# Design, Synthesis and binding studies of Trisubstitutedpyrazolo[3,4-*d*]pyrimidines

A thesis submitted in partial fulfillment
of the requirements for admission
to the degree of

Doctor of Philosophy (PhD)

By

**Khoa Quang Do**

Faculty of Science and Technology

Griffith University

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# Statement

The work described in this thesis was carried out in the Faculty of Science and Technology, Griffith University under the supervision of Professor Ron Quinn. Unless otherwise stated, it is the work of the author and has not been and is not currently being submitted for any other degree.

Signed

July 2005

# Acknowledgements

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# Synopsis

Pyrazolo[3,4-*d*]pyrimidines were known as adenosine antagonists at the rat $A_1$ and $A_{2A}$ adenosine receptors based on our previous studies. In this study, 245 pyrazolo[3,4-*d*]pyrimidines derivatives with various benzyl substitutents at N-1 and various hydrophobic side chains at C-4 and C-6 were synthesized and screened at the human $A_1$, $A_{2A}$ and $A_3$ adenosine receptors. 14 out of 245 compounds were resynthesized and purified to determine the $K_i$ values of these compounds at the human $A_1$ adenosine receptor.

Chapter 1 of the thesis is a literature review of adenosine research. It describes the physiology of adenosine and the discovery and characterization of all adenosine receptors namely $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$. It also looks at the medical application of adenosine, adenosine analogs, adenosine agonists and adenosine antagonists. The final part of the chapter discusses the discovery and development of adenosine agonists and antagonists

Chapter 2 of the thesis describes the rational design of the pyrazolo[3,4-*d*]pyrimidines template using ligand-based molecular modelling technique and describes the synthesis of the template.

Chapter 3 and chapter 4 describe the application of silicon chemistry and attempts to synthesise a series of pyrazolo[3,4-*d*]pyrimidines heterocycle by solid phase synthesis.

Chapter 5 and chapter 6 describe the synthesis of a series of pyrazolo[3,4-*d*]pyrimidines heterocycle using the solution phase parallel synthesis and the binding studies of a library of 245 compounds and the resynthesis of 14 target compounds.

Chapter 7 describes the cell culture and membrane preparation of the human $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ adenosine receptors and radioligands binding assays.

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# Abbreviations

| | |
|---|---|
| AB-MECA | $N^6$-(4-Amino-3-iodobenzyl)-adenosine-5'-N-methyl-uronamide |
| a.m.u | Atomic mass unit |
| Anal. calcd | Analysis calculated |
| APNEA | $N^6$-2-(4-Aminophenyl)ethyl-adenosine |
| Ar | Aromatic |
| ATP | Adenosine triphosphate |
| AMP | Adenosine monophosphate |
| cAMP | Adenosine-3',5'-cyclic monophosphate |
| 9-BBN | 9-borabicyclo[3.3.1]nonane |
| Bn | Benzyl |
| bp | Boiling point |
| BSA | Bovine serum albumin |
| Bu | Butyl |
| BuLi | Butyllithium |
| $^{n}$BuLi | Butyllithium |
| $^{t}$BuLi | Tertiary Butyllithium |
| 2-CADO | 2-Chloroadenosine |
| cat | Catalyst |
| CCPA | 2-Chloro- $N^6$-cyclopentyladenosine |
| CGS 15943 | 5-Amino-9-chloro-2-(2-furyl)-1,2,4-triazolo [1,5-*c*]quinazoline |
| CGS 21680 | (2-*p*-Carboxyethyl)phenylamino-5'-N-ethylcarboxamidoadenosine |
| CHA | $N^6$-Cyclohexyladenosine |
| CHO | Chinese hamster ovary |
| CNS | Central nervous system |
| COS-7 | African green monkey kidney |
| CPA | $N^6$-Cyclopentyladenosine |

| | |
|---|---|
| DABCO | 1,4-Diazabicyclo[2.2.2]octane |
| DCM | Dichloromethane |
| DIPEA | Diisopropylethylamine |
| DMBA | Dimethylbarbituric acid |
| cDNA | Complementary deoxyribonucleic acid |
| DMF | Dimethylformamide |
| DMPA | $N^6$-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)ethyl]adenosine |
| DMSO | Dimethyl sulfoxide |
| DMSO-$d_6$ | Deuterated dimethyl sulfoxide |
| $D_2O$ | Deuterium oxide |
| DPCPX | 8-Cyclopentyl-1,3-dipropylxanthine |
| EDTA | Ethylinediamine tetraacetate |
| eq | Equivalent |
| ESMS | Electrospray mass spectrometer |
| Et | Ethyl |
| g | Gram |
| GDP | Guanine diphosphate |
| GPCR | G-protein-coupled receptor |
| GTP | Guanine-5'-triphosphate |
| G-protein | Guanine nucleotide-binding regulatory protein |
| h | Hour |
| HEK 293 | Human embryonic kidney |
| Hz | Hertz |
| IB-MECA | $N^6$-(3-iodobenzyl)-adenosine-5'-N-methyl-uronamide |
| $IC_{50}$ | Concentration of test compound to cause 50% inhibition of radioligand binding to receptor |
| IR | Infrared |
| IUPHAR | International Union of Pharmacology |
| $K_i$ | Dissociation constant of test compound |

| | |
|---|---|
| $K_d$ | Dissociation constant of radioligand |
| M | Molar |
| Me | Methyl |
| MECA | 5'-N-methylcarboxamidoadenosine |
| mg | Milligram |
| min | Minute |
| ml | Millimetre |
| mm | Millilitre |
| mmol | Millimole |
| mol | Mole |
| µl | Micorlitre |
| µM | Micromolar |
| mp | Melting point |
| MRE-3008-F20 | 5-N-(4-Methoxyphenyl-carbamoyl)amino-8-Propyl-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo [1,5-*c*]pyrimidine |
| MSX-2 | 3-(3-Hydroxypropyl)-8-(*m*-methoxystyryl)-1-propargylxanthine |
| NECA | 5'-N-ethylcarboxamidoadenosine |
| NI | Negative ionisation |
| nM | Nanomolar |
| n.m.r or NMR | nuclear magnetic resonance |
| O/N | Overnight |
| PBS | Phosphate buffered saline |
| PEI | Polyethyleneimine |
| Ph | Phenyl |
| PI | Positive ionisation |
| PIA | $N^6$-phenylisopropyladenosine |
| PNS | Peripheral nervous system |
| ppm | Parts per million |
| Pyr | Pyridine |

| | |
|---|---|
| R-PIA | $N^6$-(L-2-phenylisopropyl)adenosine |
| RT | Room temperature |
| SCH 58261 | (5-Amino-7-(2-phenylethyl)-2-(2-furyl)pyrazolo [4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine |
| SDS | Sodium dodecyl sulfate |
| δ | Chemical shift |
| SM | Starting material |
| THF | Tetrahydrofuran |
| Tris | Tris[hydroxymethyl]aminomethane hydrochloric acid |
| p-TsOH | p-Toluenesulfonic acid monohydrate |
| U | Unit |
| XAC | 8-{4-[([{(2-Aminoethyl)amino}carbonyl]methyl) oxy]phenyl-1,3-dipropyl-xanthine} |
| 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 |

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# CHAPTER 1

# Introduction

## 1.1 Physiology of adenosine

The adenosine regulation of mammalian tissue function was first described in 1929 by Drury and Szent-Gyorgyi.[1] Adenosine, extracted from heart muscle, was reported to have pronounced physiological effects including heart block, arterial dilatation, lowering of blood pressure and inhibition of intestinal contraction. This discovery by Drury and Szent-Gyorgyi led Honey *et al.*[2] to explore the therapeutic potential of adenosine in 1930. The discovery of the short half-life of adenosine in the body's circulation by Honey *et al.* limited the investigation into the role of this endogenous nucleoside in the mammalian tissues for three decades.

The physiological role of endogenous adenosine was further examined following the study showing that adenosine played an important role during hypoxia.[3] In 1963, Berne hypothesized that the levels of adenosine increased during the ischaemia/hypoxia and its role in regulating the amount of oxygen supply and demand to the organ in the body. **Figure 1.1** shows adenosine's role in regulating the amount of oxygen supply/demand and in restoring the energy supply/demand balance within tissue.[4] An increase in oxygen demand of tissue during exercise or a decrease in the oxygen supply during ischaemia or hypoxia results in an increase in adenosine levels because of the degradation of released ATP. Adenosine is transported out of the cell and interacts with the cell surface receptors to produce a response and hence to restore the energy supply/demand balance within the tissue.

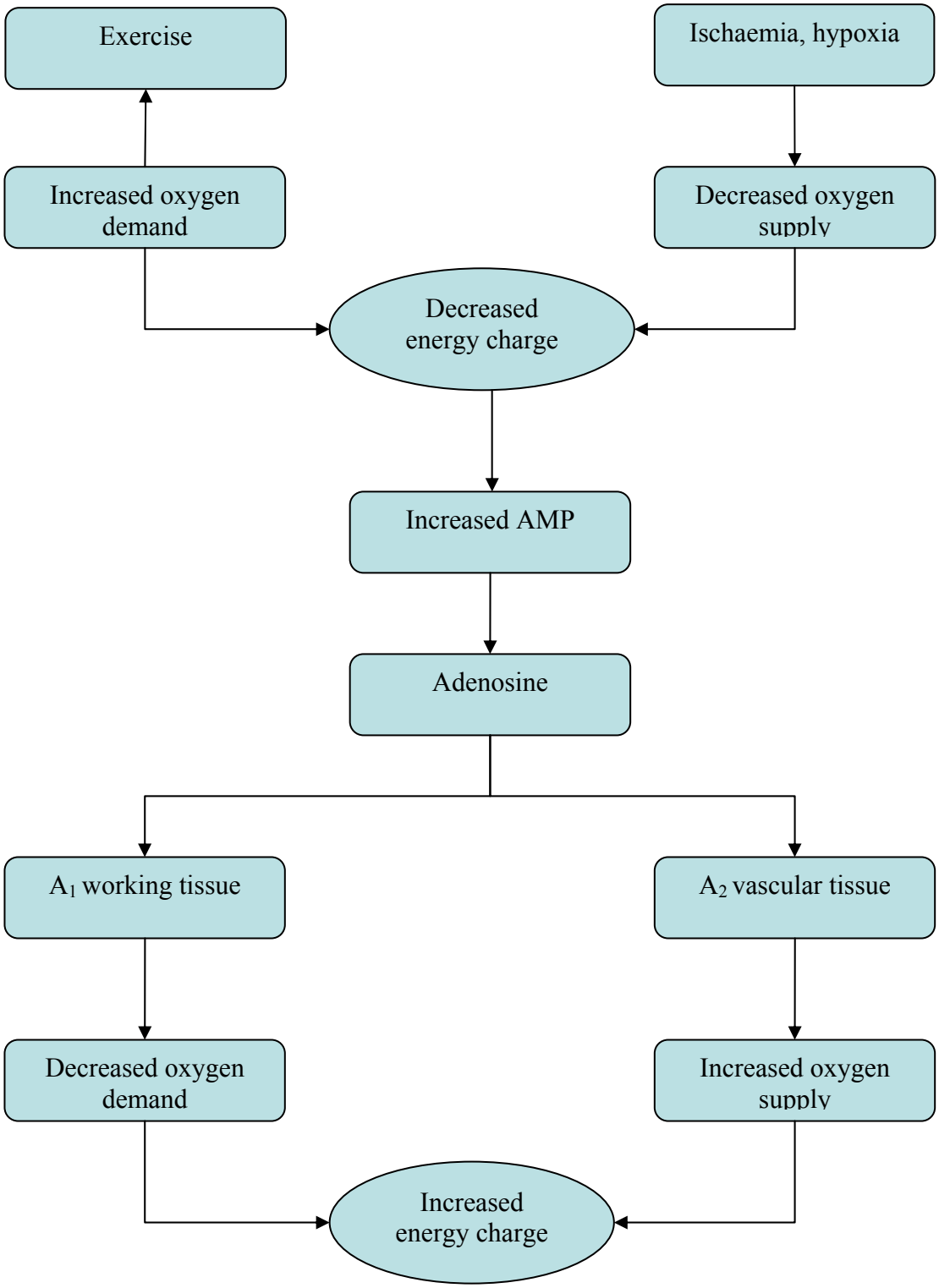



**Figure 1.1**: Adenosine's role in energy supply/demand balance.[4]

Adenosine's role has been extensively studied after the discovery of the presence of cell surface receptors[5] for adenosine and the competitive studies between adenosine and methylxanthines in the heart[6] and in the brain.[6] Some of the physiological effects of adenosine and its analogues on different tissues and organs are summarized in **Table 1.1**.

**Table 1.1**: Physiological effects of adenosine in different tissues and organs.

| Tissue/Organ | Physiological effects of adenosine and its analogues |
|---|---|
| Adipocytes | Inhibition of lipolysis[7,8] |
| Cardiovascular | Inhibition of platelet aggregation[9-12]<br>Vasodilation[13] |
| CNS | Anticonvulsant[14,15]<br>Antipsychotic[16]<br>Inhibition of neurotransmitters release[17]<br>Locomotor activity[18] |
| Heart | Antiarrhythmic[19]<br>Coronary vasodilation[3,5,20-22]<br>Ischaemic preconditioning[23,24] |
| Immune system | Antiinflammatory[25-27] |
| Kidneys | Inhibition of rennin release[28,29] |
| Liver | Stimulation of glucagon secretion[30] |
| Muscle | Relaxation[31]<br>Inhibition of tumor cell growth[32,33] |
| Stomach | Inhibition of gastric acid secretion[34,35] |

It was known that adenosine produced a large variety of effects throughout the body by interacting with the cell surface receptors. However, the mechanism of adenosine and cell surface receptor interaction which contributed to diseases was unclear. Therefore, an understanding of the molecular structure, mechanism and pharmacology of its cell surface receptor was fundamental to the realization of the

therapeutic potential for adenosine and ligands and provided a foundation for the continuation of active research in the adenosine field.

## 1.2 Adenosine receptors

Adenosine receptors are members of the G-protein-coupled family which also include many well known receptors such as dopamine receptors, adrenergic receptors, histamine receptors, serotonin receptors. They are responsible for the transduction of a diverse array of extracellular signals into the cells by activating one or more heterotrimeric G-proteins located on the cytoplasmic face of the plasma membrane and subsequently interacting with the effector systems including ion channels, phospholipases and adenylate cyclase (**Figure 1.2**). There are over 1000 members[36] of the G-protein-coupled receptor which are responsible for many diseases, hence making them important targets for drug development.[37]

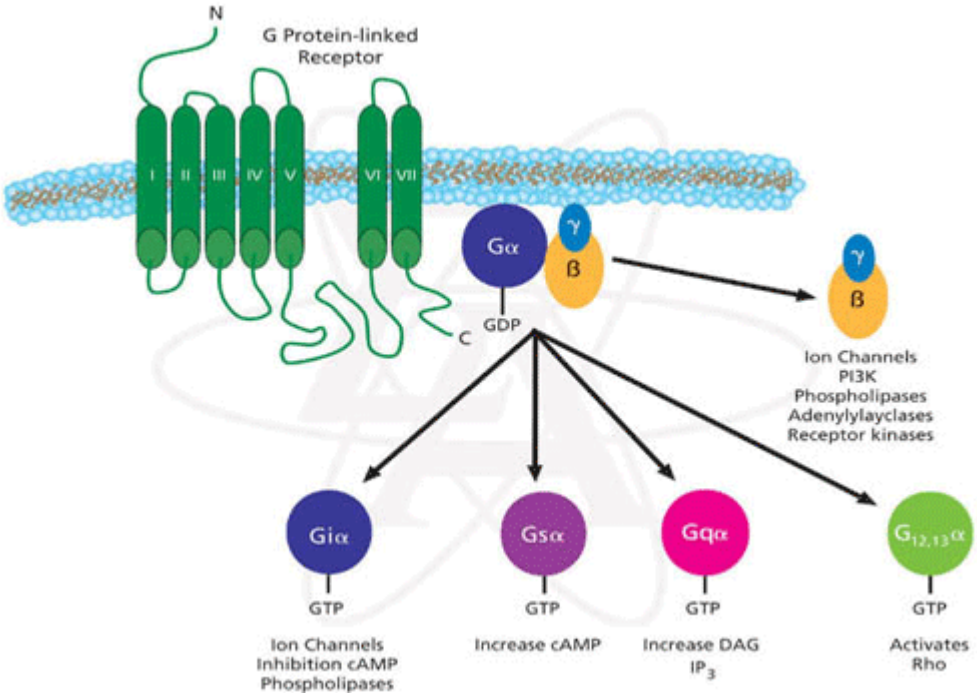



**Figure 1.2**: Diversity of GPCR signal transduction pathways. This figure was adapted from www.sigma-aldrich.com.

Similar to other G protein-coupled receptors, adenosine receptor consists of seven transmembrane helices which accommodate the binding site for ligands. Each helix is constituted by approximately 21 to 28 amino acids. The transmembrane helices are connected by three extracellular and three cytoplasmic loops of unequal size of amino acids. The N-terminal of the protein is on the extracellular side and the C-terminal on the cytoplasmic side of the membrane (**Figure 1.3**).

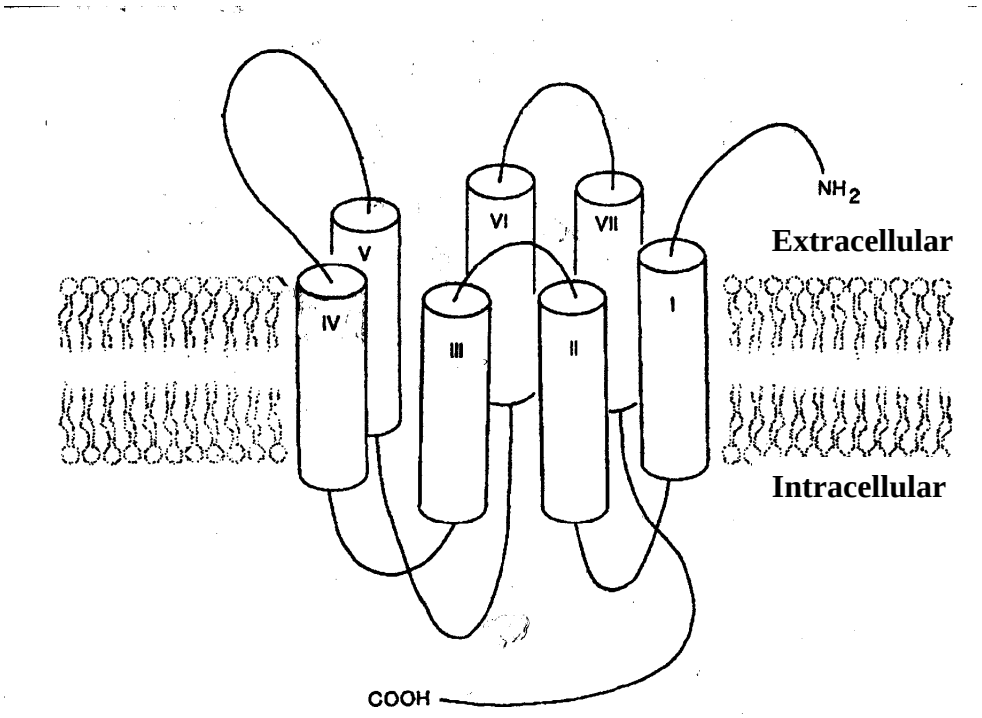



**Figure 1.3**: Schematic of the adenosine receptors. This figure was adapted from Ralevic *et al.*[38]

The existence of adenosine receptors was first suggested by Cobbin[5] *et al.* when they studied the coronary dilator actions of adenosine analogues. Burnstock[39] first proposed the receptors for adenosine and adenine nucleotides and classified them as P1 and P2 purinergic receptors respectively. The classification was based on three criteria (i) the relative potencies of adenosine and adenine nucleotides; (ii) the selective antagonism of the effects of adenosine by methylxanthine; and (iii) the modulation of activity of adenylate cyclase by adenosine and stimulation of prostaglandin synthesis by adenine nucleotides. P1 receptors were later named as

adenosine receptors by the International Union of Pharmacology (IUPHAR) committee[40,41] because the endogenous adenosine interacted with them.

Adenosine (or P1) receptors have been further subdivided into four distinct subtypes namely $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$. Each of the subtypes has been characterized by molecular, biochemical and pharmacological studies.

### 1.2.1 $A_1$ adenosine receptor

$A_1$ adenosine receptor, one of the first four adenosine receptor subtypes, was first discovered and defined on the basis of the biochemical and pharmacological studies. Van Calker[42] and Londos[43] independently proposed the presence of $A_1$ adenosine receptor based on the biochemical studies of adenylate cyclase activity and pharmacological studies of the relative potency of the two adnenosine analogues $N^6$-(phenylisopropyl)adenosine and 5'-N-ethylcarboxamidoadenosine on adenylate cyclase activity in cultured mouse brain cells, rat adipocytes and Leydig tumor cells. The classification of $A_1$ adenosine receptor was later confirmed by molecular biology studies, which allowed the determination of the primary structure of the receptor

**Molecular biology**

RDC7 was cloned from a canine thyroid cDNA library[44,45] and characterized as an $A_1$ adenosine receptor based on the binding of [$^3$H]CPA and inhibition of adenylate cyclase[45] in forskolin-stimulated CHO cells stably transfected with RDC7. $A_1$ adenosine receptors of rat brain[46,47], bovine brain[48,49], human brain[50,51] were also cloned and characterized.

Comparison of the amino acid sequences of each cloned $A_1$ adenosine receptor found small variation in the primary sequence of the $A_1$ adenosine receptor. As seen from **Figure 1.4**, there was less than 5% difference in the primary sequence of the $A_1$ adenosine receptor between human and guinea pig and less than 10% difference in the primary sequence between human, rat, dog and cow. The primary sequence for the $A_1$ adenosine receptor comprises of 326-328 amino acid residues dependant on the species.

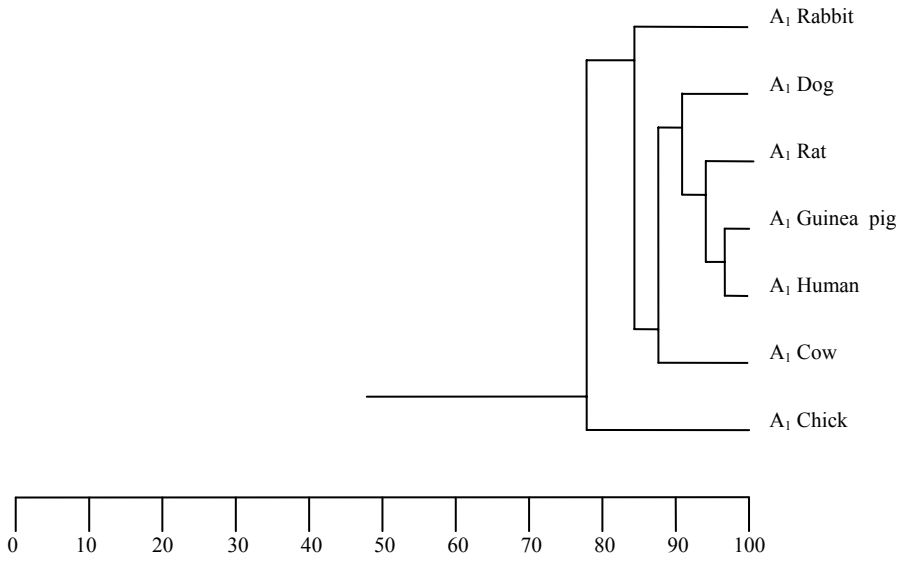



**Figure 1.4**: Sequence homology for the cloned $A_1$ adenosine receptors between species. This figure was redrawn from Fredholm *et al*.[52]

## Signal Transduction Mechanisms

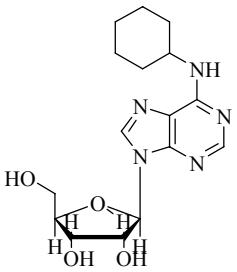
The $A_1$ adenosine receptor transduces extracelluar signals to the cell interior by activating the heterotrimeric $G_i$ proteins located on the cytoplasmic domain of the membrane. The $G_i$ proteins consist of a complex made up of $\alpha$, $\beta$ and $\gamma$ subunits. They interact with both $A_1$ adenosine receptor and the effector. When a ligand binds to the $A_1$ adenosine receptor, the ligand-receptor complex induces an exchange of GDP for GTP on the $G_i$ protein $\alpha$ subunit followed by the dissociation of the GTP-$\alpha$ subunit complex from the $\beta\gamma$ dimer. The GTP-$\alpha$ subunit either inhibits adenylate cyclase to decrease the levels of cAMP production (**Figure1.2**)[43,53]; or activates phospholipase C to increase the production of inositol 1,4,5-triphosphate[54,55]; or activates potassium[56-60] and calcium[61,62] ion channels.

## Pharmacology

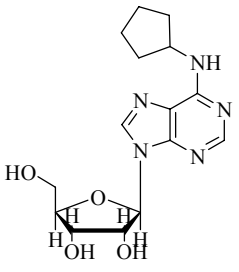
The $A_1$ adenosine receptor was first discovered[42] and classified[43] after two adenosine analogues, $N^6$-phenylisopropyladenosine and 5'-N-ethylcarboxamidoadenosine, were used to study the stimulation and inhibition of

adenylate cyclase activity in the rat adipocyte and mouse brain cells. A variety of different classes of compounds were then developed in the search for more potent and selective ligands at the $A_1$ adenosine receptor. Some of the ligands that have been developed and used in the classification of the $A_1$ adenosine receptors are shown in **Figure 1.5**.

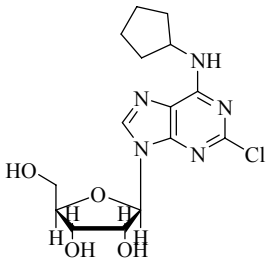
**Selective $A_1$ receptor agonists**



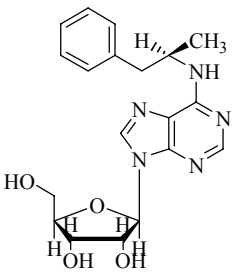
**CHA**



**CPA**

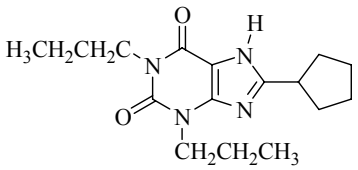


**CCPA**

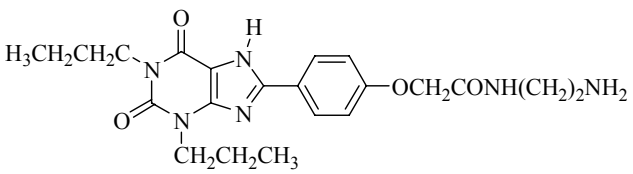


**R-PIA**

**Selective $A_1$ receptor antagonists**



**DPCPX**



**XAC**

**Figure 1.5**: The chemical structure of some agonists and antagonists at $A_1$ adenosine receptor.

Details regarding the molecular structure, signal transduction mechanisms and pharmacology of $A_1$ adenosine receptor subtypes are summarized in **Table 1.2**.

**Table 1.2**: Characterisation of $A_1$ adenosine receptor.

| | |
|---|---|
| Tissue distribution | Central nervous system[47,63]: cerebral cortex, hippocampus, cerebellum, thalamus, brain stem and spinal cord. Peripheral tissue[47,63,64]: testis, white adipose tissue, stomach, spleen, pituitary, heart, aorta, liver, eye, bladder, lung , kidney and small intestine |
| Cloning | Human[50,51], canine[44], bovine[48,49], rabbit[65], rat[46,47], mouse[66], pig[67] |
| Structural information | 326-328 amino acids |
| Structural type | G protein-coupled: $G_{i1/2/3}$ and $G_o$ |
| Effects of G Protein Coupling | $G_{i/1/2/3}$: Inhibition of adenylate cyclase → decrease in cAMP[43,53,68] $G_{i/1/2/3}$: Activation of phospholipase C → increase in $IP_3$[54,55,69] $G_{i/1/2/3}$: Activation of $K^+$ channels[56,57] $G_{i/1/2/3}$: Inactivation of $Ca^{2+}$ channels[61,62] |
| Pharmacological profile | Selective agonist[70,71]: CHA, CPA, CCPA and R-PIA Selective antagonist[70]: DPCPX and XAC |

### 1.2.2 $A_{2A}$ adenosine receptor

Like the $A_1$ adenosine receptor, the $A_2$ adenosine receptor was identified independently by two research groups Londos et al.[43] and Van Calker et al.[42] based on the relative potency of adenosine analogues $N^6$-(phenylisopropyl)adenosine and 5'-N-ethylcarboxamidoadenosine which stimulated cAMP formation in hepatocytes and cultured mouse brain cells. The $A_2$ adenosine receptor was further subdivided into two classes $A_{2A}$ and $A_{2B}$ based on high affinity binding sites and low affinity binding sites for stimulation of adenylate cyclase.[72,73] Molecular cloning of two different adenosine $A_2$ receptors, $A_{2A}$ adenosine receptor and $A_{2B}$ adenosine receptor, and pharmacological studies confirmed the existence of these two subtypes.

**Molecular biology**

RDC8 was characterized as an $A_{2A}$ adenosine receptor based on the activation of adenylate cyclase and on the binding of [$^3$H]CGS 21680 and [$^3$H]NECA in cells transfected with RDC8.[74] $A_{2A}$ adenosine receptors of rat brain[75,76], human hippocampus[77] and guinea pig brain[78] have also been cloned.

Analysis of the amino acid sequences of each cloned $A_{2A}$ adenosine receptor found that the carboxy terminal domain of the $A_{2A}$ adenosine receptor was longer than that of other adenosine receptor subtypes. $A_{2A}$ adenosine receptor therefore had a greater molecular weight compared to other adenosine receptors. In common with the other adenosine receptors, there is a variation in the primary sequence of the $A_{2A}$ adenosine receptor between species (**Fig 1.6**). For example there is 16% difference in the primary sequence between human and rat.[75-77,79] The primary sequence for the $A_{2A}$ adenosine receptor contains 409-411 amino acid residues dependant on the species (**Table 1.3**).

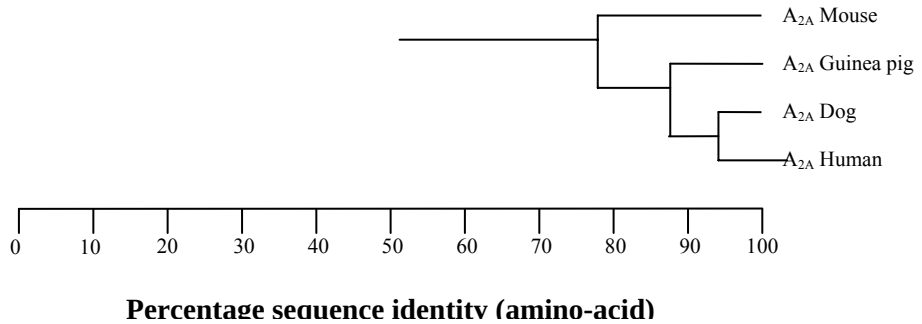



**Figure 1.6**: Sequence homology for cloned $A_{2A}$ adenosine receptors between species. This figure was redrawn from Fredholm *et al*.[52]

## Signal Transduction Mechanisms

The most recognized signal transduction mechanism for the $A_{2A}$ adenosine receptor is the activation of adenylate cyclase. The $A_{2A}$ adenosine receptor is coupled to the heterotrimeric $G_s$ proteins located on the cytoplasmic domain of the membrane. The free GTP-$\alpha$ subunit ($G_s$) stimulates the enzyme adenylate cyclase to increase the levels of cAMP productions (**Fig 1.2**).

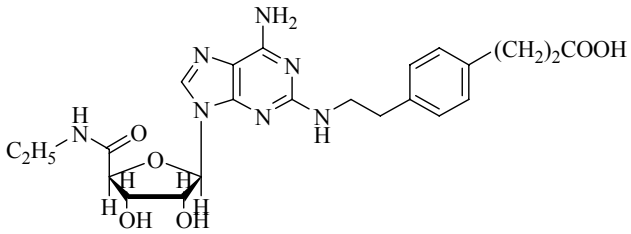
cAMP-independent signal transduction has been suggested for $A_{2A}$ adenosine receptor on striatal nerve terminals[80] and striatal cholinergic nerve terminals.[81] In striatal nerve terminals, $A_{2A}$ adenosine receptor mediated dual signaling via P- and N-type $Ca^{2+}$ channels linked to $G_s$/adenylate cyclase/PKA and cholera toxin-insensitive G protein/PKC respectively.[81]

## Pharmacology

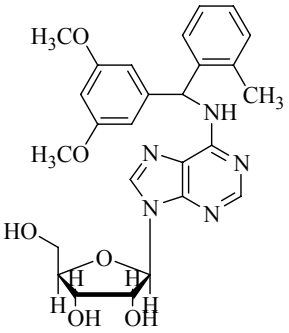
NECA, which is not a selective adenosine receptor agonist, has been used as an $A_{2A}$ receptor agonist since the discovery of the $A_{2A}$ adenosine receptor in 1979 by Van Calker[42] and Londos.[43] However, CGS 21680, a derivative of NECA, has been used to discriminate $A_{2A}$ receptor from $A_{2B}$ receptor subtype when they were coexpressed since it has a very low affinity at the $A_{2B}$ receptors.[82,83] It is 140-fold selective for the $A_{2A}$ receptor over $A_1$ receptor.[84]

Over the years, several non-xanthine $A_{2A}$ receptor antagonists were also developed for pharmacological studies. The most selective antagonists are ZM 241385[85] and SCH 58261.[86,87] Some of the ligands that have been developed and used in the classification of the $A_{2A}$ adenosine receptors are shown in **Figure 1.7**.

**Selective $A_{2A}$ receptor agonists**

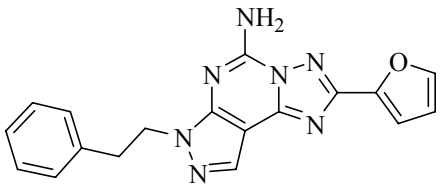


**CGS 21680**

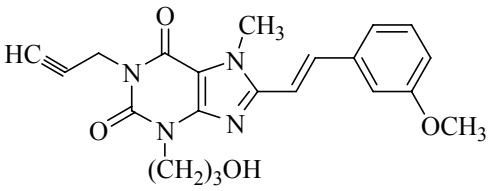


**DPMA**

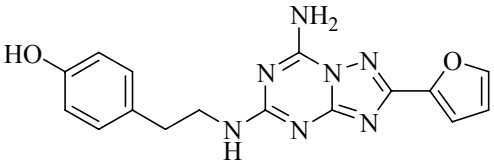
**Selective $A_{2A}$ receptor antagonists**



**SCH 58261**



**MSX-2**



**ZM 241385**

**Figure 1.7**: The chemical structure of some agonists and antagonists at $A_{2A}$ adenosine receptor.

Details regarding the molecular structure, signal transduction mechanisms and pharmacology of $A_{2A}$ adenosine receptor subtypes are summarized in **Table 1.3** below.

**Table 1.3**: Characterisation of $A_{2A}$ adenosine receptor.

| | |
|---|---|
| Tissue distribution | CNS[88]: striatum, nucleus accumbens and olfactory tubercle<br>PNS[63,89]: immune tissues, eye, skeletal muscle, heart, lung, bladder, kidney, small intestine, spleen, stomach, testis |
| Cloning | Human[77], canine[44,74], rat[75,76], guinea-pig[78] and mouse[66] |
| Structural information | 409-411 amino acids |
| Structural type | G protein-coupled: $G_s$ |
| Effects of G Protein Coupling | $G_s$: Activation of adenylate cyclase → increase in cAMP[90] |
| Pharmacological profile | Selective agonist[70,91]: CGS 21680 and DPMA<br>Selective antagonist[92,93]: SCH 58261, ZM 241385 and MSX-2 |

### 1.2.3 $A_{2B}$ adenosine receptor

There are no selective agonists or antagonists for $A_{2B}$ adenosine receptor. Activation of adenylate cyclase in membranes and accumulation of cAMP in cells was used to characterize the $A_{2B}$ adenosine receptor. Molecular cloning of the $A_{2B}$ adenosine receptor in 1992 followed by pharmacological studies confirmed the evidence for the existence of the $A_{2B}$ adenosine receptor subtype.

**Molecular biology**

RFL9 has been cloned from a rat brain cDNA library using a probe generated by polymerase chain reaction.[64,94] It was characterized as an adenosine $A_{2B}$ receptor based on the cAMP responses to drug treatments in the Chinese hamster ovary cells transfected with RFL9. The $A_{2B}$ receptor has also been cloned from human hippocampus[95] and mouse bone marrow-derived mast cells.[66] The human $A_{2B}$ adenosine receptor was also characterized by examining the activity of adenylate

cyclase in response to the treatment of [$^3$H]NECA in stably transfected Chinese hamster ovary cells.[95]

The $A_{2B}$ receptor encodes a protein of 328 to 332 amino acids dependant on the species. Like other adenosine receptor subtypes, there is a difference in the amino acid sequences of the $A_{2B}$ receptor between species; for example there is approximately 86% amino acid sequence homology between the rat and human $A_{2B}$ receptor (**Fig 1.8**).[64,95]

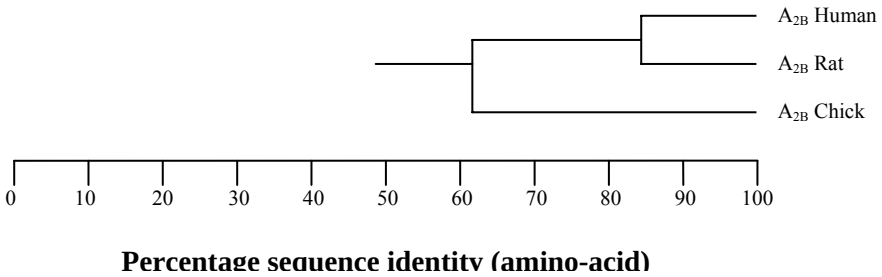



**Figure 1.8**: Sequence homology for the cloned $A_{2B}$ adenosine receptors between species. This figure was redrawn from Fredholm *et al*.[52]

**Signal Transduction Mechanisms**

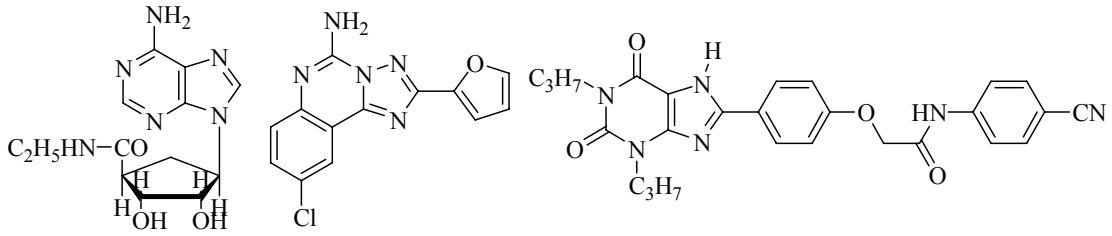
The mechanism for the $A_{2B}$ adenosine receptor coupled to G proteins to transduce the extracellular signals into the interior of cells is very similar to that of the $A_{2A}$ adenosine receptor. Activation of the $A_{2B}$ receptor stimulated the formation of cAMP accumulation and mobilization of intracellular calcium.[96,97] Activation of the $A_{2B}$ receptor also increased a chloride conductance in xenopus oocytes by stimulating phospholipase C.[98]

**Pharmacology**

There are no selective agonists or antagonists for the $A_{2B}$ adenosine receptor. NECA is currently the most potent agonist at the $A_{2B}$ adenosine receptor with low micromolar affinity.[70,99] The non-xanthine alloxazine, CGS 15943 and MRS 1754 were used as antagonists at the $A_{2B}$ adenosine receptor.[99-101] These ligands were less

useful in characterization of $A_{2B}$ adenosine receptors in cells or tissues in which $A_{2A}$ adenosine receptors were coexpressed because they are non-selective. Some of the ligands used in the classification of the $A_{2B}$ adenosine receptors are shown in **Figure 1.9**.

**$A_{2B}$ receptor agonist** **$A_{2B}$ receptor antagonists**



**NECA** **CGS-15943** **MRS-1754**

**Figure 1.9**: The chemical structure of an agonist (NECA) and antagonists (CGS-15943, MRS-1754) at $A_{2B}$ adenosine receptor.

Details regarding the molecular structure, signal transduction mechanisms and pharmacology of $A_{2B}$ adenosine receptor subtypes are summarized in **Table 1.4** below.

**Table 1.4**: Characterisation of $A_{2B}$ adenosine receptor.

| | |
|---|---|
| Tissue distribution[64] | Caecum, large intestine and urinary bladder |
| Cloning | Human[95], rat[64,94] and mouse[66] |
| Structural information | 328-332 amino acids |
| Structural type | G protein-coupled: $G_s$ and $G_{q/11}$ |
| Effects of G Protein Coupling | $G_s$: Activation of adenylate cyclase → increase in cAMP[95]<br>$G_{q/11}$: Activation of phospholipase C → increase in $IP_3$[96] |
| Pharmacological profile | Agonist[99,100]: NECA<br>Antagonist[100,101]: MRS 1754, CGS 15943 |

### 1.2.4 $A_3$ adenosine receptor

The $A_3$ receptor subtype is the youngest member of the adenosine receptor family. It was isolated by Meyerhof [102] in 1991 from a rat testis cDNA library using a PCR-amplified cDNA fragment as a hybridization probe and was later claimed to be a novel adenosine receptor subtype by Zhou[103] in accordance with the suggestions of IUPHAR committee on Receptor Nomenclature and Drug Classification.[40] In contrast to the other three adenosine receptor subtypes, the $A_3$ adenosine receptor was discovered by molecular biology studies followed by biochemical and pharmacological studies.

**Molecular biology**

The first cloned $A_3$ adenosine receptor was obtained from a rat testis cDNA libray by Meyerhof *et al.*[102] in 1991 but the authors did not characterize this novel putative G protein-coupled receptor because could not identify ligands for its binding. However, they noted that the novel cloned G protein-coupled receptor showed 47% and 42% sequence homology to the canine $A_1$ and $A_{2A}$ adenosine receptors respectively. In 1992, Zhou reported the isolation of a full length cDNA clone R226 from a rat brain cDNA library.[103] The cDNA clone R226 was identical to a clone isolated from a rat testis cDNA by Meyerhof *et al.* The cDNA clone R226 encoded for a protein of 320 amino acids that could be organized into seven transmembrane domains. It was claimed as a novel adenosine receptor and named as the $A_3$ adenosine receptor after pharmacological and biochemical studies.

$A_3$ adenosine receptors were cloned from sheep pars tuberalis[104], human striatum[105], human heart[106] and rabbit.[107] In contrast to other adenosine receptor subtypes where the homologies of the adenosine receptor between the species were very low, the interspecies differences in $A_3$ receptor structure were large. For example, the rabbit $A_3$ receptor showed only 76% and 75% sequence homology with human and sheep $A_3$ receptors respectively. The rat $A_3$ receptor shared 74% sequence homology to the sheep and human $A_3$ receptors. The homologies of the cloned adenosine $A_3$ receptors between species is summarized in **Fig 1.10**.

