Pergamon

Bioorganic & Medicinal Chemistry 8 (2000) 2581–2590

BIOORGANIC & MEDICINAL CHEMISTRY

1-Phenylpyrazolo[3,4-*d*]pyrimidines; Structure–Activity Relationships for C6 Substituents at A_1 and A_{2A} Adenosine Receptors

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Received 6 January 2000; accepted 5 July 2000

Abstract—Substitution of 1-phenylpyrazolo[3,4-*d*]pyrimidines at C6 with *N*-alkyl-2-thiopropionamide groups has resulted in a series of 18 compounds which have been evaluated for binding at A_1 and A_{2A} adenosine receptors. Introduction of an *N*-ethyl group gave increased affinity at both A_1 and A_{2A} receptors for the amino compound **7b** compared to the primary amide **7a**. An additional hydrophobic pocket exists for substituents on the amide. This pocket allows an *N*-ethyl group for increased affinity at both A_1 and A_{2A} receptors, allows larger alkyl groups at A_{2A} receptors but not at A_1 receptors and there is an H-bond interaction requiring one H-bond donor. Molecular modeling studies have also enabled a proposal of the amino acid residues involved in ligand binding at both the A_1 and A_{2A} receptors.

Introduction

Adenosine and its analogues exert physiological responses through four major subtypes of adenosine receptors, A_1, A_{2A}, A_{2B}, and A_3. The responses act via G-coupled proteins consisting of seven transmembrane helices which activate several effector systems including adenylate cyclase, potassium and calcium channels, phospholipase A2 or C and guanylate cyclase.[1–3]

The first group of AR antagonists that will enter the market appears as if it will be the A_1-selective antagonists.[4] Compounds like KFM19 and MDL 102503 are targeted for CNS disorders such as Morbus Alzheimer and as general cognition enhancers in geriatric therapy. A_1-selective antagonists, such as KW3902 and FK 453, are currently developed as potassium-saving diuretics with kidney-protective properties, and for the treatment of acute renal failure.[4] Structural requirements for selective antagonists for the A_1 and A_{2A} receptors have been reviewed.[5,6] FK 453 is a novel non-xanthine antagonist that is 657-fold selective for binding to A_1 versus A_{2A} receptors and is under development for the treatment of hypertension and renal failure.[6] FK 453, (+)-1-[(*E*)-3-(2-phenylpyrazolo[1,5-a]pyridin-3-yl)acryloyl]-2-piperidine ethanol, is a novel pyrazolopyridine derivative that was found to possess a potent diuretic activity with renal vasodilatory and uricosic effects. In addition, FK 453 markedly antagonized the negative inotropic effects of adenosine in isolated guinea-pig atria but was less potent in inhibiting the relaxation induced by adenosine in guinea pig aorta. These results suggested that FK 453 is a potent and selective adenosine A_1 antagonist that could be useful in the treatment of hypertension and renal failure. FK 453 produced dose-dependent diuretic, natriuretic and uricosuric effects in healthy human subjects and in patients with essential hypertension.[7]

Pyrazolo[3,4-*d*]pyrimidines were originally identified as adenosine A_1 antagonists during a study of a large number of nitrogen heterocycles related to caffeine and theophylline. The most active compound of the study was 4,6-bis-α-carbamoylethylthio-1-phenylpyrazolo[3,4-*d*]pyrimidine (**1**) with a K_i of 370±60 nM at the A_1 receptor (Fig. 1).[8,9] We have recently reported the synthesis and receptor binding at A_1 and A_{2A} receptors of a series of 1-phenylpyrazolo[3,4-*d*]pyrimidines substituted at C6 with thioethers containing distal amide substituents and substituted at C4 with thiol, thiomethyl and amino substituents. The most selective 1-phenylpyrazolo[3,4-*d*]pyrimidine for the A_1 receptor was 2′-(4-amino-1phenylpyrazolo[3,4-*d*]pyrimidin-6-ylthio)*N*-ethyl-ethanamide (**2**) with a K_iA_1 of 12.1±4.5 nM and a K_iA_{2A} of 131±36 nM and is a modest 10.8 times more selective for this receptor.[10,11] The most selective 1-phenylpyrazolo [3,4-*d*]pyrimidine for the A_{2A} was 3′-(4-amino-1-phenylpyrazolo

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0968-0896/00/$ - see front matter
PII: S0968-0896(00)00190-5

[3,4-*d*]pyrimidin-6-ylthio)propanamide (**3**) with a K_iA_1 of 428±25 nM and a K_iA_{2A} of 101±26 nM and is a modest 4.2 times more selective for this receptor. This was gained mainly by decreased A_1 affinity while the A_{2A} affinity remained relatively unaffected. Increasing the methylene bridge by one carbon resulted in a decrease in A_1 receptor affinity. This change was tolerated by the A_{2A} receptor. Increasing the methylene bridge further resulted in a decrease in both A_1 and A_{2A} receptor affinities.[11] The study also showed that compounds substituted with an amino group in the C4 position had higher affinity for both A_1 and A_{2A} receptors than both the thiol and thiomethyl substituents. The thiomethyl compounds had greater affinity than the thiol compounds. It was concluded that for high affinity at both A_1 and A_{2A} adenosine receptors the distal amide should be separated from the C6 thiol by only one carbon.

We now report the effect of varying the amide group and keeping the distance between the amide and the C6 thiol at one carbon. These compounds contain a thiol, thiomethyl, thioalkylamide and an amino group in the C4 position.

Results

1-Phenyl-5H,7H-pyrazolo[3,4-*d*]pyrimidine dithione (**4**) was monoalkylated as previously described[12] with the corresponding bromoamides to yield compounds **5(b–e)**, alkylated with methyl iodide to yield compounds **6(b–f)** and finally converted to the 4-amino compounds, compounds **7(b–f)**, by treatment with ethanolic ammonia in a sealed tube (Scheme 1). The bisalkylated compounds **8(b–f)** were synthesized using 2 molar equivalents of the bromoamides in aqueous sodium hydroxide and *t*-butanol (Scheme 2). Receptor bindings at rat membrane adenosine A_1 and A_{2A} receptors are given in Table 1.

Molecular Modeling

The models for both the human A_1 and the A_{2A} adenosine receptors were based on structures imported from the G-Protein Coupled Receptor Database (GPCRD).[13] These consisted of the seven transmembrane helices. The helical bundles were then minimized, with tethering, using the Powell method with Kollman force field and charges. Gradient was used as the termination criteria.

The loops (including amino- and carboxy-terminus strands) were then constructed and attached to the minimized helices. The antagonist structures modeled were then constructed and, after an initial clean-up, minimized using the conjugate gradient method. Docking experiments were then carried out using the flexidock program based on a variety of parameters such as charge and distance constraints within the general region of the receptor indicated by mutagenesis studies[14,15] and molecular modeling[16] to be important in binding. Many ligands were used for docking to explore possible binding sites and the most 'sensible' solutions were examined further and refined to leave us with the binding site models proposed here. Both receptor models were in close agreement with previous binding study results.[17] All molecular modeling was carried out using the

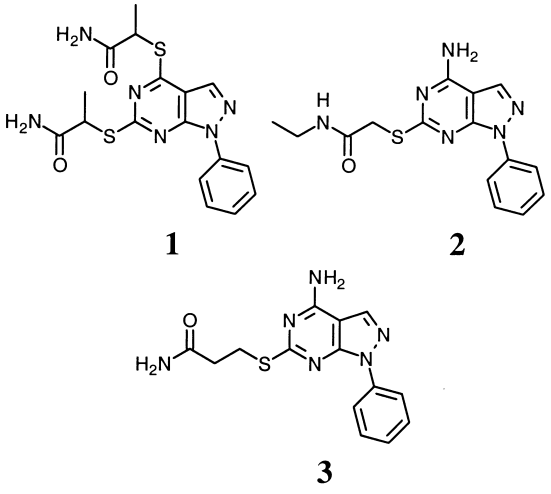
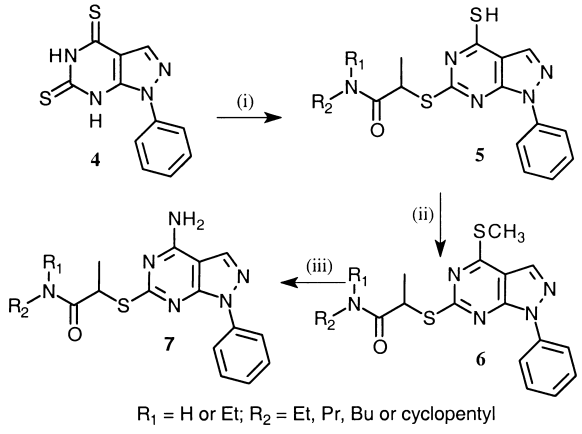
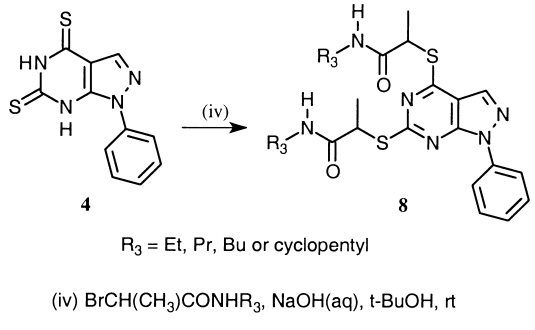


Figure 1.

