Chapter 2

7-Hydro-8-oxo-adenine derivatives as potent TLR7 ligands

Introduction

Toll-like receptor 7 (TLR7), one of the thirteen mammalian TLRs currently known, can be activated by specific imidazoquinolines. As such, TLR7 is the first TLR for which small molecule modifiers were identified, a result that has spawned great interest worldwide in the search after specific agents to either enhance a specific immune response (through activating a given TLR) in battling cancer or infectious disease, or reduce an immune response (through inactivating a TLR) in autoimmune disease therapies. TLRs appear to have evolved to recognize (partially degraded) biomolecules from microbial origin and upon recognition initiate a signaling cascade leading to a specific immune response against these microbial invaders. The natural TLR7 ligands are single strand RNA (ssRNA) oligonucleotides and the therapeutic potential of ssRNA molecules is thought to be limited. It is therefore fortuitous that TLR7 also recognizes imidazoquinolines and 7-hydro-8-oxo-adenines. Whether TLR7 activation by these adenine analogues (see for representative examples structure 1-4, Figure 2.1) occurs by binding to the ssRNA binding site or by occupation of an altogether different site of the TLR 7 receptor remains to be established. The archetypal imidazoquinoline-based TLR agonist is imiquimod, also known as R-837. Imiquimod is a component of the therapeutic ointment Aldara that is currently in use as a treatment for several skin diseases including superficial basal cell carcinoma.
Imiquimod, however is not an optimal TLR7 ligand and some side effects that could be linked to imiquimod\textsuperscript{14,15} spurned research towards alternative small molecule TLR7 agonists.

Hirota and co-workers\textsuperscript{16} conducted a study in which a compound library assembled from modified purines and pyrimidines were assessed on their TLR7 binding and agonistic properties. From these studies came compound 2 as a new lead. Ensuing optimalisation studies\textsuperscript{17-24} led, amongst others to 7-hydro-8-oxo-9-benzyl adenine derivative 3\textsuperscript{25} as the most potent TLR7 ligand in \textit{in vitro} assays, and to the analogue compound 4\textsuperscript{16} as the most potent TLR7 agonist in studies described to date. For the studies described in this Chapter, compound 3 was selected as the starting point. The aim of the study described here is two-fold: a) is it possible to enhance TLR7 binding and agonistic activity by directed modification of the alkyl substituents at C-2 in 4, and b) to introduce in derivatives of 3 a ligation handle for conjugation purposes, such as the TLR-ligand-peptide constructs as leads for vaccine development alluded to in the introduction.
Results and Discussion

Comparison of structures 3 and 4 reveals a subtle difference in the nature of the alkoxy spacer at C2 and it was decided to elaborate on this theme by the introduction of a number of different alkoxy substituents. For future investigation purposes it was decided to equip the alkoxides with a terminal azide amenable to functionalization via copper(I)-catalysed Huisgen cycloaddition\textsuperscript{26-28} with a range of acetylenes, leading to target 7-hydro-8-oxo-adenines 5a-g. Retrosynthetically (Scheme 2.1), the target compounds can be prepared from 9-benzyl-2-chloro-6-aminopurine \textit{9} by first introducing the alkoxy substituents at C2 and then installment of the oxo functionality at C8. From this retrosynthetic analysis it follows that aminochloropurine \textit{9} is the common intermediate from which the routes of synthesis towards the target compounds diverges. Aminochloropurine \textit{9} can in principle be prepared via two routes and both were investigated.

\begin{center}
\includegraphics[width=\textwidth]{Scheme2.1.png}
\end{center}

\textbf{Scheme 2.1}

\textbf{Reagents and Conditions:} \textit{i}) BnOH, PPh\textsubscript{3}, DIAD; \textit{ii}) BnCl, K\textsubscript{2}CO\textsubscript{3}, DMF, 68\% 7; \textit{iii}) TBAF, THF, BnBr, 61\% 7; \textit{iv}) NH\textsubscript{3} in MeOH, 95\% 9; \textit{v}) NH\textsubscript{3} in MeOH, 99\% 10; \textit{vi}) TBAF, THF, BnBr, 67\% 9.
In route A (Scheme 2.1) N-benzylation precedes displacement of the chlorine at C6 by ammonia. In a first attempt, reaction of 6 with benzyl alcohol under Mitsunobu conditions (PPh3, DIAD) proved troublesome. Both regio-isomers 7 and 8 were formed with concomitant formation of a number of intractable side products. In an alternative fashion, treatment of 6 with BnCl and potassium carbonate gave a clean conversion to give both regio-isomeric N-benzyl-dichloropurines 7 and 8 which were readily separated by silica gel chromatography (overall yield 86%, 7:8 = 3.4:1). A third method to obtain 7 via route A is the TBAF assisted benzylation of 6, which yields 7 as the major product (total yield: 97%, 7:8 = 2.2:1).

The desired regio-isomer 7 was treated with ammonia in methanol resulting in, after nucleophilic aromatic substitution, the formation of 2-chloroadenine derivative 9, in good (97%) yield and as the single regio-isomer. In the alternative route B, 2-chloroadenine 10, which can be obtained from 6 by treatment with NH3 in MeOH and is also commercially available, is N-benzylated at N9 giving key intermediate 9 in a single step (67%). Both routes are feasible and sufficient quantities of 9 for further modification were readily prepared.

With key intermediate 9 in hand, target compounds 5a-g were prepared in a straightforward fashion as outlined in Scheme 2.2. Nucleophilic aromatic substitution of the chlorine at C2 in 9 with a range of alcoholates proceeded uneventfully to give purine derivatives 12a-g in 38 to 69% yield. These intermediates were transformed into the target 7-hydro-8-oxo-adenines 5a-g.
by means of a reported three step procedure: 1) bromination, 2) displacement of Br with OMe and 3) acidic hydrolysis of the methoxy group. Although the yields vary for the individual steps, (see the Scheme 2.2 and Experimental for specific yields) each target compound was prepared in sufficient quantities and purity for ensuing TLR-binding studies.

The ability of the new purine derivatives to stimulate TLR7 mediated IL12 production was assessed in a comparative study in which the literature compounds 3 and 4 were included. Upon stimulation of TLR7 by its natural agonist (ssRNA) a series of events takes place resulting in, amongst others, the expression and secretion of the cytokine IL12. Enhanced IL12 production by TLR7-expressing DC cultures treated with potential TLR7 agonists therefore provides a first indication of actual TLR7 agonistic properties of the compounds at hand. It should be noted that the possibility of TLR7 independent IL12 production dictates that positive results need to be corroborated by further experiments. Briefly, the assay that was employed is as follows (see for more details the experimental section). Murine bone marrow derived cells were incubated for 24 hours with compounds 3, 4 and 5a-g at 5, 40 and 625 nM final concentrations. The supernatants were harvested and the amounts of IL12 in these were assessed using a standard sandwich ELISA assay. TLR7-agonism in this assay is evidenced by elevated IL12 production. This assay gives a reliable qualitative impression on the ability to induce TLR7 signalling, however the range of activities that can be determined is rather limited by constraints posed by the experimental limitations. In the tabulated results (Table 2.1), amounts of IL12 production that are below 5ng/ml get close to the background level. Detection of above 20 ng/ml IL12 levels should be viewed with care as the detection method is unreliable at such concentrations (because these values are out of the range of the standards).
The IL12 production stimulating properties of compounds 3, 4, and 5a-g.

Table 2.1: IL-12 production induced by compounds 3, 4 and 5a-g.

As can be seen, all compounds with the possible exception of 5g induce IL12 production at 625 nM final concentration. At this concentration no distinction between the potencies of the different compounds can be made and most compounds cluster around 20 ng/ml. At 40 nM concentrations some subtle differences become apparent, with analogues 5a and 5b expressing activities similar to that of reference compound 4. At the lowest concentration used the superior activity of 4 compared to all other compounds comes to light, but at least two of the new compounds (5a and 5b) retain some IL12 production stimulating activity.