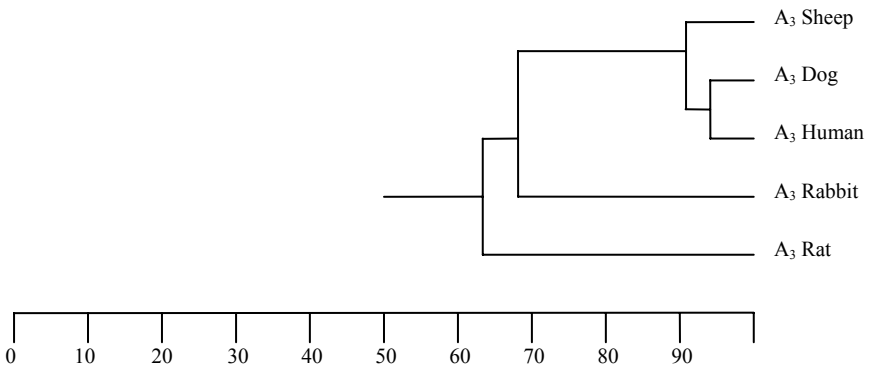



**Figure 1.10**: Sequence homology for the cloned $A_3$ adenosine receptors between species. This figure was redrawn from Fredholm *et al*.[52]

## Signal Transduction Mechanisms

The adenosine $A_3$ receptor belongs to the class of G protein-coupled receptors. The adenosine $A_3$ receptor couples to $G_{i2,3}$ and $G_{q/11}$ proteins located on the cytoplasmic domain of the membrane and hence activation of the $A_3$ receptor would result in stimulation or inhibition of different effectors. Activation of $A_3$ receptor has been shown to inhibit adenylate cyclase activity via $G_{i2}$ and $G_{i3}$ proteins[108,109] and hence results in a decrease in cAMP production (**Fig 1.2**). Activation of $A_3$ receptor also stimulated phospholipase C via $G_{q/11}$ and hence elevated $IP_3$ concentration and intracellular $Ca^{2+}$ concentratrion.[110-112]

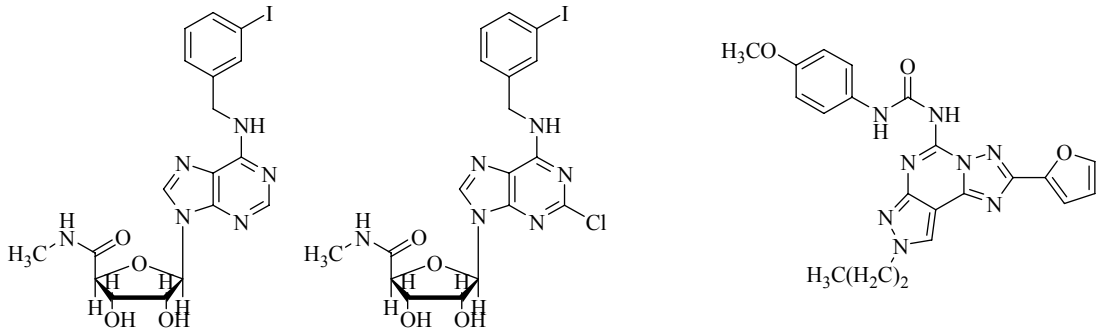
## Pharmacology

The $A_3$ receptor was first characterized pharmacologically by Zhou[103] after cloning and expression of the rat adenosine $A_3$ receptor in COS-7 and CHO cells. Many ligands including the $A_1$ selective antagonist DPCPX, the $A_2$ selective agonist CGS 21680 and the non-selective agonist NECA were used to characterize the cDNA cloned R226. Based on these observations Zhou concluded that R226 encoded an adenosine receptor with non-$A_1$ and non-$A_2$ specificity and named it the $A_3$ adenosine receptor.

It was thought that modifications at the $N^6$ and 5'-position of the adenosine structure could produce the potent and selective agonists. $N^6$-(3-iodobenzyl-adenosine-5'-N-methyl-uronamide (IB-MECA) was the first highly potent and selective $A_3$ agonist both in vitro and in vivo.[113] Substitution at the 2-position of adenosine in combination with modifications at the $N^6$ and 5'-positions further enhanced $A_3$ affinity and selectivity. 2-chloro-IB-MECA was found to be highly selective for $A_3$ versus $A_1$ and $A_{2A}$ receptors by 2500 and 1400 fold respectively.[114]

Several classes of compounds have been developed as $A_3$ receptor antagonists. One class comprised xanthines and their derivatives and the other was non-xanthine heterocycles. Xanthines tend to bind weakly to $A_3$ receptor.[104,105,115] Thus, non-xanthine structures were screened for leads and then optimized through iterative cycles of chemical synthesis, pharmacological and biochemical studies. Of all the non-xanthine heterocycles, MRE-3008-F20 was one of the most selective antagonists at the human $A_3$ receptor.[116] Some of the $A_3$ receptor agonists and $A_3$ receptor antagonists that have been developed recently are shown in **Figure 1.11**.

**Selective $A_3$ receptor agonists** **Selective $A_3$ receptor antagonist**



**IB-MECA** **Cl-IB-MECA** **MRE 3008-F20**

**Figure 1.11**: The chemical structure of agonists and antagonist at $A_3$ adenosine receptor.

Details regarding the molecular structure, signal transduction mechanisms and pharmacology of $A_3$ adenosine receptor subtypes are summarized in **Table 1.5** below.

**Table 1.5**: Characterisation of $A_3$ adenosine receptor.

| | |
|---|---|
| Tissue distribution[64] | Caecum, large intestine and urinary bladder |
| Cloning | Human[105,106], sheep[104], rabbit[107], rat[102,103] |
| Structural information | 317-320 amino acids |
| Structural type | G protein-coupled: $G_{i2,3}$ and $G_{q/11}$ |
| Effects of G Protein Coupling | $G_{i2,3}$: Inhibition of adenylate cyclase: decrease in cAMP[108]<br>$G_{q/11}$: Activation of phospholipase C: increase in $IP_3$[108] |
| Pharmacological profile | Selective agonist[70,117]: Cl-IB-MECA and IB-MECA<br>Selective antagonist[116]: MRE 3008-F20 |

## 1.3 Therapeutic potentials of ligands at the adenosine receptors

Adenosine has been known to produce a wide range of physiological effects since the initial reports on the its cardiovascular actions in 1929.[1] Adenosine, marketed as Adenocard™, was approved as a therapeutic drug in 1989 and is used in the treatment of the supraventricular tachyarrhythmias.[118] Due to the metabolic lability of adenosine, the discovery of the existence of at least 4 distinct adenosine receptors subtypes, interspecies differences in the 4 distinct adenosine receptor subtypes in terms of pharmacology and the variability of physiological responses mediated by short-lived Adenocard, there is an interest in the discovery and development of newer potential therapeutic agents.

Many therapeutic applications of adenosine agonists and antagonists of the 4 distinct adenosine receptor subtypes have been suggested. The following subsections outline the potential therapeutic uses of some ligands and some patho-physiology of the 4 distinct adenosine receptors.

**Lung**

Administered adenosine caused bronchoconstriction in asthmatic patients and promoted the release of inflammatory mediators from mast cells via stimulation of the $A_3$ receptor in the lung.[119,120] The mechanism by which adenosine produced bronchoconstriction was not fully understood. Ramkumar[111] found that the binding of the $A_3$ agonist NECA to mast cells expressing $A_3$ receptor induced secretion of inflammatory mediators. $A_3$ agonists also inhibited the lypopolysaccharide-induced stimulation of inflammatory cytokines TNF production and the release of inflammatory mediators from human macrophages and eosinophils.[121,122] These observations suggest that selective adenosine $A_3$ antagonists and agonists could be utilised as antiasthmatic and anti-inflammatory agents.[111,121-123]

**Parkinson's disease**

Parkinson's disease is an age-related disease arising from the degeneration of dopaminergic nigrostriatal neurones of the basal ganglia resulting in bradykinesia, tremor and rigidity. $A_{2A}$ receptors were found to be localized in dopamine-rich regions in the central nervous system[124,125] and mediated the inhibition of locomotor activity. Since adenosine inhibited the release of dopamine from central synaptic terminals and $A_{2A}$ receptor agonists were found to reduce locomotor activity[126], $A_{2A}$ receptor antagonists might increase the release of dopamine and consequently reduce some Parkinsonism symptoms.

**Neuroprotective**

Adenosine is present in most tissues of the body including the central nervous system and its levels increased dramatically in response to energy depletion induced by hypoxia and ischemia.[127-129] Adenosine protected tissues from ischemic brain damage by inhibiting the neurotransmitter release from presynaptic adenosine receptors during hypoxia and ischemic conditions[130-132] because the neurotransmitters were responsible for neural degeneration and neutral death and caused brain damage or death. Adenosine agonists have been shown to reduce ischemic injury to the brain.[133,134]

**Anticonvulsant**

Adenosine has been known to act as an anticonvulsant[15,135,136] and as an antiepileptic agent[14] by inhibiting the release of glutamate from excitory neurons and inhibiting neuronal firing. A non-selective $A_3$ agonist $N^6$-2-(4-Aminophenyl)ethyl-adenosine (APNEA) has been shown to enhance the anticonvulsive activity of antiepileptic drugs.[137] Adenosine agonists might therefore have potential as antiepileptic agents.

**Cognition Enhancer**

Caffeine and other adenosine antagonists stimulated the activity of the central nervous system and have proven to be effective as cognition enhancers.[138] Selective antagonists might have therapeutic potential in the treatment of memory related diseases such as dementia and Alzheimer's disease.

**Renal function**

Adenosine mediated diverse effects of renal function including renal blood flow, renin secretion, glomecular filtration rate and urine flow.[28] It plays a key role in mediating the haemodynamic changes associated with acute renal failure. Adenosine receptor antagonists like theophylline and caffeine antagonized the renal effects of adenosine; hence have potential as renal protective agents. The xanthine antagonist KW3902[139,140], 1,3-Dipropyl-8-(3-noradamantyl)xanthine and non-xanthine antagonist FK453[141], (+)-(R)-[(E)-3-(2-phenylpyrazolo[1,5-a]pyridin-3-yl) acryloyl]-2-piperidine ethanol and FK838[142], 6-Oxo-3-(2-phenylpyrazolo[1,5-a]pyridin-3-yl)-1(6H)-pyridazinebutanoic acid are used in the treatment of acute renal failure. KW3902, 1,3-Dipropyl-8-(3-noradamantyl)xanthine is undergoing clinical trials as a renal protective agent.[143]

**Cardiovascular System**

As mentioned in section 1.2, the levels of endogenous adenosine increased during hypoxia and ischaemia. One of its effects was to modulate the myocardial

oxygen supply-demand balance; hence suggesting that it played a role in the protection of the ischemic myocardium. Adenosine agonists thus might have potential as cardioprotective agents.

Adenosine is an antiarrythmic agent since adenosine has been shown to affect both sinoatrial and atrioventricular nodal conduction. It exerts protective effects in the heart through negative chronotropic, dromotropic and inotropic effects via activation of adenosine receptors.[19,144-146] Adenosine is used to treat supraventricular tachycardias[118] by slowing of the cardiac rhythms on the atrioventricular node while adenosine antagonists have been used in the treatment of bradyarrhythmias.[147]

Adenosine is also a potent regulator of coronary blood flow.[3,13,127] Many adenosine agonists act as hypotensive agents either by vasodilation or reduction of cardiac output.[148] The $A_{2A}$ selective agonist, CGS21680, acts as hypotensive agents.[149]

**Immune Function**

Adenosine was released from immune cells when the sympathetic nerve terminals in the immune organs were stimulated or during hypoxia and ischemia.[150] Adenosine decreased the antibacterial defense mechanisms of macrophages by suppressing the production of superoxide[151,152] and nitric oxide[153-156], both of which were essential in killing phagocytosed bacteria. Adenosine has been shown to suppress interleukin-2 (IL-2) production and lymphocyte proliferation.[26,157-161] Adenosine contributed to immune paralysis. However, adenosine might also have other beneficial effects outside the immune system since adenosine receptors are present in virtually every organ system in the body.[38,52,162] The development of selective blockade of immune cells expressing adenosine receptors seems to be a more rational approach.

**1.4 Structure-activity relationship of ligands at adenosine receptors**

Due to the metabolic lability of adenosine, a large number of adenosine receptor agonists and antagonists have been synthesized and evaluated for affinity at

adenosine receptors. These extensive structure-activity relationships have enhanced the understanding of the binding sites of adenosine receptors and provided information on the key structural features required for receptor affinity and subtype selectivity. These structure-activity relationships combined with molecular modeling and site-directed mutagenesis studies help to identify the pharmacophore of each adenosine receptor subtypes and hence to develop drugs targeted to a specific receptor.

### 1.4.1 Adenosine receptor agonists

Adenosine receptor agonists are derivatives of endogenous adenosine containing a ribose moiety and purine ring system. The ribose moiety, containing α-cis 2'- and 3'- hydroxyls, is required for affinity and intrinsic activity.[163,164] A number of ring systems have been studied including 7-deazaadenosines, 3-deazaadenosines, 1-deazaadenosines, 2-azaadenosines and 7-deaza-8-azaadenosine (pyrazolo[3,4-*d*]pyrimidineribonucleosides) (**Figure1.12**).[165,166]

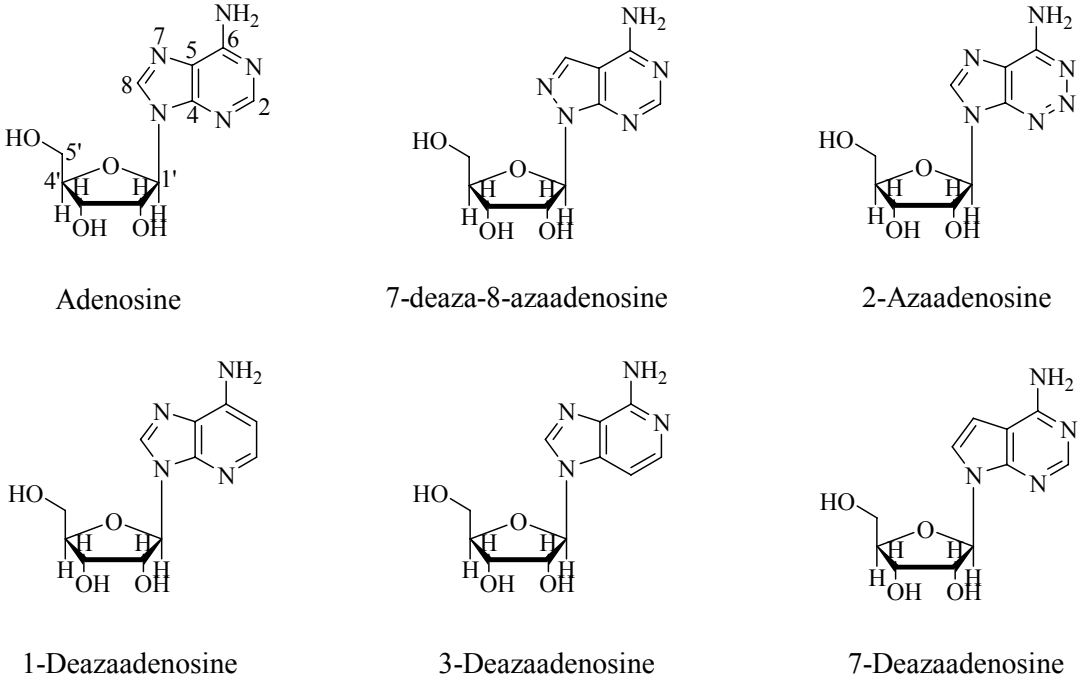



**Figure 1.12**: Adenosine and Adenosine agonists.

Modifications of the adenosine structure enhanced the stability of adenosine in biological systems. It might also lead to more or less potent and selective agonists for the 4 distinct adenosine receptor subtypes (**Figure 1.13**).[167]

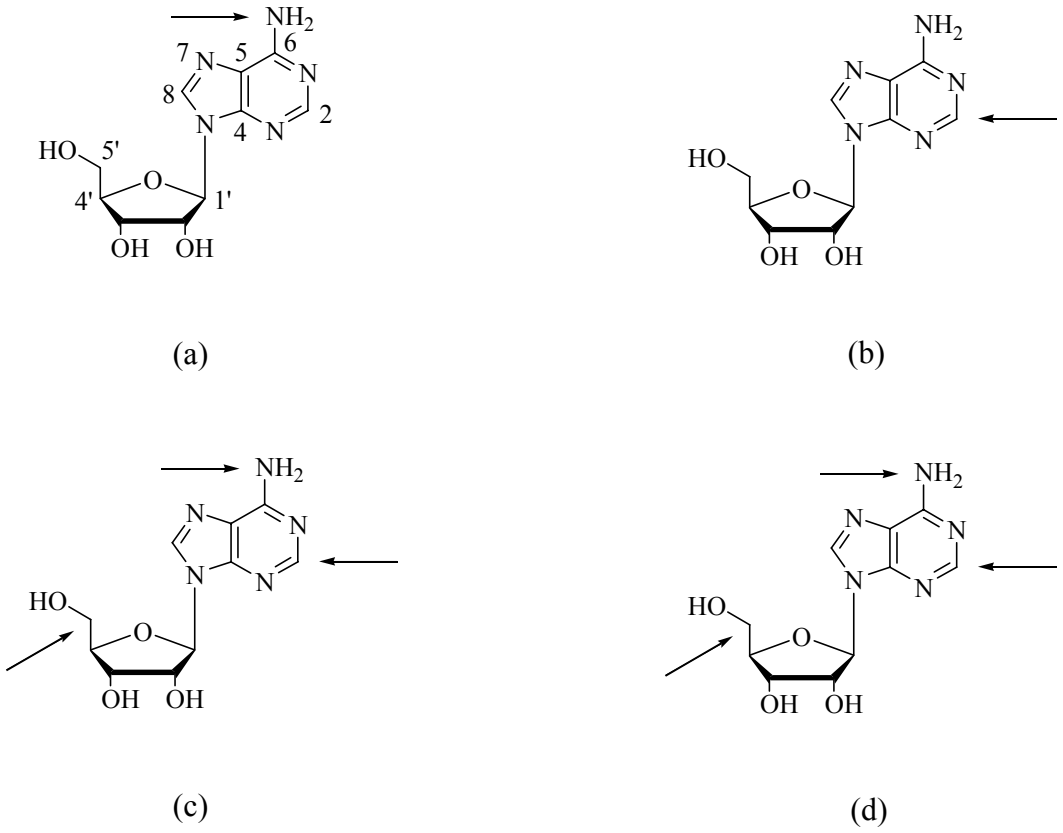



**Figure 1.13**: Sites of substitution leading to (a) $A_1$, (b) $A_{2A}$, (c) $A_{2B}$ and (d) $A_3$ agonists.

**Ribose Modifications**

Structure-activity relationships for adenosine with modifications in the ribose moiety have been studied at the $A_1$, $A_{2A}$ and $A_3$ adenosine receptor (**Table 1.6**).[72,113,164,165,168,169] Alterations of either structure or stereochemistry of the ribose moiety of adenosine were very strict. It could result in a loss of agonist activity and become an antagonist or partial agonist.

Stereochemistry

Endogenous adenosine is the β-D-adenosine isomer. Both β-L-adenosine and α-D-adenosine were virtually inactive at adenosine receptors.[164,165] Inversion of chirality at the 2'- and 3'-hydroxyl groups resulted in a loss of activity. These results showed that strict stereochemical requirements for the ribose moiety of adenosine were needed for agonist activity.

2'- And 3'-hydroxyls

Modifications of the 2'- and 3'-hydroxyls were not well tolerated. Substitution or deletion of 2'- or 3'-hydroxyls led to partial agonists with reduced affinity. 2'-deoxyadenosine, 3'-deoxyadenosine, 2'-O-methyladenosine and 3'-O-methyladenosine all exhibited low binding affinity (**Table 1.6**).[165,169]

5'-Position

Modification of the 5'-hydroxyl group of adenosine was better tolerated at adenosine receptors than modifications of the 2'- and 3'-hydroxyls group. 5-deoxyadenosine had a $K_i$ values in the 1-10 μM range at the $A_1$, $A_{2A}$, and $A_3$ receptor subtypes. Replacement of the 5'-hydroxyl group by a 5'-uronamide resulted in good affinity,[113] with the 5'-N-ethylcarboxamide derivative (NECA) having the greatest potency at the $A_1$ and $A_{2A}$ receptor subtypes and 5'-N-methylcarboxamide derivative (MECA) having the greatest potency at the $A_3$ receptor subtype (**Table 1.6**).

**Table 1.6**: Binding affinities of modified ribose adenosine expressed as $K_i$ in nM or % displacement at $10^{-4}$ M.[113,164,165,169]

| Compound | $A_1$ $K_i$[a] | $A_{2A}K_i$[b] | $A_3K_i$[c] |
|---|---|---|---|
| β-L-adenosine[164,165] | 29000 | 25.4% | 9.5% |
| α-D-adenosine[164,165] | 350000 | 128000 | 14.2% |
| 2'-deoxyadenosine[165,169] | 31% | 39% | 28% |
| 3'-deoxyadenosine[165,169] | 5.8% | 26.3% | 32.7% |
| 5'-deoxyadenosine[113] | 269 | 596 | 2830 |
| 2'-O-methyladenosine[165,169] | 29% | 49% | 43% |
| 3'-O-methyladenosine[165,169] | 0% | 8% | 11% |
| NECA[113] | 6.3 | 10.3 | 113 |
| MECA[113] | 83.6 | 66.8 | 72 |

[a] Displacement of specific [$^3$H]PIA or [$^3$H]CHA binding at rat brain membranes.

[b] Displacement of specific [$^3$H]CGS-21680 or [$^3$H]NECA binding at rat striatal membranes.

[c] Displacement of specific [$^{125}$I]AB-MECA or [$^{125}$I]APNEA binding at rat receptor in CHO cell membranes.

**$N^6$ modifications**

The structure activity relationship for this position has been well studied.[16,22,91,170-175] In general, the majority of $N^6$ substituted adenosines exhibited high $A_1$ potency and selectivity with some exceptions such as $N^6$ benzyl substitutent where introduction of a sulfonic group on the phenyl ring shifted the affinity towards better $A_3$ selectivity[168] and N,N-disubstituted derivatives lost activity at the adenosine receptor.[165]

The structure activity relationships of $N^6$ substituted adenosine showed that the $N^6$ hydrogen might act as a proton donor in the formation of a hydrogen bond with the receptor protein. They also indicated that the $N^6$ binding domain tolerated large hydrophobic groups but didn't tolerate hydrophilic groups. Finally the $N^6$ binding domain recognized the stereoselectivity as evidenced for example by the superior

potency of $N^6$-(*R*-phenylisopropyl)adenosine relative to $N^6$-(*S*-phenylisopropyl)adenosine. A variety of $N^6$ substituted adenosines with their potency are presented in **Table 1.7**.

**Table 1.7**: Binding affinities of $N^6$ substituted adenosine expressed as $K_i$ in nM.[168]

| Compound | $A_1$ $K_i$[a] | $A_{2A}K_i$[b] | $A_3K_i$[c] |
|---|---|---|---|
| $N^6$-(cyclohexyl)adenosine | 1.3 | 514 | 167 |
| $N^6$-(cyclopentyl)adenosine | 0.59 | 462 | 240 |
| $N^6$-(dimethyl)adenosine | 1000 | 28900 | 32500 |
| $N^6$-(phenethyl)adenosine | 12.7 | 161 | 240 |
| $N^6$-(phenyl)adenosine | 4.62 | 663 | 802 |
| $N^6$-(R-phenylisopropyl)adenosine | 1.2 | 124 | 158 |
| $N^6$-(S-phenylisopropyl)adenosine | 49.3 | 1820 | 920 |

[a] Displacement of specific [$^3$H]PIA or [$^3$H]CHA binding at rat brain membranes.

[b] Displacement of specific [$^3$H]CGS-21680 or [$^3$H]NECA binding at rat striatal membranes.

[c] Displacement of specific [$^{125}$I]AB-MECA or [$^{125}$I]APNEA binding at rat receptor in CHO cell membranes.

**C2 modifications**

Synthesis of C-2 substituted adenosine was more difficult than that of $N^6$-substituted adenosines, hence the structure activity relationships of C-2 substituted adenosine have not been extensively studied.[176] Several structure activity relationship studies[84,176-179] have shown that modifications of the C-2 position generally lead to $A_{2A}$ selective agonists as a result of reducing the efficacy of adenosine derivatives acting at the $A_1$ and $A_3$ adenosine receptors. **Table 1.8** shows a selection of C-2 substituted adenosine and their relative potencies at $A_1$, $A_{2A}$ and $A_3$ adenosine receptors.

**Table 1.8**: Binding affinities of C-2 substituted adenosine expressed as $K_i$ in nM or % inhibition of radioligand at 10 μM.[168,179]

| Compound | $A_1$ $K_i$[a] | $A_{2A}K_i$[b] | $A_3K_i$[c] |
|---|---|---|---|
| 2-CADO[168] | 9.3 | 63 | 1890 |
| 2-(phenylamino)adenosine[168] | 560 | 119 | 4390 |
| 2-(1-hexynyl)adenosine[179] | 63.7% | 6 | 16.9[d] |
| 2-Iodoadenosine[179] | 36.1% | 4200 | 297 |
| 2-(N'-3-Methyl-1-butylidenehydrazino)adenosine[179] | 18.9% | 20 | 38.3 |

[a] Displacement of specific [$^3$H]PIA or [$^3$H]CHA or [$^3$H]DPCPX binding at rat brain or rat cortical membranes.

[b] Displacement of specific [$^3$H]CGS-21680 or [$^3$H]NECA or [$^3$H]ZM-241385 binding at rat striatal membranes.

[c] Displacement of specific [$^{125}$I]APNEA binding at rat receptor in CHO cell membranes.

[d] Displacement of specific [$^{125}$I]AB-MECA binding at human receptor in HEK 293 cells.

**Multiple modifications**

Structural modifications at the C-2 position or $N^6$ position or ribose moiety of endogenous adenosine yielded a variety of stable adenosine analogs with different potency and selectivity for each receptor subtype. A combination of modifications at these positions might lead to more or less potent and selective ligands for each receptor subtype since each substituent may interact with independent sites on the adenosine receptor and when combined could have an additive effect on affinity.

Structure activity relationships of multiple substituted adenosines have been extensively studied.[114,117,149,180-185] Modifications at the C-2 and $N^6$ positions of adenosine resulted in a highly selective agonist CCPA at $A_1$ adenosine receptor.[182,183] Modifications at the C-2 position in combination with the 5'-position produced the $A_{2A}$ selective adenosine agonist, CGS 21680, with preferential hypotensive activity.[149]

The combination of modifications at $N^6$ and 5'- positions produced the first potent and selective agonist, IB-MECA, at $A_3$ adenosine receptor.[113] Substitution at the C-2 position in combination with modifications at $N^6$ and 5'- positions was found to enhance $A_3$ selectivity.[114]

A selection of the most potent and selective agonists at 4 distinct adenosine receptor subtypes produced by a combination of substituents are summarized in **Table 1.9**.

**Table 1.9**: Binding affinities of multiple substituted adenosine expressed as $K_i$ in nM.[113,114,186]

| Compound | $A_1$ $K_i$[a] | $A_{2A}K_i$[b] | $A_3K_i$[c] |
|---|---|---|---|
| CCPA[186] | 0.8[d] | 2300[e] | 42[f] |
| CGS-21680[186] | 290[d] | 27[e] | 67[f] |
| IB-MECA[113] | 54 | 56 | 1.1 |
| 2-Cl-IB-MECA[114] | 820 | 470 | 0.33 |

[a] Displacement of specific [$^3$H]PIA or [$^3$H]CHA binding at rat brain membranes.

[b] Displacement of specific [$^3$H]CGS-21680 or [$^3$H]NECA binding at rat striatal membranes.

[c] Displacement of specific [$^{125}$I]AB-MECA or [$^{125}$I]APNEA binding at rat receptor in CHO cell membranes.

[d] Displacement of specific [$^3$H]DPCPX or [$^3$H]CCPA binding at human receptors in CHO membranes.

[e] Displacement of specific [$^3$H]NECA binding at human receptors in CHO membranes.

[f] Displacement of specific [$^3$H]NECA binding at human receptors in CHO membranes.

**Purine ring modifications**

Modifications of the purine heterocyclic ring of endogenous adenosine have also been studied to investigate the important of the purine nitrogens on binding to

adenosine receptors (Table 1.10).[169,187-189] It was found that deletion of the purine nitrogen resulted in the loss of affinitiy. 1-deazaadenosine was less active than adenosine but still displayed the highest affinity for adenosine receptors whereas 3-deazaadenosine had very little activity. 7-deaza- and 1,3-dideazaadenosine were found to be inactive.

A series of 1-deaza analogues of 2-chloroadenosine (2-CADO), R-PIA, CHA, and NECA have also been synthesized and evaluated in radioligand binding studies for their affinity at adenosine receptors.[169,189] In general, 1-deaza analogues were less active than adenosine analogues.

**Table 1.10**: Binding affinities of modified ribose adenosine expressed as $K_i$ in nM or % inhibition of radioligand at $10^{-4}$ M.[169]

| Compound | $A_1$ $K_i$[a] | $A_{2A}K_i$[b] | $A_3K_i$[c] |
|---|---|---|---|
| 3-deazaadenosine | 21500 | 59800 | 61700 |
| 7-deazaadenosine | >100000 | 48% | 39% |
| 2-CADO | 9.3 | 63 | 1890 |
| 1-deaza-2-CADO | 226 | 163 | 2480 |
| NECA | 6.3 | 10.3 | 113 |
| 1-deaza-NECA | 51 | 580 | 703 |

[a] Displacement of specific [$^3$H]PIA binding at rat brain membranes.

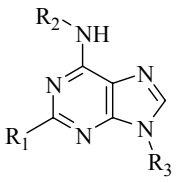
[b] Displacement of specific [$^3$H]CGS-21680 binding at rat striatal membranes.

[c] Displacement of specific [$^{125}$I]AB-MECA binding at rat receptor in CHO cell membranes.

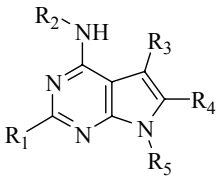
### 1.4.2 Adenosine receptor antagonists

In contrast to agonists, adenosine antagonists are diverse in structure compared to adenosine agonists which are the derivatives of physiological adenosine. Numerous lead structures of antagonists for adenosine receptors have been discovered and developed. They all shared these common structural features: (i) planar; (ii) aromatic or $\pi$ electron rich and (iii) nitrogen containing heterocycles.[71] The

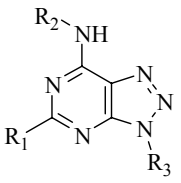
heterocycles are fused bicyclic or fused tricyclic compounds. There are some exceptions to these generalizations including flavonoid,[190,191] benzofuran[192,193] and tetrahydrobenzothiophenone[194] (**Figure 1.14**). Also, adenosine receptor antagonists lack the ribose moiety and this might limit their solubility especially for in vivo studies. Therefore, a polar moiety was needed to be attached to the antagonist to increase the water solubility of the antagonist.



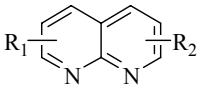
Adenine



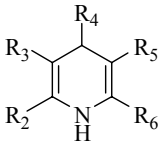
7-Deazaadenine



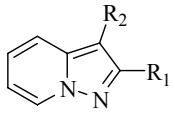
8-Azaadenine



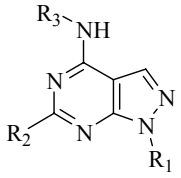
1,8-naphthyridines



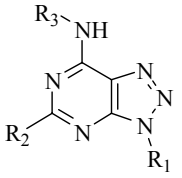
1,4-Dihydropyridines



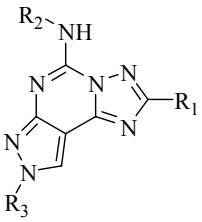
Pyrazolopyridine



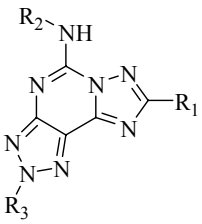
Pyrazolopyrimidine



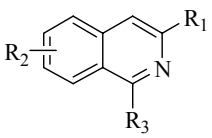
Triazolopyrimidine



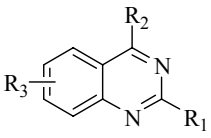
Pyrazolotriazolopyrimidine



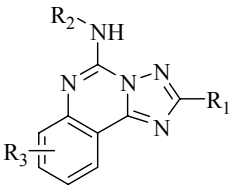
Triazolotriazolopyrimidine



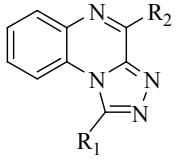
Isoquinoline



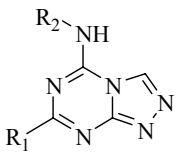
Quinazoline



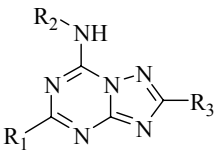
Triazoloquinazoline



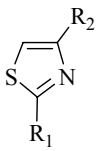
Triazoloquinoxaline



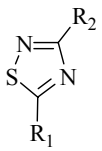
Imidazotriazine



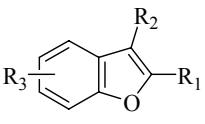
Triazolotriazine



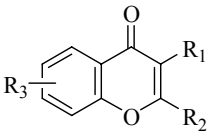
Thiazole



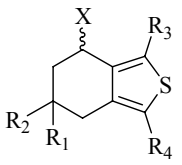
Thiadiazole



Benzo[*b*]furan



Flavonoid



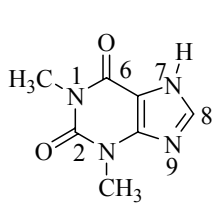
Tetrahydrobenzothiophenones

**Figure 1.14**: Chemical structure of antagonists.

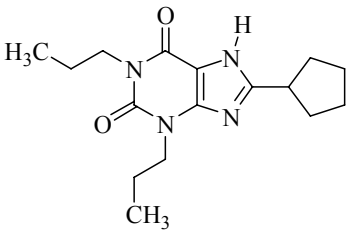
There are two types of antagonists, xanthine antagonists and non-xanthine antagonists.

**Xanthine antagonists**

The first adenosine receptor antagonists identified were the naturally occurring xanthines caffeine and theophylline.[6] These compounds are weak and non-selective at the adenosine receptors. Similar to agonists, structure activity relationships of the xanthines have been extensively studied in an attempt to improve the potency and selectivity at adenosine receptors.[139,195-205] Substitutions at N1, N3, N7 and/or C8 of the xanthines led to high affinity and selectivity at $A_1$, $A_{2A}$ and $A_{2B}$ receptors (**Figure 1.15**). A selection of the most potent and selective antagonists are summarized in **Table 1.11**.

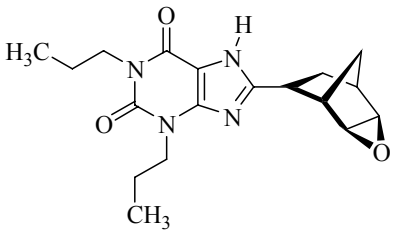


**Theophylline**[6]



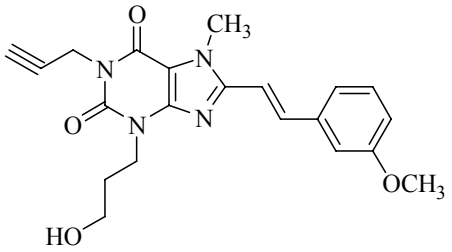
**DPCPX**[70,206-209]

**$A_1$ selective**



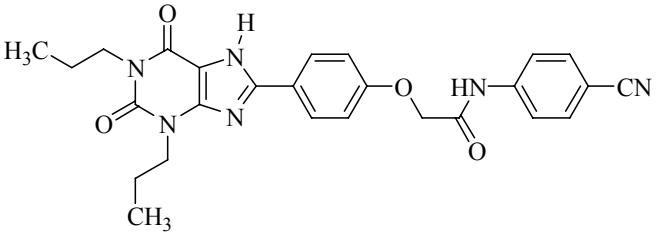
**BG-9719**[210]

**$A_1$ selective**



**MSX-2**[93]

**$A_{2A}$ selective**

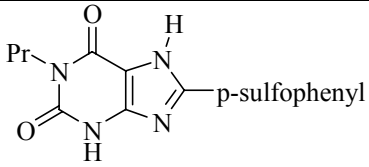
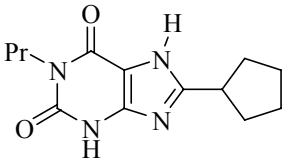
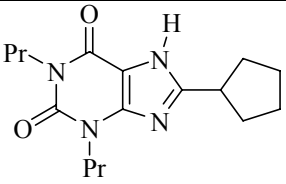
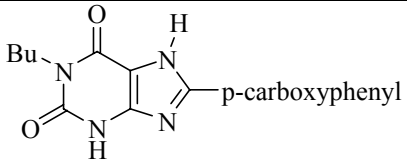
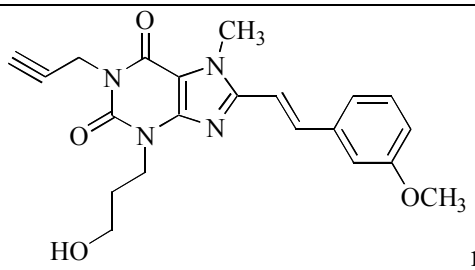
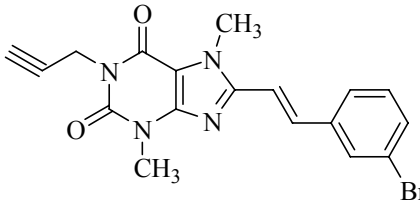


**MRS-1754**[101,211]

**$A_{2B}$ selective**

**Figure 1.15**: Theophylline and selective and potent antagonists.

**Table 1.11**: Binding affinities of xanthine derivatives expressed as $K_i$ in nM or % inhibition of radioligand at 10 μM.[93,101,210,213,215]

| Compound | $A_1$ $K_i$[a] | $A_{2A}K_i$[b] | $A_{2B}K_i$[c] | $A_3K_i$[d] |
|---|---|---|---|---|
|  ref 213 | 2200 | 24000 | ND | 1730 |
|  ref 215 | 14 | 580 | 34.4 | ND |
|  ref 215 | 0.9 | 470 | 51 | 795 |
|  ref 215 | 481 | 3800 | 24 | 4622 |
|  ref 93 | 2500[e] | 5[f] | ND | ND |
|  ref 93 | 1200 | 8.2 | ND | >10000 |
| MRS-1754[101] | 403[g] | 503[h] | 1.97[i] | 570[j] |
| BG-9719[210] | 0.5[k] | 100[k] | 200[k] | 4500[k] |

[a] Displacement of specific [$^3$H]CHA or [$^3$H]PIA or [$^3$H]MSX-2 binding at rat brain membranes.

[b] Displacement of specific [$^3$H]CGS-21680 or [$^3$H]NECA binding at rat brain membranes.

[c] Displacement of specific [$^3$H]ZM-241385 binding at human receptor in CHO cell membranes.

[d] Displacement of specific [$^3$H]NECA binding at human receptor in CHO cell membranes.

[e] Displacement of specific [$^3$H]CCPA binding at human receptor in CHO cell membranes.

[f] Displacement of specific [$^3$H]CGS-21680 binding at human receptor in CHO cell membranes.

[g] Displacement of specific [$^{125}$I]IABA binding at human receptor in HEK-293 cell membranes.

[h] Displacement of specific [$^{125}$I]iodo-ZM241385 binding at human receptor in HEK-293 cell membranes.

[i] Displacement of specific [$^{125}$I]ZM241385 or [$^{125}$I]IABOPX binding at human receptor in HEK-293 cell membranes.

[j] Displacement of specific [$^{125}$I]IAB-MECA or [$^{125}$I]IABA binding at human receptor in HEK-293 cell membranes

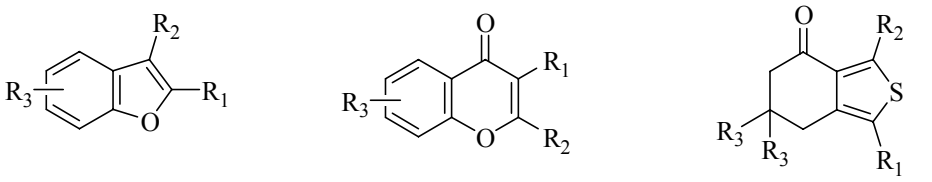
[k]The binding data studies were obtained from the abstract[210]. They were carried out in human receptors but the author didn't mention which cell membranes and radioligands were used.

ND: Not Determined.

**Non-xanthine antagonists**

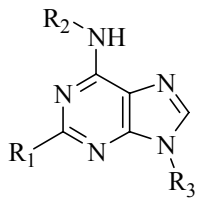
Since xanthine and its derivatives were less potent at the $A_3$ receptor, a large number of non-xanthine structures have been screened to search for $A_3$ antagonists. Numerous classes of heterocycles were identified as antagonists at the $A_3$ receptor and other receptor subtypes as well. These compounds included non-nitrogen containing heterocycles such as benzofuran derivatives,[192,193] flavonoid derivatives[190,191] and tetrahydrobenzothiophenone (**Figure 1.16**);[194] and nitrogen containing heterocycles such as adenine derivatives,[216-218] deazaadenine derivatives,[219-221] azaadenine derivatives,[218,222] naphthyridine derivatives,[223] pyridine derivatives,[224,225] pyrazolopyridine derivatives,[141,142,226] isoquinoline derivatives,[227-229] quinazoline derivatives,[230] triazoloquinazoline derivatives,[231,232] triazoloquinoxaline

derivatives,[233,234] imidazotriazine derivatives,[235] triazolotriazine derivatives,[236] and thiazole and thiadiazole derivatives[237-239] (**Figure 1.17**).

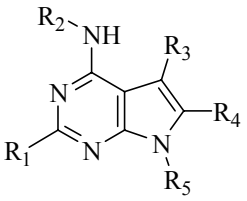



**Benzofuran**[192,193] **Flavonoid**[190,191] **Tetrahydrobenzothiophenone**[194]

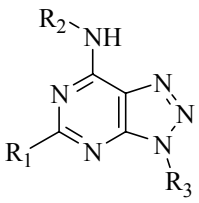
**Figure 1.16**: Non-nitrogen containing antagonists.