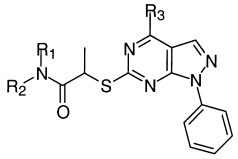


Scheme 1.



Scheme 2.

Table 1. Receptor binding at rat membrane adenosine A_1 and A_{2A} receptors



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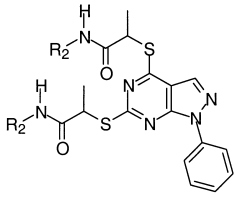

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Compound	R_1	R_2	R_3	A_1 Receptor K_i, nM	% Inhibition	A_{2A} Receptor K_i, nM	% Inhibition	K_iA_{2A}/K_iA_1
5a	H	H	SH	587±91		402±64		0.68
5b	H	Et	SH	240±22		2520±450		10.5
5c	H	Pr	SH		0		5	
5d	H	Bu	SH		5		6	
5e	H	Cyclopentyl	SH		0		17	
6a	H	H	SMe	30.3±2.9		39.5±6.4		1.30
6b	H	Et	SMe	60.8±8.2		272±33		4.47
6c	H	Pr	SMe		39		34	
6d	H	Bu	SMe		26		2	
6e	H	Cyclopentyl	SMe		21		10	
6f	Et	Et	SMe		2		3	
7a	H	H	NH_2	17.4±1.6		18.5±2.3		1.06
7b	H	Et	NH_2	7.13±1.32		10.0±2.2		1.40
7c	H	Pr	NH_2	72.4±15.2		185±29		2.56
7d	H	Bu	NH_2	329±67		196±31		0.59
7e	H	Cyclopentyl	NH_2		34		22	
7f	Et	Et	NH_2	890±170		1420±425		1.60
8a	—	H	—	229±20		146±27		0.64
8b	—	Et	—	364±64		425±49		2.39
8c	—	Pr	—		0		0	
8d	—	Bu	—		2		3	
8e	—	Cyclopentyl	—		0		0	

SYBYL 6.6 (Tripos Inc, St Louis, MO, USA) software package which was run on a Silicon Graphics Octane workstation.

Discussion

The synthetic compounds were compared to the previously synthesized primary amides **5a, 6a, 7a, 8a**.[12] The trends observed with this extended group of compounds were similar to the trends observed previously.[11] The thiol compounds **5(a–e)**, and the bisalkylated compounds **8(a–e)**, had lower affinity for the A_1 and A_{2A} receptors than the thiomethyl compounds **6(a–f)** (Table 1). The thiomethyl compounds had lower affinity for the A_1 and A_{2A} receptors than the amino compounds **7(a–f)**. Introduction of the *N*-ethyl group gave increased affinity at both A_1 and A_{2A} receptors for the amino compound **7b** compared to **7a**. As the *N*-alkyl group increased from an ethyl, compound **7b**, to a propyl group, compound **7c**, the A_1 and the A_{2A} affinity decreased. The A_1 affinity was further decreased with the butyl compounds **7d**, but the A_{2A} affinity remained relatively unchanged. There is a 46-fold loss in affinity of compound **7d** at the A_1 receptor when compared to compound **7b**, and a 19.6-fold loss in affinity at the A_{2A} receptor. This results in a 2.4-fold increase in A_{2A} receptor selectivity. This suggests that there is a tolerance for larger *N*-alkyl substituents at the C6 position for the A_{2A} receptor than the A_1 receptor. With *N,N*-dialkyl substituents, there was a loss in affinity for both the A_1 and A_{2A} receptors, suggesting possible hydrogen bonding of the amide group to the receptor. Compound **3** still remains to be the most selective compound for the A_{2A} receptor while compound **7d** is the most selective compound of this series. Although we do not observe a more selective compound for the A_{2A} receptor, it may be worth exploring *N*-alkyl substituted compounds using compound **3** as the parent structure for further optimization and development in A_{2A} selective antagonists.

Conclusion

At both the A_1 and A_{2A} receptors, the *N*-ethyl compounds have the highest affinity followed by the primary amides. As the alkyl chain increases, affinity at both receptors decreases. This study allows a further definition of the previously published model[17] as shown in Figure 2. An additional hydrophobic pocket exists for substituents on the amide. This pocket allows an *N*-ethyl group for increased affinity at both A_1 and A_{2A} receptors, allows larger alkyl groups at A_{2A} receptors but not at A_1 receptors and there is an H-bond interaction requiring one H-bond donor.

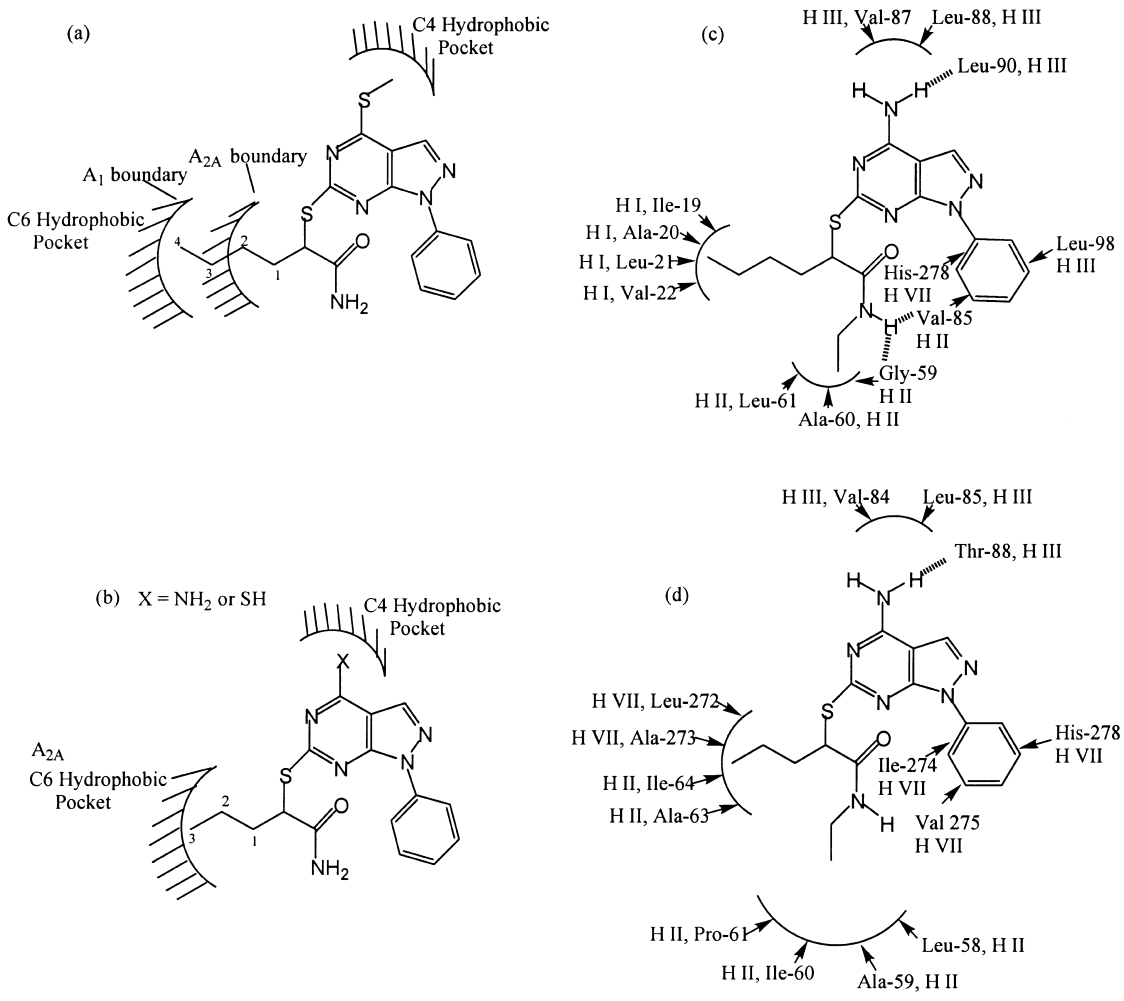

Figure 2. Diagrams (a) and (b) represent previously published work:[17] (a) the A_1 receptor with summary of hydrophobic pocket carbon tolerance and the A_{2A} boundary with a methylthio group at C4; (b) it was postulated that the A_{2A} receptor C6 hydrophobic pocket could tolerate either 2 or 3 carbons depending on whether a methylthio substituent is present at C4 or not. Diagrams (c) and (d) represent binding data and modeling results proposed by this study; (c) identification of amino acids representing the binding site for the A_1 receptor. The *N*-alkyl pocket has a limit of 2 carbons; (d) the binding site model for the A_{2A} receptor proposes a tolerance for larger substituents at the *N*-alkyl pocket. Interactions of various amino acids with the ligand are indicated with arrows. The transmembrane helix on which the amino acids are found is also shown. Dashed lines represent H-bonding.

