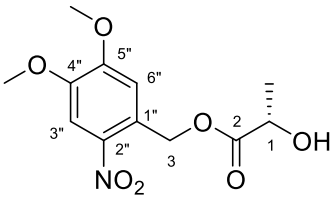
5.3.3. 4,5-dimethoxy-2-nitrobenzyl (ethoxy(phenethylamino)phosphoryl)-L-alaninate (14)



14

To a solution of the intermediate **12** was added phenethylamine (0.126 ml, 1 mmol, 1 eq), followed by Et3N (0.140 ml, 1 mmol, 1 eq) dropwise. The reaction was stirred for an hour. The solution was removed under vacuum and the product was immediately purified by preparative TLC (DCM/MeOH 95:5) with 25% yield. ^{31}P NMR (162 MHz, CDCl3) δ 14.28, 14.11.

5.4. Synthesis of L-lactate photoarm (5)



5

First approach: To a stirring solution of anhydrous L-lactic acid 98% (90.08 mg, 1 mmol, 1 eq) in MeOH (2 ml), in an ice bath, was added dropwise 1,8-diazobicyclo[5.4.0]undec-7-ene (0.149 ml, 1 mmol, 1 eq). The solution was stirred at room temperature for 30 minutes. Then, the solvent was removed under vacuum. 4,5-dimethoxy-2-nitrobenzyl bromide (231.92 mg, 0.84 mmol, 0.84 eq) in DMF (2 ml) was added dropwise to the previous solution. The solution was stirred overnight at room temperature. Then, the solvent was removed under vacuum. The product was immediately purified by chromatography column (DCM/MeOH 99:1) and repurified with a second chromatography column (pure DCM to DCM/MeOH 99:1). A white-yellow solid (145.36 mg, 0.51 mmol, 51%) was obtained.

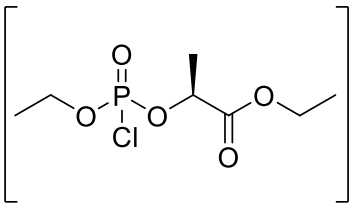
Second approach: To a solution of 1-(bromomethyl)-4,5-dimethoxy-2-nitrobenzene (50 mg, 0.18 mmol, 1 eq) in dry DMF (3ml) was added silverlactate (35.67 mg, 0.18 mmol, 1 eq). The reaction was stirred overnight at room temperature. Then, the solvent was removed under vacuum. The product was immediately purified by chromatography column (DCM/MeOH 95:5). A white-yellow solid (23.56 mg, 0.08 mmol, 46%) was obtained.

Third approach: To a solution of L-lactic acid anhydrous 98% (90.08 mg, 1 mmol, 1 eq) and 4,5-dimethoxy-2-nitrobenzyl bromide (231.92 mg, 0.84 mmol, 0.84 eq) in DMF (2 ml) was

added K_2CO_3 (69.1 mg, 0.5 mmol, 0.5 eq). The solution was stirred overnight at room temperature. Then, the solvent was evaporated under vacuum. The product was immediately purified by chromatography column (pure DCM to DCM/MeOH 99:1). A white-yellow solid (200 mg, 0.70 mmol, 70%) was obtained. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.75 (d, *J* = 8.0 Hz, 1H, 3´´), 7.20 and 6.99 (2 x s, 1H, 6´´), 5.62 (m, 2H, 3), 4.42 (q, *J* = 8.0 Hz, 1H, 1), 4.00 (m, 6H, 4´´-OMe and 5´´-OMe), 2.76 (s, 1H, O**H**), 1.52 (d, *J* = 4.0 Hz, 3H, 1-Me). ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.29 (s, HMBC, 2), 153.54 (s, HMBC, 4´´), 148.75 (s, HMBC, 5´´), 140.23 (s, HMBC, 2´´), 126.21 (s, HMBC, 1´´), 110.53 (s, 6´´), 108.18 (s, 3´´), 66.87 (s, 1), 64.28 (s, 3), 56.46 (4´´-OMe and 5´´-OMe), 20.50 (s, 1-Me).

5.5. Synthesis of model monoamidate prodrugs having simple lactate ethyl ester arm

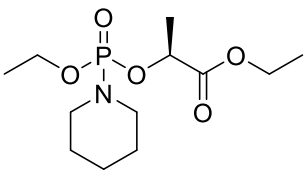
5.5.1. Ethyl (2S)-2-((chloro(ethoxy)phosphoryl)oxy)propanoate (11)



11

To a solution of (-)ethyl-L-lactate (0.171 ml, 1.5 mmol, 1.5 eq) in dry toluene (3 ml) was added ethyldichlorophosphate (0.119 ml, 1 mmol, 1 eq) followed by Et_3N (0.140 ml, 1 mmol, 1 eq) dropwise. The solution was stirred overnight. ^{31}P NMR (162 MHz, $CDCl_3$) δ 4.54, 3.62. Crude product was directly used in the next reaction step (phosphorylation of various amines).

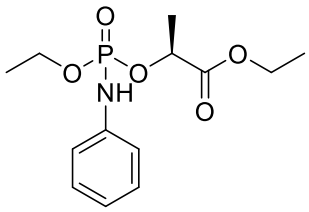
5.5.2. Ethyl (2S)-2-((ethoxy(piperidin-1-yl)phosphoryl)oxy)propanoate (20)



20

To a solution of intermediate **11** was added piperidine (0,099 ml, 1 mmol, 1 eq) followed by Et_3N (0,140 ml, 1 mmol, 1 eq) dropwise. The solution was stirred overnight. ^{31}P NMR (162 MHz, $CDCl_3$) δ 4.56, 3.64.

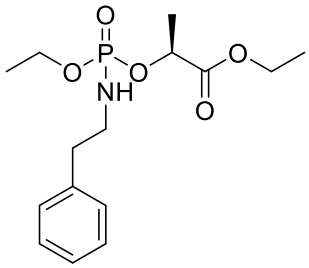
5.5.3. Ethyl (2S)-2-((ethoxy)phenylamino)phosphoryl)oxy)propanoate (21)



21

To a solution of intermediate **11** was added aniline (0.091 ml, 1 mmol, 1 eq) followed by Et_3N (0.140 ml, 1 mmol, 1 eq) dropwise. The solution was stirred overnight. ^{31}P NMR (162 MHz, $CDCl_3$) δ 1.84, 1.39.

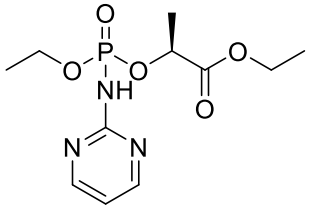
5.5.4. Ethyl (2S)-2-((ethoxy(phenethylamino)phosphoryl)oxy)propanoate (22)



22

To a solution of intermediate **11** was added phenethylamine (0.126 ml, 1 mmol, 1 eq) followed by Et_3N (0.140 ml, 1 mmol, 1 eq) dropwise. The solution was stirred overnight. ^{31}P NMR (162 MHz, $CDCl_3$) δ 9.02, 8.21.

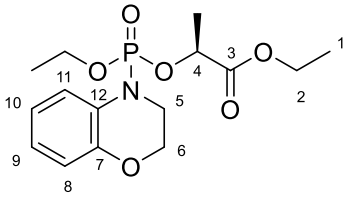
5.5.5. Ethyl (2S)-2-((ethoxy(pyrimidin-2-ylamino)phosphoryl)oxy)propanoate (23)



23

To a solution of intermediate **11** was added 2-aminopyrimidine (142.66 mg, 1.5 mmol, 1.5 eq) followed by Et_3N (0.140 ml, 1 mmol, 1 eq) dropwise. The solution was stirred overnight. ^{31}P NMR (162 MHz, $CDCl_3$) δ 4.66, 3.71.

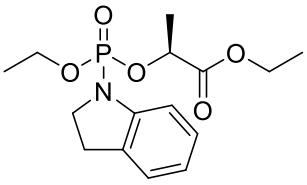
5.5.6. Ethyl (2S)-2-(((2,3-dihydro-4H-benzo[b][1,4]oxazin-4-yl)(ethoxy)phosphoryl)oxy) propanoate (24)



24

To a solution of intermediate **11** was added 3,4-dihydro-2*H*-benzo[1,4]oxazine (0.123 ml, 1 mmol, 1 eq) followed by Et_3N (0.140 ml, 1 mmol, 1 eq) dropwise. The solution was stirred overnight. Then, the reaction was heated up to 50ºC for two hours and the solvent was evaporated under vacuum. The product was immediately purified by preparative TLC (DCM/MeOH 100:1) with 43% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.53 – 7.35 (m, 1H, 8), 6.97 – 6.77 (m, 3H, 9, 10 and 11), 4.92 (m, 1H, 4), 4.33 – 4.00 (m, 8H, 2, 6 and P-O-**CH$_2$**-CH$_3$), 3.88 – 3.66 (m, 2H, 5), 1.61 – 1.49 (m, 3H, 4-Me), 1.39 – 1.16 (m, 6H, 2 and P-O-CH$_2$-**CH$_3$**). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.20 (s, 3), 145.36 (s, 7), 127.19 (s, 12), 124.04 (s, 9), 120.76 (s, 8 and 11), 116.64 (s, 10), 71.39 (d, *J* = 26.0 Hz, 4), 65.49 (s, 6), 63.42 (d, *J* = 29.0 Hz, P-O-**CH$_2$**-CH$_3$), 62.31 (s, 2), 43.43 (s, 5), 19.91 (s, 1 or 4-Me or O-CH$_2$-**CH$_3$**), 15.54 (s, 1 or 4-Me or O-CH$_2$-**CH$_3$**), 14.13 (s, 1 or 4-Me or O-CH$_2$-**CH$_3$**). ^{31}P NMR (162 MHz, $CDCl_3$) δ 2.61, 2.28.

5.5.7. Ethyl (2S)-2-((ethoxy(indolin-1-yl)phosphoryl)oxy)propanoate (25)

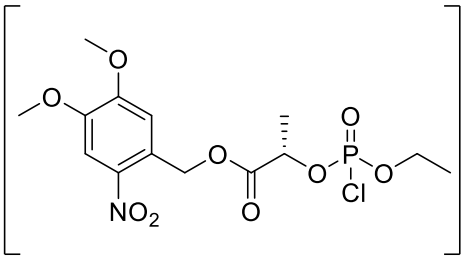


25

To a solution of intermediate **11** was added indoline (0.112 ml, 1 mmol, 1 eq) followed by Et_3N (0.140 ml, 1 mmol, 1 eq) dropwise. The solution was stirred overnight. Then, the solvent was removed under vacuum. ^{31}P NMR (162 MHz, $CDCl_3$) δ 1.35, 1.00.

5.6. Synthesis of model monoamidate prodrugs equipped with L-lactate photoarm

5.6.1. 4,5-dimethoxy-2-nitrobenzyl (2S)-2-((chloro(ethoxy)phosphoryl)oxy)propanoate (15)

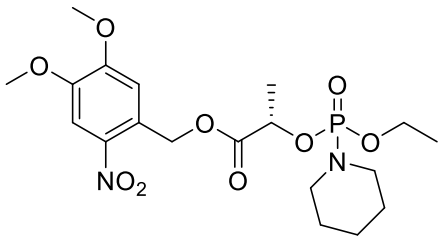


15

To a solution of L-lactate photoarm (142.63 mg, 0.5 mmol, 0.5 eq) in dry toluene (3 ml) was added ethyldichlorophosphate (0.039 ml, 0.33 mmol, 0.33 eq) followed by Et_3N (0.046 ml, 0.33 mmol, 0.33 eq). The solution was stirred overnight. It was directly used in the

next reaction step. ^{31}P NMR (162 MHz, $CDCl_3$) δ 4.52, 4.06.

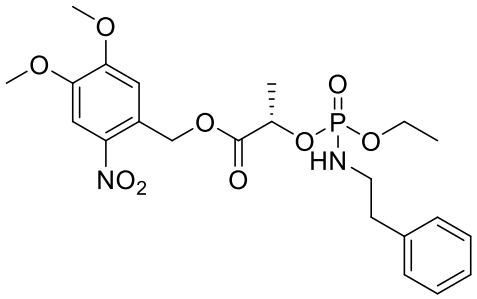
5.6.2. 4,5-dimethoxy-2-nitrobenzyl (2S)-2-((ethoxy(piperidin-1-yl)phosphoryl)oxy)propanoate (16)



16

To a solution of intermediate **15** was added piperidine (0.099 ml, 1 mmol, 1 eq) followed by Et_3N (0.140 ml, 1 mmol, 1 eq) dropwise. The solution was stirred overnight. Then, the solvent was removed under vacuum. ^{31}P NMR (162 MHz, $CDCl_3$) δ 9.16, 8.49.

5.6.3. 4,5-dimethoxy-2-nitrobenzyl (2S)-2-((ethoxy(phenethylamino)phosphoryl)oxy)propanoate (17)



17

To a solution of intermediate **15** was added phenethylamine (0.126 ml, 1 mmol, 1 eq) followed by Et_3N (0.140 ml, 1 mmol, 1 eq) dropwise. The solution was stirred overnight. Then, the solvent was removed under vacuum. ^{31}P NMR (162 MHz, $CDCl_3$) δ 8.90, 8.39.

6. Bibliography

6. Bibliography

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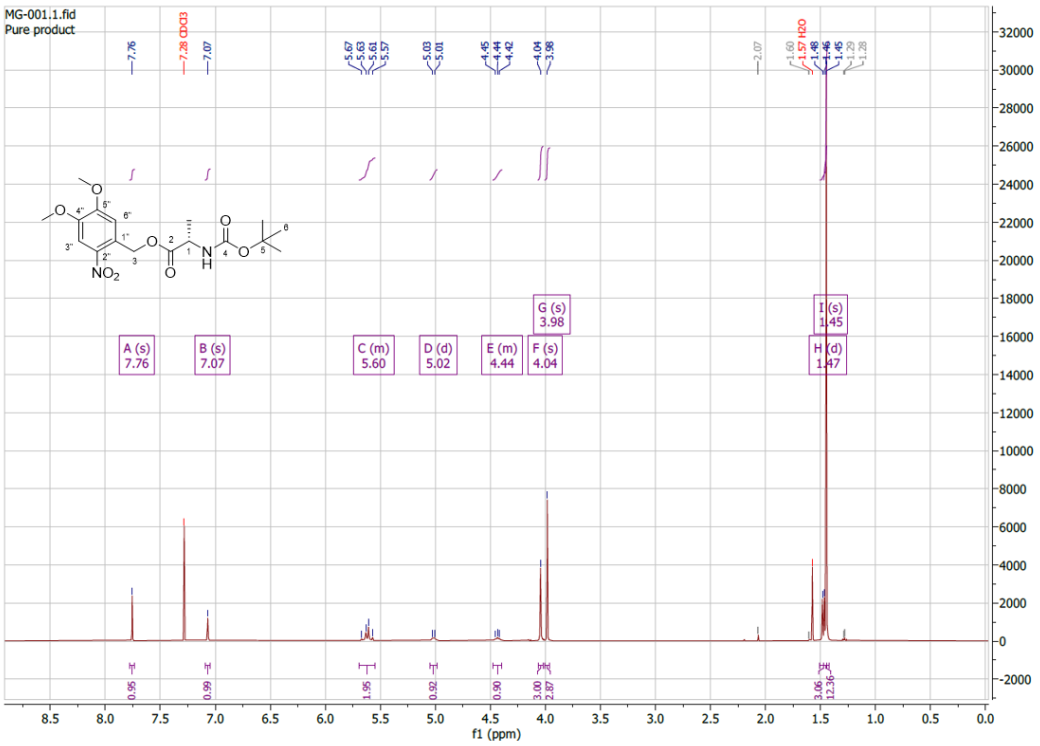
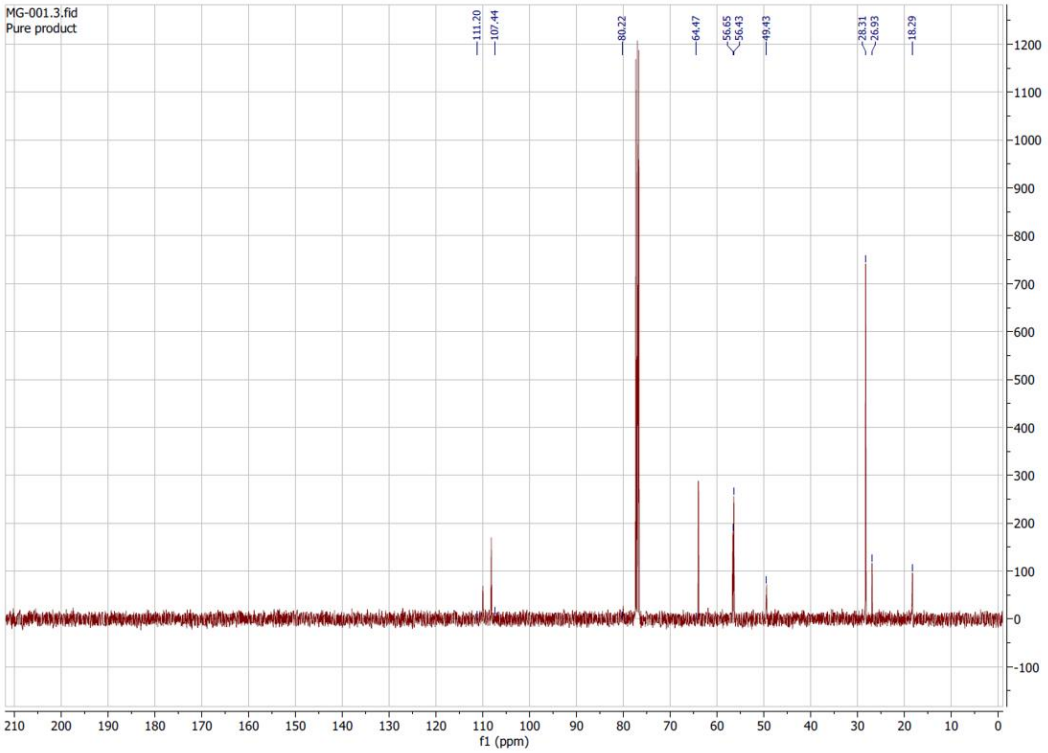
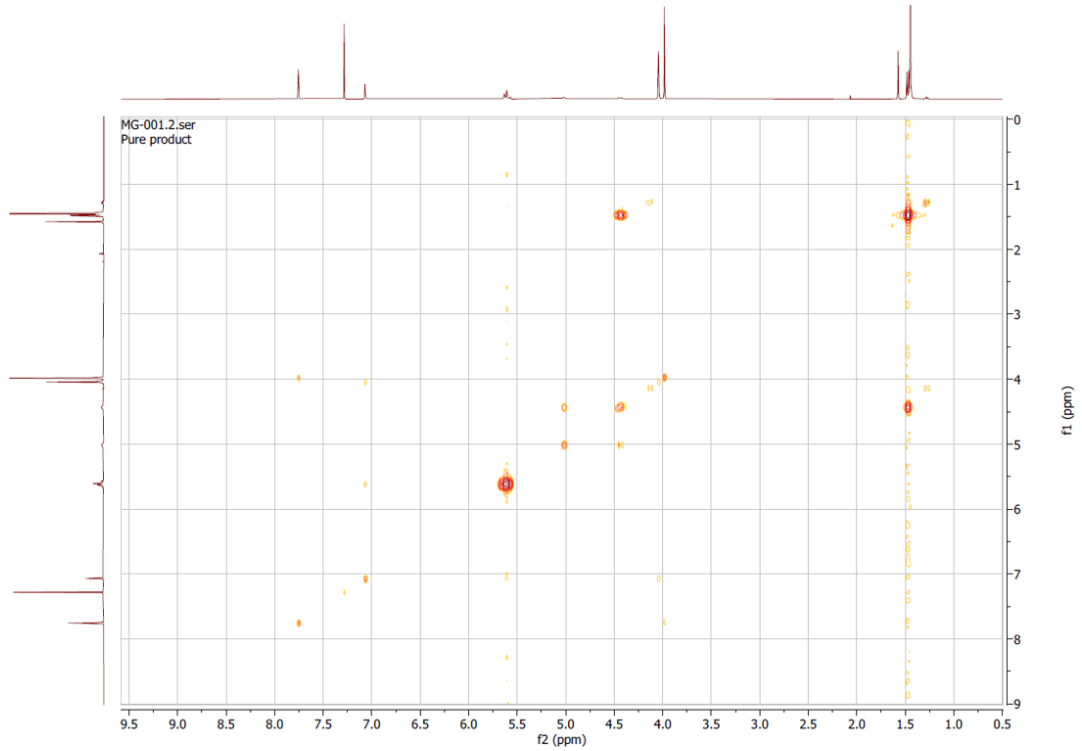
7. Supporting information

