Self adjuvanting immunopeptides : design and synthesis

Gential, G.P.P.

Chapter 5: Phosphine reactivity towards azides in water: Reduction versus hydrolysis

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Introduction

In the field of bioorganic synthesis, azides are commonly used as masked functionalities to protect amines. Azides are easily introduced by substitution reactions or can be obtained from amines by diazo transfer. Azides can withstand a variety of reaction conditions and are easily converted into amines by several types of reduction, including the Staudinger reaction[1,2]. Reaction of an azide with a trialkyl- or triarylphosphine proceeds via an iminophosphorane that upon hydrolysis leads to the corresponding amine (Figure 1). The combination of azide and Staudinger reaction was given a reappraisal by the emergence of bioorthogonal chemistry[3]. This field of research aims to the selective detection of a specific biomolecule in living or biological systems[4]. To attain this goal the biomolecule of interest should be provided with a reactive group that is inert in biological systems but selectively reacts with a reporter group under physiological conditions[5,6]. The azide function is small, relatively stable, abiotic and essentially non-cytotoxic while incorporation of an azide into the biomolecule of interest allows a number of selective reactions with probes such as reporter molecules. There are three bioorthogonal reactions of which the copper-catalyzed [3+2] Huisgen cycloaddition[7] and strain-promoted [3+2] cycloaddition[8] of azides with alkynes lead to triazoles. In contrast, the Staudinger–Bertozzi ligation, the first bioorthogonal reaction, connects the biomolecule and the reporter group via an amide linkage. These favorable properties make azides the most widely used reactive group in the field of *in vivo* bioorthogonal chemistry. On the other hand the conversion of an azide into an amine in biological systems or the use of an azide as a bioorthogonal protecting group has not been studied yet.

With the ultimate goal to develop a latent epitope that is not recognized by its cognate T-cell, an azide as an amino protecting group is incorporated in a CD8+ T-cell MHC class I peptide epitope. Upon presentation of this undetectable epitope by antigen presenting cells, Staudinger reduction of the azide in the epitope by a trialky- or triaryl phosphorus reagent would liberate the amine and produces the native MHC-I/peptide epitope that can activate the T-cell. This chapter describes a study towards the aqueous Staudinger reduction of an azide to an amine that is installed in an oligopeptide. The well-studied epitope **5** (SIINFEKL) from chicken egg white ovalbumine, was chosen as a model substrate to study the efficiency of the reduction. This model epitope has been successfully used in the field of cancer vaccines[9,10] and is relatively easy to synthesize on large scale. Moreover, the lysine in the SIINFEKL sequence is not involved in the formation of MHC complex[11,12], and can be used to replace the native amine by an azide to give target oligopeptide SIINFEK*L (K* = azido-norleucine) **4** as a model substrate to explore the Staudinger reduction[13]. For the Staudinger reaction three phosphines were selected on the basis of their solubility in water and their compatibility with the cell media. One of the most used water soluble phosphines is tris(2-carboxyethyl)phosphine (TCEP, **1**). In addition, sulfonylated triphenylphosphine ($PPh_3(SO_3Na)_3$, **2**) and sodium hypophosphite ($NaPO_2H_2$, **3**) were selected (Figure 2).

