CHEMICAL AND ANALYTICAL TOOLS TO STUDY PROTEIN-PHOSPHORYLATION

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Disputation am: 1. Dezember 2015

Declaration

Herewith I declare that I have prepared this dissertation myself and without the help of any impermissible resources. All citations are marked as such. The present thesis has neither been accepted in any previous doctorate degree procedure nor has it been evaluated as insufficient.

Berlin, October 15th 2015

Jordi Bertran-Vicente

The work on this dissertation resulted so far in the following publications, talks and posters:

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   A chemoselective tool for Lys-phosphorylation.

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Phosphorylated Lys-containing peptides by Staudinger-phosphite reactions.

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Unprotected phosphoramidates to study naturally occuring phosphorylation events.
*shared contribution

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Abbreviation

AcCoA	acetyl coenzyme A
Ar	argon
ATP	adenosine triphosphate
Boc	tert-butyloxycarbonyl
CID	collision-induced dissociation
CuAAC	copper-catalysed azide–alkyne cycloaddition
DIPEA	*N*,*N*-diisopropylethylamine
DMF	*N*,*N*-dimethylformamide
DNA	deoxyribonucleic acid
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
DTT	dithiothreitol
ECD	electron-capture dissociation
EPL	expressed protein ligation
Et_3N	triethylamine
ESI	electrospray ionization
ETD	electron-transfer dissociation
EThcD	electron-transfer/higher-energy collision dissociation
Fmoc	fluorenylmethyloxycarbonyl
GalNAc	*N*-acetylgalactosamine
h	hour
HBTU	2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HCD	higher-energy collisional dissociation
HCl	hydrochloric acid
He	helium

HF	hydrogen fluoride
HMBC	heteronuclear multiple-bond correlation
HMPT	tris(dimethylamino)phosphine
HOBt	1-hydroxybenzotriazole
HPLC	high-performance liquid chromatography
HR	high-resolution
ICAT	isotope-coded affinity tag
IMAC	immobilized metal ion affinity chromatography
iTRAQ	isobaric tag for relative and absolute quantitation
MALDI	matrix-assisted laser desorption/ionization
MeCN	acetonitrile
MgrA	regulator of autolytic activity
MHz	Megahertz
min	minute
MOAC	metal oxide affinity chromatography
MS	mass spectrometry
MSH	*O*-mesitylenesulfonylhydroxylamine
MS/MS	tandem mass spectrometry
NaOH	sodium hydroxide
NCL	native chemical ligation
NMR	nuclear magnetic resonance
ppm	parts per milion
PTM	post-translational modification
RP	reversed-phase
SAM	*S*-adenosylmethionine
SarA	staphylococcal accessory regulator A

SBL	subtilisin
SILAC	stable isotopic labeling amino acid cell culture
SPI	selective pressure incorporation
SPPS	solid-phase peptide syntesis
TFA	trifluoroacetic acid
THPTA	tris(hydroxypropyltriazolyl)methylamine
TIS	triisopropylsilane
Tris	tris(hydroxymethyl)aminomethane
UDP	uridine diphosphate glucose
UPLC	ultra-performance liquid chromatography
UV	ultraviolet
XIC	extracted ion chromatogram
1DE	one-dimensional electrophoresis
2DE	two-dimensional electrophoreses

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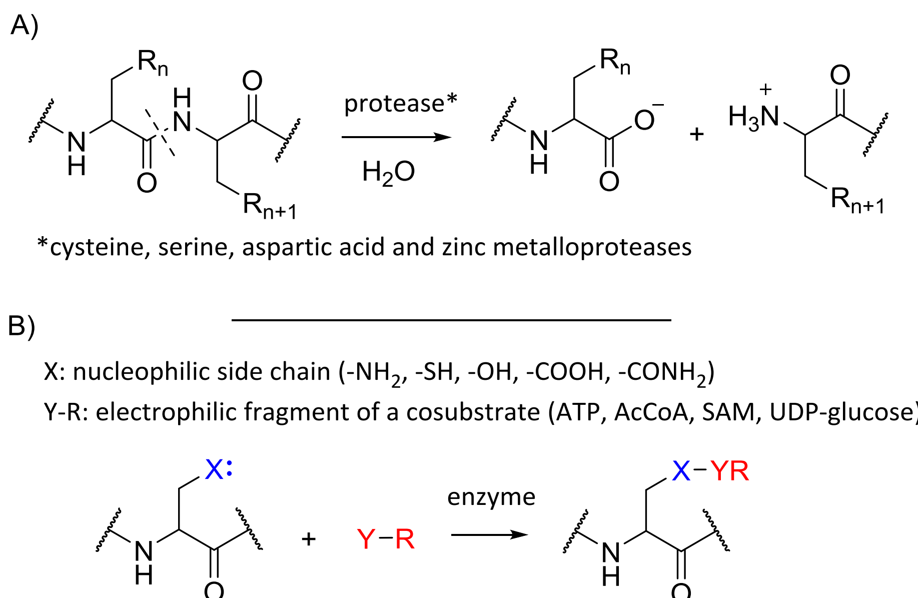
1 INTRODUCTION

1.1 Motivation: Site-specific access to post-translationally modified peptides

Most of the intra- and extracellular processes occurring in living systems are governed by interactions of proteins, which are being constantly produced, modified, and destroyed by our cellular machinery. Proteins can vary in size, structure, and function, and their roles can be very diverse depending on which context they are found. Understanding the role of each protein and thereby deciphering its role in a specific cellular process is a challenging task pursued by different scientific disciplines in the last decades. Within all these scientific disciplines, Chemical Biology has emerged as a new field to contribute to this goal by employing chemical methods and tools to the study and to manipulate biological systems.[1] Peptides and proteins prepared by chemical means have served as valuable scaffolds to be used later on in biological assays. Specifically, chemical tools designed to not address only native translated proteins but also co- and post-translationally proteins are highly needed to further advance on this field. For instance, synthetically post-translational modified peptides and proteins can be used to raise antibodies, to evaluate potential interactions partners, to study signalling events, and to develop better proteomics methods to name few applications. Within these chemical approaches, the main focus of this thesis is the development of new chemoselective strategies to deliver site-specific post-translationally modified peptides and proteins as well as the discovery of suitable analytical tools to analyze them.

1.2 Post-translational modifications (PTMs) of proteins

Proteins are not encoded by the DNA alone, as they can also be covalently modified after the translational process. This process increases the diversity of proteins and thus delivers thousands of proteins with different biological functions (>1000 000 molecular species of proteins).[2] Mainly, two types of protein PTM can occur: the covalent cleavage of peptide backbones upon the action of proteases (or less frequent by autocatalytic cleavage) and the covalent addition of a chemical group to a side chain residue in a protein (Scheme 1).[2] Proteolytic processing is an irreversible PTM involving limited and very specific peptide bond hydrolysis upon the presence of proteases (Scheme 1A). This protein degradation process results in new smaller proteins, or even peptides with new or modified biological activities.[2] On the other hand, some folded proteins can also modify themselves by using autocatalytic domains, such as inteins. This latter process is also known as protein splicing and consists on an intramolecular reaction in which a protein domain (intein) excises itself from the polypeptide, creating a new peptide bond between its two flanking regions (exteins).[2]



Scheme 1. A) proteolysis and B) covalent addition of PTMs.

The major types of PTMs that include a covalent addition to a nucleophilic side chain in a protein are phosphorylation, glycosylation, acetylation, methylation, lipidation and

ubiquitination. The PTM incorporation is catalyzed by enzymes, which are specific for each PTM (kinases, glycosyltransferases, acetyltransferases, methyltransferases, ubiquitin ligases) (Scheme 1B). Most of the amino acids are exposed to suffer such modifications (except for the non-functionalized aminoacids) and the diversity of modifications that the same side chain can harbor is in some cases very broad.[2] On the other hand the same covalent modification can occur at different amino acid residues, as it is the case for phosphorylation (*see 1.2.1*). Phosphorylation mainly occurs on the hydroxyl groups of serine, threonine, and tyrosine, or the carboxylic groups of aspartate and glutamate (*see 1.2.1.1*) but also on the nitrogen groups of histidine, arginine and lysine, and the thiol group of cysteine (*see 1.2.1.2*). The phosphorylated amino acids from the latter are known to be phosphorylated transient residues due to their lability under physiological conditions. Phosphorylated-lysine and -cysteine residues represent the biological targets of this work.

1.2.1 Protein phosphorylation

Protein phosphorylation was first reported in 1906 by Levene, who found phosphate in the protein vitellin.[3] A couple of decades afterwards, the same author identified a phosphoserine residue in casein.[4] Yey, it was not until the discovery of the first enzymatic phosphorylation that the concept of protein phosphorylation as a key regulatory mark was born.[5] To date, it is estimated that around 30% of human proteins may be phosphorylated at some point.[6] About 500 protein kinases and a fewer number of protein phosphatases have been found to regulate the phosphorylation process (Figure 1).[5,6] This reversible process is governed by kinases and phosphatases and influences most physiological processes ranging from the cardiovascular system, gastrointestinal action, neurologic mechanisms and behavior, immune response, endocrine action, and musculoskeletal regulation.[5,7] On the other hand, abnormal phosphorylation due to mutation on kinases or phosphatases can lead to a failure in these signaling events and thus be the cause of diseases.[6] The connection between phosphorylation and diseases have aroused the interest of the scientific community to investigate potential phosphorylation sites and their roles in different pathogenic pathways. For instance, many drugs have been developed as protein kinase inhibitors to treat cancer, chronic inflammatory diseases, and diabetes, among others.[6]

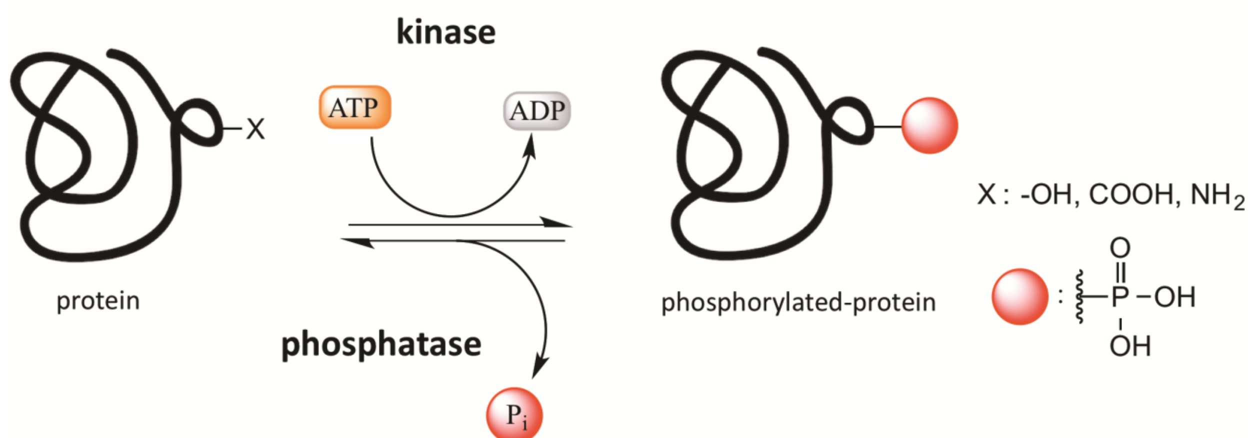
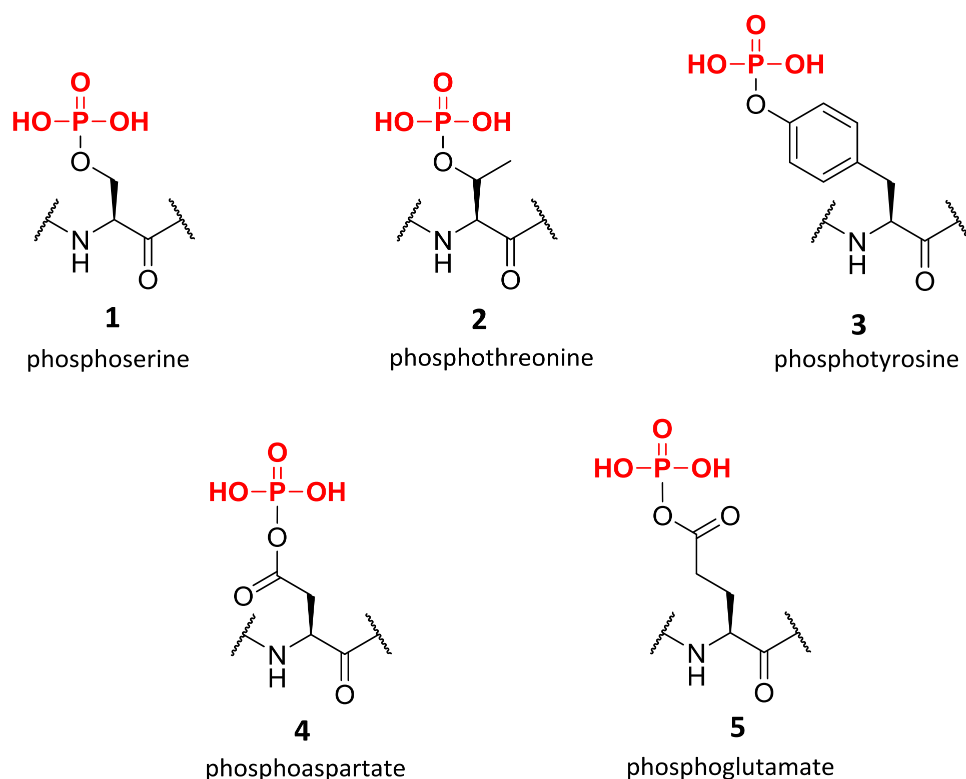


Figure 1. Protein phosphorylation governed by kinases and phosphatases.

1.2.1.1 *O*-phosphorylation

O-phosphorylation occurs on serine, threonine and tyrosine and less frequently in aspartate and glutamate. The presence of phosphoserine **1**, phosphothreonine **2** and phosphotyrosine **3** residues on proteins is more common in mammals with a ratio of 90:10 (phosphoserine:phosphothreonine/phosphotyrosine), whereas phosphoaspartate and phosphoglutamate **4** resisdues are found in bacterial and fungal phosphoproteomes (Scheme 2).[8-10]



Scheme 2. *O*-phosphorylated residues.

Phosphoserine **1** is by far the most common encountered phosphorylated residue in proteins followed by phosphothreonine **2**.[8] Among the more than 500 kinases known, 125 are serine/threonine kinases, which are responsible to install the phosphate group on the serine and threonine residues. The occurrence of serine- and threonine- phosphorylation is very broad, reflected by a vast number of examples in the literature. For instance, the abnormal phosphorylation on serines and threonines on Tau protein in neurons is thought to favor their aggregation into insoluble filaments, which in turn might underlie neuronal death as encountered in many neurodegenerative disorders like Alzheimer´s disease.[11,12] Histone

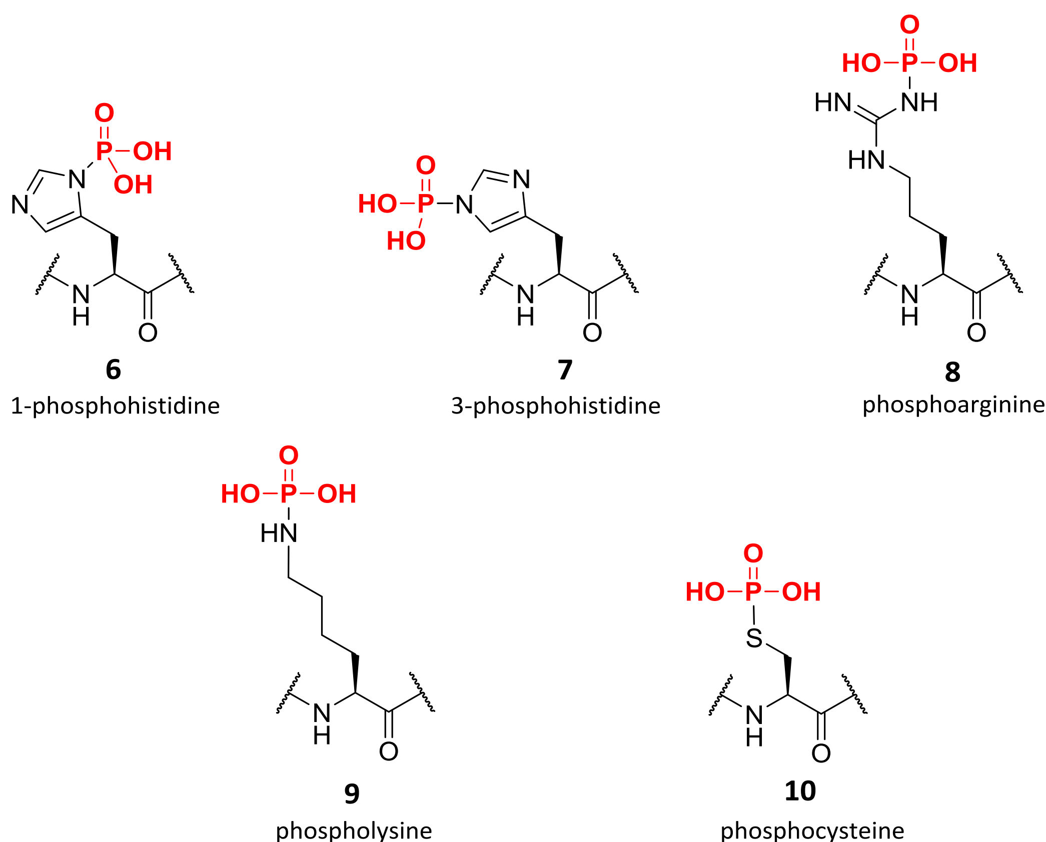
proteins, and more specifically histone H3 is another protein that is phosphorylated on serine residues. For instance, phosphorylation of S10 in histone H3 blocks the access of the adjacent site (H3K9) by specific antibodies and histone methyltransferases.[13,14] In a similar way, phosphorylation of S27 in histone H1.4 prevents the hetero-chromatin protein 1 (HP1) to bind to methylated K26, suggesting the presence of a “phospho-switch” model.[15] Considering that the serine-lysine motif is found in histone tails, phosphorylation of serine might regulate the access of methylated lysine and thus might have an important role in epigenetic regulation.[13,14]

Tyrosine phosphorylation was discovered much later than its counterparts. In 1979, a fortuitous observation in the Hunter laboratory determined that Src is a tyrosine kinase.[16,17] Following the discovery, tyrosine phosphorylation has emerged as a fundamentally important mechanism of signal transduction and regulation in all eukaryotic cells.[18] Remarkably, phosphotyrosine **3** residues in some proteins are known to bind to SH2 domain containing proteins, thus initiating downstream signaling and transduction events. The SH2 domains are commonly found in adapter proteins with the function to recruit other signaling proteins.[18] In another context, it has been recently found that tyrosine phosphorylation of the protein acetyltransferase KAT5 is a key event in the sensing of genomic and chromatin perturbations.[19]

Much less frequent in biology are aspartate- and glutamate-phosphorylation. Both are involved in signalling pathways and as phosphoenzyme intermediates.[20,21] For instance, phosphoaspartate residues **4** are found as intermediate in two-component histidine kinase signaling systems. Its role is to relay the signal from the sensor histidine kinase to the DNA and, along with transcription factors, initiate transcription of the response genes.[22,20] In addition, the free phosphoaspartate amino acid is a key intermediate in the biosynthesis of lysine, threonine and methionine.[20] Unlike phosphoaspartate, the presence of phosphoglutamate residues **5** in biological systems is less reported, and it is only found in a nuclear protein called prothymosin.[23] As with phosphoaspartate, the free phosphoglutamate has a very important role as intermediate in the biosynthesis of amino acids, namely glutamine and proline.[20]

1.2.1.2 *N*- and *S*-phosphorylation

As previously mentioned, *N*- and *S*-amino acids are also capable of being phosphorylated (Scheme 3). However, reports for *N*- and *S*-phosphorylation are less frequent than for *O*-phosphorylation proteins. One possible reason for the scarcity of literature on them is the acid-lability nature of the phosphoramidate- and phosphorothioate bond. Considering that standard proteomic approaches used acidic and other type of rather harsh conditions, it is not surprising that *N*- and *S*-phosphorylation often get missed during biochemical analysis.



Scheme 3. *N*- and *S*-phosphorylated residues.

Within this group, phosphohistidine (**6** and **7**) is by far the most studied. Histidine phosphorylation was discovered by Paul Boyer even before than tyrosine phosphorylation, in the early 60's and to date has been placed into the biological context of bacteria, fungi and plants participating in the two-component phosphorelay signaling pathways.[24-26] In contrast, reports about histidine phosphorylation in mammals are less frequent despite the fact that it's believed that its abundance in eukaryotic cells as phosphoamino acid is higher than tyrosine phosphorylation (6% phosphohistidine *vs* 1% phosphotyrosine).[25,27,28]

Similarly to phosphohistidine, phosphoarginine **8** is found mainly in bacteria on a phosphoarginine kinase and, to a less extent, in mammals as phosphoarginine-containing proteins.[29-31] Recently, the Clausen laboratory has discovered a bacterial arginine kinase (McsB), which phosphorylates arginine residues in the DNA binding domain of the heat-shock regulator (CtsR), thus inhibiting its function and pointing to have a significant role in transcriptional regulation.[32]

Much less is known about lysine phosphorylation. Consequently, to date its biological significance still remains a matter of conjecture. So far, evidence of the presence of phospholysine **9** residues have only been found in histone H1 after proteolytic digestion of phosphorylated histone fractions from a regenerating rat liver.[29] A couple of kinases and phosphatases have been isolated from living cells and have been suggested to regulate lysine phosphorylation.[33-35] Due to similar chemical properties and stability profiles, it is assumed that lysine phosphorylation might have a similar role like its counterparts, histidine- and arginine- phosphorylation.

Finally, cysteine phosphorylation is mainly known in the context of cysteine-dependent protein phosphatases (CDPs).[36] CDPs are a type of protein tyrosine phosphatases, which catalyze the hydrolysis of phosphotyrosine *via* a cysteine nucleophilic attack thus forming a phosphocysteine intermediate.[37-40] Phosphocysteine **10** residue has also been found in nature as intermediate in the phosphoenolpyruvate-dependent phosphotransferase system (PTS).[41-46] Recently, it has also been found in a couple of transcriptional regulators (SarA and MgrA), in a human pathogen Staphylococcus aureus, showing its influence in regulating virulence, and bacterial resistence to antibiotics.[47]

1.3 Chemical approaches to access post-translationally modified peptides and proteins

During the last decades, researchers in the chemistry and biochemistry field have presented different approaches to straightforward the access towards modified peptides and proteins.[48] Ranging from pure chemical methods such as solid-phase peptide synthesis (SPPS) or native chemical ligation (NCL), to semisynthetic approaches such as express protein ligation (EPL), and ending with more biotechnological techniques such as selective pressure incorporation (SPI), researchers have been able to produce biological peptides and proteins of interest.[49-51] In the context of PTMs, SPPS in combination with NCL and EPL has been the gold-standard to generate synthetic peptides and proteins with well-defined PTMs (*see 1.3.1*) (Figure 2). Nevertheless, linear peptide synthesis can be challenging for some peptide sequences and not compatible with some PTMs. Moreover, the protection of side-chain functionalities with bulky protecting groups can affect the coupling efficiency and thereby limit the accessibility of the desired peptide.[52] Alternative methods have emerged to overcome the limitations of linear peptide synthesis. For instance, the convergent assembly of previously established structures by using chemoselective reactions has gained remarkable attention in recent years (*see 1.3.2*); however, the very few chemoselective reactions available are the “bottle neck” of this strategy (Figure 2).[53] As alternative to chemical approaches, biotechnological techniques have been applied to allow the ribosomal incorporation of amino acids, which already harbor the PTMs (Figure 2). These methods based on the genetically encoding of those post-translationally modified amino acids, have allowed the site-specific and homogenous incorporation of an acetylated histone and a phosphorylated kinase (Nek7) among other examples.[54-56]

The next subchapters focus on strategies employed to introduce site-specifically PTMs or their mimetics both on peptides and proteins: First by using linear peptide synthesis alone or together with NCL and EPL (*see 1.3.1*), and second by using chemoselective reactions to assemble a previously modified peptide or protein containing the corresponding chemical tag and a small molecule reagent carrying the PTM or mimetic precursor (*see 1.3.2*). The

focus will be put mainly on examples of these strategies to access phosphorylated peptides and proteins, but without overlooking remarkable examples of other PTMs.

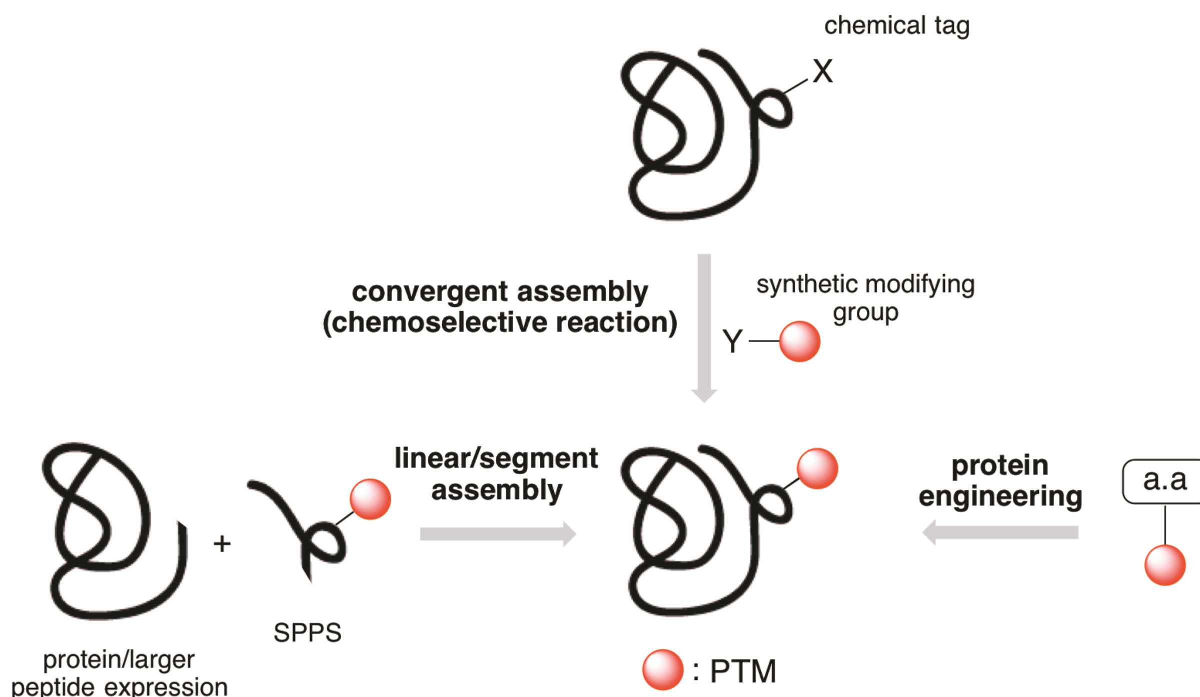
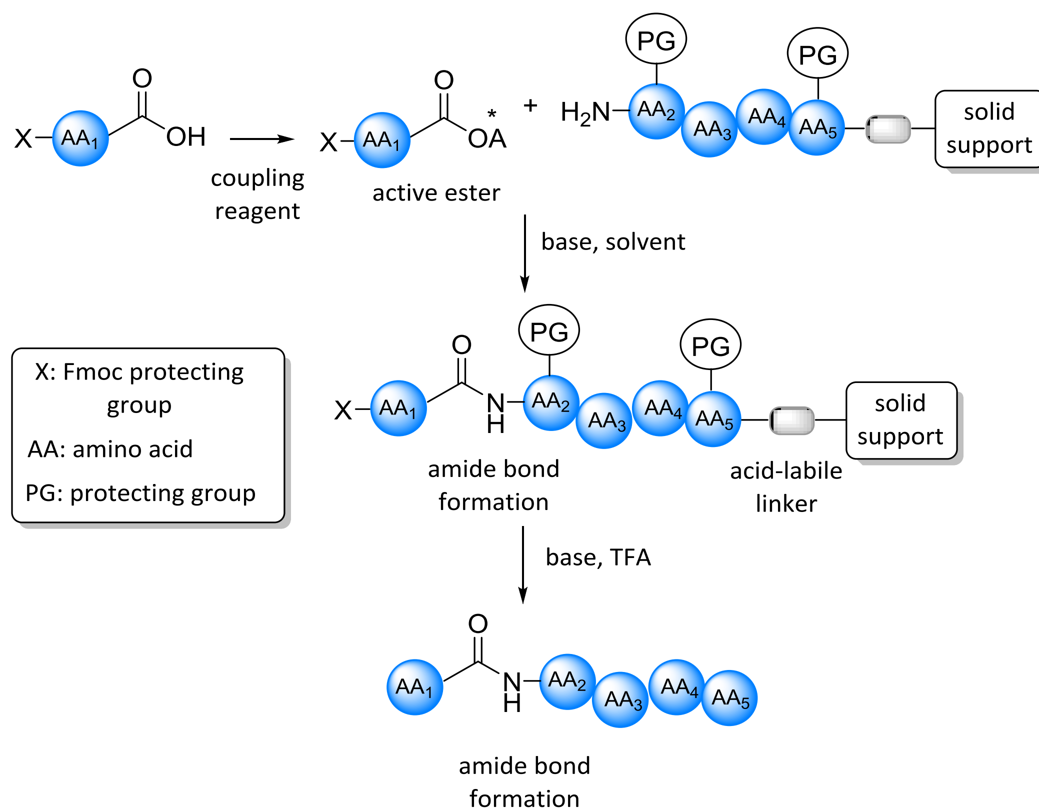


Figure 2. Chemical and biochemical strategies to incorporate well defined PTMs.[53]

1.3.1 Linear- and segment assembly approach

1.3.1.1 Solid-phase peptide synthesis (SPPS)

Since the discovery of SPPS by Merrifield in 1963,[57] the number of publications using this technique for peptide synthesis has grown dramatically. SPPS, also known as linear peptide synthesis on a solid support, is a very fast and efficient way to synthesize peptides. In a general view, SPPS consists on the following steps: First, the protected building block amino acid is loaded on to the solid support. Second, the N_α-protecting group is cleaved upon the required conditions to deliver a free *N*-terminal amino group. Third, the activated ester of the next protected amino acid reacts to the free *N*-terminal to form a new amide bond. Then, iterative N_α-deprotection and amide coupling steps generates the desired peptide. Final peptide cleavage releases the side-chain protecting groups as well as the peptide from the solid support (Scheme 4).



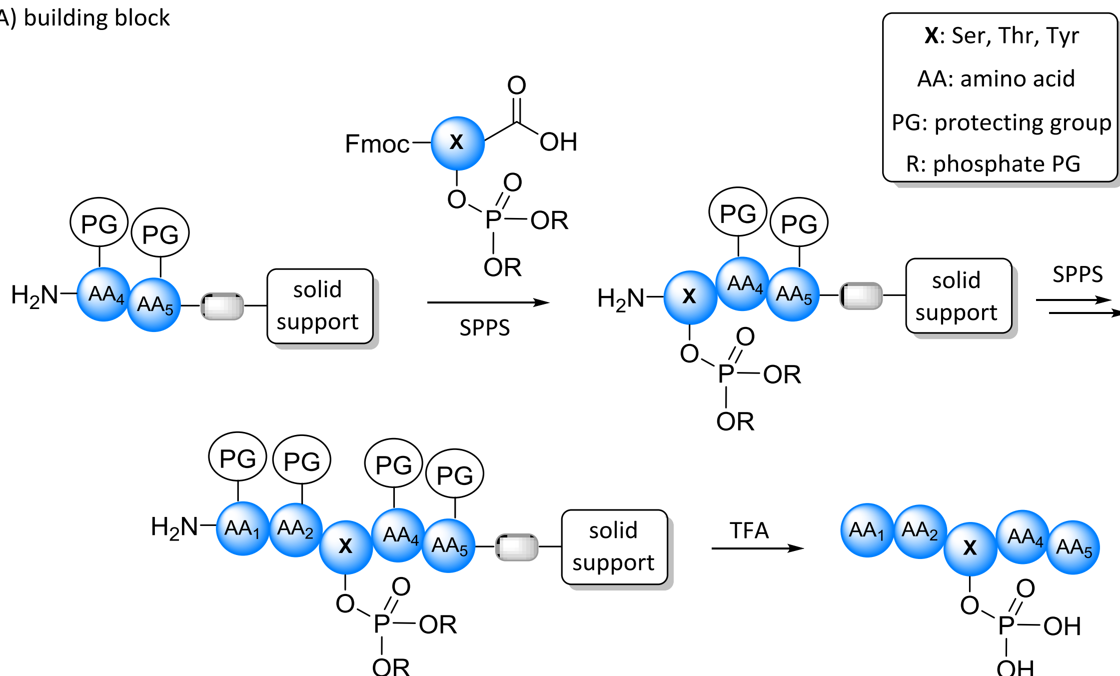
Scheme 4. Standard Fmoc-solid-phase peptide synthesis (SPPS).

In the earliest days, the acid-labile tert-butyloxycarbonyl (Boc) protecting group was employed as N_α-terminal protection. Due to the harsh conditions for final cleavage and the toxicity of the acid employed, i.e. HF, an alternative protecting group was required. The base-labile fluorenylmethyloxycarbonyl (Fmoc) protecting group appears to be a better replacement since it requires milder deprotection conditions and it is stable under acidic conditions (orthogonality).[58,59] In addition, the deprotection can be followed by UV and it is easy to automate. However, it is important to mention that the criteria to follow on choosing a particular N_α-protecting group over the other is mainly dependent on the properties of the peptide of interest.

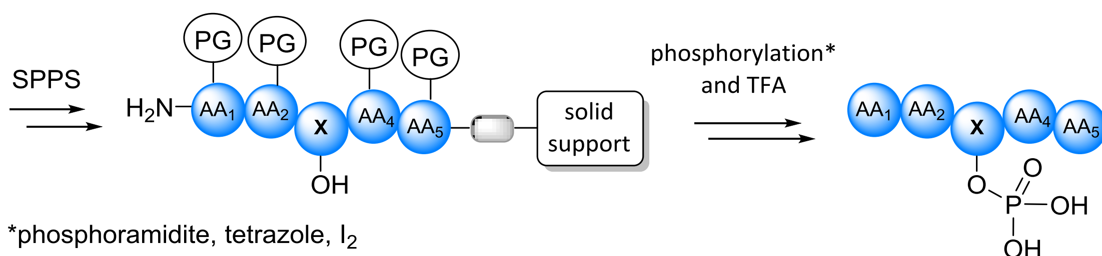
In general, the immobilization of the peptide on a solid support offers the advantage to remove the excess of reagent added, as well as side products formed during the reactions, being one of the potentials of this approach. However, SPPS has also some limitations. For instance, long peptide or difficult peptide sequences often generate truncation and deletion sequences. This fact does not only affect negatively the final peptide yield, but also makes

the separation, and thereby purification, of the final peptide more challenging. For that reason, SPPS is generally limited to the preparation of up to 50 amino acid peptides.

In the context of PTMs, SPPS is "the tool" to prepare straightforwardly post-translationally modified peptides by synthetic means. There are a vast number of examples in the literature on using SPPS to access all type of PTMs.[51,60] In the context of phosphorylation, two generic solid phase strategies can be followed to incorporate the phosphorylated residue of interest on the peptide during SPPS: a building block approach and an inter- or post-assembly strategy (Scheme 5).[61,62] In the building block approach, a protected precursor of the PTM is used as a building block in a conventional solid phase assembly (Scheme 5A). In the inter- or post-assembly approach, the protected peptide is first assembled on the solid support, leaving side chains unprotected, at which the phosphorylation will be installed (Scheme 5B).[61,62]



B) post-assembly



Scheme 5. A) building block and B) post-assembly approach.

Nowadays, the building block approach is the widely used method to access site-specifically modified phosphorylated serine, threonine and tyrosine peptides by using commercially available Fmoc mono-benzyl protected phospho-serine, -threonine, and -tyrosine building blocks. This is due to an easy incorporation into the automated SPPS and to the fact that the benzyl protecting group can be removed in the final step of SPPS by using TFA. However, for difficult peptide sequences the inter- or post-assembly approach can be a good alternative. In those cases, the phosphorylation step can be executed either by using P(V) chemistry with phosphorochloridates under alkaline conditions or by using P(III) chemistry by phosphitylation followed by oxidation.[62] Examples of these strategies have been very well-covered in an excellent review by McMurray *et al*.[62] For instance, difficulties on using the building block approach were observed by Alewood and co-workers when using Fmoc-Tyr(PO(OBu)$_2$)OH for the synthesis of a “difficult” peptide sequence.[61] The post-assembly strategy was not an alternative since only traces of phosphorylated peptide were obtained. However, by carrying out the phosphorylation step just after assembling the unprotected tyrosine building block (inter-assembly approach), the phosphorylated tyrosine-containing peptide could be accessed and delivered in good yields. It is important to note that one important drawback of using P(III) procedures is the oxidation step that can result in oxidation of cysteine, methionine and tryptophan.[62]

The application of SPPS for the site-specific synthesis of *N*-phosphorylated peptides is much more challenging. In standard SPPS, the resin is attached to the peptide through an acid-labile linker and thus the TFA cleavage is required to deliver the final peptide (Scheme 4). The TFA step is not compatible with *N*-phosphorylated peptides because of their instability under acidic conditions. Therefore, it is needed to modify the peptide synthesis strategy. For example, the development of an acid-stable bis(2,2,2-trichloroethyl) protected phosphoarginine Fmoc building block **11** allowed its incorporation on a peptide sequence during SPPS with final TFA cleavage (Figure 3). Finally, the trichloroethyl groups could be removed by hydrogenation delivering the site-specifically modified arginine phosphorylated peptide.[63] Alternatively, if it is not possible to access the native phosphorylated amino acid due to the instability under acidic conditions, the use of mimetics can be a potential alternative as demonstrated for phosphohistidine. Several groups have reported the

development of phosphorous-carbon building block mimetics of phosphohistidine (**12**-**14**) (Figure 3). Both the Boc- and the Fmoc-building blocks are assembled *via* Cu(I) catalyzed cycloaddition reaction of Fmoc-protected azidoalanine and di-ethyl or di-benzyl ethynylphosphonates. These building blocks can be used during SPPS to deliver the site-specifically phosphorylated histidine peptide mimetics after TFA or HF cleavage.[64,65]

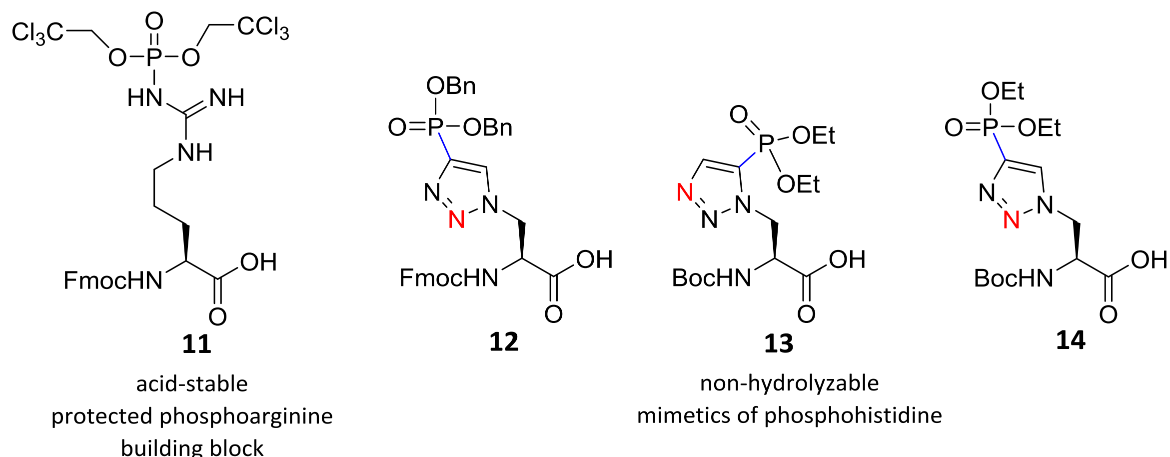
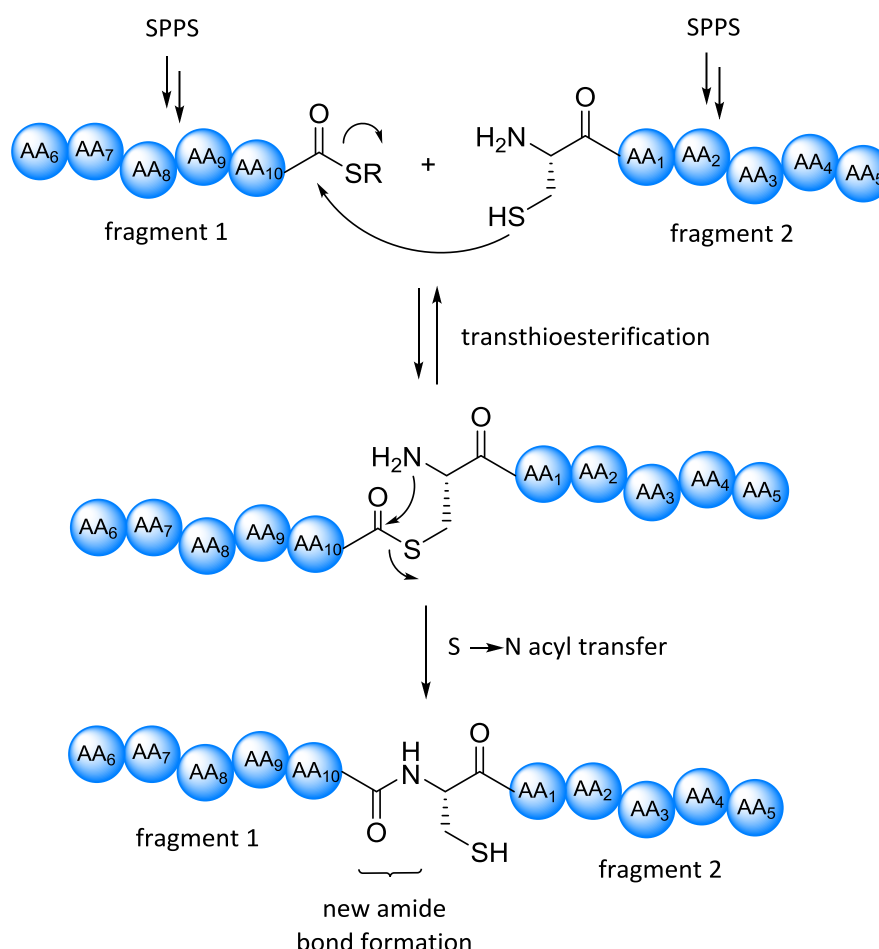


Figure 3. Compatible *N*-phosphorylated building blocks for SPPS.

