Synthetic Studies Towards Oligonucleotide Derivatives and Conjugates

Delft, P. van

Citation

Delft, P. van. (2013, September 3). *Synthetic Studies Towards Oligonucleotide Derivatives and Conjugates*. Retrieved from https://hdl.handle.net/1887/21632

Author: Delft, Pieter van
Title: Synthetic studies towards oligonucleotide derivatives and conjugates
Issue Date: 2013-09-03

7 Summary, Concluding Remarks and Future Prospects

The work described in this thesis primarily focuses on the development and application of reliable methods for the synthesis of oligonucleic acid conjugates. The strain induced alkyne-to-azide [2+3] dipolar cycloaddition was selected and found to be valuable for the synthesis of oligonucleic acid conjugates.

As one of the major classes of biopolymers nucleic acids are essential to all forms of life. As a result, nucleic acids have been subject of intensive research. Much is known about the role and function of nucleic acids in biology, however new insights are constantly evolving. Past and current research has been greatly aided by the availability of oligonucleotides from synthetic source.

Chapter 1 presents a concise historical overview of the major developments in synthetic nucleic acid methodology and describes important methods used to prepare oligonucleotides. For example, in order to develop oligonucleotide-based drugs, short RNA or DNA fragments are investigated to exploit the natural ability of certain enzymes to specifically recognize DNA-RNA and RNA-RNA duplexes and subsequently degrade these, thereby blocking the expression of the targeted genes. Additionally, fluorescently labeled oligonucleotide diagnostics have been developed and immune stimulatory oligonucleotides are being assessed for their use as adjuvants in synthetic vaccines. In line with the development of (modified) DNA or RNA fragments, a lot of research is directed to the design, synthesis and evaluation of oligonucleotide conjugates in which oligonucleotides are covalently attached to another molecular entity that append beneficial properties such as an improved cellular uptake or pharmacological profile (therapeutics/vaccines).

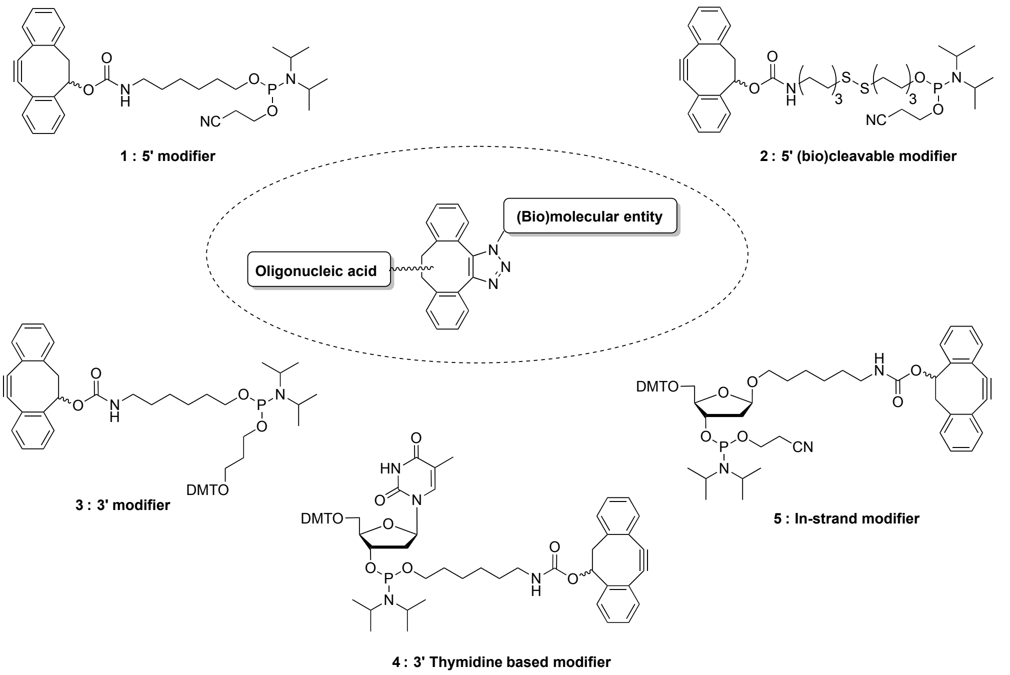
Selected recent developments in bio-orthogonal chemistry relevant for the field of chemical biology and bioorganic chemistry are reviewed in the final section of **Chapter 1**. Special attention is directed to methods that posses a high degree of selectivity, such as the Diels-Alder reaction and the Staudinger ligation. Current achievements are highlighted in the area of the inverse electron-demand Diels-Alder reaction and the strain induced azide-to-alkyne dipolar cycloaddition (*"copper free click "*). All these reactions are discussed in the context of the synthesis of oligonucleotide conjugates.

The strain induced azide-to-alkyne [2+3] dipolar cycloaddition was selected as a suitable candidate to give access to nucleic acid conjugates in a fast, selective and reagent free manner. **Chapter 2** describes the design and synthesis of a new dibenzocyclooctyne phosphoramidite (**1**, figure 7.1). The application of this reagent in solid phase DNA and RNA synthesis yielded the corresponding 5'-cyclooctyne functionalized oligonucleotides. Initial evaluation of the copper free click reaction was done by the reaction of an alkyne functionalized DNA 5-mer with an azide containing BODIPY fluorescent dye. Additionally, successful conjugation of a

RNA 16-mer was performed with an azide functionalized tetrasaccharide (a hyaluronan fragment) and azide bearing dodecameric peptide penatratin.

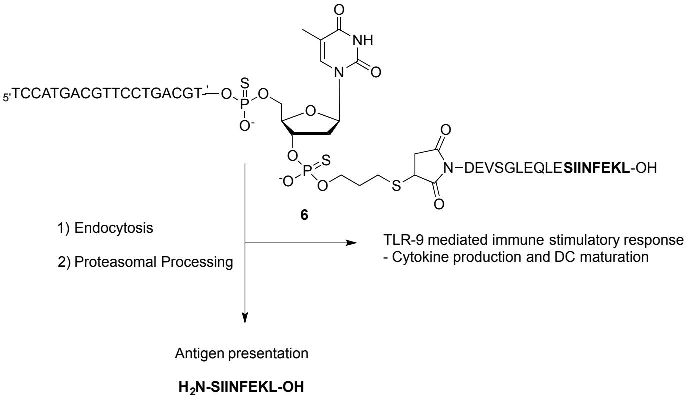
With the aim of broadening the scope of the work described in Chapter 2, **Chapter 3** continues with the development of four additional cyclooctyne-based phosphoramidite reagents (**2** – **5**, figure 7.1). Two building blocks to obtain nucleic acids conjugates functionalized at the 3'-terminus (**3** and **4**), a D-ribose based building block for intra-strand incorporation of the cyclooctyne moiety (**5**) and an additional reagent for 5' functionalization which contains a disulfide linker (**2**) that can be cleaved under mild reducing conditions. All four phosphoramidite reagents were incorporated at designated positions of short oligonucleotides. Subsequent copper free click reactions of these oligonucleotides with zwitter-ionic oligosaccharides or fluorescent dye TAMRA, both containing azides, yielded the corresponding conjugates. Having five phosphoramidites (**1** – **5**, figure 7.1) available and their compatibility with oligonucleotide solid phase chemistry established, oligonucleotides conjugated at any desired position can now be obtained. Several applications using these reagents are currently being explored.

Figure 7.1: Cyclooctyne containing phosphoramidite reagents described in chapters 2 and 3.



For instance, the stability of the cyclooctyne under sulfurizing conditions that are used to prepare oligonucleotides with phosphorothioate backbone may open the way to the synthesis of advanced conjugates useful as candidate synthetic vaccines. Specific phosphorothioate oligonucleotides rich in unmethylated CpG dinucleotides, also termed CpG DNA, act as Toll-like receptor 9 ligands, stimulating immune response in dendritic cells (DC's).[1] Several CpG DNA oligonucleotides, possessing this property have been identified, synthesized and immunological evaluated. En route to a potential synthetic vaccine, CpG DNA's were functionalized with a 3'-thiol group and were conjugated to maleimide containing oligopeptide epitope as depicted in figure 7.2. It was found that conjugates of this type led to improved antigen presentation and DC activation.[2] The copper-free click chemistry may provide a valuable alternative to the method depicted in figure 7.2.