As shown in Fig. 2, the proposed binding site from molecular modeling studies allowed amino acid residues potentially involved in ligand binding to be identified.

At the A_1 receptor an H-bond is observed between the carbonyl oxygen of Leu-90 and the C4 amino substituent. The A_{2A} receptor shows similar H-bonding but this time to Thr-88. This interaction would explain the preference for an amine over a thiol or thiomethyl substituent at C4. It was also observed that in both receptors an alkyl region or 'roof' was present far enough above the H-bonding residue to allow a thiomethyl group to interact, thereby offering a possible explanation for the preference of thiomethyl over thiol at both receptors. The residues Val-87 and Leu-88, making up this region for the A_1 receptor, have previously been postulated to have a role in ligand binding.[18]

The limitations of the C6 hydrophobic pocket had been explored in previous binding studies[17] and the receptor–ligand models were in agreement with these results. For the A_1 receptor the C6 alkyl substituent could extend into a hydrophobic area defined by residues Ile-19, Ala-20, Leu-21 and Val-22, all on helix I. This allows enough space to tolerate 4 alkyl carbons. The C6 alkyl chain at the A_{2A} receptor can extend into a smaller hydrophobic pocket defined by residues Ala-63, Ile-64, Leu-272 and Ala-273. The pocket is therefore formed between helices II and VII and can tolerate only 3 alkyl carbons.

At the A_1 receptor the C6 amide hydrogen was seen to be capable of acting as an H-bond donor to either Val-58 and Gly-59 as was predicted from the binding affinities. There was no H-bonding observed for the amide at the A_{2A} receptor, which could go some way to explaining the selectivity shown for the A_1 receptor.

The *N*-alkyl pockets investigated in this study were observed in both binding site models. For the A_1 receptor, the pocket is formed by the coiling of hydrophobic residues Val-58, Gly-59, Ala-60 and Leu-61, all of which are on helix II. This tight coiling around the alkyl chain will permit two alkyl carbons only. The corresponding hydrophobic pocket on the A_{2A} receptor is formed by

the coiling of residues Leu-58, Ala-59, Ile-60, Pro-61 and Phe-62. The *N*-alkyl pockets on the two receptors therefore correspond to very similar areas but the coiling in this region for the A_{2A} is much more relaxed accommodating up to 4 alkyl carbons.

The final region to be investigated was that around the phenyl ring. For both the A_1 and A_{2A} receptors amino acid His-278 was seen to interact with the phenyl group. In both cases the histidine imidazole ring was almost parallel to the aromatic ring with a 'face-on' arrangement, thereby maximizing π orbital interactions. This His-278 residue has previously been implicated in ligand binding from mutagenesis studies for both the A_1[19] and A_{2A}[15] receptors. For the A_1 receptor the ligand phenyl ring is further bounded by the hydrophobic residues Val-58 and Leu-98. In the A_{2A} model the opposite face of the phenyl ring (to the histidine) is flanked by hydrophobic residues Ile-274 and Val-275 of helix VII, giving an indication of how the phenyl ring juts into the coils of helix VII with the imidazole side chain of His-278 emerging from the helix to meet it.

As an overall view of the ligand binding site models, the ligand in the A_1 receptor sits in the pore formed by the helices in much closer contact with helices II and III than the others. The phenyl ring comes out almost at a right angle to the bicyclic nucleus, which itself lies in approximately the same plane as helices II and III. At the A_{2A} receptor the pyrazolo-pyrimidine core occupies a much more central position within the pore than at the A_1. The phenyl ring comes down and outwards towards helix VII while the C6 substituents are directed into the coils of helix II. The C4 thiomethyl substituent points directly from that central position into helix III.

Experimental

Melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were obtained on Bruker WM-250, Bruker CXP 300 and Varian Gemini-200. The type of carbon atom was assigned by using the DEPT pulse sequence obtained on a Varian Gemini-200; q = methyl, t = methylene, d = methine, and s = quaternary. COSYs and HMQCs were run on a Varian Unity 400 MHz instrument. Unless otherwise stated, DMSO-d_6 was used as a solvent and the solvent peak was used as the internal standard. IR spectra were recorded as KBr discs on Jasco IR-810 or Perkin-Elmer FTIR 1700 spectrometers. Starting materials were obtained from Aldrich Pty Ltd. Ethanol was dried by reflux and distillation over magnesium turnings and a catalytic amount of iodine and was stored over 3 Å molecular sieves. Pyridine was dried by reflux and distilled from potassium hydroxide and stored over 4 Å molecular sieves. DMF was dried over BaO. Hexane was distilled prior to use.

The starting compounds, (ethoxymethylene)malononitrile, 1-phenyl-5-amino-4-cyanopyrazole, and 1-phenylpyrazolo[3,4-*d*]pyrimidine-4,6(5*H*,7*H*)-dithione (**4**) were prepared following known methods.[12] The 2-bromo-*N*-alkyl-propanamides were prepared from 2-bromopropanoyl bromide. The syntheses of the primary amides **5a**, **6a**, **7a** and **8a** have been previously reported.[12]

α-(4-Mercapto-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-ylthio)amides (5). The following general procedure was used: 2-bromo-*N*-ethyl-propanamide (1.60 g, 8.84 mmol) was added to a solution of 1-phenylpyrazolo[3,4-*d*]pyrimidine-4,6(5*H*,7*H*)-dithione (**4**) (2.8 g, 0.011 mol) in pyridine (40 mL). The solution was stirred at room temperature for 3 h. Treatment with ethyl acetate (30 mL) resulted in precipitation of a white solid. The product was recrystallized from DMSO and water to afford α-(4-mercapto-1-phenylpyrazolo[3,4-d] pyrimidin-6-ylthio)-*N*-ethyl-propanamide **(5b)**. Yield 2.53 g, 80%; mp dec 272–276 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ 0.94 (t, 3H, J = 7.2 Hz, CH_2CH_3), 1.58 (d, 3H, J = 7.1 Hz, CH_3), 3.03 (m, 2H, J = 5.9 Hz, 7.1 Hz, CH_2CH_3), 4.46 (q, 1H, J = 7.0 Hz, SC*H*), 7.42 (t, 1H, J = 7.4 Hz, $C_{4'}H_{arom}$), 7.59 (t, 2H, J = 8.1 Hz, 7.4 Hz, $C_{3'}$, $C_{5'}H_{arom}$), 8.06 (d, 2H, J = 8.2 Hz, $C_{2'}$, $C_{6'}H_{arom}$), 8.21 (br t, 1H, J = 5.3 Hz, NH), 8.36 (s, 1H, C_3H), 14.10 (br s, 1H, N_5H). ^{13}C NMR (62.8 MHz, DMSO-d_6) δ 14.1 (q, CH_2CH_3), 18.2 (q, CH_3), 33.7 (t, CH_2CH_3), 44.7 (d, S*CH*), 116.4 (s, C_{3a}), 121.3 (d, $C_{2'}$, $C_{6'}$), 127.1 (s, $C_{4'}$), 129.3 (d, $C_{3'}$, $C_{5'}$), 138.0 (s, $C_{1'}$), 138.1 (d, C_3), 146.7 (s, C_{7a}), 160.1 (s, C_6), 169.6 (s, C=O), 180.5 (s, C_4). IR (KBr-disc) ν_{max} 3325, NH; 3125, NH; 1650, C=O; 1600 cm^{-1}, C=C. Anal. calcd for ($C_{16}H_{17}N_5OS_2$): C, 50.8; H, 4.2; N, 21.3; found: C, 50.7; H, 3.9; N, 21.4%.

α-(4-Mercapto-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-ylthio)*N*-propyl-propanamide (5c). Yield 80%; mp dec 257–259.5 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ 0.75 (t, 3H, J = 7.3 Hz, $CH_2CH_2CH_3$), 1.33 (sextet, 2H, J = 7.0 Hz, $CH_2CH_2CH_3$), 1.57 (d, 3H, J = 7.0 Hz, CH_3), 2.94 (m, 2H, J = 5.8 Hz, 7.0 Hz, $CH_2CH_2CH_3$), 4.46 (q, 1H, J = 7.0 Hz, SC*H*), 7.41 (t, 1H, J = 7.3 Hz, $C_{4'}H_{arom}$), 7.57 (t, 2H, J = 7.7 Hz, $C_{3'}$, $C_{5'}H_{arom}$), 8.05 (d, 2H, J = 7.6 Hz, $C_{2'}$, $C_{6'}H_{arom}$), 8.24 (br t, 1H, J = 5.5 Hz, NH), 8.35 (s, 1H, C_3H), 14.08 (br s, 1H, N_5H). ^{13}C NMR (62.8 MHz, DMSO-d_6) δ 11.0 (q, $CH_2CH_2CH_3$), 18.3 (q, CH_3), 21.9 (t, $CH_2CH_2CH_3$), 39.2 (t, $CH_2CH_2CH_3$), 44.7 (d, S*CH*), 116.3 (s, C_{3a}), 121.3 (d, $C_{2'}$, $C_{6'}$), 127.1 (s, $C_{4'}$), 129.3 (d, $C_{3'}$, $C_{5'}$), 138.0 (s, $C_{1'}$), 138.1 (d, C3), 146.4 (s, C_{7a}), 160.1 (s, C_6), 169.8 (s, C=O), 180.1 (s, C_4). IR (KBr-disc) ν_{max} 3325, NH; 1650, C=O; 1600 cm^{-1}, C=C. Anal. calcd for ($C_{17}H_{19}N_5OS_2$): C, 54.7; H, 5.1; N, 18.8; found: C, 54.6; H, 5.2; N, 18.7.

