Targeting of antigen presenting cells with mannosylated conjugates

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Chapter 3

Large scale synthesis of a conjugation-ready 2-butoxy-8-oxo-adenine analog TLR7-ligand

Introduction

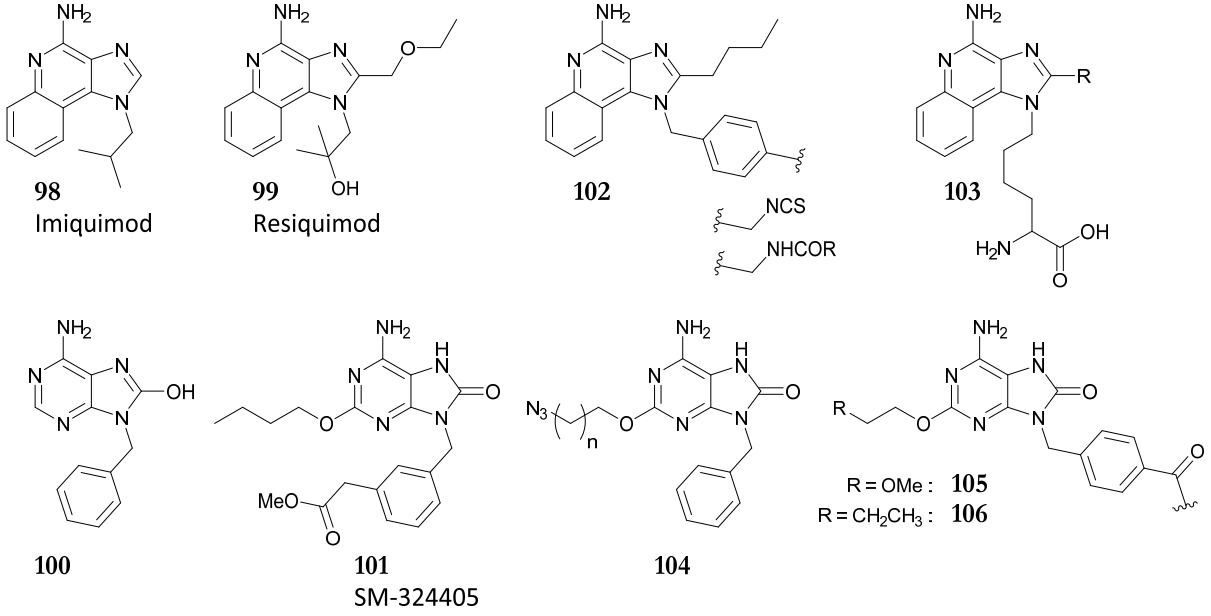
Effective vaccines require at least two functionalities, a recognizable disease-related antigen and an adjuvant. Adjuvants can be derived from pathogen-associated molecular patterns (PAMPs) that induce signaling through binding of pattern recognition receptors (PRRs) stimulating antigen-presenting cells (APCs) to mature. Maturation results in the secretion of inflammatory cytokines and upregulation of antigen processing and presentation, which are a necessity for an adequate adaptive response. The Toll-like receptor (TLR) family is intensively explored for the development of new vaccine adjuvants.[2] Ten different TLRs which recognize different types of PAMPs can be found on human immune cells. They are either expressed on the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10) or endosomal (TLR3, TLR7, TLR8, and TLR9).[3,4] Endosomal TLRs

Part of this work is published in Hogervorst & Gential et al. 2019[1]

recognize nucleic acid-based PAMPs such as viral double-stranded RNA (TLR3),[5] single-stranded bacterial and viral RNA (TLR7 and TLR8)[6–8] and bacterial and viral DNA (TLR9).[9]

Small molecule agonists have been discovered as ligands for TLR7 and TLR8. Prime examples are imidazoquinolines based structures such as imiquimod (**98**) and resiquimod (**99**, Figure 1),[10] of which the latter is used in the clinic for the treatment of an uncommon epidermal cancer[11] and is tested as vaccine adjuvant.[12] Derivatives of 8-oxo-adenine and 8-hydroxy-adenine are another class of TLR7/TLR8 agonists. Based on lead compound **100** (Figure 1), developed by Hirota et al.[13] a multitude of structure-activity relationship studies have been described resulting in agonists such as SM-324405 (**101**).[14–17]

Figure 1: TLR7/TLR8 agonists.



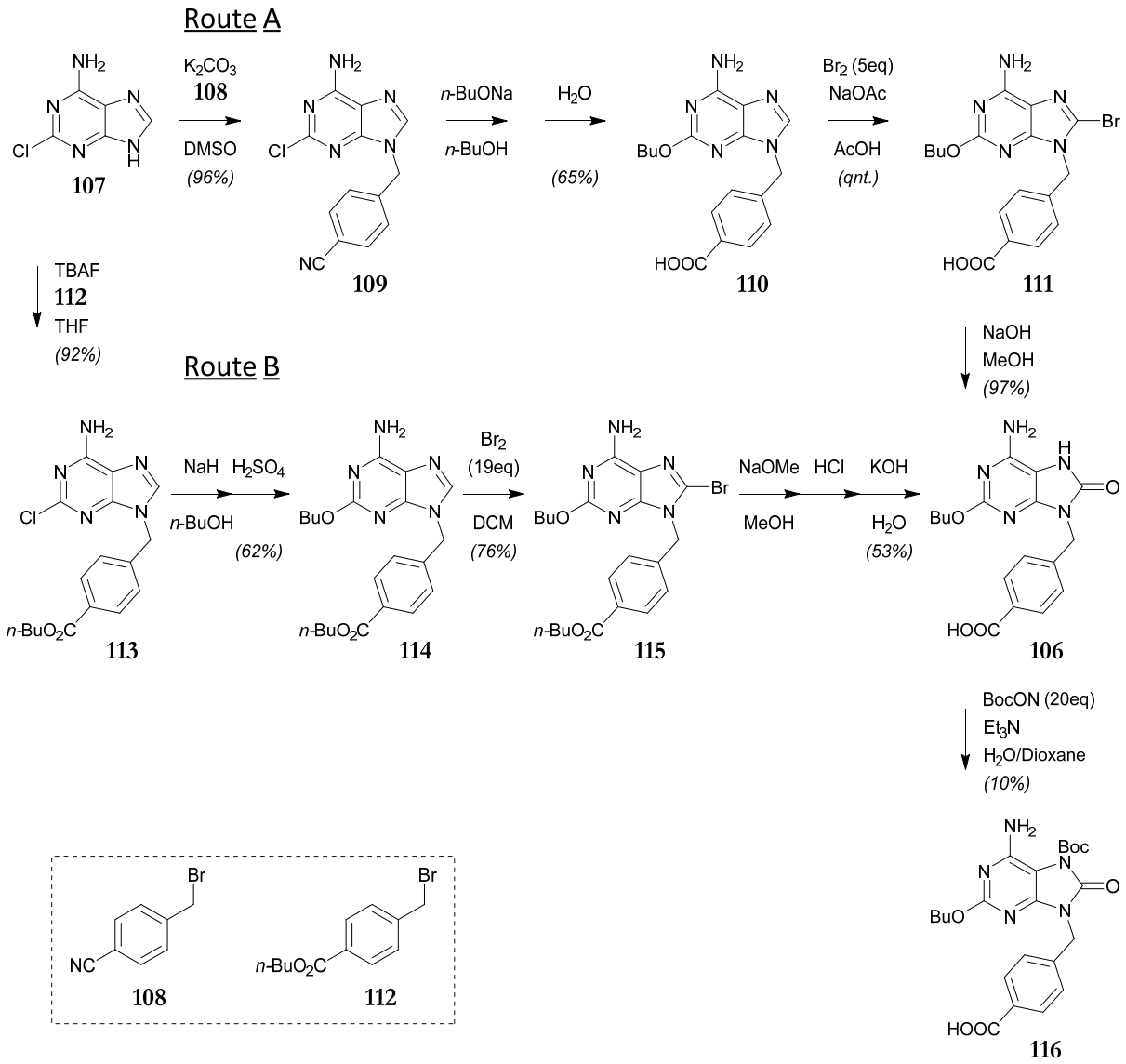
Both classes of agonists have been functionalized to give conjugation-ready compounds such as **102**, which can be conjugated *via* either an isothiocyanate or a maleimide handle.[18] Fujita et al. described the norleucine amino acid derivative **103** that can be incorporated in a peptide via solid-phase peptide synthesis.[19] Several derivatives of 8-oxo-adenine have been used for conjugation to antigens. For example, Weterings et al.[20] generated **104**, having an 2-azidoalkoxy spacer for attachment to a peptide antigen. The resulting conjugate showed enhanced antigen presentation but lost the ability to induce maturation.[20] Conjugation of **105** via its 9-benzyl function as first described by Chan et al.,[21] resulted in conjugates that maintained the maturation ability and **105** has since been successfully used in peptide-adjuvant conjugates.[22] Similar results were obtained with the 2-butoxy analog **106**.[17]

Although structures such as **106** and **105** have been successfully used in adjuvant-antigen conjugates, their synthesis and subsequent conjugation are far from ideal. Two synthetic routes towards **106** have been described. Akinbobuyi et al.[23] obtained **106** in four steps with an 61% overall yield starting from 6-amino-2-chloropurine (**107**, Scheme 1, route A). In this route, the benzoic acid was introduced with cyanobenzylbromide (**108**) to afford (**109**), which was converted to free acid **110**, after the butoxy group was installed. Subsequent bromination, followed by sodium hydroxide treatment gave ligand **106**. The authors described that the main advantage of this route is that all intermediates could be purified via precipitation in reactions with high conversion.

