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Synthesis of hypermodified adenosine derivatives as selective adenosine A_3 receptor ligands

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Abstract—We investigated the A_3AR affinity and selectivity of a series of 2-substituted 3′-azido and 3′-amino adenosine derivatives as well as some 5′-uronamide derivatives thereof. All compounds showed high A_3AR selectivity. While the 3′-azides appeared to be A_3AR antagonists with moderate A_3AR affinity, their 3′-amino congeners exhibit significantly improved A_3AR affinity and behave as partial agonists. For both the 3′-azides and the 3′-amines, the 5′-methylcarbamoyl modification improved the overall affinity. Introduction of a 2-phenylethynyl substituent provided high affinity for the A_3AR.

1. Introduction

Adenosine receptors (AR) belong to the family of G protein-coupled receptors. They are subdivided into four subtypes designated A_1, A_{2A}, A_{2B} and A_3, according to the chronological discovery of the receptors.[1] The A_3ARs are coupled to G_i proteins and, therefore, inhibit adenylate cyclase leading to a decrease in intracellular levels of cAMP.[2] The selective activation of the A_3AR is both cardioprotective and cerebroprotective in a variety of ischaemic models.[3,4] Selective A_3AR antagonists promise to be useful in the regulation of cell growth[5,6] and as anti-asthmatic,[7] cerebroprotective[4,8] and anti-inflammatory agents.[9] A_3AR antagonists appear to lower the intraocular pressure in mice and monkeys and are proposed as new potential therapeutics for the treatment of glaucoma.[10,11]

Adenosine receptors are ubiquitously distributed throughout the body. As a consequence, ligands need to be highly selective in their action with respect to receptor subtype and tissue to be of therapeutic value.[12] Numerous structure–activity studies of adenosine derivatives as receptor agonists conclude that selectivity may be provided by specific substitutions of the adenine ring.[13,1] Substitution at the 8-position of the ring is not well tolerated by any AR subtype.[14,15] The nitrogen atoms at positions 3 and 7 are required for high affinity of adenosine at all subtypes.[1] 2-Alkynyl derivatives of NECA possess high affinity at the A_3 receptor subtype. Moreover, the presence of 2-alkyne substituents enhanced the A_3AR selectivity.[16]

DeNinno et al. discovered that introduction of an amino group at the 3′ position improves the selectivity for the human A_3AR, while enhancing the water solubility. The affinity drop caused by this 3′-substitution could be overcome by elaborating the N^6-substituents.[17] The combination of a large N^6-substitituent with a 2-alkynyl group has proven to be unsuccessful because of the steric hindrance caused by the two large substituents, reflected by a decrease in A_3AR affinity.[16] Therefore, the present study investigated the effect of a 2-alkynyl substituent in concert with a small N^6-substituent on the affinity and selectivity of a series of 3′-azido and 3′-amino adenosine derivatives. In addition, we evaluated the effect of the 5′-methylcarbamoyl modification on the overall affinity and efficacy of these compounds (Fig. 1).

Keywords: Adenosine A_3 receptor; Selectivity; Affinity; Ribose and purine modifications.
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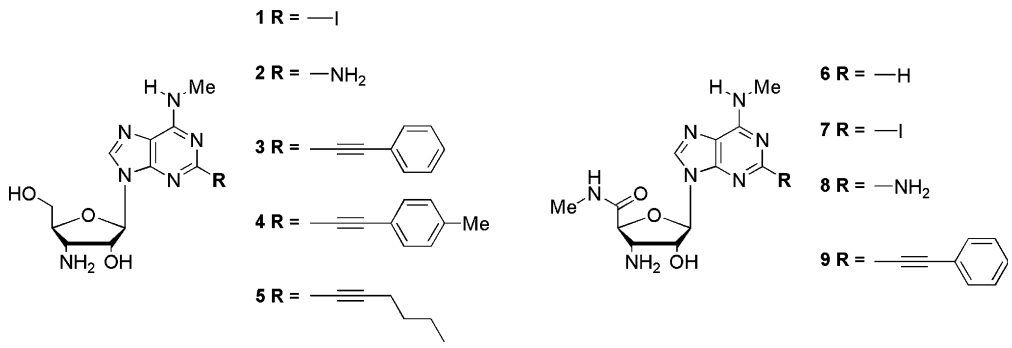


Figure 1.