Returning to the dual aim referred to in the introduction the small panel of compounds does not contain a more potent TLR-7 agonist compared to the literature compound 4, at least not in the assay employed here. On the positive side introduction of an azide moiety does not necessarily impede TLR7 activating properties, as is evidenced by the rather potent IL12 stimulating activities of derivatives 5a and 5b. This holds promise for the further derivatization of these new lead TLR7-ligands, and two separate strategies can be envisaged. In the first, the azide can be reduced to the amine which is then condensed with an acid to give an amide species. The viability of this approach was briefly investigated (Scheme 3) and although neither amine 14 (obtained after Staudinger reduction\textsuperscript{35,36} of 5a) nor acetamide 15 (obtained after N-acetylation\textsuperscript{37} of 14) showed enhanced IL12 production at 5, 40 or 625 nM final concentrations, the possibility to arrive at a range of more functionalized amides from 5a-g is obvious. In a alternative approach the azides in 5a-g may be employed in a Huisgen
[3+2] cycloaddition mediated derivatization approach. Here, a library of acetylenes should give access to a library of triazoles. Next to this, the azides can also be employed for conjugation to a peptide-derived acetylene to give potential leads for vaccine development. This approach is the subject of the next chapter, in which compound 5g features as the lead structure.

Scheme 2.3

**Reagents and Conditions:**

i) PMe$_3$, THF, H$_2$O, 31%;

ii) Ac$_2$O, NaHCO$_3$, MeOH, 49%.
Experimental Section

All chemicals and solvents used except for 2,6-dichloropurine, benzylbromide, TBAF, potassium carbonate, trimethylphosphine, sodium hydride, hydrochloric acid, dimethoxyethane and bromine were from Biosolve (The Netherlands) and used as received. Benzyl bromide (Fluka), 2,6-dichloropurine (3B Medical systems, USA), tetrabutylammonium fluoride; TBAF (Acros), potassium carbonate (Acros), hydrochloric acid (37%, Riedel-de Haën), trimethylphosphine (1M in THF, Aldrich), dimethoxyethane (Acros) and bromine (Acros) were used as received. TLC analysis was conducted on Merck 25 DC plastikfolien kieselgel 60 F254. Compounds were visualized by UV absorption (254 nm), by spraying with a solution of (NH₄)₆Mo₇O₂₄·4H₂O 25g/L and (NH₄)₄Ce(SO₄)₄·2 H₂O in 10% sulphuric acid followed by charring at ±140°C. Column chromatography was performed on Fluka silicagel (230-400 mesh). ¹H and ¹³C NMR spectra were recorded with a Bruker AC-200 (200/50.1 MHz) a Bruker AV 400 (400/100 MHz) and a Bruker DMX-600 (600/150/60.8 MHz) spectrometer. Chemical shifts (δ) are relative to tetramethylsilane unless stated otherwise. Mass spectra were recorded on a PE/SCIEX API 165 (Perkin-Elmer). IR spectra were recorded on a Shimadzu FTIR-8300 and are reported in cm⁻¹. Analytical LC/MS was conducted on a JASCO system using an Alltima C₁₈ analytical column (5μ particle size, flow: 1.0 ml/min). Absorbance was measured at 214 and 254 nm. Solvent system: A: 100% water, B: 100% acetonitrile, C: 1% TFA in water. RP-HPLC purifications were conducted on a BioCAD “Vision” automated HPLC system (PerSeptive Biosystems, inc.), supplied with a semipreparative Alltima C₁₈ column (5μ particle size, running at 4ml/min). Solvent system: A: 100% water, B: 100% acetonitrile, C: 1% TFA in water. The UV absorbance was measured with a Varian DMS 200 UV visible spectrophotometer. High resolution mass spectra were recorded by direct injection (2 μl of a 2 μM solution in water/acetonitrile; 50/50; v/v and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250°C) with resolution R = 60000 at m/z = 150-2000) and dioctylphthalate (m/z = 391.28428) as a “lock mass”³⁸. Microwave experiments were performed with an Emrys Optimizer microwave reactor (Personal Chemistry AB, Sweden), the temperature was measured by an infrared-sensor on the outer surface of the microwave vial.
**9-Benzyl-2,6-dichloropurine (7) via Route A using K$_2$CO$_3$/BnCl**

2,6-dichloropurine 6 (500 mg, 2.65 mmol) was dissolved in 25 mL DMF. Potassium carbonate (12.0 mmol, 1.65 gr.) and benzyl chloride (2 eq., 5.3 mmol, 0.62 mL) were added after which the reaction was stirred and subjected to an argon atmosphere. When TLC indicated the reaction to be complete the solvent was evaporated and the residue was dissolved in EtOAc (50 mL). The organic layer was washed twice with KH$_2$SO$_4$ (10%,aq.), dried (MgSO$_4$), filtered and concentrated. Column chromatography using a gradient of 5% to 50% EtOAc in PE yielded 500 mg of 7 (1.79 mmol, 68%).

$^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 8.11 (s, 1H, H-8), 7.38-7.30 (m, 5H, CH Arom.), 5.44 (s, 2H, CH$_2$);
$^{13}$C NMR (150 MHz, CDCl$_3$): $\delta$ 153.1, 151.7, 145.5, 11.7, 130.6 (C$_q$), 129.3-128.0 (CH Arom.), 47.9 (CH$_2$).

$^{15}$N NMR (60.8 MHz, CDCl$_3$): $\delta$ 243.8 (N7), 167.4 (N9). UV$_{max}$: 276 nm.

**9-Benzyl-2,6-dichloropurine (7) via Route A using TBAF/BnBr**

To 2,6-dichloropurine 6 (1.0 g, 5.3 mmol) in dry THF was added benzyl bromide (2 eq., 1.26 ml, 10.6 mmol) and TBAF (1M in THF, 10 mL, 10.6 mmol). The mixture was stirred at room temperature under argon for 2h. TLC analysis (PE/ EtOAc, 1/1, v/v) indicated the complete conversion of the starting compound. The solution was evaporated to dryness. The solid residue was dissolved in EtOAc and washed with sat. NaHCO$_3$. Column chromatography using a gradient of EtOAc/ PE (10 % to 40 % EtOAc in PE) yielded 7 (896 mg, 6.81 mmol, 61%) as a white solid.

**2-chloroadenine (10) via Route B from 6**

2,6-dichloropurine (750 mg, 3.70 mmol) was dissolved in saturated methanolic ammonia (3 mL) and heated to 100°C under microwave irradiation for 12 h. The mixture was concentrated and water was added. Compound 10 precipitated and was filtered off and air dried. Compound 10 was obtained as a yellow powder in 99% yield (622 mg, 3.67 mmol). $^1$H NMR (600 MHz, DMSO-$d_6$): 11.84 (bs, 1H, H$_9$ or H$_7$ due to isomerization), 8.23 (s, 1H, H$_8$), 7.61 (s, 2H, NH$_2$). IR: 3279, 3113, 1678, 1612, 1242.

**9-benzyl-2-chloroadenine (9) via Route A from 7**

Compound 7 (500 mg, 1.79 mmol) was placed in a pressure resistant glass vessel equipped with a stirring bar. Methanolic ammonia (3 mL) was added; the tube was sealed with a Teflon septum and transferred to a microwave reactor. The solution was heated at 100°C under 14 bar pressure for 8 h. with a pre-stirring time of 30 seconds. The solvent was evaporated and the residue was purified by column chromatography using a gradient of 0-5% MeOH in DCM. Yield: 441 mg (1.70 mmol, 95%) of compound 9 as a white solid. $^1$H NMR (600 MHz, DMSO-$d_6$): $\delta$ 8.24 (s, 1H, H$_8$), 7.79 (bs, 2H, NH$_2$), 7.34-7.26 (m, 5H, CH Arom.), 5.53 (s, 2H, CH$_2$); $^{13}$C NMR (50.1 MHz, DMSO-$d_6$): $\delta$ 156.9,
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153.2, 150.7, 141.5, 136.8 (Cq), 128.8-127.4 (CH Arom.), 117.8 (Cq), 46.3 (CH2Bn); 15N NMR (60.8 MHz, CDCl3): δ 243.5 (N7), 232.7 (N1), 165.8 (N9), 88.7 (NH2); IR: 3450, 3080, 1653, 1593, 1350, 1307, 1153, 929. ESI-MS: m/z 260.0 [M+H, 35Cl]+. HRMS: C12H10N535Cl + H+ calculated 260.06975, found 260.07087.

