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Self adjuvanting immunopeptides : design and synthesis

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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 chemistry³. This field of research aims to the selective detection of a specific biomolecule in living or biological systems⁴. 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 cycloaddition⁷ and strain-promoted [3+2] cycloaddition⁸ 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 trialkyl- 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** (SIINFELK) 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 SIINFELK 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 SIINFELK***L** (K* = azido-norleucine) **4** as a model substrate to explore the Staudinger reduction¹³. 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, sulfonated triphenylphosphine (PPh₃(SO₃Na)₃, **2**) and sodium hypophosphite (NaPO₂H₂, **3**) were selected (Figure 2).

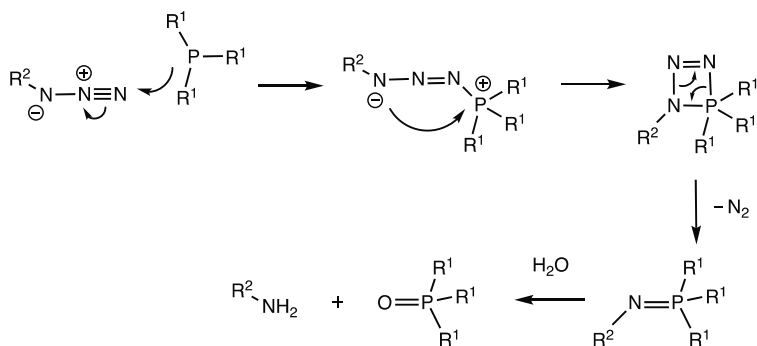


Figure 1. Staudinger reduction mechanism

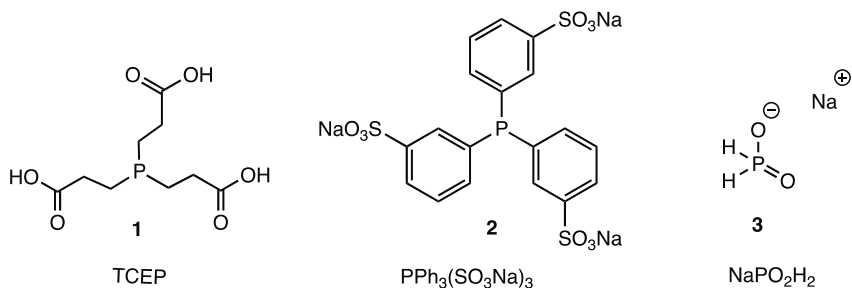


Figure 2. Reducing agents selected for this study

Results and discussion

The first step in the construction of target oligopeptide SIINFELK***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 (SIINFELK) peptide **5** were assembled by a standard solid phase peptide synthesis protocol using Fmoc chemistry, Tentagel®

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-spectra 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.