α-(4-Mercapto-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-ylthio)*N*-butyl-propanamide (5d). Yield 85%; mp dec 255–261 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ 0.79 (t, 3H, J = 7.0 Hz, $CH_2CH_2CH_2CH_3$), 1.34 (m, 4H, J = 7.0 Hz, $CH_2CH_2CH_2CH_3$), 1.58 (d, 3H, J = 7.2 Hz, C*H*3), 3.00 (m, 2H, J = 6.9 Hz, 5.8 Hz, $CH_2CH_2CH_2CH_3$), 4.48 (q, 1H, J = 7.0 Hz, SC*H*), 7.42 (t, 1H, J = 7.3 Hz, $C_{4'}H_{arom}$), 7.57 (t, 2H, J = 7.8 Hz, $C_{3'}$, $C_{5'}H_{arom}$), 8.06 (d, 2H, J = 7.7 Hz, $C_{2'}$, $C_{6'}H_{arom}$), 8.22 (br t, 1H, J = 5.5 Hz, NH), 8.36 (s, 1H, C_3H), 14.09 (br s, 1H, N_5H). ^{13}C NMR (50.3 MHz, DMSO-d_6) δ 13.6 (q, CH_2CH_2

CH_2CH_3), 18.4 (q, CH_3), 19.5 (q, $CH_2CH_2CH_2CH_3$), 31.0 (t, $CH_2CH_2CH_2CH_3$), 38.6 (t, $CH_2CH_2CH_2CH_3$), 44.8 (d, SCH), 116.4 (s, C_{3a}), 121.3 (d, $C_{2'}$, $C_{6'}$), 127.1 (s, $C_{4'}$), 129.4 (d, $C_{3'}$, $C_{5'}$), 138.0 (s, $C_{1'}$), 138.2 (d, C_3), 146.4 (s, C_{7a}), 160.1 (s, C_6), 169.7 (s, C=O), 180.0 (s, C_4). IR (KBr-disc) ν_{max} 3225, NH; 3125, NH; 1650, C=O; 1600 cm^{-1}, C=C. Anal. calcd for ($C_{18}H_{21}N_5OS_2$): C, 55.8; H, 5.5; N, 18.1; found: C, 55.9; H, 5.5; N, 17.8%.

β-(4-Mercapto-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-ylthio)*N*-cyclopentyl-propanamide (5e). Yield 84%; mp dec 260–269 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.14–1.75 (m, 8H, 4×CH_2 of cyclopentyl ring), 1.58 (d, 3H, J=7.0 Hz, CH_3), 3.92 (m, 2H, J=6.8 Hz, $C_{1'}H$), 4.48 (q, 1H, J=7.0 Hz, SCH), 7.42 (t, 1H, J=7.4 Hz, $C_{4''}H_{arom}$), 7.60 (t, 2H, J=8.1 Hz, 7.7 Hz, $C_{3''}$ $C_{5''}H_{arom}$), 8.06 (d, 2H, J=7.6 Hz, $C_{2''}$, $C_{6''}H_{arom}$), 8.35 (br d, 1H, J=7.0 Hz, NH), 8.35 (s, 1H, C_3H), 14.06 (br s, 1H, N_5H). ^{13}C NMR (75.5 MHz, DMSO-d_6) δ 18.3 (q, CH_3), 23.4 (t, $C_{3'}$, $C_{4'}$), 31.8, 31.9 (t, $C_{2'}$, $C_{5'}$), 44.7 (d, SCH), 50.7 (d, $C_{1'}H$), 116.3 (s, C_{3a}), 121.2 (d, $C_{2''}$, $C_{6''}$), 127.0 (s, $C_{4''}$), 129.3 (d, $C_{3''}$, $C_{5''}$), 137.9 (s, $C_{1''}$), 138.1 (d, C_3), 146.3 (s, C_{7a}), 160.1 (s, C_6), 169.1 (s, C=O), 179.9 (s, C_4). IR (KBr-disc) ν_{max} 3275, NH; 3100, NH; 1650, C=O; 1600 cm^{-1}, C=C. Anal. calcd for ($C_{19}H_{21}N_5OS_2$): C, 57.1; H, 5.3; N, 17.5; found: C, 57.4; H, 5.2; N, 17.3%.

α-(4-Methylthio-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-ylthio)amides (6). The following general procedure was used: δ-(4-mercapto-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-ylthio)*N*-ethyl-propanamide (2.00 g 5.57 mmol) was added to a sodium hydroxide solution (1.5 M, 20 mL). Iodomethane (0.7 mL, 1.6 g, 0.011 mol) was added, and the reaction mixture stirred at RT for 30 min. A white solid precipitated, was collected, washed with water and recrystallized from DMSO and water to afford α-(4-methylthio-1-phenylpyrazolo[3,4-*d*] pyrimidin-6-ylthio)-*N*-ethyl-propanamide (**6b**). Yield 1.7 g, 82%; mp 211–212.5 °C. ^{1}H NMR (250.1 MHz, DMSO-d_6) δ 0.93 (t, 3H, J=7.2 Hz, CH_2CH_3), 1.54 (d, 3H, J=7.1 Hz, CH_3), 2.68 (s, 3H, SCH_3), 3.03 (m, 2H, J=5.6 Hz, 7.1 Hz, CH_2CH_3), 4.48 (q, 1H, J=7.0 Hz, SCH), 7.37 (t, 1H, J=7.4 Hz, $C_{4'}H_{arom}$), 7.57 (t, 2H, J=8.3 Hz, 7.5 Hz, $C_{3'}$, $C_{5'}H_{arom}$), 8.12 (d, 2H, J=8.7 Hz, $C_{2'}$, $C_{6'}H_{arom}$), 8.22 (br t, 1H, J=5.0 Hz, NH), 8.46 (s, 1H, C_3H). ^{13}C NMR (62.8 MHz, DMSO-d_6) δ 11.5 (q, SCH_3), 14.3 (q, CH_2CH_3), 18.3 (q, CH_3), 33.7 (t, CH_2CH_3), 44.2 (d, SCH), 110.5 (s, C_{3a}), 120.9 (d, $C_{2'}$, $C_{6'}$), 126.8 (s, $C_{4'}$), 129.4 (d, $C_{3'}$, $C_{5'}$), 133.8 (d, C_3), 138.2 (s, $C_{1'}$), 151.0 (s, C_{7a}), 165.8 (s, C_4), 167.9 (s, C_6), 170.3 (s, C=O). IR (KBr-disc) ν_{max} 3320, NH; 3080, NH; 1655, C=O; 1600, cm^{-1} C=C. Anal. calcd for ($C_{17}H_{19}N_5OS_2$): C, 54.7; H, 5.1; N, 18.8; found: C, 54.3; H, 5.5; N, 18.5%.

α-(4-Methylthio-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-ylthio)*N*-propyl-propanamide (6c). Yield 82%; mp 195–196.3 °C. ^{1}H NMR (250.12 MHz, DMSO-d_6) δ 0.74 (t, 3H, J=7.4 Hz, $CH_2CH_2CH_3$), 1.34 (sextet, 2H, J=7.2 Hz, $CH_2CH_2CH_3$), 1.55 (d, 3H, J=7.0 Hz, CH_3), 2.69 (s, 3H, SCH_3), 2.98 (m, 2H, J=6.0 Hz, 7.3 Hz, $CH_2CH_2CH_3$), 4.50 (q, 1H, J=7.0 Hz, SCH), 7.38 (t, 1H, J=7.4 Hz, $C_{4'}H_{arom}$), 7.58 (t, 2H, J=7.9 Hz, $C_{3'}$, $C_{5'}H_{arom}$), 8.15 (d, 2H, J=8.1 Hz, $C_{2'}$, $C_{6'}H_{arom}$), 8.20 (br t, 1H, J=5.5 Hz, NH), 8.50 (s, 1H, C_3H). ^{13}C NMR (62.8 MHz, DMSO-d_6) δ 11.1 (q, $CH_2CH_2CH_3$), 11.5 (q, SCH_3), 18.3 (q, CH_3), 22.0 (t, $CH_2CH_2CH_3$), 39.2 (t, $CH_2CH_2CH_3$), 44.3 (d, SCH), 110.5 (s, C_{3a}), 120.9 (d, $C_{2'}$, $C_{6'}$), 126.8 (s, $C_{4'}$), 129.4 (d, $C_{3'}$, $C_{5'}$), 133.8 (d, C_3), 138.2 (s, $C_{1'}$), 151.0 (s, C_{7a}), 165.8 (s, C_4), 168.0 (s, C_6), 170.5 (s, C=O). IR (KBr-disc) ν_{max} 3310, NH; 3100, NH; 1660, C=O; 1600 cm^{-1}, C=C. Anal. calcd for ($C_{18}H_{21}N_5OS_2$): C, 55.8; H, 5.5, N, 18.1; found: C, 55.7; H, 5.2; N, 18.3%.

