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erythro- and *threo*-2-Hydroxynonyl substituted 2-phenyladenines and 2-phenyl-8-azaadenines: ligands for A_1 adenosine receptors and adenosine deaminase

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Abstract

erythro-2-Phenyl-9-(2-hydroxy-3-nonyl)adenine and its 8-aza analog were prepared and showed a very high inhibitory activity towards adenosine deaminase (ADA), with K_i 0.55 and 1.67 nM, respectively, and high affinity for A_1 adenosine receptors, with K_i 28 and 2.8 nM, respectively. To increase affinity for A_1 receptors we introduced a substituent on the N^6 position such as alkyl or cycloalkyl groups, which are present in effective agonists or antagonists. Furthermore, for some compounds, we prepared the two diastereoisomers *erythro* and *threo* to verify whether the binding with A_1 receptors is stereoselective, as in ADA. Results show that some of the synthesised compounds are good inhibitors for ADA and good ligands for A_1, and the *erythro* diastereoisomers are more active than the *threo* ones. The experimental evidence allows us to hypothesise some similarity in the three dimensional structures of the binding site of the two proteins, ADA and A_1 adenosine receptor, in spite of lacking any homologies in the aminoacid sequences.

Keywords: A_1 adenosine receptor ligands; Adenosine deaminase inhibitors; Hydroxynonyl adenines; Hydroxynonyl 8-azaadenines

1. Introduction

Adenosine is an endogenous nucleoside which mediates many important physiological effects through four receptors: A_1, A_{2A}, A_{2B}, A_3, that are involved in many central and peripheral regulatory mechanisms; they are studied for their effects on the cardiovascular, renal, immune and central nervous systems [1]. Adenosine is converted to inosine and ammonia by adenosine deaminase (ADA), an enzyme of the purine catabolic pathway. This enzyme is present in all mammalian cells and plays a central role also in maintaining immune competence [2]. Abnormal levels of ADA have been detected also in a variety of diseases including AIDS, anaemia, lymphomas and leukaemias [3]. The function of ADA is critical in controlling the effects of adenosine in the systems where adenosine receptors are present. In fact, inhibition of ADA activity has significant physiological consequences due to the accumulation of adenosine. ADA inhibition could potentiate most of the central and peripheral effects of adenosine and adenosine agonists [4,5]. Moreover, experiments with adenosine antagonists and ADA have shown that endogenous adenosine tonically inhibits the release of neurotransmitters (acetylcholine [6], norepinefrine [7], 5-hydroxytriptamine [8]) in the CNS.

During the last 10 years, ADA has been found on the surface of many cells, so it can be considered to be not only a cytosolic enzyme, but also an ecto-enzyme. Several recent reports suggested a close bind between ADA and A_1 adenosine receptors because some interactions between ecto-ADA and A_1 adenosine receptors on the surface of the brain and smooth muscle cells were discovered: ecto-ADA seems involved in coupling A_1 adenosine receptors to G-proteins [4] and in its desensitisation and internalisation; so it seems involved in modulation of ligand binding to A_1 adenosine receptors [5]. It was seen that ADA plays a key role in the regulation of A_1 adenosine receptors by accelerating

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ligand-induced desensitisation and internalisation. It was demonstrated that, during the agonist-dependent internalisation process, the two cell surface proteins internalise via the same endocytic pathway [5]. It has also been reported that there are cell types whose ADA and A1 adenosine receptors internalise in response to xanthine antagonists. This fact would lead to a decrease in adenosine deamination and, consequently, to an increase in the extracellular levels of adenosine [5]. In fact an increase in plasma adenosine concentration has been reported after antagonist administration and its reduction after antagonist withdrawal; these could alter the physiology of many organ systems and provoke bronchospasms, alter blood pressure or change cardiac rhythms [9]. A molecule which is able to interact well with the two proteins could be useful to clarify some aspects of the bind between ADA and A1 adenosine receptors. It could also be useful from the therapeutic point of view.

In the past, we synthesised many effective adenine or 8-azaadenine antagonists of A_1 receptor having three lipophilic moieties [10,11]. We hypothesised that in the binding site of A_1 receptors three hydrophobic pockets could be present, in which substituents in the positions corresponding to C(2), N^6 and N(9) of adenosine could interact [10]. We verified that a phenyl group as C(2) substituent, confers to the molecules good affinity and selectivity for A_1 receptors [12]. During our study on inhibitors of ADA we demonstrated that the introduction of a phenyl group on C(2) increases affinity of 8-azaadenosines and 8-azaadenines also for this enzyme [13]. These findings and the fact that they have the same endogenous target made us hypothesise that ADA and the adenosine receptors could be proteins having some similarities in the binding site.

N(9)-Hydroxynonyladenine is a very potent inhibitor of ADA, with high stereoselectivity being *erythro* derivative (EHNA) a better ligand than the *threo* one (THNA) [14]. In this work we prepared and assayed a new class of adenines and 8-azaadenines, having a phenyl group on C(2) and characterised by the hydroxynonyl chain on N(9), with the aim of verifying if a C(2)-phenyl group could enhance also the EHNA's activity as inhibitor of ADA. We decided that it could be interesting to assay these molecules also as ligand for A_1 adenosine receptors. A good binding could signify that the N(9)-hydroxynonyl chain could interact not only with a region of ADA but also with the pocket of A_1 adenosine receptors in front of the N(9) position. To increase affinity for the A_1 receptors we introduced a substituent on the N^6 position such as alkyl, cycloalkyl and arylalkyl groups, substituents which are present in effective agonists or antagonists. Furthermore, for some compounds we prepared the two diastereoisomers *erythro* and *threo* to verify whether the binding with A_1 receptors is stereoselective, as in ADA. Results of this work could validate our hypothesis about some significant similarity in the binding site of the two proteins, ADA and A_1 adenosine receptor, in spite of lacking any homologies in the aminoacid sequences.

Some of the prepared compounds showed good inhibitory activity for ADA and good affinity for A_1 receptors. So we decided to prepare 2-phenyladenines and 2-phenyl-8-azaadenines having the N^6 and 9 substituents inverted with respect to the previous compounds. In previous papers [12,15] we have demonstrated that some adenines and 8-azaadenines can bind A_1 receptors in different modes, varying their disposition inside the receptors; in fact some couples of compounds alternatively substituted with the same group on N^6 or N(9) showed similar K_i values: this fact has been explained hypothesising that the substituent interacted with the same lipophilic pocket, no matter where it was bound. We wanted to verify if also in the case of the hydroxynonyl chain, the N^6 or the N(9) substituted compounds could be equipotent in the binding with A_1 receptor and with ADA.

2. Chemistry

Compounds **6**–**22** (see Scheme 1) and **32**–**37** (see Scheme 2) were synthesised starting from the same material, i.e. 5-amino-4,6-dichloro-2-phenylpyrimidine **1** which was obtained by a modification of a known method [16] starting from 4,6-dichloro-5-nitro-2-phenylpyrimidine [17].

erythro-3-Aminononan-2-ol **2** was synthesised by the method described by Schaeffer and Schwender [14]; in 1988 Boschelli [18] demonstrated that this reaction gives a mixture of *erythro* and *threo* isomers 10:1 on the basis of the ponderal ratio of the benzoyl derivatives. Our crude reaction mixture was analysed by ^{1}H NMR and gas chromatographic analysis of the acetate derivatives; these analyses confirmed the obtaining of a diastereoisomeric mixture *erythro*–*threo* in an 80:20 ratio which was used for the following reactions. *threo*-3-Aminononan-2-ol was obtained by conversion of *erythro* diastereoisomer by the methods described [18,19]. ^{1}H NMR of our reaction mixture and GLC analysis of acetate derivatives demonstrated that the product obtained was pure at 95%, with the presence of only 5% *erythro* diastereoisomer.