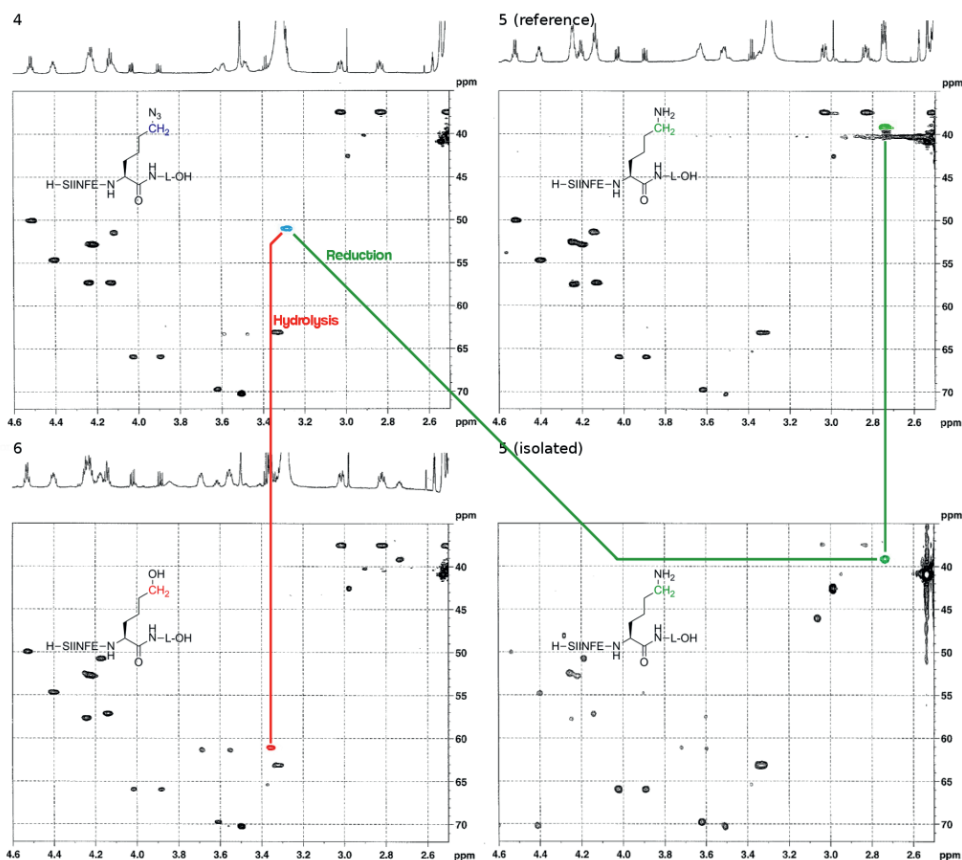


Figure 4. HSQC NMR in *d*-DMSO of isolated peptides.

Next the reduction of the azide in **4** was studied with sulfonylated triphenylphosphine ($\text{PPh}_3(\text{SO}_3\text{Na})_3$ **2** and sodium hypophosphite (NaPO_2H_2) **3**. Interestingly, $\text{PPh}_3(\text{SO}_3\text{Na})_3$ **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).

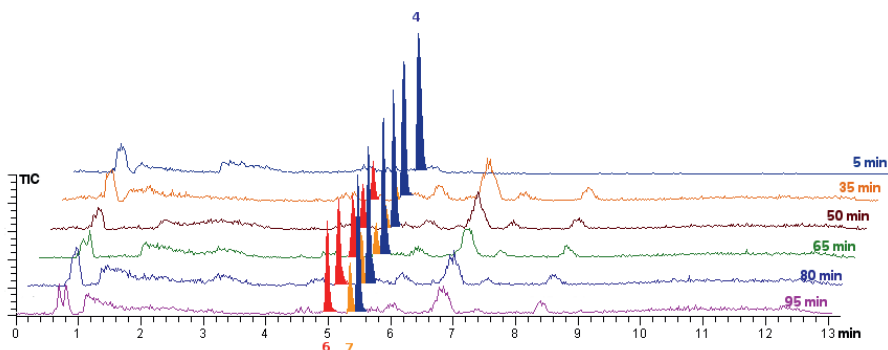


Figure 5. Reduction of azidated peptide **4** using $\text{PPh}_3(\text{SO}_3\text{Na})_3$ in PBS

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 $\text{PPh}_3(\text{SO}_3\text{Na})_3$ **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**.

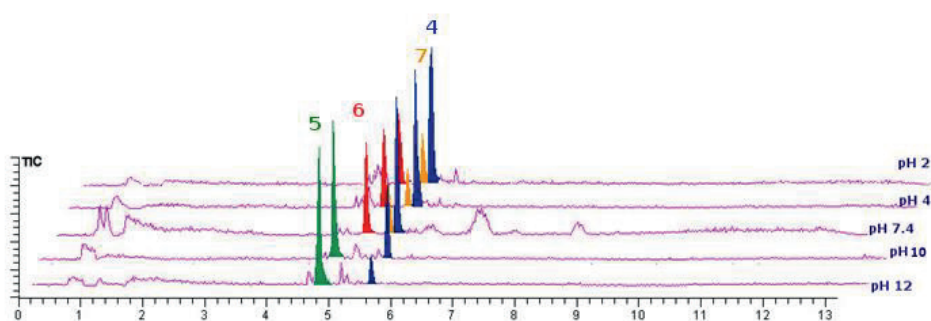


Figure 6. Reduction of **4** using $\text{PPh}_3(\text{SO}_3\text{Na})_3$ at different pH in phosphate buffer.

As not only the pH but also the type of buffer may influence the Staudinger reaction of azidated peptide **4** with $\text{PPh}_3(\text{SO}_3\text{Na})_3$, **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 either the hydrolysis product **6** or alkene **7**.