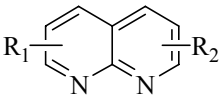
**Adenine**



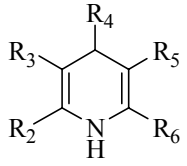
**Deazaadenine**



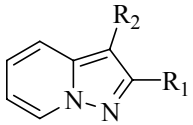
**Azaadenine**



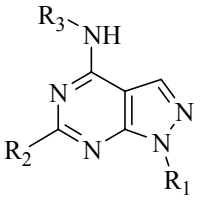
**Naphthyridine**



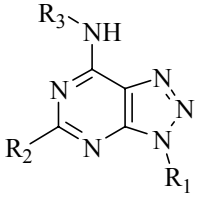
**Pyridine**



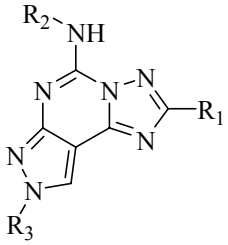
**Pyrazolopyridine**



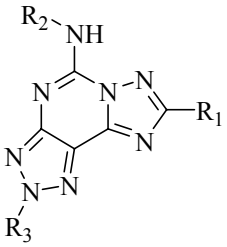
**Pyrazolopyrimidine**



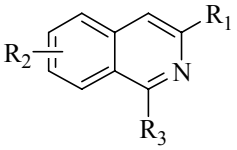
**Triazolopyrimidine**



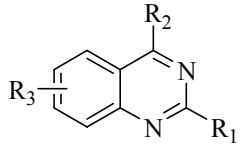
**Pyrazolotriazolopyrimidine**



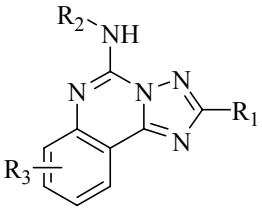
**Triazolotriazolopyrimidine**



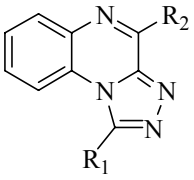
**Isoquinoline**



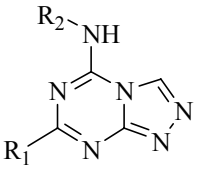
**Quinazoline**



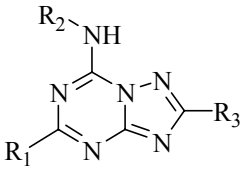
**Triazoloquinazoline**



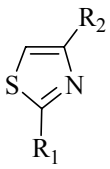
**Triazoloquinoxaline**



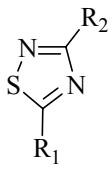
**Imidazotriazine**



**Triazolotriazine**



**Thiazole**

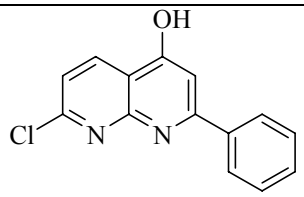
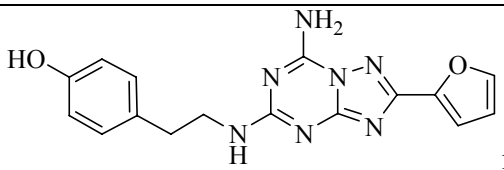
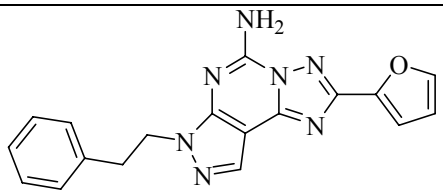
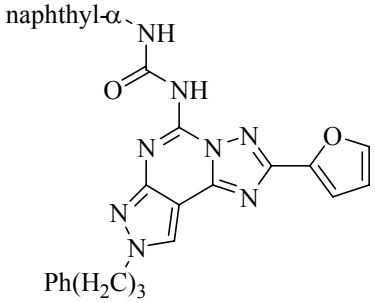
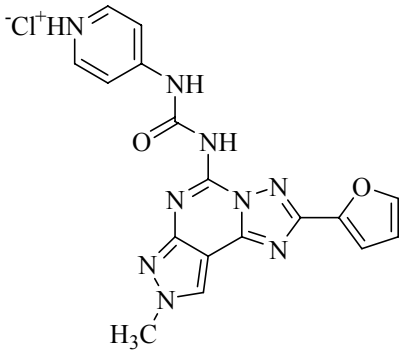


**Thiadiazole**

**Figure 1.17**: Nitrogen containing antagonists.

Structure activity relationship of those non-xanthine heterocycles has been extensively studied. A number of high potent and selective antagonists have been synthesized (**Table 1.12**)

**Table 1.12**: Binding affinities of non-xanthine derivatives expressed as $K_i$ in nM or % inhibition of radioligand at 10 μM.[92,223,240,241]

| Compound | $A_1$ $K_i$[a] | $A_{2A}K_i$[b] | $A_{2B}K_i$[c] | $A_3K_i$[d] |
|---|---|---|---|---|
|  ref 223 | 0.15 | 100 | ND | 2100 |
|  ref 92 | 255[e] | 0.8[f] | ND | >10000[g] |
|  ref 92 | 287[e] | 0.6[f] | ND | >10000[g] |
|  ref 241 | 1100[e] | 800[f] | 20 | 300[g] |
|  ref 240 | 350[e] | 100[f] | 250 | 0.01[g] |

[a] Displacement of specific [$^3$H]CHA binding at bovine membranes.

[b] Displacement of specific [$^3$H]CGS 21680 binding at bovine membranes.

[c] Displacement of specific [$^3$H]DPCPX binding at human receptors in HEK-293 cell membranes.

[d] Displacement of specific [$^3$H](R)-PIA binding at rat membranes.

[e] Displacement of specific [$^3$H]DPCPX binding at human receptor in CHO cell membranes.

[f] Displacement of specific [$^3$H]SCH 58261 or [$^3$H]ZM 241385 binding at human receptor in HEK-293 cell membranes.

[g] Displacement of specific [$^{3125}$I]AB-MECA or [$^3$H]MRE 3008-F20 binding at human receptor in HEK-293 cell membranes.

ND: Not Determined.

### 1.4.3 Adenosine receptor partial agonists

A partial agonist is a compound whose intrinsic activity is less than that of a full agonist. It might selectively induce the desired therapeutic effect while reducing side effects because of its decreased efficacy. There are several potential advantages of using adenosine receptor partial agonists compared to adenosine receptor agonists:[20,168,242-248] (i) circumvent the side effects caused by the actions of adenosine receptor agonists since the adenosine receptor was ubiquitously distributed in the body; (ii) partial agonists might be more receptor subtype selective (iii) partial agonists induced less receptor downregulation and desensitization For example, chronic administration of adenosine $A_3$ receptor agonists has been found to be cerebro and cardioprotective[249] but it also stimulated the receptors in the lungs and immune system to cause bronchoconstriciton and the release of allergic mediators respectively.

Adenosine agonist-antagonist hybrid structures (ie ribose-xanthine) was firstly reported as a partial agonists in 1990.[250] 1,3-dibutylxanthine-7-ribose was shown to act as a partial agonist at the rat adenosine $A_3$ receptor.[168] A number of partial agonists have been synthesized by substituting the 8-position of adenosine agonists; by removing the 2'- or 3'-hydroxyl groups of the ribose moiety; or by a combination of substitutions of C-2, C-8, $N^6$, 2'-, 3'- or 5'- positions (**Fig 1.18**).[244,245,251-257]

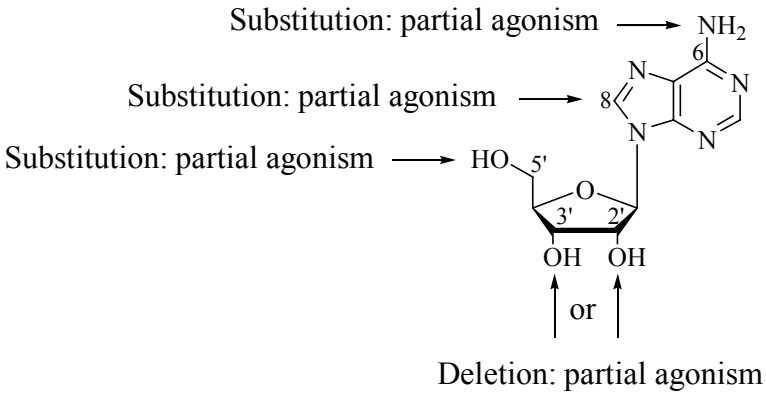



**Figure 1.18**: Sites of substitution that can cause partial agonism.

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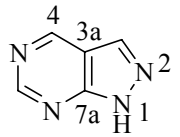
# CHAPTER 2

## Design of Template for library development

### 2.1 Introduction:

Pyrazolo[3,4-*d*]pyrimidines **(1) (Figure 2.1)** have been identified as antagonists with micromolar affinity at the rat adenosine $A_1$ receptor during a study of eleven novel nitrogen containing heterocycles structurally related to xanthine, caffeine and theophylline.[1,2] The eleven heterocyclic rings included thiazolo[5,4-*d*]pyrimidines **(A)**, pyrazolo[3,4-*d*]pyrimidines **(B)**, s-triazolo[4,3-*a*]pyrimidines **(C)**, s-triazolo[1,5-*c*]pyrimidines **(D)**, s-triazolo[4,3-*c*]pyrimidines **(E)**, s-triazolo[1,5-*a*]pyrimidines **(F)**, benzothiazoles **(G)**, pyridin-2'-ylpyrimidines **(H)**, quinazolines **(I)**, pyrazolylpyrimidiens **(J)** and s-triazolo[3,4-*d*]benzothiazoles **(K) (Figure 2.2)**.




**1**

**Figure 2.1**: Pyrazolo[3,4-*d*]pyrimidines core structure **(1)**

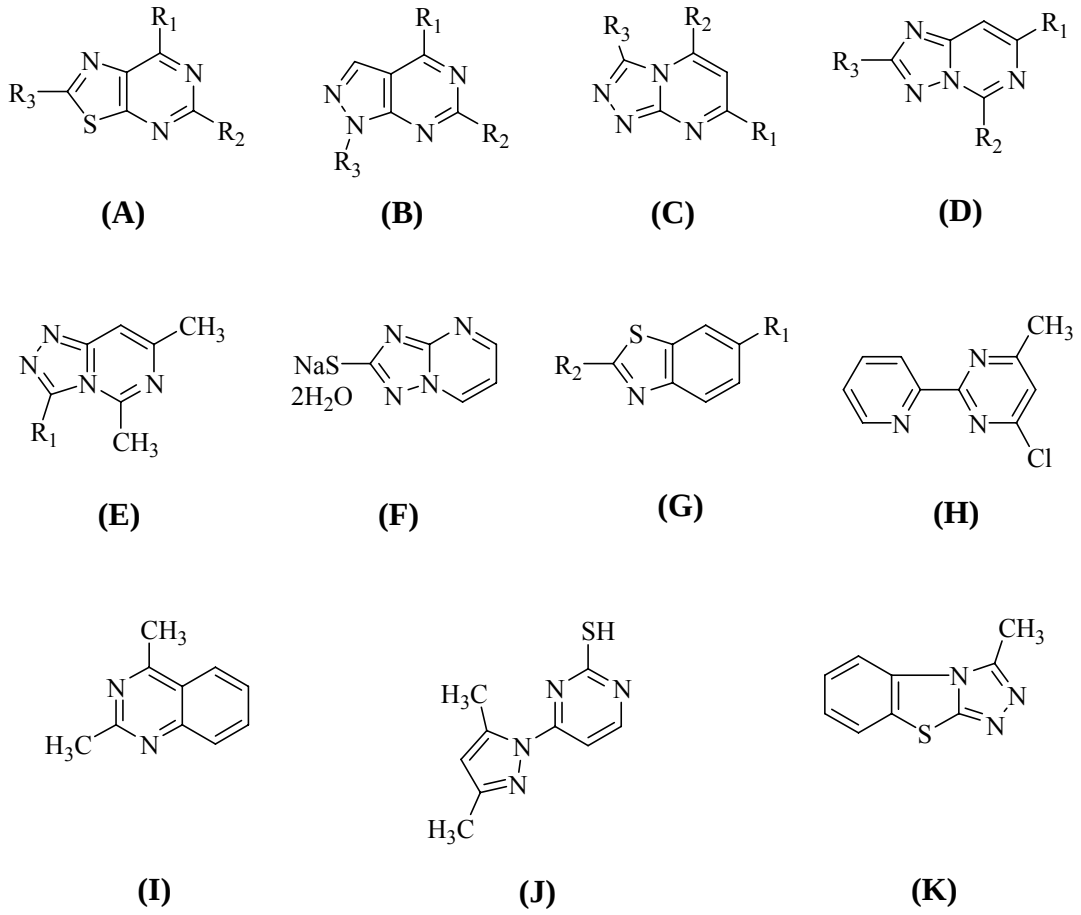
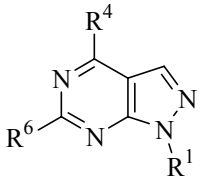



**Figure 2.2**: Structures of heterocycles tested for affinity at the $A_1$ adenosine receptor.

From the eleven heterocyclic systems, the pyrazolo[3,4-*d*]pyrimidines were found to be the most active at adenosine receptors. **Table 2.1** shows the structures of the pyrazolo[3,4-*d*]pyrimidines and their binding affinities at the rat $A_1$ adenosine receptor. The most active compounds of the pyrazolo[3,4-*d*]pyrimidines were the ones which contained the phenyl substituent at the N-1. In the phenyl series, mono-substitution at C-4 **(2 & 4)** led to poor affinity (compare with **1**) while bis-substitution at C-4 and C-6 **(3 & 5)** generated the most potent compounds (compare with **1** and mono-substitution).

Compound **5** was the most active compound in the phenyl series with a $K_i$ value of 0.37±0.06 μM and was 52-fold more potent than theophylline (19.4 μM). But when the phenyl substituent **(5)** was changed to a smaller substituent such as methyl **(6)** and hydrogen **(7)**, the affinity decreased by 38-fold and 79-fold respectively.

**Table 2.1**: Binding affinity of pyrazolo[3,4-*d*]pyrimidines expressed as $K_i$ in μM or % displacement at 10 μM.[1]

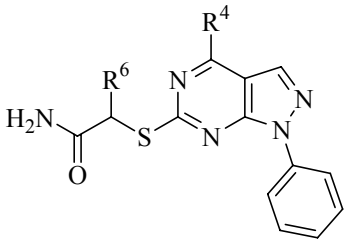


| Compound | $R^4$ | $R^6$ | $R^1$ | $K_i$ (μM) or % inhibition at10 μM |
|---|---|---|---|---|
| **1** | SH | H | Ph | 18.3 ± 2.9 |
| **2** | $SCH_2CONH_2$ | H | Ph | 47.7 ± 4.2% |
| **3** | $SCH_2CONH_2$ | $SCH_2CONH_2$ | Ph | 1.7 ± 0.5 |
| **4** | $SCH(CH_3)CONH_2$ | H | Ph | 45.6 ± 6.8% |
| **5** | $SCH(CH_3)CONH_2$ | $SCH(CH_3)CONH_2$ | Ph | 0.37 ± 0.06 |
| **6** | $SCH(CH_3)CONH_2$ | $SCH(CH_3)CONH_2$ | Me | 14.2 ± 1.6 |
| **7** | $SCH(CH_3)CONH_2$ | $SCH(CH_3)CONH_2$ | H | 29.4 ± 2.7 |
| Theophylline | | | | 19.4 ± 2.1 |

[a] Displacement of specific [$^3$H]PIA binding at the rat brain membranes.

Quinn *et al.*[3-5] expanded on the findings of Davies *et al.*[1,2] by studying the effects of the substitutions at the C-4 and C-6 positions of pyrazolo[3,4-*d*]pyrmidines on the adenosine $A_1$ and $A_{2A}$ receptor binding. The studies probed the effects of a mercaptol, methylthio, and amino substitution at C-4 for hydrogen-bonding sites and steric tolerance **(Table 2.2)**. They found that the amino substituent **(16,17,18 &19)** at C-4 was more potent than the methylthio **(12,13,14 &15)** and the mercapto **(8,9,10 & 11)** substituents. The methylthio substituent **(12,13,14 &15)** at C-4 was more potent than the mercapto substituent **(8,9,10 &11)**. Secondary amine substituent at C-4 **(20)** also led to an increase in receptor affinity and selectivity (compare **20** with **19**). These further studies have provided a useful starting point to further investigate the structure-activity relationships of pyrazolo[3,4-*d*]pyrmidines at all adenosine receptor subtypes.

**Table 2.2**: Binding affinity of C-4 and C-6 substituted 1-phenylpyrazolo[3,4-*d*]pyrimidines expressed as $K_i$ in nM or % inhibition at nM.[5]



| Compound | $R^4$ | $R^6$ | $A_1K_i$ (nM)[a] | $A_{2A}K_i$ (nM)[b] or % inhibition (nM)[c] |
|---|---|---|---|---|
| **8** | SH | Et | 155 ± 5 | 8750 ± 1100 |
| **9** | SH | *i*-Pr | 256 ± 4 | 44% (20000) |
| **10** | SH | Pr | 56.3 ± 4.5 | 4450 ± 790 |
| **11** | SH | Bu | 37.2 ± 0.8 | 52% (10000) |
| **12** | $SCH_3$ | Et | 8.4 ± 0.32 | 796 ± 135 |
| **13** | $SCH_3$ | *i*-Pr | 15.7 ± 0.6 | 37% (20000) |
| **14** | $SCH_3$ | Pr | 7.55 ± 2.32 | 4380 ± 410 |
| **15** | $SCH_3$ | Bu | 6.81 ± 0.61 | 12% (40000) |
| **16** | $NH_2$ | Et | 1.56 ± 0.09 | 44.5 ± 12.6 |
| **17** | $NH_2$ | *i*-Pr | 2.73 ± 0.12 | 147 ± 17 |
| **18** | $NH_2$ | Pr | 1.08 ± 0.30 | 35.3 ± 2.2 |
| **19** | $NH_2$ | Bu | 0.939 ± 0.341 | 88.3 ± 3.8 |
| **20** | $NHCH_3$ | Bu | 0.745 ± 0.045 | 247 ± 42 |

[a] Displacement of specific [$^3$H]PIA binding at the rat brain membranes

[b] Displacement of specific [$^3$H]CGS 21680 at the rat striatal membranes

[c] Lack of solubility at high concentrations precluded determination of $IC_{50}$

## 2.2 Results and Discussion

### 2.2.1 Rational Design of Pyrazolo[3,4-*d*]pyrimidines template

Our strategy in the design of ligands for adenosine receptors was to use ligand-based molecular modelling technique to develop a pharmacophore which then would rationally direct the future design of potent and selective ligands. The method was to superimpose the known adenosine receptor ligands and propose ligands with the assumption that adenosine agonists and antagonists bind to a common binding site of the receptor.[6-8] The superimposition of the ligands possessed good steric, hydrophobic and electrostatic overlap; and placed potential hydrogen bonding sites in close proximity to maximize ligand-receptor binding interations. There are 4 different types of ligand-based model in the literature termed the standard model, flipped model, $N^6$/C8' model and C2/$N^6$/C8' model.

The 'standard' or 'all nitrogen' model[9] had the atoms N1, N3, N7 and N9 of both xanthine antagonist and adenosine agonist map onto each other and possessed good steric, hydrophobic and electrostatic overlap **(Figure 2.3)**.

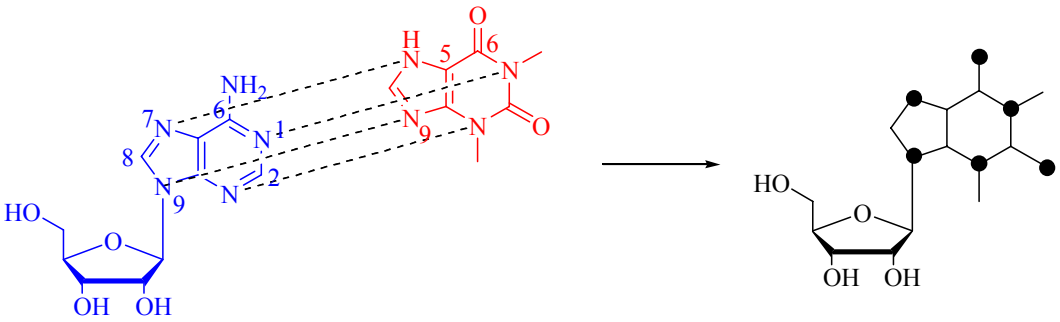
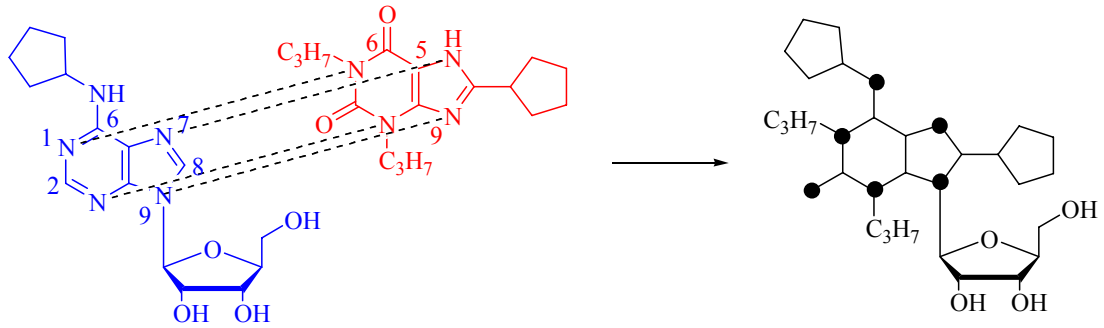



**Figure 2.3**: Superimposition of adenosine (blue) theophylline (red) according to 'standard' model.

The 'flipped' model[9] was an improved 'standard model' in which it rotated the xanthine antagonist by 180°C about its long axis relative to xanthine antagonists in the 'standard' model to improve the electrostatic overlap but at the same time maintain the steric and hydrophobic overlap of the standard model. Thus N1, N3, N7 and N9 of

adenosine agonist mapped on to C2, C6, N9 and N7 of xanthine antagonist. An example of this was when an $A_1$ selective $N^6$-substituted agonist CPA (blue) was modelled via the 'standard' model with an $A_1$ selective C8-substituted xanthine DPCPX (red), the exocyclic $N^6$- and C8- substituents occupied different spatial regions and did not enhance the steric, hydrophobic or electrostatic overlap while in the 'flipped' model rotation of the xanthine by 180°C around its long axis improved the electrostatic overlap **(Figure 2.4)**.

(a)



(b)

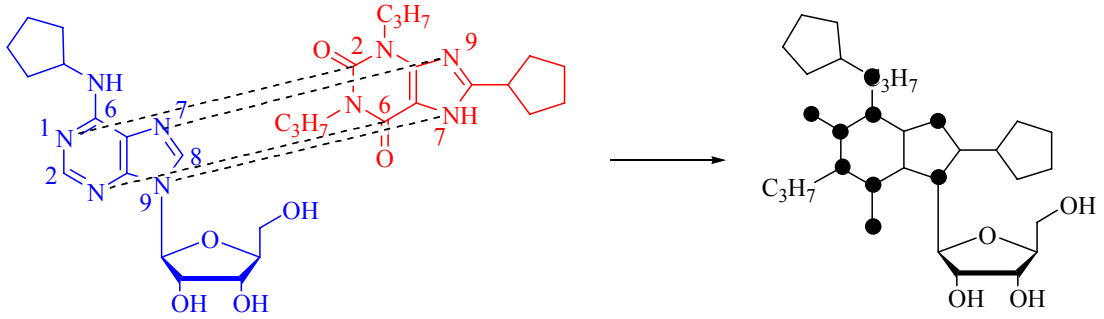


**Figure 2.4**: Superimposition of CPA (blue) and DPCPX (red) according to (a) 'standard' model and (b) 'flipped' model.

The '$N^6$/C8' model[7] hypothesized that the $N^6$-substituent of adenosine agonists and C8-substituent of xanthine antagonists bind to the same region of the receptor **(Fig 2.5)**, i.e these binding domains were not discrete. This model possessed good steric, electrostatic and hydrophobic overlap and also placed potential hydrogen

bonding sites in close proximity. Comparison of this model with the 'standard' model and 'flipped' model showed significant differences in terms of steric, electrostatic and hydrophobic overlap as well as correlation of hydrogen bonding donor and acceptor when the more potent and selective $A_1$ $N^6$-substituted adenosine derivative agonist CPA (blue) and C8-substituted xanthine derivative antagonist DPCPX (red) were modelled **(Figure 2.4 and 2.5)**.

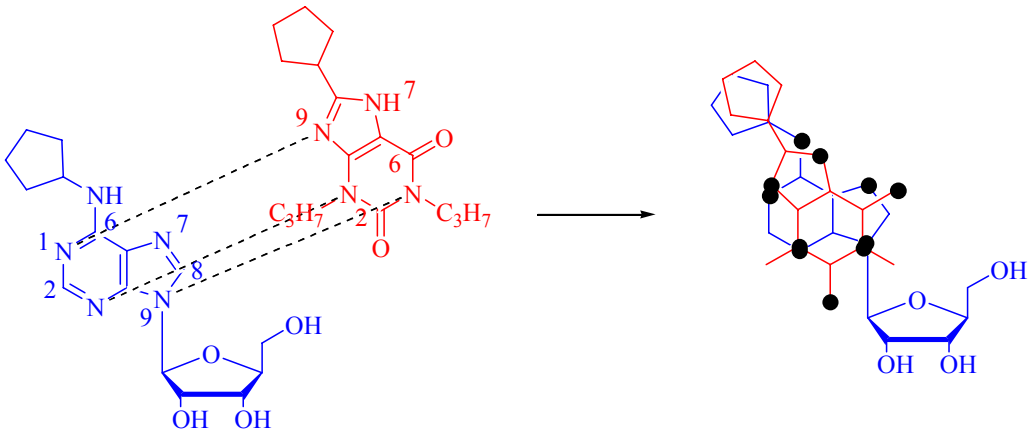



**Figure 2.5**: Superimposition of CPA (blue) and DPCPX (red) according to 'N$^6$/C8' model.

The 'C2/N$^6$/C8' model[6,10] developed by Quinn *et al.* could be seen as an extension of the 'N$^6$/C8' model by including the C2 substituent. This model proposed that the common region of the receptor was occupied by the N$^6$, C8 and C2 hydrophobic binding domains **(Figure 2.6)**. This model possessed good steric, electrostatic and hydrophobic overlap and placed potential hydrogen bonding in close proximity. In addition to the hydrophobic binding domain, the 'three binding domain' model also proposed the central aromatic binding domain and the ribose binding domain in the $A_1$ and $A_{2A}$ receptors **(Figure 2.7)**.

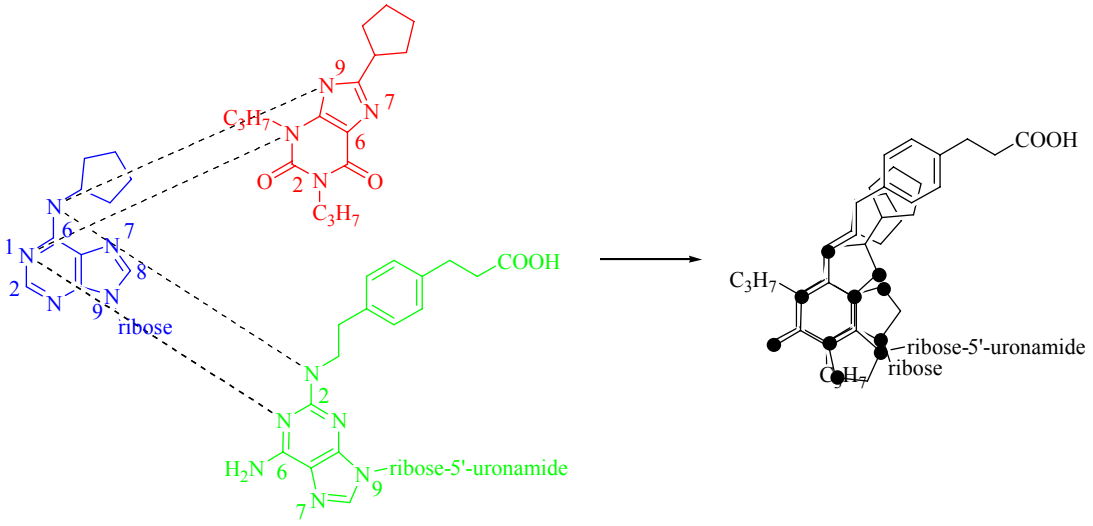



**Figure 2.6**: Superimposition of $A_1$ agonist CPA (blue), $A_1$ antagonist DPCPX (red) and $A_{2A}$ agonist CGS 21680 (green) according to 'C2/$N^6$/C8' model or 'three binding domain'.

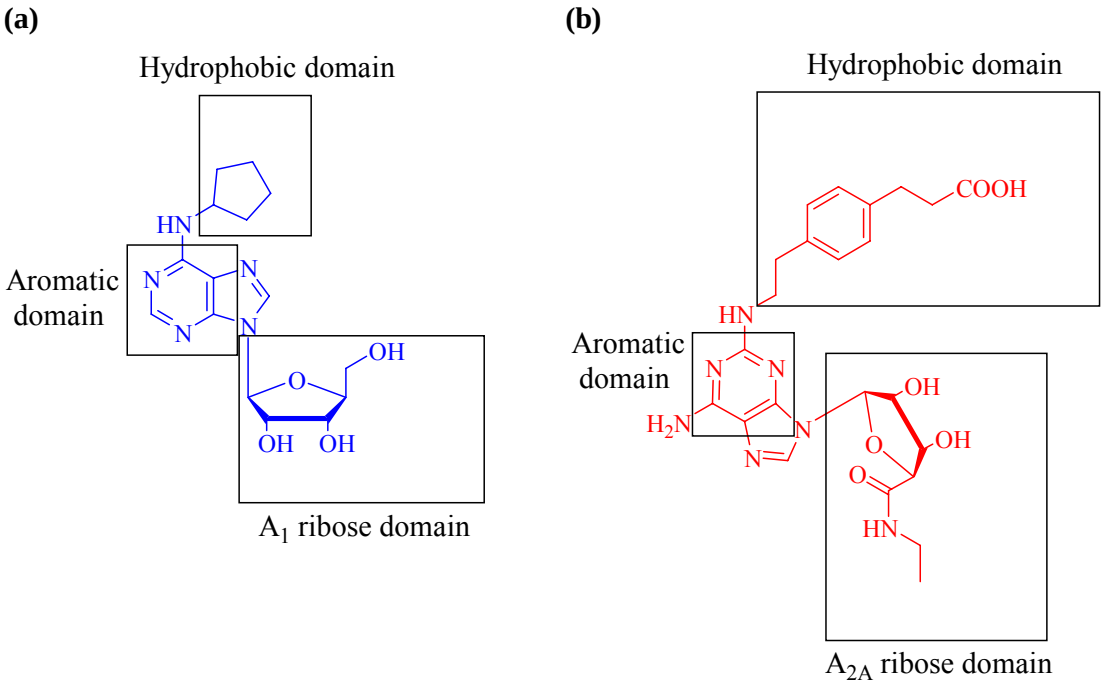



**Figure 2.7**: Hydrophobic, aromatic and ribose binding domains as proposed by the C2/$N^6$/C8' model. **(a)** $A_1$ agonist CPA (blue) and **(b)** $A_{2A}$ agonist CGS 21680 (red).

Our previous studies have used the 'C2/N$^6$/C8' model to superimpose α-((4-thioxo-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-yl)thio)propionamide (blue) and $A_1$ agonist R-PIA (red) to maximize lipophilic factors.[6] **Figure 2.8** showed the highest electrostatic, steric, and lipophilic correlations between these two ligands. Modifications at C-4 and C-6 of α-((4-thioxo-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-yl)thio)propionamide produced α–[(4-amino-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-yl)thio]hexanamide **(21) (Figure 2.9)** as one of the most potent seletive antagonist ever reported with a $K_i$ $A_1$ value of 0.745 ± 0.045 nM and 332-fold selectivity for the $A_1$ receptor over the $A_{2A}$ receptor.[5]

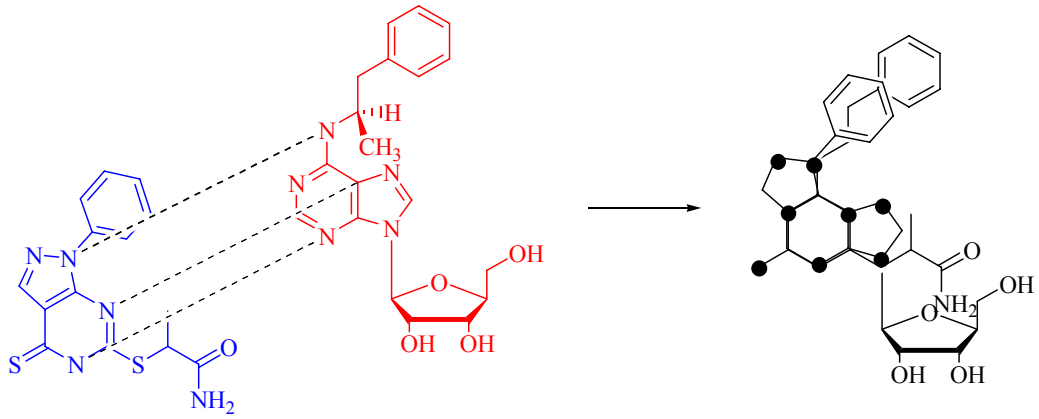
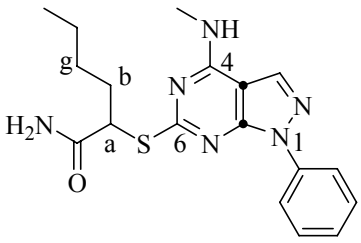


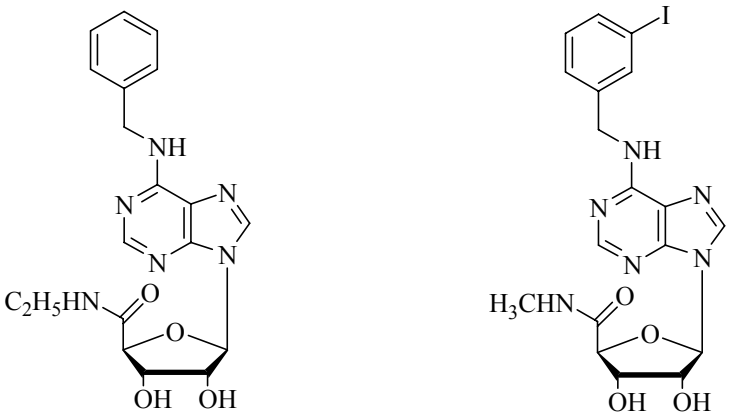
**Figure 2.8**: The superimposition of α-((4-thioxo-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-yl)thio)propionamide (blue) and $A_1$ agonist R-PIA (red).



**21**

**Figure 2.9**: α–[(4-amino-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-yl)thio]hexanamide **(21)**.

Jacobson *et al.*[11] have reported that a benzyl **(22)** or a 3-iodobenzyl **(23)** substituent at the $N^6$-position of adenosine resulted in high affinity and selective agonists for the adenosine $A_3$ receptor **(Figure 2.10)**. **(22)** had a $K_i$ $A_3$ values of 6.8 nM and was 13-fold selective for the $A_3$ receptor over the $A_1$ receptor and 14-fold selective for the $A_3$ receptor over $A_{2A}$ while **(23)** was reported to have a $K_i$ of 1.1 nM and 50-fold selectivity for the $A_3$ receptor over $A_1$ and $A_{2A}$ receptors.




**22** **23**

**Figure 2.10**: Highly selective agonist ligands for the adenosine $A_3$ receptor.

We propose that modification of the hydrophobic binding domain of phenyl substituent at the N-1 position of the α–[(4-amino-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-yl)thio]hexanamide **(21)** with bulky substituents such as benzyl and iodobenzyl could generate both potent and selective antagonist ligands for the adenosine $A_3$ receptor **(Figure 2.11 and Figure 2.12)**. The effects of substituents at the C4 and C6 positions of **(21)** will also be examined in order to determine the effects on selectivity and potency. **Figure 2.11** shows the superimposition of α–[(4-amino-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-yl)thio]hexanamide **(21)** (blue) and **(22)** or **(23)** (red)

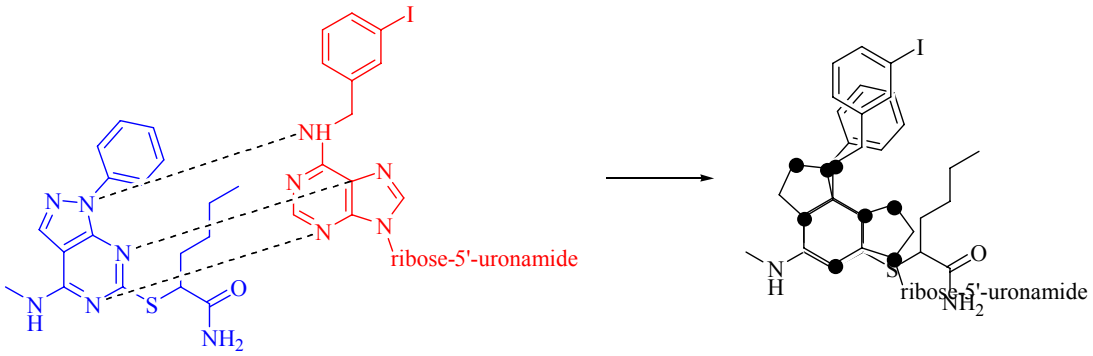



**Figure 2.11**: The superimposition of α–[(4-amino-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-yl)thio]hexanamide **(21)** and **(23)**.

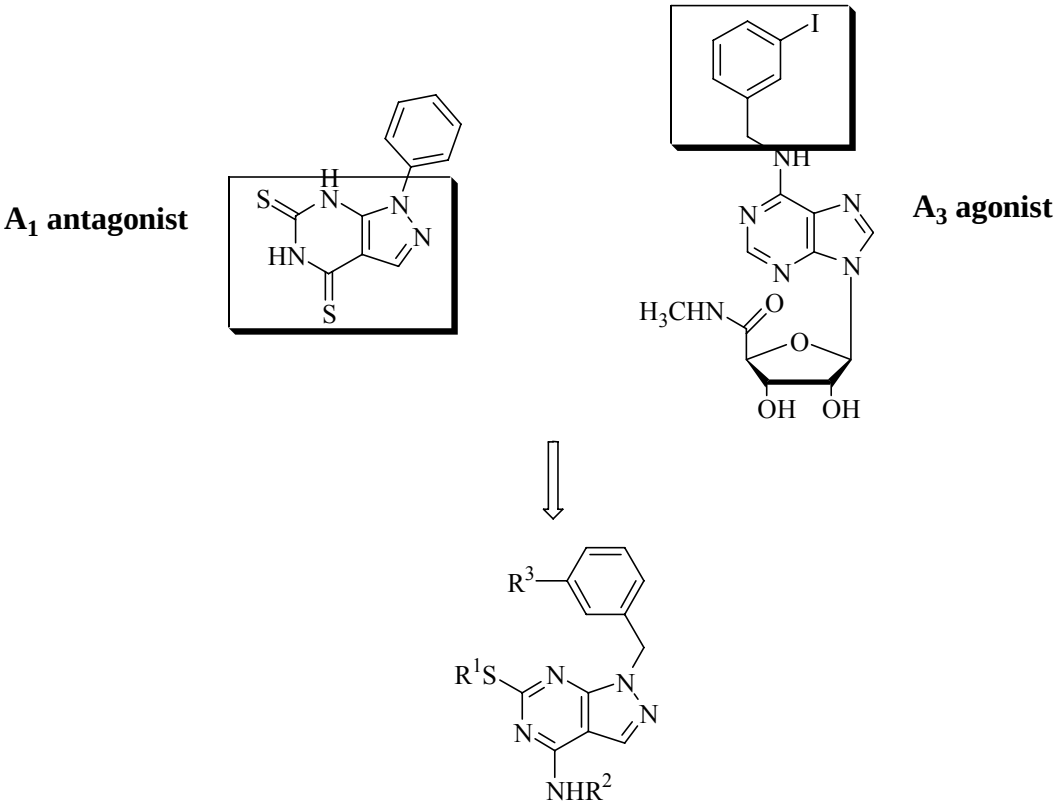
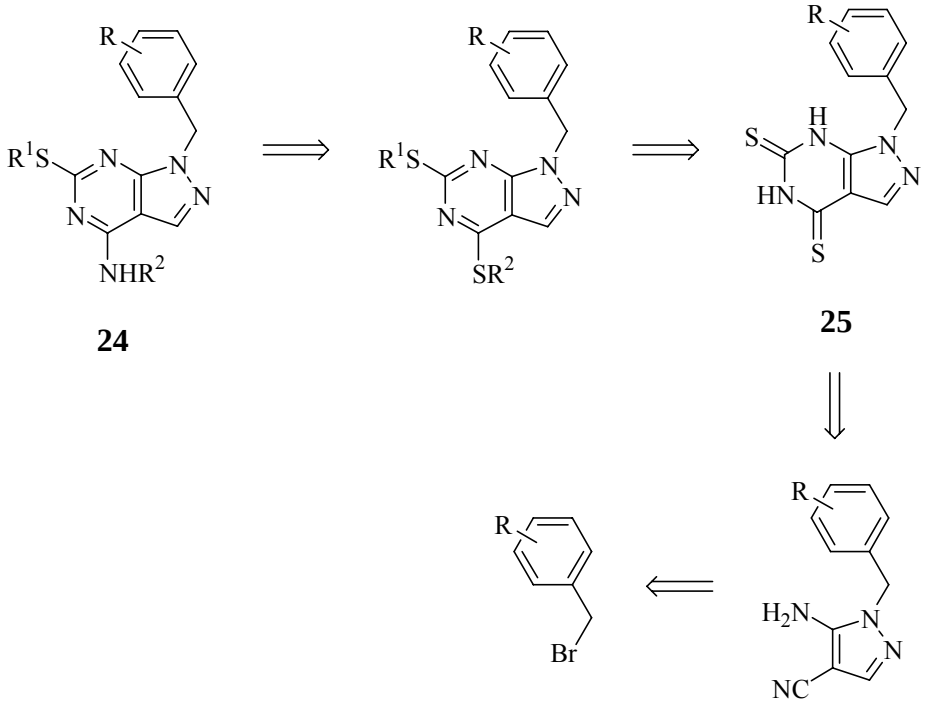



**Figure 2.12**: Rational design of $A_3$ adenosine receptor antagonist with three-point diversity.

### 2.2.2 Synthesis

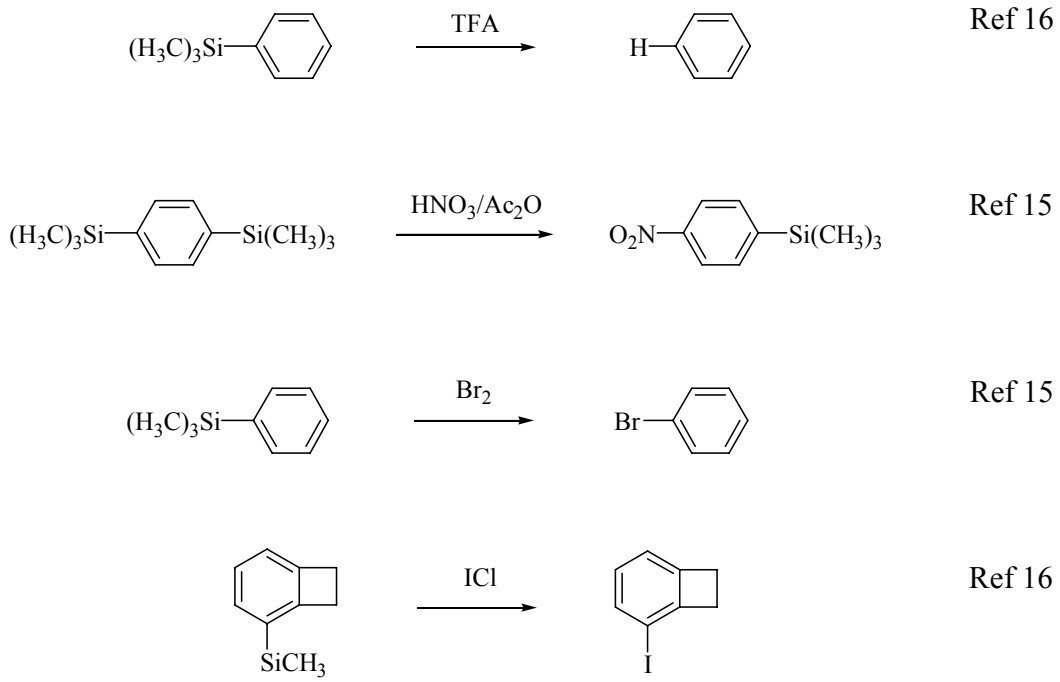
Our synthetic approach to the target molecules **(24)** was based on previous work in which the 1-benzyl-pyrazolo[3,4-*d*]pyrimidine substituted at C-4 and C-6 was

synthesized from 1-benzyl-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(25)**. Syntheses of a series of 1-phenyl-pyrazolo[3,4-*d*]pyrimidines with phenyl as the substitutent at N-1 showed that an alkylthio substituent at C-4 could act as a leaving group to allow nucleophilic substitution by amines to occur.[3-5,13,14] The core structure 1-benzyl-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(25)** could be synthesized from readily available benzyl bromide **(Scheme 2.1)**.