Reaction of **1** with *erythro* or *threo*-3-aminononan-2-ol **2** gave *erythro* or *threo* 5-amino-4-(2-hydroxy-3-nonylamino)-6-chloro-2-phenylpyrimidine (*erythro* **3** or *threo* **3**). Cyclisation of *erythro* **3** with triethyl orthoformate [20] gave the corresponding adenine *erythro* **4** formylated on the hydroxyl group; cyclisation of *threo* **3** with triethyl orthoformate gave the corresponding adenine *threo* **4**. Diazotisation of **3** with $NaNO_2$ and consequent cyclisation gave the 8-azaadenines **5** [21].

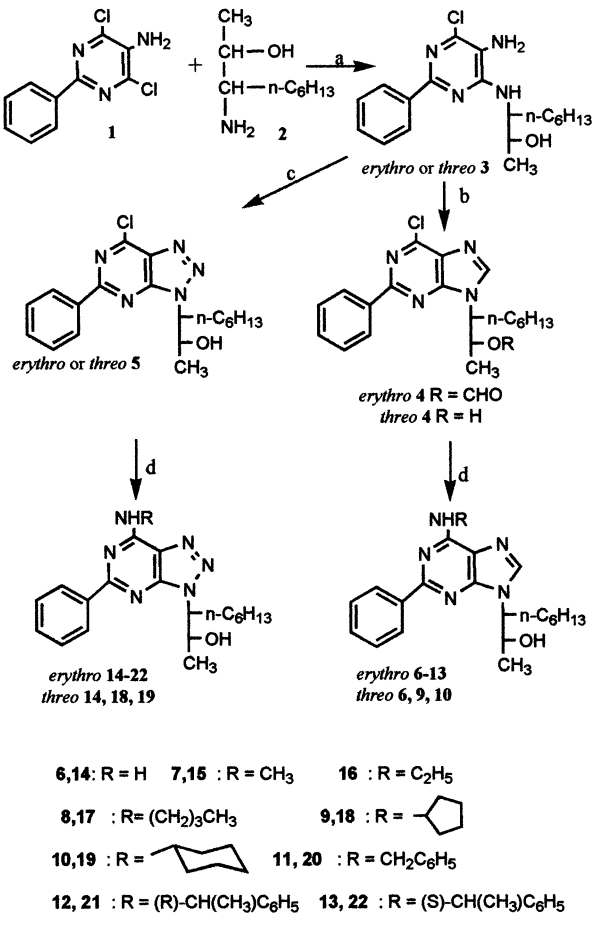
Treatment of **4** and **5** with the suitable amines gave the N^6-substituted 2-phenyl-9-(2-hydroxy-3-nonyl)adenines **6**–**13** and 8-azaadenines **14**–**22**.

Reaction of **1** with NH_3, cyclopentyl or cyclohexylamine gave pyrimidines **23**–**25** which were cyclised to purines **26**–**28** or 8-azapurines **29**–**31** as described above. Treatment with *erythro*- or *threo*-3-aminononan-2-ol gave compounds **32**–**34** and **35**–**37** of the two series *erythro* and *threo*. All final compounds were analysed by HPLC and resulted pure (*threo* isomer not superior to 5% in the *erythro* compounds, *erythro* isomer not superior to 5% in the *threo* compounds).

3. Experimental

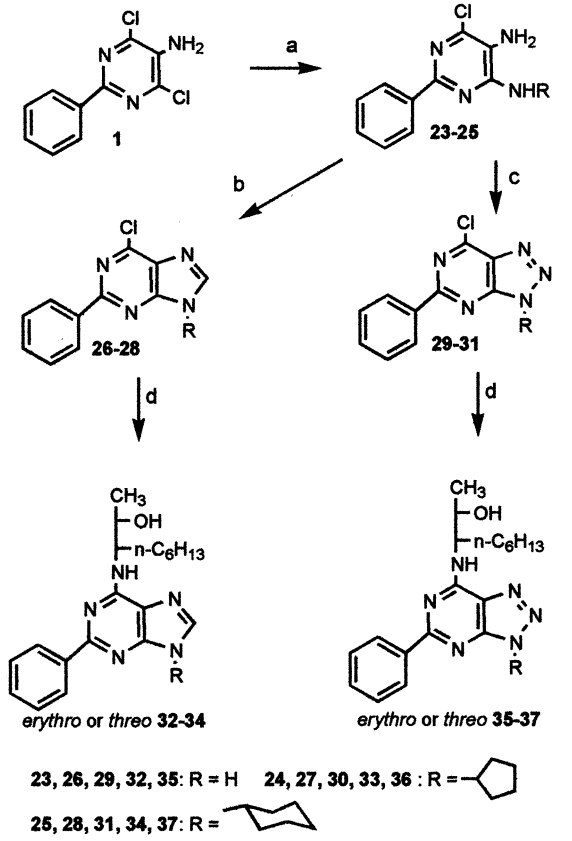
3.1. Chemistry

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. ^1H NMR spectra were recorded on a Varian Gemini 200 spectrometer in



[a]Reagents: (a) tributylamine, 1-pentanol; (b) triethyl ortoformate, HCl; (c) $NaNO_2$, CH_3COOH, THF, H_2O; (d) amine, EtOH.

Scheme 1.



[a]Reagents: (a) amine; (b) triethyl ortoformate, HCl; (c) $NaNO_2$, CH_3COOH, THF, H_2O; (d) *erythro* or *threo* 3-amino-2-hydroxynonane **2**, EtOH.

Scheme 2.

δ units using TMS as an internal standard; the compounds were dissolved in the solvent indicated in Table 3. Mass spectra were performed on a Hewlett-Packard GC/MS System 5988A. TLC was performed on precoated silica gel F_{254} plates (Merck). Flash-column chromatography was performed using Merck Kieselgel 60 (230–400 mesh). Microanalyses (C, H, N) were carried out on a Carlo Erba elemental analyser (Model 1106) and were within $\pm 0.4\%$ of the theoretical values. HPLC were carried out on a Violet PM 900 with a PH-410 UV photometer as detector, and a Nucleosil C18 column using MeOH–H_2O 95:5 as eluent. GLC analyses of compounds *erythro*- or *threo*-3-amino--nonan-2-ol were performed after transformation into the corresponding acetate derivatives prepared as follows: 10 mg of compound in anhydrous pyridine (1 ml) was treated with acetic anhydride (1 ml) at 100 °C for 20 min. An aliquot of the reaction mixture was gas chromatographed on a Carlo-Erba mod. 4200 apparatus using a glass column (1.5 m × 2.8 mm) packed with 10% neopentyl glycol succinate on 80–100 mesh silanised Chromosorb W and a FID detector.

3.2. 5-Amino-4,6-dichloro-2-phenylpyrimidine (**1**)

To a refluxing solution of 4,6-dichloro-5-nitro-2-phenylpyrimidine (1.53 g, 5.6 mmol) in 250 ml of THF, 75 ml of acetic acid and 75 ml of water, Fe powder (1.5 g, 27 mmol) was added, then the mixture was refluxed for 90 min more. After evaporation at reduced pressure, the solid residue was treated with four portions of ethyl ether which were collected and washed with 5% $NaHCO_3$, H_2O and evaporated to give compound **1** which crystallised from ethanol (m.p. 138–140 °C, 1.19 g, 5 mmol, 89% yield. Anal. ($C_{10}H_7Cl_2N_3$) C, H, N).

3.3. erythro- and threo-5-Amino-6-chloro-4-(2-hydroxy-3-nonyl)-2-phenylpyrimidine (erythro **3** and threo **3**)

A mixture of **2** (3.0 g, 12.5 mmol), *erythro*- or *threo*-3-aminononan-2-ol (**2**, 2.17 g, 13.6 mmol), tributylamine (23.1 g, 0.125 mol) in pentanol (68 ml) was heated at 170 °C for 48 h in a steel bomb. Evaporation of the mixture gave an oil which was flash-chromatographed using hexane–ethyl acetate 5:3 as eluent to give compound *erythro* **3** (3.1 g, 8.5 mmol, 68% yield) or *threo* **3** (2.0 g, 5.5 mmol, 44% yield) as a solid. ^{1}H NMR and Mass Spectra: see Table 2.