α-(4-Methylthio-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-ylthio)*N*-butyl-propanamide (6d). Yield 70%; mp 194–196 °C. ^{1}H NMR (250.12 MHz, DMSO-d_6) δ 0.75 (t, 3H, J=7.2 Hz, $CH_2CH_2CH_2CH_3$), 1.22 (m, 4H, J=7.2 Hz, $CH_2CH_2CH_2CH_3$), 1.55 (d, 3H, J=7.0 Hz, CH_3), 2.69 (s, 3H, SCH_3), 3.00 (m, 2H, J=7.0 Hz, 6.2 Hz, $CH_2CH_2CH_2CH_3$), 4.50 (q, 1H, J=7.0 Hz, SCH), 7.38 (t, 1H, J=8.0 Hz, $C_{4'}H_{arom}$), 7.57 (t, 2H, J=7.8 Hz, $C_{3'}$, $C_{5'}H_{arom}$), 8.13 (d, 3H, J=8.4 Hz, $C_{2'}$, $C_{6'}H_{arom}$, NH), 8.49 (s, 1H, C_3H). ^{13}C NMR (50.3 MHz, DMSO-d_6) δ 11.7 (q, SCH_3), 13.6 (q, $CH_2CH_2CH_2CH_3$), 18.4 (q, CH_3), 19.4 (t, $CH_2CH_2CH_2CH_3$), 31.0 (t, CH_2CH_2 CH_2CH_3), 38.5 (t, $CH_2CH_2CH_2CH_3$), 44.4 (d, SCH), 110.5 (s, C_{3a}), 120.8 (d, $C_{2'}$, $C_{6'}$), 126.7 (s, $C_{4'}$), 129.4 (d, $C_{3'}$, $C_{5'}$), 133.8 (d, C_3), 138.2 (s, $C_{1'}$), 150.9 (s, C_{7a}), 165.7 (s, C_4), 167.9 (s, C_6), 170.4 (s, C=O). IR (KBr-disc) ν_{max} 3275, NH; 3050, NH; 1675, C=O; 1650 cm^{-1}, C=O. Anal. calcd for ($C_{19}H_{23}N_5OS_2$): C, 56.8; H, 5.8; N, 17.4; found: C, 56.8; H, 5.6; N, 17.3%.

α-(4-Methylthio-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-ylthio)*N*-cyclopentyl-propanamide (6e). Yield 76%; mp 207–210 °C. ^{1}H NMR (250.12 MHz, DMSO-d_6) δ 1.22–1.78 (m, 8H, CH_2 of cyclopentyl ring), 1.56 (d, 3H, J=6.9 Hz, CH_3), 2.70 (s, 3H, SCH_3), 3.93 (m, 1H, J=6.6 Hz, 6.3 Hz, NC1′H), 4.52 (q, 1H, J=6.8 Hz, SCH), 7.38 (t, 1H, J=7.4 Hz, $C_{4''}H_{arom}$), 7.59 (t, 2H, J=7.9 Hz, $C_{3''}$, $C_{5''}H_{arom}$), 8.09 (br d, 1H, 6.7 Hz, NH), 8.14 (d, 3H, J=7.6 Hz, $C_{2''}$, $C_{6''}H_{arom}$, NH), 8.49 (s, 1H, C_3H). ^{13}C NMR (75.5 MHz, DMSO-d_6 at 60 °C) δ 11.3 (q, SCH_3), 18.0 (q, CH_3), 23.1 (t, $C_{3'}H_2$, $C_{4'}H_2$), 31.7, 31.8 (t, $C_{2'}H_2$, $C_{5'}H_2$ interchangeable), 43.9 (d, SCH), 50.4 (d, $C_{1'}$H), 110.2 (s, C_{3a}), 120.6 (d, $C_{2''}$, $C_{6''}$), 126.4 (s, $C_{4''}$), 129.0 (d, $C_{3''}$, $C_{5''}$), 133.4 (d, C_3), 137.9 (s, $C_{1''}$), 150.7 (s, C_{7a}), 165.3 (s, C_4), 167.7 (s, C_6), 169.6 (s, C=O); IR (KBr-disc) ν_{max} 3275, NH; 3050, NH; 1650, C=O; 1600 cm^{-1}, C=C. Anal. calcd for ($C_{20}H_{23}N_5OS_2$): C, 58.1; H, 5.6; N, 16.9; found: C, 58.1; H, 5.5; N, 16.7%.

α-(4-Methylthio-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-ylthio)*N*,*N*-diethyl-propanamide (6f). Yield 60%; mp 108–109 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.00 (t, 3H, J=7.1 Hz, CH_2CH_3), 1.11 (t, 3H, J=7.1 Hz, CH_2CH_3), 1.58 (d, 3H, J=6.8 Hz, CH_3), 2.69 (s, 3H, SCH_3), 3.31 (m, 4H, 2×CH_2, J=7.1 Hz, 6.5 Hz, 2×CH_2CH_3), 5.06 (q, 1H, J=6.9 Hz, SCH), 7.39 (t, 1H, J=7.4 Hz, $C_{4'}H_{arom}$), 7.58 (t, 2H, J=7.8 Hz, $C_{3'}$, $C_{5'}H_{arom}$), 8.10 (d, 2H, J=7.6 Hz, $C_{2'}$, $C_{6'}H_{arom}$), 8.48 (s, 1H, C_3H). ^{13}C NMR (50.3 MHz, DMSO-d$_6$) δ 11.6 (q, SCH_3), 12.7 (q, CH_2CH_3), 14.7 (q, CH_2CH_3), 19.4 (q, CH_3), 40.4 (t,

2×CH_2CH_3), 42.0 (d, SCH), 110.5 (s, C_{3a}), 120.9 (d, $C_{2'}$, $C_{6'}$), 126.8 (s, $C_{4'}$), 129.2 (d, $C_{3'}$, $C_{5'}$), 133.7 (d, C_3), 138.0 (s, $C_{1'}$), 150.9 (s, C_{7a}), 165.9 (s, C_4), 167.2 (s, C_6), 169.5 (s, C=O). IR (KBr-disc) ν_{max} 1660, C=O; 1600 cm^{-1}, C=C. Anal. calcd for ($C_{19}H_{23}N_5OS_2$): C, 56.8; H, 5.8; N, 17.4; found: C, 57.1; H, 5.8; N, 17.5%.

α-(4-Amino-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-ylthio) amides (7). The following general procedure was used: a saturated solution of ammonia in ethanol (15 mL) was added to α-(4-methylthio-1-phenylpyrazolo[3,4-*d*] pyrimidin-6-ylthio)-*N*-ethyl-propanamide (0.17 g, 0.44 mmol). The solution was heated at 100 °C for 72 h in a bomb. The solvent was removed to yield a white solid. This was recrystallized from DMSO and water to afford α-(4-amino-1-phenylpyrazolo[3,4-d]pyrimidin-6-ylthio)-*N*-ethyl-propanamide (**7b**). Yield 0.12 g, 79%; mp dec 261–264 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ 0.94 (t, 3H, J=7.2 Hz, CH_2CH_3), 1.53 (d, 3H, J=7.0 Hz, CH_3), 3.05 (m, 2H, J=6.0 Hz, 7.0 Hz, CH_2CH_3), 4.41 (q, 1H, J=7.1 Hz, SCH), 7.35 (t, 1H, J=7.4 Hz, $C_{4'}H_{arom}$), 7.56 (t, 2H, J=7.7 Hz, $C_{3'}$, $C_{5'}H_{arom}$), 8.19 (d, 2H, J=8.4 Hz, $C_{2'}$, $C_{6'}H_{arom}$), 7.99 (br s, 2H, NH_2), 8.11 (br t, 1H, J=5.4 Hz, NH), 8.29 (s, 1H, C_3H). ^{13}C NMR (62.8 MHz, DMSO-d_6) δ 14.2 (q, CH_2CH_3), 18.1 (q, CH_3), 33.6 (t, CH_2CH_3), 43.1 (d, SCH), 99.3 (s, C_{3a}), 120.4 (d, $C_{2'}$, $C_{6'}$), 126.0 (s, $C_{4'}$), 129.2 (d, $C_{3'}$, $C_{5'}$), 134.4 (d, C_3), 138.9 (s, $C_{1'}$), 151.1 (s, C_{7a}), 157.5 (s, C_4), 168.8 (s, C_6), 170.8 (s, C=O). IR (KBr-disc) ν_{max} 3250, NH; 3190, NH; 1665, C=O; 1600 cm^{-1}, C=C. Anal. calcd for ($C_{16}H_{18}N_6OS$): C, 56.1; H, 5.3; N, 24.5; found: C, 56.1; H, 5.4; N, 24.4%.