9-benzyl-2-chloroadenine (9) via Route B from 10 using TBAF/BnBr

2-chloroadenine 10 (5.0 gr, 29.6 mmol) was co-evapped thrice with DCE and dissolved in 59 ml of 1M TBAF in THF (2eq). Benzyl bromide (2 eq., 59.2 mmol, 7.1 mL) was added after which the reaction was subjected to an argon atmosphere. When TLC indicated the reaction to be complete the solvents were evaporated and the residue was applied to column chromatography using a gradient of 100% EtOAc to 1% MeOH/EtOAc yielding 5.20 gr. of white powder (19.9 mmol, 67%).

9-benzyl-2-azidoethoxyadenine (12a)

A solution of azidoethanol40 (83 mg, 0.96 mmol, 5 eq.), which was co-evaporated with CHCl3 twice, and NaH (60% in mineral oil, 38 mg, 0.96 mmol, 5 eq.) in 5 mL freshly distilled DME was added, after 15 minutes of stirring at r.t., to a solution of 9 (50 mg, 0.19 mmol) in DME, which was co-evaporated with CHCl3 twice. The mixture was refluxed (85°C) for 36 h. and then cooled to r.t. After evaporation of the solvent the crude was purified by means of column chromatography using a 70 to 100% EtOAc in PE gradient yielding 35 mg (0.11 mmol, 59%) of compound 12a as a white powder. 

1H NMR (200 MHz, CDCl3): δ 7.62 (s, 1H, H8) 7.36-7.27 (m, 5H, CH arom.), 6.13 (bs, 2H, NH2), 5.27 (s, 2H, CH2), 4.51 (t, 2H, OCH2), 3.64 (t, 2H, -CH2N3). 13C NMR (50.1 MHz, CDCl3): δ 161.5, 156.5, 151.7, 139.0, 135.6 (Cq), 128.9-127.8 (CH arom.), 115.8, 65.2, 50.0, 47.0 (CH3); IR: 3288, 3113, 2930, 2096, 1659, 1597. ESI-MS: m/z 311.0 [M+H]+. HRMS: C14H15N8O + H+ calculated 311.13688, found 311.13633.

9-benzyl-2-azidopropoxyadenine (12b)

A solution of azidopropanol40 (485 mg, 4.80 mmol, 5 eq.), and NaH (60% in mineral oil, 188 mg, 4.8 mmol, 5 eq.) in 5 mL freshly distilled DME was added, after 15 min of stirring at r.t., to a solution of 9 (250 mg, 0.96 mmol), which was co-evaporated with DME twice. This reaction mixture was placed in a pressure resistant glass vessel equipped with a stirring bar. The tube was sealed with a Teflon septum and transferred to a microwave reactor. The solution was heated at 80°C for 12 h. with a pre-stirring time of 30 seconds. The solvent was evaporated and the residue was purified by means of column chromatography using 70 to 100% EtOAc in PE gradient, yielding 170 mg (0.52 mmol, 55%) of compound 12b as a white powder. 

1H NMR (600 MHz, CDCl3): δ 7.60 (s, 1H, H8) 7.37-7.28 (m, 5H, CH arom.), 5.57 (bs, 2H, NH2), 5.2 (s, 2H, CH2Bn), 4.44 (t, 2H, OCH2), 3.54 (t, 2H, CH2N3), 2.09 (m, 2H, CH2CH2CH2); 13C NMR (150 MHz, CDCl3): δ 161.9 (C2), 156.2 (C6), 151.9 (C4), 139.1 (C8), 135.7 (Cq), 129.0-127.9 (CH arom.), 115.8 (C5), 64.0 (OCH2), 48.3 (CH2N3), 47.1 (CH2Bn), 28.6
9-benzyl-2-azidobutoxyadenine (12c)

Azidobutanol (3.86 mmol, 5 eq., 444 mg) which was co-evaporated thrice using DCE was added to 15 mL of freshly distilled DME. The solution was cooled to 0°C after which NaH was added (87.4 mg, 3.86 mmol, 5 eq.). After 15 min. of stirring purine (200 mg, 0.77 mmol) was added and the reaction was refluxed for 3 days at 85°C when TLC showed no further progress of the reaction. After evaporation of the solvent the residue was dissolved in DCM/MeOH and silica was added. The suspension was dried and purified with column chromatography using 100% EtOAc yielding compound 12c as a white powder (101.5 mg, 0.30 mmol, 39%).

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\begin{align*}
\text{H NMR (400 MHz, DMSO-d_6):} & \quad \delta 8.03 (s, 1H, H_8), 7.35-7.21 (m, 5H, CH arom.), 5.25 (s, 2H, CH_2Bn), 4.23 (t, 2H, CH_2), 3.36 (t, 2H, CH_2), 1.67 (m, 4H, 2x CH_2); \\
\text{13C NMR (100 MHz, DMSO-d_6):} & \quad \delta 161.4, 156.8, 151.1 (C_9), 139.6 (C_8), 128.6, 127.7, 127.6 (CH Arom.), 65.5 (CH_2Bn), 50.5 (CH_2), 45.9 (CH_2), 28.9 (CH_2), 3120, 1659, 1595, 1335, 1267. \\
\text{ESI-MS: m/z 325.0 [M+H]^+}. \\
\text{HRMS: C_{15}H_{16}N_8O + H^+ calculated 325.14471, found 325.15031.}
\end{align*}
\]

9-benzyl-2-azidopentoxyadenine (12d)

A solution of azidopentanol (260 mg, 1.90 mmol, 5 eq.), was co-evaporated with CHCl_3 twice, and NaH (60% in mineral oil, 5 eq., 76 mg, 1.9 mmol) in 10 mL freshly distilled DME was added, after 15 min. of stirring at r.t., to a solution of (100 mg, 0.38 mmol), which was also co-evaporated with CHCl_3 twice. The mixture was refluxed (85°C) for 48 h. and then cooled to r.t. After evaporation of the solvent the residue was purified by means of column chromatography using a gradient of 70 to 100% EtOAc in PE yielding compound 12d (56 mg, 0.16 mmol, 42%) as a white powder. 1H NMR (200 MHz, CDCl_3): \( \delta 7.58 \) (s, 1H, H_8), 7.32-7.27 (m, 5H, CH arom.), 5.94 (bs, 2H, NH_2), 5.26 (s, 2H, CH_2Bn), 4.34 (t, 2H, OCH_2-), 3.28 (t, 2H, CH_2N_3), 1.67-1.55 (m, 6H, 3x CH_2); 13C NMR (50.1 MHz, CDCl_3): \( \delta 210.9 \) (C_4), 162.2 (C_2), 156.4 (C_6), 138.5 (C_8), 135.8 (C_9), 128.9-127.8 (5x CH arom.), 115.7 (C_5), 66.8 (OCH_2), 51.3 (CH_2N_3), 46.9(CH_2Bn), 28.6, 28.5, 23.3 (3x CH_2); IR: 3120, 2086, 1596, 1334.  ESI-MS: m/z 353.1 [M+H]^+. HRMS: C_{17}H_{20}N_8O + H^+ calculated 353.18328, found 353.18334.

9-benzyl-2-azidohexanoxy adenine (12e)

To a solution of azidohexanol (3.86 mmol, 5 eq.), and NaH (60% in mineral oil, 146 mg, 3.86 mmol, 5 eq.) in 15 mL freshly distilled DME was added, after 15 min of stirring at 0°C 9 (200 mg, 0.77 mmol), which was co-evaporated with DME twice. The mixture was refluxed at 85°C overnight after which TLC indicated the reaction to be complete. The solvent was evaporated and the residue was dissolved in DCM, washed trice with water, brine after which the water layers were back extracted with DCM. The combined organic layers were dried, concentrated and applied to column chromatography using 1 to 4% MeOH in DCM yielding 175 mg (0.48 mmol, 62%) of compound 12e.
as a white powder. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.59 (s, 1H, H-8), 7.33-7.25 (5H, CH Arom.), 6.52 (s, 2H, NH$_2$), 5.27 (s, 2H, CH$_2$Bn), 4.32 (t, 2H, CH$_2$), 3.27 (t, 2H, CH$_2$), 1.81-1.74 (m, 2H, CH$_2$), 1.66-1.58 (m, 2H, CH$_2$), 1.56-1.54 (m, 2H, CH$_2$), 1.50-1.40 (m, 2H, CH$_2$); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 162.1, 156.6, 154.1 (C$_q$), 138.5 (C-8), 137.7 (C$_q$), 128.8-127.7 (CH Arom), 115.4 (C$_q$), 66.8 (CH$_2$Bn), 51.2, 46.7, 28.7, 28.6, 26.3, 25.5 (CH$_2$); IR: 3120, 2858, 2928, 2091, 1593, 1335, 1265. ESI-MS: m/z 367.1. [M+H]$^+$ HRMS: C$_{18}$H$_{22}$N$_8$O + H$^+$ calculated 367.19893, found 367.19897.