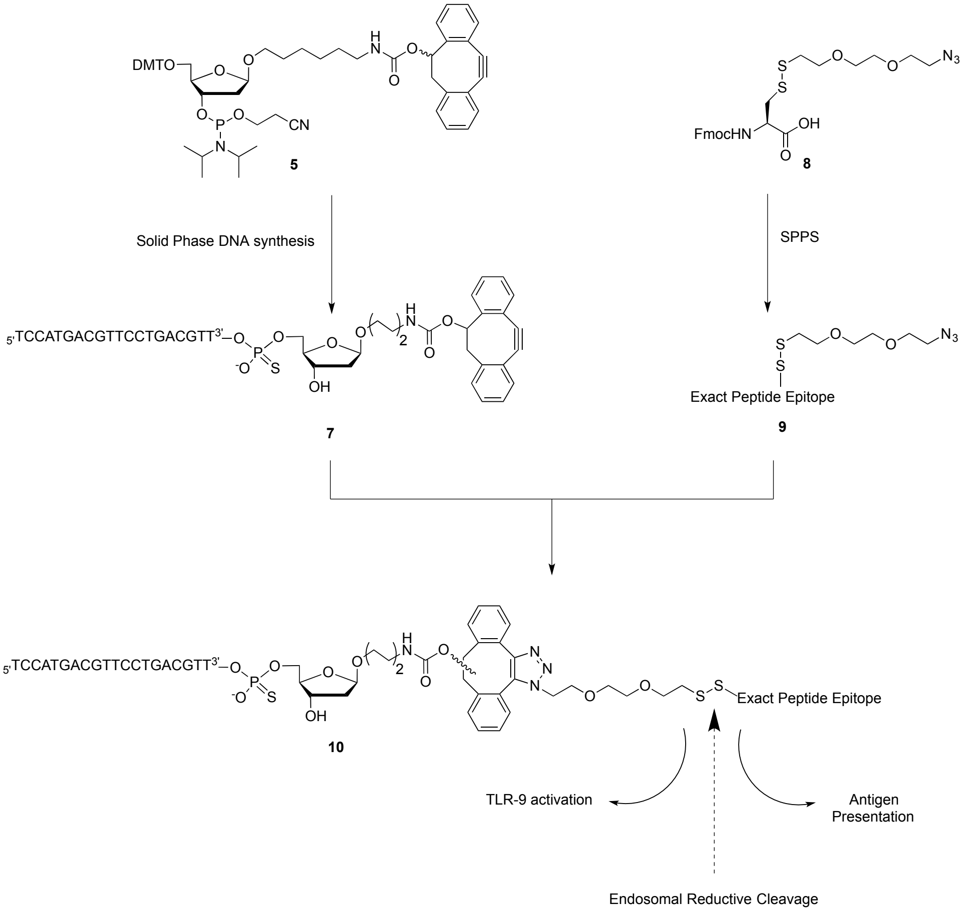
Figure 7.2: CpG DNA – OVA peptide conjugates as "synthetic vaccines" (**6** contains a phosphothioate backbone).



The newly developed cyclooctyne phosphoramidites allow the synthesis of disulfide based biodegradable conjugates containing CTL-epitopes directly attached to TLR-ligands. The synthesis of such constructs is outlined in Scheme 7.3.

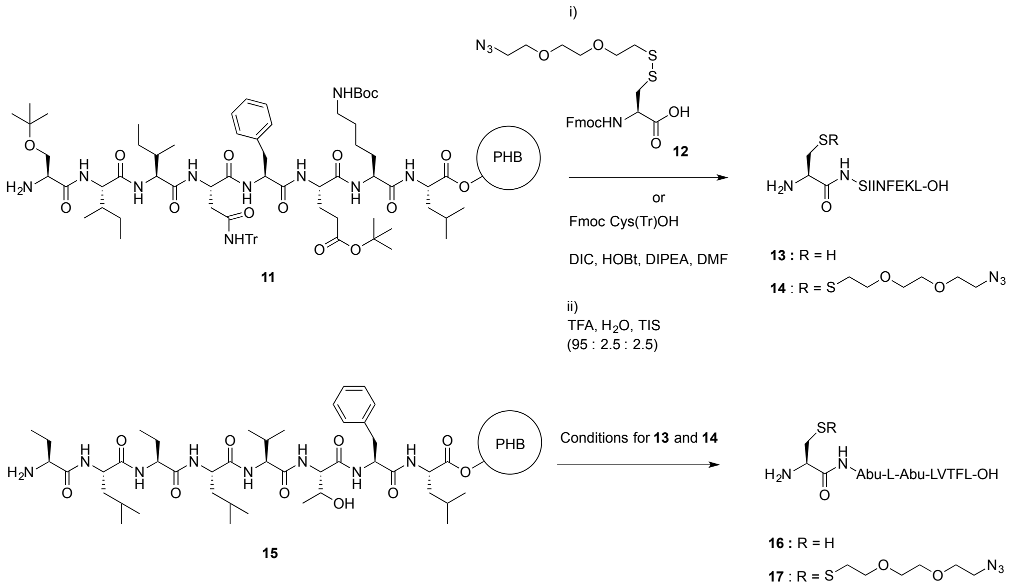
A prerequisite to this approach is a naturally occurring cysteine residue or a tolerated serine substitution in the epitope of interest. Unfortunately, preliminary results have revealed that cysteine substitution in the ovalbumin epitope SIINFEKL is not tolerated and antigen presentation was not observed. To this end it was decided to elongate the N-terminus with an additional cysteine, which is cleaved inside the cell, releasing the exact epitope (**13**, **14**). Additionally an exact epitope containing three cysteine residues presented by murine Maloney leukemia virus infected mice was selected. The cysteine residues in this epitope are known to tolerate alpha-amino butyric acid modification (**16**, **17**).[3]

Scheme 7.3: Synthetic approach to a biodegradable antigenic oligonucleotide-peptide conjugate with an exact CTL-epitope as the peptide component.



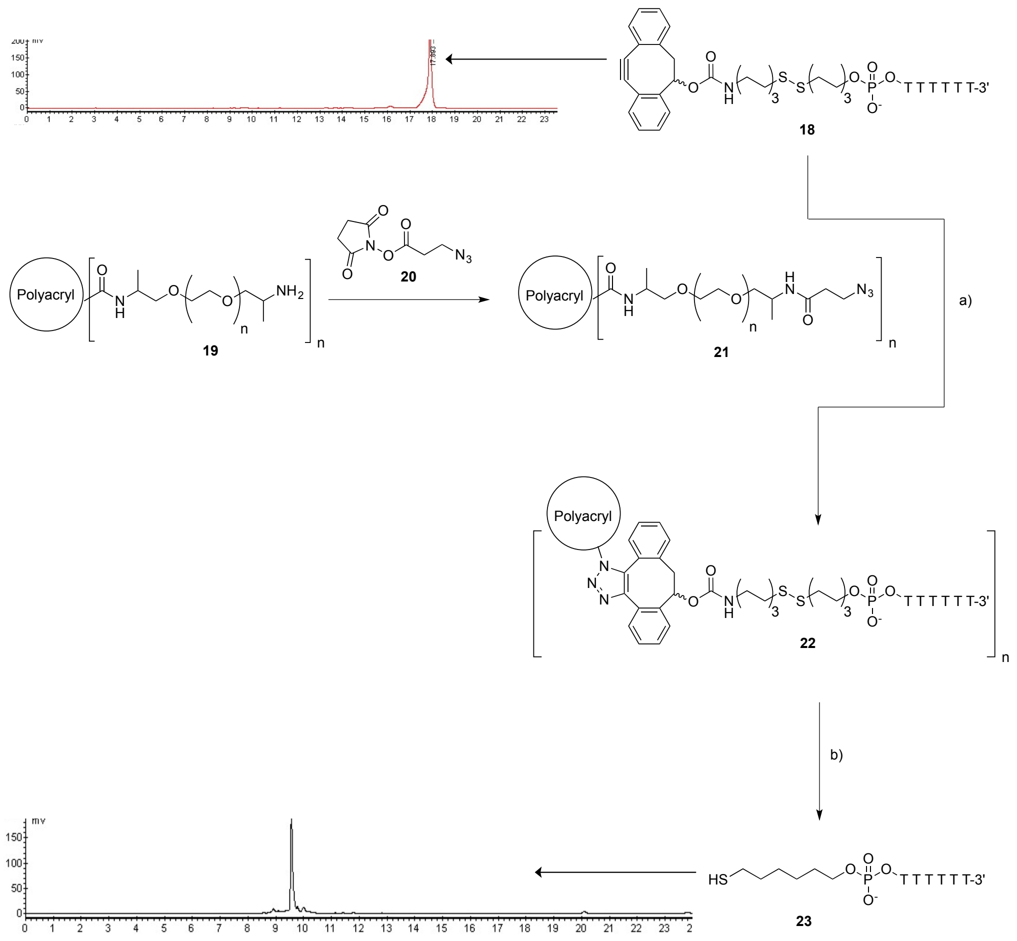
Scheme 7.4 depicts the synthesis of the target azide containing epitopes and the corresponding reference epitopes. (The synthesis of novel cysteine building block **12** is taken up in the experimental section). Future work includes the conjugation of the epitopes **14** and **17** to CpG DNA (**7**) and evaluation of the immunological properties of the conjugates, such as TLR activation and antigen presentation.

Scheme 7.4: Synthesis of azide functionalized epitopes containing a disulfide linker and the corresponding reference peptides. (corresponding yields: **13**: 5.2%, **14**: 7.6%, **16**: 14%, **17**: 44%)



The combination of the disulfide based cleavable linker and copper free click chemistry might also find valuable application in a new isolation procedure for 5'-thiol functionalized oligonucleotides via a "click and release" approach employing disulfide-containing modifier **2**. Following oligonucleotide solid phase synthesis, the unprotected crude oligonucleotide would be immobilized using the copper free click handle on the 5'-end. Washing followed by reductive cleavage from the resin, releases the pre-purified 5'-thiol oligonucleotide. To evaluate this strategy poly-acryloyl poly-ethylene glycol amine (PEGA) resin was capped with azido-propionic acid by reaction with **20** (scheme 7.5) yielding PEGA-N_3 **21**. Treatment of 5'-cyclooctyne-disulfide thymidine hexamer **18** (chapter 3) with the azide functionalized resin completely immobilized the oligonucleotide (**22**) as indicated by LC analysis of the supernatant. The resin was washed several times with water prior to treatment with Me_3P (20 eq) in 10 mM triethylammonium acetate buffer/dioxane (5 : 1).

Scheme 7.5: Click and Release approach to yield 5'-thiol functionalized oligonucleotides.



