Synthesis of chemical tools to study the immune system

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Chapter 3

Conditionally controlled intracellular TLR-ligands[1]

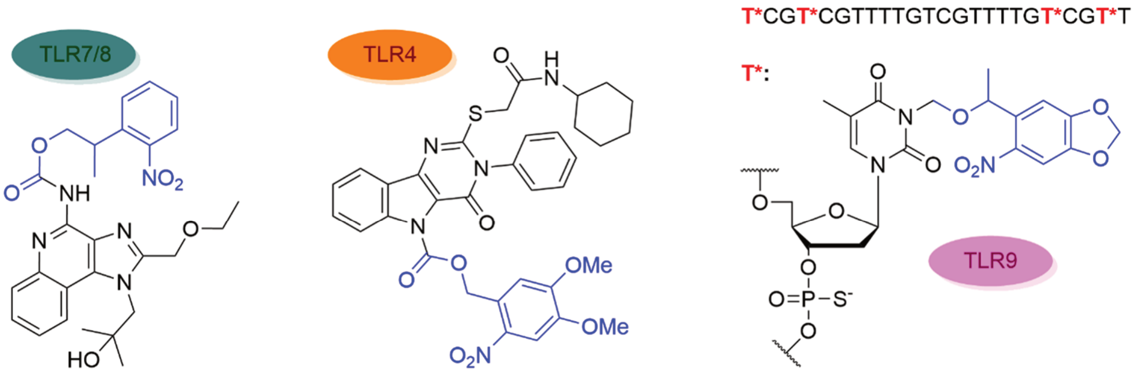
Introduction

Chapter 2 introduced the concept of bioorthogonally protected TLR ligands and revealed that a trans-cyclooctene protecting group gave control over TLR2/6 receptor dimer-mediated signaling. The aim of the research described in Chapter 2 was to make use of caged TLR2/6 ligands as tools to reveal where intracellular TLR2/6 signaling occurred, and whether TLR2/6 dimers located at the cell surface provided a pro-inflammatory signal different from that following intracellular activation. TLR2/6 is not the only receptor for which such a signaling duality has been postulated.[2] TLR4, for example, has been shown to induce NF-κB when activated at the cell surface, yet signals in an IRF-dependent manner when ligated from within the endosome.[3]

One sub-family of TLRs resides exclusively in endosomes and lysosomes. This family, encompassing TLR3, TLR7, TLR8, and TLR9, bind nucleic acids (NAs) of non-host origin. TLR3 recognizes double-stranded RNA (dsRNA)[4], TLR7 and 8 recognize single-stranded RNA (ssRNA)[5,6] and TLR9 recognizes DNA with recurring motifs of unmethylated cytosine-phosphodiester-guanine (CpG) in the 5'→3' direction.[7] For this purely endo-lysosomal family of receptors a signaling duality has also been hypothesized: namely that signaling from early endosomes and late endosomes/lysosomes is different.[8] Indeed, evidence exists that TLR9 signaling from the late endosomal compartment leads to NF-KB activation, whereas its signaling from earlier endosomal compartments induces IRF-signaling.[9,10] For TLR3, TLR7, and TLR8, no evidence supporting the existence of signaling duality has yet been reported.

Furthermore, it remains to be elucidated *how* this dichotomous TLR9 signaling arises: whether the NF-KB-activating signaling cascade occurs only when an unligated TLR9-receptor encounters its ligand in the late endosome/lysosome, or whether the signaling adapters associated with the TLR9 simply change during the maturation from an early endosome to a late endosome when the receptor encountered its ligand in this early endosome.

It was envisaged that conditional ligands could be used to probe this compartment-specific behavior. Such tools would be useful to unravel TLR9 signaling, but also to determine whether other endosomal TLRs displayed this dichotomous signaling. In an attempt to address these questions, the Esser-Kahn group has published a photocaged variant of the TLR 7/8-ligand Resiquimod (also 'R848'), namely, NPPOC-Resiquimod (Figure 1 – the photolabile groups in the caged TLR ligands depicted in Figure 1 are highlighted in blue).[11]



NPPOC-Resiquimod NVOC-pyrimido[5,4-b]indole NPOM-CpG

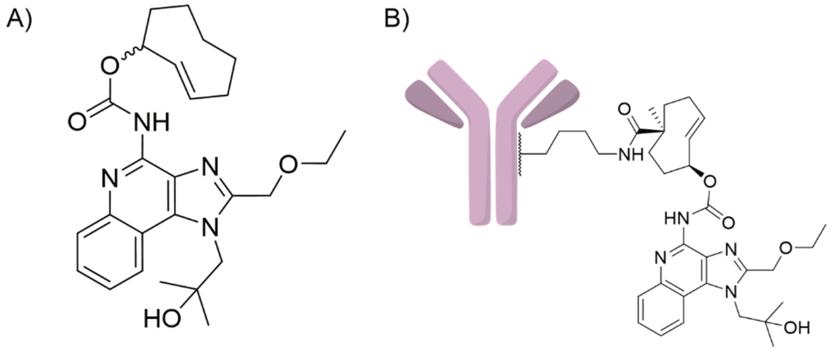
Figure 1. Previously reported conditional ligands for intracellular TLRs: a resiquimod derivative that has a 2-(2-nitrophenyl)-propoxycarbonyl (NPPOC) cage conditionally controls TLR7/8 activity,[11] a pyrimido[5,4-b]indole derivative caged with N-veratryloxycarbonyl (NVOC) conditionally controls TLR4 activity,[12] and a CpG sequence carrying four 6-nitropiperonyloxymethyl (NPOM)-modified T residues (indicated in red).[13]

Control over TLR7/8 activity can be achieved upon UV-irradiation of model cell lines and of primary dendritic cells pretreated with NPPOC-Resiquimod. The same laboratory also produced a photocaged variant of a TLR4 ligand, NVOC-pyrimido[5,4-b]indole (Figure 1).[12] The group of Deiters reported a photocaged TLR9 ligand, 24-meric CpG carrying four NPOM-groups (Figure 1).[13] Treatment of TLR9-expressing HEK293T cells with NPOM-CpG followed by UV-irradiation showed that TLR9 activity could also be controlled with light. A modest three-fold induction of TLR9 activity was observed after irradiation with 360 nm light. This is possibly due to multiple thymidines requiring caging to abolish the binding to TLR9.

The photo-deprotection experiments with the three ligands shown in Figure 1 were the first examples of the use of conditional ligands to study pattern recognition receptors. However, the experimental methods described in these papers are limited in their use for distinguishing between different endosomal compartments. The low spatial resolution of the UV-source (>7 mm^2) and the migration of cells during the 10-minute irradiation window prevents this.[11]

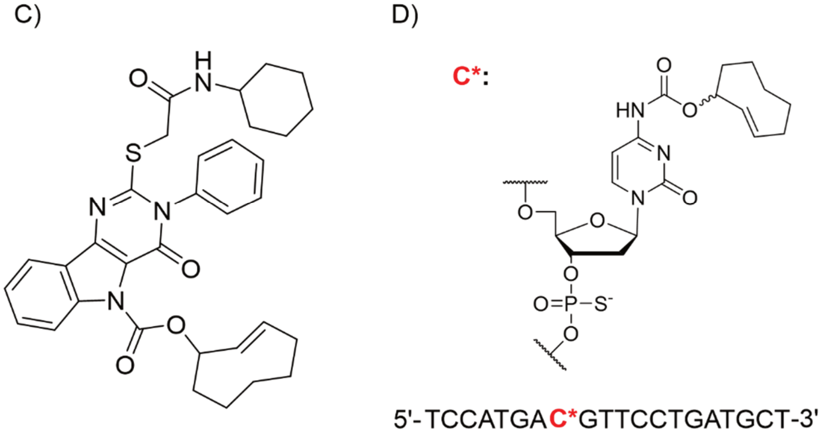
In order to achieve sub-cellular activation of TLR-ligands, it was envisaged that a chemical, rather than a photochemical, deprotection strategy would be of use. Recently, tetrazines have been reported that were targeted to specific organelles and were used for a sub-cellular ligation reaction.[14] Applying such a strategy to the study of TLR-ligands was envisaged to offer a solution for unraveling the potential signaling dichotomy of the TLRs that are present in more than one sub-cellular location: if deprotection could be limited to, for example, an early endosome, the contribution of signaling from this compartment could be isolated.

In this Chapter, the syntheses of trans-cyclooctene-protected ligands for TLR4, the TLR 7/8 combination and TLR9 are described (Figure 2). The produced reagents can be combined with the tetrazine-mediated uncaging strategy described in Chapter 2.



Tetrazine-sensitive TLR7/8 ligand

Releasable ADC with R848 payload



Tetrazine-sensitive TLR4 ligand

Tetrazine-sensitive TLR9 ligand

Figure 2. TCO-caged TLR ligands subject of the studies described in this Chapter. A) R848 caged with a TCO on its exocyclic amine. B) An antibody-drug-conjugate (ADC) bearing an R848 payload through a tetrazine-sensitive immolative linker. C) A pyrimido[5,4-b]indole-based synthetic TLR4 ligand caged with a TCO on the endocyclic amine of the indole. D) A 20-meric oligodeoxynucleotide bearing a single CpG motif, of which its deoxycytidine is caged with a TCO on the exocyclic amine.

Results and Discussion

Towards a chemo-controllable TLR9-agonist

The synthesis of a tetrazine-controllable TLR9 ligand was undertaken first. It was envisaged to first introduce a TCO-moiety on the exocyclic amine of a cytidine building block and then use this reagent in a phosphoramidite/DMTr on-resin oligonucleotide synthesis strategy.[15] This strategy was based on the observation that the cytidine in the singly-present CpG-motif (Figure 2, C*) is engaged in both strong van der Waals interactions and hydrogen bonding as revealed by the co-crystal structure of ODN1668 (a CpG class B ODN with phosphorothioate linkages) in complex with the extracellular domain of bovine TLR9.[16] It was postulated, based on the above interactions, that protection of the exocyclic amine on one of the cytidines of the TLR9-binding 12-mer fragment of ODN1668 (Scheme 2, compound **11**) would yield a compound that does not bind TLR9.

