Facilitating the Solid-phase synthesis of oligonucleotides and peptides

By

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PREFACE

The research contained in this thesis was completed by the candidate while based in the Discipline of Chemistry, School of Chemistry and Physics of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville, South Africa.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate. As the candidate's supervisor, I have approved this thesis for submission.

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DECLARATION 1: PLAGIARISM

I, Kwazi Mazwi Masuku, declare that:

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(iii) this thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons;

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DECLARATION 2: PUBLICATIONS

Publication 1: Chapter 2

1. **Kwazi Masuku**, Luis Miguel Menéndez-Méndez, Sikabwe Noki, Beatriz G. de la Torre Fernando Albericio, Susana Fernández, Miguel Ferrero, Anna Aviñó, Ramon Eritja, Carme Fàbrega. The synthesis of solid supports carrying base labile linkers to generate 3'-phosphateoligonucleotides. Bioorg. Med. Chem. Letters. 2024 (**Masuku,**

KM. contributed to the synthesis, analysis, characterization of the linker and manuscript write up). (Published on 27 May 2024)

Publication 2: Chapter 3

2. **Kwazi Masuku**, Anamika Sharma, Sikabwe Noki, Sizwe Zamisa, Ayman El-Faham, Beatriz G de la Torre, and Fernando Albericio. Synthesis and Structural Confirmation of PyHOPO and its Applicability in Racemization Reduction in Solid-Phase Peptide Synthesis. (Masuku, K.M. contributed to all synthesis, characterization and write up of manuscript). (Submitted to Tetrahedron Letters on 01 February 2025).

[redacted]

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Abstract

Oligonucleotides and peptides are important chemical entities in pharmaceutical and material industries. The synthesis of both is carried out using the solid-phase approach first developed by Merrifield for peptides. Briefly, protected monomers (nucleotides or amino acids) are incorporated into a solid support through a linker, then a protecting group is removed, and the next monomers are incorporated sequentially. At the end, the oligonucleotide or the peptide is detached from the linker-solid support. In the present thesis, we describe the synthesis of base labile linker (4-((2-hydroxyethyl)sulfonyl)benzamide) and its application in solid phase synthesis of a 3' phosphorylated oligonucleotide. The new linker is compatible with phosphoramidite chemistry and enables obtaining the desired 3' phosphate oligonucleotides in excellent yields. Secondly, we reported the synthesis of ((2-oxopyridin-1(2H)-yl)oxy)tri(pyrrolidin-1-yl)phosphonium (PyHOPO) coupling reagent for solid phase peptide synthesis (SPPS) application and racemization study. PyHOPO was produced in excellent yield, and it enabled peptide synthesis with high purity and no detectable racemization.

Furthermore, the crystal structure of PyHOPO, which is a bidentate molecule, has been solved, which has allowed to determine univocally its structure.

Acknowledgement

Firstly, I want to extend my sincere appreciation to the almighty God for carrying me through this period, for the strength and brain he gave me to push through even at my lowest self. Secondly, I want to give a big shoutout and appreciation to my supervisors Prof. Fernando Albericio and Prof. Beatriz De La Torre for their extensive support, guidance and supervision throughout my master's period, words cannot fully entail how much how much am thankful to them for their unwavering support. I'll proceed to give thanks to the entire Peptide science laboratory and school of Chemistry and Physics stuff for their unconditional assistance especially my mentor Dr Sikabwe Noki for being so patient with me and for his mentoring, am truly grateful. Not forgetting my strongest supporter (My mom), today I wouldn't be writing these acknowledgements it wasn't for you, at times where I felt like just giving up, you were always my strongest emotional and financial supporter. Lastly, I want to thank me for believing in myself.

Abbreviations

1. **Boc** - tert-butyloxycarbonyl
2. **Bzl** – Benzyl
3. **CPG** - Controlled pore glass
4. **DCC** – Dicyclohexylcarbodiimide
5. **DCA** - Dichloroacetic
6. **DCM** - Dichloromethane
7. **DIC** - Diisopropylcarbodiimide
8. **DMF** - N,N-Dimethylformamide
9. **DMT** - Dimethoxytrityl
10. **ESB linker** - 4-((2-hydroxyethyl)sulfonyl)benzoic acid
11. **FDA** - Food Drug and Administration
12. **Fmoc** - 9-fluorenylmethoxy-carbonyl
13. **HOPO** - 2-pyridinol 1-oxide
14. **iBu** - Isobutyryl
15. **PG** - Protecting group
16. **PyBOP** - Benzotriazole-1-yloxytri(pyrrolidino)phosphonium hexafluorophosphate
17. **PyCloP** - Chlorotripyrrolidinophosphonium hexafluorophosphate
18. **PyHOPO** - ((2-oxopyridin-1(2H)-yl)oxy)tri(pyrrolidin-1-yl)phosphonium
19. **PyOXIM** - O-[(Cyano-(ethoxycarbonyl)methylidene)-amino]-yloxytripyrrolidinophosphonium hexafluorophosphate
20. **SPOS** - Solid phase oligonucleotide synthesis
21. **SPPS** - Solid phase peptide synthesis
22. **SPS** - Solid-phase synthesis

23. **TCA** - Trichloroacetic

24. **TFA** - Trifluoroacetic acid

25. **TIDEs** - Oligonucleotides and peptides

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Chapter 1

1.Introduction

1.1. General introduction

Drugs are the most important tools to detect and repair physiological disorders and ultimately to save lives. As it is clear in the case of cancer, there is not just one disease, there are hundreds of diseases, and therefore, there is a necessity to have more drugs ready to face the different diseases. Drug discovery has very complex yet interconnecting parameters because a new drug first must demonstrate that it cures the disease and, in many cases, needs to show superiority over the already existing drugs. In addition, it should fulfil the safety and toxicity protocols required by the corresponding agencies, for instance the Food Drug and Administration (FDA) in USA[1]. This means that it takes between 12 and 20 years for a drug to reach the market, after it has demonstrated some biological activity in the laboratory. for the time being, the economy has become more relevant in the drug discovery pipeline due to the high costs associated with it[2]. About one billion US Dollars is needed from the laboratory until the drug reaches the market.

Drugs are generally classified into two primary categories: biologics and chemical entities. Biologics are produced using biotechnological methods, while chemical entities are created through chemical synthesis. In the past drugs were practically only small molecules, which are chemical entities with a molecular weight below to 500-700 Da. Then, two decades ago, biologic drugs, first proteins and then monoclonal antibodies, were implemented. Among the chemical entities, this is a class of important drugs, biomolecules, oligonucleotides and peptides (TIDEs), but chemically synthesized. TIDES have drawn much attention due to several characteristics such as selectivity, safety, and efficacy[3] , trying to have the best characteristics of the small molecules and the biologics[4].

As a resume of the importance of the TIDES in the pharmaceutical market, in 2024, the FDA approved 50 new drugs. Among these, 16 were biologics (compared to 17 in 2018, 10 in 2019, 13 in 2020, 14 in 2021, 15 in 2022, and 17 in 2023), 4 were TIDES (2 oligonucleotides and 2 peptides, as opposed to 3 and 1 in 2018, 2 and 3 in 2019 and 2020, 2 and 8 in 2021, 1 and 4 in 2022, and 4 and 5 in 2023), and 30 were categorized as small molecules[1,2,5,6,7]. This brought that approximately 8% of the new drugs launched in the market are TIDES.

The recent surge in the use of oligonucleotides and peptides as drugs is the result of over three decades of extensive research and investment by both academic institutions and pharmaceutical industry in these fascinating molecules. Oligonucleotides are short polymers made up of repeating nucleotide units, consisting of a deoxyribose or ribose sugar, nitrogenous bases, and a phosphate backbone[8]. The majority of oligonucleotide-based drugs have chemically modified structures to improve their stability and therapeutic potency[7]. On the other hand, peptides are formed by the joining amino acid building blocks through an amide bond[9]. Amino acids can also be modified to fine-tune the activity and gain stability, and the letter is one of the drawbacks of the TIDES.

Currently, the synthesis of oligonucleotides and peptides in research and industrial mode is carried out using the solid-phase synthesis (SPS) approach (SPOS for oligonucleotides and SPPS for peptides). The SPS was first described for peptides by R. Bruce Merrifield[10] in 1963 and just immediately after the development of this method, Letsinger and Mahadevan[11] successfully used this SPS method for the synthesis of oligonucleotides. The concept is very simple and based on the use of a functionalized solid support as a protecting group of one of the functions of the terminal moiety: 3'-hydroxy group for oligonucleotides[8] and carboxylic group for peptides[12].

The use of the solid support facilitates reagents that do not react, and the soluble side-products are removed by filtration and further washings. Thus, a large excess of reagents can be used to drive the reaction to completion. Another further advantage is that all reactions (tens if not hundred in some cases) can be carried out in the same reaction vessel, which minimizes the loss of product by mechanical manipulation. There is no doubt that without the development of the SPS, we would not have TIDES on the market today. It is important to highlight that intermediates are not isolated and therefore they can be analysed only by indirect methods. This means that the synthetic process should be very optimized for getting crudes of high purity.

In SPS, the following common parameters should be considered: the solid support, the incorporation of the first moiety (protected nucleotide or amino acid), removal of the protecting group, coupling of the remaining moieties, global deprotection with concomitant cleavage of the oligonucleotide/peptide from the solid support. Due to the development and improvements that was associated with SPS, it thereafter became the method of choice for the synthesis of both Oligonucleotides and Peptides. According to this SPS technique, this means that the TIDEs requires: solid resin, deprotection, coupling reagents, protecting group or linkers as protecting group and finally suitable solvents for the synthesis. These are pivotal essentials for a good synthesis of TIDEs.

1.2. Oligonucleotides

Currently, oligonucleotides in the laboratory settings are synthesized using SPS and they can be customized into single stranded or double stranded molecules of exact sequence. One of key characteristic of oligonucleotide is their means to specifically binds to their complementary strand, be it RNA or DNA[13]. This trait then allows the use of oligonucleotides for the identification of specific DNA or RNA sequence. Furthermore, this has led to significant interest to pharmaceutical industries to have more oligonucleotide-based molecules as potential drugs. According to recent statistics, there are about 21 oligonucleotide drugs already in the market from the year 1998-2024[8,14] .

Despite a significant increase of importance of oligonucleotides for medicinal chemistry, there are still limitations that are hindering an even much more rapid progress. Their primary setback is lack of chemical stability which often leads to short lifespan, and they are not orally available. The synthesis which for many years was also a main drawback has been optimized thanks to the efforts of researchers working in the academy and in the industry. There have been several methods applied for chemical synthesis of oligonucleotides, starting with phosphodiester, phosphotriester and lastly phosphoramidites chemistry approach.

Despite this massive improvement there were still challenges encountered in oligonucleotide synthesis, challenges that led to longer period of synthesis for each step. Despite all these challenges and improvements made, continuous research led to phosphoramidite chemistry, and it was declared as an efficient approach for oligonucleotide synthesis in SPS due to it high efficient coupling and rapid turnaround time[15].

1.2.1. Phosphoramidite chemical synthesis of oligonucleotides.

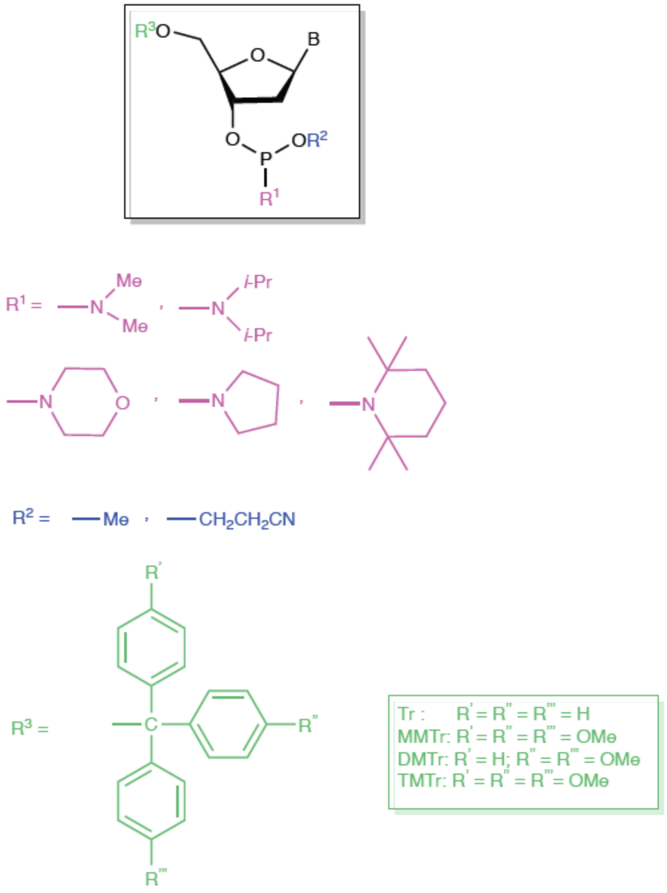
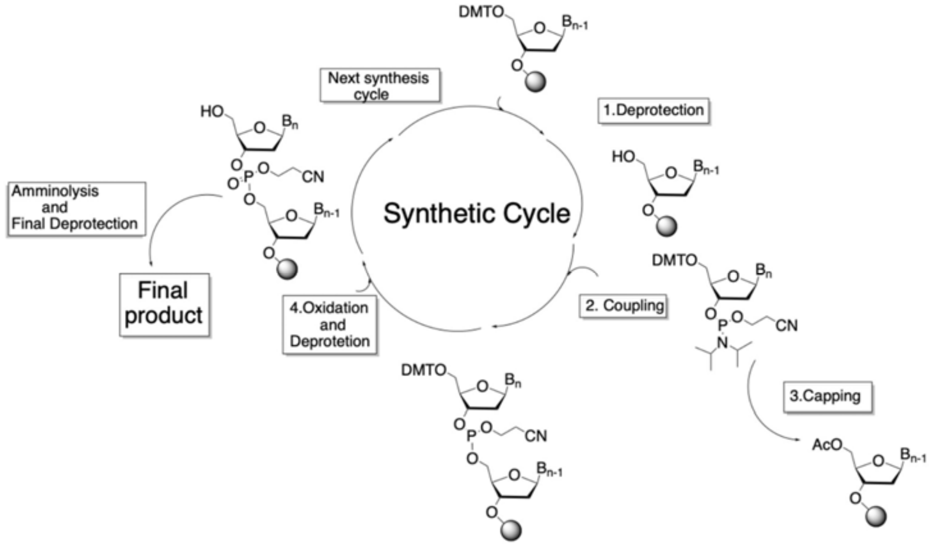


Figure 1: Representative phosphoramidites.

This synthetic approach requires nucleoside phosphoramidites (**Figure 1**) which are derivatives of natural or synthetic nucleosides and are used to synthesize not only oligonucleotides but also short fragments of nucleic acid and their analogs. This approach involves four steps namely: deprotection, coupling, capping and oxidation. **Scheme 1** below shows general cycle of oligonucleotide synthesis via phosphoramidite approach. The acid labile Dimethoxytrityl (DMT) protecting group must be firstly removed to allow reaction. Note that usage of stronger acids should be avoided because it will result in depurination which will lead to low yield production. Upon removal of DMT with 2-3% dichloroacetic (DCA) or trichloroacetic (TCA) acids in dichloromethane (DCM). The DMT cations are released from the support. The execution of the synthesis can be measured by checking the absorbance of these cation at 500 nm. The quantitative amount of DMT groups available during its deprotection is direct proportional to the amount of phosphoramidite that has been incorporated. An excess amount of the next nucleoside is brought together with a surplus of coupling activator like 1*H*-tetrazole (often used) will activate it by donating its proton to the amino group of the nucleoside phosphoramidite. The next step will be to ensure that the unreacted 5’ -OH is removed from the reaction by capping it with a solution of acetic anhydride. This is followed by oxidizing the phosphite-triester produced during the coupling thus making oligonucleotide susceptible to mild base cleavage.



Scheme 1: Schematic representation of synthesis of oligonucleotide via phosphoramidite Chemistry, where B = nucleobase with appropriate pg

1.2.2. Linkers

In general linkers are embedded on the solid support, which in this case is controlled pore glass (CPG), for facilitating the elongation of Oligonucleotide and at the end favouring the cleavage of the Oligonucleotide from the solid support. One of their primary importance is that they add stability to the growing Oligonucleotide, hence reducing possibility of side reactions and side products. oligonucleotides are connected via 3’-O terminals to a solid support resin. For example when using succinyl linker, In the presence of nucleophilic catalyst succinic anhydride react with unprotected 3’ hydroxyl group of the nucleoside to form an ester linkage and the exposed carboxy group is therefore coupled to solid support resin on its amino end, this is achieved by means of using coupling activators. The bond between the linker and the first nucleotide stays stable during the oligonucleotide synthesis and is easily removed using milder basic condition like 5% aqueous ammonia. The most currently used linkers are succinyl[16] and oxalyl[17] (**Figure 2**).

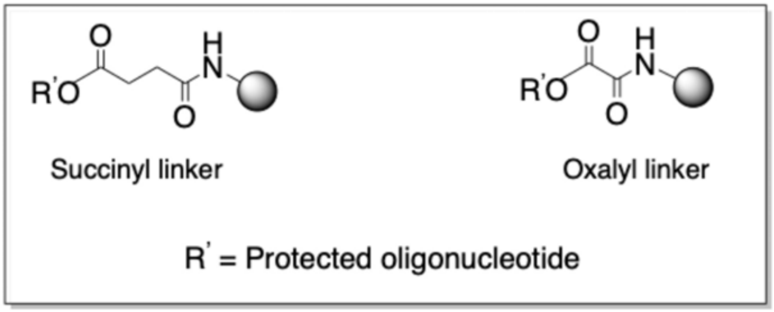


Figure 2: Linkers mostly used in oligonucleotide synthesis.

1.2.3. Coupling of nucleotides

Coupling of nucleosides requires coupling activators, 1*H*-tetrazole is most used activator. During coupling, an excess amount of the already activated phosphoramidite intermediate nucleotide protected at the 5'-position is introduced along with a significant surplus of 1*H*-tetrazole, which serves to activate the phosphoramidite. This process thereafter enables coupling of the next nucleotide and this step is followed by capping with acetonitrile.

1.2.4. Protecting groups in SPOS

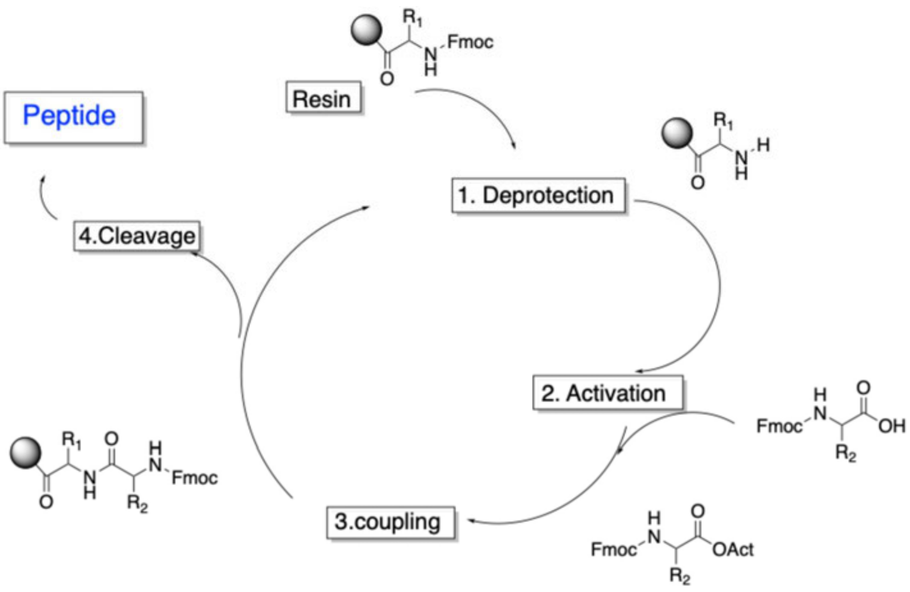
As mentioned, that protecting is mandatory for the synthesis so that the production of oligonucleotide is achieved with high purity. The nucleoside is temporally protected at the 5' hydroxyl group with the dimethoxytrityl (DMT) group, which is removed in each step with DCA or TCA. The Phosphite site is protected with 2-cyanoethyl group[18], which is removed at the end with ammonia. Since nucleobases (A, C, G) contain reactive functional groups, they require protection to prevent side reactions during chain elongation. Common protecting groups include benzoyl (Bz) for adenine (A) and cytosine (C), and isobutyryl (iBu) for guanine (G)[19,20]. All are also removed in the last step with ammonia.

2. Peptides

Peptides are highly desirable therapeutic agents due to their distinctive properties, such as high biological activity, specificity, and low toxicity, making them valuable medications[21] . The pharmaceutical industry has shown an increased interest in synthesizing more peptides with these traits for the aim of curing many diseases. The synthesis of peptides has changed a lot after Merrifield's solid phase peptide synthesis (SPPS) approach. SPPS has become a very efficient and valuable synthetic approach, this is due to an increase in yield production and reduction of tedious work that was encountered by solution phase synthesis. So it became a method of choice for the synthesis of peptides. Generally, peptides are polymers made of amino acid monomeric units. The most common amino acids, that are α-amino acids, have a carboxylic group, a side chain, and an amino group, all attached at the Cα-atom. N-terminal amino groups (and side chain reactive groups) of the amino acid to be coupled must be protected to avoid side reactions. Normally, SPPS synthesis proceeds in the C→N direction. SPPS requires a solid support[22], coupling reagents[23], protecting groups/linkers and a suitable base like piperidine for deprotection. The solid support is an insoluble polymeric resin where the peptide grows[10].