In the second route to **106**, developed by G.P.P. Gential,[24] the benzoic acid is masked as a butylester that was deprotected in the final stage of the synthesis (route B, Scheme 1). Starting from purine (**107**), adenine **106** was obtained in four steps in a 23% overall yield. In this route, the butylester protection circumvented solubility issues caused by the free benzoic acid as experienced in route A. It was described that the electrophilic aromatic bromination to obtain **115** proceeded sluggishly and could only be achieved in high yield with the aid of a large excess of bromine. Nucleophilic aromatic substitution, demethylation and ester hydrolysis resulted in **106**. The main advantage of route B is the solubility of all intermediates, allowing the purification by for example flash column chromatography. A major disadvantage is the large excess of reagents that are needed to introduce and substitute the bromine and the low overall yield of the route.

A drawback of small molecule agonists for TLR7 and TLR8 is their toxicity. Therefore, imiquimod can only be administered topically.[25] Chan et al.[21] achieved a significant reduction of toxicity by conjugation of TLR ligand **105** to phospholipids and polyethylene glycols. Unfortunately, conjugation of ligands **106** and **105** via their carboxylic acid function resulted in poor yields, due to the poor solubility of the ligand.[21] The solubility of **106** could be improved by the introduction of a *tert*-butoxycarbonyl (Boc) protecting group, but the yield of a small scale (0.15 mmol) synthesis of **116** did not exceed 10% (Scheme 1).[24]

Scheme 1: Previously described routes towards **106**.



This Chapter presents an improved route of synthesis of **116.** Because Route A could not be reproduced providing enough material in sufficient purity, Route B was optimized to enable the production of **116** on a large scale. Having sufficient amounts of **116** available, conjugates can be developed that can be targeted to the endosomes of immune cells, thereby potentially increasing their effectiveness. Conjugation of **116** to the oligomannoside clusters, described in Chapter 2, would not only increase the water solubility of the TLR7 ligand, but also allow for the active transport toward endosomes. To test this hypothesis, this Chapter also describes the synthesis of three bifunctional conjugates that target both TLR7 and trafficking receptors.

Results and Discussion

An improved synthetic route towards Boc protected TLR7 agonist **116** is described in Scheme 2. To allow for the synthesis of **116** with higher yields and on a larger scale, the synthetic procedures applied in route B (Scheme 1)[24] were modified, learning lessons from route A.[23] The main drawback in route B was the conversion of **114** to **116**, requiring a large excess of reagents for the introduction and the substitution of the bromine, hindering efficient scale-up. Furthermore, 90% of **106** was lost in the final step introducing the Boc protection group.

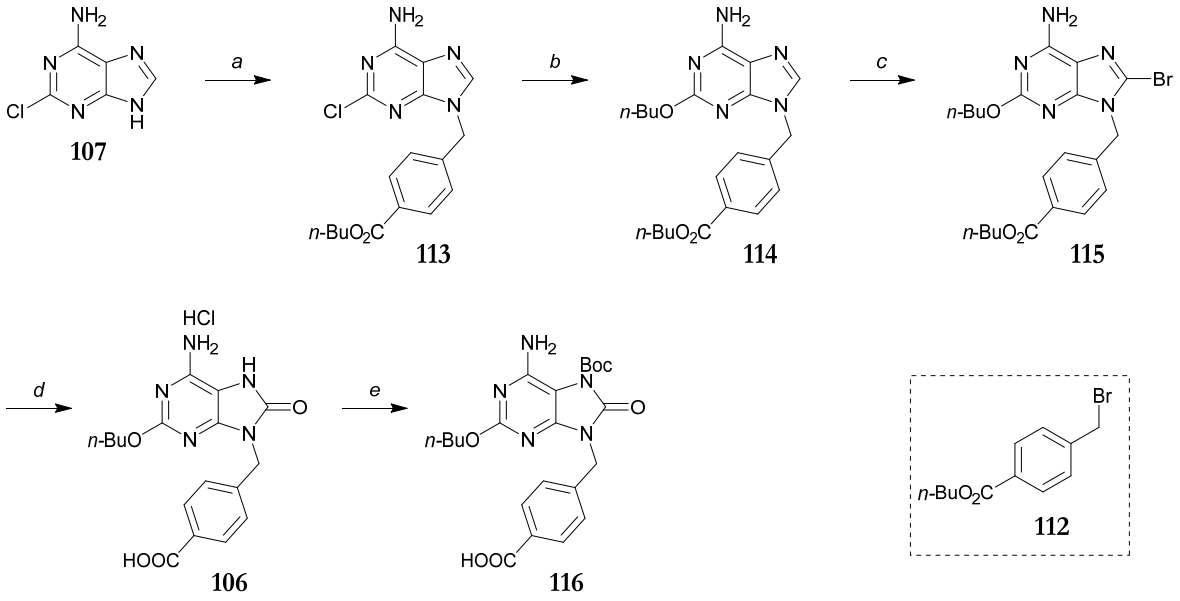
The electrophilic aromatic bromination of **114**, according to route B, required nineteen equivalents of bromine. Subsequent quenching of the excess of bromine with a sodium thiosulphate solution generated HBr that lowered the pH of the solution, leading to the formation of solid elementary sulfur.[26] On a small scale, these solids made the work-up time consuming, because they clogged the glassware and filters. On a larger scale, this problem was so prominent that it made work-up impossible. The addition of sodium acetate (as in route A)[23] improved both the efficiency of reaction and the work-up procedure. Using only five equivalents of bromine, **115** was obtained within an hour in 94% yield on a 40 mmol scale (Scheme 2). Not only did the smaller excess of bromine reduce the amount of required thiosulphate, but the presence of sodium acetate also increased the pH resulting in the formation of less solid sulfur.

Attempts to hydrolyze bromine **115** directly with NaOH were unsuccessful in route B. This was circumvented by using a more nucleophilic methoxide, followed by acidic cleavage of the resulting methyl ether and finally saponification of the remaining benzoic ester under basic condition. The large amount of acidic and basic solutions required for scale-up of the reaction made this three-step method cumbersome. The two extra steps could be circumvented by refluxing **115** for three days in a solution of NaOH in a mixture of methanol and water. In this procedure, *in situ* formed methanolate can displace the bromine while the ether and ester are also cleaved. After acidification of the resulting solution, **106** could be precipitated and filtered off to provide the free acid in 89% yield on a multigram scale.

106 proved to be insoluble in most organic solvents, which makes this building block unsuitable for conjugation. Gential described the enhancement of the solubility of the building block by the introduction of a Boc group. To introduce the *tert*-butyl carbamate, **106** was suspended in a water/dioxane mixture after which it was stirred with triethylamine and ten equivalents of 2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile (Boc-ON) for five days. The reaction proceeded sluggishly with only 10% conversion after five days. This result was explained by the poor solubility of both **106** and Boc-ON and the moderate nucleophilicity of the aromatic amine.[24] Optimization

of this reaction (Table 1) by either changing the solvent system (entries 2 and 3), changing to the more soluble di-*tert*-butyl dicarbonate (Boc_2O, entries 4-11) or changing Et_3N to DMAP (entries 2-5) improved the reaction slightly but still more than 80% of product was lost. Notably, in all entries, **106** was not completely dissolved, not even when diluted in DMSO (entries 2,4,8). In an attempt to obtain a homogenous solution, **106** was dissolved in a NaOH (1.0 M, aq.) generating its sodium salt, after which a solution of Boc_2O in dioxane was added dropwise (entry 9). Upon neutralization with HCl (1.0 M, aq.) both **106** and **116** crashed out, and subsequent centrifugation of the suspension allowed for removal of excess reagents after which extraction of the solids with chloroform gave **116** in 30-40% yield while starting compound **106** could be recovered from the residue (entries 9-11).

