Self adjuvanting immunopeptides : design and synthesis

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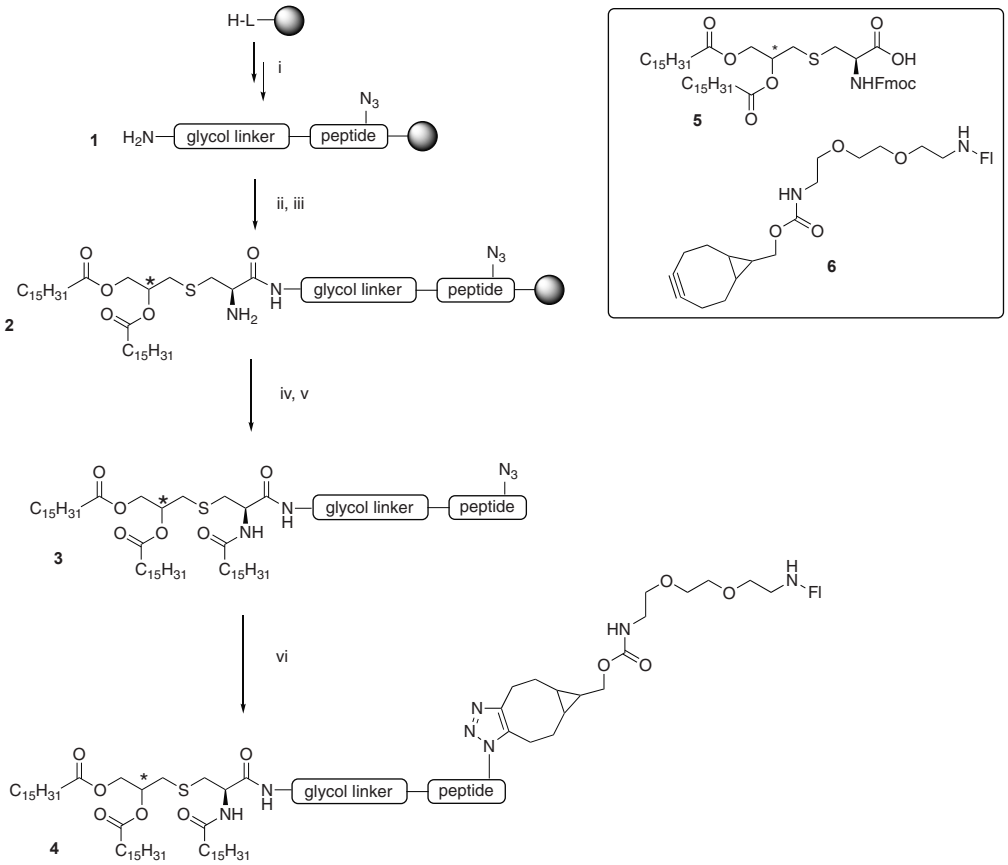
Chapter 7: Summary and future prospects

This thesis describes the design and synthesis of various bio-conjugates of a broad biological significance. All compounds share a peptide chain modified with different biomolecular entities such as nucleic acids, lipids or a small heterocyclic moiety. In the synthetic approaches described in Chapters 3 and 4, the modification could be introduced via a suitable building block using a peptide coupling on solid-phase. Alternatively, more sophisticated methods such as copper-free click chemistry (Chapter 2) could be deployed when necessary. A combined approach that involved both step-wise solid phase synthesis and block-coupling in solution was used for the synthesis of fluorescently labelled immunogenic lipopeptides (Chapter 2) and native oligoribonucleotide-peptide conjugates (Chapter 6). The well-defined conjugates described in this thesis could be applied as model compounds, references or molecular probes for the investigation of intracellular processes. For instance, TLR self-adjuvanting immunogenic peptides described in chapters 2, 3 and 4 might serve for the elucidation of antigen routing and processing within dendritic cells, while azide modified peptides (Chapter 5) could enhance the understanding of antigen presentation on cells surface.

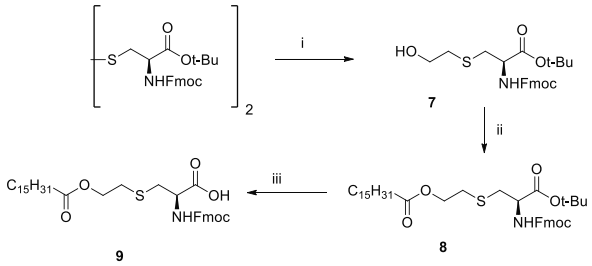
Chapter 1 represents an overview of the synthesis of selected examples of synthetic immunogenic TLR-L-peptide conjugates that were designed as vaccine candidates.