2.1. Solid phase peptide synthesis (SPPS)

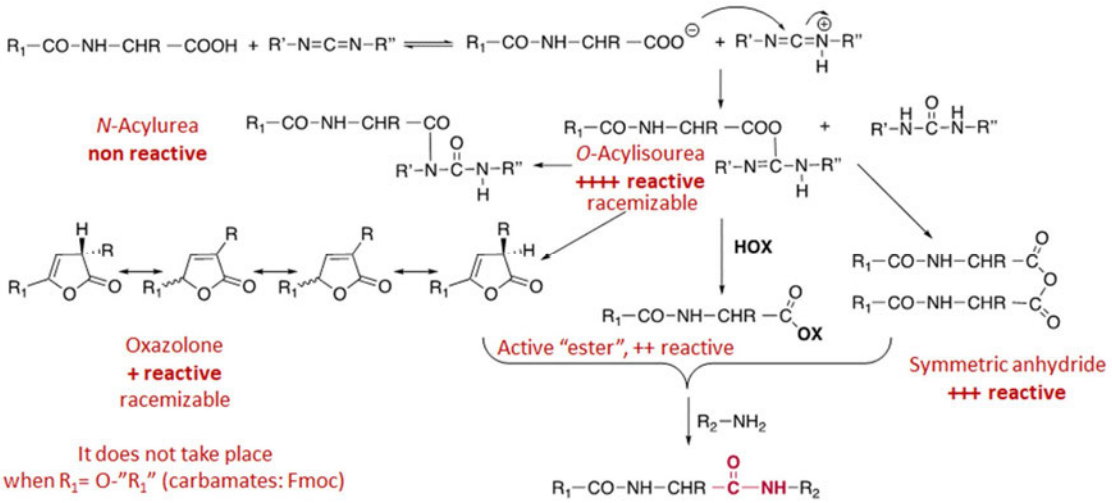
As in the case of SPOS, the growing peptide chain remains during all synthetic processes attached to the solid support. A difference between SPOS and SPPS is that SPOS is currently carried out in a rigid solid support, CPG. On the other hand, SPPS is carried out with a geltype support (polystyrene cross-linked with 1% of divinylbenzene), which should be wellsolvated (swelled) before starting the synthetic process to facilitate the accessibility of the reactive sites to the reagents[24]. So, the synthesis starts by swelling the resin in solvents like dichloromethane (DCM) or *N,N*-dimethylformamide (DMF), then the first protected amino acid [Fmoc (fluorenylmethoxycarbonyl)-tBu (*tert*-butyl) derivative] is incorporated through a linker attached to the solid support using a so-called coupling reagent[9] . Next, removal of the Fmoc group and sequentially incorporation of the rest of protected amino acids until the completion of the peptide sequence and final cleavage with trifluoroacetic acid (TFA) of the peptide from the solid support-linker with concomitant removal of the side-chain protecting groups will complete the scheme. **Scheme 2** shows SPPS cycle.



Scheme 2: General representation of SPPS, where R_1, R_2 (amino acid side chains)

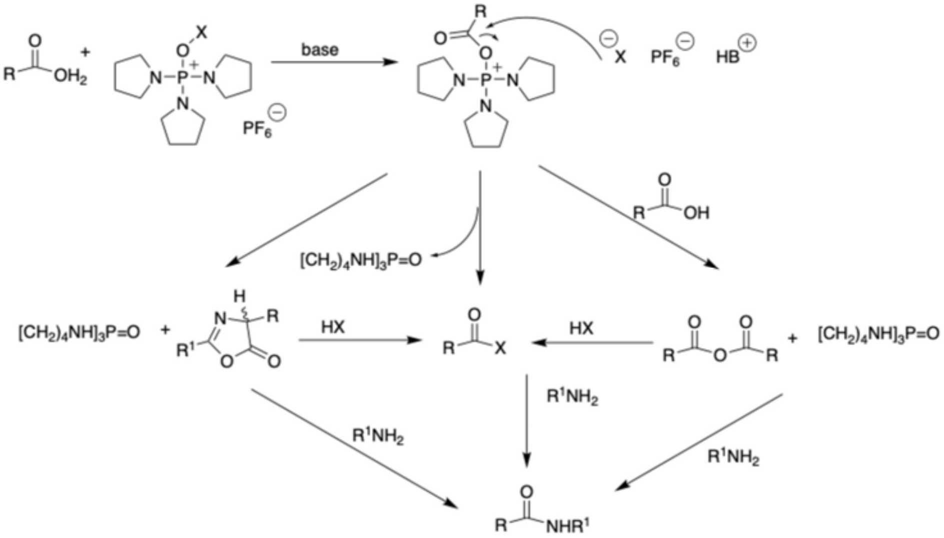
2.2.1. Coupling of amino acids in SPPS

During this stage of the synthesis, coupling reagents are required to join the carboxylic C-terminal to an N^{α} group of the resin of the amino group of the growing peptide. The mostly widely used coupling method is the use of carbodiimides like diisopropylcarbodiimide (DIC), which reacts with a carboxylic group to render *O*-acylisourea (**Scheme 3**). The *O*-acylisourea, is a very reactive active species which can undergoes aminolysis in the presence of amino function to generate a peptide. However, the *O*-acylisourea, due to its high reactivity, can undergo racemization and a rearrangement to render the *N*-acylurea, which is totally unreactive. The *O*-acylisourea can also undergo an intramoleular cyclization to oxazolone, which is less reactive and is prone to racemizaton[23] . In the presence of an extra equivalent of carboxylic acid, the *O*acylisourea render the symmetrical anhydride, which is very reactive but uses an equivalent of a non-productive carboxylic acid.



Scheme 3. Carbodiimide-based coupling step in peptide synthesis

An alternative to the use of carbodiimides is the use of stand-alone coupling reagents, phosphonium and uronium salts. Phosphonium salts such as benzotriazole-1yloxytri(pyrrolidino)phosphonium hexafluorophosphate (PyBOP)[23] or O-[(cyano(ethoxycarbonyl)methylidene)-amino]-yloxytripyrrolidinophosphonium hexafluorophosphate (PyOxim)[25] react with carboxylates to render acylphosphonium active species, which are very reactive and reacts with the leaving group of the phosphonium salt (**X** in Scheme 4, OBt in PyBOP or Oxyma in PyOxim) to render the active ester which reacts with the amino function to render the amide/peptide bond (**Scheme 4**). Phosphonium salts are known to be in general very efficient coupling reagents and present an advantage over uronium, they do not react with amino function as the uronium salts do giving guanidines (reaction not shown)[26] .



Scheme 4: Represent mechanism of phosphonium-Mediated reaction.

2.2.2. Protecting groups (PG) in SPPS

Protecting groups play a pivotal role in SPPS by ensuring stability of the peptide and hence minimizing possibility of side reactions[27]. In a typical SPPS scheme, an orthogonal scheme based in Fmoc, labile in the presence of bases (secondary amines), for the α-amino group and acid labile (tBu) for the side chain is used. The use of orthogonal protecting groups, each protecting group can be removed in the presence of the other and in any order, has the advantage that even forcing the condition of removal of one of them, the other will be stable[28].

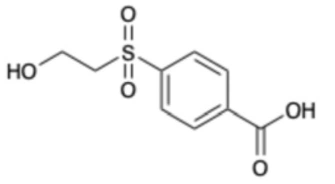
Aims and objectives

The aim of this Master thesis is to develop new tools for facilitating the synthesis of oligonucleotides and peptides using the solid-phase approach The objectives are:

1. To design, synthesize and use a base labile linker for the synthesis of 3' phosphorylated oligonucleotide via a phosphoramidite approach.
2. To synthesize a new phosphonium coupling reagent for the synthesis of peptides.

In this thesis, we are addressing two important issues regarding the synthesis of oligonucleotides and peptides.

First of all we report the synthesis and use of base labile linker 4-((2hydroxyethyl)sulfonyl)benzoic acid (ESB linker) shown on figure 1 for the synthesis of oligonucleotide carrying 3' terminal phosphate. The presence of the carboxylic acid function and the hydroxyethylsufoxide moiety allows the functionalization of solid supports with the β-eliminating linker, generating 3'-phosphate oligonucleotides.

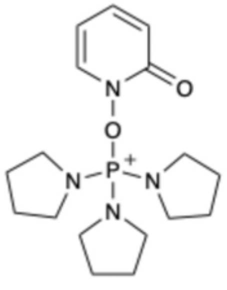


4-((2-hydroxyethyl)sulfonyl)benzoic acid

Figure 3: Structure of ESB base labile linker

ESB linker is stable under mild acidic conditions which makes it stable during the synthesis of oligonucleotide, however the linker is susceptible to basic (ammonia) cleavage. The main function of the linker was to add stability and connect the oligonucleotide to solid support. The use of this linker resulted in excellent yield of partially protected oligonucleotide.

In the second aspect of this thesis, we report the synthesis and use of a new coupling reagent for the synthesis of peptides. One of the key parameters in SPPS is having an efficient coupling reagent that is safe to use and gives minimal side products. It must also produce peptides with often little or no racemic mixtures which results in diastereomeric impurities, often hard to remove. So, we developed a new coupling reagent named ((2-oxopyridin-1(2*H*)-yl)oxy)tri(pyrrolidin-1yl)phosphonium (PyHOPO) for the use in peptide synthesis (**Figure 4**). This was prepared from using 2-pyridinol 1-oxide (HOPO) and chlorotripyrrolidinophosphonium hexafluorophosphate (PyCloP). An advantage of using phosphonium salts is that they do not react with N-amino functional group, hence terminating the peptide chain as the stand-alone additives such as amminium/uronium does and they produce active ester of the chemical additive used, which in this case is HOPO.



PyHOPO

Figure 4: structure of desired PyHOPO

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The synthesis of solid supports carrying base labile linkers to generate 3′-phosphate oligonucleotides

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ABSTRACT

Keywords: Oligonucleotides carrying 3′-terminal phosphates and conjugates are important tools in molecular biology and Oligonucleotides diagnostic purposes. We described the preparation of solid supports carrying the base labile linker 4-((2-

3Solid support Base labile linkers ′-phosphorylation hydroxyethyl)sulfonyl)benzamide for the solid-phase synthesis of 3cleotides in excellent yields. The use of mild deprotection conditions allows the generation of partially protected supports are fully compatible with the phosphoramidite chemistry yielding the desired 3′-phosphorylated oligonucleotides. These ′-phosphate oligonu-

DNA fragments.

Oligonucleotide carrying 3′-phosphates are important intermediates for the synthesis of oligonucleotide conjugates as well as for oligonucleotides that cannot be extended by DNA polymerases.[1–3] In addition, the presence of the 3′-phosphate protects oligonucleotides from degradation by exonucleases. The synthesis of these compounds can be achieved using solid supports functionalized with base labile linkers that connect the solid support to the oligonucleotides. In this way, nucleoside phosphoramidites can be used to assemble the desired oligonucleotide sequences and, ammonia deprotection will generate the desired 3′- phosphate oligonucleotides. There are several base-labile linkers used for this purpose (Scheme 1), including ethylsulfoxide linker,[1–8] 2-nitrophenylethyl linker (NPE[9]), fluorenylmethyl linker (FM[10]), substituted 2- cyanoethyl[11] and Lonnberg¨ 's chemical phosphorylation supports carrying bis(carboxymethylamido) groups.[12,13] The hydroxyethylsulfoxide (ESE) linker can be prepared from 2,2′-sulfonyldiethanol and was originally described as DMT-containing phosphoramidite for the phosphorylation of the 5′-position of oligonucleotides.[1] When this reagent is used as hemisuccinate derivative for the functionalization of solid supports,[14,15] the resulting solid supports are between the most commonly used linkers for the synthesis of oligonucleotide 3′-phosphates. In this communication, we describe a novel preparation of the β-eliminating linker [4-((2-hydroxyethyl)sulfonyl)benzoic acid, ESB, **5**] that contains the hydroxyethylsulfoxide moiety attached to a benzoic acid derivative. The presence of the carboxylic acid function and the hydroxyethylsufoxide moiety allows the functionalization of solid supports with the β-eliminating linker, generating 3′-phosphate oligonucleotides.

The synthesis of 4-((2-hydroxyethyl)sulfonyl)benzoic acid (**5**) is shown in Scheme 2. First, 4-mercaptobenzoic acid was esterified to yield methyl 4-mercapto benzoate (**2**) in 94 % yield. Next, methyl 4-mercapto benzoate (**2**) was reacted with 2-bromoethanol in the presence of cesium carbonate yielding methyl 4-((2-hydroxyethyl)thio)benzoate (**3**) in 91 % that was oxidized with *m*-chloroperoxybenzoic acid (m-CPBA) obtaining methyl 4-((2-hydroxyethyl)sulfonyl)benzoate (**4**) in 88 %. Hydrolysis of the methyl ester with

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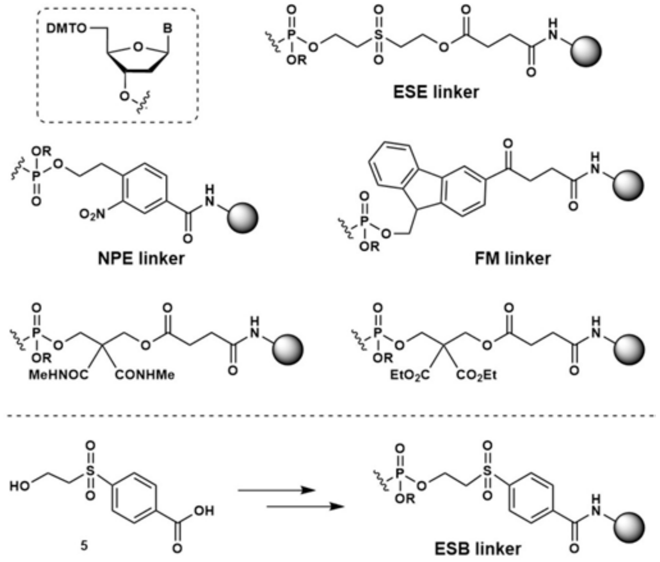
lithium hydroxide yielded the desired compound **5**. The proposed route differs from a previously described protocol [16] in the use of the methyl ester **2** instead of the benzoic acid derivative **1** in the *S*-alkylation reaction resulting in more hydrophobic intermediates which can be easily purified on silica gel (see experimental conditions and spectroscopic characterization in the Supplementary Information section).



Scheme 1. Chemical structure of some of the linkers described for the synthesis of oligonucleotide 3′-phosphates as well as the structure of 4-((2-hydroxyethyl) sulfonyl)benzoic acid developed in this communication.

Next, compound **5** was coupled to amino-controlled pore glass (CPG) using dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazol (HOBt) as coupling agents.[10] In order to study the lability of the new supports, a DMT-Thymidine 2-cyanoethylphosphoramidite was coupled and the resulting DMT-*T*-support was treated with concentrated ammonia, 10 % piperidine/acetonitrile, dimethylamine (Me_2NH) aqueous solution/acetonitrile (1:3), 10 % triethylamine (Et_3N) in acetonitrile and 0.1 M 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in acetonitrile. The percentage of DMT-T 3′-phosphate released from the support at different times was estimated by measuring the amount of DMT groups left on the support compared with the DMT group released during the treatment. From these experiments, the lability of the novel CPG support was found to be comparable to CPG functionalized with the ESE linker from commercial sources. The optimal conditions for the release were found to be: a) concentrated ammonia for 4 h at 55 °C, b) 0.1 M DBU in acetonitrile for 15 min at room temperature (rt), c) 10 % piperidine/acetonitrile for 2 h at rt, d) 40 % Me_2NH aqueous solution/ acetonitrile (1:3) at rt and e) 10 % Et_3N in acetonitrile for 2 h at rt. In all these cases, the release of the DMT-T 3′-phosphate was judged to be complete by the measure of the DMT cation remaining on the solid support.

The solid support functionalized with the ESB linker was used for the synthesis of several oligonucleotides (Table 1), which were assembled on an automatic DNA synthesizer using standard phosphoramidite synthesis cycles. In one case, the same oligonucleotide (sequence A) was prepared on commercially available CPG functionalized with the ESE linker for comparative purposes. After sequences assembly, the resulting solid supports were treated with concentrated ammonia and the resulting compounds were analyzed by analytical HPLC (Figure 1) and MALDI-TOF mass spectrometry, yielding the desired oligonucleotide 3′- phosphates with the same purity obtained using CPG functionalized with the ESE linker from commercial sources. In addition, we have

Table 1
Oligonucleotide prepared in this work.

#	Sequence (5′->3′)	Solid support	MS (expected)	MS (found)
A	TCACGTp	**ESB**	1846.3	1845.2
A	TCACGTp	**ESE**	1846.3	1845.2
B	TTTATTTp	**ESB**	2155.3	2151.6
C	TTTCTTTp	**ESB**	2131.3	2127.6
D	TTTGTTTp	**ESB**	2171.3	2167.5
E	TGTTGGp	**ESB**	1917.3	1916.1
F	CAGTTGGp	**ESB**	2519.4	2519.7
G	CAGUUGGp	**ESB**	2299.3	2299.0

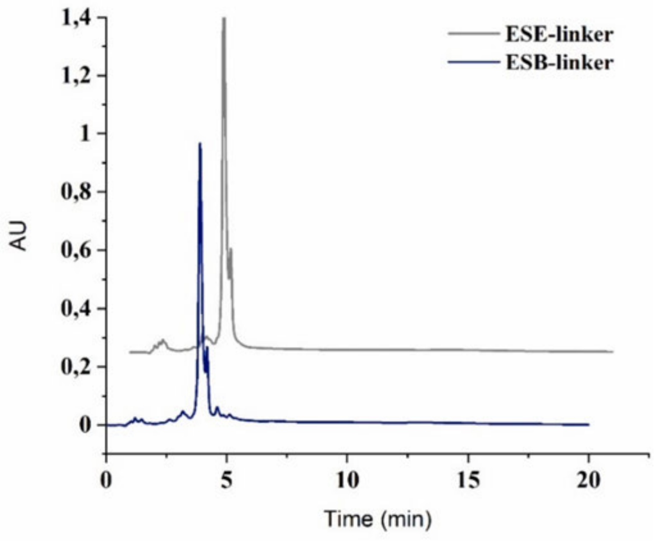
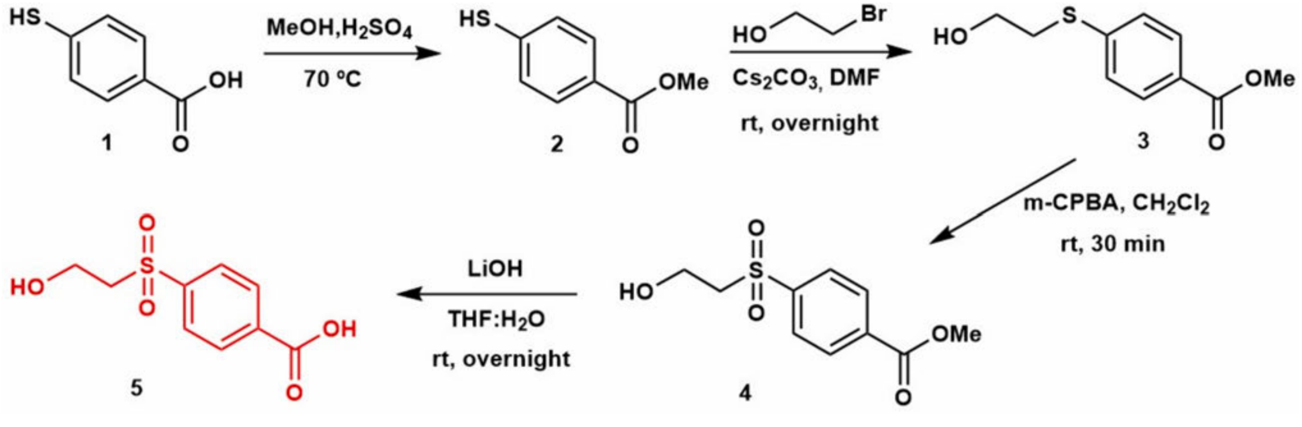


Fig 1. HPLC profiles of the 5′-TCACGTp-3′ oligonucleotide sequence prepared using the solid support functionalized with the ESE linker (grey) and the solid support functionalized with the ESB linker (blue).



Scheme 2. Synthesis of 4-((2-hydroxyethyl)sulfonyl)benzoic acid (**5**).

Table 2

Synthesis of partially-protected oligonucleotides using CPG solid supports functionalized with the ESB linker.