9-benzyl-2-(2-azidoethoxy)ethoxyadenine (12f)
Azidoethoxyethanol$^{44}$ (3.86 mmol, 5 eq) was dissolved in 10 mL freshly distilled DME and the solution was cooled to 0°C after which NaH (60% in mineral oil, 154 mg, 3.86 mmol, 5 eq.) was added, after 15 min of stirring at 0°C a solution of 9 (200 mg, 0.77 mmol, co-evapped thrice in DCE) in 5 mL DME, was added. The mixture was refluxed at 85°C overnight after which TLC indicated the reaction to be complete. The solvent was evaporated and the residue was applied to column chromatography using a two step gradient; 25% toluene in EtOAc to 100% EtOAc to 5% MeOH in EtOAC yielding 104 mg (0.29 mmol, 38%) of compound 12f as a white powder. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.60 (s, 1H, H-8), 7.34-7.22 (m, 5H, CH Arom.), 6.43 (bs, 2H, NH$_2$), 5.25 (s, 2H, CH$_2$Bn), 4.51 (t, 2H, CH$_2$), 3.86 (t, 2H, CH$_2$), 3.72 (t, 2H, CH$_2$), 3.41 (t, 2H, CH$_2$); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 161.8, 156.5, 151.6 (C$_q$), 138.7 (C-8), 135.7 (C$_q$), 128.8-127.7 (CH Arom), 115.6 (C$_q$), 69.9 (CH$_2$Bn), 69.4 (CH$_2$), 66.1 (CH$_2$), 50.6 (CH$_2$), 46.8 (CH$_2$).

9-benzyl-2-(2-[2-(2-azidoethoxy)ethoxy]ethoxy)adenine (12g)
2-(2-(2-azidoethoxy)ethoxy)ethanol$^{45}$ (7 eq, 708 mg, 4.10 mmol) and NaH (60% in mineral oil, 7 eq, 164 mg, 4.10 mmol) were added to a solution of 9 (150 mg, 0.58 mmol) in freshly distilled 1,2-dimethoxyethane (10 mL). The mixture was refluxed (85°C) overnight and then cooled to rt. After evaporation of the solvent the residue was chromatographed 0-5% MeOH in DCM yielding 12g (159 mg, 0.40 mmol, 69%) as a white solid. (12g) $^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 7.58 (s, 1H, H-8) 7.29-7.23 (m, 5H, CH arom.), 6.30 (bs, 2H, NH$_2$), 5.23 (s, 2H, CH$_2$Bn), 4.84 (t, 2H, CH$_2$), 3.84 (t, 2H, CH$_2$), 3.74-3.63 (m, 6H, 3x CH$_2$), 3.35 (t, 2H, CH$_2$); $^{13}$C NMR (50.1 MHz, CDCl$_3$): $\delta$ 161.7, 156.5, 151.4 (C$_q$), 138.5 (C$_q$), 135.7 (C$_q$), 128.7-127.6 (5x CH arom.), 115.4 (C$_q$), 70.4 (2x CH$_2$), 69.7 (CH$_2$), 69.3 (CH$_2$), 66.0 (CH$_2$), 50.4 (CH$_2$), 46.7 (CH$_2$Bn); IR: 2871, 2104, 1643, 1593, 1330, 1120. ESI-MS: m/z 399.0 [M+H]$^+$ HRMS: C$_{18}$H$_{22}$N$_8$O$_3$ + H$^+$ calculated 399.18876, found 399.18903.

9-benzyl-8-bromo-2-azidoethoxyadenine (13a)
9-benzyl-2-azidoethoxy-adenine 12a (35.0 mg, 0.11 mmol) was dissolved in 10 mL DCM after which Br$_2$ (100 μL, 1.94 mmol) was added. The mixture was shielded from light and stirred at room temperature for 5 h. TLC (eluent: 100% EtOAc) indicated the complete conversion of the starting compound into a higher running product. After concentration the residue was applied to a silica gel column and eluted with a gradient of EtOAc in PE (50 to 100%) to yield 13a (30.0 mg, 0.08 mmol,
67%) as a white solid. $^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 7.32 (s, 5H, CH arom.), 5.87 (bs, 2H, NH$_2$), 5.30 (s, 2H, CH$_2$N), 4.50 (t, 2H, CH$_2$), 3.64 (t, 2H, CH$_2$). $^{13}$C NMR (50.1 MHz, CDCl$_3$): $\delta$ 161.3, 155.4, 152.7, 135.6 (C$_q$), 128.9-127.8 (5x CH arom.), 124.8, 116.3, 65.3, 49.9, 47.3 (CH$_2$Bn); IR: 3466, 3088, 2095, 1645, 1595. ESI-MS: m/z 389.2 ($^{79}$Br) [M+H]$^+$, 391.1 ($^{81}$Br ) [M+H]$^+$. HRMS: C$_{14}$H$_{14}$N$_8$O$_7^{99}$Br + H$^+$ calculated 389.04739, found 389.04685.

9-benzyl-8-bromo-2-azidopropoxyadenine (13b)

9-benzyl-2-(3-azidopropoxy)-adenine (12b) (170 mg, 0.52 mmol) was dissolved in 10 mL DCM after which Br$_2$ (52 $\mu$L, 1.0 mmol) was added. The mixture was shielded from light and stirred at room temperature for 5 h. TLC (eluent: 100% EtOAc) indicated the complete conversion of the starting material into a higher running product. After concentration the crude product was analysed and synthesis was continued without purification. $^1$H NMR (200 MHz, acetone-$d_6$): $\delta$ 8.13 (2H, NH$_2$), 7.38-7.32 (m, 5H, CH arom.), 5.40 (s, 2H, CH$_2$Bn), 4.56 (t, 2H, OCH$_2$-), 3.55 (t, 2H, CH$_2$N$_3$), 2.06 (m, 2H, CH$_2$CH$_2$CH$_2$); IR: 2095, 1693, 1605, 1508, 1257. ESI-MS: m/z 403.1 ($^{79}$Br) [M+H]$^+$, 405.1 ($^{81}$Br ) [M+H]$^+$. HRMS: C$_{15}$H$_{15}$Br$_{79}$N$_8$O + H$^+$ calculated 403.05522, found 403.06153, C$_{15}$H$_{15}$Br$_{81}$N$_8$O + H$^+$ calcd. 405.05946.

9-benzyl-8-bromo-2-azidobutoxyadenine (13c)

Compound (63.7 mg, 0.188) was dissolved in 10 mL DCM after which 0.5 mL (9.69 mmol) of Br$_2$ was added, the reaction was shielded from light and stirred overnight. The mixture was concentrated to dryness by an airflow, after which the crude was subjected to column chromatography and purified by using a gradient of 1 to 5% MeOH in DCM. Yield 64.2 mg, 0.154 mmol, 82%. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.36-7.24 (m, 5H, CH arom.), 6.15 (s, 2H, NH$_2$), 5.27 (s, 2H, CH$_2$Bn), 4.30 (t, 2H, CH$_2$), 3.37 (t, 2H, CH$_2$), 1.76-1.62 (2x m, 4H, 2x CH$_2$); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 154.0, 151.9, 135.7 (C$_q$), 128.7, 127.8, 127.3 (CH Arom Bn), 124.6, 115.1 (C$_q$), 66.5 (CH$_2$Bn), 50.4 (CH$_2$), 46.6 (CH$_2$), 25.6 (CH$_2$), 24.9 (CH$_2$). IR: 2950, 2077, 1695, 1604, 1286. ESI-MS: m/z 418.93 ($^{81}$Br) [M+H]$^+$. HRMS: C$_{16}$H$_{17}$N$_8$OBr$_{79}$N$_9$O + H$^+$ calculated 417.07825, found 417.07825.