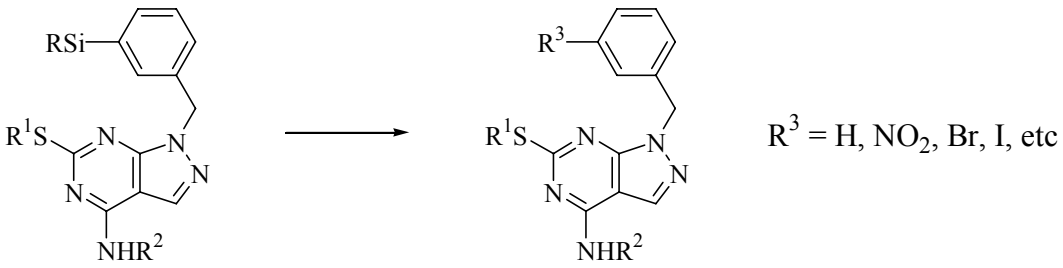

**Scheme 2.1**

Our attention focussed on the synthesis of pyrazolo[3,4-*d*]pyrimidines substituted with bulky substituents such as benzyl and iodobenzyl at N-1. As the benzyl ring was the starting material, starting from different substituted benzyl halides would require the same number of steps. The silicon directed *ipso*-substitution of aryl silanes has been extensively studied.[15] Electrophilic demetalations of aryl silanes with electrophiles[15,16] such as $H^+$, $I^+$, $Br^+$ and $NO_2^+$ would provide substituted aromatic compounds and hence allowed the generation of diversified libraries in the final cleavage step **(Scheme 2.2)**.




**Scheme 2.2**

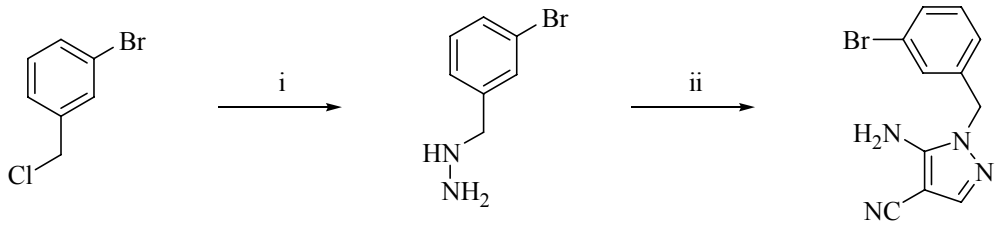
We firstly focussed on the synthesis of 1-(3-bromobenzyl)-pyrazolo[3,4-*d*]pyrimidines substituted at C-4 and C-6 from 3-bromobenzyl halide because it could later incorporate a silyl group into the phenyl ring to allow a desired diversified library **(Scheme 2.3)**.




**Scheme 2.3**

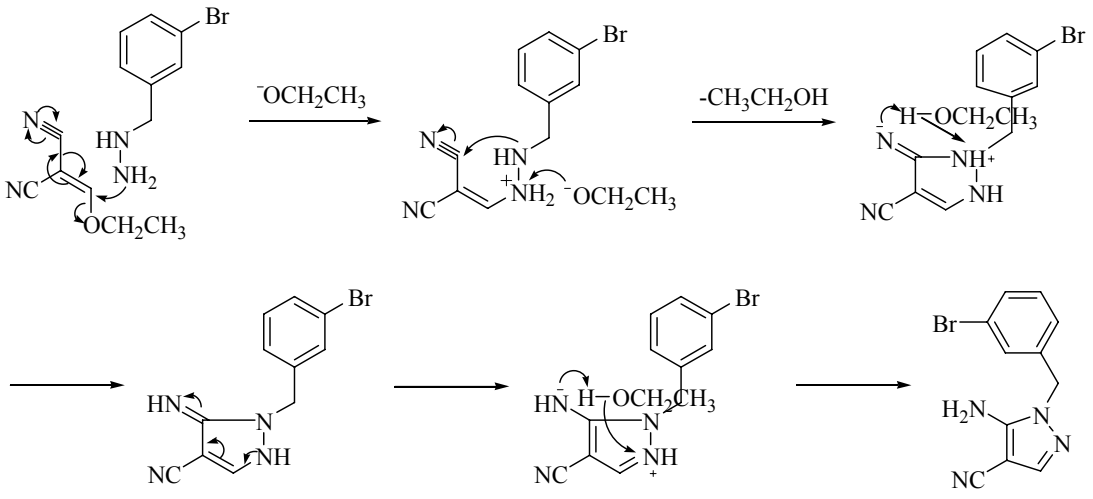
The first step of the synthesis of 1-(3-bromobenzyl)-pyrazolo[3,4-*d*]pyrimidines substituted at C-4 and C-6 involved $S_N2$ addition of hydrazine monohydrate to 3-bromobenzyl chloride in refluxing ethanol for 4h to give 3-bromobenzyl hydrazine **(26)** in 83% yield as a clear oil after purification by reduced pressure distillation. The hydrazine **(26)** was unstable and therefore it was

immediately used in the preparation of 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27) (Scheme 2.4)**. Nucleophilic addition of 3-bromobenzyl hydrazine to malononitrile followed by intramolecular cyclization gave 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27)** as white solid in 55% yield. A proposed mechanism for the formation of 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27)** is presented in **Scheme 2.5**.




**26** **27**

**Scheme 2.4**: *Reagents and conditions:* (i) $N_2H_4.H_2O$, EtOH, reflux, 4 h, 83%; (ii) $C_2H_5OCH=C(CN)_2$, EtOH, reflux, 2 h, 55%.

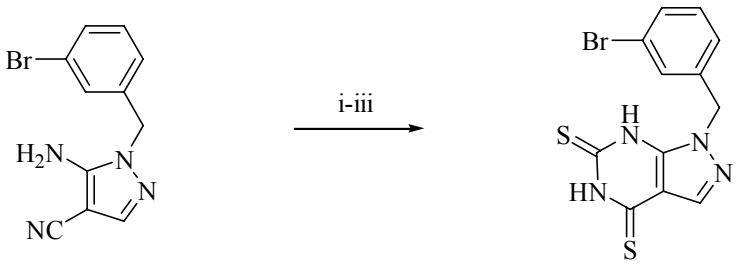



**Scheme 2.5**

The $^1H$ n.m.r spectrum of 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27)** showed a singlet at $\delta_H$ 6.78 ppm assigned to amino protons. The disappearance of this singlet upon $D_2O$ addition confirmed that there were $NH_2$ protons present in the molecule. A singlet at $\delta_H$ 5.17 ppm was assigned to benzylic proton and a singlet at $\delta_H$ 7.61 ppm was assigned to the H-3 proton. The IR spectrum showed a broad peak at

3400 $cm^{-1}$ assigned to $NH_2$ and a weak absorbance at 2200 $cm^{-1}$ assigned to CN. The ESMS (NI) of 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27)** exhibited molecular ions at *m/z* 275 and 277 a.m.u which were consistent with the molecular formulas $C_{11}H_9N_4{}^{79}Br$ and $C_{11}H_9N_4{}^{81}Br$.

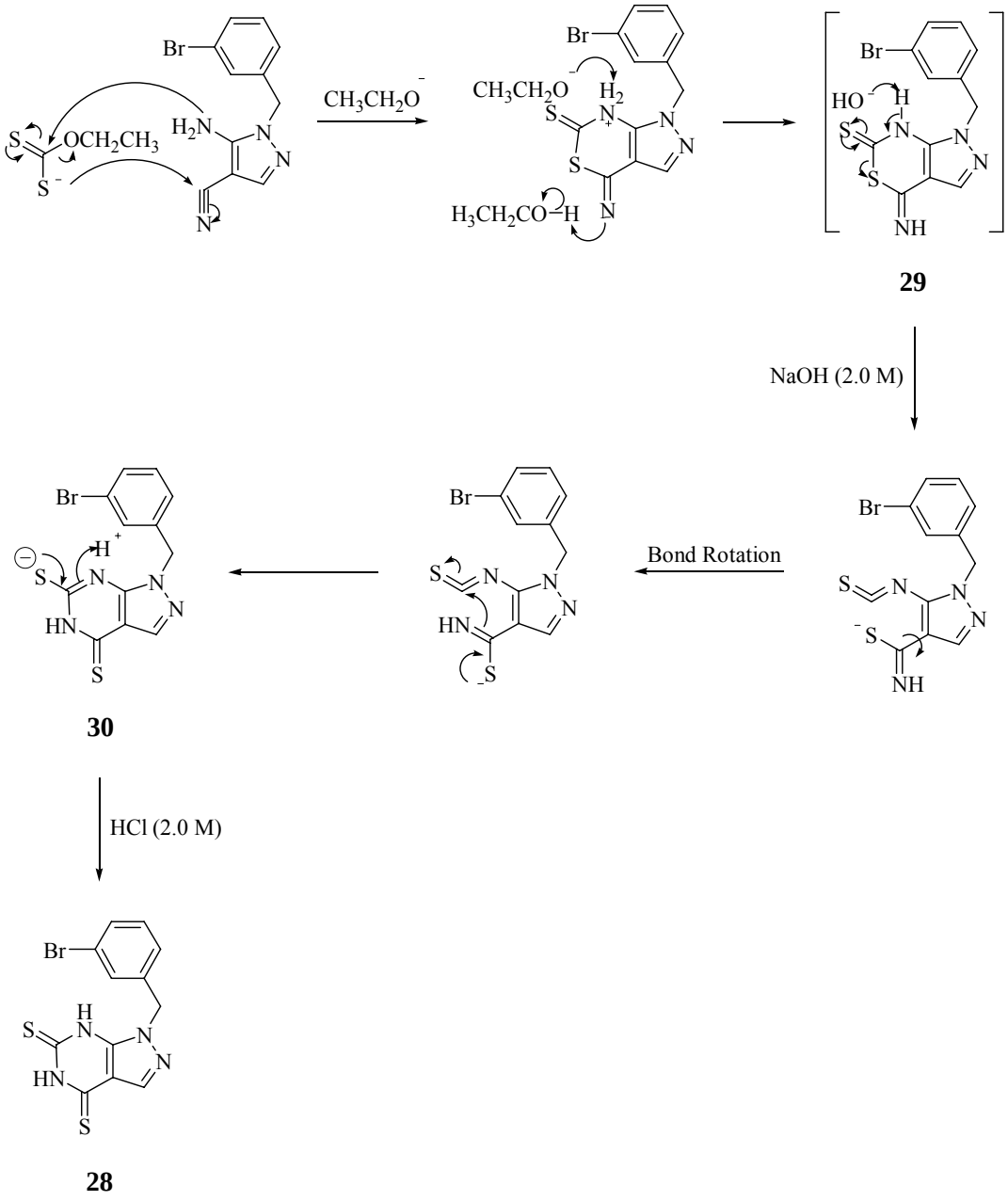
The next step in the synthesis involved refluxing 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27)** with potassium O-ethylxanthogenate in DMF at 140 $^oC$ for 2h. The intermediate was then rearranged to produce 1-(3-bromobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidines-4,6-dithione **(28)** as a light cream solid in 96% yield **(Scheme 2.6)**.




**27** **28**

**Scheme 2.6**: *Reagents and conditions:* (i) $EtOCS_2{}^-K^+$, DMF, 140 $^oC$, reflux, 2 h; (ii) NaOH (2.0 M), RT, 45 min; (iii) HCl (2.0 M), 96%.

A proposed mechanism for this reaction is shown in **(Scheme 2.7)**. Nucleophilic attack by O-xanthogenate anion at the ortho nitrile of cyanopyrazole **(27)** and nucleophilic attack by amino nitrogen of the cyanopyrazole **(27)** at the thiocarbonyl carbon of the O-xanthogenate anion formed the intermediate **(29).** Treatment of **(29)** with base, NaOH (2.0 M), at room temperature opened the ring followed by bond rotation and ring closure gave **(30)**. Protonation of **(30)** with HCl (2.0 M) gave 1-(3-bromobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(28)**. This rearrangement is called a Dimroth Rearrangment.[17,18]



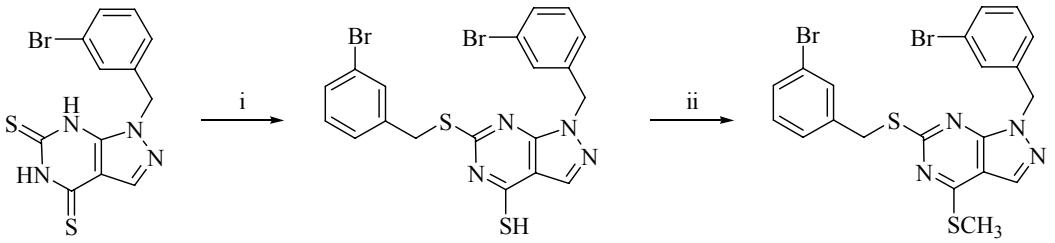

**Scheme 2.7**

The $^1$H n.m.r spectrum of 1-(3-bromobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(28)** showed the disappearance of the amino protons singlet and appearance of two broad singlets at $\delta_H$ 8.18 ppm and $\delta_H$ 11.25 ppm which were assigned to NH protons at N-7 and N-5 respectively. The H-3 proton at $\delta_H$ 7.80 ppm in 1-(3-bromobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(28)** was shifted downfield relative to the H-3 proton at $\delta_H$ 7.61 ppm in 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27)**. Two additional peaks at $\delta_C$ 176.4 ppm and $\delta_C$ 179.0 ppm in the

$^{13}C$ n.m.r spectrum of **(28)** were assigned as C-4 and C-6 respectively. The ESMS (NI) of 1-(3-bromobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(28)** exhibited molecular ions at *m/z* 351 and 353 a.m.u which were consistent with the molecular formulas $C_{12}H_9N_4S_2{}^{79}Br$ and $C_{12}H_9N_4S_2{}^{81}Br$.

1-(3-bromobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(28)** was then either alkylated at C-6 followed by methylation at C-4 or bis-alkylated at C-6 and C-4 to make a good leaving group at C-4 before the nucleophilic substitution by amines at C-4 could take place. S-methyl and S-alkyl at C-4 were both good leaving groups. The first strategy required weak base to remove the more acidic N-7 proton, hence generated the sulphur anion at C-6, to alkylate at C-6 followed by strong base to remove the N-5 proton to methylate at C-4. The second strategy required very strong base to remove both N-7 and N-5 protons to generate sulphur anion at C6 and C-4.

1-(3-bromobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(28)** was treated with a weak base, pyridine, and 1.0 equivalents of 3-bromobenzylbromide at room temperature to obtain 1-(3-bromobenzyl)-6-(3-bromobenzylthio)-4-mercaptopyrazolo[3,4-*d*]pyrimidine **(31)**. Since **(31)** was difficult to purify, the pyridine was removed from the reaction mixture and the crude product **(31)** was methylated with excess of methyl iodide in dioxane:NaOH (2.0 M) (1:1) at room temperature to afforded 1-(3-bromobenzyl)-6-(3-bromobenzylthio)-4-methylthio-pyrazolo[3,4-*d*]pyrimidine **(32)** in 49% yield and a small amount of 1-(3-bromobenzyl)-4,6-bis-methylthiopyrazolo[3,4-*d*]pyrimidine **(Scheme 2.8)**.

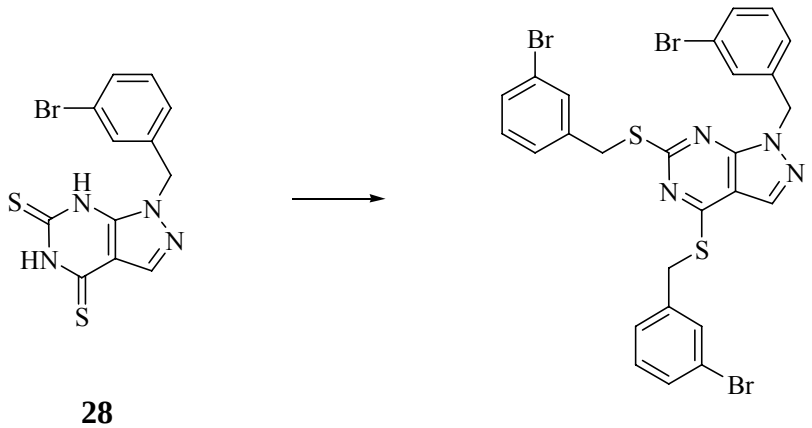



**28** **31** **32**

**Scheme 2.8**: *Reagents and conditions:* (i) 3-BrBnBr, Pyr, RT, 20 h; (ii) MeI, NaOH (2.0 M): Dioxane, RT, 1 h, 49%.

The $^{1}H$ n.m.r spectrum of 1-(3-bromobenzyl)-6-(3-bromobenzylthio)-4-methylthio-pyrazolo[3,4-*d*]pyrimidine **(32)** exhibited three singlets at $\delta_H$ 2.65 ppm, $\delta_H$ 4.41 ppm and $\delta_H$ 5.49 ppm which were assigned to $SCH_3$, $SCH_2$ and N-$CH_2$ respectively. The $^{13}C$ n.m.r spectrum of **(32)** showed two additional peaks at $\delta_C$ 12.2 ppm and $\delta_C$ 35.1 ppm, assigned to $SCH_3$ and $SCH_2$ carbons respectively, confirmed the structure of **(32)**. The ESMS (PI) of 1-(3-bromobenzyl)-6-(3-bromobenzylthio)-4-methylthio-pyrazolo[3,4-*d*]pyrimidine **(32)** exhibited molecular ions at *m/z* 534, 536 and 538 a.m.u which were consistent with the molecular formulas $C_{20}H_{16}N_4S_2{}^{79}Br^{79}Br$, $C_{20}H_{16}N_4S_2{}^{79}Br^{81}Br$ and $C_{20}H_{16}N_4S_2{}^{81}Br^{81}Br$.

Attempted bis-benzylation of 1-(3-bromobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(28)** with excess of 3-bromobenzyl bromide at room temperature using strong base, NaOH (2.0 M), afforded 1-(3-bromobenzyl)-4,6-bis-(3-bromobenzylthio)-pyrazolo[3,4-*d*]pyrimidine **(33)** in 14 h. Bis-alkylation proceeded stepwise via alkylation firstly at the C-6 position followed by the C-4 position since the base firstly removed the more acidic N-7 proton. The reaction went very slowly and was stirred very vigorously since the insoluble 1-(3-bromobenzyl)-6-(3-bromobenzylthio)-4-mercaptopyrazolo[3,4-*d*]pyrimidine **(31)** precipitated from the reaction mixture. Mix-solvent of NaOH (2.0 M) and dioxane however facilitated the reaction since it formed a homogenous reaction mixture **(Scheme 2.9)**.

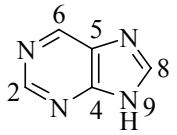



**28** **33**

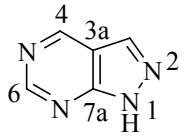
**Scheme 2.9**: *Reagents and conditions:* 3-BrBnBr, NaOH (2.0 M) : Dioxane, RT, 10 h, 50%.

The $^{1}H$ n.m.r spectrum of 1-(3-bromobenzyl)-4,6-bis-(3-bromobenzylthio)-pyrazolo[3,4-*d*]pyrimidine **(33)** exhibited three singlets at $\delta_H$ 4.39 ppm, $\delta_H$ 4.49 ppm and $\delta_H$ 5.49 ppm which were assigned to benzylic protons at C-6, C-4 and N-1 positions respectively. The $^{13}C$ n.m.r spectrum of **(33)** showed two additional peaks at $\delta_C$ 32.6 ppm and $\delta_C$ 35.1 ppm, assigned for two additional benzylic carbons at C-4 and C-6 respectively, confirming the structure of **(33)**. The ESMS (PI) of 1-(3-bromobenzyl)-4,6-bis-(3-bromobenzylthio)-pyrazolo[3,4-*d*]pyrimidine **(33)** exhibited molecular ions at *m/z* 688, 690, 692 and 694 a.m.u which were consistent with the molecular formulas $C_{26}H_{19}N_4S_2{}^{79}Br^{79}Br^{79}Br$, $C_{26}H_{19}N_4S_2{}^{79}Br^{79}Br^{81}Br$, $C_{26}H_{19}N_4S_2{}^{79}Br^{81}Br^{81}Br$ and $C_{26}H_{19}N_4S_2{}^{81}Br^{81}Br^{81}Br$.

Having successful alkylated at C-4 and C-6, the desired targer molecule could be formed by substituting S-alkyl leaving group with nucleophilic amine. Since nucleophilic substitution of purines occurred preferentially at C-6,[19,20] pyrazolop[3,4-*d*]pyrimidines would undergo nucleophilic substitution at C-4 because the C-6 position in purines corresponded to the C-4 position in pyrazolop[3,4-*d*]pyrimidines **(Figure 2.10)**. 1-(3-bromobenzyl)-6-(3-bromobenzylthio)-4-methylthio-pyrazolo[3,4-*d*]pyrimidine **(32)** and 1-(3-bromobenzyl)-4,6-bis-(3-bromobenzylthio)-pyrazolo[3,4-*d*]pyrimidine **(33)** both could undergo nucleophilic displacement with secondary amines. However, **(33)** was chosen as a starting material since it could be prepared in only one step from 1-(3-bromobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(28)** without any difficulties in comparison with the preparation of **(32)**.




**34**

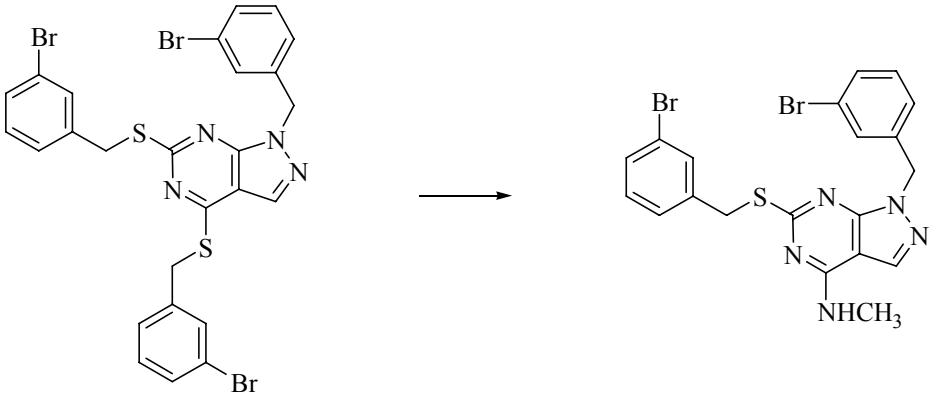



**1**

**Figure 2.13**: The imidazo[4,5-*d*]pyrimidine (purine) **(34)** and pyrazolo[3,4-*d*]pyrimidine **(1)** heterocycles.

1-(3-bromobenzyl)-4,6-bis(3-bromobenzylthio)-pyrazolo[3,4-*d*]pyrimidine **(33)** was treated with excess of methylamine in methanol for 8 h at 60$^{o}$C to produce 1-

(3-bromobenzyl)-6-(3-bromobenzylthio)-4-methylamino-pyrazolo[3,4-*d*]pyrimidine **(35)** in 52% yield. The $^{1}$H n.m.r spectrum of **(35)** showed the absence of the singlet at $\delta_H$ 4.49 ppm and appearance of doublet at $\delta_H$ 2.90 ppm ($J$ = 4.8 Hz) which confirmed the substitution of S-benzylic by N-methyl at C-4. The $^{13}$C n.m.r spectrum of **(35)** also exhibited the signal at $\delta_C$ 27.6 ppm assigned to $NCH_3$ and no signal at $\delta_C$ 32.6 ppm due to the substitution of S-3-bromobenzyl by methyl amine. The ESMS (PI) of 1-(3-bromobenzyl)-6-(3-bromobenzylthio)-4-methylamino-pyrazolo[3,4-*d*]pyrimidine **(35)** exhibited molecular ions at *m/z* 518, 520 and 522 a.m.u which were consistent with the molecular formulas $C_{20}H_{17}N_5S^{79}Br^{79}Br$, $C_{20}H_{17}N_5S^{79}Br^{81}Br$ and $C_{20}H_{17}N_5S^{81}Br^{81}Br$.




**33** **35**

**Scheme 2.10**: *Reagents and conditions:* $CH_3NH_2$ in MeOH, 60 $^{o}$C, 8 h, 52%.

## 2.3 Conclusion

The synthesis of 3-bromobenzylpyrazolo[3,4-*d*]pyrimidines substituted at C-6 and C-4 from 3-bromobenzyl chloride was achieved. It was next to incorporate a silyl group into the phenyl ring of 3-bromobenzyl substituted pyrazolo[3,4-*d*]pyrimidines to allow molecular diversity generation.

## 2.4 Experimental

Melting points were recorded on a Gallenkamp digital melting point apparatus and are uncorrected. Infra-red absorption spectra were obtained on a Perkin Elmer FT-IR spectrophotometer using sodium chloride plates. $^{1}H$ and $^{13}C$ nuclear magnetic resonance (n.m.r) spectra were recorded on a Varian Gemini-200 (200 MHz) spectrometer, Varian Unity-400 (400 MHz) spectrometer or Varian Unity Plus-600 (600 MHz) spectrometer. All samples were dissolved in deuteriochloroform ($CDCl_3$) containing tetramethylsilane (TMS) as an internal reference unless otherwise stated. The signals are recorded in terms of chemical shift in parts per million (ppm) downfield from TMS ($\delta = 0$) for protons or $CDCl_3$ ($\delta = 77$) for carbon atoms. The signals are recorded in terms of chemical shift ($\delta_H$), relative integral, multiplicity, coupling constants (*J* Hz) and assignments, in that order. The following abbreviations for multiplicity are used: s = singlet, dd = doublet of doublets, dt = doublet of triplets, m = multiplet. $^{1}H$ and $^{13}C$ spectra were assigned with the aid of HMQC (Heteronuclear Multiple-Quantum Coherence), HMBC (Heteronuclear Multiple-Bond Coherence) and $^{1}H$-$^{1}H$ COSY (Correlation Spectroscopy). Electrospray mass spectra (ESMS) were recorded on a Fisons VG Platform mass spectrometer with MassLynx Data System software.

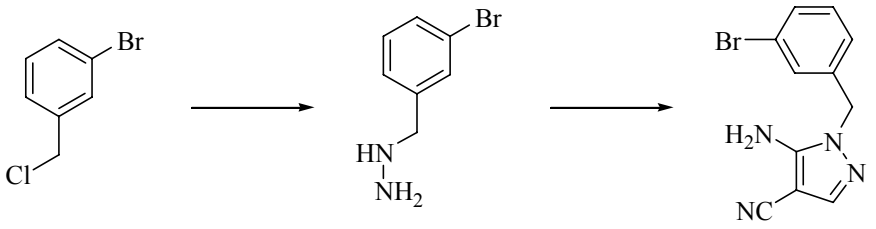
Microanalytical data was obtained from University of Queensland Microanalytical Service.

Analytical thin layer chromatography (TLC) was performed using precoated (0.2 mm) Merck silica gel plates (Merck Kieselgel 60 $F_{254}$). Flash chromatography was carried out using Merck Kieselgel 60 (230-400 mesh) with the indicated solvents.

All solvents for chromatography were distilled before use, unless otherwise stated. Ether refers to diethyl ether and hexane refers to the fraction of b.p. 60-80 °C. Mixed solvent compositions are quoted as v/v.

Solvents and reagents were purified according to the standard techniques of Perrin, Perrin and Amarego.[21]

**1-(3-bromobenzyl)-5-amino-4-cyanopyrazole (27)**



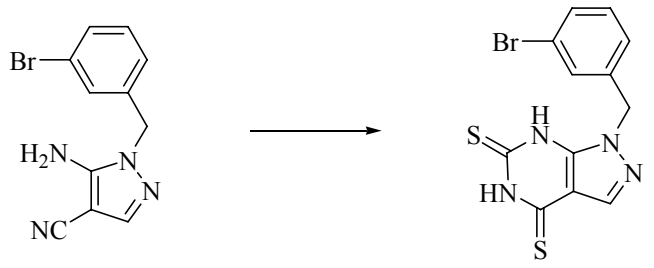

**26** **27**

To a refluxing solution of hydrazine monohydrate (46.44 g, 45.0 ml, 0.93 mol) in ethanol (150 ml), a solution of 3-bromobenzyl chloride (20.26 g, 12.6 ml, 0.099 mol) in ethanol (50 ml) was added dropwise over a period of 1 h. The reaction mixture was refluxed for additional 3 h before the solvent was removed *in vacuo*. The remaining pale yellow liquid was extracted with ether (3 x 50 ml). The combined organic layers were dried ($MgSO_4$) and concentrated *in vacuo* to yield crude oil. The crude oil was purified by distillation under reduced pressure through a Vigreux column to yield 3-bromobenzylhydrazine **(26)** (16.50 g, 83%) as a clear oil, bp 99-100 °C, at 0.6 mm Hg. The 3-bromobenzylhydrazine **(26)** was unstable and hence it was not fully characterised; $\delta_H$ (400 MHz, DMSO-$d_6$) 3.46 (br, 3H, NH-$NH_2$), 3.73 (s, 2H, $CH_2$), 7.27 (t, $J$ = 7.6 Hz, 1H, 5'-H), 7.31 (dt, $J_{ortho}$ = 7.6 Hz, $J_{meta}$ = 1.2 Hz, 1H, 6'-H), 7.42 (dt, $J_{ortho}$ = 7.6 Hz, $J_{meta}$ = 1.2 Hz, 1H, 4'-H) and 7.52 (s, 1H, 2'-H); ESMS (PI) 201 and 203. calcd for ($C_7H_9{}^{79}BrN_2$ + 1[H]) and ($C_7H_9{}^{81}BrN_2$ + 1[H]) respectively. Found 201 and 203.

To a stirred solution of ethoxymethylenemalononitrile (9.9 g, 0.081 mol) in ethanol (100 ml) under argon, a solution of 3-bromobenzylhydrazine **(26)** (16.30 g, 0.081 mol) in ethanol (20 ml) was added dropwise. The resultant mixture was refluxed over 2 h and a deep red colour solution was produced. The reaction mixture was left to cool to room temperture and yellow crystalline material precipitated. Precipitation was further enhanced by cooling in the fridge overnight before it was filtered and the solid was washed with cold ethanol. The crude solid was purified by flash chromatography (50% ethyl acetate-hexane) to afford 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27)** (12.34 g, 55%) as white solid, mp 101.5 ± 0.5 °C; $\delta_H$ (400 MHz, DMSO-$d_6$) 5.17 (s, 2H, $CH_2$), 6.78 (s, 2H, $NH_2$), 7.16 (d, $J$ = 8.0 Hz, 1H,

6'-H), 7.30 (t, $J$ = 8.0 Hz, 1H, 5'-H), 7.38 (t, $J_{meta}$ = 1.6 Hz, 1H, 2'-H), 7.49 (d, $J$ = 8.0 Hz, 1H, 4'-H) and 7.61 (s, 1H, 3-H); $\delta_C$ (100 MHz, DMSO-$d_6$) 49.3 ($CH_2$), 72.3 (C-4), 115.1 (CN), 121.7 (C-3'), 126.4 (C-6'), 130.1 (C-2'), 130.4 (C-4'), 130.7 (C-5'), 139.3 (C-1'), 140.8 (C-3) and 151.7 (C-5); $\nu_{max}$ (NaCl plates)/$cm^{-1}$ 3400 ($NH_2$) and 2200 (CN). Anal. calcd for $C_{11}H_9BrN_4$: C, 47.68; H, 3.27; N, 20.22C. Found C, 47.80; H, 3.17; N, 20.30%; ESMS (NI) 275 and 277. calcd for ($C_{11}H_9{}^{79}BrN_4$ - 1[H]) and ($C_{11}H_9{}^{81}BrN_4$ - 1[H]) respectively. Found 275 and 277.

**1-(3-bromobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione (28)**

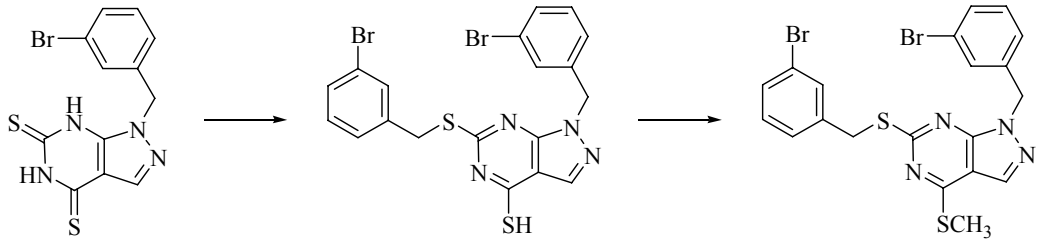



**27** **28**

To a solution of 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27)** (8.32 g, 30.0 mmol) in dry DMF (50 ml) under argon was added potassium-O-ethylxanthogenate (9.62 g, 60.0 mmol). The reaction mixture was heated to 140 °C for 2 h under argon. The initial orange and opaque solution turned to dark brown after 2 h. The reaction mixture was allowed to cool to room temperature and the solvent was removed under reduced pressure to afford crude brown oil. NaOH (2.0 M, 30 ml) was added to the remaining brown oil and stirred at room temperature for 45 min. The basic solution was filtered to give a transparent orange filtrate. HCl (2.0 M) was added dropwise to the filtrate until the neutral pH was reached. A creamy coloured precipitate was formed upon the neutralisation of the filtrate. The crude product was collected by suction filtration and recrystallised from DMSO and water to afford 1-(3-bromobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(28)** (10.18 g, 96%) as light cream solid, mp > 230 °C; $\delta_H$ (400 MHz, DMSO-$d_6$) 5.26 (s, 2H, $CH_2$), 7.08 (d, $J$ = 8.0 Hz, 1H, 6'-H), 7.22 (t, $J$ = 8.0 Hz, 1H, 5'-H), 7.29 (s, 1H, 2'-H), 7.40 (d, $J$ = 8.0 Hz, 1H, 4'-H), 7.80 (s, 1H, 3-H), 8.18 (br, 1H, NH) and 11.25 (br, 1H, NH); $\delta_C$ (100 MHz, DMSO-$d_6$) 49.2 ($CH_2$), 112.7 (C-3a), 122.4 (C-3'), 126.9 (C-6'), 130.5 (C-

2'), 130.9 (C-4'), 131.4 (C-5'), 137.4 (C-3), 140.8 (C-1'), 150.4 (C-7a), 176.4 (C-4) and 179.0 (C-6); ESMS (NI) 351 and 353. calcd for ($C_{12}H_9{}^{79}BrN_4S_2$ - 1[H]) and ($C_{12}H_9{}^{81}BrN_4S_2$ - 1[H]) respectively. Found 351 and 353.

**1-(3-bromobenzyl)-6-(3-bromobenzylthio)-4-methylthiopyrazolo[3,4-*d*]-pyrimidine (32)**

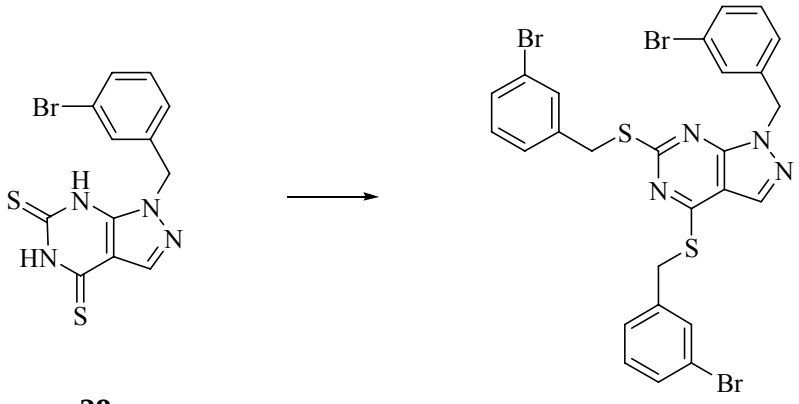



**28** **31** **32**

To a solution of 1-(3-bromobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6dithione **(28)** (0.2 g, 0.566 mmol) in dry pyridine (5 ml) under argon was added 3-bromobenzyl bromide (0.141 g, 0.564 mmol). The reaction mixture was stirred at room temperature for 20 h. The solvent was removed from reaction mixture under reduced pressure to afford the crude material, 1-(3-bromobenzyl)-6-(3-bromobenzylthio)-4-mercaptopyrazolo[3,4-*d*]pyrimidine **(31)**. The crude material **(31)** was dissolved in NaOH (2.0 M) (5ml)/Dioxane (5 ml). Methyliodide (45 µl, 0.722 mmol) was added and the reaction mixture was stirred at room temperature for 1 h. The reaction mixture was extracted with ethyl acetate (3 x 20 ml), dried ($MgSO_4$) and the solvent removed in vacuo. The resultant residue was purified by flash chromatography (10% ethyl acetate-hexane) to yield 1-(3-bromobenzyl)-6-(3-bromobenzylthio)-4-methylthiopyrazolo[3,4-*d*]-pyrimidine **(32)** (148 mg, 49%) as white solid, mp 109.5 ± 0.5 °C; $\delta_H$ (400 MHz) 2.65 (s, 3H, $CH_3$), 4.41 (s, 2H, $CH_2$), 5.49 (s, 2H, $CH_2$), 7.10-7.65 (m, 8H, Ar-H) and 7.95 (s, 1H, 3-H); $\delta_C$ (100 MHz) 12.2 ($CH_3$), 35.1 ($CH_2$), 50.3 ($CH_2$), 109.9 (C-3a), 122.6-132.6 (11 Ar-C), 138.5 (C-3), 140.4 (Ar-C), 151.9 (C-7a), 165.9 (C-4) and 167.9 (C-6); Anal. calcd for $C_{20}H_{16}N_4S_2Br_2$: C, 44.79; H, 3.01; N, 10.45. Found C, 47.92; H, 3.83; N, 9.02%; ESMS (PI) 534, 536 and 538. calcd for ($C_{20}H_{16}N_4S_2{}^{79}Br{}^{79}Br$ + 1[H]),

($C_{20}H_{16}N_4S_2{}^{79}Br^{81}Br$ + 1[H]) and $C_{20}H_{16}N_4S_2{}^{81}Br^{81}Br$ + 1[H]) respectively. Found 534, 536 and 538.

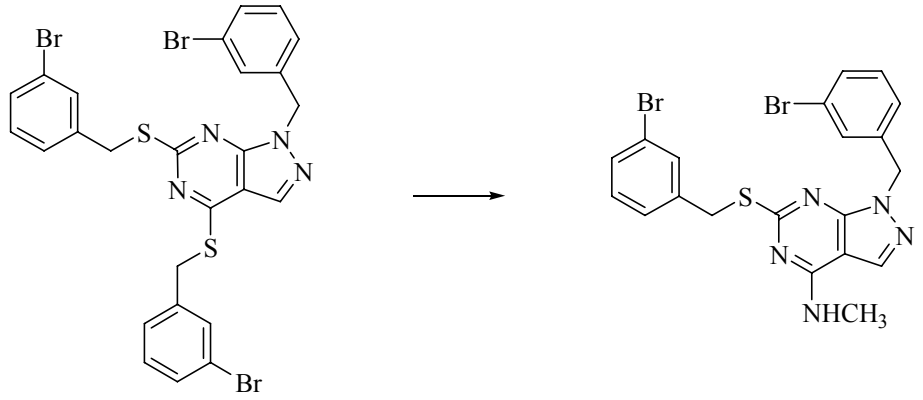
**1-(3-bromobenzyl)-4,6-bis-(3-bromobenzylthio)pyrazolo[3,4-*d*]pyrimidine (33)**



**28** **33**

To a solution of 1-(3-bromobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6dithione **(28)** (0.26 g, 0.736 mmol) in NaOH (2.0 M) (10 ml)/Dioxane (10 ml) was added 3-bromobenzyl bromide (0.46 g, 1.84 mmol). After stirring at room temperature for 10 h, the reaction mixture was extracted with ethyl acetate (3 x 30 ml), dried ($MgSO_4$) and the solvent removed in vacuo. The resultant residue was purified by flash chromatography (10% ethyl acetate-hexane) to yield 1-(3-bromobenzyl)-4,6-bis-(3-bromobenzylthio)pyrazolo[3,4-*d*]pyrimidine **(33)** (254 mg, 50%) as white solid, mp 116.5 ± 0.5 °C; $\delta_H$ (400 MHz) 4.39 (s, 2H, $CH_2$), 4.49 (s, 2H, $CH_2$), 5.49 (s, 2H, $CH_2$), 7.10-7.70 (m, 12H, Ar-H) and 7.91 (s, 1H, 3-H); $\delta_C$ (100 MHz) 32.2 ($CH_2$), 35.1 ($CH_2$), 50.4 ($CH_2$), 109.6 (C-3a), 122.7-132.5 (16 Ar-C), 138.4 (C-3), 139.5 (Ar-C), 140.2 (Ar-C), 152.1 (C-7a), 164.1 (C-4) and 167.0 (C-6); Anal. calcd for $C_{26}H_{19}N_4S_2Br_3$: C, 45.17; H, 2.77; N, 8.10. Found C, 45.05; H, 2.55; N, 7.97%; ESMS (PI) 688, 690, 692 and 694. calcd for ($C_{26}H_{19}N_4S_2{}^{79}Br^{79}Br^{79}Br$ + 1[H]), ($C_{26}H_{19}N_4S_2{}^{79}Br^{79}Br^{81}Br$ + 1[H]), ($C_{26}H_{19}N_4S_2{}^{79}Br^{81}Br^{81}Br$ + 1[H]) and ($C_{26}H_{19}N_4S_2{}^{81}Br^{81}Br^{81}Br$ + 1[H]) respectively. Found 688, 690, 692 and 694.

