Design, Synthesis, and Biological Evaluation of a Second Generation of Pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidines as Potent and Selective A_{2A} Adenosine Receptor Antagonists

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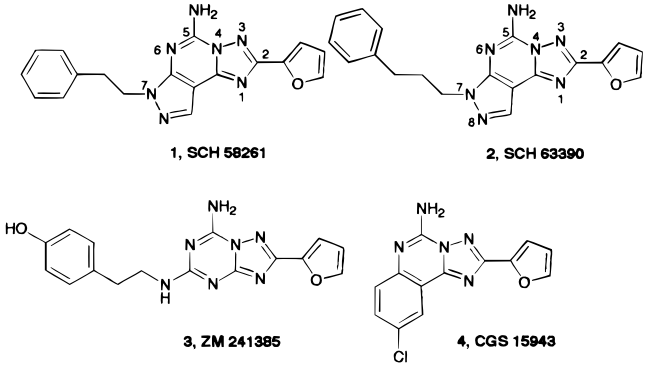
New A_{2A} adenosine receptor antagonists in the series of pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]-pyrimidines, bearing oxygenated substituents on the phenylalkyl chains on the 7-position, have been synthesized. The compounds were tested in binding and functional assays to evaluate affinity, potency, and selectivity for rat A_{2A} compared to rat A_1 and human A_3 receptor subtypes. The most interesting compounds (**5d**,**e**,**h**) were tested also in binding to human A_1 and A_{2A} adenosine receptors. They showed very good affinity (K_i = 0.94 nM for compound **5h**) and interesting selectivity with respect to both rA_1 and hA_3 (compound **5h**: rA_1/rA_{2A} = 787, hA_3/rA_{2A} > 10 000). These important findings make this new series of compounds the first really selective for A_{2A} adenosine receptors. Thermodynamic parameters were evaluated; all the tested compounds displayed an enthalpy-driven binding as expected for antagonists. Moreover, compound **5h** showed a negative entropy value. The highly negative enthalpic and entropic contributions could mean that **5h** fits very well in the binding site where, probably, an electrostatic interaction is present associated to a scarce solvent reorganization around the receptor binding site. These compounds deserve to be further developed to assess their potential for treatment of neurodegenerative disorders such as Parkinson's disease.

Introduction

Adenosine modulates physiological functions acting via specific cell surface receptors which have been identified as A_1, A_{2A}, A_{2B}, and A_3.[1,2] The A_{2A} receptor subtype which is coupled to stimulation of adenylyl cyclase activity, is a high-affinity receptor found in large amounts in the brain striatum.[3] Intensive efforts have been made over the past few years with the aim to synthesize novel compounds, either xanthine or non-xanthine, interacting selectively with the A_{2A} adenosine receptor type. Thus, the pyrazolotriazolopyrimidines SCH 58261 (5-amino-7-(2-phenylethyl)-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine, **1**) and SCH 63390 (5-amino-7-(3-phenylpropyl)-2-(2-furyl)pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine, **2**) have been found to be potent and selective adenosine A_{2A} antagonists, and SCH 58261 is widely used as a tool for characterizing the adenosine A_{2A} receptor subtype (Chart 1).[4,5] Moreover, the labeled form of SCH 58261 has been synthesized and well characterized as described in a variety of tissues and cell types.[6,7] Another compound of interest is ZM 241385 (4-[2-[[7-amino-2-(2-furyl)[1,2,4]-triazolo[2,3-*a*][1,3,5]triazin-5-yl]amino]ethyl]phenol, **3**)[8] which, like SCH 58261, has been derived from the prototype CGS 15943 (5-amino-9-chloro-2-(2-furyl)-1,2,4-triazolo[1,5-*c*]quinazoline, **4**) (Chart 1).[9] ZM 241385 is an A_{2A} adenosine antagonist endowed with good affinity and selectivity for A_{2A} vs A_1, and it is quite hydrophilic, an interesting characteristic because, usually, the A_{2A} adenosine antagonists are lipophilic compounds.

Chart 1



Starting from these observations and on the basis of data derived from an enlarged series of SCH 58261 analogues previously reported,[4] we have investigated the effects of variously substituted hydroxylic functions on the phenyl ring at the side chain in the 7-position in analogy with ZM 241385, to improve the hydrophilic character, which was the main problem of our previous series (Chart 2).

Particular attention was given to the cloned human A_3 receptor due to recent data showing that CGS 15943, the original precursor of the currently examined pyrazolotriazolopyrimidine series, has been reported to be

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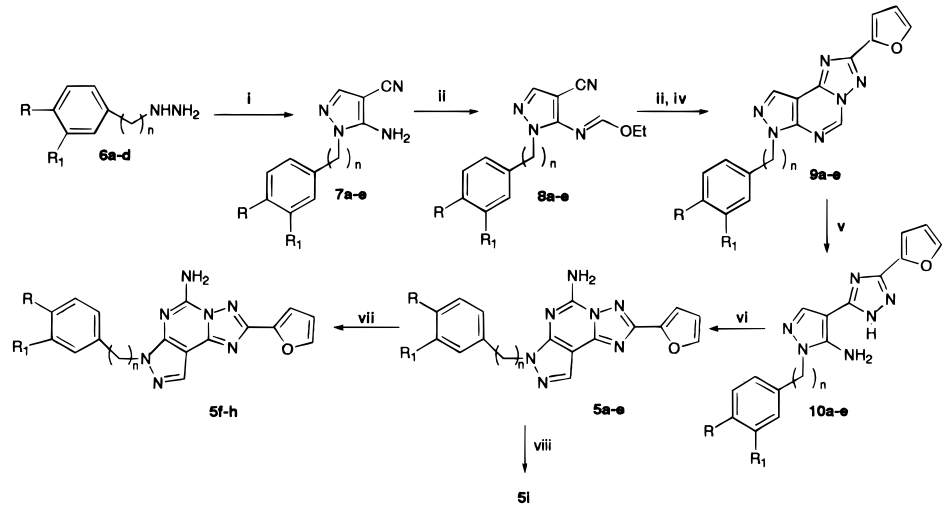
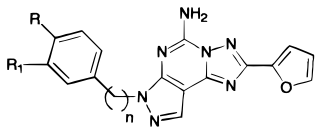
Scheme 1[a]



[a] Reagents: (i) (ethoxymethylene)malonitrile; (ii) $HC(OEt)_3$, reflux; (iii) furoic hydrazide, $MeO(CH_2)_2OH$; (iv) Ph_2O, 260 °C; (v) 10% HCl; reflux; (vi) NH_2CN, 1-methyl-2-pyrrolidone, pTsOH, 140 °C; (vii) 10% C−Pd, H_2 (70 psi), dioxane, 3 h; (viii) BCl_3.