7. Supporting information

Boc protected alanine photoarm (3)



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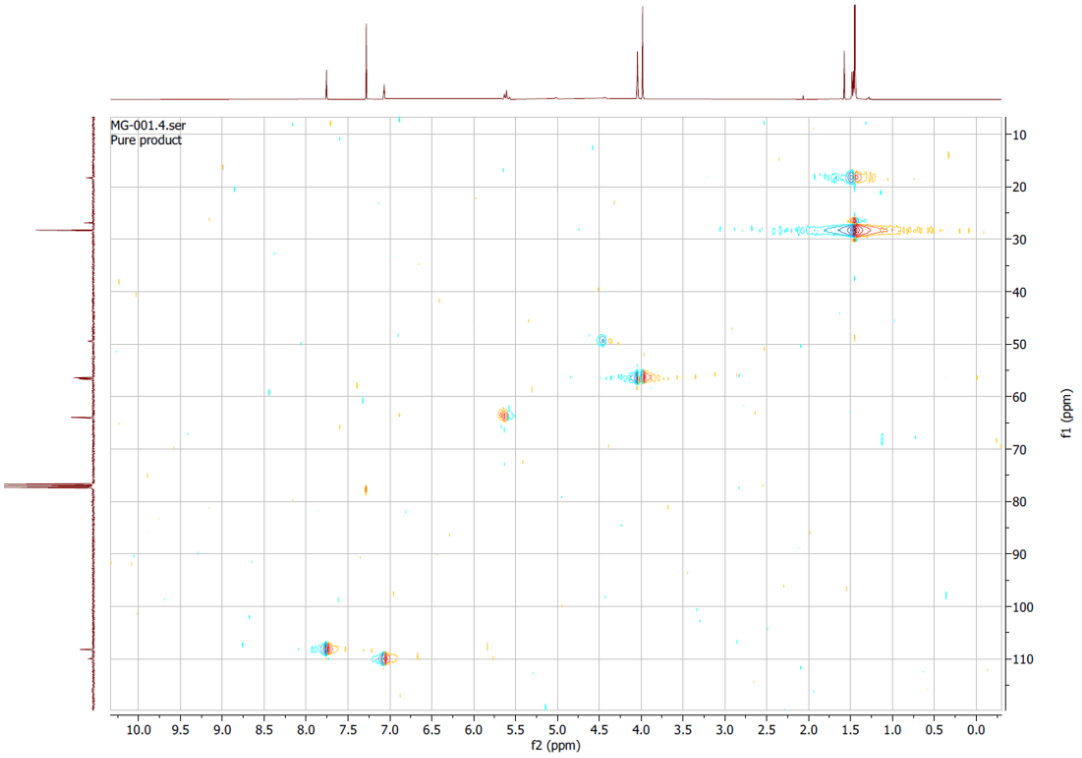
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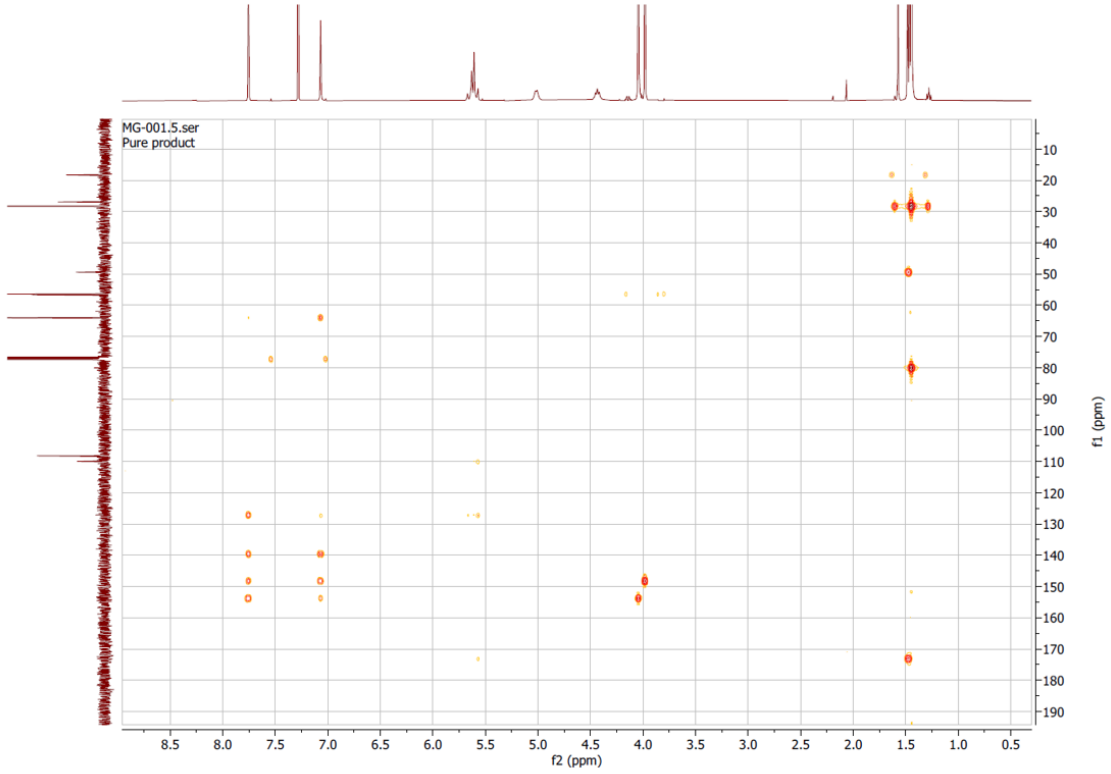
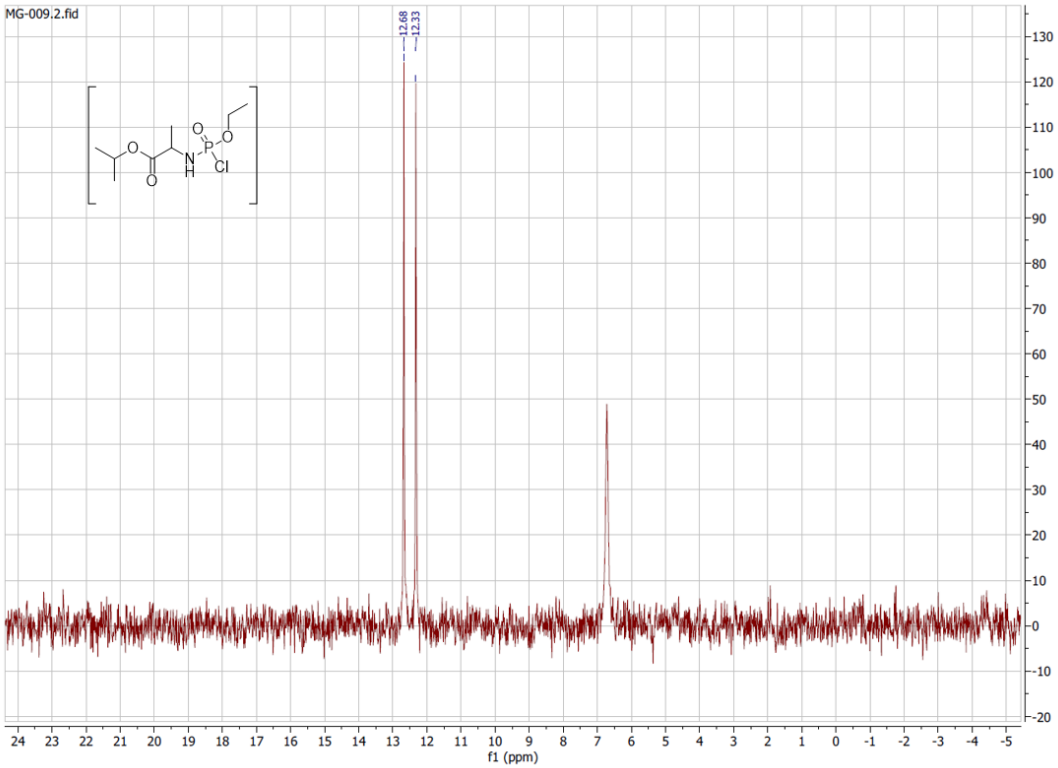
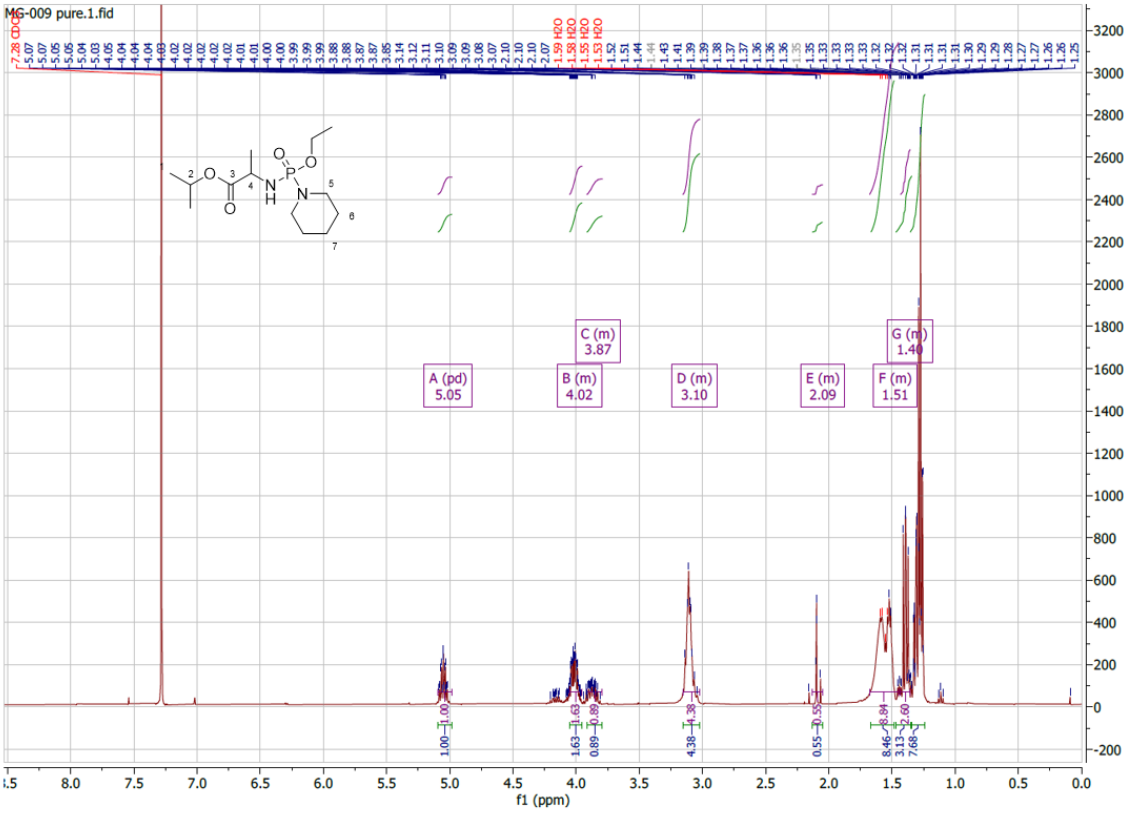
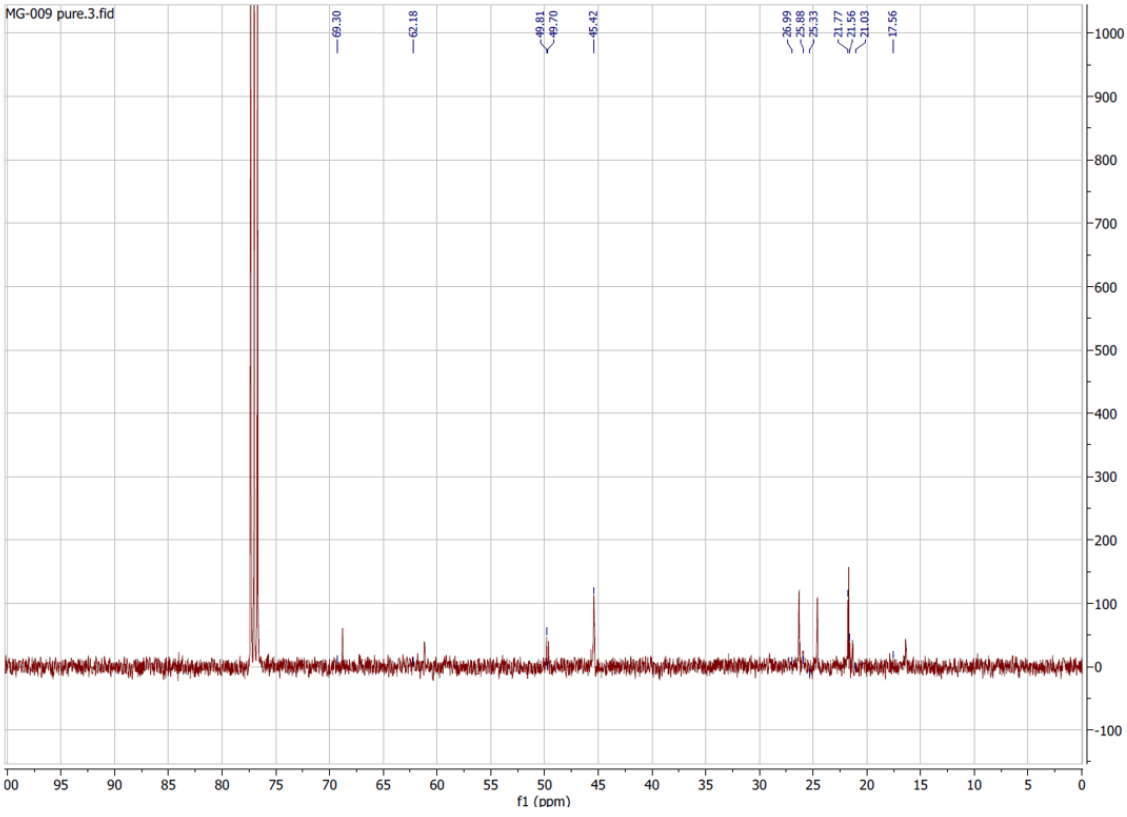


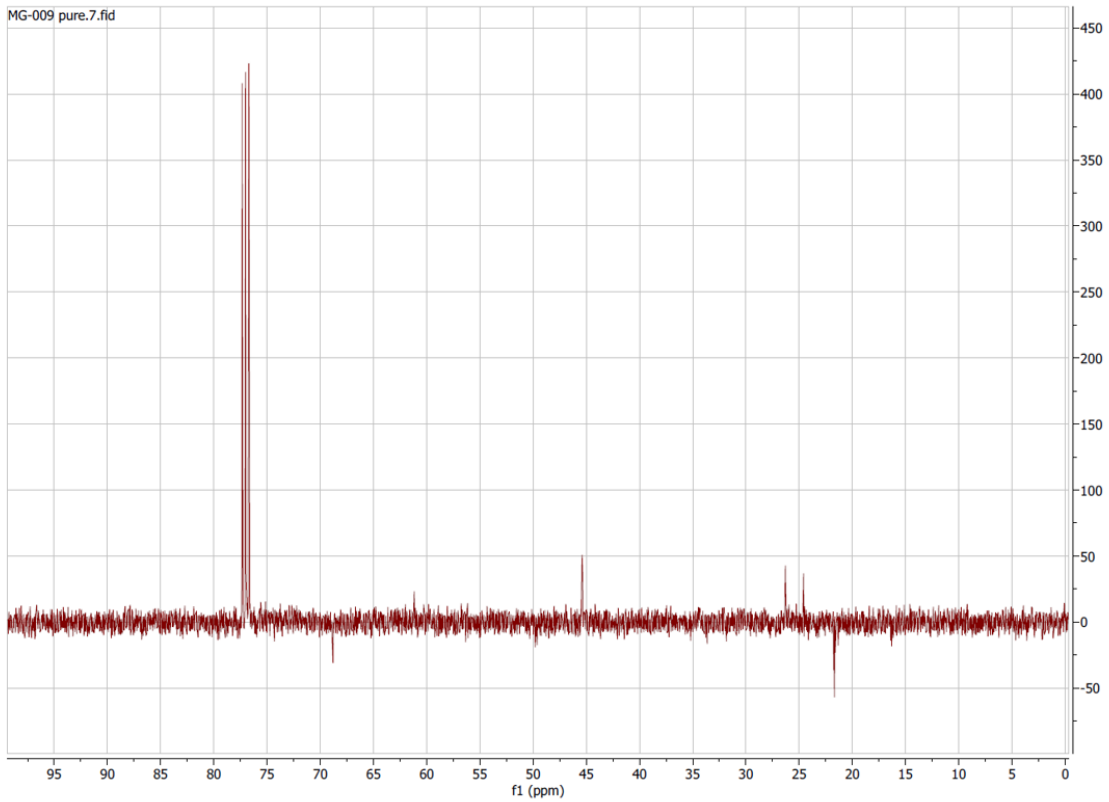
Isopropyl (chloro(ethoxy)phosphoryl)alaninate (8)