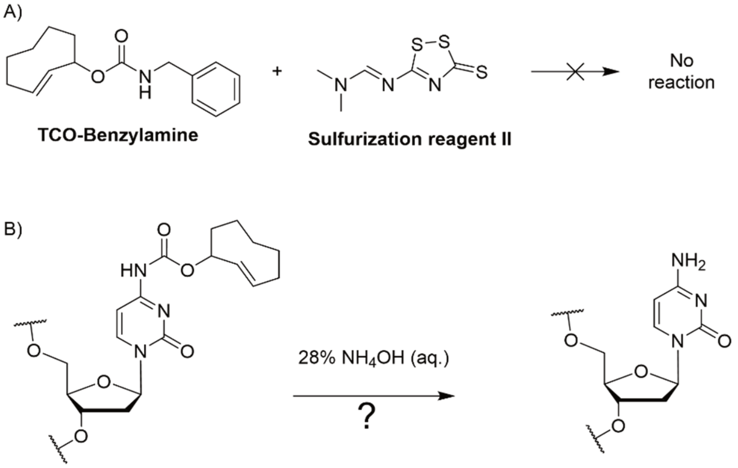
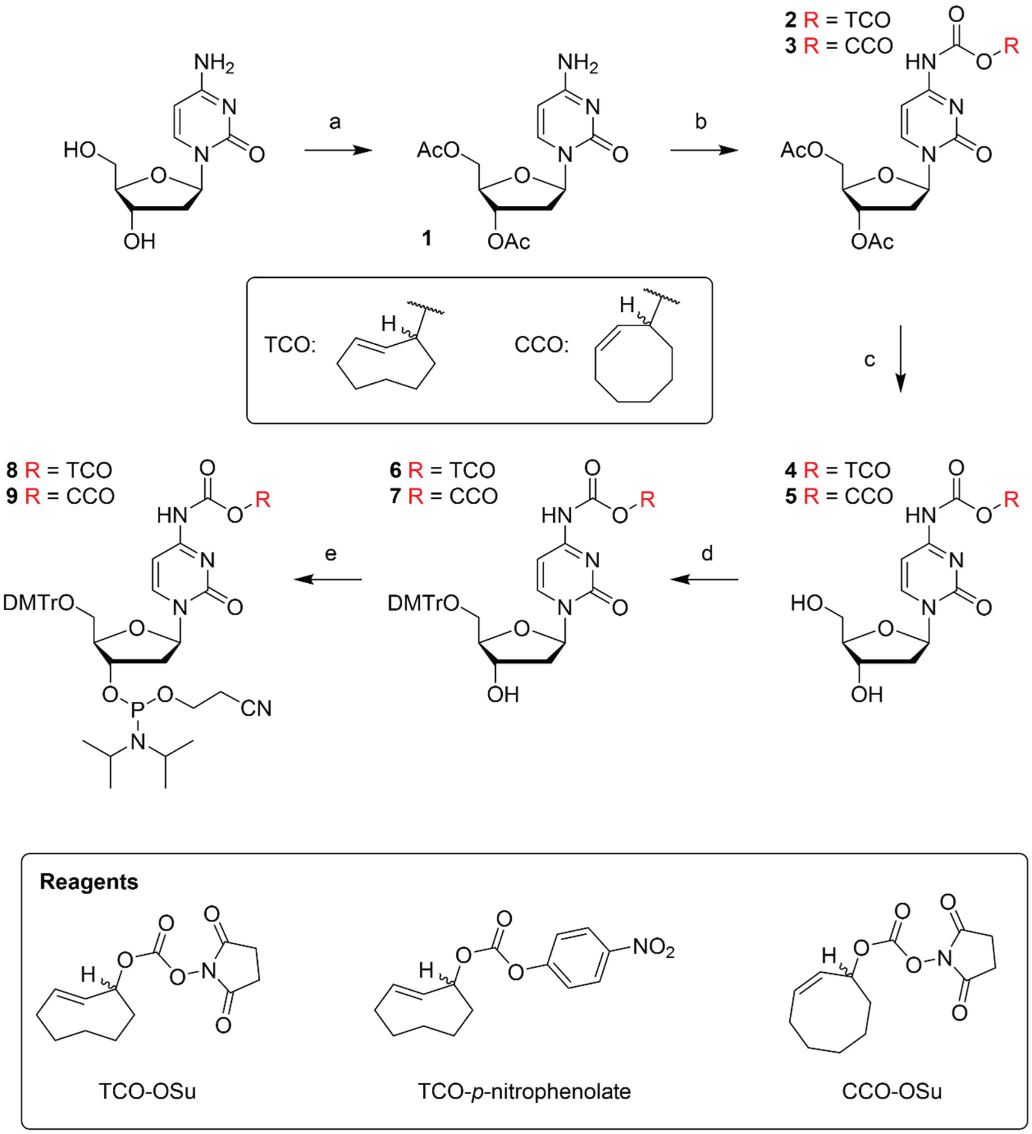


Figure 3. Potential hurdles during ODN synthesis using a TCO-caged deoxycytidine. A) Sulfurization reagents needed to oxidize the phosphites to phosphorothioates may react with the trans-configured olefin, though this was not observed when exposing TCO-benzylamine to this reagent. B) Hydrolysis of the TCO may occur during global deprotection to a significant extent.

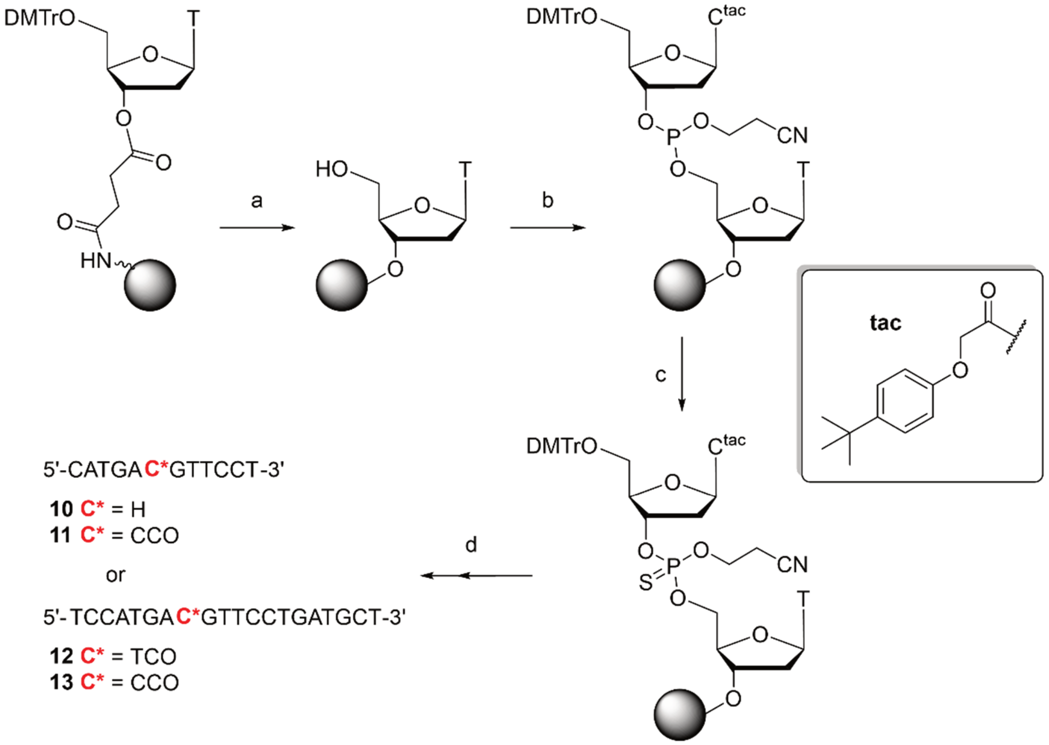
ODN1668 contains phosphorothioate esters for increased biological stability.[15] Synthesis of the TCO-protected variant **12** (Scheme 2) requires the TCO-caged cytidine to be stable to repeated cycles of sulfurization and acidic detritylation, as well as to concentrated aqueous ammonium hydroxide used in the final global deprotection step. To first assess whether this is the case, the model TCO-benzylamine was exposed to sulfurization reagent II (Figure 3A). 30-minute exposure of TCO-benzylamine to a 10-fold excess of sulfurization reagent II did not yield any degradation products and TCO-benzylamine could be recovered in >99% yield after work-up and purification.



Scheme 1. Synthesis of trans- and cis-cyclooctene protected building blocks for solid phase DNA synthesis starting from deoxycytidine. Reagents and conditions: **a)** 30% Acetyl chloride in CHCl$_3$, acetic acid, 40°C, o.n., qt.; **b)** Compound **2**: TCO-p-nitrophenolate, DiPEA, DMAP, acetonitrile, 40°C, 7 days, 90%; compound **3**: CCO-p-nitrophenolate, DiPEA, DMAP, acetonitrile, 40°C, 7 days, 84%; **c)** K$_2$CO$_3$, MeOH, r.t., 2 hrs, qt.; **d)** DMTrCl, pyridine, r.t., o.n., 77% (Compound **6**), 72% (Compound **7**); **e)** 2-cyanoethyl N,N-diisopropylchlorophosphoramidite, DiPEA, DCM, 0°C → r.t., 15 min, 71% (compound **8**), 88% (compound **9**).

Next, the synthesis of the phosphoramidite/DMTr-protected TCO-cytidine **8** was attempted. First the 3'- and 5'-hydroxyl-groups on the deoxycytidine were protected as acetates (reaction **a**, Scheme 1). Next the reaction between TCO-OSu and CCO-OSu and the exocyclic amine on the nucleobase (**1**) was attempted using various bases (DiPEA, DBU, LiHMDS) as catalysts. All failed to yield the desired product. When *p*-nitrophenolate-activated TCO carbonate was used as a TCO-donor, in presence of a catalytic amount of DMAP the reaction proceeded to yield **3** in 90% after 7 days at 40°C. H-NMR spectroscopy showed that no trans-to-cis conversion of the TCO-group had taken place during this time. Deacetylation was achieved using potassium carbonate in methanol. This resulted in the formation of product **4** in quantitative yield. Interestingly, quenching of the potassium carbonate with Amberlite H^+ prior to evaporation resulted in a loss of product. The mixture was therefore concentrated without quenching, which prevented the loss of product and did not result in hydrolysis of the carbamate.

Before proceeding with the synthesis of **8**, the stability of the TCO-carbamate to 28% NH_4OH (aq.) was assessed, as these are the conditions used in the final deprotection during solid phase oligonucleotide synthesis (Figure 3). Compound **4** was incubated for 60 minutes at room temperature, followed by concentration at 45°C. This led to partial hydrolysis (10%) of the TCO, as determined by LC-MS. It was therefore decided to employ 4-tert-butylphenoxyacetyl-protected (tac, scheme 2) nucleotide building blocks during ODN synthesis, known for their fast deprotection in comparison to standard benzoyl/isobutyl/acetyl-protected building blocks, to limit the hydro- and aminolysis of the TCO during NH_4OH (aq.) treatment.[17] Exposure to the shorter deprotection conditions required when using these protecting groups did not result in hydrolysis of **4**. Introduction of the DMTr-protecting group onto the 5-position of **4**, followed by phosphitylation proceeded to yield **8** in 41% over 5 steps. The CCO-modified control compound **9** was synthesized in the same manner in a 47% yield over 5 steps.



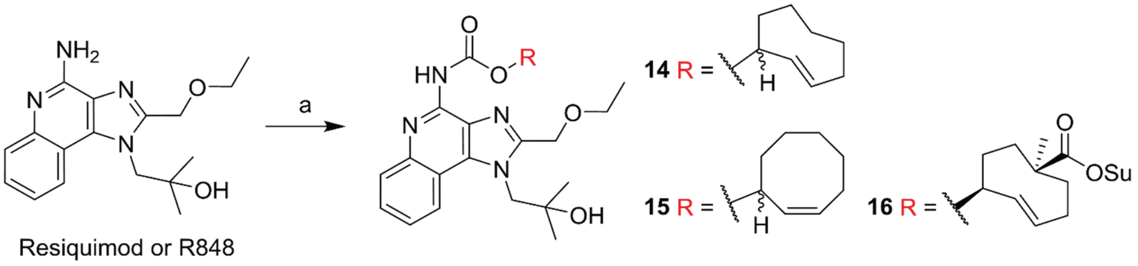
Scheme 2. Synthesis of cyclooctene-caged ODN1668 12- and 20-mer derivatives. Reagents and conditions: **a)** 5% dichloroacetic acid in toluene, r.t., 3 min (1x); **b)** (i) DMTr-dC^{tac}-PAM (3 eq.), r.t., acetonitrile, 3 min (2x) (ii) 4-t-butylphenoxyacetyl anhydride, acetonitrile, r.t., 30 sec (1x); **c)** Sulfurization reagent II (10 eq.), acetonitrile, r.t., 1 min (1x); **d)** Repeat steps **a-c** with the respective deoxynucleoside phosphoramidite building blocks: DMTr-dG^{tac}-PAM, DMTr-dT-PAM, DMTr-dA^{tac}-PAM. At position **c***, either building block **8** or **9** was used as the deoxynucleoside phosphoramidite.