Chart 2



5

n=1 R= OBn R_1= H **5a**
R= OH R_1= H **5f**
n=2 R= OBn R_1= H **5b**
R= OH R_1= H **5g**
n=3 R= OBn R_1= H **5c**
R= OH R_1= H **5h**
R= R_1= OCH_2O **5d**
R= R_1= OCH_3 **5e**
R= R_1= OH **5i**

inactive at rat A_3 receptors,[10] but its affinity was found to be high at the human A_3 receptor (K_i = 14 nM).[11]

Moreover, we have undertaken systematic investigation on the thermodynamic aspects of the binding equilibrium of this new series of compounds to adenosine A_{2A} receptors aimed at having a direct knowledge of the enthalpic ($\Delta H°$) and entropic ($\Delta S°$) contributions to the equilibrium standard free energy ($\Delta G° = \Delta H° - T\Delta S° = -RT \ln K_A$; K_A = association constant). Thermodynamic parameters of the binding equilibrium of all antagonists tested were determined by means of affinity measurements, carried out on rat striatal membranes at six different temperatures (0, 10, 20, 25, 30, 35 °C), and van't Hoff plots.[12] Affinity constants (K_A) were obtained from inhibition experiments utilizing as radioligand a selective A_{2A} agonist, [^{3}H]-2-[[p-(2-carboxyethyl)phenethyl]amino]-5′-(*N*-ethylcarboxamido)adenosine ([^{3}H]CGS 21680). In the adenylyl cyclase assay the compounds examined exhibited a rank order of potency very close to that observed in binding experiments.

Chemistry

The preparation of the compounds **5a**−**i** was performed following the general synthetic pathway depicted in Scheme 1.

To obtain only the N^1-substituted isomer of the pyrazoles **7a**−**e**, we synthesized the appropriate (phenylalkyl)hydrazines bearing different substituents, following a well-known procedure, starting from the corresponding halides.[13] Pyrazoles **7a**−**d** were prepared by reacting (ethoxymethylene)malononitrile with the appropriate hydrazines **6a**−**d**.[14,15] This strategy afforded only the N^1 isomer and allowed us to avoid tedious purification procedures. Unfortunately, the preparation of the hydrazine **6e** failed, so we got the pyrazole **7e** by alkylating 2-amino-3-cyanopyrazole with 3-(3,4-dimethoxyphenyl)propyl bromide; the two isomers were separated by chromatography, and their structure was assigned unequivocately by NOE experiment. The designed compounds **5a**−**i** were synthesized according to Gatta et al.[16] for the synthesis of pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidines which involved transformation of pyrazoles **7a**−**e** to corresponding imidates **8a**−**e** by refluxing in triethyl orthoformate. The imidates were reacted with 2-furoic acid hydrazide in refluxing 2-methoxyethanol to provide the pyrazolo[4,3-*e*]pyrimidine intermediates. The latter compounds were converted through a thermally induced cyclization in diphenyl ether to the derivatives **9a**−**e** in good overall yield.

Treatment of **9a**−**e** with dilute hydrochloric acid at reflux temperature induced pyrimidine ring opening to furnish the 5-amino-4-(1*H*-1,2,4-triazol-5-yl)pyrazoles **10a**−**e** in excellent yield. These derivatives were converted into the final compounds **5a**−**e** by reaction with an excess of cyanamide in 1-methyl-2-pyrrolidone at 140 °C. Compounds **5f**−**h** were obtained by catalytic hydrogenation of the corresponding *O*-benzyl-protected compounds **5a**−**c**. The compound **5i** was derived from the deprotection of **5d** by reaction with boron trichloride at 5 °C.[17]

Results and Discussion

Table 1 gives the receptor affinity profile of compounds **5a**−**i**, determined by receptor binding assay at rat A_1 and A_{2A} receptors using [^{3}H]-N^6-cyclohexyladenosine ([^{3}H] CHA) and [^{3}H]-2-[[4-(2-carboxyethyl)-phenethyl]amino]-5′-(*N*-ethylcarboxamido)adenosine ([^{3}H]CGS 21680), respectively.[18,19]Species differences are well-known among adenosine receptor subtypes; in particular, this evidence is really important for the A_3

Table 1. Biological Activity of a Series of Pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidines **5a**−**i**

	K_i (nM)			selectivity		
compound	rA_1[a]	rA_2[b]	hA_3[c]	rA_1/rA_{2A}	hA_3/rA_{2A}	cyclic AMP assay, IC_{50}[d] (nM)
1, SCH 58261	121 (103−123)	2.3 (2.0−2.7)	>10000	53	>4347	12 (9−17)
2, SCH 63390	504 (329−773)	2.4 (1.9−2.9)	>10000	210	>4166	18 (15−21)
4, CGS 15943	6.4 (6.2−6.6)	0.95 (0.8−1.1)	14 (9.9−18)[11]	6.7	14.5	12 (10−14)
5a	9450 (7650−11700)	246 (109−554)	>10000	38	>40	500 (484−516)
5b	>10000	59 (44−78)	>10000	>169	>169	90 (86−94)
5c	1460 (1400−1660)	45 (25−28)	>10000	32	>222	300 (282−321)
5d	1841 (1100−3060)	3.8 (2.1−6.9)	>10000	484	>2631	8 (7−10)
5e	2825 (2000−3990)	5.3 (4.1−8.3)	>10000	487	>1886	20 (19−22)
5f	13400 (11100−16100)	53 (47−70)	>10000	252	>188	149 (138−162)
5g	444 (338−582)	1.7 (1.3−2.1)	>10000	261	>5882	14 (11−17)
5h	741 (545−902)	0.94 (0.58−1.7)	>10000	787	>10638	10 (8−12)
5i	2250 (895−5640)	49 (23−104)	>10000	46	>204	60 (56−65)

[a] Data are expressed as geometric means, with 95% confidence limits. Displacement of [^{3}H]CHA binding (A_1) at rat cortical membranes. [b] Displacement of [^{3}H]CGS 21680 binding (A_{2A}) at rat striatal membranes. [c] Displacement of [^{125}I]AB-MECA binding at human A_3 adenosine receptors expressed in HEK-293 cells. K_i values, calculated from the Cheng−Prusoff equation[32] using 1.0, 18.5, and 0.9 nM as K_d values in A_1, A_{2A}, and A_3 binding assays, respectively. [d] IC_{50} values of the test compounds for inhibition of NECA (1 μM)-stimulated cyclic AMP levels in human platelets.

receptor subtype: CGS 15943 did not show affinity for the rat A_3 receptor subtype but displayed good affinity for the human A_3 receptor. For this reason the affinity of **5a**−**i** for cloned human A_3 adenosine receptors expressed in HEK-293 cells, using [^{125}I]-N^6-(4-amino-3-iodobenzyl)-5′-(*N*-methylcarbamoyl)adenosine ([^{125}I]-AB-MECA) as radioligand, was examined.[20,21]

The results show that all the tested compounds **5a**−**i** display high affinity at A_{2A} receptors: high rA_{2A} vs rA_1 and, most importantly, rA_{2A} vs hA_3 selectivity. Despite the structural similarity with CGS 15943, our series of compounds does not show affinity for the human A_3 receptor subtype; this important relevance makes **5a**−**i** the first compounds really selective versus A_{2A} adenosine receptors. Binding data indicate that the presence of the hydroxylic function at the para position on a phenyl ring increases the affinity and in particular A_{2A} receptor selectivity. In fact, comparing the derivatives **5g**,**h** with the corresponding unsubstituted analogues **1** and **2**, the affinity is slightly increased, while instead the A_{2A} receptor selectivity is highly enhanced. Furthermore, we confirmed the previous observation[4] that the best length for the side chain is three methylene groups: compound **5h** shows the highest affinity and selectivity in binding assays ($K_i(A_{2A})$ = 0.94 nM, A_1/A_{2A} = 787) and potency in the A_{2A} receptor adenylyl cyclase assay (IC_{50} = 10 nM). This statement was confirmed also for compounds **5d,e**, bearing the same side chain, which are endowed with good activity and very high selectivity. Compound **5c** was less active probably due to the presence of the bulky group at the para position of the phenyl ring.