Isopropyl (ethoxy(piperidin-1-yl)phosphoryl)alaninate (18)



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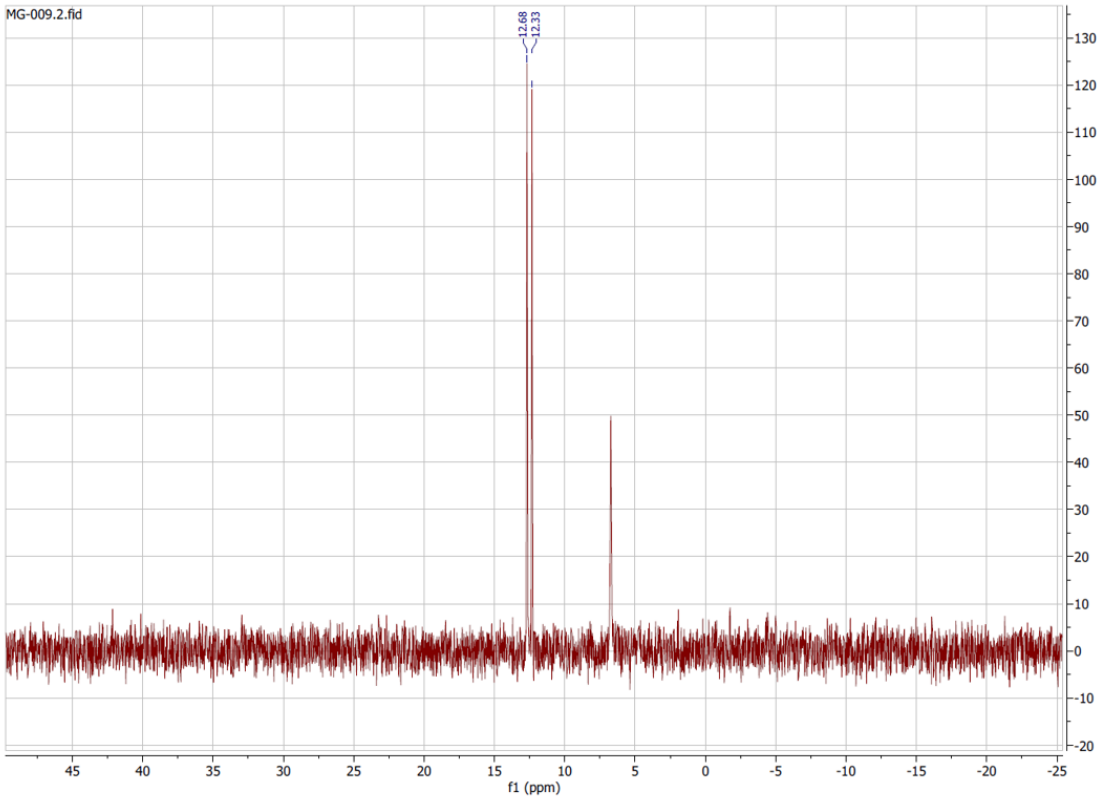
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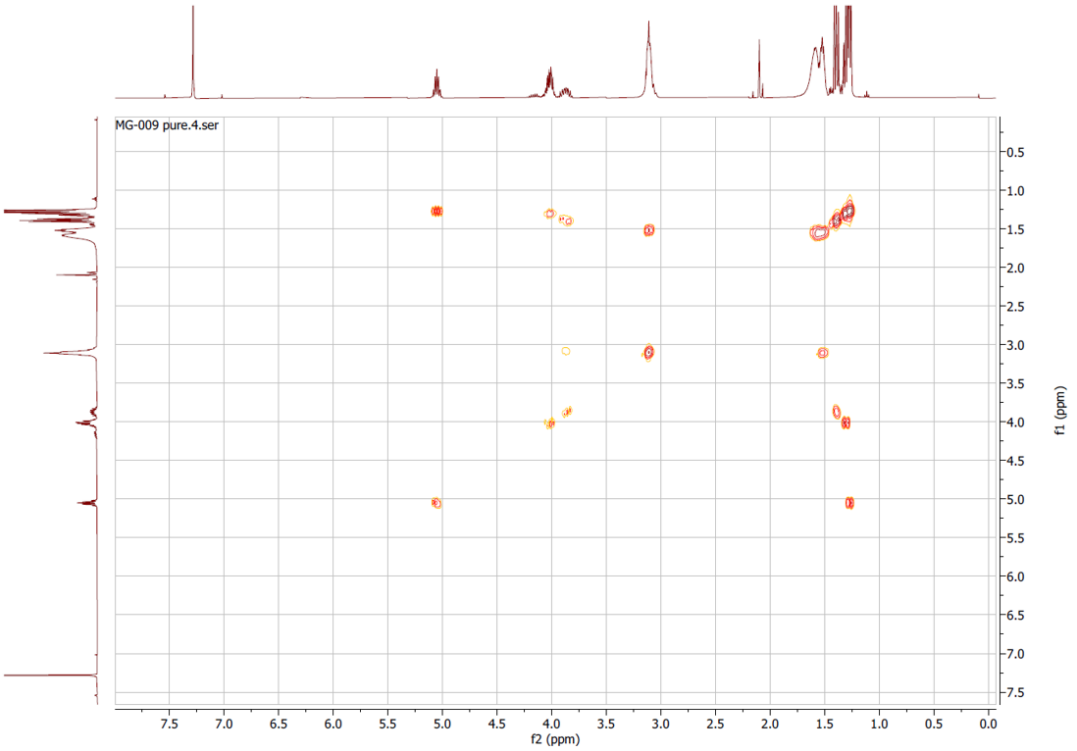
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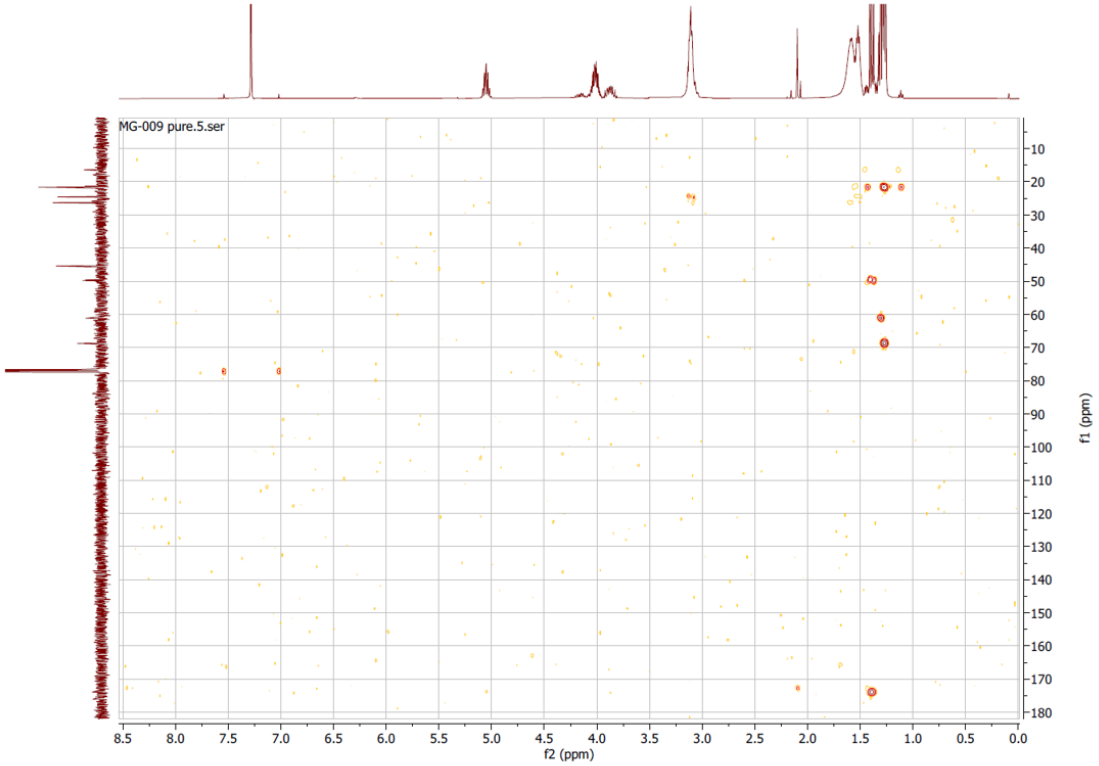
f1 (ppm)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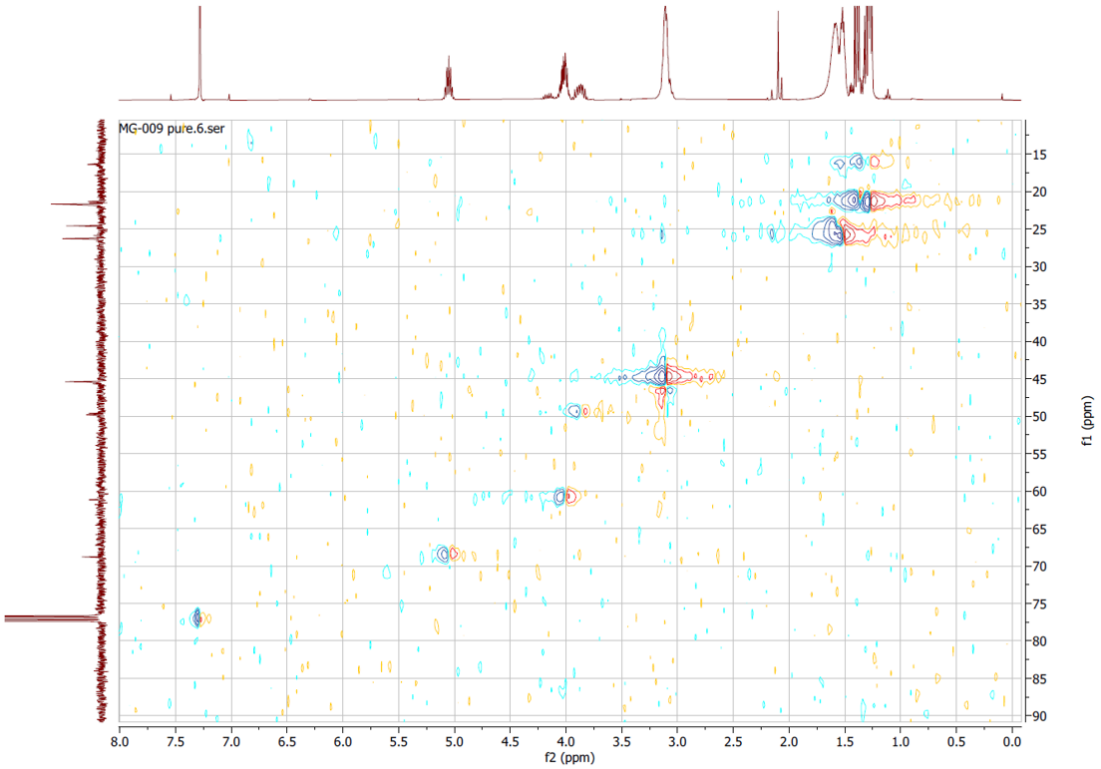
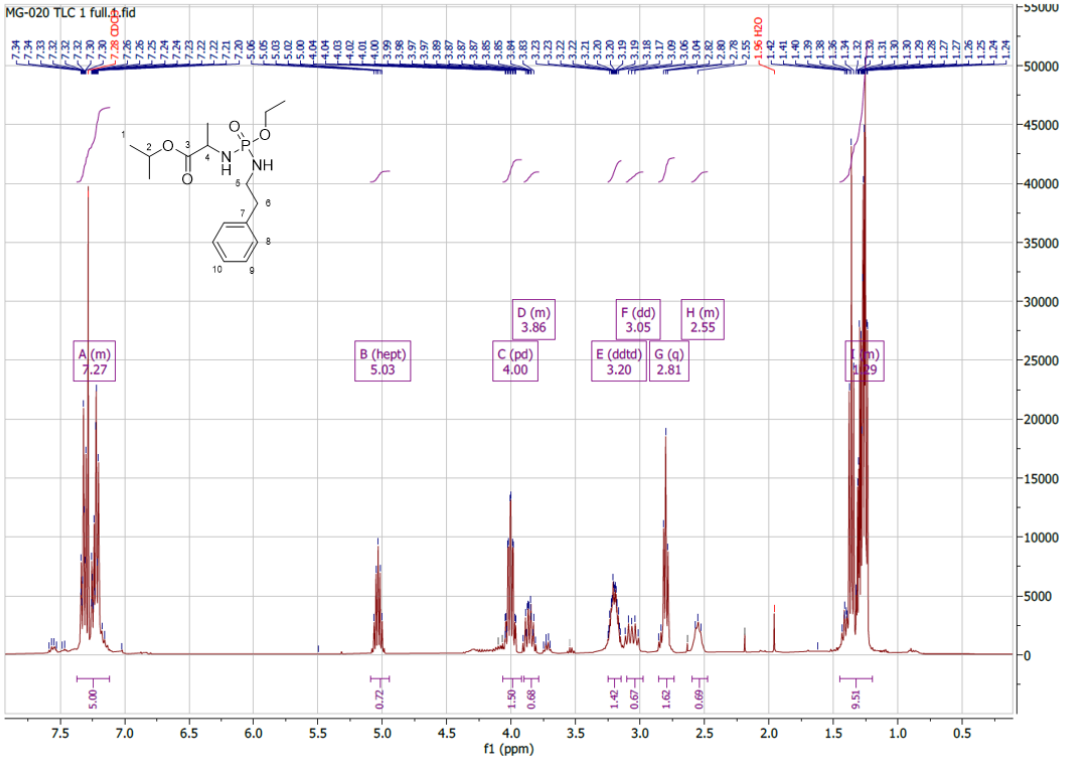
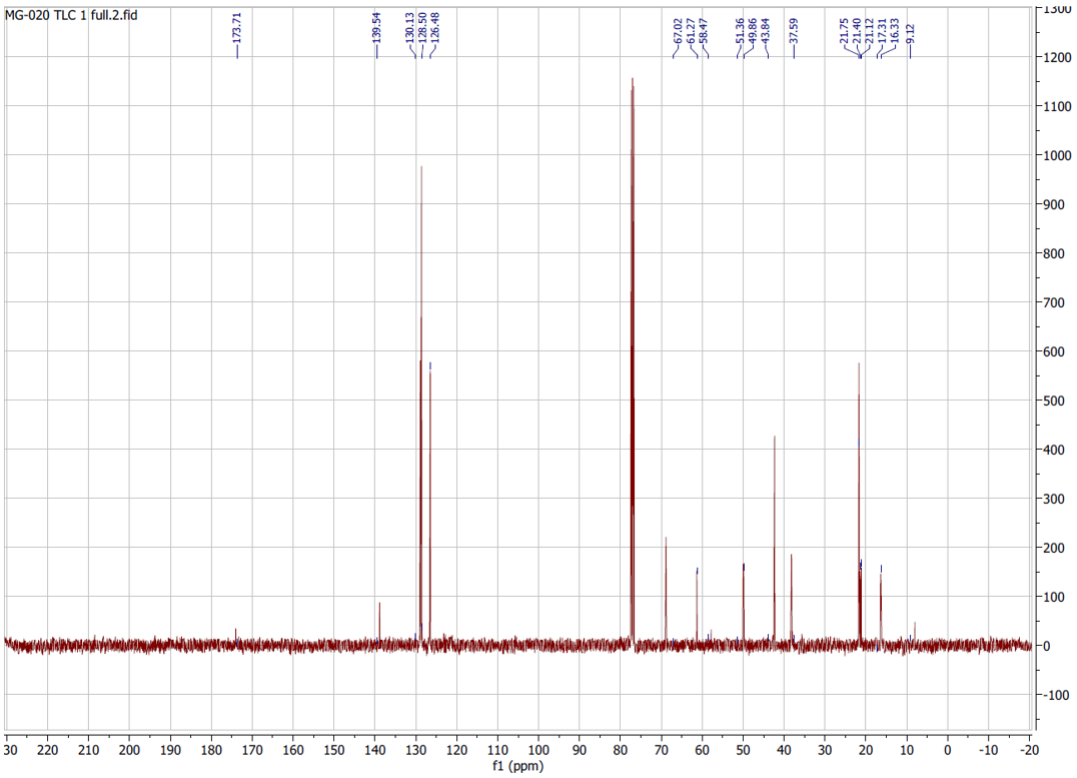


Isopropyl (ethoxy(phenethylamino)phosphoryl)alaninate (19)