Sequence (5′->3′)	Formula	MS$_{expected}$	MS$_{found}$, DBU	MS$_{found}$, Et_3N	MS$_{found}$, Me_2NH
TTTA^{Bz}TTTp	$C_{77}H_{96}N_{17}O_{49}P_7$	2260.4	2308.5 (M + 2Na$^+$)	2361.2 (M + Et_3N) + 2414.2 and 2467.2 acrylonitrile adducts (M + Et_3N + 53)	2308.6 (M + 2Na$^+$)
TTTC^{Bz}TTTp	$C_{76}H_{96}N_{15}O_{50}P_7$	2236.4	2284.7 (M + 2Na$^+$)	2336.7 (M + Et_3N) + 2390.8 and 2444.0 acrylonitrile adducts (M + Et_3N + 53)	2285.1 (M + 2Na$^+$) 2180.3 (loss of the benzoyl group)
TTTG^{Ibu}TTTp	$C_{74}H_{98}N_{17}O_{50}P_7$	2242.4	2290.2 (M + 2Na$^+$)	2343.3 (M + Et_3N) + 2396.6 and 2449.4 acrylonitrile adducts (M + Et_3N + 53)	2290.2 (M + 2Na$^+$)
TG$_{Ibu}$TTG$_{Ibu}$G$_{Ibu}$p	$C_{72}H_{95}N_{21}O_{43}P_6$	2128.5	2179.1 (M + 2Na$^+$)	2179.0 (M + 2Na$^+$) + 2232.0 and 2285.0 acrylonitrile adducts (M + 2Na$^+$+53)	2179.1 (M + 2Na$^+$)
C$_{Bz}$A$_{Bz}$TG$_{Ibu}$TTG$_{Ibu}$G$_{Ibu}$p	$C_{105}H_{127}N_{29}O_{56}P_8$	2939.1	2990.8 (M + 2Na$^+$)	2991.0 (M + Et_3N) 3043.0 and 3094.0 acrylonitrile adducts (M + Et_3N + 53)	Not determined

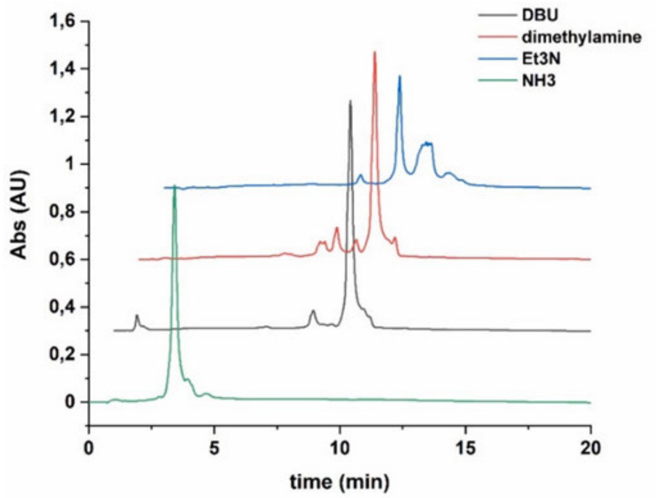


Fig 2. HPLC profiles of 5′-TGTTGG-3′ oligonucleotide E sequences prepared on a solid support functionalized with the ESB linker and treated with ammonium solution (green) and three different amines: DBU (black), Me_2NH (red) and Et_3N (blue). The profiles were obtained using a 0 to 50 %B gradient in 20 min.

prepared a short RNA sequence (sequence G) on the ESE linker support using cyanoethyl phosphoramidites protected with the *tert*-butyldimethylsilyl (TBDMS) at the 2′-position. After ammonia treatment, TBDMS groups were removed with triethylamine.HF in *N*-methylpyrrolidone and the resulting DMT-oligonucleotide was purified on RNA cartridge purification (Glen Research) following the instructions of the manufacturer giving the expected 3′-phosphate RNA oligonucleotide that was characterized by MALDI-Tof (Table 1, Figure S20).

Since the release of the oligonucleotides can be achieved under mild conditions using secondary and tertiary amines, we studied alternative methods to obtain partially-protected oligonucleotide derivatives. To this end, solid supports B-F were treated at room temperature with: i) 0.1 M DBU in acetonitrile for 15 min, ii) 40 % Me_2NH aqueous solution/ acetonitrile (1:3) and iii) 10 % Et_3N in acetonitrile for 2 h (Table 2). As the 2-cyanoethyl of the phosphate group is removed by β-elimination generating acrylonitrile, we added 7 mg of thymine to the triethylamine and DBU solutions to prevent alkylation of thymidine by acrylonitrile.[17,18] In all the cases, the deprotection solutions were concentrated and desalted with Sephadex G-25 prior HPLC analysis. Figure 2 shows the analytical HPLC of the treatment of the solid support carrying sequence E with ammonia, DBU, Et_3N and Me_2NH. The best conditions for the release of the partially-protected oligonucleotides from the ESB linker were found to be 0.1 M DBU in acetonitrile (Table 2). This is the shortest treatment (15 min) and also generates the cleanest compounds. The second optimal deprotection conditions were obtained with the use of Me_2NH solutions, which gave similar results to the DBU treatment, except for a partial release (around 30 %) of the benzoyl group of 2′- deoxycytidine (Table 2). Finally, the treatment with Et_3N solutions generated acrylonitrile adducts, as shown by the more hydrophobic impurities in the HPLC chromatograms and the presence of peaks at M + 53 and M + 106 (Table 2). These impurities are most probably generated during to the long treatment time (2 h) and cannot be avoided even with the use of thymine as a scavenger for acrylonitrile (see Figure S19).

In conclusion, we have described a novel route for the synthesis of a linker molecule for the preparation of oligonucleotide 3′-phosphates that is easy to prepare and compatible with the solid-phase phosphoramidite methodology. In addition, we have showed that under mild conditions it is possible to generate oligonucleotide 3′-phosphates carrying the benzoyl and isobutyryl groups in the nucleobases, opening up the possibility of using these linkers for the rapid preparation of protected DNA fragments.[19] To achieve this goal, phosphoramidites carrying a phosphate- protecting group different from the 2-cyanoethyl group are required.[20] Work in this direction is underway.

CRediT authorship contribution statement

Kwazi Masuku: Writing – original draft, Methodology, Investigation, Data curation. **Luis Miguel Menendez-M´ endez:** ´ Writing – review & editing, Investigation, Data curation. **Sikabwe Noki:** Writing – review & editing, Investigation, Data curation. **Beatriz G. de la Torre:** Data curation, Writing – review & editing, Supervision, Methodology. **Fernando Albericio:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Susana Fernandez:** ´ Writing – review & editing, Supervision, Methodology. **Miguel Ferrero:** Writing – review & editing, Supervision, Methodology. **Anna Avin˜o:** ´ Writing – review & editing, Supervision, Methodology. **Ramon Eritja:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization. **Carme Fabrega:** ` Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2024.129819.

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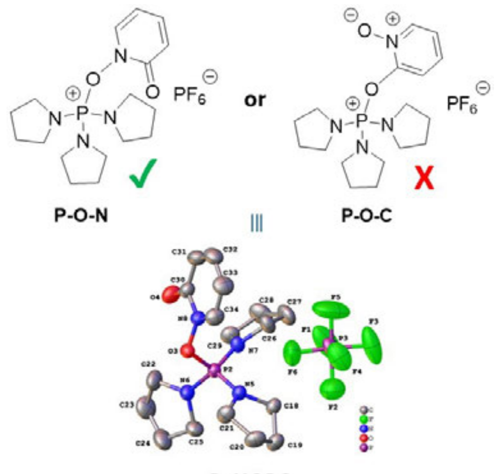
Graphical Abstract



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Abstract

In the current work, PyHOPO was synthesized and well characterized by single ray XRD. The crystal structure unambiguously confirmed the presence of a P-O-N bond rather than a P-O-C bond. The conversion of HOPO to PyHOPO elongates the N-O bond length thereby changing the electron density distribution, as evidenced by electrostatic mapping. This highlights the phosphorus atom's electron-donating nature toward the HOPO moiety. PyHOPO was used to prepare pentapeptide H-Tyr-Ser-Ser-Phe-Leu-NH_2. PyHOPO was then evaluated for racemization study. Racemization was studied in the case of Ser, His and Cys upon incorporation in a tripeptide (Gly-X-Phe-NH_2; where X = Ser, His, Cys). For the study, it was found that there was no racemization in all the three cases when PyHOPO was used as coupling reagent.

Keywords:

PyHOPO, X-ray crystallography, P-O-N bond formation, electrostatic potential mapping, pentapeptide, PyBOP, PyOxim, racemization, SPPS

Highlights:

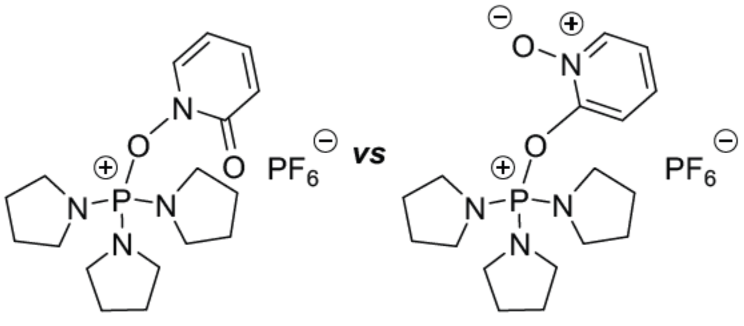
- Synthesis of PyHOPO with good yield.
- Confirmation of P-O-N bond formation instead of P-O-C by X-ray crystallography.
- Formation of PyHOPO shifts the electron density distribution of HOPO when alone.

- Successful synthesis of H-Tyr-Ser-Ser-Phe-Leu-NH_2.
- No racemization found in any case of tripeptide H-Gly-X-Phe-NH_2 (where X = Ser, His, Cys).

Introduction

Amide bond forms the core of the living system which eventually forms proteins/peptides. Peptides currently also find applications as drugs. FDA approval of peptides in the last few decades has increased enormously[1] . This led to the development of synthetic strategy for their synthesis[2] .The most crucial is the formation of amide bond[3] . The most common practical approach to form amide/peptide bond is either carbodiimide mediated coupling in the presence of a coupling additive to reduce the reactivity of O-acylisourea and therefore minimize side-reactions or use of stand-alone coupling reagents like uronium/iminium or phosphonium salts wherein the additives are already incorporated in the coupling reagent[3] . The most common additives used are N-OH derivatives like 1-hydroxy-7azabenzotriazole (HOAt), 1-hydroxybenzotriazole (HOBt), 6-chloro-1-hydroxybenzotriazole (Cl-HOBt), ethyl cyano(hydroxyimino)acetate (OxymaPure), 5-(hydroxyimino)-1,3dimethylpyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (Oxyma-B), *N*-hydroxysuccinimide (HOSu), 2hydroxypyridine-*N*-oxide (HOPO), etc. Benzotriazole derivatives remain the benchmark of the previous century[3] . However, due to their explosive nature they were substituted with milder and safer OxymaPure[4] .

Although OxymaPure is the most common coupling additive used worldwide, the choice of the coupling additive depends on the application and even the researcher or laboratory running the experiment. In this context, having more options is beneficial. For example, Oxyma-B is less reactive than OxymaPure, but renders a reduction of racemization[5] . HOSu is probably the less reactive of the most used coupling additives and for this reason is used with unprotected side chain in amino acids[6] . On similar grounds, HOPO (2-pyridinol 1-oxide) is less reactive compared to OxymaPure and hydroxybenzotriazole, but finds significant industrial and also research applications in peptide synthesis[7] . With this in mind, we prepared phosphonium derivative of HOPO [PyHOPO, ((2-oxopyridin-1(2*H*)-yl)oxy)tri(pyrrolidin-1yl)phosphonium]. When phosphonium salts are used, the final active species is the ester of the pending additive. In the case of PyHOPO, this will be the active ester of HOPO. Phosphonium salts have the advantage in front of aminium/uranium salts that they do not react with the *N*amino function, terminating the peptide chain, as the aminium/uronium do[8] . This is very beneficial for the cyclization steps and fragment coupling. As HOPO shows a bidentate structure, we were intrigued about if PyHOPO showed a N-O-P or C-O-P (**Figure 1**) similar as in the case of the aminium salt HBTU (*N*-[(1H-benzotriazol-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide), which shows an *N*-acyl structure instead of an *O*-acyl as it was expected[9] . In this context, PyHOPO was structurally characterized using single X-ray crystallography, a model peptide was synthesized and racemization was studied using Cys and His model peptides.

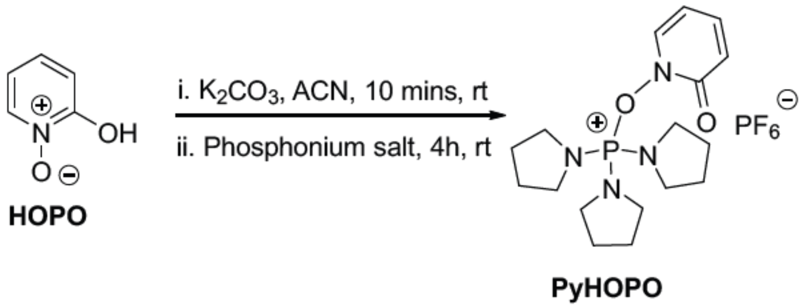


P-O-N **P-O-C**

Figure 1. Tentative structures for PyHOPO

Results and Discussion

Synthesis of PyHOPO was performed using HOPO wherein later was reacted with a base initially followed by reaction with chlorotripyrrolidinophosphonium hexafluorophosphate (PyClOP). The desired product was obtained as grey powder in excellent yield (94 %) and purity (>99%) as monitored by HPLC (*See Supporting Information,* ***Figure S1-S4***).



Scheme 1. Synthesis of PyHOPO

Crystal Structure of PyHOPO

The crystal structure of PyHOPO (*CCDC Number:* 2419347) consists of phosphonium cations and PF_6^- anions. Each cationic species constitutes of a HOPO moiety that is bonded to the phosphorus atom *via* the oxygen atom of the oxyamine functional group as shown in **Figure 2**. Moreover, the phosphorus atom adopts a distorted tetrahedral geometry with bond angles of 99.8(1)-115.1(1)°. The HOPO moiety exhibits a near orthogonal orientation with respect to the P—O bond given P2—O3—N8—C30 and P1—O1—N4—C17 torsion angles of 91.3(3) and -98.7(3), respectively. The other intramolecular bond parameters appear to be comparable with those of closely related structures in literature.[10, 11]

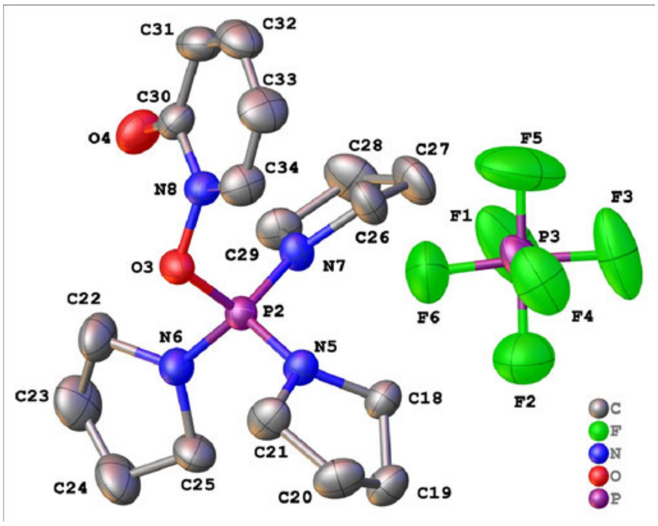


Figure 2. Molecule diagram of PyHOPO with ellipsoids drawn at 40% thermal probability. The disordered components and hydrogen atoms have been omitted for clarity.

Database survey

A search in the Cambridge Structural Database (CSD, Version 5.45, June 2024 update)[12] was done to find closely related phosphonium compounds with a substructure shown in **Figure 3**.

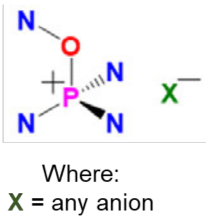


Figure 3. Representation of the substructure query used.

Only two search hits were obtained, (benzotriazole-1-yloxy)-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) (CSD ref-code: AKESAK) and tris(pyrrolidin-1-yl)[(1*H*-[1,2,3]triazolo[4,5-b]pyridin-1-yl)oxy]phosphonium hexafluorophosphate (PyAOP) (CSD ref code: MUZRUX). The latter consists of 1H[1,2,3]triazolo[4,5-b]pyridin-1-ol moiety instead of HOPO (in PyHOPO) while the former has a benzotriazole-1-yloxy and tris(dimethylamino) moieties (**Figure 4**). We extended our database survey to the hydroxyamine precursors (or additives) that were used to synthesize the phosphonium derivatives. The motivation for doing so was to investigate whether the additives undergo any structural changes after it couples with the phosphorus atom. In all the phosphonium compounds, the N—O bond distance is elongated from 1.357(2)-1.384(2) to 1.365(9)-1.404(3) (**Table 1**). This suggests that the conversion to phosphonium compounds alters the electron density distribution around additives.

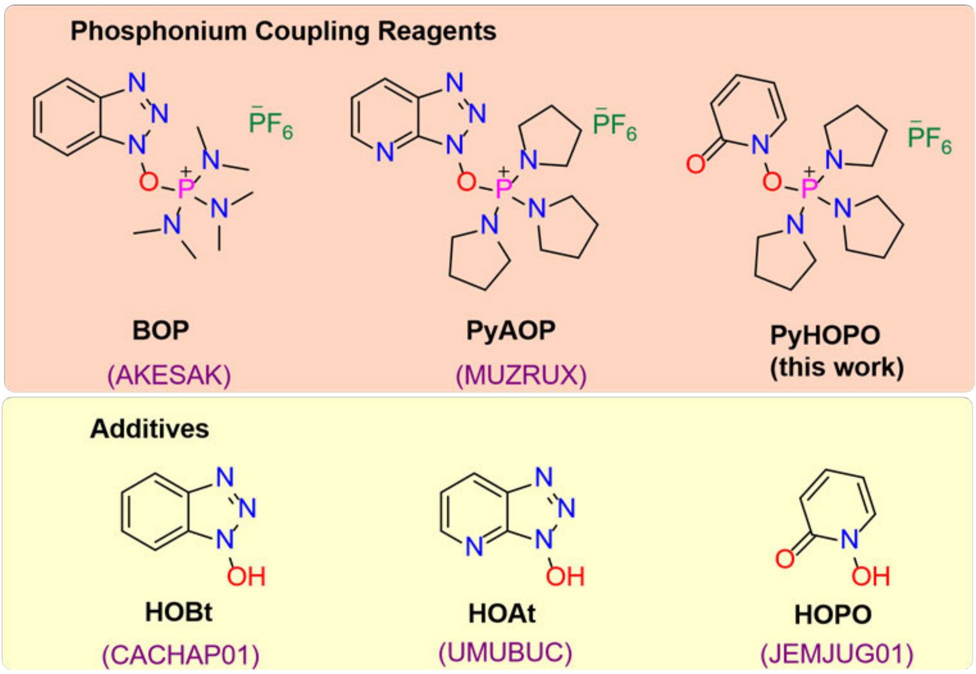


Figure 4. Structures of related phosphonium compounds and their associated additives found on the CSD. The CSD ref-codes are in purple-coloured text.

Table 1. Selected bond parameters of phosphonium derivatives and their respective additive precursors.

Compound	**N—O (Å)**	**P—O—N (°)**
Additives		
HOBt	1.357(2)	-
HOAt	1.358(3)	-
HOPO	1.384(2)	-
Phosphonium derivatives		
BOP	1.381(2)	113.0(1)
PyAOP	1.365(9)	115.4(5)
PyHOPO (this work)	1.403(4)-1.404(3)	115.7(2)-116.9(2)

Molecular electrostatic potential mapping

Figure 5 depicts the molecular electrostatic potential (MEP) mapped over the Hirshfeld surfaces of three additives (HOBt, HOAt and HOPO) and three corresponding phosphonium derivatives (BOP, PyAOP and PyHOPO). This comparison is essential for understanding the changes in electron distribution upon conversion of the additives into their respective phosphonium coupling reagents. The MEP maps of the additives reveal regions of distinct electrostatic potential. The blue regions, indicative of electropositive areas, are localized primarily around the hydrogen atoms and the aromatic rings. In contrast, the red regions, signifying electronegativity, are primarily associated with the oxygen nitrogen atoms present within these molecules. Upon formation of the phosphonium derivatives, notable changes in the MEP surfaces are observed. The once electropositive (blue) aromatic rings of the additives now appear to adopt an electronegative character. The shift in MEP patterns from the precursors to the phosphonium derivatives implies that the conversion to phosphonium alters the electron density distribution significantly. This also suggests that the phosphorus atom donates its electrons to the additives of their respective phosphonium compounds.

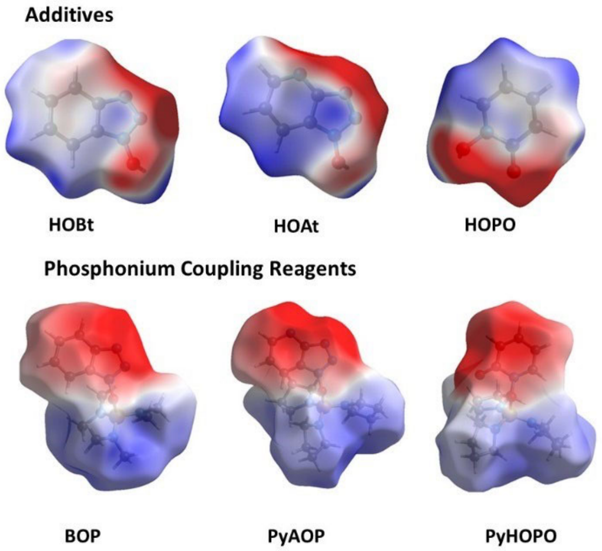


Figure 5. Molecular electrostatic potential mapped over the Hirshfeld surfaces of phosphonium derivatives and their associated additives.

SPPS of Fmoc-Tyr-Ser-Ser-Phe-Leu-NH_2 using PyHOPO and PyBOP

To understand the applicability of PyHOPO as coupling reagent, pentapeptide (FmocTyr-Ser-Ser-Phe-Leu-NH_2) was synthesized and compared with the peptide synthesized using PyBOP (*See Supporting Information, **Scheme S1***). The HPLC analysis of the two synthesis (*See Supporting Information*, **Figure S1**) shows that the synthesis of pentapeptide was efficient and quite comparable in both cases, although the purity of the peptide synthesized with PyBOP was superior to the one synthesized with PyHOPO as expected.

SPPS of H-Gly-X-Phe-NH_2 where X = Ser, His, Cys, to study racemization

Followed by these promising results, SPPS was performed to synthesize tripeptides (HGly-X-Phe-NH_2 where X = Ser, His, Cys). Three coupling reagents PyHOPO, PyBOP and PyOxim were evaluated to understand the effect of racemization. Two approaches for activation were adopted for the synthesis, i.e., *via* pre-activation or *in-situ* activation (*See Supporting Information, **Scheme S2***).