9-benzyl-8-bromo-2-azidopentoxyadenine (13d)

9-benzyl-2-azidopentoxy-adenine (12d) (56 mg, 0.16 mmol) was dissolved in 20 mL DCM after which Br$_2$ (160 $\mu$L, 3.1 mmol) was added. The mixture was shielded from light and stirred at r.t. for 5 hrs. TLC (eluent: 100% EtOAc) indicated the complete consumption of the starting compound. After concentration the residue was applied to a silica gel column and eluted with a gradient of EtOAc in PE (90 to 100%) to yield 42 mg (0.10 mmol, 61%) of compound 13d as a white solid. $^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 7.33 (s, 5H, CH arom.), 6.85 (bs, 2H, NH$_2$), 5.30 (s, 2H, CH$_2$Bn), 4.38 (t, 2H, CH$_2$), 3.29 (t, 2H, CH$_2$), 1.87-1.57 (m, 6H, 3x CH$_2$); $^{13}$C NMR (50.1 MHz, CDCl$_3$): $\delta$ 170.3, 159.2, 153.5, 152.3, 134.7 (C$_q$), 128.9-127.8 (5x CH arom.), 125.8 (C-Br), 68.2 (OCH$_2$), 51.3 (CH$_2$N$_3$), 47.6 (CH$_2$Bn), 28.5, 27.8, 23.2 (3x CH$_2$). IR: 2925, 2096, 1695, 1589, 1333. ESI-MS: m/z 431.1 [M+H]$^+$.
9-benzyl-8-bromo-2-azidohexanoyladenine (13e)
Compound 12e (135.3 mg, 0.37 mmol) was dissolved in 10 mL DCM. Bromine (0.5 mL, 9.7 mmol) was added and the reaction was shielded from light using aluminium foil and allowed to stir overnight. The crude was purified by column chromatography using a gradient of 1 to 3% MeOH in DCM. Yield: 56.1 mg (0.126 mmol, 34%). ¹H NMR (200 MHz, CDCl₃): δ 7.35-7.32 (m, 5H, CH Arom.), 5.67 (bs, 2H, NH₂), 5.30 (s, 2H, CH₂Bn), 4.32 (t, 2H, CH₂), 3.27 (t, 2H, CH₂), 1.80 (m, 2H, CH₂), 1.66 (m, 2H, CH₂), 1.47 (m, 4H, 2x CH₂). ¹³C NMR (50.1 MHz, CDCl₃): δ 155.0, 135.1 (C₉), 128.5, 127.9, 127.6 (CH Arom.), 66.9, 51.1 (CH₂), 47.0 (CH₂Bn), 28.6 (2*CH₂), 26.2, 25.4 (CH₂). HRMS: C₁₈H₂₁N₈OBr (79Br) + H⁺ calculated 445.10945, found 445.10945.

9-benzyl-8-bromo-2-(2-azidoethoxy)ethoxyadenine (13f)
Compound 12f (153 mg, 0.37 mmol) was dissolved in 10 mL of DCM and 0.5 mL (9.7 mmol) of Br₂ was added. The reaction was shielded from light using aluminium foil. After TLC indicated a completed reaction, a N₂ flow was led into the flask until an off-white solid remained. The crude was purified by means of column chromatography using a gradient of 1/1 Toluene/EtOAc to 100% EtOAc. Yield 13f: 35.5 mg, (0.08 mmol, 54%). HRMS: C₁₆H₁₇N₈OBr (81Br) + H⁺ calculated 435.07118, found 435.07071.

9-benzyl-8-bromo-2-[2-(2-azidoethoxy)ethoxy]ethoxyadenine (13g)
9-benzyl-2-[2-(2-azidoethoxy)ethoxy]ethoxyadenine 12g (159 mg, 0.40 mmol) was dissolved in 30 mL DCM after which Br₂ (400 μL, 7.75 mmol) was added. The mixture was shielded from light (aluminium foil) and stirred overnight at room temperature. TLC (eluent: 5% MeOH in DCM) indicated the complete conversion of the starting compound into a higher running product. After concentration the residue was applied to a silica gel column and eluted with a gradient of MeOH in DCM (0 to 5 %) to yield 148 mg (0.31 mmol, 78%) as a white solid. (13g) ¹H NMR (200 MHz, CDCl₃): δ 7.33-7.26 (m, 5H, CH arom.), 6.34 (bs, 2H, NH₂), 5.27 (s, 2H, CH₂Bn), 4.81 (t, 2H, CH₂), 3.72 (t, 2H, CH₂), 3.69-3.64 (m, 6H, 3x CH₂), 3.37 (t, 2H, CH₂). ¹³C NMR (50.1 MHz, CDCl₃): δ 161.6, 155.3, 152.4, 135.1 (C₉), 128.5-127.6 (5x CH arom.), 124.1 (C-Br), 115.9 (C₉), 70.4 (2x CH₂), 69.8 (CH₂), 69.2 (CH₂), 66.2 (CH₂), 50.4 (CH₂), 47.0 (CH₂Bn); IR: 3416, 3324, 2109, 1636, 1593, 1323. ESI-MS: m/z 477.1 [M+H]⁺. HRMS: C₁₈H₂₁N₈O₃Br + H⁺ calculated 479.09723(81Br), found 479.09734; calculated 477.09928 (79Br), found 477.09959.
9-benzyl-7-hydro-8-oxo-2-azidoethoxyadenine (5a)

9-benzyl-8-bromo-2-azidoethoxy-adenine \(13a\) (30 mg, 0.08 mmol) was dissolved in 5 mL MeOH after which NaOMe (0.24 mL, 30% sol. in MeOH, 1.5 mmol, 20 eq.) was added. This mixture was refluxed (65°C) over night. TLC analysis (100% EtOAc) showed complete conversion of the starting material. The mixture was transferred to a separating funnel and 1/1 DCM/H\(_2\)O was added. The aqueous layer was extracted twice with DCM after which the organic layers were combined, dried (MgSO\(_4\)) and concentrated in vacuo. The methoxide was obtained quantitatively (27.0 mg, 0.08 mmol) as a white solid and used in the next step.\(^1\)H NMR (200 MHz, CDCl\(_3\)): \(\delta\) 7.30 (m, 5H, CH arom.), 5.54 (bs, 2H, NH\(_2\)), 5.09 (s, 2H, CH\(_2\)Bn), 4.48 (t, 2H, OCH\(_2\)), 4.09 (s, 3H, OMe), 3.63 (t, 2H, CH\(_2\)N\(_3\)); \(^{13}\)C NMR (50.1 MHz, CDCl\(_3\)): \(\delta\) 159.9 (C\(_2\)), 154.6 (C\(_8\)), 153.9 (C\(_6\)), 151.4 (C\(_4\)), 136.1 (C\(_q\)), 128.6 -127.9 (5x CH Arom.), 111.2 (C\(_5\)), 65.0 (OCH\(_2\)), 56.8 (OMe), 50.0 (CH\(_2\)N\(_3\)), 44.6 (CH\(_2\)Bn); IR: 3325, 3170, 2931, 2338, 2098, 1605, 1396, 1327. ESI-MS: \(m/z\) 341.0 [M] +. HRMS: C\(_{15}\)H\(_{17}\)N\(_8\)O\(_2\) + H + calculated 341.14715, found 341.14690.