**1-(3-bromobenzyl)-6-(3-bromobenzylthio)-4-methylamino-pyrazolo[3,4-*d*]-pyrimidine (35)**




**33** **35**

To a solution of methylamine in methyl alcohol (10 ml, 2.0 M, 10 mmol) was added 1-(3-bromobenzyl)-4,6-bis-(3-bromobenzylthio)pyrazolo[3,4-*d*]pyrimidine **(33)** (0.150 g, 0.217 mmol) and the mixture was heated to 60 °C for 8 h. The reaction mixture was concentrated and purified by flash chromatography (2.5% methanol-DCM) to yield 1-(3-bromobenzyl)-6-(3-bromobenzylthio)-4-methylamino-pyrazolo[3,4-*d*]pyrimidine **(35)** (59 mg, 52%) as white solid, mp 161.5 ± 0.5 °C; $\delta_H$ (400 MHz, DMSO-$d_6$) 2.90 (d, $J$ = 4.8 Hz, 3H, $NCH_3$), 4.34 (s, 2H, $CH_2$), 5.41 (s, 2H, $CH_2$), 7.05-7.45 (m, 7H, Ar-H), 7.59 (s, 1H, 2'-H at C-6), 7.97 (s, 1H, 3-H), and 8.37 (q, $J$ = 4.8 Hz, 1H, NH); $\delta_C$ (100 MHz, DMSO-$d_6$) 27.6 ($NCH_3$), 34.1 ($SCH_2$), 49.6 ($CH_2$), 99.1 (C-3a), 122.0-132.2 (10 Ar-C + C-3), 140.6 (Ar-C), 142.8 (Ar-C), 153.8 (C-7a), 156.6 (C-4) and 168.2 (C-6); Anal. calcd for $C_{20}H_{17}N_5SBr_2$: C, 46.26; H, 3.30; N, 13.49. Found C, 45.64; H, 3.26; N, 12.98%; ESMS (PI) 518, 520 and 522. calcd for ($C_{20}H_{17}N_5S^{79}Br^{79}Br$ + 1[H]), ($C_{20}H_{17}N_5S^{79}Br^{81}Br$ + 1[H]) and ($C_{20}H_{17}N_5S^{81}Br^{81}Br$ + 1[H]) respectively. Found, 518, 520 and 522.

## 2.5 References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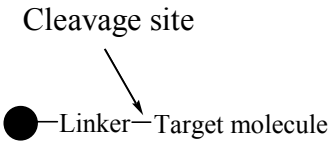
# CHAPTER 3

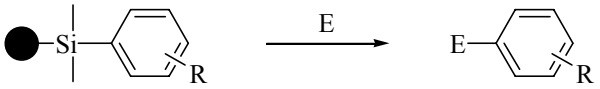
## Application of Silicon Chemistry in synthesis of diversified library

### 3.1 Introduction:

Introduced in the early 1990's, combinatorial chemistry and parallel synthesis techniques[1] were regarded as an important tools in lead generation and lead optimization in the drug discovery process because they had the potential to synthesize compounds faster than classical organic synthesis. The aims of the project were to synthesize a series of pyrazolo[3,4-*d*]pyrimidines with bulky substituents such as benzyl and iodobenzyl at N-1. They could be synthesized by parallel solution synthesis but required the same number of steps. In the last 10 years, silicon was developed as a linkage element to the solid support in the solid phase synthesis[2,3] since it could be cleaved with a variety of electrophiles such as $H^+$, $I^+$, $Br^+$, $Cl^+$, $Ac^+$, $NO_2^+$, etc to produce diversified libraries **(Scheme 3.2 and Scheme 3.3)**. [4-15]




**Scheme 3.1**



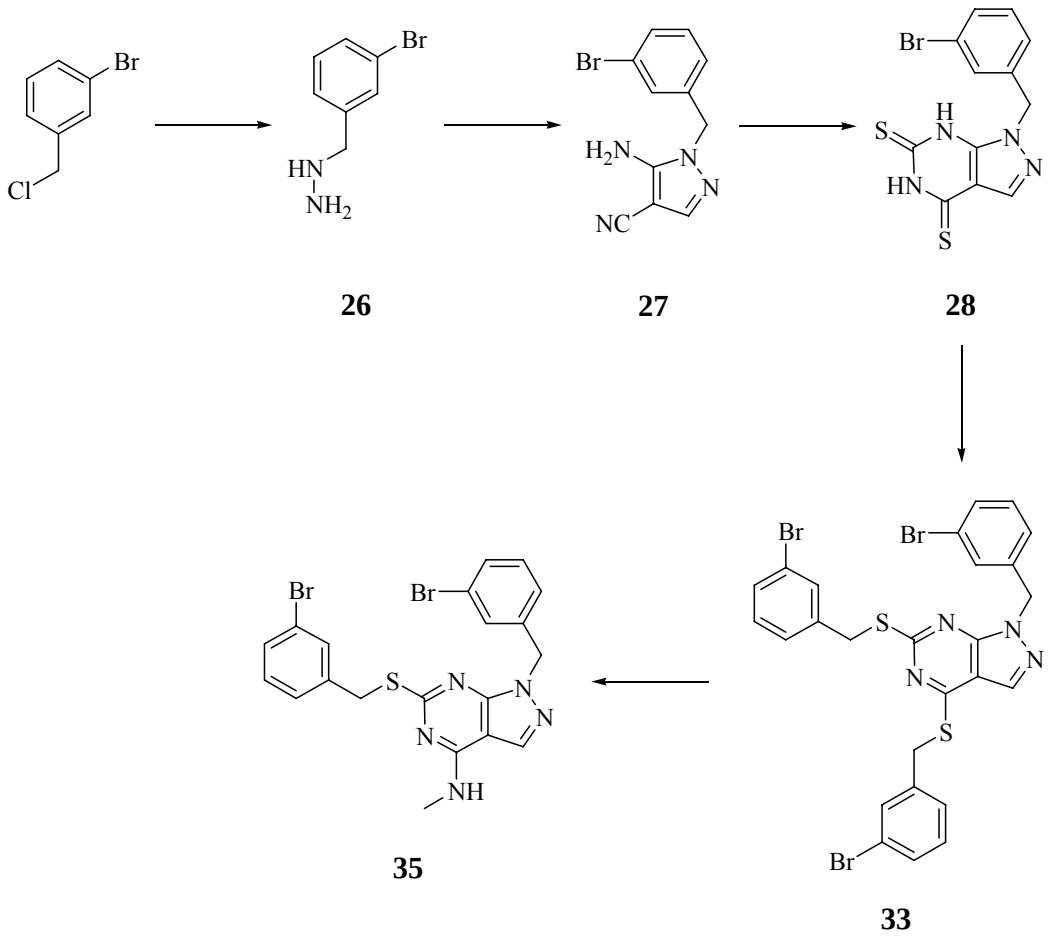

**Scheme 3.2**: *E is the electrophiles* such as $H^+$, $I^+$, $Br^+$, $Cl^+$, $Ac^+$, $NO_2^+$, etc.

Having successful synthesized pyrazolo[3,4-*d*]pyrimidines with 3-bromobenzyl substituted at N-1, we attemped to synthesize a series of pyrazolo[3,4-*d*]pyrimidines heterocylce by solid phase synthesis because solid phase synthesis had several advantages over solution phase synthesis such as easy work-up procedures; high yields by employing excess reagents; and amenability to automation. In order to undertake a solid phase synthesis of pyrazolo[3,4-*d*]pyrimidines, a silyl group was firstly incorporated into the phenyl ring of 3-bromobenzyl substituted pyrazolo[3,4-*d*]pyrimidines.

## 3.2 Results and Discussion

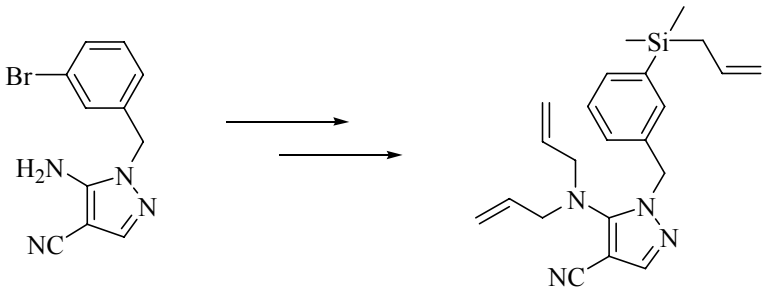
### 3.2.1 Protection and deprotection of the intermediate (27)

Solution phase synthesis of 1-(3-bromobenzyl)-pyrazolo[3,4-*d*]pyrimidines substituted at C-4 and C-6 had been successfully completed as a feasibility study of the combinatorial synthetic work **(Scheme 3.3)**. An intermediate 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27)** in this pathway was considered a suitable intermediate for incorporation of a silyl group into the phenyl ring **(Scheme 3.4)**.



**26** **27** **28** **35** **33**

**Scheme 3.3**

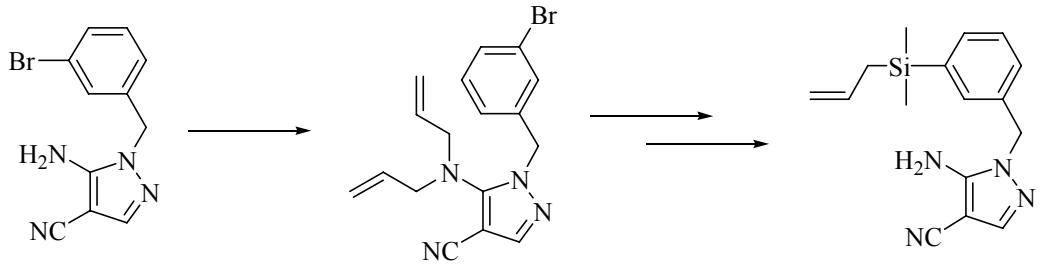


**27**

**Scheme 3.4**

The 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27)** was first protected before incorporation of the silyl into the phenyl ring via lithium-halogen exchange with butyl lithium. The allyl group was chosen as a protecting group for the amino

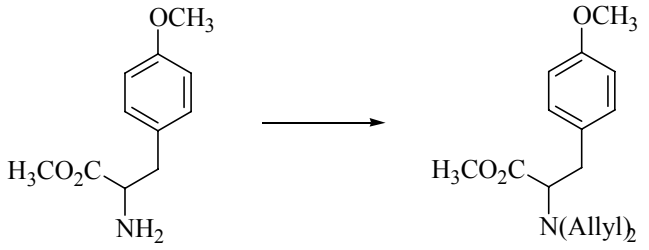
functional group since it was very stable to acid and base conditions; inert to nucleophiles such as butyllithium reagent or Grignard reagent and could be readily removed by isomerization to the enanmine[16] or by rhodium-catalyzed isomerization[17] **(Scheme 3.5)**.



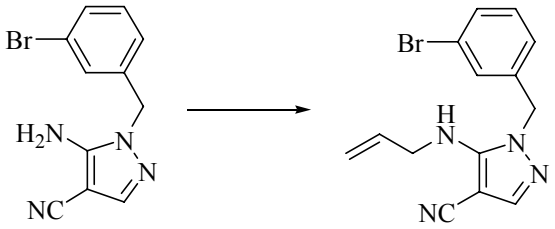

**27** **36**

**Scheme 3.5**

The protection of 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27)** was attempted with diisopropylethylamine and allyl bromide using Laguzza's procedure in the synthesis of anticapsin **(Scheme 3.6)** but was unsuccessful. However, 5-allylamino-1-(3-bromobenzyl)-4-cyanopyrazole **(37)** was produced as a major product **(Scheme 3.7)**. A stronger base, sodium hydride, was used to generate amide anion which was then added to allylbromide. A mixture of 5-allylamino-1-(3-bromobenzyl)-4-cyanopyrazole **(37)** and 1-(3-bromobenzyl)-N,N-bisallyl-4-cyanopyrazole **(36)** were produced when 2.3 equivalents of sodium hydride was used. The 1-(3-bromobenzyl)-N,N-bisallyl-4-cyanopyrazole **(36)** was produced as only major product when 4.4 equivalents of sodium hydride was used **(Scheme 3.8) (Table 3.1)**.

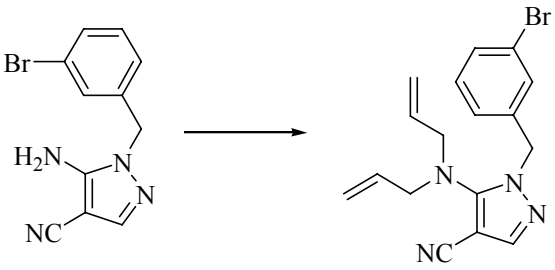



**Scheme 3.6**: *Reagents and conditions:* DIPEA, Allylbromide, Toluene, reflux, 84%.




**27** **37**

**Scheme 3.7**: *Reagents and conditions:* DIPEA, Allylbromide, THF, reflux, 24 h, 78%.




**27** **36**

**Scheme 3.8**: *Reagents and conditions:* NaH, Allylbromide, THF, RT, 3 h, 97%.

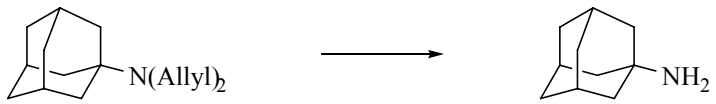
**Table 3.1**: Comparison of protection methods of 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27)**.

| Solvent | Reagents | Conditions | Yields |
|---|---|---|---|
| THF | DIPEA (6.0 eq)<br>Allyl bromide | 85 °C, 24 h | 78 % Monoallylated product **(37)** |
| THF | NaH (2.3 eq)<br>Allyl bromide | RT, 3 h | 38 % Monoallylated product **(37)**<br>57 % Diallylated product **(36)** |
| THF | NaH (4.4 eq)<br>Allyl bromide | RT, 3 h | 97 % Diallylated product **(36)** |

The structure of 1-(3-bromobenzyl)-N,N-bisallyl-4-cyanopyrazole **(36)** was confirmed by the absence of the amino protons singlet at $\delta_H$ 6.78 ppm and the

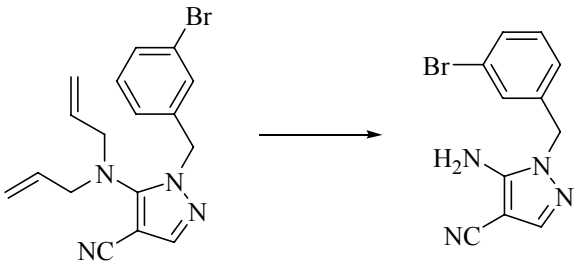
addition of allyl protons at $\delta_H$ 3.60-3.65 ppm, $\delta_H$ 5.10-5.20 ppm and $\delta_H$ 5.59-5.71 ppm in the $^1H$ n.m.r spectrum. $^{13}C$ n.m.r spectrum of **(36)** contained three additional peaks for allyl carbons at $\delta_C$ 56.3, $\delta_C$ 119.9 and $\delta_C$ 132.8. The ESMS (NI) of 1-(3-bromobenzyl)-N,N-bisallyl-4-cyanopyrazole **(36)** exhibited molecular ions at *m/z* 355 and 357 a.m.u which were consistent with the molecular formulas $C_{17}H_{17}N_4{}^{79}Br$ and $C_{17}H_{17}N_4{}^{81}Br$.

Having achieved the protection of the 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27)**, the deprotection of diallylamine was investigated using mild conditions so that it wouldn't cleave the proposed aromatic silicon-carbon bond. Laguzza[17] reported a procedure wherein 1-adamantyldiallylamine was deallylated to 1-adamantylamine via a isomerisation of the double bond in the present of the transition metals **(Scheme 3.9)**. Laguzza's procedure was used to attempt to deprotect 1-(3-bromobenzyl)-N,N-bisallyl-4-cyanopyrazole **(36)** but only starting material was recovered from the reaction.




**Scheme 3.9**: *Reagents and conditions:* $(Ph_3P)_3RhCl$, $CH_3CN$ : $H_2O$, reflux, 2 h, 65%.

Palladium catalyst was also used but it was unsuccessful. However, Palladium catalysts did remove the allyl protecting group from 1-(3-bromobenzyl)-N,N-bisallyl-4-cyanopyrazole **(36)** in the present of dimethyl barbituric acid (DMBA), a carbon nucleophile which acted as an allyl scavenger **(Scheme 3.10) (Table 3.2)**.[18-20] The structure of the product obtained from the deprotection reaction was confirmed by the n.m.r data of the mixed sample of the product obtained from the reaction and the synthesized 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27)**.




**36** **27**

**Scheme 3.10**: *Reagents and conditions:* $Pd(PPh_3)_4$ (2% eq), DMBA (6 eq), DCM, 40 °C, 3 h, 94%.

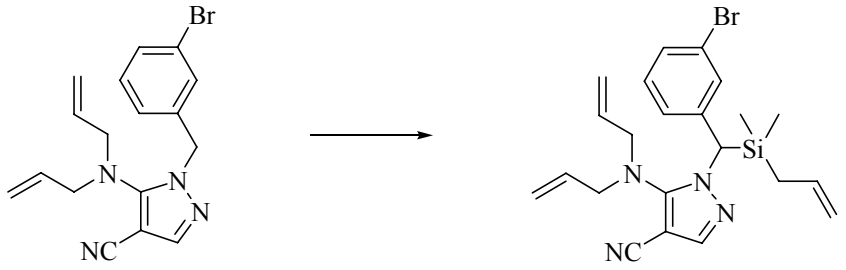
**Table 3.2:** Comparison of deprotection methods to generate 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27)**.

| Solvents | Reagents | Conditions | Comments |
|---|---|---|---|
| $H_2O$:THF (8:2) | Wilkinson cat (8% eq) | 75 °C, 15 h | Recovered SM |
| $H_2O$:$CH_3CN$ (8:2) | Wilkinson cat (8% eq) | 95 °C, 16 h | Recovered SM |
| $H_2O$:$CH_3CN$ (8:2) | Wilkinson cat (8% eq)/ DABCO (0.4 eq) | 95 °C, 15 h | Recovered SM |
| THF | $Pd(PPh_3)_4$ (10% eq) | 75 °C, 14 h | Recovered SM |
| DCM | $Pd(PPh_3)_4$ (2% eq)/ DMBA (6 eq) | 40 °C, 3 h | 94 % **(27)** |

### 3.2.2 1st attempted incorporation of silyl group into phenyl ring

Having successfully achieved the protection and deprotection of the intermediate 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27)**, a silyl group was then incorporated into the phenyl ring so that the diversified libraries of pyrazolo[3,4-*d*]pyrimidines could be generated in the final stage of the synthesis. Attempts to treat the 1-(3-bromobenzyl)-N,N-bisallyl-4-cyanopyrazole **(36)** with nBuLi or Mg followed by quenching with allylchlorodimethylsilane were unsuccessful. However,

silylation occurred at the benzylic carbon rather than at lithium-halogen exchange position when $^{t}$BuLi was used **(Scheme 3.11)**.




**36** **38**

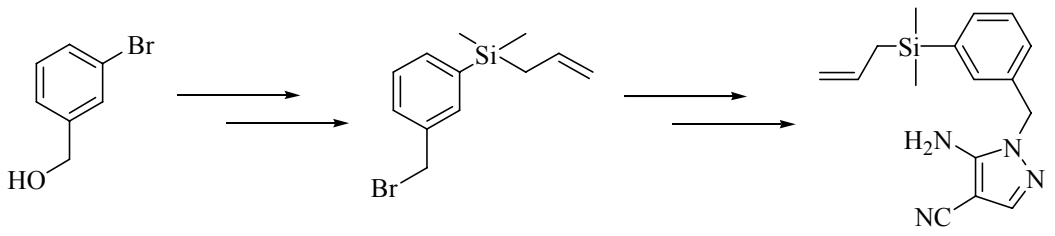
**Scheme 3.11**: *Reagents and conditions:* $^{t}$BuLi, THF, -78 $^{o}$C, allylchlorodimethylsilane, 70%.

The $^{1}$H n.m.r spectrum of 1-(1-allyldimethylsilyl-1-(3-bromophenyl)methyl)-N,N-bisallyl-4-cyanopyrazole **(38)** showed the addition of peaks corresponding to the allyldimethylsilane. Two methyl proton singlets at $\delta_H$ 0.030 ppm and $\delta_H$ 0.035 indicated that the two methyl groups attached to silicon were present. Two doublet of doublets with $J_{gem}$ = 13.6 Hz and $J$ = 8.0 Hz at $\delta_H$ 1.51 ppm and $\delta_H$ 1.61 ppm were assigned to the methylene protons next to the silicon carbon. Two multiplets at $\delta_H$ 4.73-4.85 ppm and $\delta_H$ 5.47-5.66 ppm were assigned to two vinylic protons ($CH_2$) and the remaining vinylic proton of the allyl group attached to silicon atom. The benzylic proton singlet at $\delta_H$ 4.95 confirmed the position of the silylation. The ESMS (PI) of 1-(1-allyldimethylsilyl-1-(3-bromophenyl)methyl)-N,N-bisallyl-4-cyanopyrazole **(38)** exhibited molecular ions at *m/z* 455 and 457 a.m.u which were consistent with the molecular formulas $C_{22}H_{27}N_4Si^{79}Br$ and $C_{22}H_{27}N_4Si^{81}Br$.

### 3.2.3 2$^{nd}$ attempted incorporation of silyl group into phenyl ring

The first attempted incorporation of a silyl group in an intermediate 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27)** to obtain 1-(3-allyldimtheylsilylbenzyl)-5-amino-4-cyanopyrazole **(39)** was unsuccessful. An alternative synthetic route was devised with the incorporation of a silyl group at the early stages of a longer

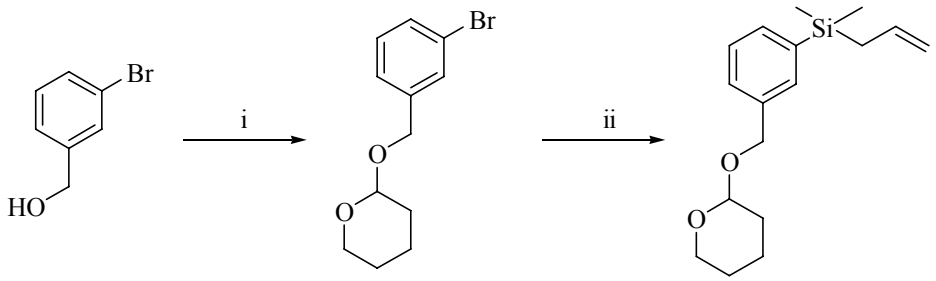
synthesis. The new proposed route for the synthesis of 1-(3-allyldimethysilyllbenzyl)-5-amino-4-cyanopyrazole **(39)** is outlined in **Scheme 3.12**.




**39**

**Scheme 3.12**

The synthetic route required the protection of the hydroxyl group prior to incorporation of the desired silyl side chain. Tetrahydropyranyl (THP) was chosen as a protecting group since it is very cheap and generally proceeds in very high yield. It is stable to alkali and lithium alkyl and can be removed very easily. The protection of 3-bromobenzyl alcohol **(40)** was achieved by mixing the alcohol with dihydropyran in the presence of acid catalysis. Once the hydroxyl group was protected, a silyl side chain was introduced into a phenyl ring by reacting with nBuLi followed by quenching with allylchlorodimethylsilane to produce O-tetrahydropyranyl-3-allyldimethylsilylbenzyl ether **(41) (Scheme 3.13)**.

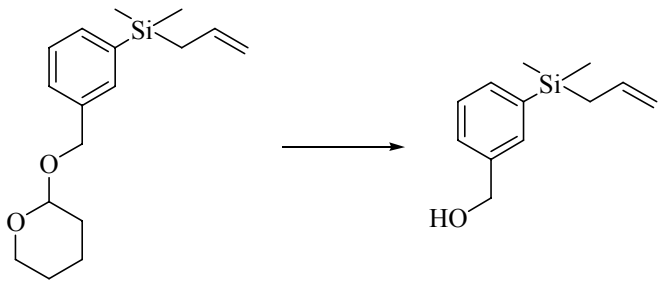



**40** **41**

**Scheme 3.13**: *Reagents and conditions:* (i) 3,4-dihydro-2H-pyran, p-TsOH, DCM, RT, 5 h, 99%; (ii) *n*-BuLi, THF, -78 $^{o}$C, allylchlorodimethylsilane, 70%.

The $^{1}H$ n.m.r spectrum of O-tetrahydropyranyl-3-allyldimethylsilylbenzyl ether **(41)** showed the addition of peaks corresponding to the allyldimethylsilyl side chain. A methyl protons singlet at $\delta_H$ 0.030 ppm indicated that the two methyl groups attached to silicon were symmetrical. A multiplet at $\delta_H$ 4.72-5.00 ppm was assigned to two vinylic protons ($CH_2$) and a multiplet at $\delta_H$ 5.68-5.96 ppm was assigned to the remaining vinylic proton of the allyl group. The $^{13}C$ n.m.r spectrum of **(41)** showed a signal at $\delta_C$ -3.1 ppm confirmed the presence of the two methyl groups attached to silicon. The ESMS (PI) of O-tetrahydropyranyl-3-allyldimethylsilylbenzyl ether **(41)** exhibited molecular ions at *m/z* 291 a.m.u which was consistent with the molecular formula $C_{17}H_{26}O_2Si$.

Having successful incorporated a desired silyl side chain into a phenyl ring, tetrahydropyranyl (THP) was then removed to obtain an alcohol which could then be converted into a corresponding bromide using a mild reagent of triphenylphosphine and carbon tetrabromide. O-tetrahydropyranyl-3-allyldimethylsilylbenzyl ether **(41)** was treated with *p*-toluenesulfonic acid in methanol at room temperature for 14 h to obtain 3-allyldimethylbenzyl alcohol **(42)**. It was found that leaving the deprotection reaction for more than 20 h led to the loss of 3-allyldimethylbenzyl alcohol **(42)** **(Scheme 3.14)**.



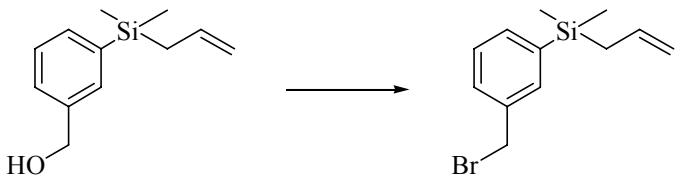

**41** **42**

**Scheme 3.14**: *Reagents and conditions:* TsOH, MeOH, RT, 14 h, 80%.

The $^{1}H$ n.m.r spectrum of 3-allyldimethylbenzyl alcohol **(42)** showed the loss of peaks corresponding to the tetrahydropyranyl protecting group. A singlet at $\delta_H$ 0.26 ppm, a doublet of triplets with $J$ = 8.0 Hz and $J$ = 1.2 Hz at $\delta_H$ 1.73 ppm and two

multiplets at $\delta_H$ 4.79-4.87 ppm and $\delta_H$ 5.68-5.80 ppm were assigned to methyl protons, methylene protons and vinylic protons of the allyl group respectively. A triplet with $J$ = 6.0 Hz at $\delta_H$ 1.65 ppm was assigned to the hydroxyl proton and a doublet with $J$ = 6.0 Hz at $\delta_H$ 4.67 ppm was assigned to the benzylic protons. The $^{13}C$ n.m.r spectrum of **(42)** showed a signal at $\delta_C$ -3.3 ppm confirmed the presence of the two methyl groups attached to silicon and a signal at $\delta_C$ 65.8 ppm confirmed the loss of the tetrahydropyranyl protecting group. The ESMS (PI) of 3-allyldimethylbenzyl alcohol **(42)** exhibited molecular ions at *m/z* 207 a.m.u which was consistent with the molecular formula $C_{12}H_{18}OSi$.

3-allyldimethylbenzyl alcohol **(42)** was then converted into the corresponding bromide using a mild reagent of triphenylphosphine and carbon tetrabromide in dichloromethane. The crude 3-allyldimethylbenzyl bromide **(43)** was produced as a gum after removal of dicholoromethane. This gum was first triturated with 1:1 ethyl acetate/hexane and filtered off. The combined filtrate was then concentrated and purified by flash chromatography to give 3-allyldimethylsilylbenzyl bromide **(43)** as clear oil in 62% yield **(Scheme 3.15)**.

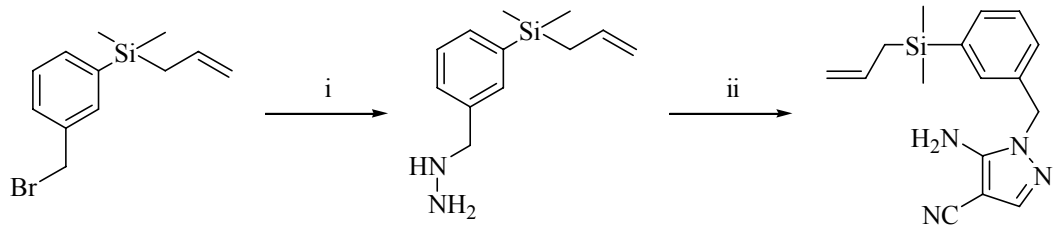



**42** **43**

**Scheme 3.15**: *Reagents and conditions:* $PPh_3$, $CBr_4$, DCM, 2 h, 62%.

The $^1H$ n.m.r spectrum of 3-allyldimethylsilylbenzyl bromide **(43)** showed the disappearance of a hydroxyl proton triplet with $J$ = 6.0 Hz at $\delta_H$ 1.65 ppm and a benzylic protons singlet at $\delta_H$ 4.48 ppm indicates the successful conversion of an alcohol into a corresponding bromide. The $^{13}C$ n.m.r spectrum of **(43)** also exhibited the signal at $\delta_C$ 34.0 ppm and no signal at $\delta_C$ 65.8 ppm due to the conversion of an alcohol into a corresponding bromide.

Having successful synthesized the 3-allyldimethylsilylbenzyl bromide, 1-(3-allyldimethylsilylbenzyl)-5-amino-4-cyanopyrazole **(39)** could then be synthesized using the established conditions for the synthesis of 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27)**. Since the 3-allyldimethylsilylbenzyl bromide **(43)** was an air and moisture sensitive, the prepared hydrazine in THF solvent was substituted for hydrazine monohydrate in ethanol. 3-allyldimethylsilylbenzyl bromide **(43)** and an excess of hydrazine in THF was refluxed for 4 h under the argon to yield 3-allyldimethylsilylbenzyl hydrazine as light yellow oil upon the removal of THF and a quick extraction with diethyl ether. The crude unstable 3-allyldimethylsilylbenzyl hydrazine was then refluxed with 1 equivalent of ethoxymethylenemalononitrile in dried ethanol for 2 h to produce 1-(3-allyldimethylsilylbenzyl)-5-amino-4-cyanopyrazole **(39)** as white solid in 25% yield over 2 steps **(Scheme 3.16)**.




**43** **39**

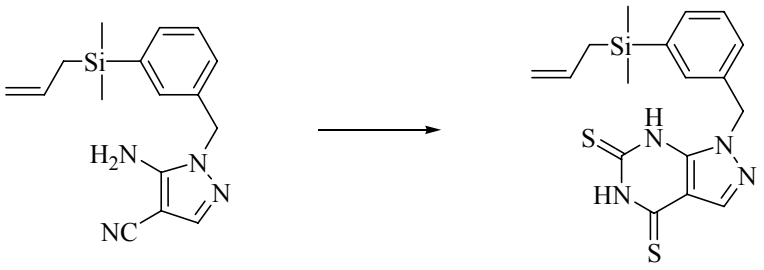
**Scheme 3.16**: *Reagents and conditions:* (i) $N_2H_4$ in THF, reflux, 3 h; (ii) $C_2H_5OCH=C(CN)_2$, EtOH, reflux, 2 h, 25%.

The $^1H$ n.m.r spectrum of 1-(3-allyldimethylsilylbenzyl)-5-amino-4-cyanopyrazole **(39)** showed a singlet at $\delta_H$ 0.23 ppm which was assigned to methyl protons. The doublet with $J$ = 8.0 Hz at $\delta_H$ 1.69 ppm was assigned to methylene protons. The broad singlet at $\delta_H$ 4.39 ppm was assigned to amino protons. It was confirmed by its disappearance upon $D_2O$ addition. A singlet at $\delta_H$ 5.11 ppm was assigned to the benzylic protons and a singlet at $\delta_H$ 7.46 ppm was assigned to the H-3 proton. The $^{13}C$ n.m.r spectrum of **(39)** exhibited methyl carbons at $\delta_C$ -3.3 ppm, a methylene carbon at $\delta_C$ 23.7 ppm, a benzylic carbon at $\delta_C$ 52.5 ppm and aromatic carbons in the region $\delta_C$ 127-141 ppm. The ESMS (PI) of 1-(3-

allyldimethysilyllbenzyl)-5-amino-4-cyanopyrazole **(39)** exhibited molecular ions at *m/z* 297 a.m.u which was consistent with the molecular formula $C_{16}H_{20}N_4Si$.

1-(3-allyldimethylsilylbenzyl)-5-amino-4-cyanopyrazole **(39)** had been successfully synthesized but in very low yield. The yield might improve if the reaction was carried out in different solvents and/or at different temperature since 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27)** was obtained in a 46% yield over 2 steps from 3-bromobenzyl chloride **(Section 2.2)**. Although the yield has not optimized, 1-(3-allyldimethylsilylbenzyl)-5-amino-4-cyanopyrazole **(39)** was used to complete the synthesis of 1-(3-allyldimethylsilylbenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(44)** using the same procedure as in the preparation of 1-(3-bromobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(28)**.

The mixture of 1-(3-allyldimethysilyllbenzyl)-5-amino-4-cyanopyrazole **(39)** and potassium O-ethylxanthogenate in DMF was initially heated at 110 °C to avoid loosing a precious 1-(3-allyldimethylsilylbenzyl)-5-amino-4-cyanopyrazole **(39)**. The reaction mixture was monitored by the electrospray mass spectrometer which indicated that 1-(3-allyldimethysilyllbenzyl)-5-amino-4-cyanopyrazole **(39)** was still present after heating for more than 3 h. However, it disappeared when the reaction mixture was raised to 135 °C after 1.5 h. This indicated that the nucleophilic attack between the O-ethylxanthogenate anion of the potassium O-ethylxanthogenate and the amino of the cyanopyrazole **(39)** would not occur at low temperature. Therefore, the 1-(3-allyldimethysilyllbenzyl)-5-amino-4-cyanopyrazole **(39)** and potassium O-ethylxanthogenate in DMF was heated at 135 °C for 2 h under the argon to produce an intermediate which was rearranged upon treatment with base followed by neutralization with acid at room temperature to produce 1-(3-allyldimethylsilylbenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(44)** as a light cream solid in 78% yield **(Scheme 3.17)**.



**39** **44**

**Scheme 3.17**: *Reagents and conditions:* (i) $EtOCS_2^-K^+$, DMF, 135 $^oC$, reflux, 2 h; (ii) NaOH (2.0 M), RT, 45 min; (iii) HCl (2.0 M), 78%.

The $^1H$ n.m.r spectrum of 1-(3-allyldimethylsilylbenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidines-4,6-dithione **(44)** showed the disappearance of an amino protons $\delta_H$ 4.39 ppm and appearance of the two broad singlets at $\delta_H$ 7.00-7.40 ppm and $\delta_H$ 10.99 ppm which were assigned to NH protons at N-7 and N-5 respectively. H-3 protons at $\delta_H$ 7.78 ppm in 1-(3-allyldimethylsilylbenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidines-4,6-dithione **(44)** were shifted downfield relative to H-3 protons at $\delta_H$ 7.46 ppm in 1-(3-allyldimethylsilylbenzyl)-5-amino-4-cyanaopyrazole **(39)**. The ESMS (NI) of 1-(3-allyldimethylsilylbenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidines-4,6-dithione **(44)** exhibited molecular ions at *m/z* 371 a.m.u which was consistent with the molecular formulas $C_{17}H_{20}N_4S_2Si$.

## 3.3 Conclusion

The protection and deprotection of the intermediate 1-(3-bromobenzyl)-5-amino-4-cyanaopyrazole **(27)** had been achieved. However, incorporation of a silyl group into the phenyl ring of 3-bromobenzyl substituted pyrazolo[3,4-*d*]pyrimidines was unsuccessful. An alternative synthetic route was devised with the incorporation of a silyl group at the early stages of a longer synthesis. It took 7 steps to achieve the silicon-containing scaffolds. Some intermediates were unstable and produced in a low yield. The solid phase model reactions with silicon-containing intermediates were next to being investigated.

## 3.4 Experimental

Melting points were recorded on a Gallenkamp digital melting point apparatus and are uncorrected. Infra-red absorption spectra were obtained on a Perkin Elmer FT-IR spectrophotometer using sodium chloride plates. $^{1}H$ and $^{13}C$ nuclear magnetic resonance (n.m.r) spectra were recorded on a Varian Gemini-200 (200 MHz) spectrometer, Varian Unity-400 (400 MHz) spectrometer or Varian Unity Plus-600 (600 MHz) spectrometer. All samples were dissolved in deuteriochloroform ($CDCl_3$) containing tetramethylsilane (TMS) as an internal reference unless otherwise stated. The signals are recorded in terms of chemical shift in parts per million (ppm) downfield from TMS ($\delta = 0$) for protons or $CDCl_3$ ($\delta = 77$) for carbon atoms. The signals are recorded in terms of chemical shift ($\delta_H$), relative integral, multiplicity, coupling constants (*J* Hz) and assignments, in that order. The following abbreviations for multiplicity are used: s = singlet, dd = doublet of doublets, dt = doublet of triplets, m = multiplet. $^{1}H$ and $^{13}C$ spectra were assigned with the aid of HMQC (Heteronuclear Multiple-Quantum Coherence), HMBC (Heteronuclear Multiple-Bond Coherence) and $^{1}H$-$^{1}H$ COSY (Correlation Spectroscopy). Electrospray mass spectra (ESMS) were recorded on a Fisons VG Platform mass spectrometer with MassLynx Data System software.

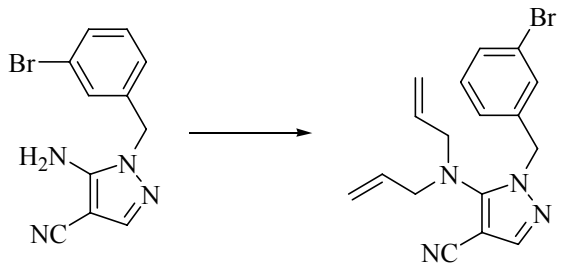
Microanalytical data was obtained from University of Queensland Microanalytical Service.

Analytical thin layer chromatography (TLC) was performed using precoated (0.2 mm) Merck silica gel plates (Merck Kieselgel 60 $F_{254}$). Flash chromatography was carried out using Merck Kieselgel 60 (230-400 mesh) with the indicated solvents.

All solvents for chromatography were distilled before use, unless otherwise stated. Ether refers to diethyl ether and hexane refers to the fraction of b.p. 60-80 °C. Mixed solvent compositions are quoted as v/v.

Solvents and reagents were purified according to the standard techniques of Perrin, Perrin and Amarego.[21]

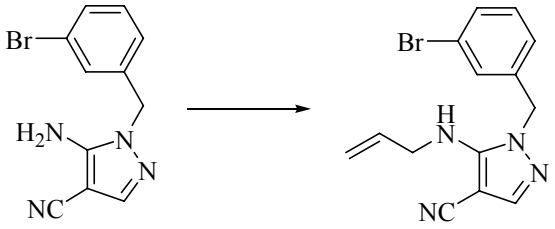
**1-(3-bromobenzyl)-N,N-bisallyl-4-cyanopyrazole (36)**



**27** **36**

To a solution of 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27)** (0.35 g, 1.26 mmol) in THF (10 ml) under nitrogen was added slowly sodium hydride (133 mg, 5.54 mmol). Allyl bromide (0.96 ml, 11.09 mmol) was added and the reaction mixture was stirred at room temperature for 3 h. The initial yellowish solution turned to red solution after 3 h. The reaction mixture was quenched with methanol (5 ml) and diluted with water (10 ml). The mixture was then extracted with DCM (2 x 20 ml), washed with water (2 x 10 ml), dried ($MgSO_4$) and the solvent removed in vacuo. The resultant residue was purified by flash chromatography (100% DCM) to yield 1-(3-bromobenzyl)-N,N-bisallyl-4-cyanopyrazole **(36)** (436 mg, 97%) as yellow oil; $\delta_H$ (400 MHz) 3.63 (dt, *J* = 6.4 Hz, *J* = 1.2 Hz, 4H, 2 x $CH_2$), 5.10-5.20 (m, 6H, $CH_2$ + 2 x $CH_2$), 5.60-5.70 (m, 2 x 1H, CH), 7.06 (d, *J* = 8.0 Hz, 1H, 6'-H), 7.15 (t, *J* = 8.0 Hz, 1H, 5'-H), 7.28 (s, 1H, 2'-H), 7.37 (d, *J* = 8.0 Hz, 1H, 4'-H) and 7.66 (s, 1H, 3-H); $\delta_C$ (100 MHz) 51.2 ($CH_2$), 56.3 ($CH_2$), 86.4 (C-4), 114.3 (CN), 119.9 (2 x $CH_2$), 123.0 (C-3'), 126.2 (C-6'), 130.5 (C-2'), 130.7 (C-4'), 131.4 (C-5'), 132.8 (2 x CH), 138.2 (C-1'), 142.2 (C-3) and 153.7 (C-5); Anal. calcd for $C_{17}H_{17}N_4Br$: C, 57.15; H, 4.80; N, 15.68. Found C, 57.25; H, 4.83; N, 15.68%; ESMS (NI) 355 and 357. calcd for ($C_{17}H_{17}{}^{79}BrN_4$ - 1[H]) and ($C_{17}H_{17}{}^{81}BrN_4$ - 1[H]) respectively. Found 355 and 357.

