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Synthesis of novel purine nucleosides towards a selective inhibition of human butyrylcholinesterase

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ABSTRACT

The search for new and potent cholinesterase inhibitors is an ongoing quest mobilizing many organic chemistry groups around the world as these molecules have been shown to treat the late symptoms of Alzheimer's disease as well as to act as neuroprotecting agents. In this work, we disclose the synthesis of novel 2-acetamidopurine nucleosides and, for the first time, regioselective N^7-glycosylation with 2-acetamido-6-chloropurine, promoted by trimethylsilyl triflate, was accomplished by tuning the reaction conditions (acetonitrile as solvent, 65 °C, 5 h) starting from 1-acetoxy bicyclic glycosyl donors, or by direct coupling of a methyl glucopyranoside with the nucleobase to obtain only N^7 nucleosides in reasonable yield (55–60%). The nucleosides as well as their sugar precursors were screened for acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition. While none of the compounds tested inhibited AChE, remarkably, some of the N^7 nucleosides and sugar bicyclic derivatives showed potent inhibition towards BChE. Nanomolar inhibition was obtained for one compound competing well with rivastigmine, a drug currently in use for the treatment of Alzheimer's disease. Experimental results showed that the presence of benzyl groups on the carbohydrate scaffold and the N^7-linked purine nucleobase were necessary for strong BChE inactivation. A preliminary evaluation of the acute cytotoxicity of the elongated bicyclic sugar precursors and nucleosides was performed indicating low values, in the same order of magnitude as those of rivastigmine.

1. Introduction

There are two major forms of cholinesterases, acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8) in mammalian tissues. Both AChE and BChE belong to the group of serine hydrolases and are responsible for the breakdown of the neurotransmitter acetylcholine (ACh) and of butyrylcholine (BCh), respectively.[1] In Alzheimer's disease (AD) the loss of cholinergic neurotransmission in the brain is accompanied by a reduced concentration of ACh and contributes to the salient cognitive and behavioral disturbances characteristic of AD.[2–4] This is the most common type of dementia in western societies, which has been causing profound economic and social impact as the aging population increases.[5] The cholinergic hypothesis[6] represents one of the most promising approaches involving the design of new agents for the treatment of AD. This strategy is based on the development of drugs with an AChE inhibition profile in order to rectify the deficit of cerebral ACh. However, in advanced AD, AChE levels in the brain have already decreased, while BChE activity is still high, suggesting that ACh hydrolysis may occur to a greater extent via BChE catalysis.[7] In fact, it has been reported that the specific inhibition of BChE is important in raising acetylcholine levels and improving cognition.[8,9]

Cholinesterase inhibitors such as tacrine,[10] rivastigmine,[11] donepezil[12,13] and galanthamine,[14] currently in use to treat AD, inhibit both AChE and BChE,[15] and it is difficult to determine whether the positive effects observed result from inhibition of AChE or BChE enzymes or both. For this reason, it is important to design selective, potent and well-tolerated inhibitors of each cholinesterase in order to determine which enzyme needs to be targeted for maximum effect in treating AD.[16]

2-Aminopurine nucleosides are rarely found in Nature. However such N^9 nucleosides are key structural features of Amypurimycin and Miharamycins A and B, three natural products which are potent inhibitors of *Pyricularia oryzae*, a fungus responsible for the rice blast disease. We have previously reported the first total synthesis of the core of Miharamycin B, which includes the synthesis of several structural fragments, such as N^9 nucleosides

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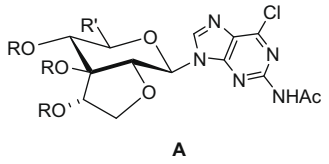
incorporating a complex bicyclic carbohydrate skeleton.[17,18] Herein we report the synthesis of novel 2-acetamidopurine N^7 nucleosides, in which this unusual bicyclic sugar moiety is present (compounds type A, Fig. 1). Our interest in the development of selective cholinesterase inhibitors prompted us to evaluate these molecules, as well as their precursors, as AChE and BChE inhibitors. In addition, a preliminary screening of the acute cytotoxicity of this family of compounds completed the biological studies.

2. Results and discussion

A total of fourteen compounds including nucleosides and their bicyclic sugar precursors were synthesized and evaluated for their ability to inhibit human serum BChE and bovine erythrocyte AChE (BoAChE). The free bicyclic sugar, its acyl and benzyl protected derivatives, the sugar azido esters and the two regioisomeric nucleosides resulting from N-glycosylation of the purine base were screened. A strong and selective inhibition for BChE was observed for some of the products, and compared to rivastigmine. The N^7 nucleosides were the most promising compounds and for that purpose, stereo- and regioselective coupling conditions were devised in order to obtain only the N^7 regioisomers. A preliminary toxicity screening of closely related elongated sugar precursors and purine nucleosides was also accomplished.

2.1. Chemistry

The synthesis of the bicyclic sugar moiety **1** was carried out according to the strategy previously developed by Fairbanks and Sinaÿ.[19] Benzylidene hydrolysis cleanly afforded the unprotected bicyclic scaffold **2**, which was peracetylated and perbenzoylated to give compounds **3** and **4** in good yield, respectively (Scheme 1).



A

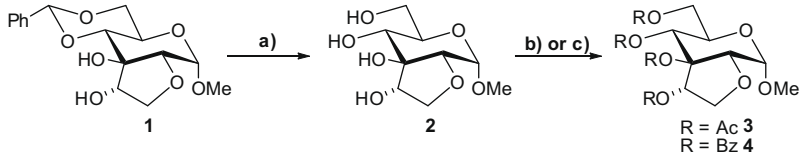
Figure 1. General structure of the new synthetic 2-acetamidopurine nucleosides.

As part of an ongoing program to exploit the total synthesis of the natural Miharamycins antibiotics,[17] we devised a non-stereoselective chain extension at C-6 of the corresponding primary alcohol **5**. Its Swern oxidation proceeded efficiently to give the crude aldehyde, which was used directly without further purification (Scheme 2). In order to obtain both diastereoisomers at C-6′, vinylation of the aldehyde in the absence of chelating species proved to be the method of choice to obtain both allylic alcohols **6a** and **6b**.[17] Their conversion into the corresponding azido esters was then studied. Since the azido group is stable in the presence of oxidants, it could be either introduced early in the synthesis, before the oxidative cleavage of the vinyl group, or after the introduction of the carboxylic acid function. Preliminary studies showed that azide displacement of the triflate derived from the allylic alcohol **6a** mainly afforded the regioisomeric azide resulting from an allylic rearrangement. Czernecki's group also reported an allylic rearrangement when he tried to introduce the azide via Mitsunobu reaction on a hexodialdo-1,5-pyranose.[20] These results prompted us to synthesize the hydroxy esters first.

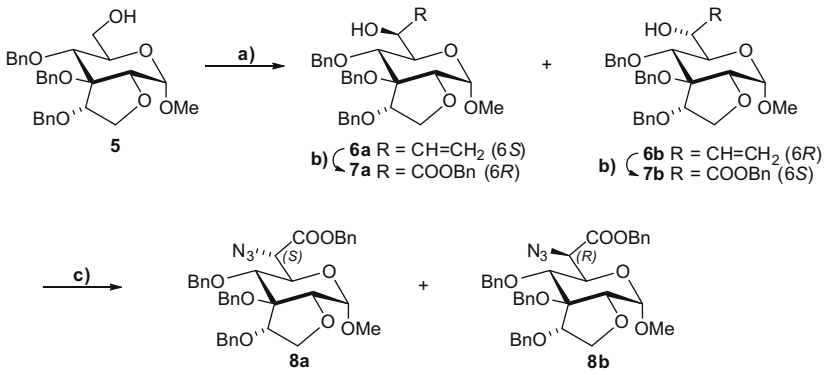
Ozonolysis of the allylic alcohols **6a** and **6b** followed by sodium chlorite oxidation in *t*BuOH/H_2O/2-methylbut-2-ene (2:2:1) afforded the corresponding carboxylic acids, which were not isolated. Esterification with benzyl bromide in the presence of potassium hydrogenocarbonate gave the corresponding benzyl esters **7a** and **7b** (Scheme 2) which were converted to the corresponding triflates. Azide displacement with sodium azide in DMF at room temperature afforded the inverted azido esters in good yield (Scheme 2).

In order to obtain both N^9 and N^7 nucleosides **11** and **12** our gained knowledge concerning N-glycosylation with glycosyl donors such as **9** was exploited,[17,18] and their coupling with the persilylated 2-acetamido-6-chloropurine **10** was accomplished using the conditions described in Scheme 3.

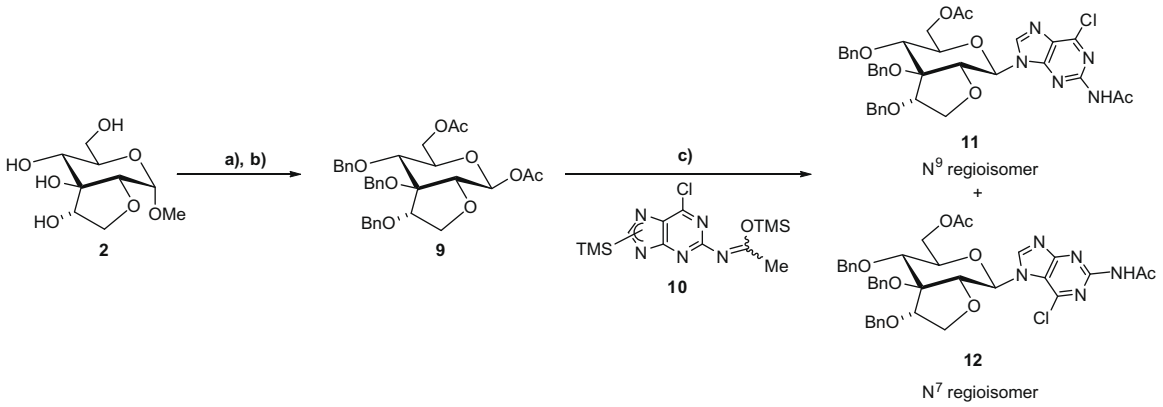
Since the N^7 regioisomer **12** was the most promising compound to inhibit the BChE (Table 2), regioselective coupling conditions were developed to furnish only N^7 nucleosides. In order to obtain



Scheme 1. Synthesis of the bicyclic sugar moieties **2**, **3** and **4**. Reagents and conditions: (a) AcOH (80% aq), 50 °C, quant; (b) Ac_2O, pyridine, DMAP, rt, 92% for **3**; (c) BzCl, pyridine, DMAP, rt→80 °C, 72% for **4**.