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173.71
139.54
130.13
128.50
126.48
67.02
61.27
58.47
51.36
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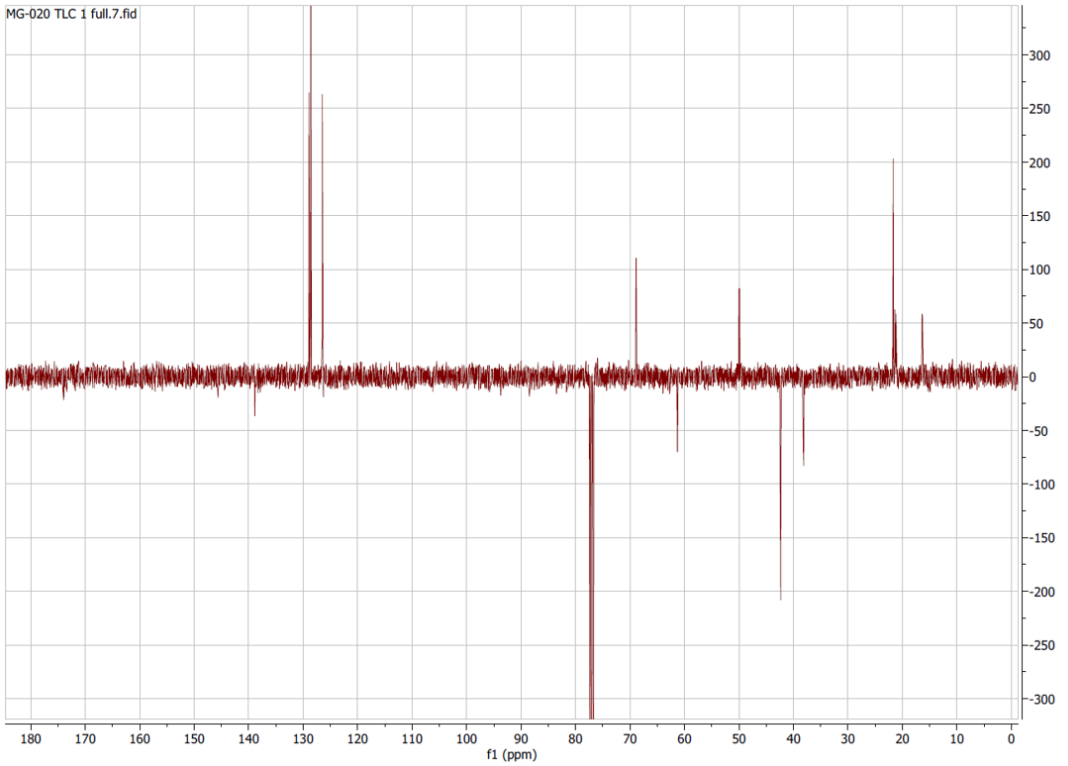
21.75
21.40
21.12
17.31
16.33
9.12
f1 (ppm)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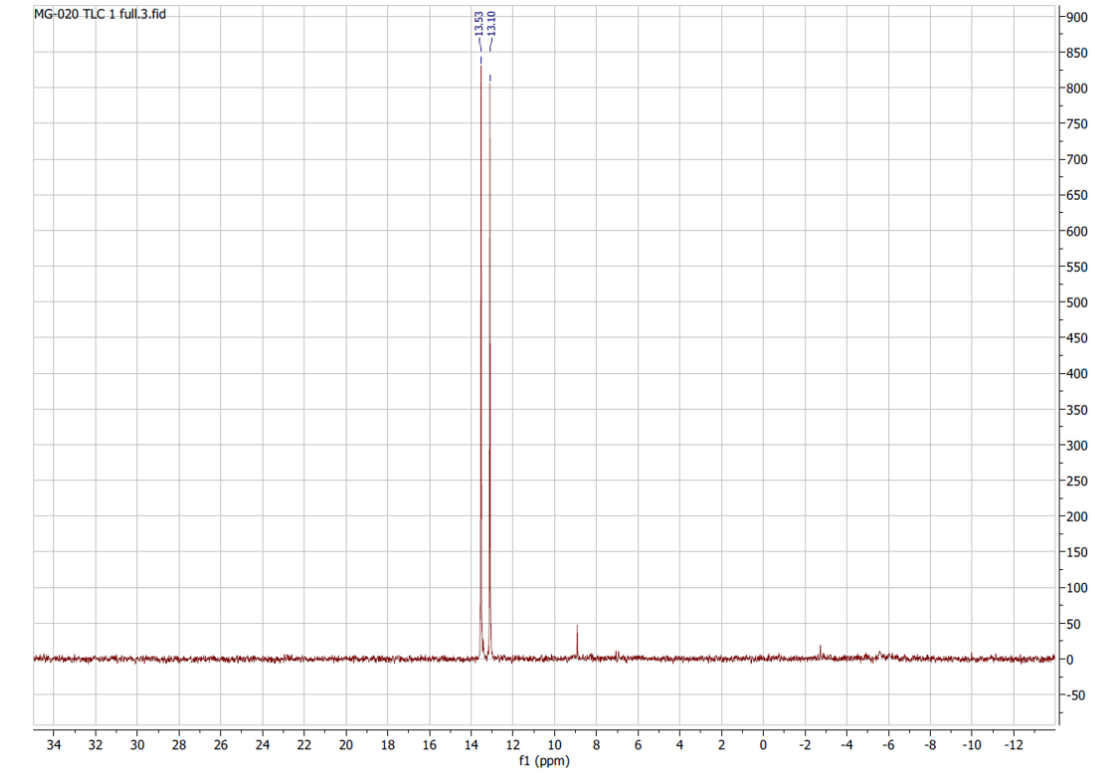
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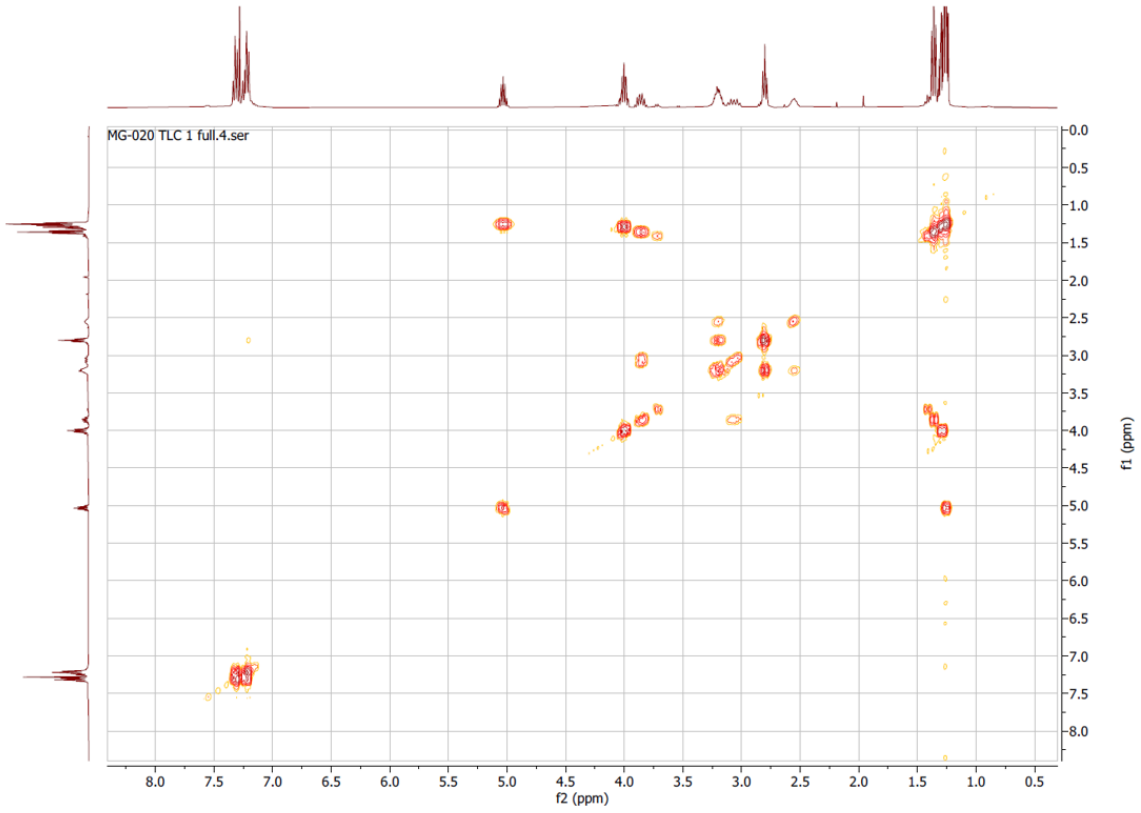
13.53
13.10
f1 (ppm)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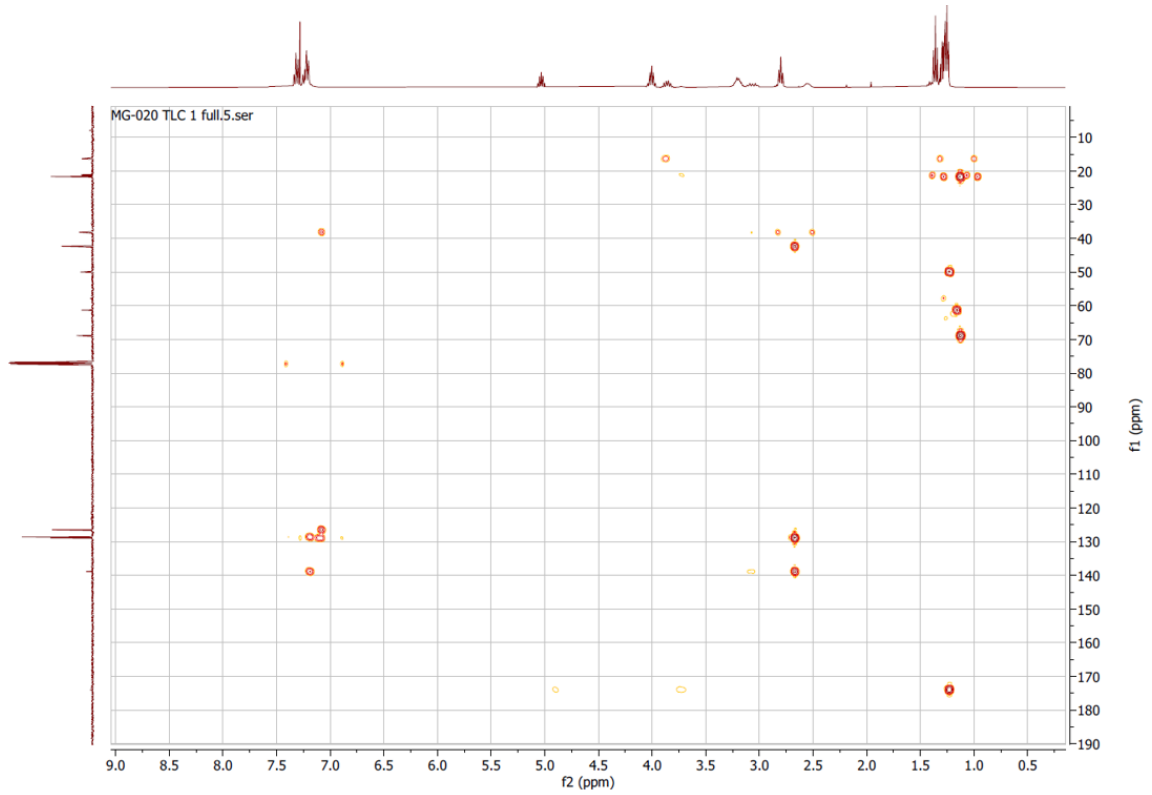
f2 (ppm)
f1 (ppm)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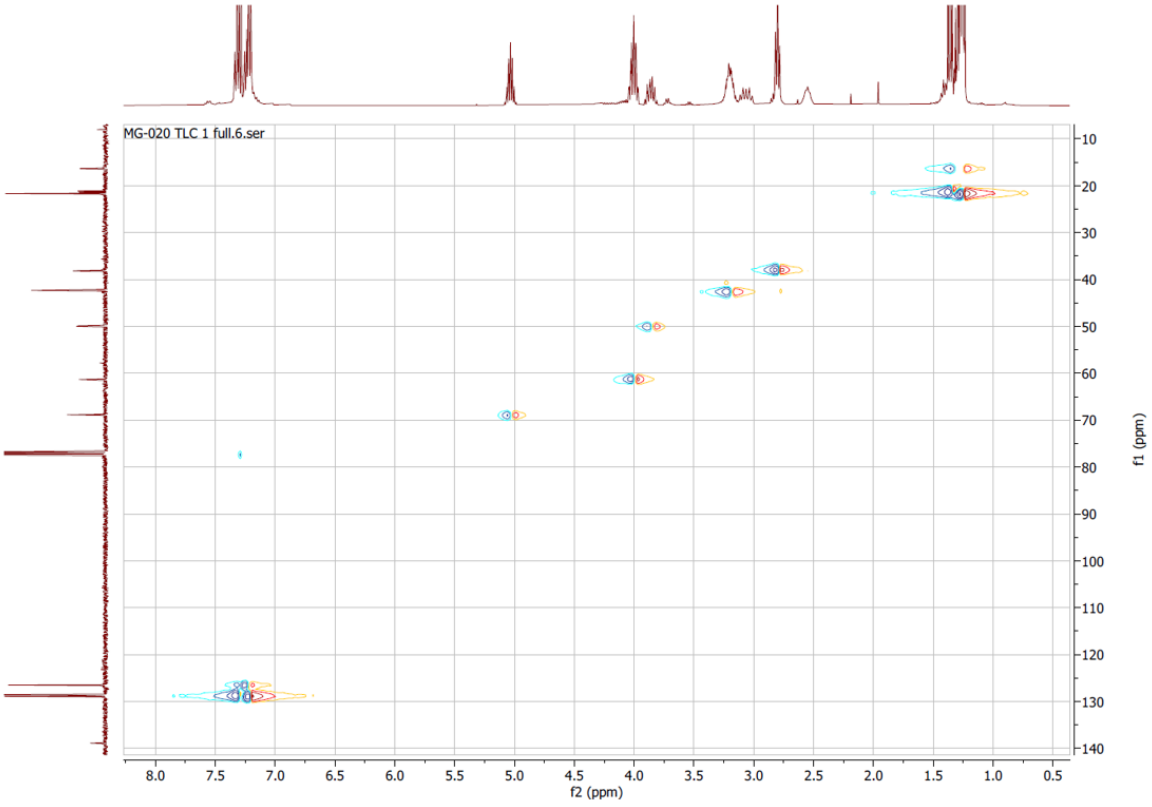
f2 (ppm)
f1 (ppm)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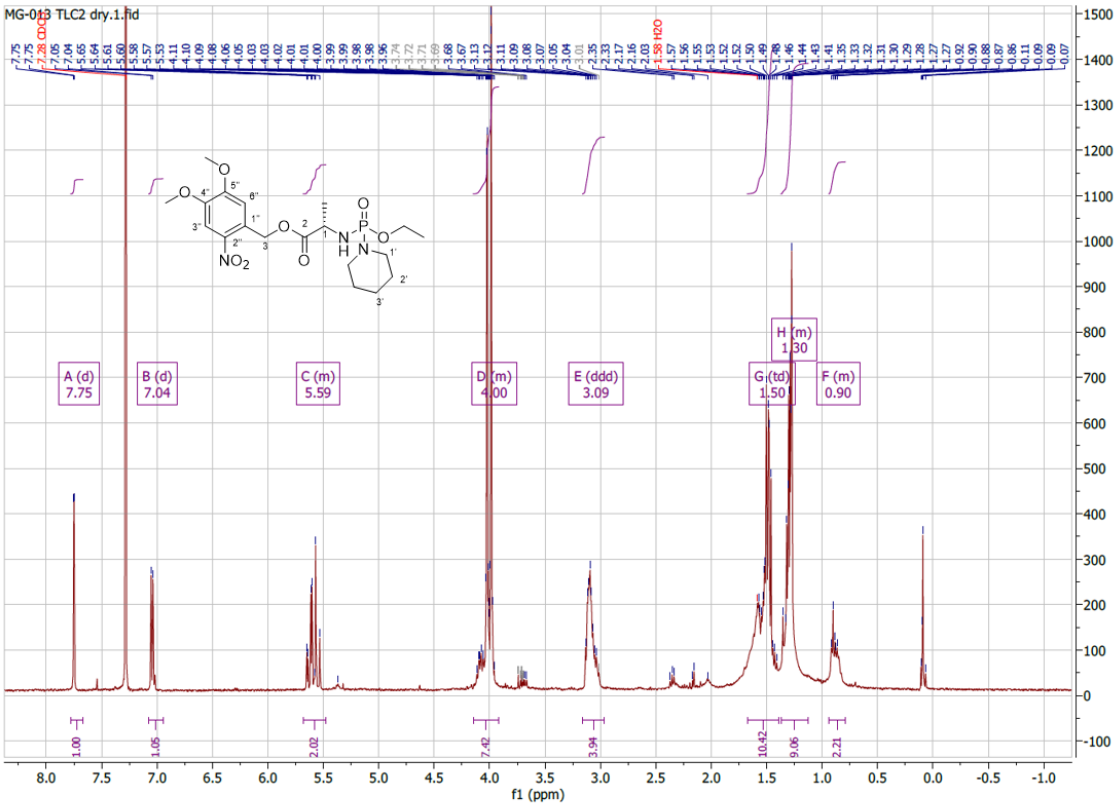
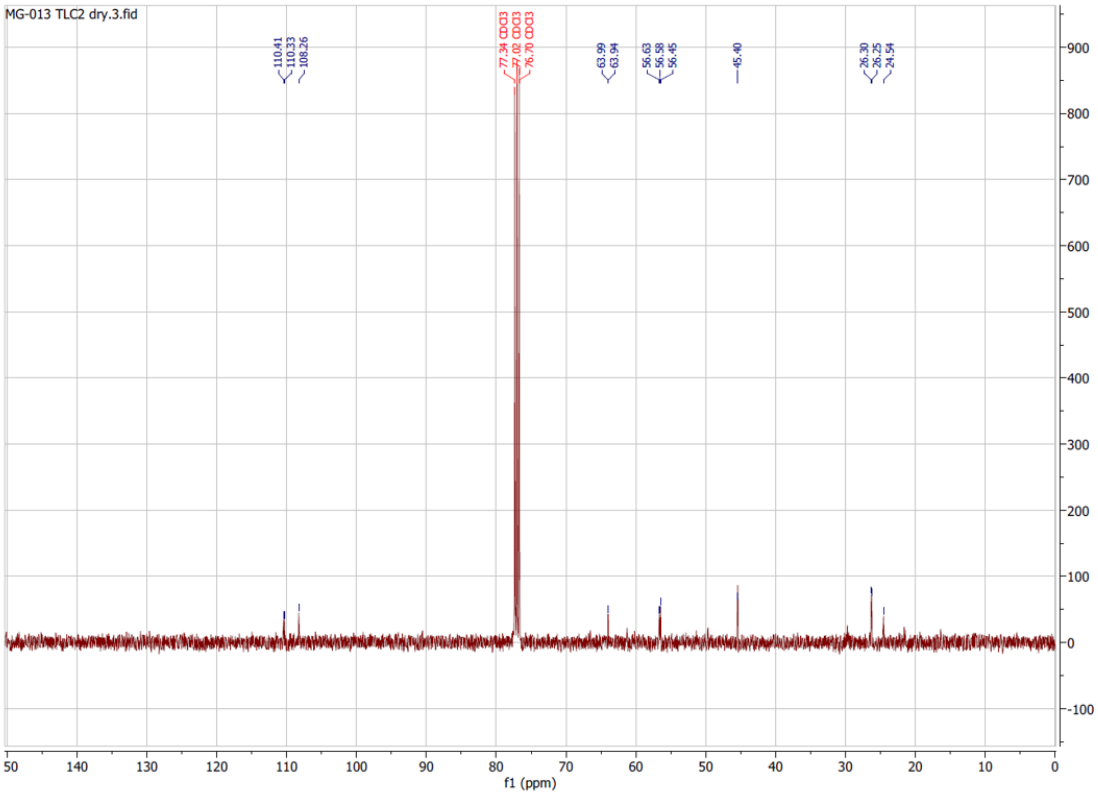
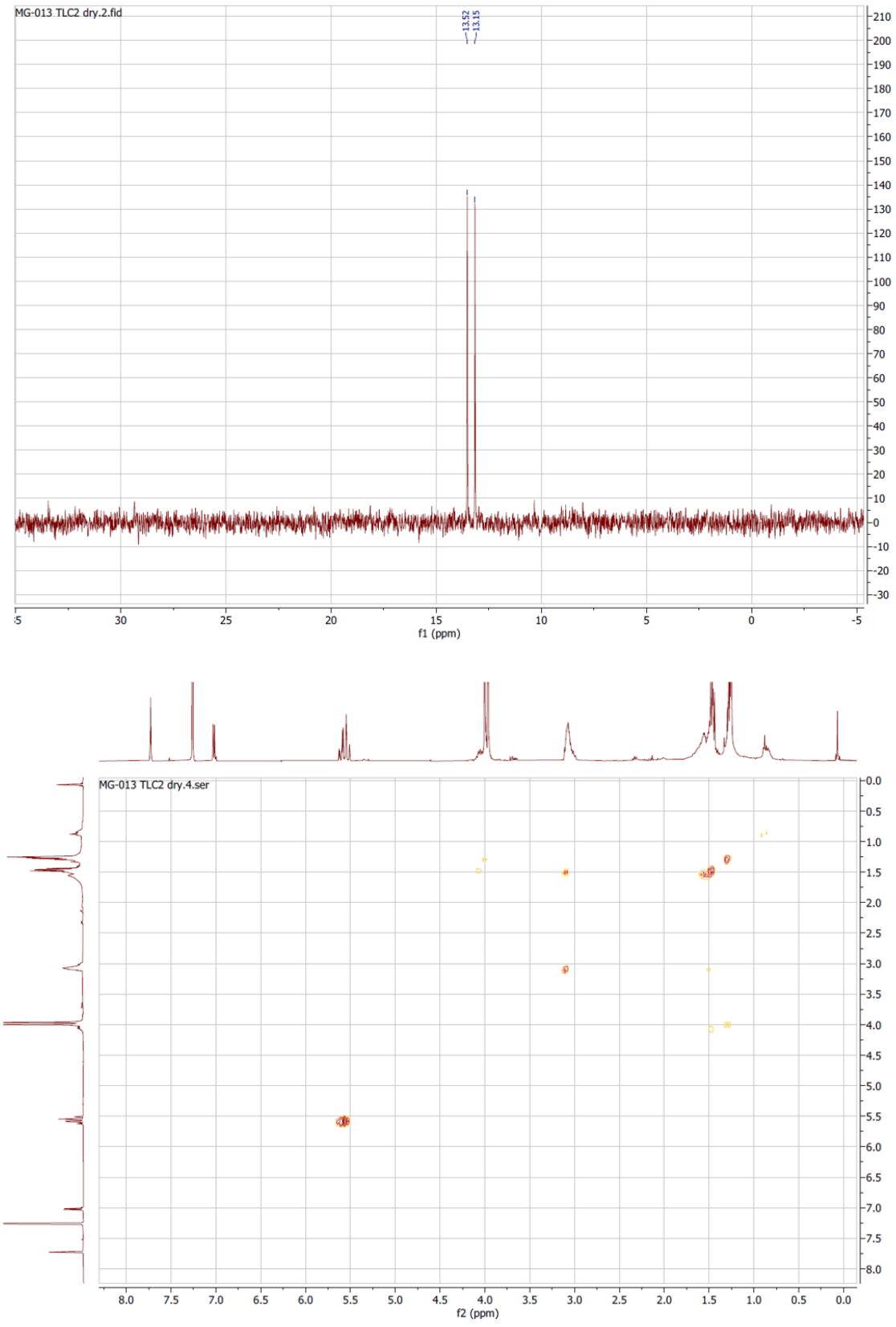
f1 (ppm)