3.4. erythro-6-Chloro-9-(2-formyloxy-3-nonyl)-2-phenylpurine (erythro **4**)

A solution of *erythro* **3** (300 mg, 0.83 mmol), triethyl orthoformate (4 ml) and 12 N HCl (0.14 ml) was stirred at room temperature for 12 h; then the reaction mixture was evaporated and flash-chromatographed using hexane–ethyl acetate 5:3 as eluent to obtain the title compound as a solid (100 mg, 0.25 mmol, 30% yield). ^{1}H NMR and Mass Spectra: see Table 2.

3.5. threo-6-Chloro-9-(2-hydroxy-3-nonyl)-2-phenylpurine (threo **4**)

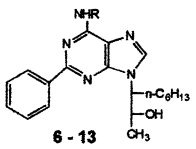
A solution of *threo* **3** (300 mg, 0.83 mmol), triethyl orthoformate (4 ml) and 12 N HCl (0.14 ml) was stirred at room temperature for 12 h; then water was added to the reaction mixture to precipitate the title compound as a solid (200 mg, 0.54 mmol, 65% yield). ^{1}H NMR and Mass Spectra: see Table 2.

3.6. erythro- and threo-6-Chloro-9-(2-hydroxy-3-nonyl)-2-phenyl-8-azapurine (erythro **5** and threo **5**)

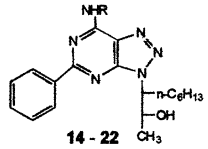
To an ice-cold solution of *erythro* or *threo* **3** (0.4 g, 1.1 mmol), H_2O (3.6 ml), glacial acetic acid (1.6 ml) and

Table 1
Biological results

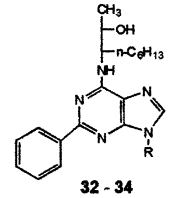
a) A_1 adenosine receptors binding.



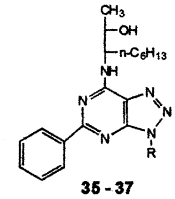
6 - 13



14 - 22



32 - 34



35 - 37

R	Comp	Ki ± SEM (nM)	Comp	Ki ± SEM (nM)	Comp	Ki ± SEM (nM) or % inhib 1 μM	Comp	Ki ± SEM (nM) or % inhib 1 μM
H	*Erythro* **6**	28 ± 2	*Erythro* **14**	2.8 ± 0.3	*Erythro* **32**	93 ± 8	*Erythro* **35**	583 ± 50
	Threo **6**	347 ± 35	*Threo* **14**	239 ± 25	*Threo* **32**	66 ± 7.2	*Threo* **35**	62%
CH_3	*Erythro* **7**	38.7 ± 4	*Erythro* **15**	52 ± 5				
C_2H_5			*Erythro* **16**	31 ± 3				
n-C_4H_9	*Erythro* **8**	18.5 ± 2	*Erythro* **17**	22 ± 2.5				
	*Erythro* **9**	5.5 ± 0.5	*Erythro* **18**	4.3 ± 5	*Erythro* **33**	60%	*Erythro* **36**	140 ± 12
	Threo **9**	54 ± 6	*Threo* **18**	33 ± 3.2	*Threo* **33**	60%	*Threo* **36**	65%
	*Erythro* **10**	116 ± 10	*Erythro* **19**	40.5 ± 4	*Erythro* **34**	456 ± 51	*Erythro* **37**	659 ± 70
	Threo **10**	2454 ±2 51	*Threo* **19**	118 ± 12	*Threo* **34**	60%	*Threo* **37**	65%
$CH_2C_6H_5$	*Erythro* **11**	649 ± 71	*Erythro* **20**	156 ± 17				
(R)-$CH(CH_3)C_6H_5$	*Erythro* **12**	155 ± 13	*Erythro* **21**	221 ± 23				
(S)-$CH(CH_3)C_6H_5$	*Erythro* **13**	89.7 ± 10	*Erythro* **22**	541 ± 61				

b) Adenosine Deaminase inhibition.

	Erythro **6**	*Threo* **6**	*Erythro* **7**	*Erythro* **14**	*Threo* **14**	*Erythro* **15**	*Erythro* **32**	*Threo* **32**	*Erythro* **35**	*Threo* **35**
Ki (nM) ±SEM	0.55 ± 0.04	1.30 ± 0.1	0.68 ± 0.07	1.67 ± 0.2	14.4 ± 1.5	4.51 ± 0.34	inactive	inactive	inactive	inactive