Scheme 2. Synthesis of the benzyl azido esters **8a** and **8b**. Reagents and conditions: (a) $(COCl)_2$, DMSO, Et_3N, −78 °C then CH_2=CHMgBr, THF, −78 °C, 65% over two steps;[17] (b) O_3, DMS, CH_2Cl_2, −78 °C, then $NaClO_2$, $NaH_2PO_4.H_2O$, *t*BuOH/H_2O/2-methylbut-2-ene (2:2:1), then BnBr, $KHCO_3$, Bu_4NI, DMF, 68% over three steps; (c) Tf_2O, pyridine, CH_2Cl_2, −78 °C, then NaN_3, DMF 72% for **8a** over two steps, 86% for **8b** over two steps.



Scheme 3. Synthesis of the N^9- and N^7 nucleosides **11** and **12**. Reagents and conditions: (a) NaH (60% w/w), BnBr, DMF, 85%; (b) 5% concd H_2SO_4 in AcOH, Ac_2O, −20 °C→ 0 °C, 48%; (c) TMSOTf, $(CH_2Cl)_2$, 85 °C, 25% for **11** and 37% for **12**.

the suitable glycosyl donors, methyl glycosides **8a** and **8b** were transformed into their more reactive 1-acetoxy counterparts **13a** and **13b** (Scheme 4). Acetolysis of the tribenzylated 6*S* diastereoisomer **8a** took place under the conditions previously optimized to avoid the formation of ring contraction products,[17,18] namely in the presence of catalytic concentrated sulfuric acid 5% in acetic acid, at low temperature, while acetolysis of its 6*R* epimer occurred smoothly and in high yield under catalysis of concentrated sulfuric acid, emphasizing the acidic sensitivity of the 6S isomer. Regioselective synthesis of N^7 nucleosides under kinetic control ($SnCl_4$ as Lewis acid, CH_3CN as polar solvent and at low temperature) has been previously reported by Garner.[21] When applying these conditions, unsatisfactory yields were obtained for the β-N^7-nucleosides **14a** and **14b** (Scheme 4) which could be related to the presence of the benzyl ethers, which are sensitive to the strong acidic conditions[22] required for the N-glycosylation. In order to improve the yield various conditions were applied and it was found that the use of the milder TMSOTf Lewis acid as promoter at 65 °C furnished the β-N^7-nucleosides **14a** and **14b** in satisfactory yield. Considering the problematic acetolysis of the 6*S* epimer, the direct coupling of the methyl glycoside **8a** with the persilylated base was successfully attempted using TMSOTf (20 equiv) in CH_3CN at 65 °C for 5 h to give the target N^7 nucleoside in 60% yield. When these conditions were applied to the methyl glycoside **8b** the N^7 nucleoside was also isolated as single product in 55% yield.

2.2. Enzymatic studies

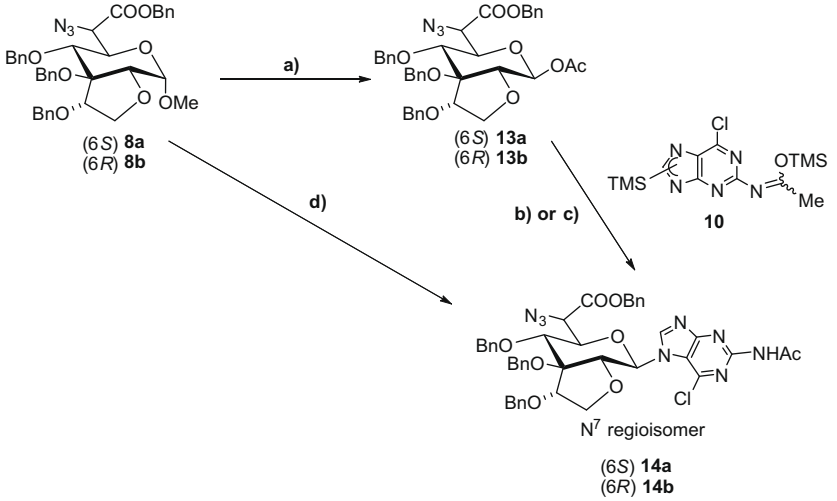
The inhibitory activity of the new synthetic compounds against both AChE (bovine erythrocytes) and BChE (human serum) was evaluated using Ellman's spectrophotometric method[23] to determine the rate of hydrolysis of acetylthiocholine and butyrylthiocholine, respectively, in the presence of the inhibitor. The activity of the enzymes produces a yellow compound which was monitored spectrophotometrically (λ = 410 nm) along the reaction time. The enzyme activity (%) and the enzyme inhibition (%) were calculated from the rate of absorbance change with time ($V = \Delta Abs/\Delta t$) data as follows:

$$\text{Enzyme Inhibition } (\%) = 100 - \text{Enzyme Activity } (\%)$$

$$\text{Enzyme Activity } (\%) = 100 \times V/V_{max}$$

Maximum rates (V_{max}) are obtained when no inhibitor is used while V is the rate obtained in the presence of the inhibitor.

The *t*-test (one-sided) was carried out in order to evaluate if the average inhibition of the enzymes with compounds tested and the positive control rivastigmine (drug available on the market), are significantly higher than the average inhibition (0%) obtained in the assay without any inhibitor. The *t*-test gives a probability between 0.00 and 1.00. When the probability ⩽0.05 or 5%, this means that the inhibition obtained with the compound at a certain concentration is significant. The IC_{50} is an estimate value of the



Scheme 4. Synthesis of the (6′*S*)- and (6′*R*)-configurated N^7 nucleosides **14a** and **14b**. Reagents and conditions: (a) 5% concd H_2SO_4 in AcOH, Ac_2O, −20 °C→0 °C, 36% for **13a** and concd H_2SO_4, Ac_2O, 0 °C, 63% for **13b**; (b) $SnCl_4$, CH_3CN, rt 32% for **14a** and 28% for **14b**; (c) TMSOTf, CH_3CN, 65 °C, 65% for **14a** and 58% for **14b**; (d) TMSOTf, CH_3CN, 65 °C, 60% for **14a** and 55% for **14b**.

compound concentration which inhibits 50% of the enzyme activity. IC_{50} values were determined for the active compounds and rivastigmine using non-linear regression analysis (Statistica 8.0 by StatSoft, Inc., USA) toward a dose–response curve (enzyme inhibition vs substance concentration) and fitting an appropriate mathematical model.

The BoAChE and human AChE (HuAChE) display identical structure characteristics. Mendelson's et al. have mapped onto the three-dimensional model of HuAChE, where all divergent amino acids between BoAChE and HuAChE were shown.[24] The authors reported that all the 34 divergent amino acids are located at the protein surface and these structural differences could only lead to functional manifestations not related to the cholinergic activities. For this reason identical kinetic behavior of BoAChE and HuAChE with respect to hydrolysis and binding with different inhibitors should be expected.[24]

None of the compounds tested showed a significant inhibition of BoAChE. Noteworthy, inhibition of BChE (human serum) was detected for most of the studied compounds (Table 1). The presence of the benzyl groups seems to improve BChE inhibition if we compare the results obtained for the free and acylated derivatives (**2**–**4**) and for the benzylated derivative **5**. The azido group also appears to play a role for the inhibition of the BChE. Nevertheless the inhibition results obtained do not allow to establish a structure–activity relationship regarding the configuration at C-6.

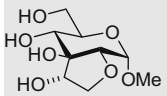
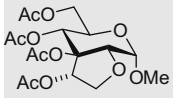
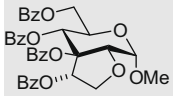
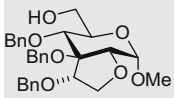
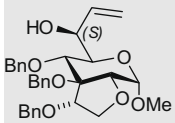
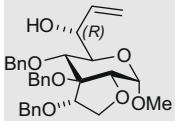
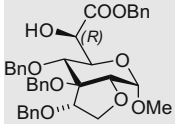
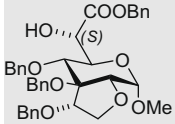
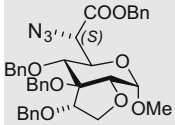
Regarding the BChE inhibitory profile of nucleosides **11** and **12**, only N^7 regioisomer proved to be a good inhibitory agent (Table 2). Nevertheless, the two diastereoisomers at C-6′ **14a** and **14b** showed a remarkable BChE inhibition and especially compound **14b** with *R* configuration at C-6′ showed an IC_{50} of the same order of magnitude as that of the control, rivastigmine.

Interestingly, experimental results showed a good and selective inhibition of BChE vs AChE for the N^7 nucleosides (**12**, **14a,b**) and some of the bicyclic sugar precursors (**7a**, **8a,b**). These two enzymes have a significant homology and they mainly differ by the volume of their active site gorge. In AChE the estimated volume is relatively small (302 $Å^3$), being lined with 14 bulky aromatic amino acid residues, while that of BChE, with only 8 aryl residues, is considerably larger (502 $Å^3$).[25,26] In addition AChE active site has an elevated electronegative potential and an anionic peripheral site while BuChE has a lower electronegative potential and a non-anionic peripheral site. Since the experiments were run at pH 8, protonation of the studied compounds is not expected. Hence, the volume of the molecules' active moiety may play an important role in the observed selectivity. Alternatively, it can be hypothesized that the binding site of the new ligands may be different from the catalytic gorge. The bioactivity shown by the benzylated derivatives also indicates that the binding site at BChE seems highly lipophilic, a characteristic known for its catalytic site as reported by other authors.[27] Additionally, π–π interaction has been reported to take place between the heterocyclic ring system of known inhibitors and the aromatic amino acid residues F329 and Y332 of BChE.[28,29] Thus, π–π interaction could explain the better inhibition found for the compounds containing the heterocyclic purine base. Nevertheless, it should be noted that the pattern of activity displayed by N^7 and N^9 regioisomers is undoubtedly divergent. Apparently, the structural differences between these two regioisomers play an important role in BChE profile inhibition.