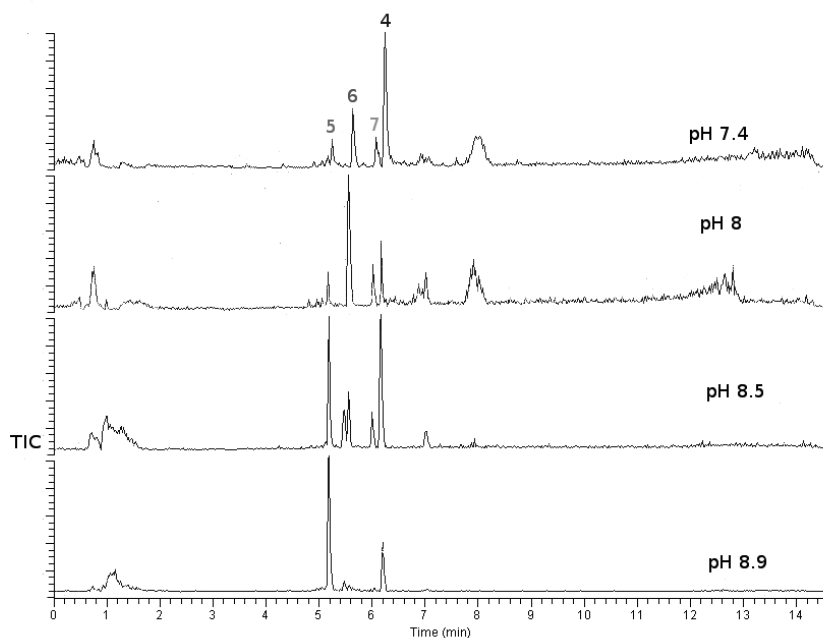


Figure 7. pH dependency using **2** as reductive agent for TRIS-buffer.

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 Raines¹⁵. 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).

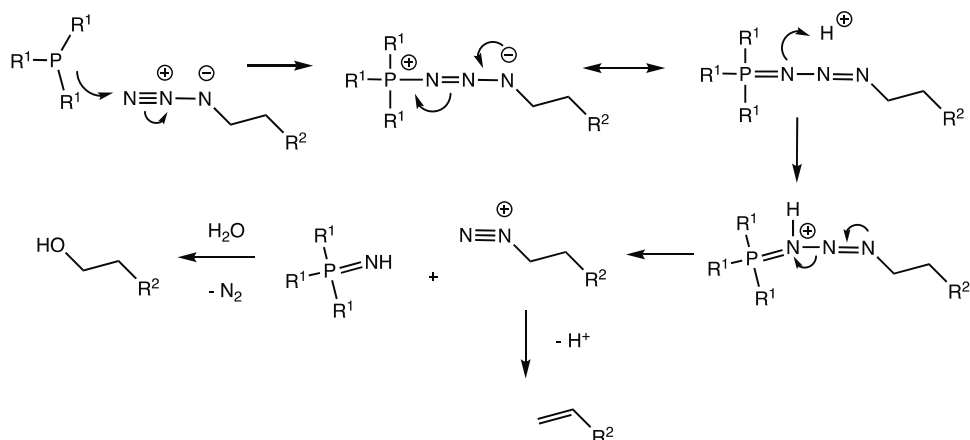


Figure 8. Proposed mechanism for the hydrolysis/elimination

Conclusion

As part of a study to explore azides as bioorthogonal protecting groups the azided 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 C₁₈ column (4.6 mmD × 50 mmL, 3 μ particle size) in combination with buffers A: H₂O, B: MeCN and C: 0.5% aq. TFA. A PE/SCIEX API 165 single quadrupole 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 Finnigan) was used. An analytical Gemini C₁₈. HPLC purification were performed on a Gilson GX-281 HPLC system. A preparative Gemini C₁₈ (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. ¹H and ¹³C were recorded on Bruker Ascend 850. Chemical shift (δ) of ¹H and ¹³C 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 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/H₂O/TIS (95%/2.5%/2.5%). The solution was separated from the resin by filtration and transferred into cold Et₂O followed by centrifugation (4400 rpm, 5 min). Decantation of the supernatant afforded the crude peptide which was purified by RP HPLC (10% $\rightarrow$ 50% ACN in 0.1% aq TFA) yielding **4** and **5**.