Building block **9** was next used in the synthesis of a the TCO-protected 12-mer oligonucleotide **11**.[16] The parent unprotected motif **10** was also synthesized (Scheme **2**). The oligonucleotides were subsequently cleaved from the resin, purified and desalted as reported.[15] MALDI-analysis confirmed the molecular weight of the products.

Next, the ability of **10** and **11** to activate TLR9 was assessed on murine bone marrow-derived dendritic cells (BMDCs). Neither the protected nor unprotected 12-mers induced cytokine expression in these cells. This could be due to the lower levels of TLR9 (and its trafficking adapter Unc93B1) expression in BMDCs, compared to the transfected HEK293T-cell lines used in the aforementioned publication that shows the activation of TLR9 by non-caged 12-mer **10**.[16] In an attempt to achieve a higher NF-kB activity in BMDCs, the full length 20-mer CpG was synthesized with either a TCO (**12**) or CCO (**13**) on the deoxycytidine indicated in red in Scheme **2**. These compounds are currently awaiting their biological evaluation.

A Chemo-controllable TLR7/8-Agonist

Next, the synthesis of the TCO-modified TLR7/8 ligand Resiquimod was attempted (Scheme **3**). Previous studies using the *O*-nitrobenzyl-protected variant of the same ligand (NPPOC-Resiquimod, Figure **1**) had shown the that modification of the exocyclic amine could block TLR7/8 ligation.[11]



Scheme 3. Synthesis of trans- and cis-cyclooctene protected resiquimod. Reagents and conditions: **a**) Compound **14**: TCO-OSu, DiPEA, 1:1 DMF:DCM, r.t., 5 days, 43%; Compound **15**: CCO-OSu, DiPEA, 1:1 DMF:DCM, r.t., 5 days, 71%; Compound **16**: $TCO-(OSu)_2$, DiPEA, 1:2 DMF:DCM, r.t., 10 days, 24%

14 Was synthesized by reacting Resiquimod with TCO-OSu to yield the TCO-carbamate **14** (Scheme **3**) in 14% yield after 5 days reaction time. The CCO-carbamate-modified control compound **15** was made in the same manner in a 71% yield. Resiquimod modified with a more soluble bifunctional TCO-carbamate (**16**) was made in a 24% yield. The ability of **14** (R848-TCO) and **15** (R848-CCO) to activate TLR7 and/or TLR8 was assessed by quantifying their ability to induce TNF-a and IL-6 production in murine BMDMs (Figure **4**).

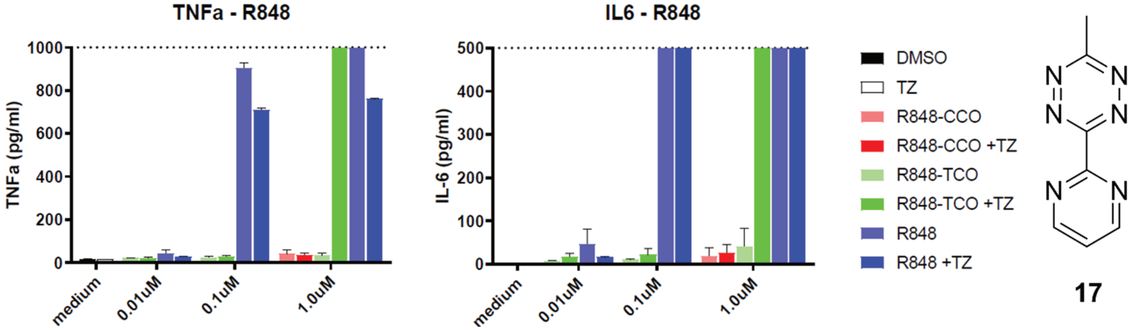


Figure 4. Biological evaluation of compounds 14 and 15: R848-TCO and R848-CCO, respectively. TNF-α and interleukin 6 (IL6) concentrations were measured in medium containing murine bone marrow-derived macrophages (BMDMs). R848-TCO only elicits a response when co-administering tetrazine 17 at 1 μM, whereas R848-CCO is irresponsive at the same concentration.

14 Did not induce cytokine production at 0.01-1 μM (light green bars). Upon addition of tetrazine 17 (Figure 4), activity was restored to a 1 μM ligand concentration for both secretion of TNF-α and IL6. Future work includes testing this ligand at >1 μM concentrations in conjunction with tetrazines directed to either the extracellular environment or the endo-lysosomal pathway to selectively activate the populations of TLR7/8 that are residing in various locations in or on the cell.

Synthesis of R848-TCO Conjugates

TLR-ligands have been suggested as chemotherapeutics against numerous cancers by inducing a robust immune response against tumor-associated antigens. Resiquimod is clinically approved for the treatment of cutaneous T cell lymphoma as well as herpes-derived skin lesions. These treatments are, however, all limited by to topical application as systemic application of TLR ligands can induce cytokine storms and carry the potential to induce septic shock.[18] It was therefore envisaged that the second OSu-functionality present in 16 (Scheme 3) could be used to conjugate the ligand to an antibody to produce an antibody-drug conjugate (ADC). The ligand's activity would be blocked by the TCO, and 'released' *in vivo* after targeting it to a tumour via the antibody.[19–22]

In order produce this R848-based ADC, the protocol described by Rossin *et al.*[23] for modifying antibodies with TCO-modified chemotherapeutics TCO-OSu was used to modify the model protein, egg white protein ovalbumin (OVA). OVA was reacted with compound 16 (Figure 5) for 2 hours in a mixture of PBS, propyleneglycol, and DMF (7:2:1). After size-based separation over a 30 kDa cutoff filter ($m_{ovalbumin}$ = 42.7 kDa), the product was analyzed using a fluorogenic BODIPY-labeled tetrazine (synthesized in-house by Alexi Sarris).[24] A single band in the BODIPY channel was observed indicating successful TCO-conjugation, although the modification yield remains to be determined (Figure 5A).

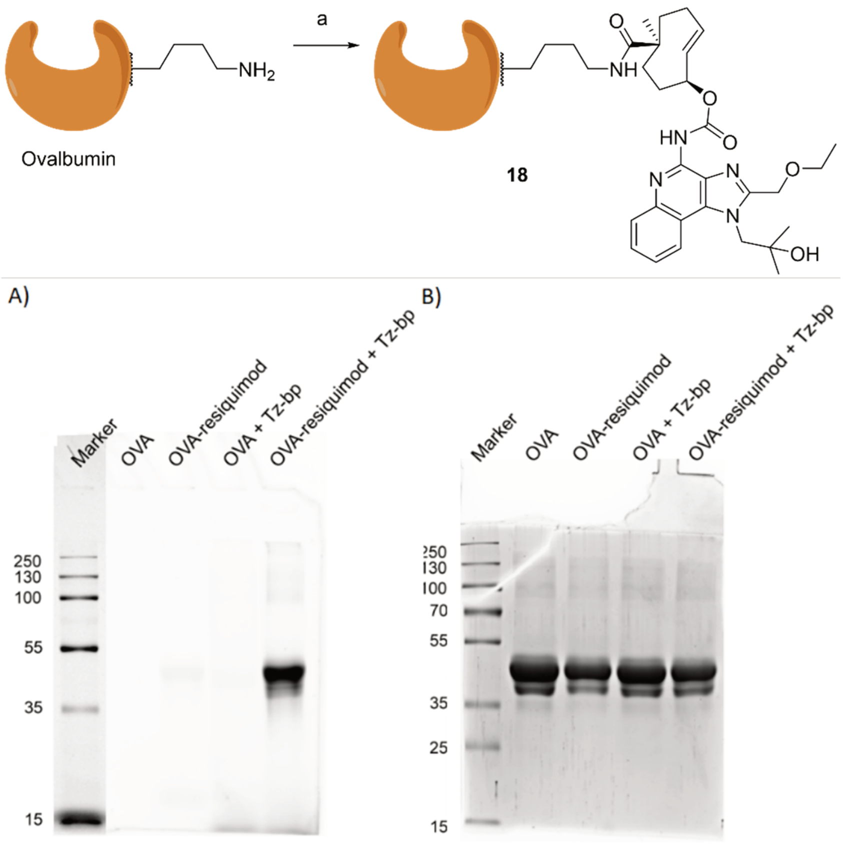
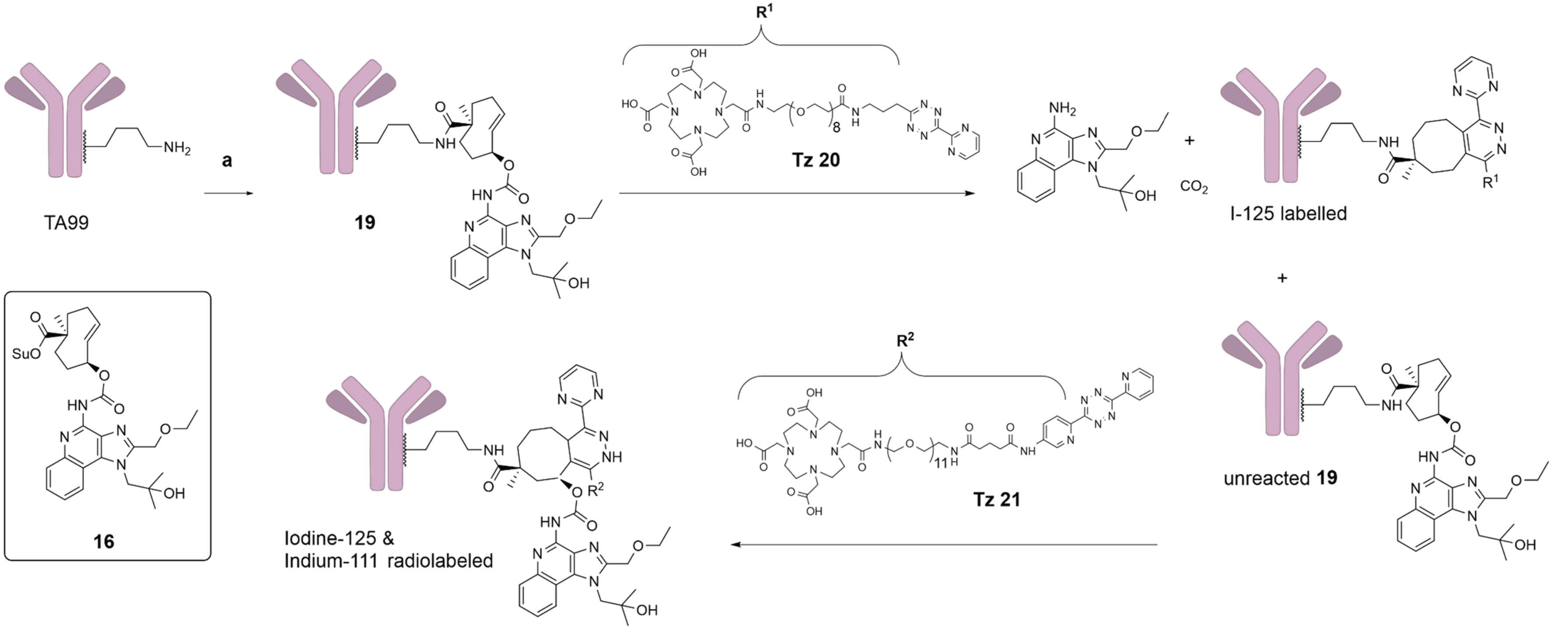


Figure 5. Conjugation of ovalbumin to R848 through a bis-functionalized TCO linker. SDS-PAGE analysis of caged ovalbumin (OVA) was used to verify conjugation as well as trans-configuration of the TCO. A) Fluorescence in the BODIPY channel. Only OVA-TCO-R848 gives rise to a signal when treated with a BODIPY-tetrazine (Tz-bp), indicating product formation. B) Coomassie staining indicating near-equal loading of proteins in each lane. Reagents and conditions: **a**) Compound **16**, 1M Na_2CO_3 - (aq.), 7:2:1 PBS:propylene glycol:DMF (v:v:v).