2.3. Preliminary acute toxicity screening

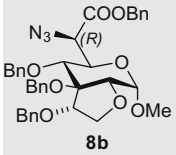
Considering the inhibition activity of some of the synthetic compounds towards BChE, it became interesting to explore further application in the treatment or palliation of Alzheimer's disease symptoms. The first concern in this matter would be the potential toxicity of such compounds family, and therefore the sugar precursors **7a**, **8a**, **8b** and the readily available nucleosides **14a**, **15a**[17] and

Table 1
Inhibition (%) of BChE for different concentrations of the compounds tested and IC_{50} values

Compound tested	Concentration (μg/mL)	BChE inhibition (%)	BChE IC_{50} ± SEM QiM)[a]
 **2**	100	<5	–
 **3**	100	<5	–
 **4**	100	7	–
 **5**	100.00 10.00	60*** <5	148.00 ± 43.00
 **6a**	100.00 10.00 1.00	76*** 54*** 4	31.00 ± 2.90
 **6b**	100.00 10.00	73*** 23***	50.00 ± 4.60
 **7a**	100.00 10.00 1.00	95*** 56*** 7	16.10 ± 0.90
 **7b**	100.00 10.00	55*** 8	133.00 ± 27.00
 **8a**	100.00 10.00 1.00 0.10	91*** 64*** 36*** 15*	4.20 ± 0.40

(*continued on next page*)

Table 1 (*continued*)

Compound tested	Concentration (μg/mL)	BChE inhibition (%)	BChE IC_{50} ± SEM QiM)[a]
 **8b**	100.00	98***	13.40 ± 0.80
	10.00	73***	
	1.00	14**	
Rivastigmine[b]	100.00	100***	0.17 ± 0.01
	10.00	100***	
	1.00	100***	
	0.10	76***	
	0.01	NI	

[a] IC_{50} is an estimate of the compound concentration which inhibits 50% of the enzyme activity; values are expressed as mean ± standard error of the mean (SEM).
[b] Rivastigmine is a standard drug to treat AD patients and was used as positive control. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

15b[18] (Table 3) were selected as probe compounds for a preliminary screening of acute in vitro toxicity. Cytotoxicity was assessed by the quantification of viable and metabolically active continuous culture human cells following a 24 h exposure to the drug by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cell proliferation assay.[30,31] A similar toxicity profile was exhibited by these probe compounds, namely **8a** and **8b** showed the same behavior, suggesting that configuration at C-6 does not influence the toxic effect. There was a subtle trend for a slightly higher toxicity of the N^7 nucleoside **14a**, while the less toxic molecule appeared to be **7a**, with a free OH-6. These data only give an estimate of the potential toxicity of this family of compounds. However the toxicity shown can still be considered low when compared to the one exhibited by rivastigmine, a standard drug currently used in the treatment of patients suffering from Alzheimer's disease.

3. Conclusion

Novel and non-natural N^7 purine nucleosides, as well as some of their bicyclic sugar counterparts demonstrated a high potential for the selective inhibition of butyrylcholinesterase, with IC_{50} values of the same order of magnitude as that of the positive control rivastigmine. Interestingly, for the nucleobase-free derivatives the presence of benzyl protecting groups and the azido group seem to be significant for BChE inhibition. Regarding nucleosides synthesis, an efficient regioselective N^7-glycosylation procedure of the 2-acetamido-6-chloropurine was developed starting from 1-acetoxy glycosyl donors, in acetonitrile at 65 °C and using TMSOTf as promoter. Noteworthy, the increase of the number of equivalents of the Lewis acid and reaction time (3.5 h) allowed the direct coupling of a methyl glucopyranoside with a 2-acetamido purine base, in reasonable yields. To the best of our knowledge, this is the first direct synthesis of a nucleoside starting from an unactivated methyl glycopyranoside. The low values for acute cytotoxicity of the probe molecules tested encourage further investigation of this family of compounds for the control of neurodegenerative disorders such as Alzheimer's disease.

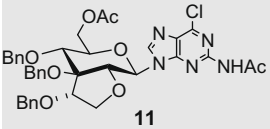
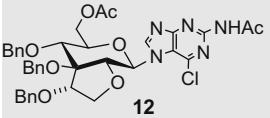
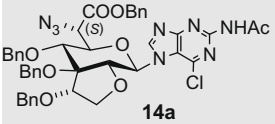
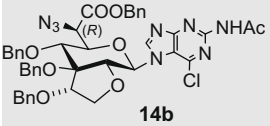
4. Experimental

4.1. Chemistry

4.1.1. General methods

Solvents and reagents were purchased from Fluka, Aldrich or Acros Organics. Solvents were freshly distilled under argon from

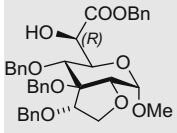
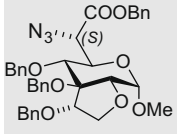
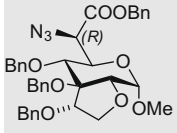
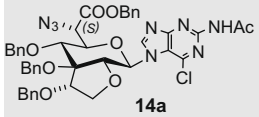
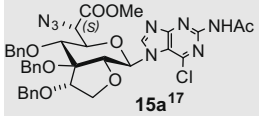
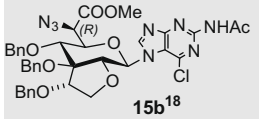
Table 2
Inhibition (%) of BChE for different concentrations of the compounds tested and IC_{50} values

Compound tested	Concentration (μg/mL)	BChE inhibition (%)	BChE IC_{50} ± SEM[a] (μM)
 **11**	100.00	48***	–
	10.00	11	
 **12**	100.00	84***	0.76 ± 0.05
	10.00	85***	
	1.00	70***	
	0.10	20***	
 **14a**	100.00	81***	22.00 ± 1.60
	10.00	34***	
	1.00	9	
 **14b**	100	91***	0.14 ± 0.01
	10	79***	
	1	77***	
	0.1	63***	
	0.01	10	
Rivastigmine[b]	100	100***	0.17 ± 0.01
	10	100***	
	1	100***	
	0.1	76***	
	0.01	NI	

[a] IC_{50} is an estimate of the compound concentration which inhibits 50% of the enzyme activity; values are expressed as mean ± standard error of the mean (SEM).
[b] Rivastigmine is a standard drug to treat AD patients and was used as positive control. ***$P < 0.001$.

Na/benzophenone (THF) or P_2O_5 [CH_2Cl_2, $(CH_2Cl)_2$]. Other solvents were dried over a bed of activated molecular sieves (MS) 4 Å (DMF) or KOH (pyridine). Solvents for column chromatography (cyclohexane, EtOAc, CH_2Cl_2) were distilled before use. Reactions were monitored by thin-layer chromatography (TLC) on a precoated plate of Silica Gel 60 F_{254} (layer thickness 0.2 mm; E. Merck, Darmstadt, Germany) and detection by UV light (254 nm) and by charring with H_2SO_4 10% in EtOH or with 0.2% w/v cerium sulfate and 5% ammonium molybdate in 2 M H_2SO_4. Flash column chromatography was performed on silica gel 60 (230–400 mesh, E. Merck). Melting points (mp) were determined with a Büchi B-510 capillary apparatus and are uncorrected. Optical rotations ($[\alpha]_D$) were measured at 20 ± 2 °C with a Perkin Elmer Model 241 digital polarimeter, using a 10 cm, 1 mL cell. High resolution mass spectra (HRMS) using chemical ionization (CI, ammonia) or fast atom bombardment (FAB) were obtained with a JMS-700 spectrometer at École Normale Supérieure de Paris (ENS-Paris). Elemental analyses were performed by the service d'analyse of UPMC-Paris VI. NMR experiments were recorded on Brüker spectrometers: ^1H NMR spectra were recorded at 400 MHz with a DRX 400 at ENS-Paris,

Table 3
Preliminary screening of the acute toxicity of compounds **7a**, **8a**, **8b**, **14a**, **15a**[17] and **15b**[18] in immortalized human fibroblasts (A549 cells)

Compound no.	Concentration (μg/mL)	Cell viability ± SEM (% of control)
 **7a**	2000	35.95 ± 3.84
	200	63.58 ± 10.37
	20	67.24 ± 12.68
	2	81.56 ± 12.49
	0.2	91.88 ± 13.98
 **8a**	2000	24.97 ± 2.49
	200	47.94 ± 3.84
	20	73.90 ± 16.91
	2	71.23 ± 10.38
	0.2	90.22 ± 9.42
 **8b**	2000	15.98 ± 4.61
	200	64.25 ± 17.87
	20	63.58 ± 21.72
	2	76.23 ± 10.18
	0.2	90.54 ± 15.75
 **14a**	2000	7.32 ± 1.92
	200	51.26 ± 4.61
	20	46.94 ± 16.72
	2	82.22 ± 2.88
	0.2	88.54 ± 3.46
 **15a**[17]	2000	15.05 ± 3.56
	200	19.54 ± 0.81
	20	40.84 ± 0.18
	2	77.70 ± 5.88
	0.2	102.90 ± 0.84
 **15b**[18]	2000	21.27 ± 15.16
	200	52.27 ± 3.62
	20	69.27 ± 4.29
	2	99.90 ± 0.50
	0.2	102.91 ± 2.80
DMSO	2000	60.25 ± 11.01
	200	89.54 ± 0.58
	20	86.22 ± 5.19
	2	104.19 ± 13.64
	0.2	90.54 ± 6.15
H_2O_2% (positive control)		44.51 ± 0.65
Rivastigmine, 100 μg/mL		89.02 ± 5.76
Blank sample		100.00 ± 7.39

or with an Avance 400 (fitted with a QNP probe) at FCUL for soln in $CDCl_3$, or D_2O at room temperature. Assignments were confirmed by COSY experiments. ^{13}C NMR spectra were recorded at 100 MHz with a DRX 400 spectrometer at ENS-Paris, or with an Avance 400 (fitted with a QNP probe) at FCUL. Assignments were confirmed by J-mod technique, HMQC and HMBC. Chemical shifts (δ) are given in ppm relative to tetramethylsilane (TMS) or to the residual solvent signal. Coupling constants (J) are reported in hertz (Hz).