Racemization was studied initially for the synthesis of H-Gly-Ser-Phe-NH_2. In this case the tripeptide was synthesized by PyHOPO as coupling reagent using two approaches. In one case, pre-activation was performed wherein, Fmoc-Phe-OH-PyHOPO-DIEA (1:1:1; 3 eq) in DMF were mixed and pre-activated for 3 mins and then the coupling cocktail was added to the resin. In both cases, the coupling was allowed to take place for 1 h at rt. Fmoc was then removed and Fmoc-Ser(*t*Bu)-OH was coupled using both approaches as explained earlier. In this way, tripeptide H-Gly-Ser-Phe-NH_2 was synthesized. The synthesis was repeated replacing PyHOPO with PyBOP and PyOxim for comparison. In parallel, H-Gly-D-Ser-Phe-NH_2 was also synthesized to determine the amount of racemization in each case. It can be seen from **Table 1** that racemization was not found in any of case for H-Gly-Ser-Phe-NH_2.

Table 1. Analysis of HPLC for determination of percentage of racemization in tripeptides.

Peptide (HPLC method)	**Coupling Method**	**PyHOPO**	**PyBOP**	**PyOxim**
H-Gly-Ser-Phe-NH_2 (0-30)*	Preactivation**	-	-	-
	In-Situ activation	-	-	-
H-Gly-His-Phe-NH_2 (5-20)*	Preactivation**	-	0.4	2.9
	In-Situ activation	-	1.0	3.0
H-Gly-Cys-Phe-NH_2 (0-40)*	Preactivation**	-	0.8	-
	In-Situ actovation	-	0.8	-

* HPLC ACN (0.1% TFA) - H_2O (0.1% TFA) over 15 min; ** 3 min preactivation

Further, tripeptide H-Gly-His-Phe-NH_2 and H-Gly-Cys-Phe-NH_2 containing Cys and His (amino acids more prone to racemization) were synthesized using the above two protocol of pre-activation and *in-situ* activation. It is evident that no racemization was observed in case of PyHOPO in both the tripeptides containing His and Cys. Peptide synthesized using PyBOP showed racemization in both cases of Cys and His whereas PyOxim lead to racemization in case of His but not in the case of Cys (**Table 1**).

Conclusion

In conclusion, PyHOPO was synthesized in good yield and well characterized by Mass and NMR. Further, X-ray crystallography confirms the formation of P-O-N instead of P-O-C bond. Formation of PyHOPO results in slight elongation of N-O bond length. This could be a contributing factor in altering the electronic distribution of PyHOPO when compared to HOPO alone. The phosphorous atom donates the electron density towards HOPO. After successful confirmation of the structure, PyHOPO was used to synthesize H-Tyr-Ser-Ser-Phe-Leu-NH_2. The results were compared to that of synthesis performed using PyBOP as coupling reagent. The results were comparable in both cases. Following this, racemization study was performed using Ser, His and Cys when present in a tripeptide H-Gly-X-Phe-NH_2 (where X = Ser, His and

Cys). For comparison, PyOxim, PyBOP were also used in parallel to carry out the synthesis. It was found that there was no racemization in any case when PyHOPO was used as coupling reagent.

Acknowledgments: We thank Mr. Yoav Luxembourg from Luxembourg Bio Technologies for encouragement this work and provide free samples of coupling reagents.

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Conclusion

In conclusion, we have demonstrated the successful synthesis of a base labile linker and its application for the synthesis of the desired 3' phosphate oligonucleotide in excellent yield. The linker was compactible with phosphoramidite chemistry. Regarding the second part of my research associated peptide synthesis, we managed to synthesize PyHOPO from HOPO additive with high purity and excellent yield (**94%**). An advantage PyHOPO is that its synthesis is just a two-step process and only takes 4 hours to prepare. XRD enabled us to confirm the structure of PyHOPO coupling reagent, which was later applied in SPPS, and racemization study was conducted successfully. The use of PyHOPO was not associated with any racemization in the synthesis of several model peptides. Furthermore, the resolution of the crystal structure has allowed to find out that PyHOPO has a P-O-N atom connectivity and not a P-O-C one. Finally, this work has showed once again the importance of the solid phase approach for the synthesis of both oligonucleotides and peptides. In future this linker could be used for rapid preparation of protected DNA fragments however, this would be achieved by having a phosphoramidites carrying a phosphate protecting group, different from the 2-cyanoethyl group.

Appendix

Supplementary information

The synthesis of solid supports carrying base labile linkers to generate 3’-phosphate oligonucleotides.

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[#]Both authors have contributed equally to this work

1- Material and Methods

Standard phosphoramidites, the DMT-phosphate-ESE linker-CPG (Chemical phosphorylating reagent 3’-CPR-CPG) and solvents for oligonucleotide synthesis were purchased from Applied

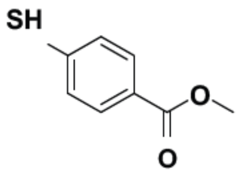
Biosystems (USA) and LGC Biosearch Technologies. NAP-10 column (GE Healthcare, UK)). All other common chemical reagents were purchased from Sigma-Aldrich (Spain). Oligonucleotides were synthesized on an H-8 DNA synthesizer (K&A Laboratories, Germany). Analytical high-performance liquid chromatography was performed on a Xbridge^{TM} OST C18 2.5 µm column (Waters, Spain), with a linear gradient from 0% to 50% B for 20 min and a flow rate of 1 mL/min were used. NMR spectra were measured in a 600 MHz Bruker Biospin (USA). Mass spectra were recorded on a MALDI Voyager DE RP time-of-flight (TOF) spectrometer (Applied Biosystems). A Jasco 1500 spectrophotometer was used to quantify the oligonucleotides and to obtain the molecular absorption spectra between 220 and 320 nm.

2- Experimental

2.1. Preparation of the linker.

2.1.1. Preparation of methyl 4-mercaptobenzoate (2).

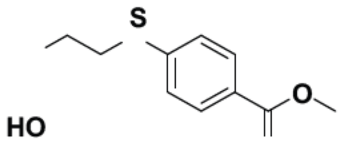
A mixture of 4 mercaptobenzoic acid (1) (5 g, 1 eq), H_2SO_4 (0.159 mL, 0.1 eq) in methanol (30 mL) was heated to 70°C with stirring for 2 hours, the reaction mixture was monitoring by TLC (SiO_2) (MeOH/EtOAc, 5:95). The reaction mixture was cooled to 0°C in ice bath, and then neutralized with $NaHCO_3$ to pH = 7. Solvent was concentrated to about 10 mL. Then DCM (30 mL) and water (20 mL) was added. The aqueous phase was extracted with DCM (20 mL x 3). The combined organics were dried over $MgSO_4$, filtered, and concentrated to render methyl 4-mercaptobenzoate (**2**) (4.7 g). (94% yield).



HPLC [5-95% of MeCN (0.1% TFA/ H_2O (0.1% TFA) over 15min] tR = 8.49 min; **^{1}H NMR** (600MHz, DMSO): δ=7.81 (d, *J*= 7.9 Hz, 2H; ArH), 7.43 (d, *J*= 7.8 Hz, 6.01 (s, 2H, SH), 3.83 (s, 3H, CH_3), **^{13}C{^{1}H}NMR** (150 MHz, DMSO-d$_6$): 166.3, 140.8, 130.5, 130.1, 128.3, 126.7, 126.3, 52.4.

2.1.2. Preparation of methyl 4-((2-hydroxyethyl) thio) benzoate (3).

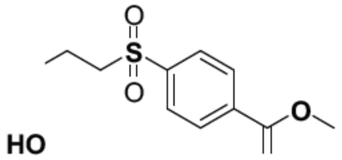
A mixture of methyl 4-mercaptobenzoate (**2**) (4.7 g), 2-bromoethanol (2.4 g) and Cs_2CO_3 (4 g) in N, N - dimethylformamide (DMF) (70 mL) was stirred at room temperature overnight. The mixture was filtered, and DCM (70 mL) was added. The solution was washed with water (50 mL x 5), brine (50 mL x 2), dried over $MgSO_4$ filtered, and concentrated. The crude product was purified by flash chromatography (silica gel; Hexane:Ethyl acetate = 20:1 to 2:1), to render methyl 4-((2-hydroxyethyl) thio) benzoate (**3)** (4,3 g) as a white solid. (91% yield).



HPLC [5-95% of MeCN (0.1% TFA/ H_2O (0.1% TFA) over 15min] tR = 7.07 min; **^{1}H NMR** (600MHz, DMSO): δ=7.85 (dd, *J*= 6.3 Hz, 2H; ArH), 7. (dd, *J*= 6.3 Hz, 2H, ArH), 5.04 (s, 1H, OH), 3.83 (s, 3H, CH_3), 3.62 (t, *J*= 5.1 Hz, 2H), 3.14 (m, *J*= 6.4 Hz, 2H) **^{13}C{^{1}H}NMR** (150 MHz, DMSO): 166.4, 144.6, 130.1, 126.5, 126.3, 60.1, 52.4, 34.2. **HRMS**: m/z: calcd. for $C_{10}H_{13}O_3S^+$: 213.0580 [M+H] $^+$; found: 213.0577.

2.1.3. Preparation of methyl 4-((2-hydroxyethyl) sulfonyl) benzoate (4).

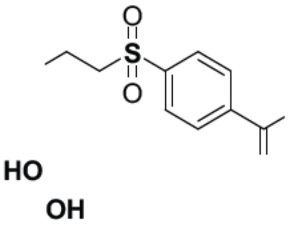
To a stirred solution of methyl 4-((2-hydroxyethyl) thio) benzoate (**3**) (4.3 g) in DCM (50 mL) at 0°C, m-chloroperoxybenzoic acid (7.1 g) in DCM (30 mL) was added dropwise upon which the mixture was allowed to warm to room temperature, and the reaction proceed until judge complete (30 min) by TLC. Upon completion, a saturated solution of $NaHCO_3$ was poured into the mixture followed by a solution of 0.1M ascorbic acid. The organic phase was washed with 1M $NaHCO_3$ (2x) then H_2O and dried over $MgSO_4$. The solvent was removed under reduced pressure to give methyl 4-((2-hydroxyethyl) sulfonyl) benzoate (**4**) (3.8 g, 88%).



HPLC [5-95% of MeCN (0.1% TFA/ H_2O (0.1% TFA) over 15min] tR = 5.26 min; **^{1}H NMR** (600MHz, DMSO): δ=8.17 (dd, *J*= 8.2 Hz, 2H; ArH), 8.06 (dd, *J*= 8.3 Hz, 2H, ArH), 4.91 (s, 1H, OH), 3.91 (s, 3H, CH_3), 3.71 (t, *J*= 5.3 Hz, 2H), 3.54 (t, *J*= 5.9 Hz, 2H) **^{13}C{^{1}H}NMR** (150 MHz, DMSO): 165.6, 144.5, 134.4, 130.3, 128.6, 54.9, 55.4, 53.1. **HRMS**: m/z: calcd. for $C_{10}H_{12}NaO_5S^+$: 267.0300 [M+Na] $^+$ found: 267.0305.

2.1.4. Preparation of 4-((2-hydroxyethyl) sulfonyl) benzoic acid (5).

Lithium hydroxide monohydrate (2 g) in water (70 mL) was added into a solution of methyl 4((2-hydroxyethyl) sulfonyl) benzoate (**4**) (3.8 g) in tetrahydrofuran (THF) (70.0 mL). The reaction mixture was stirred at room temperature overnight. Solvent was removed under reduced pressure, and the mixture was cooled in an ice bath and acidified to pH = 1-2 with conc. HCl. The aqueous phase was extracted with EtOAc (50 mL x 5). The combined organic phases were washed with brine (50 mL x 2), dried over $MgSO_4$, filtered, and concentrated, to render 4-((2-hydroxyethyl) sulfonyl) benzoic acid (**5**) with low yield.



HPLC [5-95% of MeCN (0.1% TFA/ H_2O (0.1% TFA) over 15min] tR = 3.83 min; **^{1}H NMR** (600MHz, DMSO): δ=13.5 (s, 1H, COOH), 8.15 (dd, *J*= 8.2 Hz, 2H; ArH), 8.02 (dd, *J*= 8.2 Hz, 2H, ArH), 4.89 (s, 1H, OH), 3.70 (t, *J*= 6.1 Hz, 2H, CH_2), 3.52 (t, *J*= 6.1 Hz, 2H, CH_2) **^{13}C{^{1}H}NMR** (150 MHz, DMSO): 166.7, 144.1, 135.8, 130.5, 128.5, 57.9, 55.4. **HRMS**: m/z: calcd. for $C_9H_9O_5S^-$: 229.0200 [M-H]$^-$ found: 228.9988.

2.2. Functionalization of amino-controlled-pore glass supports with 4-((2-hydroxyethyl)sulfonyl)benzoic acid.

To a solution of 4-((2-hydroxyethyl)sulfonyl)benzoic acid (23 mg, 0.1 mmol) in *N,N*dimethylformamide (DMF) (1 mL), 13. 5 mg (0.1 mmol) of 1-hydroxybenzotriazol (10% water) and 20.6 mg of dicyclohexylcarbodiimide (0.1 mmol) were added and the mixture was incubated at 0 ºC for 10 minutes. Then, the mixture was added to 0.4 g of amino-controlled pore glass (CPG) (0.1 mmol/gr, 0.04 mmol) and the mixture was left overnight at room temperature with occasional manual stirring. The suspension was filtrate and washed with DMF, CH_2Cl_2 and CH_3CN and dried over vacuum.

2.3. Oligonucleotide synthesis.

Oligonucleotide sequences A-F were assembled on the CPG solid supports functionalized with

4-((2-hydroxyethyl)sulfonyl)benzoic acid and DMT-2’-hydroxyethyl sulfoxide-2-hydroxyethyl hemisuccinate (DMT-Phosphate-CPG, LGC Biosearch Technologies). After the assembly of the oligonucleotides, the solid supports were treated with concentrated ammonia overnight at 50 ºC. The ammonia solutions were concentrated to dryness and desalted by NAP-10 columns (Sephadex G-25). Analysis by reversed-phase HPLC gave a major peak that coeluted. Mass spectrometry analysis of both products (MALDI-Tof) gave the expected molecular mass. MS (Maldi-Tof, negative): sequence A, expected for $C_{58}H_{76}N_{20}O_{38}P_6$ 1846.3 found 1845.2 ($M-H^+$); sequence B, expected for $C_{70}H_{92}N_{17}O_{48}P_7$ 2155.3 found 2151.6 ($M-H^+$); sequence C, expected for $C_{69}H_{92}N_{15}O_{49}P_7$ 2131.3 Found 1845.2 ($M-H^+$); sequence D, expected for $C_{70}H_{92}N_{17}O_{49}P_7$ 2171.3 found 2167.5 ($M-H^+$); sequence E, expected for $C_{60}H_{77}N_{21}O_{40}P_6$ 1917.3 found 1916.1 ($M-H^+$); sequence F: expected for $C_{79}H_{101}N_{29}O_{51}P_8$ 2519.4 found 2519.7 ($M-H^+$).

RNA sequence G was synthesized on 1-µmol scale using CPG solid support functionalized with 4-((2-hydroxyethyl)sulfonyl)benzoic acid. The oligonucleotide was synthesized on an H-8 DNA synthesizer (K&A Laboratories, Germany) using commercially available reagents and 2’-O-TBDMS-5’-O-DMT-protected phosphoramidites (A^{Bz}, G^{dmf}, C^{Ac} and U). We used 5-benzylthio1H-tetrazole (BTT) as coupling agent and the coupling time was 15 min. The last DMT was left on the 5’-end (DMT on mode). After the assembly of the RNA sequence the phosphate and base-protecting groups as well as the oligonucleotide-solid support linkage were removed by treatment of the support with ammonia/ethanol (1:1) at 55 ºC for 2 hours. The support was filtered and the solution was concentrated to dryness. The residue was dissolved in 115 µl of N-methylpyrrolidone, 75 µL of triethylamine-trishydrofluoride and 60 µL of triethylamine and incubated at 55 °C for 1.5 hours (to remove the 2’-O-silyl groups). The solution was cooled to room temperature and the resulting solution was purified on RNA cartridge purification (Glen Research) following the instructions of the manufacturer giving the expected 3’-phosphate RNA oligonucleotide that was characterized by MALDI-Tof. MS (Maldi-Tof, negative): sequence

G: expected for $C_{67}H_{84}N_{27}O_{51}P_7$ 2299.3 found 2299.0 ($M-H^+$).

2.4. Oligonucleotide deprotection to obtain based-protected oligonucleotides.

Oligonucleotide sequences B) TTTA^{bz}TTTp, C) TTTC^{bz}TTTp, D) TTTG^{ibu}TTTp, E) TG^{ibu}TTG^{ibu}G^{ibu}p, and F) A^{bz}C^{bz}TG^{ibu}TTG^{ibu}G^{ibu}p were assembled on the CPG solid supports functionalized with 4-

((2-hydroxyethyl)sulfonyl)benzoic acid. After the assembly of the oligonucleotides, the solid supports were treated with different basic solutions: a) concentrated ammonia solution for 4 hr at 55 ºC (Table 1); b) 0.1 M DBU (15 µl) in acetonitrile (ACN) in the presence of 7 mg of thymine for 15 min at rt; c) 40% dimethylamine aqueous solution in ACN (1:3) for 2 hr rt and d) 10% Et3N solution in (ACN) in the presence of 7 mg of thymine for 2 hr rt. Then, the resulting oligonucleotides solutions were filtered and were concentrated to dryness. In the case of the oligonucleotide solid support treated whit DBU and Et_3N the solutions were neutralized with 1 eq of acid acetic before concentration. The products were dissolved in 1 mL of water and desalted by NAP-10 columns (Sephadex G-25) using a solution of 20% acetonitrile in water. Then, the products were quantified and analysis by HPLC chromatography. The major products were characterized by Mass spectrometry analysis (Table 2).

NMR Spectra, HPLC Chromatograms and HRMS Spectra

Methyl 4-mercaptobenzoate (2).

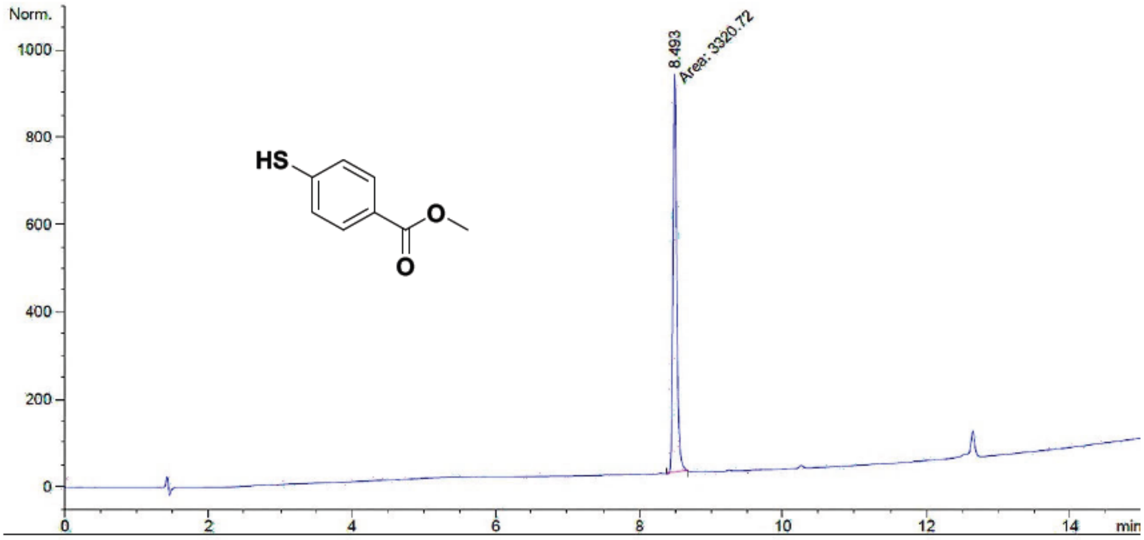


Figure S1. HPLC for methyl 4-mercaptobenzoate.

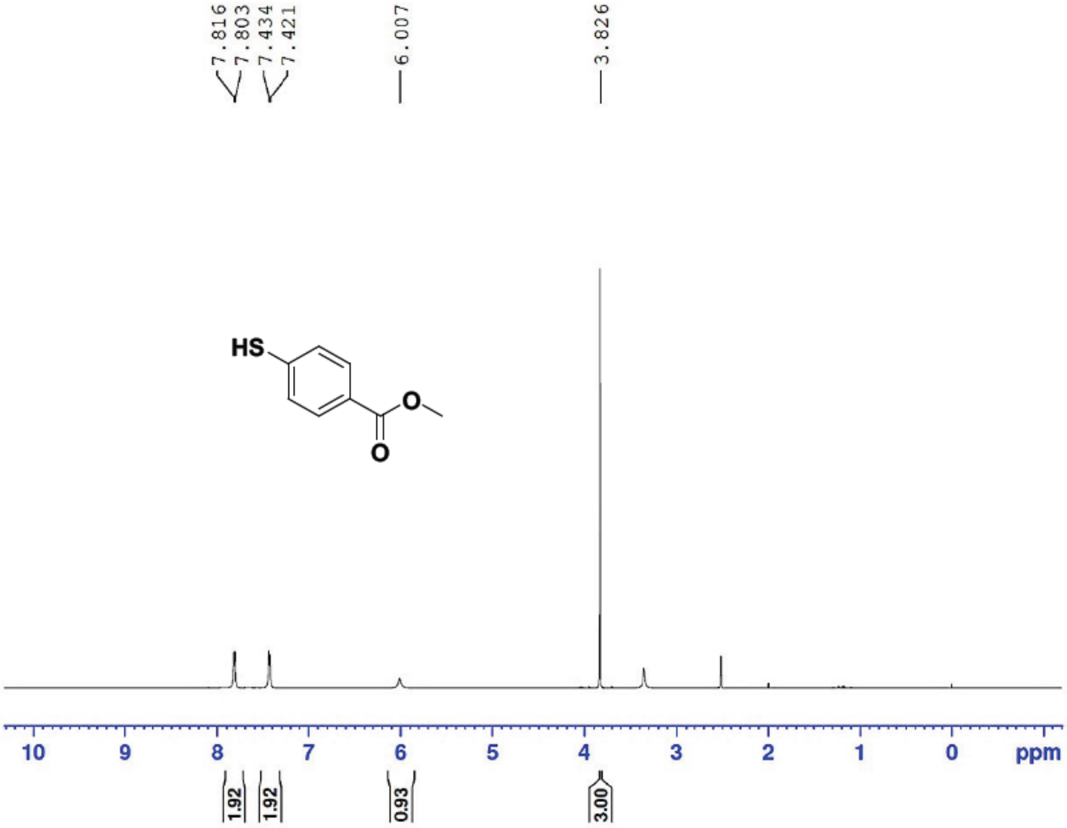


Figure S2. ^{1}H NMR, DMSO, 600 MHz for methyl 4-mercaptobenzoate.