Scheme 2: Synthesis of building block **116**



Reagents and conditions a) ***112****, K_2CO_3, DMSO, 63%; b) i. NaH, n-BuOH, 120 °C; ii. H_2SO_4, 80 °C, 71%; c) Br_2, NaOAc, DCM, 94%; d) NaOH, H_2O, MeOH, reflux, 89%; e) NaOH, Boc_2O, H_2O, Dioxane, 35% (95% recovery of* ***106****).*

When the reaction was performed on a 30 mmol scale (entry 11), the final extraction proved to be more difficult due to large amounts of solids. However, using a Soxhlet extraction apparatus, **116** could be isolated in high yield, delivering 10.5 mmol of product (35%). By extraction of the remaining residue in the sock using a NaOH solution (1 M, aq.) and subsequent acidification of the filtrate, the precipitated **106** could be recovered in high yield (18.5 mmol, 95% recovery) and it could be re-subjected to the bocylation reaction. Together these optimizations improved the

conversion of **114** into **116** from 4% to 29%. The synthesis of **116** on a multi-mmol scale makes it a feasible conjugation-ready building block to be used in SPPS (as described in Chapter 4 and 5) or solution-based synthesis (below).

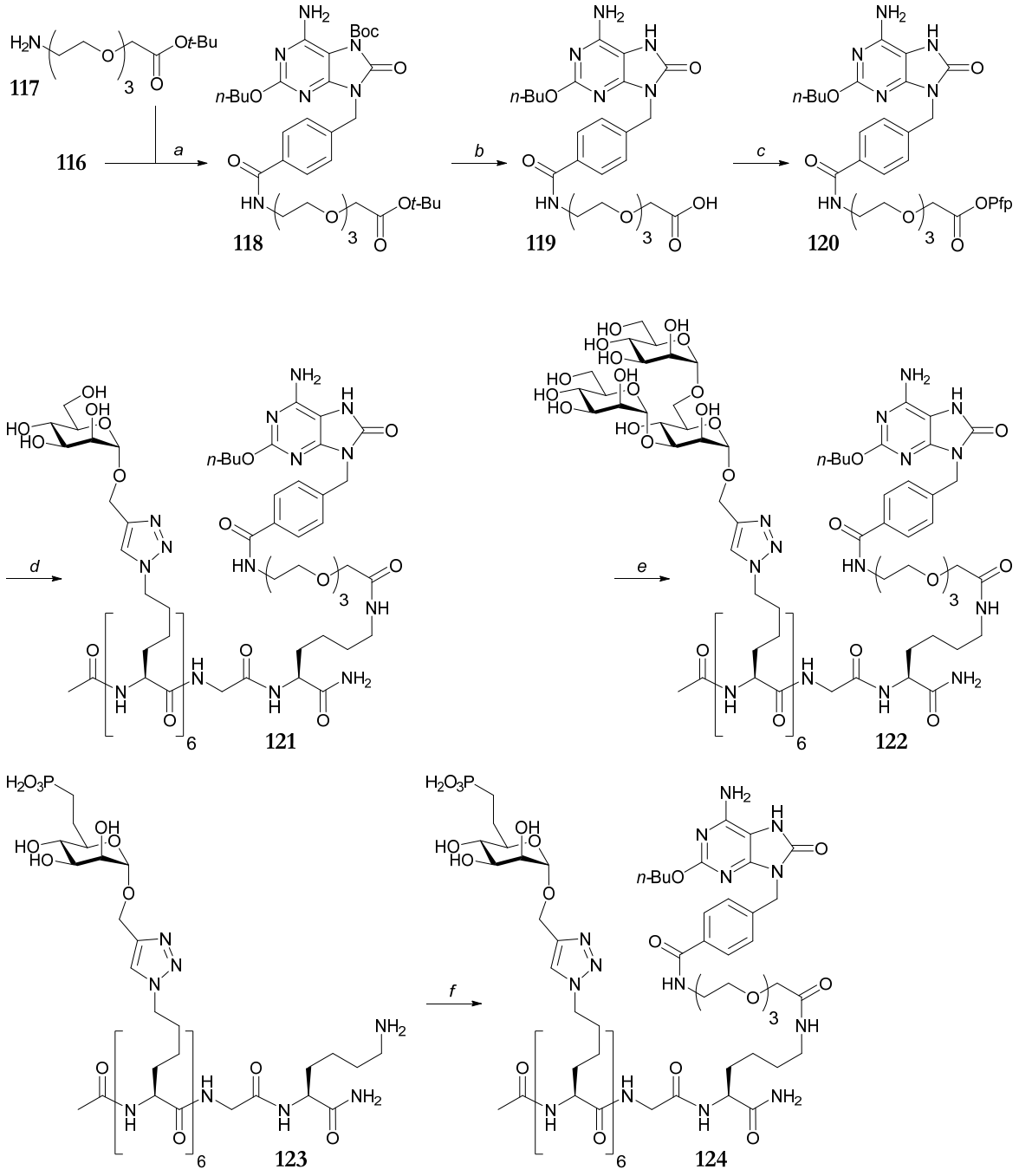
Before conjugation of **116** to the projected glycoside clusters, a spacer was introduced (Scheme 3). Condensation of **116** with known triethylene glycol **117**,[27,28] obtained via previously described procedures resulted in *tert*-butyl ester protected **118**. Simultaneous acid-mediated removal of the Boc group and butyl ester[29] resulted in a compound that caused gelation in water and DMSO, thereby hindering its purification. In the end, the compound could be dissolved using NH_4OAc (0.033 M) in a mixture of H_2O/ACN/*t*-BuOH/AcOH (4/1/1/3, v/v/v/v) allowing the purification of **119** via RP-HPLC. Conversion of carboxylic acid **119** into the pentafluorophenol (Pfp) ester **120** improved the solubility in water and DMSO which enabled the synthesis of three bi-functional conjugates (Scheme 3). The mannoside clusters **50** and **54** (mono- or tri-mannoside clusters on a hexavalent scaffold), described in Chapter 2, were conjugated to Pfp ester **120** resulting in clusters **121** and **122**. Both clusters should be able to target DC-SIGN (see Chapter 2) and could route the bi-functional conjugates toward endosomes. A similar hexavalent scaffold **123** containing six mannose-6-phosphates, described by R.N.M. Reintjes,[30] was conjugated to Pfp ester **120** forming cluster **124**. This conjugate was designed to target the mannose-6-phospate receptor which could result in endo-/lysosomal routing. Unlike the previously described 9-benzyl-8-oxo adenine analog, all these bi-functional conjugates are highly water-soluble. All three conjugates are being tested for their ability to mature APCs and this evaluation is ongoing.

Table 1: Optimization of Boc introduction

Entry	Boc source	Solvent	Base	[106]	Yield
1[24]	Boc-ON (13 eq)	H_2O/dioxane (1/1)	Et_3N (3 eq)	0.5 M	10%[a]
2	Boc-ON (2 eq)	DMSO	DMAP (0.1 eq)	0.5 M	8%[a]
3	Boc-ON (2 eq)	THF	DMAP (0.1 eq)	0.5 M	7%[a]
4	Boc_2O (4 eq)	DMSO	DMAP (0.1 eq)	0.5 M	14%
5	Boc_2O (4 eq)	THF	DMAP (0.1 eq)	0.5 M	13%
6	Boc_2O (4 eq)	MeOH/H_2O/Et_3N (10/10/7)	Et_3N	0.5 M	0%
7	Boc_2O (8 eq)	H_2O/dioxane (1/1)	Et_3N (3 eq)	0.5 M	13%
8	Boc_2O (8 eq)	DMSO	Et_3N	0.15 M	15%
9	Boc_2O (1.5 eq)	H_2O/dioxane (1/1)	NaOH (2 eq)	0.5 M	24%
10	Boc_2O (1.2 eq)	H_2O/dioxane (3/1)	NaOH (3 eq)	0.25 M	41%
11	Boc_2O (1.2 eq)	H_2O/dioxane (3/1)	NaOH (3 eq)	0.25 M	35%