4.1.2. Methyl 3-*C*,2-*O*-[(1*S*)-1-hydroxyethylene]-α-D-glucopyranoside (2)

The bicyclic derivative **1** (0.73 g, 2.25 mmol) was dissolved in 80% aqueous solution of acetic acid (25 mL) and warmed to 50 °C over a period of 2 h. The solvent was removed under reduced pressure and the residue obtained co-evaporated with toluene (3 × 50 mL). Purification by flash column chromatography (CH_2Cl_2/MeOH 8:2) afforded the miharamycins bicyclic unit **2** (0.53 g, quant. yield) as a white solid. R_f 0.23 (EtOAc/MeOH, 9:1); mp 142–144 °C (CH_2Cl_2/MeOH); $[\alpha]_D$ +125.3 (*c* 1.0, MeOH); ^1H NMR (D_2O, 400 MHz): δ 4.95 (d, 1H, $J_{1,2}$ = 5.4 Hz, H-1), 4.36 (dd, 1H, $J_{8a,7}$ = 5.0 Hz, $J_{8a,8b}$ = 10.1 Hz, H-8a), 4.25 (d, 1H, $J_{7,8a}$ = 5.0 Hz, H-7), 4.04 (d, 1H, $J_{2,1}$ = 5.4 Hz, H-2), 3.93 (d, 1H, $J_{4,5}$ = 10.0 Hz, H-4), 3.93–3.83 (m, 3H, H-5, H-6a, H-8b), 3.72 (dd, 1H, $J_{6b,5}$ = 5.5 Hz, $J_{6b,6a}$ = 12.3 Hz, H-6b), 3.46 (s, 3H, OCH_3); ^{13}C NMR (D_2O, 100 MHz): δ 98.13 (C-1), 82.30 (C-3), 81.74 (C-2), 77.29 (C-8), 76.78 (C-7), 71.68 (C-5), 70.75 (C-4), 61.15 (C-6), 55.62 (OCH_3); HRMS (CI) *m/z*: $[M+NH_4]^+$ calcd for $C_9H_{20}O_7N$, 254.1234, found: 254.1241; Anal. Calcd for $C_9H_{16}O_7$: C, 45.76; H, 6.83. Found: C, 45.80; H, 6.90.

4.1.3. Methyl 3,4,6-tri-*O*-acetyl-3-*C*,2-*O*-[(1*S*)-1-(acetoxy)ethylene]-α-D-glucopyranoside (3)

To the bicyclic compound **2** (0.27 g, 1.14 mmol) in dry pyridine (5 mL) were added acetic anhydride (2.5 mL) and a catalytic amount of DMAP. The reaction mixture was stirred overnight at room temperature, then co-evaporated with toluene and concentrated. The crude residue was dissolved in CH_2Cl_2 (80 mL) and washed with a 1 M aqueous solution of HCl (20 mL). The organic layer was separated, dried ($MgSO_4$) and concentrated under reduced pressure. Purification by flash column chromatography (cyclohexane/EtOAc, 1:1) afforded the peracetylated compound **3** (0.43 g, 92%) as a colorless oil. R_f 0.32 (cyclohexane/EtOAc, 1:1); $[\alpha]_D$ +95.5 (*c* 0.7, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz): δ 5.91 (d, 1H, $J_{4,5}$ = 10.3 Hz, H-4), 5.51 (dd, 1H, $J_{7,8b}$ = 2.2 Hz, $J_{7,8a}$ = 5.7 Hz, H-7), 4.92 (d, 1H, $J_{1,2}$ = 5.2 Hz, H-1), 4.77 (d, 1H, $J_{2,1}$ = 5.2 Hz, H-2), 4.22 (ddd, 1H, $J_{5,6b}$ = 2.4 Hz, $J_{5,6a}$ = 5.4 Hz, $J_{5,4}$ = 10.3 Hz, H-5), 4.17–4.13 (m, 2H, H-6, H-8a), 4.04 (dd, 1H, $J_{6b,5}$ = 2.4 Hz, $J_{6b,6a}$ = 12.6 Hz, H-6b), 3.91 (dd, 1H, $J_{8b,7}$ = 2.2 Hz, $J_{8b,8a}$ = 10.0 Hz, H-8b), 3.36 (s, 3H, OCH_3), 2.03, 1.99, 1.94, 1.93 (4 × s, 12H, 4 × OAc); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 170.31, 169.92, 169.55, 169.12 (4 × C=O), 98.20 (C-1), 89.49 (C-3), 79.03 (C-2), 76.58 (C-7), 73.18 (C-8), 66.22 (C-5), 65.93 (C-4), 62.41 (C-6), 55.12 (OCH_3), 21.74, 20.73, 20.35, 20.16 (4 × OAc); HRMS (FAB) *m/z*: $[M+Na]^+$ calcd for $C_{17}H_{28}O_{11}Na$, 422.1662, found: 422.1671.

4.1.4. Methyl 3,4,6-tri-*O*-benzoyl-3-*C*,2-*O*-[(1*S*)-1-(benzoyloxy)ethylene]-α-D-glucopyranoside (4)

To the bicyclic compound **2** (0.16 g, 0.67 mmol) in anhydrous pyridine (2.5 mL) were added benzoyl chloride (0.78 mL, 6.71 mmol) and a catalytic amount of DMAP. The reaction mixture was stirred over 8 h at room temperature and then warmed to 80 °C. After stirring for 48 h, TLC revealed no trace of starting material; then the reaction mixture was co-evaporated with toluene and concentrated. The crude residue was dissolved in CH_2Cl_2 (80 mL) and washed with a 1 M aqueous solution of HCl (20 mL). The organic layer was separated, dried ($MgSO_4$) and concentrated under reduced pressure. Purification by flash column chromatography (cyclohexane/EtOAc, 4:1) afforded the perbenzoylated compound 4 (0.32 g, 72%) as a colorless oil. R_f 0.58 (cyclohexane/EtOAc, 1:1); $[\alpha]_D$ +105 (*c* 0.8, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz): δ 8.23–7.26 (m, 20H, H arom.), 6.83 (d, 1H, $J_{4,5}$ = 10.3 Hz, H-1), 6.09 (dd, 1H, $J_{7,8b}$ = 1.8 Hz, $J_{7,8a}$ = 5.3 Hz, H-7), 5.31 (d, 1H, $J_{2,1}$ = 5.4 Hz, H-2), 5.27 (d, 1H, $J_{1,2}$ = 5.4 Hz, H-1), 4.85 (ddd, 1H, $J_{5,6a}$ = 3.2 Hz, $J_{5,6b}$ = 5.3 Hz, $J_{5,4}$ = 10.3 Hz, H-5), 4.61 (dd, 1H, $J_{6a,5}$ = 3.2 Hz, $J_{6a,6b}$ = 12.0 Hz, H-6a), 4.56 (dd, 1H, $J_{8a,7}$ = 5.3 Hz,

$J_{8a,8b}$ = 10.3 Hz, H-8a), 4.43 (dd, 1H, $J_{6b,5}$ = 5.3 Hz, $J_{6b,6a}$ = 12.0 Hz, H-6b), 4.29 (dd, 1H, $J_{8b,7}$ = 1.8 Hz, $J_{8b,8a}$ = 10.3 Hz, H-8b), 3.64 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 166.51, 165.97, 165.90, 165.76 (4x C=O), 134.11–133.46 (5CH arom.), 130.60, 130.57, 130.03, 129.14 (4C arom. quat.), 130.31–128.70 (15CH arom.), 99.42 (C-1), 91.20 (C-3), 79.92 (C-2), 78.54 (C-7), 74.92 (C-8), 68.22 (C-4), 67.20 (C-5), 64.34 (C-6), 56.31 (OCH_3); HRMS (FAB) *m/z*: $[M+Na]^+$ calcd for $C_{37}H_{32}O_{11}Na$, 675.1842, found: 675.1849.

4.1.5. Benzyl {methyl 3,4-di-*O*-benzyl-3-*C*,2-*O*-[(1*S*)-1-(benzyloxy)ethylene]-6-hydroxy-L-*glycero*-α-D-*gluco*-heptopyranosid}uronate (7a) and benzyl {methyl-3,4-di-*O*-benzyl-3-*C*,2-*O*-[(1*S*)-1-(benzyloxy)ethylene]-6-hydroxy-D-*glycero*-α-D-*gluco*-heptopyranosid}uronate (7b)