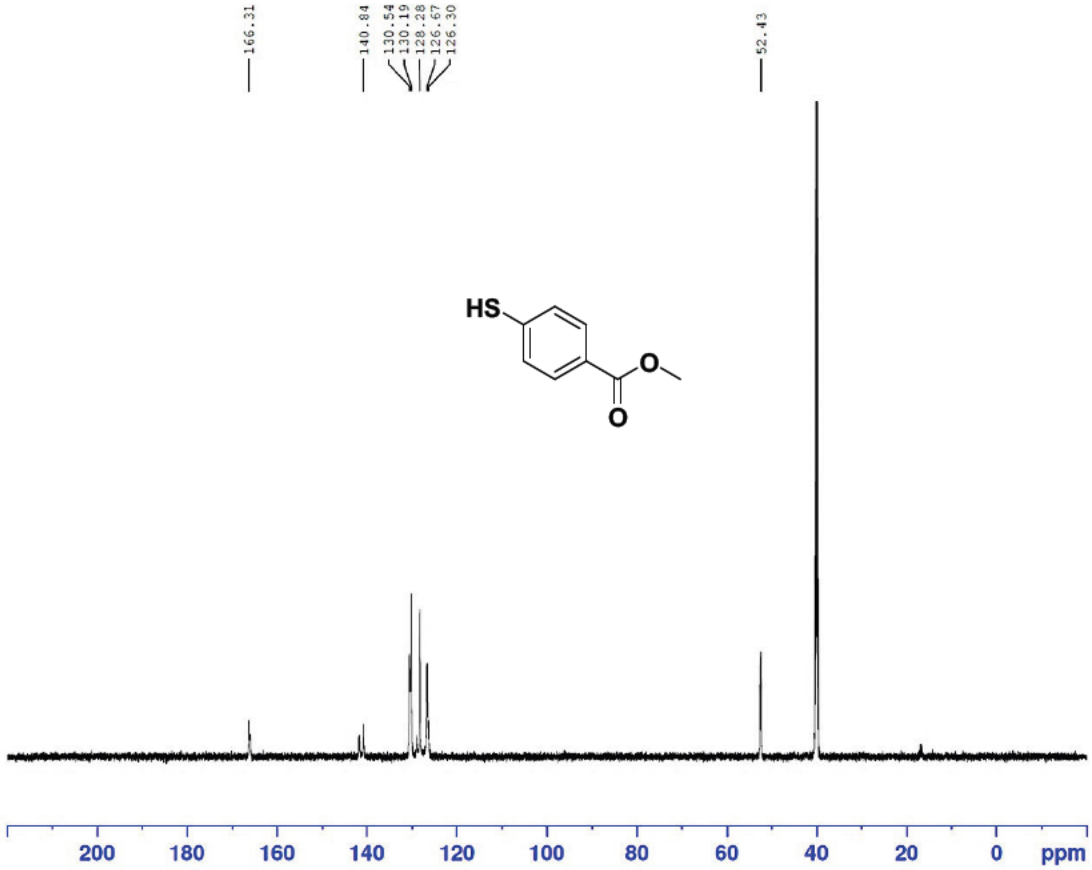


Figure S3. ^{13}C NMR, DMSO, 600 MHz for methyl 4-mercaptobenzoate.

Methyl 4-((2-hydroxyethyl) thio) benzoate (3).

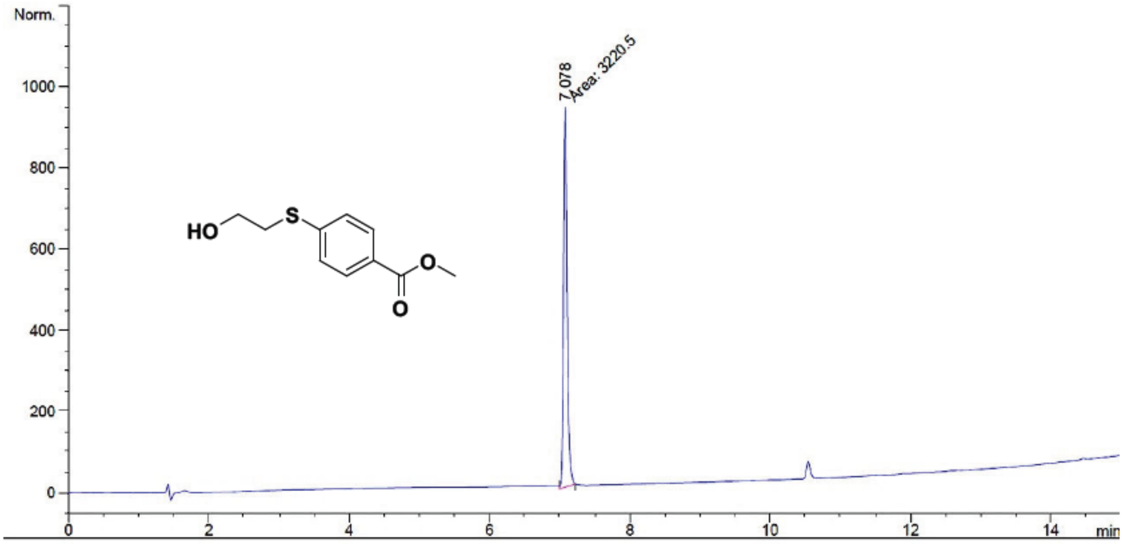


Figure S4. HPLC for methyl 4-((2-hydroxyethyl) thio) benzoate.

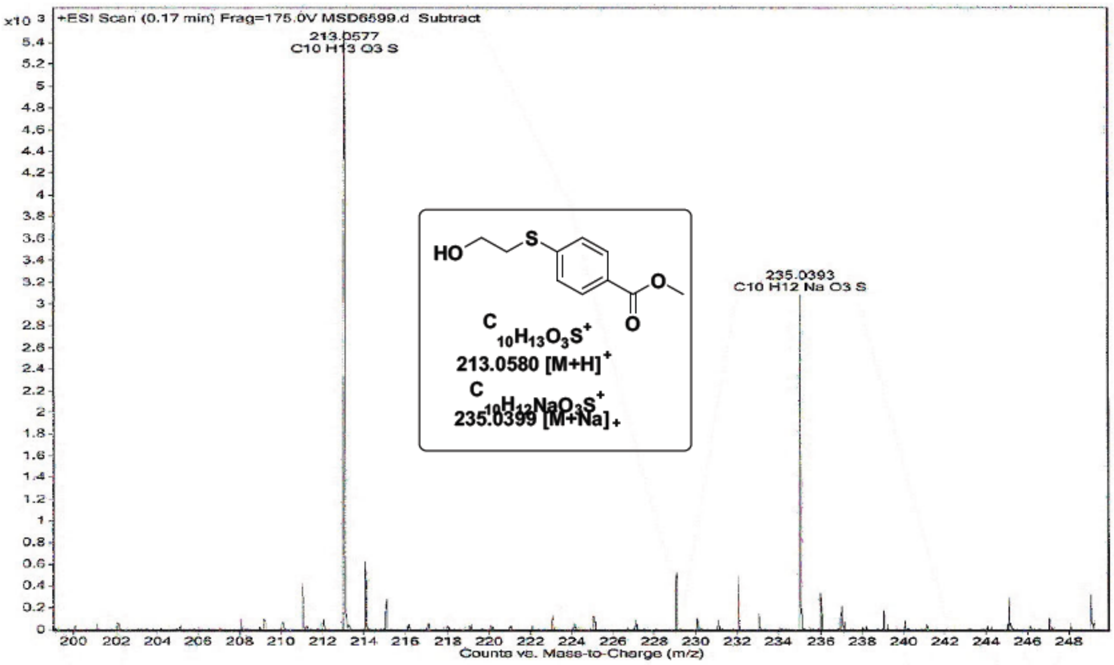


Figure S5. HRMS for methyl 4-((2-hydroxyethyl) thio) benzoate (**3)**

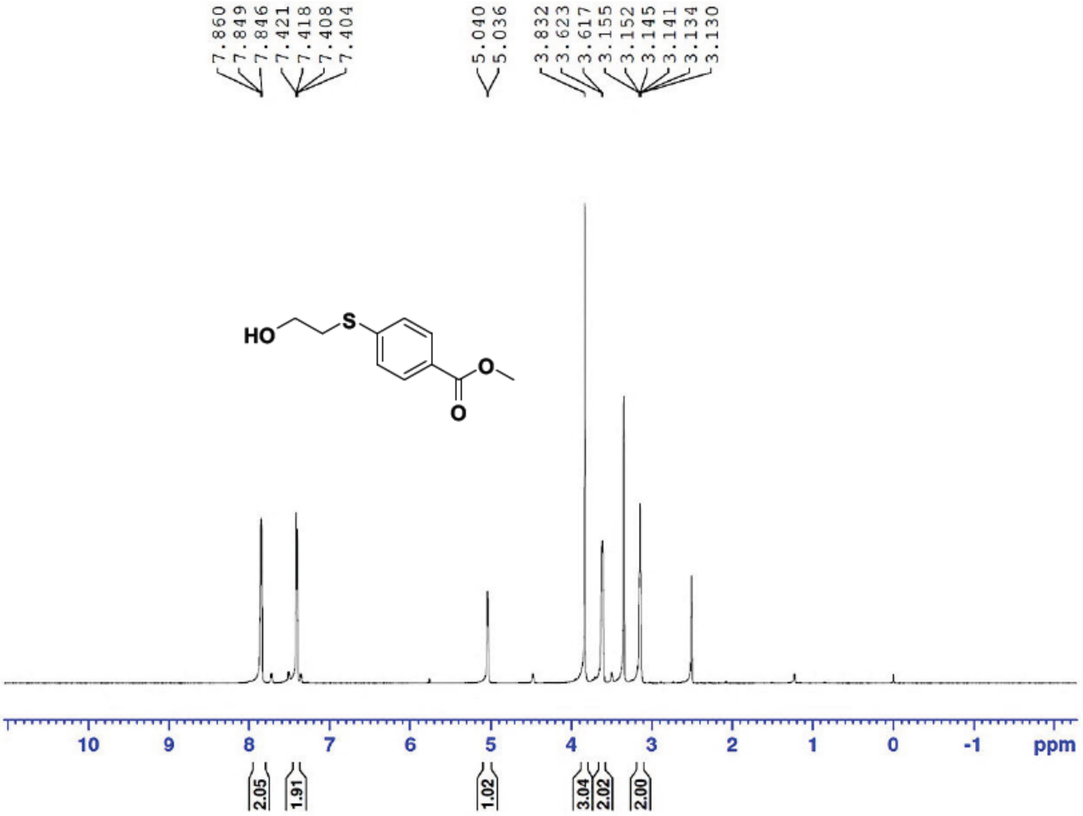


Figure S6. ^{1}H NMR, DMSO, 600 MHz for methyl 4-((2-hydroxyethyl) thio) benzoate.

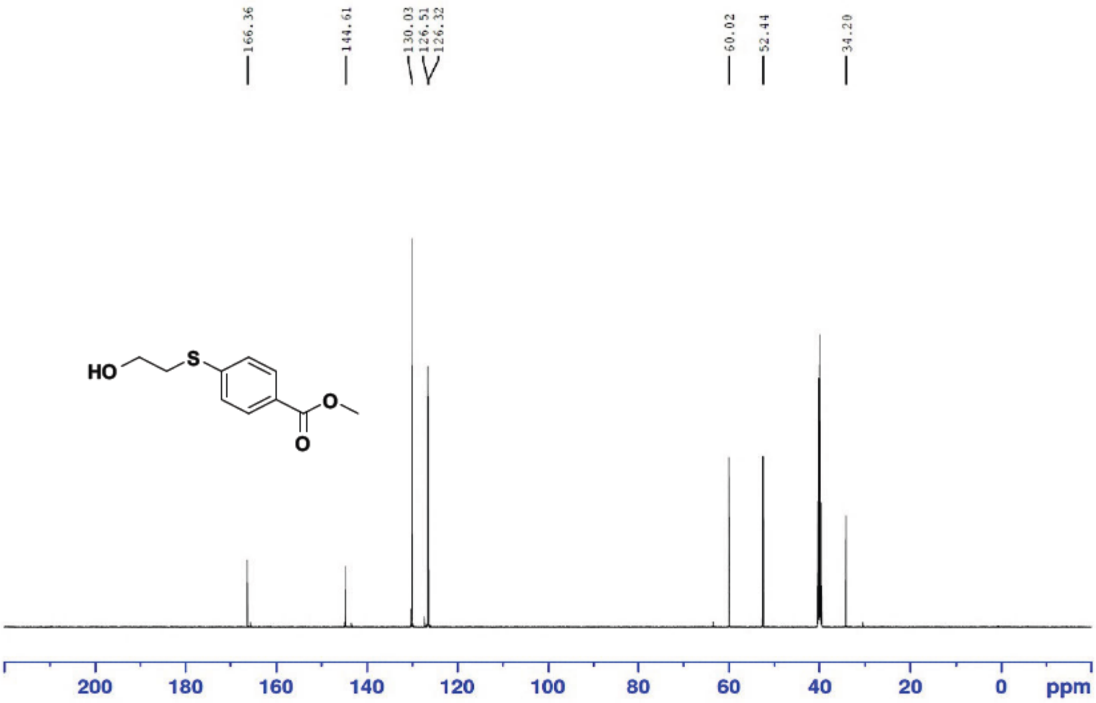


Figure S7. ^{13}C NMR, DMSO, 600 MHz for methyl 4-((2-hydroxyethyl) thio) benzoate.

Methyl 4-((2-hydroxyethyl) sulfonyl) benzoate (4).

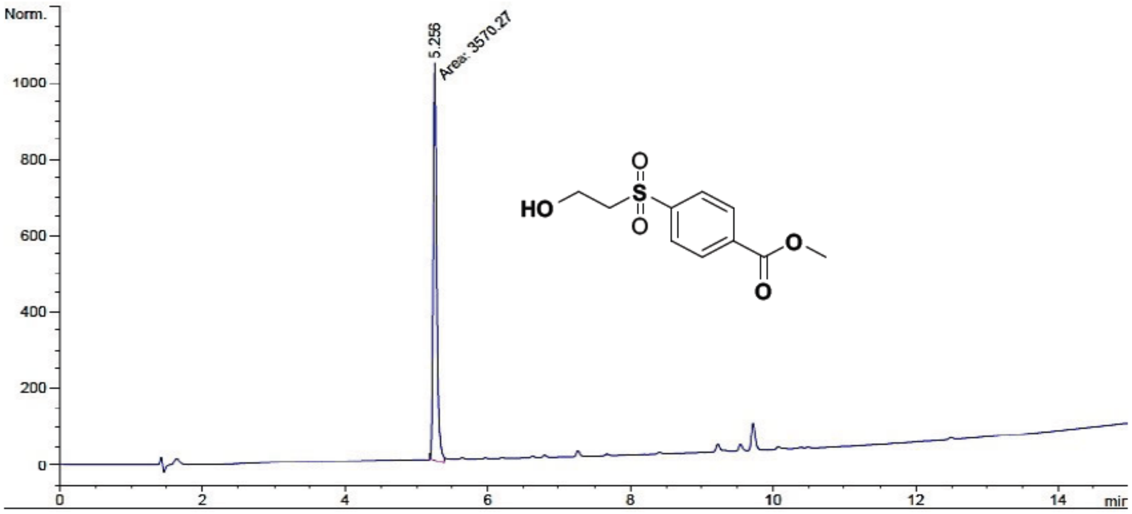


Figure S8. HPLC for methyl 4-((2-hydroxyethyl) sulfonyl) benzoate.

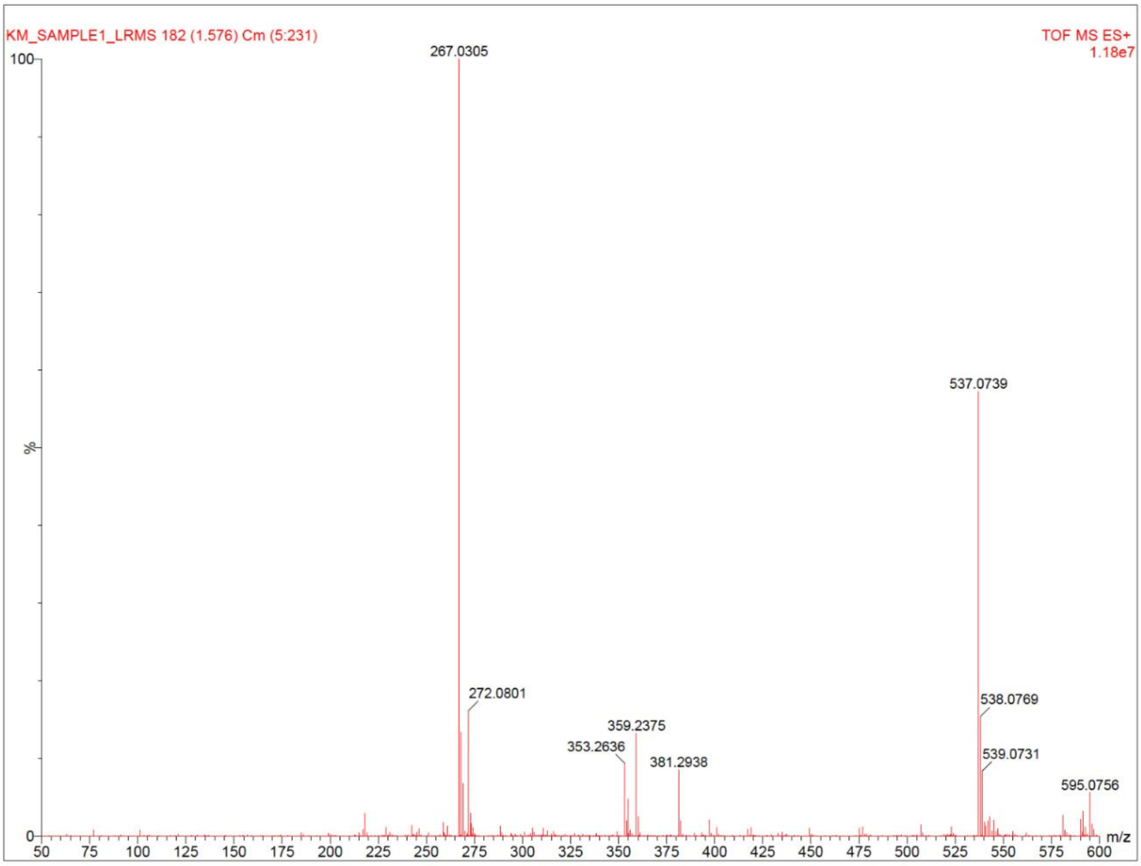


Figure S9. HRMS for methyl 4-((2-hydroxyethyl) sulfonyl) benzoate.

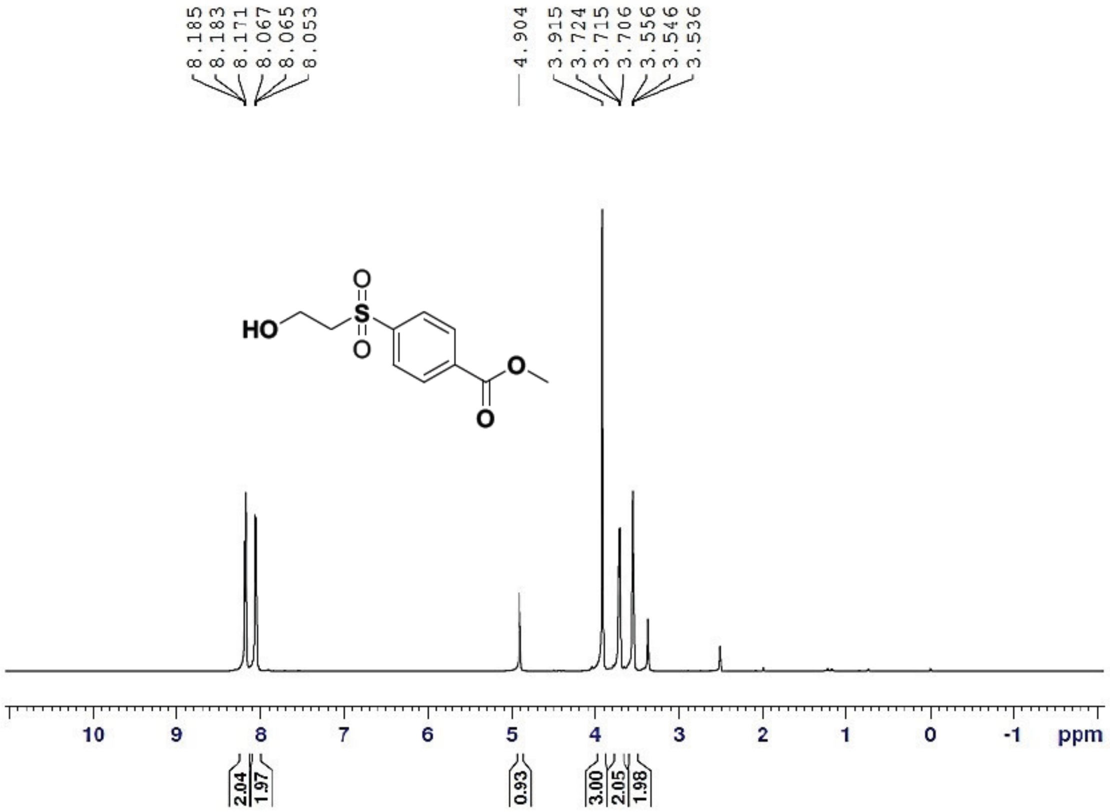


Figure S10. ^{1}H NMR, DMSO, 600 MHz for methyl 4-((2-hydroxyethyl) sulfonyl) benzoate.