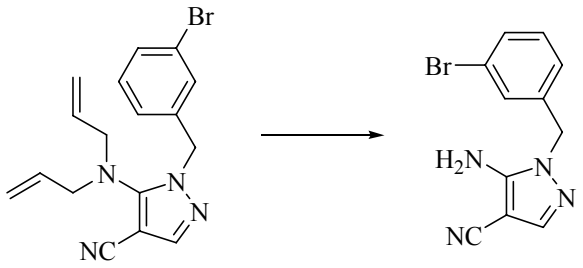
**5-allylamino-1-(3-bromobenzyl)-4-cyanopyrazole (37)**



**27** **37**

To a solution of 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27)** (1.90 g, 6.86 mmol) in THF (15 ml) under argon at 0 °C was added diisopropylamine (3.2 ml, 18.4 mmol) followed by allyl bromide (1.5 ml, 17.3 mmol). The resulting solution was refluxed for 4 h at 85 °C. The reaction mixture was monitored by TLC and mass spectrometer. Allyl bromide (3.0 ml, 34.7 mmol) was added and the reaction mixture was refluxed for 16 h at 85 °C. The initial orange solution turned to yellow solution and white precipitate was formed in the solution. Diisopropylamine (2.8 ml, 16.1 mmol) was added to the solution followed by allyl bromide (3.0 ml, 17.3 mmol). The resulting mixture was refluxed for additional 4 h at 85 °C. After cooling, the precipitate was filtered and washed several time with DCM. The combined organic filtrate was concentrated in vacuo. The resultant residue was purified by flash chromatography (2.5:7.5 ethyl acetate/hexane) to yield 5-allylamino-1-(3-bromobenzyl)-4-cyanopyrazole **(37)** (1.90 g, 78%) as yellow oil; $\delta_H$ (400 MHz) 4.00 (dt, *J* =5.2 Hz, *J* = 1.6 Hz, 2H, $CH_2$), 5.05-5.17 (m, 4H, $CH_2$ + $CH_2$), 5.26 (s, 1H, NH), 5.74-5.85 (m, 1H, CH), 7.02 (d, *J* = 8.0 Hz, 1H, 6'-H), 7.21 (t, *J* = 8.0 Hz, 1H, 5'-H), 7.26 (s, 1H, 2'-H), 7.43 (d, *J* = 8.0 Hz, 1H, 4'-H) and 7.53 (s, 1H, 3-H); $\delta_C$ (100 MHz) 47.2 ($CH_2$), 51.5 ($CH_2$), 94.6 (C-4), 115.3 (CN), 117.5 ($CH_2$), 123.5 (C-3'), 125.5 (C-6'), 130.1 (C-2'), 131.0 (C-4'), 131.9 (C-5'), 133.7 (CH), 137.0 (C-1'), 142.0 (C-3) and 150.1 (C-5); Anal. calcd for $C_{14}H_{13}N_4Br$: C, 53.01; H, 4.13; N, 17.66. Found C, 51.31; H, 4.39; N, 16.29%; ESMS (NI) 315 and 317. calcd for ($C_{14}H_{13}{}^{79}BrN_4$ - 1[H]) and ($C_{14}H_{13}{}^{81}BrN_4$ - 1[H]) respectively. Found 315 and 317.

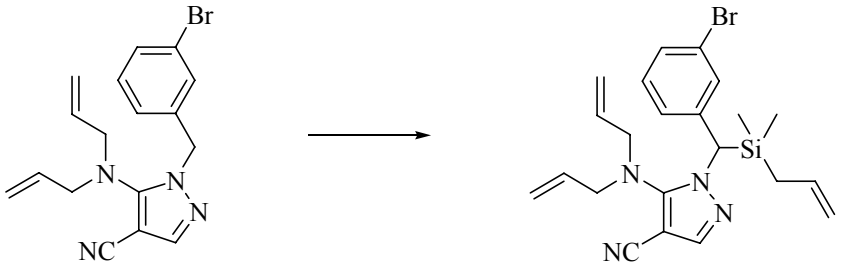
**Deprotection of 1-(3-bromobenzyl)-N,N-bisallyl-4-cyanaopyrazole (36)**



**36** **27**

A solution of 1-(3-bromobenzyl)-N,N-bisallyl-4-cyanopyrazole **(36)** (305 mg, 0.85 mmol) in dry DCM (5 ml) was added dropwise to tetrakis(triphenylphosphine)palladium (0) (22 mg, 1.76% mmol) and N,N-dimethylbarbituric acid (0.8 g, 5.13 mmol) under argon. The reaction mixture was stirred for 3 h at 40 °C. After cooling, the solvent was removed from the reaction mixture and dissolved in 1:1 ether/ethyl acetate (3 x 30 ml). The resulting mixture was extracted with aqueous $Na_2CO_3$ (2 x 20 ml), dried ($MgSO_4$) and the solvent removed in vacuo. The resultant residue was purified by flash chromatography (50% ethyl acetate-hexane) to afford 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27)** (222 mg, 94%) as white solid, mp 101.5 ± 0.5 °C; $\delta_H$ (400 MHz, DMSO-$d_6$) 5.17 (s, 2H, $CH_2$), 6.78 (s, 2H, $NH_2$), 7.16 (d, $J$ = 8.0 Hz, 1H, 6'-H), 7.30 (t, $J$ = 8.0 Hz, 1H, 5'-H), 7.38 (t, $J_{meta}$ = 1.6 Hz, 1H, 2'-H), 7.49 (d, $J$ = 8.0 Hz, 1H, 4'-H) and 7.61 (s, 1H, 3-H); $\delta_C$ (100 MHz, DMSO-$d_6$) 49.3 ($CH_2$), 72.3 (C-4), 115.1 (CN), 121.7 (C-3'), 126.4 (C-6'), 130.1 (C-2'), 130.4 (C-4'), 130.7 (C-5'), 139.3 (C-1'), 140.8 (C-3) and 151.7 (C-5); $\nu_{max}$ (NaCl plates)/$cm^{-1}$ 3400 ($NH_2$) and 2200 (CN). Anal. calcd for $C_{11}H_9BrN_4$: C, 47.68; H, 3.27; N, 20.22C. Found C, 47.80; H, 3.17; N, 20.30%; ESMS (NI) 275 and 277. calcd for ($C_{11}H_9{}^{79}BrN_4$ - 1[H]) and ($C_{11}H_9{}^{81}BrN_4$ - 1[H]) respectively. Found 275 and 277.

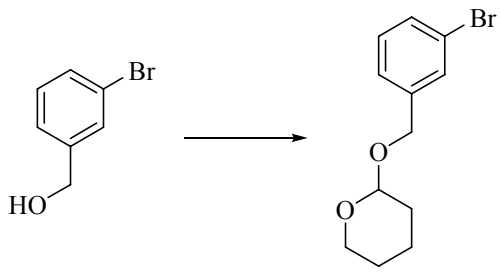
**1-(1-allyldimethylsilyl-1-(3-bromophenyl)methyl)-N,N-bisallyl-4-cyanopyrazole (38)**




**36** **38**

To a solution of 1-(3-bromobenzyl)-N,N-bisallyl-4-cyanopyrazole **(36)** (1.99 g, 5.57 mmol) in dried THF (50 ml) under argon at -78 °C was added dropwise t-butyllithium (3.7 ml, 1.6 M, 5.92 mmol) over a period of 5 min. The initial yellow solution turned to dark orange solution. After 15 min of further stirring at -78 °C, allylchlorodimethylsilane (0.88 ml, 0.78 g, 5.79 mmol) in dried THF (5 ml) was added dropwise over a period of 5 min. After stirring for 15 min, the reaction mixture was allowed to warm to room temperature. After 30 min of further stirring, the reaction mixture was quenched with MeOH and solvent removed in vacuo. The resultant residue was purified by flash chromatography (15% ethyl acetate/hexane) to yield 1-(1-allyldimethylsilyl-1-(3-bromophenyl)methyl)-N,N-bisallyl-4-cyanaopyrazole **(38)** (1.77 g, 70%) as yellow oil; $\delta_H$ (400 MHz) 0.03 (s, 3H, $CH_3$), 0.035 (s, 3H, $CH_3$), 1.51 (dd, $J_{gem}$ = 13.6 Hz, $J$ = 8.0 Hz, 1H, $CH_2$), 1.61 (dd, $J_{gem}$ = 13.6 Hz, $J$ = 8.0 Hz, 1H, $CH_2$), 3.42-3.56 (m, 4H, 2 x $CH_2$), 4.73-4.85 (m, 2H, $CH_2$), 4.95 (s, 1H, CH), 5.03-5.12 (m, 4H, 2 x $CH_2$ ), 5.47-5.66 (m, 3H, 3 x CH), 7.00 (d, $J$ = 8.0 Hz, 1H, 6'-H), 7.11 (t, $J$ = 8.0 Hz, 1H, 5'-H), 7.23 (s, 1H, 2'-H), 7.30 (d, $J$ = 8.0 Hz, 1H, 4'-H) and 7.68 (s, 1H, 3-H); $\delta_C$ (100 MHz) -4.2 ($CH_3$), -4.0 ($CH_3$), 21.9 (Si-$CH_2$), 54.1 (N-$CH_2$), 55.9 (2 x $CH_2$), 85.9 (C-4), 114.5 ($CH_2$), 114.6 (CN), 119.4 (2 x $CH_2$), 122.8 (C-3'), 125.0 (C-6'), 129.3 (C-2'), 129.8 (C-4'), 130.2 (C-5'), 133.1 (2 x CH), 133.7 (CH), 141.1 (C-3), 141.8 (C-1') and 153.7 (C-5); Anal. calcd for $C_{22}H_{27}BrN_4Si$: C, 58.01; H, 5.98; N, 12.30 Found C, 58.10; H, 6.02; N, 12.26%; ESMS (PI) 455 and 457. calcd for ($C_{22}H_{27}{}^{79}BrN_4Si$ + 1[H]) and ($C_{22}H_{27}{}^{81}BrN_4Si$ + 1[H]) respectively. Found 455 and 457.

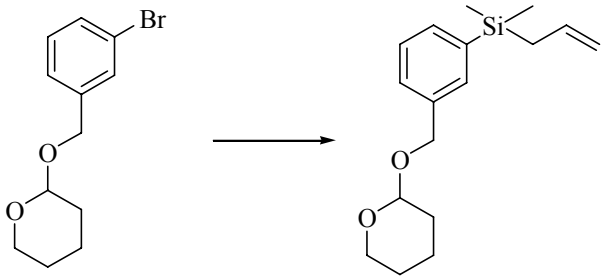
**O-tetrahydropyranyl-3-bromobenzyl ether (40)**




**40**

To a solution of 3-bromobenzyl alcohol (9.90g, 52.9 mmol) and 3,4-dihydro-2H-pyran (5.3 ml, 4.89 g, 58.1 mmol) in DCM (150 ml) was added p-TsOH (200 mg). After stirring at room temperature for 5 h, the reaction mixture was concentrated and the resultant residue was purified by flash chromatography (1:6 ethyl acetate/hexane) to yield O-tetrahydropyranyl-3-bromobenzyl ether **(40)** (14.0 g, 99%) as clear oil; $\delta_H$ (400 MHz) 1.40-1.90 (m, 6H, 3 x $CH_2$), 3.48-3.56 (m, 1H, $CH_2$), 3.82-3.91 (m, 1H, $CH_2$), 4.44 (d, $J_{gem}$ = 12.4 Hz, 1H, $CH_2$), 4.67 (t, $J$ = 3.2 Hz, 1H, CH), 4.72 (d, $J_{gem}$ = 12.4 Hz, 1H, $CH_2$), 7.18 (t, $J$ = 7.6 Hz, 1H, 5'-H), 7.25 (d, $J$ = 7.6 Hz, 1H, 6'-H), 7.38 (d, 1H, 4'-H) and 7.45 (s, 1H, 2'-H); $\delta_C$ (100 MHz) 19.5 ($CH_2$), 25.6 ($CH_2$), 30.7 ($CH_2$), 62.3 ($CH_2$), 68.2 ($CH_2$), 98.1 (CH), 122.7 (C-3'), 126.4 (Ar-C), 130.1 (Ar-C), 130.7 (Ar-C), 130.8 (Ar-C) and 140.9 (C-1'); Anal.calcd for $C_{12}H_{15}BrO_2$: C, 53.15; H, 5.58. Found C, 52.87; H, 5.53%.

**O-tetrahydropyranyl-3-allyldimethylbenzyl ether (41)**

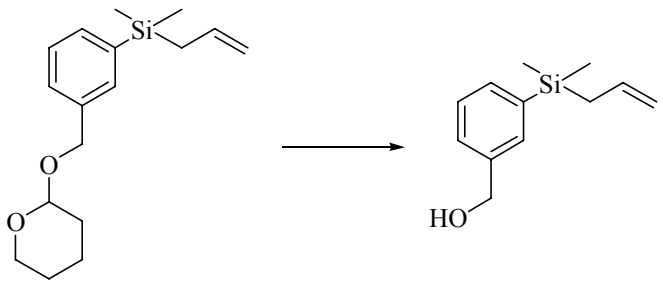



**40** **41**

To a solution of O-tetrahydropyranyl-3-bromobenzyl ether **(40)** (10.11 g, 37.3 mmol) in dried THF (50 ml) under argon at -78 °C was added dropwise n-

butyllithium (15.6 ml, 2.4 M, 37.4 mmol) over a period of 10 min. After 30 min of further stirring at -78 °C, allylchlorodimethylsilane (5.65 ml, 5.03 g, 37.3 mmol) in dried THF (20 ml) was added dropwise over a period of 30 min and the reaction mixture was allowed to warm to room temperature. After 30 min of further stirring, the reaction mixture was quenched with saturated $NH_4Cl$ (1 ml) and solvent removed in vacuo. The resultant residue was purified by flash chromatography (1:10 ethyl acetate/hexane) to yield O-tetrahydropyranyl-3-allyldimethylsilylbenzyl ether **(41)** (7.58 g, 70%) as clear oil; $\delta_H$ (400 MHz) 0.03 (s, 6H, 2 x $CH_3$), 1.40-2.00 (m, 8H, 4 x $CH_2$), 3.50-3.70 (m, 1H, $CH_2$), 3.90-4.10 (m, 1H, $CH_2$), 4.54 (d, $J_{gem}$ = 11.8 Hz, 1H, $CH_2$), 4.72-5.00 (m, 4H, $CH_2$ + 1 H of $CH_2$ + CH), 5.68-5.96 (m, 1H, CH) and 7.28-7.58 (m, 5H, Ar-H); $\delta_C$ (100 MHz) -3.1 (2 x $CH_3$), 19.7 ($CH_2$), 24.0 ($CH_2$), 25.8 ($CH_2$), 30.9 ($CH_2$), 62.5 ($CH_2$), 69.3 ($CH_2$), 98.0 (CH), 113.6 ($CH_2$) and 128.0-140.0 (6 x Ar-C + CH); Anal. calcd for $C_{17}H_{26}O_2Si$: C, 70.29; H, 9.02 Found C, 70.40; H, 9.15%; ESMS (PI) 291. calcd for ($C_{17}H_{26}O_2Si$ + 1[H]). Found 291.

**3-allyldimethylsilylbenzyl alcohol (42)**

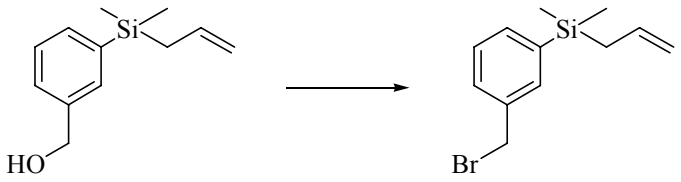



**41** **42**

To a solution of O-tetrahydropyranyl-3-allyldimethylsilylbenzyl ether **(41)** (6.65 g, 22.89 mmol) in methanol (150 ml) under nitrogen was added p-TsOH (120 mg). After stirring at room temperature for 14h, the reaction mixture was concentrated and purified by flash chromatography (1.5:4.5 ethyl acetate/ hexances) to yield 3-allyldimethylsilylbenzyl alcohol **(42)** (3.77 g, 80%) as clear oil; $\delta_H$ (400 MHz) 0.26 (s, 6H, 2 x $CH_3$), 1.65 (t, $J$ = 6.0 Hz, 1H, OH), 1.73 (dt, $J$ = 8.0 Hz, $J$ = 1.2 Hz 2H, $CH_2$), 4.67 (d, $J$ = 6.0 Hz, 2H, $CH_2$), 4.79-4.87 (m, 2H, $CH_2$), 5.68-5.80 (m, 1H, CH) and 7.22-7.48 (m, 4H, Ar-H); $\delta_C$ (100 MHz) -3.3 (2 x $CH_3$), 23.8 (Si-$CH_2$), 65.8 ($CH_2$), 113.7 ($CH_2$) and 128.0-140.2 (6 x Ar-C + CH); Anal. calcd for

$C_{12}H_{18}OSi$: C, 69.84; H, 8.79 Found C, 69.78; H, 9.02%; ESMS (PI) 207. calcd for ($C_{12}H_{18}OSi$ + 1[H]). Found 207.

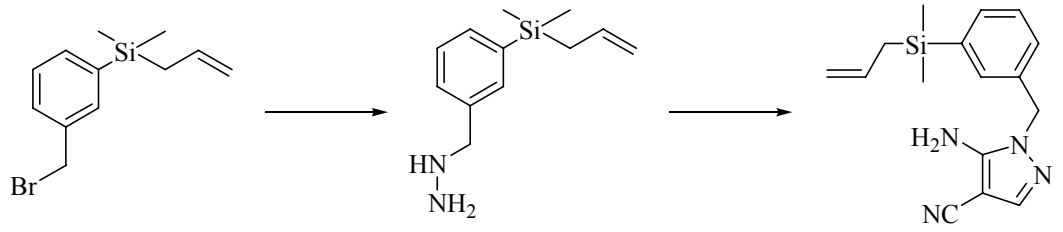
**3-allyldimethylsilylbenzyl bromide (43)**




**42** **43**

To a solution of 3-allyldimethylsilylbenzyl alcohol **(42)** (12.83 g, 62.2 mmol) and triphenylphosphine (16.33 g, 62.2 mmol) in DCM (150 ml) at 0 °C was added carbon tetrabromide (20.63 g, 62.2 mmol) portionwise over a period of 10 min. The reaction mixture was allowed to warm to room temperature. After stirring at room temperature for 2h, the reaction mixture was concentrated. The resultant residue was triturated with 1:1 ethyl acetate/hexane (2 x 200 ml). The combined filtrate was concentrated and purified by flash chromatography (1:30 ethyl acetate/hexanes) to yield 3-allyldimethylsilylbenzyl bromide **(43)** (6.53 g, 62%) as clear oil; $\delta_H$ (400 MHz) 0.26 (s, 6H, 2 x $CH_3$), 1.73 (dt, $J$ = 8.0 Hz, $J$ = 1.2 Hz, 2H, $CH_2$), 4.48 (s, 2H, $CH_2$), 4.82 (t, $J$ = 1.2Hz, 1H, $CH_2$), 4.83-4.87 (m, 1H, $CH_2$), 5.68-5.80 (m, 1H, CH), 7.28-7.50 (m, 4H, Ar-H); $\delta_C$ (100 MHz) -3.3 (2 x $CH_3$), 23.7 (Si-$CH_2$), 34.0 ($CH_2$), 113.8 ($CH_2$) and 128.0-140.0 (6 x Ar-C + CH); Anal. calcd for $C_{12}H_{17}BrSi$: C, 53.53; H, 6.36 Found C, 53.58; H, 6.44%.

**1-(3-allyldimethylsilylbenzyl)-5-amino-4-cyanopyrazole (39)**

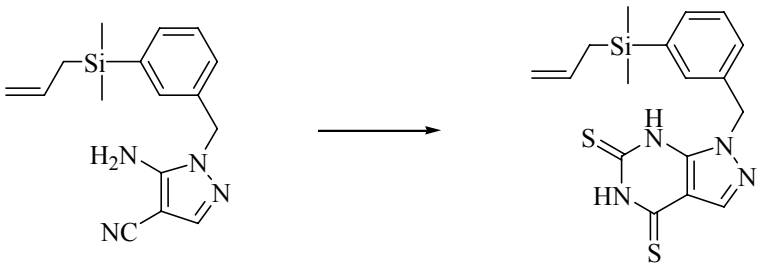



**43** **39**

To a refluxing solution of hydrazine in THF (150 ml, 1.0 M solution in THF, 150 mmol) under argon was added dropwise a solution of 3-allyldimethylsilylbenzyl bromide **(43)** (5.40 g, 0.020 mol) in dried THF (10 ml) over a period of 10 min. The reaction mixture was refluxed for 3h before the solvent was removed *in vacuo*. The remaining pale yellow liquid was extracted with ether (2 x 20 ml). The combined organic layers were dried ($MgSO_4$) and concentrated *in vacuo* to yield the 3-allyldimethylsilylbenzylhydrazine (4.01 g, 91%) as a crude oil. The crude product was used in the preparation of 1-(3-allyldimethylsilylbenzyl)-5-amino-4-cyanopyrazole **(39)** without further purification.

To a stirred solution of ethoxymethylenemalononitrile (2.22 g, 18.2 mol) in dried ethanol (25 ml) under argon was added dropwsie a solution of 3-allyldimethylsilylbenzylhydrazine (4.01 g, 18.2 mmol) in dried ethanol (5 ml). The resultant mixture was refluxed for 2 h and a deep red colour solution was produced. The reaction mixture was left to cool to room temperture and yellow crystalline material precipitated. Precipitation was further enhanced by cooling in the fridge overnight before it was filtered and the solid was washed with cold ethanol. The crude solid was purified by flash chromatography (40% ethyl acetate-hexane) to afford 1-(3-allyldimethylsilylbenzyl)-5-amino-4-cyanopyrazole **(39)** (1.34 g, 25%) as white solid, mp 91.0 ± 0.5 °C; $\delta_H$ (400 MHz) 0.23 (s, 6H, 2 x $CH_3$), 1.69 (d, $J$ = 8.0 Hz, 2H, $CH_2$), 4.39 (s, 2H, $NH_2$), 4.76-4.83 (m, 2H, $CH_2$), 5.11 (s, 2H, $CH_2$), 5.63-5.75 (m, 1H, CH), 7.04-7.44 (m, 4H, Ar-H) and 7.46 (s, 1H, 3-H); $\delta_C$ (100 MHz) -3.3 (2 x $CH_3$), 23.7 (Si-$CH_2$), 52.5 ($CH_2$), 113.9 ($CH_2$), 114.6 (CN), 127.0-140.1 (6 x Ar-C + CH + C-3) and 150.4 (C-5); Anal. calcd for $C_{16}H_{20}N_4Si$: C, 64.83; H, 6.80; N, 18.90. Found C, 64.69; H, 7.02; N, 18.41%; ESMS (PI) 297. calcd for ($C_{16}H_{20}N_4Si$ + 1[H]). Found 297.

**1-(3-allyldimethylsilylbenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione (44)**



**39** **44**

To a solution of 1-(3-allyldimethylsilylbenzyl)-5-amino-4-cyanopyrazole **(39)** (1.05 g, 3.54 mmol) in dry DMF (15 ml) under argon was added potassium-O-ethylxanthogenate (1.14 g, 7.11 mmol). The reaction mixture was heated to 135 °C for 2 h under argon. The initial orange and opaque solution turned to dark brown after 2 h. The reaction mixture was allowed to cool to room temperature and the solvent was removed under reduced pressure to afford the crude brown oil. 2.0 M NaOH (3.6 ml) was added to the remaining brown oil and stirred at room temperature for 45 min. The basic solution was filtered to give a transparent orange filtrate. 2.0 M HCl was added dropwise to the filtrate until the neutral pH was reached. A creamy coloured precipitate was formed upon the neutralisation of the filtrate. The crude product was collected by suction filtration and recrystallised from DMSO and water to afford 1-(3-allyldimethylsilylbenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(44)** (1.03 g, 96%) as light cream solid, mp > 230 °C; $\delta_H$ (400 MHz, DMSO-$d_6$) 0.17 (s, 6H, 2 x $CH_3$), 1.67 (d, $J$ = 8.0 Hz, 2H, $CH_2$), 4.70-4.80 (m, 2H, $CH_2$), 5.25 (s, 2H, $CH_2$), 5.60-5.75 (m, 1H, CH), 7.00-7.40 (m, 5H, 4 x Ar-H + NH), 7.78 (s, 1H, 3-H) and 10.99 (br s, 1H, NH); $\delta_C$ (100 MHz, DMSO-$d_6$) -2.8 (2 x $CH_3$), 23.6 (Si-$CH_2$), 50.0 ($CH_2$), 112.6 (C-3a), 114.2 ($CH_2$), 128.0-140.0 (6 x Ar-C + CH + C-3), 150.6 (C-7a), 176.4 (C-4) and 178.8 (C-6); ESMS (NI) 371. calcd for ($C_{17}H_{20}N_4S_2Si$ - 1[H]). Found 371.

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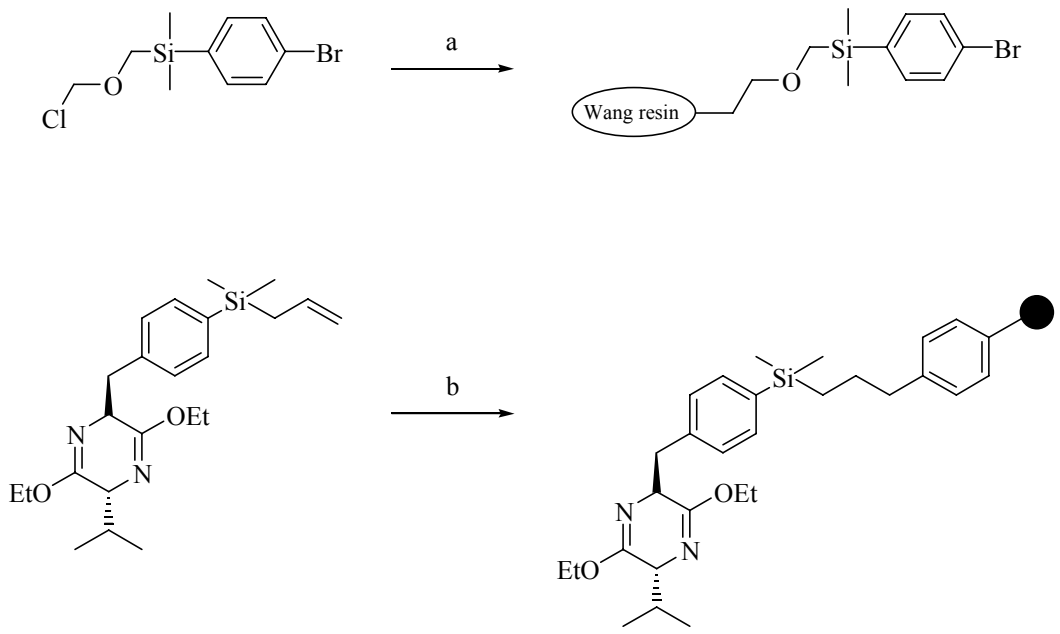
# CHAPTER 4

## Attempted attachment of silyl intermediates to the polymeric support

### 4.1 Introduction

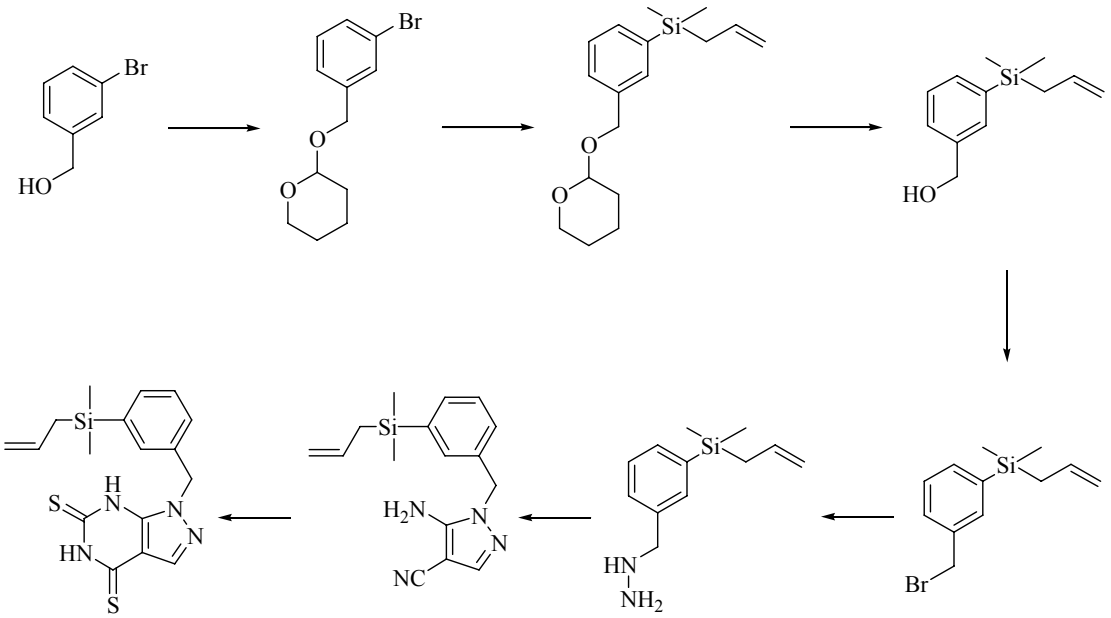
The use of silicon as a linkage element for solid support in solid phase synthesis had been extensively studied.[1-9] The first silicon-based cleavable linker was reported in 1996 in a solid phase synthesis of 1,4-benzodiazepine derivatives[7]. The silylaromatic intermediates were either attached to a solid support via a silyl ether bond[3] or via a silylalkyl bond[9] **(Scheme 4.1)**. Herein, the silicon-containing scaffolds were attempted to link to a solid support via an alkylsilane bond.




**Scheme 4.1**: *Reagents and conditions:* (a)[3] DIPEA, Wang resin, DMF, 40-50 $^{o}$C, O/N; (b)[9] 9-BBN, THF, 5 h, then bromopolystyrene, DMF, $Pd(PPh_3)_4$, $K_2CO_3$, 60 $^{o}$C, 24 h.

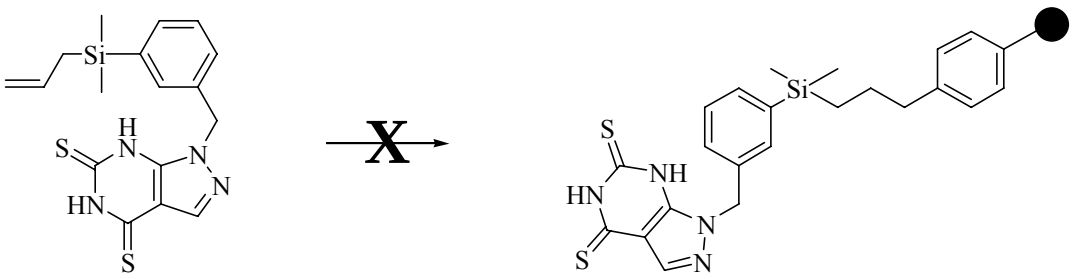
## 4.2 Results and Discussion

The synthesis of silicon-containing scaffold, 1-(3-allyldimethylsilylbenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(44)** has been discussed in Chapter 3 **(Scheme 4.2)**. This scaffold needs to be attached to a polystyrene resin to carry a solid phase synthesis of pyrazolo[3,4-*d*]pyrimidines. Suzuki coupling has been reported[10] to proceed cleanly on solid support for aryl halides. Attempts to attach 1-(3-allyldimethylsilylbenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(44)** to bromopolystyrene resin via Hydroboration-Suzuki coupling using the literature procedures[9] was unsuccessful and only starting material was recovered from the reaction (**Scheme 4.3)**. The hydroboration did not proceed even when an excess amount of 9-BBN (3 eq) was used. To the best of our knowledge, the hydroboration reaction has not yet been investigated on a system with thioamide bonds.




**44** **39**

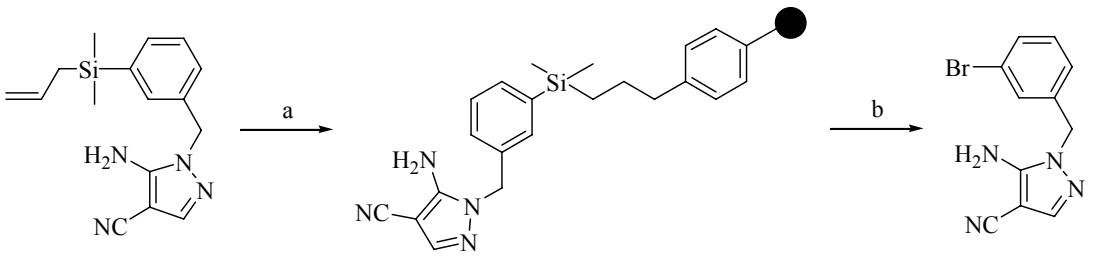
**Scheme 4.2**



**44** **45**

**Scheme 4.3**: *Reagents and conditions:* 9-BBN, THF, RT, 5 h, then bromopolystyrene resin, DMF, $Pd(PPh_3)_4$, $K_2CO_3$, 65 $^oC$, 24 h.

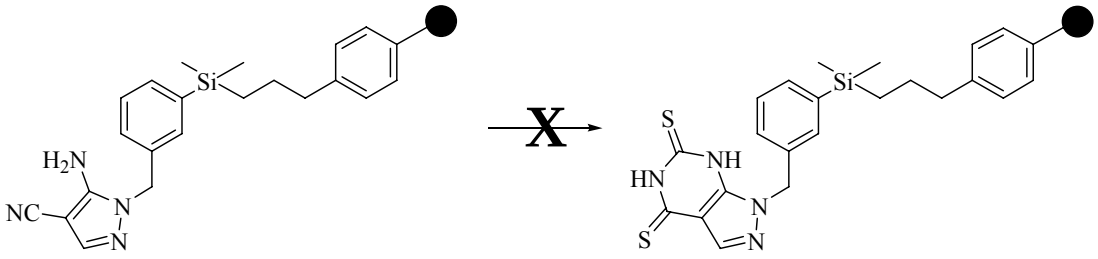
However, the intermediate 1-(1,1-dimethylallylsilylbenzyl)-5-amino-4-cyanopyrazole **(39)** underwent Hydroboration-Suzuki coupling with bromopolystyrene resin[11] to give the resin-bound 1-(1,1-dimethylallylsilylbenzyl)-5-amino-4-cyanopyrazole **(46)**. The resin-bound cyanopyrazole **(46)** was prepared by hydroboration of the cyanopyrazole **(39)** with 9-BBN in THF followed by in situ Suzuki coupling[12] of the borane complex with bromopolystyrene resin[11], Pd(0), and $K_2CO_3$, in THF. In order to prove **(39)** had been successfully loaded on to the resin via Hydroboration-Suzuki coupling, the resin-bound cyanopyrazole **(46)** was cleaved with $Br_2$/Pyridine in $CH_2Cl_2$ to produce 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27) (Scheme 4.4)** which was prepared in solution studies **(Section 2.2.2).** The loading level of support was also determined from brominative cleavage of resins with $Br_2$/Pyridine. The loading level was 0.4 mmol/g.



**39** **46** **27**

**Scheme 4.4**: *Reagents and conditions:* (a) 9-BBN, THF, RT, 5 h, then bromopolystyrene, DMF, $Pd(PPh_3)_4$, $K_2CO_3$, 65 $^oC$, 24 h; (b) $Br_2$/Pyr, DCM, 3 h.

The resin-bound 1-(1,1-dimethylallylsilylbenzyl)-5-amino-4-cyanopyrazole **(46)** was then swelled in DMF and refluxed with potassium O-ethylxanthogenate using the established solution phase synthesis of 1-(3-allyldimethylsilylbenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(44) (Scheme 4.5)**. Only starting material was recovered upon brominative cleavage of resin-bound cyanopyrazole **(46)** with $Br_2$/Pyridine. The reaction was also carried out at 155 °C for several hours but only starting material was recovered.




**46** **47**

**Scheme 4.5**: *Reagents and conditions:* (i) $EtOCS_2^-K^+$, DMF, 140 °C, reflux, 2 h; (ii) NaOH (2.0 M); RT, 45 min (iii) HCl (2.0 M).

## 4.3 Conclusion

The synthesis of resin-bound 1-(3-allyldimethylsilylbenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(47)**, a resin-bound scaffold, was not successful either by direct attachment or by reaction from resin-bound cyanopyrazole **(46)**. The hydroboration of **(44)** did not proceed possibly due to the presence of thioamide bonds. The synthetic route to 1-(3-allyldimethylsilylbenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(44)** was lengthy, difficulty and had low yields. This prompted us to go back to the parallel solution phase synthesis of pyrazolo[3,4-*d*]pyrimidines derivatives.

## 4.4 Experimental

Melting points were recorded on a Gallenkamp digital melting point apparatus and are uncorrected. Infra-red absorption spectra were obtained on a Perkin Elmer FT-IR spectrophotometer using sodium chloride plates. $^{1}H$ and $^{13}C$ nuclear magnetic resonance (n.m.r) spectra were recorded on a Varian Gemini-200 (200 MHz) spectrometer, Varian Unity-400 (400 MHz) spectrometer or Varian Unity Plus-600 (600 MHz) spectrometer. All samples were dissolved in deuteriochloroform ($CDCl_3$) containing tetramethylsilane (TMS) as an internal reference unless otherwise stated. The signals are recorded in terms of chemical shift in parts per million (ppm) downfield from TMS ($\delta = 0$) for protons or $CDCl_3$ ($\delta = 77$) for carbon atoms. The signals are recorded in terms of chemical shift ($\delta_H$), relative integral, multiplicity, coupling constants (*J* Hz) and assignments, in that order. The following abbreviations for multiplicity are used: s = singlet, dd = doublet of doublets, dt = doublet of triplets, m = multiplet. $^{1}H$ and $^{13}C$ spectra were assigned with the aid of HMQC (Heteronuclear Multiple-Quantum Coherence), HMBC (Heteronuclear Multiple-Bond Coherence) and $^{1}H$-$^{1}H$ COSY (Correlation Spectroscopy). Electrospray mass spectra (ESMS) were recorded on a Fisons VG Platform mass spectrometer with MassLynx Data System software.

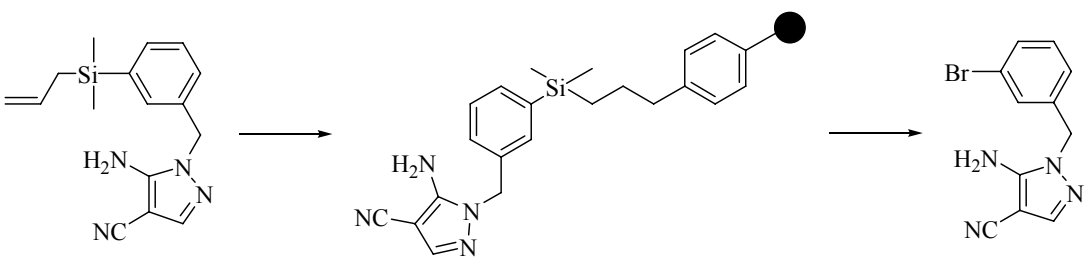
Microanalytical data was obtained from University of Queensland Microanalytical Service.

Analytical thin layer chromatography (TLC) was performed using precoated (0.2 mm) Merck silica gel plates (Merck Kieselgel 60 $F_{254}$). Flash chromatography was carried out using Merck Kieselgel 60 (230-400 mesh) with the indicated solvents.

All solvents for chromatography were distilled before use, unless otherwise stated. Ether refers to diethyl ether and hexane refers to the fraction of b.p. 60-80 °C. Mixed solvent compositions are quoted as v/v.

Solvents and reagents were purified according to the standard techniques of Perrin, Perrin and Amarego.[13]

**Hydroboration and Suzuki coupling of (39) to the bromopolystyrene resin.**



**39** **46** **27**

To a solution of 1-(1,1-dimethylallylsilylbenzyl)-5-amino-4-cyanopyrazole **(39)** (111 mg, 374.4 mmol) in dry THF (3 ml) at 0 °C was added 9-BBN (0.8 ml, 0.5 M solution in THF, 400.0 mmol) dropwise. The reaction mixture was gradually warmed to room temperature and stirred for 5 h. $Pd(PPh_3)_4$ (13 mg, 1.1 mmol %), 4-bromopolystyrene resin (180 mg, 1.95 mmol/g, 351.0 mmol), powdered $K_2CO_3$ (155 mg, 1.12 mmol), dry DMF (3 ml), and $H_2O$ (0.5 ml) were then added to the reaction mixture and stirred for 24 h at 65 °C. The resin was filtered and washed with THF (2 x 5ml), 1:1 THF/water (2 x 5ml), water (2 x 5ml), methanol (2 x 5ml) and dried under the vacuum. The loading level of support was determined as follows: To the dried resin-bound 1-(1,1-dimethylallylsilylbenzyl)-5-amino-4-cyanopyrazole **(46)** in DCM (4 ml) was added $Br_2$ (100 µl) and pyridine (90 µl). The reaction mixture was stirred for 3 h. The cleavage solution was filtered and rinsed with DCM (5 ml). The combined solution was concentrated to give 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27)** (20.0 mg, 0.072 mmol, loading level = 0.4 mmol/g) as solid, mp 101.5 ± 0.5 °C; $\delta_H$ (400 MHz, DMSO-$d_6$) 5.17 (s, 2H, $CH_2$), 6.78 (s, 2H, $NH_2$), 7.16 (d, $J$ = 8.0 Hz, 1H, 6'-H), 7.30 (t, $J$ = 8.0 Hz, 1H, 5'-H), 7.38 (t, $J_{meta}$ = 1.6 Hz, 1H, 2'-H), 7.49 (d, $J$ = 8.0 Hz, 1H, 4'-H) and 7.61 (s, 1H, 3-H); $\delta_C$ (100 MHz, DMSO-$d_6$) 49.3 ($CH_2$), 72.3 (C-4), 115.1 (CN), 121.7 (C-3'), 126.4 (C-6'), 130.1 (C-2'), 130.4 (C-4'), 130.7 (C-5'), 139.3 (C-1'), 140.8 (C-3) and 151.7 (C-5); $\nu_{max}$ (NaCl plates)/$cm^{-1}$ 3400 ($NH_2$) and 2200 (CN). Anal. calcd for $C_{11}H_9BrN_4$: C, 47.68; H, 3.27; N, 20.22C. Found C, 47.80; H, 3.17; N, 20.30%; ESMS (NI) 275 and 277. calcd for ($C_{11}H_9{}^{79}BrN_4$ - 1[H]) and ($C_{11}H_9{}^{81}BrN_4$ - 1[H]) respectively. Found 275 and 277.