With these conditions in hand, the conjugation of **16** to the tumor-targeting antibody TA99 was attempted.[25] This monoclonal antibody binds tyrosinase-related protein 1 (TYPR1) that is often upregulated in melanoma cells.[26] The conjugation procedure yielded conjugate **19** after purification using a PD-10 column (Scheme **4**, reaction a). Release of Resiquimod from conjugate **19** was assessed in an *in vitro* experiment, in which THP1-Dual cells were treated with the conjugate either in the presence or absence of tetrazine **20** (Figure **6**A). Pure Resiquimod was used as a positive control, and PBS buffer as a negative control. After overnight incubation the production of secreted embryonic alkaline phosphatase (SEAP) was quantified as a measure of NF-κB activity (Figure **6**). Activity of Resiquimod was fully recovered upon co-incubation with tetrazine.



Scheme 4. Conjugation of antibody TA99 with **16** and consequent release with tetrazine (Tz) **20**. Tz **20** is able to both click and release R848. Tz **21** is first radiolabeled with Indium-111 and, due to the two pyridine substituents, clicks very efficiently but releases R848 poorly. Thus Tz **20** is used release R848 from the ADC and Tz **21** is used to quantify unreacted ADC using radiolabelling. Reagents and condition: **a**) TA99 in PBS (5 mg/mL), compound **16** (10 mM in DMF) in 7:2:1 PBS:propylene glycol:DMF at pH=9 as adjusted with 1M Na_2CO_3, 2h, RT.

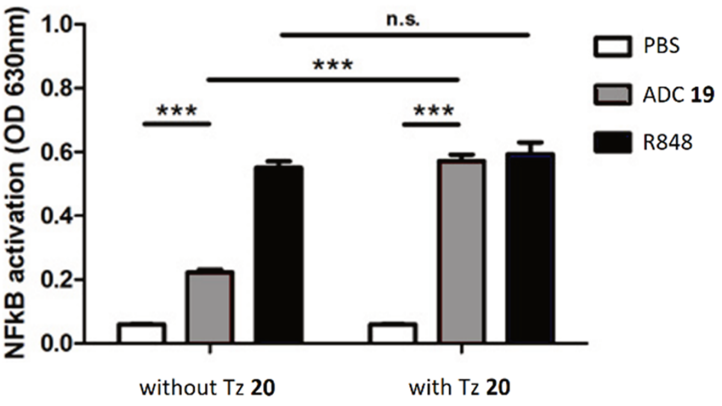


Figure 6. THP1-Dual cells were treated with antibody-drug conjugate (ADC) 19 either in the presence or absence of Tz activator 20. PBS buffer was used as a negative control and free R848 agonist as a positive control. After overnight incubation NF-κB activity was measured through quantification of SEAP production. Experiment was carried out by Raffaella Rossin (Tagworks Pharmaceuticals).

Behavior of conjugate 19 was next assessed *in vivo* by the company Tagworks. Native TA99 and conjugate 19 were radiolabeled through acylation with radio-iodinated N-succinimidyl-3[4-hydroxyphenyl]propionate (Bolton-Hunter method).[27] One group (n=4) of female C57BL/6 mice bearing a subcutaneous B16-F10 melanoma was injected with the radiolabelled native TA99 (5 mg/kg, 0.3 MBq/mouse) and euthanized 48h post-mAb injection (Figure 7A). Three more groups (n=4) of mice were treated with radiolabelled conjugate 19, of which one group was euthanized 48h post-mAb injection, and two groups were injected with a clearing agent (CA, Figure 7C) 48h post-mAb injection. This clearing agent, a conjugate of tetrazine with galactose-albumin, is known to increase the tumour-to-blood ratio of TCO-bearing antibodies by reacting only with freely-circulating antibodies.[28] Two hours after CA injection one group was injected with activating tetrazine 20. Three hours after CA injection both groups were injected with Indium-111 labelled tetrazine probe 21 (Figure 7B). This probe reacts very efficiently with TCO-groups, but releases drug payloads very poorly (Scheme 4) and was therefore used to quantify the conjugates 19 that have not reacted with tetrazine 20.

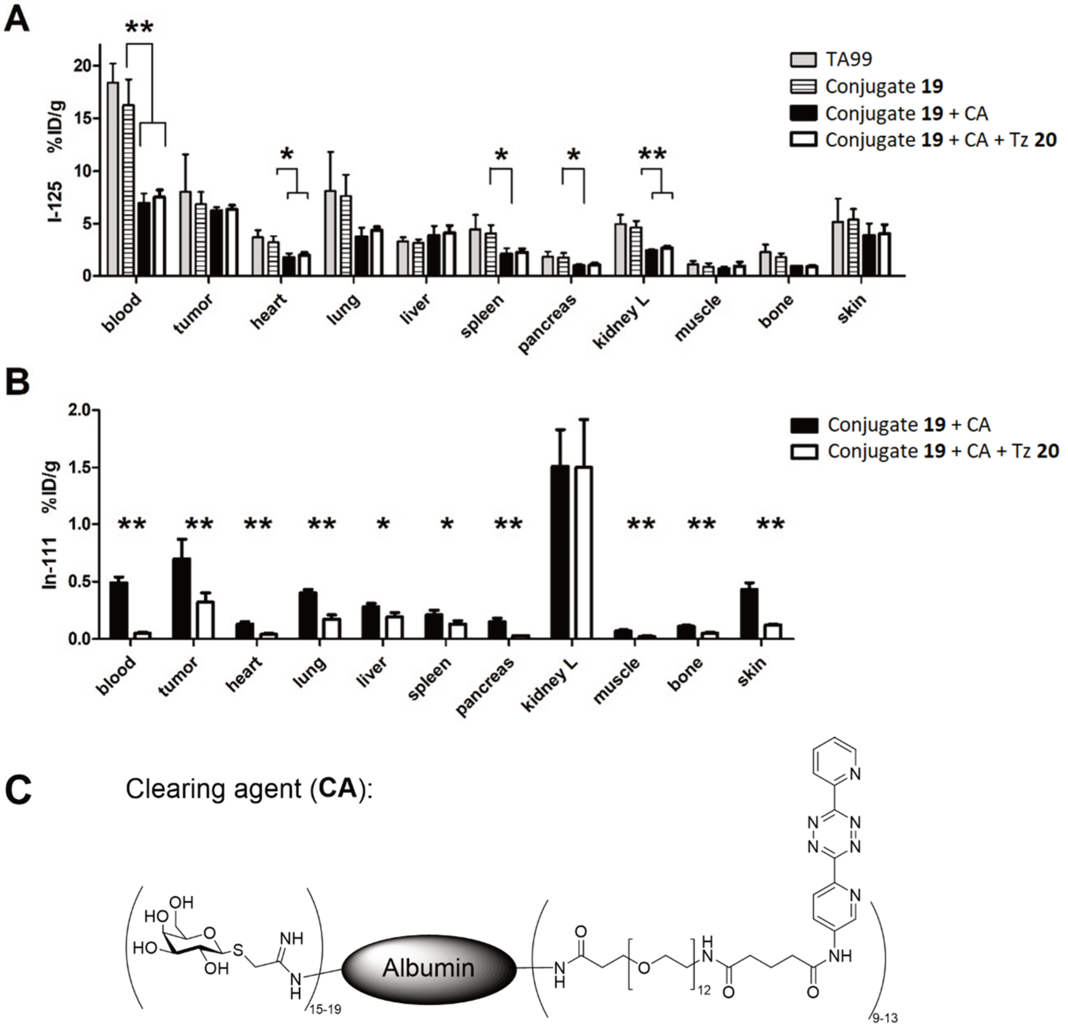
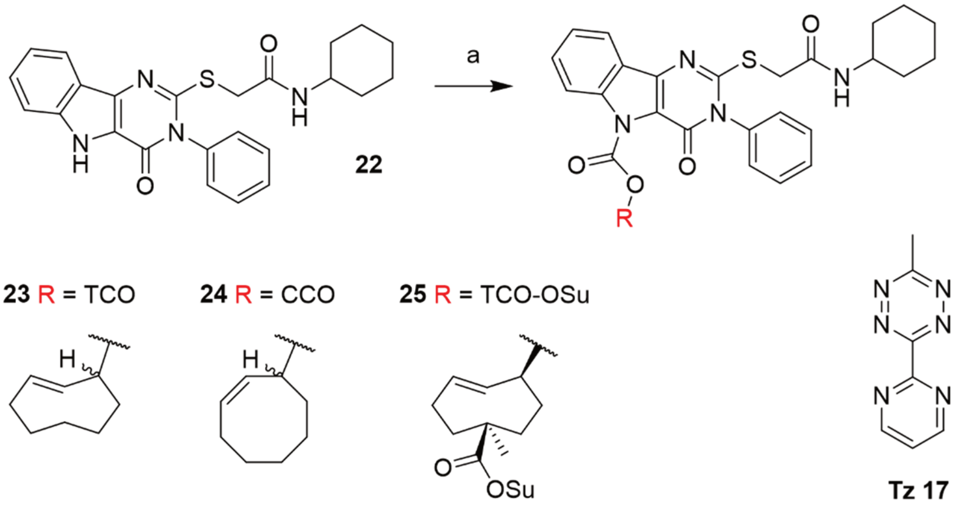


Figure 7. In vivo performance of native TA99 and conjugate **19** and its distribution throughout female C57BL/6 mice bearing a subcutaneous B16-F10 melanoma. A) Native TA99 and conjugate **19** were radiolabelled with ^{125}I, whereas tetrazine **21** was radiolabelled with ^{111}In. The study contained four groups of mice each subject to the order of reagents as presented in the key. ^{125}I isotope signal indicates presence of the TA99 antibody in the specified organ. B) The groups of mice receiving both the conjugate and clearing agent were also injected with ^{111}In-radiolabelled tetrazine **21** one hour after receiving activating tetrazine **20**. ^{111}In isotope signal indicates presence of unreacted ADC conjugate **19** prior to the second injection. Significance calculated through a one-way ANOVA Bonferroni post-hoc test, *:$p<0.05$; **:$p<0.01$. Experiment was carried out by Raffaella Rossin (Tagworks Pharmaceuticals). C) Clearing agent (CA) consisting of a polygalactosylated albumin-polytetrazine conjugate.

The results outlined in Figure 7A show that conjugate **19** exerts similar target affinity compared to native TA99. A high uptake in blood and blood-rich organs was observed as well as tumoral tissue and the skin. Application of the clearing agent significantly reduced the conjugate uptake in blood and blood-rich organs, whilst maintaining target-specific uptake in the skin and tumoral tissue. Figure 7B shows a measure of reaction inefficiency between Tz activator **20** and conjugate **19**, as unreacted conjugates will react with Indium-111-labelled Tz probe **21**. Uptake of the probe in all tissues, except for the kidney, is significantly lower (Student's t-test) after administration of Tz activator **20**, confirming that reaction between Tz activator **20** and mAb conjugate **19** has taken place. The strong uptake of the probe in the kidney can be explained by the clearance of circulating **19** due to addition of the clearing agent (**CA**). These results suggest the approach is feasible, and further immunological evaluations are required to test immune activating properties of the constructs.