Post-assembly approaches in the solution-phase using potassium phosphoramidate as phosphorylating reagent have been described for phosphorylated histidine and lysine-containing peptides.[66,67] Nevertheless, due to the acid-lability of the P(═O)–N bonds, those approaches are not applicable on an acid-labile solid-phase resin by using a post-assembly approach and that is why the site-specific incorporation of the phosphorylated residue is not possible.

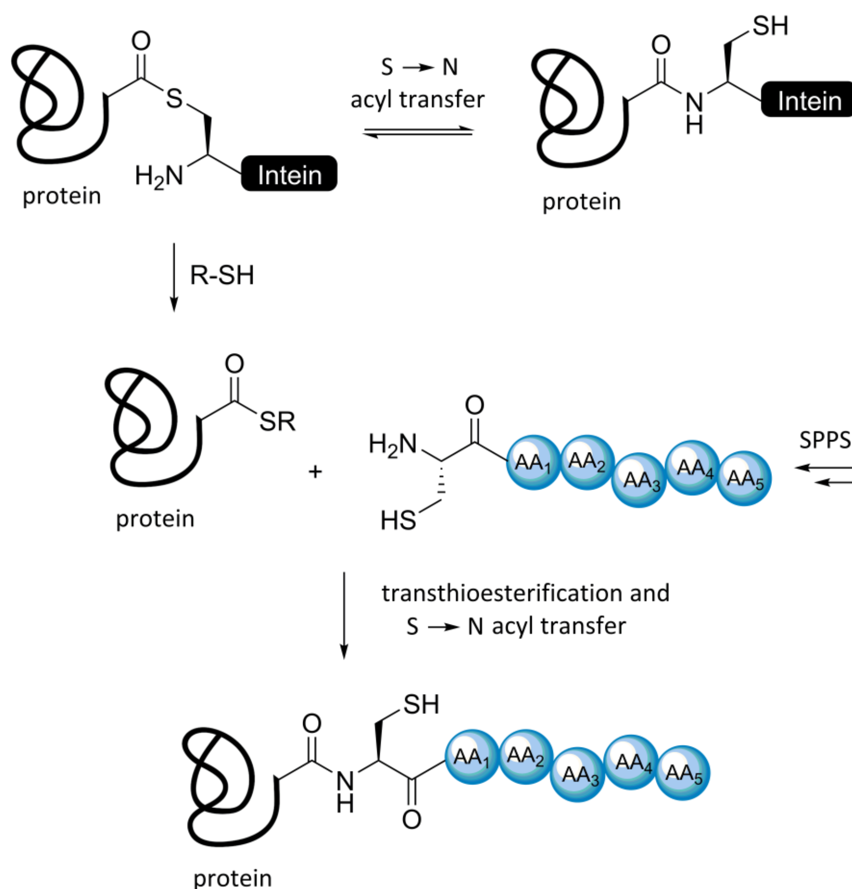
1.3.1.2 Native chemical ligation (NCL) and expressed protein ligation (EPL)

NCL appears as the method of choice to generate full-length proteins by synthetic means.[68] The method, developed in 1994 by Kent and co-workers, relies on assembling two unprotected fragment peptides that have been synthesized previously by SPPS either using Boc- or Fmoc-SPPS (Scheme 6). To ligate both fragments together, one of the fragments must carry a *C*-terminal thioester while the other must carry an *N*-terminal cysteine residue. Then, the thiolate from the *N*-terminal cysteine attacks the *C*-terminal thioester to allow the transthioesterification. Finally, after an *S*-->*N* acyl transfer, the amide bond is formed. Since the reaction is chemoselective, it can be carried out with unprotected peptides and in aqueous buffers.[69]



Scheme 6. Native chemical ligation (NCL).

Without any doubt, NCL overcomes the size limitation of SPPS as it is evident from the literature available about proteins made by this method. However, the corresponding fragments from larger proteins up to 150-200 residues may be challenging to synthesize by SPPS. In order to increase the range of proteins available with NCL, Cole and co-workers developed the so-called expressed protein ligation (EPL) technique.[70] The chemistry behind EPL is the same as in NCL, being the only difference that one of the fragments is an expressed protein (Scheme 7). Since one of the fragments is required to contain a thioester, the protein is expressed as intein construct which after thiolysis forms the corresponding *C*-terminal thioester. Its reaction with the *N*-terminal cysteine fragment, synthesized previously by SPPS, delivers the protein of interest. EPL is known as a semisynthetic approach since it combines biochemistry with organic synthesis.



Scheme 7. Expressed protein ligation (EPL).

Several examples showed the potential of NCL and EPL to introduce PTMs on proteins.[71] In the context of phosphorylation, Cole and co-workers demonstrated the utility of EPL by ligating a phosphotyrosine peptide to the *C*-terminus of the protein kinase *C*-terminal Src kinase (Csk).[70] The phosphotyrosine peptide was prepared by Boc SPPS while the 50 KDa Csk protein bearing a *C*-terminal thioester was expressed using intein splicing. It is important to note that neither the size of the protein, nor the site-specific incorporation of the phosphorylation would have been possible to make by chemical or enzymatic methods. Muir and co-workers undertook a more challenging task by preparing a tetraphosphorylated TGFβ receptor protein using EPL.[72] In that case, the fragment containing the thioester was the tetraphosphorylated *N*-terminal peptide (175-195) that was synthesized by Fmoc-SPPS, while the protein fragment (196-503) TGFβ was expressed containing a mutated cysteine at the *C*-terminus (L196C). The access to a homogenous preparation of tetraphosphorylated TGFβ receptor protein has allowed to study the molecular mechanism behind this receptor.[72,73]

1.3.2 Convergent assembly using chemoselective reactions

In principle, the convergent assembly strategy using chemoselective reactions offers the advantage of a more ready and flexible modification of a well-defined and fully-folded peptide or protein structure.[53] The concept behind this strategy relies on the potential of chemoselective reactions. Generally, a chemical tag is incorporated in the amino acid to be modified. This step can be performed either on a peptide level by using a building block approach as well as on proteins by amber suppression or selective pressure incorporation (SPI) methods. Then, a small molecule reagent carrying the PTM reacts in a chemoselective manner towards the chemical tag on the peptide or protein structure to form the native or mimetic PTM, leaving the other side chains intact (Figure 4). Ideally, the incorporation of the native PTM is desired. However, this is not always feasible and in some cases the installation of a mimetic is a good compromise. Chemists have looked at chemical reactions to exploit them as powerful chemoselective tools to incorporate PTMs.[74] To date, mainly two chemoselective reactions have been exploited to generate well defined native or mimetic post-translationally modified peptides and proteins: The copper-catalysed azide–alkyne

cycloaddition (CuAAC) reaction and the Staudinger-type reactions (*see 1.3.2.1*). The former targets unnatural substrates on proteins such as azides and alkynes while the latter only azides. In addition, other site-selective approaches targeting different substrates have also been developed to cover the complex access to post-translationally modified peptides (*see 1.3.2.2 and 1.3.2.3*). For instance, the scarcity of cysteine in nature (accounting for 2% in eukaryotic organisms) and its unique reactivity has been exploited as substrate to perform site-selective conjugate additions of mimetics to cysteine and dehydroalanine residues (Figure 4).

The next subchapters focus on these chemoselective and site-selective strategies to generate post-translationally modified peptides and proteins, putting the focus on the chemical reactions used and their application to generate site-specifically phosphorylated peptides among other PTMs.

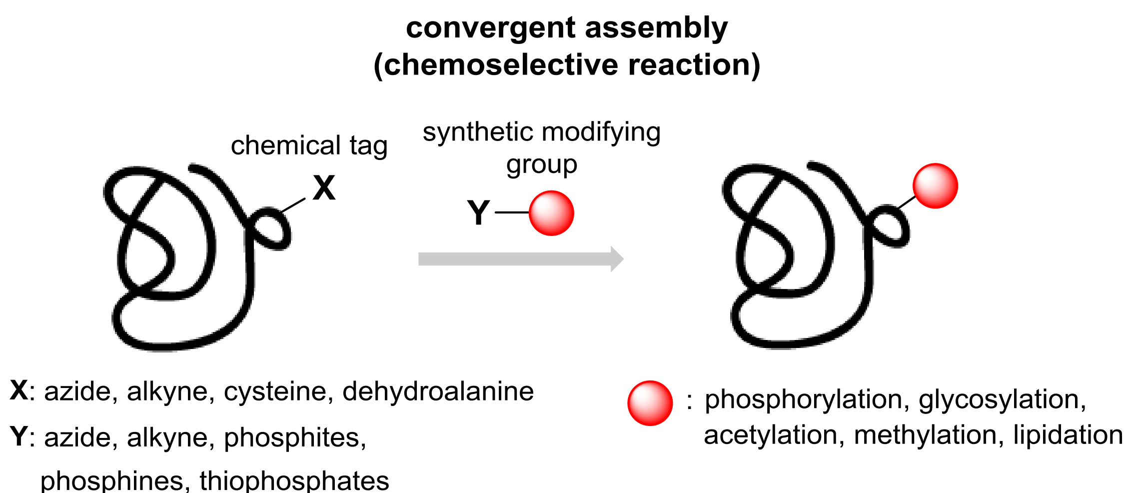
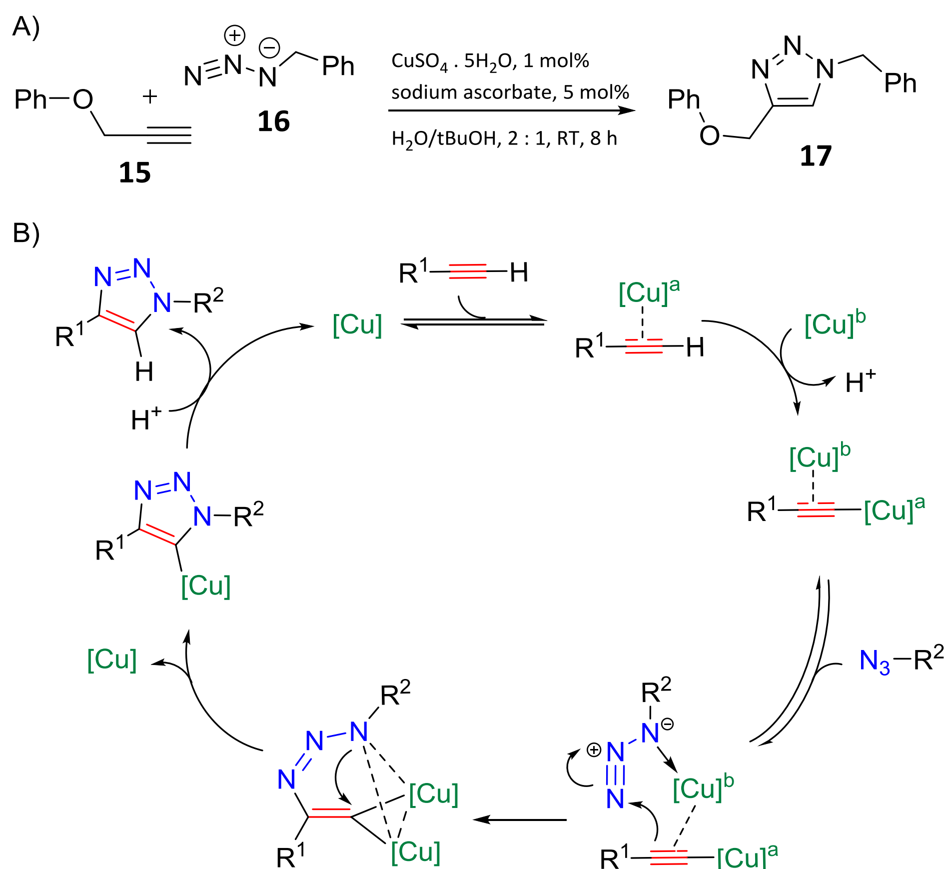


Figure 4. Convergent assembly using chemoselective and site-selective strategies.[53]

1.3.2.1 Site-selective strategies based on the modification on azides and alkynes

1.3.2.1.1 Copper-catalyzed azide-alkyne cycloaddition (CuAAC)

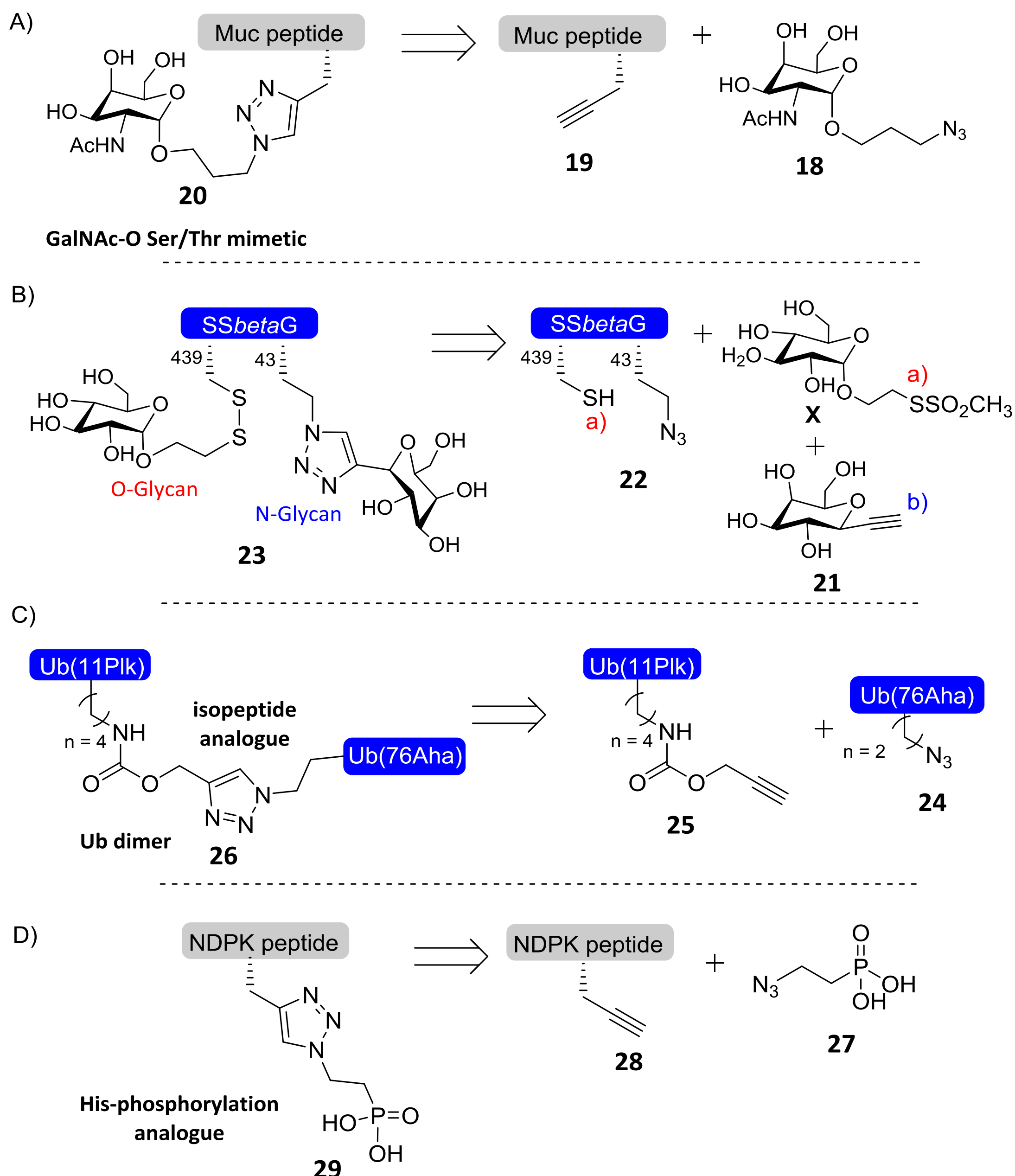
In the CuAAC an azide **16** reacts with a terminal alkyne **15** catalyzed by Cu(I) to form 1,4-substituted 1H-1,2,3-triazoles **17** at room temperature and in aqueous solution (Scheme 8).[75,76] The reaction was discovered in parallel by Meldal and Sharpless, and it is a variant of the Huisgen 1,3-dipolar cycloaddition that proceed at high temperatures and without Cu(I).[77] To date, many researchers have worked in deciphering the mechanism of the CuAAC. Early hypothesis suggest a stepwise catalytic cycle where the formation of the acetylide-azide-copper complex was believed to proceed *via* the formation of a monomeric copper acetylide complex.[75] Nevertheless, subsequent studies have suggested and demonstrated that more than one copper may be involved in the formation of the chemical complex, leading to, at least, a binuclear pathway for this reaction.[78]



Scheme 8. A) CuAAC reaction and B) the proposed catalytic model.[78]

The CuAAC is the most well-known “click” chemistry reaction, a concept introduced by Sharpless, who defined “click chemistry” as a set of reactions that are high-yielding, modular, stereospecific, use easy accessible starting materials/reagents and generate inoffensive byproducts. Along these lines, these byproducts must be easily removed by non-chromatographic methods. Ideally the use of a friendly solvent such as water is preferred and the final product isolation must be performed using simple methods such as crystallization or distillation. CuAAC has been widely used as a bioconjugation reaction for *in vitro* and to a far lesser extent for *in vivo* labeling of living cells.[79] The toxicity caused by the Cu(I) catalyst makes it incompatible for this latter application. To circumvent the Cu(I) toxicity alternative approaches have been developed. For instance, strained cyclooctynes react with azides without the requirement of Cu(I) but with slower kinetics.[80] Another approach is the use of water-soluble ligands for Cu(I) such as THPTA. The Cu(I)-THPTA ligand catalyst acts as a sacrificial reductant for oxidative species generated by the Cu(I)-catalyzed reduction of oxygen by ascorbate.[81]

The CuAAC has been used to address the incorporation of different type of PTMs mimetics on peptides and proteins. For instance, Brimble and co-workers have used unprotected sugar azides **18** and peptides containing one or more propargyl groups **19** to access MUC1 neoglycopeptides **20** with a variable number of GalNAcα1-*O*-threonine/serine mimics for biological evaluation (Scheme 9A).[82] Davis and co-workers have also used the CuAAC to install a *N*-glycan mimetic **23** in a SSβG enzyme to study their ability to bind to known protein readers (Scheme 9B).[83,84] Others have also developed strategies to install carbohydrate derivatives on proteins by exploting the CuAAC.[85-87] Rubini and co-workers have employed a very easy and efficient method to access site-specifically linked ubiquitin dimers **26**, which were further used to confirm their feasibility as analogues through several enzymatic assays (Scheme 9C).[88-91] In the context of phosphorylation, Brimble and co-workers have used the CuAAC reaction to click propargylated unprotected peptides **28** with 2-azidoethyl phosphonic acid **27** to provide site-specifically phosphorylated histidine mimetic-containing peptides **29** (Scheme 9D).[92]



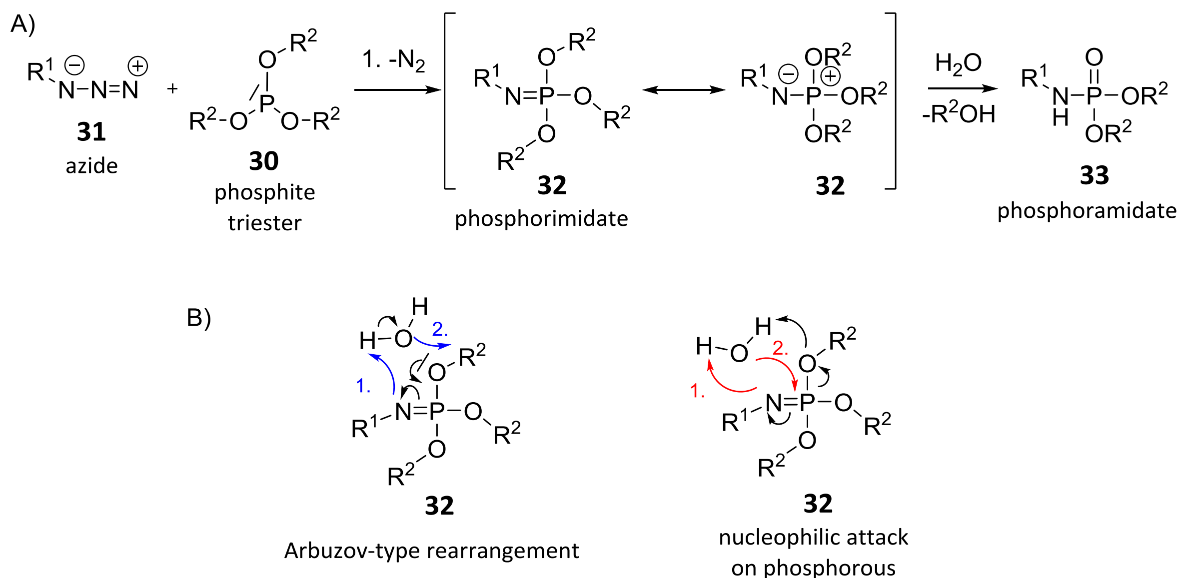
Scheme 9. Applications of the CuAAC to install mimics of PTMs.

1.3.2.1.2 Staudinger-type reactions

1.3.2.1.2.1 Staudinger-phosphite reactions

The Staudinger-phosphite reaction is a Staudinger-type reaction that is based on the reaction of phosphites esters **30** and azides derivatives **31** to form phosphoramidates **33** and it was first described by Kabachnik and co-workers.[93] However, its application as a

chemoselective tool for site-specific modification of biomolecules was recently discovered by Hackenberger and co-workers.[94-96]

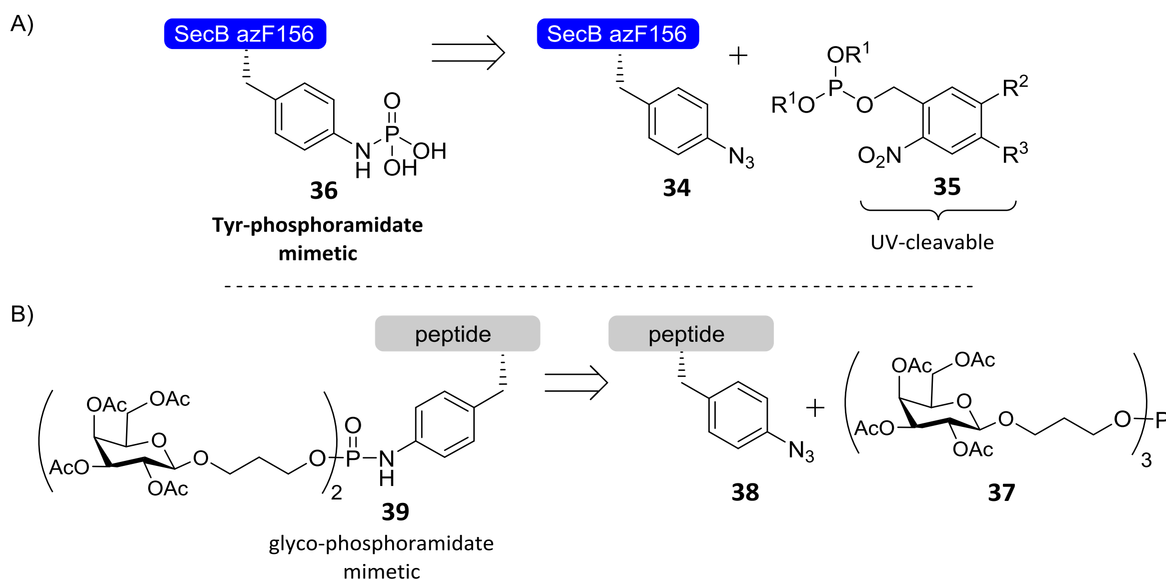


Scheme 10. A) Staudinger-phosphite reaction mechanism and B) hydrolysis pathways of phosphorimidate

The mechanistic pathway of the Staudinger-phosphite reaction is analogues to the classical Staudinger reduction with the difference that the hydrolysis of the phosphorimidate **32** leads to a phosphoramidate product **33** instead of the amine reduction product (Scheme 10A). This is due to the fact that phosphites **30** contain an alkoxy group that is a better leaving group than the corresponding amine. Mechanistically speaking, the hydrolysis of the phosphor-imidate **32** to the –amidate **33** can occur *via* different routes (Scheme 10B): The Arbuzov-type rearrangement where water attacks at the alkyl group or a purely nucleophilic attack of water at the phosphorous atom.[97,98] Both mechanisms seem to compete and to be dependent on the pH of the solution, as it has been demonstrated by ^{18}O-labeling experiments.[98] However, the chemical identity of the alkoxy R^2 group plays also an important role on which mechanism is favoured over the other as it has been demonstrated by Hackenberger and co-workers.[99]

In the context of PTMs, the first use of the Staudinger-phosphite has installed a PTM mimetic, in the chemical phosphorylation of tyrosine residues.[94,100] To do so, Hackenberger and co-workers installed first the *p*-azido phenylalanine amino acid in **34** either on a peptide

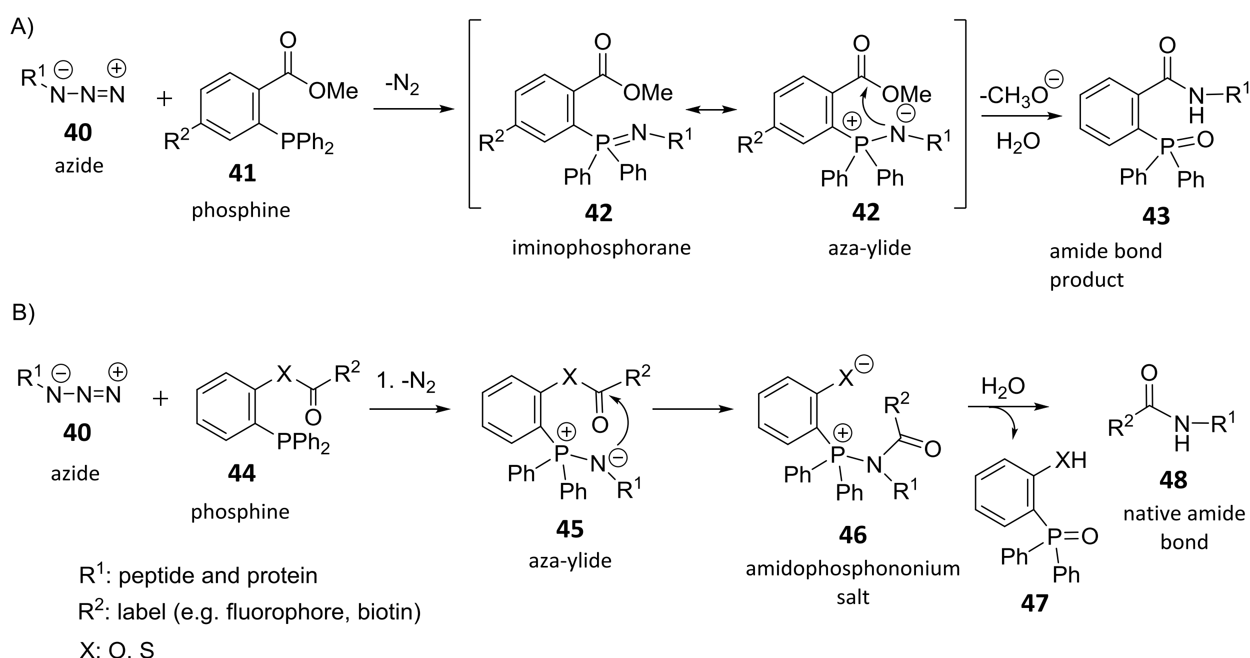
or protein level. Then, its reaction with *o*-nitrobenzyl based phosphite triester **35** led to the formation of a UV-cleavable phosphoramidate, which upon UV-irradiation delivered a phosphoramidate tyrosine mimetic **36** (Scheme 11A). Remarkably, the reaction proceeded quantitatively on proteins delivering the phosphotyrosine mimetic **36**, which was further identified by using a well-known phosphotyrosine antibody.[94] It is important to note, the importance of this synthetic approach to study tyrosine phosphorylation since provide a more direct access to phosphotyrosine residues compare to the combination of other synthetic methods such as SPPS and EPL. In addition, the incorporation of phosphotyrosine residues on proteins by engineering methods is not possible yet. The Staudinger-phosphite reaction has also been applied to introduce two carbohydrate residues as mimetics of glycol-phosphodiesters by reaction of azido containing peptides **38** with glycosyl phosphites **37** (Scheme 11B).[101] It is important to indicate that none of both cases targeted any natural substrate where the modification occured. Yet, the methodology reported might be applicable to a potential target where either phosphotyrosine or phosphoglycosylation is present.[102]



Scheme 11. Applications of the Staudinger-phosphite reaction to A) install a mimic of phosphotyrosine and B) a mimic of phosphoglycosylation.

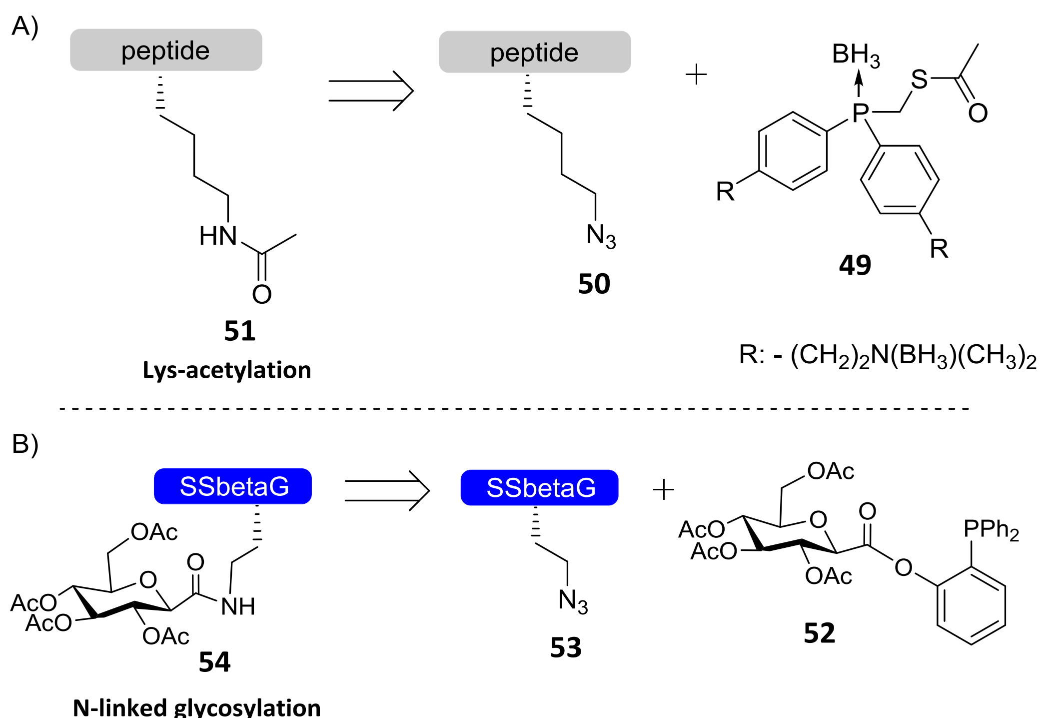
1.3.2.1.2.2 Traceless Staudinger ligation

The traceless Staudinger ligation is an evolution of the non-traceless Staudinger ligation and was discovered simultaneously by the laboratories of Bertozzi and Raines in 2000.[103-105] Both reactions proceed similarly and are based on the reaction of an azide derivative **40** and a phosphine **41** containing an electrophilic trap to finally form an amide bond compound **43**. The electrophilic trap in phosphine **41**, i.e. a methoxycarbonyl group, serves as a precursor of this amide bond, being attacked by the nucleophilic nitrogen in the aza-ylide intermediate **42** (Scheme 12A). The key difference is that in the traceless Staudinger ligation, the final hydrolysis of the amidophosphonium salt **46** releases the phosphine oxide **47**, allowing the formation of native amide bond **48** without harboring any non-natural derivative (Scheme 12B). This is due to the design of the phosphine **44**, which contains two key elements: An ester cleavable linker between the acyl group and the aryl ring (X: S, O), and two aromatic groups as phosphine substituents to impart stability towards the oxidation of the phosphine **47** (Scheme 12B). Both reactions have extensively used as a bioconjugation reaction to label proteins both *in vivo* and *in vitro*.[106,107] The main advantage of the Staudinger ligations over the CuAAC is that the azide and the phosphine, as well as the by-products generated, are non-toxic.



Scheme 12. A) non-traceless Staudinger ligation and B) traceless Staudinger ligation.

The chemoselective potential of the traceless Staudinger ligation has been applied to address peptide acetylation and *N*-glycosylation.[108,109] Hackenberger and co-workers designed a phosphinothioester **49** which contains an acetyl group as the acyl group to be transferred to the nitrogen in the aza-ylide intermediate (Scheme 13A). In order to probe the feasibility of the phosphine as an acetylating reagent for peptide and proteins, a water-soluble phosphine **49** was reacted with an azidonorleucine-containing peptide **50**. Formation of site-specifically acetylated lysine peptide **51** was observed together with amine formation (Scheme 13A).[108] Despite the formation of the amine as byproduct, this protocol is expected to be very useful to access native acetylated peptides both on peptides and proteins. Regarding *N*-glycosylation, Davis and co-workers have explored the installation of *N*-linked glycans on proteins by reacting a carbohydrate phosphine **52** and an azidohomoalanine-containing protein **53** (Scheme 13B).[109] The *N*-linked glycoprotein mimic **54** contains a “reverse amide” linkage instead of the natural *N*-linked glycans that provides a key advantage in the context of stability towards hydrolases.



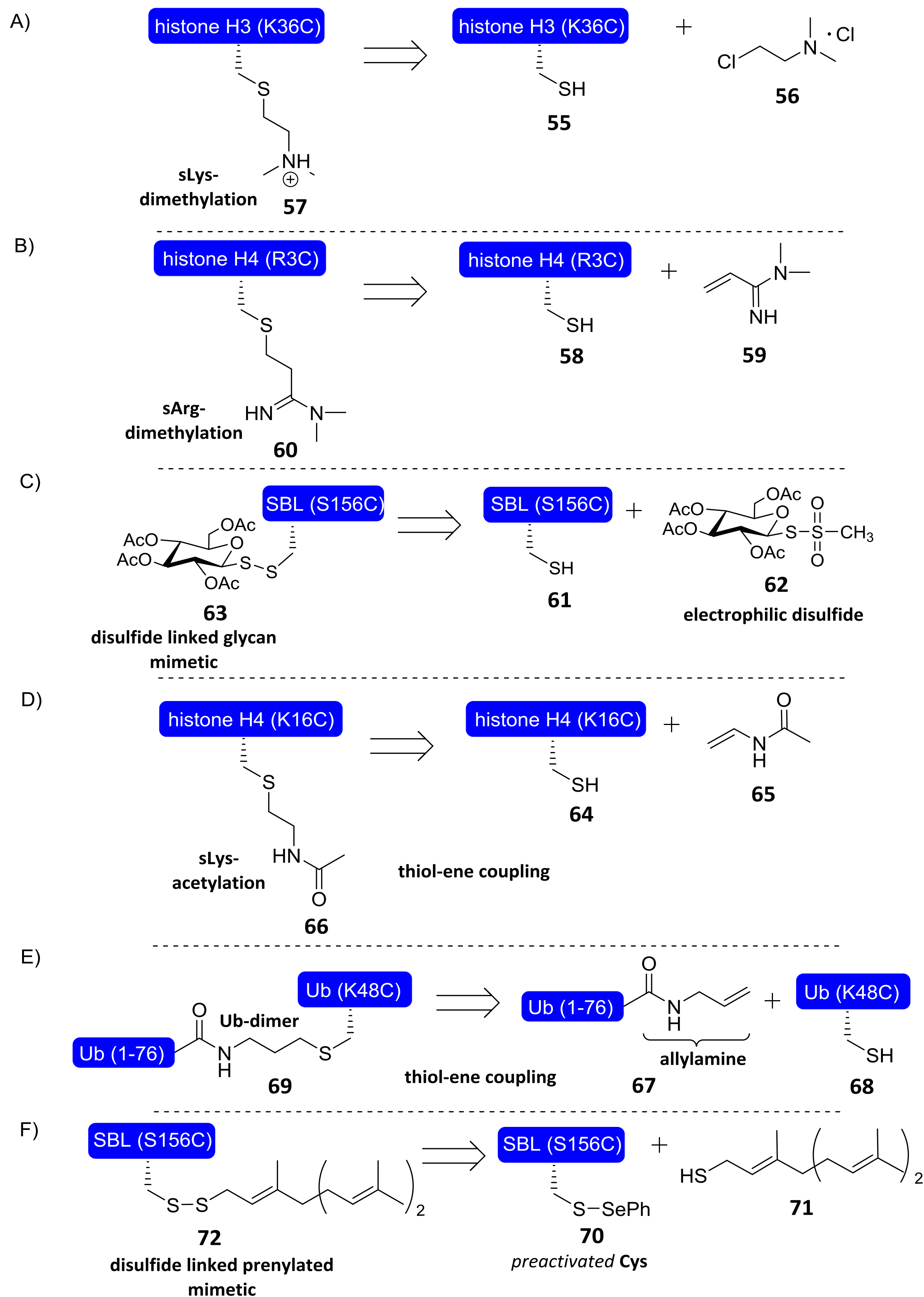
Scheme 13. Applications of the traceless Staudinger ligation for A) lysine-acetylation and B) *N*-glycosylation.

1.3.2.2 Site-selective strategies based on the modification on cysteine and dehydroalanine residues

For site-selective modification of proteins, cysteine is a very interesting target due to its strong nucleophilicity and its low natural abundance in eukaryotic organisms. On top of that, cysteine residues can be incorporated by standard site-directed mutagenesis at a predetermined site on a protein. This fact is exploited by chemists and biochemists to react the nucleophilic thiol towards different kinds of soft electrophiles to install different kinds of chemical tags.[110,111] In such a way, a cysteine residue can be alkylated, oxidized to form a disulfide, desulfurize to alanine, and allow metal-mediated modifications such as olefin metathesis.[112] In addition, cysteine residues can be converted to dehydroalanine residues *via* different elimination procedures. For instance, cysteine residues can be converted first to electrophilic disulfides upon reaction with 5,5'-dithiobis-2-nitrobenzoic acid (Ellman´s reagent or DTNB) and then eliminated to dehydroalanine after addition of tris(dimethylamino)phosphine (HMPT) or upon incubation with a base such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU).[113] Alternatively, dehydroalanine can also be obtained upon oxidative elimination of cysteine using *O*-mesitylenesulfonylhydroxylamine (MSH).[113] Thus, dehydroalanine can also be used as a Michael acceptor for thiol nucleophiles and thereby it is another target residue to incorporate different chemical tags.

In the area of PTMs, both cysteine and dehydroalanine have been used as precursors for a site-selective installation of different PTM mimetics. For instance, Shokat and co-workers have developed a chemical approach which is based on the aminoethylation of cysteine residues to install analogs of mono-, di- or tri- methyl lysine into recombinant histones (Scheme 14A).[114] Site directed mutagenesis allowed the incorporation of a cysteine mutant at K36C from histone H3 (**55**), which after reaction, for instance, with a commercially available (2-haloethyl) amine **56**, afforded a dimethyl-lysine mimetic **57**. With the corresponding analogues in hand, they demonstrated that those mimetics are functionally similar to their natural counterparts *via* recognition with known antibodies and *via* binding to HP1 chromodomain, a very well-known reader of methylated K9 in histone H3. In a very similar fashion, Fujimori and co-workers have been able to incorporate methyl arginine into

recombinant histones as well (Scheme 14B).[115] As before, the site of methylarginine analogue installation in **60** was directed by chemoselective reaction with a mutant cysteine residue of histone H4 (R3C) **58** and synthetically prepared α,β-unsaturated amidine scaffolds **59** with various degrees of *N*-methylation. Antibodies to endogenous methylarginine, as well as a native methylarginine-specific binding domain, were used to recognize the feasibility of those amidine analogues. The Jones and Davis laboratories have also used the reactivity of cysteine to introduce carbohydrate mimetics through the formation of disulfide bonds. The active glycoform of the protease Subtilisin (SBL) was accessed by reacting a SBL cysteine mutant **61** towards glycosyl methane- **62** or phenylthiosulfonates as thiol selective electrophile agents thus generating the corresponding disulfide linkages mimetic **63** (Scheme 14C).[116-118] It is important to note that, by combining this method with the CuACC, dual labeling can be performed, allowing the incorporation of two different type of glycosylations, *N*-glycans and *O*-glycans (Scheme 9B).[83] Liu and co-workers have described another direct method for site-specific peptide and protein acetylation by using a thiol-ene radical addition (Scheme 14D).[119] The reaction between a mutant cysteine protein **64** and *N*-vinyl-acetamide **65** formed straightforwardly the acetyl-thialysine protein **66** upon addition of a radical initiator and UV light. The corresponding sLys(Ac) analogue **66** has shown to be a good functional mimic of the natural Lys(Ac) as it was recognized by a specific anti-H4K16Ac antibody. By using the same thiol-ene reaction, Strieter and co-workers have shown how to merge in a site specific manner two ubiquitin proteins (Scheme 14E).[120] The final *N*ε-Gly-L-homothialysine isopeptide bond in **69** is only one bond longer than the native linkage, which they have demonstrated not to affect the ubiquitin-ubiquitin dimer function. A method to target prenylation has also been developed by the Davis and co-workers (Scheme 14F). The method relies on a preactiviation of a cysteine mutant protein to its phenyl selenyl sulfide intermediate **70** after addition of phenylselenenyl bromide.[121] Then, its reaction with a prenylated thiol **71** delivered the prenylated disulfide mimetic **72**. Although this method was demonstrated only for a non-prenylated protein, it should be compatible with real prenylated protein targets.



Scheme 14. Applications of the cysteine reactivity for A) - B) lysine-, arginine-methylation respectetively, C) glycosylation, D) and E) lysine-acetylation and -ubiquitination, F) prenylation.