4,5-dimethoxy-2-nitrobenzyl (ethoxy(piperidin-1-yl)phosphoryl)-L-alaninate (13)



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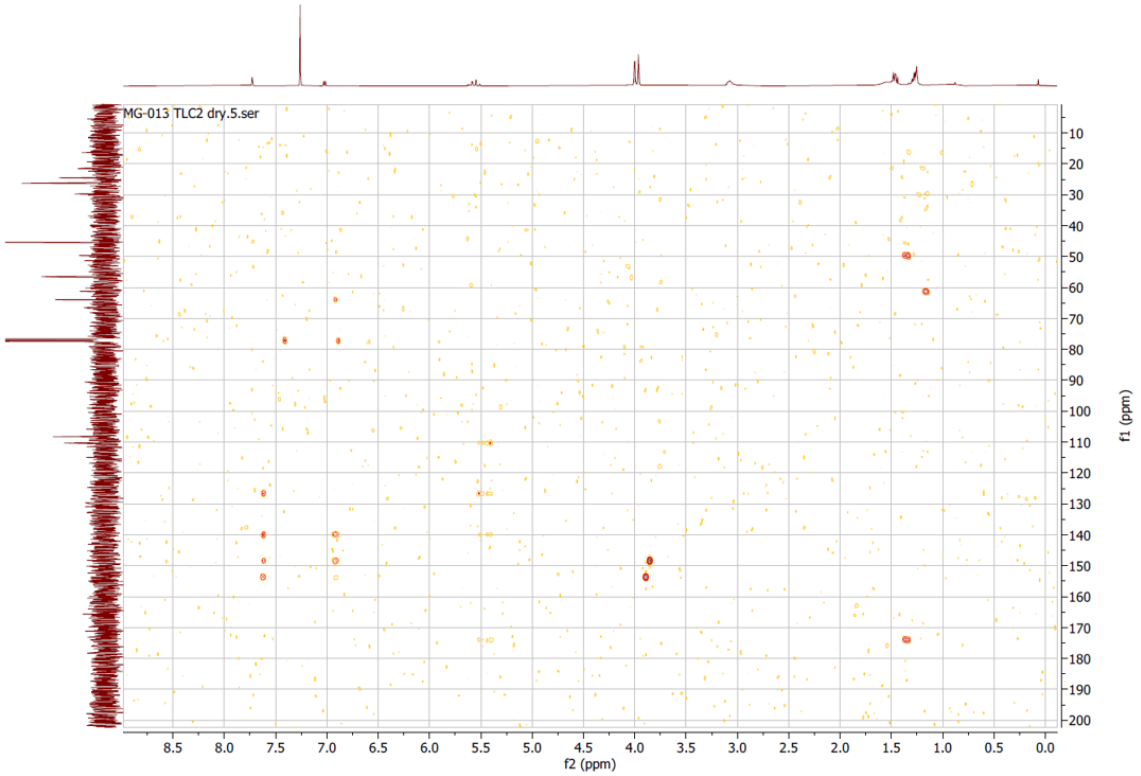
13.52
13.15
f1 (ppm)
MG-013 TLC2 dry.4.ser
f2 (ppm)
f1 (ppm)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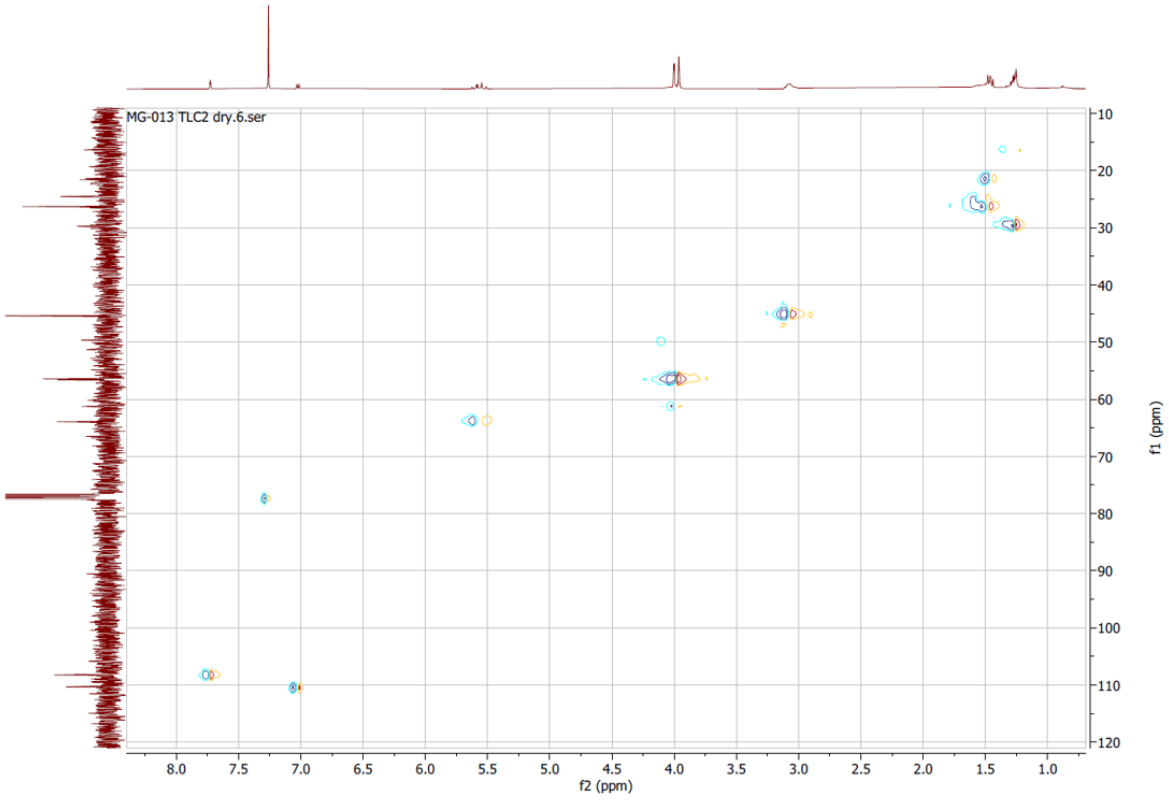
f1 (ppm)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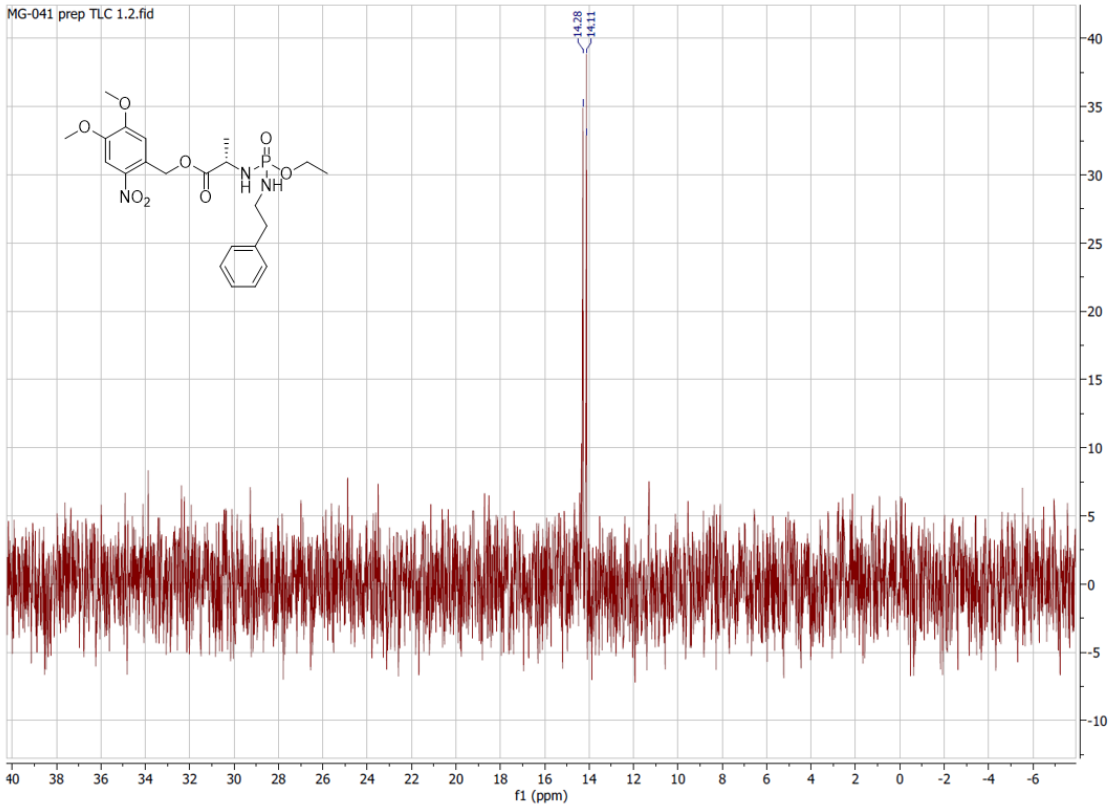
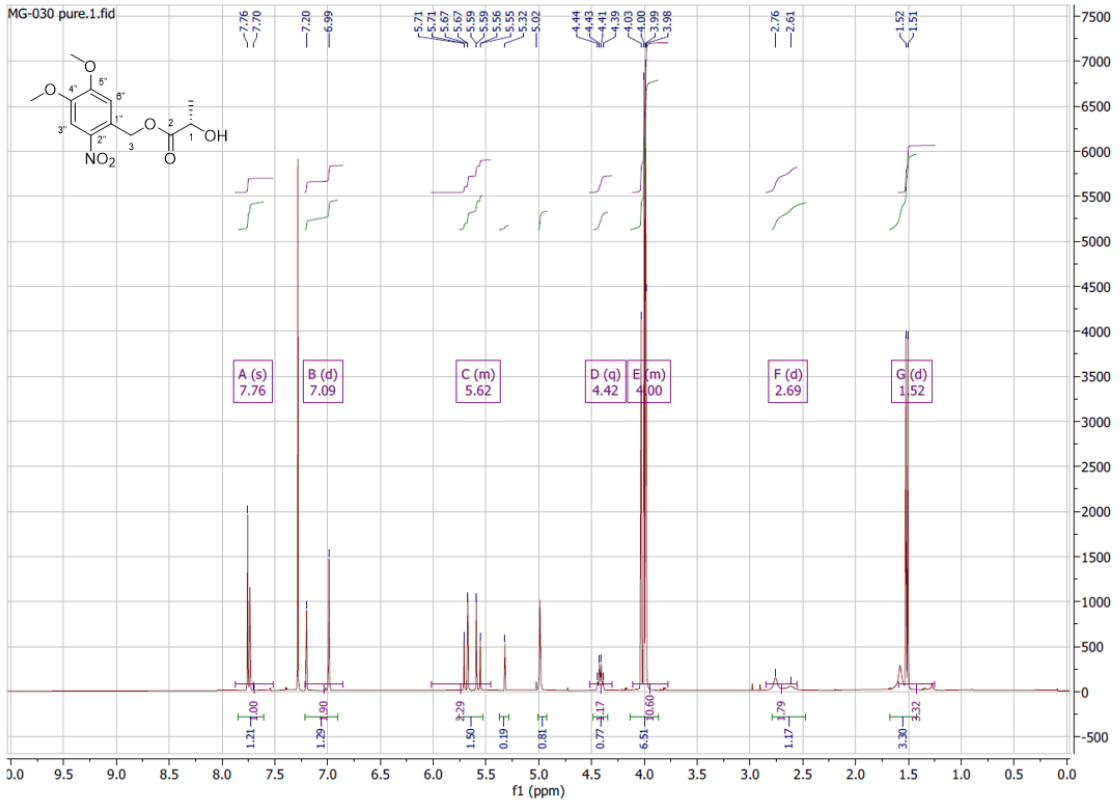
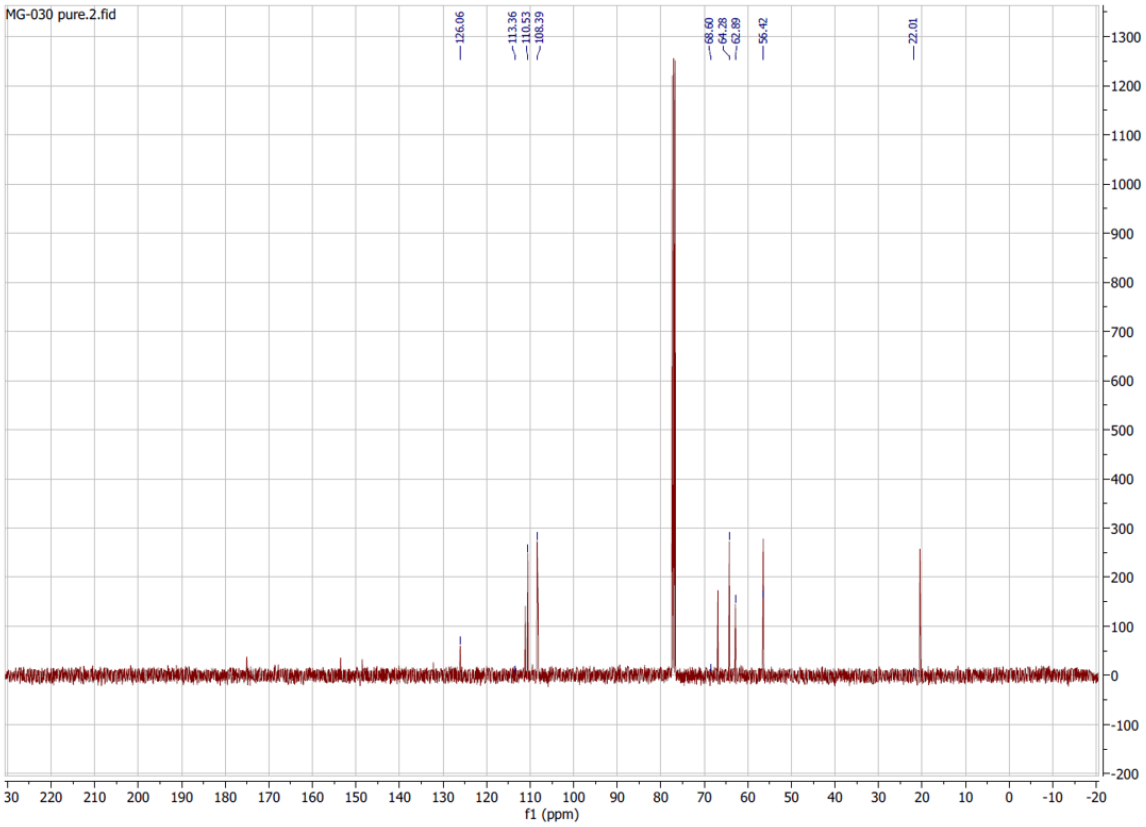
f1 (ppm)

4,5-dimethoxy-2-nitrobenzyl (ethoxy(phenethylamino)phosphoryl)-L-alaninate (14)



L-lactate photoarm (5)