A mixture of both epimeric allylic alcohols **6a** and **6b** (ratio 1:1, 0.36 g, 0.67 mmol) was dissolved in CH_2Cl_2 (80 mL) and cooled to −78 °C. Ozone was bubbled through the reaction mixture until persistence of a blue color (typically 5 min). Dimethylsulfide (0.1 mL) was added and the reaction mixture was allowed to reach the room temperature. The solvent was removed under reduced pressure and the mixture of epimeric aldehydes was used in the next step without further purification. $NaH_2PO_4.H_2O$ (1.20 g, 8.71 mmol) and $NaClO_2$ (1.09 g, 12.06 mmol) were added to a solution of the crude aldehydes in 2-methylbut-2-ene (1.5 mL), *t*BuOH (3 mL) and water (3 mL). After stirring overnight at room temperature the reaction mixture was diluted with EtOAc (50 mL) and water (20 mL). The aqueous layer was extracted with EtOAc (3 × 50 mL). The organic layers were combined, dried ($MgSO_4$), filtered and concentrated. The carboxylic acids obtained were directly engaged in the next step. To a solution of both epimeric carboxylic acids in DMF (8 mL) was added sequentially Bu_4NI (0.99 g, 2.68 mmol) and $KHCO_3$ (0.37 g, 3.68 mmol) followed by slowly addition of benzyl bromide (0.32 mL, 2.68 mmol). After stirring overnight the solvent was removed under reduced pressure and the crude residue diluted with Et_2O (80 mL) and water (30 mL). The aqueous layer was extracted with ether (3 × 80 mL), the organic layers were combined, dried ($MgSO_4$), filtered and concentrated under reduced pressure. Purification by flash column chromatography (cyclohexane/EtOAc, 5:1 then cyclohexane/EtOAc, 3:1) afforded the benzyl ester 6*R* **7a** (154 mg, 36% over three steps) as a colorless oil. R_f 0.61 (cyclohexane/EtOAc 2:1); $[\alpha]_D$ +76 (*c* 1.0, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz): δ 7.38–7.22 (m, 20H, H arom.), 5.36 (d, 1H, *J* = 12.2 Hz, CHPh), 5.13 (d, 1H, *J* = 12.2 Hz, CHPh), 4.99 (d, 1H, *J* = 11.2 Hz, CHPh), 4.96 (d, 1H, *J* = 11.8 Hz, CHPh), 4.89 (d, 1H, $J_{1,2}$ = 5.8 Hz, H-1), 4.82 (d, 1H, *J* = 11.8 Hz, CHPh), 4.78 (dd, 1H, $J_{5,6}$ = 1.7 Hz, $J_{5,4}$ = 10.0 Hz, H-5), 4.69 (d, 1H, *J* = 11.8 Hz, CHPh), 4.67 (d, 1H, *J* = 11.2 Hz, CHPh), 4.60 (d, 1H, $J_{6,5}$ = 1.7 Hz, H-6), 4.57 (d, 1H, *J* = 11.8 Hz, CHPh), 4.39 (d, 1H, $J_{4,5}$ = 10.0 Hz, H-4), 4.35–4.31 (m, 3H, H-2, H-8, H-9a), 4.21 (dd, 1H, $J_{9b,8}$ = 4.1 Hz, $J_{9b,9a}$ = 7.2 Hz, H-9b), 3.16 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 173.10 (C=O), 138.86, 138.56, 138.16, 134.82 (4C arom. quat.), 129.58–126.98 (20CH arom.), 99.53 (C-1), 88.82 (C-3), 85.31 (C-2 or C-8), 77.82 (C-2 or C-8), 75.19 (C-9), 74.76 (CH_2Ph), 74.38 (C-4), 72.95 (CH_2Ph), 69.60 (C-6), 69.32 (C-5), 67.45 (CH_2Ph), 64.74 (CH_2Ph), 55.01 (OCH_3); HRMS (CI) *m/z*: $[M+NH_4]^+$ calcd for $C_{38}H_{44}O_9N$, 658.3016, found: 658.3022; Anal. Calcd for $C_{38}H_{44}NO_9$: C, 71.23; H, 6.29. Found: C, 69.27; H, 6.41. Further elution afforded the benzyl ester 6*S* **7b** (140 mg, 32%, over three steps) as a colorless oil. R_f 0.51 (cyclohexane/EtOAc 2:1); $[\alpha]_D$ +50.5 (*c* 0.5, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz): δ 7.38–7.15 (m, 20H, H arom.), 5.02 (d, 1H, *J* = 12.0 Hz, CHPh), 4.99 (d, 1H, $J_{1,2}$ = 5.8 Hz, H-1), 4.96 (d, 1H, *J* = 10.1 Hz, CHPh), 4.83 (dd, 1H, $J_{5,6}$ = 2.1 Hz, $J_{5,4}$ = 10.2 Hz, H-5), 4.58 (d, 1H, *J* = 10.1 Hz, CHPh), 4.56 (s, 2H, CH_2Ph), 4.55 (d, 1H, *J* = 12.5 Hz, CHPh), 4.40 (m, 2H, H-6, CHPh), 4.40 (d, 1H, $J_{4,5}$ = 10.2 Hz, H-4), 4.35 (d, 1H, $J_{2,1}$ = 5.8 Hz, H-2), 4.31 (dd, 1H, $J_{9a,8}$ = 5.4 Hz, $J_{9a,9b}$ = 8.8 Hz, H-9a), 4.26 (dd, 1H, $J_{8,9a}$ = 5.4 Hz, $J_{8,9b}$ = 1.5 Hz, H-8), 4.19 (dd, 1H, $J_{9b,8}$ = 1.5 Hz, $J_{9b,9a}$ = 8.8 Hz, H-9b), 3.50 (s, 3H, OCH_3), 3.29 (d, 1H, $J_{OH,6}$ = 3.7 Hz, OH); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 172.29 (C=O), 139.04, 138.19, 137.93, 135.05 (4C arom. quat.), 128.59 -126.80 (20CH arom.), 99.34 (C-1), 90.06 (C-3), 85.63 (C-8), 77.28 (C-2), 75.34 (C-9), 74.37 (CH_2Ph), 72.80 (C-4), 72.72 (CH_2Ph), 70.52 (C-6), 69.97 (C-5), 67.32 (CH_2Ph), 64.49 (CH_2Ph), 55.31 (OCH_3); HRMS (CI) *m/z*: $[M+NH_4]^+$ calcd for $C_{38}H_{44}O_9N$, 658.3016, found: 658.3010; Anal. Calcd for $C_{38}H_{44}NO_9$: C, 71.23; H, 6.29. Found: C, 70.98; H, 6.18.

4.1.6. Benzyl {methyl 6-azido-3,4-di-*O*-benzyl-3-*C*,2-*O*-[(1*S*)-1-(benzyloxy)ethylene]-6-deoxy-D-*glycero*-α-D-*gluco*-heptopyranosid}uronate (8a)

To a solution of benzyl ester **7a** (175 mg, 0.27 mmol) in dry CH_2Cl_2 (5.5 mL), anhydrous pyridine (0.35 mL, 4.37 mmol) was added followed by slow addition of Tf_2O (0.37 mL, 2.19 mmol) at −78 °C under argon. After stirring 2 h at room temperature, water (10 mL) and CH_2Cl_2 (20 mL) were added. The organic layer was separated, dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude triflate compound was directly engaged in the next step. Sodium azide (106 mg, 1.64 mmol) was added to a solution of the crude triflate product in anhydrous DMF (6 mL) at room temperature. After stirring overnight the reaction mixture was concentrated under reduced pressure. The residue was then diluted with ether (80 mL) and water (30 mL). The aqueous layer was extracted with ether (3 × 80 mL), the organic layers were combined, dried ($MgSO_4$), filtered and concentrated under reduced pressure. Purification by flash column chromatography (cyclohexane/EtOAc, 5:1) afforded the benzyl azido ester 6*S* **8a** (131 mg, 72% over two steps) as a colorless oil. R_f 0.66 (cyclohexane/EtOAc 1:1); $[\alpha]_D$ +85.0 (*c* 0.6, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz): δ 7.42–7.28 (m, 20H, H arom.), 5.08 (d, 1H, *J* = 12.1 Hz, CHPh), 5.00 (d, 1H, $J_{1,2}$ = 5.8 Hz, H-1), 4.98 (dd, 1H, $J_{5,6}$ = 1.7 Hz, $J_{5,4}$ = 10.1 Hz, H-5), 4.85 (d, 1H, *J* = 12.1 Hz, CHPh), 4.69 (d, 1H, *J* = 10.5 Hz, CHPh), 4.61 (d, 1H, *J* = 12.4 Hz, CHPh), 4.53 (s, 2H, CH_2Ph), 4.52 (d, 1H, *J* = 12.4 Hz, CHPh), 4.40 (d, 1H, $J_{6,5}$ = 1.7 Hz, H-6), 4.34 (d, 1H, $J_{2,1}$ = 5.8 Hz, H-2), 4.35 (d, 1H, $J_{4,5}$ = 10.1 Hz, H-4), 4.34–2.28 (m, 2H, H-8, H-9a), 4.23 (dd, 1H, $J_{9b,8}$ = 1.4 Hz, $J_{9b,9a}$ = 8.8 Hz, H-9b), 3.56 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 167.49 (C=O), 138.79, 138.12, 137.74, 134.86 (4C arom. quat.), 128.64–126.86 (20CH arom.), 99.47 (C-1), 89.60 (C-3), 85.40 (C-8), 77.11 (C-2), 75.27 (C-9), 74.39 (CH_2Ph), 74.08 (C-4), 72.74 (CH_2Ph), 69.71 (C-5), 66.98 (CH_2Ph), 64.49 (CH_2Ph), 62.49 (C-6), 55.69 (OCH_3); HRMS (CI) *m/z*: $[M+NH_4]^+$ calcd for $C_{38}H_{43}O_8N_4$, 683.3081, found: 683.3073.

4.1.7. Benzyl {methyl 6-azido-3,4-di-*O*-benzyl-3-*C*,2-*O*-[(1*S*)-1-(benzyloxy)ethylene]-6-deoxy-L-*glycero*-α-D-*gluco*-heptopyranosid}uronate (8b)

To a solution of benzyl ester **7b** (137 mg, 0.21 mmol) in dry CH_2Cl_2 (5 mL), anhydrous pyridine (0.28 mL, 3.42 mmol) was added followed by slow addition of Tf_2O (0.29 mL, 1.71 mmol) at −78 °C under argon. After stirring 2 h at room temperature, water (10 mL) and CH_2Cl_2 (20 mL) were added. The organic layer was separated, dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude triflate compound was directly engaged in the next step. Sodium azide (83 mg, 1.28 mmol) was added to a solution of the crude triflate product in anhydrous DMF (5 mL) at room temperature. After stirring overnight the reaction mixture was concentrated under reduced pressure. The residue was then diluted with ether (80 mL) and water (30 mL). The aqueous layer was extracted with ether (3 × 80 mL), the organic layers were combined, dried ($MgSO_4$), filtered and concentrated under reduced pressure. Purification by flash column chromatography (cyclohexane/EtOAc, 5:1) afforded the benzyl azido ester 6*R* **8b** (122 mg, 86%

over two steps) as a colorless oil. R_f 0.68 (cyclohexane/EtOAc 1:1); $[\alpha]_D$ +19.1 (*c* 0.2, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz): δ 7.42–7.26 (m, 20H, H arom.), 5.34 (d, 1H, *J* = 12.2 Hz, CHPh), 5.19 (d, 1H, *J* = 12.2 Hz, CHPh), 5.05 (d, 1H, *J* = 11.3 Hz, CHPh), 5.02 (dd, 1H, $J_{5,6}$ = 2.2 Hz, $J_{5,4}$ = 10.4 Hz, H-5), 4.92 (d, 1H, $J_{1,2}$ = 5.9 Hz, H-1), 4.74 (d, 1H, *J* = 11.3 Hz, CHPh), 4.70 (d, 1H, *J* = 12.0 Hz, CHPh), 4.69 (s, 2H, CH_2Ph), 4.58 (d, 1H, *J* = 12.0 Hz, CHPh), 4.38 (d, 1H, $J_{2,1}$ = 5.9 Hz, H-2), 4.37–4.33 (m, 2H, H-8, H-9a), 4.31 (d, 1H, $J_{6,5}$ = 2.2 Hz, H-6), 4.28 (d, 1H, $J_{4,5}$ = 10.4 Hz, H-4), 4.24 (dd, 1H, $J_{9b,8}$ = 4.6 Hz, $J_{9b,9a}$ = 9.8 Hz, H-9b), 3.19 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 168.80 (C=O), 138.68, 138.16, 137.95, 134.81 (4C arom. quat.), 128.53–127.06 (20CH arom.), 99.47 (C-1), 89.56 (C-3), 85.30 (C-8), 77.56 (C-2), 75.24 (C-9), 75.21 (C-4), 74.90 (CH_2Ph), 72.96 (CH_2Ph), 69.49 (C-5), 67.53 (CH_2Ph), 64.79 (CH_2Ph), 61.37 (C-6), 55.23 (OCH_3); HRMS (CI) *m/z*: $[M+NH_4]^+$ calcd for $C_{38}H_{43}O_8N_4$, 683.3081, found: 683.3076.