The dehydroalanine moiety has also been addressed as a chemical tag to install PTM mimetics. One of these examples is the site-specific incorporation of phosphocysteine residues in proteins.[122] The method developed by Davis and co-workers relies on the conjugate addition of thiophosphate **74** to a dehydroalanine containing protein **75** to deliver a racemic phosphocysteine protein **76** (Scheme 15A). The corresponding dehydroalanine protein **75** was generated *via* oxidative elimination of a cysteine residue **73** previously incorporated by mutagenesis at the site of interest. By employing this method, different phosphocysteine proteins were generated to demonstrate its ability to mimic phosphoserine and phosphothreonine in different scenarios.[122-125] Although its power as mimetic seems to be promising, the chemical lability of the phosphorothioate linkage at physiological pH can be considered as a drawback for future applications. Similarly, Schultz and co-workers have installed an acetylated mimic (H3KAc9) into histone H3 **80** upon reaction of an acetylated alkyl thiol **78** and a dehydroalanine-containing histone protein **79** (Scheme 15B).[126] Interestingly, here the dehydroalanine residue in **79** was generated upon oxidation of a phenylselenocysteine residue **77** incorporated previously by amber suppression at the position of interest, i.e., K9. Despite that the final product lacks the correct stereochemistry, the chemically modified H3 protein could be recognized by the histone deacetylase enzyme (HDAC3). Analogously to using cysteine as substrate, dehydroalanine has also been employed as a potential residue to introduce cysteine prenylation. Van der Donk and co-workers have focused on the *N*-Ras protein, which is known to be prenylated at cysteine residues. The methodology involved the introduction of a dehydroalanine into peptides **83** *via* oxidation of phenylselenocysteine **81**, which was first introduced by standard SPPS on a peptide level (Scheme 15C).[127] Subsequent chemoselective conjugate addition of a nucleophilic thiol precursor, such as a triisopropylsilyl protected farnesylthiol **82**, led to the formation of the corresponding racemic prenylated *N*-Ras peptide **84**.

In general, despite the versatility that dehydroalanine residues possess as potential site to incorporate different chemical tags to mimic PTMs, it is constrained by lacking diastereoselectivity and thereby it does not deliver the PTM in its right stereochemistry. In addition, cysteine and phenylselenocysteine mutations as well as chemical transformations

on proteins are not always feasible or not easy to perform, which in some cases result in low-yielding reactions.[128]

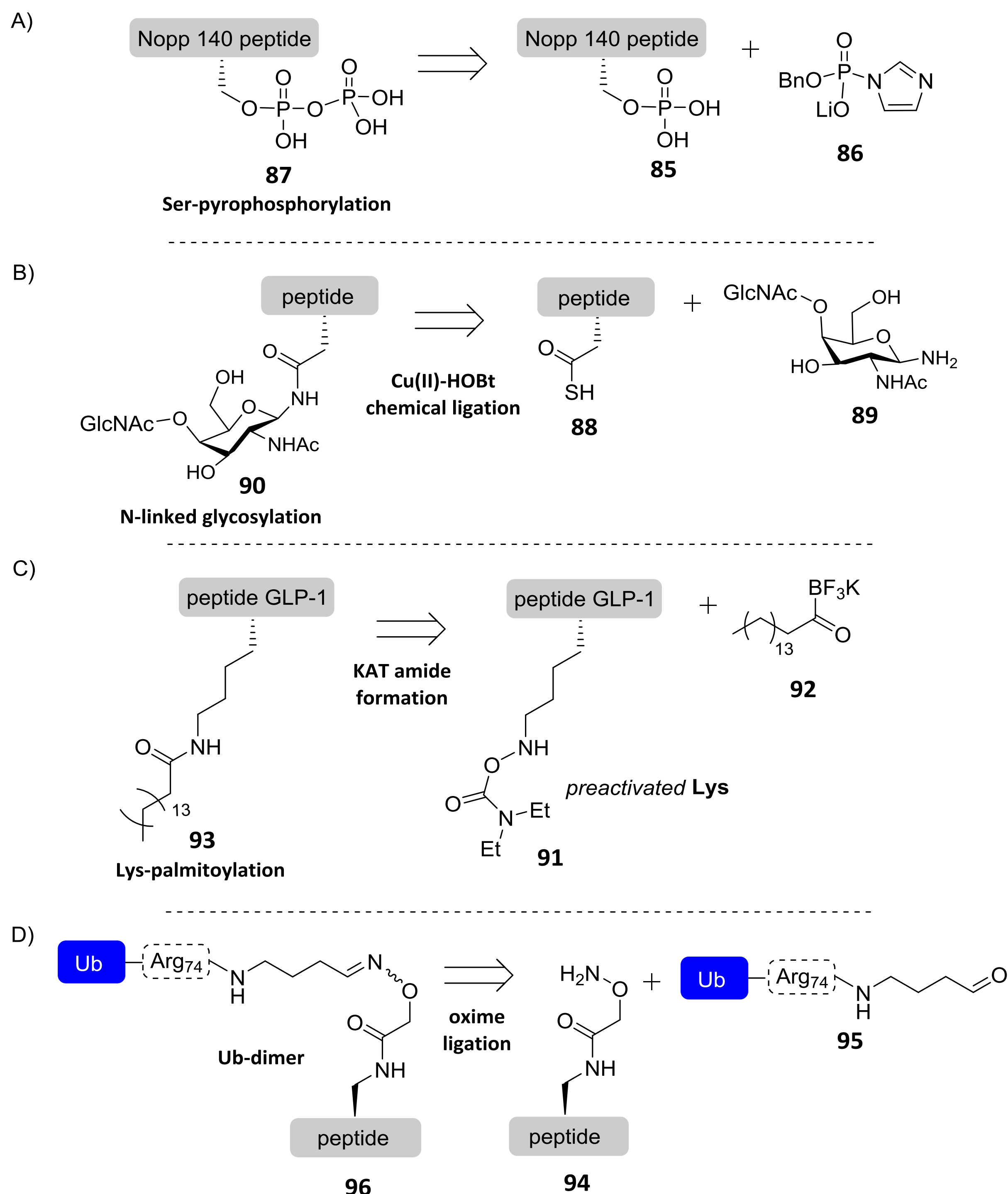
Scheme 15. Applications of the dehydroalanine reactivity for A) cysteine-phosphorylation, B) lysine-acetylation, and C) cysteine-prenylation.

1.3.2.3 Other chemoselecitve and site-selective chemical approaches

In addition to the well-known chemical approaches explained in the previous chapters, there are other site-selective and chemoselective strategies, which have been developed for the synthesis of post-translationally modified peptide and proteins. This is the case for serine pyrophosphorylation, a poorly understood PTM.[129,130] The acid-lability of pyrophosphorylated serines made SPPS-based methods not feasible for the incorporation of pyrophosphorylated residues on peptides. Thus, Fiedler and co-workers have developed a method that provides easy access to pyrophosphopeptites **87** by exploiting the nucleophilicty of phosphorylated serine peptides **85** with phosphorus (V) electrophiles reagents **86**, without side-reactivity with other amino acid side chains (Scheme 16A).[131] With this chemical tool in hand, they have synthesized a variety of site-specifically modified serine pyrophosphorylated peptides to probe the specificity of different phosphatases as well as to develop affinity reagents towards serine pyrophosphorylated peptides.[132,133] The combination of the "Fiedler approach", together with the site-specific incorporation of phosphoserine residues on proteins, might also allow the incorporation of pyrophosphorylation residues into proteins in the future.[54] A significant improvement to access *N*-glycosylated peptides in a site-specific manner was reported recently by Garner and co-workers by developing a chemoselective Cu(II)-HOBt promoted chemical ligation method (Scheme 16B).[134] Instead of using aspartate as substrate, they used a thioacid derivative in peptide **88**, which can be incorporated in three steps from an orthogonal protected aspartate – containing peptide. Then, its reaction with different glycosyl amine substrates **89** gives a native *N*-linked glycopeptide fragment **90**, which resembles the native asparagine *N*-linked glycosylated fragments. It is worth noting that no aspartimide formation is observed, a common problem with reported *N*-glycosylation procedures.

Bode and co-workers have developed to date the only available chemoselective approach to install palmitoylated residues on peptides such as in **93** (Scheme 16C). To achieve that, they reported a chemoselective amide-forming ligation using potassium acyltrifluoroborates (KATs) **92** and *O*-carbamoylhydroxylamines containing peptides **91**, which proceed in the presence of all unprotected functional groups.[135] Ovaa and co-workers have used the

chemoselective oxime ligation to form site-specifically linked ubiquitin-isopeptide isosteres **96**, which are stable upon incubation with deubiquitinating enzymes (Scheme 16D).[136]



Scheme 16. A) serine-pyrophosphorylation, B) *N*-linked glycosylation, C) lysine-palmitoylation, and D) ubiquitination.

1.4 Mass spectrometry-based tools for studying PTMs

After the discovery of most human genes, understanding the vast complexity of the protein expression has become the main goal to gain information about the biological processes occurring in a cell, tissue or organism. The fact that proteins can be modified after translation, increases this complexity and makes its analysis much more complex. To understand the relation of gene expression and phenotypes, a new area in science has emerged: Proteomics.[137] Per definition, proteomics is the large-scale study of proteins, usually by biochemical methods.[137] In the first era of proteomics, researchers started to build databases of proteins using two-dimensional gel electrophoresis.[137] Despite this methodology provided the first insights into the complexitiy of the expressed proteins existing in the proteome, it lacked sensitivity and precision, but most importantly it could not be used alone as a characterization tool. The big step forward on this field occurred when mass spectrometry (MS) evolved to be the “tool” to not only identify but also quantify the proteome of living cells or organisms.[138,139] All this was possible because of the great contributions that techniques like ESI and MALDI made in the field of peptide and protein analysis (*see 1.4.1*).[140,141] Along these lines, it is worth recalling that the the Nobel Prize in chemistry in 2002 was awarded to J.B. Fenn and K. Tanaka for their work in the development of ESI and MALDI, respectively. MS alone did not solve the technical issues present when analyzing such diverse and complex samples. Liquid chromatography (LC) systems coupled to MS as well as additional separation techniques together with enrichment strategies for specific proteins or PTMs have reduced the complexity and thereby made possible to perform proteome analysis (*see 1.4.2*).

The quantification of the proteome is also an essential information to gain knowledge about its dynamic nature and how this is influenced by different external or internal changes. Thereby, the combination of MS with different labeling techniques has allowed researchers to quantify the abundance of desired target proteins in different biological systems (*see 1.4.3*). Among all these MS-based strategies, peptide fragmentation by tandem MS (MS/MS) is probably the most crucial step because it is needed to identify and localize the PTM site (*see 1.4.4*). All these technical contributions are discussed in more detail in the next sub-

chapters. It is worth noting that excellent reviews about MS-based proteomics can be found in the literature.[138,142,143]

1.4.1 Ionization techniques

MALDI and ESI are considered soft ionization techniques. Both differ from each other in the ionization process as well as in sample preparation, which make the analysis of a wide range of different type of samples possible. MALDI ionization requires that the analyte of interest is mixed previously with an excess of UV absorbing- and acidic-matrix to aid the protonation of the molecule. After the mixture of analyte and matrix crystallized under high vacuum, the sample is irradiated with UV light, which excites the matrix structure and allows the sample to reach the gas phase and drift towards the detector (Figure 5A).[141] In ESI, the analyte is mixed with a mixture of polar and volatile solvent. Then, the sample is put through a capillary in which a high voltage is applied, charging the proteins and peptides in the solvent. This mixture is guided through to an evaporation chamber under high vacuum causing solvent evaporation and thereby formation of ionized droplets which are finally driven towards the MS detector (Figure 5B).[140] MALDI is very useful for peptide and protein analysis due to its rapid analysis, high sensitivity, high accuracy and its tolerance to heteregenous samples like proteolytic digests.[144] On top of that, it generates mostly MS spectra of singly charged molecules. Nevertheless, limitations concern the analysis of peptides with a molecular weigh lower than 600 Da, the analysis of peptide/proteins containing fluorophores and UV-cleavable moieties, and the difficulty to deal with samples containing high salt concentration.

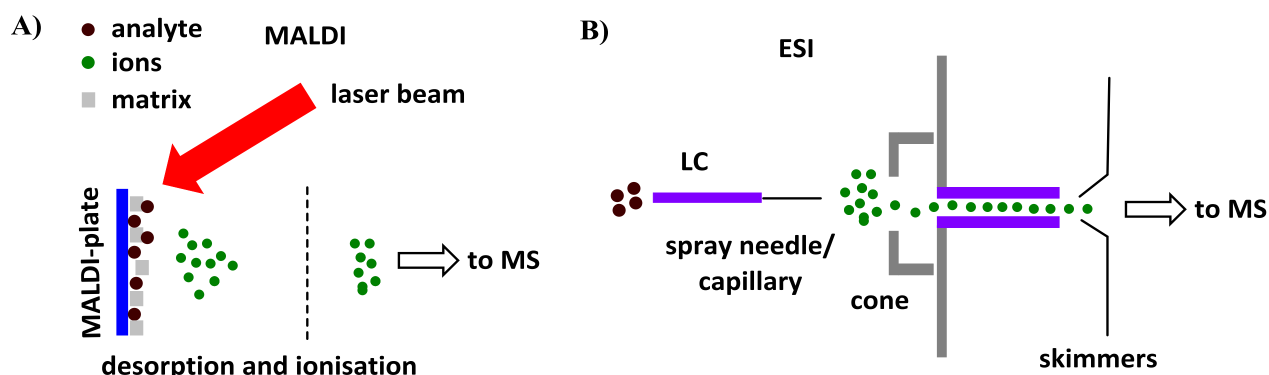


Figure 5. Schematic of a A) MALDI and B) ESI interface.

The advantages of ESI are fairly similar to MALDI: High accuracy, rapid analysis and high sensitivity. A significant advantage is the possibility to generate MS spectra with multiple charging states, which allows the analysis of proteins with a limited *m/z* range for MALDI. Another key advantage of ESI over MALDI is the straightforward assembling with LC systems. This is due to the significant improvements in MS and specially with the emerging of quadrupole-TOF and Orbitrap based instruments equipped with ESI.[145] This fact has made LC-MS (ESI) instruments much more convenient for proteomic analysis basically because of their speed. Another advantage of ESI LC-MS systems is that they can implement different types of fragmentation techniques like electron-transfer dissociation (ETD) or electron-capture dissociation (ECD), whereas MALDI LC-MS is only limited to the standard collision method, i.e., collision-induced dissociation (CID). It is important to note that for ETD and ECD fragmentation, analysis of multiple charging peptides is required to obtain a complete fragmentation in tandem MS experiments (*see 1.4.4 chapter*).

1.4.2 Separation and PTM enrichment strategies

In shotgun proteomic approaches simple LC separation does not always provide sufficient peak capacity to separate the complex mixture of peptides originated from the proteolytic digestion of cellular lysates.[138] Therefore, an additional separation step has to be performed prior LC-MS analysis. For instance, substantial progress has been made both on the protein and peptide level, by combining one- or two-dimensional gel electrophoresis (1DE and 2DE) and strong cation exchange/reversed phase prior LC-MS analysis, respectively.[146-148] Another limiting factor in proteomics is the abundance of the target peptide/protein in the complex biological sample, which is even more problematic when looking at low abundant proteins that are post-translationally modified. Therefore, there is a high need to provide methods to specifically enriched post-translationally modified peptides *vs* their unmodified counterparts.[149-151] Researchers have circumvent this issue by developing enrichment strategies like immobilized metal ion affinity chromatography (IMAC), and metal oxide affinity chromatography (MOAC) such as TiO_2, among others which are based on electrostatic interactions of the PTM against a solid support (Table 1). For instance, those methods have been exploited to enrich phosphorylated serine, thereonine

and arginine peptides.[152-154] Generally, the recovery of phosphorylated peptides over unphosphorylated peptides after the enrichment is rather low, which points towards the necessity to develop novel and more efficient strategies. Other more harsh enrichment methods rely on the chemical derivatization of the post-translationally modified peptide to attach it afterwards covalently to a solid-support (Table 1).[155-158] Along these lines, hydrazide chemistry is often used to isolate *N*-glycopeptides from cell lysates upon oxidation of the carbohydrate groups and then their coupling to hydrazide containing beads.[156] Obviously, the glycan structure is broken upon oxidation which renders impossible to elucidate its structure. *O*-glycosylation enrichment by chemical derivatization is also possible by using biotin alkynes and azide containing GalNAc carbohydrates, which can be transferred to the *O*-glycan by enzymatic coupling. However, due to the low enzymatic transfer reaction, the coverage of the *O*-glycans is rather limited.[157] Another chemical strategy relies on the removal of *O*-GlcNAc from serine and threonine containing-peptides/proteins by mild β-elimination to their dehydrated forms, i.e., dehydroalanine for serine *O*-GlcNAc and α-amino butyric acid for threonine *O*-GlcNAc.[158] The corresponding dehydrated derivatives can, for example, be enriched using thiol-based resins. The drawback of this approach is the β-elimination of phosphorylated serine and threonine peptides, which can give false-positive results.

Alternatively, biochemical approaches such as the enzymatic- and the antibody-based enrichment approach have been developed to contribute on the PTM enrichment process (Table 1). For instance, an engineered enzyme called subtiligase was used to mediate the tagging of free *N*-terminal cellular substrates generated during intracellular proteolysis. By using this enrichment method, more than 800 proteolytics sites were identified in human cells.[159] Antibody-based enrichment applications have been used for the detection of tyrosine- and histidine-phosphorylation, arginine- and lysine-methylation, lysine-acetylation and ubiquitin-like proteins (Table 1).[160-165] In the context of this thesis, Muir and co-workers have recently generated a specific phosphohistidine antibody that bears a high specificity for phosphohistidine proteins over phosphotyrosine in a sequence-independent fashion. Importantly, they have exploited the use of stable phosphohistidine mimetics for antibody generation.[163,164]

Table 1. Chemical and biochemical approaches for PTM enrichment.[149]

enrichment strategy	PTM
ionic interation-based	
IMAC, TiO_2 SIMAC	serine/threonine/arginine phosphorylation
chemical derivatization	
hydrazide chemsitry biotin alkyne-azide	*N*-glycosylation *O*-glycosylation
enzymatic-based	
subtiligase PNGase phospholipase	proteolysis N-glycosylation GPI-anchor
antibody-based	tyrosine/histidine phosphorylation arginine/lysine methylation lysine acetylation ubiquitin-like

1.4.3 Quantitative proteomics by MS

Another issue in MS-based proteomics is the direct quantification from the ion intensity of two peptides from biological samples treated under different conditions. Since the ion intensity is related to the ionization behavior of each peptide and thereby to its composition, simple direct quantification using label free methods have been considered for long as inaccurate.[166] This fact has changed recently since the application of the MaxLFQ algorithms to solve the main problems of label-free protein quantification.[167] Delayed normalization solves the problem of how to compare sample fractions that have been handled in a different manner and analyzed with different MS performance. Furthermore MaxLFQ algorithms allows to extract the maximum ratio information from peptide signals intensities to achieve the highest possible accuracy of quantification.

Alternatively, other approaches rely on the alteration of one of the biological set without influencing the ionization behavior of the peptides in MS.[143] For instance, incorporation of stable isotopes by enzymatic, chemical or metabolic labeling have emerged as a simple yet

powerful approaches which have been applied to address different biological questions across a variety of systems.[168,169] These approaches take advantage of the fact that chemically identical analytes with different stable-isotope composition can be distinguished by MS due to its mass difference. Isotope-coded affinity tags (ICAT), isobaric tag for relative and absolute quantitation (iTRAQ), ^{18}O labeling, and stable isotopic labeling amino acid cell culture (SILAC) are just some examples of *in vitro* and in *vivo* labeling methods that rely on chemical, enzymatic, and metabolic labeling (Figure 6).[170-172,175]

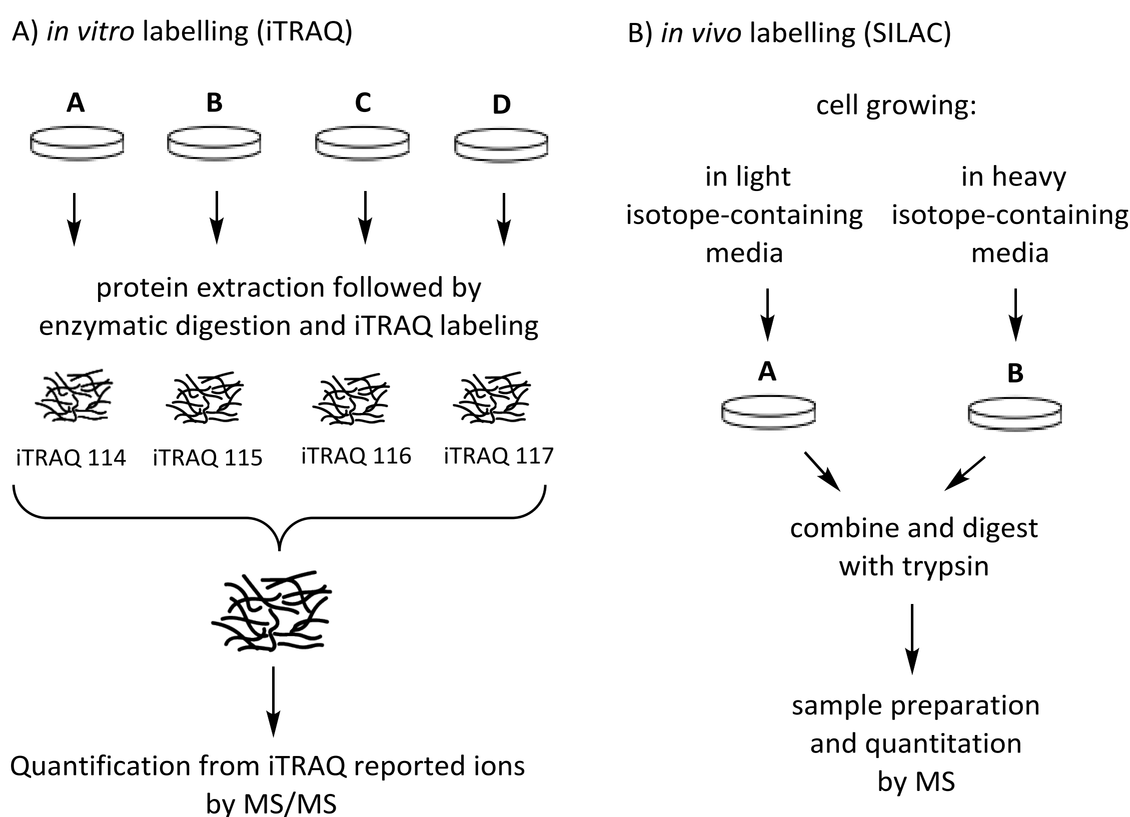


Figure 6. A) *In vitro* and B) *in vivo* labeling.[178]

ICAT consists on the incorporation of chemical tags *via* a chemical reaction occurring mainly on cysteine residues.[170] Combination of ICAT with enrichment strategies has been successfully used for quantification of low level of threonine and serine phosphoproteins.[173] Yet, the fact that only cysteine residues are targeted, limits the coverage of the proteome and specially of the PTMs.[174] iTRAQ appears to overcome the limitations of ICAT. It consists on the incorporation of isobaric mass labels *via* a chemical reaction on the *N*-terminal or lysine side chains of peptides thus potentially labeling all peptides in the proteome.[171] The

iTRAQ reagents consists of a *N*-methyl piperazine reporter group (mass 114-117), a so-called balance group (mass 31-28) and *N*-hydroxy succinimide ester. The balance group keeps the combined mass constant (isobaric) and final quantification is performed through the analyisis of the reporter groups that are generated upon MS/MS (Figure 6A). Another advantage of iTRAQ is that it allows comparision of more than two samples. In vitro labeling can also be performed by enzymatic reactions. ^{18}O labeling involves the enzymatic incorporation of two ^{18}O isotopes at the *C*-terminus of peptides generated after tryptic digestion.[172] One very important aspect of the chemical and enzymatic labeling approaches is the unlimited application to any biological sample since the labeling step occurs after cell lysis and digestion. However, this fact can also lead to quantification problems due to external influences during preparation.

The labeling step can also be introduced *in vivo* by growing cells in the presence of heavy isotopic labeled amino acids (Figure 6B). Stable isotopic labeling amino acid cell culture (SILAC) is the most representative example of metabolic labeling and it has been highly used in the study of functional proteomics and PTMs.[174,175] Generally, metabolic labeling is highly preferred over chemical or enzymatic labeling because the label incorporation occurs in the earliest stage of the experiment, and thereby minimizes the error in quantification.[176] However, not all cell lines are feasible with a metabolic labeling or with the cell medium required for the incorporation of the isotopes. On top of that, SILAC is costly in contrast to the chemical or enzymatic labeling methods.[174] Interestingly, both ^{18}O and SILAC labeling techniques have been recently compared in the identification of interaction partners of phosphorylated adhesion and degranulation-promoting adapter protein (ADAP). The data obtained showed that ^{18}O labeling is as good as SILAC for distinguishing ADAP phosphorylated-specific binding partners.[177]

1.4.4 MS fragmentation methods

Another crucial step in MS-based proteomics is the localization of the modification site. In general, MS fragmentation strategies provide sufficient peptide fragmentation to cover and sequence the whole peptide. Yet, when analyzing post-translationally modified peptides, the

analysis can be more challenging.[149] CID has become the standard method to fragment peptides and thereby elucidate the peptide sequence of the precursor ions selected. In CID fragmentation, an ion population is first accelerated by some electrical potential in a gas chamber with the presence of an inert gas such He, N_2 or Ar (Figure 7). Due to this ion acceleration, the ions collide against the gas molecules until peptide dissociation occurs, resulting in the formation of *b*- (*N*-terminus) and *y*- (*C*-terminus) type ions.[179] Generally, CID gives enough peptide coverage and even some PTMs such as methylation or acetylation can be clearly identified.[180] However, for others such as glycosylation and phosphorylation, the applicability of CID is limited due to the harsh conditions of the fragmentation method.[181] In the case of phosphorylation of serine, threonine, tyrosine, histidine, arginine, lysine, and cysteine residues, the phosphate group is usually lost during the fragmentation, which results in a MS Spectra dominated by a neutral loss of H_3PO_4 or HPO_3, depending on which phosphoamino acid is fragmented. The instability of the phosphate group under CID not only makes the phosphorylated site-assignment impossible but also leads in some cases to gas-phase rearrangements which questions the reliability of site-localization using CID in classical phosphoproteomics.[182] For *O*-glycosylated peptides the problem is similar. The glycan attached to the amino acid can be deciphered but not the glycosylated site due to the glycosidic cleavage during CID fragmentation.[183]

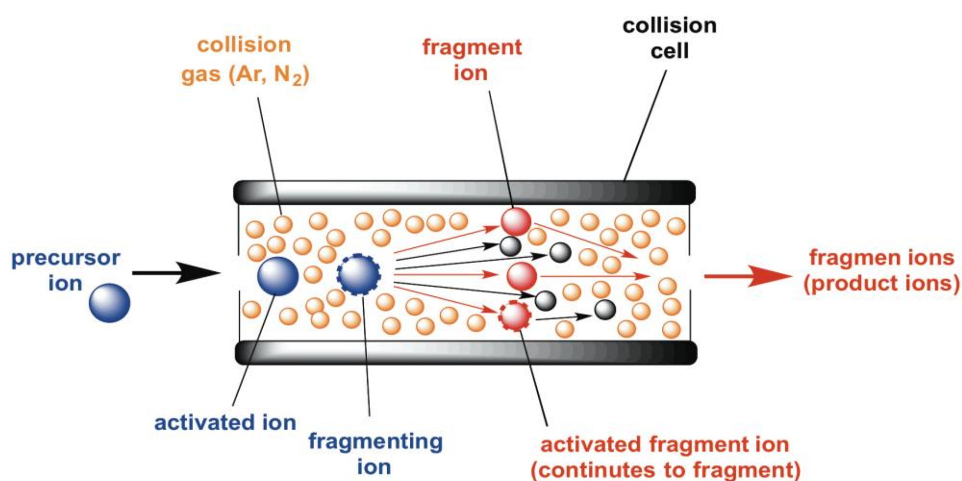


Figure 7. Schematic of the CID fragmentation.[184]

Recently, electron-based fragmentation techniques such as ECD and ETD have strongly emerged as potential alternatives for standard fragmentation methods, and specifically in the field of CID-labile PTMs.[185-187] In ECD fragmentation, near-thermal electrons are

bombarded towards the precursor ion which has been trapped previously in the gas-phase. The precursor ion captures electrons and then it finally leads to an excited radical cation that rapidly undergoes N-C_α bond cleavage.[188] ETD is very similar but here the electron is transfer to the peptide from a radical anion, i.e. fluoranthene anions (Figure 8).[189] Both techniques display mainly N-C_α cleavage fragments, which are commonly labeled as *c*-(*N*-terminus) and *z*-(*C*-terminus) type ions.[190] As previously mentioned, the power of ECD and ETD has been demonstrated with CID-labile modifications such as glycosylation and phosphorylation. For instance, both techniques outperform CID for unambiguous localization of serine- and threonine- phosphorylated sites on peptides, showing complete peptide coverage without observation of neutral loss.[181] More interestingly, is the application of ECD and ETD to *N*-phosphorylated peptides. Generally, the application of CID to *N*-phosphorylated peptides leads to a complete neutral loss without the chance to get any indicative phosphorylated fragment, which can unambiguously characterize the phosphorylated site.[186,191] In contrast, as shown for phosphorylated arginine and histidine peptides, ETD and ECD provide MS spectra with a full coverage of the peptide sequence including the fragment indicative for the phosphorylated site. A significant limitation of ETD and ECD over CID is the higher amount of time required to fragment peptides which limits the number of peptides that can be analyzed simulteonausly. In addition, it is not always possible to perform ETD and ECD straightforwardly. Since both techniques are based on an electron transfer to a charged peptide, small peptides or peptides, which do not provide a higher charge state than +2, are not well fragmented upon ETD and ECD process, leading to an incomplete peptide fragmentation.

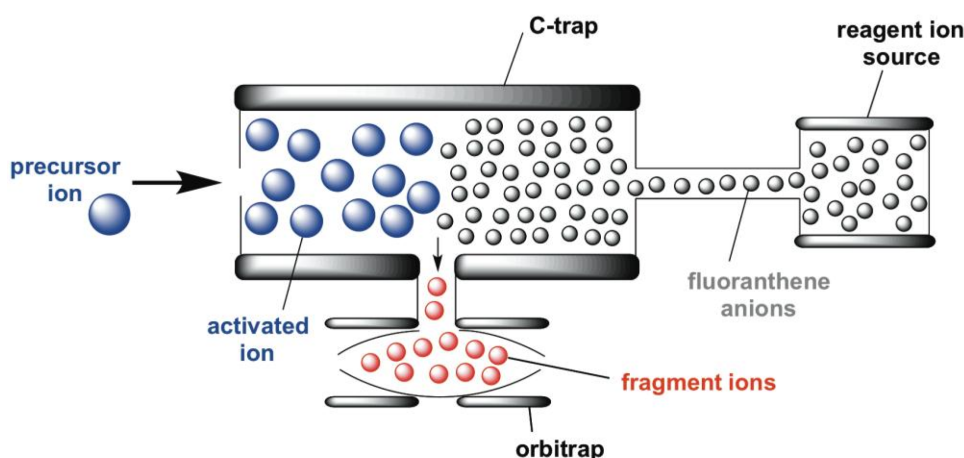


Figure 8. Schematic of the ETD fragmentation.

2 OBJECTIVE

Chemoselective reactions are very valuable tools for introducing different chemical entities (fluorophores, purification-affinity tags, PTMs) on peptides and proteins in a site-specific manner. Recently, chemoselective reactions have gained interest as chemical tools to introduce PTMs due to its power to not interfere with natural substrates. In addition, they have certain advantages compared to other synthetic methods such as SPPS and NCL or protein engineering methods. Nevertheless, very few examples exist so far compared to the wide variety of PTMs available in nature and in most of the cases, chemoselective reactions only allow to introduce a mimetic instead of the natural PTM. Moreover, the need of a chemical tool to access PTMs is of high interest not only for chemists but also for biologists. By solving the accessibility issue, the biological role of PTMs can be studied in more detail and their biological significance can be understood. On top of that, the peptides can be used to develop and improve existing proteomic methods, which can definitely help to uncover the roles of PTMs in for example cell signaling.

The aim of this work is to develop chemoselective strategies to introduce natural PTMs into peptides and proteins. In particular, we focus on two existing, yet rare, PTMs: lysine and cysteine phosphorylation. Both are not well-studied mainly because of the lack of suitable chemical and analytical tools. Along these lines, we also aim to establish analytical methods to characterize in both cases the phosphorylation sites.

GOAL 1 – ACCESSING PTMs ON PEPTIDES *VIA* CHEMOSELECTIVE REACTIONS

Project 1: A chemoselective synthetic tool for studying lysine phosphorylation

So far, only non site-specific methods have been reported to introduce lysine phosphorylation on peptides and proteins, delivering heterogeneous mixtures of phosphorylated lysine peptides. This study aims to use a recent reported chemoselective reaction, the Staudinger-phosphite reaction, in order to introduce phosphorylated lysine residues in a site-specific manner into peptides. With the peptides in hand, we plan to study their stability profiles in different contexts as well as their characterization by MS.

Project 2: A solid-supported approach to streamline the site-specific synthesis of phosphorylated lysine peptides

After successful site-specific installation of phosphorylated lysine on peptides, in this second study we aim to streamline the previous synthetic protocol by developing a compatible solid-phase approach which directly delivers the site-specifically phosphorylated lysine peptides.

Project 3: A chemoselective synthetic tool for studying cysteine phosphorylation

To date, there is only one method available to introduce phosphorylated cysteine residues on proteins in a site-specific manner (*see chapter 3.3*). A significant limitation of the reported method is that it delivers a racemic mixture due to the fact that dehydroalanine is the precursor substrate. In this study we aim to make use of electron deficient disulfides and nucleophilic phosphite triesters to incorporate phosphorylated cysteine residues into peptides in a site-specific manner. We plan to test different nucleophilic phosphites under different reaction conditions as well as different uncaging systems to deliver the final phosphorylated cysteine peptides. With these peptides in hand, we also intend to demonstrate the unambiguous characterization of the phosphorylation site by ETD MS, being an essential step for studying this rare PTM.

GOAL 2 – UNAMBIGUOUS CHARACTERIZATION OF LYSINE PHOSPHORYLATION

Project 4: Phosphate migration in phosphorylated lysine peptides during ETD MS analysis.

In the course of the characterization of the synthetic phosphorylated lysine peptides, we have observed that the phosphate group migrates to other phosphoacceptors during the ETD fragmentation process. Thus, we have decided to undertake a more detailed study of the fragmentation behavior of phosphorylated lysine peptides. Some key parameters as well as different peptides containing key substitutions are analyzed. Importantly, this research might be relevant to optimize suitable MS phosphoproteomics methods to study lysine phosphorylation and to its unambiguous characterization.

3 RESULTS AND DISCUSSION

3.1 A chemoselective synthetic tool for studying lysine phosphorylation

This chapter was published in the following journal:

Jordi Bertran-Vicente, Remigiusz Serwa, Michael Schümann, Peter Schmieder, Eberhard Krause, Christian P. R. Hackenberger*

"Site-specifically phosphorylated lysine peptides"

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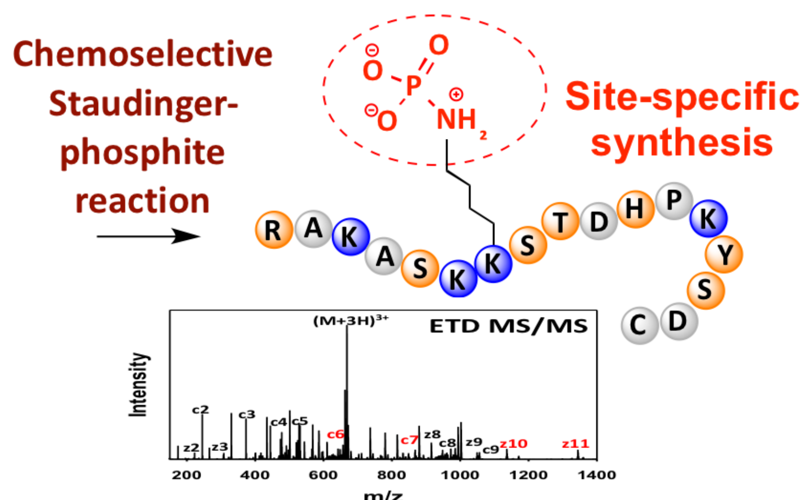

Figure 9. Site-specific synthesis of phospholysine peptides *via* the chemoselective Staudinger-phosphite reaction and its characterization by ETD MS/MS.

Abstract:

Protein phosphorylation controls major processes in cells. Although phosphorylation of serine, threonine, and tyrosine and also recently histidine and arginine are well-established, the extent and biological significance of lysine phosphorylation has remained elusive. Research in this area has been particularly limited by the inaccessibility of peptides and proteins that are phosphorylated at specific lysine residues, which are incompatible with solid-phase peptide synthesis (SPPS) due to the intrinsic acid lability of the P(=O)–N

phosphoramidate bond. To address this issue, we have developed a new synthetic route for the synthesis of site-specifically phosphorylated lysine (pLys)-containing peptides by employing the chemoselectivity of the Staudinger-phosphite reaction. Our synthetic approach relies on the SPPS of unprotected ε-azido lysine-containing peptides and their subsequent reaction to phosphoramidates with phosphite esters before they are converted into the natural modification *via* UV irradiation or basic deprotection. With these peptides in hand, we demonstrate that electron-transfer dissociation tandem mass spectrometry can be used for unambiguous assignment of phosphorylated lysine residues within histone peptides and that these peptides can be detected in cell lysates using a bottom-up proteomic approach. This new tagging method is expected to be an essential tool for evaluating the biological relevance of lysine phosphorylation.

Responsibility assignment

The design of the project was provided by Christian P. R. Hackenberger. Remigiusz A. Serwa performed initial reagent synthesis. The author synthesized reagents, peptides, evaluated alternative routes for the synthesis, performed the stability studies and the identification of phosphorylated lysine peptides in a proteomic context. Eberhard Krause designed and guided the MS characterization of the phosphorylated lysine peptides and Michael Schümann performed the ETD-MS analysis. Peter Schmieder performed the NMR experiments of the phosphorous-containing peptides as well as the NMR experiments for the stability studies. Furthermore, the publication was planned and written by the author under supervision of Christian P. R. Hackenberger.

Summary of content

While *O*-phosphorylation like serine-, threonine- and tyrosine-phosphorylation are well studied, little is known about *N*-phosphorylation in histidine-, arginine- and lysine-phosphorylation due to the lack of appropriate chemical and analytical methods to decipher its biological significance. One possible reason for the scarcity of literature about *N*-phosphorylation is the different stability profiles of the phosphoramidate bonds. The phosphoramidate bond is acid-labile and thereby very challenging to be studied with established synthetic and analytical protocols. Within this latter group, phospholysine is by far the least studied. Due to the acid-lability, accessibility by solid phase peptide synthesis

(SPPS) is hindered. Other strategies employing phosphorylating agents are not chemoselective delivering a heteregenous mixtures of phosphorylated species. With this accessibility issue in mind, the goal of this study was to provide a synthetic approach to access site-specifically phosphorylated lysine peptides. We have taken advantage of the chemoselectivity of the Staudinger-phosphite reaction to convert unprotected azidornorleucine peptides and phosphites triesters into phosphoramidate ester peptides in solution. In this way, the TFA-cleavage was performed before the installation of the desired phosphoramidate moiety. Then, by using UV- or base-cleavable linkers at the phosphorous substituents the final phosphorylated lysine peptides can be released. As a proof of concept, we chose two peptides derived from the histone H1 protein which is known to be a phosphorylated lysine-containing protein. Firstly, we confirmed that harsh acidic conditions are not compatible with phosphoramidate bonds as demonstrated with the studies performed on a small molecule level and at peptide level. Then, we tested the reactivity of the phosphite triesters with the azidonorleucine peptide in the Staudinger-phosphite reaction. After reaction optimization, we synthesized different phosphoramidate ester peptides containing two different UV-cleavable linkers and a base cleavable linker. Both, UV irradiation and alkaline incubation, released the phosphorylated peptides in a quantitative manner. Isolation of the phosphorylated lysine peptides was performed by a semi-preparative HPLC using an alkaline gradient to avoid phosphoramidate hydrolysis. Another crucial step in the study of lysine phosphorylation was the characterization of the phosphorylation site by MS. The phosphoramidate bond form is not stable upon CID fragmentation, the common fragmentation technique to perform peptide sequencing. Thereby, a more soft fragmentation method such as ETD is required to enable not only the characterization of our peptides but also to perform reliable phosphoproteomics studies of lysine phosphorylation. With the phosphorylated lysine peptides synthesized in hand, we performed ETD-MS analysis obtaining the corresponding MS fragments ions that confirmed the phosphorylation site. Finally we added one of the synthetic phosphorylated lysine peptide to a cell lysate. We demonstrated that by using a bottom-up proteomic approach the synthetic phosphorylated lysine peptide can be detected in the presence of a complex cell lysate, after digestion with chymotrypsin and analysis by LC-MS and its corresponding characterization by ETD. With this new chemical approach in hand plus the relevant

information here obtained about the stability of phosphorylated lysine peptides, we expect to obtain more knowledge about the biochemical role of lysine phosphorylation in proteins.

Outlook

In this study we have established a synthetic strategy and an analytical method to ease the identification of lysine phosphorylation in proteins. Despite the significance of this work, other strategies are needed to establish an apropiate phosphoproteomic method for lysine phosphorylation, e.g. phosphoenrichment, antibody generation, etc.

3.2 A solid-supported approach to streamline the site-specific synthesis of phosphorylated lysine peptides

This chapter was published in the following journal:

Jordi Bertran-Vicente, Michael Schümann, Eberhard Krause,
Christian P. R. Hackenberger*

`*Direct access of site-specifically phosphorylated-lysine peptides from solid-support*"
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Publication date (Web): May 7, 2015
The original article is available at:
http://dx.doi.org/10.1039/C5OB00734H

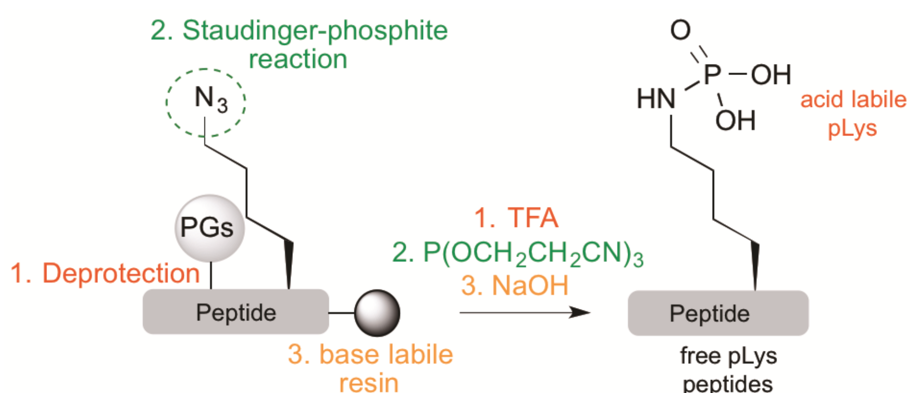


Figure 10. Solid-phase synthetic approach to synthesize site-specifically phosphorylated lysine peptides.