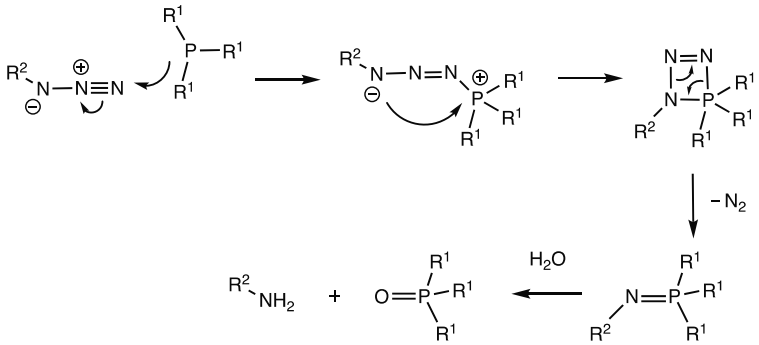


Figure 1. Staudinger reduction mechanism

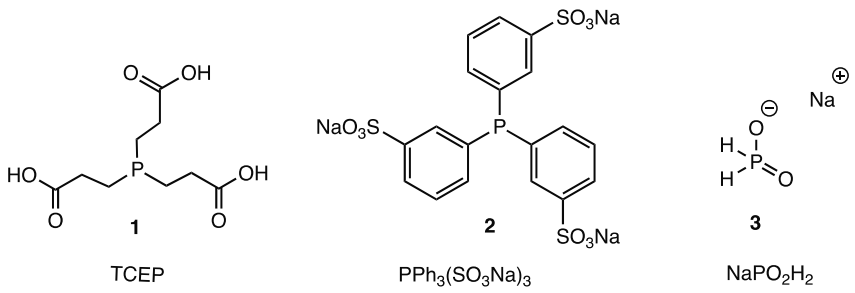


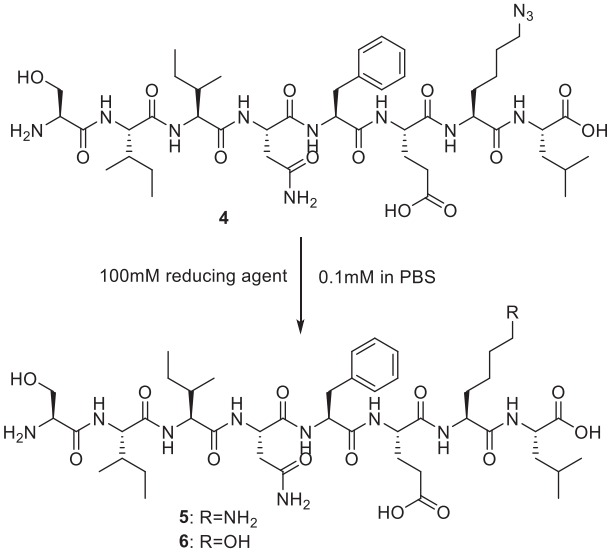
Figure 2. Reducing agents selected for this study

Results and discussion

The first step in the construction of target oligopeptide SIINFEK*L **4** involves the synthesis of Fmoc protected azido lysine (K* or 6-azido-norleucine) as described in Chapter 2. Having all protected amino acids available, azido containing peptide **4** and reference (SIINFEKL) peptide **5** were assembled by a standard solid phase peptide synthesis protocol using Fmoc chemistry, Tentagel®

S PHB Resin preloaded with leucine and HCTU as condensating agent. After cleavage from the solid support and purification by RP HPLC oligopeptide **4** and **5** were obtained in 21% and 19 % yield, respectively.

To explore the Staudinger reduction event of the azide in **4** into amine in **5**, conditions required for on-cell-surface reduction were mimicked by selecting a ratio of 1:1000 for peptide **4** and phosphine reagent (Scheme 1). Although the concentration of the reductive agent looks extreme, it was shown that cells can tolerated it with an acceptable level of survivability[14].



Scheme 1. Azide reduction in PBS is accompanied by hydrolysis

In practice this means that the use of 0.1 µM azido-containing peptide **(4)** and 100mM of the respective phosphine (**1**, **2** or **3**, Figure 2) allowed an easy monitoring of the progress of reaction by standard LC-MS analysis. In the first experiment peptide (**4**) was treated with TCEP (**1**) in PBS buffer (pH=7.4-7.6). Starting peptide (**4**, blue) disappeared within 95 min as depicted in Figure 3. Surprisingly, apart from native peptide (**5**, green) another major (**6**, red) product and a minor product (**7**, yelllow) were formed. Repetition of this experiment on large scale to allow HPLC purification led to the isolation of homogenous **5** and **6**.

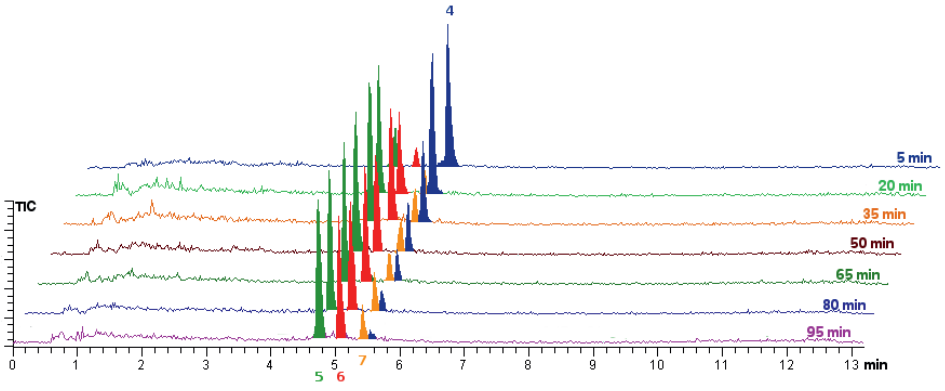


Figure 3. Reduction of peptide **4** by TCEP in PBS (1:1000). Peak intensity corresponds to the amount of compound present in the reaction mixture. While starting material (**4,** blue) disappears as the reaction proceeds, several new species: **5** (green), **6** (red) and **7** (yellow) are formed.