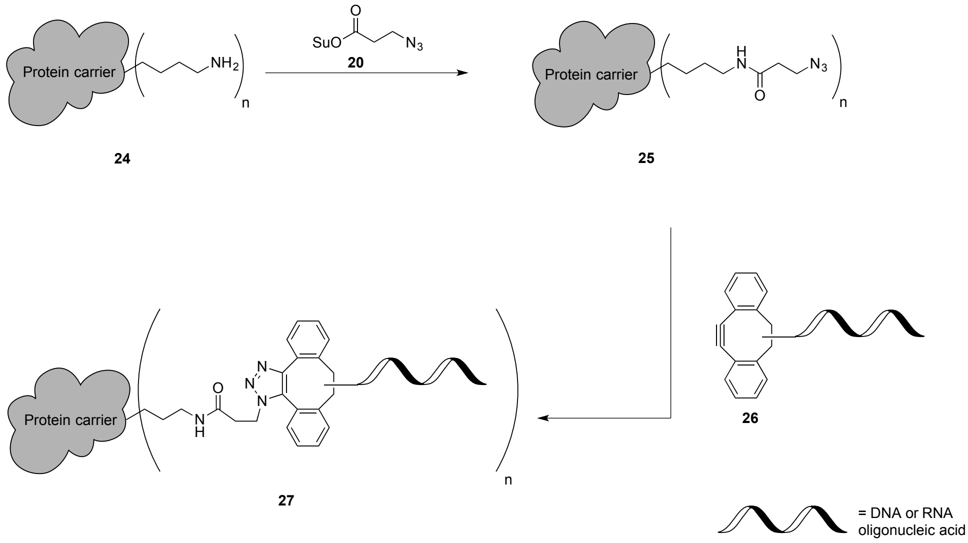
Reagents and conditions : a) oligonucleotide immobilization;1.25 mM TEAA. b) oligonucleotide release; Me_3P, 10 mM TEAA / dioxane (5 : 1).

Next, three cycles of washing (TEAA buffer), centrifugation and decantation followed by lyophilization led to the isolation of the 5'-thiol functionalized thymidine hexamer **23**. Based on these results it can be concluded that immobilization of the oligonucleotide by "copper-free click" chemistry and the reductive release of the oligonucleotide proceed efficient resulting in a high sample recovery (UV absorbance). The full scope and benefit however needs to be determined by applying this protocol on a crude oligonucleotide.[4] Ultimately, the pre-purification might be beneficial for the synthesis and isolation of oligonucleotides which would otherwise be difficult to purify (e.g. due to RNA folding).

In **Chapter 4** the copper free click chemistry presented in Chapters 2 and 3 is applied to the synthesis of protein-nucleic acid conjugates. With the aim of raising antibodies against the naturally occurring RNA modification mcm^5S^2U, found at the wobble position (U34) of $tRNA^{lys}$, three different bovine serum albumin conjugates were synthesized. To this end BSA was first reacted with 3-azidopropionic acid *O*-succinylester and then either with 5'-dibenzocyclooctyne mononucleotides mcm^{5}U and mcm^{5}S^{2}U or with the full-length tRNA^{lys} anticodon stem loop containing the mcm^{5}S^{2}U modification (Scheme 7.6, **27** as illustrative example). The BSA conjugates were obtained in a fast, mild and clean fashion and are currently under evaluation for their ability to generate antibodies against the mcm^{5}S^{2}U RNA modification. In this respect, it has to be addressed if and to what extent the large dibenzocyclooctyne moiety hampers the antibody generation against the modified nucleoside.

Eventually this conjugation strategy might be a valuable addition to the existing methods given several advantages. For example, the azide moiety is stable whereas maleimide, for instance, is prone to hydrolysis resulting in less efficient conjugation. Moreover both the azidopropionylation and "click" reaction proceed under neutral conditions and do not require any other reagents or chemical conversions, making this strategy reliable in an otherwise difficult to analyze reaction. This is especially beneficial for proteins that are more difficult to handle than BSA, such as a highly immunogenic protein Keyhole Limphet Hemocyanin (KLH), which is known to easily aggregate.

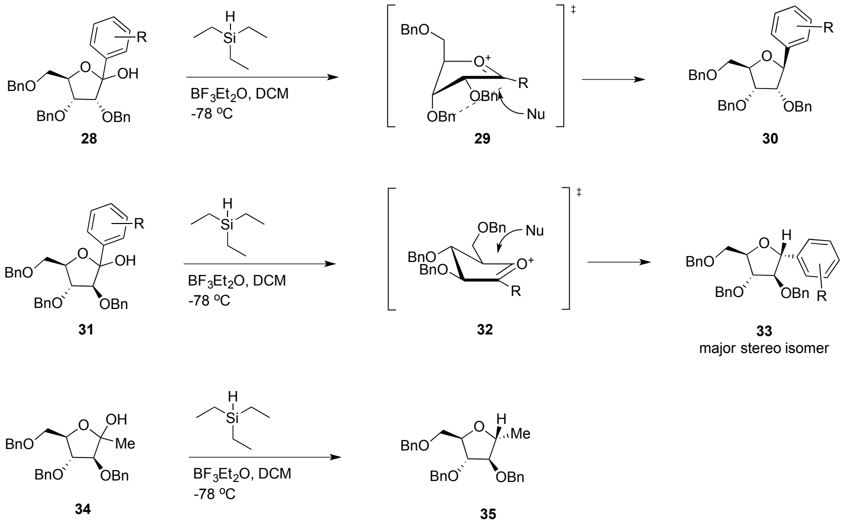
Figure 7.6 : general approach to protein oligonucleic acid conjugates



It would also be interesting to establish the usability of such constructs for the isolation of antibodies against a certain secondary structure rather than the mononucleosides. Besides the stem-loop described in Chapter 4, there are several different naturally abundant types of RNA secondary structures such as RNA G-quadruplexes and RNA pseudoknots that might also be interesting moieties for this approach

Chapter 5 describes a study on the reductive conversion of furanose derived hemiketals into *C*-glycosides. As depicted in Scheme 7.7 2,3,5-tri-*O*-benzyl protected furanose hemiketals yield the corresponding *C*-glycosides in a BF_3•OEt_2 mediated triethylsilane reduction. D-Ribose hemiketals commonly yielded the corresponding β-*C*-glycosides (**30**) in these reductions. Contrary to these findings, arabinose hemiketal analogues (**31**) are reported[5,6] to yield anomeric mixtures with the exception of the methyl-substituted congener, which was found[6,7] to give a single isomer in the reduction. To elucidate the stereochemical outcome of these reductions several 2,3,5-tri-*O*-benzyl protected arabinose and ribose hemiketals were synthesized and reduced to the C-glycosides. As expected, all ribose derived hemiketals yielded β-products. Reductions performed on the aromatic substituted arabinose hemiketals at -78 ^{o}C gave mostly mixtures, with the α-C-glycoside **(33)** being the major isomer (α > 75%). The methyl-substituted arabinose *C*-glycoside was indeed isolated as a single isomer (**35**), which was found to have the α-configuration. This stereochemical outcome can be adequately explained using a model, proposed by the group of Woerpel.[8–10] As depicted in Scheme 7.7, ribose **29** reacts from the E_3-oxocarbenium ion by an inside attack of the hydride forming β-*C*-glycosides. This is proposed to be a result of the favorable interaction between the C-3 benzyloxy substituent with the positively charged anomeric center.

Scheme 7.7: Reductions of 2,3,5-tri-*O*-benzyl protected ribo- and arabinofuranose hemiketals and the proposed oxocarbenium-ion-like transition states.

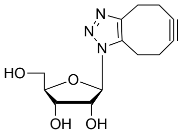


Alternatively, the arabinose-derived oxocarbenium ion was proposed to react from the ^{3}E-conformer (**32**). Here all substituents are placed in the favorable equatorial position avoiding the destabilizing 1,3-diaxal interaction between the C-2 and C-5 substituents that would occur in E_3 conformer. To establish this, quantum mechanical calculations were performed to determine the conformational preferences of arabinose- and ribose-derived oxocarbenium ions. It was found that ribose indeed favors (3-4 Kcal/mol) the E_3 conformer whereas the arabinose favors the proposed ^{3}E-conformer albeit to a lesser extend of 1-1.5 Kcal/mol. The reduced preference for the phenyl substituted arabinose-derived oxocarbenium ions to adopt the ^{3}E conformer meets the experimental findings. With the aromatic ring forced in planarity with the sugar pucker there might be a form of allylic strain partially sabotaging the preference for adopting the ^{3}E-conformer leading the formation of a minor amount of β-product. The latter explanation was supported in an experiment in which a secondary oxocarbenium-ion generated from Acetyl-2,3,5-tri-*O*-benzyl-D-arabinose was reduced with deuterated triethylsilane. This yielded the expected β-deutered product. The latter insight might also prove useful in the field of carbohydrate synthesis since these secondary oxocarbenium-ions play a crucial role in glycosylating reactions. Although quantum mechanical calculations for the ground state of the intermediate oxycarbenium ions supports the postulated reaction pathway it would be of interest to execute calculations on transition states of all reduction reactions.

Future work on the synthesis of the xylo- and lyxo furanose hemiketals, their behavior in ensuing reductions (Scheme 7.8) and *in silico* calculations will clarify whether the model also applies to explain the stereochemical outcome for these hemiketals.

Chapter 6 describes the synthesis and application of a newly developed D-ribosyltriazole-annulated cyclooctyne (RITRICO). With the triazole linked to the anomeric position of D-ribose bridging the cyclooctyne moiety, RITRICO can be viewed as a purine mimic. A RITRICO phosphoramidite was synthesized and subsequently incorporated in a pentameric oligonucleotide. This pentamer (5'-TT-RITRICO-TT-3') was treated with the azide containing fluorescent label TAMRA and a fluorogenic azidocoumarine derivative, which fluoresces upon triazole formation in the click reaction. In these initial strain induced azide-to-alkyne [2+3] dipolar cycloadditions it was found that RITRICO reacts rapidly. When mixed in stoichiometric amounts, the azides and the RITRICO containing DNA-pentamer formed the corresponding conjugates quantitatively within 3 hours.