Abstract:
Phosphorylation is a key process for changing the activity and function of proteins. The impact of phospho-serine (pSer), -threonine (pThr) and –tyrosine (pTyr) is certainly understood for some proteins. Recently, peptides and proteins containing *N*-phosphorylated amino acids such as phosphoarginine (pArg), phosphohistidine (pHis) and phospholysine (pLys) have gained interest because of their different chemical properties and stability profiles. Due to its high intrinsic lability, lysine phosphorylation is within this latter group the least studied. In order to gain insight into the biological role of lysine phosphorylation, chemical and analytical tools which are compatible with the labile P(═O)–N bond are highly

sought-after. We recently reported an in solution synthetic approach to incorporate phosphorylated lysine residues in a site-specific manner on peptides by taking advantage of the chemoselectivity of the Staudinger-phosphite reaction. While the in solution approach allows us to circumvent the critical TFA cleavage, it still requires several transformations and purification steps to finally deliver phosphorylated lysine peptides. Here we report the synthesis of site-specifically phosphorylated lysine peptides directly from the solid support by using a base labile resin. This straightforward and highly efficient approach facilitates the synthesis of various site-specifically phosphorylated lysine-containing peptides and lays the groundwork for future studies about this elusive protein modification.

Responsibility assignment

The design of the project was provided by Christian P. R. Hackenberger. The author synthesized reagents and peptides, evaluated alternative routes for the synthesis, and performed the stability studies. Eberhard Krause designed and guided the MS characterization of the phosphorylated lysine peptides and Michael Schümann performed the ETD-MS analysis. Peter Schmieder performed the NMR experiments of the phosphorous-containing peptides as well as the NMR experiments for the stability studies. Furthermore, the publication was planned and written by the author under supervision of Christian P. R. Hackenberger.

Summary of content

The synthetic accessibility by SPPS of site-specifically modified *N*-phosphorylated peptides is very challenging due to the acid-lability of the phosphoramidate bond. Since the last step in standard SPPS to release the peptides require harsh acidic conditions (TFA), alternative methods to provide *N*-phosphorylated peptides from solid-supported resins are highly sought after. To fill this gap, we envisioned a straightforward approach that combines the use of a base-labile resin, the chemoselectivity of the Staudinger-phosphite reaction and the use of a base-cleavable phosphite ester. The key step was to perform the TFA protecting group deprotection on the resin, delivering an immobilized unprocted azidonorleucine-containing peptide. To probe the feasibility of the approach, we first confirmed that phosphoramidate bond was stable under the basic conditions required to release peptides from the base-labile resin. As a proof of concept, we synthesized different *o*-nitrobenzyl

based phosphoramidate ester lysine peptides directly from the solid support. Albeit, at this point, the approach already improves the recent in solution synthetic approach reported previously (*see chapter 3.1*), it still demanded a last UV-uncaging step to deliver site-specifically phosphorylated lysine peptides. In order to streamline the solid-phase approach, we then used a base-cleavable phosphite which allows us to deliver the phosphorylated lysine peptides directly from the base-labile solid support. Furthermore, we confirmed that ETD-MS is the method of choice for an unambiguous analysis of the phosphorylation site in lysine phosphorylated peptides and that basic conditions employed in this protocol do not lead to phosphorylation transfer within these peptides. Altogether this protocol allows the very first direct synthesis of site-specifically phosphorylated lysine peptides from solid-supported azidonorleucine-containing peptides. Thereby, this method constitutes a significant improvement to our recent published synthetic strategy (*see chapter 3.1*), in which several intermediate purification steps were required.

Outlook

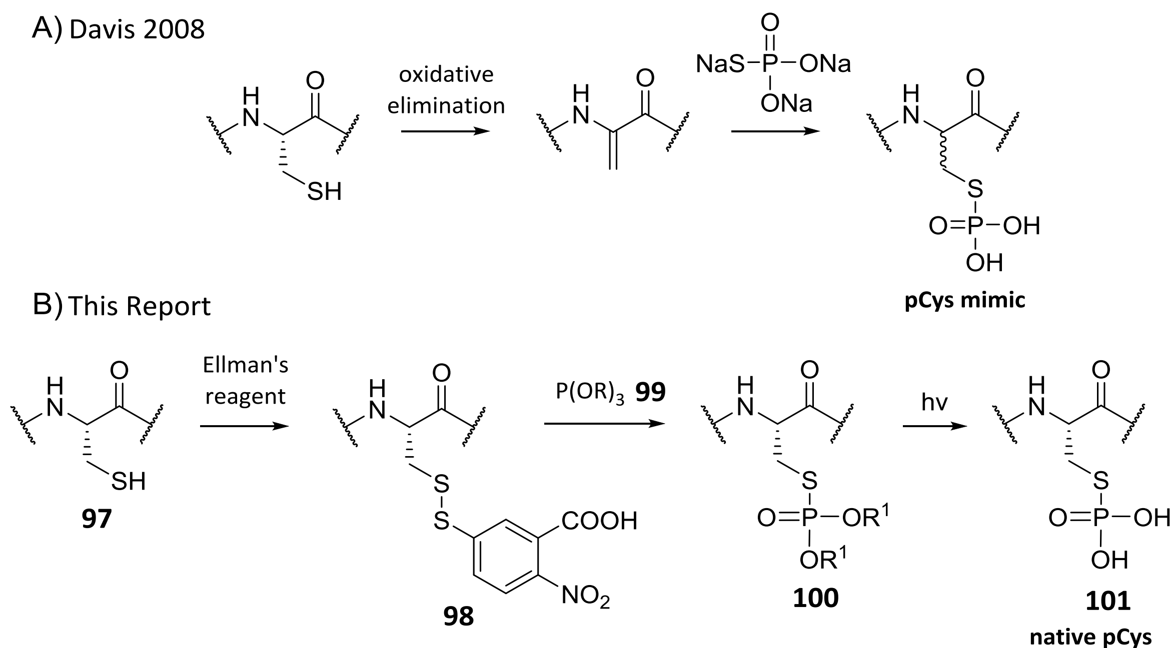
With this established synthetic approach, we aim to synthesize a diverse variety of site-specific modified phosphorylated lysine peptides in order to perform biological assays as well as stability studies. For instance we envision to access peptide sequences containing phosphorylated lysine residues, which are potential substrates of a known phospholysine phosphatase. We can also access other potential phosphorylated lysine peptides from different regions of histone H1, which can be further used in protein-protein interactions studies to study whether potential binding partners interact with phosphorylated lysine peptides. Finally we want to study whether at physiological pH, transfer of phosphate residues from phosphorylated lysine to other phosphoacceptors occurs.

3.3 A chemoselective synthetic tool for studying cysteine-phosphorylation

3.3.1 Introduction

In addition to the well-known and studied phosphorylated-serine, -threonine and -tyrosine proteins, other phosphorylated proteins like phospho-histidine, -arginine, -lysine and -cysteine have gained interest due to its different chemical properties and stability profiles.[8,31,32,163,164] Within this latter group, phosphocysteine is known to have a biological significance in nature as intermediate in the phosphoenolpyruvate-dependent phosphotransferase system (PTS), in the dephosphorylation of phosphotyrosine residues by protein tyrosine phosphatases and in bacterial signaling and regulation.[36-47] Yet, the isolation and characterization of phosphorylated-cysteine peptides is challenging due to the acid-lability of the phosphorothioate bond (half-life at pH 4, 30min; at pH 6, 2h; at pH 7.5, 30h) which has prevented this modification from being studied extensively by using standard phosphoproteomic approaches.[36,41] Besides its role as PTM, phosphocysteine has recently been used as phosphoserine and phosphothreonine mimetic[123-125] as a better alternative of other established strategies such as glutamate and aspartate substitution.[192] Both to study phosphocysteine as a PTM or to use it as mimetic, it is crucial to have chemical accessibility to site-specific phosphorylated cysteine peptides and proteins. Along these lines, Davis and co-workers have developed a two-step method for installing phosphocysteine residues on a protein level (Scheme 17A). The method relies on generating dehydroalanine from cysteine residues which have been previously incorporated by site-directed mutagenesis and then its reaction with sodium thiophosphate to finally form a racemic mixture of phosphocysteine proteins.[122,123]

Although this strategy allowed to access phosphocysteine proteins in different biological contexts, the lack of stereoselectivity and harsh elimination conditions may impose certain limitations on the general applicability of this chemical tool for the functional analysis of phosphorylated proteins.



Scheme 17. A) conjugate addition of thiophosphate to dehydroalanine to deliver racemic phosphocysteine. B) Chemoselective phosphorylation approach.

3.3.2 Outline of the project

To circumvent the imposed limitation of the already published protocols to access phosphocysteine peptides and proteins, we aim to develop a novel chemoselective and racemization-free phosphorylation strategy of cysteine residues on peptides (Scheme 17B). The key step in the envisioned strategy is the conversion of cysteine-containing peptides **97** in electrophilic disulfide bonds **98** by Ellman´s reagent addition, which is followed by nucleophilic attack of nucleophilic phosphites **99** to generate phosphocysteine residues in **101** after UV-irradiation of the phosphorothioate ester **100**. The use of phosphite esters to open disulfides bonds has been reported before in a small molecule level but to the best of our knowledge this reaction has never been reported with peptide or protein substrates.[193-195] Early reports reacted diethyl sulfides with triethyl phosphites at high temperatures to afford triethyl monothiophosphate.[193] *S*-phenyl nucleoside phosphorothioates have been synthesized by using nucleoside silyl phosphites and phenyl disulfides.[194] More recently, electron-deficient aryl disulfides have reacted with phosphites to form phosphorothioate oligoribonucleotide analogues.[195] Along these lines, the use of electron-deficient aryl disulfides such as 2,2´-dithiobis(5-nitropyridine) (DTNP) has been exploited to activate a cysteine thiol of a histone H2B for subsequent ligation with a cysteine ubiquitin

fragment.[196] The Ellman´s reagent has also been used for activating cysteine-containing proteins which serves as precursor of dehydroalanine upon elimination using phosphines or DBU.[113]

In addition to the synthetic accessibility, we aim to gain information about the analytical characterization of phosphocysteine peptides under NMR and MS techniques as well as their fragmentation behavior under MS/MS fragmentation methods (HCD, CID, ETD). As for phospholysine, phosphocysteine is also considered a CID-labile modification, pointing towards the need to have a suitable MS fragmentation technique in order to identify potential phosphocysteine peptides during phosphoproteomics approaches.

3.3.3 Responsibility Assignment

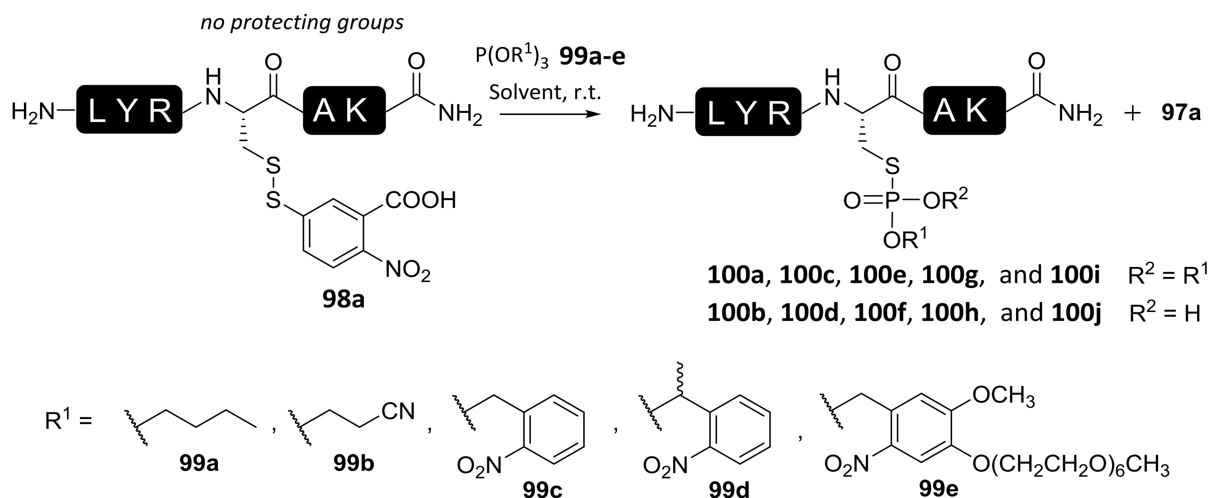
The design of the project was provided by Christian P. R. Hackenberger. The author synthesized reagents, peptides, evaluated alternative routes for the synthesis, and delivered the compounds for NMR and ETD-MS analysis. Eberhard Krause designed and guided the MS characterization of the phosphorylated cysteine peptides and Martin Penkert performed the ETD-MS analysis. Peter Schmieder performed the NMR experiments of the phosphorous-containing peptides.

3.3.4 Results and Discussion

3.3.4.1 Site-specific synthesis of phosphorothioate ester peptides

To show the feasibility of this chemical strategy, we performed initial experiments with available phosphite triesters from our laboratory (**99a-e**). Previous attempts using oxidized glutathione (GSSG) and comercially available trimethyl phosphite in DMF did not show formation of phosphorothioate ester **100** (data not shown). The reactivity of GSSG towards phosphites was also tested in water (25 mM Tris Buffer-HCl at pH 8.0) by using the water-soluble phosphite **99e** without indication of phosphorothioate ester **100** formation. Based on the previous reported work on small molecule level, we decided to probe the reactivity with an electron-deficient disulfide peptide (Scheme 18). Thereby, we first synthesized by solid-phase peptide synthesis (SPPS) a small cysteine-containing peptide (sequence LYRCAK) **97a**. Peptide **97a** was then reacted with Ellman´s reagent following standard protocols to

form peptide **98a**.[113] With the isolated peptide **98a** in hand, we reacted it with tributyl phosphite **99a** in DMF (Scheme 18, Table 2). Analysis of the reaction crude by HPLC-UV coupled to MS, showed mainly formation of cysteine peptide **97a** together with minor amount of phosphorothioate ester peptide **100a** (Table 2, entry 1). Then, we decided to check the reactivity of peptide **98a** in DMF towards different phosphites having different electronic properties. The base-cleavable phosphite **99b** showed good conversion by MS as it was also indicated after isolation using semi-preparative HPLC yielding peptide **100c** in 37% yield (Table 2, entry 2). However, again formation of peptide **97a** was also observed. *O*-nitrobenzyl-based phosphites were also employed to study the reactivity of the electrophilic disulfide peptide **98a**. Remarkably, phosphite **99d** showed quantitative conversion to phosphorothioate ester peptide **100g** with minor formation of hydrolysis product **97a** based on HPLC-UV-MS, and with an isolated yield of 55% after semi-preparative HPLC (Table 2, entry 4, Figure 11). Interestingly, when using a water-soluble electron-rich phosphite such as **99e**, we observed formation of monoprotected phosphorothioate ester **100j** (34% isolated yield) in addition to the expected product **100i** (27% isolated yield) and peptide **97a** (Table 2, entry 5). The same result was observed when using *o*-nitrobenzyl phosphite **99c** (Table 2, entry 3). We speculated that the formation of monoprotected phosphorothioate esters is due to a nucleophilic attack on the electrophilic benzylic position of the *o*-nitrobenzyl substrates by water or by the thiolate nitrobenzoic acid byproduct (Scheme 19). As expected, when using phosphite **99d** the monoprotected ester was not formed due to the lack of electrophilicity of the benzylic position (Table 2, entry 4).



Scheme 18. Reaction of peptide **98a** with phosphites **99a**-**e**.

Table 2. Conversion and isolated yields for phosphorothioate esters peptides **100**.

Entry	phosphite	Solvent	Product	% conversion[a]	% yield[b]	Product	% conversion[a]	% yield[b]
1	**99a**	DMF	**100a**	20	-	**100b**	-	-
2	**99b**	DMF	**100c**	54	37	**100d**	-	-
3	**99c**	DMF	**100e**	6	-	**100f**	32	-
4	**99d**	DMF	**100g**	97	55	**100h**	-	-
5	**99e**	DMF	**100i**	22	27	**100j**	35	34
6	**99a**	MeCN:buffer	**100a**	18	-	**100b**	-	-
7	**99b**	MeCN:buffer	**100c**	59	43	**100d**	-	-
8	**99c**	MeCN:buffer	**100e**	7	-	**100f**	18	-
9	**99d**	MeCN:buffer	**100g**	36	27	**100h**	-	-
10	**99e**	buffer, pH 7.0	**100i**	2	-	**100j**	25	-
11	**99e**	buffer, pH 8.0	**100i**	3	-	**100j**	30	38

[a]Conversion was determined by LC-MS analysis after **98a** was depleted. The sum of areas under the XIC peaks corresponding to **97a**, **98a**, oxidized **97a**, and **100** for each entry were counted as a 100%. All experiments were performed at least in duplicate, and the average values are presented. [b]Isolated yields of **100c**, **100g**, **100i**, and **100j** products after semi-preparative HPLC. For further details see Experimental Part (6.2).

Since we aimed for a chemical method to be used on proteins, we decided to probe the reactivity in aqueous solvents. Reactivity of peptide **98a** with phosphites **99a** and **99b** in a mixture of MeCN and 25 mM Tris Buffer-HCl at pH 8.0 gave similar conversions than previous observed in DMF (Table 2, entry 6,7). Surprisingly, for phosphite **99c** we observed less conversion to the desired product **100g** and an increase in the formation of peptide **97a** and its corresponding oxidized disulfide product (Table 2, entry. 9) The same effect was observed when using the water-soluble phosphite **99e**, in which mainly formation of monoprotected phosphorothioate ester **100j** was observed (Table 2, entry 10, 11). These observations implied that the reactivity of phosphites **99** with peptide **98a** in aqueous solutions seem to be low-yielding compare to using organic solvents such as DMF.

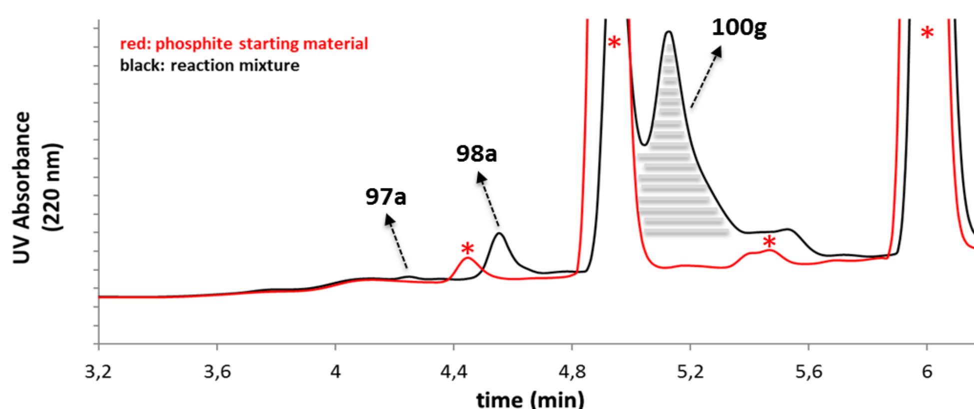
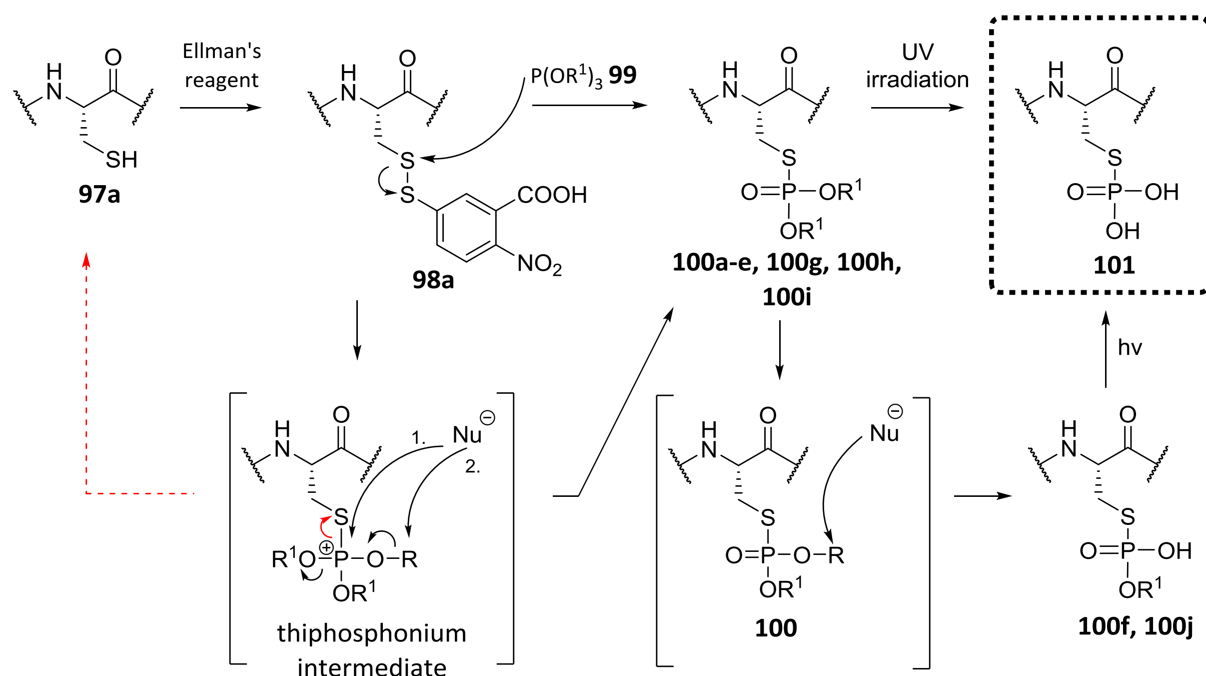


Figure 11. Overlapping LC-UV chromatograms of reaction mixture and phosphite. Red asterisk mark the decomposition byproducts of phosphite.

In general, we hypothesize that the hydrolysis of the phosphorothioate bond occurs in the first intermediate formed during the reaction between phosphites and electrophilic disulfides, i.e., the triester thiophosphonium cation (Scheme 19). The tendency to hydrolyze the P-S bond than the P-O bond at this stage seems to be highly dependent on the nucleophilic substances around. This would explain that the formation of **97a** is more abundant in reactions performed in H_2O/MeCN mixtures than in DMF, due to the higher nucleophilicity of water.



Scheme 19. Proposed mechanism for the formation of phosphorothioate esters **100a-j**.

3.3.4.2 UV- and alkaline-deprotection of phosphorothioate ester peptides

With the phosphorothioate ester peptides in hand, we aimed to find suitable methods to deliver a site-specific modified phosphorylated cysteine peptide. To do so, we considered both the UV-irradiation to uncage the *O*-nitrobenzyl-based phosphorothioate esters peptides **100g** and **100i**, and the alkaline deprotection of the base-labile phosphorothioate cyanoethyl ester peptide **100c**. For instance, the uncaging of peptide **100g** upon UV-irradiation delivered phosphorylated cysteine peptide **101a** successfully, with minor formation of the hydrolyzed P-S bond byproduct (Figure 12). After semi-preparative HPLC, peptide **101a** was isolated in 65% yield and with a purity criteria ≥ 90% based on UPLC-UV. To avoid major hydrolysis of the P-S bond a mixture of MeCN/alkaline aqueous buffer (pH 8.2) was used instead of the standard TFA gradient.

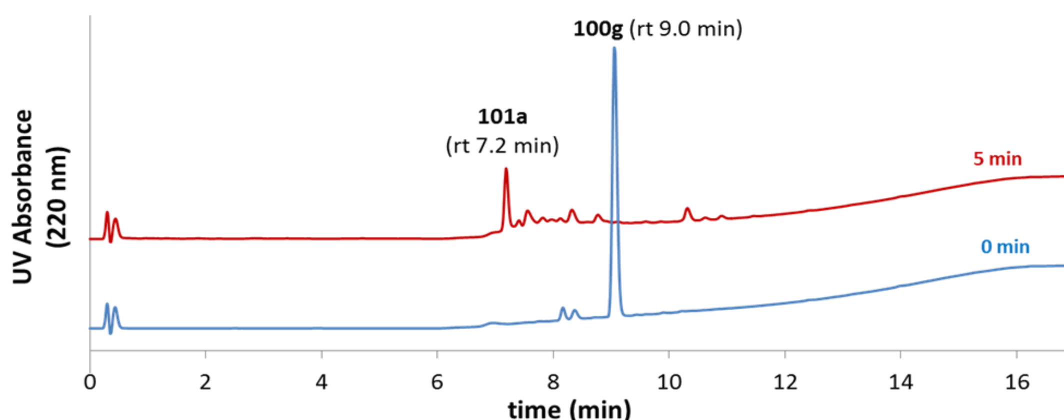


Figure 12. UPLC-trace before (blue) and after 5 min irradiation (red) of **100g**.

With the cyanoethyl phosphorothioate ester peptide **100c** we followed standard procedures to allow the *β*-elimination of the protecting group. The base-labile cyanoethyl group is commonly used as protecting group in phosphorimidites for solid-phase synthesis of oligonucleotides.[197] Unfortunately, the incubation of peptide **100c** with 250 mM NaOH formed not only the phosphorylated cysteine peptide **101a** but also quantitative amounts of dehydroalanine-containing peptide (Figure 13). Similar as phosphoserine, phosphocysteine undergoes also *β*-elimination to dehydroalanine under basic conditions. Thereby, we discarded the use of cyanoethyl as protecting group to deliver phosphorylated cysteine-containing peptides.

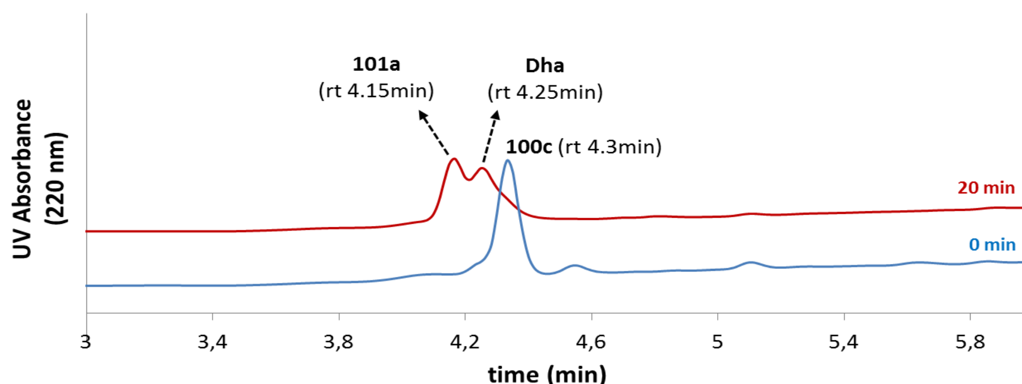


Figure 13. UPLC-trace before (blue) and after (red) 20 min incubation of peptide **100c** with NaOH.

3.3.4.3 Analytical characterization of the phosphorylation site in phosphorylated cysteine peptides

The analytical characterization of the phosphorylation site is a very important step because is the only evidence to confirm the site-specific modification of the peptide. By doing this, we can also confirm that there is not phosphate-transfer or migration processes involved during the preparation of the phosphorylated cysteine-containing peptides. In addition, the development of analytical methods for the phosphorylation site-assignment is a very important contribution in the proteomic field when analyzing unknown phosphorylation events of biological samples. Generally, MS and NMR among other techniques can fill this gap, being the former the most widely used for this task.[181,187,198]

First, we used NMR to identify by ^{31}P-NMR the characteristic signal for the phosphorylated cysteine peptide **101a**. A major signal was observed in the ^{31}P-NMR spectra at 12.02 ppm (Figure 14), which is in accordance to the literature.[42,44] Additionally, a minor peak at -1.39 ppm was found which belong to inorganic phosphate as it was confirmed upon addition of external sodium phosphate (data not shown). To demonstrate that the ^{31}P signal was attached to a cysteine residue, ^{1}H-^{31}P HMBC NMR experiment was performed. The α-methylene hydrogen atoms from the cysteine side-chain at 3.13 ppm showed coupling to the phosphorous signal peak at 12.02 ppm, thus confirming that the phosphate was attached to a cysteine side chain (Figure 14). However, with additional presence of cysteine residues on the peptide sequence and when analyzing biological samples it would be cumbersome if not impossible to distinguish by NMR the phosphorylated site among the cysteine side chains.

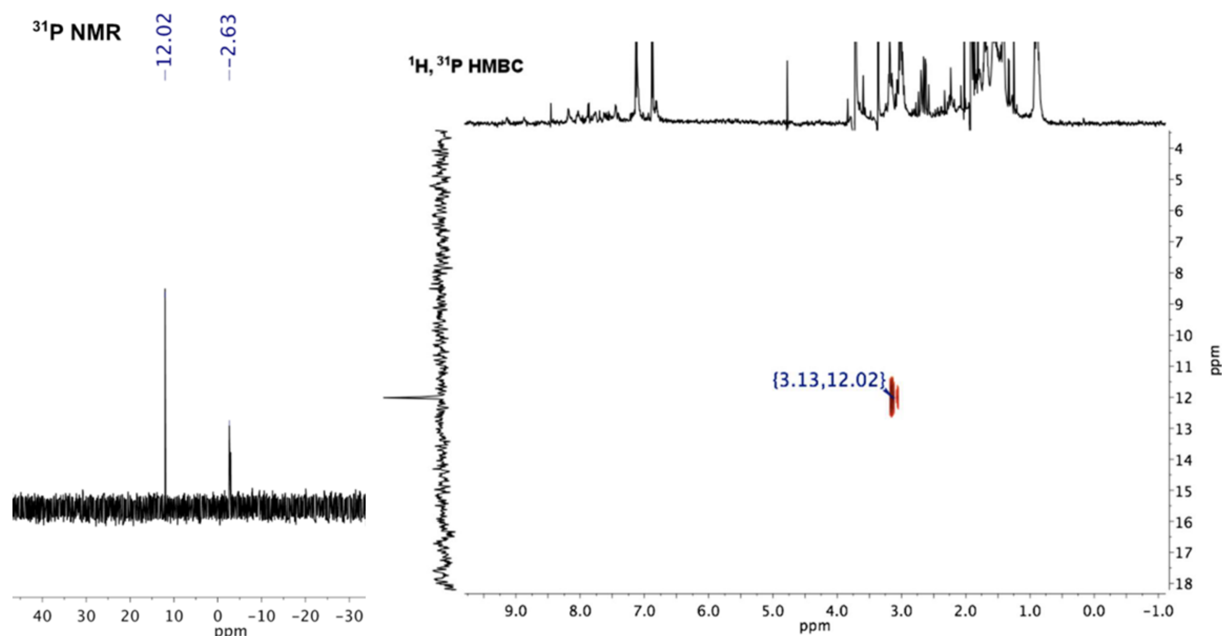


Figure 14. ^{31}P-NMR and ^{1}H, ^{31}P HMBC NMR spectra of peptide **101a**

We therefore decided to use mass spectrometry (MS), with the intention of unambiguous localization of the phosphorylation site. Tandem mass spectrometry (MS/MS) has become the method of choice for analysis of PTMs on peptides because of the high sensitivity and high-throughput capabilities when coupled to nanoliquid chromatography (nLC).[137] CID is the most common fragmentation technique to allow a full peptide coverage; however, its applicability to analyze peptides containing PTMs is in some cases problematic.[181] Electron-based techniques such as ECD or ETD have recently emerged as a better fragmentation method for CID-labile modification such as glycosylation and phosphorylation.[181,186,191,199,200] Examples in the literature include the phosphorylated site-assignment by ETD or ECD of phosphoarginine, and phospholysine as well as the glycosylated site-assignment of *N*-glycosylation and *O*-glycosylation. In the case of phosphorylated cysteine peptides, CID was applied recently to analyze two potential transcriptional regulators, SarA and MgrA, which are believed to harbor phosphorylation cysteine residues within its sequence.[47] Although CID provided an uncomplete coverage of the peptide sequence, it was sufficient to assign the phosphate moiety to the cysteine residue.

With this in mind we tried first to fragment peptide **101a** using higher-energy collisional dissociation (HCD), a beam type CID fragmentation that occur in the C-trap.[201] Unfortunately, a complete neutral loss of phosphate was observed and only unphosphorylated *b*- and *y*-type fragments were detected (*see Experimental Part 6.2.6*). In contrast, when using ETD combined with HCD, i.e. EThcD, we observed some *c* and *z*-type phosphorylated fragment ions, which however allows us to confirm the phosphorylation at cysteine (*see Experimental Part 6.2.6*). The reason of the uncomplete coverage of the peptide sequence was due to the small size of the peptide and thereby the impossibility to generate by ESI a charge state higher than +2. It is known that lower charge states do not fragment well upon ETD process. For this reason, we decided to synthesize a bigger peptide with the aim to make a fully peptide sequence coverage using ETD. Two different phosphorylated cysteine peptides were synthesized, both from real biological targets: EQLpCFSLYNAQR (**101b**), a tryptic peptide fragment from the MrgA regulator,[47] and IGIHSGSpCVAGVVGLTMPR (**101c**), a tryptic fragment of guanylate cyclase identified after a proteomic approach of sea urchin sperma.[202] Both peptides, were synthesized following the above mentioned protocol, using phosphite **99d** and UV irradiation to deliver **101b** and **101c** in 5% and 2% overall yield, respectively (*see Experimental Part 6.2*). EThcD fragmentation of peptide **101b** provided a MS spectra containing an almost full coverage of the peptide sequence (Figure 15). Importantly, the phosphorylated fragment *c4* was identified together with the unphosphorylated fragment *z8* that confirmed the unambiguous phosphorylation at cysteine residue.

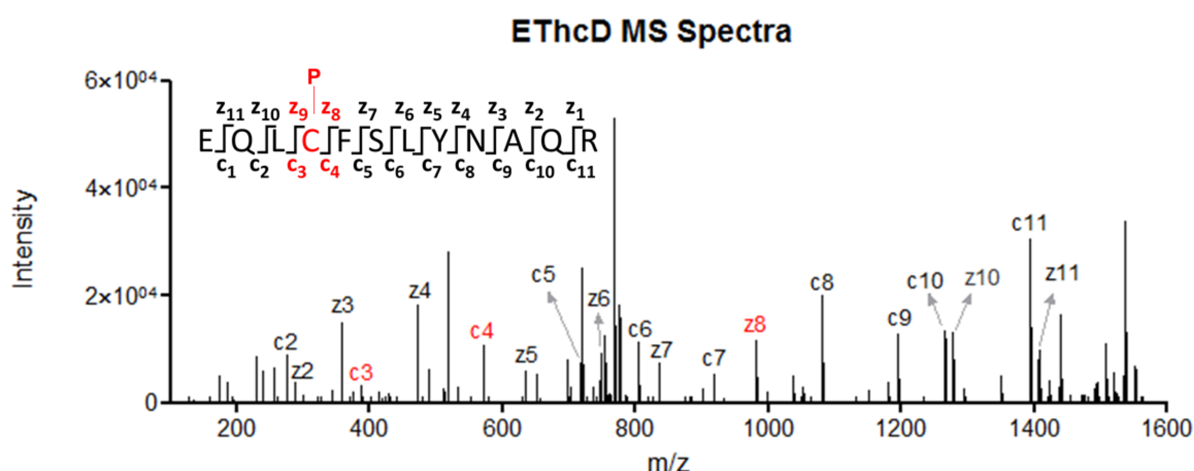


Figure 15. EThcD MS Spectra of phosphorylated cysteine peptide **101b**

Peptide **101c** was also submitted to EThcD fragmentation showing similar results (Figure 16). The unambiguous phosphorylated site-assignment was confirmed by the presence of the *z11* unphosphorylated fragment and the phosphorylated *c8*. In addition, for peptide **101b** and **101c**, presence of fragments indicating phosphate transfer to other phosphoacceptors was not observed.

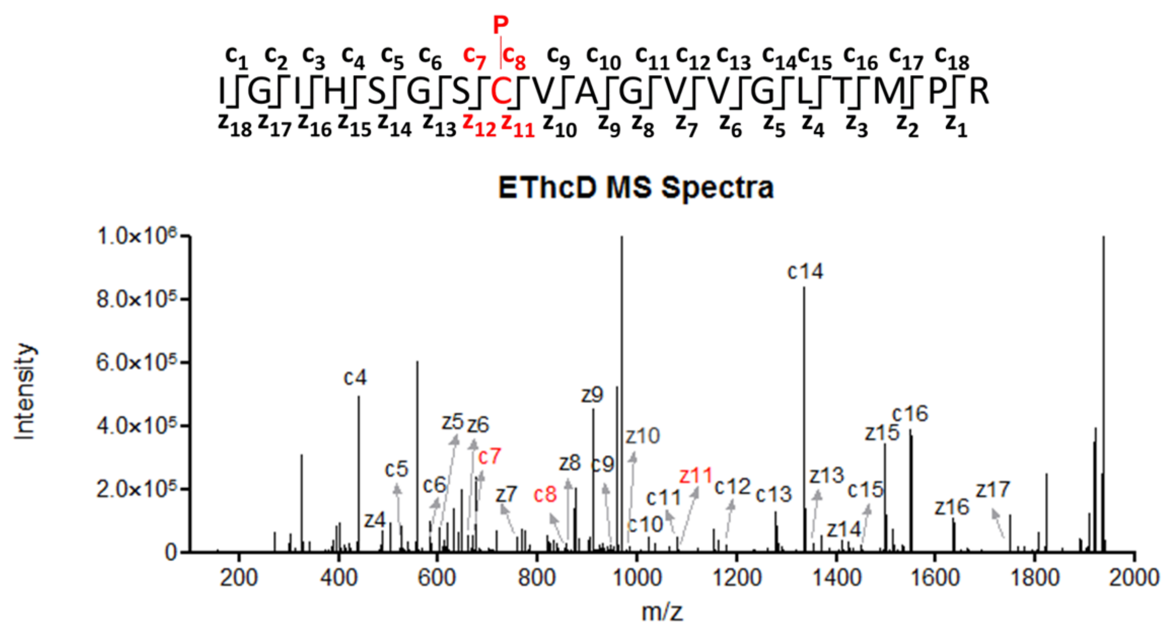


Figure 16. EThcD MS Spectra of phosphorylated cysteine peptide **101c**

3.3.5 Conclusions and outlook

In summary, we have developed a new chemoselective modification strategy that allows the racemization-free conversion of cysteine into phosphorylated cysteine residues on peptides. The synthetic route takes advantage of the chemoselectivity reaction of nucleophilic phosphite esters towards electrophilic disulfides peptides to form phosphorothioate esters peptides. UV irradiation delivers straightforwardly the final phosphorylated cysteine peptide, which can be easily isolated with minor hydrolysis. On top of that, we have demonstrated for the first time that EThcD fragmentation of phosphorylated cysteine peptides outperforms CID or HCD for peptide sequencing and for phosphorylated-site assignment. We strongly believed that by using this chemical and analytical tool, the understanding of the biological significance of cysteine-phosphorylation would be easily approachable.

In the near future, the stability of phosphorylated cysteine peptides in different scenarios (pH, thermal and cell lysate stability) will be evaluated. For further applicability on proteins, the synthetic method has to be optimized in aqueous buffer to reduce the hydrolysis of the P-S bond during the reaction and thereby to increase the conversion to the desired phosphorothioate ester cysteine-containing proteins. It is also envisioned to used ETD-MS for characterization of an already known phosphorylated-cysteine protein by performing a bottom-up proteomic approach.[44]

3.4 Phosphate migration in phosphorylated lysine peptides during ETD MS analysis.

This chapter was published in the following journal:

Jordi Bertran-Vicente,* Michael Schümann, Christian P. R. Hackenberger,
Eberhard Krause*

‶*Gas-Phase Rearrangement in Lysine Phosphorylated Peptides During ETD Tandem Mass Spectrometry*“

Anal. Chem., **2015**, 87, 6990-6994

Publication date (Web): June 25, 2015

The original article is available at:

http://dx.doi.org/10.1021/acs.analchem.5b01389

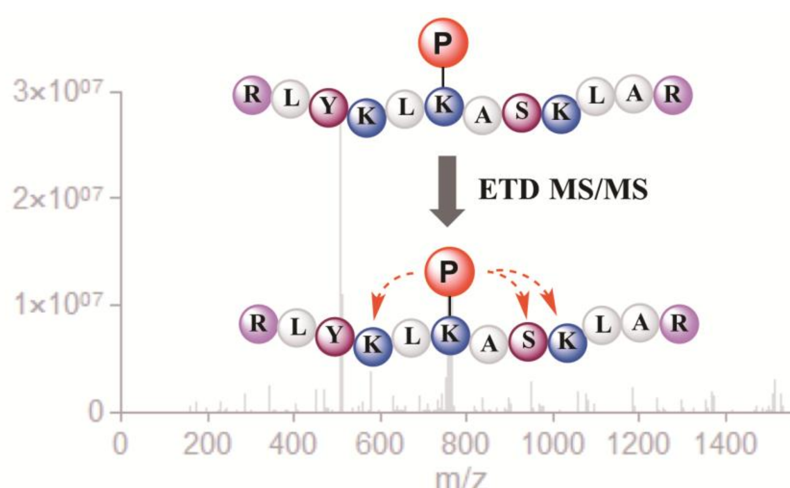


Figure 17. Gas-phase phosphate tranfer in phospholysine peptides during ETD fragmentation.

Abstract:

Tandem mass spectrometry (MS/MS) strategies coupled with collision-induced dissociation (CID) or radical-driven fragmentation techniques such as electron-capture dissociation (ECD) or electron-transfer dissociation (ETD) have been successfully used for comprehensive phosphoproteome analysis. However, the unambiguous characterization of the phosphorylation site remains a significant challenge due to phosphate-related neutral losses and gas-phase rearrangements, which have been observed during CID. In particular, for the analysis of labile *N*-phosphorylated peptides ECD and ETD are emerging as a complementary

method. In contrast to CID, the phosphorylation site of histidine, arginine, and lysine phosphorylated peptides can be characterized by ETD. Here, we present a study on the application of ETD for analysis of phospholysine (pLys) peptides. We show that depending on the charge state of the precursor ion as well as the presence of basic amino acid side chains, phosphate transfer reactions during the ETD process can be observed leading to ambiguous fragment ion spectra. Basically, pLys is stable under ETD conditions allowing an unambiguous assignment of the site of phosphorylation but some factors/parameters have to be considered to avoid gas-phase rearrangement which would lead to false positive results in phosphoproteomic studies.