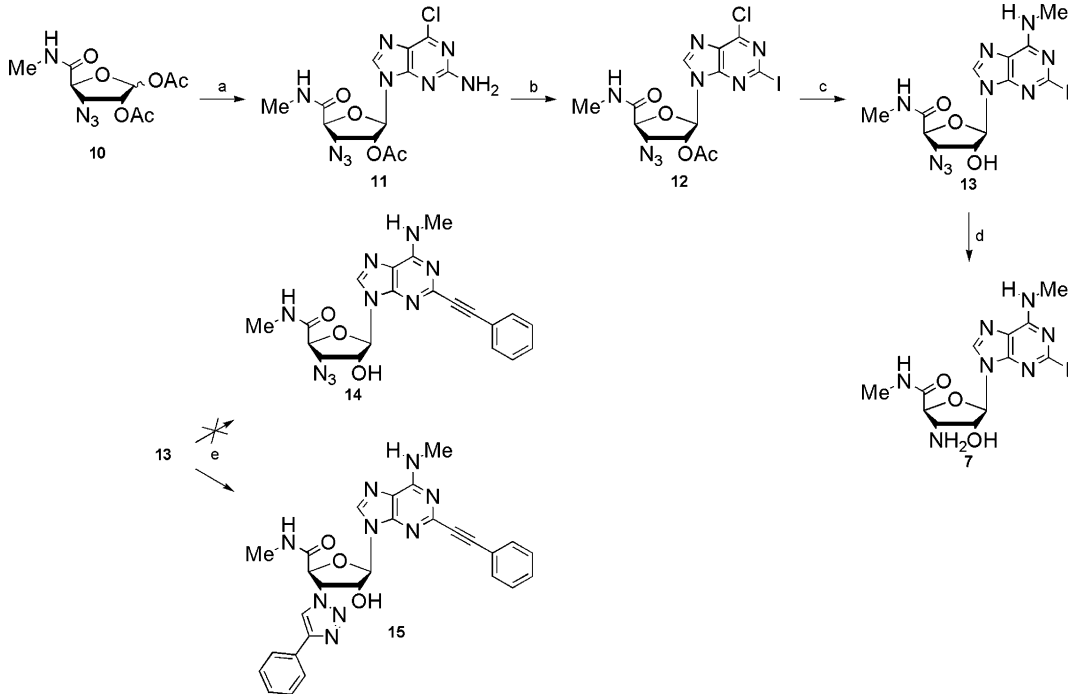
2. Results and discussion

2.1. Chemistry

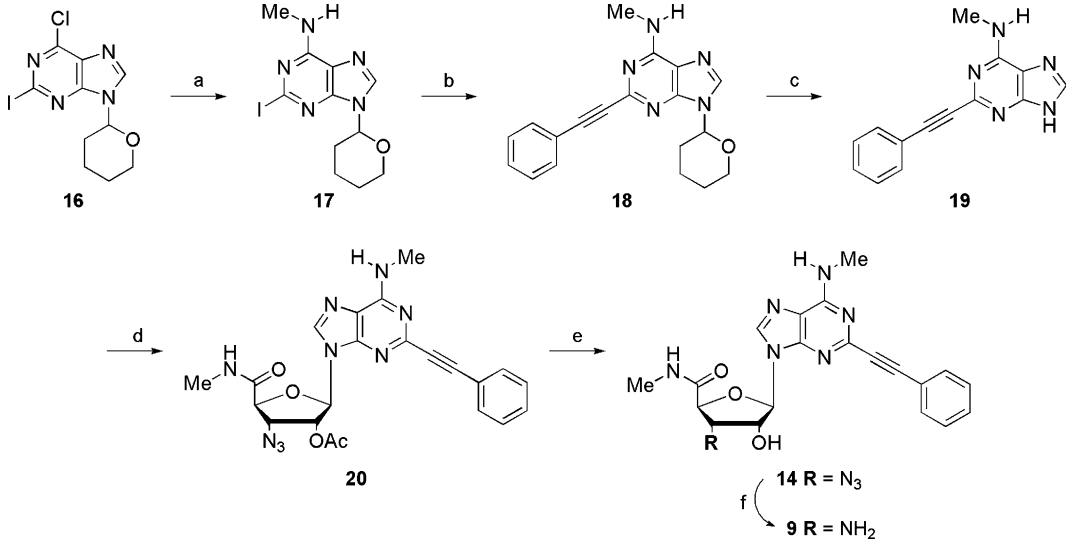
3-Azido-3-deoxy-1,2-di-*O*-acetyl-α-D-ribofuronamide (**10**) was prepared from the commercially available 1,2-*O*-isopropylidene-α-D-xylofuranose as described by us previously.[18] It was coupled under Vorbrüggen conditions with silylated 2-amino-6-chloropurine to give **11** in 79% yield. Classical procedures allowed a straightforward conversion of **11** to **13**. Triphenylphosphine reduction of the azido moiety yielded the corresponding amine **7**.

Based on the results of Cristalli et al.[19] we have chosen phenylethynyl as the most promising C2-substituent. Reaction conditions used to perform a Sonogashira coupling[20] of **13** with phenylacetylene yielded the 3′-(4-phenyl-1,2,3-triazol-1-yl) derivative (**15**) of the 2-alkynylated compound (Scheme 1). This result was due to a Cu^{+}-catalysed Huisgen dipolar cycloaddition[21] of the 3′-azide with phenylacetylene. Consequently, another strategy was used to gain access to compound **14** (Scheme 2), starting from 6-chloro-2-iodo-(9-tetrahydropyran-2-yl)purine (**16**), obtained from the 2-unsubstituted analogue via a lithiation-mediated stannyl transfer process followed by 2-tributylstannyl-iodine exchange.[22] Sonogashira coupling of **17**, followed by deprotection, provided **19**. Unfortunately, classical Vorbrüggen coupling,[23] as described for the synthesis of **11**, did not give satisfying results. By using *N*,*O*-bis(trimethylsilyl)acetamide (BSA) as silylating agent,[24] 9-(2-acetyl-3-azido-3-deoxy-5-methylcarbamoyl-β-D-ribofuranosyl)-N^6-methyl-2-phenylethynyladenine (**20**) was obtained in poor yield.

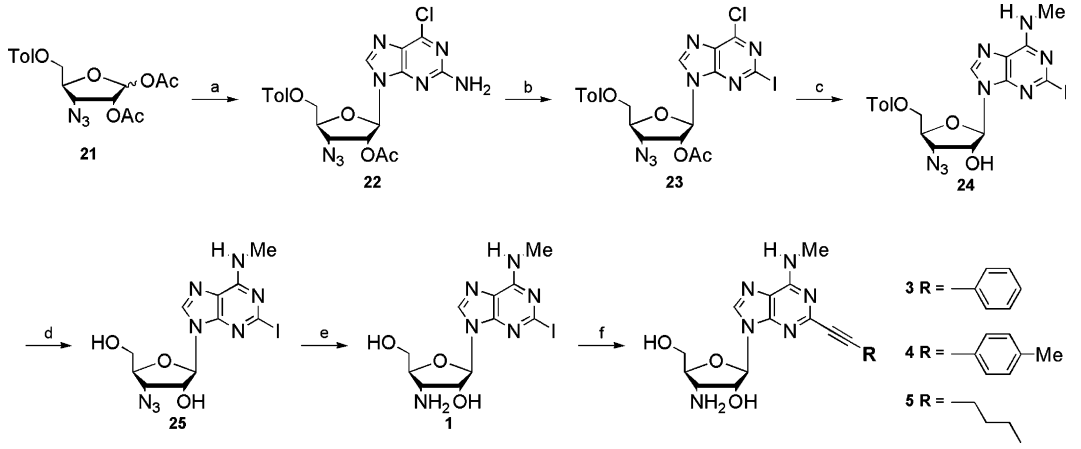
During the course of this work it became clear that the 3′-amino-analogues generally exhibit much better A_3AR affinities than their 3′-azide precursors. Consequently, we focussed only on the 3′-amino derivatives for further



Scheme 1. Reagents and conditions: (a) i—HMDS, $(NH_4)_2SO_4$, reflux, 20 h, ii—2-amino-6-chloropurine, TMSOTf; (b) isoamyl nitrite, I_2, CuI, CH_2I_2 in THF, reflux; (c) CH_3NH_3Cl, Et_3N, EtOH, reflux; (d) Ph_3P, H_2O, THF, 2 days; (e) phenylacetylene, $(PPh_3)_2PdCl_2$, CuI, Et_3N, DMF.



Scheme 2. Reagents and conditions: (a) CH_3NH_3Cl, DMAP, EtOH, reflux; (b) phenylacetylene $(PPh_3)_2PdCl_2$, CuI, Et_3N, DMF; (c) TFA, CH_2Cl_2; (d) **10**, BSA, TMSOTf, CH_3CN reflux; (e) 7 N NH_3 in MeOH; (f) Ph_3P, H_2O, THF, 2 days.



Scheme 3. Reagents and conditions: (a) 2-amino-6-chloropurine, BSA, TMSOTf, CH_3CN, reflux; (b) isoamylnitrite, I_2, CuI, CH_2I_2 in THF, reflux; (c) i—CH_3NH_3Cl, Et_3N, EtOH, reflux, ii—7 N NH_3 in MeOH; (d) Na° in MeOH; (e) Ph_3P, H_2O, THF, 2 days; (f) alkyne, $(PPh_3)_2PdCl_2$, CuI, Et_3N, DMF.

synthesis. This direction permitted us to reduce the 3′-azide to a 3′-amine before the Sonogashira coupling, avoiding the unwanted cycloaddition.

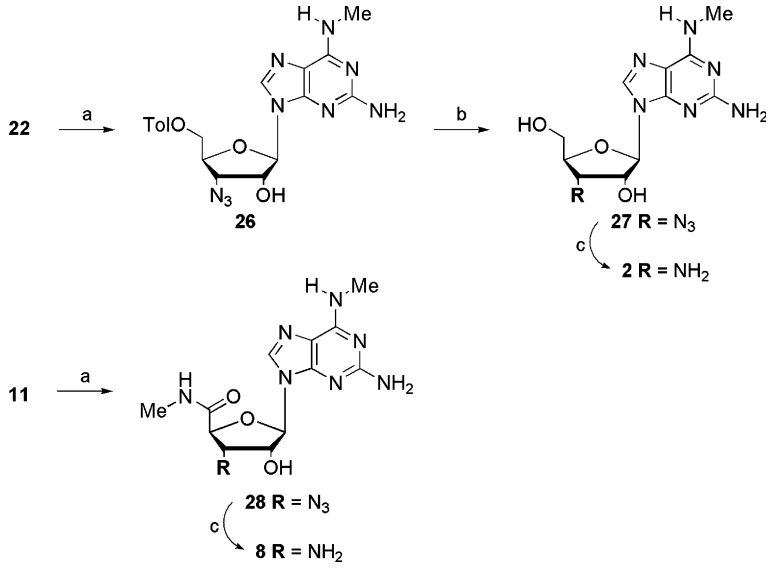
9-(3-Amino-3-deoxy-β-D-ribofuranosyl)-N^6-methyl-2-iodopurine (**1**) served as a suitable synthon for the synthesis of the 2-alkynylated 3′-amino-adenosines **3**–**5** (Scheme 3). It was obtained by coupling of sugar **21** with 2-amino-6-chloropurine. Elaboration of the base moiety to yield **25** was essentially accomplished as for **13**. Staudinger reduction allowed the unmasking of the amine group. Finally, Sonogashira coupling on amine **1** provided the alkynylated analogues **3**–**5** in 80–82% yield.