4.1.8. 1,6-Di-*O*-acetyl-3,4-di-*O*-benzyl-3-*C*,2-*O*-[(1*S*)-1-(benzyloxy)ethylene]-β-D-glucopyranose (9)

Sodium hydride (275 mg, 6.86 mmol, 60% w/w) was added to a solution of the bicyclic compound **2** (270 mg, 1.14 mmol) in anhydrous DMF (8 mL) at 0 °C. After 30 min, BnBr (1.1 mL, 9.15 mmol) was added at 0 °C. After stirring for 2.5 h at room temperature, MeOH (10 mL) was added and the mixture was concentrated under reduced pressure. The residue was then diluted with ether (80 mL) and water (30 mL). The aqueous layer was extracted with ether (3 × 80 mL), the organic layers were combined, dried ($MgSO_4$), filtered and concentrated under reduced pressure. Purification by flash column chromatography (cyclohexane then cyclohexane/EtOAc, 4:1) afforded the fully protected benzylated compound (579 mg, 85%) as a colorless oil. R_f 0.56 (cyclohexane/EtOAc, 2:1); $[\alpha]_D$ +115.8 (*c* 1.0, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz): δ 7.54–7.43 (m, 20H, H arom.), 5.18 (d, 1H, $J_{1,2}$ = 5.8 Hz, H-1), 5.07 (d, 1H, *J* = 11.2 Hz, CHPh), 4.86–4.77 (m, 5H, 3 × CHPh, CH_2Ph), 4.72–4.67 (m, 3H, 2 × CHPh, H-5), 4.56 (d, 1H, $J_{2,1}$ = 5.8 Hz, H-2), 4.53–4.42 (m, 4H, H-4, H-7, H-8a, H-8b), 3.99 (dd, 1H, $J_{6a,5}$ = 3.6 Hz, $J_{6a,6b}$ = 10.8 Hz, H-6a), 3.90 (dd, 1H, $J_{6b,5}$ = 1.9 Hz, $J_{6b,6a}$ = 10.8 Hz, H-6b), 3.85 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 138.73, 138.39, 138.12, 137.66 (4C arom. quat.), 128.61–126.63 (20CH arom.), 98.86 (C-1), 89.38 (C-3), 84.96 (C-2), 77.90 (C-7), 74.76 (C-8), 74.57 (C-4), 74.08 (CH_2Ph), 72.98 (CH_2Ph), 72.36 (CH_2Ph), 68.55 (C-6), 67.48 (C-5), 64.37 (CH_2Ph), 54.60 (OCH_3); HRMS (FAB) *m/z*: $[M+Na]^+$ calcd for $C_{37}H_{40}O_7Na$, 619.2666, found: 619.2668; Anal. Calcd for $C_{37}H_{40}O_7$: C, 74.47; H, 6.76. Found: C, 74.45; H, 6.69. To a solution of the perbenzylated derivative (450 mg, 0.76 mmol) in acetic anhydride (15 mL) was added dropwise sulfuric acid 5% in AcOH (0.4 mL) at −20 °C. The reaction mixture was stirred at this temperature over 25 min and then neutralized by slowly addition of a $NaHCO_3$ saturated aqueous solution. The organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2 (3 × 150 mL). The organic layers were combined, dried ($MgSO_4$), filtered and concentrated. Purification by flash column chromatography (cyclohexane/CH_2Cl_2/EtOAc, 8:1:1) afforded the suitable glycosyl donor **9** (209 mg, 48%) as a colorless oil. R_f 0.54 (cyclohexane/CH_2Cl_2/EtOAc, 2:1:1); $[\alpha]_D$ +33.9 (*c* 1.0, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz): δ7.40–7.28 (m, 15H, H arom.), 6.10 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1), 4.91 (d, 1H, *J* = 11.0 Hz, CHPh), 4.71–4.60 (m, 5H, CHPh, 2 × CH_2Ph), 4.38–4.25 (m, 5H, H-2, H-5, H-7, H-6a, H-6b), 4.20 (dd, 1H, $J_{8a,7}$ = 3.5 Hz, $J_{8a,8b}$ = 9.3 Hz, H-8a), 4.10–4.04 (m, 2H, H-4, H-8b), 2.18, 2.09 (2 × s, 6H, OAc); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 171.22, 169.78 (2 × C=O), 138.27, 137.97, 137.75 (C arom. quat.), 128.99–127.72 (15CH arom.), 93.90 (C-1), 90.35 (C-3), 84.84 (C-2 or C-7), 78.36 (C-2 or C-7), 75.03 (CH_2Ph), 74.33 (C-4), 74.01 (C-8), 73.89 (C-5), 73.25 (CH_2Ph), 65.39 (CH_2Ph), 63.80 (C-6), 21.57 (OAc), 21.35 (OAc); HRMS (FAB) *m/z*: $[M+Na]^+$ calcd for $C_{33}H_{36}O_9Na$, 599.2257, found: 599.2252.

4.1.9. 2-(Acetylamino)-6-chloro-9-{6-*O*-acetyl-3,4-di-*O*-benzyl-3-*C*,2-*O*-[(1*S*)-1-benzyloxyethylene]-D-glucopyranosyl}purine (11) and 2-(acetylamino)-6-chloro-7-{6-*O*-acetyl-3,4-di-*O*-benzyl-3-*C*,2-*O*-[(1*S*)-1-benzyloxyethylene]-D-glucopyranosyl}purine (12)

BSA (70.7 μL, 286 μmol) was added to a suspension of the 2-acetamido-6-chloropurine (30.3 mg, 143 μmol) in anhydrous 1,2-dichloroethane (1 mL) under argon. The mixture was heated to 80 °C for 45 min. To this solution was added a solution of the glycosyl donor **9** (55 mg, 95.4 μmol) in dry 1,2-dichloroethane (1 mL) was added, followed by slow addition of TMSOTf (138.1 μL, 763 μmol) under argon. The reaction mixture was stirred at 85 °C for 4 h, cooled to room temperature, diluted with CH_2Cl_2 (10 mL). The organic layer was neutralized with a $NaHCO_3$ saturated aqueous solution (2 × 5 mL). The aqueous layer was extracted with CH_2Cl_2 (3 × 15 mL), and the organic layers were combined, dried ($MgSO_4$), filtered and concentrated under reduced pressure. Purification by flash column chromatography (cyclohexane/EtOAc, 2:1 then cyclohexane/EtOAc, 1:1) afforded the N^9 regioisomer **11** (17 mg, 25%) as a colorless oil. R_f 0.63 (cyclohexane/EtOAc, 1:1); $[\alpha]_D$ +18.2 (*c* 1.0, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz): δ 8.14 (s, 1H, H-8), 8.05 (br s, 1H, NH), 7.42–7.29 (m, 15H, H arom.), 6.10 (d, 1H, $J_{1',2'}$ = 8.9 Hz, H-1′), 5.00 (m, 2H, H-2′, CHPh), 4.82–4.64 (m, 5H, CHPh, 2 × CH_2Ph), 4.47–4.38 (m, 3H, H-5′, H-7′, H-6′a), 4.29–4.21 (m, 3H, H-4′, H-6′b, H-8′a), 4.12 (app d, 1H, $J_{8'b,8'a}$ = 9.5 Hz, H-8′b), 2.49 (s, 3H, NHAc), 2.05 (s, 3H, OAc); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 170.51, 171.06 (2 × C=O), 153.01 (C-4), 152.49 (C-2 or C-6), 152.00 (C-2 or C-6), 143.35 (C-8), 137.94, 137.70, 137.39 (3C arom. quat.), 129.15–127.63 (15CH arom.), 128.70 (C-5), 90.02 (C-3′), 85.19 (C-7′), 83.31 (C-1′), 77.58 (C-2′), 76.27 (C-5′), 75.32 (CH_2Ph), 74.58 (C-8′), 74.46 (C-4′), 73.97 (CH_2Ph), 65.73 (CH_2Ph), 63.85 (C-6′), 25.62 (NHAc), 21.29 (OAc); HRMS (CI) *m/z*: $[M+H]^+$ calcd for $C_{38}H_{39}O_8N_5{}^{35}Cl$, 728.2487, and $C_{38}H_{39}O_8N_5{}^{37}Cl$, 730.2458, found: 728.2515 and 730.2465, respectively. Further elution (cyclohexane/EtOAc, 1:2 then cyclohexane/EtOAc, 1:4) afforded the N^7 regioisomer **12** (26 mg, 37%) as a colorless oil. R_f 0.14 (cyclohexane/EtOAc, 1:1); $[\alpha]_D$ +28.8 (*c* 1.0, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz): δ 8.47 (s, 1H, H-8), 8.37 (br s, 1H, NH), 7.41–7.29 (m, 15H, H arom.), 6.45 (d, 1H, $J_{1',2'}$ = 9.1 Hz, H-1′), 5.00 (d, 1H, *J* = 10.9 Hz, CHPh), 4.80–4.60 (m, 6H, H-2′, CHPh, 2 × CH_2Ph), 4.45–4.41 (m, 3H, H-5′, H-6′a, H-7′), 4.34 (dd, 1H, $J_{8'a,7'}$ = 3.3 Hz, $J_{8'a,8'b}$ = 9.4 Hz, H-8′a), 4.24 (dd, 1H, $J_{6'b,5'}$ = 5.6 Hz, $J_{6'b,6'a}$ = 12.6 Hz, H-6′b), 4.21–4.10 (m, 2H, H-4′, H-8′b), 2.60 (s, 3H, NHAc), 2.06 (s, 3H, OAc); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 171.50, 170.63 (2 × C=O), 163.18 (C-4), 152.53 (C-2 or C-6), 146.36 (C-8), 143.95 (C-2 or C-6), 137.50, 137.11, 136.80 (3C arom. quat.), 128.71–127.24 (15CH arom.), 118.97 (C-5), 89.73 (C-3′), 84.56 (C-7′), 82.95 (C-1′), 77.55 (C-2′), 75.74 (C-5′), 74.80 (CH_2Ph), 74.37 (C-8′), 73.86 (C-4′), 73.60 (CH_2Ph), 65.37 (CH_2Ph), 63.23 (C-6′), 25.26 (NHAc), 20.89 (OAc); HRMS (FAB) *m/z*: $[M+Na]^+$ calcd for $C_{38}H_{38}O_8N_5{}^{35}ClNa$, 750.2307, and $C_{38}H_{38}O_8N_5{}^{37}ClNa$, 752.2277, found: 750.2310 and 752.2309, respectively.