[a] Formation of butyl ester was observed on LC-MS

Scheme 3: Synthesis of mannosylated - TLR7 agonist - conjugates



Reagents and conditions: a) HCTU, DIPEA, DCM/DMF, 84%; b) H_3PO_4, H_2O/toluene, 43%; c) pentafluorophenol, DIC, DMAP, DMSO; d) ***50****,* ***120****, DIPEA, DMSO/H_2O, 73%; e)* ***54****,* ***120****, DIPEA, DMSO/H_2O, 46%; f)* ***120****, DIPEA, DMSO/H_2O, 2.8%.*

Conclusion

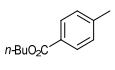
A major improvement in the synthesis of 2-butoxy-8-oxo-adenine analog **116** was achieved. In particular, the overall total yield for the conversion of **114** into **116**, comprising electrophilic aromatic bromination, bromine hydrolysis, and introduction of a Boc group, was increased from 4% to 29%. Furthermore, it was possible to regenerate unprotected adenine **106** during the introduction of the Boc group, by which the Boc-protection could be repeated, increasing the total overall yield further. The new synthetic procedures improved the workability of the synthetic route, in terms of work-up and purification, allowing for a significant increment of reaction scale. Which resulted in the isolation of **116** on a 10.5 mmol scale, making this building block readily available for further conjugations. Together with the improvement in solubility due to the introduction of the Boc-group, makes **116** a suitable building block for application in both solution and solid-phase peptide syntheses. The applicability of **116** was successfully demonstrated by the solution phase conjugation to three mannoside clusters forming three highly water-soluble bi-functional conjugates of which their effectiveness to induce DC-maturation is under current evaluation.

Experimental

General procedures:

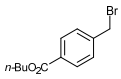
All reactions, purifications, and analyses were performed as described in the general procedures of Chapter 2.

Butyl 4-methylbenzoate (125).



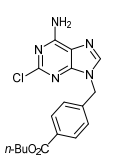
p-Toluic acid (53.3 g, 390 mmol) was dissolved in dry n-BuOH (500 mL, 0.8 M). H_2SO_4 (1.9 mL 35 mmol, 0.09 eq) was added and the mixture was refluxed for 5 hours, after which the reaction mixture was diluted with DCM (1 L) and washed with $NaHCO_3$ (sat. aq.) twice. The organic layer was collected, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The mixture was co-evaporated with toluene (2x) yielding ester **125** as a clear oil (56.13 g, 370 mmol, 95%). TLC R_f 0.63 (Et_2O/PE, 5/95, v/v); IR (neat, cm^{-1}): 2957, 1713; ^{1}H NMR (400 MHz, $CDCl_3$, HH-COSY, HSQC): δ 7.93 (d, J = 8.3 Hz, 2H, H-3/H-4), 7.20 (d, J = 8.0 Hz, 2H, H-3/H-4), 4.30 (t, J = 6.6 Hz, 2H, H-7), 2.37 (s, 3H, H-1), 1.80 - 1.66 (m, 2H, H-8), 1.53 - 1.39 (m, 2H, H-9), 0.97 (t, J = 7.4 Hz, 3H, H-10); ^{13}C NMR (100 MHz, $CDCl_3$, HSQC): δ 166.7 (C-6), 143.4 (C-5), 129.6, 129.0 (C-3, C-4), 127.8 (C-2), 64.6 (C-7), 30.8 (C-8), 21.6 (C-1), 19.3 (C-9), 13.8 (C-10); HRMS [$C_{12}H_{16}O_2$ +H]$^+$: 193.12226 found, 193.12231 calculated.

Butyl 4-(bromomethyl) benzoate (112).



Compound **125** (54.45 g, 283 mmol) was dissolved in CCl_4 (283 mL, 1.0 M) in a three liter flask equipped with a bump-trap and a large cooler loosely stoppered with a septum under nitrogen. NBS (86.5 g, 311 mmol, 1.1 eq) and α,α'-Azoisobutyronitrile (AIBN, 2.4 g, 14.6 mmol, 0.05 eq) were added and the mixture was carefully heated to 90 °C for six hours. After TLC analysis showed full conversion of the starting material the reaction mixture was concentrated *in vacuo*, diluted in EtOAc and washed with H_2O. The organic layer was collected, dried over $MgSO_4$, filtered and concentrated. Compound **112** was obtained after purification by silica gel chromatography (absorbed on Celite → 1/199 → 2/98, Et_2O/PE, v/v) as a yellow oil (49.62 g, 183 mmol, 65%). TLC R_f 0.55 (Et_2O/PE, 5/95, v/v); IR (neat, cm^{-1}): 3197, 2957, 1713; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.04 (d, J = 8.3 Hz, 2H, H-3/H-4), 7.48 (d, J = 8.3 Hz, 2H, H-3/H-4), 4.52 (s, 2H, H-1), 4.35 (t, J = 6.6 Hz, 2H, H-7), 1.83 - 1.72 (m, 2H, H-8), 1.56 - 1.44 (m, 2H, H-9), 1.01 (t, J = 7.4 Hz, 3H, H-10); ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.2 (C-6), 142.6 (C-2), 130.6 (C-5), 130.1, 129.1 (C-3, C-4), 65.1 (C-7), 32.4 (C-1), 30.9 (C-8), 19.4 (C-9), 13.9 (C-10); HRMS [$C_{12}H_{15}BrO_2$ + H]$^+$: 271.11816 found, 271.03282 calculated.

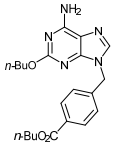
Butyl 4-((6-amino-2-chloro-9H-purin-9-yl)methyl)benzoate (113).



To a suspension of 6-amino-2-chloropurine (**107**) (22.11 g, 130 mmol) in DMSO (300 mL, final 0.3 M) at 0 °C, K_2CO_3 (54 g, 390 mmol, 3 eq) and a solution of compound **112** (42.3 g, 156 mmol, 1.2 eq) in DMSO (133 mL) were added successively and the mixture was stirred for a day. After complete consumption of **107** the mixture was poured in H_2O (3 L) and stored at 4 °C overnight. The suspension was filtered off, washed with H_2O (3x) and the powder was dried *in vacuo*. Purification by

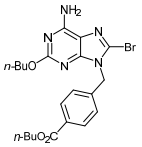
silica gel column chromatography (absorbed on Celite → 1/99 → 6/94, MeOH/DCM, v/v) yielded title compound **113** as an off-white solid (29.88 g, 83.0 mmol, 63%). TLC R_f 0.34 (MeOH/DCM, 5/95, v/v); IR (neat, cm^{-1}): 3297, 3122, 2957, 1736, 1597; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.03 (d, J = 8.3 Hz, 2H, H-12/H-13), 7.72 (s, 1H, H-8), 7.33 (d, J = 8.3 Hz, 2H, H-12/H-13), 6.06 (s, 2H, NH_2), 5.39 (s, 2H, H-10), 4.32 (t, J = 6.7 Hz, 2H, H-16), 1.80 - 1.71 (m, 2H, H-17), 1.51 - 1.42 (m, 2H, H-18), 0.97 (t, J = 7.4 Hz, 3H, H-19); ^{13}C NMR (101 MHz, $CDCl_3$): δ166.1, 156.3 (C-2, C-4, C-5, C-6, C-11, C-14), 140.7 (C-8), 140.0 (C-2, C-4, C-5, C-6, C-11, C-14), 130.5, 127.8 (C-12, C-13), 65.2 (C-16), 47.1 (C-10), 30.9 (C-17), 19.4 (C-18), 13.9 (C-19); HRMS $[C_{17}H_{18}ClN_5O_2 + H]^+$: 360.12143 found, 360.12218 calculated.

Butyl 4-((6-amino-2-butoxy-9H-purin-9-yl)methyl)benzoate (114).