Figure 7.9



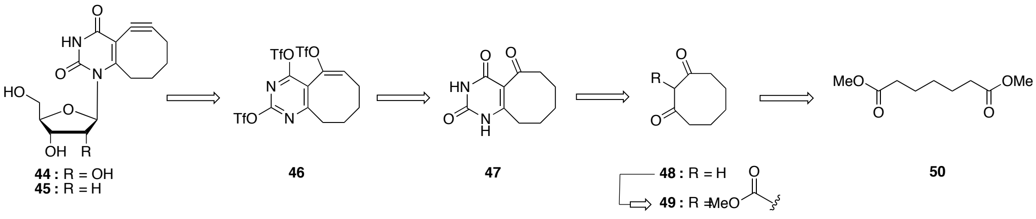
Ribose-Triaza-Cyclooctyne
RITRICO

To evaluate the RITRICO as a purine mimic the corresponding phosphoramidite was used in the solid phase synthesis of a RNA 13-mer. The synthesis of the complementary DNA strand allowed the formation of RNA-DNA duplex, which was evaluated for its reactivity in the strain induced click reaction. Upon addition of the 3-azidocoumarine label the duplex was quickly converted to the corresponding fluorescently labeled conjugate. Additionally a reference duplex, comprising of the unmodified RNA 13-mer in combination with the DNA fragment, was synthesized. The latter allowed for a comparison of the structural characteristics and stabilities of these three duplexes. Circular dichroism measurements showed A-type double helix for all three duplexes as expected for regular RNA-DNA hybrids. Nevertheless, the stability of both the RITRICO modified and the fluorescently labeled duplexes significantly decreased as determined by melting experiment (T_m depression of 15 ^oC). A probable cause for this effect might be the orientation of the cyclooctyne towards the helix rather than the major groove and the ability of cyclooctyne to neither sustain hydrogen bonding nor engage in stacking interactions. .

The favorable conjugation kinetics and the small size of RITRICO prompted the investigation of the synthesis of uridine analogue **44**. The retrosynthesis of **44** and its 2'-deoxy analogue **45** is depicted in Scheme 7.10 Starting from pimelic acid dimethyl ester, 1,3-cyclooctadione can be obtained via described methods.[11] Acylation at C-2 was proposed to lead to **49**. Cyclisation using urea procedures similar to those used in Chapter 4 would give bicyclic **47**. Triflation followed by KO*t*Bu mediated substitution of the pyrimidyl triflates and formation of the alkyne by elimination yields a 2,4-di-*tert*-butoxy pyrimidine-annulated cyclooctyne.

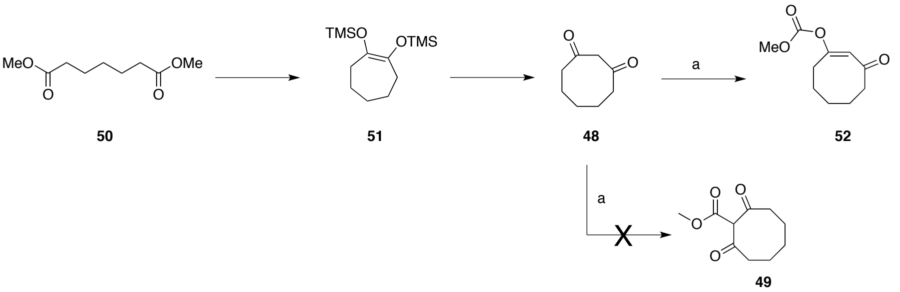
Deprotection of the *tert*-butyl ethers followed by Vorbruggen type[12] coupling would yield **44**. Alternatively Hoffer's chlorosugar[13] can be used as the starting material to obtain **45**.[14]

Scheme 7.10: Proposed synthesis towards an uridine or thymidine derived cyclooctyne.



Hence, as depicted in Scheme 7.11, pimelic acid dimethyl ester was converted into cyclic **51** by the silyl acyloin reaction as described.[11] Next, Simmons-Smith cyclopropanation introduced the eighth carbon and the following periodate oxidation yielded di-ketone **48**.[11]

Scheme 7.11: efforts towards the synthesis of 1,3-diketo-intermediate **49**.

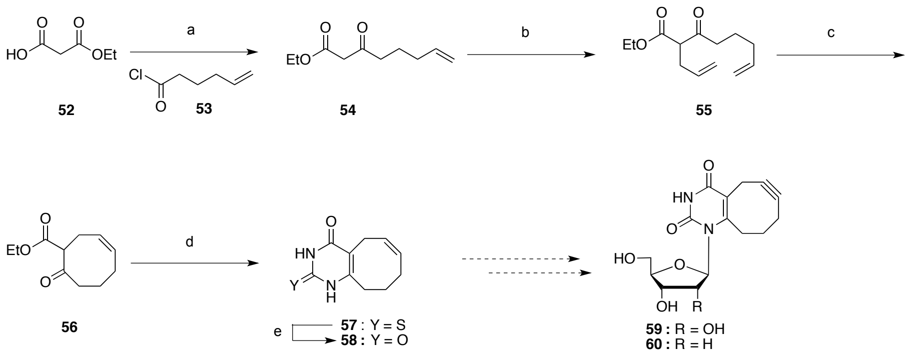


Reagents and conditions : a) NaH, THF/Toluene (1 : 1, v/v), methyl chloroformate; or NaOMe, dimethylcarbonate, MeOH; or Lutidine, $MgCl_2$, methyl chloroformate, DCM; or $SmCl_3$, TEA, methyl chloroformate, toluene.

As envisioned, acylation of **48** C-2 would lead to the desired key intermediate **49**. Unfortunately, none of the procedures applied led to product formation. In fact, under all the tried conditions, carbonate **52** was isolated as the main product. The literature procedure that was suggested to give exclusive C-acylation of 1,3-diketones applying $SmCl_3$ as catalyst[15] provided **52** rather than desired **49** as well.

An alternative approach using ethyl 2-diazo acetate in the cyclopropanation of **51** followed by periodic acid oxidation was swiftly evaluated but also did not lead to the desired product. Therefore pyrimidine annulated **59** and **60** (scheme 7.12) were selected as suitable alternatives.

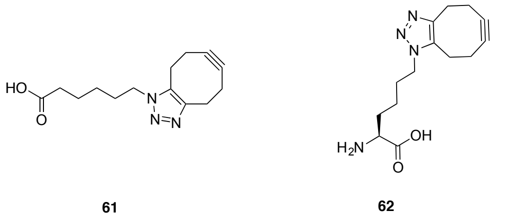
Scheme 7.12: Progress towards a second pyrimidine annulated cyclooctyne nucleoside derivative.



Reagents and conditions : a) i) *i*PrMgBr, THF ii) 5-hexenoyl chloride (**53**) iii) aq HCl, 69%; b) NaH (0 °C), allyl bromide (0 °C – r.t.), 70%; c) Hoveyda-Grubbs II, DCM (reflux), 62%; d) thio urea, NaOMe, MeOH (reflux), 46%; e) chloro acetic acid, dioxane/H_2O (2 : 1, v/v), 88%.

Towards **59** and **60** the current achievements (Scheme 7.12) includes the successful synthesis of uracil-annulated **58**. The synthesis started with the preparation of β-ketoester **54** via a described method. Deprotonation of ethyl malonate using isopropyl magnesium chloride directs C-acylation with acyl chloride **53**,[16] subsequent decarboxylation with aqueous HCl yielded **54** in good yields. Next, straightforward allylation using allyl bromide gave diene **55**. Ring closing metathesis mediated by Hoveyda-Grubbs II catalyst yielded **56** in 62% yield. To form the pyrimidine ring, thioureum and base were added to yield, under refluxing conditions, bicyclic **57** and upon desulfurization uracil derivative **58** was obtained. Future efforts would involve bromination of the alkene followed by elimination and subsequent nucleoside bond. Eventually **59** or **60** can be used for chemical or enzymatic introduction into nucleic acid oligomers as small ligation handles.

Figure 7.13: Functionalized triazole-annulated cyclooctynes.



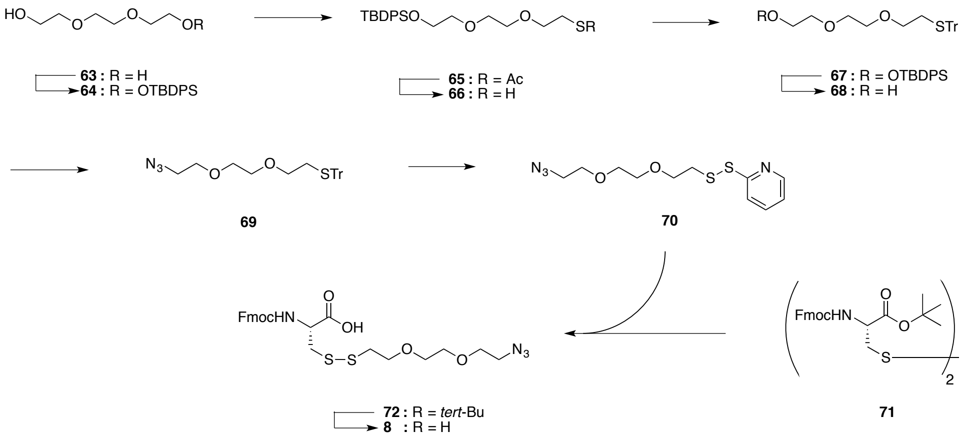
Additionally, based on the apparent applicability of triazole-annulated cyclooctynes in strain induced alkyne azide cycloadditions, other building blocks might also be considered. For instance carboxylic acid **61** (Figure 7.13), which can be coupled to any amine containing molecule or azido-lysine derivative **62,** which can be used to functionalize peptides and proteins.