## 4.5 References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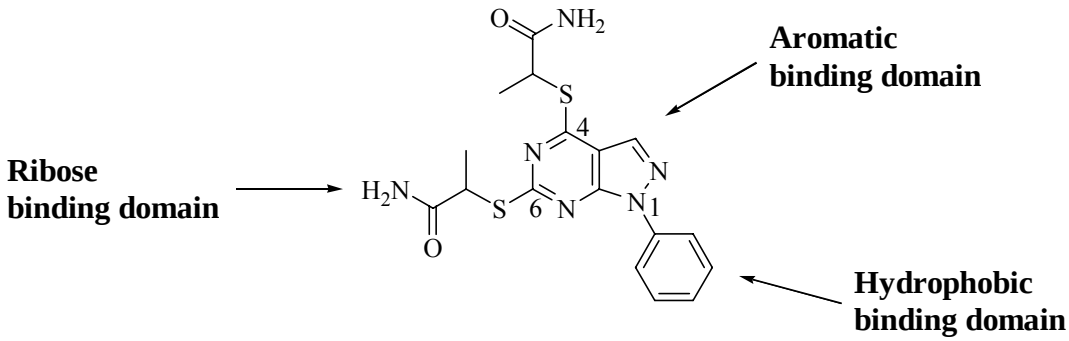
# CHAPTER 5

## Parallel synthesis of pyrazolopyrimidine derivatives

### 5.1 Introduction:

A potent and selective ligand often has several discrete domains so that a receptor can have an energetically favorable interaction. As discussed in chapter 2 **(Section 2.1)**, compound **(5)**, the pyrazolo[3,4-*d*]pyrimidine derivative, was a good lead compound for further development of adenosine antagonists. Molecular modeling studies[1,2] have shown that compound **(5)** fitted the three binding domain model of the adenosine receptors in such a way that the phenyl ring at N-1 occupied the hydrophobic binding domain, the purine ring system occupied the aromatic binding domain and the amide side chain at C-6 occupied the ribose domain **(Figure 5.1)**.

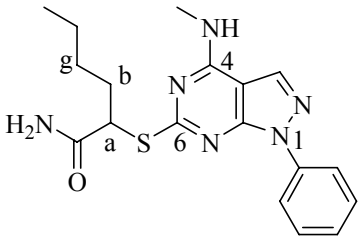



**5**

**Figure 5.1**: Proposed binding sites of **(5)**.

Quinn's previous studies[3-5] have concentrated on modifying the length of the amide side chain at C-6, varying the substituents at C-4 and leaving the phenyl hydrophobic domain at N-1 constant to optimize the receptor binding affinity and

subtype selectivity. A major outcome from these studies was the production of amino-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-yl)thio]hexanamide **(21)**, a highly potent and selective antagonist at the rat adenosine $A_1$ receptor **(Figure 5.2)**.[5]




**21**

**Figure 5.2**: Highly potent and selective ligands at the rat adenosine $A_1$ receptor, α–[(4-amino-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-yl)thio]hexanamide **(21)**.

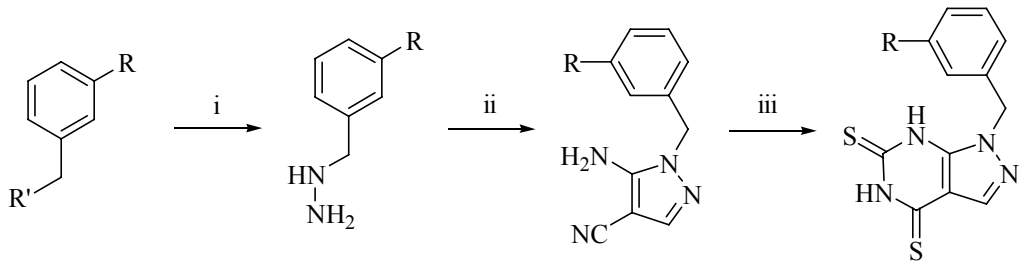
In this study, a library of pyrazolo[3,4-*d*]pyrimidines derivatives with different substituents at C-4, C-6 and N-1 was synthesized by parallel synthesis. Each crude mixture of a library was evaluated for receptor binding at the human adenosine $A_1$, $A_{2A}$ and $A_3$ receptors was checked with mass spectrometer to confirm the presence of expected compound in the crude mixture.

## 5.2 Results and Discussion

### 5.2.1 Synthesis of pyrazolo[3,4-*d*]pyrimidine dithione scaffolds

The four benzyl-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione scaffolds **(26), (28), (51) and (52)** were synthesized from the corresponding benzyl halides. The synthesis of these dithione scaffolds used the procedures established in chapter 2 **(section 2.2.2)**. For the synthesis of 1-(3-nitrobenzyl)-5-amino-4-cyanopyrazole **(49)**, it was found that heating the reaction mixture above 120 °C would decompose the product or the corresponding starting material. The reaction was successfully accomplished by heating the reaction mixture slowly and monitoring the reaction mixture by thin layer chromatography and mass spectrometry. When the expected

mass of the product was detected by mass spectrometry, the reaction mixture was heated at this temperature until no starting material was detected by the mass spectrometry. It was observed that the product was obtained when the reaction mixture was heated at 100 °C. The same problem happened to the synthesis of 1-(3-nitrobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(51)** from corresponding 1-(3-nitrobenzyl)-5-amino-4-cyanopyrazole **(49)**, the compound **(51)** was lost when the reaction mixture was heated at 135 °C. Using the same technique as in obtaining its corresponding starting material, 1-(3-nitrobenzyl)-5-amino-4-cyanopyrazole **(49)**, the product was successful obtained by heating the reaction mixture at 110 °C. The 1-(3-nitrobenzyl)-5-amino-4-cyanopyrazole **(49)** seemed to decompose in the solution at around 120 °C. The general synthetic route for the syntheses of four benzyl-5H,7H-pyrazolo[3,4-*d]*pyrimidine-4,6-dithione scaffolds was outlined in **Scheme 5.1**.




R' = halide

R = H **(48)**, $NO_2$ **(49)**
Br **(27)**, I **(50)**

R = H **(26)**, $NO_2$ **(51)**
Br **(28)**, I **(52)**

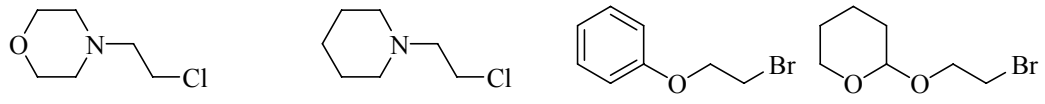
**Scheme 5.1**: *Reagents and conditions:* (i) $N_2H_4.H_2O$, EtOH, reflux, 3-5 h; (ii) $C_2H_5OCH=C(CN)_2$, EtOH, reflux, 2 h (45-65% over 2 steps); (iii) (a) $EtOCS_2^-K^+$, DMF, 135 $^oC$ (110 $^oC$ for R = $NO_2$), reflux, 2 h; (b) NaOH (2.0 M), RT, 45 min; (c) HCl (2.0 M), 85-96%.

The NMR assignments for these synthesized compounds are recorded in the experimental section **(Section 5.4)**. The $^1H$ n.m.r spectra of 5-amino-4-cyanopyrazole structures **(48, 49, 27 and 50)** showed the presence of amino protons at $\delta_H$ 6.70-6.80 ppm, which disappeared upon $D_2O$ addition, and benzylic protons at $\delta_H$ 5.00-5.30 ppm. The $^1H$ n.m.r spectra of their corresponding 5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione scaffolds **(26, 51, 28 and 52)** showed the absence of amino protons at $\delta_H$ 6.70-6.80 ppm and the presence of two broad singlets at $\delta_H$ 7.00-8.20 ppm and at $\delta_H$ 11.00-13.50 ppm which were assigned to NH protons at N-7 and N-5 respectively.

### 5.2.2 Synthesis of pyrazolo[3,4-*d*]pyrimidine derivatives

As discussed in **Section 2.2.2**, S-methyl and S-alkyl at C-4 of pyrazolo[3,4-*d*]pyrimidines are both good leaving groups and could undergo nucleophilic substitution reactions with secondary amines. Since some alkyl halides were expensive and some secondary amines were very bulky or had no carbon spacer, S-alkyl at C-6 and S-methyl at C-4 for all four 5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione scaffolds **(26, 51, 28 and 52)** were synthesized and chosen as a starting material for nuclephilic substitution reactions with amines.

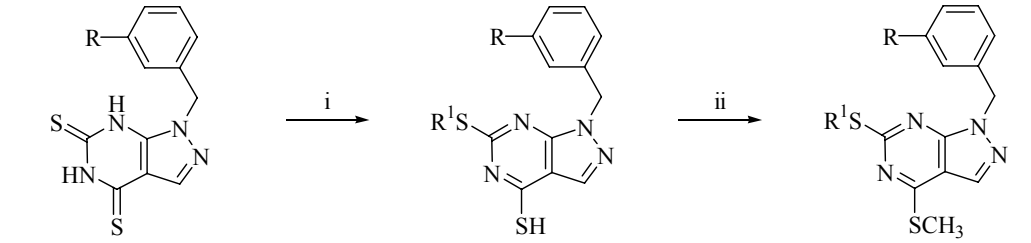
Therefore, each of the four 5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione scaffolds **(26, 51, 28 and 52)** was reacted with 1.0 equivalent of eight different alkyl halides **(Figure 5.3)** in a pyridine. The reaction mixtures were left to stir at room temperature for 48 h and each of these thirty two reaction mixtures was monitored by electrospray mass spectrometry to make sure each of the four scaffolds **(26, 51, 28 and 52)** was alkylated. It was found that the halides containing 1 carbon spacer such as 2-(bromomethyl)tetrahydro-2H-pyran reacted very slowly compared to the halides with 2 or more carbon spacer such as 4-(2-chloroethyl)morpholine hydrochloride. After stirring for 48 h at room temperature, each of these 32 reaction mixtures was concentrated and methylated with an excess of methyl iodide in dioxane:NaOH (2.0 M) (1:1) at room temperature. After stirring for 2 h at room temperature, each of 32 reaction mixtures was extracted with ethyl acetate, dried over anhydrous magnesium sulphate and concentrated to give thirty two crude methylated mixtures **(Scheme 5.2)**.




4-(2-Chloroethyl)mopholine hydrochloride | 1-(2-Chloroethylpiperidine monohydrochloride | 2-Bromoethyl phenyl ether | 2-(2-Bromoethoxy) tetrahydro-2H-pyran


2-(Bromomethyl) tetrahydro-2H-pyran | Phenethyl bromide | 1-(2-Chloroethyl)pyrrol idine hydrochloride | Methyl iodide

**Figure 5.3**: Alkylating Agents.




R = H **(26)**, $NO_2$ **(51)**
Br **(28)**, I **(52)**

R = H, $NO_2$, Br, I
$R^1$ = Alkyl halides

R = H, $NO_2$, Br, I
$R^1$ = Alkyl halides

**Scheme 5.2**: *Reagents and conditions:* (i) Alkyl halides, Pyr, RT, 48 h; (ii) MeI, NaOH (2.0 M): Dioxane, RT, 2 h.

Having synthesized the 32 desired crude starting materials for nucleophilic substitution reaction, a preliminary study of eight 1-(3-bromobenzyl)-6-(alkylthio)-4-methylthio-pyrazolo[3,4-*d*]pyrimidines was undertaken. The compounds were reacted with 12 different secondary amines **(Figure 5.4)** in n-butanol. These 96 modeled reactions were monitored by electrospray mass spectrometry. It found that the amines with low boiling points such as cyclopropyl amine were lost during the reactions and the amine with bulky group or no carbon spacer such as 4-amino-1-benzyl-piperidine underwent the nucleophilic substitution reaction extremely slow. Low boiling point amine was added every 12 h until no starting material was detected by electrospray mass spectrometry. The products and starting materials were found very stable after heating at 92 $^{o}$C for 4 days. After the conditions were established, each of 32 crude methylated mixtures was reacted 12 different secondary amines in dimethylsulfoxide (DMSO) for 4 days at 92 $^{o}$C **(Scheme 5.3)**. DMSO was chosen as a substituting solvent for n-butanol because several crude methylated mixtures didn't dissolve in the protic solvents such as butanol and methanol and DMSO was used to dissolve tested compounds for screening with a final DMSO concentration of 1% or 2%. DMSO also had a high boiling point and was less corrosive to the sealing gaskets than chloroform.

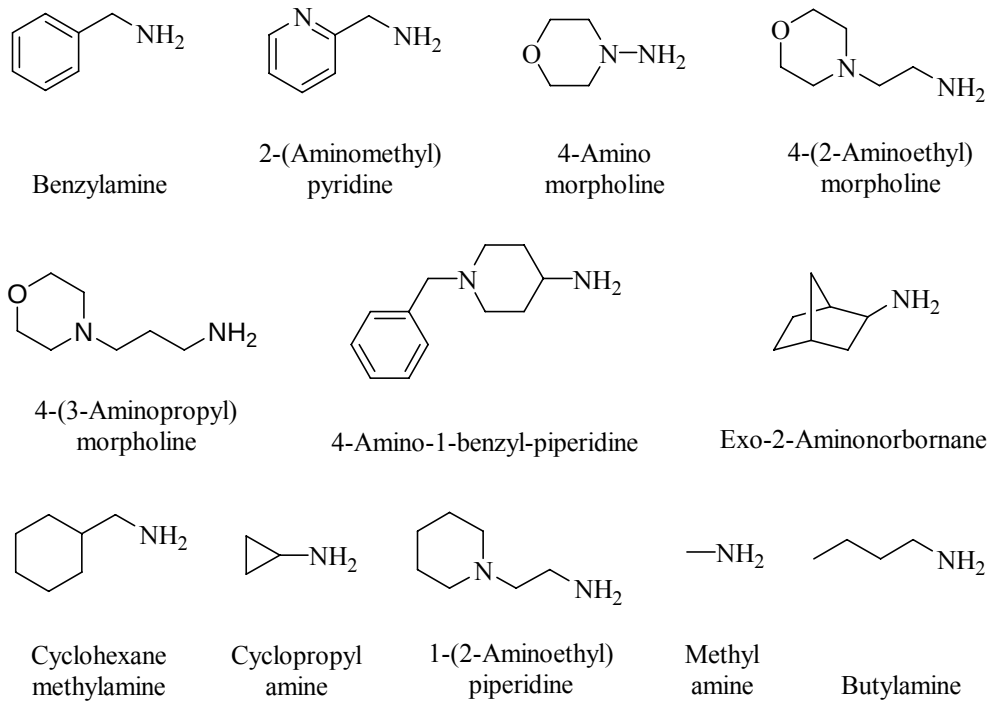
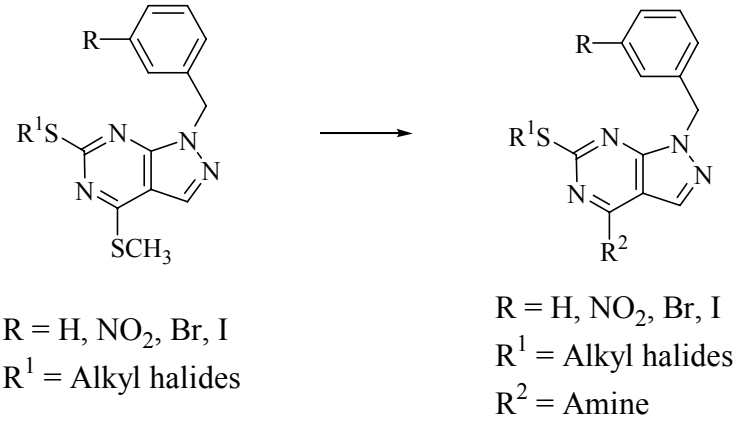



**Figure 5.4**: Nucleophilic Substituting Agents.

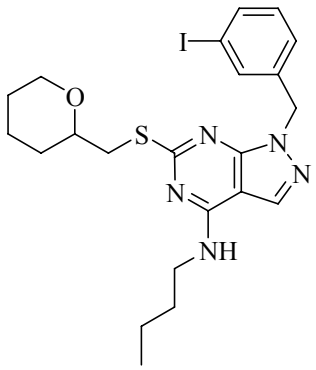



**Scheme 5.3**: *Reagents and conditions:* Amino agents, DMSO, 92 $^{o}$C, 4 days.

### 5.2.3 Radio-Ligand Binding Results

The yields for synthesis of 1-(3-bromobenzyl)-6-(3-bromobenzylthio)-4-methylthio-pyrazolo[3,4-*d*]pyrimidine **(32)** and 1-(3-bromobenzyl)-6-(3-bromobenzylthio)-4-methylaminopyrazolo[3,4-*d*]pyrimidine **(35) (section 2.4)** were 49% and 52% respectively. Assumed that the yields for each step leading to the

desired target pyrazolo[3,4-*d*]pyrimidines were 50 % each. 384 crude mixtures were screened at an assumed 1.0 μM to determine the inhibition of these compounds at the human $A_1$, $A_{2A}$ and $A_3$ receptors. Each crude mixture of the library was then checked with mass spectrometry to confirm the presence of the expected compound in the crude mixture. One representative mass spectrum of the crude compound **(421)** with correct mass is presented in **Figure 5.5**. 384 mass spectra of the crude pyrazolo[3,4-*d*]pyrimidines are recorded on the CD-rom (attachment). The binding results of these 384 crude compounds at human $A_1$, $A_{2A}$ and $A_3$ receptors are presented in **Table 5.1, Table 5.2 and Table 5.3** respectively. The crude mixtures having compounds with the correct mass are not shaded.




**(421)**

*m/z*: 538 a.m.u

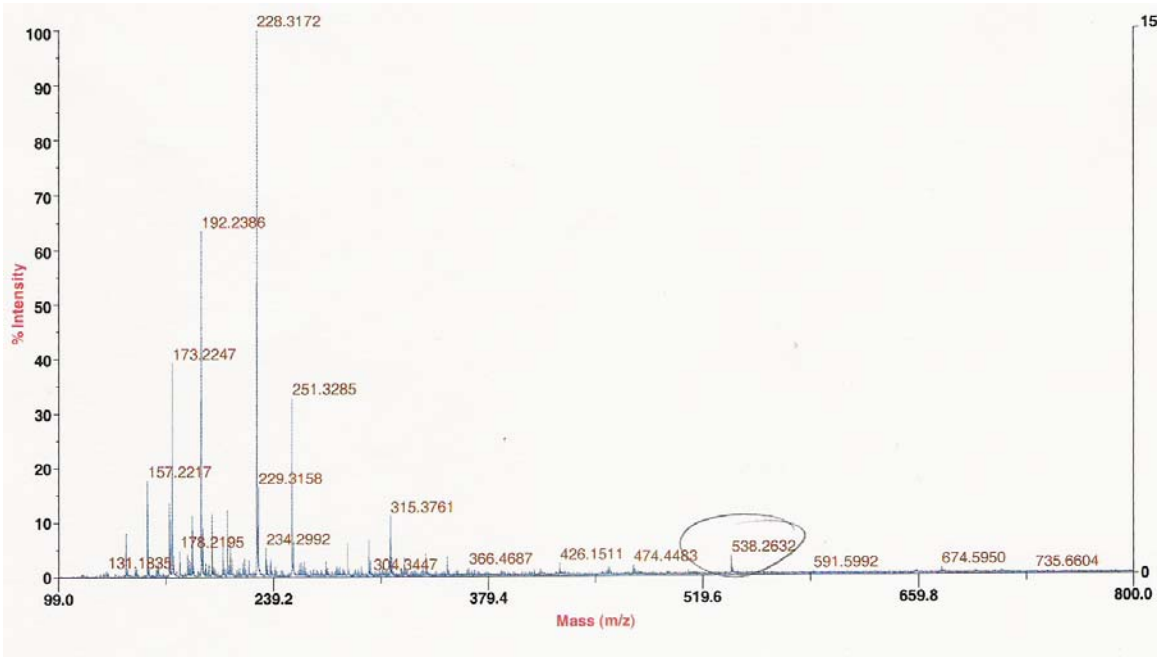
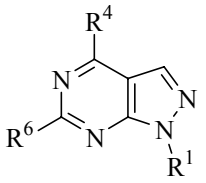
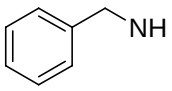
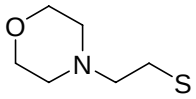
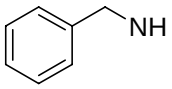
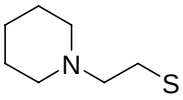
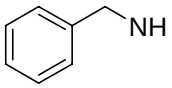
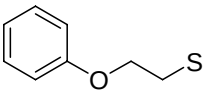
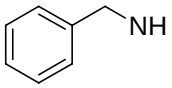
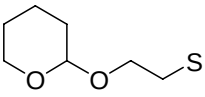
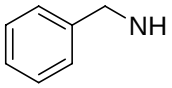
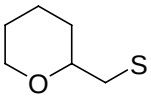
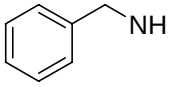
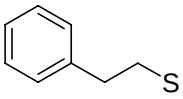
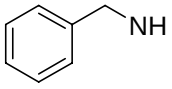
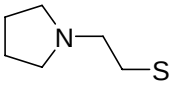
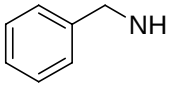



**Figure 5.5**: Mass spectrum of crude **(421)**.

**Table 5.1**: Binding affinity of the crude pyrazolo[3,4-*d*]pyrimidines expressed as % displacement at 1.0 μM. Displacement of specific [$^3$H]DPCPX binding from CHO cells transfected with $A_1$ human adenosine receptor. The crude compounds with the corrected masses were not shaded.



| Comp | Substituents | | %Activity (1.0 μM) | | | |
|---|---|---|---|---|---|---|
| | | | $R^1$ | | | |
| | $R^4$ | $R^6$ | 3-I Bn | 3-H Bn | 3-Br Bn | 3-$NO_2$ Bn |
| **53-56** |  |  | 51 | 66 | 60 | 35 |
| **57-60** |  |  | 63 | 71 | 46 | 20 |
| **61-64** |  |  | 44 | 51 | 30 | 23 |
| **65-68** |  |  | 19 | 64 | 49 | 25 |
| **69-72** |  |  | 62 | 75 | 53 | 74 |
| **73-76** |  |  | 28 | 59 | 17 | 42 |
| **77-80** |  |  | 64 | 85 | 19 | 38 |
| **81-84** |  |  | 64 | 61 | 29 | 46 |

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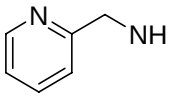
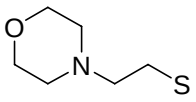
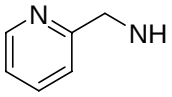
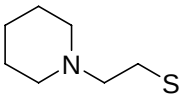
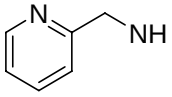
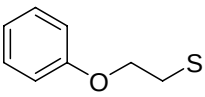
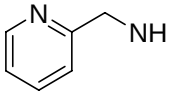
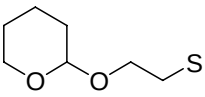
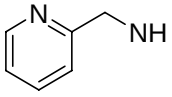
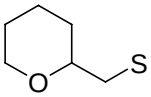
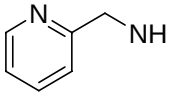
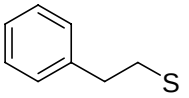
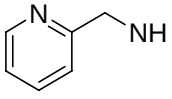
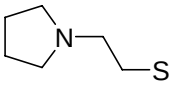
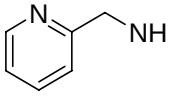
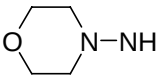
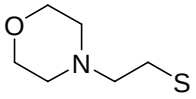
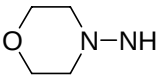
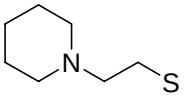
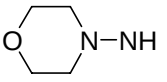
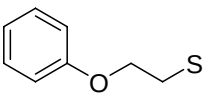
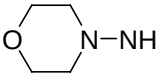
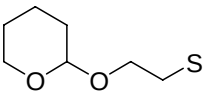
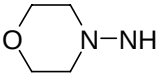
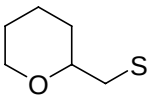
| **85-88** |  |  | 72 | 57 | 72 | 20 |
| **89-92** |  |  | 46 | 81 | 31 | 14 |
| **93-96** |  |  | 51 | 66 | 26 | 22 |
| **97-100** |  |  | 88 | 61 | 48 | 25 |
| **101-104** |  |  | 85 | 64 | 52 | 39 |
| **105-108** |  |  | 72 | 40 | 20 | 31 |
| **109-112** |  |  | 62 | 42 | 59 | 17 |
| **113-116** |  |  | 58 | 65 | 52 | 24 |
| **117-120** |  |  | 49 | 44 | X | 3 |
| **121-124** |  |  | 92 | 68 | X | 24 |
| **125-128** |  |  | 73 | 36 | X | 26 |
| **129-132** |  |  | 66 | 32 | X | 22 |
| **133-136** |  |  | 79 | 43 | X | 24 |

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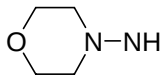
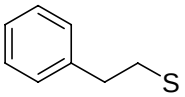
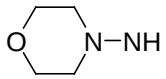
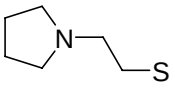
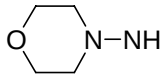
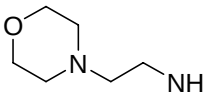
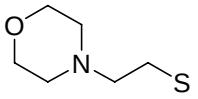
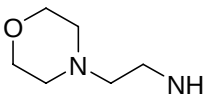
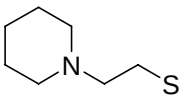
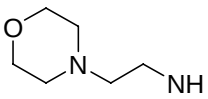
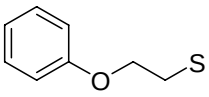
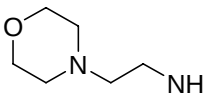
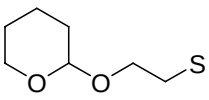
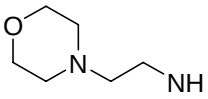
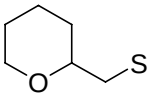
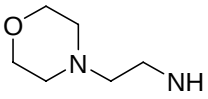
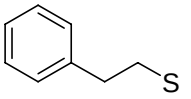
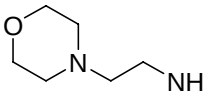
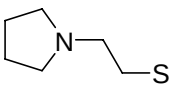
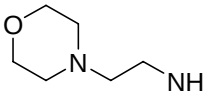
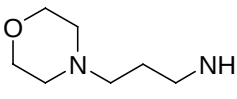
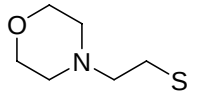
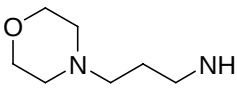
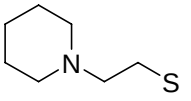
| **137-140** |  |  | 47 | 29 | X | 8 |
| **141-144** |  |  | 76 | 73 | X | 0 |
| **145-148** |  |  | 76 | 74 | X | 28 |
| **149-152** |  |  | 64 | 30 | 42 | 22 |
| **153-156** |  |  | 56 | 51 | 35 | 17 |
| **157-160** |  |  | 27 | 39 | 13 | 30 |
| **161-164** |  |  | 19 | 20 | 53 | 8 |
| **165-168** |  |  | 83 | 36 | 57 | 21 |
| **169-172** |  |  | 55 | 27 | 39 | 17 |
| **173-176** |  |  | 55 | 37 | 41 | 10 |
| **177-180** |  |  | 55 | 32 | 43 | 28 |
| **181-184** |  |  | 40 | 30 | 38 | 23 |
| **185-188** |  |  | 54 | 35 | 29 | 28 |

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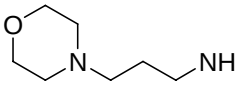
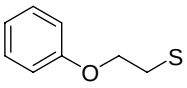
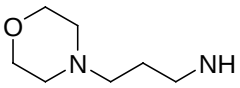
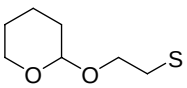
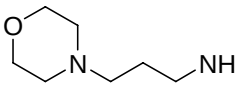
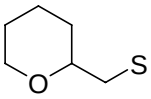
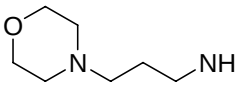
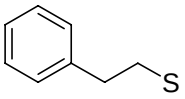
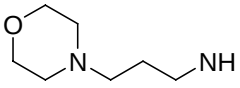
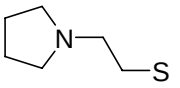
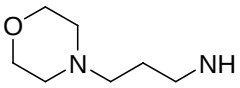
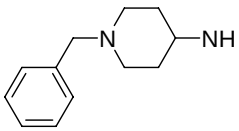
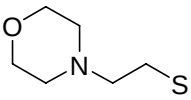
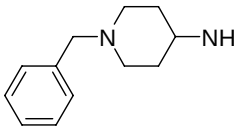
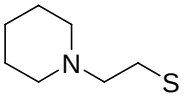
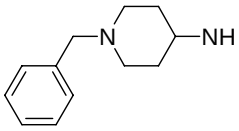
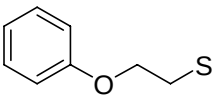
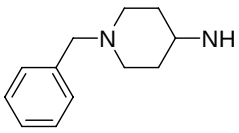
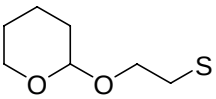
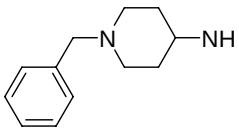
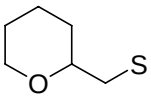
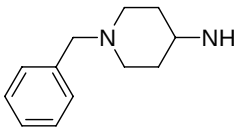
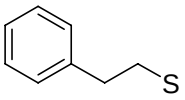
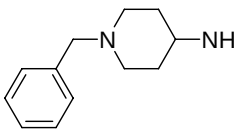
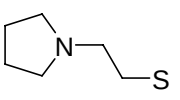
| **189-192** |  |  | 58 | 37 | 33 | 0 |
| **193-196** |  |  | 56 | 39 | 41 | 4 |
| **197-200** |  |  | 73 | 45 | 63 | 24 |
| **201-204** |  |  | 46 | 53 | 29 | 33 |
| **205-208** |  |  | 39 | 48 | 10 | 26 |
| **209-212** |  |  | 14 | 45 | 20 | 32 |
| **213-216** |  |  | 95 | 42 | 44 | 0 |
| **217-220** |  |  | 85 | 24 | 27 | 0 |
| **221-224** |  |  | 78 | 60 | 30 | 13 |
| **225-228** |  |  | 68 | 43 | 65 | 18 |
| **229-232** |  |  | 89 | 52 | 26 | 28 |
| **233-236** |  |  | 61 | 36 | 14 | 31 |
| **237-240** |  |  | 76 | 69 | 43 | 33 |

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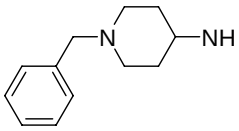
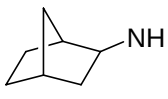
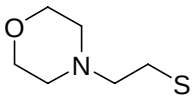
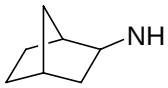
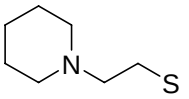
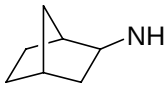
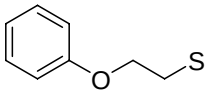
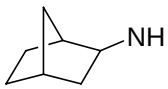
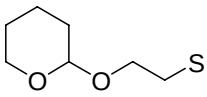
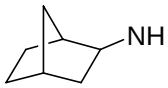
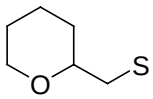
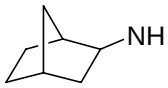
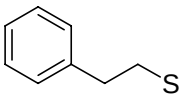
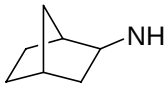
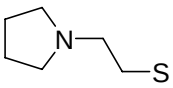
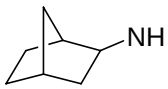
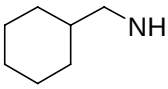
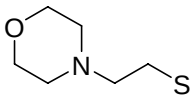
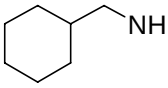
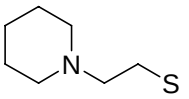
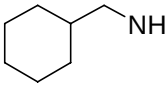
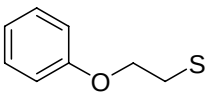
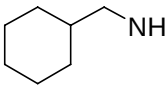
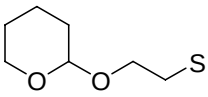
| **241-244** |  | —S | 73 | 53 | 42 | 31 |
| **245-248** |  |  | 70 | 92 | 75 | 38 |
| **249-252** |  |  | 87 | 100 | 78 | 29 |
| **253-256** |  |  | 60 | 66 | 26 | 22 |
| **257-260** |  |  | 58 | 50 | 48 | 30 |
| **261-264** |  |  | 75 | 70 | 38 | 56 |
| **265-268** |  |  | 45 | 67 | 38 | 29 |
| **269-272** |  |  | 77 | 100 | 55 | 25 |
| **273-276** |  | —S | 91 | 100 | 72 | 44 |
| **277-280** |  |  | 56 | X | 43 | 19 |
| **281-284** |  |  | 60 | X | 43 | 26 |
| **285-288** |  |  | 48 | X | 0 | 39 |
| **289-292** |  |  | 53 | X | 37 | 27 |

| | | | | | | |
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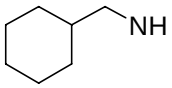
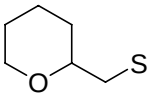
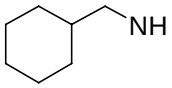
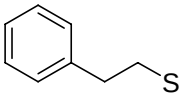
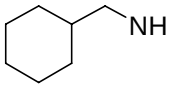
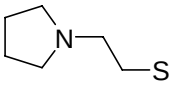
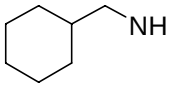
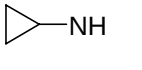
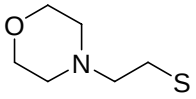
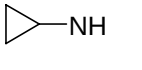
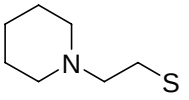
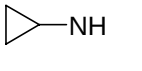
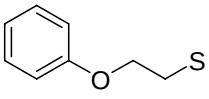
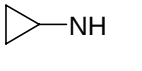
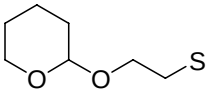
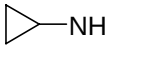
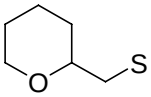
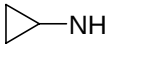
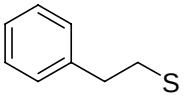
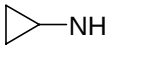
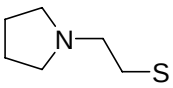
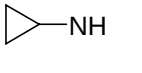
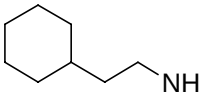
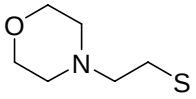
| **293-296** |  |  | 68 | X | 41 | 51 |
| **297-300** |  |  | 52 | X | 4 | 31 |
| **301-304** |  |  | 41 | X | 33 | 19 |
| **305-308** |  |  | 62 | X | 33 | 43 |
| **309-312** |  |  | 72 | 89 | 77 | 39 |
| **313-316** |  |  | 94 | 100 | 94 | 45 |
| **317-320** |  |  | 78 | 64 | 55 | 29 |
| **321-324** |  |  | 69 | 46 | 42 | 14 |
| **325-328** |  |  | 70 | 75 | 59 | 27 |
| **329-332** |  |  | 61 | 71 | 55 | 24 |
| **333-336** |  |  | 88 | 99 | 96 | 42 |
| **337-340** |  |  | 95 | 97 | 93 | 48 |
| **341-344** |  |  | 69 | 31 | 28 | 21 |

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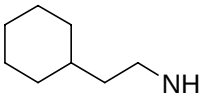
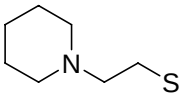
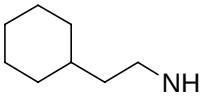
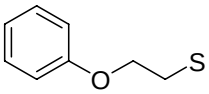
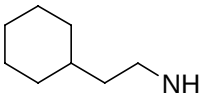
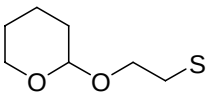
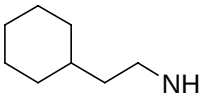
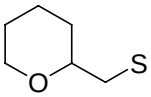
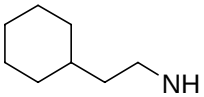
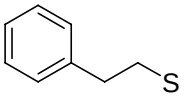
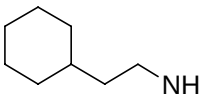
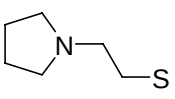
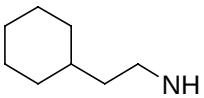
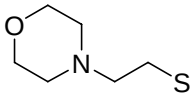
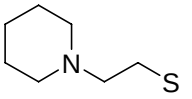
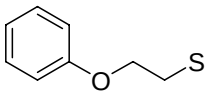
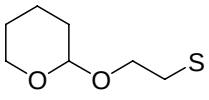
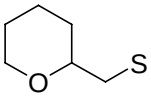
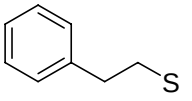
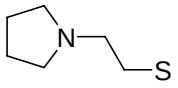
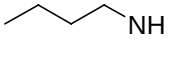
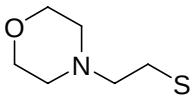
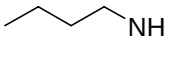
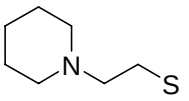
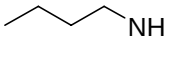
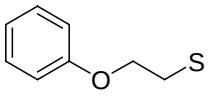
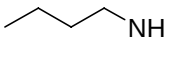
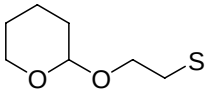
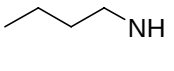
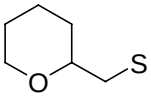
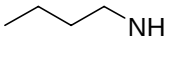
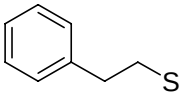
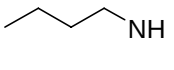
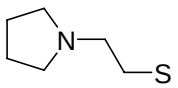
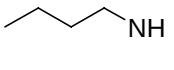
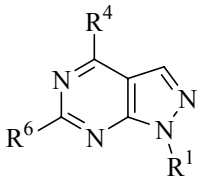
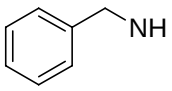
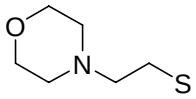
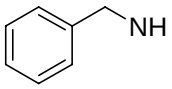
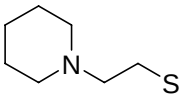
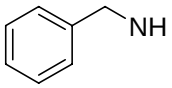
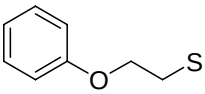
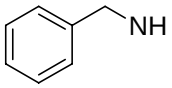
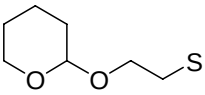
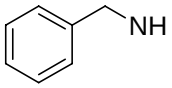
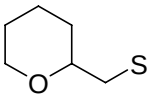
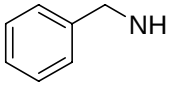
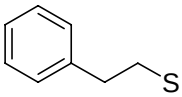
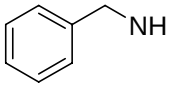
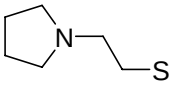
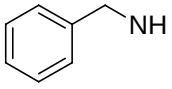
| **345-348** |  |  | 58 | 35 | 4 | 28 |
| **349-352** |  |  | 28 | 56 | 19 | 19 |
| **353-356** |  |  | 38 | 30 | 53 | 10 |
| **357-360** |  |  | 69 | 46 | 41 | 18 |
| **361-364** |  |  | 61 | 40 | 4 | 21 |
| **365-368** |  |  | 58 | 35 | 19 | 26 |
| **369-372** |  |  | 44 | 34 | 21 | 11 |
| **373-376** |  |  | 68 | 68 | 63 | 0 |
| **377-380** |  |  | 70 | 84 | 90 | 25 |
| **381-384** |  |  | 45 | 30 | 28 | 35 |
| **385-388** |  |  | 69 | 20 | 41 | 32 |
| **389-392** |  |  | 79 | 50 | 10 | 30 |
| **393-396** |  |  | 65 | 36 | 8 | 36 |

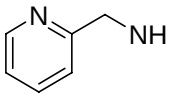
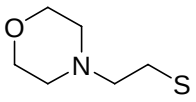
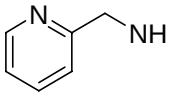
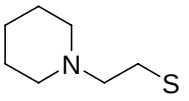
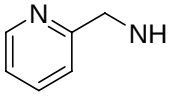
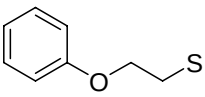
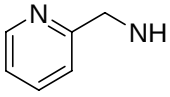
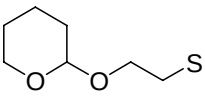
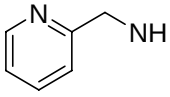
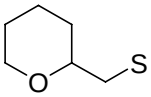
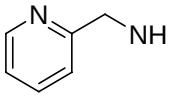
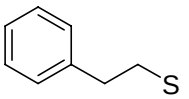
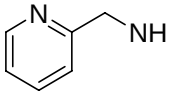
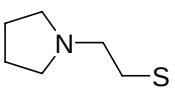
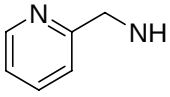
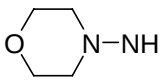
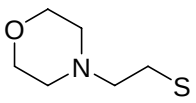
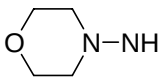
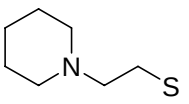
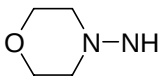
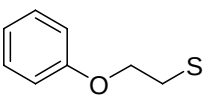
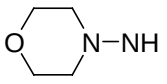
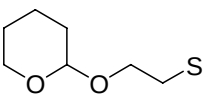
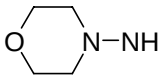
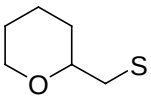
| | | | | | | |
|---|---|---|---|---|---|---|
| **397-400** | —NH |  | 63 | 88 | 81 | 11 |
| **401-404** | —NH | —S | 70 | 60 | 82 | 15 |
| **405-408** |  |  | 63 | 64 | 63 | 74 |
| **409-412** |  |  | 82 | 83 | 61 | 75 |
| **413-416** |  |  | 83 | 62 | 0 | 71 |
| **417-420** |  |  | 72 | 58 | 31 | 70 |
| **421-424** |  |  | 91 | 85 | 72 | 77 |
| **425-428** |  |  | 78 | 50 | 50 | 49 |
| **429-432** |  |  | 83 | 76 | 67 | 84 |
| **433-436** |  | —S | 85 | 87 | 77 | 42 |

NB: X denotes the crude mixture was not tested.