To continue the exploration of the 2-position, we synthesized the 2-I and the 2-NH_2 derivatives of the 5′-OH and the 2-H and the 2-I derivatives of the 5′-methylcarbamoyl 3′-amino-N^6-aminomethyl adenosine analogues (Scheme 4).

3′-Amine **6** (Fig. 1) was prepared by catalytic hydrogenation of the 3′-azide precursor which has been described.[18]

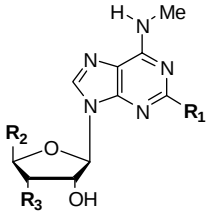
2.2. Biological evaluation

For the adenosine derivatives prepared in this study (**1**–**9**, **13**, **14**, **15**, **25**, **27** and **29**) we measured both the binding affinities at the hA_1, hA_{2A} and hA_3AR and their degrees of activation of the A_3AR subtype. The results are reported in Table 1. The ability of each of these adenosine derivatives to compete for radioligand binding at each of these hARs was evaluated at a fixed concentration of 10 μM, and full competition curves were determined at the A_3AR. Six different 2-substituents were included: H, I, NH_2, Ph-C≡C, *p*MePh-C≡C and *n*Bu-C≡C. The choice of the methyl group as a small N^6 substituent was based on the results of Cristalli et al., who demonstrated that it



Scheme 4. Reagents and conditions: (a) i—CH_3NH_3Cl, Et_3N, EtOH, reflux, ii—7 N NH_3 in MeOH; (b) Na° in MeOH; (c) Ph_3P, H_2O, THF, 2 days.

Table 1. Binding affinities of adenine derivatives at human A_1, A_{2A} and A_3ARs expressed in CHO cells[a]



Compound	R_1	R_2	R_3	% Inhibition (hA_3AR)		K_i (nM)	% Activation[d] (hA_3AR)
				(hA_1AR) (%)	($hA_{2A}AR$) (%)		
1	I	CH_2OH	NH_2	39	12	879 ± 346	67 ± 6
2	NH_2	CH_2OH	NH_2	16	4	654 ± 42	57 ± 2
3	Ph-C≡C	CH_2OH	NH_2	50	14	126 ± 4	36 ± 6
4	*p*MePh-C≡C	CH_2OH	NH_2	33	0	145 ± 35	23 ± 3
5	*n*Bu-C≡C	CH_2OH	NH_2	4	3	389 ± 112	66 ± 11
6	H	CONHMe	NH_2	18	12	32.3 ± 3.9	72 ± 10
7	I	CONHMe	NH_2	16	24	71.4 ± 12.8	18 ± 10
8	NH_2	CONHMe	NH_2	−14	10	536 ± 247	92 ± 3
9	Ph-C≡C	CONHMe	NH_2	9	9	15.6 ± 3.6	67 ± 11
13	I	CONHMe	N_3	16	0	2530[b]	−8 ± 5
14	Ph-C≡C	CONHMe	N_3	31	16	78.9 ± 12.4	−11 ± 8
15	Ph-C≡C	CONHMe	4-Ph-1,2,3-triazol-1-yl	14	2	1820 ± 770	0
25	I	CH_2OH	N_3	85[b,c]	27%	6540 ± 320	−3 ± 2
27	NH_2	CH_2OH	N_3	49	16	28,800[b]	0
29[18]	H	CONHMe	N_3	12	10	1140 ± 300	38 ± 4

[a] All A_3AR experiments were performed using adherent CHO cells stably transfected with cDNA encoding one of the human adenosine receptors. Binding at human A_1, A_{2A} and A_3ARs in this study was carried out as described in methods using [^{3}H]PIA, [^{3}H]CGS 21680 or [^{125}I]AB-MECA as radioligand. Values from the present study are expressed as K_i values (means ± SEM, $n = 3$, unless noted) or as percent displacement of radioligand at 10 μM.

[b] $n = 1$.

[c] K_i (A_1 AR) = 1850 nM.

[d] % Inhibition at 100 μM of forskolin-stimulated cAMP production at 10 μM, in CHO cells expressing the hA_3AR, as a percentage of the response of the full angonist CI-IB-MECA ($n = 3$).

increased the affinity for the human A_3AR and significantly enhanced the A_3AR selectivity.[19]

Results from the competition experiments showed that the A_3AR affinities of the 3′-amines were much higher than those of the 3′-azides. For all derivatives studied, the 5′-methylcarbamoyl modification, in general, enhanced the affinity at the A_3AR in comparison to 5′-CH_2OH. Except for **25**, all evaluated compounds showed a very high selectivity for the A_3AR compared to the other

ARs. The most potent compound (**9**) displayed a K_i value of 16 nM at the A_3AR. The C-2 substituent of this compound, a phenylethynyl moiety, was previously shown to enhance A_3AR affinity and selectivity,[19] and proved to have a superior contribution to A_3AR affinity than a *p*-methyl-phenylethynyl (**4**) or a 1-hexynyl (**5**) moiety. Furthermore, the 2-phenylethynyl modification appeared to overcome the reduction of affinity caused by the 3′-azide (cf. K_i = 78.9 nM for **14** vs 1140 nM for **29**). Consequently, this C-2 substituent was selected to be combined with a 3′-amino and a 5′-methylcarbamoyl modification.

Previous studies showed that 3′-amino derivatives exhibit a decreased affinity at the A_3AR compared to their 3′-hydroxy analogues. The affinity reduction associated with this 3′ modification could be overcome by elaborating the N^6-substituents, for example with a substituted benzyl group.[17] The high affinity of compound **9** (K_i = 15.6 nM) demonstrated that a 2-phenylethynyl modification in concert with a small N^6-substituent was likewise capable of overcoming this reduction in affinity. Note that in our experiments the affinity of derivative **6** for the A_3AR (K_i = 32.3 nM) was 4-fold higher than that reported by DeNinno et al.[15] The 2-I analogue **7** also showed appreciable A_3AR affinity (K_i = 71.4 nM). Conversely, the 2-NH_2 analogue **8** exhibited weak A_3AR affinity (K_i = 536 nM).

The results of the cyclic cAMP-assay (Table 1) indicated that all 3′-azides were A_3AR antagonists, except for compound **29** which showed partial agonist activity. Also the 3′-(4-phenyl-1,2,3-triazol-1-yl) derivative **15** appeared to be an A_3AR antagonist. All other compounds were partial agonists, except for compound **8**, which manifested full agonist activity.

3. Conclusions

The 2,3′,5′-trisubstituted and 2,3′-disubstituted N^6-methyl adenosine derivatives described in the present study were synthesized in good overall yields. All the compounds had A_3AR affinities in the low micromolar or nanomolar range and showed very high A_3AR selectivity. The 3′-azides appeared to be A_3AR antagonists with a moderate A_3AR affinity. The 3′-amino modification significantly improved the A_3AR affinity and resulted in partial A_3AR agonists. For both the 3′-azido and the 3′-amino derivatives, the 5′-methylcarbamoyl modification improved the overall affinity. Curiously, the presence of a 5′-uronamide did not restore full A_3AR efficacy in 2-position derivatives, as was demonstrated in the case of N^6-substituents that reduced efficacy.[26] The 2-phenylethynyl derivative **9** demonstrated high A_3AR receptor affinity with a K_i value of 15.6 nM and >1000-fold selectivity. Previous studies revealed that 3′-amines exhibit a decreased affinity compared to their 3′-hydroxy analogues. This study demonstrated that introduction of a 2-phenylethynyl substituent in concert with the N^6-methyl group is capable of overcoming this affinity drop.