SIINFEK*L **4**

LCMS:RT: (C₁₈column, 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: (C₁₈column, 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: (C₁₈column, 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 ₂ PO 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

References

- Gololobov YG, Kasukhin LF. Recent advances in the Staudinger reaction. *Tetrahedron*. 1992;48(8):1353-1406. doi:10.1016/S0040-4020(01)92229-X
- Gololobov YG, Zhmurova IN, Kasukhin LF. Sixty years of staudinger reaction. *Tetrahedron*.

1981;37(3):437-472. doi:10.1016/S0040-4020(01)92417-2

3. Lin FL, Hoyt HM, Van Halbeek H, Bergman RG, Bertozzi CR. Mechanistic investigation of the Staudinger ligation. *J Am Chem Soc.* 2005;127(8):2686-2695. doi:10.1021/ja044461m
4. Sletten EM, Bertozzi CR. Bioorthogonal Reactions. *Acc Chem Res.* 2011;44(9):666-676. doi:10.1021/ar200148z
5. Debets MF, van Hest JCM, Rutjes FPJT. Bioorthogonal labelling of biomolecules: new functional handles and ligation methods. *Org Biomol Chem.* 2013;11(38):6439-6455. doi:10.1039/c3ob41329b
6. Zheng M, Zheng L, Zhang P, Li J, Zhang Y. Development of Bioorthogonal Reactions and Their Applications in Bioconjugation. *Molecules.* 2015;20(2):3190-3205. doi:10.3390/molecules20023190
7. Wang Q, Chan TR, Hilgraf R, Fokin V V., Sharpless KB, Finn MG. Bioconjugation by copper(I)-catalyzed azide-alkyne [3 + 2] cycloaddition. *J Am Chem Soc.* 2003;125(11):3192-3193. doi:10.1021/ja021381e
8. Agard NJ, Prescher J a, Bertozzi CR. A strain-promoted [3 + 2] azide-alkyne cycloaddition for covalent modification of biomolecules in living systems. *J Am Chem Soc.* 2004;126(46):15046-15047. doi:10.1021/ja044996f
9. Zom GG, Khan S, Britten CM, et al. Efficient Induction of Antitumor Immunity by Synthetic Toll-like Receptor Ligand-Peptide Conjugates. *Cancer Immunol Res.* 2014;2(August):756-764. doi:10.1158/2326-6066.CIR-13-0223
10. Zom GGP, Khan S, Filippov D V., Ossendorp F. *TLR Ligand-Peptide Conjugate Vaccines. Toward Clinical Application.* Vol 114. 1st ed. Elsevier Inc.; 2012. doi:10.1016/B978-0-12-396548-6.00007-X
11. Jameson, Stephen C, Bevan, Michael J. Dissection of major histocompatibility complex (MHC) and Tcell receptor contact residues in a Kb-restricted ovalbumin peptide and an assessment of the predictive power of MHC- binding motifs. *Eur J Immunol.* 1992;22:2663-2667.
12. Fremont DH, Stura E a, Matsumura M, Peterson P a, Wilson I a. Crystal structure of an H-2Kb-ovalbumin peptide complex reveals the interplay of primary and secondary anchor positions in the major histocompatibility complex binding groove. *Proc Natl Acad Sci U S A.* 1995;92(7):2479-2483. doi:10.1073/pnas.92.7.2479
13. Walseng E, Bakke O, Roche PA. Major Histocompatibility Complex Class II-Peptide Complexes Internalize Using a Clathrin- and Dynamin-independent Endocytosis Pathway. *J Biol Chem.* 2008;283(21):14717-14727. doi:10.1074/jbc.M801070200
14. Pawlak JB, Gentile GPP, Ruckwardt TJ, et al. Bioorthogonal Deprotection on the Dendritic Cell Surface for Chemical Control of Antigen Cross-Presentation. *Angew Chemie Int Ed.* 2015;54:5628-5631. doi:10.1002/anie.201500301
15. Myers EL, Raines RT. A Phosphine-Mediated Conversion of Azides into Diazo Compounds. *Angew Chemie Int Ed.* 2009;48(13):2359-2363. doi:10.1002/anie.200804689