Mass spectroscopic analysis showed that, the mass of **6** (red) is one dalton higher than native peptide **5** (green) suggesting that product **6** is provided with 6-hydroxy-norleucine instead of lysine. To confirm this assumption NMR studies were performed. Comparison of HSQC-specta of **4**, **5** and **6** showed the following features (Figure 4). The blue cross-peak in the spectrum of SIINFEK*L **4** corresponds to the ε-hydrogen/carbon coupling in the 6-azido-norleucine. In the similar spectra of both reference and isolated SIINFEKL peptide (**5**), this signal is lacking and shifted to the new cross-peak (green), which corresponds to ε-hydrogen of lysine. Also in the NMR spectra of **6** the ε-hydrogen/carbon (red) is shifted to the range of 60-65 ppm in carbon NMR confirming that **6** is provided with a 6-hydroxyl-norleucine instead of lysine. The mass spectrometry and NMR spectroscopic data point to the fact that TCEP (**1**) not only reduces the azide into an amine but also hydrolyses the azide to an alcohol under the conditions applied.

Although the isolated amount of minor impurity **7** was not enough for NMR analysis, it is hypothesized (on the basis of LC MS data) that the hydrolysis is accompanied by elimination to give **7**, having a double bond between the δ- and ε-carbon.

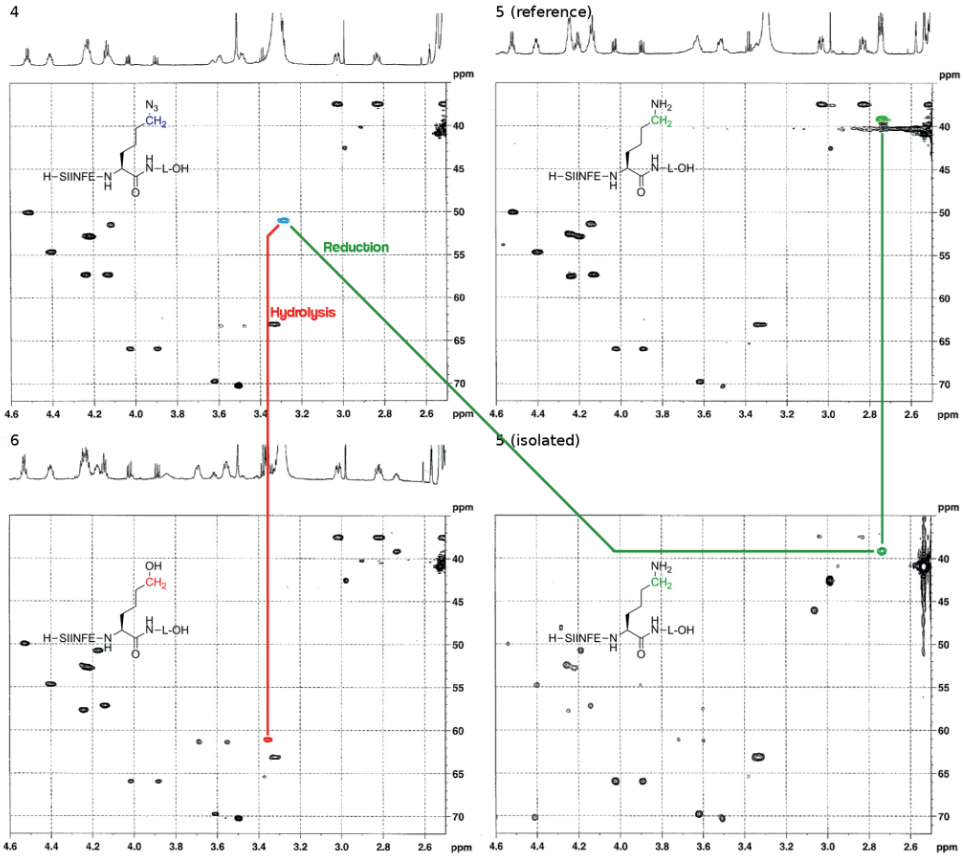


Figure 4. HSQC NMR in *d*-DMSO of isolated peptides.