Responsibility assignment

The design of the project was provided by Eberhard Krause together with the author. The author synthesized the peptides and evaluated the data obtained. Michael Schümann performed the ETD-MS analysis of the phosphorylated peptides. Christian P. R. Hackenberger made valuable contributions toward the analysis of the peptides as well as in the manuscript preparation. The publication was planned and written by the author under supervision of Eberhard Krause.

Summary of content

The characterization of lysine phosphorylation sites on peptides is a crucial step to improve the phosphoproteomic analysis of phospholysine in proteins. One of the bottle-neck at present in phosphoproteomics is to find reliable MS fragmentation methods which deliver unambiguous information about the localization of the phosphorylation site. In the case of phosphorylated lysine we demonstrated that ETD is the method of choice (*see chapter 3.1 and 3.2*), however, as it occurs with serine-, threonine- and tyrosine-phosphorylation careful data analysis should be applied to avoid false phosphorylated site assignments. To understand better the ETD fragmentation behavior of phosphorylated lysine peptides we synthesized several peptides, which match the amino acid composition of histone H1 (peptide sequence: X^{1}LYKLKASKLAX^{12}) varying the position of lysine phosphorylation as well as the entity of the *C*- and *N*-terminal amino acids. We also studied the influence of the charge state of the precursor on the ETD fragmentation process. First, we observed phosphate transfer to other phosphate acceptor residues when analyzing the 3+ charge

state precursor ion of a peptide with the following sequence: R^{1}LYKLpKASKLAR^{12}. When, selecting the 4+ charge state precursor ion, the phosphate transfer was not observed and the phosphoramidate bond seemed to be stable upon ETD process. A similar observation was reported for *O*-phosphorylation peptides and it was suggested that proton mobility plays an important role in rearrangement reactions. Ion mobility in the gas phase is mainly influence by the presence of basic amino-acid side chains such as arginine and lysine. Thereby, we decided to exchange the arginine residue in both *C*- and *N*-terminal sides to a more hydrophobic residue like alanine (A^{1}LYKLpKASKLAA^{12}). We selected again the 3+ charge state precursor ion for ETD fragmentation. Analysis of the MS spectra obtained showed no phosphate transfer to any of the other phosphate acceptor residues on the peptide. Thus, by reducing the proton mobility, the ability of the phosphate residue to migrate to other phosphate acceptors was suppressed.

Outlook

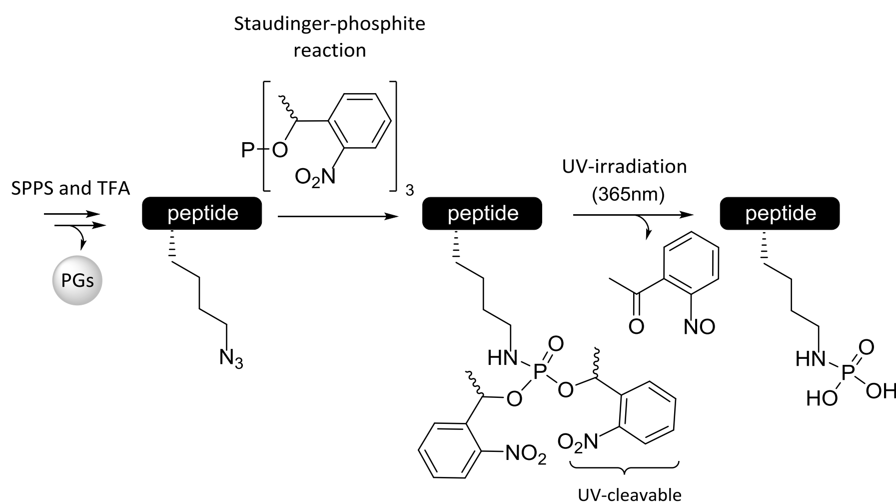
This study represents the first in detail analysis of the fragmentation of phosphorylated lysine peptides using ETD. However, the phosphate transfer effect might depend on other parameters, which were not considered in this preliminary study. For instance, strategies envisioned to study in more detail the phosphate transfer effect include the synthesis of multiple phosphorylated lysine peptides, in which the phosphate acceptor residues are placed at different positions. It is also envisioned to get more insight into relevant ETD parameters (mixing time, activation time, etc.) in order to see their influence on phosphate rearrangement processes.

4 SUMMARY

The results presented here from this work showed the power of chemoselective reactions to obtain access towards challenging post-translationally modified peptides. Specifically, two different chemoselective phosphorylation strategies were developed to synthesize site-specifically phosphorylated lysine and cysteine peptides. In addition to solving the synthetic accessibility issue, the site-specifically phosphorylated peptides were used to establish ETD tandem mass spectrometry as a reliable analytical tool to characterize both lysine- and cysteine-phosphorylation sites in a proteomic context.

Project 1: A chemoselective synthetic tool for studying lysine phosphorylation.[203]

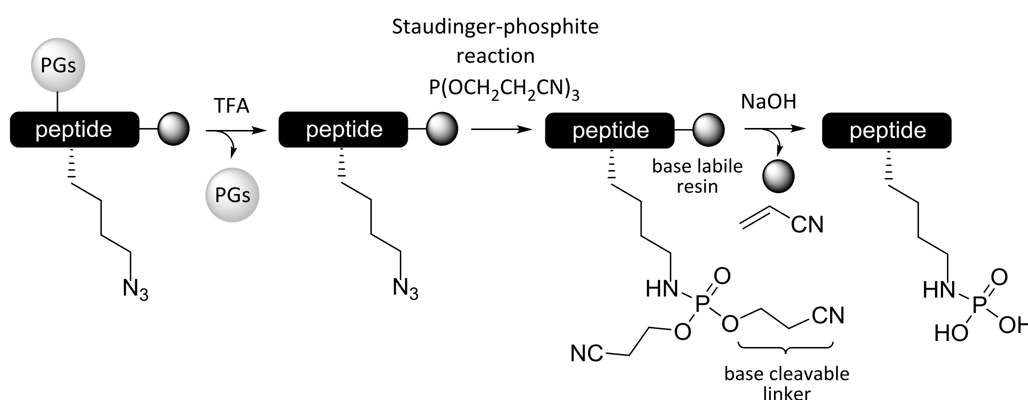
In this study, the site-specific incorporation of phosphorylated lysine residues into peptides by the Staudinger-phosphite reaction was developed and demonstrated with various peptide sequences containing different side chain functionalities. In order to release the phosphorylated lysine residues from its corresponding phosphoramidate esters, two different release systems were developed based on: (i) Photolysis of *o*-nitrobenzyl phosphoramidate esters peptides and (ii) alkaline treatment of base-cleavable cyanoethyl phosphoramidate ester peptides. In addition, the site-specifically modified phosphorylated lysine peptides turned out to be very useful for the unambiguous characterization by ETD MS and to assess their stability under different conditions (pH, temperature, cell lysate).



Scheme 20. Site-specific synthesis of phosphorylated lysine peptides in solution.

Project 2: A solid-supported approach to streamline the site-specific synthesis of phosphorylated lysine peptides.[204]

Having successfully applied the Staudinger-phosphite reaction to synthesize site-specifically phosphorylated lysine peptides in solution, the feasibility and compatibiliy of a solid-support approach was probed by combining a (i) base-labile resin with (ii) a base-cleavable phosphite ester and as previously the (iii) chemoselectivity of the Staudinger-phosphite reaction. This strategy successfully yielded site-specifically phosphorylated lysine peptides directly from a solid-supported resin, which proved to be a significant improvement to our recent published synthetic strategy.[203]

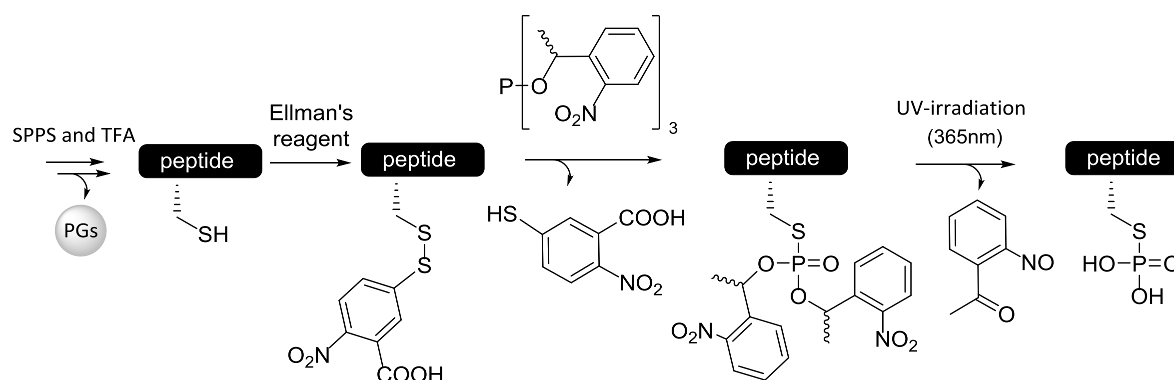


Scheme 21. Site-specific synthesis of phosphorylated lysine peptides directly from a solid-support.

Project 3: A chemoselective synthetic tool for studying cysteine-phosphorylation.

In this study, the site-specific incorporation of phosphorylated cysteine residues into peptides was demonstrated by reaction of electrophilic cysteine-disulfides peptides and phosphite triesters. The key step of this novel chemoselective tool was the preactivation of a cysteine residue with an electrophilic disulfide such as the Ellman´s reagent. The thereby formed electrophilic disulfide peptide enhanced the reactivity of the sulphur atom towards nucleophilic phosphites, allowing the formation of a protected phosphorothioate ester cysteine-containing peptide. Finally, UV-irradiation of the UV-cage phosphorothioate ester peptide delivered the phosphorylated cysteine peptide. Owing to the relatively low abundance of cysteine residues on proteins and their ability to form disulfide bonds, this novel synthetic strategy is expected to be a powerful tool to study cysteine phosphorylation. The phosphorylated cysteine peptides were also used to compare their behavior under different fragmentation techniques to finally establish an ETD MS/MS method as tool for the

unambiguous characterization of phosphorylation sites. In the future, in order to assess the synthetic strategy on protein level, the conversion in buffer has to be improved by chemically tunning the electrophilicity of the disulfides as well as the phosphite esters.



Scheme 22. Site-specific synthesis of phosphorylated cysteine peptides.

Project 4: Phosphate migration in phosphorylated lysine peptides during ETD MS analysis.[205]

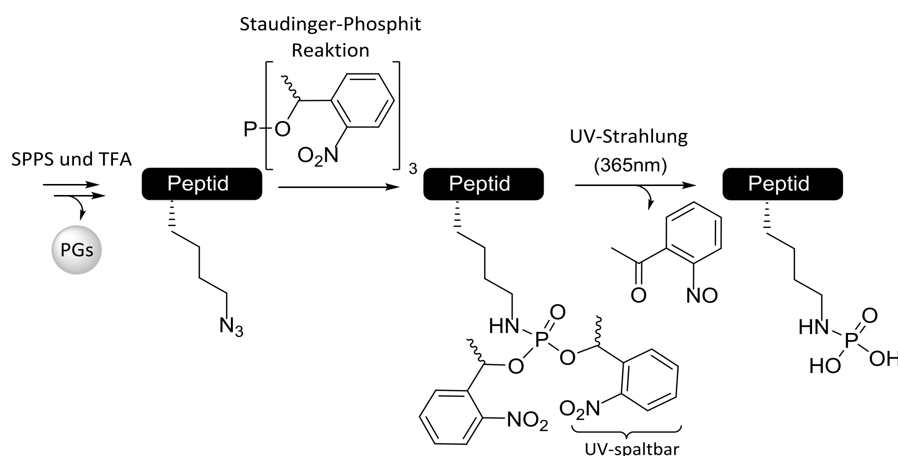
In this project, a more detailed analysis of the ETD fragmentation of phosphorylated lysine peptides was studied. Specifically, gas-phase phosphate rearrangements were observed during the analysis of different synthetic phosphorylated lysine peptides by ETD mass spectrometry. Similar phosphate transfer reactions have been previously reported to occur during the often used CID fragmentation of *O*-phosphorylated peptides. We hypothesized that the phosphate transfer occurs under the same conditions than in CID, i.e., under non- or partially- mobile protonation conditions. As ETD is a soft fragmentation technique, the different phosphate rearrangement products remain stable during ETD process and can thereby be observed in the ETD fragment ion spectra. We concluded that even if ETD is used, some factors and parameters, such as proton mobility which is determined by the sequence and charge-state of the peptide ion, have to be considered to avoid false-positive phosphorylated site assigments in phosphoproteomic studies.

5 ZUSAMMENFASSUNG

Die Ergebnisse der hier vorgestellten Dissertation zeigen die Leistungsfähigkeit der untersuchten chemoselektiven Reaktionen, vor allem für die Synthese von anspruchsvollen posttranslational modifizierten Peptiden, auf. Im Rahmen der Arbeit wurden zwei unterschiedliche chemoselektive Phosphorylierungsreaktionen, die den Einbau von ortsspezifisch phosphorylierten Lysinen und Cysteinen in Peptiden ermöglichen, entwickelt. Mit Hilfe der synthetisierten Phospholysin und –cystein Peptide konnten ETD-MS/MS Methoden zur zuverlässigen analytischen Charakterisierung solcher Peptid- und Proteinmodifizierung entwickelt werden.

Projekt 1: Ein chemoselektives synthetisches Werkzeug zur Erforschung der Lysin-Phosphorylierung.[203]

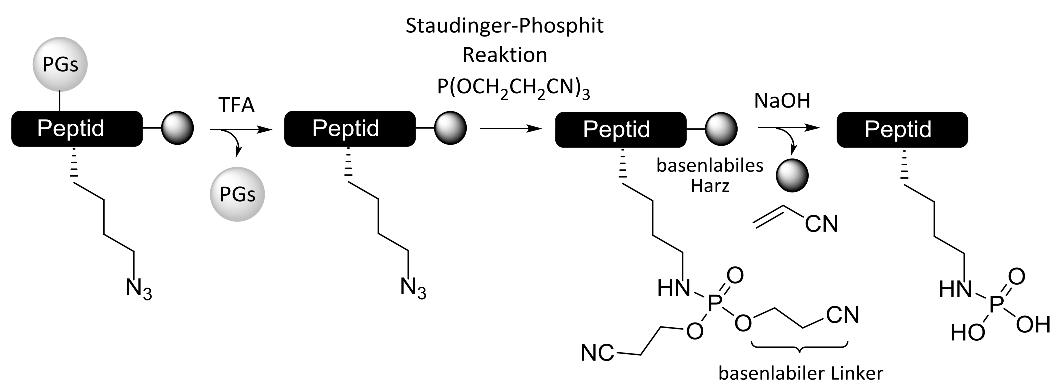
In diesem Projekt wurden zwei Varianten der Staudinger-Phosphit Reaktion für den ortsspezifischen Einbau von Phosphatgruppen an Lysinen in Peptiden entwickelt. Die Methoden wurden mit verschiedenen Peptidsequenzen unterschiedlicher Aminosäurenzusammensetzung verifiziert. Der Fokus lag hier auf zwei Strategien: Die erste Variante basierte auf der Photolyse von peptidischen *o*-Nitrobenzyl Phosphoramidatestern, während die zweite Strategie über basisch labile Cyanoethylphosphoramidatester führte. Die ortsspezifisch phosphorylierten Peptide konnten anschliessend mithilfe von ETD-MS/MS charakterisiert und darüber hinaus für Untersuchungen zur Stabilität der N-Phosphorylierung unter verschiedenen Bedingungen (pH, Temperatur, Zellysat) verwendet werden.



Scheme 23. Ortsspezifischer Einbau von Phosphatgruppen in Lysinseitenketten von Peptiden.

Projekt 2: Eine Festphasensynthese zur Optimierung des ortsspezifischen Einbaus von Phosphatgruppen an Lysinen in Peptiden.[204]

Nach der erfolgreichen Anwendung der Staudinger-Phosphit Reaktion für die ortsspezifische Synthese von Lysinphosphorylierten Peptiden in Lösung, wurde eine Synthesevariante über Festphasen-peptidsynthese (SPPS) als mögliche Alternative entwickelt. In diesem Ansatz wurde die chemoselektive Staudinger-Phosphit Reaktion in unter Anwendung eines basenlabilen Syntheseharzes und basenlabilen Phosphitester durchgeführt. Es konnte gezeigt werden, dass ortsspezifisch am Lysin mit einer Phosphatgruppe modifizierte Peptide auch über SPPS efektiv hergestellt werden können. Dieses Ergebnis ist eine wesentliche Verbesserung der erst kurz zuvor veröffentlichte Synthesestrategie in Lösung.[203]

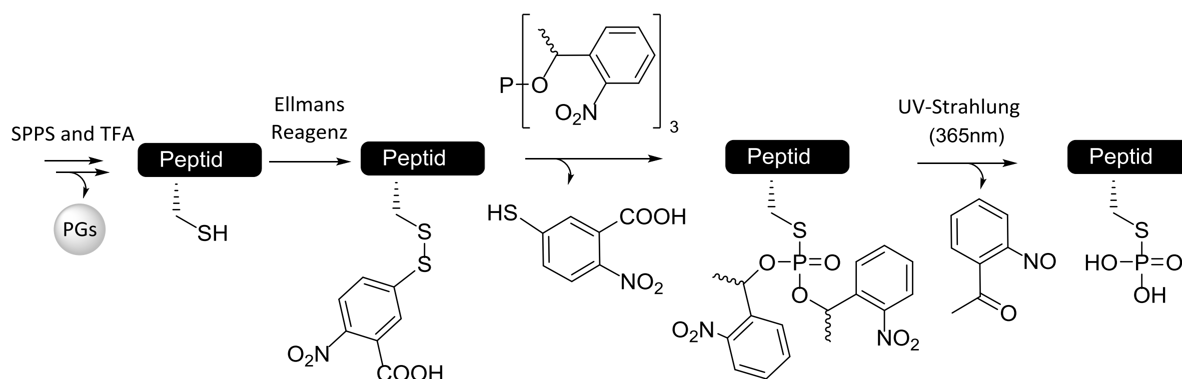


Scheme 24. Festphasen-Synthese von ortsspezifische phosphorylierten Lysin Peptiden.

Projekt 3: Ein chemoselektives synthetisches Werkzeug zur Erforschung der Cystein-Phosphorylierung.

In diesem Teil der Arbeit wurden Cysteinseitenketten in Peptiden über eine Reaktion von elektrophilen Cysteindisulfiden und Phosphitester ortsspezifisch mit Phosphatresten modifiziert. Der wichtigste Schritt ist hier die vorherige Aktivierung des Cysteins durch ein elektrophiles Disulfid wie z. B. dem Ellmans Reagenz. Dies erhöht die Reaktivität des Schwefelatoms gegenüber einem nukleophilen Angriff von Phosphiten und ermöglicht so den Einbau eines geschützten Phosphorothioatesters am Cystein. Durch UV-Strahlung kann der Phosphorothioatester gespalten werden und liefert das gewünschte phosphorylierte Cystein. Aufgrund der relativ geringen Häufigkeit von Cystein in Proteinen und der Bildung einiger Disulfidbrücken ist diese neuartige Synthesestrategie ein wichtiges Werkzeug, um Cysteinphosphorylierungen zu untersuchen. Die modifizierten Peptide wurden auch

verwendet um ihr Verhalten bei unterschiedlichen massenspektrometrischen Fragmentierungstechniken zu vergleichen. So konnte gezeigt werden, dass die ETD Fragmentierung als analytisches Werkzeug für die eindeutige Charakterisierung von den Cys-Phosphorylierungen geeignet ist. In naher Zukunft sollten die Reaktionsbedingungen (eingesetzte Puffer, Disulfid, Phosphitester) optimiert werden um spezifische Modifizierungen von Cysteinen auch in Proteinen zu ermöglichen.



Scheme 25. Ortsspezifische Synthese von phosphorylierte Cystein Peptiden.

Project 4: Phosphat-Migration bei der ETD MS Analyse von Lysin-phosphorylierten Peptiden.[205]

In diesem Projekt wurde der Einfluß der Peptidsequenz und von ETD MS Parametern auf die Fragmentierung von Lysin-phosphorylierten Peptiden untersucht. Es konnte gezeigt werden, dass eine Migration von Phosphatresten in der Gasphase, welche bisher nur bei CID MS/MS von *O*-Phosphopeptiden beobachtet wurden, auch bei der ETD Fragmentierung auftreten kann. Eine Phosphatübertragung auf andere Lysin- oder auch Serinseitenketten konnte dabei bevorzugt unter Bedingungen der eingeschränkten Protonenmobilität nachgewiesen werden. Da ETD eine sanfte Fragmentierungstechnik ist, bleiben die Migrationsprodukte während des ETD Prozesses stabil und können so in den MS/MS Spektren gesehen werden. Die Ergebnisse zeigen, dass die ETD Experimente hinsichtlich des Auftretens von Phosphatmigrationsprodukten kritisch bewertet werden müssen, um falsch-positive Ergebnisse, d.h. falsche Zuordnungen von Phosphorylierungsposition in Phosphoproteomprojekten zu vermeiden.

6 EXPERIMENTAL PART

6.1 Material and Methods

Materials: All reagents, starting materials, amino acids, and solvents were purchased from commercial suppliers and used without further purification if not further mentioned.

NMR: ^{1}H and ^{31}P-NMR spectra were recorded on a Bruker Ultrashield AV 600 MHz at ambient temperature. The chemical shifts are reported in ppm relatively to the residual solvent peak.

Analytical UPLC: UPLC-UV traces were obtained using a Waters H-class instrument, equipped with a Quaternary Solvent Manager, a Waters autosampler, a Waters TUV detector connected to a 3100 mass detector using a Acquity UPLC-BEH C18 1.7 µM 2.1x50mm RP column with a flow rate of 0.6 mL/min. The following solvent and gradients were applied for all peptides if not further mentioned: **Method 1** (A = H_2O + 0.1% TFA, B = MeCN + 0.1% TFA) 1% B 0-5 min, 1-99% 5-15 min, 99% B 15-17 min. **Method 2** (A = H_2O + 0.1% TFA, B = MeCN + 0.1% TFA) 1% B 0-3 min, 1-99% 3-8 min, 99% B 8-10 min. UPLC-UV chromatograms were recorded at 220 nm.

Solid-phase peptide synthesis: Peptides were synthesized with a Tribute Peptide Synthesizer (Protein Technologies, Inc) and on automated parallel petide synthesizer Syro II *via* standard Fmoc-based conditions with HOBt/HBTU/DIPEA activation and piperidine Fmoc deprotection in DMF.

Semi-preparative HPLC purification: Phosphorothioate ester peptides were purified on a Dionex 580 HPLC system using a reversed phase Nucleodur C18 HTec column (10 x 250 mm) using a TFA gradient (0% B 0-5 min; 0-50% B 5-60 min; 50-100% 60-80 min; 100% B 80-90 min) in H_2O/MeCN system (A = H_2O + 0.1% TFA, B = 85% MeCN + 15% A + 0.1% TFA) **(Method A)**. Phosphorylated cysteine peptides were purified on a Shimadzu HPLC system using a reversed phase Nucleodur C18 HTec column (10 x 250 mm) at a flow rate of 3 mL/min using an MeCN/H_2O gradient system (0% B 0-5 min; 0-40% B 5-55 min; 40-100% B 55-85 min) in alkaline aqueous buffer (pH 8.2) as a mobile phase (A = 10 mM ammonium acetate in H_2O, B = MeCN + 10 % of sol. A) (**Method B**).

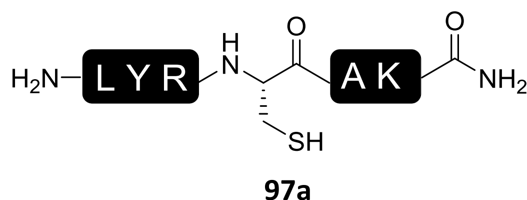
High-resolution mass spectra (HRMS): Characterization of peptides were done with an Agilent 6210 ToF LC/MS system.

ETD Tandem MS analysis: For LC-MS analysis, peptides were dissolved in H_2O (1 pM/µL) and analyzed by a reversed-phase capillary liquid chromatography system (Dionex Ultimate 3000 NCS-3500RS Nano, Thermo Scientific) connected to an Orbitrap Fusion mass spectrometer (Thermo Scientific). LC separations were performed on an in-house packed 75 µm inner diameter PicoTip column containing 25 cm of ReproSil-Pur C18AQ resin (3 µm, 120 Å, Dr. Maisch GmbH Ammerbuch-Enttringen, Germany) at an eluent flow rate of 300 nl/min using a gradient of 2–50% B in 40 min. Mobile phase A contained 0.1% formic acid in H_2O, and mobile phase B contained 0.1% formic acid in MeCN. FT survey scans were acquired in a range from 350 to 1500 *m/z*, with a resolution of 60000, an automatic gain control (AGC) target of 100000 and a max injection time of 50 ms. In data dependent mode monoisotopic precursor ions with charge states between 2 and 5 were selected, preferential choosing higher charge states prior to fragmentation. FTMS2 spectra were measured in the orbitrap with a resolution of 15000, an AGC target of 100000 and max injection time of 200 ms. For HCD the normalized collision energy was set to 30%. ETD spectra were collected using calibrated charge dependent ETD parameters and HCD supplemental activation (SA). For EThcD fragmentation the SA Collisions Energy was to 10 or 30%, respectively. MS/MS spectra were manually verified and compared with the theoretical fragment ions of the peptides considering all possible phosphorylation sites.

6.2 A chemoselective tool for studying cysteine-phosphorylation

6.2.1 Synthesis of cysteine-containing peptides 97a-c

Cysteine peptide **97a**



Procedure: Rink Amide AM Resin (0.2 mmol; 0.73 mmol/g; 100-200 mesh) was loaded with Fmoc-Lys(Boc)-OH and applied to SPPS using Fmoc-couplings with HOBt/HBTU/DIPEA in DMF on a Tribute Peptide Synthesizer (Protein Technologies, Inc). The peptide was cleaved off the resin by addition of TFA/DTT/TIS/thioanisol (95/2/2/1), followed by precipitation of the peptide in cold ether. Peptide **97a** was used without further purification.

Molar mass (peptide) = 751.94 Da; molar mass (TFA_3 salt) = 1094.02 Da

ESI-MS (positive mode) = 376.7138 $[M+2H]^{2+}$ (calcd. m/z: 376.7154)

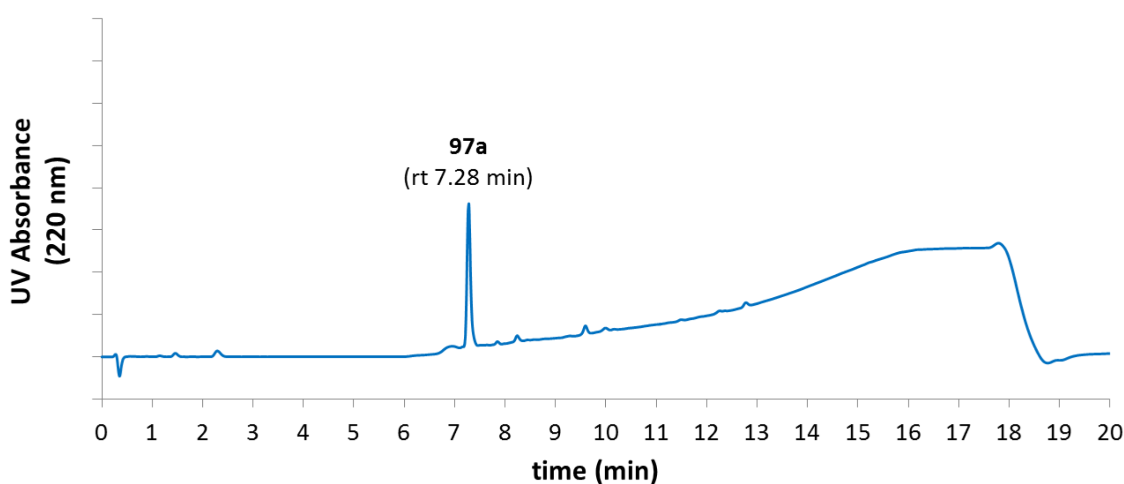
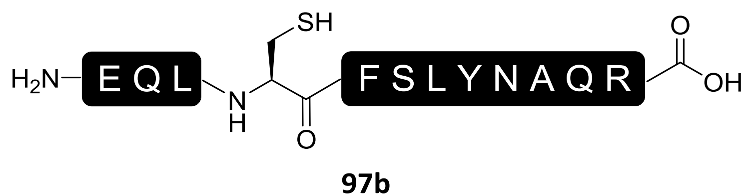


Figure 18. UPLC-trace of pure peptide **97a** using chromatographic method 1.

Cysteine peptide **97b**



Procedure: A TentaGel S PHB resin (0.05 mmol; 0.25 mmol/g; 90 mesh) preloaded with Fmoc-Arg(Pbf)-OH was used and applied to SPPS using Fmoc-coupling with HOBt/HBTU/DIPEA in DMF on a automated parallel petide synthesizer Syro II. The peptide was cleaved off the resin by addition of TFA/DTT/TIS/thioanisol (95/2/2/1), followed by precipitation of the peptide in cold ether. Purification *via* semi-preparative HPLC (method A) yielded peptide **97b** (18 mg, 10.6 µmol) in 21% yield.

Molar mass (peptide) = 1471.65 Da; molar mass (TFA_2 salt) = 1699.70 Da

ESI-MS (positive mode) = 736.3504 $[M+2H]^{2+}$ (calcd. m/z: 736.3535)

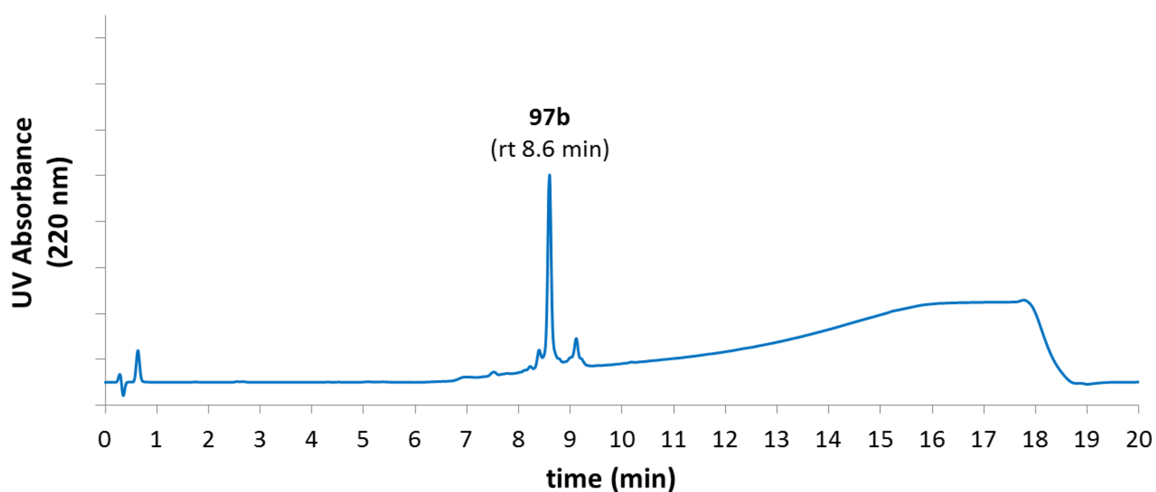
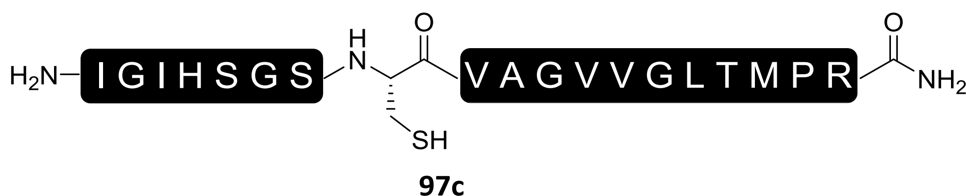


Figure 19. UPLC-trace of pure peptide **97b** using chromatographic method 1.

Cysteine peptide **97c**



Procedure: Rink Amide AM Resin (0.2 mmol; 0.73 mmol/g; 100-200 mesh) was loaded with Fmoc-Arg(Pbf)-OH and applied to SPPS using Fmoc-couplings with HOBt/HBTU/DIPEA in DMF on a Tribute Peptide Synthesizer (Protein Technologies, Inc). The peptide was cleaved off the resin by addition of TFA/DTT/TIS/thioanisol (95/2/2/1), followed by precipitation of the peptide in cold ether. Purification *via* semi-preparative HPLC (method A) yielded peptide **97c** (48.7 mg, 22.2 µmol) in 11% yield.

Molar mass (peptide) = 1853.24 Da; molar mass (TFA_3 salt) = 2195.31 Da

ESI-MS (positive mode) = 618.3322 $[M+3H]^{3+}$ (calcd. m/z: 618.3343)

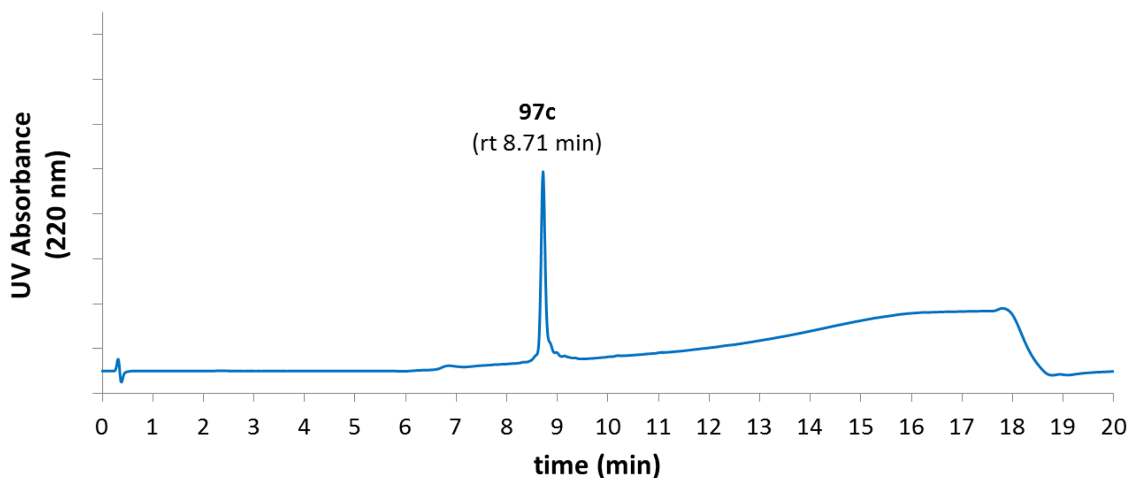
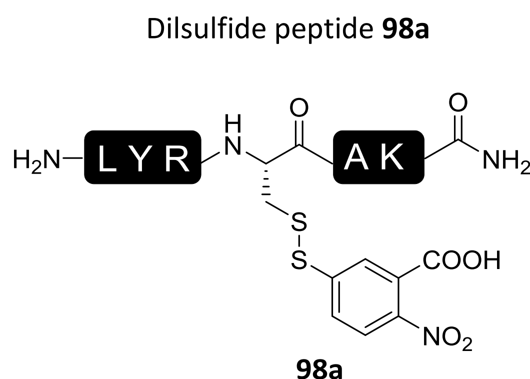


Figure 20. UPLC-trace of pure peptide **97c** using chromatographic method 1.

6.2.2 Synthesis of electrophilic disulfides containing peptides 98a-c

Dilsulfide peptide **98a**



Procedure: Cysteine peptide **97a** as white trifluoroacetate salt (25 mg, 22.9 µmol) was dissolved in DMF (0.8 mL) along with Et_3N (63.8 µL, 457 µmol, 20 eq). To the stirred solution was added a solution of Ellman´s reagent (18.1 mg, 45.8 µmol, 2 eq) in DMF (0.8 mL). Final peptide concentration was 28.6 mM. The reaction mixture was incubated at room temperature for 15 min. Purification *via* semi-preparative HPLC (method A) yielded peptide **98a** (9.8 mg, 7.6 µmol) in 33% yield.

Molar mass (peptide) = 949.11 Da; molar mass (TFA_3 salt) = 1291.18 Da

ESI-MS (positive mode) = 475.2047 $[M+2H]^{2+}$ (calcd. m/z: 475.2045)

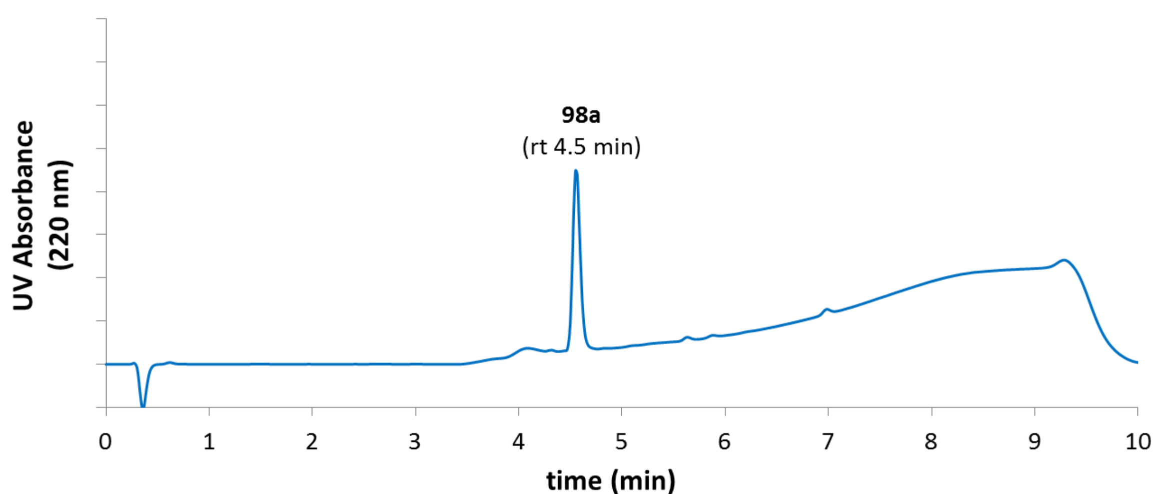
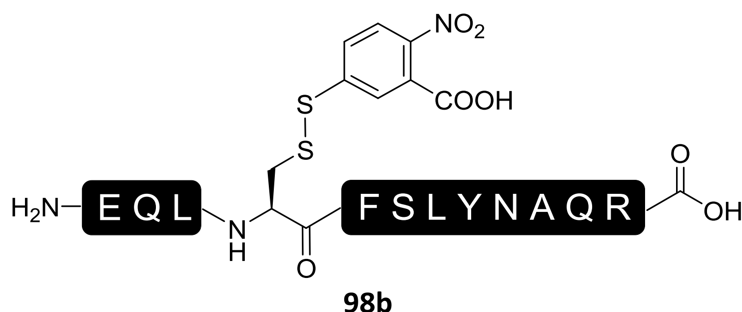


Figure 21. UPLC-trace of pure peptide **98a** using chromatographic method 2.

Disulfide peptide **98b**



Procedure: Cysteine peptide **97b** as white trifluoroacetate (18 mg, 10.6 µmol) was dissolved in DMF (0.4 mL) along with Et_3N (29.6 µL, 212 µmol, 20 eq). To the stirred solution was added a solution of Ellman´s reagent (8.7 mg, 21.2 µmol, 2 eq) in DMF (0.4 mL). Final peptide concentration was 13.3 mM. The reaction mixture was incubated at room temperature for 15 min. Purification *via* semi-preparative HPLC (method A) yielded peptide **98b** (12.2 mg, 6.4 µmol) in 61% yield.

Molar mass (peptide) = 1668.82 Da; molar mass (TFA_2 salt) = 1896.86 Da

ESI-MS (positive mode) = 834.8374 $[M+2H]^{2+}$ (calcd. m/z: 834.8427)

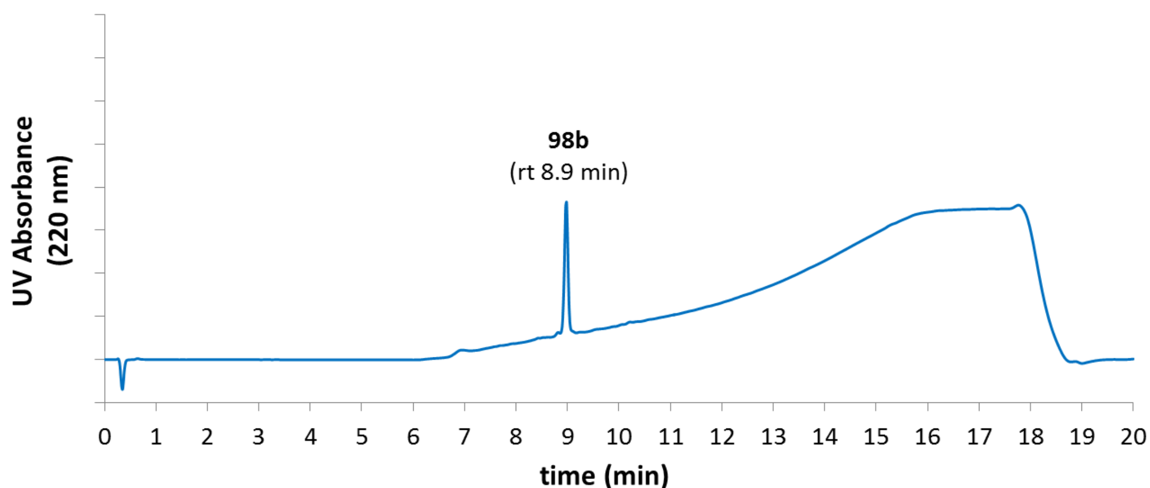
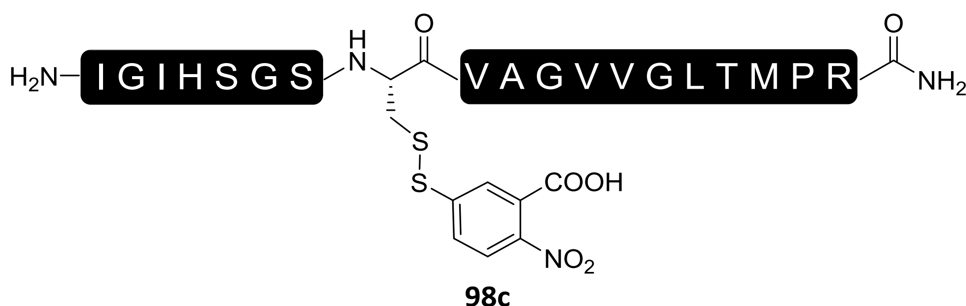


Figure 22. UPLC-trace of pure peptide **98b** using chromatographic method 1.