4. Experimental

4.1. Chemicals and solvents

All reagents were from standard commercial sources and of analytic grade.

4.2. Chromatography

Precoated Merck silica gel F254 plates were used for TLC and spots were examined under UV light at 254 nm and further visualized by sulfuric acid-anisaldehyde spray. Column chromatography was performed on Uetikon silica (0.2–0.06 mm).

4.3. Instruments and analyses

NMR spectra were obtained with a Varian Mercury 300 MHz spectrometer. Chemical shifts are given in ppm (δ) relative to the residual solvent signal, in the case of DMSO-d_6 2.54 ppm for ^{1}H and in the case of $CDCl_3$ 7.26 ppm for ^{1}H. All signals assigned to amino, amide hydrogen and hydroxyl groups were exchangeable with D_2O. Exact mass measurements were performed on a quadrupole/orthogonal-acceleration time-of-flight (Q/oaTOF) tandem mass spectrometer (qToF 2, Micromass, Manchester, UK) equipped with a standard electrospray ionization (ESI) interface. Samples were infused in a 2-propanol/water (1:1) mixture at 3 μL/min.

4.4. 9-(3-Amino-3-deoxy-β-D-ribofuranosyl)-2-iodo-N^6-methyladenine (1)

This compound was synthesized from 30 mg (0.069 mmol) of **25** by the procedure described for the synthesis of **7**; yield: 26 mg (92%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.66 (br s, 2H, NH_2), 2.87 (d, 3H, J = 4 Hz, N^6-CH_3), 3.41 (app t, 1H, J = 6.0 Hz, H3′), 3.54–3.71 (m, 3H, H4′ and H5′A and H5′B), 4.10 (br s, 1H, 2′-OH), 4.19 (dd, 1H, J = 2.6 and 4.4 Hz, H2′), 4.98 (br s, 1H, 5′-OH), 5.82 (d, 1H, J = 2.6 Hz, H1′), 8.11 (d, 1H, J = 4 Hz, N^6H), 8.30 (s, 1H, H8); Exact Mass (ESI-MS, *i*-PrOH/H_2O): Calcd for $C_{11}H_{16}N_6O_3I[M+H]^+$: 407.0330. Found: 407.0332.

4.5. 9-(3-Amino-3-deoxy-β-D-ribofuranosyl)-2-amino-N^6-methyladenine (2)

This compound was synthesized from **27** (35 mg, 0.11 mmol) by the procedure described for the synthesis of **7**; yield: 30 mg (93%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.70 (br s, 2H, 3′-NH_2), 2.86 (s, 3H, N^6-CH_3), 3.34 (app t, 1H, J = 6.0 Hz, H3′), 3.52–3.70 (m, 3H, H4′ and H5′A and H5′B), 4.16 (br s, 2H, 2′-OH and H2′), 5.16 (br s, 1H, 5′-OH), 5.74 (d, 1H, J = 2.8 Hz, H1′), 5.83 (s, 2H, 2-NH_2), 7.24 (br s, 1H, N^6H), 7.91 (s, 1H, H8); Exact Mass (ESI-MS, *i*-PrOH/H_2O): Calcd for $C_{12}H_{18}N_7O_3$ $[M+H]^+$: 296.1471. Found: 296.1470.

4.6. General procedure for the synthesis of alkynes 3, 4 and 5 from 1

Compound **1** (50 mg, 0.12 mmol) was dissolved in Et_3N (1.5 mL) and DMF (1 mL). After purging the solution with N_2, $(PPh_3)_2PdCl_2$ (8.6 mg, 0.012 mmol) and CuI (2.3 mg, 0.012 mmol) were added. The appropriate alkyne (2 equiv) was subsequently added dropwise and the mixture was stirred at room temperature for 3 h. The solvents were removed under reduced pressure, the residue was taken up in ethyl acetate and the solution was filtered over a Celite pad. The residue remaining after solvent evaporation was purified on silica gel column (CH_2Cl_2/MeOH, 90:10).

4.7. 9-(3-Amino-3-deoxy-β-D-ribofuranosyl)-N^6-methyl-2-phenylethynyladenine (3)

The reaction of **1** (50 mg, 0.12 mmol) with phenylacetylene (27 μL, 0.24 mmol) gave compound **3** in 81% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.07 (br s, 2H, 3′-NH_2), 2.94 (s, 3H, N^6-CH_3), 3.44 (app t, 1H, J = 5.3 Hz, H3′), 3.56–3.75 (m, 3H, H4′ and H5′A and H5′B), 4.09 (br s, 1H, 2′-OH), 4.24 (dd, 1H, J = 2.6 and 4.4 Hz, H2′), 5.11 (br s, 1H, 5′-OH), 5.94 (d, 1H, J = 2.3 Hz, H1′), 7.46 (m, 3H, Ph), 7.61 (m, 2H, Ph), 7.95 (br s, 1H, N^6H), 8.47 (s, 1H, H8); Exact Mass (ESI-MS, i-PrOH/H_2O): Calcd for $C_{19}H_{21}N_6O_3[M+H]^+$: 381.1675. Found: 381.1675.

4.8. 9-(3-Amino-3-deoxy-β-D-ribofuranosyl)-N^6-methyl-2-(4-methyl-phenyl)ethynyladenine (4)

The reaction of **1** (50 mg, 0.12 mmol) with 4-methylphenylacetylene (31 μL, 0.24 mmol) gave compound **4** in 82% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.33 (s, 3H, CH_3Ph), 2.94 (s, 3H, N^6-CH_3), 3.50–3.79 (m, 4H, H3′, H4′, H5′A and H5′B), 4.31 (dd, 1H, J = 2.4 and 4.5 Hz, H2′), 5.15 (br s, 1H, 5′-OH), 5.74 (s, 1H, 2′-OH), 5.96 (d, 1H, J = 2.4 Hz, H1′), 7.25 (m, 2H, Ph), 7.50 (m, 2H, Ph), 7.95 (br s, 1H, N^6H), 8.46 (s, 1H, H8); Exact Mass (ESI-MS, i-PrOH/H_2O): Calcd for $C_{20}H_{23}N_6O_3[M+H]^+$: 395.1831. Found: 395.1823.

4.9. 9-(3-Amino-3-deoxy-β-D-ribofuranosyl)-N^6-methyl-2-(1-hexyn-1-yl)adenine (5)

The reaction of **1** (50 mg, 0.12 mmol) with 1-hexyn (28 μL, 0.24 mmol) gave compound **5** in 80% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.90 (t, 3H, J = 7.03 Hz, CH_2CH_3), 1.37–1.54 (m, 4H, $CH_2CH_2CH_3$), 2.41 (t, 2H, J = 7.04 Hz, C≡CCH_2), 2.88 (s, 3H, N^6-CH_3), 3.48–3.73 (m, 4H, H3′, H4′, H5′A and H5′B), 4.10–4.23 (m, 2H, H2′ and 2′-OH), 5.10 (br s, 1H, 5′-OH), 5.90 (d, 1H, J = 2.5 Hz, H1′), 7.83 (br s, 1H, N^6H), 8.41 (s, 1H, H8); Exact Mass (ESI-MS, i-PrOH/H_2O): Calcd for $C_{17}H_{25}N_6O_3[M+H]^+$: 361.1988. Found: 361.1982.