Compound **113** (29.88 g, 83.0 mmol) was dissolved in anhydrous n-BuOH (500 mL, 0.17 M) and cooled to 0 °C. To this solution NaH (33.2 g, 830 mmol, 10 eq) was added in small portions over three hours. The mixture was stirred at 120°C overnight, after which LC-MS analysis showed full conversion of the starting material into the product and a small portion hydrolyzed product. The mixture was cooled to 0°C and H_2SO_4 (53 mL, 1 mol, 1.2 eq, 98%) was added dropwise over three hours under vigorous stirring. After addition the reaction mixture was stirred at 80 °C for two hours. The mixture was diluted in DCM (1.5 L), washed with $NaHCO_3$ (sat. aq., 3x) after which the organic layer was collected, dried over $MgSO_4$, filtered and concentrated *in vacuo*. Remaining *n*-BuOH was removed by co-evaporating with toluene (3x). Purification by silica gel column chromatography (applied in $CHCl_3$ → 1/99 → 1/1, MeOH/DCM, v/v) followed by crystallization ($CHCl_3$/toluene, 1/1, v/v) yielding title compound **114** as an off-white solid (23.46 g, 59.0 mmol, 71%). TLC R_f 0.26 (MeOH/DCM, 5/95, v/v); IR (neat, cm^{-1}): 3282, 3110, 2955, 1736, 1597; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.01 (d, J = 8.3 Hz, 2H, H-12/H-13), 7.61 (s, 1H, H-8), 7.34 (d, J = 8.3 Hz, 2H, H-12/H-13), 5.69 (s, 2H, NH2), 5.33 (s, 2H, H-10), 4.35 - 4.25 (m, 4H, H-16, H-20), 1.83 - 1.69 (m, 4H, H-17, H-21), 1.56 - 1.40 (m, 4H, H-22, H-18), 0.97 (td, J = 7.4, 2.0 Hz, 6H, H-19, H-23); ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.2, 162.6, 156.6, 152.1, 140.8, 138.8, 130.6 (C-2, C-4, C-5, C-6, C-11, C-14), 130.3, 127.8 (C-12, C-13), 67.2, 65.1 (C-16, C-20), 46.7 (C-10), 31.2, 30.8 (C-17, C-21), 19.4, 19.4, 14.0, 13.9; HRMS $[C_{21}H_{27}N_5O_3 + H]^+$: 398.21702 found, 398.21867 calculated.

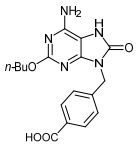
Butyl 4-((6-amino-8-bromo-2-butoxy-9H-purin-9-yl)methyl)benzoate (115).



Compound **114** (16.93 g, 42.5 mmol) was dissolved in DCM (430 mL, 0.1 M), cooled to 0 °C and NaOAc (13.95 g, 85 mmol, 2 eq) and Br_2 (10.9 mL, 212.5 mmol, 5 eq) were added successively. After one hour the reaction was quenched with $Na_2S_2O_3$ (sat. aq.). The mixture was transferred to a separation funnel and the organic layer was washed with $Na_2S_2O_3$ (sat. aq. 3x) and H_2O (1x), dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by silica gel column chromatography (1/99, MeOH/DCM, v/v) yielded title compound **115** as an orange solid (19.0 g, 39.9 mmol, 94%). TLC R_f 0.52 (MeOH/DCM, 5/95, v/v); IR (neat, cm^{-1}): 3320, 3196, 2957, 1722, 1652, 1589; ^{1}H NMR (400 MHz, CDCl3): δ 8.00 (d, J = 8.3 Hz, 2H, H-12/13), 7.37 (d, J = 8.4 Hz, 2H, H-12/13), 5.89 (s, 2H, NH_2), 5.35 (s, 2H, H-10), 4.31 (td, J = 6.1, 0.9 Hz, 4H, H-16, H-20), 1.81 - 1.68 (m, 4H, H-17, H-21), 1.55 - 1.40 (m, 4H,

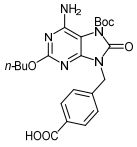
H-18, H-22), 0.96 (td, *J* = 7.4, 1.1 Hz, 6H, H-19, H-23); ^{13}C NMR (100 MHz, CDCl3): *δ* 166.2, 162.4, 155.4, 153.1,140.2, 130.5 (C-2, C-4, C-5, C-6, C-11, C-14, C-15), 130.2, 127.8, (C-12, C-13), 124.5 (C-8), 116.2 (C-2, C-4, C-5, C-6, C-11, C-14, C-15), 67.4, 65.1 (C-16, C-20), 47.1 (C-10), 31.1, 30.9 (C-17, C-21), 19.4, 19.2 (C-18, C-22), 14.0, 13.9 (C-19, C-23); HRMS [$C_{21}H_{26}BrN_5O_3$ + H]$^+$: 478.12507 measured, 478.12740 calculated.

4-((6-Amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)benzoic acid (106).



Compound **115** (13.03 g, 27.3 mmol) was dissolved in MeOH (182 mL, 0.15 M) and NaOH (240 mL, 10 M, aq., 2.4 mol, 88 eq) was added dropwise after which the cream white suspension was refluxed for three days. After LC-MS analysis showed full conversion of the starting material the clear brown solution was cooled to 0 °C and quenched with HCl (420 mL, 6 M, aq., 2.52 mol, 92 eq). The solution changed to a white suspension and the reaction volume was reduced by halve under reduced pressure. The solids were filtered off and washed with water and dried *in vacuo* yielding compound **106** as an orange solid (8.71 g, 24.4 mmol, 89%). LC-MS: Rt = 5.09 min (0 - 50% ACN; 13 min); IR (neat, cm^{-1}): 3418, 3168, 1689; ^{1}H NMR (400 MHz, DMSO-d6): *δ* 12.94 (s, 1H, CO_2H), 10.93 (s, 1H, NH-7), 7.88 (d, *J* = 8.1 Hz, 2H, H-12/H-13), 7.36 (d, *J* = 8.1 Hz, 2H, H-12/H-13), 6.98 (s, 2H, NH_2), 4.92 (s, 2H, H-10), 4.11 (t, *J* = 6.6 Hz, 2H, H-16), 1.67 - 1.51 (m, 2H, H-17), 1.42 - 1.27 (m, 2H, H-18), 0.87 (t, *J* = 7.3 Hz, 3H, H-19); ^{13}C NMR (101 MHz, DMSO-d6): *δ* 167.0, 160.0, 152.0, 148.9, 148.0, 142.2, 129.8 (C-2, C-4, C-5, C-6, C-11, C-14, C-15), 129.6, 127.3 (C-13, C-12), 98.4 (C-2, C-4, C-5, C-6, C-11, C-14, C-15), 65.9 (C-16), 42.1 (C-10), 30.6 (C-17), 18.7 (C-18), 13.7 (C-19); HRMS [$C_{17}H_{19}N_5O_4$ + H]$^+$: 358.15009 found, 358.15098 calculated.

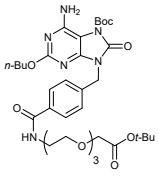
4-((6-amino-2-butoxy-7-(tert-butoxycarbonyl)-8-oxo-7,8-dihydro-9H-purin-9-yl) methyl) benzoic acid (116).



Compound **106** (10.7 g, 30.0 mmol) was dissolved in a NaOH solution (1.0 M, 100 mL, 100 mmol, 3.3 eq). To this clear dark-brown solution a solution of Boc_2O in dioxane (1.2 M, 33 mL, 39.6 mmol, 1.3 eq) was added dropwise over 10 minutes under vigorous stirring. After 2.5 hours the reaction mixture was diluted with $CHCl_3$ (200 mL) and acidified with HCl (1.0 M, 100 mL, 100 mmol, 3.3 eq) dropwise over 20 minutes in which a clouded suspension formed. Separation of layers was achieved by spinning the mixture down at 4000 rpm for 5 min. The aqueous layer was removed and the solid and organic layer were combined and transferred to a soxhlet extractor. This solid was extracted with $CHCl_3$ in the soxhlet apparatus until no clear color emerged from the sock (7 times). The organic layer was dried over $MgSO_4$, filtered and concentrated. Purification by silica gel column chromatography (1/99 → 4/96, MeOH/DCM, v/v) yielded title compound **116** as an off-white solid. (4.83 g, 10.5 mmol, 35%) Starting material was recovered by rinsing the soxhlet with NaOH (1 M, aq.) followed by acidification of the aqueous layer (HCl, 1 M, aq.), results in starting material **106** as a solid that could be obtained by filtration (6.60 g, 18.5 mmol, 95% recovery). TLC R_f 0.27 (MeOH/DCM, 1/9, v/v); IR (neat, cm^{-1}): 3431, 3163, 2958, 1753, 1720, 1637; ^{1}H NMR (400 MHz, DMSO-d6): *δ* 12.96 (s, 1H, CO_2H), 7.90 (d, *J* = 8.0 Hz, 2H,

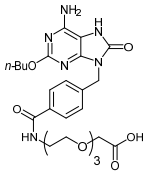
H-12/H-13), 7.41 (d, *J* = 8.0 Hz, 2H, H-12/H-13), 7.06 (s, 2H, NH_2), 4.94 (s, 2H, H-10), 4.16 (t, *J* = 6.5 Hz, 2H, H-16), 1.66 - 1.57 (m, 2H, H-17), 1.54 (s, 9H, *t*-Bu), 1.35 (dq, *J* = 14.7, 7.4 Hz, 2H, H-18), 0.89 (t, *J* = 7.3 Hz, 3H, H-19); ^{13}C NMR (101 MHz, DMSO-d6): *δ* 167.0, 161.2, 150.5, 150.2, 149.6, 149.1, 141.1, 130.0 (C-2, C-4, C-5, C-6, C-11, C-14, C-15), 129.6, 127.6 (C-13, C-12), 96.4 (C-2, C-4, C-5, C-6, C-11, C-14, C-15), 85.2 (C_q *t*-Bu), 66.2 (C-16), 42.7 (C-10), 30.5 (C-17), 27.6 (*t*-Bu), 18.7 (C-18), 13.7 (C-19); HRMS $[C_{22}H_{27}N_5O_6 + H]^+$: 458.2046 found, 458.2034 calculated.

tert-butyl 6-amino-2-butoxy-9-(4-((13,13-dimethyl-11-oxo-3,6,9,12-tetraoxatetradecyl) carbamoyl) benzyl)-8-oxo-8,9-dihydro-7H-purine-7-carboxylate (118).