A Chemo-controllable TLR4-Agonist

In a final application of TCO-chemistry to TLR-activation studies, a TCO-modified TLR4 ligand was synthesized. The canonical ligand for TLR4 is lipopolysaccharide[29], which is only accessible through isolation from the outer membrane of gram-negative bacteria as a highly heterogeneous mixture. This – and the absence of a unique reactive site in the molecule – precluded its use as starting material for this approach. Our attention was therefore directed to a synthetic pyrimido[5,4-*b*]indole based TLR4 ligand **22** (Scheme **5**).[30] The activity of this ligand is dependent on a critical heterocyclic amine.[30] It was envisaged that this amine could be protected as a TCO-carbamate to allow tetrazine-mediated control over ligand activity.



Scheme 5. Synthesis of a TCO- or CCO-protected pyrimido[5,4-b]indole derivative. Reagents and conditions: a) Compound **23**: (i) NaH, THF, r.t., 30 min (ii) TCO-OSu, THF, r.t., o.n., 51%; Compound **24**: (i) NaH, THF, r.t., 30 min (ii) CCO-OSu, THF, r.t., o.n., 63%; Compound **25**: (i) NaH, THF, r.t., 30 min (ii) TCO-(OSu)$_2$, THF, r.t., o.n.

As endocyclic indolamines are poor nucleophiles, deprotonation with strong bases was performed prior to reaction with the TCO-OSu reagent. Suspending the starting material in THF followed by addition of an excess of sodium hydride led to the formation of a clear yellow solution. Addition of TCO-OSu to this solution resulted in near-quantitative conversion to the product within minutes. Purification was achieved using silica gel column chromatography and yielded TCO-, CCO- and OSu-TCO-modified indoles **23**-**25** in 50-63% yield (Scheme **5**).

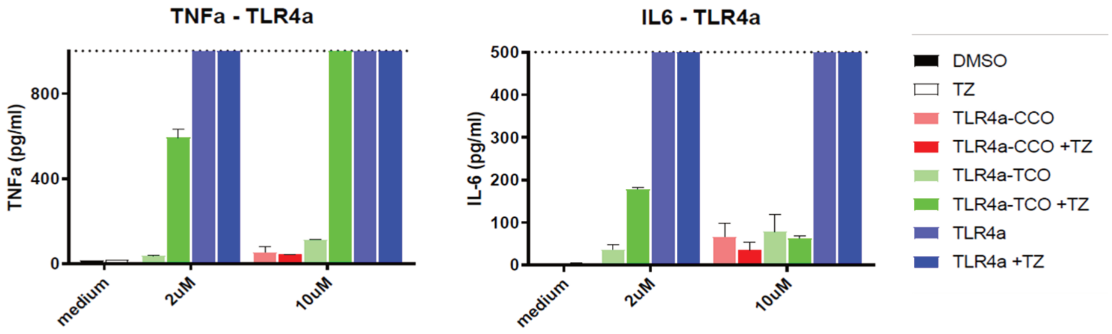
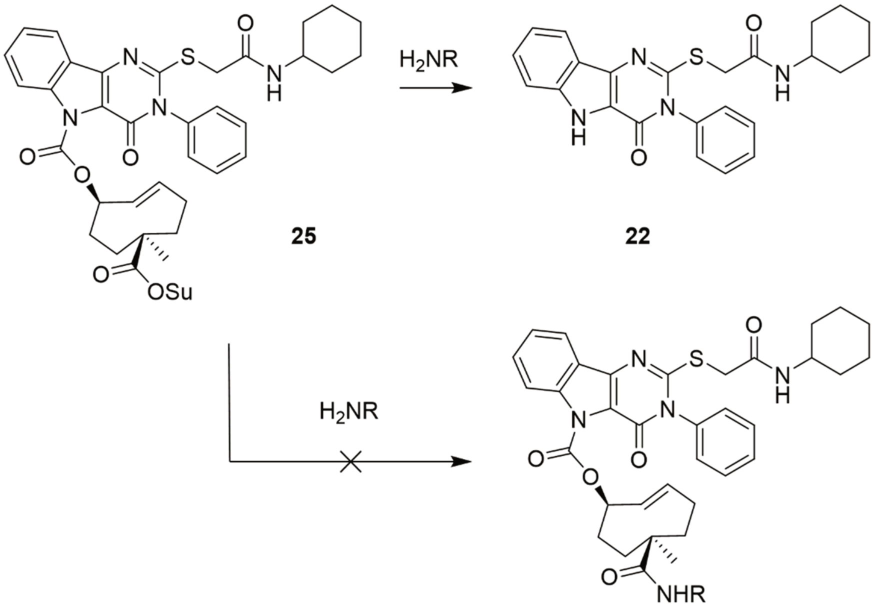


Figure 8. Biological evaluation of compounds **23** and **24**: TLR4a-TCO and TLR4a-CCO, respectively. TNF-α and interleukin 6 (IL6) concentrations were measured in medium containing murine bone marrow-derived macrophages (BMDMs) after treatment with the indicated compounds. TLR4a-TCO **23** only elicits a response when co-administering tetrazine **17**, whereas TLR4a-CCO **24** is irresponsive at the same concentrations. IL6 production appears hampered at higher concentrations.

To assess the suitability of **23**-**25** as reagents to study TLR4-activation, murine bone marrow-derived macrophages (BMDMs) were treated with either free ligand **22**, TCO-caged ligand **23**, or CCO-caged ligand **24** at 2 or 10 μM in the presence or absence of tetrazine (Figure **8**). **23** Showed a strongly diminished (<10%) production of TNF-α and IL-6 in absence of the deprotecting tetrazine. Addition of tetrazine **17** resulted in the production of both cytokines, albeit at lower levels than for the unmodified ligand (60 and 20% respectively). Future work could involve employing this ligand at the window-of-activity concentration in conjunction with tetrazines directed to the endo-lysosomal pathway to selectively activate the intracellular population of TLR4.



Scheme 6. Aminolysis of compound **25**. Addition of amine-containing linkers resulted in regeneration of the free ligand **22**, as opposed to substitution of the succinimidyl ester.

The synthesis of a conjugatable variant of this caged ligand was also attempted using the previously reported bis-functionalized TCO-$(OSu)_2$ reagent.[23] Using the procedure outlined in Scheme 5, compound 25 was observed by LC-MS, whilst keeping the succinimidyl ester at position 5 intact. Unfortunately, addition of various amine-containing linkers (e.g. ethanol amine, dodecaethylene glycolamine) resulted in quantitative aminolysis of the TCO from the indole (Scheme 6).

This lability of the indole-carbamate could prevent the biological use of 23 as it might give rise to premature TLR4 activation. The use of an ether-based conjugation-strategy could potentially solve this issue.[31–33]

Conclusion

In this Chapter the concept of *trans*-cyclooctene as bioorthogonal protecting groups was applied to ligands specific for TLRs residing in intracellular compartments. Deoxycytidine was caged with a CCO or a TCO on the exocyclic amine of the nucleobase. These building blocks were made amenable for DMTr/phosphoramidite solid-phase oligonucleotide synthesis and used to procure a caged 12-meric and 20-meric caged CpG ODN. Although the 12-mer was unable to induce TLR9 activation on BMDMs, it is known that the 20-mer is, and the caged 20-mer is currently awaiting biological evaluation.

The same TCO-caging strategy was applied on Resiquimod, which showed a recovery of functionality after treatment with tetrazine in BMDMs marked by the production of TNF-α and IL6. Substitution of a second functionality on TCO was feasible when present on Resiquimod, enabling the synthesis of an antibody-drug conjugate able to release Resiquimod in sites where the antibody accumulates, for example the tumoral environment. This was assessed and confirmed in an *in vivo* murine experiment employing TA99 as the antibody targeting melanocytes.

Lastly, a synthetic TLR4 ligand was successfully caged with a TCO. This yielded a compound of which its activity towards TLR4 could be controlled by the addition of tetrazine.

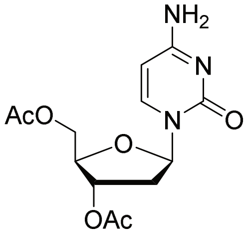
Acknowledgements

Timo Oosenbrug is acknowledged for the biological evaluation of compounds 14 and 15 (Figure 4) and compounds 23 and 24 (Figure 9) described in this Chapter. Nico Meeuwenoord is acknowledged for his aid in the synthesis and purification of DNA fragments. Thuur van Onzen, Raffaella Rossin and Marc Robillard (Tagworks Pharmaceuticals) are acknowledged for the synthesis, purification, and the biological evaluation of the antibody-drug conjugate 19.

Experimental Section

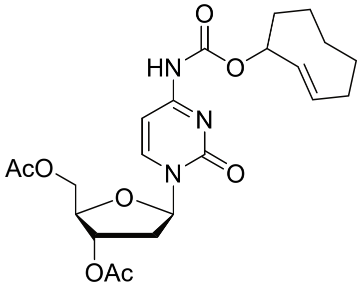
General reagents were obtained from Sigma Aldrich, Fluka, and Acros. All reagents were of commercial grade and used as received unless stated otherwise. Solvents used in synthesis were dried and stored over 4Å molecular sieves, except MeOH and ACN which were stored over 3Å molecular sieves. Triethylamine (Et_3N) and Diisopropylethylamine (DiPEA) were stored over KOH pellets. LC-MS analysis was performed on a Finnigan Surveyor HPLC system with a Nucleodur C18 Gravity 3um 50 x 4.60 mm column (detection at 200-600 nm) coupled to a Finnigan LCQ Advantage Max mass spectrometer with ESI or coupled to a Thermo LCQ Fleet Ion mass spectrometer with ESI. The Method used was 10→90% 13.5 min (0→0.5 min: 10% MeCN; 0.5→8.5 min: 10% to 90% MeCN; 8.5→ 11 min: 90% MeCN; 11→13.5 min: 10% MeCN) unless otherwise stated. High resolution mass spectra (HRMS) were recorded on the following machines: Thermo Scientific Q Exactive HF Orbitrap mass spectrometer equipped with an electrospray ion source in positive-ion mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 275 °C) with resolution R = 240.000 at m/z 400 (mass range of 150-6000) correlated to an external calibration, or on a Waters Synapt G2-Si (TOF) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV) and LeuEnk (m/z = 556.2771) as internal lock mass. HPLC purification was performed on a Gilson HPLC system coupled to a Magerey-Nagel Nucleodur C18 Gravity 5 µm 250 ×10mm column, or on an Agilent 1200 HPLC/6130 MS system coupled to a Magerey-Nagel Nucleodur C18 Gravity 5µm 250×10 mm column or on a Waters autopurifier HPCL/MS system coupled to a Phenomenex Gemini 5µm 150×21.2 mm column.