Chapter 2 describes the synthesis of fluorescently labelled conjugates of TLR2 agonist Pam_3Cys and antigenic peptides. Using Fmoc-azidonorleucine in place of Fmoc-Lysine(Boc) in SPPS, unlabelled precursor-conjugates could be isolated. Copper-free click chemistry was then applied to attain a number of fluorescent conjugates. Although the constructs performed as expected during biological assays, they suffer from very poor solubility. The water solubility of such constructs could be improved in the future through the incorporation of an ethylene glycol moiety (PEG-linker) in the peptide sequence to enhance water solubility of the overall construct (Scheme 1).



Scheme 1. Convergent synthesis of labelled Pam_3Cys-lipopeptides. Reagents and conditions: i) SPPS Fmoc automated synthesis, ii) **5**, HCTU, D*i*PEA, NMP, iii) 20% piperidine, NMP, iv) PamCl, Pyridine/DCM, v) 95% TFA, 2.5% TIS, 2.5% H_2O, vi) **6**, DMSO

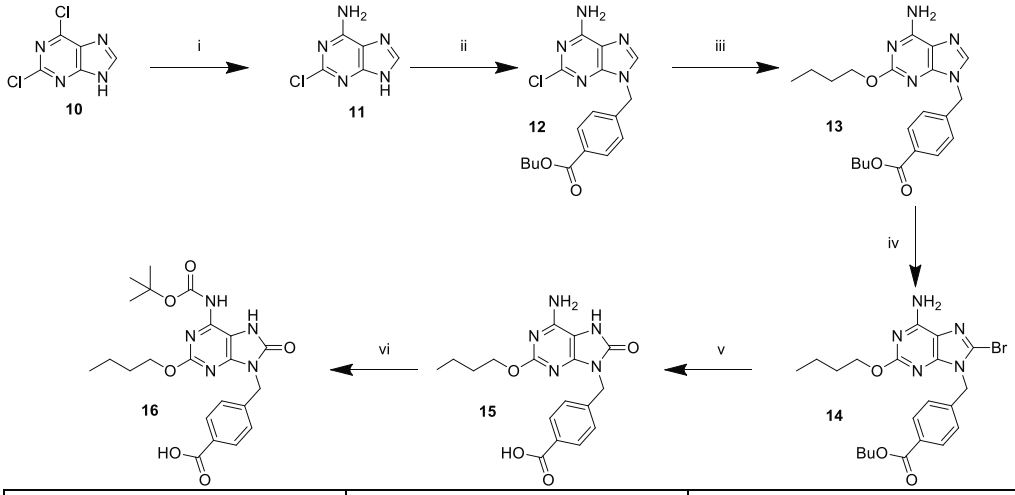
It is known that an ethylene glycol moiety ("glycol linker" Scheme 1) can be introduced between Pam_3Cys-Ser and the peptide sequence via standard peptide coupling conditions and using a commercially available ethylene glycol Fmoc-amino acid.[1] Using different R-groups as labels, a wide variety of labelled immunogenic peptide may be obtainable via this synthesis route. **Chapter 3** describes the design and synthesis of a peptide conjugate containing a simplified TLR-2 agonist ("mini-Pam"). Compared to the original ligand Pam_3Cys, two palmitic chains are removed, while the immunogenic activity of the agonist is retained. (see **9**, Scheme 2). Fluorescently labelled conjugates based on the "mini-Pam" connected to the peptide via a short PEG-linker could be obtained without constrains with regard to solubility in aqueous media. If this agonist is further adopted as a drug candidate, a transfer from lab-scale synthesis to industrial process would be necessary.



	Current	Potential substitutes
Step ii, Coupling agent/base	DIC/DMAP(cat)	T3P, EDCI, CDI / DiPEA, TEA, NMO
Step ii, Solvent	DCM	THF, MeTHF, EtOAc, iPrOAc
Step iii, Acid	TFA	pTSA
Step iii, Solvent	None	Toluene

Scheme 2. Synthesis of simplified TLR-2 ligand building block, Reagents and conditions: i) 1) Zn, H_2SO_4, HCl, MeOH 2) Oxirane, 50% ii) Palmitic acid, DIC, DMAP, DCM, 88% iii) TFA, 90%