Amine **117** (313.9 mg, 1.19 mmol 1.03 eq), Boc protected adenine **116** (528.3 mg, 1.15 mmol, 1 eq) and HCTU (475 mg, 1.15 mmol, 1 eq) were combined and dissolved in DCM/DMF (4 mL, 0.28 M, 3/1, v/v) to which DIPEA (400 µL, 2.30 mmol, 2eq) was added dropwise. After two hours the mixture was diluted with $CHCl_3$ washed with HCl (1 M, aq., 1x) dried over $MgSO_4$ (s), filtered, concentrated *in vacuo* and purified by silica gel column chromatography (1/19 → 3/22, acetone/DCM, v/v) to yield title compound **118** as a yellow oil (678 mg, 0.96 mmol, 84%). TLC R_f 0.65 (MeOH/DCM, 1/49, v/v); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.01 (s, 1H), 7.75 (d, *J* = 8.3 Hz, 2H, H-12/H-13), 7.47 (d, *J* = 8.2 Hz, 2H, H-12/H-13), 7.00 (s, 2H, NH_2), 5.01 (s, 2H, H-10), 4.27 (t, *J* = 6.7 Hz, 2H, H-16), 3.99 (s, 2H, *O*-CH_2-CO), 3.69 - 3.62 (m, 12H, CH_2 PEG), 1.77 - 1.68 (m, 2H, H-17), 1.62 (s, 9H, *t*-Bu), 1.47 - 1.39 (m, 11H, *t*-Bu, H-18), 0.95 (t, *J* = 7.4 Hz, 3H, H-19); ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.2, 167.9, 161.9 (C=O), 150.9, 150.3, 150.1, 149.6, 139.2, 134.0, 128.9 (C_q), 128.7, 127.6 (C-12/C-13), 125.9, 120.3, 109.7, 97.2 (C_q), 86.4 (C_q *t*-Bu), 82.5, 70.8, 70.2, 70.1, 70.0, 69.9(CH_2 PEG), 68.7 (O-*C*H_2-CO), 67.5 (C-16), 55.6 (C_q), 43.4 (C-10), 31.0 (C-17), 28.1, 28.1 (*t*-Bu), 18.6 (C-18), 13.9 (C-19); HRMS $[C_{34}H_{50}N_6O_{10} + H]^+$: 703.36517 found, 703.36612 calculated.

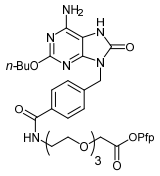
1-(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)phenyl)-1-oxo-5,8,11-trioxa-2-azatridecan-13-oic acid (119).



Butyl ester **118** (170.7 mg, 0.243 mmol, 1 eq) was dissolved in toluene (0.4 mL, 0.5 M) and H_3PO_4 (0.1 mL, 85-90% wt) was added. After LCMS analysis indicated complete conversion the mixture was concentrated *in vacuo*, dissolved in a solution of NH_4OAc (0.033 M) in a mixture of AcOH/ACN/*t*-BuOH/H_2O (3/1/1/4, v/v/v/v, 6 mL) and purified via RP-HPLC (linear gradient 25 - 40% B in A, 10 min, Gemini-NX 5µm C18, 110 Å, 250 x 10.0 mm, 5 mL/min) to yield title compound **119** as a white powder after lyophilization (57.54 mg, 105 µmol, 43%). LC-MS: R_t = 4.80 min (10 - 90% ACN; 13 min); ^{1}H NMR (400 MHz, DMSO) δ 10.17 (s, 1H, COOH), 8.50 (t, *J* = 5.6 Hz, 1H, NH), 7.79 (d, *J* = 8.3 Hz, 2H, H-12/H-13), 7.34 (d, *J* = 8.3 Hz, 2H, H-12/H-13), 6.56 (s, 2H, NH_2), 4.90 (s, 2H, H-10), 4.12 (t, *J* = 6.6 Hz, 2H, H-16), 4.00 (s, 2H, *O*-CH_2-CO), 3.57 - 3.48 (m, 11H, (CO)NH, CH_2 PEG), 3.39 (q, *J* = 5.8 Hz, 2H, CH_2-NH), 1.65 - 1.56 (m, 2H, H-17), 1.35 (dq, *J* = 14.7, 7.4

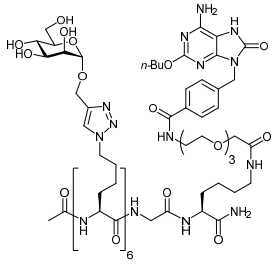
Hz, 2H, H-18), 0.89 (t, *J* = 7.4 Hz, 3H, H-19); <u>^{13}C NMR</u> (101 MHz, DMSO) δ 171.7, 166.0, 160.1 (C=O), 152.3, 149.1, 147.8, 140.2, 133.5 (C_q), 127.4, 127.2 (C-12/C-13), 98.3 (C_q), 69.8, 69.7, 69.7, 69.6, 68.9 (CH_2 PEG), 67.6 (O-*C*H_2-CO), 65.9 (C-16), 42.1 (C-10), 39.2 (CH_2-NH), 30.6 (C-17), 18.8 (C-18), 13.7 (C-19); <u>HRMS</u> $[C_{25}H_{34}N_6O_8 + H]^+$: 547.25105 found, 547.25109 calculated.

perfluorophenyl 1-(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)phenyl)-1-oxo-5,8,11-trioxa-2-azatridecan-13-oate (120).



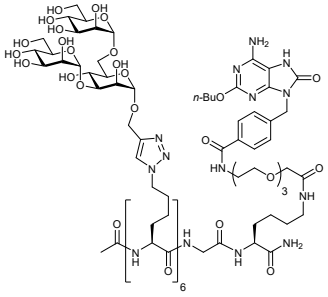
Carboxylic acid **119** (11.04 mg, 20.1 μmol, 1 eq) was dissolved in a solution of pentafluorophenol (120 μL, 0.34 M, 40.8 μmol, 2 eq) and *N,N′*-Diisopropylcarbodiimide (3.42 μL, 22.1 μmol, 1.1 eq) was added. After two hours, this solution was used without further workup.

Ac-Lys(Man$_1$)-Lys(Man$_1$)-Lys(Man$_1$)-Lys(Man$_1$)-Lys(Man$_1$)-Lys(Man$_1$)-Gly-Lys[1-(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)phenyl)-1-oxo-5,8,11-trioxa-2-azatridecan-13-oic amide]-NH_2 (121).