4.10. 9-(3-Amino-3-deoxy-5-methylcarbamoyl-β-D-ribofuranosyl)-N^6-methyl-2-iodoadenine (7)

Compound **13** (50 mg, 0.11 mmol) and PPh_3 (57 mg, 0.21 mmol) were dissolved in THF (2 mL). After stirring for 10 min, H_2O was added (270 μL, 15 μmol) and the reaction mixture was stirred for 2 days. The residue obtained after solvent evaporation was purified by column chromatography (CH_2Cl_2/MeOH, 95:5) to yield 82% of compound **7**. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.79 (s, 2H, 3′-NH_2), 2.69 (d, 3H, J = 4.7 Hz, CH_3NHCO), 2.88 (d, 3H, J = 4.1 Hz, N^6-CH_3), 3.54 (t, 1H, J = 11.1 Hz, H3′), 4.10 (d, 1H, J = 6.2 Hz, H4′), 4.30 (app t, 1H, J = 8.8 Hz, H2′), 5.91 (br s, 1H, 2′-OH), 5.93 (d, 1H, J = 3.81 Hz, H1′), 8.03 (d, 1H, J = 4,7 Hz, NHCO), 8.13 (d, 1H, J = 4.1 Hz, N^6H), 8.48 (s, 1H, H8); Exact Mass (ESI-MS, i-PrOH/H_2O): Calcd for $C_{12}H_{17}ClIN_7O_3[M+H]^+$: 434.0439. Found: 434.0445.

4.11. 9-(3-Amino-3-deoxy-5-methylcarbamoyl-β-D-ribofuranosyl)-2-amino-N^6-methylpurine (8)

Compound **28** (40 mg, 0.12 mmol) and PPh_3 (66 mg, 0.25 mmol) were dissolved in THF (2 mL). After stirring for 10 min, H_2O was added (310 μL, 17 mmol) and the mixture was stirred for 2 days. The residue obtained after solvent evaporation was purified by column chromatography (CH_2Cl_2/MeOH, 80:20) to give compound **8** in 80% yield. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.95 (br s, 2H, 3′-NH_2), 2.66 (d, 3H, J = 4.7 Hz, CH_3NHCO), 2.86 (s, 3H, N^6-CH_3), 3.54 (app t, 1H, J = 5.4 Hz, H3′), 4.05 (d, 1H, J = 5.6 Hz, H4′), 4.37 (app t, 1H, J = 4.7 Hz, H2′), 5.82 (d, 1H, J = 4.1 Hz, H1′), 5.88 (s, 2H, 2-NH_2), 7.31 (s, 1H, N^6H), 8.04 (s, 1H, H8), 8.27 (d, 1H, J = 4.4 Hz, NHCO); Exact Mass (ESI-MS, i-PrOH/H_2O): Calcd for $C_{12}H_{19}N_8O_3[M+H]^+$: 323.1580. Found: 323.1579.

4.12. 9-(3-Amino-3-deoxy-5-methylcarbamoyl-β-D-ribofuranosyl)-N^6-methyl-2-phenylethynyladenine (9)

This compound was synthesized by the procedure described for the synthesis of **7** from 20 mg (0.046 mmol) of **14** in 96% yield (18 mg). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.78 (s, 2H, 3′-NH_2), 2.71 (d, 3H, J = 4.4 Hz, CH_3NHCO), 2.94 (s, 3H, N^6-CH_3), 4.33 (dd, 1H, J = 5.0 and 5.3 Hz, H3′), 4.13 (d, 1H, J = 5.3 Hz, H4′), 4.36 (br s, 1H, H2′), 5.94 (s, 1H, 2′-OH), 6.02 (d, 1H, J = 4.40, H1′), 7.46 (m, 3H, Ph), 7.61 (m, 2H, Ph), 8.04 (d, 1H, J = 4.4 Hz, N^6H), 8.41 (d, 1H, J = 4.7 Hz, NHCO), 8.62 (s, 1H, H8); Exact Mass (ESI-MS, i-PrOH/H_2O): Calcd for $C_{20}H_{22}N_7O_3[M+H]^+$: 408.1784. Found: 408.1787.

4.13. 9-(2-O-Acetyl-3-azido-3-deoxy-5-methylcarbamoyl-β-D-ribofuranosyl)-2-amino-6-chloropurine (11)

4.13.1. Silylation of the base. 2-Amino-6-chloropurine (462 mg, 2.7 mmol) was treated with 1,1,1,3,3,3-hexamethyldisilazane (HMDS, 40 mL) and $(NH_4)_2SO_4$ (0.27 mmol, 36 mg) and refluxed for 20 h. The silylated compound was concentrated and used without further purification.

4.13.2. Vorbrüggen coupling. 3-Azido-3-deoxy-1,2-di-O-acetyl-α-D-ribofuronamide (**10**, 600 mg, 2.09 mmol) in dry 1,2-dichloroethane (25 mL) was added to the silylated

2-amino-6-chloropurine (462 mg, 2.7 mmol). The solution was gently refluxed, and after 5 min TMSOTf (417μL, 2.3 mmol) was added dropwise. After 4 h, the mixture was cooled to room temperature, quenched with a cold saturated $NaHCO_3$ solution (80 mL) and extracted with CH_2Cl_2 (40 mL). The organic layer was dried with $MgSO_4$, filtered and evaporated to dryness. The residue was purified by column chromatography (CH_2Cl_2/MeOH, 98:2) to give 650 mg (79%) of compound **11**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.15 (s, 3H, CH_3CO), 2.90 (d, 3H, J = 5.0 Hz, CH_3N), 4.53 (d, 1H, J = 3.2 Hz, H4′), 4.90 (dd, 1H, J = 3.5 and 5.0 Hz, H3′), 5.14 (s, 2H, NH_2), 5.94 (t, 1H, J = 5.3 Hz, H2′), 5.97 (d, 1H, J = 6.2 Hz, H1′), 7.09 (br s, 1H, NHCO), 7.82 (s, 1H, H8); Exact Mass (ESI-MS, i-PrOH/H_2O): Calcd for $C_{13}H_{15}ClN_9O_4[M+H]^+$: 396.0935. Found: 396.0932.

4.14. 9-(2-*O*-Acetyl-3-azido-3-deoxy-5-methylcarbamoyl-β-D-ribofuranosyl)-6-chloro-2-iodopurine (12)

Isoamylnitrite (681 μL, 4.98 mmol) was added to a mixture of **11** (650 mg, 1.65 mmol), I_2 (418 mg, 1.65 mmol), CH_2I_2 (1.37 mL, 16.5 mmol) and CuI (330 mg, 1.72 mmol) in 15 mL THF. The mixture was refluxed for 45 min and then cooled to room temperature. Insoluble materials were removed by filtration, and the filtrate was concentrated to dryness. The residue was purified by means of a silica gel column, which was washed with CH_2Cl_2 until the iodine colour disappeared and then eluted with CH_2Cl_2/MeOH, 98:2. Compound **12** was obtained in 79% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.13 (s, 3H, CH_3CO), 3.06 (d, 3H, J = 4.98 Hz, CH_3N), 4.56 (d, 1H, J = 2.9 Hz, H4′), 4.80 (dd, 1H, J = 2.9 and 5.6 Hz, H3′), 5.75 (dd, 1H, J = 5.9 and 7.0 Hz, H2′), 6.06 (d, 1H, J = 7.3 Hz, H1′), 7.09 (br s, 1H, NHCO), 8.11 (s, 1H, H8); Exact Mass (ESI-MS, i-PrOH/H_2O): Calcd for $C_{13}H_{13}ClIN_8O_4[M+H]^+$: 506.9794. Found: 506.9822.