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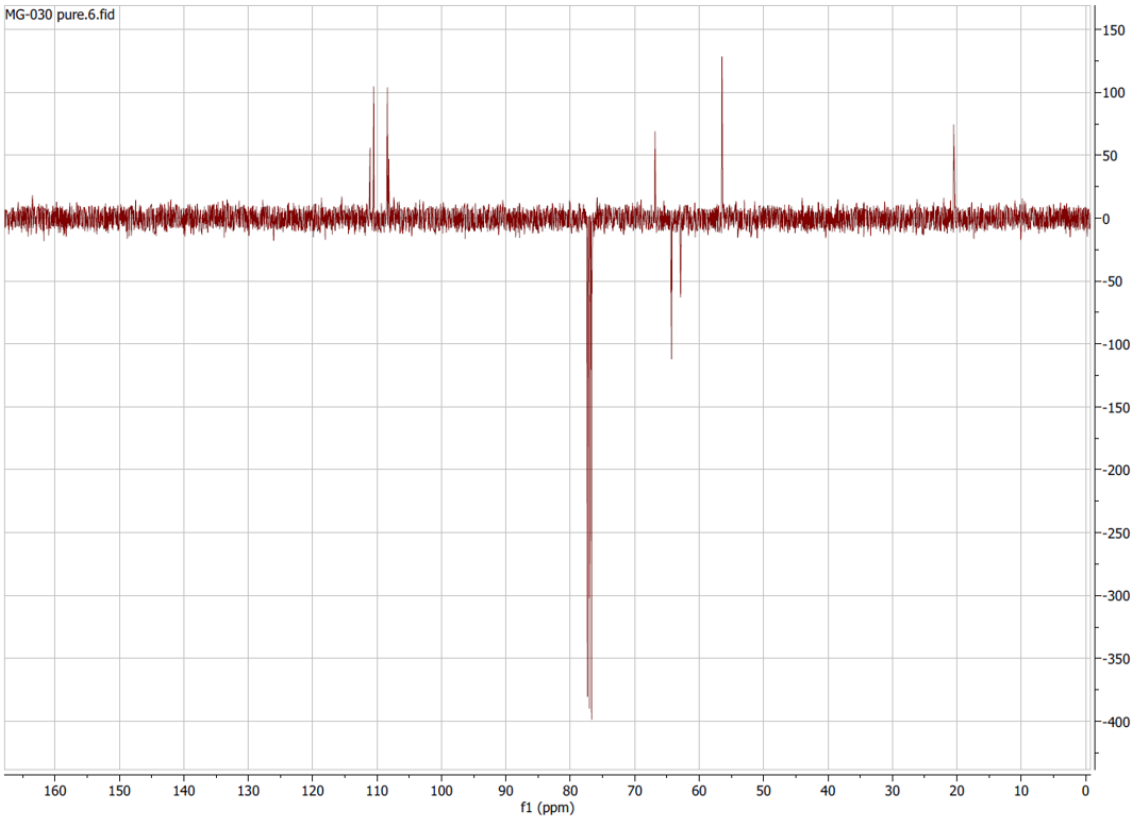
113.36
110.53
108.39
68.60
64.28
62.89
56.42
22.01
f1 (ppm)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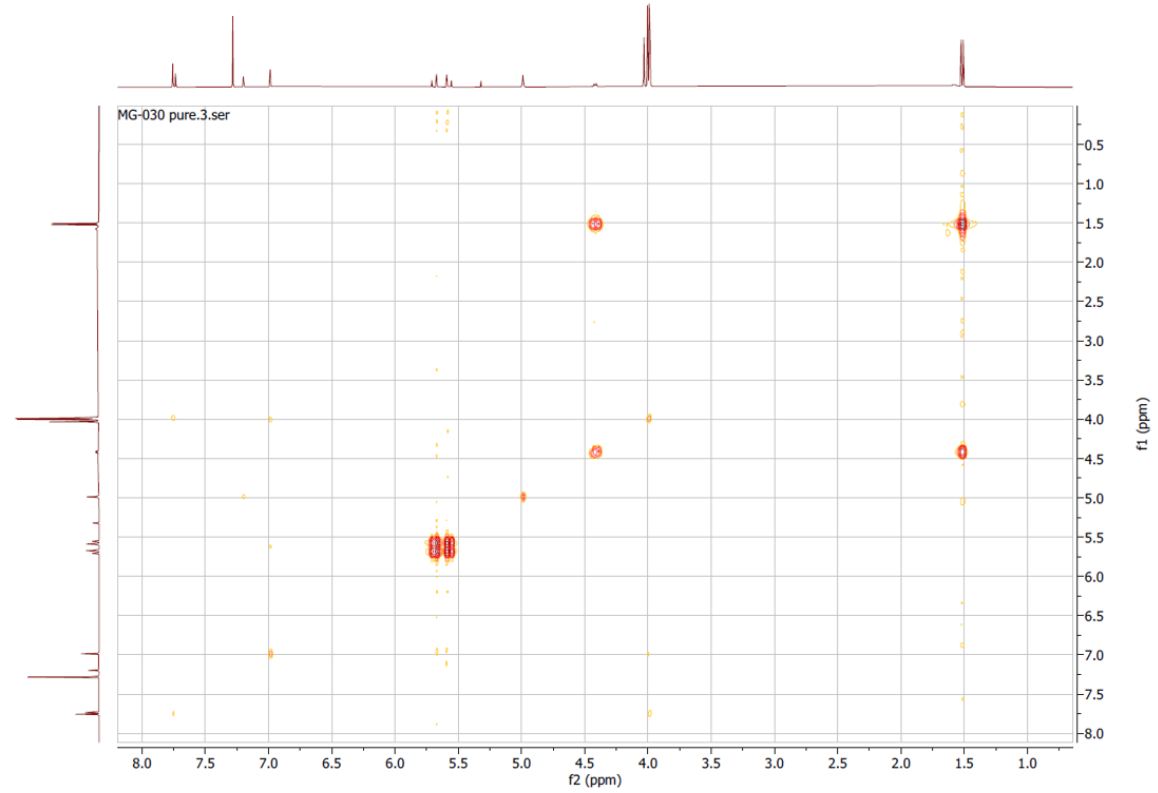
f1 (ppm)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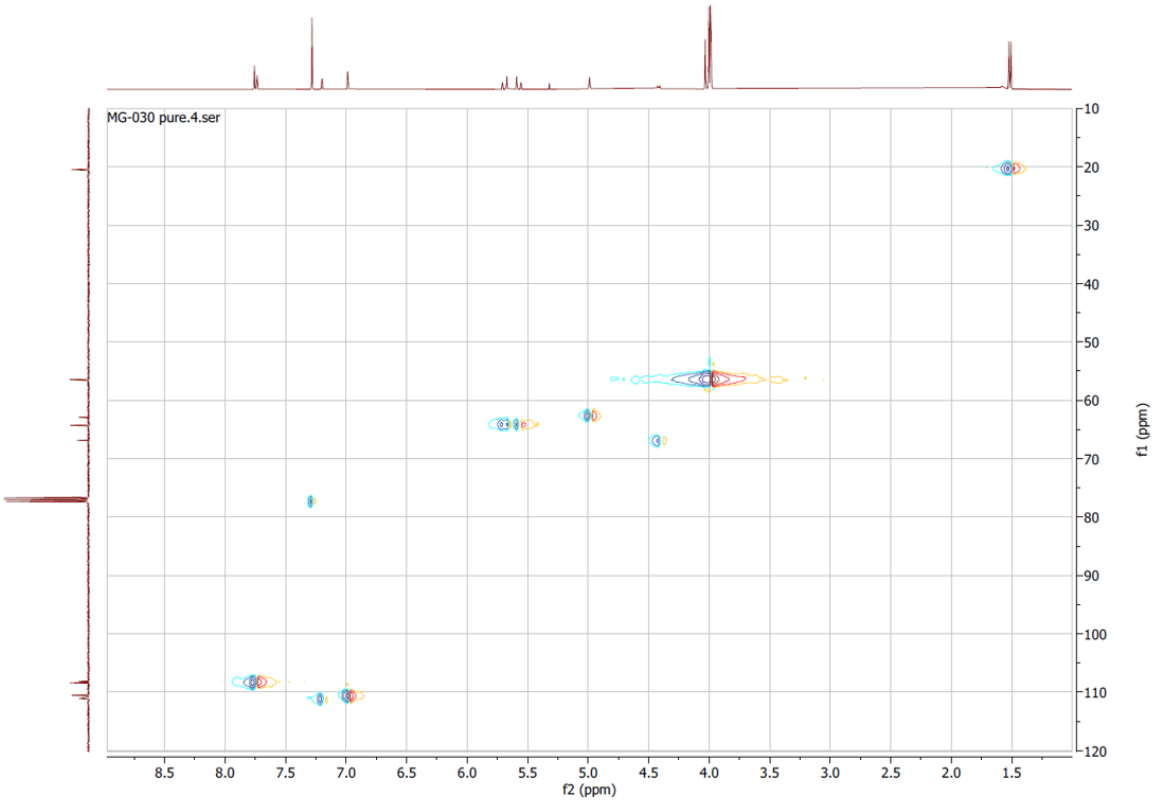
f1 (ppm)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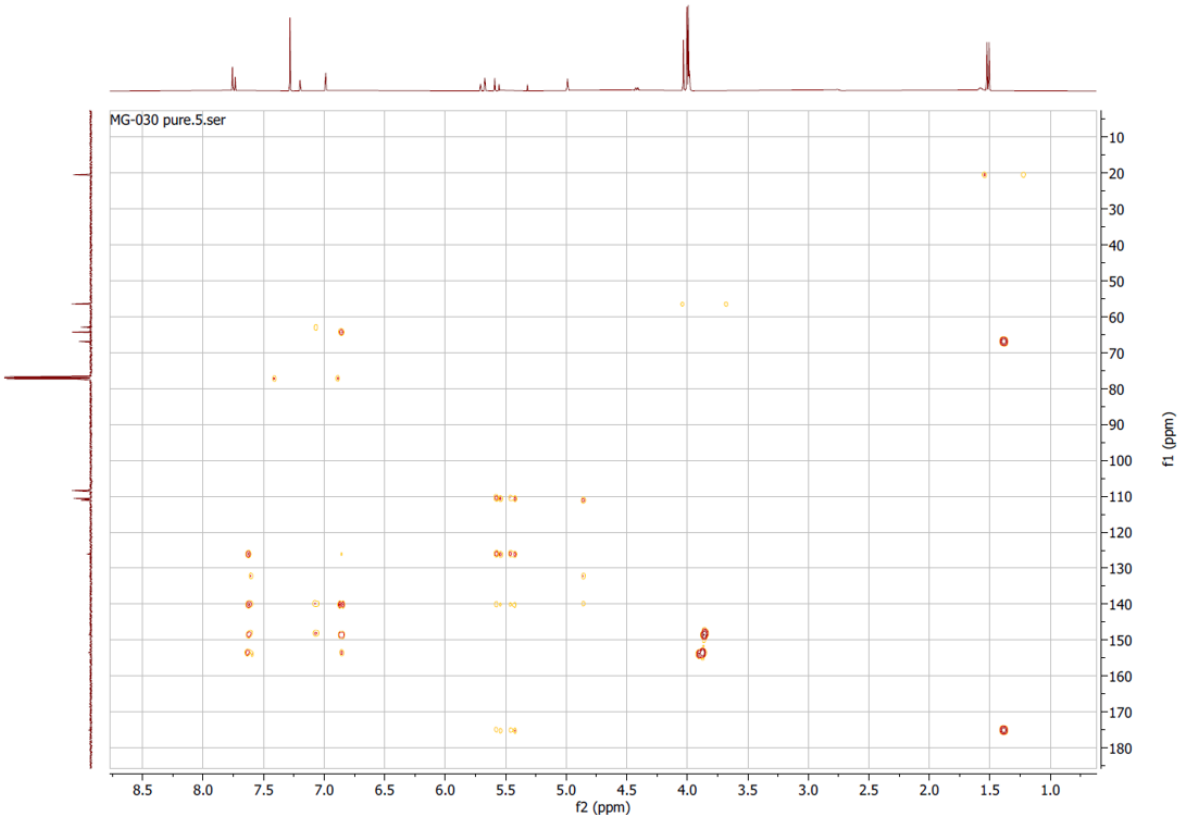
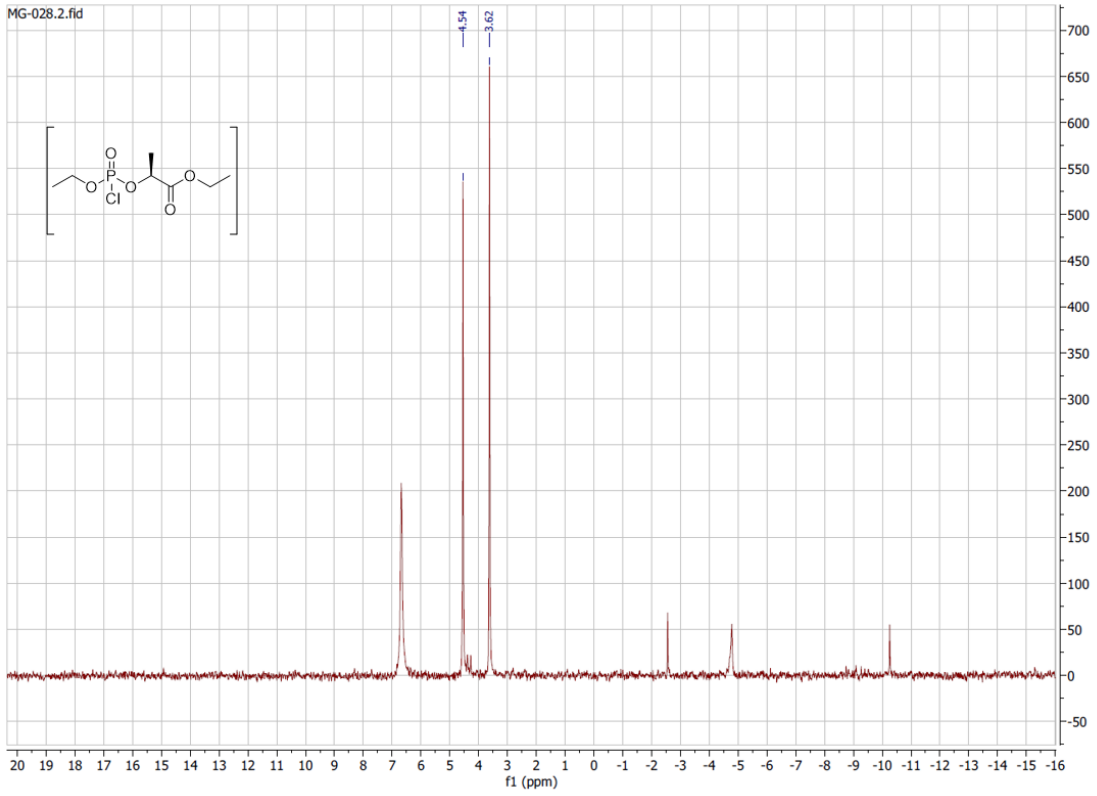
f1 (ppm)



Ethyl (2S)-2-((chloro(ethoxy)phosphoryl)oxy)propanoate (11)



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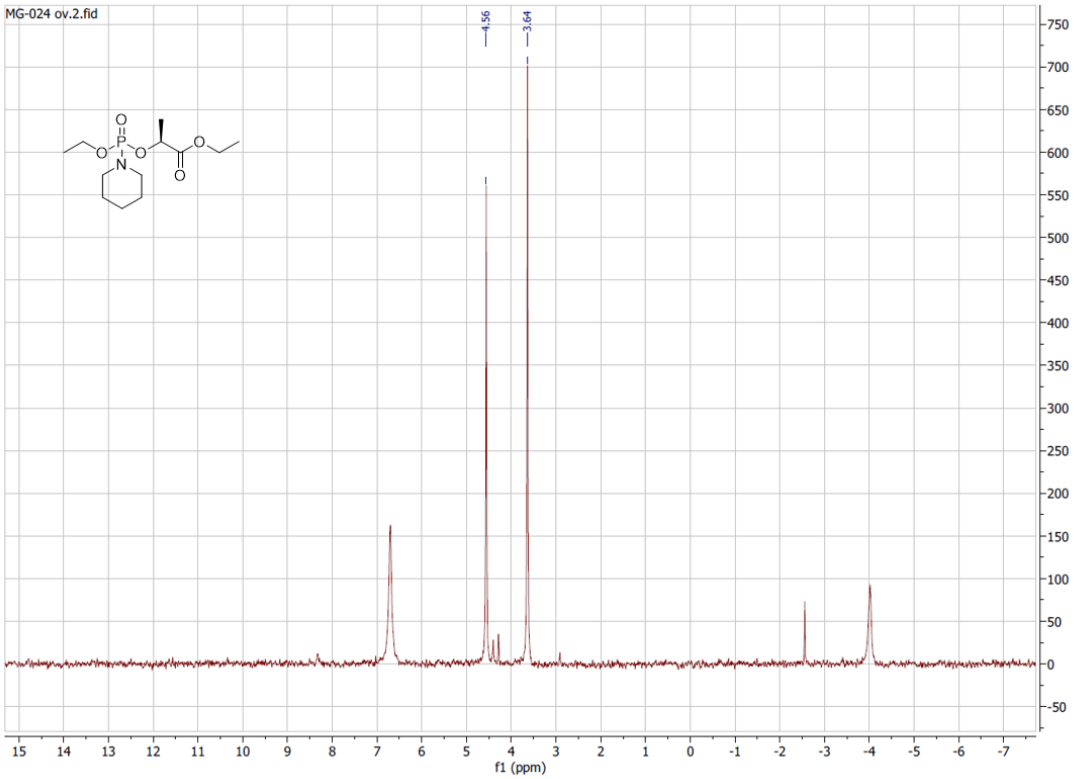

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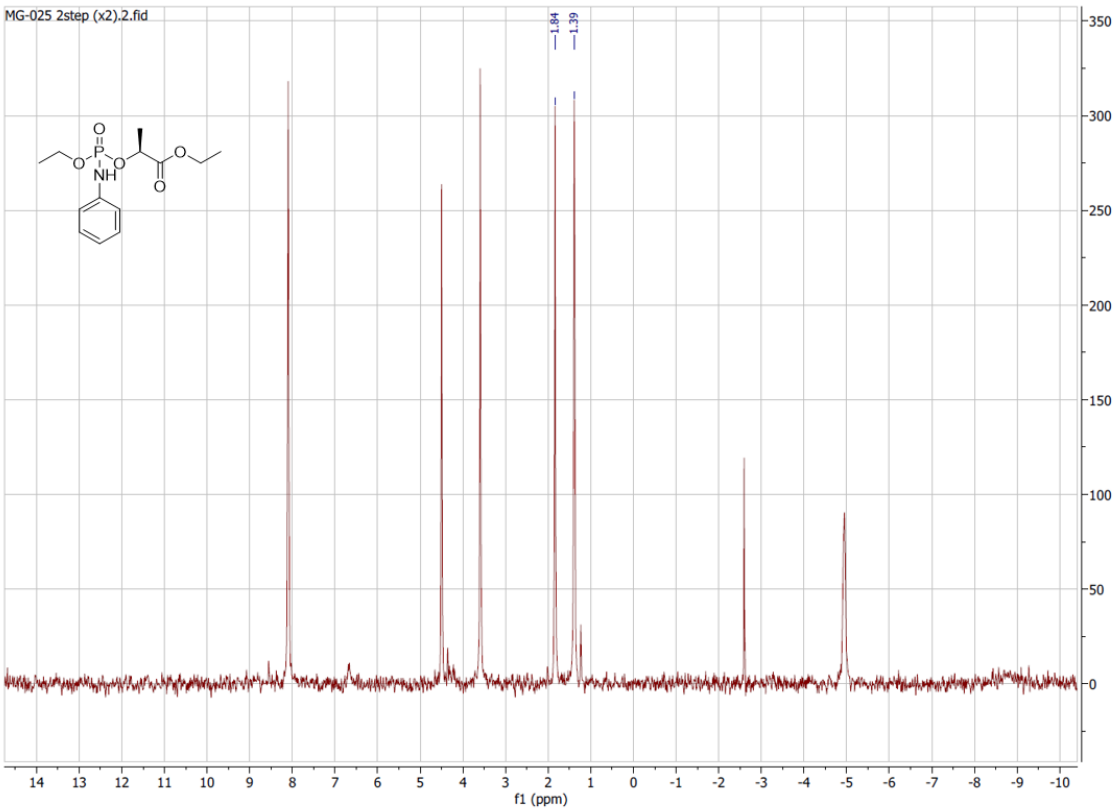