Table 2
^{1}H NMR and mass spectra of synthesised compounds

Comp.	^{1}H NMR				MS m/z (%)
	Aromatic H	Other H	Aliphatic H	Exchang H	
erythro **3** ($CDCl_3$)	8.20 (m, 2H); 7.38 (m, 3H)		4.32 (m, 1H); 4.07 (m, 1H); 1.31 (m 10H); 1.20 (d, 3H); 0.86 (t, 3H)	4.95 (1H), 3.20 (2H)	362 (M^+, 5); 317 (100); 233 (14); 104 (13)
threo **3** ($CDCl_3$)	8.28 (m, 2H); 7.42 (m, 3H)		4.32 (m, 1H); 4.03 (m, 1H); 2.15–1.24 (m, 13H); 0.86 (t, 3H)	5.22 (1H)	362 (M^+, 8); 317 (100); 233 (24); 104 (11)
erythro **4** ($CDCl_3$)	8.52 (m, 2H); 8.09 (s, 1H); 7.50 (m,3H)	8.03 (s, 1H, OCHO)	5.46 (m, 1H); 4.93 (m,1H); 2.40–2.00 (m, 2H); 1.32–1.22 (m, 11H); 0.82 (t, 3H)		400 (M^+, 29); 402 (M^++2, 10); 327 (30); 231 (100); 233 (33); 45 (59)
threo **4** ($CDCl_3$)	8.48 (m, 2H); 8.23 (s, 1H); 7.52 (m, 3H)		4.57 (m, 1H); 4.34 (m, 1H); 2.10–1.20 (m, 10H); 1.11 (d, 3H); 0.83 (t, 3H)	5.01 (1H)	372 (M^+, 6); 374 (M^++2, 2); 231 (24); 233 (11); 77 (7.68); 45 (100)
erythro **5** ($CDCl_3$)	8.55 (m, 2H); 7.52 (m, 3H)		5.04 (m, 1H); 4.40 (m,1H); 2.40–2.10 (m, 2H); 1.30–1.20 (m, 11H); 0.81 (t, 3H)	3.15 (1H)	373 (M^+, 1.5); 375 (M^++2, 0.5); 328 (9.5); 330 (4); 244 (63); 246 (22); 231 (24); 233 (11); 45 (100)
threo **5** ($CDCl_3$)	8.54 (m, 2H); 7.54 (m, 3H)		5.01 (m, 1H); 4.44 (m, 1H); 2.31 (m, 1H); 2.11 (m, 1H); 1.43–1.20 (m, 11H); 0.83 (t, 3H)	2.98 (1H)	373 (M^+, 1.3); 375 (M^++2, 0.4); 328 (2.3); 244 (9.7); 246 (3.2); 231 (4); 233 (2.2); 45 (100)
erythro **6** ($CDCl_3$)	8.20 (m, 2H); 7.63 (s, 1H); 7.33 (m, 3H)		4.22 (m, 2H); 2.01 (m, 2H); 1.34 (d, 3H); 1.17 (m, 8H); 0.76 (t, 3H)	5.68 (2H)	353 (M^+, 10); 308 (13); 238 (31); 212 (100); 104 (57); 45 (89)
threo **6** ($CDCl_3$)	8.34 (m, 2H); 7.78 (s, 1H); 7.46 (m, 3H)		4.29 (m, 2H); 2.10 (m, 2H); 1.48–1.23 (m, 8H); 1.08 (d, 3H); 0.84 (t, 3H)	5.78 (2H); 5.42 (1H)	353 (M^+, 3); 308 (6); 238 (22); 212 (60); 104 (83); 45 (100)
erythro **7**·HCl ($CDCl_3$)	8.50 (m, 2H); 8.08 (s, 1H); 7.60 (m, 3H)		4.60 (m, 1H); 4.26 (m, 1H); 3.68 (d, 3H); 2.10 (m, 2H); 1.33 (d, 3H); 1.22 (m, 8H); 0.84 (t, 3H)	10.42 (1H); 2.77 (1H)	367 (M^+, 2); 238 (8); 226 (28); 45 (100)
erythro **8** (DMSO)	8.36 (m, 2H); 8.14 (s, 1H); 7.45 (m, 3H)		4.31 (m, 1H); 4.09 (m, 1H); 2.08–0.9 (m, 22 H); 0.74 (t, 3H)	5.21 (1H)	409 (M^+, 7.7); 353 (2.5); 238 (28); 211 (23); 45 (100)
erythro **9** ($CDCl_3$)	8.37 (m, 2H); 7.65 (s, 1H); 7.46 (m, 3H)		4.35 (m, 1H); 4.15 (m, 1H); 2.3–1.69 (m, 6H); 1.36 (d, 3H); 1.22 (m, 12H); 0.80 (t, 3H)	6.01 (1H); 4.78 (1H)	421 (M^+, 7.6); 353 (8.9); 238 (16); 211 (35); 45 (100)
threo **9**·HCl ($CDCl_3$)	8.51 (m, 2H); 8.12 (s, 1H); 7.59 (m, 3H)		5.23 (m, 1H); 4.62 (m, 1H); 4.33 (m, 1H); 2.18–1.20 (m, 18H); 1.11 (d, 3H); 0.83 (t, 3H)	10.65 (1H); 8.32 (1H)	421 (M^+, 2); 353(4); 238 (9); 211 (20); 104 (43); 45 (100)
erythro **10** ($CDCl_3$)	8.52 (m, 2H); 8.04 (s, 1H); 7.60 (m, 3H)		4.57 (m, 1H); 4.27 (m, 1H); 2.20–1.57 (m, 6H); 1.34 (d, 3 H); 1.23 (m, 14H); 0.84 (t, 3H)	4.78 (1H)	435 (M^+, 20); 353 (36); 211 (100); 45 (90)
threo **10**·HCl ($CDCl_3$)	8.53 (m, 2H); 8.12 (s, 1H); 7.56 (m, 3H)		4.83 (m, 1H); 4.62 (m, 1H); 4.33 (m, 1H); 2.18–1.20 (m, 20H); 1.10 (d, 3H); 0.83 (t, 3H)	10.60 (1H); 8.42 (1H)	435 (M^+, 2); 353 (4.4); 211 (23); 104 (42); 45 (100)
erythro 11 ($CDCl_3$)	8.39 (m, 2H); 7.66 (s, 1H); 7.45 (m, 3H); 7.38 (m, 5H)	5.03 (d, 2H, benzylic H)	4.38 (m, 1H); 4.18 (m, 1H); 2.43–1.91 (m, 2H); 1.36 (d, 3H); 1.22 (m, 8H); 0.84 (t, 3H)	6.53 (1H); 6.14 (1H)	443 (M^+, 7.5); 398 (3); 301 (14); 238(9); 91 (100); 45 (57)
erythro **12** ($CDCl_3$)	8.31 (m, 2H); 8.18 (s, 1H); 7.65–7.28 (m, 8H)	5.23 (m, 1H, benzylic H)	4.32 (m, 1H); 4.16 (m, 1H); 2.18–1.09 (m, 10H); 1.72 (d, 3H); 1.35 (d, 3H); 0.84 (t, 3H)	6.34 (1H); 5.91 (1H)	457 (M^+, 12); 315 (11); 239 (39); 238 (12); 105 (45); 45 (100)
erythro **13** ($CDCl_3$)	8.31 (m, 2H); 8.18 (s, 1H); 7.65–7.28 (m, 8H)	5.23 (m, 1H, benzylic H)	4.32 (m, 1H); 4.16 (m, 1H); 2.18–1.09 (m, 10H); 1.72 (d, 3H); 1.35 (d, 3H); 0.84 (t, 3H)	6.34 (1H); 5.91 (1H)	457 (M^+, 8); 315 (9); 238 (5); 105 (57); 45 (100)
erythro **14**·HCl ($CDCl_3$)	8.40 (m, 2H); 7.44 (m, 3H)		4.95 (m, 1H); 4.40 (m, 1H); 2.20 (m, 2H); 1.34 (d, 3H); 1.23 (m, 8H); 0.82 (t, 3H)	6.02 (2H)	354 (M^+, 2); 239 (19); 213 (54); 104 (89); 45 (100)
threo **14** ($CDCl_3$)	8,40 (m, 2H); 7.51 (m, 3H)		4.96 (m, 1H); 4.35 (m, 1H); 2.19 (m, 2H); 1.68–1.16 (m, 8H); 1.12 (d, 3H); 0.85 (t, 3H)	6.20 (1H); 4.80 (1H)	354 (M^+, 1); 239 (2.5); 213 (7,5); 104 (51); 45 (100)

Table 2 (*Continued*)