As already mentioned, marked species differences have been found in the affinity of antagonists at rat A_3 receptors;[22,23] to this purpose, we have tested the compounds **5a**−**i** on human A_3 receptors. All the tested compounds were unable to displace [^{125}I]AB-MECA binding at human A_3 adenosine receptors up to the highest concentration examined (K_i > 10 μM), thus indicating the absence of interaction with this receptor subtype.

Table 2. Affinity and Selectivity of Selected A_{2A} Receptor Antagonists at Human A_1, A_{2A}, and A_3 Adenosine Receptor Subtypes

	K_i nM			selectivity	
compound	hA_1[a]	hA_{2A}[b]	hA_3[c]	hA_1/hA_{2A}	hA_3/hA_{2A}
SCH 58261	549 (322−987)	1.1 (0.75−1.6)	>10000	499	>9090
5d	2170 (1260−3740)	3.3 (2.9−3.8)	>10000	658	>3030
5e	1650 (1530−1780)	2.7 (1.8−4.0)	>10000	611	>3700
5h	253 (110−585)	1.5 (1.2−1.9)	>10000	169	>6670

[a] Displacement of [^{3}H]CHA binding at human A_1 adenosine receptors expressed in CHO cells. [b] Displacement of [^{3}H]SCH 58261 binding at human A_{2A} adenosine receptors expressed in CHO cells. [c] Displacement of [^{125}I]AB-MECA binding at human A_3 adenosine receptors expressed in HEK-293 cells.

We thought that it could be interesting to test some compounds of this new series also at human A_1 and A_{2A} adenosine receptors. The results are shown in Table 2; surprisingly, the best compound so far evaluated, **5h**, at human receptors displays lower selectivity for A_{2A} vs A_1, whereas the former SCH 58261 and **5d**,**e** show very high selectivity.

The A_{2A} vs A_{2B} selectivity of these compounds was not evaluated. However, in CHO cells transfected with the human A_{2B} receptor, SCH 58261 at micromolar concentrations was unable to antagonize NECA-induced cAMP accumulation.[24] Moreover, SCH 58261 and its prototype 8FB-PTP (5-amino-8-(4-fluorobenzyl)-2-(2-furyl)pyrazolo-

Table 3. R_m Values of Compounds 5**a**−**i** Measured at pH 7.0 by TLC in Methanol−Water System

compound	$R_m(0)$[a]
1, SCH 58261	3.5 ± 0.2
2, SCH 63390	3.6 ± 0.2
5a	3.7 ± 0.2
5b	3.6 ± 0.2
5c	3.4 ± 0.2
5d	3.6 ± 0.2
5e	3.5 ± 0.2
5f	2.8 ± 0.1
5g	2.5 ± 0.1
5h	2.9 ± 0.1
5i	3.2 ± 0.2

[a] The R_m values of **1**, **2**, and **5a**−**i** were measured with a mobile phase of different concentrations of CH_3OH/H_2O. R_m values are reported as theoretical at 0% organic solvent in the mobile phase ($R_m(0)$).

[4,3-*e*]-1,2,4-triazolo[1,5-*c*] pyrimidine)[16] were found to have little or no activity in CHO cells transfected with human A_{2B} receptors[25] and in the guinea pig aorta,[5] which is a functional model specific for the evaluation of A_{2B}-mediated effects.

The functional A_{2A} receptor-mediated antagonist properties of compounds **5a**−**i** were tested on NECA (1 μM)-induced cAMP stimulation in human platelets (Table 1), which are known to represent a reliable assay to examine potency of adenosine antagonists.[26,27] Most of the tested compounds showed activity in the low-nanomolar range, with **5d** being the most potent compound.

Furthermore, the new series did not show, as we hoped, a remarkable increase in water solubility, measured by evaluation of R_m values ($R_m = \log(1/R_f - 1)$) through reversed-phase TLC experiments (Table 3).[28]

If we compared compounds SCH 58261 and SCH 63390 with the corresponding compounds bearing the hydroxy moiety at the para position on the phenyl ring in the side chain (**5g**,**h**), it is possible to notice an appreciable improvement in hydrophilic properties, unfortunately not enough to make our compounds water-soluble. However, these data prove that our hypothesis to introduce oxygenated functions was correct.

In thermodynamic studies, inhibitory binding constants (K_i) were measured at the six chosen temperatures for the selected 11 ligands by displacement of [^{3}H]CGS 21680 from rat striatum adenosine A_{2A} receptors. All adenosine receptor antagonists studied display K_i values that depend on the temperature showing a lower affinity at room temperature. This behavior is in perfect agreement with that already reported in rat striatum for xanthine antagonists.[12] The temperature dependence of the affinity constants, $K_A = 1/K_i$, is exemplified by the van't Hoff plots, ln K_A versus $1/T$, of Figure 1 which report typical results for all antagonists studied.

The van't Hoff plots are linear in the range 0−30 °C, and the slopes are consistently positive with affinities decreasing with the increase in temperature. Final thermodynamic parameters calculated for the binding equilibria of the different compounds investigated are reported in Table 4. $\Delta G°$ values range from −50.2 to −37.8 kJ/mol; equilibrium standard enthalpy $\Delta H°$ and entropy $\Delta S°$ values show that binding of antagonists is

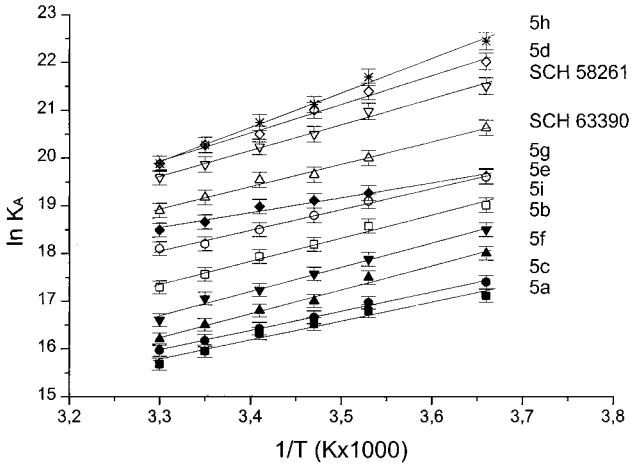


Figure 1. Representative van't Hoff plots showing the effect of temperature on the equilibrium association constants, K_A, for tested compounds **5a**−**i**. All plots are essentially linear (r = 0.95) in the temperature range from 0 to 30 °C.