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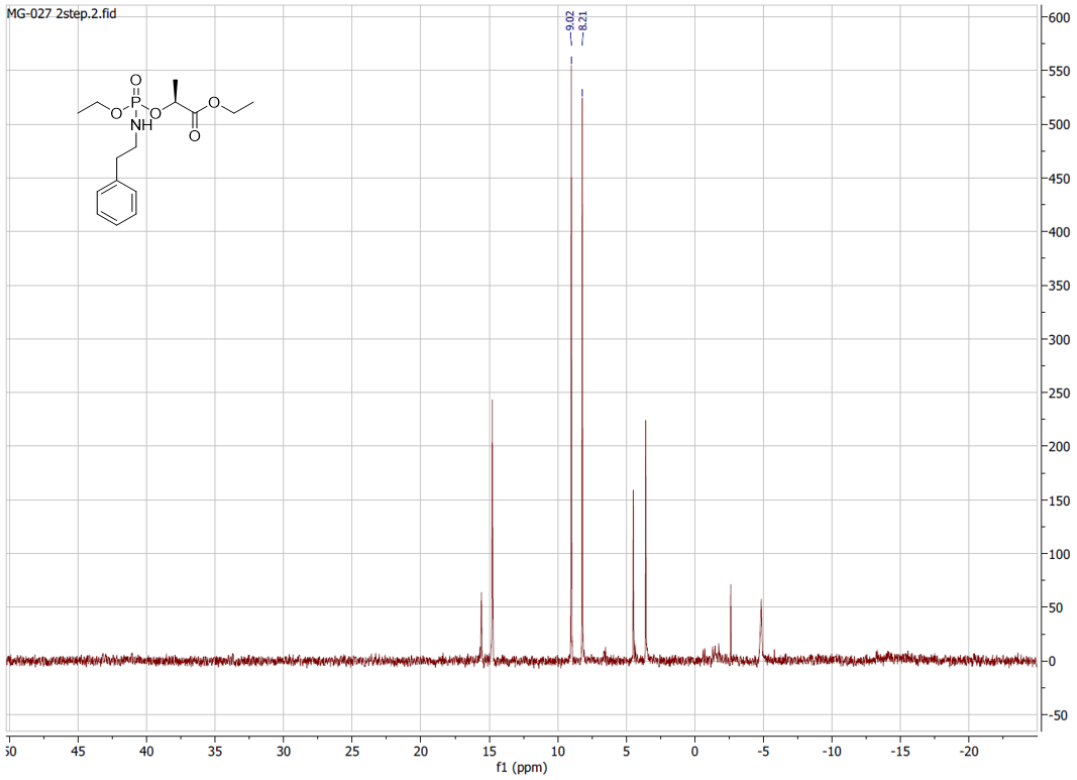

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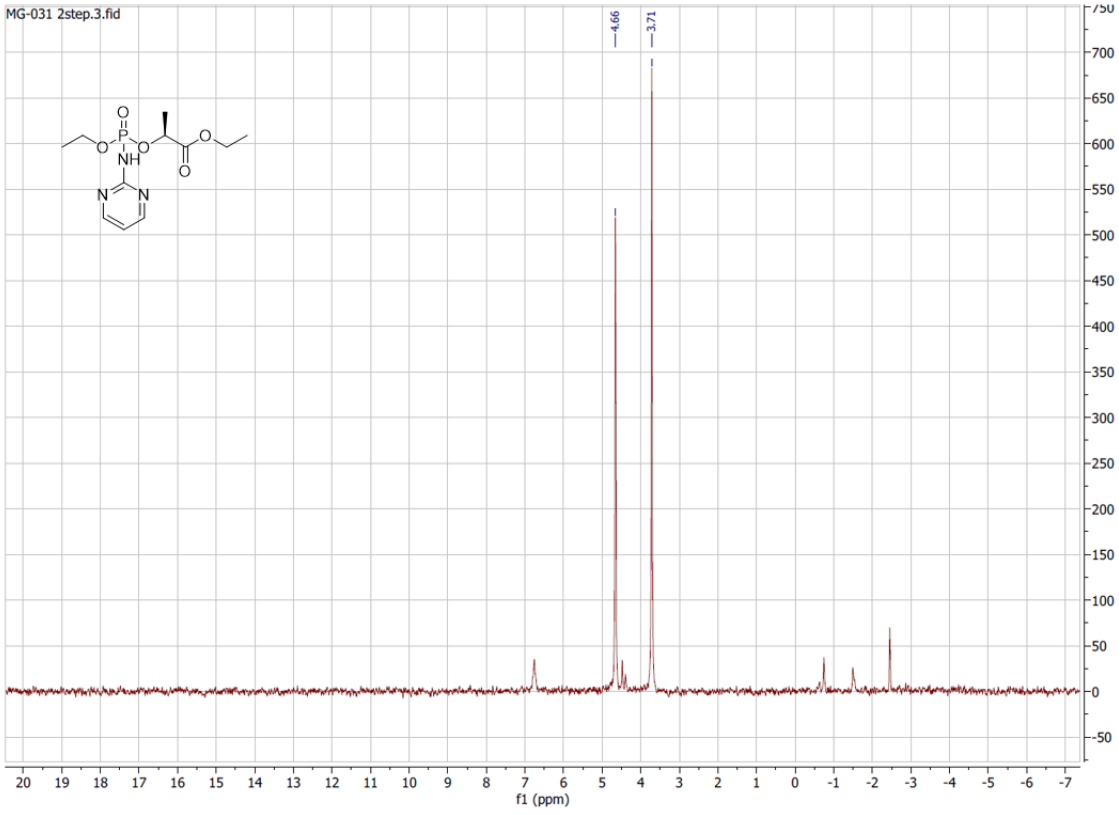
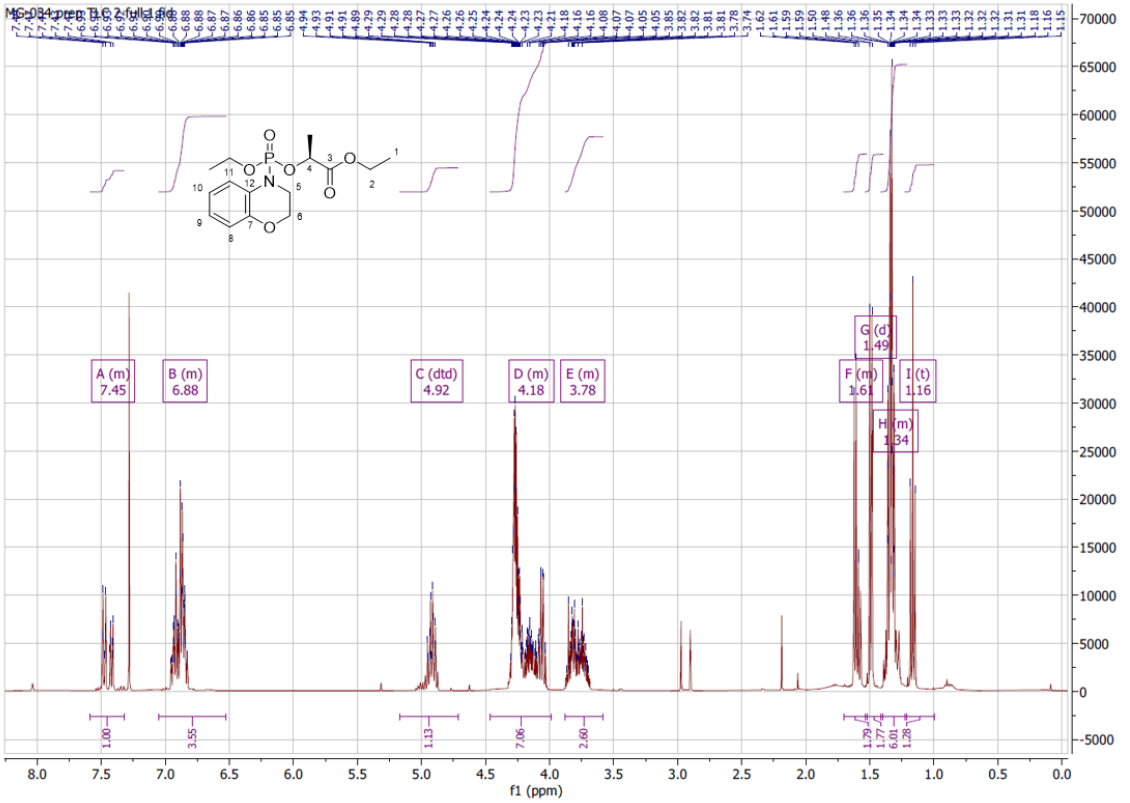
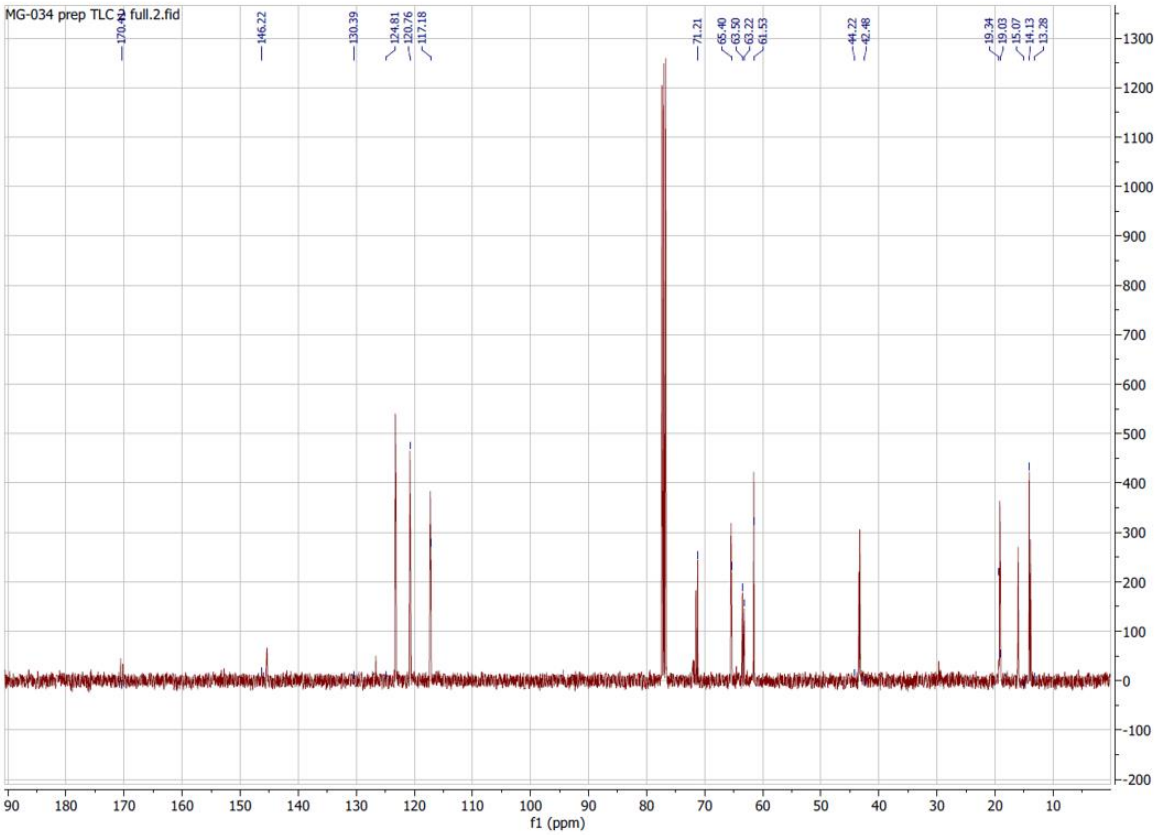
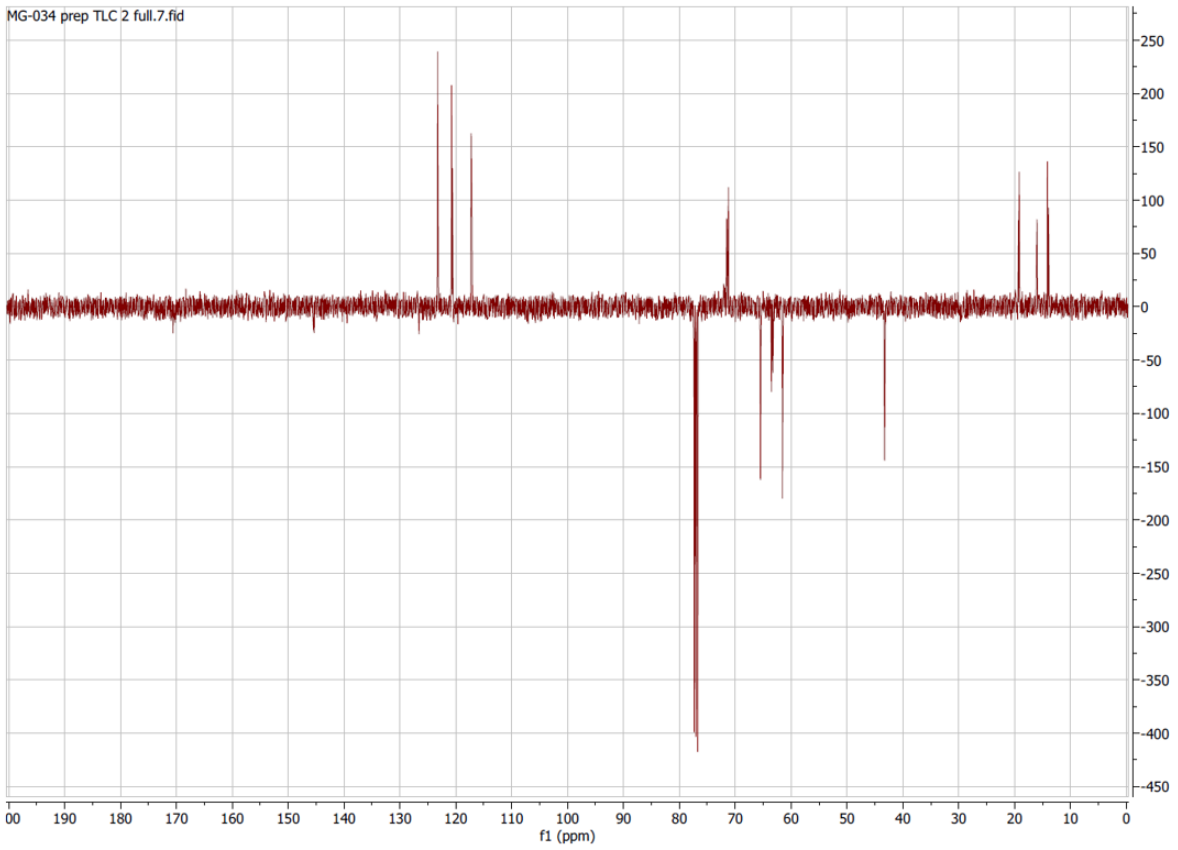


Ethyl (2S)-2-(((2,3-dihydro-4H-benzo[b][1,4]oxazin-4-yl)(ethoxy)phosphoryl)oxy)propanoate (24)