4.15. 9-(3-Azido-3-deoxy-5-methylcarbamoyl-β-D-ribofuranosyl)-N^6-methyl-2-iodoadenine (13)

Compound **12** (460 mg, 0.91 mmol) was dissolved in EtOH (10 mL). Methylammonium chloride (92 mg, 1.36 mmol) and Et_3N (158 μL, 1.13 mmol) were added, and the solution was refluxed overnight. The mixture was concentrated to dryness, dissolved in 7 N NH_3 in methanol and stirred at room temperature for 2 h to deprotect the 2′-hydroxyl group. The volatiles were removed under reduced pressure, and the residue was purified by silica gel column (CH_2Cl_2/MeOH, 98:2). The product, **13**, was realized, in 77% yield. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.71 (d, 3H, J = 4.7 Hz, CH_3NHCO), 2.89 (d, 3H, J = 4.4 Hz, N^6-CH_3), 4.33 (d, 1H, J = 3.5 Hz, H4′), 4.47 (dd, 1H, J = 3.5 and 5.0 Hz, H3′), 4.92 (dd, 1H, J = 5.6 and 11.4 Hz, H2′), 5.89 (d, 1H, J = 6.5 Hz, H1′), 6.33 (d, 1H, J = 5.6 Hz, 2′-OH), 8.10 (d, 1H, J = 4,7 Hz, NHCO), 8.17 (d, 1H, J = 4.7 Hz, N^6H), 8.36 (s, 1H, H8); Exact Mass (ESI-MS, i-PrOH/H_2O): Calcd for $C_{12}H_{15}IN_9O_3[M+H]^+$: 460.0344. Found: 460.0350.

4.16. 9-(3-Azido-3-deoxy-5-methylcarbamoyl-β-D-ribofuranosyl)-N^6-methyl-2-phenylethynyladenine (14)

A solution of compound **20** (30 mg, 0.06 mmol) and 10 mL of 7 N NH_3 in methanol was kept at room temperature for 2 h to allow deprotection of the 2′-hydroxyl group. The mixture was concentrated to dryness and purified on a silica gel column (CH_2Cl_2/MeOH, 97:3). A 91% yield of compound **14** was obtained. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.73 (d, 3H, J = 3.5 Hz, CH_3NHCO), 2.96 (s, 3H, N^6-CH_3), 4.33 (d, 1H, J = 2.6 Hz, H4′), 4.51 (dd, 1H, J = 3.0 and 5.27 Hz, H3′), 4.96 (app t, 1H, J = 5.3 Hz, H2′), 5.98 (d, 1H, J = 6.7 Hz, H1′), 7.46 (m, 3H, Ph), 7.64 (m, 2H, Ph), 8.12 (s, 1H, N^6H), 8.51 (s, 1H, H8), 8.53 (d, 1H, J = 4.4 Hz, NHCO); Exact Mass (ESI-MS, i-PrOH/H_2O): Calcd for $C_{20}H_{20}N_9O_3[M+H]^+$: 434.1688. Found: 434.1686.

4.17. Attempted synthesis of compound 14

Attempted conversion of **13** to **14** using the procedure as described for **3**, **4** and **5** failed to give **14**, but resulted in the formation of triazole **15** as the sole reaction product. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.78 (d, 3H, J = 4.6 Hz, N^6-CH_3), 3.0 (br s, 3H, CH_3NHCO), 5.15 (app q, 1H, J = 6.3 Hz, H2′), 5.17 (d, 1H, J = 3.4 Hz, H4′), 5.61 (dd, 1H, J = 3.6 and 6.3 Hz, H3′), 6.26 (d, 1H, J = 5.4 Hz, 2′OH), 6.27 (d, 1H, J = 6.3 Hz, H1′), 7.36 (t, 1H, J = 7.3 Hz, 4″-Ph), 7.46–7.51 (m, 2H, 4″-Ph and 3H, C≡CPh), 7.65 (br d, 2H, J = 6.8 Hz, C≡CPh), 7.90 (d, 2H, J = 7.6 Hz, 4″-Ph), 8.59 (s, 1H, H8), 8.71 (m, 2H, H5″ and NHCO); Exact Mass (ESI-MS, i-PrOH/H_2O): Calcd for $C_{28}H_{25}N_9O_3[M+H]^+$: 536.2158. Found: 536.2166.

4.18. 2-Iodo-N^6-methyl-(9-tetrahydropyran-2-yl)adenine (17)

Methylammonium chloride (28 mg, 0.41 mmol) and DMAP (67 mg, 0.54 mmol) were added to 100 mg (0.27 mmol) of compound **16** in EtOH (6 mL), and the solution was refluxed overnight. The mixture was concentrated to dryness and the residue was purified on a silica gel column (pentane/ethyl acetate, 50:50). The title compound was obtained in 81% yield. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.52 (m, 6H, H2A′ and H2B′, H3A′ and H3B′, H4A′ and H4B′), 2.87 (s, 3H, NH CH_3), 3.68 (t, 1H, J = 11.4 Hz, H5′A), 3.96 (app d, 1H, J = 11.4 Hz, H5′B), 5.43 (dd, 1H, J = 2.3 and 10.9 Hz, H1′), 7.26 (br s, 1H, N$H$$CH_3$), 7.87 (s, 1H, H8); Exact Mass (ESI-MS, i-PrOH/H_2O): Calcd for $C_{11}H_{15}N_5O_3I[M+H]^+$: 360.0323. Found: 360.0333.

4.19. N^6-Methyl-2-phenylethynyl-tetrahydropyranyladenine (18)

Compound **17** (200 mg, 0.557 mmol) was dissolved in a mixture of Et_3N (3 mL) and DMF (1 mL) and the solution was purged with N_2. $(PPh_3)_2PdCl_2$ (39 mg, 0.056 mmol) and CuI (10.6 mg, 0.056 mmol) were added. Phenyl acetylene (112 μL, 1.11 mmol) was subsequently added dropwise, and the mixture was stirred at room temperature for 3 h. The solvents were removed under

reduced pressure, the residue was taken up in ethyl acetate and the resulting solution was filtered over a pad of Celite. After solvent evaporation, the residue was purified on a silica gel column (pentane/ethyl acetate, 50:50) to give compound **18** in 91% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.18–2.25 (m, 6H, H2A′ and H2B′, H3A′ and H3B′, H4A′ and H4B′), 3.21 (s, 3H, NH *CH*$_3$), 3.72 (app t, 1H, J = 11.4 Hz, H5′A), 3.96 (app d, 1H, J = 10.3 Hz, H5′B), 5.79 (d, 1H, J = 9.1 Hz, H1′), 7.20 (s, 1H, H8), 7.30 (d, 3H, J = 5.3 Hz, H-Ph), 7.61 (m, 2H, H-Ph); Exact Mass (ESI-MS, *i*-PrOH/H_2O): Calcd for $C_{19}H_{20}N_5O$[M+H]$^+$: 334.1667. Found: 334.1671.

4.20. N^6-Methyl-2-phenylethynylpurine (19)

To a solution of **18** (170 mg, 0.510 mmol) in 10 mL of CH_2Cl_2 was added slowly a solution of 0.78 mL TFA (10.2 mmol) and 0.78 mL CH_2Cl_2. After stirring at room temperature for 1 h, the solvent was evaporated, and the residue was taken up in ethyl acetate, and the solution was washed with 7% $NaHCO_3$. After silica gel chromatography (CH_2Cl_2/MeOH, 97:3), pure **19** was obtained in a 72% yield. ^{1}H NMR (300 MHz, DMSO): δ 2.97 (s, 3H, NH *CH*$_3$), 7.30 (m, 3H, H-Ph), 7.61 (m, 2H, H-Ph), 7.88 (br s, 1H, N*H*CH$_3$), 8.24 (s, 1H, H8); Exact Mass (ESI-MS, *i*-PrOH/H_2O): Calcd for $C_{14}H_{12}N_5$[M+H]$^+$: 250.1092. Found: 250.1073.