The methoxide was dissolved in HCl (7 mL, 37%). The mixture was stirred until LCMS revealed complete disappearance of the starting material. After removal of the solvent the compound was taken up in water and brought to high pH using sat NH\(_4\)OH. The mixture was taken up in water which was extracted with DCM (thrice), after which the organic layers were combined, dried (MgSO\(_4\)) and concentrated in vacuo. 21.0 mg of compound \(5a\) (0.07 mmol, 85%) was obtained as a white solid.\(^1\)H NMR (600 MHz, DMSO-\(d_6\)): \(\delta\) 7.33-7.28 (m, 5H, arom.), 6.71 (bs, 2H, NH\(_2\)), 4.86 (s, 2H, CH\(_2\)Bn), 4.31 (t, 2H, OCH\(_2\)), 3.60 (t, 2H, CH\(_2\)N\(_3\)); \(^{13}\)C NMR (150 MHz, DMSO-\(d_6\)): \(\delta\) 159.5 (C\(_2\)), 152.2 (C\(_6\)), 148.9 (C\(_8\)), 147.9 (C\(_4\)), 137.2 (C\(_q\)), 128.5-127.4 (CH arom.), 64.9, 49.6, 42.4 (CH\(_2\)Bn); IR: 3421, 3348, 2924, 2102, 1647, 1608, 1412, 1361. ESI-MS: \(m/z\) 327.1 [M+H] +. HRMS: C\(_{14}\)H\(_{15}\)N\(_8\)O\(_2\) + H + calculated 327.13180, found 327.13125.

9-benzyl-7-hydro-8-oxo-2-azidopropyloxyadenine (5b)

Bromo compound \(13b\) was dissolved in 15 mL MeOH after which 1.3 mL NaOMe solution (30% sol. in MeOH, 4.3 mmol, 8 eq.) was added. This mixture was refluxed (65°C) overnight. TLC analysis (100% EtOAc) showed complete conversion of the starting material. The mixture was separated between H\(_2\)O and DCM, after which the organic layer was dried using MgSO\(_4\) and concentrated. The residue was chromatographed using 50 to100% EtOAc in PE yielding 173 mg (0.48 mmol) compound as a white powder, which was used in the next step. (yield: 94% over two steps).\(^1\)H NMR (600 MHz, CDCl\(_3\)): \(\delta\) 7.33-7.26 (m, 5H, CH arom.), 5.29 (s, 2H, NH\(_2\)), 5.01 (s, 2H, CH\(_2\)Bn), 4.40 (t, 2H, OCH\(_2\)), 4.09 (s, 3H, OMe), 3.52 (t, 2H, CH\(_2\)N\(_3\)), 2.11 (m, 2H, CH\(_2\)CH\(_2\)CH\(_2\)); \(^{13}\)C NMR (150 MHz, CDCl\(_3\)): \(\delta\) 160.4 (C\(_2\)), 154.7 (C\(_4\)), 153.8 (C\(_6\)), 151.6 (C\(_8\)), 136.2 (C\(_q\)), 128.7-127.9 (CH arom.), 111.0 (C\(_5\)), 63.8 (OCH\(_2\)), 56.8 (OMe), 48.4 (CH\(_3\)), 44.5 (CH\(_2\)Bn), 28.6 (CH\(_2\)CH\(_2\)); IR: 2087, 1647, 1601, 1396, 1334, 1245. ESI-MS: \(m/z\) 354.7 [M] +. HRMS: C\(_{16}\)H\(_{18}\)N\(_8\)O\(_2\) + H + calculated 355.15527, found 355.16069. The methoxide was dissolved in HCl (30 mL, 37%) and the mixture was stirred overnight. After in vacuo removal of the solvent the compound was taken up in water and brought to high pH using sat. NH\(_4\)OH. The mixture was taken up in H\(_2\)O after which it was extracted with DCM (thrice).
The organic layers were dried using MgSO$_4$ and concentrated in vacuo. Compound 5b (127 mg (0.37 mmol, 78%) was obtained as a white solid. $^1$H NMR (600 MHz, DMSO-$d_6$): δ 9.99 (bs, 1H, OH), 7.33-7.24 (m, 5H, arom.), 6.49 (bs, 2H, NH$_2$), 4.85 (s, 2H, CH$_2$Bn), 4.21 (t, 2H, OCH$_2$), 3.56 (t, 2H, CH$_2$N$_3$), 1.93 (m, 2H, CH$_2$CH$_2$CH$_2$). $^{13}$C NMR (150 MHz, DMSO-$d_6$): δ 159.8 (C2), 152.2 (C4), 149.1 (C6), 147.8 (C8), 137.1 (Cq), 128.5-127.3 (CH arom.), 98.3 (C5), 63.3 (OCH$_2$), 47.7 (CH$_2$N$_3$), 42.4 (CH$_2$Bn), 27.9 (CH$_2$CH$_2$CH$_2$); IR: 2095, 1682, 1608, 1439, 1350, 1246. ESI-MS: m/z 341.0 [M+H]$^+$. HRMS: C$_{15}$H$_{16}$N$_8$O$_2$ + H$^+$ calculated 341.13962, found 341.14531.

9-benzyl-7-hydro-8-oxo-2-azidobutoxyadenine (5c)

Compound 13c (64.2 mg, 0.154 mmol) was dissolved in 10 mL MeOH and NaOMe (20 eq., 3.08 mmol, 166 mg) was added. The mixture was refluxed at 65°C overnight. TLC (5% MeOH in DCM) revealed complete conversion of the starting material into the desired methoxide. The crude was purified by means of column chromatography, 1 to 3% MeOH in DCM. Yield 38.4 mg (0.10 mmol, 68%). $^1$H NMR (400 MHz, DMSO-$d_6$): δ 7.34-7.22 (m, 5H, CH arom.), 6.84 (s, 2H, NH$_2$), 5.03 (s, 2H, CH$_2$), 4.20-4.17 (t, 2H, CH$_2$), 4.03 (s, 3H, OCH$_3$), 1.73-1.61 (m, 4H, 2x CH$_2$). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 160.6, 155.1, 153.9, 151.4, 137.2 (Cq), 129.1-127.7 (CH Arom Bn), 65.9 (CH$_2$Bn), 57.3 (CH$_3$), 50.9 (CH$_2$), 44.3 (CH$_2$), 26.4 (CH$_2$), 25.6 (CH$_2$); IR: 3150, 2087, 1665, 1597, 1564, 1348, 995. ESI-MS: m/z 369.0 [M+H]$^+$. HRMS: C$_{17}$H$_{20}$N$_8$O$_2$ + H$^+$ calculated 369.17820, found 369.17832. The methoxide (35.5 mg, 0.10 mmol) was dissolved in HCl (10 mL, 37%) and allowed to stir overnight at rt. The solvent was evaporated. Milipore (10 mL) was added, after which the solution was basified using sat. NH$_4$OH. The solution was centrifuged and decanted from the precipitate. The crude was coevapped with CHCl$_3$, three times and subjected to column chromatography using a gradient of 5 to 20% MeOH in DCM. Further purification by RP-HPLC (gradient: 10 to 75 %B in 3CV) yielded 3.57 mg (0.01 mmol, 11%) of white solid. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 10.0 (s, 1H, 8-OH), 7.31-7.22 (m, 5H, CH arom.), 6.46 (s, 2H, NH$_2$), 4.84 (s, 2H, CH$_2$Bn), 4.16 (t, 2H, CH$_3$), 3.38 (t, 2H, CH$_2$); 13C NMR (50.1 MHz, CDCl$_3$): δ 160.7, 154.6, 153.8, 146.8, 136.3 (Cq), 128.6-127.8 (CH arom.), 66.7 (OCH$_2$), 56.7 (OCH$_3$), 51.4 (CH$_2$N$_3$), 44.6 (CH$_2$Bn), 28.6 (2x CH$_2$), 23.4 (CH$_2$); IR: 3418, 2097, 1699, 1684, 1636, 1352, 1190, 1142. ESI-MS: m/z 355.1 [M+H]$^+$. HRMS: C$_{16}$H$_{18}$N$_8$O$_2$ + H$^+$ calculated 355.16255, found 355.16260.