Table 4. Thermodynamic Parameters (Given at 298.15 K) for the Binding Equilibrium of Antagonists to Adenosine A_{2A} Receptors

ligand	$\Delta G°$ (kJ/mol)	$\Delta H°$ (kJ/mol)	$\Delta S°$ (J/mol/deg)
1, SCH 58261	−49.07	−44.9	14
2, SCH 63390	−46.52	−32.0	49
5a	−37.79	−24.8	44
5b	−42.11	−39.1	10
5c	−39.42	−33.0	21
5d	−50.15	−47.9	7
5e	−46.11	−25.8	68
5f	−41.17	−43.4	−2
5g	−47.23	−38.8	28
5h	−49.99	−59.5	−32
5i	−43.38	−40.3	10

mostly enthalpy-driven (−59.5 = $\Delta H°$ = −24.8 kJ/mol; −32 = $\Delta S°$ = 68 J/mol/K).

From the equilibrium thermodynamic parameters, binding of all compounds resulted to be mostly enthalpy-driven as expected for adenosine receptor antagonists;[12] moreover, the enthalpic contribution was greater for **5h** with respect to the corresponding derivative lacking the hydroxylic group (**2**), while the entropic contribution was practically negligible.

It is remarkable that **5h** has a higher binding equilibrium enthalpy value than all the other compounds and, in particular, of compound **2** which is similar but lacks the hydroxyl group. This could be indicative of the presence of an electrostatic interaction (most probably a hydrogen bond) formed by the hydroxyl moiety with the recognition of the binding site. In light of this observation, it is possible to understand the affinity and selectivity of **5d**,**e**. Moreover, the entropic contribution to the binding of compound **5h** is low with respect to that of other compounds (**1**,**2**,**5a**−**g**), which could be explained by hypothesizing either scarce or null solvent reorganization around the receptor binding site. This, speculatively, might be indicative of the presence of a pocket where the aromatic ring of **5h** fits well via electrostatic forces with hydrogen bond acceptors or donors present on the receptor binding site.

Conclusions

In the present study we have described the affinity and selectivity at adenosine receptors of a series of SCH

58261 derivatives, in which different substituents, such as hydroxy, methoxy, or methylenedioxy groups, have been introduced on the phenyl ring. Considerable progress has been made with the introduction of the hydroxylic function at the para position, together with the presence of three methylene groups at the side chain. In fact, all the tested compounds **5a**−**i** showed high A_{2A} affinity and, in particular, the very high selectivity for both human and rat A_{2A} vs A_1 and A_{2A} vs A_3 adenosine receptors. This is particularly important because of the affinity of CGS 15943, structurally correlated to our compounds, for the human A_3 adenosine receptor. These new compounds **5a**−**i** are the first A_{2A} adenosine antagonists really selective versus the human A_3 adenosine receptor subtype. Since it is now clear that the stimulation of A_{2A} adenosine receptors decreases the affinity of D_2 dopamine receptor agonists in striatopallidal neurons, potent and selective A_{2A} receptor antagonists may be a novel therapeutic approach to Parkinson's disease.[29,30]

Experimental Section

Chemistry. Reaction courses and product mixtures were routinely monitored by thin-layer chromatography (TLC) on silica gel (precoated F_{254} Merck plates) and visualized with iodine or aqueous potassium permanganate. Infrared spectra (IR) were measured on Perkin-Elmer 257 instruments. ^{1}H NMR were determined in $CDCl_3$ or DMSO-d_6 solutions with a Bruker AC 200 spectrometer, peak positions are given in parts per million (δ) downfield from tetramethylsilane as internal standard, and J values are given in Hz. Light petroleum refers to the fractions boiling at 40−60 °C. Melting points were determined on a Buchi-Tottoli instrument and are uncorrected. Chromatography was performed with Merck 60−200 mesh silica gel. All products reported showed IR and ^{1}H NMR spectra in agreement with the assigned structures. Organic solutions were dried over anhydrous magnesium sulfate. Elemental analyses were performed by the microanalytical laboratory of Dipartimento di Chimica, University of Ferrara, and were within ±0.4% of the theoretical values for C, H. and N.

General Procedure for the Preparation of *N*-Substituted-5-amino-4-cyanopyrazoles 7a−e. The appropriate hydrazine (65 mmol) was dissolved in EtOH (150 mL), and (ethoxymethylene)malononitrile (8 g, 65 mmol) was added in small portions. Then the mixture was heated at 70 °C for 18 h before evaporating the solvent. The solid residue was purified by chromatography (EtOAc/light petroleum, 1:3) to afford the product as a solid in good yield. The following spectral data are reported as examples.

5-Amino-4-cyano-1-[4-(benzyloxy)benzyl]pyrazole (7a): yield 72%, yellow solid; mp 205−207 °C (EtOAc−light petroleum); IR (KBr) 3450−2950, 2200, 1670, 1580, 1420 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 5.07 (s, 4H), 6.73 (bs, 2H), 6.96 (d, 2H, J = 8), 7.14 (d, 2H, J = 8),7.3−7.44 (m, 5H), 7.56 (s, 1H). Anal. ($C_{18}H_{16}N_4O$) C, H, N.

5-Amino-1-[2-[4-(benzyloxy)phenyl]ethyl]-4-cyanopyrazole (7b): yield 82%, yellow solid; mp 189−190 °C (EtOAc−light petroleum); IR (KBr) 3350−3150, 2210, 1670, 1470 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.97 (t, 2H, J = 7), 4.09 (t, 2H, J = 7), 5.03 (s, 2H), 5.7 (bs, 2H), 6.88 (d, 2H, J = 8), 7.09 (d, 2H, J = 8), 7.3−7.41 (m, 6H). Anal. ($C_{19}H_{18}N_4O$) C, H, N.

5-Amino-4-cyano-1-[3-[4-(benzyloxy)phenyl]propyl]-pyrazole (7c): yield 79%, pale-yellow solid; mp 160−162 °C (EtOAc−Et_2O); IR (KBr) 3350−3170, 2215, 1650, 1440 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.9−2.1 (m, 2H), 2.56 (t, 2H, J = 7), 3.91 (t, 2H, J = 7), 5.02 (s, 2H), 5.93 (bs, 2H), 6.87 (d, 2H, J = 8), 7.09 (d, 2H, J = 8), 7.33−7.39 (m, 6H). Anal. ($C_{20}H_{20}N_4O$) C, H, N.

5-Amino-4-cyano-1-[3-[3,4-(methylenedioxy)phenyl]propyl]pyrazole (7d): yield 83%, yellow solid; mp 156 °C (EtOAc−Et_2O); IR (KBr) 3350−3160, 2200, 1655, 1460 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.04−2.15 (m, 2H), 2.57 (t, 2H, J = 8), 3.85 (t, 2H, J = 8), 4.23 (bs, 2H), 5.93 (s, 2H), 6.63−6.76 (m, 3H), 7.5 (s, 1H). Anal. ($C_{14}H_{14}N_4O_2$) C, H, N.