Despite a very straightforward synthesis of compound **9**, each intermediate is purified via column chromatography, and evaporated to dryness. For an industrial process a crystallization procedure would need to be developed for each intermediate. When not achievable, using the crude as a stock solution in a suitable solvent for the next step might be a back-up solution. Because DMAP is toxic and DCM is a class 3 solvent, alternative conditions should be investigated. Alternative for DCM could be THF, Me-THF or EtOAc, depending on solubility, and condensing agent DIC catalysed with DMAP may be substituted with propylphosphonic anhydride (T3P) and NMO. The Fmoc stability under those conditions is yet to be assessed. Although, very efficient at small scale, neat TFA is not preferred at large scale. Different organic acids such as p-TSA could be employed. Chapter **4** describes the synthesis of a derivative of a known TLR-7 agonist, 2-alkoxy-9-benzyl-8-oxoadenine, that would be suitable for the conjugation to immunogenic peptides. The original structure of the pharmacofore was not alerted and the anchor site known not to disrupt the biological activity of the agonist was chosen. As depicted in scheme **3** compound **16** can be synthesized from compound **10** in 6 steps. Due to the overall poor solubility of this construct, substituting column chromatography for crystallizations appears a feasible option in an industrial process. Step i condition are readily scalable, yet, lower temperature of reaction would allow standard reactor to be used rather than an autoclave. In step ii, although TBAF is not preferred on scale, it appears difficult to substitute it for a different base without putting at risk the regioselectivity of the alkylation. In step iii, due to safety concern, it would be preferable to use commercially available sodium n-butoxide in butanol rather generating it *in-situ* by the use of sodium hydride. Step iv, consisting of a bromination in DCM, would benefit from a different source of bromine. However, alernatives for these conditions are difficult to find. Despite a long and tedious work up in step v, compound **15** should be easily isolable by crystallization due to its very poor solubility. Other options using Boc_2O rather than BocON could be explored in step vi. Homogenous conditions in THF/Water, or Schotten-Baumann conditions could perform as an alternative for the Boc protection.

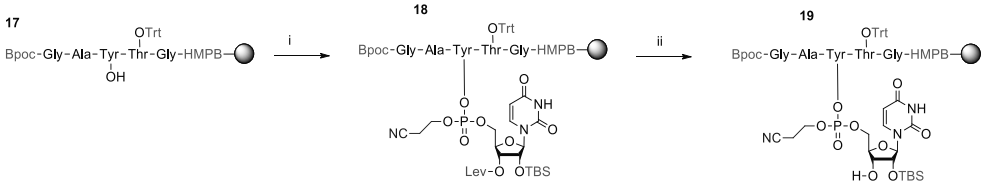


	Current	Potential substitutes
Step i, temperature	120°C	60-120°C
Step iii, reagent	NaH, n-BuOH	n-BuONa in butanol
Step vi, Reagent	BocO**N**	Boc_2O
Step vi, solvents	H_2O/Dioxane	H_2O/THF, H_2O/MeTHF, H_2O/EtOAc

Scheme 3. Synthesis of compound **16**. *Reagent and conditions:* i) Sat. NH_3 in MeOH, 100°C. 88% ii), 1M TBAF in THF, Bromo methyl, butyl benzoate, RT, 92% iii) 1) NaH, n-BuOH, 120°C 2) H_2SO_4, 80 C°, 62% iv) Br_2, DCM, RT, 76% v) 1) NaOMe, MeOH, 65°C 2) 37% HCl, RT 3) 1M KOH, H_2O, RT, 53% vi) BocON, TEA, H_2O/Dioxane 1:1, RT 10%.