9-benzyl-7-hydro-8-oxo-2-azidopentoxyadenine (5d)

9-benzyl-8-bromo-2-azidopentoxy-adenine 13d (42.0 mg, 0.10 mmol) was dissolved in 5 mL MeOH after which 0.38 mL (30% sol. in MeOH, 2.0 mmol) NaOMe was added. This mixture was refluxed (65°C) for 16h. TLC analysis (100% EtOAc) showed complete conversion of the starting material. The mixture was separated between H$_2$O and DCM, after which the organic layer was dried using MgSO$_4$ and concentrated. 22.0 mg of the methoxide (0.06 mmol, 59%) was obtained as a white solid. $^1$H NMR (200 MHz, CDCl$_3$): δ 7.30 (m, 5H, CH arom.), 5.27 (bs, 2H, NH$_2$), 5.10 (s, 2H, CH$_2$Bn), 4.30 (t, 2H, OCH$_2$), 4.09 (s, 3H, OMe), 3.28 (t, 2H, CH$_2$N$_3$), 1.84-1.55 (m, 6H, 3x CH$_2$); $^{13}$C NMR (50.1 MHz, CDCl$_3$): δ 160.7, 154.6, 153.8, 146.8, 136.3 (Cq), 128.6-127.8 (CH arom.), 66.7 (OCH$_2$), 56.7 (OCH$_3$), 51.4 (CH$_2$N$_3$), 44.6 (CH$_2$Bn), 28.6 (2x CH$_2$), 23.4 (CH$_2$); IR: 3318, 3157, 2955, 2090, 1598, 1400,
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1344. ESI-MS: \[m/z\] 382.2 [M]+. HRMS: C_{18}H_{23}N_{8}O_{2} + H^+ calculated 383.19387, found 383.19385. The methoxy compound (22.0 mg, 0.06 mmol) was dissolved in 10 mL 37% HCl. The mixture was stirred until LCMS revealed complete disappearance of the starting material. After concentration the compound was taken up in water and basified with sat. NH₄OH. The mixture was taken up in H₂O and extracted with DCM (thrice), after which the organic layers were pooled, dried (MgSO₄) and concentrated, yielding 10.0 mg (0.03 mmol, 48%) of compound 5d as a white solid. 

1H NMR (600 MHz, DMSO-d₆ + drop of MeOD): \(\delta\) 7.32-7.23 (m, 5H, arom.), 6.40 (bs, 2H, NH₂), 4.84 (s, 2H, CH₂Bn), 4.18 (t, 2H, OCH₂), 3.29 (t, 2H, CH₂N₃), 1.68-1.36 (m, 6H, 3x CH₂). 13C NMR (150 MHz, DMSO-d₆ + drop of MeOD): \(\delta\) 177.8, 160.2, 152.3, 149.3, 147.8, 137.22 (Cₜ), 128.5-127.4 (5x CH arom.), 65.0 (OCH₂), 50.7 (CH₂N₃), 42.4 (CH₂Bn), 28.1 (2x CH₂), 22.88 (-CH₂-).

IR: 2950, 2098, 1705, 1612, 1415, 1346. ESI-MS: \[m/z\] 369.1 [M+H]+, HRMS: C₁₇H₂₁N₈O₂ + H+ calculated. C₁₇H₂₁N₈O₂ + H+ calculated.

9-benzyl-7-hydro-8-oxo-2-azidohexanoyladenine (5e)

Bromide 13e was dissolved in MeOH (10 mL) and NaOMe (20 eq., 2.52 mmol, 136 mg) was added. The mixture was stirred overnight at 65°C after which the solvent was removed. The crude was purified by column chromatography using a gradient of 1 to 5% MeOH in DCM. Yield: 38.4 mg, 0.10 mmol, 77%. 1H NMR (600 MHz, DMSO-d₆): \(\delta\) 9.9 8-OH, 7.31-7.24 (5H, CH Arom.), 6.45 (s, 2H, NH₂), 4.84 (s, 2H, CH₂Bn), 4.12 (t, 2H, CH₂), 3.30 (t, 2H, CH₂), 1.63 (m, 2H, CH₂), 1.52 (m, 2H, CH₂), 1.35-1.33 (m, 4H, 2x CH₂) 13C NMR (150 MHz, DMSO-d₆): \(\delta\) 160.1, 152.3, 147.8, 137.2 128.5 (Cₜ), 127.5, 127.4 (CH Arom.), 66.0, 50.6 (CH₂), 42.4 (CH₂Bn), 28.4, 28.2, 25.9, 25.1 (CH₂). IR: 3418, 3152, 2935, 2855, 2091, 1701, 1682, 1632, 1612, 1346. ESI-MS: \[m/z\] 383.1 [M+H]+. HRMS: C₁₇H₂₁N₈O₂ + H⁺ calculated. 383.19385, found 383.19411. The methoxide (35.5 mg, 0.09 mmol) was dissolved in 10 mL concentrated HCl and allowed to stir overnight at r.t. The mixture was concentrated \textit{in vacuo} and 10 mL of milipore was added. The mixture was basified using sat. NH₄OH after which the water layer was extracted with CHCl₃ (thrice, 20 mL). The organic layer was dried (MgSO₄), filtered and concentrated. Yield: 20.8 mg (0.05 mmol, 61%) 5e as a white compound. 

1H NMR (400 MHz, DMSO-d₆): \(\delta\) 9.90 (s, 1H, 8-OH), 7.31-7.24 (5H, CH aro.), 6.45 (s, 2H, NH₂), 4.84 (s, 2H, CH₂Bn), 4.14-4.10 (t, 2H, CH₂), 3.32-3.28 (t, 2H, CH₂), 1.63 (m, 2H, CH₂), 1.52-1.51 (m, 2H, CH₂), 1.35-1.33 (m, 4H, 2x CH₂); 13C NMR (150 MHz, DMSO-d₆): \(\delta\) 160.1, 152.3, 147.8 (Cₜ), 128.5, 127.5, 127.4 (CH Arom.), 66.0 (CH₂Bn), 50.6 (CH₂), 42.4 (CH₂Bn), 28.4, 28.2, 25.9, 25.1 (CH₂). IR: 3418, 3152, 2935, 2855, 2091, 1701, 1682, 1632, 1612, 1346. ESI-MS: \[m/z\] 383.1 [M+H]+. HRMS: C₁₇H₂₁N₈O₂ + H⁺ calculated. 383.19385, found 383.19411.

9-benzyl-7-hydro-8-oxo-2-(2-azidoethoxy)ethoxyadenine (5f)

Bromide 13f (35.5 mg, 0.083 mmol) was dissolved in 20 mL MeOH and NaOMe (40 eq., 3.33 mmol, 180 mg) was added. The mixture was refluxed at 65°C overnight. After cool down and concentration TLC (5% MeOH/DCM) indicated a near complete reaction. The solid was dissolved again in MeOH
and NaOMe (40 eq., 3.33 mmol, 180 mg) was added. Reflux overnight completed the reaction according to TLC. After concentration, the crude was purified by means of column chromatography using a gradient of 1 to 4% MeOH in DCM. Yield: 31.9 mg, 0.08 mmol (100%). $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 7.34-7.22 (m, 5H, CH Arom.), 6.87 (s, 2H, NH$_2$), 5.03 (s, 2H, CH$_2$), 4.31-4.28 (t, 2H, CH$_2$), 4.03 (s, 3H, OCH$_3$), 3.73-3.71 (t, 2H, CH$_2$), 3.64-3.62 (t, 2H, CH$_2$), 3.41-3.38 (t, 2H, CH$_2$). $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ 159.9, 154.5, 153.5, 150.9, 136.7 (C q), 128.6-127.2 (CH Arom. Bn), 110.1 (C$_q$), 69.3 (CH$_2$), 68.7 (CH$_2$), 65.4 (CH$_2$), 56.8 (CH$_2$), 49.9 (CH$_2$), 43.8 (CH$_2$); IR: 3161, 2085, 1663, 1607, 1564, 1373, 1121, 991. ESI-MS: $m/z$ 385.1 [M+H]$^+$. HRMS: C$_{17}$H$_{20}$N$_8$O$_3$ + H$^+$ calculated. 385.17311, found 385.17347.