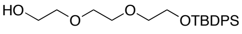
Experimental Section

General methods and materials

Chemicals were purchased from Acros Organics, Sigma Aldrich, Proligo and Jena Bioscience and used as received. Dichloromethane was distilled over CaH_2 and stored on 4 Å molecular sieves. DIPEA was distilled and stored on KOH pellets. Compounds used in reactions requiring anhydrous conditions were co-evaporated with 1,4-dioxane, pyridine or toluene three times. All reactions were performed at ambient temperature under an argon atmosphere unless stated otherwise. Oligonucleotides were synthesized on an ÄKTA Oligopilot Plus oligonucleotide synthesizer (GE Healthcare Life Sciences). Reactions were monitored by TLC on Kieselgel 60 F254 (Merck). Compounds were visualized by using UV light (254 nm) or applying a solution of $(NH_4)_6Mo_7O_{24}\cdot 4\ H_2O$ 25 g/L, $(NH_4)_4Ce(SO_4)_4\cdot 2\ H_2O$ 10 g/L, 10% H_2SO_4 in H_2O followed by charring (+/- 150 °C). LC/MS analysis was performed on a Jasco HPLC system (UV detection simultaneously at 214 and 254 nm) coupled to a PE/SCIEX API 165 single quadruple mass spectrometer (Perkin-Elmer). An analytical Gemini C_{18} column (Phenomex, 50 x 4.60 mm, 3 micron) was used in combination with eluents A: H_2O; B: MeCN and C: 0.1 M aq. NH_4OAc as the solvent system. Analytical anion-exchange was performed on a GE ÄKTAexplorer 10 using a Dionex DNA-PAC PA-200 4x250 mm column with eluents A: 500 mM NaOAc and 50 mM $NaClO_4$ and B: 500 mM NaOAc and 500 mM $NaClO_4$ using a linear gradient (0 - 20%). Anion exchange purification was performed on a GE ÄKTAexplorer 10 using a GE Q-Sepharose HR 26 x 10 column with eluents A: 500 mM NaOAc and 50 mM $NaClO_4$ and B: 500 mM NaOAc and 500 mM $NaClO_4$ followed by a desalting procedure using a Sephadex G25 column with 150 mM NH_4OAc as the solvent system. Preperative RP HPLC was performed on a Gilson GX-281 HPLC system. A semipreparative Altima C_{18} column (Phenomex, 250 x 10 mm, 5 micron) was used in combination with eluents A: 50 mM aq. NH_4OAc and B: MeCN as the solvent system. ^1H, ^{13}C and ^{31}P NMR were recorded on a Bruker AV-400 instrument. Chemical shifts (δ) of ^1H and ^{13}C spectra are relative to tetramethylsilane. ^{31}P chemical shifts are relative to phosphoric acid, reaction mixture aliquots measured by means of an acetone-d6 capillary. $CDCl_3$ was neutralized by filtration over neutral Al_2O_3 (Merck). HRMS spectra were recorded by direct injection (2 µL of a µM solution in H_2O or MeCN and 0.1% formic acid) on a Thermo Finnigan LTQ Orbitrap equipped with an electro spray ion source in positive mode. IR spectra were recorded on a Shimadzu FT-IR 8300 and are reported in cm^{-1}. The yields of the oligonucleotides were determined spectrophotometrically by Optical Density measurements on a Varian Cary 50 Bio UV-VIS Spectrophotometer at 260 nm.

Scheme 7.14 : Synthesis of cysteine building block **8**.

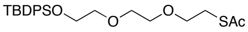




2-(2-(tert-butyl(2-ethoxy)diphenylsilane)ethoxy)ethanol (64).

To a stirred solution of polyethylene glycol (23.00 mL, 195 mmol) in pyridine (150ml) was added TBDPSCl (17.03 mL, 65 mmol, 0.33 eq) drop wise and stirred for 4hrs. The solvent was evaporated and the concentrated product was diluted with H_2O (100 mL) and extracted with EtOAc (3 x 150 mL). The combined organic layers where dried ($MgSO_4$) and concentrated under reduced pressure. The resulting oil was purified by silica column chromatography (petroleum ether / ethyl acetate, 10 : 0 → 4 : 6) that afforded the title compound **64** as oil (19.57 g, 50.36 mmol, 78%). $R_f = 0.2$ (petroleum ether / ethyl acetate, 8 : 2).

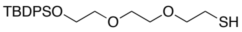
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68 (dd, 4H), 7.47 – 7.31 (m, 6H), 3.81 (t, *J* = 5.3 Hz, 2H), 3.72 (t, 2H), 3.68 – 3.63 (m, 4H), 3.63 – 3.58 (m, 4H), 2.48 (s, 1H), 1.05 (s, 9H).^{13}C NMR (101 MHz, $CDCl_3$) δ 135.7, 133.7, 129.8, 127.8, 72.6, 72.6, 70.9, 70.6, 63.5, 61.9, 26.9, 19.3. IR (neat) : 3426, 2931, 2858, 1472, 1428, 1105, 954, 822, 734, 701, 614, 504. HRMS calculated for $[C_{22}H_{32}O_4Si + Na]^+$: 411.1962 found: 411.1952 $[M + Na]^+$



2-(2-(tert-butyl(2-ethoxy)diphenylsilane)ethoxy)S-ethylthioate (65).

DIAD (11.11 mL, 56 mmol, 1.2 eq) and PPh_3 (13.48 g, 51.4 mmol, 1.1 eq) where dissolved in anhydrous toluene (50 mL) and stirred for 30 min at 0°C. A solution of PEG compound **64** (18.15 g, 46.7 mmol) and HSAc (4.0 mL, 56.0 mmol, 1.2 eq) in anhydrous toluene (100 mL) was pre cooled to 0°C. The solution of the PEG compound **64** was added dropwise to the stirred and cooled solution of the activated phosphine and stirred for 16 hrs at r.t. The solvent was evaporated under reduced pressure. The resulting slurry was taken up in toluene (50mL), filtrated and the solvent was removed under reduced pressure. The resulting oil was taken up in a small amount of toluene and purified by silica gel column chromatography (petroleum ether / ethyl acetate, 9.8 : 0.2 → 9.0 : 1.0) to afford the title compound **65** as an oil (19.00 g, 42.5 mmol, 91%). $R_f = 0.75$ (petroleum ether / ethyl acetate, 8 : 2).

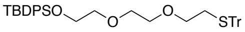
^{1}H NMR (400 MHz, CDCl3) δ 7.68 (dd, *J* = 7.8, 1.6 Hz, 4H), 7.45 – 7.34 (m, 6H), 3.81 (t, *J* = 5.3 Hz, 2H), 3.66 – 3.55 (m, 8H), 3.08 (t, *J* = 6.4 Hz, 2H), 2.32 (s, 3H), 1.05 (s, 9H).^{13}C NMR (101 MHz, CDCl3) δ 195.7, 135.7, 133.8, 129.7, 127.8, 72.6, 70.8, 70.6, 69.9, 63.6, 30.7, 29.0, 27.0, 19.3. IR (neat) : 2930, 2858, 1691, 1472, 1428, 1353, 1104, 951, 823, 738, 701, 614, 504. HRMS calculated for $[C_{24}H_{34}O_4SSi + Na]^+$: 469.1839 found: 469.1831$[M + Na]^+$



2-(2-(tert-butyl(2-ethoxy)diphenylsilane)ethoxy)ethylthiol (66).

Thioacetate **65** (19.00 g, 42.5 mmol) was dissolved in a solution of NaOH in MeOH (425 mL, 0.1M) and stirred for 4 hours. The solution was quenched by the addition of AcOH (5 mL, pH = 7). EtOAc (200 mL) and H_2O (200 mL) were added and the organic layer was removed. The aqueous layer was extracted with EtOAc (200 mL). The combined organic layers were washed with Brine (200 mL) and the organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The resulting oil was purified by means of silica gel column chromatography (petroleum ether / ethyl acetate, 9 : 1) to afford the title thiol **66** as an oil (12.2 g, 30.2 mmol, 71%). $R_f = 0.8$ (petroleum ether / ethyl acetate, 8 : 2).

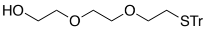
^{1}H NMR (400 MHz, CDCl3) δ 7.70 – 7.66 (m, 4H), 7.42 – 7.34 (m, 6H), 3.82 (t, *J* = 5.3 Hz, 2H), 3.68 – 3.64 (m, 2H), 3.64 – 3.58 (m, 6H), 2.68 (t, *J* = 8.2, 6.5 Hz, 2H), 1.57 (t, *J* = 8.2 Hz, 1H), 1.05 (s, 9H).^{13}C NMR (101 MHz, CDCl3) δ 135.7, 133.8, 129.8, 127.8, 73.1, 72.6, 70.8, 70.5, 63.6, 27.0, 24.4, 19.3. IR (neat) : 2930, 2859, 1473, 1427, 1354, 1108, 951, 823, 739, 700, 614, 504.HRMS calculated for [$C_{22}H_{32}O_3SSi$ + Na]$^+$: 427.1734 found: 427.1726[M + Na]$^+$



2-(2-(tert-butyl(2-ethoxy)diphenylsilane)ethoxy)ethyl(trityl)thiol (67).

Thiol **66** (283 mg, 0.7 mmol) was dissolved in DCM (3.5 mL) and TrCl (215 mg, 0.77mmol, 1.1 eq) was added. To the stirred solution TEA (300 µL, 1.55 mmol, 2.2 eq) was added. The reaction was stirred for 16 hrs. The solution was dissolved in EtOAc (10 mL) and Brine (10 mL) was added. The organic layer was separated, dried (MgSO$_4$) and concentrated under reduced pressure. The resulting oil was taken up in a small amount of toluene and purified by means of silica gel column chromatography (hexane / DCM, 8 : 2 → 2 : 8) to afford the title compound **67** as an oil (421 mg, 0.65 mmol, 93%). R$_f$ = 0.7 (hexane / DCM, 2 : 8).