Next the reduction of the azide in **4** was studied with sulfonylated triphenylphosphine ($PPh_3(SO_3Na)_3$ **2** and sodium hypophosphite ($NaPO_2H_2$) **3**. Interestingly, $PPh_3(SO_3Na)_3$ **2** in PBS buffer (pH 7.2-7.4) did not reduce the azide to an amine and yielded predominantly hydrolysis product **6** and putative elimination product **7** (Figure 5). In addition, the reaction rate has also significantly decreased and more impurities were found (peaks with higher retention time, Figure 5).

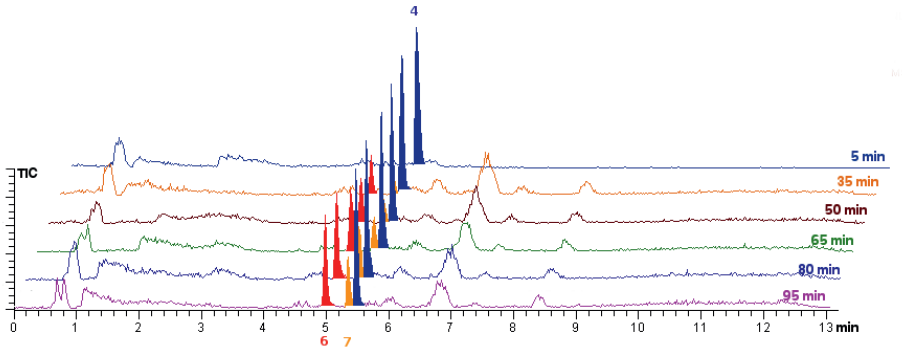


Figure 5. Reduction of azidated petide **4** using $PPh_3(SO_3Na)_3$ in PBS

No conversion was observed upon treatment of peptide **4** with $NaPO_2H_2$ (**3**) in PBS buffer for 1.5 h. With these results in hand the influence of the pH on reactions with both TCEP **1** and sulfonylated triphenylphosphine **2** was studied, using the procedure described above and different buffers (from pH 2 to pH 12).

The rate of the reaction of TCEP **1** with peptide **4** proved to be pH dependent without affecting product ratios. Contrary, the pH dramatically influenced the product distribution in the reaction of $PPh_3(SO_3Na)_3$ **2** with azidated peptide **4**. As depicted in Figure 6, at pH 12, only reduction of azide **4** into amine **5** was observed while at pH 7.4 and below, mainly hydrolysis occurred to give **6** along with the formation of minor amounts of elimination product **7**.

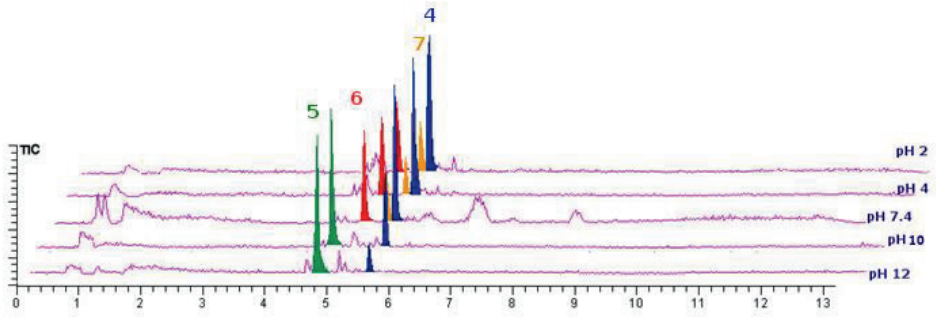


Figure 6. Reduction of **4** using $PPh_3(SO_3Na)_3$ at different pH in phosphate buffer.

As not only the pH but also the type of buffer may influence the Staudinger reaction of azidated peptide **4** with $PPh_3(SO_3Na)_3$, **2** similar experiments were executed using TRIS/HCl based buffer and a pH-range from 8.9 to 7.4. As depicted in figure 7, at pH 7.4 and pH 8, hydrolysis product **6** is still the major product, along with minor amount of **7**. At pH 8.5, amine **5** has become the major product, while minor amounts of **6** and **7** can still be observed. Increasing the pH to 8.9 shows only formation of amine **5** without any detectable amount of ether the hydrolysis product **6** or alkene **7**.