Comp.	^{1}H NMR				MS *m/z* (%)
	Aromatic H	Other H	Aliphatic H	Exchang H	
erythro **15** ($CDCl_3$)	8.34 (m, 2H); 7.44 (m, 3H)		4.85 (m, 1H); 4.40 (m, 1H); 3.38 (d, 3H); 2.05 (m, 2H); 1.35 (d, 3H); 1.24 (m, 8H); 0.82 (t, 3H)	6.35 (1H); 5.28 (1H)	351 (2.8); 239 (34); 227 (61); 104 (38); 45 (100)
erythro **16** ($CDCl_3$)	8.43 (m, 2H); 7.49 (m, 3H)		4.88 (m, 1H); 4.42 (m, 1H); 3.89 (m, 2H); 2.30–1.98 (m, 2H); 1.61–1.37 (m, 6H); 1.23 (m, 8H); 0.83 (t, 3H)	6.39 (1H); 5.66 (1H)	382 (M^+, 2.3); 310 (29); 253 (31); 241 (100); 104 (50); 45 (60)
erythro **17** ($CDCl_3$)	8.43 (m, 2H); 7.48 (m, 3H)		4.86 (m, 1H); 4.42 (m, 1H); 3.84 (m, 2H); 1.80–1.51 (m, 2H); 1.38 (d, 3H); 1.23 (m, 8H); 1.06 (t, 3H); 0.82 (t, 3H)	6.55 (1H); 5.67 (1H)	410 (5); 338 (13); 269 (35); 45 (100)
erythro **18** ($CDCl_3$)	8.42 (m, 2H); 7.49 (m, 3H)		4.81 (m, 2H); 4.43 (m, 1H); 2.20–1.66 (m, 6H); 1.38 (d, 3H); 1.21 (m, 12H); 0.83 (t, 3H)	6.28 (1H); 5.66 (1H)	422 (M^+, 3); 211 (13); 45 (100)
threo **18**·HCl ($CDCl_3$)	8.56 (m, 2H); 7.64 (m, 3H)		5.30 (m, 1H); 4.89 (m, 1H); 4.43 (m, 1H); 2.27–1.23 (m, 18H); 1.08 (d, 3H); 0.84 (t, 3H)	11.53 (1H)	422 (M^+, 5); 211 (18); 45(100)
erythro **19** ($CDCl_3$)	8.40 (m, 2H); 7.49 (m, 3H)		4.86 (m, 1H); 4.42 (m, 2H); 2.24–1.21 (m, 23H); 0.83 (t, 3H)	6.30 (1H); 5.72 (1H)	364 (1.2); 295(3.2); 213 (1); 104 (3); 45 (100)
threo **19**·HCl ($CDCl_3$)	8.56 (m, 2H); 7.64 (m, 3H)		4.89 (m, 2H); 4.20 (m, 1H); 2.19–1.26 (m, 20H); 1.08 (d, 3H); 0.84 (t, 3H)	11.48 (1H)	364 (6); 295(4.5); 213 (9); 104 (8); 45 (100)
erythro **20** (DMSO)	8.43 (m, 2H); 7.50 (m, 3H); 7.32 (m, 5H)	5.05 (d, 2H, benzylic H)	4.88 (m, 1H); 4.45 (m,1H); 2.40–1.98 (m, 2H); 1.39 (d, 3H); 1.24 (m, 8H); 0.83 (t, 3H)	6.78 (1H); 5.43 (1H)	444 (M^+, 3.3); 303 (35); 91 (100); 45 (54)
erythro **21** ($CDCl_3$)	8.34 (m, 2H); 7.54–7.27 (m, 8H)	5.74 (m, 1H, benzylic H)	4.87 (m, 1H); 4.41 (m, 1H); 2.00–1.80 (m, 2H); 1.77 (d, 3H); 1.37 (d, 3H); 1.30–0.95 (m, 8H); 0.83 (t, 3H)	6.73 (1H); 5.56 (1H)	458 (M^+, 2.5); 317 (14); 105 (58); 45 (34); 43 (100)
erythro **22** ($CDCl_3$)	8.34 (m, 2H); 7.54–7.27 (m, 8H)	5.74 (m, 1H, benzylic H)	4.87 (m, 1H); 4.41 (m, 1H); 2.00–1.80 (m, 2H); 1.77 (d, 3H); 1.37 (d, 3H); 1.30–0.95 (m, 8H); 0.83 (t, 3H)	6.73 (1H); 5.56 (1H)	458 (M^+, 6); 317 (3); 105 (100); 45 (56); 43 (95)
23 (DMSO)	8.13 (m, 2H); 7.40 (m, 3H)			6.90 (2H); 5.19 (2H)	220 (M^+, 54); 220 (M^++2, 18); 204 (12); 104 (100); 77 (71)
24 ($CDCl_3$)	8.38 (m, 2H); 7.47 (m, 3H)		4.59 (m,1H); 2.24 (m, 2H); 1.80–1.55 (m, 6H)	4.82 (1H); 3.38 (2H)	288 (M^+, 3); 290 (1.2); 220 (15); 69 (22); 41 (100)
25 ($CDCl_3$)	8.32 (m, 2H); 7.44 (m, 3H)		4.14 (m, 1H); 2.19–1.26 (m, 10H)	4.77 (1H)	302 (M^+, 17); 304 (6); 220 (100)
26 ($CDCl_3$)	8.81 (s, 1H); 8.48 (m, 2H); 7.53 (m, 2H)				230 (M^+, 94); 195 (69); 104 (19); 77 (21); 43 (100)
27 ($CDCl_3$)	8.55 (m, 2H); 8.13 (s, 1H); 7.50 (m, 3H)		5.06 (m,1H); 2.38 (m, 2H); 2.18–1.55 (m, 6H)		
28 ($CDCl_3$)	8.53 (m, 2H); 8.19 (s, 1H); 7.54 (m, 3H)		4.62 (m, 1H); 2.25 (m, 2H); 2.03–1.55 (m, 8H)		
29 (DMSO)	8.44 (m, 3H); 7.57 (m, 3H)				231 (M^+, 8); 168 (13); 104(32); 53 (100)
30 ($CDCl_3$)	8.57 (m, 2H); 7.55 (m. 3H)		5.49 (m, 1H); 2.45–1.58 (m, 8H)		
31 ($CDCl_3$)	8.58 (m, 2H); 7.55 (m, 3H)		4.98 (m, 1H); 2.36–1.51 (m, 10H)		
erythro **32** ($CDCl_3$)	8.17 (m, 2H); 7.67 (s, 1H); 7.51 (m, 3H)		4.11 (m, 1H); 3.90 (m, 1H); 1.72–1.26 (m, 10H); 1.35 (d, 3H); 0.82 (t, 3H)	6.19 (1H); 5.51 (1H)	308 (5); 212 (2); 195 (4.5); 104 (8); (8); 45 (38); 43 (100)

Table 2 (*Continued*)

Comp.	^{1}H NMR				MS *m/z* (%)
	Aromatic H	Other H	Aliphatic H	Exchang H	
threo **32** (DMSO)	8.36 (m, 2H); 8.11 (s, 1H); 7.45 (m, 3H)		4.43 (m, 1H); 3.87 (m, 1H); 1.79–1.10 (m, 10H); 1.07 (d, 3H); 0.77 (t, 3H)	7.10 (1H)	308 (7); 212 (3); 195 (5); 104 (15); 45 (89); 43 (89); 41 (100)
erythro **33** ($CDCl_3$)	8.38 (m, 2H); 7.82 (s, 1H); 7.46 (m, 3H)		4.99 (m, 1H); 4.50 (m, 1H); 4.08 (m, 1H); 2.36–1.27 (m, 18H); 1.20 (d, 3H); 0.86 (t, 3H)	5.68 (1H)	376 (5); 195 (1); 45 (100)
threo **33** ($CDCl_3$)	8.45 (m, 2H); 7.80 (s, 1H); 7.47 (m, 3H)		5.00 (m, 1H); 4.36 (m, 1H); 4.04 (m, 1H); 2.36–1.24 (m, 21H); 0.83 (t, 3H)	6.01 (1H)	376 (2.52); 279 (1.0); 45 (100)
erythro **34** ($CDCl_3$)	8.38 (m, 2H); 7.84 (s, 1H); 7.46 (m, 3H)		4.53 (m, 2H); 4.09 (m, 1H); 2.26–1.28 (m, 20H); 1.20 (d, 3H); 0.87 (t, 3H)	5.75 (1H)	390 (3); 195 (1); 45 (100)
threo **34** ($CDCl_3$)	8.43 (m, 1H); 7.82 (s, 1H); 7.45 (m, 3H)		4.53 (m, 1H); 4.33 (m,1H); 4.01 (m, 1H); 2.21–1.24 (m, 23 H); 0.84 (t, 3H)	5.99 (1H)	390 (1); 195 (4); 45 (100)
erythro **35** (DMSO)	8.39 (m, 2H); 7.50 (m, 3H)		4.47 (m, 1H); 3.74 (m, 1H); 1.95–1.00 (m, 10H); 0.98 (d, 3H); 0.74 (t, 3H)	8.13 (1H); 4.79 (1H)	309 (5); 213 (6); 104 (18); 45 (100)
threo **35** ($CDCl_3$)	8.40 (m, 2H); 7.49 (m, 3H)		4.54 (m, 1H); 3.88 (m, 1H); 1.75–1.15 (m, 10H); 1.10 (d, 3H); 0.77 (t, 3H)	8.13 (1H); 4.76 (1H)	309 (6); 213 (9); 104 (54); 45 (100)
erythro **36** ($CDCl_3$)	8.43 (m, 2H); 7.47 (m, 3H)		5.36 (m, 1H); 4.61 (m, 1H); 4.06 (m, 1H); 2.37–1.24 (m, 18H); 1.21 (d, 3H); 0.84 (t, 3H)	6.88 (1H); 4.10 (1H)	377 (3); 309 (1); 69 (9); 45 (100)
threo **36** ($CDCl_3$)	8.46 (m, 2H); 7.48 (m, 3H)		5.38 (m, 1H); 4.52 (m, 1H); 4.11 (m, 1H); 2.39–1.26 (m, 21H); 0.84 (t, 3H)	6.64 (1H)	377 (2.52); 45 (100)
erythro **37** ($CDCl_3$)	8.44 (m, 2H); 7.49 (m, 3H)		4.89 (m, 1H); 4.61 (m, 1H); 4.12 (m, 1H); 2.29–1.24 (m, 23H); 0.86 (t, 3H)	6.40 (1H)	391 (3); 309 (1); 213 (1); 83 (3); 45 (100)
threo **37** ($CDCl_3$)	8.47 (m, 2H); 7.49 (m, 3H)		4.86 (m, 1H); 4.52 (m, 1H); 4.11 (m, 1H); 2.29–1.25 (m, 23H); 0.82 (t, 3H)	6.64 (1H)	391 (2.02); 295 (1.0); 83 (3); 45 (100)

THF (7 ml), a solution of KNO_2 (103 mg, 1.22 mmol) in 3.6 ml of H_2O was added dropwise. The mixture was stirred for 3 h at 0 °C and for 2 h at room temperature, then was concentrated in vacuo. The residue was diluted with water and the solid precipitated was crystallised from ethanol–H_2O to obtain compound *erythro* **5** (336 mg, 0.9 mmol, 82% yield) or *threo* **5** (260 mg, 0.7 mmol, 63% yield). ^{1}H NMR and Mass Spectra: see Table 2.