α-(4-amino-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-ylthio)-*N*-propyl-propanamide (7c). Purified by chromatography on silica gel (230–400 mesh, 60 Å) using ethyl acetate:hexane (1:1) then ethyl acetate (100%), R_f (ethyl acetate, 100%)=0.75. Yield 79%; mp 248–249 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ 0.72 (t, 3H, J=7.3 Hz, $CH_2CH_2CH_3$), 1.29 (sextet, 2H, J=7.1 Hz, $CH_2CH_2CH_3$), 1.50 (d, 3H, J=6.9 Hz, CH_3), 2.96 (br q, 2H, J=6.0 Hz, 6.9 Hz, $CH_2CH_2CH_3$), 4.43 (q, 1H, J=7.0 Hz, SCH), 7.32 (t, 1H, J=7.3 Hz, $C_{4'}H_{arom}$), 7.54 (t, 2H, J=7.9 Hz, $C_{3'}$, $C_{5'}H_{arom}$), 7.94 (br s, 2H, NH2), 8.07 (br t, 1H, J=5.5 Hz, NH_{amide}), 8.16 (d, 2H, J=7.4 Hz, $C_{2'}$, $C_{6'}H_{arom}$), 8.26 (s, 1H, H_3). ^{13}C NMR (62.8 MHz, DMSO-d_6) δ 11.0 (q, $CH_2CH_2CH_3$), 18.0 (q, CH_3), 39.8 (t, $CH_2CH_2CH_3$), 43.0 (d, SCH), 99.3 (s, C_{3a}), 120.5 (d, $C_{2'}$, $C_{6'}$), 126.0 (s, $C_{4'}$), 129.2 (d, $C_{3'}$, $C_{5'}$), 134.4 (d, C_3), 138.9 (s, $C_{1'}$), 153.6 (s, C_{7a}), 157.5 (s, C_4), 168.8 (s, C_6), 171.0 (s, C=O). IR (KBr-disc) ν_{max} 3525, NH; 3325, NH; 3150, NH; 3100, NH; 1665, C=O; 1600 cm^{-1}, C=C. Anal. calcd for ($C_{17}H_{20}N_6OS$): C, 57.3; H, 5.7; N, 23.6; found: C, 57.2; H, 5.6; N, 23.4%.

α-(4-Amino-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-ylthio)-*N*-butyl-propanamide (7d). Yield 75%; mp 213–216 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ 0.72 (t, 3H, J=7.3 Hz, $CH_2CH_2CH_2CH_3$), 1.21 (m, 4H, J=7.2 Hz, $CH_2CH_2CH_2CH_3$), 1.48 (d, 3H, J=7.0 Hz, CH_3), 2.98 (br q, 2H, J=6.5 Hz, 6.0 Hz, $CH_2CH_2CH_2CH_3$), 4.42 (q, 1H, J=7.0 Hz, SCH), 7.31 (t, 1H, J=7.4 Hz, $C_{4'}H_{arom}$), 7.53 (t, 2H, J=7.7 Hz, $C_{3'}$, $C_{5'}H_{arom}$), 7.93 (br s, 2H, NH_2), 8.01 (br t, 1H, J=5.5 Hz, NH_{amide}), 8.16 (d, 2H, J=8.2 Hz, $C_{2'}$, $C_{6'}H_{arom}$), 8.27 (s, 1H, C_3H). ^{13}C NMR (50.3 MHz, DMSO-d_6) δ 13.6 (q, $CH_2CH_2CH_2CH_3$), 18.2 (q, CH_3), 19.4 (t, $CH_2CH_2CH_2CH_3$), 31.0 (t, $CH_2CH_2CH_2CH_3$), 38.4 (t, $CH_2CH_2CH_2CH_3$), 43.1 (d, SCH), 99.4 (s, C_{3a}), 120.4 (d, $C_{2'}$, $C_{6'}$), 126.0 (s, $C_{4'}$), 129.2 (d, $C_{3'}$, $C_{5'}$), 134.4 (d, C_3), 138.9 (s, $C_{1'}$), 153.5 (s, C_{7a}), 157.4 (s, C_4), 168.7 (s, C_6), 170.9 (s, C=O). IR (KBr-disc) ν_{max} 3525, NH; 3300, NH; 3050, NH; 1660 C=O; 1595 cm^{-1}, C=C. Anal. calcd for ($C_{18}H_{22}N_6OS$): C, 58.4; H, 6.0; N, 22.7; found: C, 58.2; H, 6.3; N, 22.9%.

α-(4-Amino-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-ylthio)-*N*-cyclopentyl-propanamide (7e). Yield 80%; mp dec 252–257 °C. ^{1}H NMR (300.1 MHz, DMSO-d_6) δ 1.05–1.70 (m, 8H, 4×CH_2 of cyclopentyl group), 1.49 (d, 4H, J=7.0 Hz, CH_3), 1.48 (d, 3H, J=7.0 Hz, CH_3), 3.92 (m, 1H, J=7.1 Hz, 5.9 Hz, $C_{1'}H$), 4.39 (q, 1H, J=7.0 Hz, SCH), 7.32 (t, 1H, J=7.4 Hz, C4″H_{arom}), 7.55 (t, 2H, J=7.8 Hz, C3″, C5″H_{arom}), 7.98 (br d, 2H, J=7.3 Hz, NH_{amide}, NH), 8.09 (br s, 1H, NH), 8.12 (d, 2H, J=8.1 Hz, C2″, C6″H_{arom}), 8.27 (s, 1H, C_3H). ^{13}C NMR (75.5 MHz, DMSO-d_6) δ 17.6 (q, CH_3), 23.1 (t, $C_{3'}H_2$, $C_{4'}H_2$), 31.7, 31.8 (t, $C_{2'}H_2$, $C_{5'}H_2$ interchangeable), 42.5 (d, SCH), 50.3 (d, $C_{1'}$H), 99.1 (s, C_{3a}), 120.3 (d, $C_{2''}$, $C_{6''}$), 125.8 (s, $C_{4''}$), 129.0 (d, $C_{3''}$, $C_{5''}$), 134.1 (d, C_3), 138.6 (s, $C_{1''}$), 153.2 (s, C_{7a}), 157.2 (s, C_4), 168.6 (s, C_6), 170.2 (s, C=O). IR (KBr-disc) ν_{max} 3325, NH; 3250, NH; 3175, NH; 1660 C=O; 1595 cm^{-1} C=C. Anal. calcd for ($C_{19}H_{22}N_6OS$): C, 59.7; H, 5.8; N, 22.0; found: C, 59.6; H, 5.9; N, 22.2%.

α-(4-Amino-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-ylthio)-*N,N*-diethyl-propanamide (7f). Yield 65%; mp dec 222–225 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ 0.98 (t, 3H, J=7.0 Hz, CH_2CH_3), 1.11 (t, 3H, J=7.0 Hz, CH_2CH_3), 1.54 (d, 3H, J=6.6 Hz, CH_3), 3.35 (m, 4H, J=7.2 Hz, 6.8 Hz, 2×CH_2CH_3), 5.02 (q, 1H, J=6.9 Hz, SCH), 7.32 (t, 1H, J=7.4 Hz, $C_{4'}H_{arom}$), 7.57 (t, 2H, J=7.3 Hz, $C_{3'}$, $C_{5'}H_{arom}$), 7.99 (br s, 1H, NH), 8.16 (d, 3H, J=8.5 Hz, $C_{2'}$, $C_{6'}H_{arom}$, NH), 8.29 (s, 1H, C_3H). ^{13}C NMR (50.3 MHz, DMSO-d_6) δ 12.8 (q, CH_2CH_3), 14.8 (q, CH_2CH_3), 19.8 (q, CH_3), 39.2 (t, CH_2CH_3), 40.0 (t, CH_2CH_3), 42.0 (d, SCH), 99.5 (s, C_{3a}), 120.5 (d, $C_{2'}$, $C_{6'}$), 126.2 (s, $C_{4'}$), 129.2 (d, $C_{3'}$, $C_{5'}$), 134.4 (d, C_3), 138.9 (s, $C_{1'}$), 153.5 (s, C_{7a}), 157.6 (s, C_4), 168.0 (s, C_6), 170.1 (s, C=O). IR (KBr-disc) ν_{max} 3325, NH; 3150, NH; 1660, C=O; 1635, C=O; 1600 cm^{-1}, C=C. Anal. calcd for ($C_{18}H_{22}N_6OS$): C, 58.4; H, 6.0; N, 22.7; found: C, 58.2; H, 6.0; N, 23.1%.