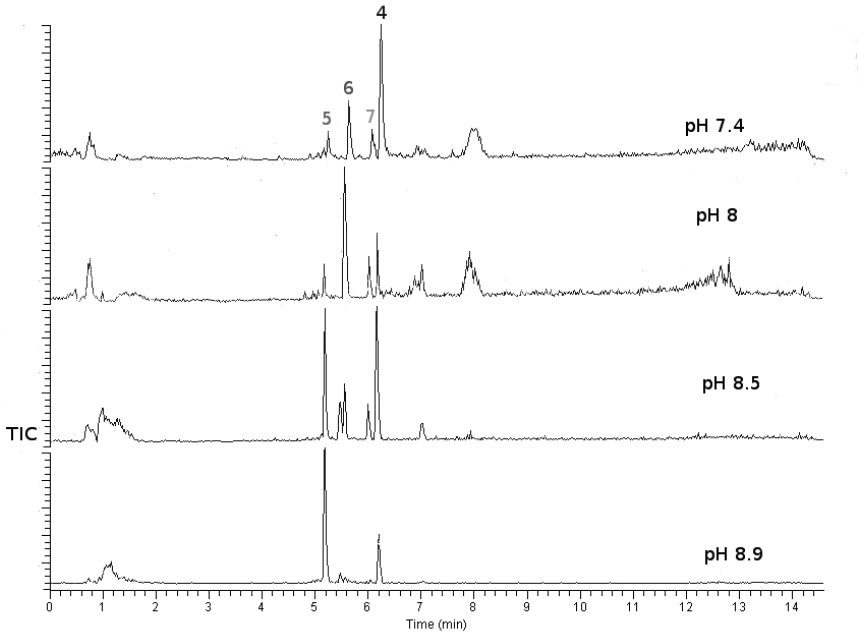


Figure 7. pH dependency using **2** as reductive agent for TRIS-buffer.

Concurrent hydrolysis during the Staudinger reduction of azides to amines with trialkyl- or triarylphosphines has not been reported. An indication for a possible mechanism is given by the group of Raines[15]. Guided by the versatility of diazo compounds they develop a procedure to prepare diazo compounds from azides and specific phosphines. Adopting their proposed mechanism, the putative formation of diazo compounds under acidic conditions, subsequent hydrolysis and elimination is a reasonable assumption and explains the formation of peptides **6** and **7** (Figure 8). The absence of **6** at high pH points to the same mechanism by which the standard Staudinger reduction of an azide to an amine is explained. Under basic conditions iminophosphorane is formed from the initial phosphazene intermediate via nitrogen elimination and subsequent hydrolysis produces an amine (Figure 8).

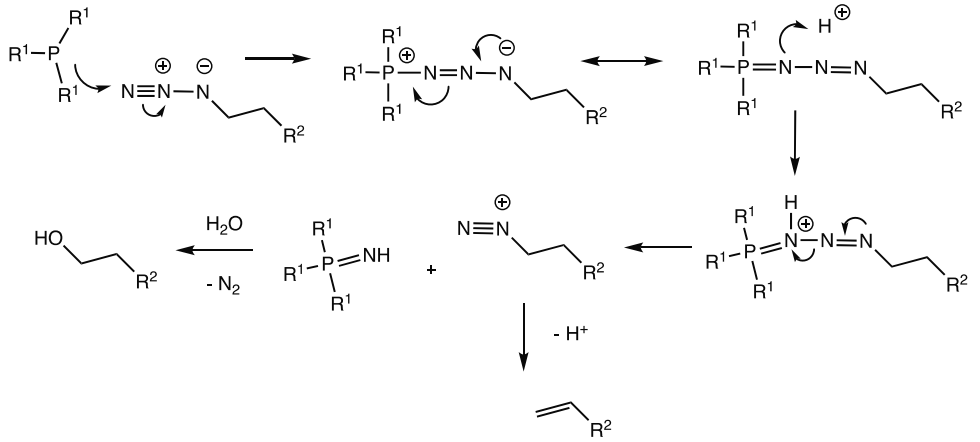


Figure 8. Proposed mechanism for the hydrolysis/elimination

Conclusion

As part of a study to explore azides as bioorthogonal protecting groups the azidated peptide epitope **4** was prepared and subjected to a reaction with three different phosphines in aqueous media. Tris(2-carboxyethyl)phosphine **1** and sulfonylated triphenylphosphine **2** transformed the azide in **4** into the expected amine **5**, but surprisingly also into several side products (**6** and **7**). Using spectroscopic analysis the major side product was identified as hydroxyl compound **6**. By exploring the reaction at different pH it was shown that using phosphine **1**, the product ratio could not be influenced. Contrary to this, phosphine **2** at either acidic or neutral pH favored hydrolysis /elimination (**6** / **7**). Under basic conditions the reduction of the azide into the amine prevailed. A mechanism is proposed explaining the formation of side products **6** and **7**.