*3.7. erythro-6-Aminosubstituted-9-(2-hydroxy-3-nonyl)-2-phenylpurine (erythro **6–13**)*

A mixture of *erythro* **4** (100 mg, 0.27 mmol), 5 ml of anhydrous ethanol and the suitable amine was heated in a sealed tube at 120 °C for 24 h. After evaporation, the residue was diluted with chloroform, washed with 10% HCl and evaporated. The residue was crystallised or chromatographed to give pure products (Tables 2 and 3).

Table 3
Reaction data of synthesised compounds

Comp.	Amine	Yield (%)	M.p.	R_f	Purification methods
erythro **6**	NH_3	32	170	0.28 [a]	A (isopropanol–ethyl ether)
threo **6**	NH_3	63	134	0.34 [b]	A (isopropanol–ethyl ether)
erythro **7**·HCl	methylamine	29	>300	0.30 [a]	A (isopropanol–ethyl ether)
erythro **8**	butylamine	86	oil	0.32 [a]	B [a]
erythro **9**	cyclopentylamine	62	163	0.26 [a]	B [a]
threo **9**·HCl	cyclopentylamine	45	>300	0.37 [c]	A (ethyl ether)
erythro **10**	cyclohexylamine	85	166	0.33 [d]	B [d]
threo **10**·HCl	cyclohexylamine	44	>300	0.37 [c]	A (ethyl ether)
erythro **11**	benzylamine	28	163	0.34 [d]	A (isopropanol)
erythro **12**	(*R*)-1-phenyl-ethylamine	73	oil	0.29 [d]	B [d]
erythro **13**	(*S*)-1-phenyl-ethylamine	57	oil	0.29 [d]	B [d]
erythro **14**·HCl	NH_3	83	>300	0.35 [a]	A (isopropanol–ethyl ether)
threo **14**	NH_3	53	128	0.34 [e]	A (isopropanol–ethyl ether)
erythro **15**	methylamine	38	165	0.40 [a]	A (isopropanol–ethyl ether)
erythro **16**	ethylamine	52	160	0.28 [f]	B [f]
erythro **17**	butylamine	70	132	0.37 [f]	A (isopropanol)
erythro **18**	cyclopentylamine	71	oil	0.37 [f]	B [f]
threo **18**·HCl	cyclopentylamine	71	>300	0.28 [g]	A (chloroform–hexane)
erythro **19**	cyclohexylamine	35	203	0.42 [f]	B [f]
threo **19**·HCl	cyclohexylamine	54	>300	0.28 [g]	A (chloroform–hexane)
erythro **20**	benzylamine	90	182	0.40 [d]	B [d]
erythro **21**	(*R*)-1-phenyl-ethylamine	63	oil	0.40 [f]	B [f]
erythro **22**	(*S*)-1-phenyl-ethylamine	55	oil	0.40 [f]	B [f]
erythro **32**	NH_3	57	182	0.18 [h]	B [h]
threo **32**	NH_3	30	oil	0.14 [h]	B [h]
erythro **33**	cyclopentylamine	22	117	0.31 [i]	B [i]
threo **33**	cyclopentylamine	32	119	0.30 [i]	B [i]
erythro **34**	cyclohexylamine	55	143	0.29 [i]	B [i]
threo **34**	cyclohexylamine	64	137	0.27 [i]	B [i]
erythro **35**	NH_3	69	185	0.15 [h]	B [h]
threo **35**	NH_3	45	176 [h]	0.18 [h]	B [h]
erythro **36**	cyclopentylamine	44	128	0.26 [g]	B [g]
threo **36**	cyclopentylamine	52	125	0.28 [g]	B [g]
erythro **37**	cyclohexylamine	61	120	0.23 [g]	B [g]
threo **37**	cyclohexylamine	42	127	0.23 [g]	B [g]

A, crystallisation (solvent); B, column chromatography.
[a] Eluent: hexane–ethyl acetate, 5:3.
[b] Eluent: chloroform–methanol, 2:0.1.
[c] Eluent: chloroform–methanol, 9:0.1.
[d] Eluent: hexane–ethyl acetate, 5:4.
[e] Eluent: chloroform–methanol, 4:0.1.
[f] Eluent: petroleum ether–ethyl acetate, 5:1.
[g] Eluent: petroleum ether–ethyl acetate, 4:0.5.
[h] Eluent: chloroform–methanol, 9.5:0.5.
[i] Eluent: petroleum ether–ethyl acetate, 4:1.

*3.8. threo-6-Aminosubstituted-9-(2-hydroxy-3-nonyl)-2-phenylpurine (threo **6**, **9** and **10**)*

Starting from *threo* **4**, the title compounds were obtained and purified as described above for *erythro* **6**–**13** (see Tables 2 and 3).

*3.9. erythro-6-Aminosubstituted-9-(2-hydroxy-3-nonyl)-2-phenyl-8-azapurine (erythro **14**–**22**)*

Starting from *erythro* **5**, the title compounds were obtained and purified as described above for compounds *erythro* **6**–**13** (see Tables 2 and 3).

*3.10. threo-6-Aminosubstituted-9-(2-hydroxy-3-nonyl)-2-phenyl-8-azapurine (threo **14**, **18** and **19**)*

Starting from *threo* **5**, the title compounds were obtained and purified as described above for the corresponding *erythro* compounds (Tables 2 and 3).

*3.11. 6-Chloro-4,5-diamino-2-phenylpyrimidine (**23**)*

A mixture of **2** (0.5 g, 2.08 mmol) and absolute ethanol (15 ml) saturated with gaseous NH_3 was heated at 110 °C in a steel bomb for 72 h. Evaporation at reduced pressure gave a residue which was diluted with chloroform. The solution was washed with 10% HCl and evaporated to give a residue which crystallised from petroleum ether–ethyl ether (0.38 g, 1.7 mmol, 82% yield). ^{1}H NMR and Mass Spectra: see Table 2.

*3.12. 5-Amino-6-chloro-4-cycloalkylamino-2-phenylpyrimidine (**24**, **25**)*

A mixture of **2** (500 mg, 2.08 mmol) and proper cycloalkylamine (0.4 mol) was heated in a sealed tube at 110 °C for 90 min. After evaporation, the residue was diluted with chloroform, washed with 10% HCl, 5% $NaHCO_3$, H_2O and evaporated. The residue was crystallised from ethanol–water to give **24** (m.p. 198–199 °C, 320 mg, 1.12 mmol, 54% yield) or **25** (m.p. 182–183 °C, 450 mg, 1.49 mmol, 72% yield). ^{1}H NMR and Mass Spectra: see Table 2.

*3.13. 6-Chloro-2-phenylpurine (**26**)*

A solution of **23** (150 mg, 0.68 mmol), triethyl orthoformate (3 ml) and 12 N HCl (0.14 ml) was stirred at room temperature for 12 h. Evaporation of the mixture gave an oil which was flash-chromatographed using petroleum ether–ethyl acetate 4:1 as eluent to give the title compound (127 mg, 0.55 mmol, 81% yield). ^{1}H NMR and Mass Spectra: see Table 2.