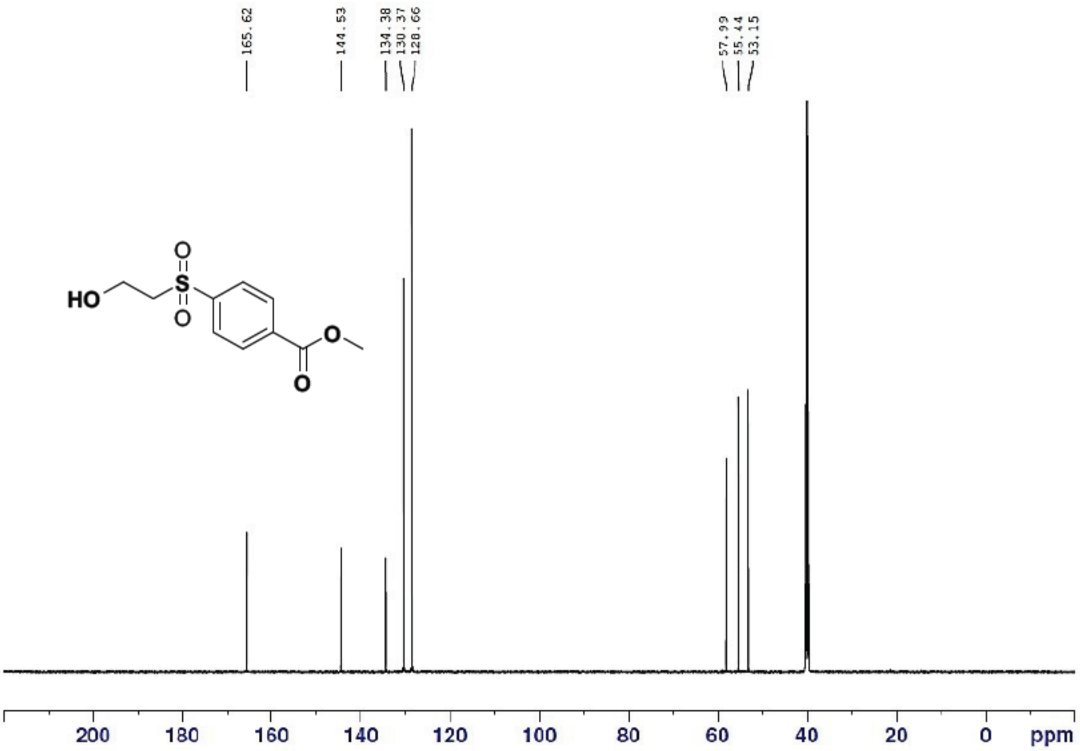


Figure S11. ^{13}C NMR, DMSO, 600 MHz for methyl 4-((2-hydroxyethyl) sulfonyl) benzoate. **4-((2-hydroxyethyl) sulfonyl) benzoic acid** (**5**).

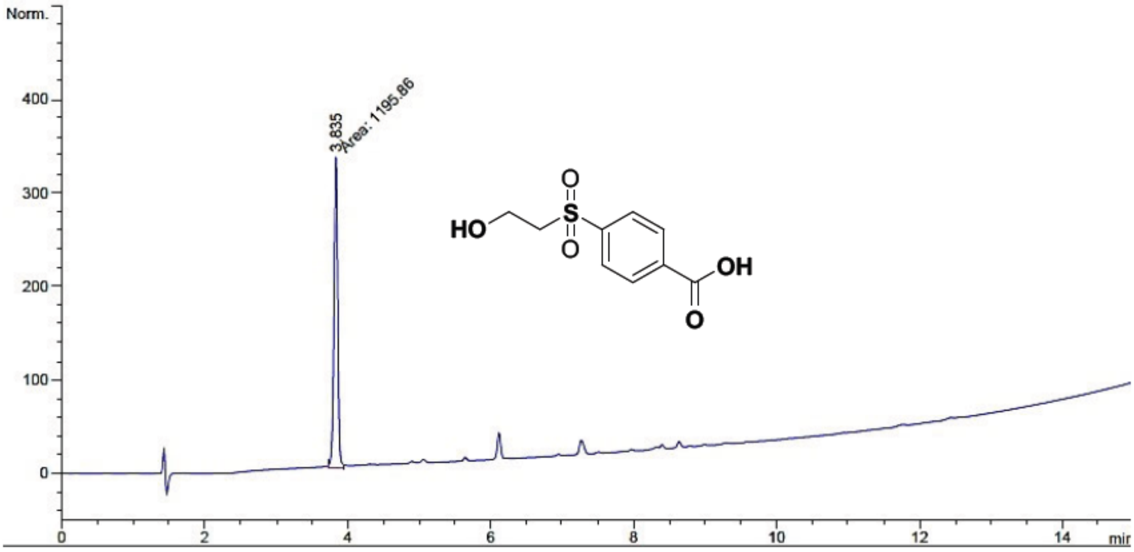


Figure S12. HPLC for 4-((2-hydroxyethyl) sulfonyl) benzoic acid.

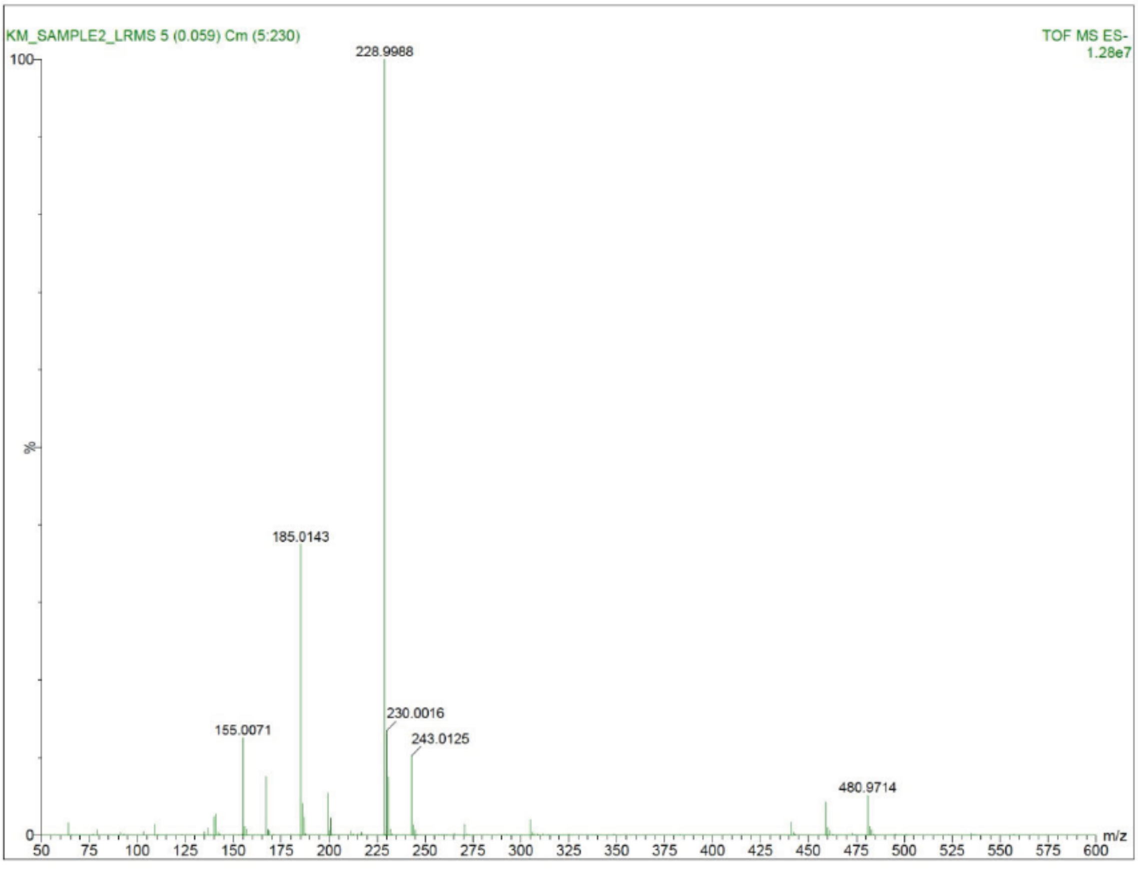


Figure S13. HRMS for 4-((2-hydroxyethyl) sulfonyl) benzoic acid.

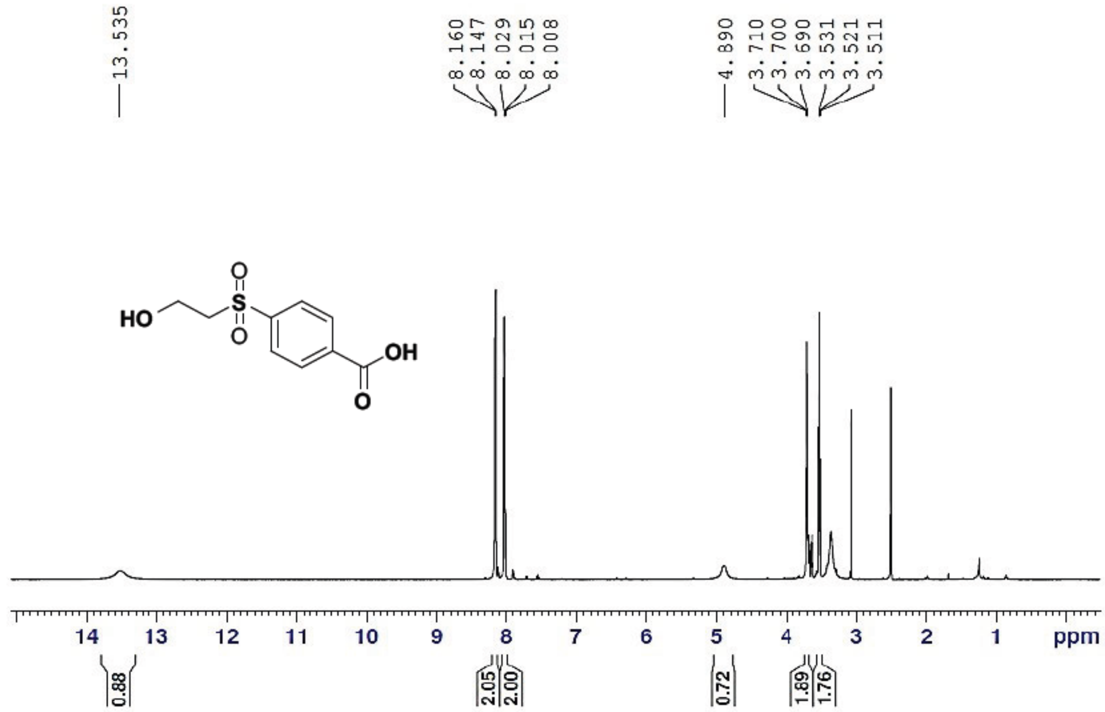


Figure S14. ^{1}H NMR, DMSO, 600 MHz for 4-((2-hydroxyethyl) sulfonyl) benzoic acid.

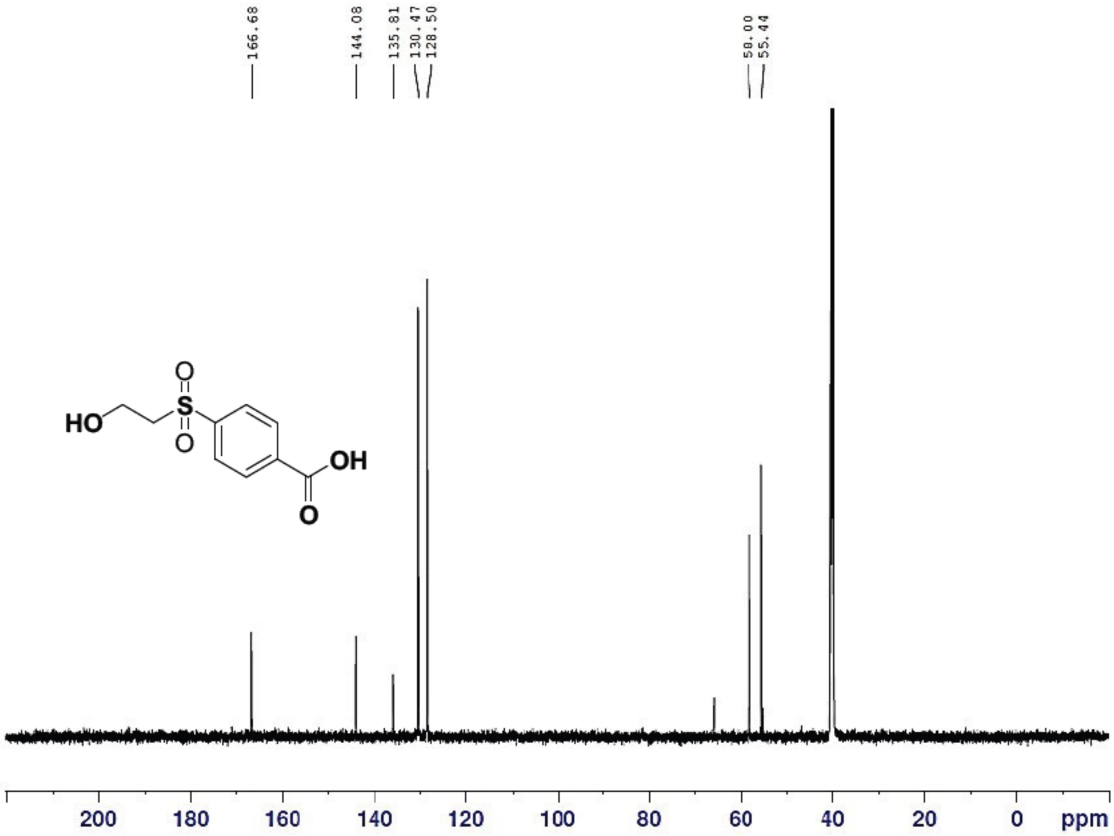


Figure S15. ^{13}C NMR, DMSO, 600 MHz for 4-((2-hydroxyethyl) sulfonyl) benzoic acid.

HPLC profiles of the different oligonucleotide sequences.

All the sequences were prepared using the solid support functionalized with the ESB linker and deprotected three different amines: DBU (black), Me_2NH (red) and Et_3N (blue). The profiles were obtained using a 0 to 50%B gradient in 20 minutes.

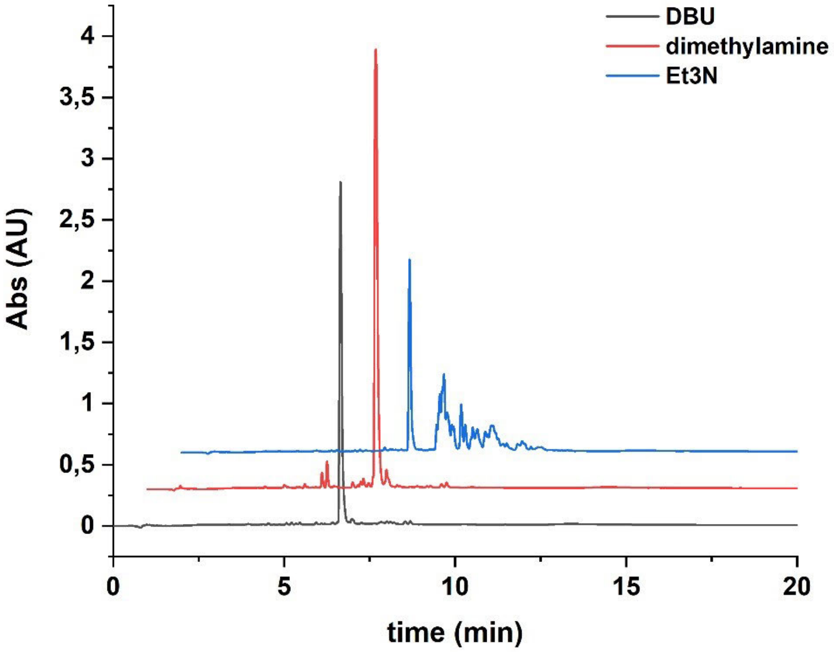


Figure S16. HPLC profiles of the TTTTATTT oligonucleotide sequences.

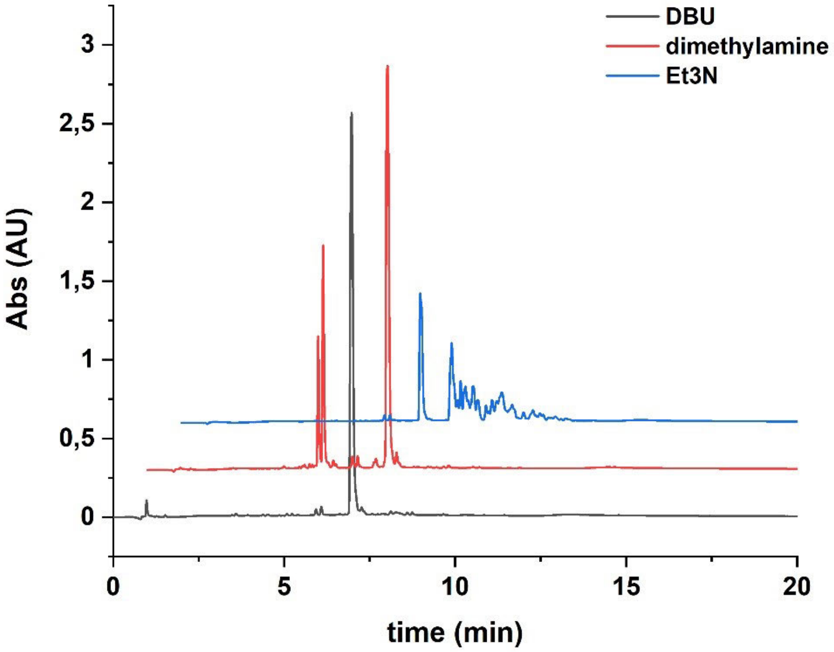


Figure S17. HPLC profiles of the TTTTCTTT oligonucleotide sequences.

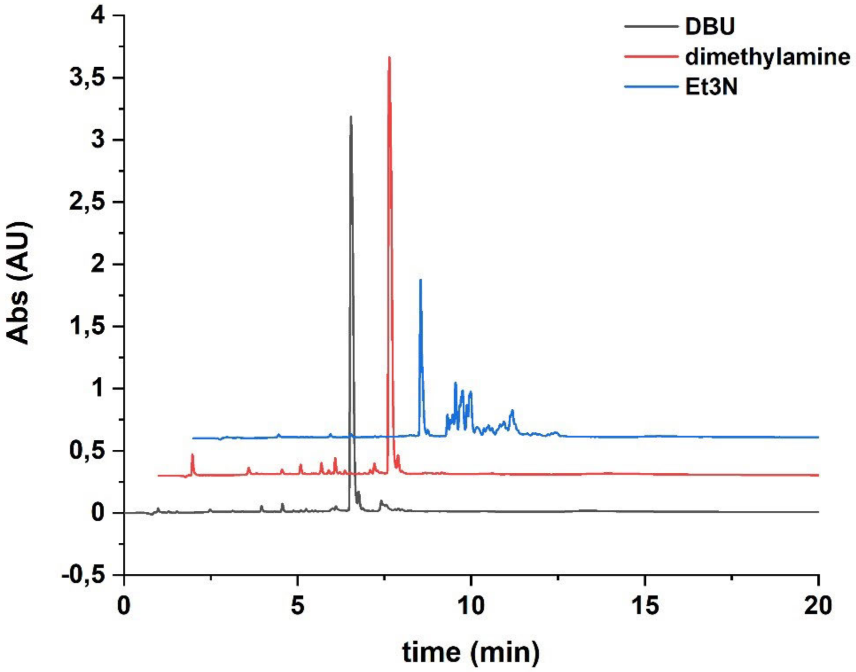


Figure S18. HPLC profiles of the TTTTGTTT oligonucleotide sequences.

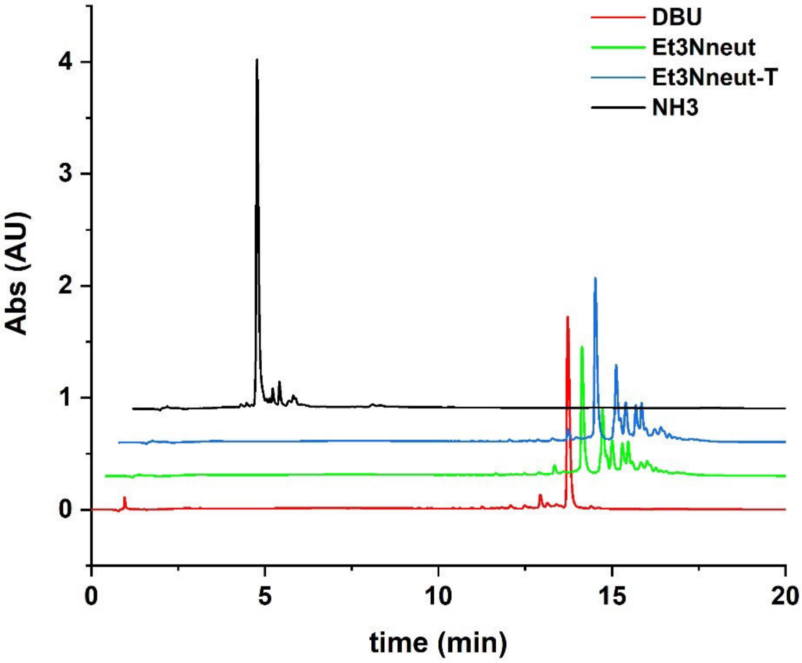


Figure S19. HPLC profiles of the CAGTTGG oligonucleotide sequences. The treatment with triethylamine has been done in the presence of thymine (Et_3Nnuet-T) and in the absence of thymine (Et_3Nneut) as scavenger of acrylonitrile. In both cases the triethylamine solution was neutralized (neut) with 1 eq of acetic acid before concentration of the sample to avoid the formation of acrylonitrile adducts.