Preparation of 5-Amino-4-cyano-1-[3-(3,4-dimethoxyphenyl)propyl]pyrazole (7e). Potassium carbonate (1.4 g, 1.1 equiv) and 3-amino-4-cyanopyrazole (1 g, 9.25 mmol) were suspended in dry DMF (20 mL), and 3-(3,4-dimethoxyphenyl)-propyl bromide (2.63 g, 1.1 equiv) was added. The reaction was stirred at room temperature for 12 h; then the solvent was removed and the mixture of the two isomers purified by flash chromatography (EtOAc/light petroleum, 1:4) to afford **7e** as a yellow solid (yield 68%): mp 153−155 °C (EtOAc−Et_2O); IR (KBr) 3355−3050, 2220, 1665, 1440 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.04−2.17 (m, 2H), 2.62 (t, 2H, J = 7), 3.67 (t, 2H, J = 7), 3.85 (s, 3H), 3.86 (s,3H), 4.38 (bs, 2H), 6.66−6.82 (m, 3H), 7.5 (s, 1H), the 2D ^{1}H−^{1}H NOE NMR ($CDCl_3$) did not display a cross-peak for C1′−H and C3−H. Anal. ($C_{15}H_{18}N_4O_2$) C, H, N.

General Procedure for the Preparation of 1-Substituted-4-cyano-5-[(ethoxymethylene)amino]pyrazoles 8a−e. The 4-cyano-5-aminopyrazoles **7a**−**e** (20 mmol) were dissolved in triethyl orthoformate (40 mL), and the solution was refluxed under nitrogen for 8 h. Then the solvent was removed under reduced pressure, and the oily residue was dissolved in ether and roughly purified on silica gel (EtOAc−light petroleum, 1:9) to afford the corresponding imino ethers. The following spectral data are reported as examples.

1-[4-(Benzyloxy)benzyl]-4-cyano-5-[(ethoxymethylene)amino]pyrazole (8a): yield 85%, pale-yellow oil; IR (neat) 2230, 1650 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.43 (t, 3H, J = 7), 4.43 (q, 2H, J = 7), 5.05 (s, 2H), 5.2 (s, 2H), 6.94 (d, 2H, J = 8), 7.2 (d, 2H, J = 8), 7.32−7.44 (m, 5H), 7.66 (s, 1H), 8.4 (s, 1H). Anal. ($C_{21}H_{20}N_4O_2$) C, H, N.

1-[2-[4-(Benzyloxy)phenyl]ethyl]-4-cyano-5-[(ethoxymethylene)amino]pyrazole (8b): yield 87%, pale-yellow oil; IR (neat) 2215, 1640, 1420 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.33 (t, 3H, J = 7), 2.97 (t, 2H, J = 7), 4.18−4.26 (m, 4H), 5.01 (s, 2H), 6.82 (s, 4H), 7.33−7.41 (m, 5H), 7.64 (s, 1H), 7.77 (s, 1H). Anal. ($C_{22}H_{22}N_4O_2$) C, H, N.

1-[3-[4-(Benzyloxy)phenyl]propyl]-4-cyano-5-[(ethoxymethylene)amino]pyrazole (8c): yield 92%, yellow oil; IR (neat) 2210, 1650, 1520 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.36 (t, 3H, J = 7), 2.03−2.2 (m, 2H), 2.54 (t, 2H, J = 7), 4.06 (t, 2H, J = 7), 4.28 (q, 2H, J = 7), 5.02 (s, 2H), 6.88 (d, 2H, J = 8), 7.07 (d, 2H, J = 8), 7.33−7.40 (m, 5H), 7.64 (s, 1H), 8.38 (s, 1H). Anal. ($C_{23}H_{24}N_4O_2$) C, H, N.

4-Cyano-5-[(ethoxymethylene)amino]-1-[3-[3,4-(methylenedioxy)phenyl]propyl]pyrazole (8d): yield 93%, yellow oil; IR (neat) 2225, 1630, 1510 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.39 (t, 3H, J = 7), 2.01−2.19 (m, 2H), 2.52 (t, 2H, J = 7), 4.05 (t, 2H, J = 7), 4.25 (q, 2H, J = 7), 5.91 (s, 2H), 6.62−6.73 (m, 3H), 7.65 (s, 1H), 8.39 (s, 1H). Anal. ($C_{17}H_{18}N_4O_3$) C, H, N.

4-Cyano-1-[3-[3,4-(dimethoxy)phenyl]propyl]-5-[(ethoxymethylene)amino]pyrazole (8e): yield 89%, pale-yellow oil; IR (neat) 2210, 1640 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.37 (t, 3H, J = 7), 2.02−2.23 (m, 2H), 2.57 (t, 2H, J = 7), 3.85 (s, 3H), 3.86 (s, 3H), 4.08 (t, 2H, J = 7), 4.35 (q, 2H, J = 7), 6.66−6.78 (m, 5H), 7.66 (s, 1H), 8.23 (s, 1H). Anal. ($C_{18}H_{22}N_4O_3$) C, H, N.

General Procedures for the Preparation of 7-Substituted-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidines 9a−e. Imino ethers **8a**−**e** (20 mmol) were dissolved in 2-methoxyethanol (50 mL), and 2-furoic acid hydrazide (2.5 g, 22 mmol) was added. The mixture was refluxed for 5−10 h, then, after cooling, the solvent was removed under reduced pressure, and the dark oily residue was cyclized without any other purification in diphenyl ether (50 mL) at 260 °C using a Dean−Stark trap for the azeotropic elimination of water produced in the reaction. After 1.5 h, the mixture was poured onto hexane (200 mL) and cooled. The precipitate was filtered off and purified by chromatography (EtOAc/ hexane, 1:1). In this way, different compounds were obtained. The following spectral data are reported as examples.

7-[4-(Benzyloxy)benzyl]-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*] pyrimidine (9a): yield 65%, white solid; mp 168−170 °C (EtOAc); IR (KBr) 1530, 1420 cm^{-1}; ^1H NMR (DMSO-d_6) δ 5.06 (s, 2H), 5.65 (s, 2H), 6.71−6.74 (m, 1H), 6.93−7.01 (m, 3H), 7.1 (d, 2H, J = 8), 7.3−7.4 (m, 6H), 7.55 (s, 1H), 8.37 (s, 1H). Anal. ($C_{24}H_{18}N_6O_2$) C, H, N.

7-[2-[4-(Benzyloxy)phenyl]ethyl]-2-(2-furyl)pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (9b): yield 72%, white solid; mp 193−194 °C (EtOAc−light petroleum); IR (KBr) 1620, 1510, 1440 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.21 (t, 2H, J = 8), 4.72 (t, 2H, J = 8), 4.97 (s, 2H), 6.58 (dd, 1H, J = 2, 4), 6.81 (d, 2H, J = 8), 6.87 (d, 1H, J = 2), 7.03 (d, 2H, J = 8), 7.28−7.36 (m, 5H), 7.62 (s, 1H), 8.37 (s, 1H), 9.0 (s, 1H). Anal. ($C_{25}H_{20}N_6O_2$) C, H, N.

7-[3-[4-(Benzyloxy)phenyl]propyl]-2-(2-furyl)pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (9c): yield 74%, mp 183−186 °C (EtOH); IR (KBr) 1650, 1510, 1430 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.23−2.36 (m, 2H), 2.62 (t, 2H, J = 7), 4.57 (t, 2H, J = 7), 5.01 (s, 2H), 6.6−6.63 (m, 1H), 6.87 (d, 2H, J = 8), 7.05 (d, 2H, J = 8), 7.3−7.46 (m, 6H), 7.66 (s, 1H), 8.38 (s, 1H), 9.1 (s, 1H). Anal. ($C_{26}H_{22}N_6O_2$) C, H, N.