*3.14. 6-Chloro-9-cyclopentyl-2-phenylpurine (**27**)*

Compound **27** (160 mg, 0.54 mmol, 78% yield) was obtained starting from **24** (200 mg, 0.69 mmol) as described above for **26**. ^{1}H NMR: Table 2.

*3.15. 6-Chloro-9-cyclohexyl-2-phenylpurine (**28**)*

Compound **28** (150 mg, 0.48 mmol, 73% yield) was obtained starting from **25** (200 mg, 0.66 mmol) as described above for **26**. ^{1}H NMR: see Table 2.

*3.16. 6-Chloro-2-phenyl-8-azapurine (**29**)*

To an ice-cold solution of **23** (240 mg, 1.1 mmol), H_2O (3.6 ml), glacial acetic acid (1.6 ml) and THF (7 ml) was added dropwise to a solution of KNO_2 (103 mg, 1.22 mmol) in 3.6 ml of H_2O. The mixture was stirred for 3 h at 0 °C, 2 h at room temperature and then was concentrated in vacuo. The residue was diluted with water and the solid precipitated was filtered to obtain compound **29** (135 mg, 0.58 mmol, 53% yield). ^{1}H NMR and Mass Spectra: Table 2.

*3.17. 6-Chloro-9-cyclopentyl-2-phenyl-8-azapurine (**30**)*

Compound **30** (120 mg, 0.40 mmol, 58% yield) was obtained starting from **24** (200 mg, 0.69 mmol) as described above for **29**. ^{1}H NMR: Table 2.

*3.18. 6-Chloro-9-cyclohexyl-2-phenyl-8-azapurine (**31**)*

Compound **31** (130 mg, 0.41 mmol, 63% yield) was obtained starting from **25** (200 mg, 0.66 mmol) as described above for **29**. ^{1}H NMR: Table 2.

*3.19. erythro- and threo-6-(2-Hydroxy-3-nonylamino)-2-phenylpurine (erythro **32** and threo **32**)*

A mixture of **26** (260 mg, 1.13 mmol), *erythro*- or *threo*-3-aminononan-2-ol (520 mg, 3.27 mmol) and anhydrous EtOH (4 ml) was heated in a closed tube at 120 °C for 15 h. After evaporation, the residue was flash-chromatographed obtaining *erythro* **32** (220 mg, 0.62 mmol) or *threo* **32** (134 mg, 0.38 mmol) (Tables 2 and 3).

*3.20. erythro- and threo-6-(2-Hydroxy-3-nonylamino)-2-phenyl-9-cyclopentylpurine (erythro **33** and threo **33**)*

Compounds *erythro* **33** (50 mg, 0.12 mmol) and *threo* **33** (72 mg, 0.17 mmol) were obtained starting from **27** (160 mg, 0.54 mmol) as described above for **32** (Tables 2 and 3).

*3.21. erythro- and threo-6-(2-Hydroxy-3-nonylamino)-2-phenyl-9-cyclohexylpurine (erythro **34** and threo **34**)*

Compounds *erythro* **34** (100 mg, 0.23 mmol) and *threo* **34** (70 mg, 0.16 mmol) were obtained starting from **27** (130 mg, 0.41 mmol) as described above for **32** (see Tables 2 and 3).

*3.22. erythro- and threo-6-(2-Hydroxy-3-nonylamino)-2-phenyl-8-azapurine (erythro **35** and threo **35**)*

Compounds *erythro* **35** (110 mg, 0.31 mmol) and *threo* **35** (110 mg, 0.31 mmol) were obtained starting from **29** (160 mg, 0.69 mmol) as described above for **32** (see Tables 2 and 3).

*3.23. erythro- and threo-6-(2-Hydroxy-3-nonylamino)-2-phenyl-9-cyclopentyl-8-azapurine (erythro **36** and threo **36**)*

Compounds *erythro* **36** (50 mg, 0.12 mmol) and *threo* **36** (60 mg, 0.14 mmol) were obtained starting from **30** (80 mg, 0.27 mmol) as described above for **32** (see Tables 2 and 3).

*3.24. erythro- and threo-6-(2-Hydroxy-3-nonylamino)-2-phenyl-9-cyclohexyl-8-azapurine (erythro **37** and threo **37**)*

Compounds *erythro* **37** (51 mg, 0.12 mmol, 61% yield) and *threo* **37** (33 mg, 0.08 mmol) were obtained starting from **30** (60 mg, 0.19 mmol) as described above for **32** (see Tables 2 and 3).

4. Biochemical assays

4.1. A_1 Receptor binding

Bovine cerebral cortex was homogenised in ice-cold 0.32 M sucrose containing protease inhibitors, as previously described [22]. The homogenate was centrifuged at 1000*g* for 10 min at 4 °C and the supernatant again centrifuged at 48 000*g* for 15 min at 4 °C. The final pellet was dispersed in ten volumes of fresh buffer, incubated with adenosine deaminase (2 units/ml), to remove endogenous adenosine, at 37 °C for 60 min, and then recentrifuged at 48 000*g* for 15 min at 4 °C. The pellet was suspended in buffer and used in the binding assay.

The [^{3}H]CHA binding assay was performed in triplicate by incubating aliquots of the membrane fraction (0.2–0.3 mg of protein) at 25 °C for 45 min in 0.5 ml of Tris–HCl, pH 7.7, containing 2mM $MgCl_2$, with approximately 1.2 nM [^{3}H]CHA. Non-specific binding was defined in the presence of 50 μM *R*-PIA. Binding reactions were terminated by filtration through Whatman GF/C filters under reduced pressure. Filters were washed three times with 5 ml of ice-cold buffer and placed in scintillation vials.

4.2. A_{2a} Receptor binding

Bovine striatum was homogenised in 20 volumes of ice-cold 50 mM Tris–HCl, pH 7.5, containing 10 mM $MgCl_2$ and protease inhibitors. The membrane homogenate was centrifuged at 48 000*g* for 10 min at 4 °C. The resulting pellet was resuspended in buffer containing 2 units/ml of adenosine deaminase and incubated at 37 °C for 30 min. The membrane homogenate was centrifuged, and the final pellet frozen at − 80 °C. Routine assays were performed in triplicate by incubating an aliquot of striatal membranes (0.2–0.3 mg of protein) in cold 50 mM Tris–HCl, pH 7.5, containing 10 mM $MgCl_2$ with approximately 5 nM [^{3}H]CGS 21680 in a final volume of 0.5 ml. Incubation was carried out for 90 min at 25 °C. Non-specific binding was defined in the presence of 50 μM CGS 21680. Binding reactions were terminated by filtration through Whatman GF/C filters under reduced pressure. Filters were washed three times with 5 ml of ice-cold buffer and placed in scintillation vials.

The radioactivity was counted in a 4 ml Beckman Ready-Protein scintillation cocktail in a scintillation counter. The compounds were dissolved in DMSO and added to the assay mixture to make a final volume of 0.5 ml. Blank experiments were carried out to determine the effect of the solvent (2%) on binding. The concentrations of the tested compounds to produce 50% inhibition of specific [^{3}H]CHA binding (IC_{50}) were determined from semilog plots of data from experiments of binding inhibition. The K_i values were calculated from the IC_{50} values using the equation $IC_{50}/(1 + L/K_d)$ [23] ([^{3}H]CHA $K_d = 10.5$ nM and $L = 1.2$ nM); protein estimation was based on the method reported [24], using bovine serum albumin as standard.

4.3. ADA inhibition

Calf intestinal ADA (sp. act. ca. 200 units/mg protein) was purchased from Sigma Chemical Co. (St. Louis, Mo, USA) (Type VIII). Adenosine deaminase assay was performed according to a modified procedure of the method described by Colowick and Kaplan [25]. All reactions were in 0.1 M phospate buffer pH 7.2 at 30 °C in a final volume of 1 ml. The substrate concentration was 40 μM. The reaction was started by the addition of ADA (7.5 mU) and the absorbance at 265 nm was followed in phosphate buffer to the proper concentration. In the assay the DMSO final concentration never exceeded 4% which did not interfere with the enzymatic activity.