Compound 1



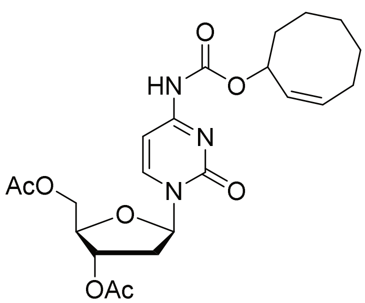
The following synthesis was adopted from literature procedure. [28] Acetic acid (20mL) was added to a flask charged with deoxycytidine (10 mmol, 2.3 g) and stirred vigorously. A solution of acetyl chloride (6 mL) in $CHCL_3$ (14 mL) was added to the suspension and the reaction was stirred overnight. The reaction mixture was concentrated *in vacuo* to yield the title compound as a white solid (10 mmol, 3.1 g, qt.) **^{1}H NMR (400 MHz, DMSO-*d*$_6$)** δ = 9.98 (d, *J*=13.7, 1H), 8.86 (d, *J*=9.7, 1H), 7.99 (dd, *J*=7.9, 1.3, 1H), 6.27 – 6.19 (m, 1H), 6.08 (t, *J*=6.8, 1H), 5.19 (dt, *J*=6.3, 3.3, 1H), 4.31 – 4.20 (m, 3H), 2.50 – 2.36 (m, 2H), 2.05 (d, *J*=9.9, 6H). **^{13}C NMR (101 MHz, DMSO-*d*$_6$)** δ = 170.47, 160.02, 147.38, 144.77, 94.74, 86.66, 82.39, 74.13, 63.97, 37.01, 21.21, 21.06.

Compound 2



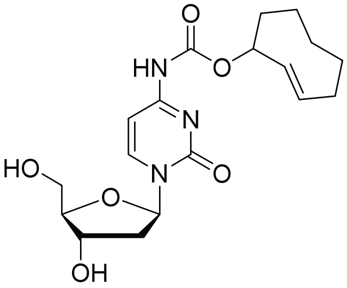
Compound 1 (1.3 mmol, 0.40 g) was dissolved in acetonitrile (2 mL). *Trans*-cyclooctene-2-oxycarbonyl paranitrophenolate (0.67 mmol, 0.20 g), DiPEA (2.6 mmol, 0.45 mL) and DMAP (0.67 mmol, 82 mg) were added and the mixture was stirred for 7 days at 40°C. TLC analysis (10% MeOH in DCM, R_f=0.8) indicated complete conversion of the starting material. The reaction mixture was cooled to room temperature, diluted with EtOAc and washed with 0.1 M HCl (aq.) (3x). The combined aqueous layers were back-extracted with EtOAc (1x). The organic layers were combined and washed with a saturated solution of $NaHCO_3$ (aq.) (3x). The combined aqueous layers were back-extracted with EtOAc (1x). The organic layers were combined, washed with brine (1x), dried over $MgSO_4$ and concentrated *in vacuo*. Silica gel column chromatography (DCM → 1% MeOH in DCM → 2% MeOH in DCM) yielded the pure title compound (0.60 mmol, 0.28 g, 90%). **^{1}H NMR (400 MHz, CDCl$_3$)** δ = 7.97 (d, *J*=7.5, 1H), 6.26 (ddd, *J*=8.2, 5.6, 2.9, 1H), 5.86 (ddd, *J*=15.9, 11.0, 3.6, 1H), 5.55 (dt, *J*=16.5, 2.1, 1H), 5.42 (s, 1H), 5.31 (s, 1H), 5.22 (d, *J*=6.2, 1H), 4.37 (s, 3H), 2.86 – 2.76 (m, 1H), 2.50 (dd, *J*=9.9, 4.6, 1H), 2.10 (d, *J*=11.4, 8H), 2.03 (d, *J*=10.5, 1H), 2.02 – 1.91 (m, 1H), 1.89 (dq, *J*=11.1, 5.0, 1H), 1.82 – 1.63 (m, 2H), 1.50 (td, *J*=12.8, 4.2, 1H), 1.31 (s, 1H), 1.26 (d, *J*=2.7, 1H), 1.16 – 1.03 (m, 1H), 0.92 – 0.75 (m, 1H). **^{13}C NMR (101 MHz, CDCl$_3$)** δ = 170.52, 132.88, 129.96, 87.38, 83.07, 75.96, 74.31, 74.26, 63.79, 40.55, 39.15, 36.05, 35.99, 29.07, 24.15, 21.02, 20.92.

Compound 3



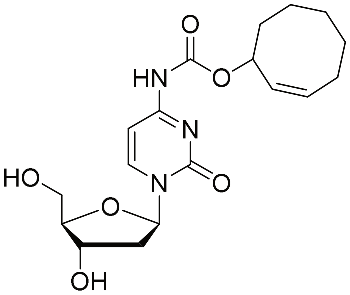
Compound 1 (1.0 mmol, 0.31 g) was dissolved in acetonitrile (2 mL). *Cis*-cyclooctene-2-oxycarbonyl paranitrophenolate (0.54 mmol, 0.16 g), DiPEA (2.0 mmol, 0.35 mL) and DMAP (0.50 mmol, 61 mg) were added and the mixture was stirred for 7 days at 40°C. TLC analysis (10% MeOH in DCM, R_f=0.8) indicated complete conversion of the starting material. The reaction mixture was cooled to room temperature, diluted with EtOAc and washed with 0.1 M HCl (aq.) (3x). The combined aqueous layers were back-extracted with EtOAc (1x). The organic layers were combined and washed with a saturated solution of $NaHCO_3$ (aq.) (3x). The combined aqueous layers were back-extracted with EtOAc (1x). The organic layers were combined, washed with brine (1x), dried over $MgSO_4$ and concentrated *in vacuo*. Silica gel column chromatography (DCM → 1% MeOH in DCM → 2% MeOH in DCM) yielded the pure title compound (0.45 mmol, 0.21 g, 84%). **^{1}H NMR (400 MHz, CDCl$_3$)** δ = 7.96 (d, *J*=7.6, 1H), 7.87 (s, 1H), 7.29 (dd, *J*=7.7, 1.5, 1H), 6.26 (ddd, *J*=7.5, 5.5, 1.7, 1H), 5.79 – 5.60 (m, 2H), 5.57 – 5.48 (m, 1H), 5.22 (dd, *J*=6.4, 2.3, 1H), 4.36 (d, *J*=1.8, 3H), 2.82 (ddd, *J*=14.4, 5.7, 2.3, 1H), 2.33 – 2.23 (m, 1H), 2.23 – 2.12 (m, 1H), 2.16 – 2.04 (m, 7H), 1.98 (qd, *J*=8.1, 5.1, 2H), 1.77 – 1.48 (m, 2H), 1.42 (tdt, *J*=9.5, 6.5, 3.4, 1H). **^{13}C NMR (101 MHz, CDCl$_3$)** δ = 170.52, 170.33, 162.60, 154.88, 152.03, 143.01, 130.51, 129.87, 95.00, 87.35, 83.02, 82.99, 75.08, 74.29, 74.23, 63.77, 39.10, 35.05, 28.81, 26.46, 25.85, 23.28, 20.99, 20.88.

Compound 4



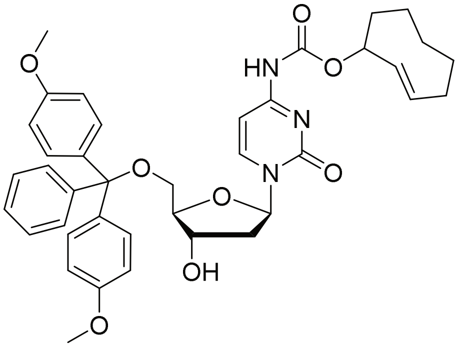
Compound **2** (0.60 mmol, 0.28 g) was dissolved in MeOH (5 mL). K_2CO_3 (0.30 mmol, 41 mg) was added and the reaction was stirred at room temperature for 30 minutes. TLC analysis (5% MeOH in DCM, R_f=0.2) indicated complete conversion of the starting material. The reaction mixture was concentrated *in vacuo* and was used *as is* in the following reaction.

Compound 5



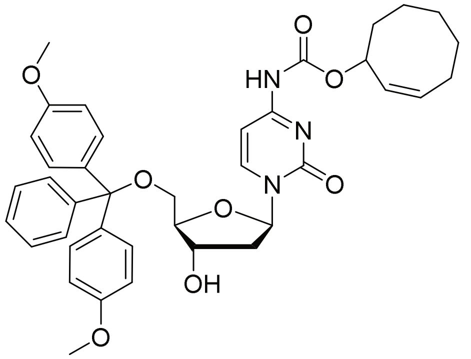
Compound **3** (0.41 mmol, 0.19 g) was dissolved in MeOH (4 mL). K_2CO_3 (0.20 mmol, 28 mg) was added and the reaction was stirred at room temperature for 30 minutes. TLC analysis (5% MeOH in DCM, R_f=0.2) indicated complete conversion of the starting material. The reaction mixture was concentrated *in vacuo* and was used *as is* in the following reaction.

Compound 6



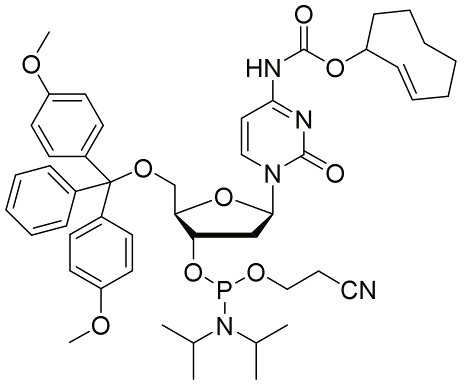
Compound **4** (0.60 mmol, 0.23 g) was dissolved in pyridine (4 mL). DMTr-Cl (1.80 mmol, 610 mg) was added and the reaction mixture was stirred overnight at room temperature. TLC analysis (10% MeOH in DCM, R_f=0.7) indicated complete conversion of the starting material. The mixture was concentrated *in vacuo*, diluted with EtOAc and consequently washed with H_2O (3x). The combined aqueous layers were back-extracted with EtOAc (1x). The organic layers were combined, washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. Silica gel column chromatography (DCM → 0.5% MeOH in DCM → 2% MeOH in DCM) yielded the pure title compound (0.46 mmol, 0.31 g, 77% over two steps).**^{1}H NMR (400 MHz, $CDCl_3$)** δ = 8.21 (dd, *J*=7.5, 1.2, 1H), 7.75 (s, 1H), 7.43 – 7.35 (m, 2H), 7.34 – 7.26 (m, 7H), 7.26 – 7.19 (m, 1H), 7.02 (dd, *J*=7.5, 2.0, 1H), 6.89 – 6.80 (m, 4H), 6.27 (t, *J*=5.8, 1H), 5.89 – 5.77 (m, 1H), 5.53 (dd, *J*=16.4, 2.5, 1H), 5.39 (d, *J*=5.2, 1H), 4.50 (d, *J*=5.4, 1H), 4.14 (q, *J*=3.7, 1H), 3.83 – 3.77 (m, 6H), 3.49 (dd, *J*=10.8, 3.2, 1H), 3.44 – 3.36 (m, 1H), 2.72 (dt, *J*=11.9, 5.7, 1H), 2.48 (d, *J*=10.7, 1H), 2.25 (ddd, *J*=13.6, 6.8, 4.3, 1H), 2.15 – 1.95 (m, 3H), 1.95 – 1.83 (m, 1H), 1.75 (d, *J*=13.6, 1H), 1.71 (s, 4H), 1.28 (s, 1H), 1.26 (d, *J*=2.6, 1H), 1.13 – 1.00 (m, 1H), 0.82 (td, *J*=19.0, 18.6, 9.8, 1H).**^{13}C NMR (101 MHz, $CDCl_3$)** δ = 144.26, 128.26, 128.24, 128.16, 130.22, 127.22, 94.82, 113.47, 87.24, 132.86, 129.92, 130.15, 75.69, 71.08, 71.15, 86.44, 55.44, 62.85, 62.73, 42.36, 36.04, 41.97, 36.10, 35.36, 29.85, 23.97, 29.21.