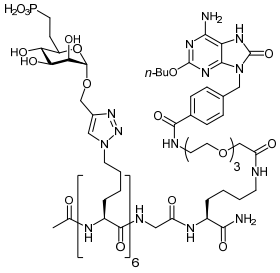
To a solution of **50** in water (250uL, 4.0 mM, 1.0 μmol, 1 eq) a stock solution of Pfp ester **120** (30 μL, 0.17 M, 5.0 μmol, 5 eq) and DIPEA (0.5 μL, 3 μmol, 3 eq) were added. The mixture was further diluted with DMSO (250 μL) until the mixture was a homogenous solution, and shaken overnight. After purification via RP-HPLC (linear gradient 7 - 55% B in A, 10 min, Gemini-NX 5μm C18, 110 Å, 250 x 10.0 mm, 5 mL/min) compound **121** was isolated as a white powder after lyophilization (2.19 mg, 727 nmol, 73%). <u>LC-MS:</u> R_t = 3.68 min (10 - 90% ACN; 13 min); <u>^{1}H NMR</u> (500 MHz, D_2O) δ 8.01 - 7.91 (m, 6H, trzl), 7.71 (d, *J* = 8.3 Hz, 2H, H-12*/H-13*), 7.40 (d, *J* = 8.2 Hz, 2H, H-12*/H-13*), 5.01 (s, 2H, H-10*), 4.92 (s, 6H, H-1), 4.77 - 4.70 (m, 6H, O-C*H*H-trzl), 4.65 - 4.56 (m, 6H, O-CH*H*-trzl), 4.37 - 4.25 (m, 12H, CH_2-trzl), 4.24 - 4.15 (m, 8H, H-16*, CH (K)), 4.13 - 4.08 (m, 1H, CH (K)), 3.93 - 3.50 (m, 52H, H-2, H-3, H-4, H-5, H-6, CH_2 (G), CH_2 (PEG, 7x)), 3.04 - 2.99 (m, 2H, C*H*$_2$-NH(CO) (K)), 1.97 (s, 3H, Ac), 1.87 - 1.62 (m, 24H, CH_2 (K)), 1.61 - 1.53 (m, 2H, H-17*), 1.37 - 1.18 (m, 20H, H-18*, CH_2 (K)), 0.82 (t, *J* = 7.4 Hz, 3H, H-19*); <u>HRMS</u> $[C_{125}H_{196}N_{34}O_{52} + 3H]^{3+}$: 1003.13252 found, 1003.13283 calculated.

Ac-Lys(Man$_3$)-Lys(Man$_3$)-Lys(Man$_3$)-Lys(Man$_3$)-Lys(Man$_3$)-Lys(Man$_3$)-Gly-Lys(1-(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)phenyl)-1-oxo-5,8,11-trioxa-2-azatridecan-13-oic amide)-NH$_2$ (122).



To a solution of **54** in water (250uL, 3.74 mM, 0.935 μmol, 1 eq) a stock solution of Pfp ester **120** (30 μL, 0.17 M, 5.0 μmol, 5.3 eq) and DIPEA (0.5 μL, 3 μmol, 3.2 eq) were added. The mixture was further diluted with DMSO (250 μL) until the mixture was a homogenous solution, and shaken overnight. After purification via RP-HPLC (linear gradient 7 - 55% B in A, 10 min, Gemini-NX 5μm C18, 110 Å, 250 x 10.0 mm, 5 mL/min) compound **122** was isolated as a white powder after lyophilization (2.15 mg, 429 nmol, 46%). LC-MS: R_t = 3.33 min (10 - 90% ACN; 13 min); ^{1}H NMR (500 MHz, D_2O) δ 8.02 - 7.92 (m, 6H, trzl), 7.72 (d, *J* = 8.1 Hz, 2H, H-12*/H-13*), 7.40 (d, *J* = 8.2 Hz, 2H, H-12*/H-13*), 5.06 (s, 6H, H-1/H-1'/H-1"), 5.02 (s, 2H, H-10*), 4.90 (s, 6H), 4.88 (s, 6H, H-1/H-1'/H-1"), 4.69 - 4.59 (m, 6H, O-CH*H*-trzl), 4.42 - 4.27 (m, 12H, CH_2-trzl), 4.27 - 4.16 (m, 8H, H-16*, CH (K)), 4.14 (dd, *J* = 5.5, 1.8 Hz, 1H, CH (K)), 4.10 - 3.50 (m, 124H, H-2, H-2', H-2", H-3, H-3', H-3", H-4, H-4', H-4", H-5, H-5', H-5", H-6, H-6', H-6", CH_2 (G), CH_2 (PEG, 7x)), 3.02 (d, *J* = 6.8 Hz, 2H, C*H*$_2$-NH(CO) (K)), 1.98 (s, 3H, Ac), 1.91 - 1.62 (m, 24H CH_2 (K)), 1.62 - 1.52 (m, 2H, H-17*), 1.42 - 1.16 (m, 20H, H-18*, CH_2 (K)), 0.83 (t, *J* = 7.4 Hz, 3H, H-19*); HRMS $[C_{197}H_{316}N_{34}O_{112} + 4H]^{4+}$: 1239.01239 found, 1239.01069 calculated.

Ac-Lys(M6P)-Lys(M6P)-Lys(M6P)-Lys(M6P)-Lys(M6P)-Lys(M6P)-Gly-Lys(1-(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)phenyl)-1-oxo-5,8,11-trioxa-2-azatridecan-13-oic amide)-NH$_2$ (124).



Mannose-6-phospate clusters **123**[30] (2.45 mg, 8.3 μmol, 1 eq) was dissolved in a stock solution of Pfp ester **120** (90 μL, 0.17 M, 15.0 μmol, 1.9 eq) and DIPEA (2 μL, 12 μmol, 1.5 eq) was added. After overnight shaking the mixture was purified via gel filtration (Toyopearl HW-40S, 1.6x60 cm, 150 mM NH_4OAc, 20% ACN, 1mL/min) (25 - 37 mL) followed by RP-HPLC (linear gradient 5 - 55% B in A, 10 min, Gemini-NX 5μm C18, 110 Å, 250 x 10.0 mm, 5 mL/min) to isolate compound **124** as a white powder after lyophilization (0.80 mg, 231 nmol, 2.8%). LC-MS: R_t = 5.68 min (0 - 50% ACN; 13 min); HRMS $[C_{131}H_{208}N_{34}O_{64}P_6^{6-} + 4NH_4^+ + 2\,Na^+ + 3H^+]^{3+}$: 1197.05295 found, 1197.13554 calculated.

References

(1) Gential, G. P. P.; Hogervorst, T. P.; Tondini, E.; van de Graaff, M. J.; Overkleeft, H. S.; Codée, J. D. C.; van der Marel, G. A.; Ossendorp, F.; Filippov, D. V. Peptides Conjugated to 2-Alkoxy-8-Oxo-Adenine as Potential Synthetic Vaccines Triggering TLR7. *Bioorg. Med. Chem. Lett.* **2019**, *29*, 1340–1344. https://doi.org/10.1016/j.bmcl.2019.03.048.

(2) Apostólico, J. D. S.; Lunardelli, V. A. S.; Coirada, F. C.; Boscardin, S. B.; Rosa, D. S. Adjuvants: Classification, Modus Operandi , and Licensing. *J. Immunol. Res.* **2016**, *2016*, 1–16. https://doi.org/10.1155/2016/1459394.

(3) Zom, G. G. P.; Khan, S.; Filippov, D. V.; Ossendorp, F. *TLR Ligand-Peptide Conjugate Vaccines. Toward Clinical Application.*, 1st ed.; Elsevier Inc., 2012; Vol. 114. https://doi.org/10.1016/B978-0-12-396548-6.00007-X.

(4) Muñoz-Wolf, N.; Lavelle, E. C. Innate Immune Receptors. In *Nod Like Receptors*; Pelegrin, P., Virgilio, F. Di, Eds.; Springer, 2016; pp 1–43. https://doi.org/10.1007/978-1-4939-3566-6_9.

(5) Gay, N. J.; Gangloff, M. Structure and Function of Toll Receptors and Their Ligands. *Annu. Rev. Biochem.* **2007**, *76*, 141–165. https://doi.org/10.1146/annurev.biochem.76.060305.151318.

(6) Jung, S.; von Thülen, T.; Laukemper, V.; Pigisch, S.; Hangel, D.; Wagner, H.; Kaufmann, A.; Bauer, S. A Single Naturally Occurring 2'-O-Methylation Converts a TLR7- and TLR8-Activating RNA into a TLR8-Specific Ligand. *PLoS One* **2015**, *10*, e0120498. https://doi.org/10.1371/journal.pone.0120498.

(7) Leslie, A. J.; Pichulik, T.; Khatamzas, E.; Mayer, A.; McMichael, A.; Simmons, A. Early Phosphorylation Events Induced in Dendritic Cells by the HIV Derived TLR8 Ligand SsRNA40. **2011**.

(8) Gantier, M. P.; Tong, S.; Behlke, M. A.; Xu, D.; Phipps, S.; Foster, P. S.; Williams, B. R. G. TLR7 Is Involved in Sequence-Specific Sensing of Single-Stranded RNAs in Human Macrophages. *J. Immunol.* **2008**, *180*, 2117–2124. https://doi.org/10.4049/jimmunol.180.4.2117.

(9) Krieg, A. M.; Yi, A.-K.; Matson, S.; Waldschmidt, T. J.; Bishop, G. A.; Teasdale, R.; Koretzky, G. A.; Klinman, D. M. CpG Motifs in Bacterial DNA Trigger Direct B-Cell Activation. *Nature* **1995**, *374*, 546–549. https://doi.org/10.1038/374546a0.