4.1.10. Benzyl 1-*O*-acetyl-6-azido-3,4-di-*O*-benzyl-3-*C*,2-*O*-[(1*S*)-1-(benzyloxy)ethylene]-6-deoxy-D-*glycero*–D-*gluco*-heptopyranuronate (13a)

A solution of concd sulfuric acid (5% in AcOH, 70 μL) was added dropwise at −20 °C to a solution of benzyl azido ester **8a** (170 mg, 255 μmol) in acetic anhydride (6 mL). The reaction mixture was allowed to warm to 0 °C over a period of 15 min, and then neutralized by slow addition of a $NaHCO_3$ saturated aqueous solution. The organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL). The organic layers were combined, dried ($MgSO_4$), filtered and concentrated. Purification by flash column chromatography (cyclohexane/CH_2Cl_2/EtOAc, 10:1:1) afforded the glycosyl donor **13a** (64 mg, 36%) as a colorless

oil. R_f 0.46 (cyclohexane/CH_2Cl_2/EtOAc, 6:1:1); $[\alpha]_D$ +23.8 (*c* 0.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.50–7.00 (m, 20H, H arom.), 6.13 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1), 5.01 (d, 1H, *J* = 12.0 Hz, CHPh), 4.99 (d, 1H, *J* = 9.8 Hz, CHPh), 4.79 (dd, 1H, $J_{5,6}$ = 2.4 Hz, $J_{5,4}$ = 9.8 Hz, H-5), 4.74 (d, 1H, *J* = 12.0 Hz, CHPh), 4.64–4.58 (m, 3H, CH_2Ph, CHPh), 4.51 (d, 1H, $J_{6,5}$ = 2.4 Hz, H-6), 4.45–4.42 (m, 3H, H-4, CH_2Ph), 4.26 (d, 1H, $J_{2,1}$ = 7.8 Hz, H-2), 4.21–4.16 (m, 2H, H-8, H-9a), 3.95 (d, 1H, $J_{9b,9a}$ = 9.0 Hz, H-9b), 2.21 (s, 3H, OAc); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 169.37, 169.09 (2 × C=O), 137.71, 137.33, 137.19, 134.58 (4C arom. quat.), 128.70–127.45 (20CH arom.), 93.29 (C-1), 89.98 (C-3), 84.25 (C-8), 77.31 (C-2), 74.98 (C-5), 74.56 (CH_2Ph), 74.03 (C-9), 72.85 (C-4), 72.74 (CH_2Ph), 67.64 (CH_2Ph), 64.84 (CH_2Ph), 62.85 (C-6), 21.05 (OAc); HRMS (CI) *m/z*: $[M+NH_4]^+$ calcd for $C_{39}H_{43}O_9N_4$, 711.3030, found: 711.3025.

4.1.11. Benzyl 1-*O*-acetyl-6-azido-3,4-di-*O*-benzyl-3-*C*,2-*O*-[(1*S*)-1-(benzyloxy)ethylene]-6-deoxy-L-*glycero*–D-*gluco*-heptopyranuronate (13b)

To a solution of benzyl azido ester **8b** (200 mg, 0.30 mmol) in acetic anhydride (7 mL) was added dropwise sulfuric acid (21 μL) at 0 °C. The reaction mixture was stirred at this temperature over 30 min and then neutralized by slow addition of a $NaHCO_3$ saturated aqueous solution. The organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL). The organic layers were combined, dried ($MgSO_4$), filtered and concentrated. Purification by flash column chromatography (cyclohexane/CH_2Cl_2/EtOAc, 10:1:1) afforded the glycosyl donor **13b** (131 mg, 63%) as a colorless oil. R_f 0.50 (cyclohexane/CH_2Cl_2/EtOAc, 6:1:1); $[\alpha]_D$ +30.8 (*c* 0.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.36–7.14 (m, 20H, H arom.), 5.96 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1), 5.28 (d, 1H, *J* = 12.1 Hz, CHPh), 5.08 (d, 1H, *J* = 12.1 Hz, CHPh), 4.94 (d, 1H, *J* = 11.1 Hz, CHPh), 4.73 (dd, 1H, $J_{5,6}$ = 2.5 Hz, $J_{5,4}$ = 9.9 Hz, H-5), 4.67 (d, 1H, *J* = 11.1 Hz, CHPh), 4.64 (d, 1H, *J* = 11.1 Hz, CHPh), 4.62 (d, 1H, *J* = 11.1 Hz, CHPh), 4.56 (s 2H, CH_2Ph), 4.30 (d, 1H, $J_{4,5}$ = 9.9 Hz, H-4), 4.25 (d, 1H, $J_{2,1}$ = 7.8 Hz, H-2), 4.23–4.21 (m, 2H, H-6, H-8), 4.18 (dd, 1H, $J_{9a,8}$ = 3.3 Hz, $J_{9a,9b}$ = 9.4 Hz, H-9a), 4.03 (d, 1H, $J_{9b,9a}$ = 9.4 Hz, H-9b), 2.10 (s, 3H, OAc); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 169.09, 169.02 (2 × C=O), 137.79, 137.36, 137.14, 135.06 (4C arom. quat.), 128.55–127.80 (20CH arom.), 93.63 (C-1), 89.96 (C-3), 84.33 (C-8), 77.65 (C-2), 75.19 (C-5), 74.74 (CH_2Ph), 74.09 (C-4), 73.53 (C-9), 72.82 (CH_2Ph), 67.96 (CH_2Ph), 65.03 (CH_2Ph), 62.28 (C-6), 21.01 (OAc); HRMS (CI) *m/z*: $[M+NH_4]^+$ calcd for $C_{39}H_{43}O_9N_4$, 711.3030, found: 711.3021.

4.1.12. 2-(Acetylamino)-6-chloro-7-(benzyl {6-azido-3,4-di-*O*-benzyl-3-*C*,2-*O*-[(1*S*)-1-(benzyloxy)ethylene]-6-deoxy-D-*glycero*–D-*gluco*-heptopyranosyl}urinate)purine (14a)

Method A: BSA (54 μL, 216.3 μmol) was added to a suspension of the nucleobase 2-acetamido-6-chloropurine (23 mg, 108.2 μmol) in anhydrous CH_3CN (1 mL) under argon. The mixture was heated to 60 °C for 45 min to afford the crude silylated base. To this solution was added the glycosyl donor 6*S* **13a** (50 mg, 72.1 μmol) dissolved in anhydrous CH_3CN (1 mL) followed by slow addition of TMSOTf (104 μL, 576.8 μmol) under argon. The solution was stirred at 65 °C for 90 min. The mixture was cooled to room temperature, CH_2Cl_2 (20 mL) was added and the organic layer was washed with $NaHCO_3$ saturated aqueous solution (2 × 10 mL). The aqueous layer was extracted with CH_2Cl_2 (3 × 30 mL), the organic layers were combined, dried ($MgSO_4$), filtered and concentrated under reduced pressure. Purification by flash column chromatography (cyclohexane/EtOAc, 1:1) afforded the N^7 nucleoside 6*S* **14a** (39 mg, 65%) as a colorless oil. *Method B*: BSA (32 μL, 129.6 μmol) was added to a suspension of the nucleobase 2-acetamido-6-chloropurine (14 mg, 64.8 μmol) in anhydrous CH_3CN (1 mL) under argon. The mixture was heated to 60 °C for 45 min to afford the crude silylated base. To this solution was added the glycosyl donor 6*S* **13a** (30 mg, 43.2 μmol) dissolved in anhydrous CH_3CN (1 mL) followed by slow addition of TMSOTf (156 μL, 864 μmol) under argon. The solution was stirred at 65 °C for 90 min. The mixture was cooled to room temperature, CH_2Cl_2 (20 mL) was added and the organic layer was washed with $NaHCO_3$ saturated aqueous solution (2 × 10 mL). The aqueous layer was extracted with CH_2Cl_2 (3 × 30 mL), the organic layers were combined, dried ($MgSO_4$), filtered and concentrated under reduced pressure. Purification by flash column chromatography (cyclohexane/EtOAc, 1:1) afforded the N^7 nucleoside 6*S* **14a** (22 mg, 60%) as a colorless oil. R_f 0.22 (cyclohexane/EtOAc, 1:1); $[\alpha]_D$ +33 (*c* 0.3, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.33 (s, 1H, H-8), 8.06 (br s, 1H, NH), 7.42–7.21 (m, 20H, H arom.), 6.49 (d, 1H, $J_{1',2'}$ = 8.9 Hz, H-1′), 5.10 (d, 1H, *J* = 10.1 Hz, CHPh), 5.04 (d, 1H, *J* = 12.0 Hz, CHPh), 4.87 (dd, 1H, $J_{5',6'}$ = 2.4 Hz, $J_{5',4'}$ = 9.8 Hz, H-5′), 4.78 (d, 1H, *J* = 12.0 Hz, CHPh), 4.72–4.65 (m, 4H, H-4′, CH_2Ph, CHPh), 4.61–4.58 (m, 2H, H-2′, CHPh), 4.55 (d, 1H, $J_{6',5'}$ = 2.4 Hz, H-6′), 4.48 (d, 1H, *J* = 10.8 Hz, CHPh), 4.39 (d, 1H, $J_{8',9'a}$ = 3.2 Hz, H-8′), 4.32 (dd, 1H, $J_{9'a,8'}$ = 3.2 Hz, $J_{9'a,9'b}$ = 9.5 Hz, H-9′a), 4.09 (d, 1H, $J_{9'b,9'a}$ = 9.5 Hz, H-9′b), 2.59 (s, 3H, NHAc); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 171.80, 166.61 (2 × C=O), 162.99 (C-4), 152.47 (C-2 or C-6), 146.30 (C-8), 143.77 (C-2 or C-6), 137.48, 136.71, 136.61, 134.46 (4C arom. quat.), 128.66–127.82 (20CH arom.), 118.69 (C-5), 89.72 (C-3′), 84.51 (C-8′), 82.76 (C-1′), 77.44 (C-5′), 77.37 (C-2′), 74.60 (CH_2Ph), 74.48 (C-9′), 73.46 (CH_2Ph), 72.67 (C-4′), 67.80 (CH_2Ph), 65.40 (CH_2Ph), 62.81 (C-6′), 25.20 (NHAc); HRMS (FAB) *m/z*: $[M+Na]^+$ calcd for $C_{44}H_{41}O_8N_8{}^{35}ClNa$, 867.2634, and $C_{44}H_{41}O_8N_8{}^{37}ClNa$, 869.2604, found: 867.2618 and 869.2678, respectively.