Disulfide peptide **98c**



Procedure: Cysteine peptide **97c** as white trifluoroacetate (15 mg, 6.8 µmol) was dissolved in DMF (0.3 mL) along with Et_3N (19.0 µL, 136.65 µmol, 20 eq). To the stirred solution was added a solution of Ellman´s reagent (5.4 mg, 13.6 µmol, 2 eq) in DMF (0.3 mL). Final peptide concentration was 11.3 mM. The reaction mixture was incubated at room temperature for 15 min. Purification *via* semi-preparative HPLC (method A) yielded peptide **98c** (7.0 mg, 2.9 µmol) in 43% yield.

Molar mass (peptide) = 2050.40 Da; molar mass (TFA_3 salt) = 2392.45 Da

ESI-MS (positive mode) = 683.9949 $[M+3H]^{3+}$ (calcd. m/z: 683.9938)

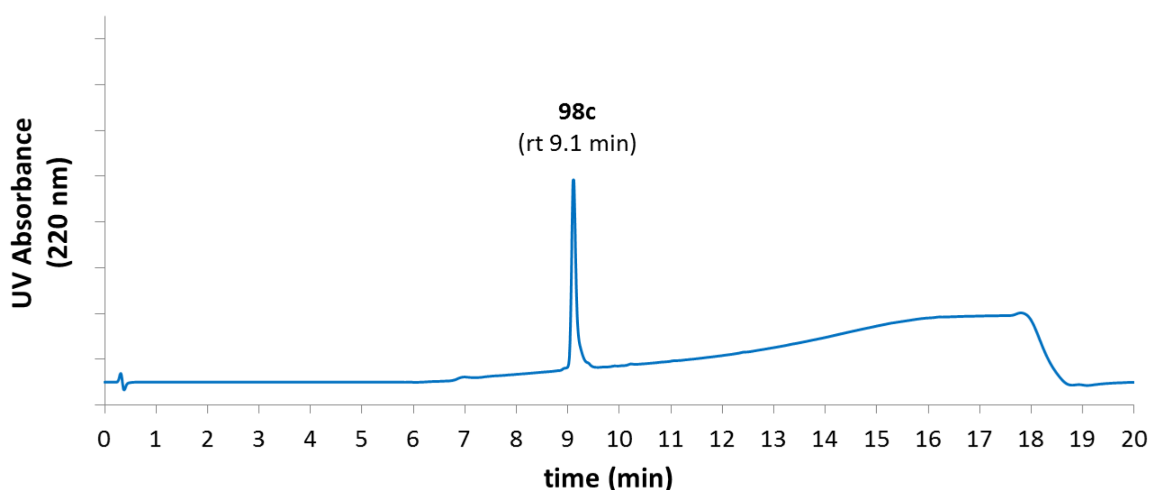


Figure 23. UPLC-trace of pure peptide **98c** using chromatographic method 1.

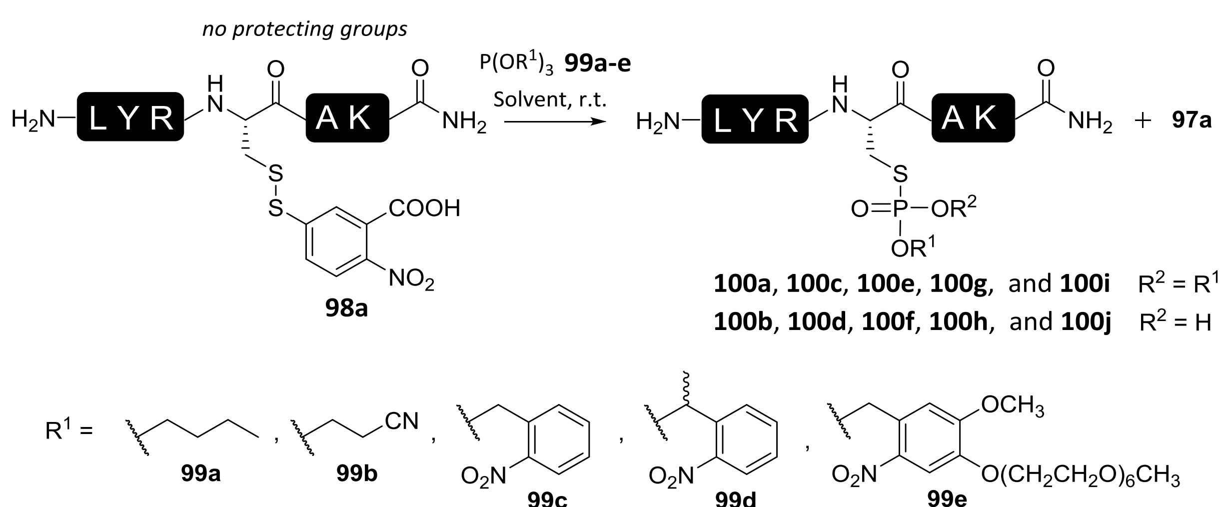
6.2.3 Synthesis of phosphorothioate ester containing peptides 100a-l

6.2.3.1 Reactivity of electrophilic disulfides towards different phosphites

General procedure: A stock solution of peptide **98a** (2.5 mM) in DMF and in 25 mM Tris-NaOH buffer (pH 8.0) was prepared. All phosphites employed (**99a**-**e**) were added in excess (5 eq.). Reactions in DMF and Tris buffer were run until peptide **98a** was fully or almost depleted (aprox. 16h in DMF and 3h in Tris buffer) at room temperature. After the removing of solvents, the crude reaction mixtures were diluted in a mixture of H_2O/MeCN (8:2) and injected in a UPLC-UV and –MS detector. Identity of the product was confirmed by HR-MS (*for MS spectra see appendix*).

Quantification of the reaction by MS: The reaction conversions were determined on LC-MS analysis after the starting material, peptide **98a**, was depleted. The sum of the XIC areas corresponding to the expected products was counted as 100%. All experiments were performed at least in duplicates and the conversion values were taken from the average values of each replicate.

Scheme 26. Synthesis and reactivity of different phosphites (**99a-e**) towards peptide **98a**



6.2.3.2 Monitoring the reaction by HPLC-UV and –MS

Entry 4:

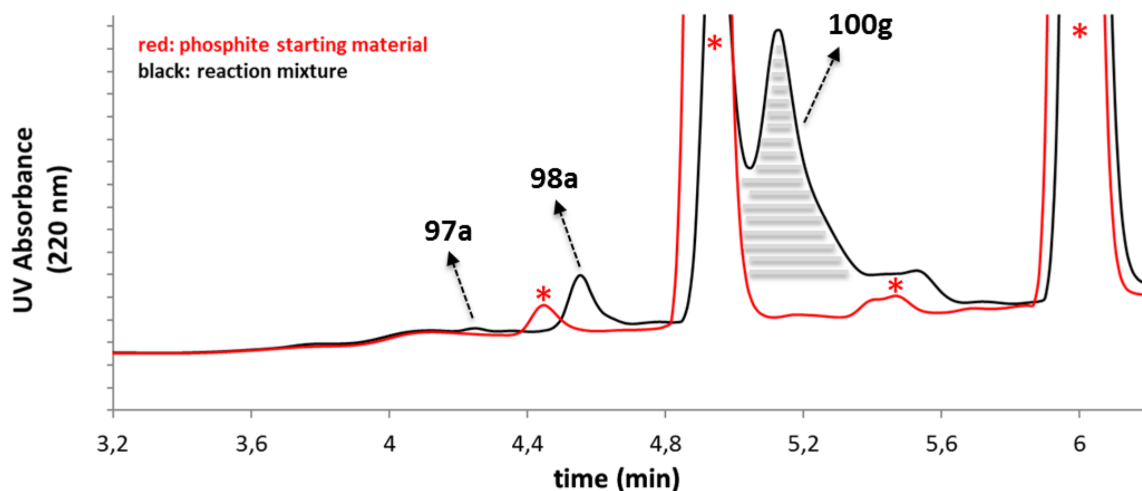


Figure 24. Overlapping LC-UV chromatograms of reaction mixture and phosphite after 16h. Red asterisk mark the decomposition by-products of phosphite.

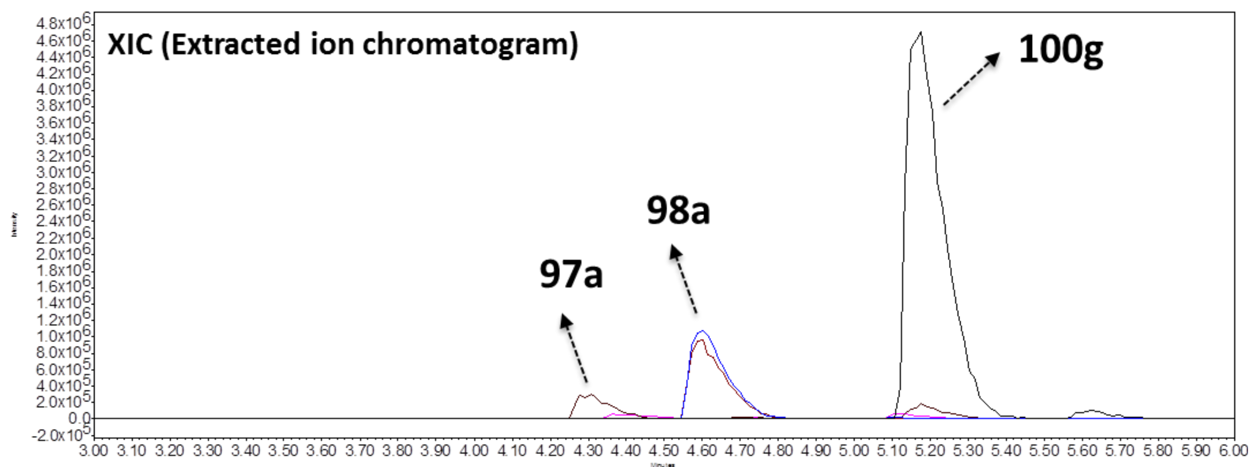


Figure 25. XIC chromatogram with the extracted masses for peptide **98a**, **100g**, and **97a**

Entry 9:

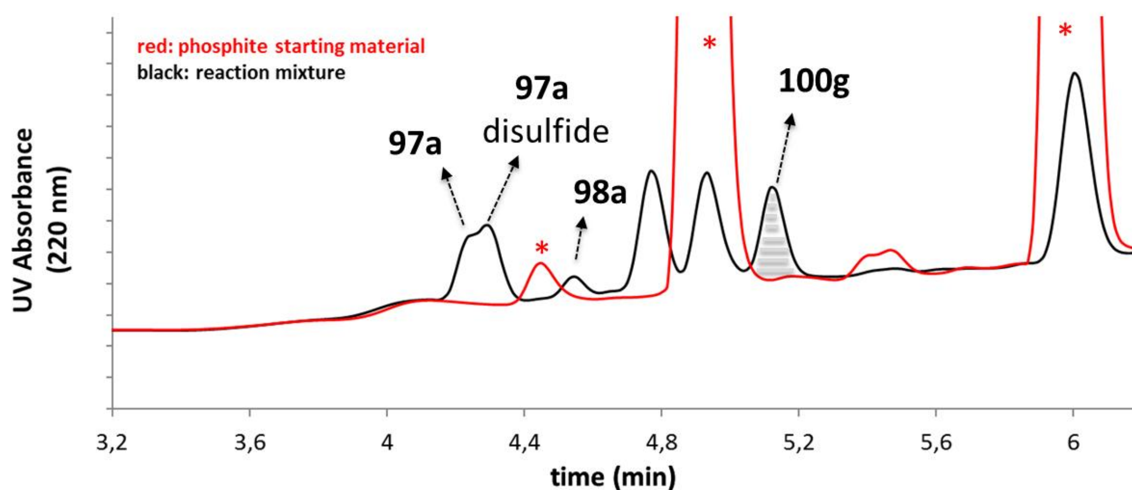


Figure 26. Overlapping LC-UV chromatograms of reaction mixture and phosphite after 3h. Red asterisk mark the decomposition by-products of phosphite.

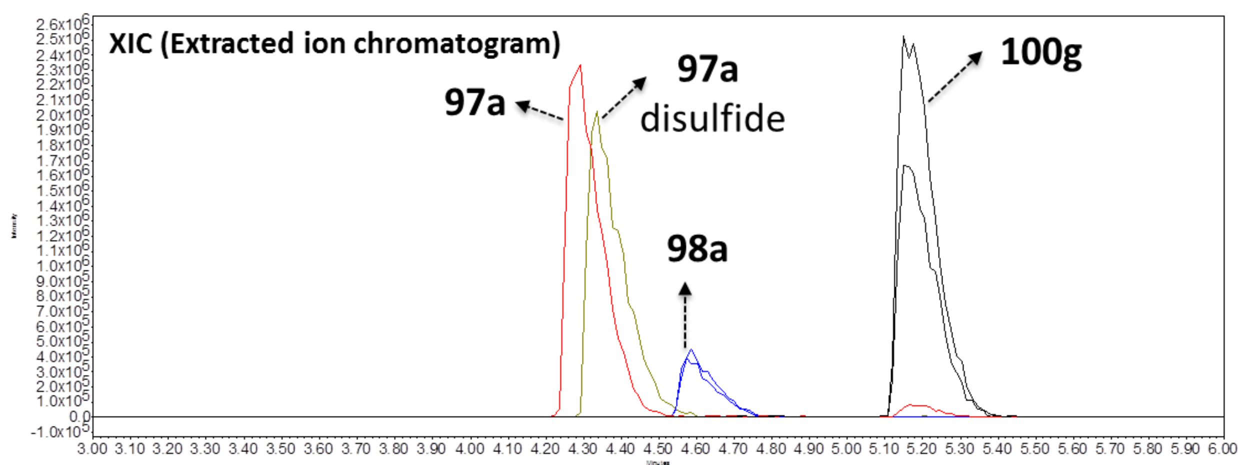


Figure 27. XIC chromatogram with the extracted masses for peptide **97a**, **100g**, **98a** and disulfide **97a**.

Entry 5:

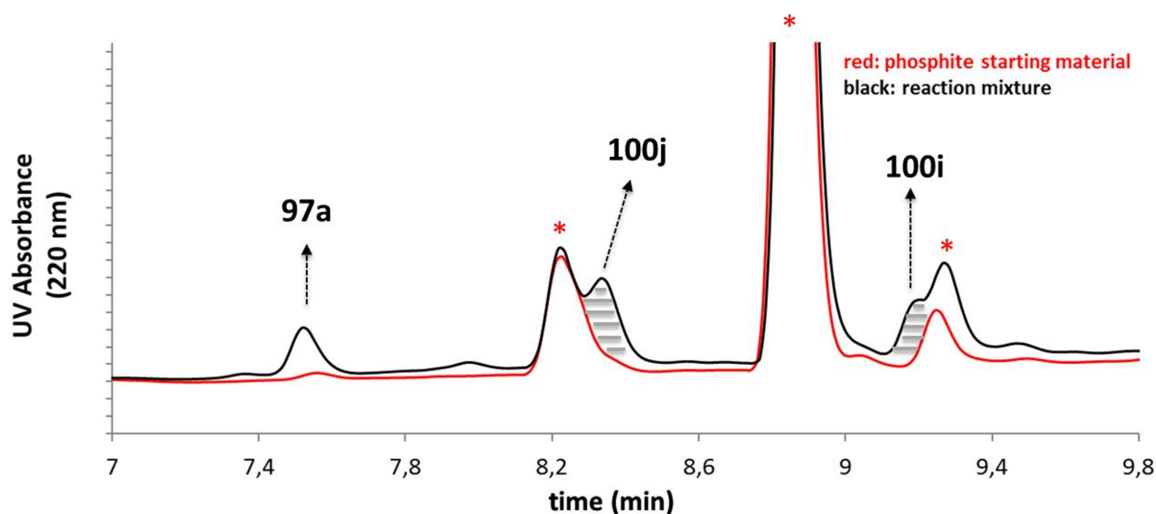


Figure 28. Overlapping LC-UV chromatograms of reaction mixture and phosphite after 16h. Red asterisk mark the decomposition by-products of phosphite.

Entry 11:

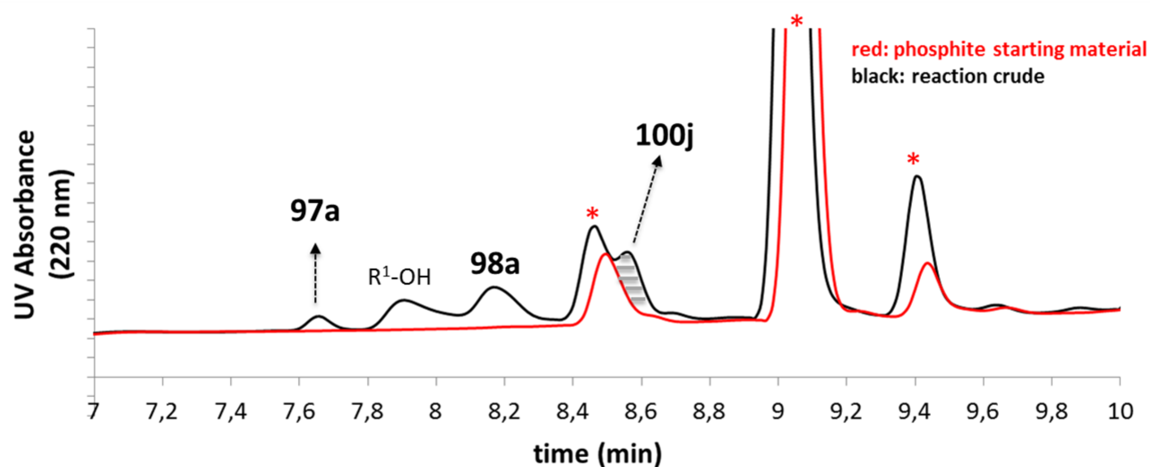
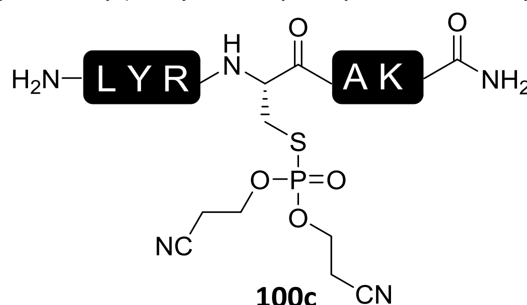


Figure 29. Overlapping LC-UV chromatograms of reaction mixture and phosphite after 3h. Red asterisk mark the decomposition by-products of phosphite.

6.2.3.3 Synthesis of phosphorothioate protected containing peptides

O,*O*-bis(2-cyanoethyl) *S*-cysteine phosphorothioate peptide **100c**



In DMF as solvent:

Procedure: Ellman´s labelled cysteine peptide **98a** (1.9 mg, 1.5 µmol) was dissolved in DMF (150 µL) and a solution of phosphite **99b** (1.8 mg, 7.5 µmol, 5 eq) in DMF (150 µL) was added. Final peptide concentration was 5 mM. The reaction mixture was incubated at room temperature for 16 h. Purification *via* semi-preparative HPLC (method A) yielded peptide **100c** (0.7 mg, 0.55 µmol) in 37% yield.

Molar mass (peptide) = 938.05 Da; molar mass (TFA_3 salt) = 1280.13 Da

ESI-MS (positive mode) = 469.7230 $[M+2H]^{2+}$ (calcd. m/z: 469.7251)

^{31}**P-NMR** (243 MHz, D_2O at pH 7.5) δ = 29.88 ppm.

In 50 mM Tris Buffer pH 8.0 / MeCN (2:3) as a solvent:

Procedure: Ellman´s labelled cysteine peptide **98a** (1.9 mg, 1.5 µmol) was dissolved in 50 mM Tris Buffer at pH 8.0 (150 µL) and a solution of phosphite **99c** (1.8 mg, 7.5 µmol, 5 eq) in MeCN (150 µL) was added. Final peptide concentration was 5 mM. The reaction mixture was incubated at room temperature for 3 h. Purification *via* semi-preparative HPLC (method A) yielded peptide **100c** (0.8 mg, 0.62 µmol) in 43% yield.

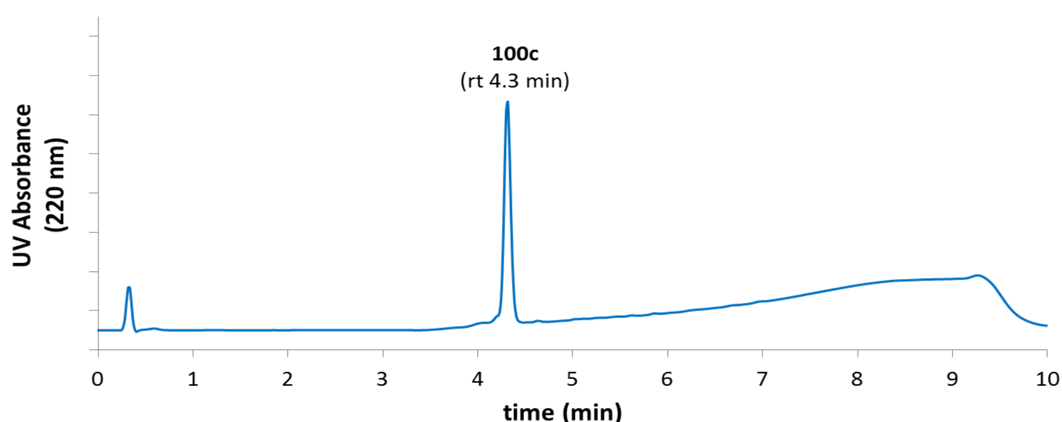
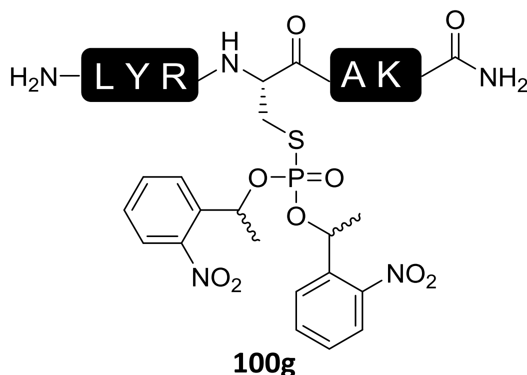


Figure 30. UPLC-trace of pure peptide **100c** using chromatographic method 2.

O,*O*-bis(1-(2-nitrophenyl)ethyl) *S*-cysteine phosphorothioate peptide **100g**



In DMF as a solvent:

Procedure: Ellman´s labelled cysteine peptide **98a** (1 mg, 0.8 µmol) was dissolved in DMF (200 µL) and a solution of phosphite **99c** (2.0 mg, 3.9 µmol, 5 eq) in DMF (200 µL) was added. Final peptide concentration was 1.9 mM. The reaction mixture was incubated at room temperature for 16 h. Purification *via* semi-preparative HPLC (method A) yielded peptide **100g** (0.6 mg, 0.4 µmol) in 55% yield.

Molar mass (peptide) = 1130.22 Da; molar mass (TFA_3 salt) = 1472.30 Da

ESI-MS (positive mode) = 565.7439 $[M+2H]^{2+}$ (calcd. m/z: 565.7463)

^{31}**P-NMR** (243 MHz, D_2O at pH 7.5) δ = 28.40, 27.55, 27.50, 26.79 ppm.

In 50 mM Tris Buffer pH 8.0 / MeCN (2:3) as a solvent:

Procedure: Ellman´s labelled cysteine peptide **98a** (1 mg, 0.8 µmol) was dissolved in 50 mM Tris Buffer at pH 8.0 (175 µL) and a solution of phosphite **99c** (2.0 mg, 3.9 µmol, 5 eq) in MeCN (225 µL) was added. Final peptide concentration was 1.9 mM. The reaction mixture was incubated at room temperature for 3 h. Purification *via* semi-preparative HPLC (method A) yielded peptide **100g** (0.3 mg, 0.2 µmol) in 27% yield.

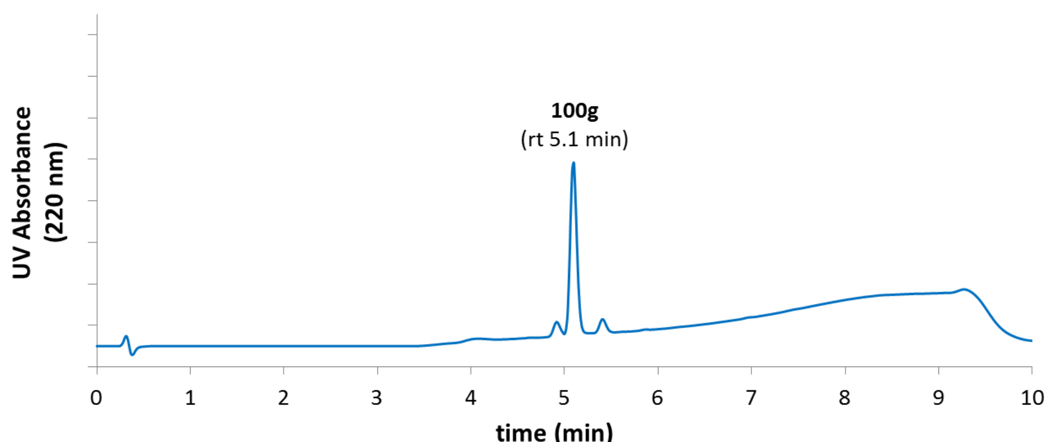
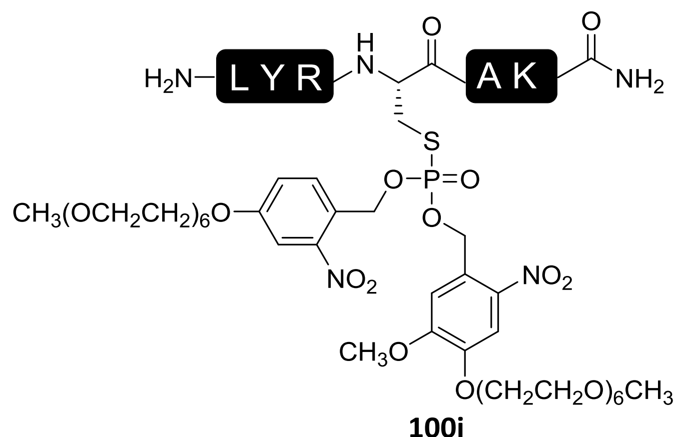


Figure 31. UPLC-trace of pure peptide **100g** using chromatographic method 2.

O,*O*-bis((4-((2,5,8,11,14,17-hexaoxanonadecan-19-yl)oxy)-5-methoxy-2-nitrobenzyl) *S*-cysteine phosphorothioate peptide **100i**



In DMF as a solvent:

Procedure: Ellman´s labelled cysteine peptide **98a** (1.5 mg, 1.2 µmol) was dissolved in DMF (300 µL) and a solution of phosphite **99e** (8.7 mg, 6.0 µmol, 5 eq) in DMF (300 µL) was added. Final peptide concentration was 2.0 mM. The reaction mixture was incubated at room temperature for 16 h. Purification *via* semi-preparative HPLC (method A) yielded peptide **100i** (0.65 mg, 0.31 µmol) in 27% yield.

Molar mass (peptide) = 1750.91 Da.; molar mass (TFA_3 salt) = 2092.98 Da

ESI-MS (positive mode) = 875.9098 $[M+2H]^{2+}$ (calcd. m/z: 875.9091)

^{31}**P-NMR** (243 MHz, D_2O at pH 7.5) δ = 30.34 ppm.

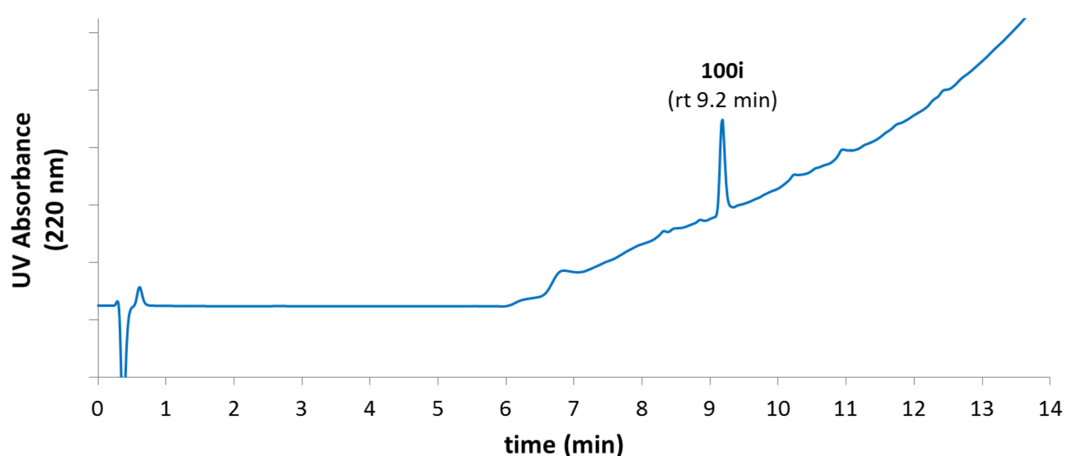
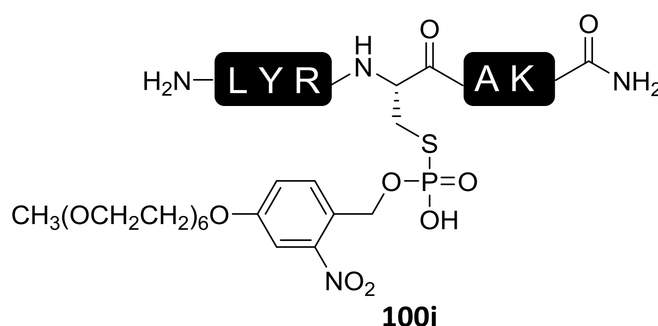


Figure 32. UPLC-trace of pure peptide **100i** using chromatographic method 1.

O-(4-((2,5,8,11,14,17-hexaoxanonadecan-19-yl)oxy)-5-methoxy-2-nitrobenzyl) *S*-cysteine phosphorothioate peptide **100j**



In DMF as a solvent:

Ellman´s labelled cysteine peptide **98a** (1.5 mg, 1.2 µmol) was dissolved in DMF (300 µL) and a solution of phosphite **99e** (8.7 mg, 6.0 µmol, 5 eq) in DMF (300 µL) was added. Final peptide concentration was 2.0 mM. The reaction mixture was incubated at room temperature for 16 h. Purification *via* semi-preparative HPLC (method A) yielded peptide **100j** (0.64 mg, 0.39 µmol) in 34% yield.

Molar mass (peptide) = 1291.42 Da.; molar mass (TFA_3 salt) = 1633.49 Da

ESI-MS (positive mode) = 646.3013 $[M+2H]^{2+}$ (calcd. m/z: 646.3038)

^{31}**P-NMR** (243 MHz, D_2O at pH 7.5) δ = 18.81 ppm.

In 50 mM Tris Buffer pH 8.0:

Ellman´s labelled cysteine peptide **98a** (2 mg, 1.5 µmol) was dissolved in 50 mM Tris Buffer at pH 8.0 (400 µL) and a solution of phosphite **99e** (10.9 mg, 7.5 µmol, 5 eq) in the same buffer (400 µL) was added. Final peptide concentration was 1.9 mM. The reaction mixture was incubated at room temperature for 3 h. Purification *via* semi-preparative HPLC (method A) yielded peptide **100j** (0.92 mg, 0.56 µmol) in 38% yield.

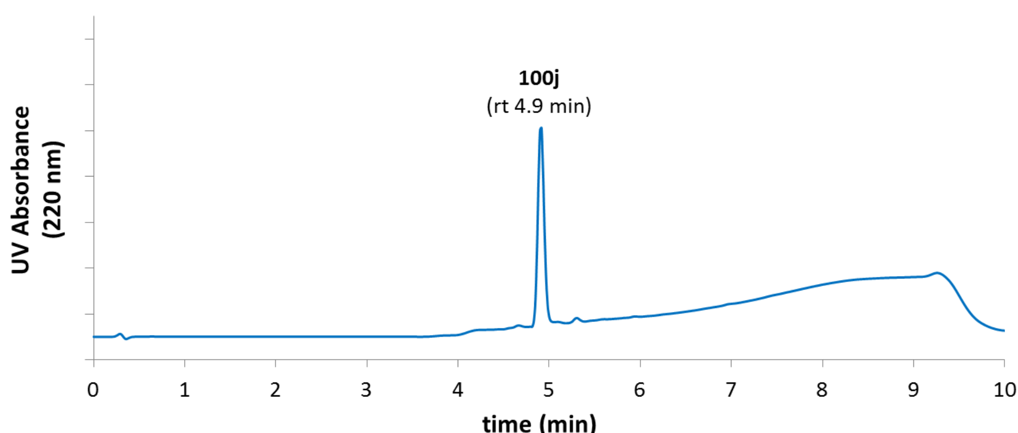
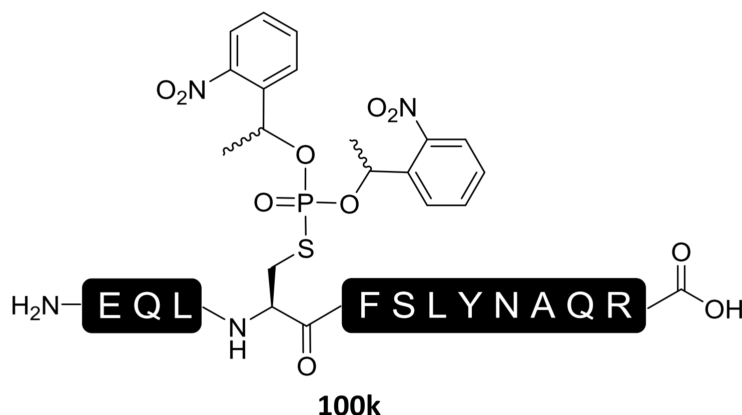


Figure 33. UPLC-trace of pure peptide **100j** using chromatographic method 2.

O,*O*-bis(1-(2-nitrophenyl)ethyl) *S*-cysteine phosphorothioate peptide **100k**



In DMF as a solvent:

Ellman´s labelled cysteine peptide **98b** (1.7 mg, 0.9 µmol) was dissolved in DMF (225 µL) and a solution of phosphite **99c** (2.4 mg, 4.5 µmol, 5 eq) in DMF (225 µL) was added. Final peptide concentration was 1.8 mM. The reaction mixture was incubated at room temperature for 16 h. Purification *via* semi-preparative HPLC (method A) yielded peptide **100k** (1.0 mg, 0.48 µmol) in 53% yield.

Molar mass (peptide) = 1849.93 Da.; molar mass (TFA_3 salt) = 2077.97 Da

ESI-MS (positive mode) = 925.3800 $[M+2H]^{2+}$ (calcd. m/z: 925.3844)

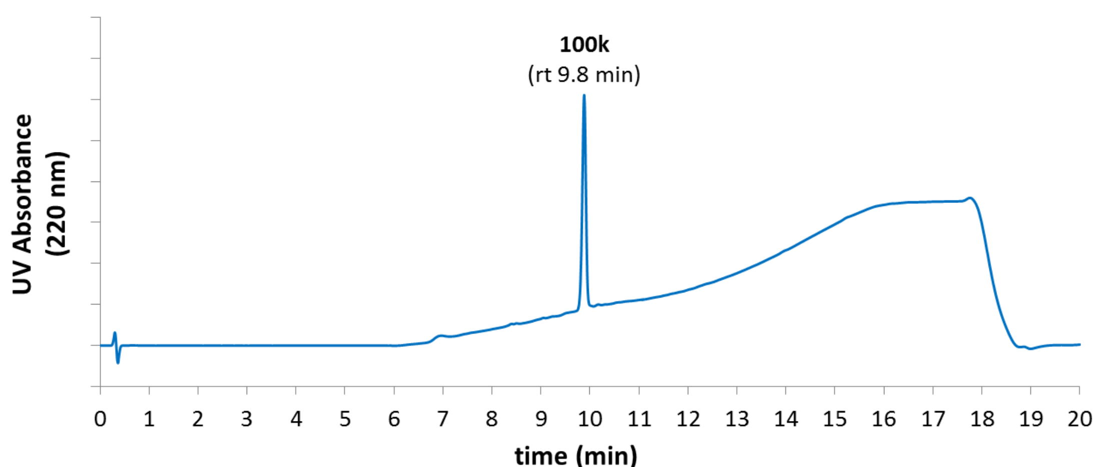
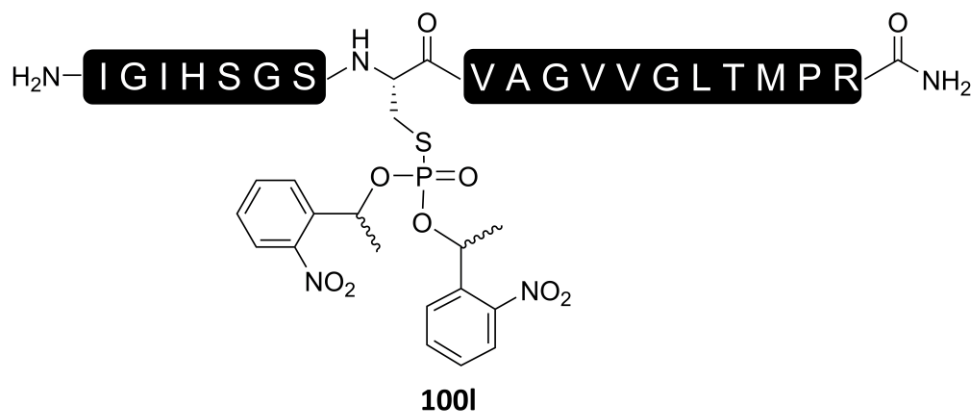


Figure 34. UPLC-trace of pure peptide **100k** using chromatographic method 1.

O,*O*-bis(1-(2-nitrophenyl)ethyl) *S*-cysteine phosphorothioate peptide **100l**



In DMF as a solvent:

Ellman´s labelled cysteine peptide **98c** (2.0 mg, 0.83 µmol) was dissolved in DMF (200 µL) and a solution of phosphite **99c** (2.2 mg, 4.15 µmol, 5 eq) in DMF (175 µL) was added. Final peptide concentration was 2.2 mM. The reaction mixture was incubated at room temperature for 16 h. Purification *via* semi-preparative HPLC (method A) yielded peptide **100l** (1.2 mg, 0.46 µmol) in 56% yield.

Molar mass (peptide) = 2231.51 Da.; molar mass (TFA_3 salt) = 2573.58 Da

ESI-MS (positive mode) = 744.6866 $[M+3H]^{3+}$ (calcd. m/z: 744.6893)

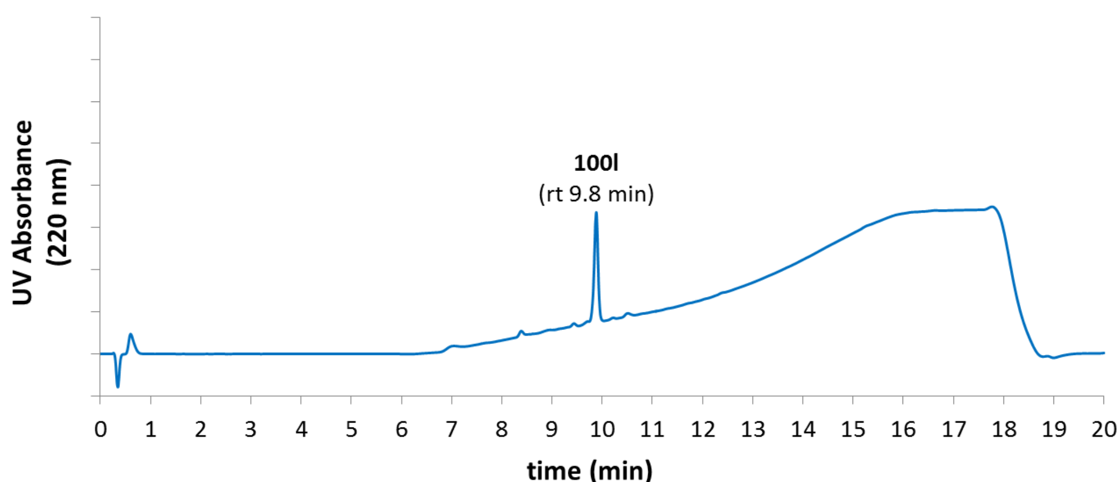
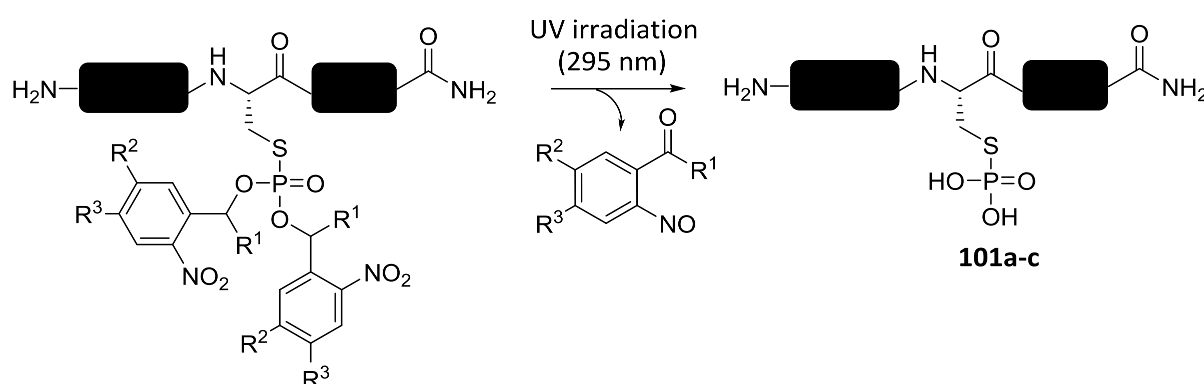


Figure 35. UPLC-trace of pure peptide **100l** using chromatographic method 1.