7-[3-[3,4-(Methylenedioxy)phenyl]propyl]-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (9d): yield 82%, pale-yellow solid; mp 145 °C (EtOAc); IR (KBr) 1640, 1510, 1445 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.05−2.2 (m, 2H), 2.57 (t, 2H, J = 8), 4.56 (t, 2H, J = 8), 5.92 (s, 2H), 6.58−6.76 (m, 4H), 7.29 (d, 1H, J = 4), 7.66 (d, 1H, J = 2), 8.39 (s, 1H), 9.1 (s, 1H). Anal. ($C_{20}H_{16}N_6O_3$) C, H, N.

7-[3-[3,4-(Dimethoxy)phenyl]propyl]-2-(2-furyl)pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (9e): yield 78%, pale-yellow solid; mp 181 °C (EtOAc); IR (KBr) 1610, 1520 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.2−2.4 (m, 2H), 2.63 (t, 2H, J = 8), 3.84 (s, 3H), 3.86 (s, 3H), 4.58 (t, 2H, J = 8), 6.6−6.63 (m, 1H), 6.71−6.76 (m, 3H), 7.28 (d, 1H, J = 2), 7.66 (s, 1H), 8.39 (s, 1H), 9.1 (s, 1H). Anal. ($C_{21}H_{20}N_6O_3$) C, H, N.

General Procedure for the Preparation of *N*-Substituted-4-[3-(2-furyl)-1,2,4-triazol-5-yl]-5-aminopyrazoles 10a−e. A solution of the mixture of **9a**−**e** (10 mmol) in aqueous 10% HCl (20 mL) and dioxane (30 mL) was refluxed for 3 h. Then the solution was cooled and basified with concentrated ammonium hydroxide at 0 °C. The compounds were extracted with EtOAc (3 × 20 mL); the organic layers were dried with Na_2SO_4 and evaporated under vacuum. The residue was purified by chromatography (EtOAc/light petroleum, 2:1) to afford the desired compound as a solid. The following spectral data are reported as examples.

5-Amino-1-[4-(benzyloxy)benzyl]-4-[3-(2-furyl)-1,2,4-triazol-5-yl]pyrazole (10a): yield 75%, yellow oil; IR (neat) 3340−3150, 1610 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.01 (bs, 4H), 5.15 (s, 2H), 5.46−5.49 (m, 1H), 6.87−6.95 (m, 3H), 7.12 (d, 2H, J = 8), 7.32−7.41 (m, 5H), 7.46 (s, 1H), 7.77 (s, 1H), 14.51 (bs, 1H). Anal. ($C_{23}H_{20}N_6O_2$) C, H, N.

5-Amino-1-[2-[4-(benzyloxy)phenyl]ethyl]-4-[3-(2-furyl)-1,2,4-triazol-5-yl]pyrazole (10b): yield 79%, yellow oil; IR (neat) 3345−3140, 1630 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.95 (t, 2H, J = 7), 4.08 (t, 2H, J = 7), 4.86 (bs, 2H), 4.91 (s, 2H), 6.38−6.40 (m, 1H), 6.77 (d, 2H, J = 8), 6.90−6.94 (m, 3H), 7.24−7.37 (m, 6H), 7.79 (s, 1H), 14.41 (bs, 1H). Anal. ($C_{24}H_{22}N_6O_2$) C, H, N.

5-Amino-1-[3-[4-(Benzyloxy)phenyl]propyl]-4-[3-(2-furyl)-1,2,4-triazol-5-yl]pyrazole (10c): yield 83%, yellow oil; IR (neat) 3320−3160, 1635 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.1−2.25 (m, 2H), 2.55 (t, 2H, J = 7), 3.92 (t, 2H, J = 7), 5.01 (s, 2H), 5.2 (bs, 2H), 6.48−6.55 (m, 1H), 6.88 (d, 2H, J = 8), 6.99 (d, 1H, J = 4), 7.08 (d, 2H, J = 8), 7.31−7.4 (m, 5H), 7.48 (d, 1H, J = 2), 7.8 (s, 1H), 13.81 (bs, 1H). Anal. ($C_{25}H_{24}N_6O_2$) C, H, N.

5-Amino-1-[3-[3,4-(methylenedioxy)phenyl]propyl]-4-[3-(2-furyl)-1,2,4-triazol-5-yl]pyrazole (10d): yield 82%, yellow oil; IR (neat) 3460−3120, 1650, 1460 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.99−2.09 (m, 2H), 2.49 (t, 2H, J = 7), 3.87 (t, 2H, J = 7), 5.22 (bs, 2H), 5.85 (s, 2H), 6.43−6.45 (m, 1H), 6.5−6.66 (m, 3H), 6.93 (d, 1H, J = 4), 7.42 (d, 1H, J = 2), 7.73 (s, 1H), 13.6 (bs, 1H). Anal. ($C_{19}H_{18}N_6O_3$) C, H, N.

5-Amino-1-[3-(3,4-dimethoxyphenyl)propyl]-4-[3-(2-furyl)-1,2,4-triazol-5-yl]pyrazole (10e): yield 69%, yellow solid; mp 73 °C (EtOAc); IR (KBr) 3295−3155, 1640, 1480 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.1−2.22 (m, 2H), 2.59 (t, 2H, J = 7), 3.82 (s, 3H), 3.83 (s, 3H), 3.93 (t, 2H, J = 7), 5.08 (bs, 2H), 6.48−6.51 (m, 1H), 6.67−6.74 (m, 3H), 6.97 (d, 1H, J = 4), 7.48 (d, 1H, J = 2), 7.74 (s, 1H), 13.51 (bs, 1H). Anal. ($C_{20}H_{22}N_6O_3$) C, H, N.

General Procedure for the Preparation of 5-Amino-7-substituted-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidines 5a−e. To a solution of pyrazole derivatives **10a**−**e** (10 mmol) in *N*-methylpyrrolidone (40 mL) were added cyanamide (0.42 g, 60 mmol) and *p*-toluenesulfonic acid (2.85 g, 15 mmol), and the mixture was heated at 160 °C for 4 h. Then cyanamide (0.42 g, 60 mmol) was added again and the solution was heated overnight. Then the solution was diluted with EtOAc (80 mL) and the precipitate (excess of cyanamide) was filtered off; the filtrate was concentrated under reduced pressure and washed with water (3 × 30 mL). The organic layer was dried (Na_2SO_4) and evaporated under vacuum. The residue was purified by chromatography (EtOAc/light petroleum, 4:1) to afford the final products **5a**−**e** as solids.

5-Amino-7-[4-(benzyloxy)benzyl]-2-(2-furyl)pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (5a): white solid; mp 188−190 °C; IR (KBr) 3490−3050, 1660, 1640, 1610, 1555, 1440 cm^{-1}; ^1H NMR (DMSO-d_6) δ 5.06 (s, 2H), 5.4 (s, 2H), 6.7−6.74 (m, 1H), 6.95 (d, 2H, J = 8), 7.1−7.2 (m, 3H), 7.32−7.42 (m, 5H), 7.94 (s, 1H), 8.13 (bs, 2H), 8.17 (s, 1H). Anal. ($C_{24}H_{19}N_7O_2$) C, H, N.