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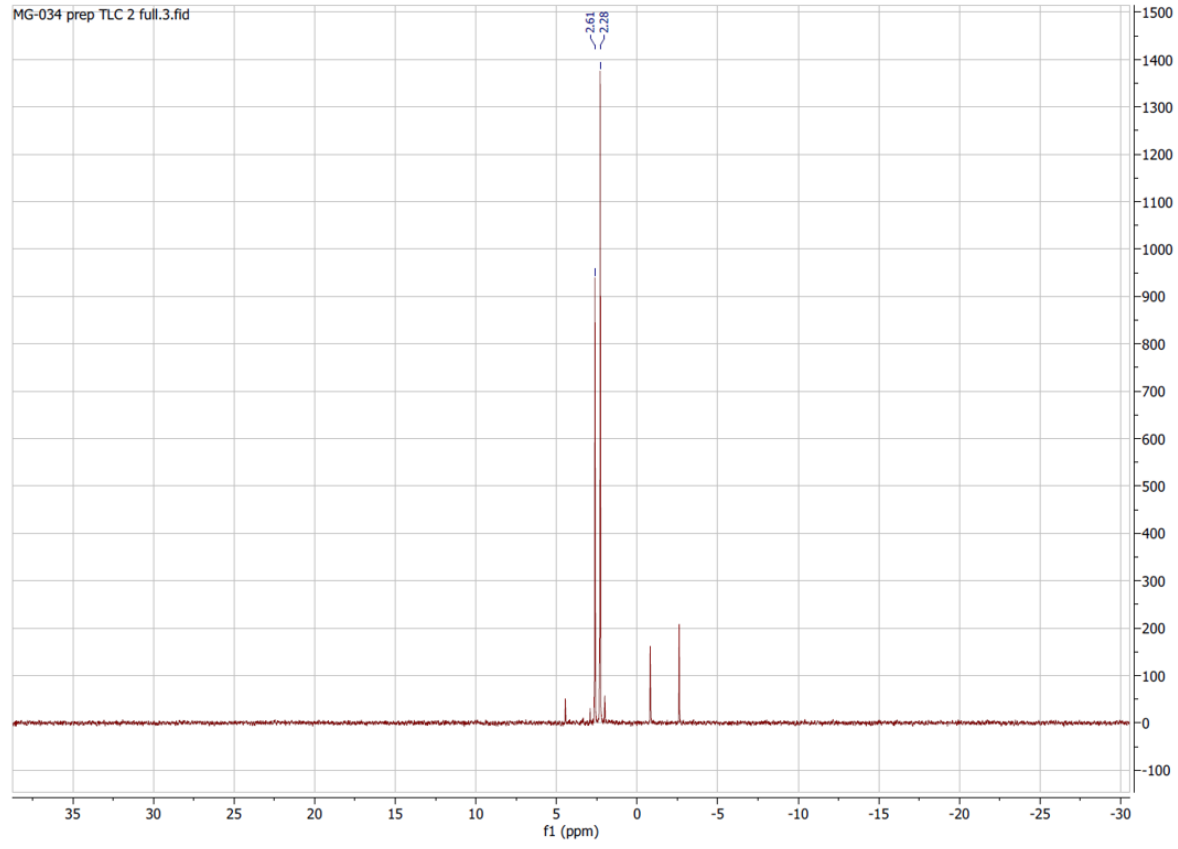
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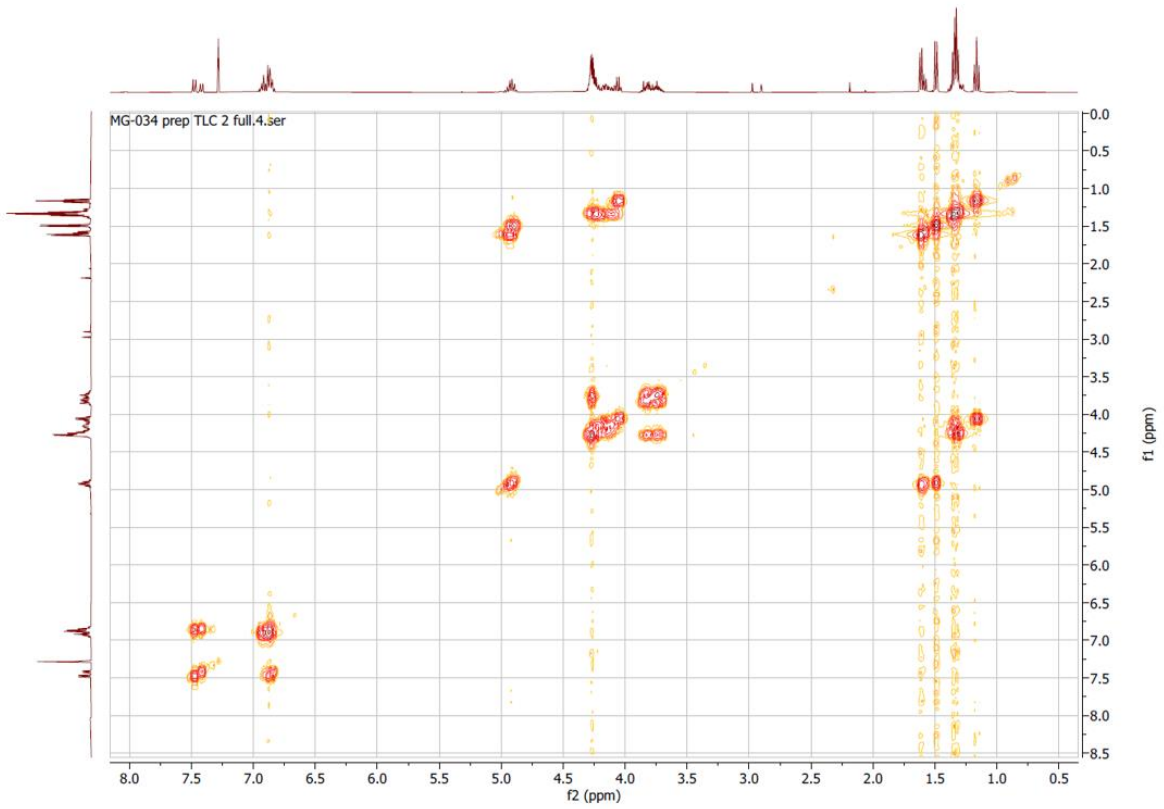
f1 (ppm)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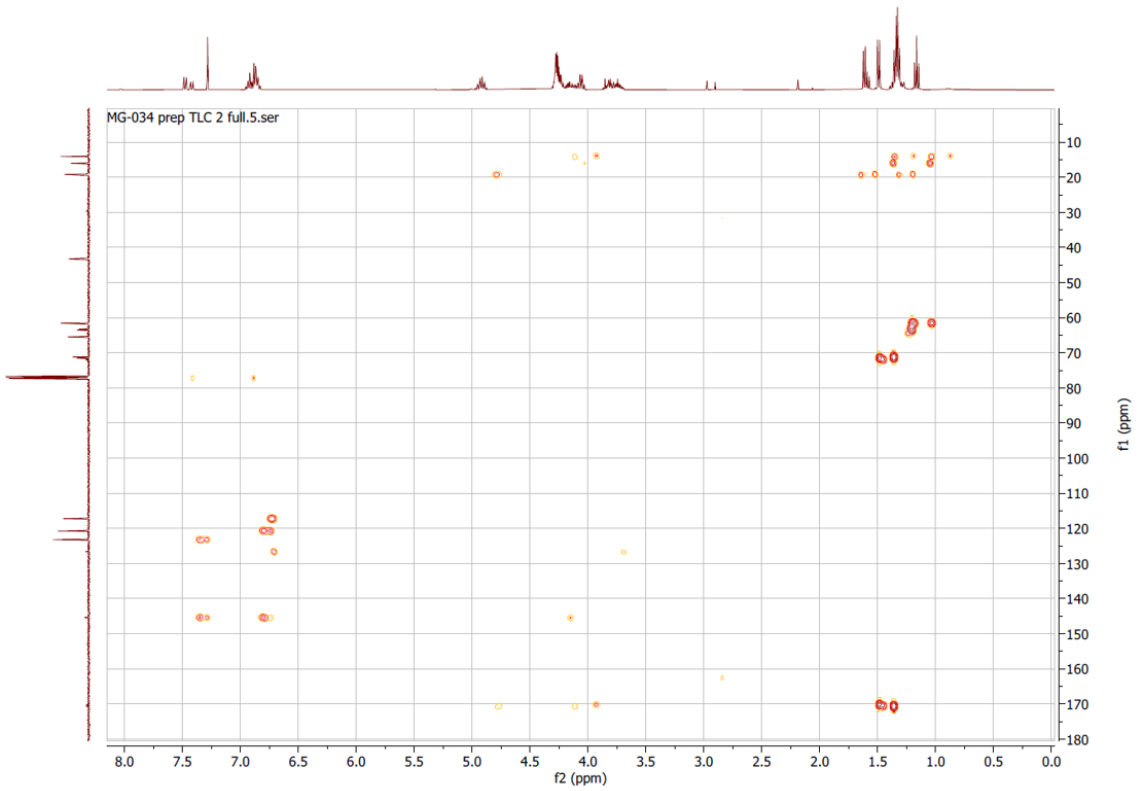
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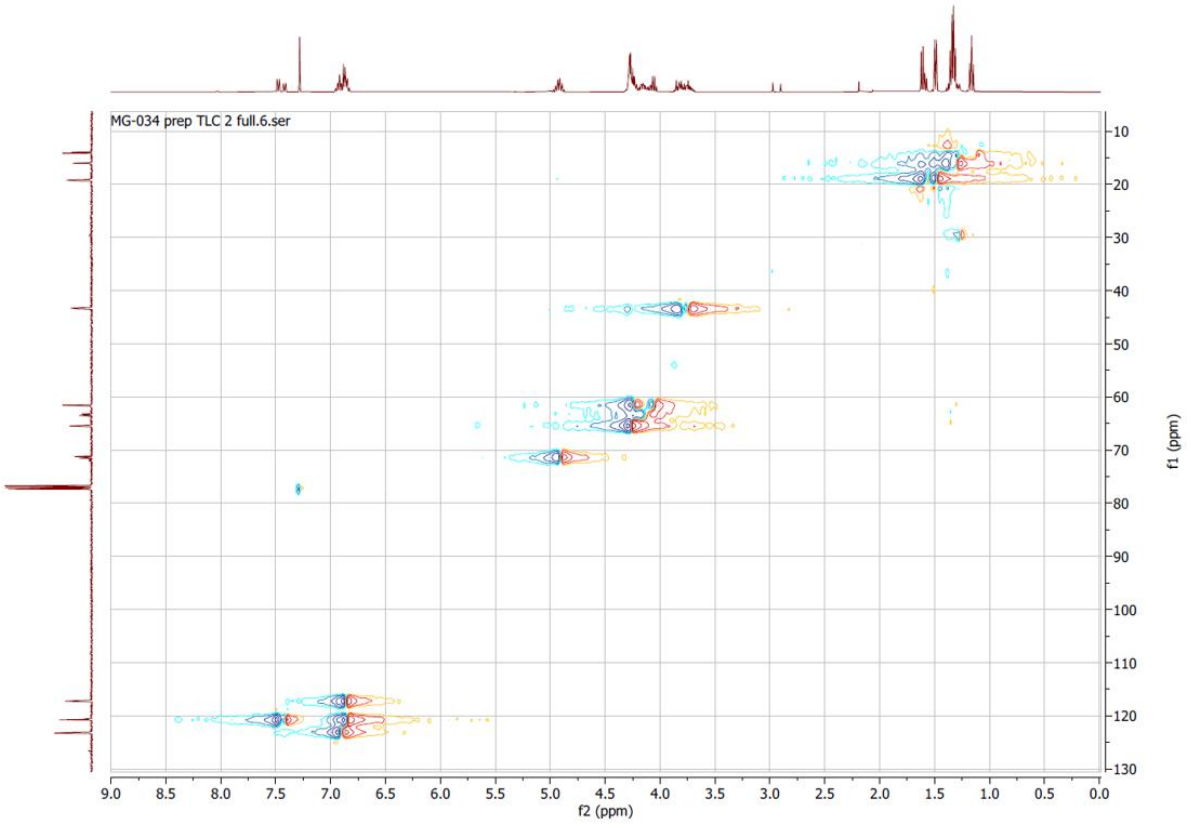
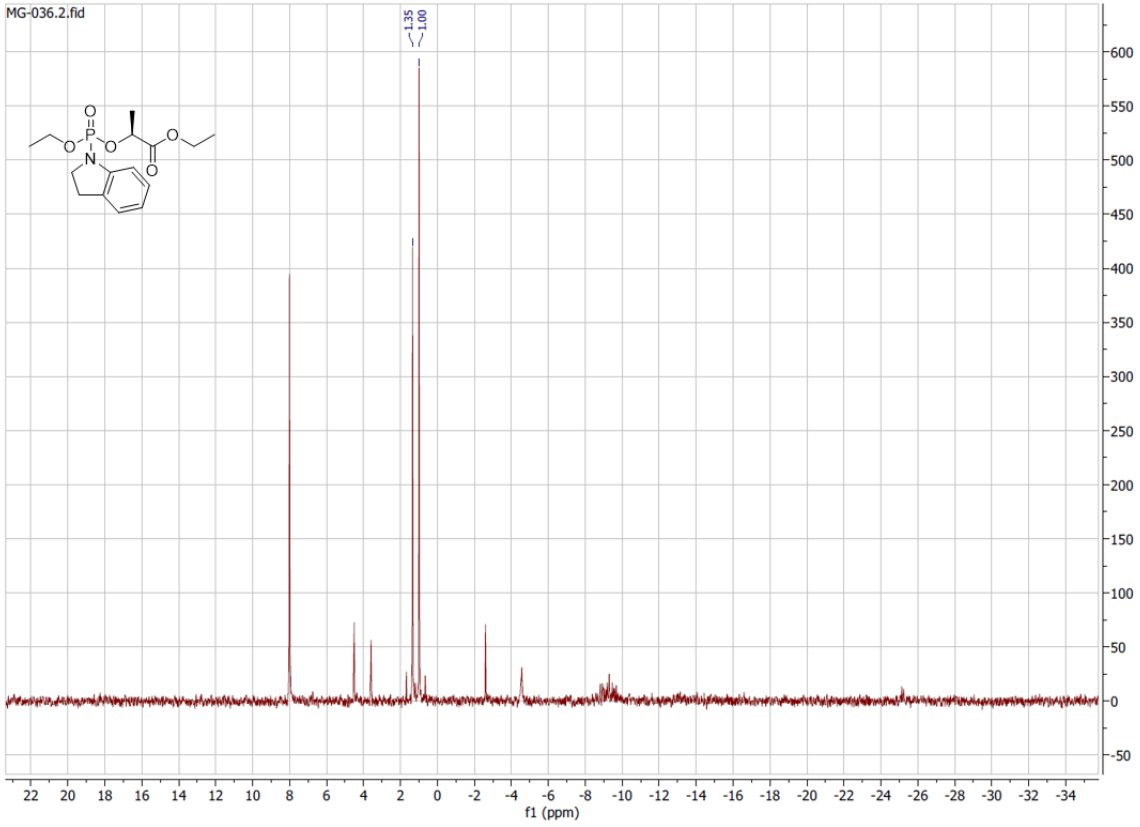
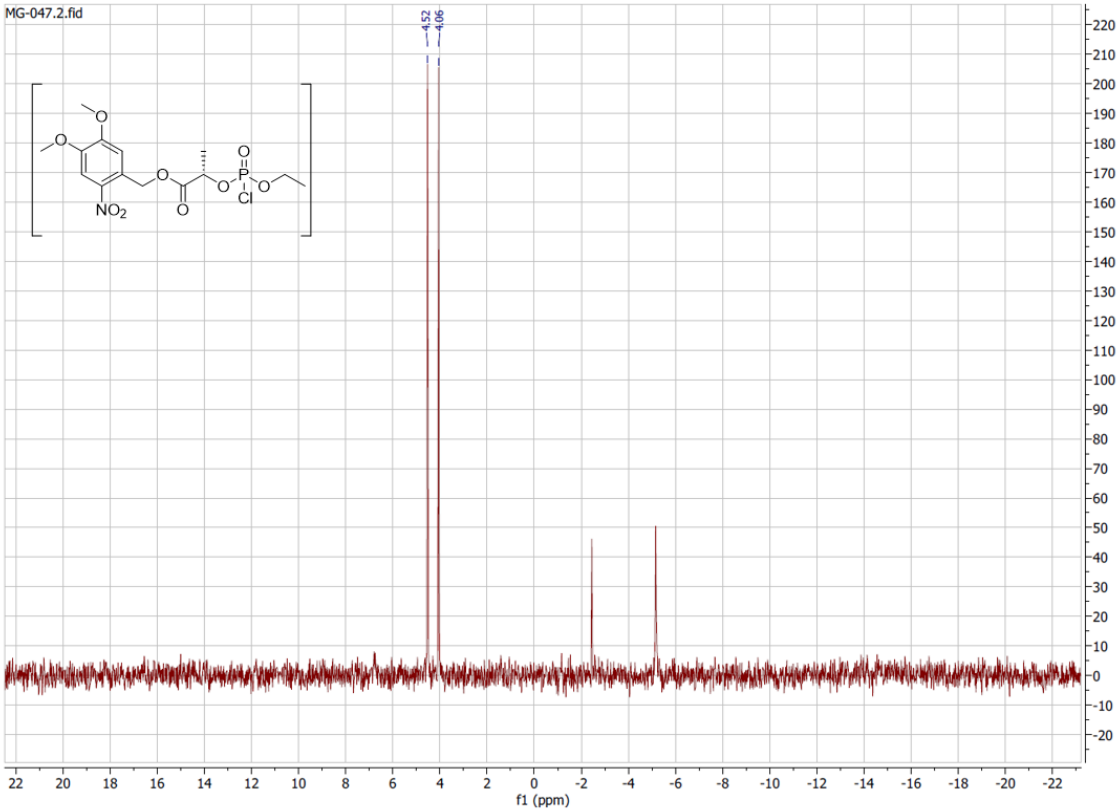
f1 (ppm)



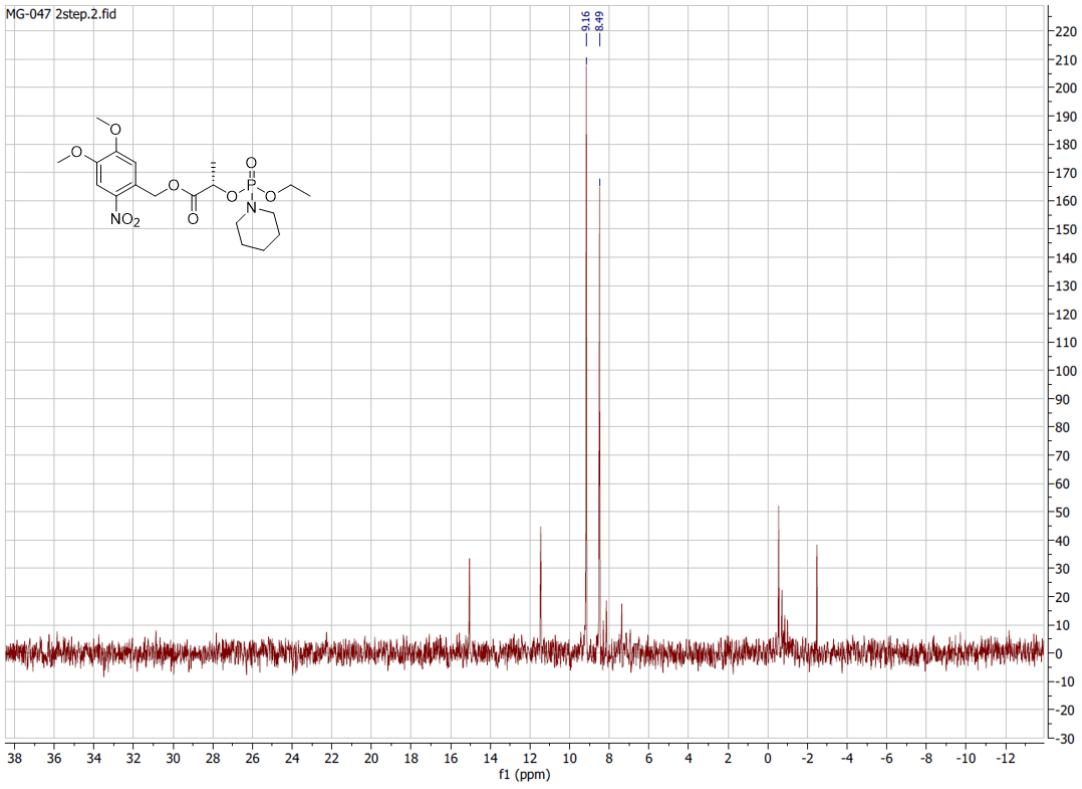
Ethyl (2S)-2-((ethoxy(indolin-1-yl)phosphoryl)oxy)propanoate (25)



4,5-dimethoxy-2-nitrobenzyl (2S)-2-((chloro(ethoxy)phosphoryl)oxy)propanoate (15)



4,5-dimethoxy-2-nitrobenzyl (2S)-2-((ethoxy(piperidin-1-yl)phosphoryl)oxy)propanoate (16)



4,5-dimethoxy-2-nitrobenzyl (2S)-2-((ethoxy(phenethylamino)phosphoryl)oxy)propanoate (17)