6.2.4 UV-irradiation of *o*-nitrobenzyl protected phosphorothioate peptides

General procedure: Tris-NaOH buffered (20 mM, pH 7.8) solution (500 µL) of peptide **100g** (1.8 mM), **100j** (1.4 mM), **100k** (0.96 µM) and **100l** (0.78 µM) were irradiated at 295 nm with a UV lamp for 5 min for peptide **100g**, **100k** and **100l**, and for 15 min for peptide **100j**. The samples were injected into the HPLC-UV and the identification of the peaks was confirmed by MS.

Scheme 27. Uncaging of peptides **100g**, **100j, 100k,** and **100l** upon UV-irradiation.



Peptide **100g, 100k, 100l**:
$R^1 = CH_3$
$R^2 = H$
$R^3 = H$

Peptide **100j**:
$R^1 = H$
$R^2 = OCH_3$
$R^3 = (CH_2CH_2O)_6CH_3$

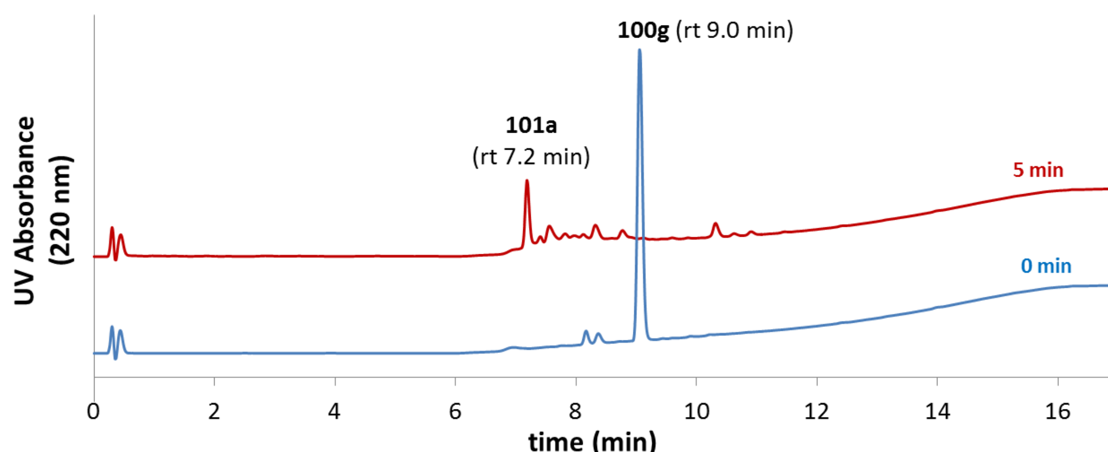


Figure 36. UPLC-trace before (blue) and after 5 min irradiation (red) of **100g** using chromatographic method 1.

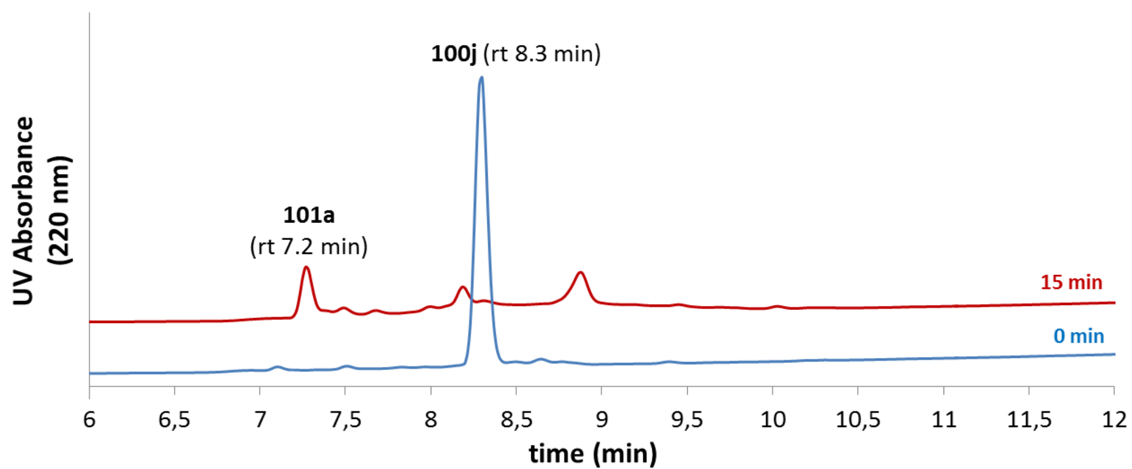
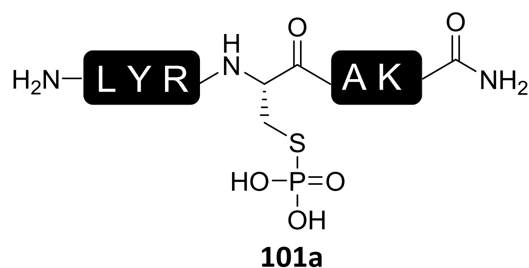


Figure 37. UPLC-trace before (blue) and after 15 min irradiation (red) of **100j** using chromatographic method 1.

S-cysteine phosphorothioate peptide **101a**



Phosphorothioate peptide **100g** (1.3 mg, 0.88 µmol) was dissolved in Tris-NaOH buffer solution (500 µL). Final peptide concentration was 1.8 mM. The sample was irradiated at 295 nm with a UV lamp for 5 min. Purification *via* semi-preparative HPLC (method B) yielded peptide **101a** as an ammonium acetate salt (0.6 mg, 0.57 µmol) with a purity criteria ≥ 90% based on UPLC-UV and in 65% yield.

Molar mass (peptide) = 831.93 Da.; molar mass (NH_4Ac salt) = 1046.15 Da

ESI-MS (positive mode) = 416.6968 $[M+2H]^{2+}$ (calcd. m/z: 416.6986)

^{31}**P-NMR** (243 MHz, D_2O at pH 7.5) δ = 12.02 ppm.

Side product. The side product detected by UPLC-UV and –MS at 7.2 min retention time, corresponds to the hydrolysis product of the phosphorothioate cysteine-containing peptide, i.e., the unphosphorylated peptide. Integration of the unphosphorylated peptide by UV accounts for 9% relative to the phosphorothioate cysteine-containing peptide.

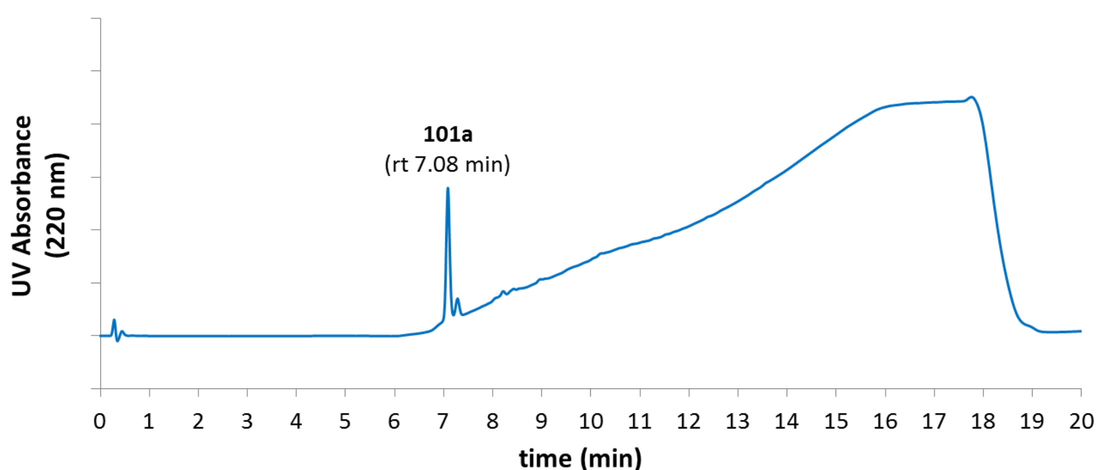
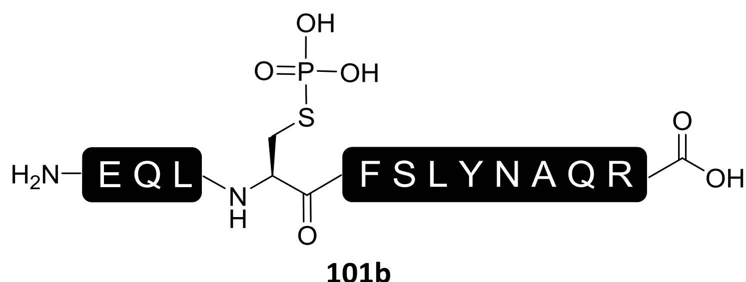


Figure 38. UPLC-trace of pure peptide **101a** using chromatographic method 1.

S-cysteine phosphorothioate peptide **101b**



Phosphorothioate peptide **100k** (1.0 mg, 0.48 µmol) was dissolved in Tris-NaOH buffer solution (500 µL). Final peptide concentration was 0.96 mM. The sample was irradiated at 295 nm with a UV lamp for 5 min. Purification *via* semi-preparative HPLC (method B) yielded peptide **101b** as an ammonium acetate salt (0.6 mg, 0.34 µmol) with a purity criteria ≥ 74% based on UPLC-UV and in 73% yield.

Molar mass (peptide) = 1551.63 Da.; molar mass (NH_4Ac salt) = 1705.7988 Da

ESI-MS (positive mode) = 776.3347 $[M+2H]^{2+}$ (calcd. m/z: 776.3367)

Side product. The side product detected by UPLC-UV and –MS at 8.7 min retention time, corresponds to the hydrolysis product of the phosphorothioate cysteine-containing peptide, i.e., the unphosphorylated peptide. Integration of the unphosphorylated peptide by UV accounts for 26% relative to the phosphorothioate cysteine-containing peptide.

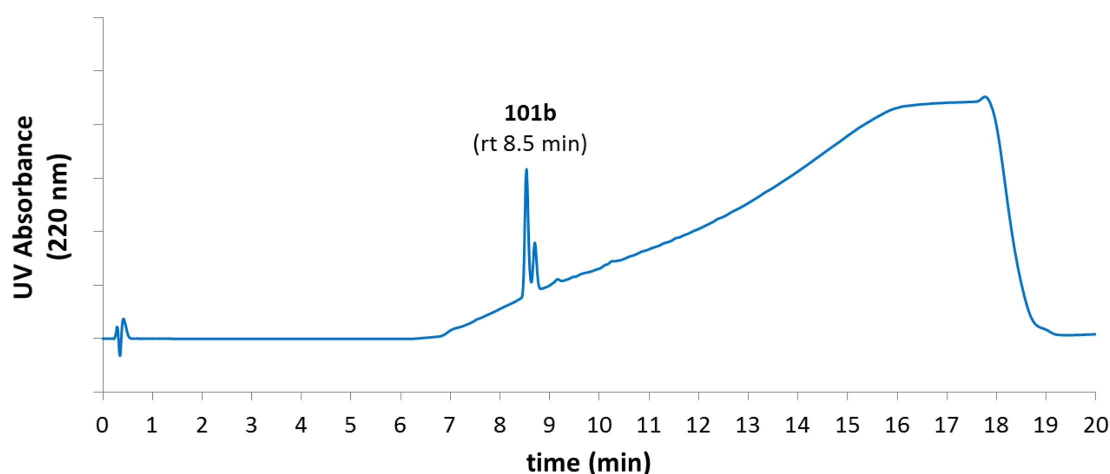
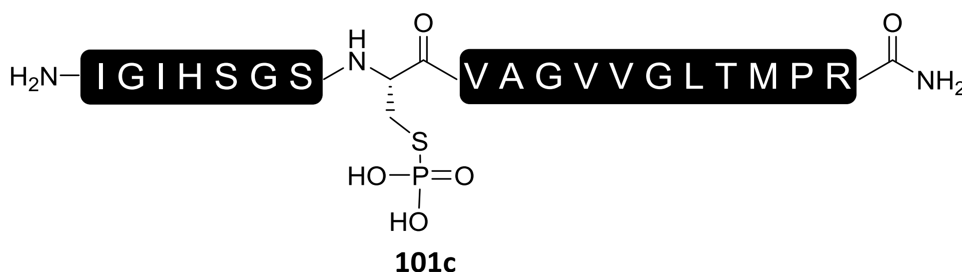


Figure 39. UPLC-trace of pure peptide **101b** using chromatographic method 1.

S-cysteine phosphorothioate peptide **101c**



Phosphorothioate peptide **100l** (1.0 mg, 0.39 µmol) was dissolved in Tris-NaOH buffer solution (500 µL). Final peptide concentration was 0.78 mM. The sample was irradiated at 295 nm with a UV lamp for 5 min. Purification *via* semi-preparative HPLC (method B) yielded peptide **101c** as an ammonium acetate salt (0.5 mg, 0.23 µmol) with a purity criteria ≥ 76% based on UPLC-UV and in 60% yield.

Molar mass (peptide) = 1933.22 Da.; molar mass (NH_4Ac salt) = 2147.43 Da

ESI-MS (positive mode) = 644.9812 $[M+3H]^{3+}$ (calcd. m/z: 644.9898)

Side product. The side product detected by UPLC-UV and –MS at 8.8 min retention time, corresponds to the hydrolysis product of the phosphorothioate cysteine-containing peptide, i.e., the unphosphorylated peptide. Integration of the unphosphorylated peptide by UV accounts for 24% relative to the phosphorothioate cysteine-containing peptide.

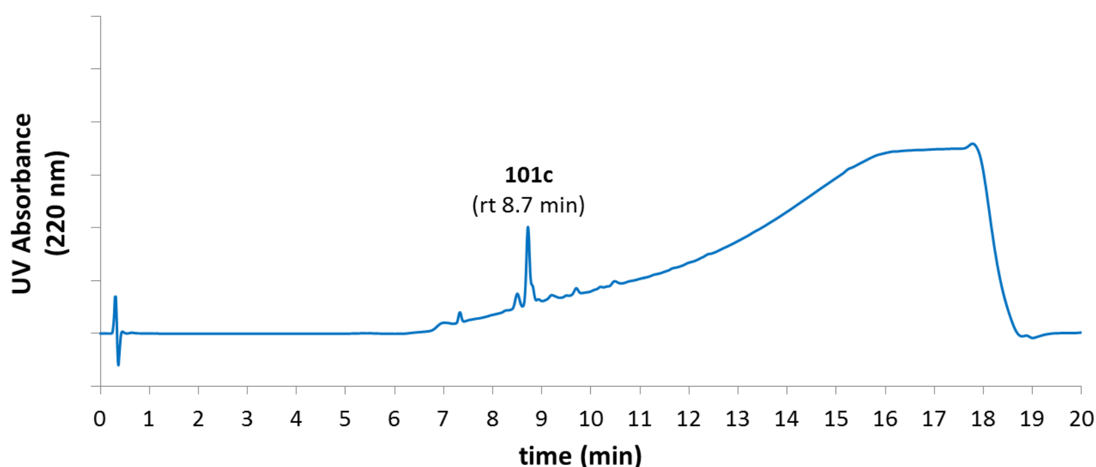


Figure 40. UPLC-trace of pure peptide **101c** using chromatographic method 1.

6.2.5 Alkaline deprotection of cyanoethyl protected phosphorothioate peptide

Procedure: An ammonium acetate solution (250 µL) of peptide **100c** (0.7 mg, 0.55 µmol) was incubated with 500 mM NaOH (250 µL) for 20 min. Final peptide concentration was 1.1 mM. After neutralization with HCl, the samples were injected into the HPLC-UV and the identification of the peaks was confirmed by MS.

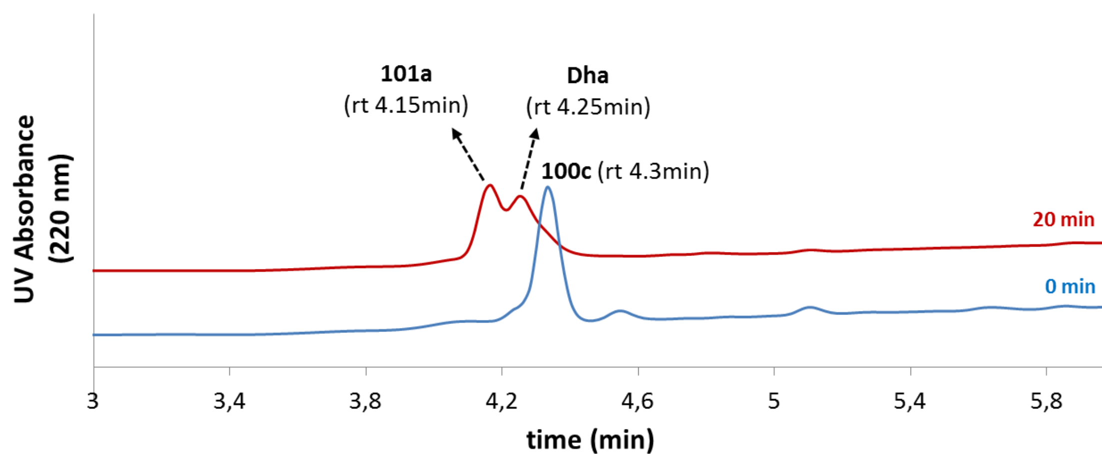


Figure 41. UPLC-trace before (blue) and after (red) 20 min incubation using chromatographic method 2.

6.2.6 Characterization of the phosphorothioate site by ETD MS

6.2.6.1 Analysis by ETD MS of peptide **101a**

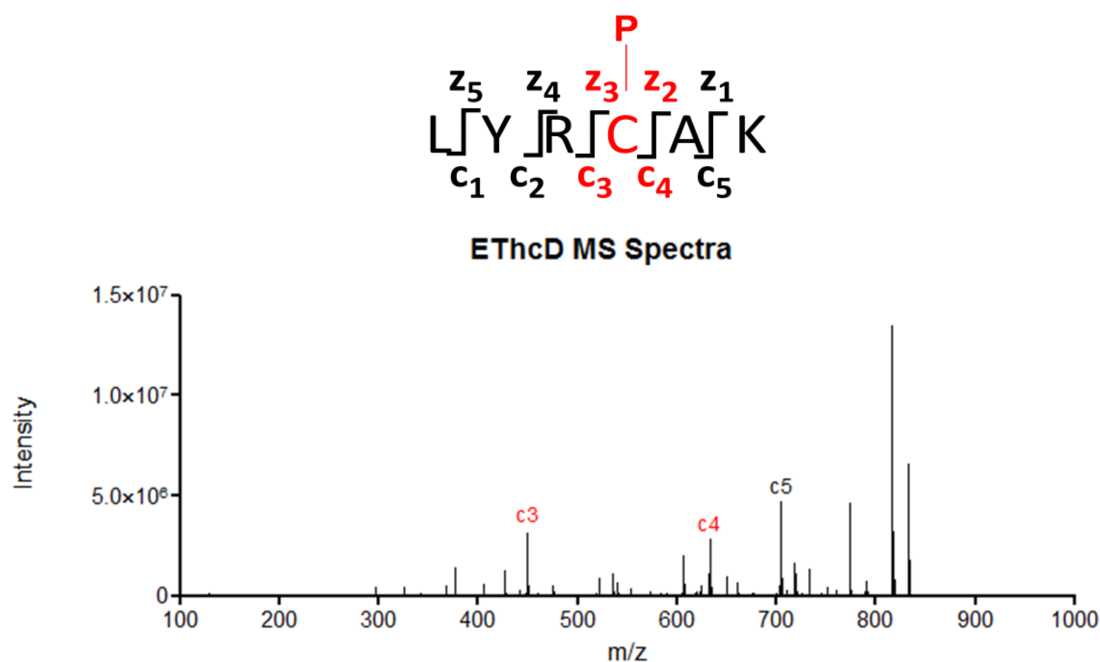


Figure 42. EThcD MS Spectra of phosphorylated cysteine peptide **101a**

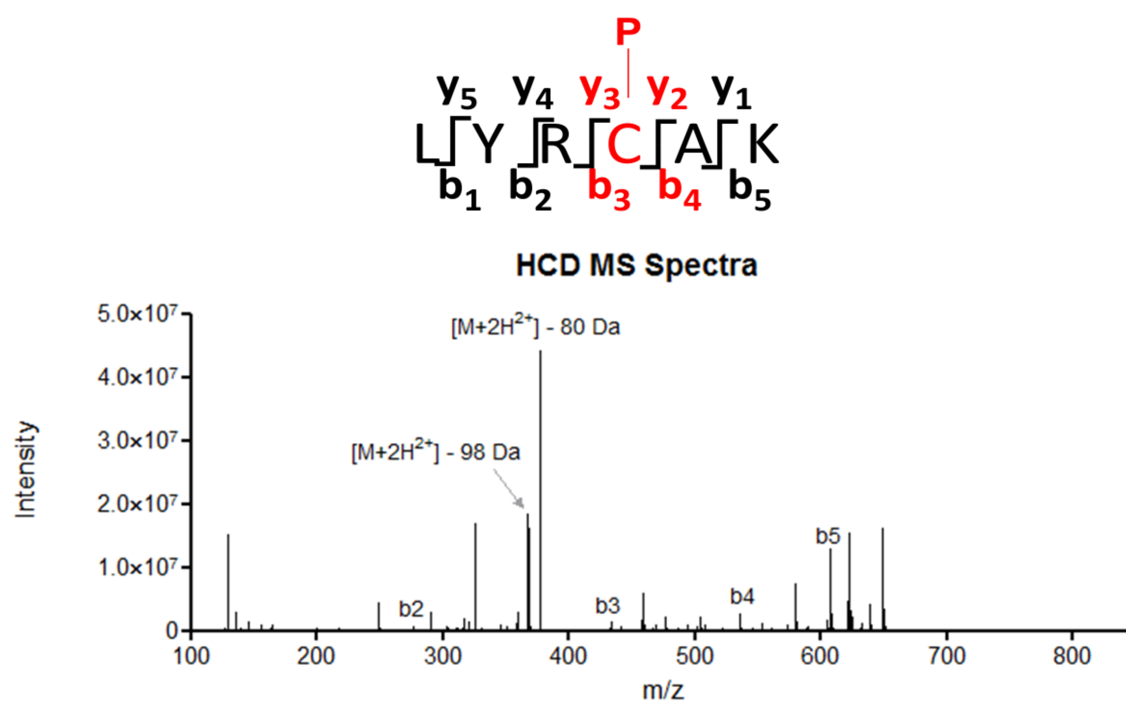
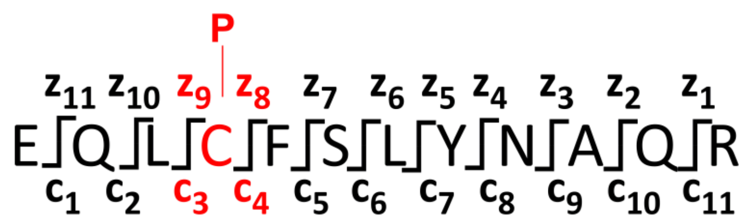
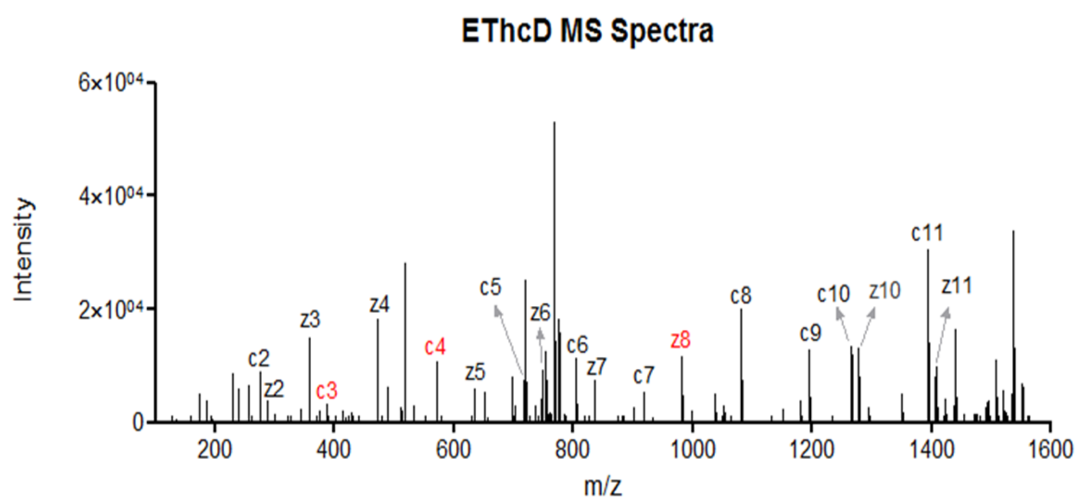


Figure 43. HCD MS Spectra of phosphorylated cysteine peptide **101a**

6.2.6.2 Analysis by ETD MS of peptide **101b**





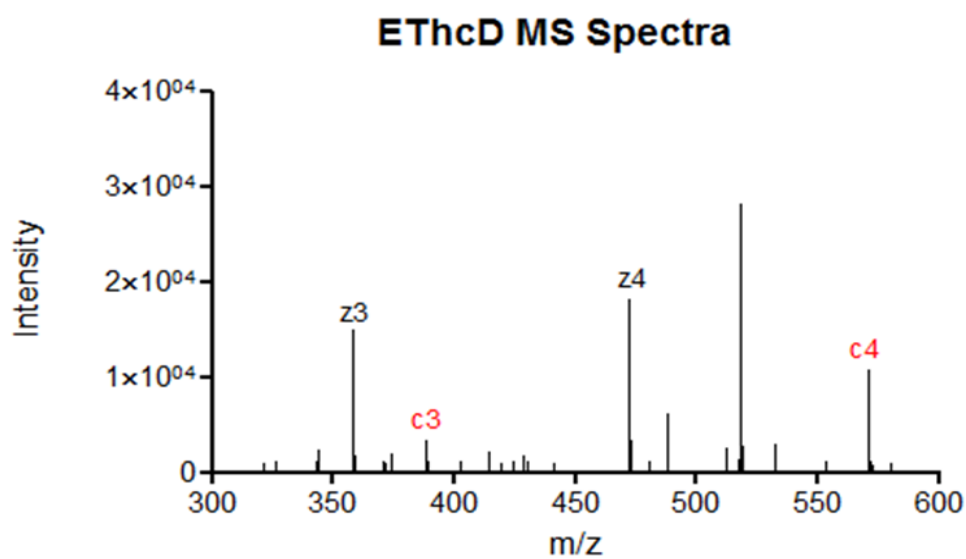
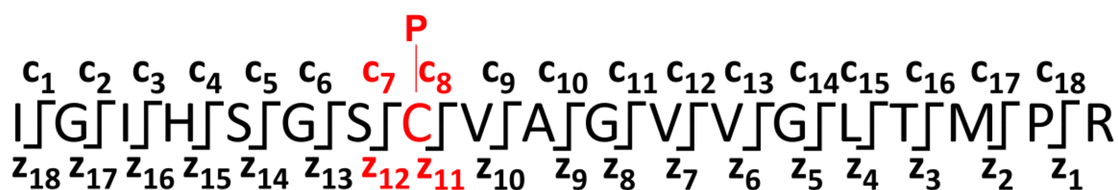
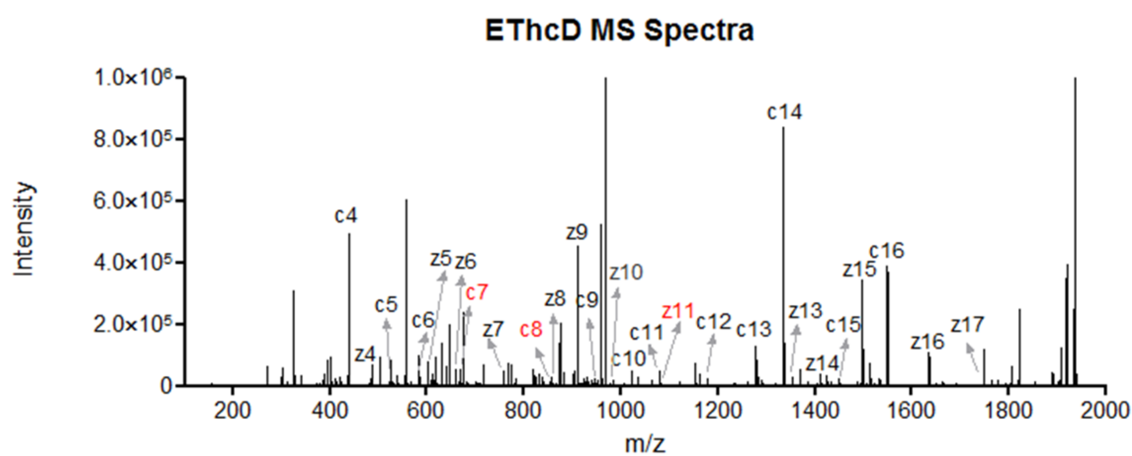


Figure 44. EThcD MS Spectra of phosphorylated cysteine peptide **101b**

6.2.6.3 Analysis by ETD MS of peptide **101c**





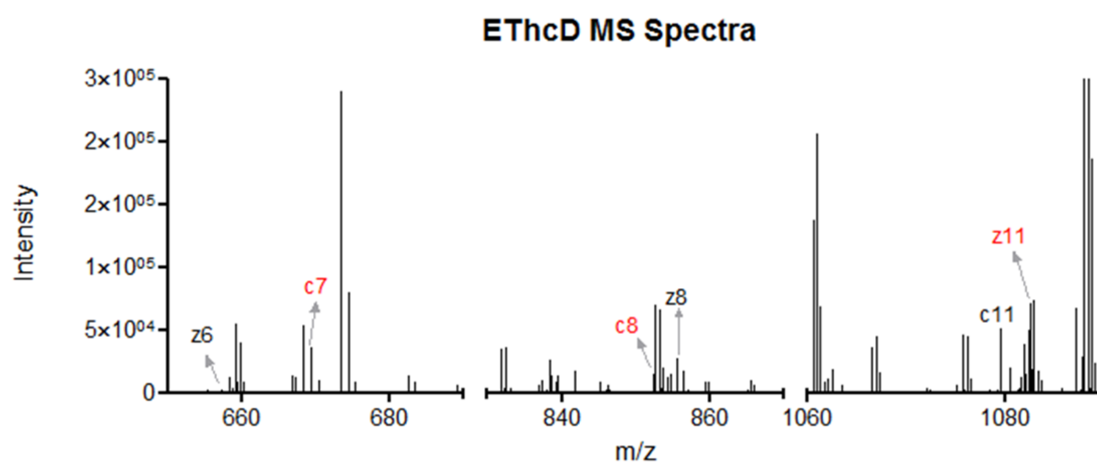


Figure 45. EThcD MS Spectra of phosphorylated cysteine peptide **101c**

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8 APPENDIX

8.1 A chemoselective tool for studying cysteine-phosphorylation

8.1 Spectra of peptides

Cysteine peptide **97a**

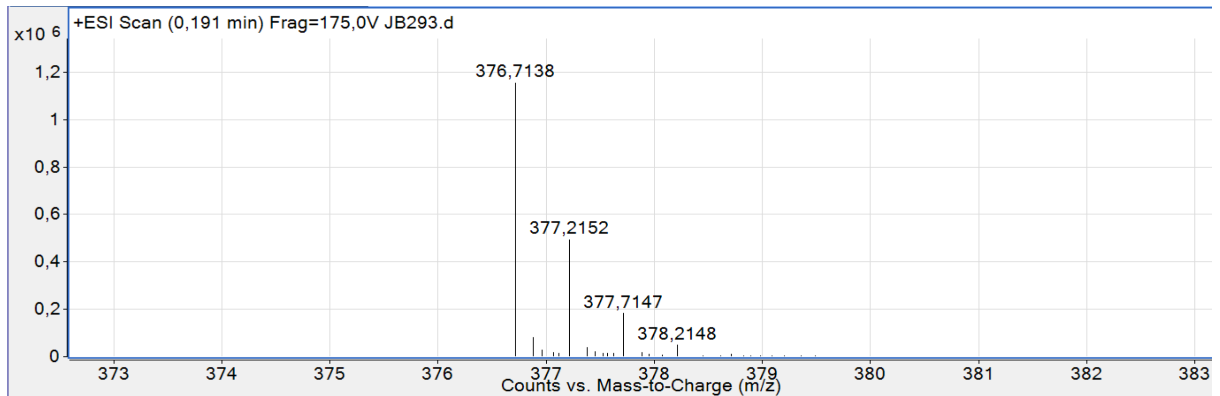

Figure 46. ESI-MS spectrum of cysteine peptide **97a**.

Cysteine peptide **97b**

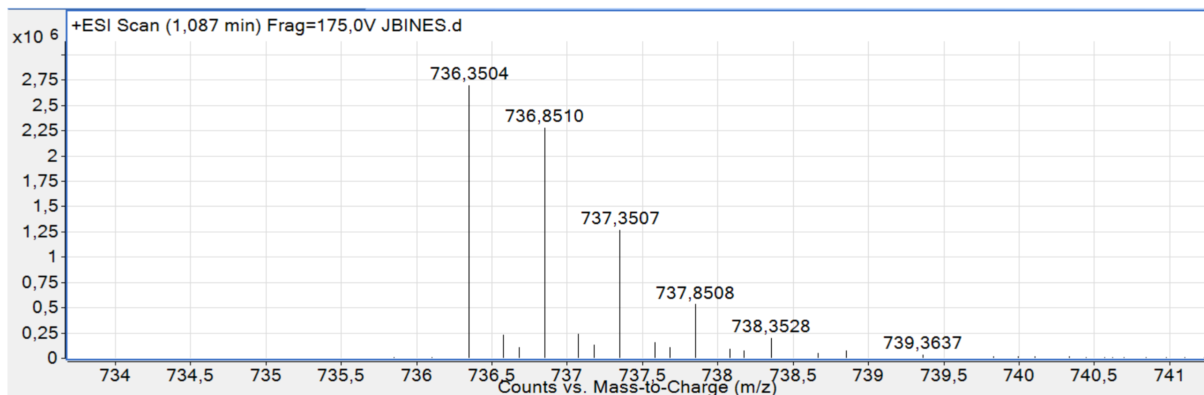

Figure 47. ESI-MS spectrum of cysteine peptide **97b**.

Cysteine peptide **97c**

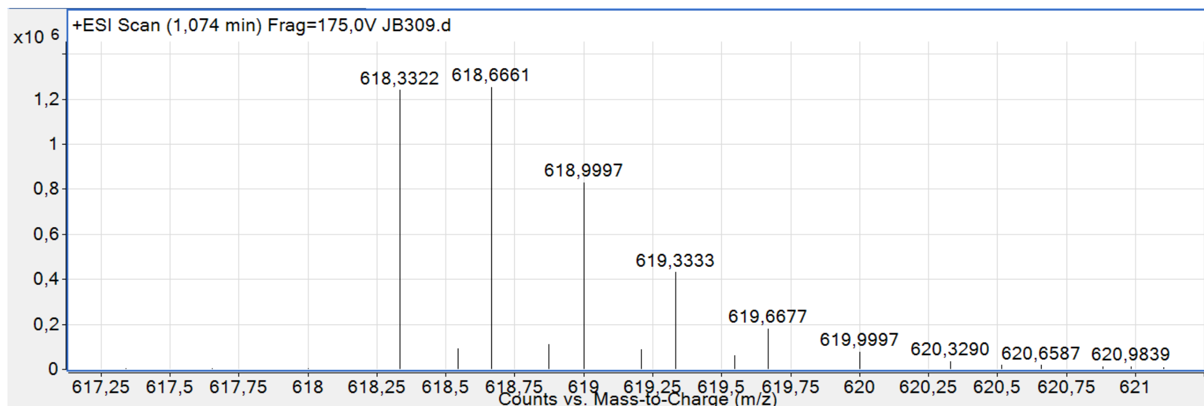

Figure 48. ESI-MS spectrum of cysteine peptide **97c**.

Ellman´s disulfide peptide **98a**

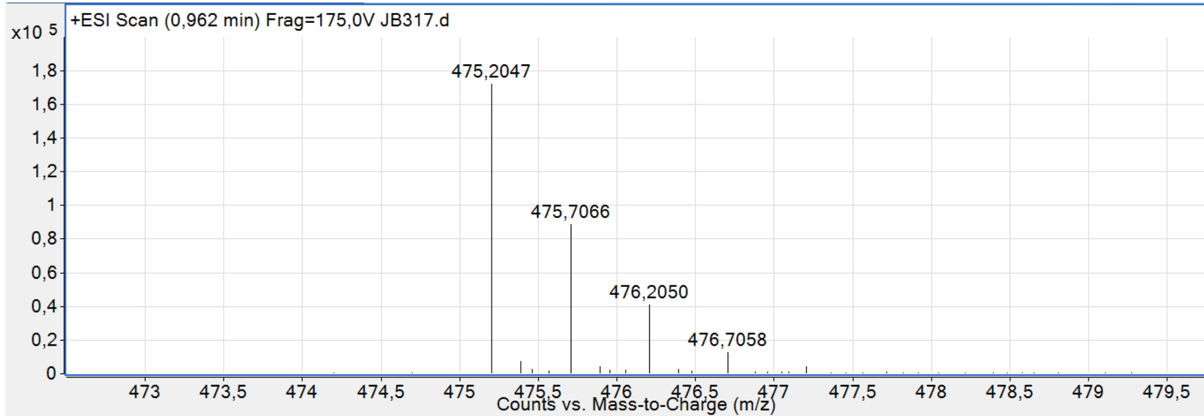

Figure 49. ESI-MS spectrum of Ellman's disulfide peptide **98a**.

Ellman's disulfide peptide **98b**

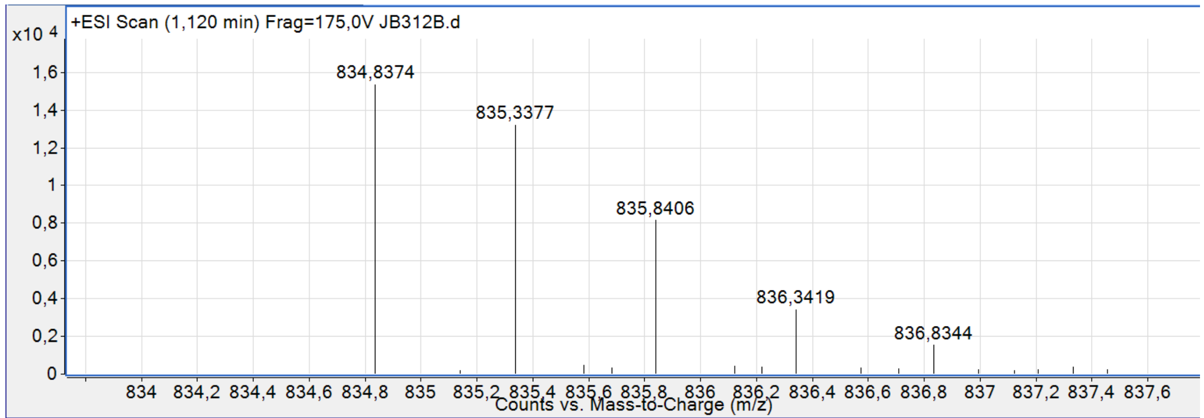

Figure 50. ESI-MS spectrum of Ellman's disulfide peptide **98b**.

Ellman´s disulfide peptide **98c**

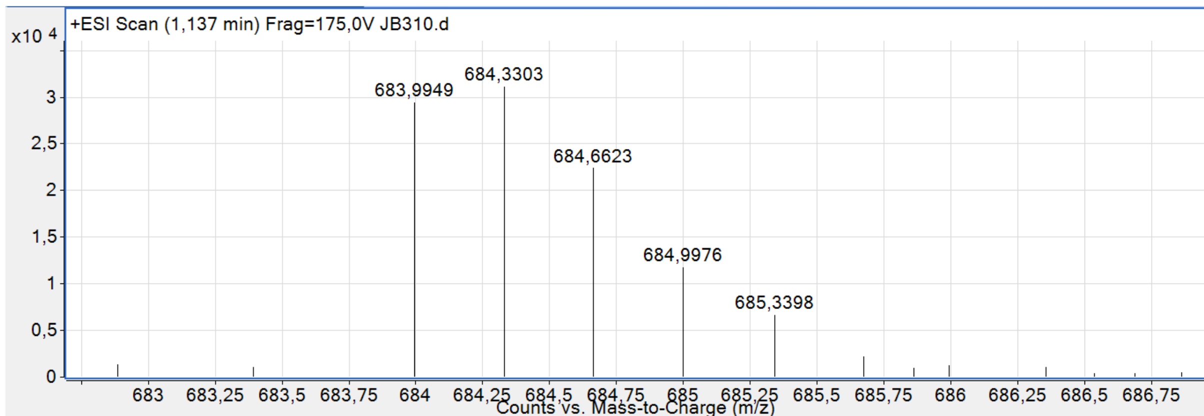

Figure 51. ESI-MS spectrum of Ellman's disulfide peptide **98c**.

<u>*O*,*O*-bis(2-cyanoethyl) *S*-cysteine phosphorothioate peptide **100c**</u>

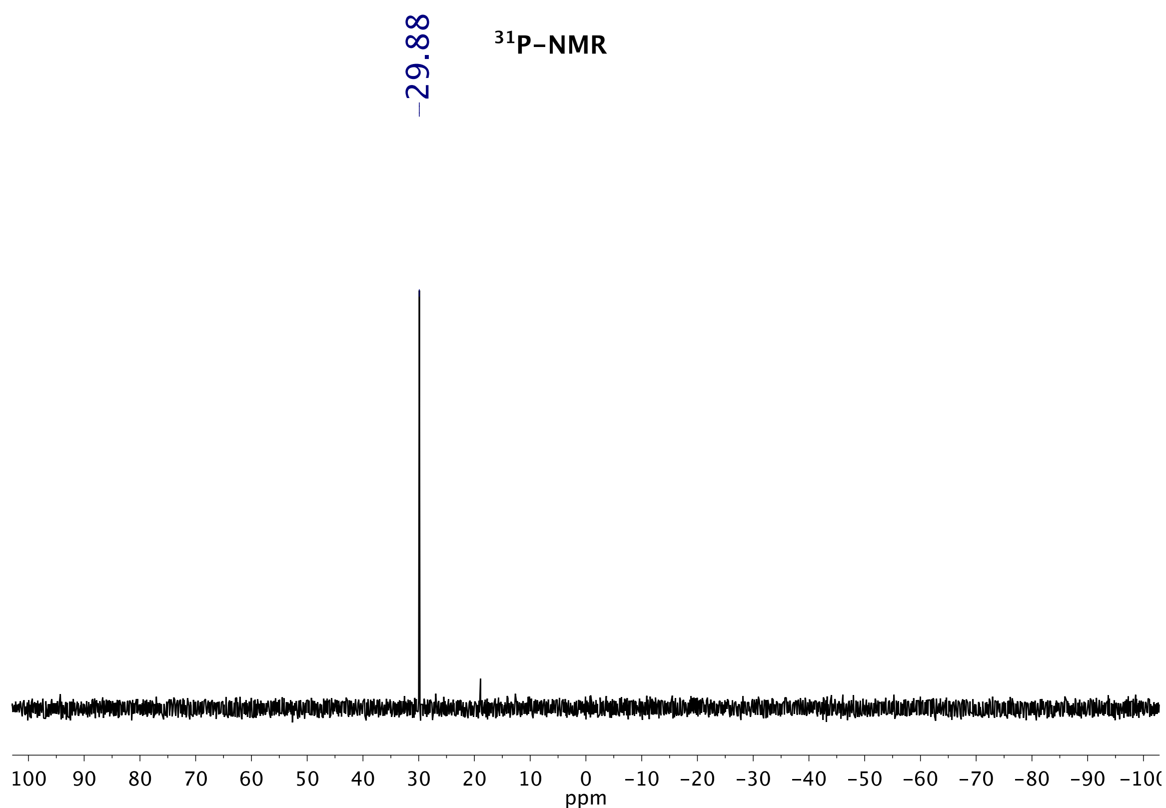

Figure 52. ^{31}P-NMR spectrum of phosphorothioate ester peptide **100c**.