Compound 7



Compound **5** (0.41 mmol, 0.16 g) was dissolved in pyridine (4 mL). DMTr-Cl (1.23 mmol, 420 mg) was added and the reaction mixture was stirred overnight at room temperature. TLC analysis (10% MeOH in DCM, R_f=0.7) indicated complete conversion of the starting material. The mixture was concentrated *in vacuo*, diluted with EtOAc and consequently washed with H_2O (3x). The combined aqueous layers were back-extracted with EtOAc (1x). The organic layers were combined, washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. Silica gel column chromatography (DCM → 0.5% MeOH in DCM → 2% MeOH in DCM) yielded the pure title compound (0.29 mmol, 0.20 g, 72% over two steps).**^{1}H NMR (400 MHz, $CDCl_3$)** δ = 8.25 (d, *J*=7.5, 1H), 7.43 – 7.36 (m, 2H), 7.33 – 7.16 (m, 7H), 7.04 – 6.98 (m, 1H), 6.87 – 6.79 (m, 4H), 6.28 (t, *J*=5.8, 1H), 5.75 – 5.61 (m, 1H), 5.60 (q, *J*=5.5, 1H), 5.53 – 5.43 (m, 1H), 4.54 (q, *J*=5.2, 1H), 4.19 (q, *J*=3.7, 1H), 3.77 (dd, *J*=2.5, 1.3, 6H), 3.45 (dd, *J*=12.4, 2.0, 1H), 3.39 (dd, *J*=10.7, 3.9, 1H), 2.81 – 2.71 (m, 1H), 2.30 – 2.19 (m, 1H), 2.23 – 2.07 (m, 1H), 1.92 (td, *J*=11.4, 10.5, 6.2, 1H), 1.74 – 1.45 (m, 5H), 1.40 (dddd, *J*=12.3, 9.5, 6.4, 3.2, 1H), 1.04 (d, *J*=6.7, 3H).**^{13}C NMR (101 MHz, $CDCl_3$)** δ = 162.45, 158.63, 158.60, 155.43, 151.98, 144.38, 144.16, 144.12, 135.69, 135.50, 130.19, 130.08, 129.97, 128.22, 128.03, 127.10, 113.32, 94.95, 87.32, 86.85, 86.56, 77.37, 74.69, 70.74, 62.80, 55.25, 42.13, 35.02, 34.97, 28.79, 26.41, 25.83, 23.26, 20.45.

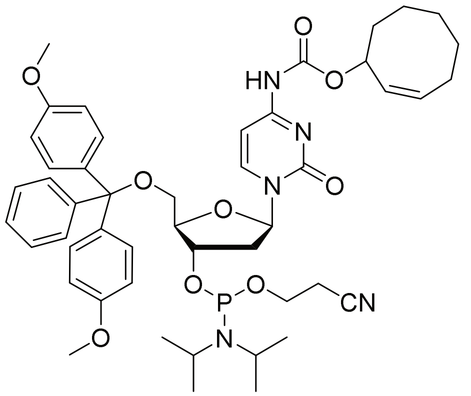
Compound 8



Compound **6** (0.11 mmol, 74 mg) was dissolved in anhydrous DCM (1 mL) and cooled to 0°C. DiPEA (0.50 mmol, 87 µL) and then 2-Cyanoethyl N,N-diisopropylchloro-phosphoramidite (0.16 mmol, 36 µL) was added and the mixture was stirred for 10 minutes at room temperature. TLC analysis (5% MeOH in DCM, R_f=0.7) indicated complete conversion of the starting material. The reaction mixture was diluted with DCM and washed with a 5% solution of $NaHCO_3$ (aq.) (3x). The organic layers were combined, dried (Na_2SO_4) and concentrated *in vacuo*. Silica gel column chromatography (Si neutralized with 2% TEA in DCM before adsorbing; DCM → 0.5% MeOH in DCM) yielded the pure title compound (78 µmol, 69 mg, 71%).**^{1}H NMR (400 MHz, $CDCl_3$)** δ = 8.28 – 8.18 (dd, *J*=7.6, 1H), 7.69 (s, 1H), 7.44 – 7.35 (m, 2H), 7.35 – 7.21 (m, 13H), 7.20 – 7.11 (m, 8H), 6.94 (ddd, *J*=7.1, 4.3, 2.3, 1H), 6.84 (ddd, *J*=8.9, 5.3, 1.8, 5H), 6.31 – 6.22 (m, 1H), 5.89 – 5.75 (m, 1H), 5.52 (d, *J*=16.5, 1H), 5.41 – 5.36 (m, 1H), 5.28 (s, 1H), 4.61 (s, 1H), 4.25 – 4.18 (m, 1H), 3.89 – 3.69 (m, 8H), 3.68 –

3.53 (m, 2H), 3.58 (s, 3H), 3.55 – 3.43 (m, 1H), 3.38 (td, *J*=10.7, 3.6, 1H), 2.63 (dt, *J*=12.9, 6.3, 1H), 2.46 (dt, *J*=16.6, 5.8, 2H), 2.35 (s, 8H), 2.28 (ddd, *J*=13.8, 6.8, 4.1, 1H), 2.15 – 1.94 (m, 2H), 1.87 (d, *J*=9.8, 2H), 1.79 – 1.70 (m, 1H), 1.52 (t, *J*=15.1, 1H), 1.27 (s, 3H), 1.36 – 1.13 (m, 14H), 1.07 (d, *J*=6.8, 4H), 0.91 – 0.73 (m, 2H).**^{31}P NMR (162 MHz, CDCl$_3$)** δ = 148.74, 148.68, 148.45, 148.10, 148.07.

Compound 9

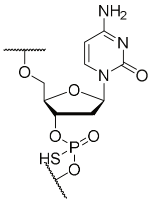


Compound 7 (0.17 mmol, 0.12 g) was dissolved in anhydrous DCM (2 mL) and cooled to 0°C. DiPEA (0.50 mmol, 87 µL) and then 2-Cyanoethyl N,N-diisopropylchlorophosphoramidite (0.21 mmol, 48 µL) was added and the mixture was stirred for 10 minutes at room temperature. TLC analysis (5% MeOH in DCM, R$_f$=0.7) indicated complete conversion of the starting material. The reaction mixture was diluted with DCM and washed with a 5% solution of NaHCO$_3$ (aq.) (3x). The organic layers were combined, dried (Na$_2$SO$_4$) and concentrated *in vacuo*. Silica gel column chromatography (Si neutralized with 2% TEA in DCM before adsorbing; DCM → 0.5% MeOH in DCM) yielded the pure title compound (0.15 mmol, 0.13 g, 88%).**^{1}H NMR (400 MHz, CDCl$_3$)** δ = 8.30 – 8.20 (dd, *J*=7.5, 2.2, 1H), 7.52 (s, 1H), 7.40 (dt, *J*=8.2, 1.3, 2H), 7.35 – 7.21 (m, 7H), 6.95 (d, *J*=7.5, 1H), 6.89 – 6.81 (m, 4H), 6.25 (t, *J*=5.7, 1H), 5.77 – 5.65 (m, 1H), 5.62 (d, *J*=7.9, 1H), 5.55 – 5.45 (m, 1H), 4.64 (dq, *J*=10.2, 5.5, 4.9, 1H), 4.20 (dt, *J*=5.4, 3.1, 1H), 3.83 – 3.78 (m, 6H), 3.71 – 3.48 (m, 4H), 3.39 (dd, *J*=10.8, 3.3, 1H), 2.71 (dtd, *J*=12.1, 6.6, 1.8, 1H), 2.44 (t, *J*=6.4, 2H), 2.36 – 2.20 (m, 1H), 2.20 – 2.10 (m, 1H), 1.96 (s, 1H), 2.02 – 1.88 (m, 1H), 1.69 (dtd, *J*=14.3, 10.3, 9.4, 5.2, 1H), 1.58 (h, *J*=6.3, 5.9, 3H), 1.41 (dddd, *J*=12.6, 9.9, 6.7, 3.2, 1H), 1.25 (dd, *J*=6.8, 4.6, 1H), 1.17 (t, *J*=6.5, 12H). **^{31}P NMR (162 MHz, CDCl$_3$)** δ = 149.31, 149.28, 148.66.

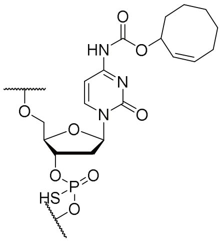
General method for synthesizing oligonucleotides:

5'-CATGAC*GTTCCT-3'

10 C* =

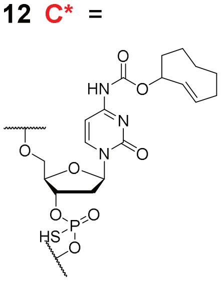


11 C* =

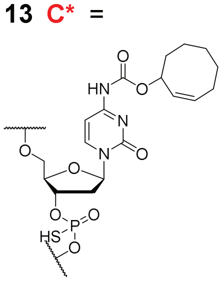


5'-TCCATGAC*GTTCCTGATGCT-3'

12 C* =

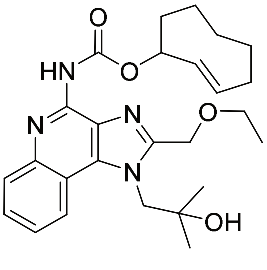


13 C* =



Resin: Controlled Pore Glass (CPG) resin pre-loaded with a deoxythymidine via a succinyl linker (bought from Proligo® Reagents). For a typical oligonucleotide synthesis, 10 µmol of preloaded nucleotide was started with. **Solvent:** Anhydrous acetonitrile (DNA synthesis grade) **Atmosphere:** All steps are carried out under an argon atmosphere. **Reagents:** 2-Cyanoethyl N,N-diisopropyl phosphoramidites protected with 4-*t*-butylphenoxyacetyl (tac) on exocyclic amines of the nucleobases. Bought as a 0.1 M solution in acetonitrile from Proligo® Reagents. IUPAC name for ''Sulfurization Reagent II'' is 3-((N,N-dimethylaminomethylidene)amino)-3H-1,2,4-dithiazole-5-thione. A 0.25 M solution of 5-(Ethylthio)-1H-tetrazole (ETT) was used as the activator in the coupling steps. **Detritylation:** For the first detritylation, the resin was washed three times with acetonitrile. Then, the resin was treated with a solution of 5% dichloroacetic acid in toluene (3 min, 1x). The resin was washed with acetonitrile (3x). **Coupling:** Two cycles were carried out consisting of the following: a solution of 0.1 M phosphoramidite in acetonitrile (30 µmol, 300 µL, 3 eq.) was added to the resin. A solution of 0.25 M ETT in acetonitrile (120 µmol, 480 µL, 12 eq.) was added and the resin was shaken for 3 min. After two cycles were carried out and the resin was washed with acetonitrile, a solution of 0.1 M sulfurization reagent II in 6:4 pyridine:acetonitrile (100 µmol, 1 mL, 10 eq.) was added and the resin was shaken for 1 min. (1x). **Deprotection:** The final DMTr group was removed according to the detritylation procedure. The resin was then treated with a solution of 28% NH_4OH (aq.) and shaken for 30 min. The suspension was filtered and the resin was washed with H_2O (3x). The filtrates were combined and concentrated *in vacuo*. The resulting residu was purified using an anion exchange column followed by a desalting procedure.