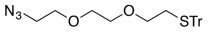
^{1}H NMR (400 MHz, CDCl$_3$) δ 7.68 (dd, *J* = 7.7, 1.6 Hz, 4H), 7.44 – 7.39 (m, 6H), 7.38 – 7.30 (m, 6H), 7.27 – 7.20 (m, 6H), 7.18 – 7.13 (m, 3H), 3.78 (t, *J* = 5.3 Hz, 2H), 3.58 – 3.51 (m, 4H), 3.41 (t, *J* = 5.7, 3.9 Hz, 2H), 3.30 (t, *J* = 7.0 Hz, 2H), 2.43 (t, *J* = 7.0 Hz, 2H), 1.05 (s, 9H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 144.9, 135.6, 133.7, 129.7, 127.9, 127.7, 126.7, 72.5, 70.6, 70.3, 69.7, 66.6, 63.5, 31.7, 26.9, 19.2. IR (neat) : 2930, 2857, 1488, 1472, 1444, 1428, 1361, 1111, 1034, 954, 823, 742, 700, 616, 532, 504. HRMS calculated for [$C_{41}H_{46}O_3SSi$ + Na]$^+$: 669.2829 found: 669.2822[M + Na]$^+$



2-(2-(-2-ethoxyethanol)ethoxy)ethyl(trityl)thiol (68).

To a stirred solution of **67** (18.17 g, 28.1 mmol) in THF (480 ml) was added drop wise a solution of TBAF (1M in THF, 60 mL, 2 eq). The reaction mixture was stirred for 16 hours after which the solvent was removed under reduced pressure. The resulting oil was taken up in a small amount of toluene and purified by means of silica gel column chromatography (petroleum ether / ethyl acetate, 9 : 1 → 5 : 5) to afford the title alcohol **68** as a crystalline white solid (10.79 g, 26.4 mmol).

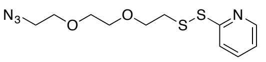
^{1}H NMR (400 MHz, CDCl$_3$) δ 7.42 (d, *J* = 7.7 Hz, 6H), 7.27 (t, *J* = 7.7 Hz, 6H), 7.20 (t, *J* = 7.2 Hz, 3H), 3.69 (s, 2H), 3.60 – 3.52 (m, 4H), 3.47 – 3.41 (m, 2H), 3.29 (t, *J* = 6.9 Hz, 2H), 2.47 – 2.34 (m, 3H).^{13}C NMR (101 MHz, CDCl$_3$) δ 144.9, 129.7, 128.0, 126.8, 72.6, 70.4, 70.3, 69.8, 66.8, 61.9, 31.7. IR (neat) : 3434, 3056, 2933, 2862, 1489, 1444, 1350, 1108, 1033, 887, 742, 697, 616, 504. HRMS calculated for [$C_{25}H_{28}O_3S$ + Na]$^+$: 431.1651 found: 431.1644 [M + Na]$^+$



2-(2-(-2-azidoethoxy)ethoxy)ethyl(trityl)thiol (69).

A biphasic mixture of sodiumazide (1.2 g, 18.45 mmol) in H_2O (3 mL) and toluene (15 mL) was stirred and cooled to 0°C. Sulfonic acid (1.0 mL, 6.15 mmol, 1 eq) was added drop wise and the mixture was stirred for 30 min**. Caution HN_3 is explosive, volatile and highly toxic!** The organic layer was removed and dried (Na_2SO_4) to obtain a solution of HN_3 in toluene (1.23 M). Compound **68** (4.90 g, 12mmol) was dissolved in toluene (120 mL) and stirred at 0°C. To the stirred and cooled solution was added PPh_3 (3.78 g, 14.4 mmol, 1.2 eq) and DEAD (≈40% in tol., 7.2 mL, 15.6 mmol, 1.3 eq) and stirred for 30 min. The prepared solution HN_3 (9.76 mL) was added and the reaction mixture was stirred for 16 hrs (0°C → 20°C). The solution was quenched with sat. aq. $NaHCO_3$ (200 mL). The organic layer was separated, dried (Na_2SO_4) and concentrated under reduced pressure. The resulting oil was taken up in toluene and was purified by means of silica gel column chromatography (toluene / acetone, 100 : 0 → 98 : 2) to afford the title compound **69** as an oil (4.30 g, 9.9 mmol, 83%).

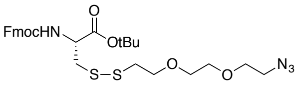
^1H NMR (400 MHz, $CDCl_3$) δ 7.42 (d, *J* = 7.8 Hz, 6H), 7.26 (t, *J* = 7.6 Hz, 6H), 7.19 (t, *J* = 7.2 Hz, 3H), 3.62 (t, *J* = 5.1 Hz, 2H), 3.56 (t, 2H), 3.45 (t, 2H), 3.37 – 3.27 (m, 4H), 2.43 (t, *J* = 6.9 Hz, 2H).^{13}C NMR (101 MHz, $CDCl_3$) δ 144.9, 129.7, 128.0, 126.7, 70.6, 70.3, 70.1, 69.8, 66.8, 50.7, 31.8. IR (neat) : 3057, 2932, 2866, 2102, 1489, 1444, 1354, 1184, 1112, 1033, 924, 886, 850, 742, 698, 676, 616, 506. HRMS calculated for $[C_{25}H_{27}N_3O_2S + Na]^+$: 456.1716 found: 456.1709 $[M + Na]^+$.



2-(2-(-2-azidoethoxy)ethoxy) ethyldisulfanylpyridine (70).

To a stirred and cooled solution of azide **69** (262 mg, 0.6 mmol) in EtOH (3 mL) was added commercially available 2,2-dithio-*bis*-pyridine (132 mg, 0.66 mmol, 1.1 eq). TFA (5 mL) was added dropwise and the reaxtion mixture was stirred for 16 hrs at r.t. The solution was quenched with sat. aq. $NaHCO_3$ (10 mL) and DCM (30 mL) was added. The organic layer was separated and the aqueous layer was extracted with DCM (3x 50 mL). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. The resulting oil was taken up in a small amount of toluene and purified by means of silica gel column chromatography (petroleum ether / ethyl acetate, 9: 1 → 6 : 4) to afford the title compound **70** as oil (156 mg, 0.52 mmol, 87%).

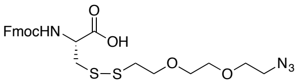
^1H NMR (400 MHz, $CDCl_3$) δ 8.40 (d, 1H), 7.72 (d, *J* = 8.1 Hz, 1H), 7.61 (td, *J* = 7.8, 1.8 Hz, 1H), 7.04 (ddd, *J* = 7.4, 4.8, 0.9 Hz, 1H), 3.69 (t, *J* = 6.3 Hz, 2H), 3.65 – 3.57 (m, 4H), 3.57 – 3.51 (m, 2H), 3.34 (t, 2H), 2.95 (t, *J* = 6.3 Hz, 2H).^{13}C NMR (101 MHz, $CDCl_3$) δ 160.3, 149.5, 137.1, 120.6, 119.6, 70.6, 70.4, 70.1, 69.0, 50.6, 38.4. IR (neat) : 3048, 2932, 2864, 2094, 1575, 1446, 1414, 1282, 1112, 1043, 986, 926, 851, 760, 718, 639, 618, 555, HRMS calculated for $[C_{11}H_{16}N_4O_2S_2 + H]^+$: 301.0787 found : 301.0788 $[M + Na]^+$ and calculated $[C_{11}H_{16}N_4O_2S_2 + Na]^+$: 323.0607 found: 323.0606 $[M + Na]^+$



***tert*-butyl *N*-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-*S*-((2-(2-(2-azidoethoxy)ethoxy)ethyl)thio)-*L*-cysteinate (72)**

(Fmoc-Cys-tBu ester)$_2$ disulfide bond (1195 mg, 1.5 mmol) was taken up in THF : H_2O (9 : 1, v/v, 50 mL) and treated with a solution of Me_3P in toluene (1 M, 7.5 mL, 7.5 mmol, 5 eq). The reaction mixture was stirred for 3 hours and quenched by the addition of H_2O (20 mL). The reaction mixture was dilluted with DCM (200 mL), the aqueous phase was separated and the organic phase was washed with brine (2 x 50 mL), dried ($MgSO_4$) and concentrated under reduced pressure. The crude thiol was co-evaporated twice using deoxygenated 1,4-dioxane and taken up in deoxygenated 1,4-dioxane (10 mL). Activated di-sulfide **70** (635 mg, 2.14 mmol, 2.14 mmol) in deoxygenated 1,4-dioxane (10 mL) was added and the mixture was stirred for 16 hours. The reaction mixture was concentrated under reduced pressure and the crude product was purified by means of silica gel column chromatography (petroleum ether / ethyl acetate, 9: 1 → 8 : 2) to afford the title compound **72** as an oil (1.3 g, 2.2 mmol, quant).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.61 (d, *J* = 7.6 Hz, 2H), 7.41 (d, *J* = 7.2 Hz, 2H), 7.38 – 7.31 (m, 4H), 5.72 (d, *J* = 7.6 Hz, 1H), 4.57 – 4.56 (m, 1H), 4.44 – 4.34 (m, 2H), 4.24 (t, *J* = 7.2 Hz, 1H), 3.72 (t, *J* = 6.4 Hz), 3.65 – 3.54 (m, 6H), 3.52 – 3.46 (m, 1H), 3.37 – 3.34 (m, 2H), 3.26 (dd, *J* = 14.0, 4.4 Hz, 1H), 3.14 (dd, *J* = 13.2, 6 Hz, 1H), 2.90 (t, *J* = 6.4 Hz, 2H), 1.49 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.0, 155.6, 143.8, 141.2, 127.7, 127.0, 125.1, 119.9, 82.9, 70.5, 70.4, 70.0, 69.5, 67.1, 54.2, 50.6, 47.1, 41.8, 38.6, 29.6, 28.0. IR (neat) cm^{-1} : 3329, 2978, 2926, 2866, 2099, 1717, 1510, 1450, 1244, 1223. HRMS calculated for $[C_{28}H_{36}N_4O_6S_2 + H]^+$: 589.21490 found: 589.21564 $[M+H]^+$.