4.21. 9-(2-Acetyl-3-azido-3-deoxy-5-methylcarbamoyl-β-D-ribofuranosyl)-N^6-methyl-2-phenylethynyladenine (20)

To a mixture of **19** (150 mg, 0.602 mmol) and methyl 3-azido-3-deoxy-1,2-di-*O*-acetyl-α-D-ribofuronamide (**10**) (207 mg, 0.722 mmol) in 3 mL CH_3CN were successively added 223 μL (0.903 mmol) of *N*,*O*-bis(trimethylsilyl)-acetamide (BSA) and 131 μL (0.722 mmol) TMSOTf. The suspension was refluxed for 10 h. After being cooled to room temperature, the reaction was quenched with 7% $NaHCO_3$ and extracted with CH_2Cl_2. The organic layer was washed with brine, filtered through a short pad of Celite and evaporated to dryness. The crude material was purified by column chromatography (CH_2Cl_2/MeOH, 99:1), and compound **20** was obtained in 24.4% yield. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.12 (s, 3H, CH_3CO), 3.02 (d, 3H, J = 4.69 Hz, *CH*$_3$NHCO), 3.25 (s, 3H, N^6-CH_3), 4.58 (d, 1H, J = 2.6 Hz, H4′), 4.78 (dd, 1H, J = 2.1 and 5.3 Hz, H3′), 5.78 (dd, 1H, J = 7.3 and 12.9 Hz, H2′), 5.92 (s, 1H, N^6H) 6.02 (d, 1H, J = 7.6 Hz, H1′), 7.40 (app d, 3H, J = 7.0 Hz H-Ph), 7.63 (app d, 2H, J = 9.1 Hz, H-Ph), 7.81 (s, 1H, H8), 8.83 (s, 1H, NHCO); Exact Mass (ESI-MS, *i*-PrOH/H_2O): Calcd for $C_{22}H_{22}N_9O_4$[M+H]$^+$: 476.1794. Found: 476.1800.

4.22. 9-(2-Acetyl-3-azido-3-deoxy-5-*O*-toluoyl-β-D-ribofuranosyl)-2-amino-6-chloropurine (22)

To a mixture of 2-amino-6-chloropurine (90 mg, 0.53 mmol) and 3-azido-3-deoxy-1,2-di-*O*-acetyl-5-*O*-toluoyl-α-D-ribofuranose (**21**) (240 mg, 0.64 mmol) in 3 mL CH_3CN were successively added 196 μL (0.79 mmol) BSA and 115 μL (0.64 mmol) TMSOTf. The suspension was heated at 80 °C for 3 h. After being cooled to room temperature, the reaction was quenched with 7% $NaHCO_3$ and extracted with CH_2Cl_2. The organic layer was washed with brine, filtered through a short Celite pad and evaporated to dryness. The residue was purified by column chromatography (CH_2Cl_2/MeOH, 99:1) to yield 200 mg (65%) of compound **22**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.19 (s, 3H, CH_3CO), 2.41(s, 3H, CH_3-Ph), 4.40 (m, 1H, H4′), 4.52–4.58 (m, 1H, H5′A′), 4.76–4.82 (m, 2H, H3′ and H5′B), 5.13 (br s, 2H, NH_2), 5.94 (d, 1H, J = 3.8 Hz, H1′), 5.80 (dd, 1H, J = 3.8 and 5.6 Hz, H2′), 7.23 (d, 2H, J = 7.63 Hz, Ph), 7.79 (s, 1H, H8), 7.86 (d, 2H, J = 8.2 Hz, Ph); Exact Mass (ESI-MS, *i*-PrOH/H_2O): Calcd for $C_{20}H_{19}N_8O_5Cl$[M+H]$^+$: 487.1245. Found: 487.1246.

4.23. 9-(2-Acetyl-3-azido-3-deoxy-5-*O*-toluoyl-β-D-ribofuranosyl)-6-chloro-2-iodopurine (23)

This compound was prepared by the procedure described for the synthesis of **12** from **22** (200 mg, 0.41 mmol); yield: 200 mg (81%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.15 (s, 3H, CH_3CO), 2.35 (s, 3H, CH_3-Ph), 4.37 (dd, 1H, J = 4.1 and 7.6 Hz, H4′), 4.52 (dd, 1H, J = 4.4 and 12.3 Hz, H5′A′), 4.65 (dd, 1H, J = 3.2 and 12.3 Hz, H5′B), 4.96 (dd, 1H, J = 5.8 and 7.6 Hz, H3′), 6.03 (dd, 1H, J = 2.6 and 5.28 Hz, H2′), 6.29 (d, 1H, J = 2.9 Hz, H1′), 7.24 (d, 2H, J = 7.9 Hz, Ph), 7.68 (d, 2H, J = 8.2 Hz, Ph), 8.74 (s, 1H, H8); Exact Mass (ESI-MS, *i*-PrOH/H_2O): Calcd for $C_{20}H_{17}N_7O_5ClINa$[M+Na]$^+$: 619.9924. Found: 619.9920.

4.24. 9-(3-Azido-3-deoxy-2-hydroxyl-5-*O*-toluoyl-β-D-ribofuranosyl)-2-iodo-N^6-methyladenine (24)

The title compound was prepared as described for the synthesis of **13** from **23** (200 mg, 0.335 mmol); yield: 127 mg (69%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.36 (s, 3H, *CH*$_3$-Ph), 2.87 (d, 3H, J = 3.5 Hz, N^6-CH_3), 4.29 (dd, J = 5.3 and 9.7 Hz, H4′), 4.46–4.60 (m, 3H, H3′, H5′A and H5′B), 5.01 (t, 1H, J = 4.7 Hz, H2′), 5.87 (d, 1H, J = 4.7 Hz, H1′), 6.43 (d, 1H, J = 5.3 Hz, 2′-OH), 7.29 (d, 2H, J = 8.2 Hz, Ph), 7.78 (d, 2H, J = 8.2 Hz, Ph), 8.17 (d, 1H, J = 4.4 Hz, N^6H), 8.21 (s, 1H, H8); Exact Mass (ESI-MS, *i*-PrOH/H_2O): Calcd for $C_{19}H_{20}N_8O_4I$ [M+H]$^+$: 551.0653. Found: 551.0649.

4.25. 9-(3-Azido-3-deoxy-β-D-ribofuranosyl)-2-iodo-N^6-methyl-adenine (25)

Ester **24** (127 mg, 0.23 mmol) was dissolved in 2.5 mL MeOH. Na° (11.28 mg, 0.32 mmol) was added and the mixture was stirred at room temperature for 1 h. The reaction was quenched by adding a mixture of CH_3COOH/H_2O (9:1, v/v) to pH 7. The solution was concentrated to dryness, and the residue was purified by column chromatography (CH_2Cl_2/MeOH, 98:2) to yield 100 mg (95%) of compound **25**. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.88 (d, 3H, J = 4 Hz, N^6-CH_3), 3.52–3.67 (m, 2H, H5′A and H5′B), 3.94 (dd, 1H, J = 7.26 and 3.81 Hz, H4′), 4.28 (app t, 1H, J = 4.5 Hz, H3′), 4.89 (app t, 1H, J = 5.4 Hz, H2′), 5.20 (br s, 1H, 5′-OH), 5.80 (d, 1H, J = 6.16 Hz, H1′), 6.25 (br s, 1H, 2′-OH), 8.18 (d, 1H, J = 4 Hz, N^6H), 8.28 (s, 1H, H8); Exact Mass (ESI-MS, *i*-PrOH/H_2O):

Calcd for $C_{11}H_{14}N_8O_3I$ $[M+H]^+$: 433.0235. Found: 433.0237.