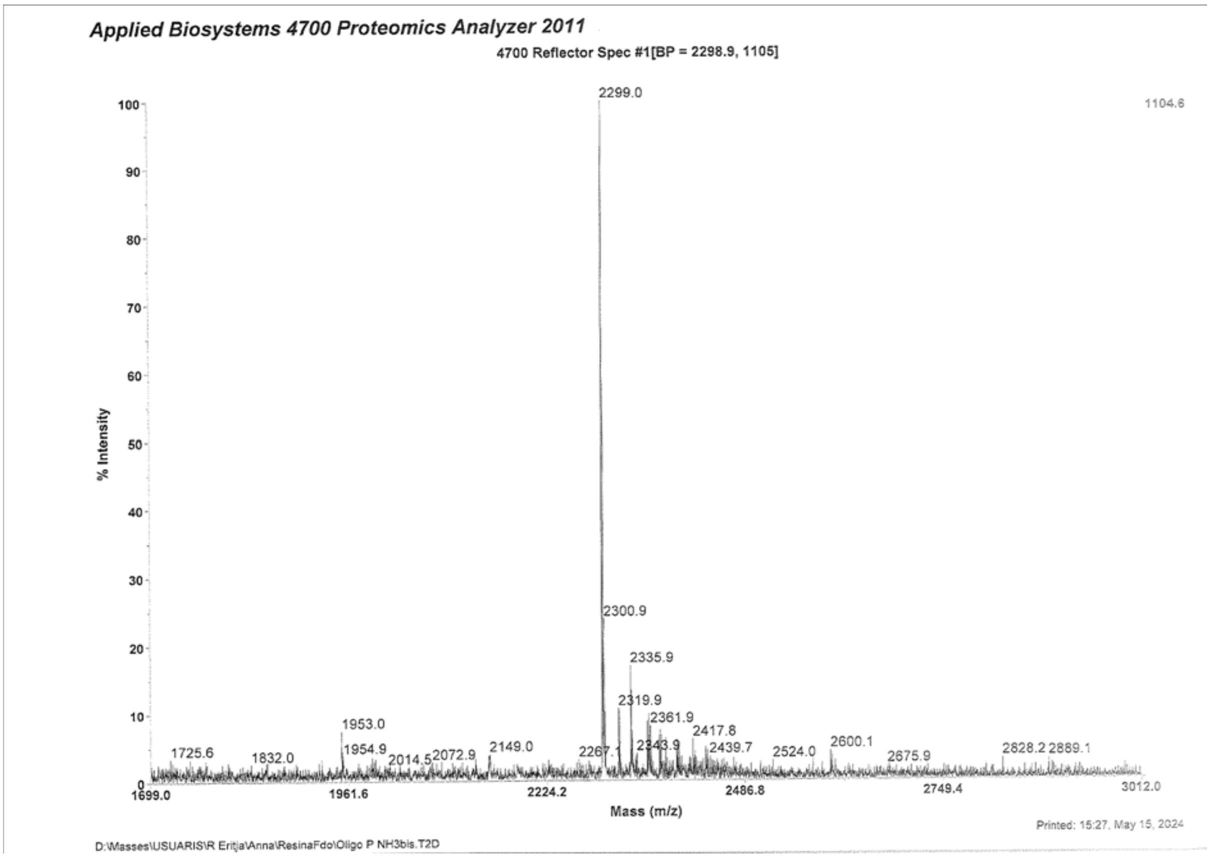


Figure S20. MS (MALDI-TOF) of 7mer RNA 3’-phosphate (sequence G).

Synthesis and Structural Confirmation of PyHOPO and its Applicability in Racemization Reduction in Solid-Phase Peptide Synthesis

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Experimental

General

All reagents and solvents were purchased from commercial suppliers and used without further purification. Fmoc amino acids, Fmoc-RinkAmide-AM-PS resin (loading 0.69 mmol/g), were purchased from Iris Biotech. HOPO, PyBOP and PyOxim were gifted from Luxembourg Biotech. Organic solvents, dimethylformamide (DMF) and HPLC quality acetonitrile (CH_3CN), were purchased from Merck. Milli-Q water was used for RP-HPLC. Analytical HPLC was performed on an Agilent 1100 system using a Phenomenex AerisTMC18 (3.6 µm, 4.6 × 150 mm) column, with a flow rate of 1.0 mL/min and UV detection at 220 nm. Chemstation software was used for data processing. Buffer A: 0.1% TFA in H_2O; buffer B: 0.1% TFA in CH_3CN. LC-MS was performed on a Thermo Fisher Scientific UltiMate 3000 UHPLC-ISQTM EC single quadrupole mass spectrometer in positive ion mode using a Phenomenex AerisTM C18 (3.6 µm, 4.6 × 150 mm) column. Buffer A: 0.1% formic acid in H_2O; buffer B: 0.1% formic acid in CH_3CN.

Synthesis of PyHOPO

In a round bottom flask, 2-hydroxypyridine-1-oxide (HOPO) (1.0 g, 9.0 mmol, 1eq.) was dissolved in ACN (20 mL). K_2CO_3 (0.6 g, 4.5 mmol, 0.5 eq.) was added, and the reaction was stirred for 10 mins at rt. Chlorotripyrrolidinophosphonium hexafluorophosphate (PyCLOP) (2.5 g, 9.0 mmol, 1 eq.) was added to the stirring reaction mixture and was allowed to react for 4 h at rt. The completion of the reaction was monitored by TLC using n-Hex:EtOAc (7:3). The reaction mixture was then filtered to remove unreacted K_2CO_3 and concentrated the filtrate under high vacuum. The residue was further washed with ACN (10 mL). The filtrate was concentrated as the residue was dissolved in around 10 mL of DCM:*n*-Hex (1:3) and placed the mixture in ice bath undisturbed for precipitation. After precipitation the residue was filtered and dried to afford PyHOPO in 94 % yield (2.97 g). **HPLC** [5-95% of ACN (0.1% TFA/ H_2O (0.1% TFA) over 15 min] t_R = 6.05 min; **^{1}H NMR** (600 MHz, $CDCl_3$): δ 7.70 (d, *J*= 7.4 Hz; ArH), 7.45-7.42 (m, ArH), 6.69 (d, *J*= 9.1 Hz, ArH), 6.47-6.45 (m, ArH), 3.40-3.38 (m, 12H), 2.00-1.95 (m, 12H), **^{13}C NMR** (150 MHz, $CDCl_3$): 157.5, 140.7, 136.1, 122.4, 107.3, 48.2, 26.1.

Physical Characterization of PyHOPO

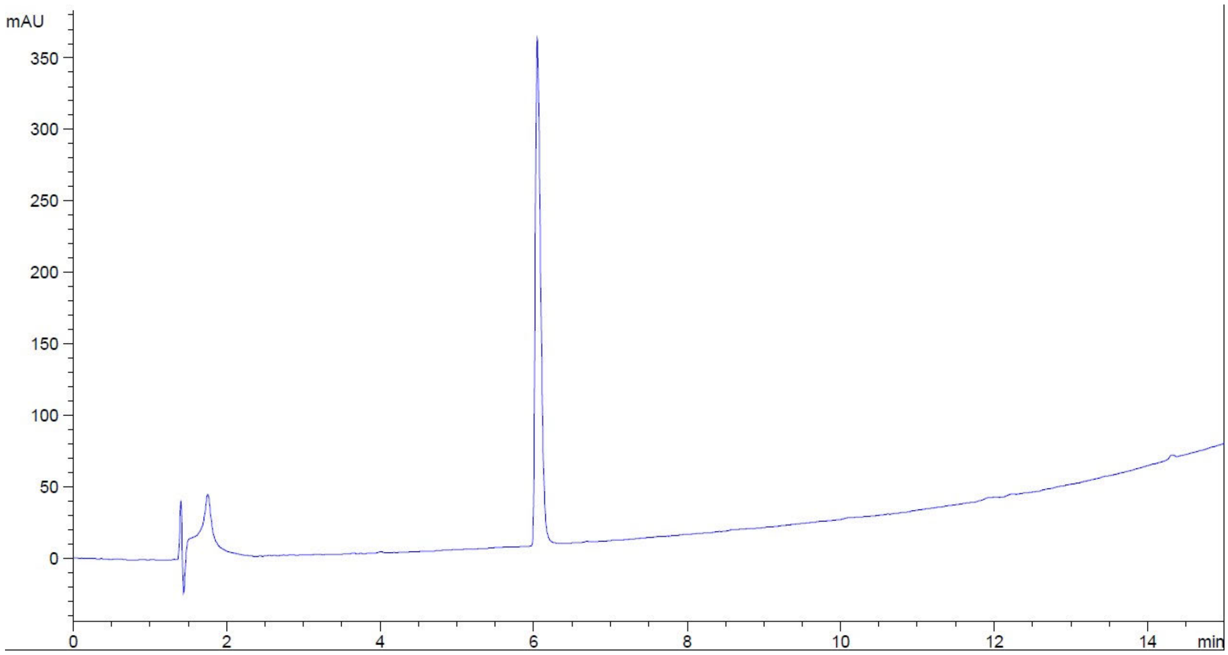
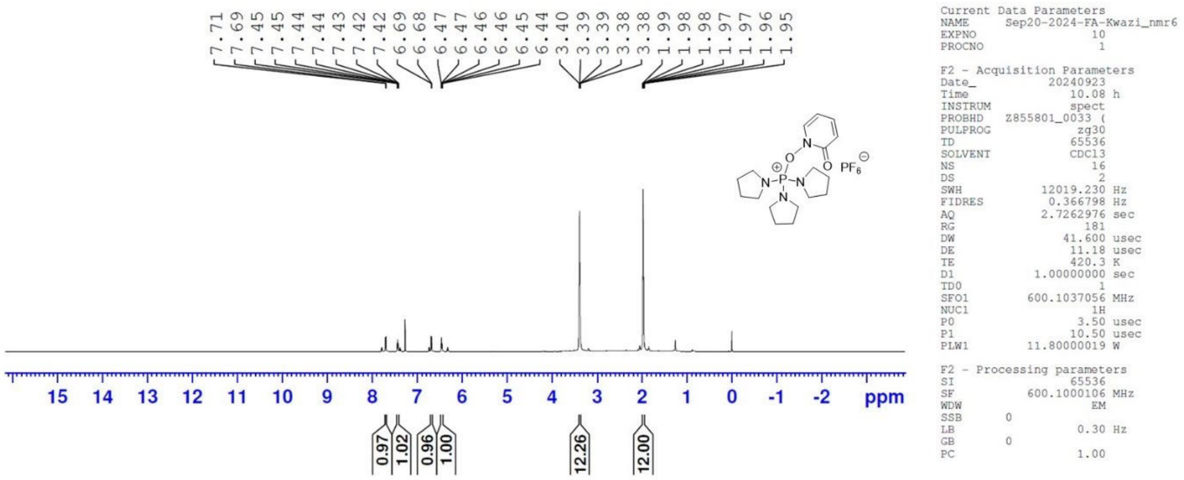


Figure S1. HPLC of PyHOPO



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TE                   420.3 K
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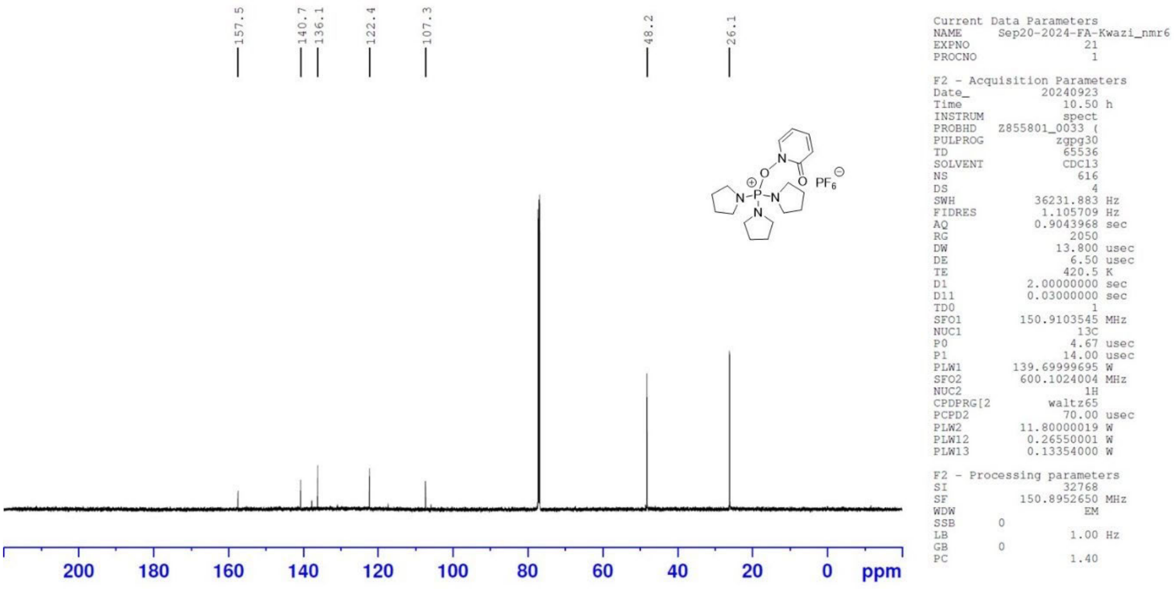
Figure S2. ^{1}H NMR of PyHOPO



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NUC2                    1H
CPDPRG[2           waltz65
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PLW13           0.13354000 W

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Figure S3. ^{13}C NMR of PyHOPO

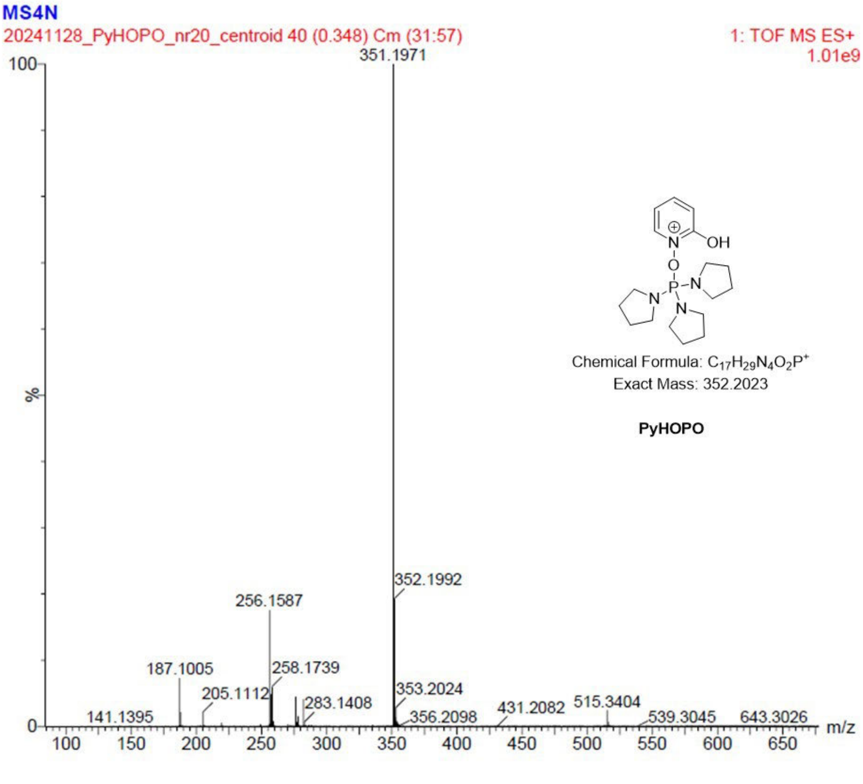


Figure S4. HRMS of PyHOPO

X-Ray Crystallography

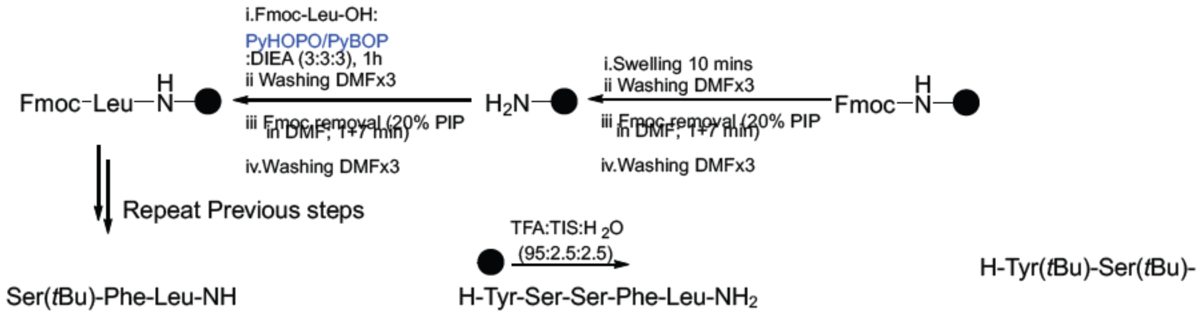
Colourless trapezoid-shaped crystals of PyHOPO were obtained by slow evaporation technique of a methanolic solution at ambient temperatures. The X-ray crystallographic investigations were recorded on a Bruker Smart APEXII diffractometer with a MoKα radiation source.[1] The crystal was kept at 293(2) K during data collection. Using *Olex2*, the structure was solved with the *SHELXS* structure solution program using Direct Methods and refined with the *SHELXL* refinement package using Least Squares minimisation.[2-5] The crystallographic data and structure refinement details are summarized in **Table 1**. The disordered PF_6 and pyrrolidine moieties were modelled using PART instructions with the major component having site occupancy of 58%.

Table S1: Crystal data and structure refinement details for compound **PyHOPO.**

Empirical formula	$C_{17}H_{28}F_6N_4O_2P_2$
CCDC number	
Formula weight	496.37
Crystal system Orthorhombic	Space group *Pbca*
a/Å	13.6740(4)
b/Å	24.9821(7)
c/Å	26.5484(8)
α/°	90
β/°	90
γ/°	90
Volume/Å^3	9069.1(5)
Z	16
ρ_{calc}g/cm$_3$	1.454
μ/mm^{-1}	0.262
F(000)	4128.0
Crystal size/mm^3	0.32 × 0.24 × 0.21
2Θ range for data collection/°	3.068 to 51.998
Index ranges	$-16 \le h \le 16$, $-30 \le k \le 30$, $-32 \le l \le 32$
Reflections collected	266290
Independent reflections	8906 [R_{int} = 0.0590, R_{sigma} = 0.0159]
Data/restraints/parameters	8906/13/641
Goodness-of-fit on F^2	1.027
Final R indexes [I>=2σ (I)]	R_1 = 0.0641, wR_2 = 0.1762
Final R indexes [all data]	R_1 = 0.0894, wR_2 = 0.2020
Largest diff. peak/hole / e Å^{-3}	0.53/-0.35

Peptide Synthesis using Solid Phase Peptide Synthesis (SPPS)

All peptides were assembled manually in plastic syringes fitted with a porous polypropylene disk. The Fmoc/*t*Bu strategy was used for synthesis using PyHOPO/PyBOP/PyOxim in DMF as a coupling cocktail. Fmoc removal was carried out using 20% piperidine in DMF (PIP/DMF). All the peptides were synthesized using the protocol explained below.



Scheme S1. SPPS of Fmoc-Tyr-Ser-Ser-Phe-Leu-NH_2 using PyHOPO and PyBOP as coupling reagent.

SPPS of H-Tyr-Ser-Ser-Phe-Leu-NH_2

Fmoc-Rink-Amide AM-PS resin (88 mg, 0.69 mmol/g resin loading) was washed with DMF (3 times). Deprotection of the Fmoc group was achieved by the treatment of the resin with 20% PIP/DMF (1 x 1 min; 1 × 7 min) followed by washing DMF (3 times). The protected Fmocamino acids (3.0 eq.), PyHOPO/PyBOP (3.0 eq.) and DIEA (3 eq.) were dissolved in DMF and added to the resin. Upon addition and after 1 h of coupling, filtration was done to remove excess coupling cocktail. Washing was then performed with DMF (3 times). Coupling and deprotection were repeated until the peptide was assembled onto the resin. The peptidyl resin was dried, and the peptide was cleaved from the resin by treating it with TFA/TIS/H_2O (95:2.5:2.5) for 90 min at rt. After that, the peptide was precipitated using chilled diethyl ether. The precipitate was centrifuged to afford the desired peptide, as confirmed by HPLC and LCMS for purity and mass, respectively.