***N*-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-*S*-((2-(2-(2-azidoethoxy)ethoxy) ethyl)thio)-*L*-cysteine (8)**

Tert-butyl ester **72** (1.06 mg, 2 mmol) was taken up in DCM/TFA (8 : 2, v / v, 22 mL) and stirred for 5 hours. Toluene (10 mL) was added and the reaction mixture was concentrated under reduced pressure to ~ 5 mL. Toluene (20 mL) was added and the mixture was concentrated under reduced pressure. The resulting oil was taken up in a small amount of DCM and purified by silica gel column chromatography (DCM / EtOAc / AcOH, 9: 1 : 0.1 → 75 : 25 : 0.1) to afford the title compound **8** as an oil (590 mg, 1.1 mmol, 55%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.50 (d, *J* = 4.0 Hz, 2H), 7.62 – 7.60 (m, 2H), 7.41 – 7.37 (m, 2H), 7.32 – 7.29 (m, 2H), 5.94 (d, *J* = 7.6 Hz, 1H), 4.70 (dd, *J* = 12.4, 6 Hz, 1H), 4.42 – 4.40 (m, 2H), 4.25 – 4.15 (m, 1H), 3.75 – 3.71 (m, 2H), 3.66 – 3.62 (m, 6H), 3.37 (t, *J* = 5.2, 2H), 3.27 (dd, *J* = 12.8, 4.4 Hz, 1H), 3.19 (dd, *J* = 14.0, 6.8 Hz, 1H), 2.92 – 2.89 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.1, 174.4, 155.9, 143.6, 141.2, 127.6, 127.0, 125.0, 124.8, 119.8, 71.6, 71.1, 70.4, 70.1, 69.9, 69.3, 67.2, 53.2, 50.5, 46.9, 40.4, 38.5, 20.7. IR (neat) cm^{-1} : 2916, 2887, 2860, 2100, 1717, 1520, 1449, 1254. HRMS calculated for $[C_{24}H_{28}N_4O_6S_2 + H]^+$: 533.15230 found: 533.15253 $[M+H]^+$.

Solid Phase Peptide Synthesis of epitope peptides 13 and 14

Solid phase peptide synthesis was performed on tentagel PHB resin (RAPP Polymer, 0.24 mmol/g, 208 mg, 50 µmol) using standard Fmoc protected amino acid building blocks (Nova Biochem, 0.25 mmol, 5 eq), HCTU as the activating agent and Fmoc cleavage as a final step after the incorporation of the before last amino acid residue. The resin was split in two equal batches of 25 µmol each which were treated with azido-disulfide Fmoc cysteine (**8**) or Fmoc-Cys(Tr)-OH (0.1 mmol, 4eq), HOBt (0.1 mmol, 4 eq) and DIC (0.1 mmol, 4 eq) in DMF (pre-activation 5 minutes) and shaken for 1 hour. The resin was washed with DMF (3 x 2 mL). Piperidine / DMF (1 : 4, v / v, 1 mL) deprotection was repeated twice (10 minutes). The resin was washed with DMF (3 x 2 mL) and DCM (3 x 2 mL). Final deprotection was performed using a solution of TFA/H_2O/TIS (95 : 2.5 : 2.5, v/v/v, 1.5 mL). After shaking for 90 minutes the resin was filtered and washed with TFA (3 x 1 mL). Concentration of the TFA solution yielded the crude peptides. RP-HPLC (C_{18}) purification (0.1% TFA in H_2O and MeCN as the solvent sytems) yielded the oligopeptides.

The same sequence of reactions was applied for epitopes **16** and **17** 50 µmol scale followed by 20 µmol final couplings.

H-CSIINFEKL-OH (13)

1.39 mg, 1.3 µmol, 5.2%, LC/MS: (MeCN : 0.1% TFA 10 → 90 v/v) retention time 5.13 min : **13** ($[M + H]^+$ = 1066.5), HRMS calculated for $[C_{48}H_{79}N_{11}O_{14}S_2 + H]^+$: 1066.56014 found: 1066.55908 $[M+H]^+$.

H-(N_3-diethyleneglycol-disulfide)CSIINFEKL-OH (14)

2.4 mg, 1.9 µmol, 7.6%, LC/MS: (MeCN : 0.1% TFA 10 → 90 v/v) retention time 5.71 min : **14** ($[M + H]^+$ = 1255.4), HRMS calculated for $[C_{54}H_{90}N_{14}O_{16}S_2 + H]^+$: 1255.61734 found: 1255.61853 $[M+H]^+$.

H-C Abu L Abu LVTFL-OH (16)

2.82 mg, 14%, LC/MS: (MeCN : 0.1% TFA 10 → 90 v/v) retention time 6.05 min : **16** ($[M + H]^+$ = 977.5)

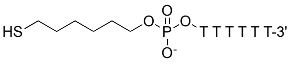
H-(N_3-diethyleneglycol-disulfide)CAbuLAbuLVTFL-OH (17)

10.4 mg, 44%, LC/MS: (MeCN : 0.1% TFA 10 → 90 v/v) retention time 6.5 min : **17** ($[M + H]^+$ = 1167.7)

PEGA-N_3 resin (21)

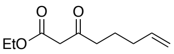
Poly[acryloyl-bis(aminopropyl)polyethylene glycol], PEGA-amine resin (Sigma-aldrich, 0.4 mmol/g loading, 0.25 g) was treated with 2-azido propionic-OSu ester **x** (127 mg, 0.6 mmol, 6 eq) in NMP (6 mL) and shaken o.n. The resin was was washed with NMP (4 mL) and DCM (2 x 4 mL).

IR (neat) : 2101 cm^{-1}



5’-thiol oligonucleotide (23)

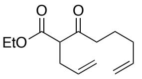
To a solution of 5’ dibenzocyclooctyne-disulfide containing oligonucleotide **18** (100 nmol) in aqueous TEAA (10 mM, 50 µL) was added a suspension of PEGA-N_3 in MeCN (40 µL, 100 µmolar eq/ mL) and the mixture was shaken for 3 hours. A second addition of **21** (20 µL) was added and the mixture was shaken for an additional 2 hours. LC-analysis of the liquid phase showed complete disappearance and the resin was washed with aqueous TEAA (20 mM, 100 µL) and H_2O (2 x 100 mL). The resin was treated with a solution of Dioxane : Me_3P (1 M) in THF (10 µL, 8 : 2, v / v), H_2O (25 µL) and aqueous TEAA (20 mM, 25 µL) and shaken for 90 minutes. The resin was centrifuged and the supernatant was removed, this was repeated three times washing the resin with H_2O (150 µL). The supernatants were combined and lyophilized. LC-analyis of the reconstituted solid revealed recovery of the title thiol **23** (UV, A_{260}), JASCO HPLC system using a ReproSil-Pur C_{18}-Aq (Dr. Maisch, 150 x 4.6 mm, 5 micron) in combination with eluents A: H_2O; B: MeCN and C 0.1 M aq. NH_4OAc, 0 → 50 v/v) retention time 9.6 min. (25 min. run).



Ethyl 3-oxooct-7-enoate (54)

Mono-ethylmalonate (0.94 mL, 8.0 mmol, 2 eq.) was dissolved in dry dichloromethane (3.0 mL). A solution of isopropylmagnesium bromide in dry tetrahydrofuran (22 mL, 1 M, 22 mmol, 5.5 eq) was added dropwise over 15 minutes. The reaction mixture was cooled to -15 ºC using an ice/salt bath and a solution of 5-hexenoic acid chloride (4.0 mmol) in dry dichloromethane was added dropwise. The reaction mixture was stirred for 3 hours at room temperature. The reaction mixture was quenched by the addition of aqueous HCl (10%) at 0 ºC. The mixture was transferred to a seporatory funnel and diluted with $CHCl_3$ (50 mL). The layers were separated and the organic layer was washed with sat. aq. $NaHCO_3$ (15 mL), H_2O (15 mL), Brine (15 mL), dried over $MgSO_4$, filtrated and concentrated *in vacuo*. Silicagel column chromatography (EtOAc/Petroleum ether, 2/98) yielded the title compound as a colorless oil (548 mg, 2.97 mmol, 74%).

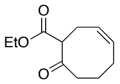
^1H NMR (400 MHz, $CDCl_3$) δ 5.89 – 5.65 (ddt, *J* = 17.0, 10.2, 6.7 Hz, 1H), 5.09 – 4.92 (m, 2H), 4.20 (q, *J* = 7.1 Hz, 2H), 3.43 (s, 2H), 2.55 (t, *J* = 7.3 Hz, 2H), 2.14 – 2.00 (m, 2H), 1.78 – 1.65 (m, 2H), 1.28 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.7, 167.2, 137.7, 115.4, 61.4, 49.4, 42.1, 32.8, 22.4, 14.1. IR (neat) cm^{-1}: 2934; 1741; 1718; 1642; 1368; 1313; 1236; 1031; 913. Calculated for $[C_{10}H_{16}O_3 + H]^+$: 185.11777; found: 185.11729 $[M + H]^+$



Ethyl 2-allyl-3-oxooct-7-enoate (55)

Compound **54** (1.73 g. 9.38 mmol) was dissolved in tetrahydrofuran and cooled to 0ºC. NaH (236 mg, 9.38 mmol, 1 eq.) was added and the mixture was stirred for 15 min. Allyl bromide (0.817 mL, 9.38 mmol, 1 eq.) was added and the mixture was allowed to warm to r.t. The mixture was stirred for 2 days, extra allyl bromide (0.050 mL, 0.57 mmol, 0.06 eq.) was added and the mixture was stirred for 2.5 h. The reaction mixture was diluted with EtOAc (100 mL) and washed with H_2O (50 mL) and brine (50 mL). The organic layer was dried over $MgSO_4$, filtrated, concentrated *in vacuo* and purified by means of silica gel column chromatography (EtOAc/hexane 0.5/99.5 – 10/90) yielding the title compound as a pale yellow oil (1.45 g, 6.51 mmol, 69%).