The methoxide (30.9 mg, 0.08 mmol) was dissolved in concentrated HCl (10 ml) and allowed to stir at rt overnight. The solvent was removed after which milipore was added (5-10 mL). The solution was basified using sat. NH$_4$OH. The water layer was extracted three times with DCM (20 mL) after which the organic layers were combined, dried (MgSO$_4$), filtered and concentrated. Yield: 16.8 mg (0.05 mmol, 57%) $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 9.97 (s, 1H, 8-OH), 7.33-7.23 (m, 5H, CH arom.), 6.48 (s, 2H, NH$_2$), 4.84 (s, 2H, CH$_2$Bn), 4.27-4.25 (t, 2H, CH$_2$), 3.71-3.69 (t, 2H, CH$_2$), 3.63-3.60 (t, 2H, CH$_2$), 3.40-3.38 (t, 2H, CH$_2$). $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ 159.8, 152.2, 149.1, 147.7, 137.1 (C$_q$), 128.4, 127.5, 127.3 (CH Arom.), 98.3 (C$_q$), 69.2 (CH$_2$), 68.6 (CH$_2$), 65.5 (CH$_2$), 49.9 (CH$_2$), 42.4 (CH$_2$); IR: 3437, 3163, 3043, 2924, 2855, 2098, 1701, 1670, 1636, 1616, 1366. ESI-MS: $m/z$ 370.9 [M+H]$^+$. HRMS: C$_{16}$H$_{18}$N$_8$O$_3$ + H$^+$ calculated 371.15746, found 371.15731.

9-benzyl-7-hydro-8-oxo-(2-[2-(2-azidoethoxy)ethoxy]ethoxy)adenine (5g)

9-benzyl-8-bromo-2-(2-[2-(2-azidoethoxy)ethoxy]ethoxy)adenine 13g (40.0 mg, 0.08 mmol) was dissolved in MeOH (6.5 mL) and NaOMe (90 mg, 1.68 mmol, 20 eq.) was added. The mixture was stirred and heated overnight. TLC analysis (EtOAc / MeOH, 19/1, v/v) showed total conversion into a lower running product. The mixture was cooled to room temperature, concentrated, and dissolved in DCM/H$_2$O. The organic layer was separated and the aqueous layer was washed with DCM. The organic layers were combined, dried (MgSO$_4$), filtered, and concentrated. Column chromatography (MeOH/EtOAc, 0/1- 12/1, v/v) afforded the product as a white solid in 97% (35.0 mg, 0.08 mmol). $^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 7.29 (m, 5H, CH Arom.), 5.26 (bs, 2H, NH$_2$), 5.08 (s, 2H, CH$_2$Bn), 4.47 (t, 2H, CH$_2$), 4.08 (s, 3H, OCH$_3$), 3.85 (t, 2H, CH$_2$), 3.68 (m, 6H, 3x CH$_2$), 3.37 (t, 2H, CH$_3$); IR: 2920, 2102, 1600, 1396, 1330. ESI-MS: $m/z$ 419.1 [M]$^+$. HRMS: C$_{19}$H$_{24}$N$_8$O$_4$ + H$^+$ calculated 429.19931, found 429.19933. The methoxide compound (35.0 mg, 0.08 mmol) was dissolved in 3 M HCl (1.2 mL, 3.6 mmol, 45 eq.) and stirred at r.t. for 3 days. TLC analysis (EtOAc/MeOH, 19/1, v/v) showed the total conversion of starting material. The mixture was brought to high pH with sat. NH$_4$OH during which a white solid precipitated. After filtration, the product was dissolved in water and extracted with DCM (thrice), dried over MgSO$_4$, filtered and concentrated. The product was isolated as a white solid in 80% yield (27.0 mg, 0.07 mmol). (5g) $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 9.96 (s, 1H, 8-OH), 7.33-7.25 (m, 5H, CH Arom.), 6.46 (s, 2H, NH$_2$), 4.84 (s, 2H, CH$_2$Bn), 4.26-4.23
(t, 2H, CH₂), 3.68-3.66 (t, 2H, CH₂), 3.59-3.56 (m, 6H, 3x CH₂), 3.37-3.35 (t, 2H, CH₂); ¹³C NMR (150 MHz, DMSO-d₆): δ 159.8, 152.1, 148.9, 147.9, 137.22 (C₆), 128.4-127.3 (5x CH Arom.), 98.4 (C₆), 69.8-65.4 (5x CH₂), 49.9 (CH₂N₃); IR: 3431, 3167, 2864, 2108, 1703, 1670, 1634, 1612, 1364, 1105. ESI-MS: m/z 415.2. LCMS: 10-70% B, Rt = 12.2 min; ESI-MS: [M+H]⁺: 415.1; HRMS: C₁₈H₂₂N₈O₄ + H⁺ calculated. 415.18368, found 415.18497.

9-benzyl-7-hydro-8-oxo-2-aminoethoxy (14)

Azide 5a (20.0 mg, 0.06 mmol) was dissolved in 293 μL of 1 M PMe₃ in THF which was cooled via icebath to 0°C. After 15 min. of stirring 10 μL of milipore water was added. LCMS indicated complete disappearance of starting material. The mixture was concentrated and dissolved in 2 mL of ¹BuOH/H₂O/ACN. After lyophilisation the crude was purified by HPLC using a gradient of 20 to 50% ACN in water (0.1%TFA). Lyophilisation of fractions containing the product yielded white 14. Yield: 5.56 mg, 0.02 mmol (31%). ¹H NMR (400 MHz, DMSO-d₆): δ 10.70 (s, 1H, 8-OH), 8.15 (bs, 2H, NH₂), 7.25 (m, 5H, CH Arom.), 6.90 (bs, 2H, NH₂), 4.85 (s, 2H, CH₂Bn), 4.34-4.31 (t, 2H, CH₂), 3.14-3.13 (m, 2H, CH₂). ¹³C NMR (100 MHz, DMSO-d₆): δ 159.3, 158.3, 152.1, 149.0, 147.8 (C₆), 128.5, 127.3, 127.3 (CH Arom.), 98.7 (C₆), 63.1 (CH₂), 45.4 (CH₂Bn), 42.3 (CH₂). ESI-MS: m/z 301.3 [M+H]⁺. HRMS: C₁₄H₁₄N₆O₂ + H⁺ calculated 301.14075, found 301.14086.

9-benzyl-7-hydro-8-oxo-2-N-Acetyl-aminoethoxy (15)

Crude compound 14 (18.0 mg, 0.06 mmol) was added to a saturated NaHCO₃ in MeOH (300 μL) solution. Acetic anhydride (5 eq., 0.31 mmol, 29 μL) was added and the mixture was stirred overnight. The reaction was filtered and the solid was washed with MeOH after which the filtrate was concentrated. Column chromatography; 5% to10% MeOH in DCM yielded 15 as a white solid. Yield: 9.97 mg, 0.029 mmol (49%). ¹H NMR (400 MHz, DMSO-d₆): δ 10.0 (bs, 1H, 8-OH), 8.05 (t, 1H, NH₂), 7.31-7.25 (m, 5H, CH Arom.), 6.49 (s, 2H, NH₂), 4.84 (s, 2H, CH₂Bn), 4.14-4.11 (t, 2H, CH₂), 3.44 (m, 2H, CH₂), 1.88 (s, 3H, CH₃). ¹³C NMR (100 MHz, DMSO-d₆): δ 169.4 (C=0), 159.8, 152.2, 149.2, 147.7, 137.2 (C₆), 128.5, 127.4, 127.4 (C Arom.), 98.3 (C₆), 65.1 (CH₂Bn), 42.4, 38.1 (CH₂), 22.5 (CH₃). IR: 3155, 2918, 1705, 1643, 1609, 945. ESI-MS: m/z 343.2 [M+H]⁺. HRMS: C₁₆H₁₈N₆O₃ + H⁺ calculated 343.15131, found 343.15134.

Dendritic cells and IL12 ELISA

Freshly isolated DC were cultured from bone marrow cells of C57BL/6 mice as described. BMDC (4x10⁶) were plated into 96-well round bottom plate, and incubated for 24hrs or 48hrs with the indicated compounds. Supernatants were harvested, and tested for IL-12 p40/p70 content using a standard sandwich ELISA. Coating Ab: rat anti-mouse IL-12 p40/p70 mAb (clone C15.6; BD PharMingen). Detection Ab: biotinylated rat anti-mouse IL-12 p40/p70 mAb (clone C17.8; BD PharMingen). Streptavidin-HRP and ABTS (Sigma-Aldrich) were used as enzyme and substrate, respectively.
References and Notes


34. See experimental section.


43. Prepared similar to the procedure used for azidopentanol.