4.1.13. 2-(Acetylamino)-6-chloro-7-(benzyl {6-azido-3,4-di-*O*-benzyl-3-*C*,2-*O*-[(1*S*)-1-(benzyloxy)ethylene]-6-deoxy-L-*glycero*–D-*gluco*-heptopyranosyl}urinate)purine (14b)

Method A: BSA (65 μL, 259.5 μmol) was added to a suspension of the nucleobase 2-acetamido-6-chloropurine (28 mg, 129.8 μmol) in anhydrous CH_3CN (1 mL) under argon. The mixture was heated to 60 °C for 45 min to afford the crude silylated base. To this solution was added the glycosyl donor 6*R* **13b** (60 mg, 86.5 μmol) dissolved in anhydrous CH_3CN (1 mL) followed by slow addition of TMSOTf (125 μL, 692 μmol) under argon. The solution was stirred at 65 °C for 90 min. The mixture was cooled to room temperature, CH_2Cl_2 (20 mL) was added and the organic layer was washed with $NaHCO_3$ saturated aqueous solution (2 × 10 mL). The aqueous layer was extracted with CH_2Cl_2 (3 × 30 mL), the organic layers were combined, dried ($MgSO_4$), filtered and concentrated under reduced pressure. Purification by flash column chromatography (cyclohexane/EtOAc, 1:1) afforded the N^7 nucleoside 6*R* **14b** (44 mg, 58%) as a colorless oil. *Method B*: BSA (32 μL, 129.6 μmol) was added to a suspension of the nucleobase 2-acetamido-6-chloropurine (14 mg, 64.8 μmol) in anhydrous CH_3CN (1 mL) under argon. The mixture was heated to 60 °C for 45 min to afford the crude silylated base. To this solution was added the glycosyl donor 6*R* **13b** (30 mg, 43.2 μmol) dissolved in anhydrous CH_3CN (1 mL) followed by slow addition of TMSOTf (156 μL, 864 μmol) under argon. The solution was stirred at 65 °C for 90 min. The mixture was cooled to room temperature, CH_2Cl_2 (20 mL) was added and the organic layer was washed with $NaHCO_3$ saturated aqueous solution (2 × 10 mL). The aqueous layer was extracted with CH_2Cl_2 (3 × 30 mL), the organic layers were combined, dried ($MgSO_4$), filtered and concentrated under reduced pressure. Purification by flash column chromatography (cyclohexane/EtOAc, 1:1) afforded the N^7 nucleoside 6*R* **14b** (20 mg, 55%) as a colorless oil. R_f 0.31 (cyclohexane/EtOAc, 1:1); $[\alpha]_D$ +51.3 (*c* 0.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.31 (s, 1H, H-8), 8.30 (br s, 1H, NH), 7.44–7.08 (m, 20H, H arom.), 6.35 (d, 1H, $J_{1',2'}$ = 8.8 Hz, H-1′), 5.22 (d, 1H, *J* = 12.0 Hz, CHPh), 5.09 (d, 1H, *J* = 11.2 Hz, CHPh), 5.05 (d, 1H, *J* = 12.0 Hz, CHPh), 4.83 (dd, 1H, $J_{5',6'}$ = 2.2 Hz,

$J_{5',4'}$ = 9.8 Hz, H-5′), 4.77 (d, 1H, J = 11.2 Hz, CHPh), 4.75 (s, 2H, CH_2Ph), 4.70 (d, 1H, J = 10.4 Hz, CHPh), 4.61 (d, 1H, J = 10.4 Hz, CHPh), 4.51 (d, 1H, $J_{4'5'}$ = 9.8 Hz, H-4), 4.44–4.42 (m, 2H, H-2′, H-8′), 4.31 (dd, 1H, $J_{9'a,8'}$ = 3.3 Hz, $J_{9'a,9'b}$ = 9.5 Hz, H-9′a), 4.22–4.19 (m, 2H, H-6′, H-9′b), 2.65 (s, 3H, NHAc); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 171.61, 167.63 (2 × C=O), 162.84 (C-4), 152.36 (C-2 or C-6), 145.90 (C-8), 143.62 (C-2 or C-6), 137.48, 136.84, 136.65, 134.35 (4C arom. quat.), 128.69–127.27 (20CH arom.), 118.69 (C-5), 89.38 (C-3′), 84.42 (C-8′), 82.78 (C-1′), 78.96 (C-2′), 78.14 (C-5′), 74.96 (CH_2Ph), 74.19 (C-9′), 73.85 (C-4′), 73.60 (CH_2Ph), 67.66 (CH_2Ph), 65.42 (CH_2Ph), 61.02 (C-6′), 25.19 (NHAc); HRMS (FAB) m/z: $[M+Na]^+$ calcd for $C_{44}H_{41}O_8N_8{}^{35}ClNa$, 867.2634, and $C_{44}H_{41}O_8N_8{}^{37}ClNa$, 869.2604, found: 867.2642 and 869.2612, respectively.

4.2. Enzymatic studies

4.2.1. Spectrophotometer and chemicals

A double beam spectrophotometer Shimadzu® equipped with thermostatic cell holders was used on visible range and operated on the kinetic mode. The absorbance data were acquired in a computer by means of UV Probe software. Appropriate disposable plastic cuvettes Plastibrand® were used in the kinetic experiments. The following materials were purchased from Aldrich: enzymes acetylcholinesterase (AChE) from bovine erythrocytes and butyrylcholinesterase (BChE) from human serum, substrates acetylthiocholine iodide (ATchI) and *S*-butyrylthiocholine iodide (BTchI) and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). Other Aldrich reagents used for the preparation of buffers and solutions were KH_2PO_4, KOH and $NaHCO_3$. Deionized water was used to prepare the buffer pH 8.0, the substrate and DTNB solutions.

4.2.2. Solutions preparation

Preparation of 0.1 M phosphate buffer pH 8.0: KH_2PO_4 (136.1 mg) was dissolved in water (10 mL) and adjusted with KOH to a pH of 8.0 ± 0.1. Buffer was freshly prepared and stored in the refrigerator.

AChE solution 1.32 U/mL: the enzyme (1.02041U, 10 μL, 4.4 mg) was dissolved in freshly prepared buffer pH 8.0 (1.0 mL).

BChE solution 0.44 U/mL: The enzyme (2.9762 U, 1.0 mg) was dissolved in freshly prepared buffer pH 8.0 (6.764 mL).

DTNB solution 0.01 M: DTNB (3.96 mg) was dissolved in water (1 mL) containing sodium hydrogen carbonate (1.5 mg).

ATchI solution 0.022 M: ATchI (6.4 mg) was dissolved in water (1 mL).

BTchI solution 0.022 M: BTchI (7.0 mg) was dissolved in water (1 mL).

All solutions were stored in eppendorf caps (100 μL aliquots) in the refrigerator. The pure compounds and the control (rivastigmine) were initially dissolved in DMSO and diluted with distilled water until concentrations decrease to values between 4.4 mg/mL and 0.00044 mg/mL, to yield the final concentrations for the enzymatic test between 100 μg/mL and 0.01 μg/mL. No inhibition was detected by residual DMSO (<0.5%) at the reaction cuvette.

4.2.3. AChE and BChE activity assay

A mixture of the buffer (200 μL), enzyme (5 μL), DTNB (5 μL) and new synthetic compounds (5 μL) solutions was prepared and kept for 15 min at 30 °C in a heated water bath, and then the substrate reagent (5 μL) was added to start the enzymatic reaction. The absorbance data along reaction time was taken for 4 min under a controlled temperature of 30 °C. At least four replicates were made. Several blank assays without the new synthetic compounds were carried out in order to determine the average V_{max}. Also assays without the enzyme and the inhibitor compound were carried out to check for any non-enzymatic hydrolysis of the substrate. The final concentrations of chemicals in the test were as follows: [AChE] = 0.03 U/mL, [BChE]=0.01 U/mL, [compound] = 0.01–100 μg/mL, [DTNB] = 0.0002273 M, [ATchI] = [BTchI] = 0.0005 M.

4.3. Preliminary acute toxicity screening

Acute cytotoxicity measurements were performed by the MTT method.[29,30] The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to quantify metabolically viable cells in all samples. Adherent cells (A549 human lung fibroblasts) were seeded onto 96-well plates, allowed to attach for 24 h and exposed to the test compound for the following 24 h. Positive control (hydrogen peroxide) and negative control (pure solvent) were also included. At 48 h of culture MTT was added to the cells at a final concentration of 0.5 mg/mL, followed by an incubation period of 3 h to allow the formazan crystals to form. After incubation, medium was removed, cells were washed twice to remove traces of medium and un-metabolized MTT, and DMSO (100 μL) was added to each well. Solubilization of formazan crystals was performed by agitation in a 96-well plate shaker for 20 min at room temperature. Absorbance of each well was quantified at 550 nm using 620 nm as reference wavelength on a scanning multiwell spectrophotometer (automated plate reader).

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