α-{6-(1′-*N*-Ethylcarbamoylethylthio)-1-phenylpyrazolo [3,4-*d*]pyrimidin-4-ylthio}-*N*-ethyl-propanamide (8b). 2-Bromo-*N*-ethyl-propanamide (1.19 g, 0.007 mol) was added to a solution of 1-phenylpyrazolo[3,4-*d*]pyrimidine-4,6(5*H*,7*H*)-dithione (0.67 g, 0.0026 mol) in sodium hydroxide solution (1 M, 6 mL). The mixture was stirred at room temperature for 90 min. A white solid precipitated and was recrystallized from DMSO and water to yield α-{6-(1′-*N*-ethylcarbamoylethylthio)-1-phenylpyrazolo [3,4-*d*]pyrimidin-4-ylthio}-*N*-ethyl-propanamide (**8b**). Yield 1.0 g, 85%; mp 191–192 °C. Diastereomers—all peaks are duplicated: ^{1}H NMR (250.1

MHz, DMSO-d_6) δ 0.95 (2×t, 6H, $J=7.2$ Hz, 2×CH_2CH_3), 1.05 (2×t, 6H, $J=7.2$ Hz, 2×CH_2CH_3), 1.58 (2×d, 6H, $J=7.0$ Hz, 2×CH_3), 3.08 (m, 4H, $J=5.5$ Hz, 7.1 Hz, 2×CH_2CH_3), 4.50 (2×q, 1H, $J=7.0$ Hz, C_6SCH), 4.78 (2×q, 1H, $J=7.0$ Hz, C_4SCH), 7.38–8.18 (m, 5H, CH_{arom}), 8.24 (2×br t, 1H, $J=5.0$ Hz, NH), 8.37 (2×br t, 1H, $J=5.0$ Hz, NH), 8.52 (s, 1H, C3H). ^{13}C NMR (62.8 MHz, DMSO-d_6) δ 14.2 (q, 4×CH_2CH_3), 18.1 (q, 2×CH_3), 18.9 (q, 2×CH_3), 33.6 (t, 2×CH_2CH_3), 33.7 (t, 2×CH_2CH_3), 42.7 (d, 2×SCH), 44.0, 44.1 (d, 2×SCH), 110.1 (s, C_{3a}), 120.9 (d, $C_{2'}$, $C_{6'}$), 126.8 (s, $C_{4'}$), 129.4 (d, $C_{3'}$, $C_{5'}$), 133.8 (d, C_3), 138.2 (s, $C_{1'}$), 151.0 (s, C7a), 165.4 (s, C_4), 166.0 (s, C_6), 169.9, (s, 2×C=O), 170.4 (s, 2×C=O). IR (KBr-disc) ν_{max} 3250, NH; 3080, NH; 1675, C=O; 1665, C=O; 1655, C=O; 1645, C=O; 1600 cm^{-1}, C=C. Anal. calcd for ($C_{21}H_{26}N_6O_2S_2$): C, 55.0; H, 5.7; N, 18.3; found: C, 55.3; H, 5.8; N, 18.7%.

α-{6-(1′-*N*-Alkylcarbamoylethylthio)-1-phenylpyrazolo [3,4-*d*]pyrimidin-4-ylthio}amides (8c–e). The following general procedure was used for the remaining compounds: 2-bromo-*N*-propyl-propanamide (3.04 g, 0.016 mol) was added to a solution of 1-phenylpyrazolo [3,4-*d*] pyrimidine-4,6(5*H*,7*H*)-dithione (2.20 g, 0.0085 mol) in sodium hydroxide (1.5 M, 20 mL) with 2-methylpropan-2-ol (20 mL). The mixture was stirred at room temperature for 5 h. A white solid precipitated and was recrystallized from DMSO and water to yield α-{6-(1′-*N*-propylcarbamoylethylthio)-1-phenylpyrazolo [3,4-*d*] pyrimidin-4-ylthio}-*N*-propyl-propanamide (**8c**). Yield 2.6 g, 70%; mp 164.5–169 °C. Diastereoisomers—all peaks are duplicated: ^1H NMR (250.1 MHz, DMSO-d_6) δ 0.73 (t, 6H, $J=7.4$ Hz, 2×$CH_2CH_2CH_3$), 0.82 (2×t, 6H, $J=7.4$ Hz, 2×$CH_2CH_2CH_3$), 1.38 (m, 4H, $J=7.3$ Hz, 2×$CH_2CH_2CH_3$), 1.58 (2×d, 6H, $J=6.9$ Hz, 2×CH_3), 3.00 (m, 4H, $J=5.5$ Hz, 6.9 Hz, 2×CH_2CH_3), 4.49 (2×q, 1H, $J=7.0$ Hz, C_6SCH), 4.79 (2×q, 1H, $J=6.9$ Hz, C_4SCH), 7.34–8.14 (m, 5H, CH_{arom}), 8.21 (2×br t, 1H, NH), 8.35 (2×br t, 1H, $J=5.1$ Hz, NH), 8.48 (s, 1H, H_3). ^{13}C NMR (62.8 MHz, DMSO-d_6) δ 10.6 10.7 (q, 2×$CH_2CH_2CH_3$), 17.8, 18.0, 18.6, 18.7 (q, 4×CH_3), 21.8 (t, 4×$CH_2CH_2CH_3$), 40.5, 40.6 (t, 4×$CH_2CH_2CH_3$), 42.7 (d, 2×SCH), 44.0, 44.1 (d, 2×SCH), 111.0 (s, C_{3a}), 121.8 (d, $C_{2'}$, $C_{6'}$), 127.8 (s, $C_{4'}$), 130.4 (d, $C_{3'}$, $C_{5'}$), 134.9 (d, C_3), 139.3 (s, $C_{1'}$), 152.3 (s, C_{7a}), 165.8 (s, 2×C_4), 169.5, 169.6 (s, 2×C_6), 171.7, 172.2 (s, 4×C=O). IR (KBr-disc) ν_{max} 3300, NH; 3125, NH; 1650, C=O; 1600 cm^{-1}, C=C. Anal. calcd for ($C_{23}H_{30}N_6O_2S_2$): C, 56.8; H, 6.2; N, 17.3; found: C, 57.2; H, 6.3; N, 17.2%.

α-{6-(1′-*N*-Butylcarbamoylethylthio)-1-phenylpyrazolo [3,4-*d*]pyrimidin-4-ylthio}-*N*-butyl-propanamide (8d). Yield 72%; mp 148–149 °C, 165–167 °C. Diastereoisomers—all peaks are duplicated: ^1H NMR (250.1 MHz, DMSO-d_6) δ 0.76 (t, 6H, $J=7.3$ Hz, 2×$CH_2CH_2CH_2CH_3$), 0.84 (2×t, 6H, $J=7.3$ Hz, 2×$CH_2CH_2CH_2CH_3$), 1.29 (m, 8H, $J=7.2$ Hz, 2×$CH_2CH_2CH_2CH_3$), 1.57 (2×d, 6H, $J=6.9$ Hz, 2×CH3), 3.03 (m, 4H, $J=7.4$ Hz, 6.2 Hz, 2×CH_2CH_3), 4.50 (2×q, 1H, $J=7.0$ Hz, C_6SCH), 4.80 (2×q, 1H, $J=7.0$ Hz, C_4SCH), 7.36–8.16 (m, 6H, 5CH_{arom}, NH), 8.28 (2×br t, 1H, $J=5.5$ Hz, N*H*), 8.50 (s, 1H, H_3). ^{13}C NMR (75.5 MHz, DMSO-d_6) δ 13.2 13.3 (q, 2×$CH_2CH_2CH_2CH_3$), 18.0, 18.1, 18.7, 18.8 (q, 4×CH_3), 19.1, 19.2 (t, 4×$CH_2CH_2CH_2CH_3$), 30.8 (t, 4×$CH_2CH_2CH_2CH_3$), 38.3, 38.4 (t, 4×$CH_2CH_2CH_2CH_3$), 42.6 (d, 2×SCH), 43.9, 44.0 (d, 2×SCH), 109.9, 110.0 (s, 2×C_{3a}), 120.7 (d, $C_{2'}$, $C_{6'}$), 126.5 (s, $C_{4'}$), 129.1 (d, $C_{3'}$, $C_{5'}$), 133.5 (d, C_3), 137.9 (s, $C_{1'}$), 150.8 (s, C_{7a}), 164.0, 164.1 (s, 2×C_4), 167.7, 167.8 (s, 2×C_6), 169.8, 170.3 (s, 4×C=O). IR (KBr-disc) ν_{max} 3285, NH; 3100, NH; 1650, C=O; 1600 cm^{-1}, C=C. Anal. calcd for ($C_{25}H_{34}N_6O_2S_2$): C, 58.3; H, 6.7; N, 16.3; found: C, 58.7; H, 6.9; N, 16.2%.