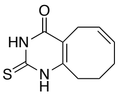
^{1}H NMR (400 MHz, $CDCl_3$) δ 5.82 – 5.65 (m, 2H), 5.15 – 4.91 (m, 4H), 4.18 (q, *J* = 7.1 Hz, 2H), 3.52 (t, *J* = 7.4 Hz, 1H), 2.69 – 2.42 (m, 4H), 2.05 (q, *J* = 7.3 Hz, 2H), 1.75 – 1.64 (m, 2H), 1.26 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 204.5, 169.2, 137.8, 134.3, 117.4, 115.3, 61.4, 58.5, 41.3, 32.2, 32.8, 22.4, 14.1. IR (neat) cm^{-1}: 3079; 2982; 2930; 1742; 1714; 1642; 1442; 1368; 1231; 1180; 998; 914. Calculated for $[C_{13}H_{20}O_3 + H]^+$: 225.14907; found: 225.14863 $[M + H]^+$



(Z)-ethyl 8-oxocyclooct-3-enecarboxylate (56)

Compound **55** (325 mg, 1.45 mmol) was coevaporated twice with 1,4-dioxane, dissolved in dichloromethane (145 mL) and exposed to ultrasound while argon was led trough the solution for 15 min. Hoveyda-Grubbs second generation catalyst (27 mg, 0.047 mmol, 0.03 eq.) was added. The mixture was stirred for 2 h at r.t. and refluxed for another 2 h untill full conversion of the starting material was observed by TLC. *tris*(2-carboxyethyl)phosphine (630 mg, 2.2 mmol, 1.5 eq) in dichloromethane (40 mL) was added with 4 mL triethylamine. The organic layer was washed with H_2O (4 x 50 mL), and 4 mL triethylamine was added to each new water layer. The water layers turned green. The organic phase was dried over $MgSO_4$, filtrated and concentrated in vacuo. silicagel column chromatography purification (dichloromethane/toluene 80/20) yielded compound **56** in 62% (176 mg) Rf 0.1 (dichloromethane/toluene 80/20)

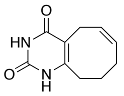
^{1}H NMR (400 MHz, $CDCl_3$) δ 5.85 – 5.63 (m, 2H), 4.30 – 4.07 (m, 2H), 3.47 (dd, *J* = 10.7, 4.5 Hz, 1H), 2.94 – 2.80 (m, 1H), 2.76 – 2.62 (m, 1H), 2.56 – 2.44 (m, 1H), 2.44 – 2.32 (m, 1H), 2.32 – 2.05 (m, 2H), 1.81 – 1.67 (m, 1H), 1.64 – 1.49 (m, 1H), 1.26 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 208.3, 169.3, 132.2, 127.7, 62.2, 61.1, 39.5, 26.3, 24.8, 24.7, 14.0.



5,6-(3-cycloocteno)-thiouracil (57)

Thiourea (45 mg, 0.59 mmol, 1.4 eq.) was coevaporated with dioxane and MeOH and a freshly prepared solution NaOEt (2.25 mL, 0.4M, 0.90 mmol, 2 eq.) in dry EtOH was added. Compound **56** (87 mg, 0.42 mmol) in 2.25 mL dry EtOH and molsieves were added, the reaction mixture was refluxed for 3.5 h (incomplete conversion), and extra NaOEt solution (0.50 mL, 0.4M, 0.20 mmol, 0.5 eq.) was added. The reaction mixture was refluxed for 1.5 h, diluted with EtOAc (10 mL), quenched with sat. aq. NH_4Cl (5 mL) and the layers were separated. The water layer was extracted with EtOAc (3 x 8 mL). The combined organic layers were dried over $MgSO_4$, filtrated and concentrated *in vacuo*. The residue was dissolved in 1mL H_2O and 120 mL MeOH and concentrated on silica and coevaporated with 1,4-dioxane three times. silicagel column chromatography (MeOH/DCM 0.25/99.75- 0.75/99.25) yielded compound **57** (40 mg, 19 mmol, 46%).

^{1}H NMR (400 MHz, DMSO-d6) δ 12.38 (s, 1H, NH), 12.15 (s, 1H), 5.70 – 5.59 (m, 1H), 5.53 – 5.43 (m, 1H), 3.12 (d, *J* = 4.3 Hz, 2H), 2.65 (t, *J* = 6.2 Hz, 2H), 2.05 (q, *J* = 7.5 Hz, 2H), 1.66 – 1.56 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d6) δ 174.2, 161.1, 151.5, 130.6, 127.2, 114.3, 28.3, 24.6, 24.1, 23.5.



5,6-(3-cycloocteno)-uracil (58)

Compound **57** (34 mg, 0.16 mmol) was dissolved in H_2O (3 mL) and dioxane (6mL). Chloroacetic acid (77 mg, 0.81 mmol, 5 eq.) was added and the mixture was stirred for 21 h. Nearly complete conversion was shown on TLC and an additional 40 mg of chloroacetic acid was added. The mixture was stirred for 1.5 h and diluted with H_2O (20 mL) and EtOAc (40 mL). The layers were separated and the aqueous phase was extracted with EtOAc (2 x 40 mL). The combined organic layers were dried over $MgSO_4$, filtrated and concentrated *in vacuo*. The crude product was purified by flash column chromatography (MeOH/DCM 0.5/99.5 – 3/97) to yield compound **58** (27 mg, 0.14 mmol, 86%).

^{1}H NMR (400 MHz, CD_3OD-d4) δ 7.72 (m, 1H), 7.62 (m, 1H), 5.71 (m, 1H), 5.59 – 5.47 (m, 1H), 3.21 (d, *J* = 5.1 Hz, 2H), 2.73 – 2.63 (m, 2H), 2.16 (q, *J* = 7.6 Hz, 2H), 1.77 – 1.64 (m, 2H).

References and notes:

(1) Zom, G. G. P.; Khan, S.; Filippov, D. V; Ossendorp, F.; Melief, C. J. M. *Synthetic Vaccines*, **2012**, 114, 177–201.

(2) Khan, S.; Bijker, M. S.; Weterings, J. J.; Tanke, H. J.; Adema, G. J.; Van Hall, T.; Drijfhout, J. W.; Melief, C. J. M.; Overkleeft, H. S.; Van der Marel, G. A.; Filippov, D. V; Van der Burg, S. H.; Ossendorp, F. *J. Biol. Chem.* **2007**, *282*, 21145–59.

(3) Schepers, K.; Toebes, M.; Sotthewes, G.; Vyth-Dreese, F. A.; Dellemijn, T. A. M.; Melief, C. J. M.; Ossendorp, F.; Schumacher, T. N. M. *J. Immun.* **2002**, *169*, 3191–9.

(4) . The additional satalites in the LC trace of **23** are attributed to the lyophilisation and reconstitution. When **23** was injected on the HPLC directly after the reductive cleavage, these small satelites were not observed.

(5) Aslam, T.; Fuchs, M. G. G.; Le Formal, A.; Wightman, R. H. *Tetrahedron Lett.* **2005**, *46*, 3249–3252.

(6) Calzada, E.; Clarke, C. a.; Roussin-Bouchard, C.; Wightman, R. H. *J. Chem. Soc. Perkin Trans. 1* **1995**, 517–518.

(7) Sharma, G. V. M.; Subash Chander, A.; Krishnudu, K.; Radha Krishna, P. *Tetrahedron Lett.* **1997**, *38*, 9051–9054.

(8) Larsen, C. H.; Ridgway, B. H.; Shaw, J. T.; Smith, D. M.; Woerpel, K. A. *J. Am. Chem. Soc.* **2005**, *127*, 10879–84.

(9) Smith, D. M.; Woerpel, K. A. *Org. Lett.* **2004**, *6*, 2063–2066.

(10) Larsen, C. H.; Ridgway, B. H.; Shaw, J. T.; Woerpel, K. A. *J. Am. Chem. Soc.* **1999**, *18*, 12208–12209.

(11) Pirrung, M. C.; Webster, N. J. G. *J. Org. Chem.* **1987**, *52*, 3603–3613.

(12) Kawashima, E.; Umabe, K.; Sekine, T. *J. Org. Chem.* **2002**, *67*, 5142–5151.

(13) Hoffer, M. *Chem. Ber.* **1960**, *93*, 2777–2781.

(14) Schiesser, S.; Hackner, B.; Pfaffeneder, T.; Müller, M.; Hagemeier, C.; Truss, M.; Carell, T. *Angew. Chem. Int. Ed.* **2012**, *51*, 6516–20.

(15) Shen, Q.; Huang, W.; Wang, J.; Zhou, X. *Org. Lett.* **2007**, *9*, 4491–4.

(16) Ahrendt, K. a; Williams, R. M. *Org. Lett.* **2004**, *6*, 4539–4541.

* Contributions to the experimental part of this chapter were made by E. van Schie and W. de Witte