4.26. 9-(3-Azido-3-deoxy-5-*O*-toluoyl-β-D-ribofuranosyl)-2-amino-N^6-methyl-adenine (26)

Derivative **22** (100 mg, 0.21 mmol) was solubilized in EtOH (5 mL). Methylammonium chloride (35 mg, 0.515 mmol) and Et_3N (72 μL, 0.515 mmol) were added, and the solution was refluxed overnight. The mixture was concentrated to dryness, the residue redissolved in methanolic NH_3 and the solution stirred at room temperature for 2 h to allow deprotection of the 2′-hydroxyl group. The mixture was concentrated to dryness and purified on a silica gel column (CH_2Cl_2/MeOH, 97:3). Compound **26** was obtained in 68% yield. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.36 (s, 3H, CH_3-Ph), 2.84 (br s, 3H, N^6-CH_3), 4.23 (dd, J = 5.27 and 9.38 Hz, H4′), 4.43 (dd, 1H, J = 5.6 and 11.6, H5′A), 4.55 (m, 2H, H3 and H5′B), 4.48 (app t, 1H, 4.7 Hz, H2′), 5.78 (d, 1H, J = 4.7 Hz, H1′), 5.95 (br s, 2H, 2-NH_2), 6.34 (d, 1H, J = 7.9 Hz, 2′-OH), 7.28 (br s, 1H, N^6H), 7.29 (d, 2H, J = 7.9 Hz, Ph), 7.79 (s, 1H, H8), 7.82 (d, 2H, J = 8.2 Hz, Ph); Exact Mass (ESI-MS, *i*-PrOH/H_2O): Calcd for $C_{19}H_{22}N_9O_4$ $[M+H]^+$: 440.1794. Found: 440.1789.

4.27. 9-(3-Azido-3-deoxy-β-D-ribofuranosyl)-2-amino-N^6-methyladenine (27)

The title compound was synthesized from **26** (60 mg, 0.013 mmol) by the procedure described for the synthesis of **25**; yield: 40 mg (91%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.85 (br s, 3H, N^6-CH_3), 3.48–3.64 (m, 2H, H5′A and H5′B), 3.89 (dd, 1H, J = 3.2 and 7.0 Hz, H4′), 4.25 (dd, 1H, J = 3.2 and 5.6 Hz, H3′), 4.88 (app t, 1H, J = 5.3 Hz, H2′), 5.58 (t, J = 6.8 Hz, 1H, 5′-OH), 5.71 (d, 1H, J = 6.2 Hz, H1′), 5.85 (s, 2H, NH_2), 6.16 (s, 1H, 2′-OH), 7.31 (br s, 1H, N^6H), 7.89 (s, 1H, H8); Exact Mass (ESI-MS, *i*-PrOH/H_2O): Calcd for $C_{11}H_{16}N_9O_3[M+H]^+$: 322.1376. Found: 322.1365.

4.28. 9-[3-Azido-3-deoxy-5-(methylcarbamoyl)-β-D-ribofuranosyl]-2-amino-6-chloropurine (28)

Derivative **11** (120 mg, 0.30 mmol) was dissolved in EtOH (6 mL). Methylammonium chloride (30 mg, 0.46 mmol) and Et_3N (53 μL, 0.38 mmol) were added, and the solution was refluxed overnight. The mixture was concentrated to dryness, the remaining solid dissolved in 7 N NH_3 in methanol and the solution stirred at room temperature for 2 h to deprotect the 2′-hydroxyl group. The mixture was concentrated to dryness and the residue was purified on a silica gel column (CH_2Cl_2/MeOH, 95:5). Compound **28** was obtained in 79% yield. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.68 (d, 3H, J = 4.7 Hz, CH_3NHCO), 2.87 (s, 3H, N^6-CH_3), 4.24 (d, 1H, J = 2.9 Hz, H-4′), 4.42 (dd, 1H, J = 2.9 and 5.3 Hz, H3′), 4.97 (dd, 1H, J = 5.6 and 11.7 Hz, H2′), 5.81 (d, 1H, J = 6.8 Hz, H1′), 5.91 (s, 2H, NH_2), 6.26 (d, 1H, J = 5.3 Hz, 2′-OH), 7.31 (s, 1H, N^6H), 7.96 (s, 1H, H8), 8.35 (d, 1H, J = 4.7 Hz, NHCO); Exact Mass (ESI-MS, *i*-PrOH/H_2O): Calcd for $C_{12}H_{17}N_{10}O_3[M+H]^+$: 349.1484. Found: 349.1491.

4.29. Biological assays

4.29.1. Cell culture and membrane preparation. CHO cells expressing recombinant human A_3ARs were cultured in DMEM (Dulbecco's modified Eagle's medium) and F12 (1:1) supplemented with 10% foetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 μmol/mL glutamine and 800 μL geneticin. After harvest and homogenization, the cells were centrifuged at for 10 min. The pellet was resuspended in 50 mM Tris–HCl buffer (pH 8.0) containing 10 mM $MgCl_2$ and 1 mM EDTA. The suspension was homogenized with an electric homogenizer for 10 s and was then recentrifuged at 20,000*g* for 20 min at 4 °C. The resulting pellets were resuspended in buffer containing 3 U/mL of adenosine deaminase, and the suspension was stored at −80 °C prior to the binding experiments. The protein concentration was measured using the Bradford assay.[25]

4.29.2. Radioligand binding studies. For the A_3AR binding experiments, the procedures were similar to those previously described.[26] Briefly, each tube contained 100 μL of membrane suspension, 50 μL [^{125}I]I-AB-MECA (final concentration 0.5 nM) and 50 μL of increasing concentrations of compounds in Tris–HCl buffer (50 mM, pH 7.4) containing 10 mM $MgCl_2$, 1 mM EDTA. Non-specific binding was determined using 10 μM NECA. The mixtures were incubated at 25 °C for 60 min. Binding reactions were terminated by filtration through Whatman GF/B filters under reduced pressure using a MT-24 cell harvester (Brandel, Gaithersburg, MD). Filters were washed three times with ice-cold buffer. Radioactivity was determined in a Beckman 5500B γ-counter. The binding of [^{3}H]R-PIA to the recombinant hA_1AR and the binding of [^{3}H]CGS21680 to the recombinant hA_{2A}AR were performed as previously described.[27,28]

4.29.3. Cyclic AMP accumulation assay. Intracellular cyclic AMP levels were measured by the competitive protein binding method.[29] CHO cells expressing recombinant human[30] ARs were harvested by trypsinization. After resuspension in the medium, cells were plated in 24-well plates in 0.5 mL medium/well. After 24 h, the medium was removed and cells were washed three times with 1 mL/well DMEM containing 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4. Cells were then treated with agonists and/or test compounds in the presence of rolipram (10 μM) and adenosine deaminase (3 U/mL) and incubated at 37 °C. For A_3AR, after 45 min forskolin (10 μM) was added to the medium, and incubation was continued for an additional 15 min. The reaction was terminated upon removal of the medium, and the cells were lysed with 200 μL/well of 0.1 M ice-cold HCl. The cell lysate was resuspended and stored at −20 °C. For determination of cyclic AMP production, protein kinase A (PKA) was incubated with [^{3}H]cyclic AMP (2 nM) in K_2HPO_4/EDTA buffer (K_2HPO_4, 150 mM; EDTA, 10 mM), 20 μL of the cell lysate and 30 μL of 0.1 M HCl. Bound radioactivity was separated by rapid filtration through Whatman GF/C filters under reduced pressure and washed once with cold buffer. Bound radioactivity was subsequently measured by scintillation spectrometry.

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