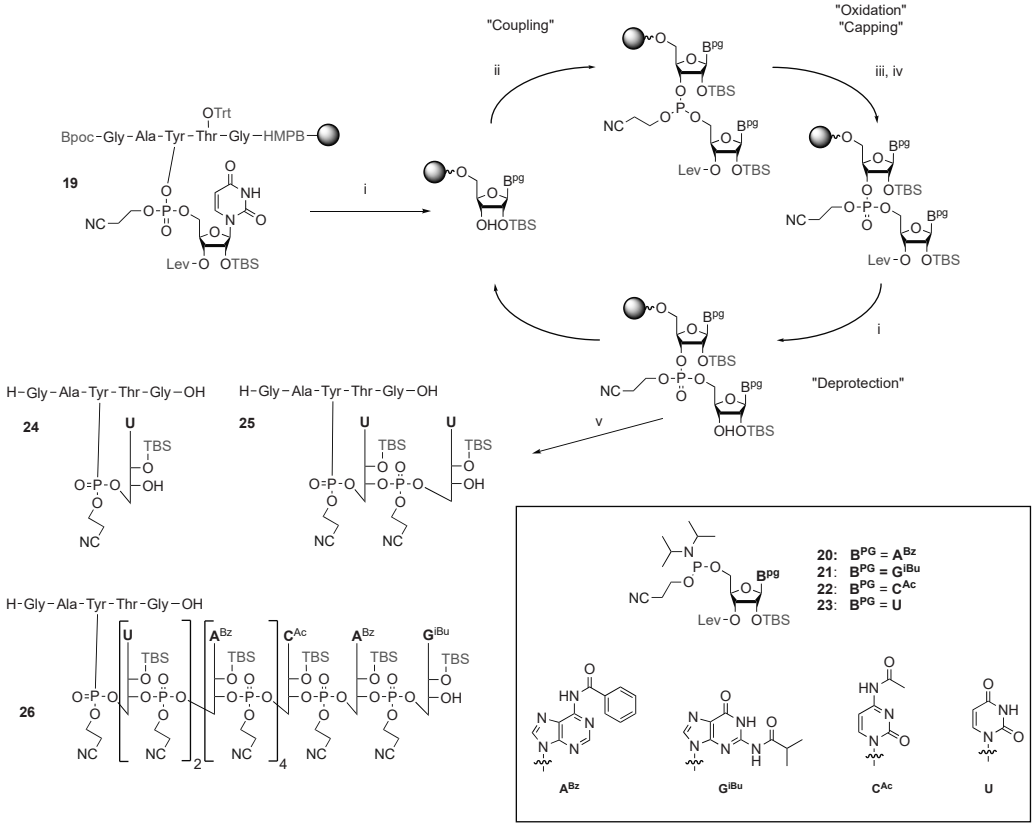
Chapter 5 describes the synthesis of an azidated peptide and the influence of the type of phosphine and the pH on the outcome of the Staudinger reduction in aqueous conditions. With a careful choice of the phosphine and the pH, it appears possible to control the obtained product. High pH resulted in reduction of the azide to the corresponding amine, while low pH resulted in hydrolysis to a product bearing an alcohol function. The structures of both products could be determined by 2D HSQC NMR. A negligible amount of the postulated eliminated product could be detected as well. As shown by Pawlak *et al.*[2], Staudinger reduction could be used to deprotect an epitope on cell surface, making it available for recognition by other immune cells. In order to expand this technology to other amino acids, azidoalanine could be used as a protected serine. Although, a larger amount of the eliminated product is to be expected, it would broaden the range of epitopes that can be masked by such process. **Chapter 6** describes the automated synthesis of a natural nucleopeptide, occurring in the genome of Coxsackie virus. The four necessary building blocks were made in a similar fashion, using a 7-step synthesis. Despite being non-optimized, the proposed synthesis gave access to sufficient material to initiate automated solid support synthesis of the targeted oligonucleopeptide. Although the first phosphoramidite coupling on the side chain of the immobilized peptide **17** proved to be troublesome, the use of a large excess of the nucleoside phosphoramidite in combination of BMT as a base yielded after oxidation the pentapeptide **18** as depicted in scheme **4**. Cleavage of the Lev group without migration of the TBDMS group could be achieved after extended screening of conditions. Although, performed in near neutral buffered condition, TBDMS migration has been observed in many occasions in such cis diol systems. Complete deprotection on the 3'

hydroxyl could be achieved using 75 equivalent excess of hydrazine in AcOH, pyridine and THF for 20 min to give nucleopeptide **19**.



Scheme 4. Synthesis of partially protected nucleopeptide **19** *Reagent and conditions:* i) 1) BMT, 5'-PAM-3'-Lev-2'-TBDMS uridine, ACN/Dioxane, 2) I_2, THF/Pyr/H2O, rt, 1.5 min ii) (TEA/TEA*3HF/DMF (2:3:4, v:v:v), rt, o.n

By repetitive cycles, as depicted in Scheme **5**, nucleopeptides of various sizes, **24-26,** could be synthesized and isolated. Subsequently, **24** and **25** were successfully coupled to the C-terminal peptide of the native VPg fragment to generate the rudimentary native nucleopeptides VPgpU and VPg-pUpU. The future coupling of nucleopeptide **26** to VPg-derived sequence will provide a synthetic Picornavirus nucleopeptide of unprecedented complexity.



Scheme 5. Synthesis of compounds **24** -**26**. *Reagents and conditions:* (i) Hydrazine, THF/Pyr/AcOH, rt, 20 min; (ii) **20**, **21**, **22**, **23,** BMT, dioxane/ACN, rt, 20/30/30/20 min; (iii) I_2, THF/Pyr/H2O, rt, 1.5 min; (iv) Ac_2O, MeIm, 2,6-lutidine, NMP, rt, 30 sec (v) 3% TFA/DCM, rt, 5 min.

References

1. Wilkinson, B. L., Day, S.; Malins, L. R.; Apostolopoulos, V.; Payne, R. J. *Angew. Chem. Int. Ed.* **2011**, *50,* 1635-1639.
2. Pawlak, J. B.; Gential, G. P. P.; Ruckwardt, T. J.; Bremmers, J. S.; Meeuwenoord, N. J.; Ossendorp, F. a.; Overkleeft, H. S.; Filippov, D. V.; van Kasteren, S. I. *Angew. Chem. Int. Ed.* **2015**, *54*, 5628–5631.