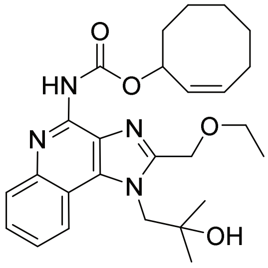
Compound 14



Resiquimod (32 µmol, 10 mg) was dissolved in DMF (200 µL) in an eppendorf tube (1.5 mL). DiPEA (70 µmol, 12 µL), TCO-OSu (35 µmol, 9 mg) and DMAP (3 µmol, 0.4 mg) were added. The tube was shaken for 7 days at 50°C when no further conversion was observed by LC-MS analysis. A solution of 50% brine in H_2O was added to the reaction mixture and washed with DCM (3x). The combined organic layers were evaporated *in vacuo* and reconstituted in DMSO. Purification with RP-HPLC yielded the title compound (13 µmol, 5.9 mg, 41%) as well as unreacted Resiquimod (17 µmol, 5.3 mg, 53%). **LC-MS (1090, C18, H_2O/AcN + TFA):** 467.27 (z=1, simulated), 467.07

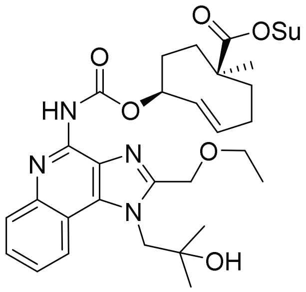
(z=1, observed), 932.67 (z=1, dimer) **HRMS:** $[M+H]^+$ calculated for $C_{26}H_{35}N_4O_4$ 467.26528; found 467.26530

Compound 15



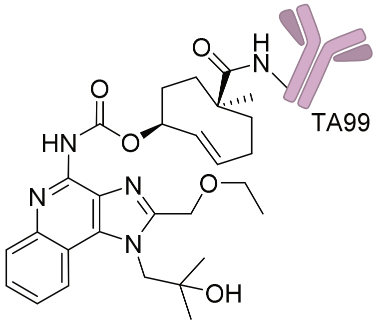
Resiquimod (18 µmol, 5.7 mg) was dissolved in DMSO (180 µL) in an eppendorf tube (1.5 mL). DiPEA (27 µmol, 4.7 µL) and CCO-OSu (27 µmol, 7.2 mg) were added. The tube was shaken for 7 days at room temperature when no further conversion was observed by LC-MS analysis. A solution of 50% brine in H_2O was added to the reaction mixture and washed with DCM (3x). The combined organic layers were evaporated *in vacuo* and reconstituted in DMSO. Purification with silica gel column chromatography (loaded in a Pasteur pipette, DCM→0.5% MeOH in DCM→1% MeOH in DCM) yielded the title compound (5.6 µmol, 2.6 mg, 31%). **LC-MS (1090, C18, H_2O/AcN + TFA):** 467.27 (z=1, simulated), 467.25 (z=1, observed), 932.67 (z=1, dimer) **HRMS:** $[M+H]^+$ calculated for $C_{26}H_{35}N_4O_4$ 467.26528; found 467.26522 **^{1}H NMR (400 MHz, $CDCl_3$)** δ = 8.31 (s, 1H), 8.23 (d, *J*=8.5, 1H), 8.15 (d, *J*=8.3, 1H), 7.63 (t, *J*=7.7, 1H), 7.49 (t, *J*=7.6, 1H), 5.85 (s, 1H), 5.79 – 5.68 (m, 1H), 5.67 – 5.59 (m, 1H), 5.33 (s, 1H), 4.94 (s, 2H), 4.82 (s, 2H), 3.70 (q, *J*=7.0, 2H), 3.20 (s, 1H), 2.64 (s, 1H), 2.35 (d, *J*=11.9, 1H), 2.15 (s, 3H), 1.73 (s, 1H), 1.44 (s, 1H), 1.36 (s, 7H), 1.33 – 1.25 (m, 6H).

Compound 16



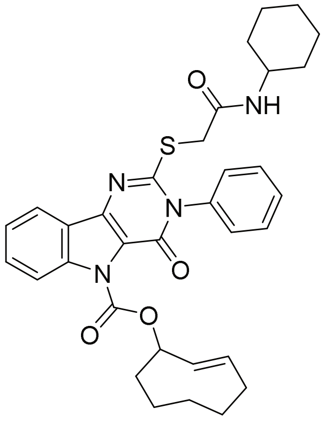
Resiquimod (32 µmol, 10 mg) was dissolved in anhydrous DMF (200 µL) in an eppendorf tube. DiPEA (153 µmol, 28 µL) and TCO-$(OSu)_2$ (40 µmol, 17 mg) were added and the tube was shaken for 10 days at room temperature. LC-MS analysis indicated >50% conversion of the starting material. Water was added to the reaction mixture and the product was extracted with DCM (3x). The organic layers were combined, dried ($MgSO_4$) and concentrated *in vacuo*. The product was purified using silica gel column chromatography (loaded in a Pasteur pipette, 50% EtOAc in Pnt → 80% EtOAc in Pnt) yielding the title compound (7.8 µmol, 3.6 mg, 24%). **HRMS:** $[M+H]^+$ calculated for $C_{32}H_{40}N_5O_8$ 662.28714; found 622.28713. **^{1}H NMR (400 MHz, $CDCl_3$)** δ = 8.15 (t, 2H, Ar*H*), 7.61 (t, 1H, Ar*H*), 7.48 (t, 1H, Ar*H*), 6.20 (m, 1H, N*H*), 5.70 (m, 1H, NHCOOC*H*), 5.48 (s, 1H, trans-alkene *H*), 5.34 (s, 1H, trans-alkene *H*), 4.95 (s, 2H, CCH_2N), 4.81 (s, 2H, CCH_2O), 3.68 (q, 2H, OCH_2CH_3), 2.83 (d, 4H, CCH_2CH_2C), 2.50-0.77 (m, 21H, aliphatic protons).

Compound 19



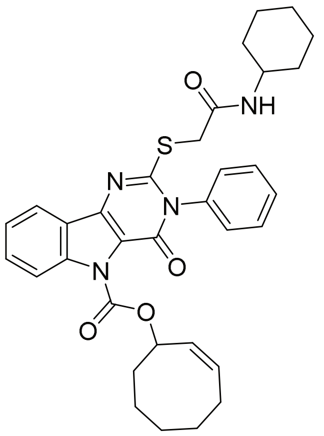
Anti-glycoprotein 75 (gp-75) mAb TA99 (1.25 mg, 5 mg/mL in PBS) was reacted with compound **16** (30 mM in DMF, 80 eq.) in a solution of 7:2:1 (PBS:propylene glycol:DMF, total volume of 330 µL). A pH of 9 was maintained by careful adjustment with a 1 M solution of Na_2CO_3 (aq.). The reaction vessel was wrapped in aluminium foil and shaken for 2 hours. The reaction was transferred to an Amicon Ultra-15 centrifugal device (50 kDa cut-off), washed with a solution of 7.5:2.5 (PBS:propylene glycol) (3x) and purified using a PD-10 column. A ratio of 1.7 R848-TCO per antibody was determined by a tetrazine titration and SDS-PAGE analysis.

Compound 23



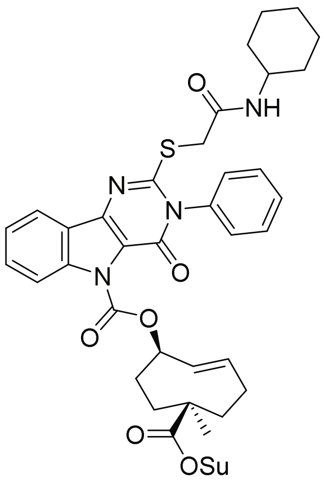
The following procedure was modified from literature[12]: *N*-Cyclohexyl-2-((4-oxo-3-phenyl-4,5-dihydro-3*H*-pyrimido[5,4-*b*]indol-2-yl)thio)acetamide.HCl (As received from the laboratory of Prof. A.P. Esser-Kahn) (10 µmol, 4.3 mg) was suspended in THF (100 µL) in an eppendorf tube (1.5 mL). NaH (50 µmol, 2 mg) was added which dissolved the indole and turned the solution yellow. After shaking for 30 minutes at room temperature, TCO-OSu (12.5 µmol, 3.34 mg) was added and the mixture was shaken for another 10 minutes. LC-MS analysis indicated near quantitative conversion of the starting material. H_2O was added to the mixture and the product was extracted with DCM (5x). The combined organic layers were dried ($MgSO_4$) and concentrated *in vacuo*. Purification with silica gel column chromatography (loaded in a Pasteur pipette; DCM→5% acetonitrile in DCM;TLC analysis R_f=0.7 in 10% acetonitrile in DCM) yielded the title compound (5.1 µmol, 3.0 mg, 51%). **LC-MS (5090, C18, H_2O/AcN + TFA):** 585.25 (z=1, simulated), 585.17 (z=1, observed), 1191.00 (z=1, dimer with sodium cation). **HRMS:** $[M+H]^+$ calculated for $C_{33}H_{37}N_4O_4S$ 585.25300; found 585.25280

Compound 24



The following procedure was modified from literature[12]: *N*-Cyclohexyl-2-((4-oxo-3-phenyl-4,5-dihydro-3*H*-pyrimido[5,4-*b*]indol-2-yl)thio)-acetamide.HCl[30] (As received from the laboratory of Prof. A.P. Esser-Kahn) (10 µmol, 4.3 mg) was suspended in THF (100 µL) in an eppendorf tube (1.5 mL). NaH (50 µmol, 2 mg) was added which dissolved the indole and turned the solution yellow. After shaking for 30 minutes at room temperature, CCO-OSu (12.5 µmol, 3.34 mg) was added and the mixture was shaken for another 10 minutes. LC-MS analysis indicated near quantitative conversion of the starting material. H_2O was added to the mixture and the product was extracted with DCM (5x). The combined organic layers were dried ($MgSO_4$) and concentrated *in vacuo*. Purification with silica gel column chromatography (loaded in a Pasteur pipette, DCM→2.5% acetonitrile in DCM→5% acetonitrile in DCM;TLC analysis R_f=0.7 in 10% acetonitrile in DCM) yielded the title compound (6.3 µmol, 3.7 mg, 63%). **LC-MS (5090, C18, H_2O/AcN + TFA):** 585.25 (z=1, simulated), 585.13 (z=1, observed), 1191.27 (z=1, dimer with sodium cation), 1776.00 (z=1, trimer with sodium cation). **HRMS:** $[M+H]^+$ calculated for $C_{33}H_{37}N_4O_4S$ 585.25300; found 585.25254

Compound 25



The following procedure was modified from literature[12]: *N*-Cyclohexyl-2-((4-oxo-3-phenyl-4,5-dihydro-3*H*-pyrimido[5,4-*b*]indol-2-yl)thio)acetamide.HCl[30] (As received from the laboratory of Prof. A.P. Esser-Kahn) (5 µmol, 2 mg) was suspended in THF (100 µL) in an eppendorf tube (1.5 mL). NaH (25 µmol, 1 mg) was added which dissolved the indole and turned the solution yellow. After shaking for 30 minutes at room temperature, TCO-$(OSu)_2$ (12.5 µmol, 5.3 mg) was added and the mixture was shaken for another 60 minutes. LC-MS analysis indicated near quantitative conversion of the starting material. The crude mixture was used *as is* to conjugate amine-containing linkers. **LC-MS (5090, C18, H_2O/AcN + TFA):** 740.27 (z=1, simulated), 740.07 (z=1, observed), 1478.93 (z=1, dimer), 1502.00 (z=1, dimer with sodium cation)

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