5-Amino-7-[2-[4-(benzyloxy)phenyl]ethyl]-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (5b): white solid; mp 225−228 °C; IR (KBr) 3400−2955, 1680, 1650, 1620, 1570, 1440 cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.1 (t, 2H, J = 7), 4.44 (t, 2H, J = 7), 1.80 (m, 2H), 5.02 (s, 2H), 6.71−6.72 (m, 1H), 6.87 (d, 2H, J = 8), 7.07 (d, 2H, J = 8), 7.22 (d, 1H, J = 4), 7.32−7.39 (m, 5H), 7.94 (s, 1H), 8.08 (bs, 2H), 8.16 (s, 1H). Anal. ($C_{25}H_{21}N_7O_2$) C, H, N.

5-Amino-7-[3-[4-(benzyloxy)phenyl]propyl]-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (5c): white solid; mp 187 °C; IR (KBr) 3520−2960, 1660, 1645, 1610, 1560, 1430 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.08−2.16 (m, 2H), 2.5−2.57 (m, 2H), 4.25 (t, 2H, J = 7), 5.03 (s, 2H), 6.72−6.74 (m, 1H), 6.9 (d, 2H, J = 8), 7.13 (d, 2H, J = 8), 7.21 (d, 1H, J = 4), 7.3−7.43 (m, 5H), 7.94 (s, 1H), 8.09 (bs, 2H), 8.16 (s, 1H). Anal. ($C_{26}H_{23}N_7O_2$) C, H, N.

5-Amino-7-[3-[3,4-(methylenedioxy)phenyl]propyl]-2-(2-furyl)pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*] pyrimidine (5d): white solid; mp 210−211 °C; IR (KBr) 3520−2950, 1670, 1630, 1620, 1545, 1450 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.01−2.12 (m, 2H), 2.49−2.51 (m, 2H), 4.24 (t, 2H, J = 7), 5.94 (s, 2H), 6.68−6.82 (m, 4H), 7.23 (d, 1H, J = 4), 7.94 (s, 1H), 8.09 (bs, 2H), 8.17 (s, 1H). Anal. ($C_{20}H_{17}N_7O_3$) C, H, N.

5-Amino-7-[3-(3,4-dimethoxyphenyl)propyl]-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*] pyrimidine (5e): white solid; mp 148 °C; IR (KBr) 3500−2950, 1655, 1640, 1630, 1550, 1440 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.03−2.2 (m, 2H), 2.48−2.54 (m, 2H), 3.69 (s, 3H), 3.71 (s, 3H), 4.26 (t, 2H, J = 7), 6.72−6.85 (m, 4H), 7.23 (d, 1H, J = 4), 7.95 (s, 1H), 8.08 (bs, 2H), 8.17 (s, 1H). Anal. ($C_{21}H_{21}N_7O_3$) C, H, N.

General Procedure for *O*-Benzyl Deprotection (5f−h). A solution of the benzyl-protected compounds (0.25 mmol, **5a**−**c**) in a mixture of CH_3OH/dioxane (1:3) (40 mL) was hydrogenated over 10% palladium on charcoal (100 mg) at room temperature (50 psi, 4 h). Then the catalyst was filtered off on Celite and the filtrate concentrated under reduced pressure to afford compounds **5f**−**h** in very good yield.

5-Amino-7-(4-hydroxybenzyl)-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*] pyrimidine (5f): off-white solid; mp >280 °C (CH_3OH); IR (KBr) 3580−2950, 1665, 1620, 1610, 1545, 1450 cm^{-1}; ^1H NMR (DMSO-d_6) δ 5.35 (s, 2H), 6.66−6.72 (m, 3H), 7.08 (d, 2H, J = 8), 7.22 (d, 1H, J = 4), 7.95 (s, 1H), 8.16 (bs, 2H), 8.24 (s, 1H), 9.4 (s, 1H). Anal. ($C_{17}H_{13}N_7O_2$) C, H, N.

5-Amino-7-[2-(4-hydroxyphenyl)ethyl]-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (5 g): white solid; mp 265 °C with dec (EtOH); IR (KBr) 3570−2910, 1660, 1620, 1605, 1540, 1460 cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.04 (t, 2H, J = 7), 4.41 (t, 2H, J = 7), 6.62 (d, 2H, J = 8), 6.72−6.75 (m, 1H), 6.95 (d, 2H, J = 8), 7.22 (d, 1H, J = 4), 7.94 (s, 1H), 8.07 (bs, 2H), 8.16 (s, 1H), 9.22 (s, 1H). Anal. ($C_{18}H_{15}N_7O_2$) C, H, N.

5-Amino-7-[3-(4-hydroxyphenyl)propyl]-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (5h): white solid; mp 189−191 °C (EtOH); IR (KBr) 3550−2930, 1670, 1640, 1625, 1535, 1440 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.99−2.11 (m, 2H), 2.45−2.50 (m, 2H), 4.24 (t, 2H, J = 7), 6.66 (d, 2H, J = 8), 6.72−6.74 (m, 1H), 7.0 (d, 2H, J = 8), 7.22 (d, 1H, J = 4), 7.94 (s, 1H), 8.09 (bs, 2H), 8.17 (s, 1H), 9.17 (s, 1H). Anal. ($C_{19}H_{17}N_7O_2$) C, H, N.

Preparation of 5-Amino-7-[3-(3,4-dihydroxyphenyl)-propyl]-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]-pyrimidine (5i): To a solution of **5d** (0.12 mmol) in methylene chloride (5 mL) at 0 °C was added a solution of 1 M boron trichloride in CH_2Cl_2 (0.25 mL), and the mixture was stirred at 5 °C for 5 h. Then methanol was added (1 mL), and the solvent was evaporated under reduced pressure to afford **5i** as a white solid (92%): mp 272 °C with dec (CH_3OH); IR (KBr) 3570−2920, 1650, 1635, 1620, 1535, 1440 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.01−2.15 (m, 2H), 2.43 (t, 2H, J = 7), 4.23 (t, 2H, J = 7), 6.43 (d, 1H, J = 3), 6.47 (d, 1H, J = 3), 6.57−6.6 (m, 3H), 6.71−6.74 (m, 1H), 7.22 (d, 1H, J = 4), 7.94 (s, 1H), 8.08 (bs, 2H), 8.16 (s, 1H). Anal. ($C_{19}H_{17}N_7O_3$) C, H, N.

Determination of R_m Values by C_{18} RP-HPTLC. The HPTLC determinations were carried out on Whatman $KC_{18}F$ plates as previously described.[28] Solvent mixtures of methanol−water buffer at pH 7.0 were used as mobile phase. The methanol concentration ranged from 60% to 90%. The solutes were detected under UV 254-nm light.

Biological Studies. 1. Rat A_1 and A_{2A} Adenosine Receptor Binding Assays. Male Wistar rats (200−250 g) were decapitated and the whole brain and striatum dissected on ice. The tissues were disrupted in a Polytron homogenizer at a setting of 5 for 30 s in 25 volumes of 50 mM Tris HCl, pH 7.4, containing 10 mM $MgCl_2$. The homogenate was centrifuged at 48000*g* for 10 min, and the pellet was resuspended in the same buffer containing 2 IU/mL adenosine deaminase. After 30-min incubation at 37 °C, the membranes were centrifuged and pellets were stored at −80 °C. Prior to freezing, an aliquot of homogenate was removed for protein assay according to a Bio-Rad method[31] with bovine albumin as reference standard.