Experimental

General: Chemicals were purchased from sigma Aldrich and Acros Organics; HPLC solvent were purchased from Biosolve. Endotoxin-free PBS was Gibco-brand purchased from Life Technologies. Peptide synthesis was performed on Applied Biosystem 433A Peptide synthesizer. LC-MS analysis was performed on a JASCO HPLC-system (detecting simultaneously at 214 and 254 nm) equipped with an analytical Gemini C_{18} column (4.6 mmD × 50 mmL, 3 µ particle size) in combination with buffers A: H_2O, B: MeCN and C: 0.5% aq. TFA. A PE/SCIEX API 165 single quadruple mass spectrometer (Perkin-Elmer) was used as the mass detector. Alternatively, a Thermo Finnigan LCQ Advantage MAX ion-trap mass spectrometer with an electrospray ion source coupled to surveyor HPLC system (Thermo Finnegan) was used. An analytical Gemini C_{18}. HPLC purification were performed on a Gilson GX-281 HPLC system. A preparative Gemini C_{18} (Phenomex, 150 x 21.2 mm, 5 micron) was used in combination with eluents A: 0.1M aq TFA and B: ACN as a solvent system. ^{1}H and ^{13}C were recorded on Bruker Ascend 850. Chemical shift (δ) of ^{1}H and ^{13}C spectra are relative to tetramethylsilane.

General procedure for reduction assay:

In 700 µL of PBS was added 100 µL of 1mM SIINFEK*L solution in PBS. Then 200 µL of 0.5 M solution of either desired phosphine or sodium hypophosphite was added and the reaction was performed at room temperature without stirring. Monitoring the reaction was done using LCMS every 15 min.

General procedure pH dependency assay:

In 700 µL of desired buffer was added 100 µL of 1mM SIINFEK*L solution in milliQ water. Then 200 µL of 0.5 M solution of of either desired phosphine or sodium hypophosphite was added and the reaction was performed at room temperature without stirring. Monitoring the reaction was done using LCMS after 3hours.

Peptide Synthesis:

Tentagel® S PHB Resins Preloaded with leucine was subjected to solid phase Fmoc peptide synthesis using standard Fmoc protected amino acid building block (NovaBiochem, 0.25 mmol, 5 eq), HCTU as an activating agent, and Fmoc cleavage as the final step. The resin was washed with DCM, and shaken in a solution of TFA/H_2O/TIS (95%/2.5%/2.5%). The solution was separated from the resin by filtration and transferred into cold Et_2O followed by centrifugation (4400 rpm, 5 min). Decantation of the supernatant afforded the crude peptide which was purified by RP HPLC (10% → 50% ACN in 0.1% aq TFA) yielding **4** and **5**.

SIINFEK*L **4**

LCMS:RT: (C_{18}column, 10%B-90%B, 13min grad): 6.1 min

[M+H]$^+$: 989.4 ; 21% yield

HRMS:[M+1]$^+$: 989.54146 found: 989.53982

SIINFEKL **5**

LCMS:RT: (C_{18}column, 10%B-90%B, 13min grad): 5.2 min

[M+H]$^+$: 963.6 Yield 19% yield

HRMS:[M+1]$^+$: 963.55096 found: 963.54944

SIINFEOL **6**

LCMS:RT: (C_{18}column, 10%B-90%B, 13min grad): 5.5 min

[M+H]$^+$: 964.3

Buffers:

Table 1 Compositions of phosphate buffers for different pH

pH	Solution A	Solution B
2	0.2M KCl (0.5mL)	0.2M HCl (0.13mL)
6	0.1M KH_2PO_4 (10mL)	0.1M HCl (0.02mL)
10	0.05M $NaHCO_3$ (1mL)	0.1M NaOH (0.214mL)
12	0.2M KCl (0.5mL)	0.2M NaOH (0.12mL)

Table 2 Compositions of TRIS buffer for different pH

pH	0.1M Tris	0.1M HCl	MilliQ water
7.4	0.5mL	0.42mL	0.08mL
8	0.5mL	0.292mL	0.208mL
8.5	0.5mL	0.147mL	0.253mL
8.9	0.5mL	0.007mL	0.497mL

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