α-{6-(1′-*N*-Cyclopentylcarbamoylethylthio)-1-phenylpyrazolo[3,4-*d*]pyrimidin-4-ylthio}-*N*-cyclopentyl-propanamide (8e). Yield 72%; mp 186–188 °C, 196–199 °C. Diastereoisomers—all peaks are duplicated: ^1H NMR (200 MHz, DMSO-d_6) δ 1.15–1.82 (m, 16H, 8×CH_2 of cyclopentyl groups), 1.57 (2×d, 6H, $J=6.9$ Hz, 2×CH_3), 3.97 (m, 2H, $J=6.6$ Hz, 2×$C_{1'}H$), 4.50 (2×q, 1H, $J=6.9$ Hz, C_6SCH), 4.75 (2×q, 1H, $J=7.0$ Hz, C_4SCH), 7.35–8.17 (m, 6H, $J=7.0$ Hz, 5CH_{arom}, 1NH), 8.31 (2×br t, 1H, $J=6.9$ Hz, NH), 8.50 (s, 1H, H_3). ^{13}C NMR (50.3 MHz, DMSO-d_6) δ 18.1, 18.4 (q, 2×CH_3), 18.9, 19.1 (q, 2×CH_3), 23.3, 23.4 (t, 8×CH_2), 42.8, 42.9 (d, 2×SCH), 44.0, 44.2, (d, 2×SCH), 42.6 (d, 2×SCH), 50.6 (d, 2×$C_{1'}H$), 100.0, 110.1 (s, 2×C_{3a}), 120.8 (d, $C_{2''}$, $C_{6''}$), 126.7 (s, $C_{4''}$), 129.4 (d, $C_{3''}$, $C_{5''}$), 133.8 (d, C_3), 138.0 (s, $C_{1''}$), 150.8 (s, C_{7a}), 164.2, 164.3 (s, 2×C_4), 168.0, 168.1 (s, 2×C_6), 169.4, 169.9 (s, 4×C=O). IR (KBr-disc) ν_{max} 3300, NH; 3100, NH; 1650, C=O; 1600 cm^{-1}, C=C. Anal. calcd for ($C_{27}H_{34}N_6O_2S_2$): C, 60.2; H, 6.4; N, 15.6; found: C, 60.4; H, 6.5; N, 15.4%.

Preparation of membranes from rat cerebral cortex and rat cerebellum

This followed a simplified version of the Gray and Whittaker method.[12,20] Whole brains from Wistar rats (25 rats, 300–350 g) were immersed in 0.32 M ice-cold sucrose. The striata were explanted and placed in 10 mL ice-cold buffer (50 mM Tris–HCl (Sigma), 10 mM $MgCl_2$, pH 7.4), to be used for the preparation of A_2 adenosine receptors. The cerebellum and remainder of the cerebral cortex were placed in 15 mL of ice-cold buffer (50 mM Tris–HCl, 1 mM $MgCl_2$, pH 7.4). The tissue was homogenized (Brinkman polytron) and the volume adjusted to 48.0 g. Homogenates were centrifuged (1000 *g* for 10 min at 4 °C) and the supernatant (S_1) recentrifuged (35,000 *g* for 15 min at 4 °C). The supernatant (S_2) was discarded and every eight pellets (P_2) pooled and resuspended in ice-cold distilled water (30 mL) and the volume was adjusted to 48.00 g. The homogenates were recentrifuged (35,000 g for 15 min at 4 °C). The pellets (P_3) were pooled and resuspended in ice-cold buffer (60 mL of 50 mM Tris–HCl, 1 mM $MgCl_2$, pH 7.4). When stored at −30 °C, binding to the synaptosomal membranes remained unchanged for 1 month. The protein concentration was estimated using Pierce BCA protein assay reagent using a modified Lowry protein assay.[21] Tissue samples were solubilized using 10 μL of 10% SDS and were made up to 100 μL. The standards contained 0 to 120 mg of protein. Five dilutions of tissue from 4 to 75% were sampled. The absorbance was read using a Titertek Multiscan MC

Microtiter Plate Reader at 540 nm absorbance. The average concentration of A_1 tissue was 2.63 μg/μL.

Preparation of membranes from rat striata

The buffer was drained from the striata and weighed. The striata were placed in 20 mL ice-cold buffer (50 mM Tris–HCl (Sigma), 10 mM $MgCl_2$, pH 7.4) and homogenized using the polytron, placed into centrifuge tubes and the volume adjusted to 48.00 g, and centrifuged (35 000 *g* for 15 min at 4 °C). The supernatant was discarded and the pellet resuspended in ice-cold buffer (10 mL of 50 mM Tris–HCl, 10 mM $MgCl_2$, pH 7.4). The homogenate was recentrifuged (35 000 *g* for 15 min at 4 °C). The supernatant was discarded and the pellets pooled and resuspended in ice-cold buffer (1 g of striata/10 mL of 50 mM Tris–HCl, 10 mM $MgCl_2$, pH 7.4). This was stored at −30 °C and remained unchanged for 1 month. The protein concentration was estimated using Pierce BCA protein assay reagent. The average concentration of A_{2A} tissue was 3.78 μg/μL.

[^{3}H]-(*R*)-N^6-(Phenylisopropyl)adenosine binding assay[22]

Aliquots of A_1 tissue were thawed and preincubated with adenosine deaminase (1 mg/mL for 20 min at 37 °C; ADA, EC 3.5.4.4; Sigma) to remove endogenous adenosine. Between 0.3 and 0.5 mg of protein per replicate was incubated with 2 nM [^{3}H]-(*R*)-N^6-(phenylisopropyl) adenosine (60 Ci/mmol, 1 mCi/mL; New England Nuclear) for 1 h at 37 °C in an incubation volume of 0.5 mL. Radioligand binding assays were performed over 10 concentrations in duplicates. Each compound was tested in two different experiments. Total volume of the A_1 assay was 0.5 mL. The compounds proved insoluble in buffer and hence the stock concentrations were made up in DMSO. The final concentration of DMSO in the assay was 1%. The assay was monitored against a control of total binding and non-specific binding each containing 1% DMSO. Non-specific binding was determined using cold CPA (0.1 mM final concentration). Reactions were terminated by the addition of 3 mL of ice-cold incubation buffer. This was filtered over Whatman GF/B Glass fiber filters and washed with two more 3-mL ice-cold buffer washes. The filters were transferred to vials, thoroughly shaken with 3 mL of BCS scintillant fluid. Samples were equilibrated in the dark for a minimum of 6 h before counting for 1 min in a Packard Tricarb 2000CA series liquid scintillation Analyzer at 40% efficiency.

The results from concentration–inhibition studies were analyzed with non-linear-least-squares curve-fitting program.[23] A_1K_i values were calculated using the Cheng–Prusoff equation[24] using the average K_d value of [^{3}H]*R*-PIA as 2.35 nM and a final ligand concentration of 2 nM. A_1K_i values are geometric means from two determinations, run in duplicate ± standard error.

[^{3}H]-CGS21680 binding assay[25]

Aliquots of A_{2A} tissue were thawed and preincubated with adenosine deaminase (1 mg/mL for 20 min at 37 °C; ADA, EC 3.5.4.4) to remove endogenous adenosine. Between 0.3 and 0.5 mg of protein per replicate was incubated with 5 nM [^{3}H]-CGS21680 (48.6 Ci/mmol, 1 mCi/mL; New England Nuclear) for 95 min at room temperature. Radioligand binding assays were performed over 10 concentrations in duplicates. Each compound was tested in two different experiments. Total volume of the A_{2A} assay was 0.5 mL. The compounds proved insoluble in buffer and hence the stock concentrations were made up in DMSO. The final concentration of DMSO in the assay was 1 percent. The assay was monitored against a control of total binding and non-specific binding each containing 1 percent DMSO. Non-specific binding was determined using cold 2-CADO (0.1 mM final concentration). Reactions were terminated by the addition of 3 mL of ice-cold incubation buffer. This was filtered over presoaked (6 h) Whatman GF/B Glass fiber filters and washed with two more 3 mL ice-cold buffer washes. The filters were transferred to vials, thoroughly shaken with 3 mL of BCS scintillant fluid. Samples were equilibrated in the dark for a minimum of 6 h before counting for 1 min in a Packard Tricarb 2000CA series liquid scintillation Analyzer at 40% efficiency.

The results from concentration–inhibition studies were analyzed with non-linear-least-squares curve-fitting program.[23] $A_{2A}K_i$ values were calculated using the Cheng Prusoff equation[24] using the average K_d value of [^{3}H]-CGS21680 as 14.9 nM and a final ligand concentration of 5 nM. $A_{2A}K_i$ values are geometric means from two determinations, run in duplicate±standard error.

Acknowledgements

We thank the National Health and Medical Research Council for support of this research.

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