Binding assays[3,19] were performed on rat brain and striatum membranes, respectively, in the presence of 10 mM $MgCl_2$ at 0, 10, 15, 20, 25, and 30 °C. All buffer solutions were adjusted to maintain a constant pH of 7.4 at the desired temperature.

Displacement experiments were performed in 500 μL of Tris HCl buffer containing 1 nM of the selective adenosine A_1 receptor ligand [^{3}H]CHA (N^6-cyclohexyladenosine) and membranes of rat brain (150−200 μg of protein/assay).

Displacement experiments were performed in 500 μL of Tris HCl buffer containing 10 mM $MgCl_2$, 5 nM of the selective adenosine A_{2A} receptor ligand [^{3}H]CGS 21680 (2-[[4-(2-carboxyethyl)phenethyl]amino]-5′-(*N*-ethylcarbamoyl)adenosine) and membranes of rat striatum (80−100 μg of protein/assay). To determine IC_{50} values (where IC_{50} is the inhibitor concentration displacing 50% of labeled ligand) the test compounds were added in triplicate to binding assay samples at a minimum of six different concentrations. Separation of bound from free radioligand was performed by rapid filtration through Whatman GF/B filters which were washed three times with ice-cold buffer. Filter-bound radioactivity was measured by scintillation spectrometry after addition of 5 mL of Aquassure. Nonspecific binding was defined as binding in the presence of 10 μM R-PIA (N^6-(phenylisopropyl)adenosine) and 10 μM NECA (5′-(*N*-ethylcarboxamido)adenosine), respectively, and was always 10% of the total binding. Incubation time ranged from 150 min at 0 °C to 75 min at 30 °C according to the results of previous time-course experiments. K_i values were calculated from the Cheng−Prusoff equation.[32] All binding data were analyzed using the nonlinear regression curve-fitting computer program LIGAND.[33]

2. Human Cloned A_1 and A_{2A} Adenosine Receptor Binding Assays. Chinese hamster ovary cells (CHO cells) stably expressing the human A_1 adenosine receptor were used for binding experiments. Thawed membranes were resuspended in the same buffer to a final protein concentration of 2.5 mg/mL. Adenosine deaminase (2 IU/mL) was added for 30 min at 37 °C, before radioligand binding assays, to remove endogenous adenosine. Binding of [^{3}H]CHA to human A_1 adenosine receptor was performed as described by Bruns et al.[18] Assays were carried out in duplicate and in a final volume of 0.5 mL, containing 1.0 nM [^{3}H]CHA, 50 mM Tris HCl buffer, pH 7.4, and CHO membrane suspension (0.25 mg of protein/assay). In competition studies, at least seven different concentrations of the tested compounds were used. Nonspecific binding was determined in the presence of 10 μM CHA.

CHO cells stably expressing the human A_{2A} adenosine receptor were used for binding experiments. Binding of [^{3}H]-SCH 58261 to human A_{2A} adenosine receptors was performed as described in rat striatal membranes.[7] Assays were carried out in duplicate and in a final volume of 0.5 mL, containing 0.5 nM [^{3}H]SCH 58261, 50 mM Tris HCl buffer, pH 7.4, and CHO membrane suspension (0.1 mg of protein/assay). Nonspecific binding was determined in the presence of 10 μM NECA.

After 30-min incubation at 25 °C, samples were filtered through Whatman GF/B filters using a Brandel cell harvester (Gaithersburg, MD). Radioactivity was determined in a LS-6000IC Beckman liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA), at an efficiency of 50−60%. Protein concentration was determined by the method of Lowry et al.[34] with bovine serum albumin used as standard.

Human Cloned A_3 Adenosine Receptor Binding Assays. An aliquot of membranes (8 mg of protein/mL) from HEK-293 cells transfected with the human recombinant A_3 adenosine receptor was used for binding assays.[35] Inhibition experiments were carried out in duplicate in a final volume of 100 μL in test tubes containing 0.3 nM [^{125}I]AB-MECA (N^6-(4-amino-3-iodobenzyl)-5′-(*N*-methylcarbamoyl)adenosine), 50 mM Tris HCl buffer, 10 mM $MgCl_2$, pH 7.4, 20 μL of diluted membranes (12.4 mg of protein/mL), and at least 6−8 different concentrations of typical adenosine receptor antagonists. Nonspecific binding was defined in the presence of 50 μM R-PIA and was about 30% of total binding. Incubation time was 60 min at 37 °C, according to the results of previous time-course experiments. Bound and free radioactivity was separated by filtering the assay mixture through Whatman GF/B glass-fiber filters using a Brandel cell harvester.

4. Thermodynamic Parameter Determination. The standard free energy was calculated as $\Delta G° = -RT \ln K_A$ (where K_A is the association constant of the equilibrium drug + receptor $\rightleftharpoons$ drug−receptor) at 298.15 K. The standard enthalpy, $\Delta H°$, was obtained from the van't Hoff plot of $\ln K_A$ versus $1/T$ (whose slope is $-\Delta H°/R$), and the standard entropy was $\Delta S° = (\Delta H° - \Delta G°)/T$, with T = 298.15 K and R = 8.314 J/mol/K.[36]

5. Cyclic AMP Assay. Washed human platelets from peripheral blood of healthy volunteers were prepared as previously described by Korth et al.[37] The final suspending medium was a Tyrode's buffer, pH 7.4, of the following composition (mM): NaCl, 137; KCl, 2.68; $NaHCO_3$, 11.9; $MgCl_2$, 1.0; $NaHPO_4$, 0.4; glucose, 5.5. Platelets (6−8 × 10^4 cells) were suspended in 0.5 mL of incubation mixture (Tyrode's buffer containing 0.25% BSA, 1 IU/mL adenosine deaminase, and 0.5 mM Ro 20-1724 as a phosphodiesterase inhibitor) and preincubated for 10 min in a shaking bath at 37 °C. Then, the synthesized compounds, NECA (1 μM), and forskolin (1 μM) were added, and the incubation was continued for a further 5 min. The reaction was terminated by the addition of cold 6% trichloroacetic acid (TCA). TCA suspension was centrifuged at 2000*g* for 10 min at 4 °C, and the supernatant was extracted four times with water-saturated

diethyl ether. The final aqueous solution was tested for cAMP levels by a competitive protein binding assay carried out essentially according to Brown et al.[38] and Nordstedt and Fredholm.[39] Samples of cAMP standards (0−10 pM) were added to each test tube containing Brown buffer, Brown pH 7.4 (trizma base, 0.1 M; aminophylline, 8.0 mM; 2-mercaptoethanol, 6.0 mM), and [^{3}H]cAMP, in a total volume of 0.5 mL. Binding protein, previously prepared from bovine adrenals, was added to the samples which were incubated at 4 °C for 150 min and, after the addition of charcoal, centrifuged at 2000*g* for 10 min (clear supernatant in a LS-1800 Beckman scintillation counter). IC_{50} values were obtained from concentration−response curves by linear regression analysis after log transformation.

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