The IC_{50} values were obtained by plotting $\Delta A_i/\Delta A_0 \times 100$ against the concentration of the inhibitor using a sigmoidal dose–response equation (GRAPHPAD Prism program, Version 3.0). Competitive inhibition was revealed by measuring $1/V$ versus $1/[S]$ in the presence of various inhibitor concentrations. The K_i values were calculated from IC_{50} values by using the Cheng and Prusoff equation [23], considering $[S] = 40$ μM, and $K_D = 27$ μM.

5. Results and discussion

All synthesised compounds were tested as ligands for A_1 and A_{2A} adenosine receptors and compounds **6**, **7**, **14**, **15**, **32** and **35** were tested as inhibitors of Adenosine deaminase; the results of A_1 adenosine receptor binding and ADA inhibition assays are reported in Table 1. In the A_2 adenosine receptor binding assays, all the compounds showed inhibition values $< 60\%$ at 1 μM, so were considered inactive. The results of ADA assays showed that all tested 9-(2-hydroxy-3-nonyl) substituted compounds were very active ($K_i < 15$ nM). Compounds *erythro* **6** ($K_i = 0.55$ nM) and *threo* **6** ($K_i = 1.30$ nM) were more active than the corresponding 2-unsubstituted compounds, EHNA ($K_i = 4$ nM) [26] or THNA ($K_i \approx 100$ nM) [26]. The *erythro* isomers were more potent than the corresponding *threo* isomers, according to the profile of EHNA itself. So we can hypothesise that the new compounds bind at the enzyme at the same site as EHNA, with a strong interaction with the alkyl chain, and that a further interaction between the enzyme and the C-2 phenyl group must be present. This confirms our previous finding about substitution on C-2 of adenine or 8-azaadenine derivatives: a phenyl group increases binding of these molecules towards ADA [13]. Compounds *erythro* or *threo* **32** and **35**, having the alkyl chain on N^6, were inactive according to what has been reported in the past by Schaeffer [27].

Biological results on adenosine receptors showed a generally high affinity and selectivity for A_1 subtype, compared with A_{2A}, confirming that the presence of a 2-phenyl group gives to adenine derivatives this biological profile and demonstrates that the pocket II (Fig. 1a) of A_1 adenosine receptors can bind a long alkyl chain like the 2-hydroxy-3-nonyl one very well. The most active compounds were the N^6-alkyl adenine and 8-azaadenine derivatives: cycloalkyl derivatives *erythro* **9** and *erythro* **18** had a K_i of 5.5 and 4.3 nM, respectively. This fact could indicate that also for these compounds the receptor interaction site is the same as other high-affinity ligands like CPA. Also N^6-unsubstituted-8-azaadenine **14** showed a very high affinity (2.8 nM). N^6-aralkyl substituted compounds (**11**–**13** and **20**–**22**) showed a lower affinity. Differently from the corresponding 8-azaderivative **35**, compounds *erythro* and *threo* **32**, having the hydroxyalkyl chain on N^6, maintain a good affinity compared with *erythro* and *threo* **6**; this result indicates that these molecules could bind the receptors in a position rotated 180° in respect of adenosine, inserting the N^6 substituent in pocket II. For trisubstituted adenines and 8-azaadenines this hypothesis is not verified, as previously reported [8]. In fact the other N^6-2-hydroxy-3-nonyl derivatives **33**, **34**, **36**, **37** bind receptors very weakly.

These results show that both ADA and A_1 adenosine receptors can bind the same groups substituted on C(2) or N(9) positions of adenine ligands and could indicate in the ADA the presence of two lipophilic pockets similar to the pockets I and II of A_1 adenosine receptors (Fig. 1).

It is very interesting to note that, comparing affinity of *erythro* and *threo* isomers, all the *erythro* isomers are more potent than the *threo* corresponding compounds,

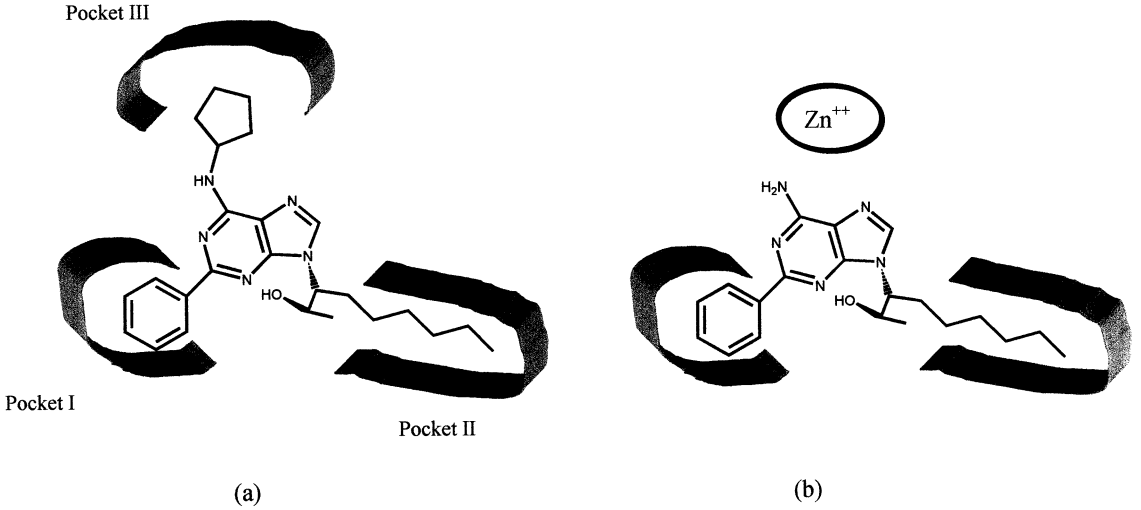


Fig. 1. (a) Hypothesised lipophilic pocket of A_1 adenosine receptor binding site. (b) Hypothesised lipophilic pocket of ADA binding site.

not only towards ADA, but also towards A_1R (K_i *erythro* $\leq 1/10$ K_i *threo*). Comparing the stereoselectivity of the two diastereoisomers of compounds **6**, **9**, **10**, **14**, **18** and **19** for A_1 adenosine receptors and the stereoselectivity of compounds **6**, **14** (K_i *erythro* **6**/K_i *threo* **6** 0.42, K_i *erythro* **14**/K_i *threo* **14** 0.16) and of other 2-hydroxynonyl compounds reported in the literature (for example, EHNA and THNA [26], 1-(2-hydroxy-3-nonyl)-1,2,4-triazole-3-carboxamides [28] or 2-hydroxy-3-nonylderivatives of azoles [29]) for ADA, we can observe that, towards the two proteins, *erythro* compounds are more active than *threo* ones.

This indicates that the moieties of the two proteins which bind the N(9) substituent (pocket II) could have similar stereochemical requirements.

6. Conclusions

Adenosine is the endogenous ligand of ADA protein and of A_1 receptor protein. In the past we have observed that these proteins could recognise some analogues of adenine and adenosine through favourable interactions with them, specially with reference to ADA obtained from cow milk and A_1 protein from cow brain.

Results in this work show that two similar zones and one different zone between the three dimensional structures of the binding site of the two proteins could be present.

In fact the zones facing both the 2 position and the 9 position of the adenosine nucleus presented some analogies. Introduction of an aromatic group, in particular a phenyl one, on the 2 position and of the 3-(2′-hydroxynonyl) chain on the 9 position, leads to products with improved affinities towards ADA and A_1 receptor proteins. Therefore, these two zones should present similar binding characteristics, in spite of having different aminoacidic sequences. Instead, the third zone facing the N^6 position presents great differences: ADA, as is known, does not accept any substituent except methyl [30], whereas A_1 protein can bind bulky groups such as cycloalkyl ones.

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