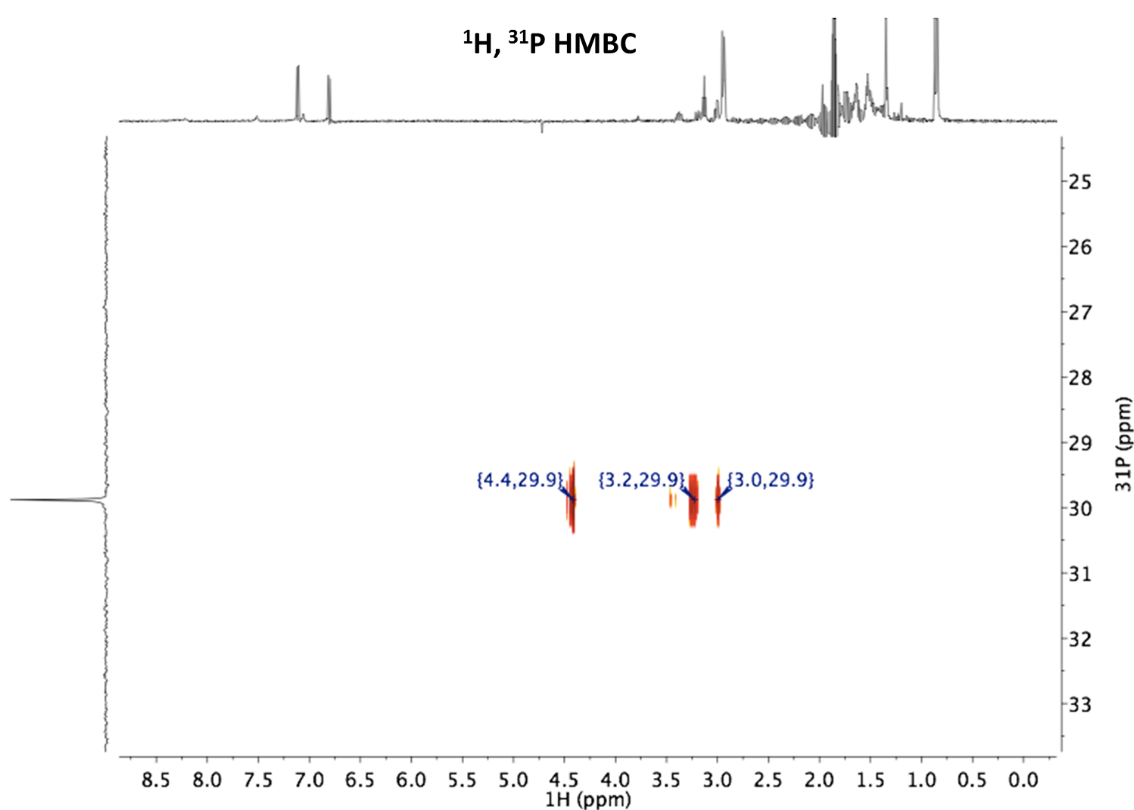

Figure 53. ^{1}H,^{31}P HMBC NMR spectrum of phosphorothioate ester peptide **100c**.

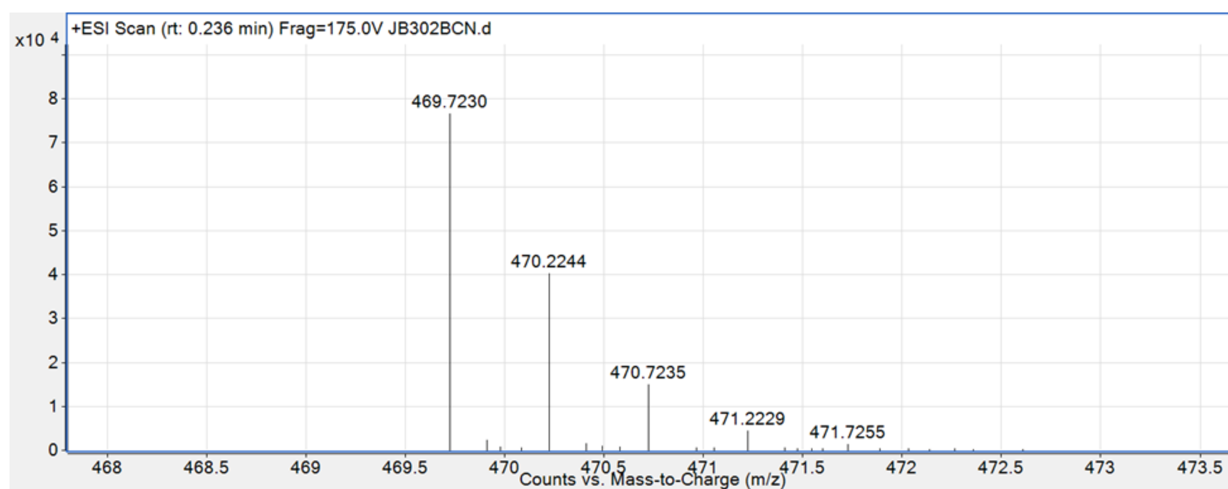

Figure 54. ESI-MS spectrum of phosphorothioate ester peptide **100c**.

O,*O*-bis(1-(2-nitrophenyl)ethyl) *S*-cysteine phosphorothioate peptide **100g**

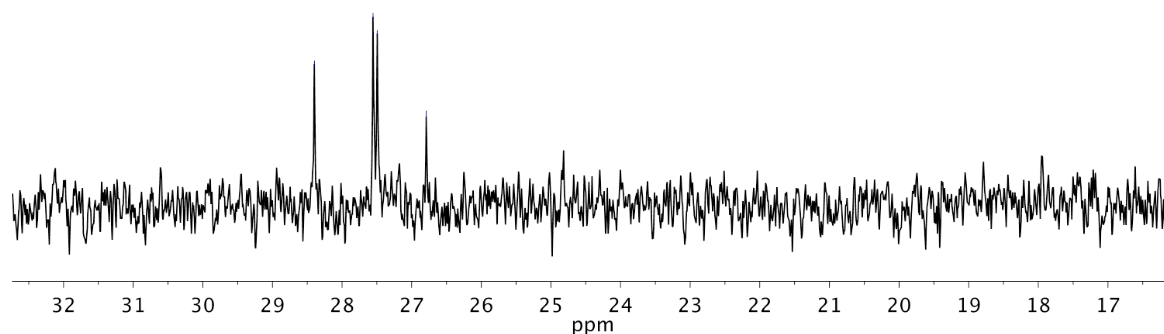

Figure 55. ^{31}P-NMR spectrum of phosphorothioate ester peptide **100g**.

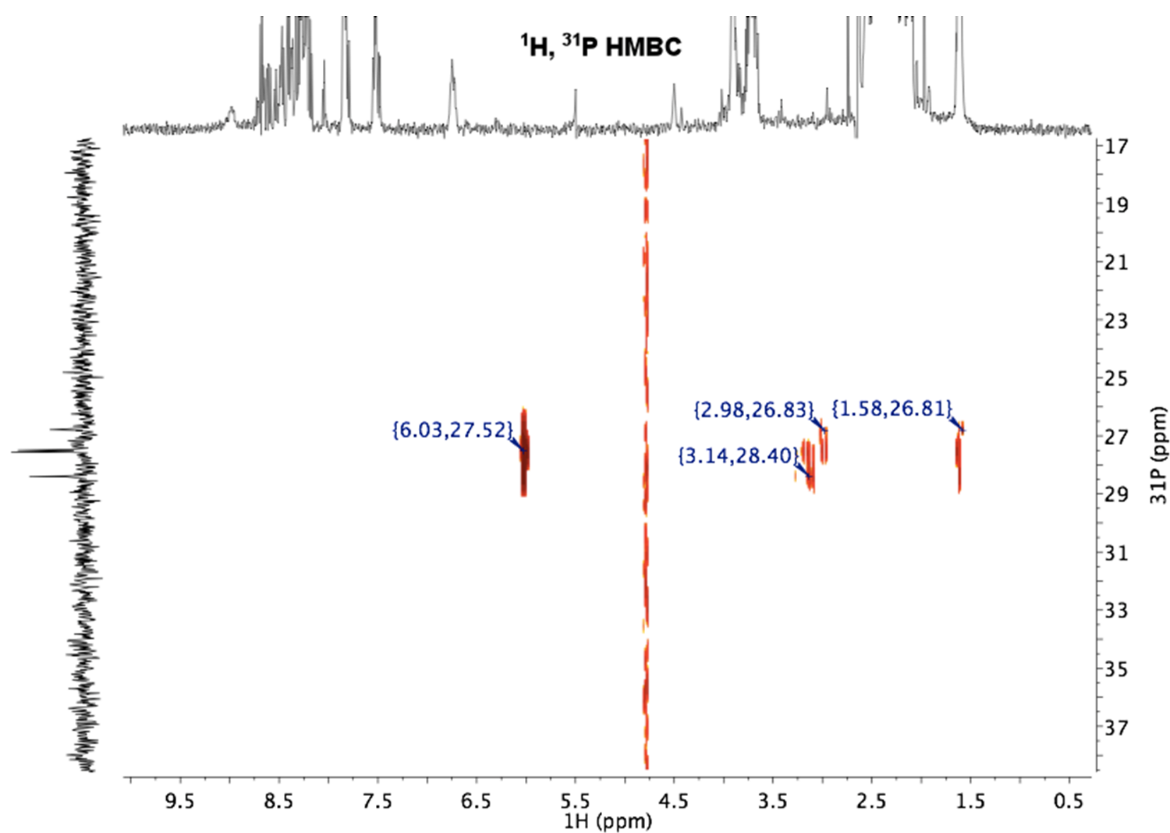

Figure 56. $^{1}H,^{31}P$ HMBC NMR spectrum of phosphorothioate ester peptide **100g**.

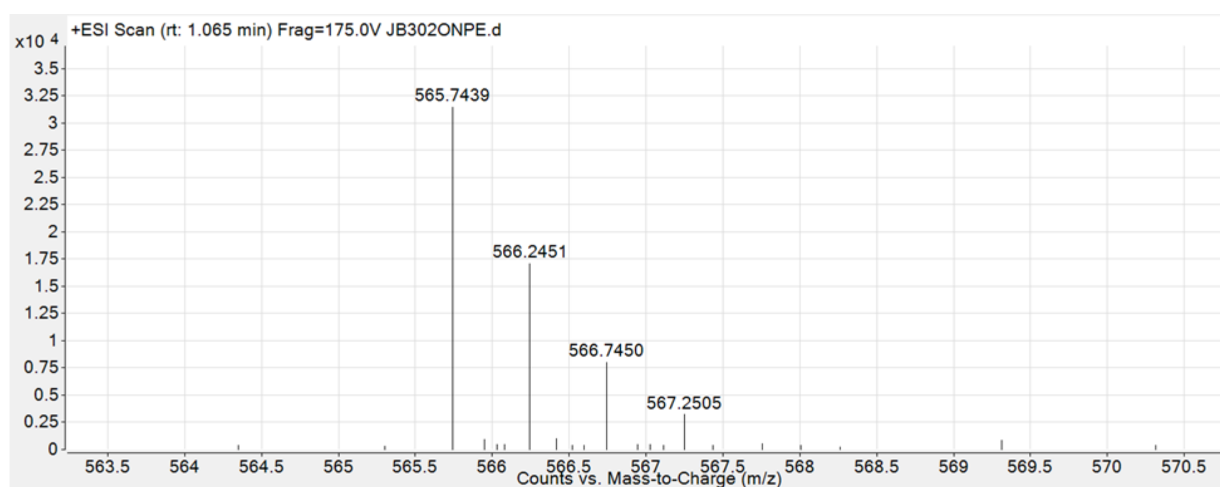

Figure 57. ESI-MS spectrum of phosphorothioate ester peptide **100g**.

O,O-bis((4-((2,5,8,11,14,17-hexaoxanonadecan-19-yl)oxy)-5-methoxy-2-nitrobenzyl) *S*-cysteine phosphorothioate peptide **100i**

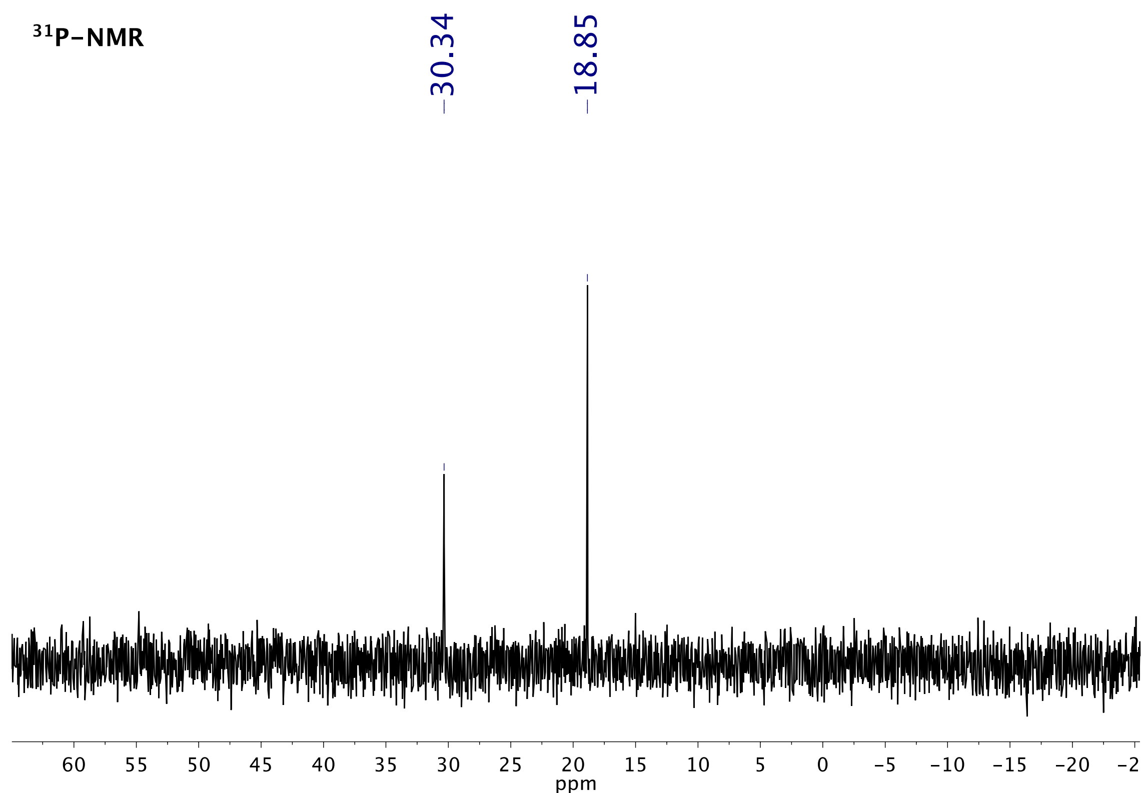

Figure 58. ^{31}P-NMR spectrum of phosphorothioate ester peptide **100i**.

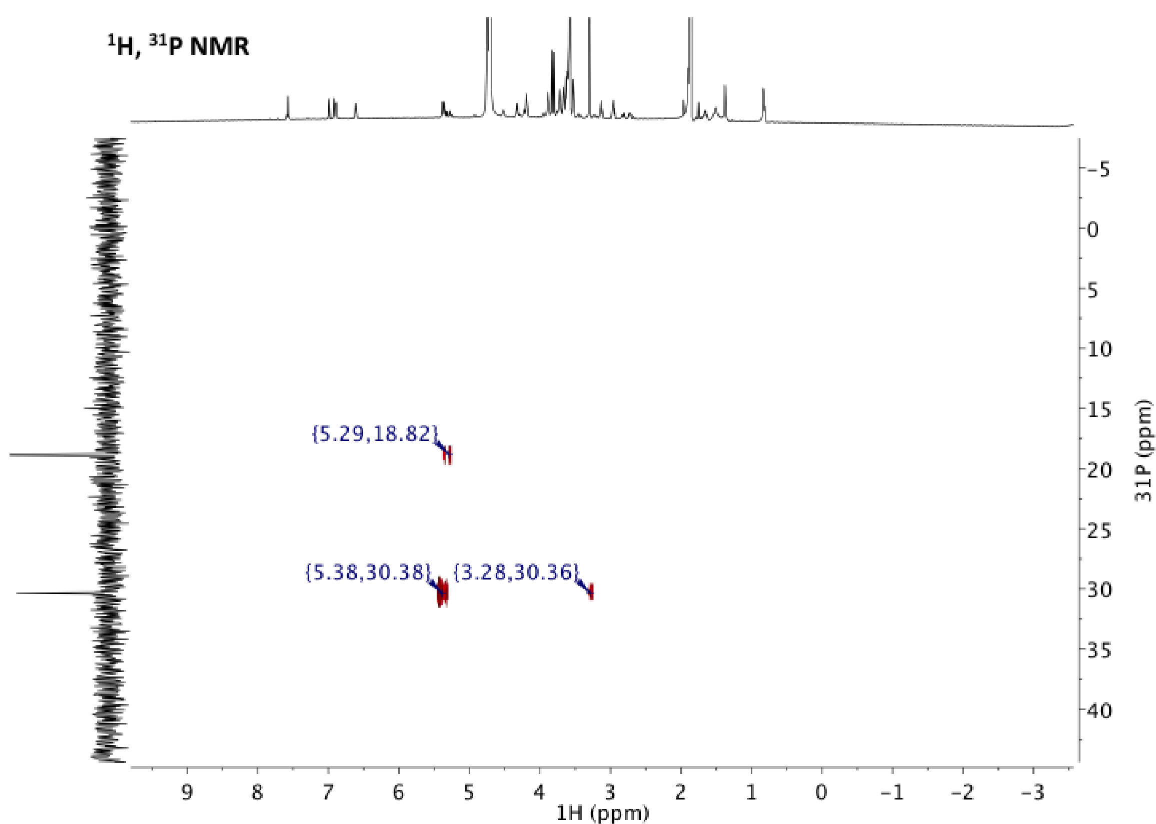

Figure 59. ^{1}H,^{31}P HMBC NMR spectrum of phosphorothioate ester peptide **100i**.

O-(4-((2,5,8,11,14,17-hexaoxanonadecan-19-yl)oxy)-5-methoxy-2-nitrobenzyl) *S*-cysteine phosphorothioate peptide **100j**

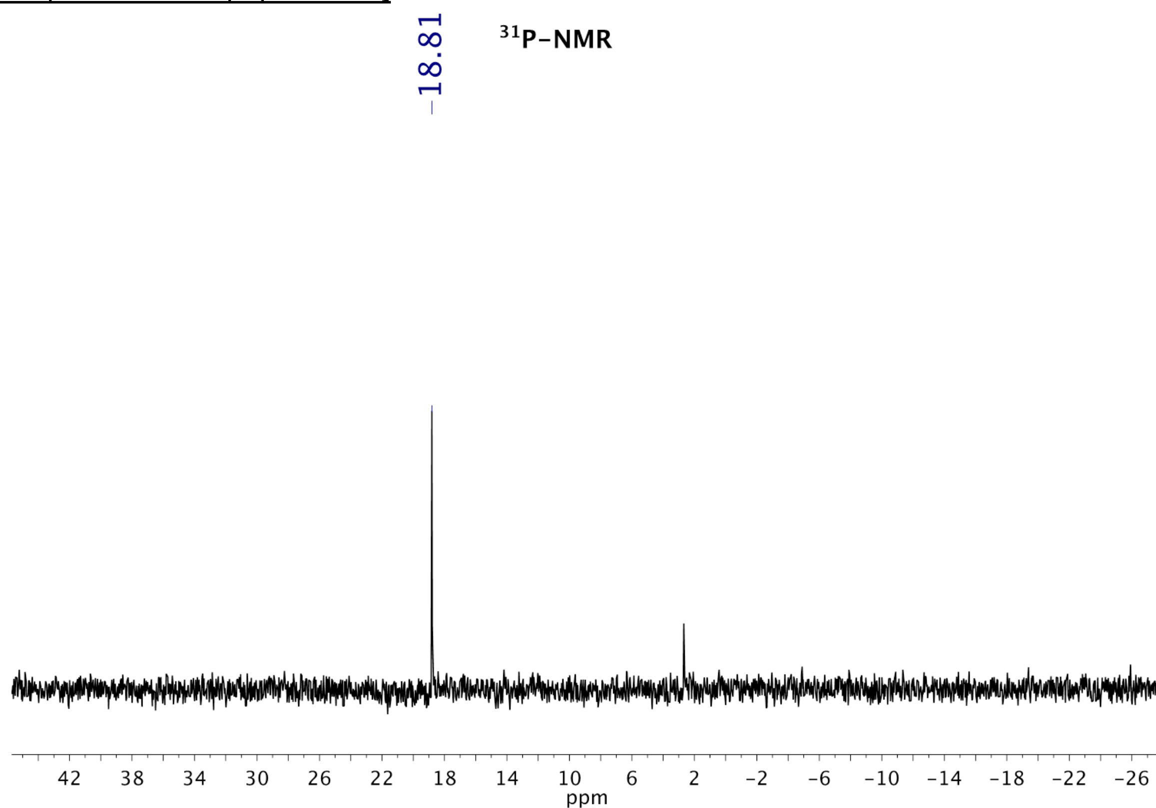

Figure 60. ^{31}P-NMR spectrum of phosphorothioate ester peptide **100j**.

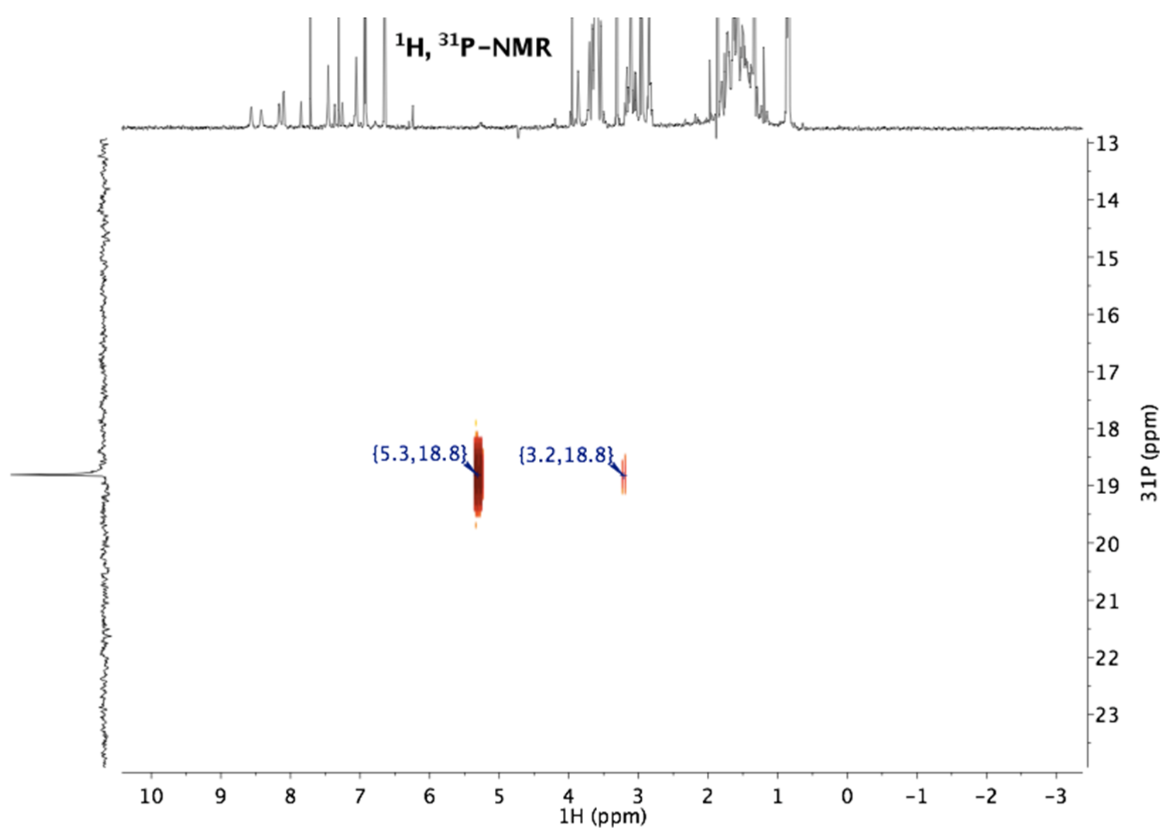

Figure 61. ^{1}H,^{31}P HMBC NMR spectrum of phosphorothioate ester peptide **100j**.

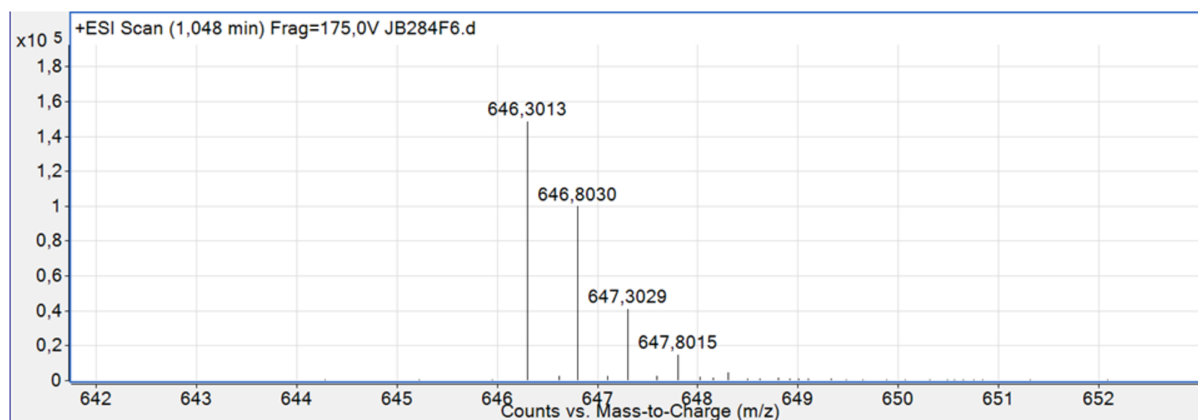

Figure 62. ESI-MS spectrum of phosphorothioate ester peptide **100j**.

<u>*S*-cysteine phosphorothioate peptide **101a**</u>

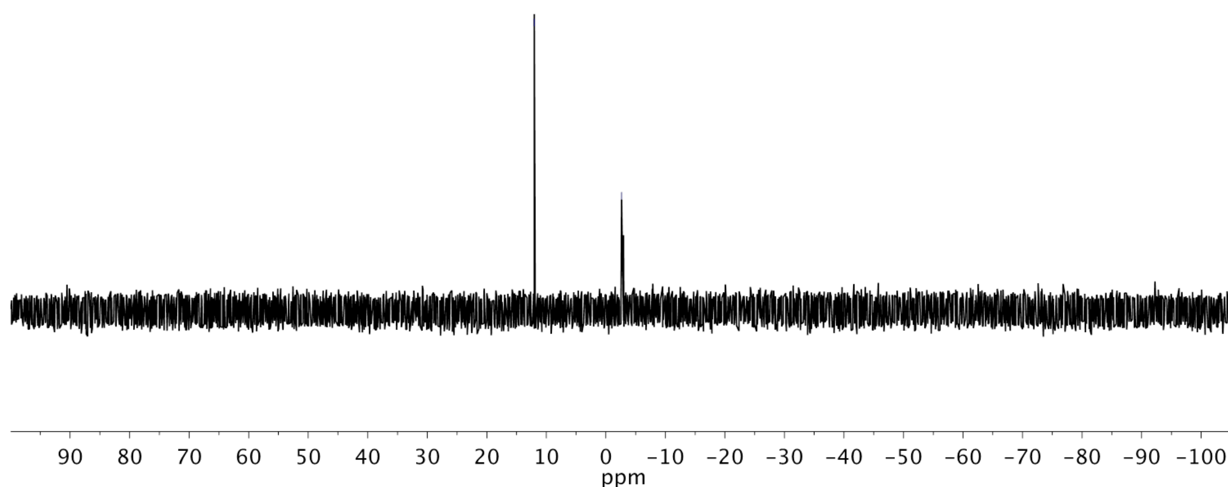

Figure 63. ^{31}P-NMR spectrum of phosphorothioate peptide **101a**.

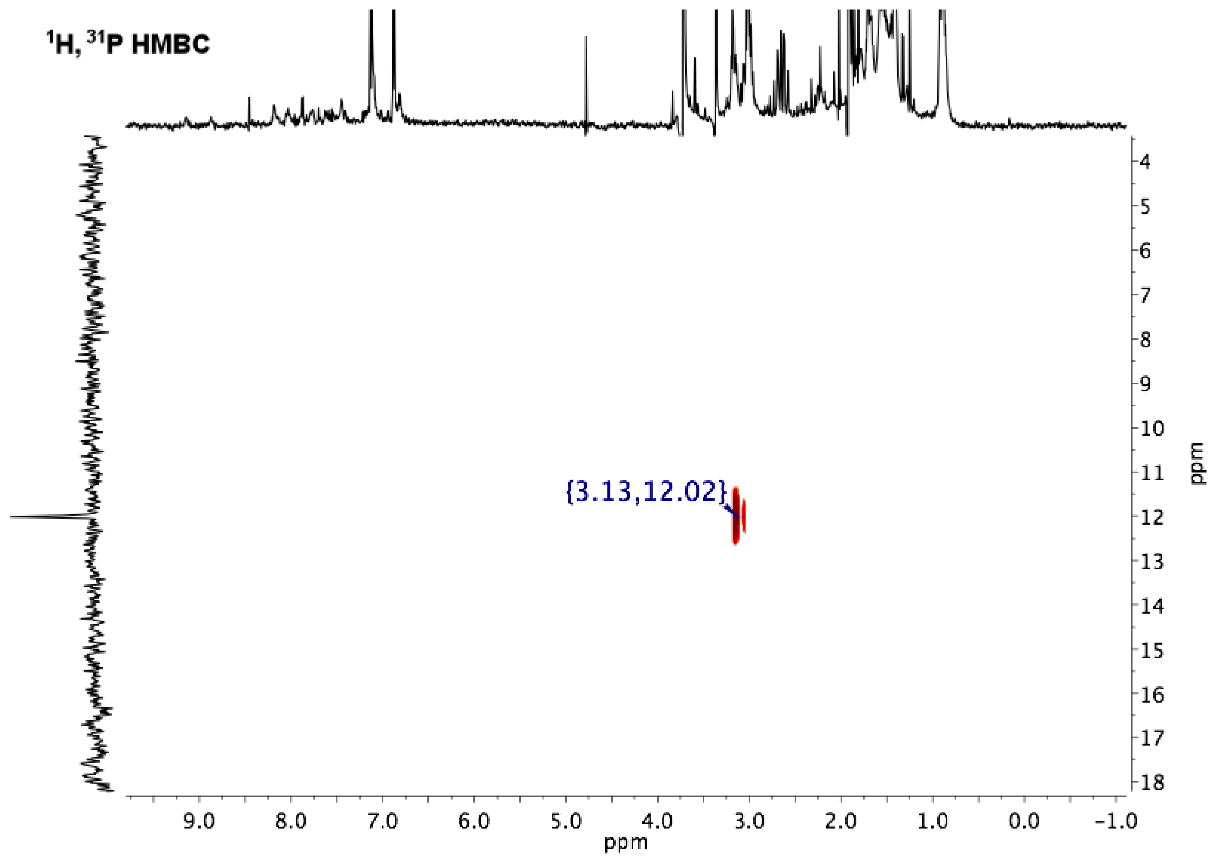

Figure 64. $^{1}H,^{31}P$ HMBC NMR spectrum of phosphorothioate peptide **101a**.

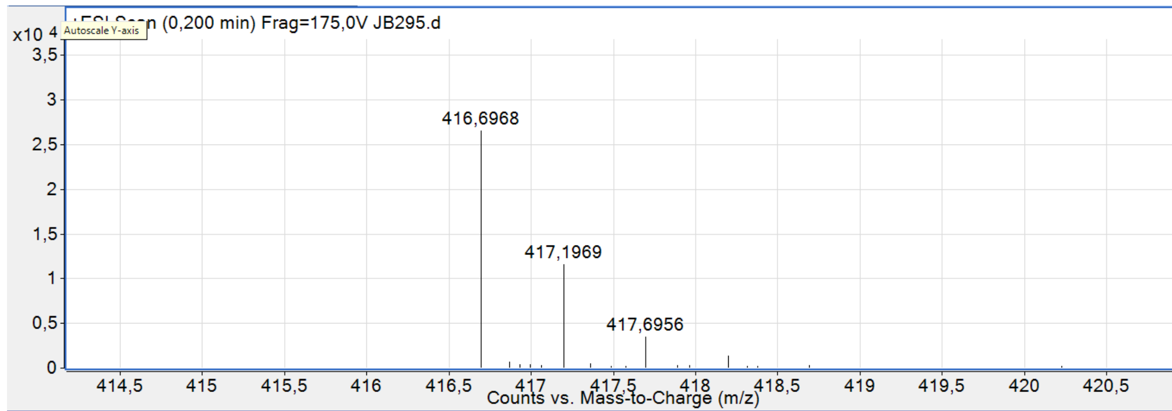

Figure 65. ESI-MS spectrum of phosphorothioate peptide **101a**.

O,O-bis(1-(2-nitrophenyl)ethyl) *S*-cysteine phosphorothioate peptide **100k**

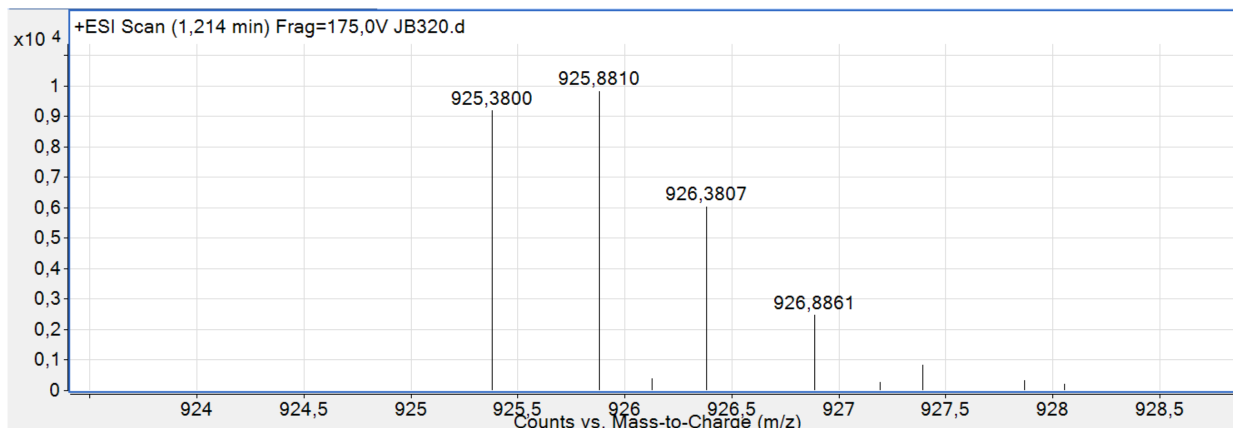

Figure 66. ESI-MS spectrum of phosphorothioate ester peptide **100k**.

O,O-bis(1-(2-nitrophenyl)ethyl) *S*-cysteine phosphorothioate peptide **100l**

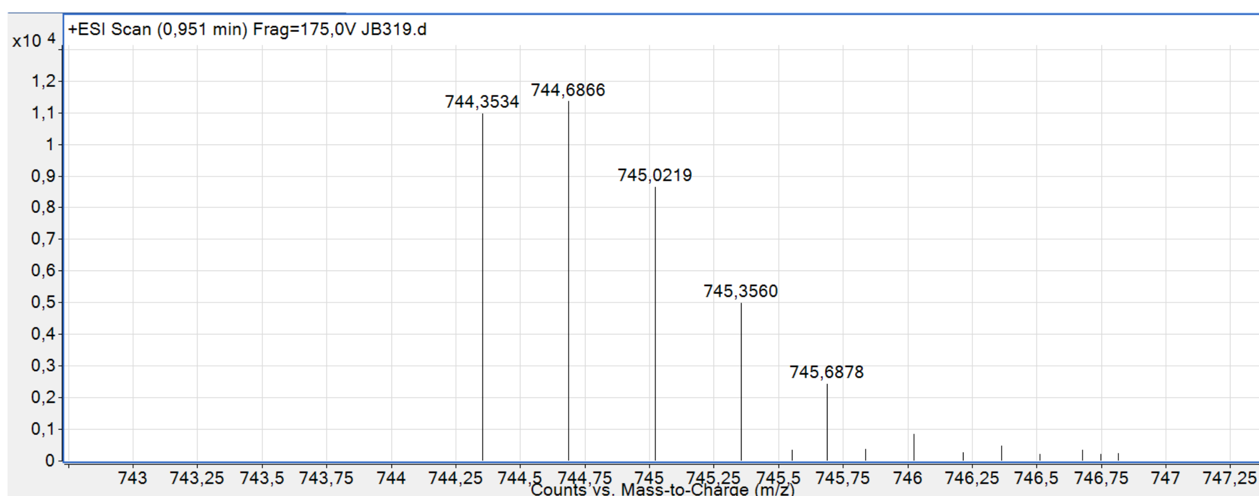

Figure 67. ESI-MS spectrum of phosphorothioate ester peptide **100l**.

S-cysteine phosphorothioate peptide **101b**

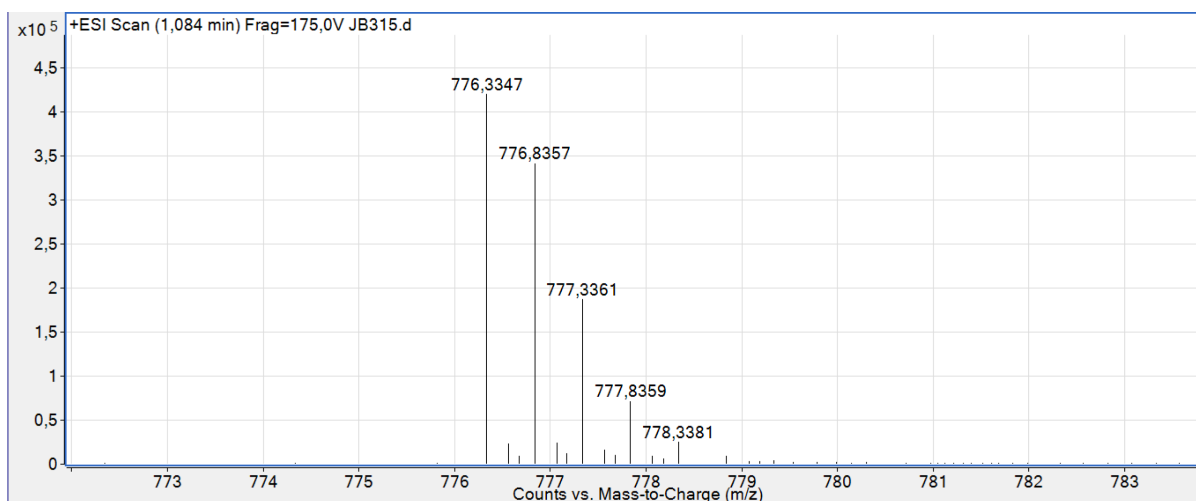

Figure 68. ESI-MS spectrum of phosphorothioate peptide **101b**.

S-cysteine phosphorothioate peptide **101c**

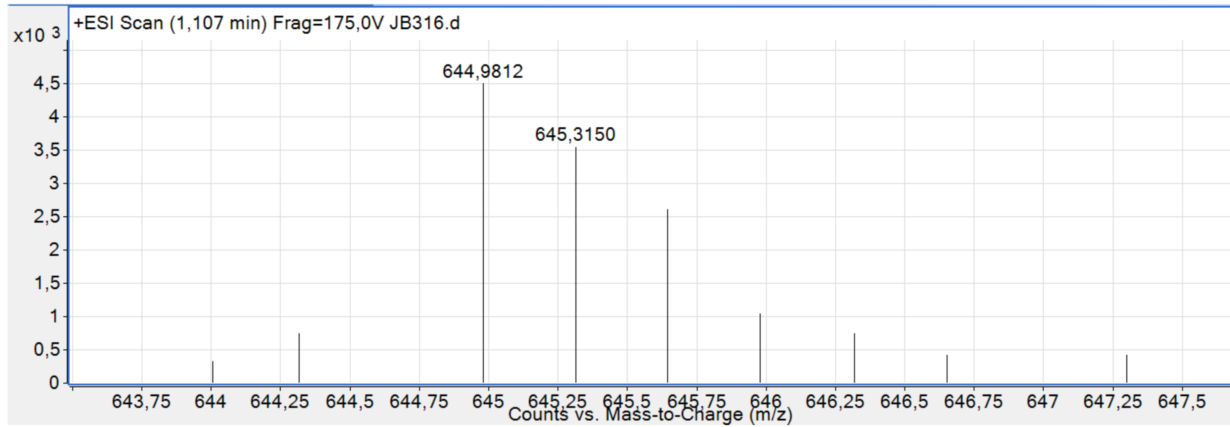

Figure 69. ESI-MS spectrum of phosphorothioate peptide **101c**.

9 CURRICULUM VITAE

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.