(10) Gibson, S. J.; Lindh, J. M.; Riter, T. R.; Gleason, R. M.; Rogers, L. M.; Fuller, A. E.; Oesterich, J. L.; Gorden, K. B.; Qiu, X.; McKane, S. W.; Noelle, R. J.; Miller, R. L.; Kedl, R. M.; Fitzgerald-Bocarsly, P.; Tomai, M. A.; Vasilakos, J. P. Plasmacytoid Dendritic Cells Produce Cytokines and Mature in Response to the TLR7 Agonists, Imiquimod and Resiquimod. *Cell. Immunol.* **2002**, *218*, 74–86. https://doi.org/10.1016/S0008-8749(02)00517-8.

(11) Mirer, E.; El Sayed, F.; Ammoury, A.; Lamant, L.; Messer, L.; Bazex, J. Treatment of Mammary and Extramammary Paget's Skin Disease with Topical Imiquimod. *J. Dermatolog. Treat.* **2006**, *17*, 167–171. https://doi.org/10.1080/09546630600788877.

(12) Bernhardt, S. L.; Buanes, T. A.; Møller, M.; Eriksen, J. A.; Geudernack, G. Imiquimod a New Adjuvant for Telomerase Peptide Vaccine: A Phase I Trial in Patients with Inoperable Pancreatic Cancer. *J. Clin. Oncol.* **2005**, *23*, 9623–9623. https://doi.org/10.1200/jco.2005.23.16_suppl.9623.

(13) Hirota, K.; Kazaoka, K.; Niimoto, I.; Kumihara, H.; Sajiki, H.; Isobe, Y.; Takaku, H.; Tobe, M.; Ogita, H.; Ogino, T.; Ichii, S.; Kurimoto, A.; Kawakami, H. Discovery of 8-Hydroxyadenines as a Novel Type of Interferon Inducer. *J. Med. Chem.* **2002**, *45*, 5419–5422. https://doi.org/10.1021/jm0203581.

(14) Moyle, P. M.; Toth, I. Modern Subunit Vaccines: Development, Components, and Research Opportunities. *ChemMedChem* **2013**, *8*, 360–376. https://doi.org/10.1002/cmdc.201200487.

(15) Jin, G.; Wu, C. C. N.; Tawatao, R. I.; Chan, M.; Carson, D. A.; Cottam, H. B. Synthesis and Immunostimulatory Activity of 8-Substituted Amino 9-Benzyladenines as Potent Toll-like Receptor 7 Agonists. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 4559–4563. https://doi.org/10.1016/j.bmcl.2006.06.017.

(16) Yoo, E.; Crall, B. M.; Balakrishna, R.; Malladi, S. S.; Fox, L. M.; Hermanson, A. R.; David, S. A. Structure-Activity Relationships in Toll-like Receptor 7 Agonistic 1H-Imidazo[4,5-c]Pyridines. *Org. Biomol. Chem.* **2013**, *11*, 6526–6545. https://doi.org/10.1039/c3ob40816g.

(17) Kurimoto, A.; Hashimoto, K.; Nakamura, T.; Norimura, K.; Ogita, H.; Takaku, H.; Bonnert, R.; McInally,

T.; Wada, H.; Isobe, Y. Synthesis and Biological Evaluation of 8-Oxoadenine Derivatives as Toll-like Receptor 7 Agonists Introducing the Antedrug Concept. *J. Med. Chem.* **2010**, *53*, 2964–2972. https://doi.org/10.1021/jm100070n.

(18) Shukla, N. M.; Lewis, T. C.; Day, T. P.; Mutz, C. A.; Ukani, R.; Hamilton, C. D.; Balakrishna, R.; David, S. A. Toward Self-Adjuvanting Subunit Vaccines: Model Peptide and Protein Antigens Incorporating Covalently Bound Toll-like Receptor-7 Agonistic Imidazoquinolines. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 3232–3236. https://doi.org/10.1016/j.bmcl.2011.04.050.

(19) Fujita, Y.; Hirai, K.; Nishida, K.; Taguchi, H. 6-(4-Amino-2-Butyl-Imidazoquinolyl)-Norleucine: Toll-like Receptor 7 and 8 Agonist Amino Acid for Self-Adjuvanting Peptide Vaccine. *Amino Acids* **2016**, *48*, 1319–1329. https://doi.org/10.1007/s00726-016-2190-7.

(20) Weterings, J. J.; Khan, S.; van der Heden van Noort, G. J.; Melief, C. J. M.; Overkleeft, H. S.; van der Burg, S. H.; Ossendorp, F.; van der Marel, G. A.; Filippov, D. V. 2-Azidoalkoxy-7-Hydro-8-Oxoadenine Derivatives as TLR7 Agonists Inducing Dendritic Cell Maturation. *Bioorganic Med. Chem. Lett.* **2009**, *19*, 2249–2251. https://doi.org/10.1016/j.bmcl.2009.02.095.

(21) Chan, M.; Hayashi, T.; Kuy, C. S.; Gray, C. S.; Wu, C. C. N.; Corr, M.; Wrasidlo, W.; Cottam, H. B.; Carson, D. A. Synthesis and Immunological Characterization of Toll-Like Receptor 7 Agonistic Conjugates. *Bioconjug. Chem.* **2009**, *20*, 1194–1200. https://doi.org/10.1021/bc900054q.

(22) Wang, X.-D.; Gao, N.-N.; Diao, Y.-W.; Liu, Y.; Gao, D.; Li, W.; Wan, Y.-Y.; Zhong, J.-J.; Jin, G.-Y. Conjugation of Toll-like Receptor-7 Agonist to Gastric Cancer Antigen MG7-Ag Exerts Antitumor Effects. *World J. Gastroenterol.* **2015**, *21*, 8052. https://doi.org/10.3748/wjg.v21.i26.8052.

(23) Akinbobuyi, B.; Byrd, M. R.; Chang, C. A.; Nguyen, M.; Seifert, Z. J.; Flamar, A.-L.; Zurawski, G.; Upchurch, K. C.; Oh, S.; Dempsey, S. H.; Enke, T. J.; Le, J.; Winstead, H. J.; Boquín, J. R.; Kane, R. R. Facile Syntheses of Functionalized Toll-like Receptor 7 Agonists. *Tetrahedron Lett.* **2015**, *56*, 458–460. https://doi.org/10.1016/j.tetlet.2014.11.126.

(24) Gential, G. P. P. Self Adjuvanting Immunopeptides : Design and Synthesis (Dissertation), Leiden University, 2018.

(25) Savage, P.; Horton, V.; Moore, J.; Owens, M.; Witt, P.; Gore, M. A Phase I Clinical Trial of Imiquimod, an Oral Interferon Inducer, Administered Daily. *Br. J. Cancer* **1996**, *74*, 1482–1486. https://doi.org/10.1038/bjc.1996.569.

(26) Xiang, Y.; Caron, P.-Y.; Lillie, B. M.; Vaidyanathan, R. Sulfur Contamination Due to Quenching of Halogenation Reactions with Sodium Thiosulfate: Resolution of Process Problems via Improved Quench Protocols. *Org. Process Res. Dev.* **2008**, *12*, 116–119. https://doi.org/10.1021/op700227p.

(27) Heller, K.; Ochtrop, P.; Albers, M. F.; Zauner, F. B.; Itzen, A.; Hedberg, C. Covalent Protein Labeling by Enzymatic Phosphocholination. *Angew. Chemie Int. Ed.* **2015**, *54*, 10327–10330. https://doi.org/10.1002/anie.201502618.

(28) Peterse, E. [To Be Determined] (Dissertation), Leiden University, 2020.

(29) Li, B.; Berliner, M.; Buzon, R.; Chiu, C. K. F.; Colgan, S. T.; Kaneko, T.; Keene, N.; Kissel, W.; Le, T.; Leeman, K. R.; Marquez, B.; Morris, R.; Newell, L.; Wunderwald, S.; Witt, M.; Weaver, J.; Zhang, Z.; Zhang, Z. Aqueous Phosphoric Acid as a Mild Reagent for Deprotection of Tert -Butyl Carbamates, Esters, and Ethers. *J. Org. Chem.* **2006**, *71*, 9045–9050. https://doi.org/10.1021/jo061377b.

(30) Reintjens, N. R. M. Synthetic Carbohydrate Ligands for Immune Receptors (Dissertation), Leiden University, 2020.