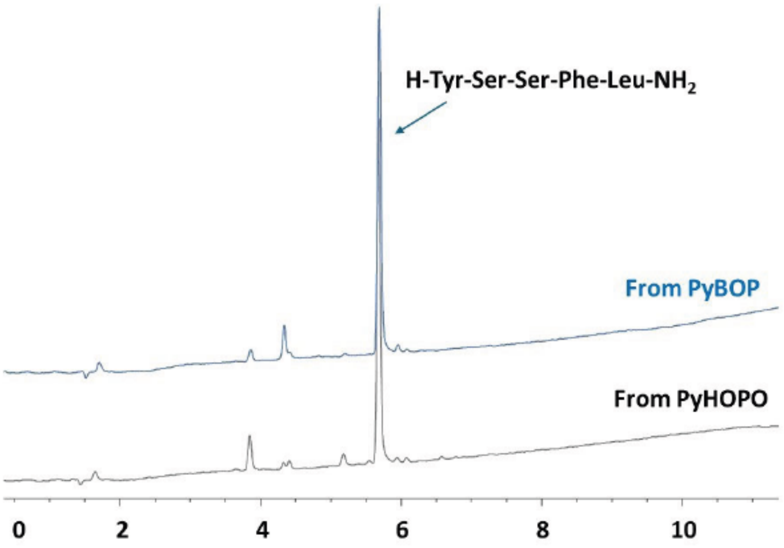
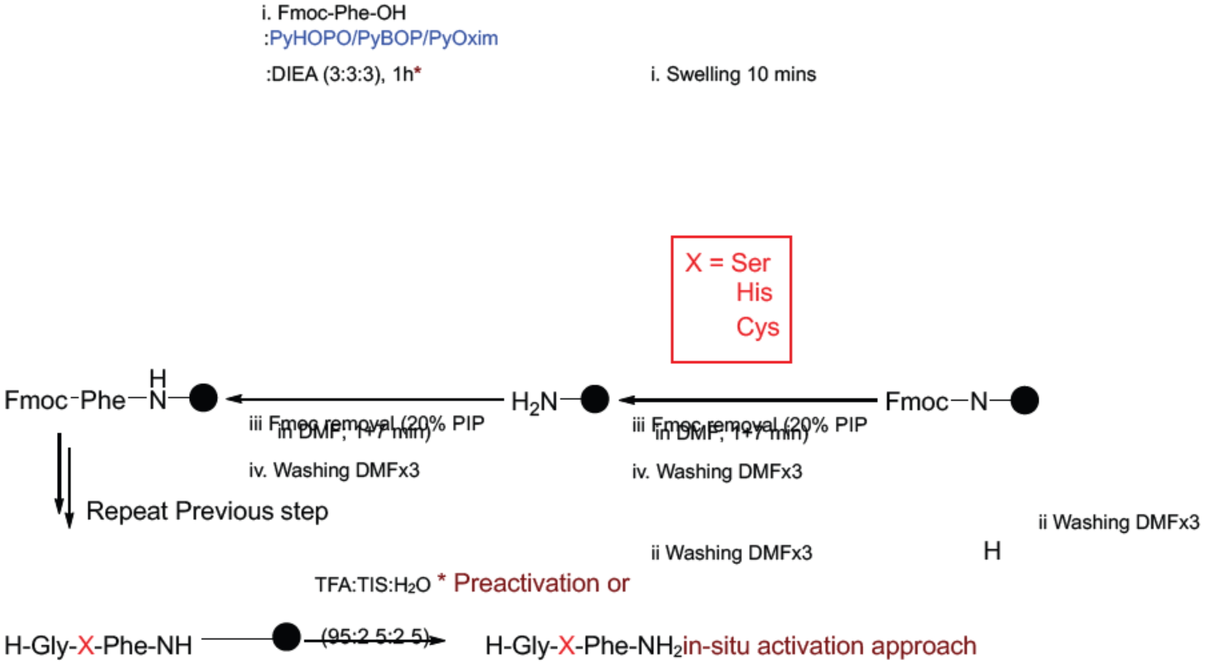


Figure S1. HPLC comparison of H-Tyr-Ser-Ser-Phe-Leu-NH_2 synthesized using PyHOPO and PyBOP.

SPPS of H-Gly-X-Phe-NH_2 (where X = Ser, His, Phe)

Similar protocol was adopted as above to synthesize H-Gly-X-Phe-NH_2 (where X = Ser, His, Phe). Different coupling reagents *viz*., PyHOPO, PyBOP and PyOxim were used in parallel for synthesis of tripeptide H-Gly-X-Phe-NH_2 (where X = Ser, His, Phe). *D*-Amino acids (Ser, His and Phe) were also used to synthesize the above tripeptides for comparison to calculate percentage of racemization.



Scheme S2. Synthetic protocol for racemization study.

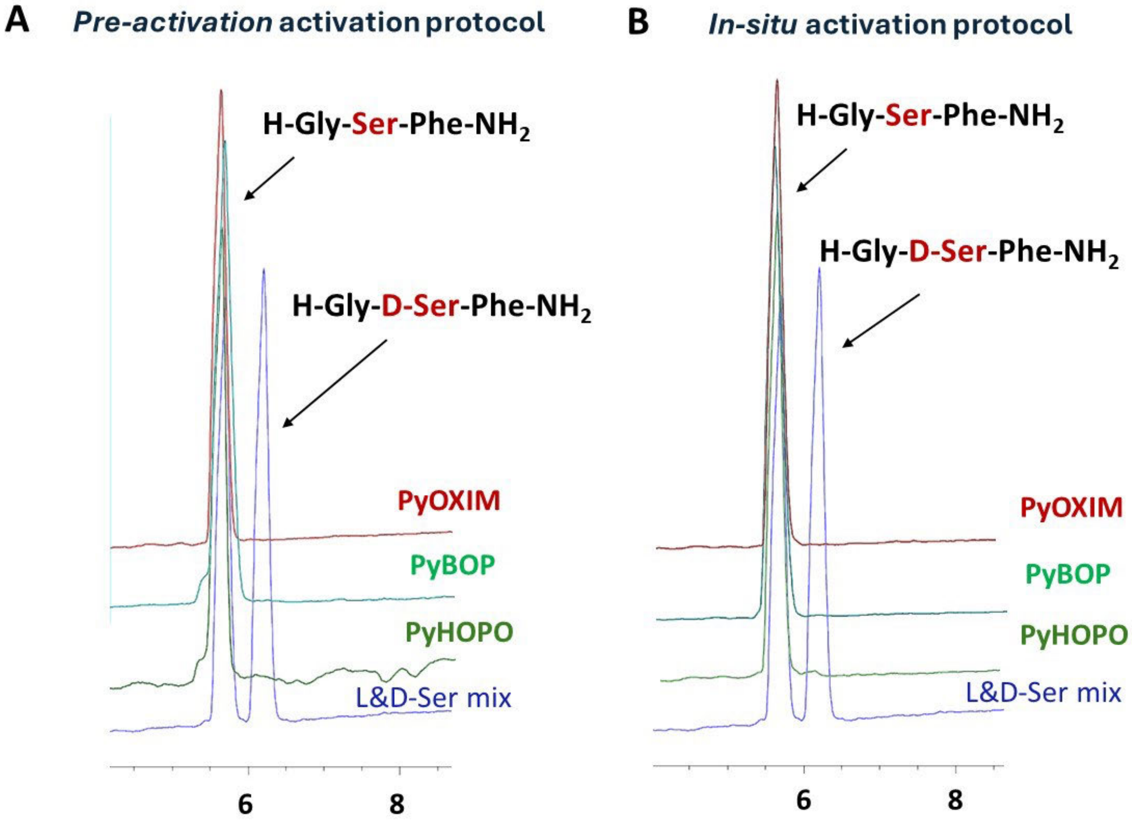


Figure S2. HPLC comparison of the SPPS of H-Gly-Ser-Phe-NH_2 to study racemization.

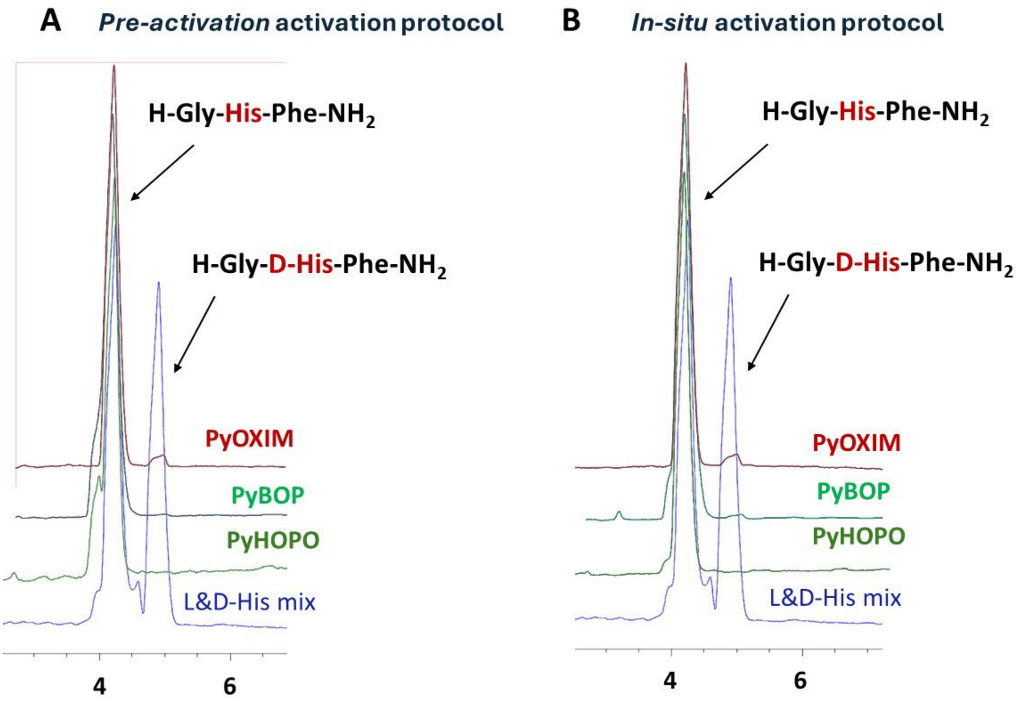


Figure S3. HPLC comparison of the SPPS of H-Gly-His-Phe-NH$_2$ to study racemization.

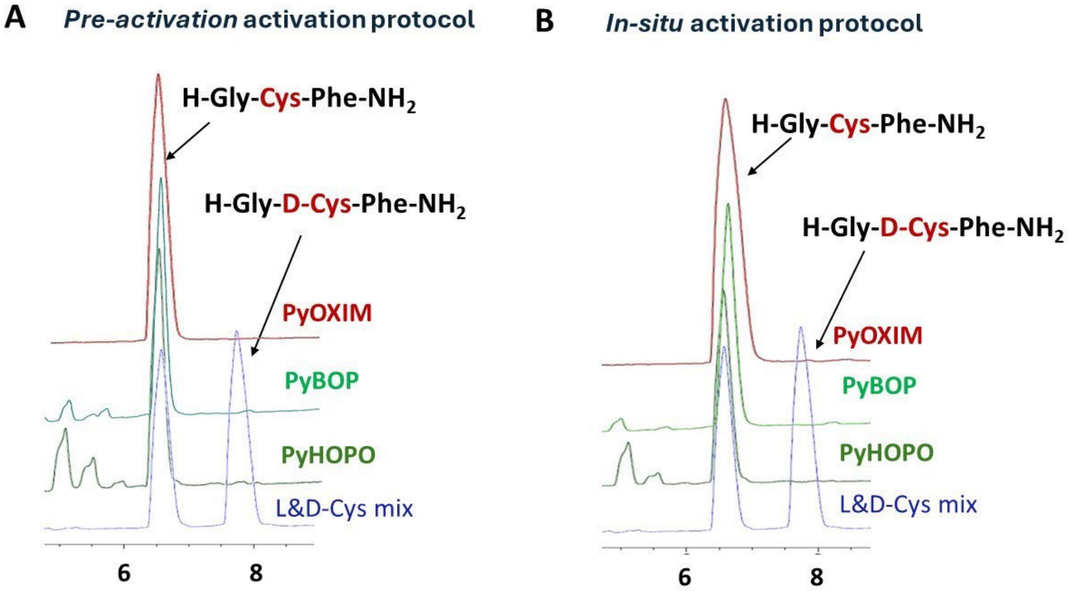


Figure S4. HPLC comparison of the SPPS of H-Gly-Cys-Phe-NH$_2$ to study racemization.

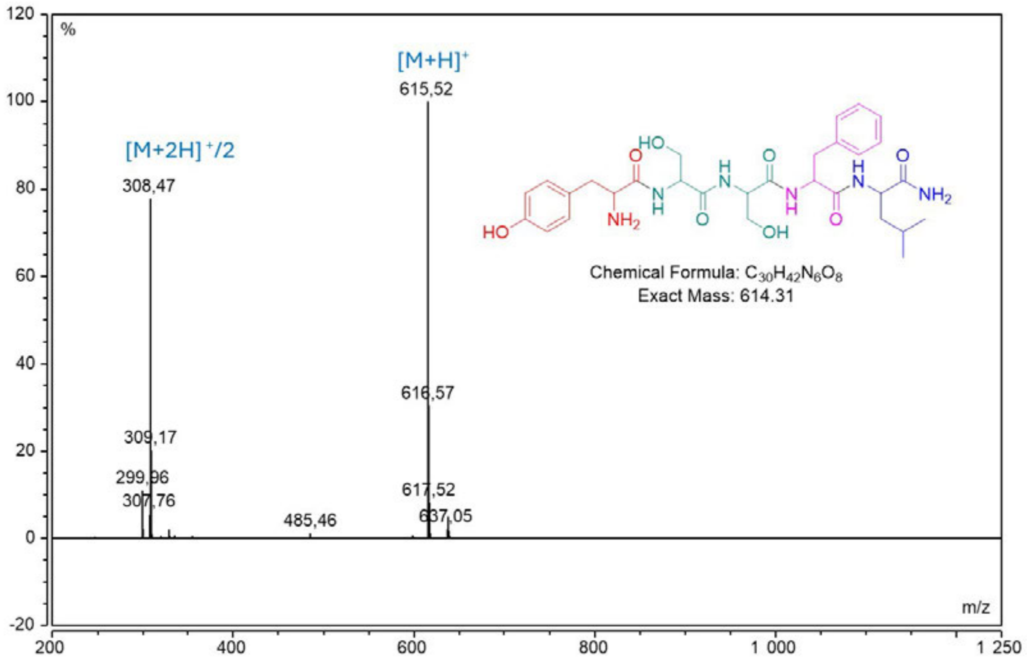


Figure S5. Mass results of H-Tyr-Ser-Ser-Phe-Leu-NH$_2$

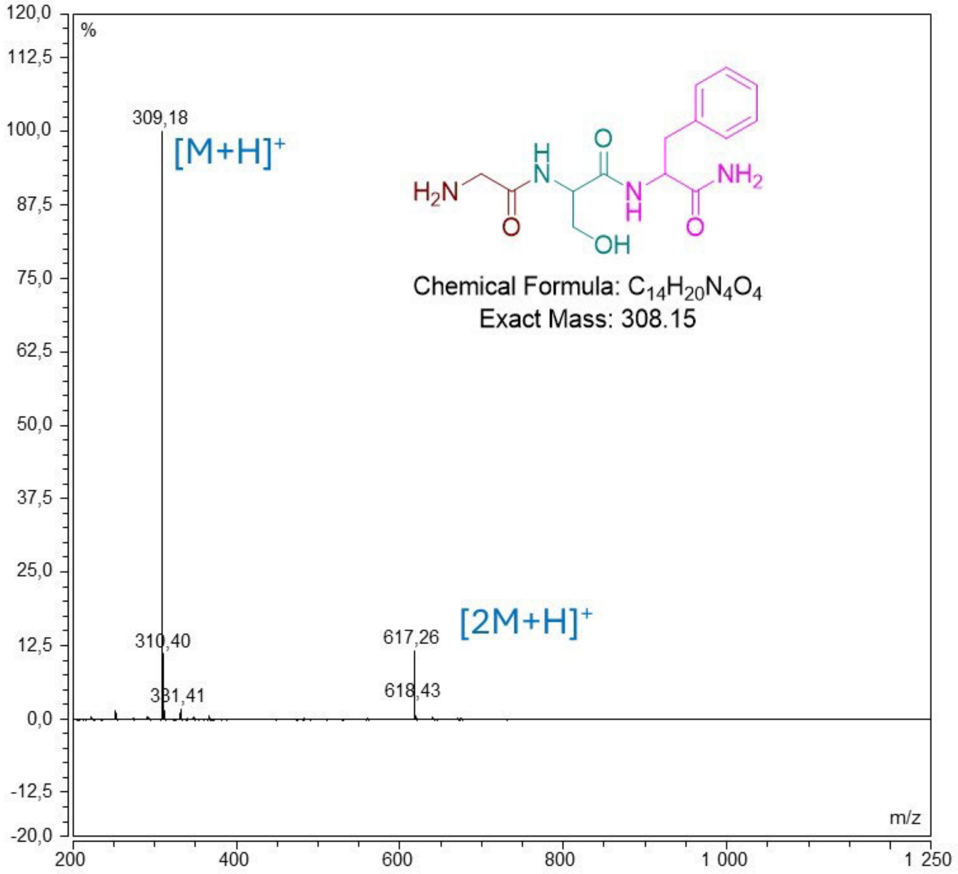


Figure S6. Mass of H-Gly-Ser-Phe-NH$_2$

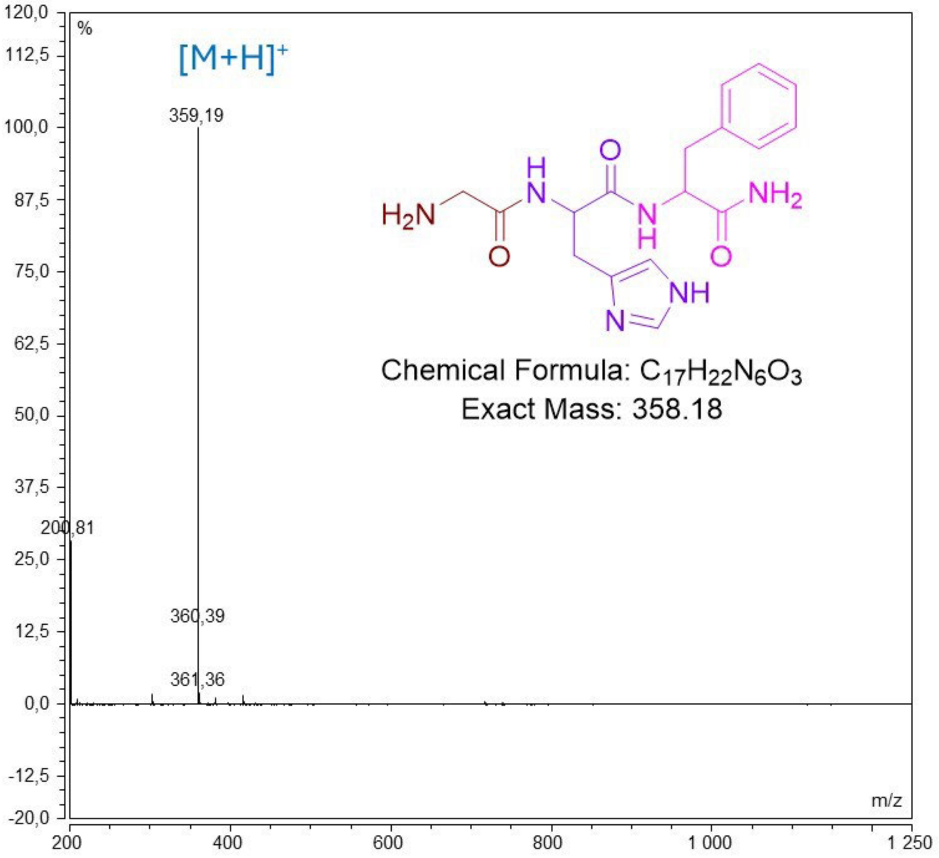


Figure S7. Mass of H-Gly-His-Phe-NH_2

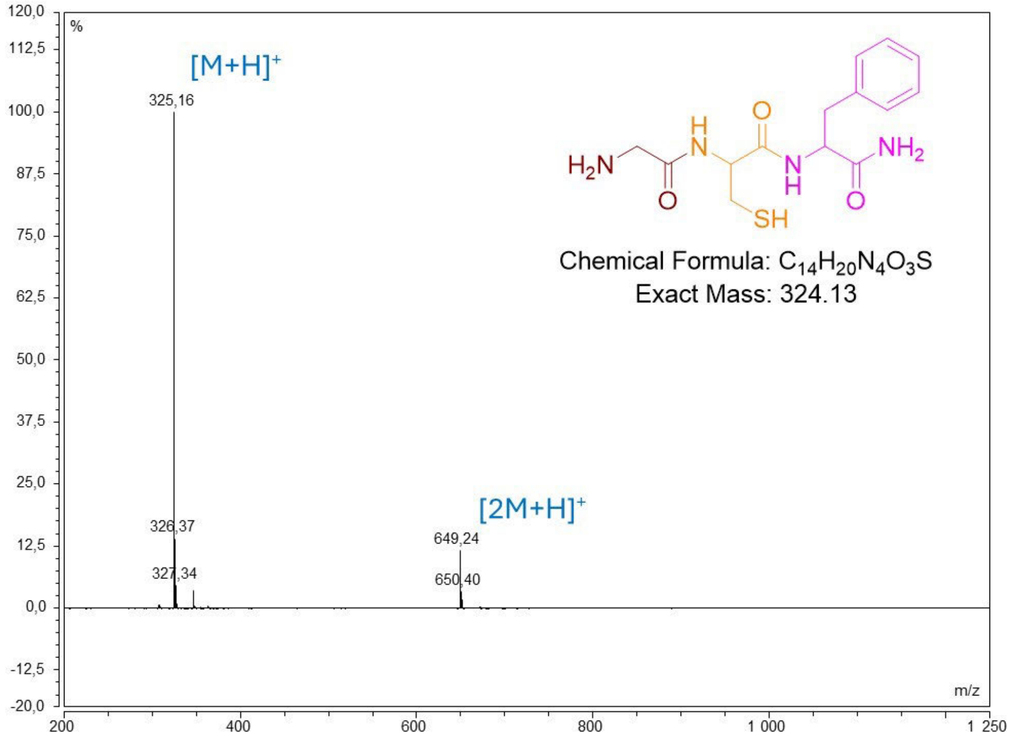


Figure S8. Mass of H-Gly-Cys-Phe-NH_2

References

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