**Table 5.2**: Binding affinity of the crude pyrazolo[3,4-*d*]pyrimidines expressed as % displacement at 1.0 μM. Displacement of specific [$^3$H]NECA binding from CHO cells transfected with $A_{2A}$ human adenosine receptor. The crude compounds with the corrected masses were not shaded.




| Comp | Substituents | | %Activity (1.0 μM) | | | |
|---|---|---|---|---|---|---|
| | | | $R^1$ | | | |
| | $R^4$ | $R^6$ | 3-I Bn | 3-H Bn | 3-Br Bn | 3-$NO_2$ Bn |
| **53-56** |  |  | 30 | 23 | 0 | 54 |
| **57-60** |  |  | 27 | 23 | 7 | 54 |
| **61-64** |  |  | 0 | 6 | 8 | 18 |
| **65-68** |  |  | 0 | 0 | 15 | 27 |
| **69-72** |  |  | 0 | 0 | 0 | 17 |
| **73-76** |  |  | 0 | 9 | 0 | 24 |
| **77-80** |  |  | 16 | 25 | 0 | 66 |
| **81-84** |  | —S | 13 | 24 | 3 | 54 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **85-88** |  |  | 15 | 4 | 0 | 35 |
| **89-92** |  |  | 6 | 1 | 12 | 36 |
| **93-96** |  |  | 14 | 0 | 0 | 22 |
| **97-100** |  |  | 13 | 22 | 0 | 58 |
| **101-104** |  |  | 0 | 22 | 29 | 53 |
| **105-108** |  |  | 1 | 8 | 0 | 53 |
| **109-112** |  |  | 0 | 7 | 0 | 50 |
| **113-116** |  |  | 0 | 16 | 0 | 41 |
| **117-120** |  |  | 35 | 14 | 53 | 35 |
| **121-124** |  |  | 10 | 13 | 56 | 62 |
| **125-128** |  |  | 0 | 6 | 51 | 47 |
| **129-132** |  |  | 6 | 0 | 27 | 43 |
| **133-136** |  |  | 5 | 0 | 23 | 18 |

| | | | | | | |
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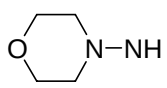
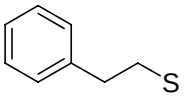
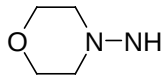
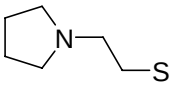
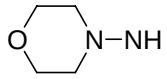
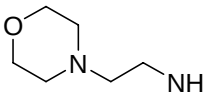
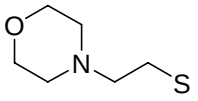
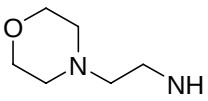
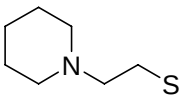
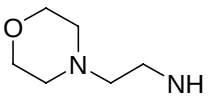
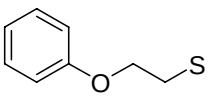
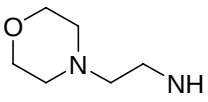
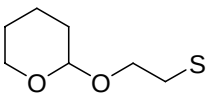
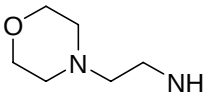
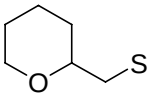
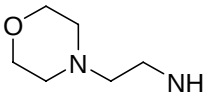
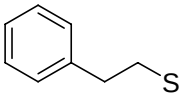
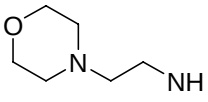
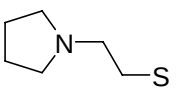
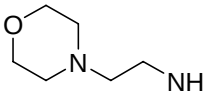
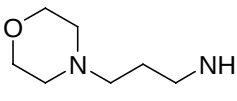
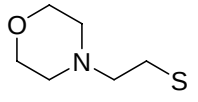
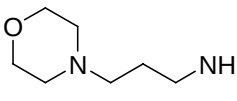
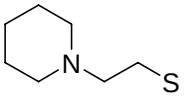
| **137-140** |  |  | 0 | 6 | 25 | 32 |
| **141-144** |  |  | 28 | 32 | 70 | 85 |
| **145-148** |  |  | 0 | 31 | 43 | 64 |
| **149-152** |  |  | 2 | 0 | 0 | 46 |
| **153-156** |  |  | 5 | 17 | 0 | 59 |
| **157-160** |  |  | 0 | 4 | 0 | 57 |
| **161-164** |  |  | 40 | 4 | 0 | 49 |
| **165-168** |  |  | 15 | 31 | 0 | 99 |
| **169-172** |  |  | 12 | 22 | 0 | 66 |
| **173-176** |  |  | 1 | 3 | 0 | 63 |
| **177-180** |  |  | 15 | 18 | 8 | 57 |
| **181-184** |  |  | 0 | 6 | 0 | 31 |
| **185-188** |  |  | 8 | 16 | 0 | 39 |

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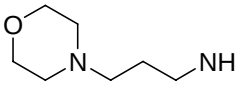
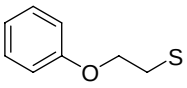
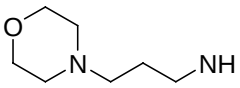
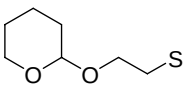
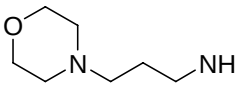
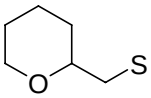
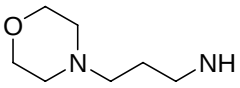
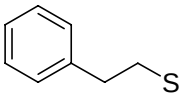
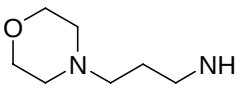
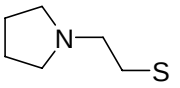
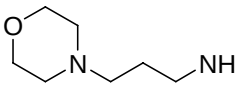
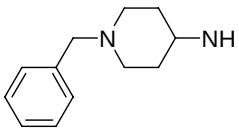
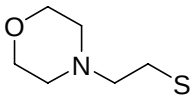
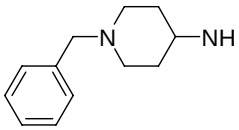
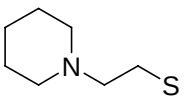
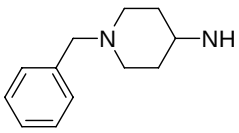
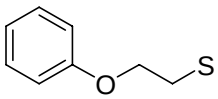
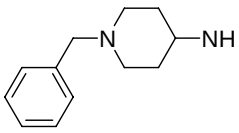
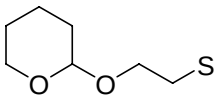
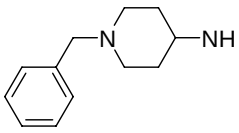
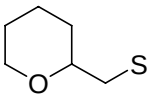
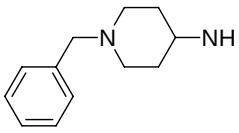
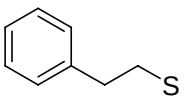
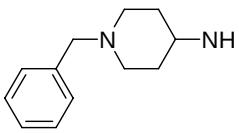
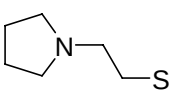
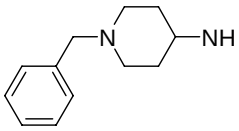
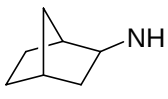
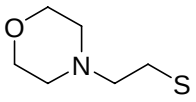
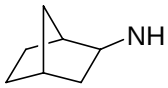
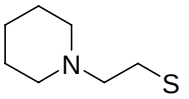
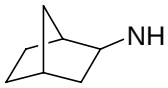
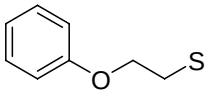
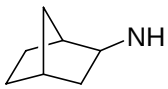
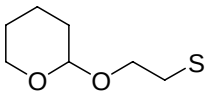
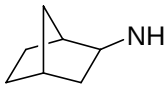
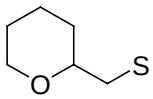
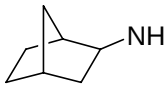
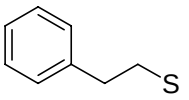
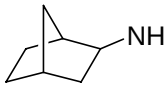
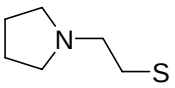
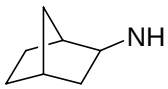
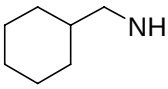
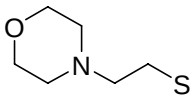
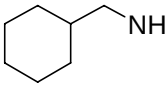
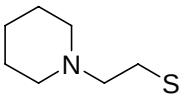
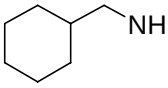
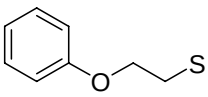
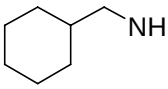
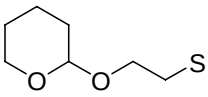
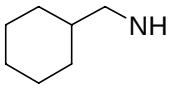
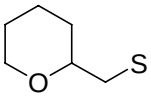
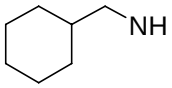
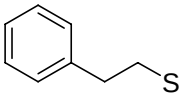
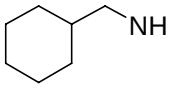
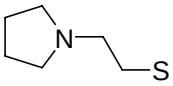
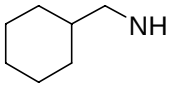
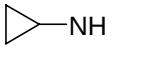
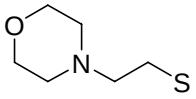
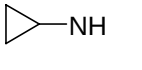
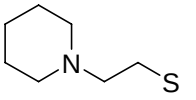
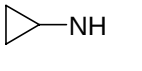
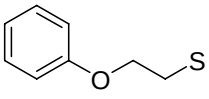
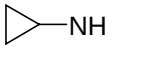
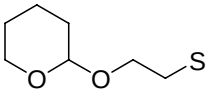
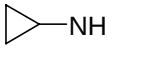
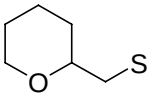
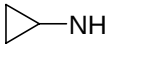
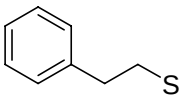
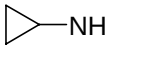
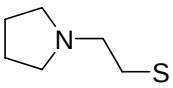
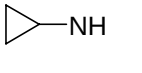
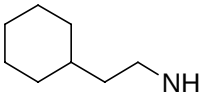
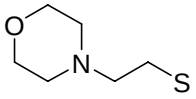
| **189-192** |  |  | 35 | 56 | 0 | 98 |
| **193-196** |  |  | 18 | 34 | 0 | 82 |
| **197-200** |  |  | 22 | 24 | 0 | 64 |
| **201-204** |  |  | 19 | 31 | 6 | 69 |
| **205-208** |  |  | 16 | 18 | 1 | 60 |
| **209-212** |  |  | 25 | 26 | 16 | 61 |
| **213-216** |  |  | 35 | 54 | 3 | 99 |
| **217-220** |  |  | 22 | 31 | 15 | 95 |
| **221-224** |  |  | 23 | 30 | 20 | 76 |
| **225-228** |  |  | 9 | 38 | 35 | 61 |
| **229-232** |  |  | 17 | 9 | 38 | 64 |
| **233-236** |  |  | 20 | 25 | 23 | 57 |
| **237-240** |  |  | 57 | 100 | 14 | 63 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **241-244** |  | —S | 39 | 88 | 1 | 0 |
| **245-248** |  |  | 28 | 71 | 25 | 30 |
| **249-252** |  |  | 8 | 29 | 54 | 0 |
| **253-256** |  |  | 1 | 0 | 25 | 0 |
| **257-260** |  |  | 13 | 64 | 16 | 0 |
| **261-264** |  |  | 25 | 100 | 25 | 55 |
| **265-268** |  |  | 27 | 64 | 29 | 27 |
| **269-272** |  |  | 22 | 42 | 0 | 9 |
| **273-276** |  | —S | 8 | 0 | 0 | 0 |
| **277-280** |  |  | 12 | 36 | 29 | 0 |
| **281-284** |  |  | 18 | 13 | 10 | 53 |
| **285-288** |  |  | 5 | 3 | 0 | 35 |
| **289-292** |  |  | 0 | 38 | 1 | 24 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **293-296** |  |  | 0 | 0 | 0 | 9 |
| **297-300** |  |  | 0 | 15 | 24 | 19 |
| **301-304** |  |  | 24 | 30 | 7 | 84 |
| **305-308** |  |  | 13 | 0 | 0 | 21 |
| **309-312** |  |  | 10 | 19 | 24 | 9 |
| **313-316** |  |  | 0 | 85 | 11 | 28 |
| **317-320** |  |  | 0 | 54 | 13 | 24 |
| **321-324** |  |  | 0 | 68 | 4 | 46 |
| **325-328** |  |  | 17 | 26 | 0 | 54 |
| **329-332** |  |  | 1 | 0 | 11 | 44 |
| **333-336** |  |  | 0 | 0 | 0 | 20 |
| **337-340** |  |  | 0 | 85 | 0 | 46 |
| **341-344** |  |  | 0 | 48 | 15 | 27 |

| | | | | | | |
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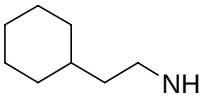
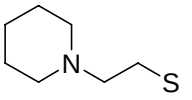
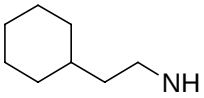
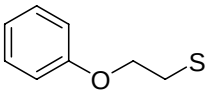
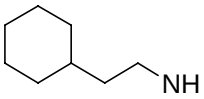
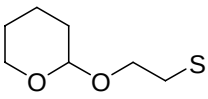
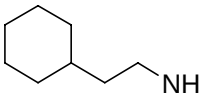
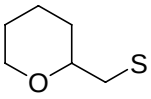
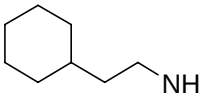
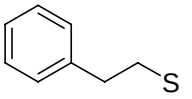
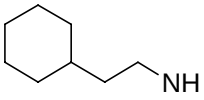
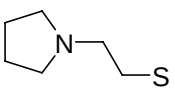
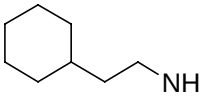
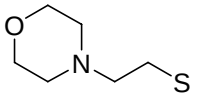
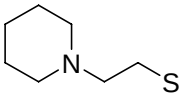
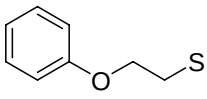
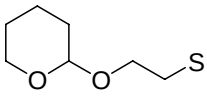
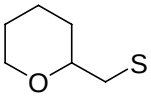
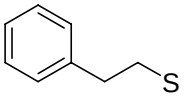
| **345-348** |  |  | 0 | 9 | 13 | 57 |
| **349-352** |  |  | 14 | 15 | 0 | 63 |
| **353-356** |  |  | 0 | 0 | 8 | 23 |
| **357-360** |  |  | 0 | 100 | 17 | 1 |
| **361-364** |  |  | 0 | 60 | 6 | 51 |
| **365-368** |  |  | 0 | 0 | 16 | 8 |
| **369-372** |  |  | 0 | 28 | 15 | 16 |
| **373-376** |  |  | 36 | 0 | 8 | 46 |
| **377-380** |  |  | 11 | 0 | 24 | 55 |
| **381-384** |  |  | 0 | 97 | 7 | 33 |
| **385-388** |  |  | 12 | 34 | 8 | 25 |
| **389-392** |  |  | 2 | 0 | 45 | 19 |
| **393-396** |  |  | 0 | 17 | 33 | 19 |

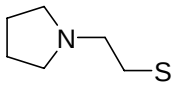
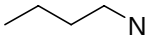
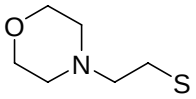
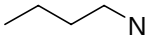
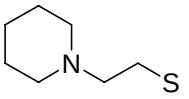
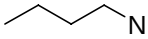
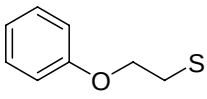
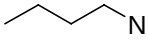
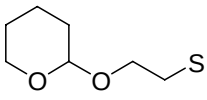
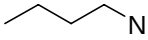
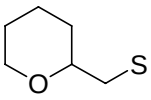
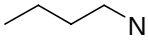
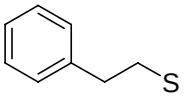
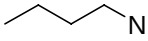
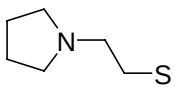
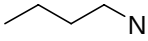
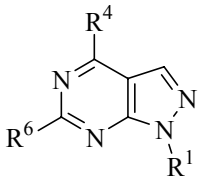
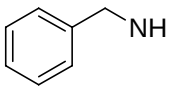
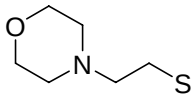
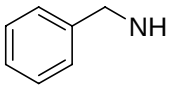
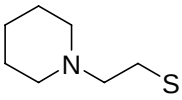
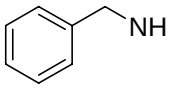
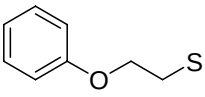
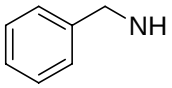
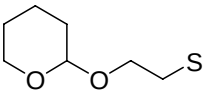
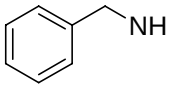
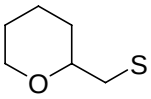
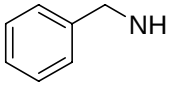
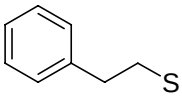
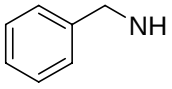
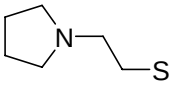
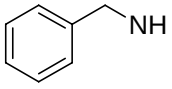
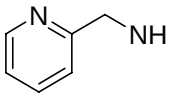
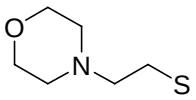
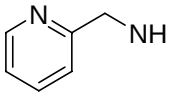
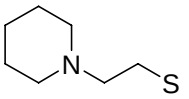
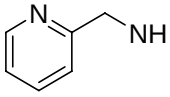
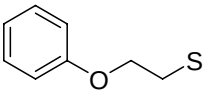
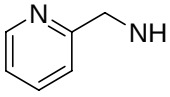
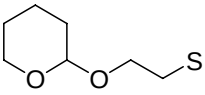
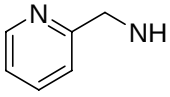
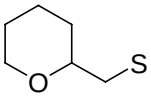
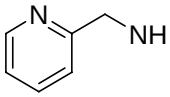
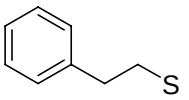
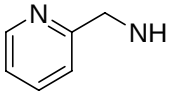
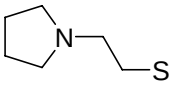
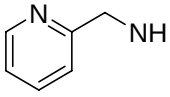
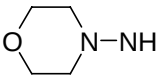
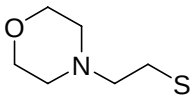
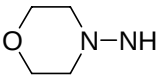
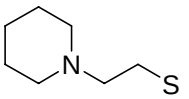
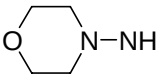
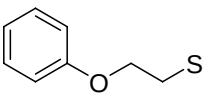
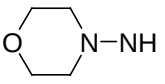
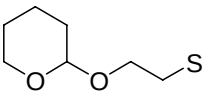
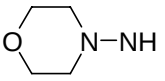
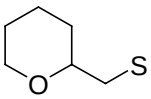
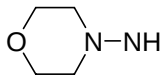
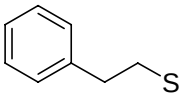
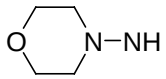
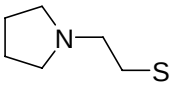
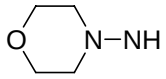
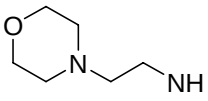
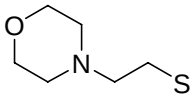
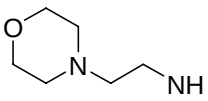
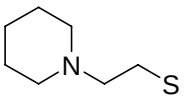
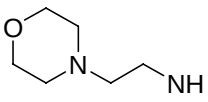
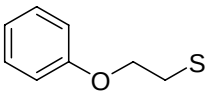
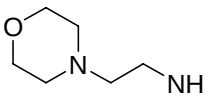
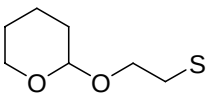
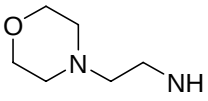
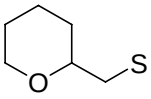
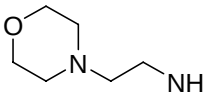
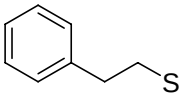
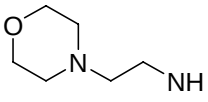
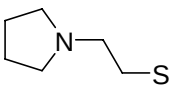
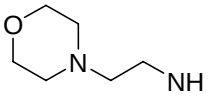
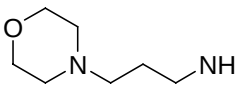
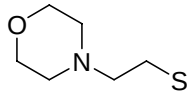
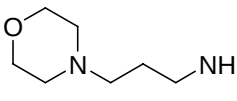
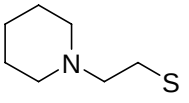
| | | | | | | |
|---|---|---|---|---|---|---|
| **397-400** | —NH |  | 41 | 13 | 0 | 79 |
| **401-404** | —NH | —S | 37 | 0 | 32 | 53 |
| **405-408** |  |  | 20 | 100 | 7 | 25 |
| **409-412** |  |  | 30 | 68 | 32 | 38 |
| **413-416** |  |  | 15 | 14 | 46 | 42 |
| **417-420** |  |  | 12 | 45 | 28 | 29 |
| **421-424** |  |  | 0 | 26 | 23 | 62 |
| **425-428** |  |  | 0 | 1 | 17 | 73 |
| **429-432** |  |  | 0 | 100 | 13 | 52 |
| **433-436** |  | —S | 0 | 66 | 37 | 66 |

**Table 5.3**: Binding affinity of the crude pyrazolo[3,4-*d*]pyrimidines expressed as % displacement at 1.0 μM. Displacement of specific [$^3$H]NECA binding from CHO cells transfected with $A_3$ human adenosine receptor. The crude compounds with the corrected masses were not shaded.



| Comp | Substituents | | %Activity (1.0 μM) | | | |
|---|---|---|---|---|---|---|
| | | | $R^1$ | | | |
| | $R^4$ | $R^6$ | 3-I Bn | 3-H Bn | 3-Br Bn | 3-$NO_2$ Bn |
| **53-56** |  |  | 47 | 0 | 38 | 50 |
| **57-60** |  |  | 44 | 0 | 40 | 39 |
| **61-64** |  |  | 38 | 0 | 33 | 58 |
| **65-68** |  |  | 31 | 0 | 16 | 29 |
| **69-72** |  |  | 38 | 7 | 3 | 41 |
| **73-76** |  |  | 34 | 0 | 8 | 26 |
| **77-80** |  |  | 48 | 0 | 27 | 12 |
| **81-84** |  | —S | 46 | 0 | 6 | 5 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **85-88** |  |  | 37 | 0 | 0 | 15 |
| **89-92** |  |  | 32 | 0 | 24 | 1 |
| **93-96** |  |  | 43 | 0 | 0 | 0 |
| **97-100** |  |  | 55 | 0 | 19 | 55 |
| **101-104** |  |  | 44 | 0 | 5 | 51 |
| **105-108** |  |  | 51 | 0 | 12 | 57 |
| **109-112** |  |  | 44 | 1 | 5 | 48 |
| **113-116** |  |  | 50 | 2 | 19 | 47 |
| **117-120** |  |  | 34 | 0 | 28 | 30 |
| **121-124** |  |  | 40 | 4 | 29 | 0 |
| **125-128** |  |  | 34 | 0 | 34 | 0 |
| **129-132** |  |  | 36 | 0 | 22 | 20 |
| **133-136** |  |  | 34 | 0 | 19 | 4 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **137-140** |  |  | 33 | 0 | 9 | 16 |
| **141-144** |  |  | 52 | 15 | 19 | 36 |
| **145-148** |  |  | 45 | 6 | 20 | 47 |
| **149-152** |  |  | 47 | 14 | 0 | 54 |
| **153-156** |  |  | 45 | 2 | 21 | 45 |
| **157-160** |  |  | 43 | 15 | 0 | 1 |
| **161-164** |  |  | 37 | 17 | 0 | 19 |
| **165-168** |  |  | 33 | 20 | 11 | 17 |
| **169-172** |  |  | 35 | 12 | 0 | 24 |
| **173-176** |  |  | 39 | 0 | 23 | 10 |
| **177-180** |  |  | 41 | 27 | 14 | 16 |
| **181-184** |  |  | 43 | 28 | 4 | 13 |
| **185-188** |  |  | 20 | 28 | 3 | 0 |

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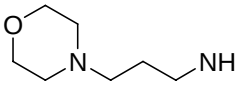
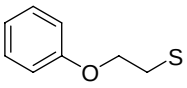
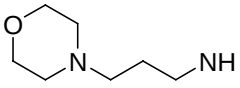
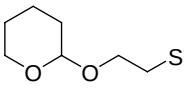
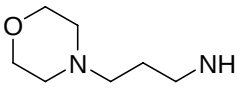
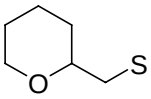
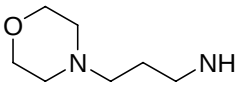
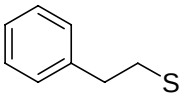
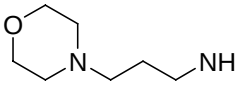
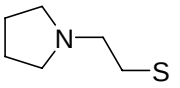
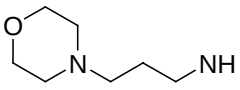
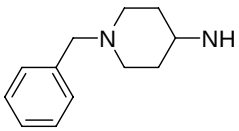
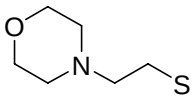
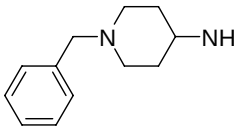
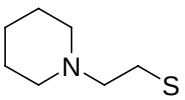
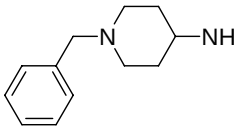
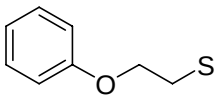
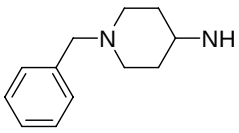
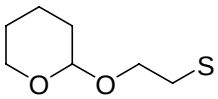
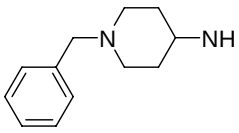
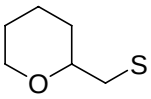
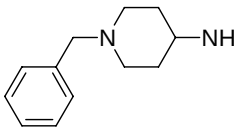
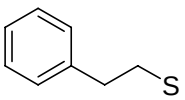
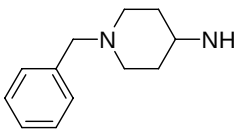
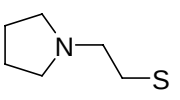
| **189-192** |  |  | 47 | 0 | 5 | 71 |
| **193-196** |  |  | 48 | 0 | 6 | 56 |
| **197-200** |  |  | 54 | 14 | 13 | 38 |
| **201-204** |  |  | 47 | 29 | 6 | 45 |
| **205-208** |  |  | 51 | 28 | 16 | 20 |
| **209-212** |  |  | 45 | 39 | 8 | 4 |
| **213-216** |  |  | 43 | 25 | 1 | 23 |
| **217-220** |  |  | 40 | 27 | 0 | 8 |
| **221-224** |  |  | 44 | 23 | 0 | 23 |
| **225-228** |  |  | 45 | 20 | 7 | 19 |
| **229-232** |  |  | 48 | 26 | 23 | 21 |
| **233-236** |  |  | 37 | 32 | 2 | 9 |
| **237-240** |  |  | 30 | 8 | 26 | 0 |

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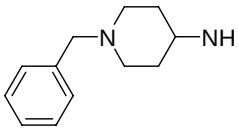
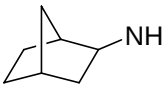
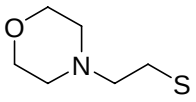
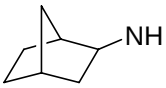
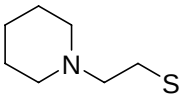
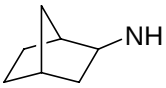
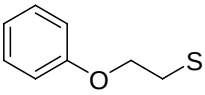
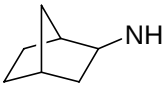
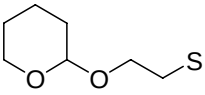
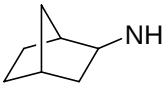
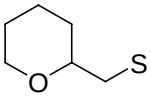
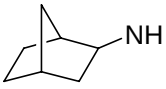
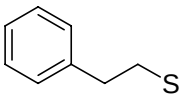
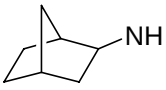
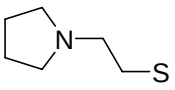
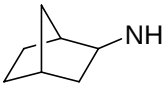
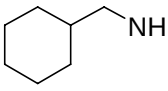
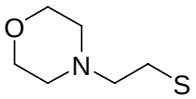
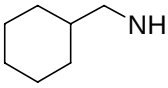
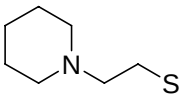
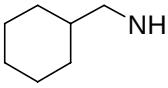
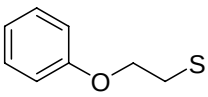
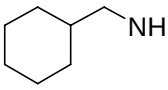
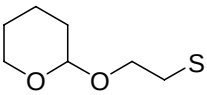
| **241-244** |  |  | 14 | 8 | 4 | 0 |
| **245-248** |  |  | 30 | 15 | 15 | 23 |
| **249-252** |  |  | 39 | 18 | 15 | 11 |
| **253-256** |  |  | 35 | 3 | 0 | 11 |
| **257-260** |  |  | 38 | 0 | 0 | 6 |
| **261-264** |  |  | 40 | 2 | 0 | 18 |
| **265-268** |  |  | 27 | 0 | 0 | 19 |
| **269-272** |  |  | 36 | 0 | 0 | 23 |
| **273-276** |  |  | 30 | 0 | 1 | 31 |
| **277-280** |  |  | 38 | 29 | 9 | 8 |
| **281-284** |  |  | 27 | 29 | 0 | 6 |
| **285-288** |  |  | 14 | 20 | 0 | 0 |
| **289-292** |  |  | 16 | 18 | 0 | 7 |

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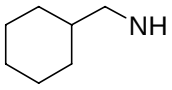
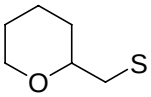
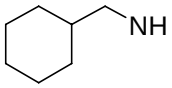
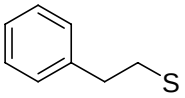
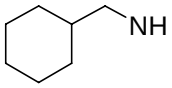
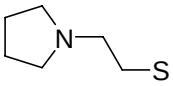
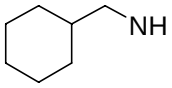
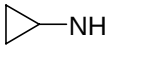
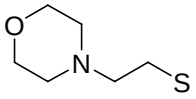
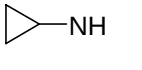
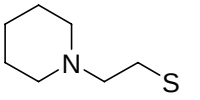
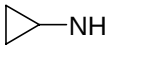
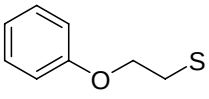
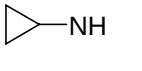
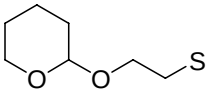
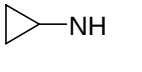
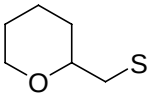
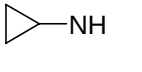
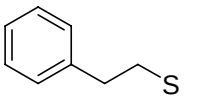
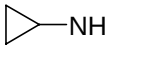
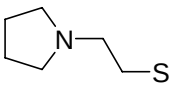
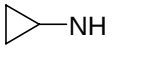
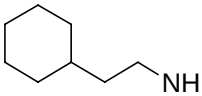
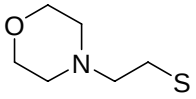
| **293-296** |  |  | 11 | 15 | 0 | 6 |
| **297-300** |  |  | 16 | 19 | 0 | 13 |
| **301-304** |  |  | 9 | 40 | 0 | 0 |
| **305-308** |  |  | 19 | 27 | 0 | 1 |
| **309-312** |  |  | 6 | 0 | 0 | 8 |
| **313-316** |  |  | 36 | 29 | 23 | 27 |
| **317-320** |  |  | 25 | 25 | 23 | 18 |
| **321-324** |  |  | 8 | 31 | 0 | 20 |
| **325-328** |  |  | 39 | 30 | 0 | 0 |
| **329-332** |  |  | 42 | 0 | 9 | 0 |
| **333-336** |  |  | 41 | 8 | 19 | 26 |
| **337-340** |  |  | 44 | 11 | 28 | 17 |
| **341-344** |  |  | 21 | 0 | 0 | 23 |

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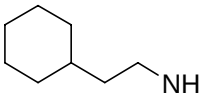
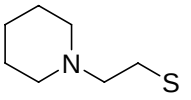
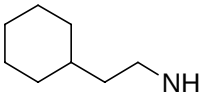
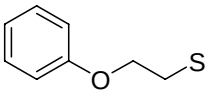
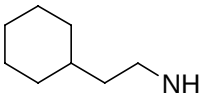
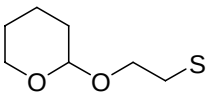
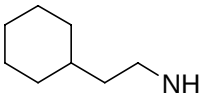
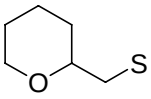
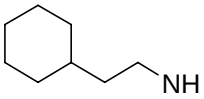
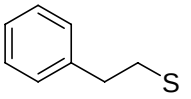
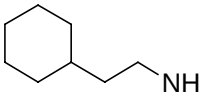
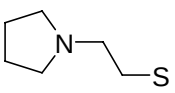
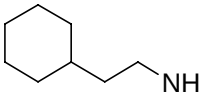
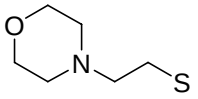
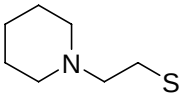
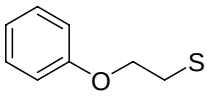
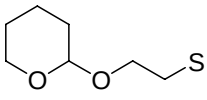
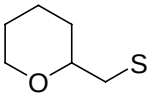
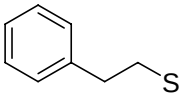
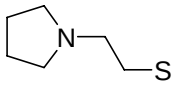
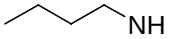
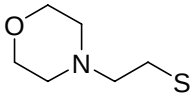
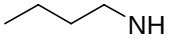
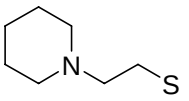
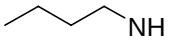
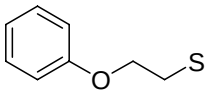
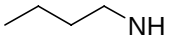
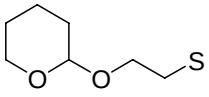
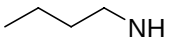
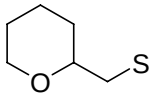
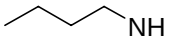
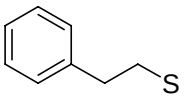
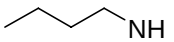
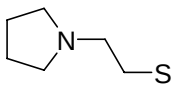
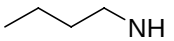
| **345-348** |  |  | 34 | 0 | 0 | 24 |
| **349-352** |  |  | 37 | 0 | 0 | 25 |
| **353-356** |  |  | 29 | 0 | 0 | 30 |
| **357-360** |  |  | 25 | 42 | 2 | 19 |
| **361-364** |  |  | 27 | 36 | 9 | 20 |
| **365-368** |  |  | 19 | 45 | 0 | 20 |
| **369-372** |  |  | 17 | 37 | 0 | 30 |
| **373-376** |  |  | 34 | 19 | 1 | 7 |
| **377-380** |  |  | 33 | 15 | 6 | 0 |
| **381-384** |  |  | 56 | 21 | 11 | 17 |
| **385-388** |  |  | 40 | 28 | 16 | 21 |
| **389-392** |  |  | 36 | 3 | 4 | 25 |
| **393-396** |  |  | 36 | 0 | 0 | 33 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **397-400** | —NH |  | 59 | 21 | 3 | 35 |
| **401-404** | —NH | —S | 54 | 5 | 0 | 30 |
| **405-408** |  |  | 56 | 28 | 0 | 31 |
| **409-412** |  |  | 46 | 48 | 16 | 29 |
| **413-416** |  |  | 50 | 34 | 17 | 39 |
| **417-420** |  |  | 61 | 33 | 0 | 35 |
| **421-424** |  |  | 25 | 6 | 4 | 0 |
| **425-428** |  |  | 10 | 9 | 0 | 0 |
| **429-432** |  |  | 32 | 1 | 15 | 3 |
| **433-436** |  | —S | 13 | 0 | 14 | 23 |

Benzyl substituted pyrazolo[3,4-*d*]pyrimidines with the same substituents at C-4 and C-6 were expected to be more potent than their corresponding 3-nitrobenzyl substituted pyrazolo[3,4-*d*]pyrimidines due to their smaller size. Similarly, 3-nitrobenzyl substituted pyrazolo[3,4-*d*]pyrimidines were expected to be more potent than their corresponding 3-bromobenzyl substituted pyrazolo[3,4-*d*]pyrimidines due

to the size of the bromo substituent in comparison to the nitro substituent. Based on the crude mixtures with the correct molecular weights in **Tables 5.1**, the binding data obtained showed that 3-bromobenzyl substituted pyrazolo[3,4-*d*]pyrimidines and 3-nitrobenzyl substituted pyrazolo[3,4-*d*]pyrimidines were less potent than both benzyl substituted pyrazolo[3,4-*d*]pyrimidines and 3-iodobenzyl substituted pyrazolo[3,4-*d*]pyrimidines **(**for examples **57-60 and 77-80)** at the $A_1$ receptor. The results also showed that 3-bromobenzyl substituted pyrazolo[3,4-*d*]pyrimidines were less potent than 3-iodobenzyl substituted pyrazolo[3,4-*d*]pyrimidines and less than 30% of 3-nitrobenzyl substituted pyrazolo[3,4-*d*]pyrimidines were successfully synthesized. For these reasons, only binding data of benzyl substituted pyrazolo[3,4-*d*]pyrimidines and 3-iodobenzyl substituted pyrazolo[3,4-*d*]pyrimidines were compared.

Benzyl substituted pyrazolo[3,4-*d*]pyrimidines were more potent at the $A_1$ receptor than 3-iodobenzyl substituted pyrazolo[3,4-*d*]pyrimidines when benzylamine was at the C-4 position **(53-84)**, the only exception being when a small substitutent was at the C-6 position.

When benzylamine at C-4 was replaced with 2-(aminomethyl)pyridine, the nitrogen on the phenyl ring may influence activity at the $A_1$ receptor. When benzylamine was at C-4, benzyl substituted pyrazolo[3,4-*d*]pyrimidines **(70, 78)** were more potent than their corresponding 3-iodobenzyl substituted pyrazolo[3,4-*d*]pyrimidines **(69, 77)** and benzyl substituted pyrazolo[3,4-*d*]pyrimidine **(82)** was less potent than 3-iodobenzyl substituted pyrazolo[3,4-*d*]pyrimidine **(81)**. However, when 2-(aminomethyl)pyridine was at C-4, 3-iodobenzyl substituted pyrazolo[3,4-*d*]pyrimidines **(101 and 109)** were more potent than benzyl substituted pyrazolo[3,4-*d*]pyrimidines **(102 and 110)** and 3-iodobenzyl substituted pyrazolo[3,4-*d*]pyrimidine **(114)** was more potent than benzyl substituted pyrazolo[3,4-*d*]pyrimidine **(113)**.

When the amine at C-4 had two or more carbon spacers, 3-iodobenzyl substituted pyrazolo[3,4-*d*]pyrimidines **(149, 153, 165, 169, 173, 177, 181, 185, 189, 193, 197, 341, 345, 353, 357, 361, 365 and 369)** were generally more potent at the $A_1$ receptor than benzyl substituted pyrazolo[3,4-*d*]pyrimidines **(150, 154, 166, 170, 174, 178, 182, 186, 190, 194, 198, 342, 346, 354, 358, 362, 366 and 370)**. Several benzyl substituted pyrazolo[3,4-*d*]pyrimidines **(158, 162, 202, 206, 210 and 350)** were more

potent at the $A_1$ receptor than 3-iodobenzyl substituted pyrazolo[3,4-*d*]pyrimidines **(157, 161, 201, 205, 209 and 349)**.

When the amine at C-4 didn't have a carbon spacer, benzyl substituted pyrazolo[3,4-*d*]pyrimidines **(246, 250, 270, 274, 310, 314, 334 and 338)** were more potent than 3-iodobenzyl substituted pyrazolo[3,4-*d*]pyrimidines **(245, 249, 269, 273, 309, 313, 333 and 338)**. When the amine at C-4 was a straight chain with one or three carbon side chain, benzyl substituted pyrazolo[3,4-*d*]pyrimidines **(382, 386, 390, 414, 418, 422, 425 and 430)** were generally less potent than 3-iodobenzyl substituted pyrazolo[3,4-*d*]pyrimidines **(381, 385, 389, 413, 417, 421, 424 and 429)**.

The binding data obtained showed that the percentage activity of these crude trisubstituted pyrazolo[3,4-*d*]pyrimidines were generally low at 1.0 micromolar concentration at human $A_{2A}$ adenosine receptor **(Tables 5.2)** . Also, the goal of the project was to replace the hydrophobic phenyl substituent at N-1 of pyrazolo[3,4-*d*]pyrimidines, $A_1$ antagonist, with benzyl substituents to generate antagonist for $A_3$ receptor. Several crude benzyl substituted pyrazolo[3,4-*d*]pyrimidines with high potency at $A_{2A}$ were less active at the $A_1$ or $A_3$ receptors. Therefore, the binding data for these crude trisubstituted pyrazolo[3,4-*d*]pyrimidines at $A_{2A}$ receptor were not considered.

Benzyl substituted pyrazolo[3,4-*d*]pyrimidines with the same substituents at C-4 and C-6 were expected to be less potent than their corresponding 3-nitrobenzyl substituted pyrazolo[3,4-*d*]pyrimidines due to the size. Similarly, 3-nitrobenzyl substituted pyrazolo[3,4-*d*]pyrimidines were expected to be less potent than the bigger 3-bromobenzyl substituted pyrazolo[3,4-*d*]pyrimidines at the $A_3$ receptor. However, the binding data obtained showed benzyl substituted pyrazolo[3,4-*d*]pyrimidines to be more potent than both 3-nitrobenzyl substituted pyrazolo[3,4-*d*]pyrimidines and 3-bromobenzyl substituted pyrazolo[3,4-*d*]pyrimidines **(**for example **205-208 and 377-380 in Table 5.3)**. Therefore, only binding data of benzyl substituted pyrazolo[3,4-*d*]pyrimidines and 3-iodobenzyl substituted pyrazolo[3,4-*d*]pyrimidines were compared.

Based on the crude mixtures with the correct molecular weights in **Tables 5.3**, 3-iodobenzyl substituted pyrazolo[3,4-*d*]pyrimidines had greater affinity for the $A_3$ receptor than benzyl substituted pyrazolo[3,4-*d*]pyrimidines. However, when the amine at C-4 was cyclohexane methylamine, benzyl substituted pyrazolo[3,4-*d*]pyrimidines **(282, 286, 294, 298 and 302)** were more potent than the corresponding 3-iodobenzyl substituted pyrazolo[3,4-*d*]pyrimidines **(281, 285, 293, 297 and 301)**.

Based on the crude mixtures that were tested at the same concentrations, these compounds had a higher potency at the $A_1$ receptor than at the $A_3$ receptor. For example compare the binding affinities of compounds **165, 166, 273, 274, 333, 334, 417 and 418** at the $A_1$ receptor in **Table 5.1** with the binding affinities of those compounds at the $A_3$ receptor in **Table 5.3**.

## 5.3 Conclusion

A library of 384 compounds has been synthesized and each of these crude compounds were tested at the human $A_1$, $A_{2A}$ and $A_3$ adenosine receptors at an assumed concentration of 1.0 μM. In general, 3-iodobenzyl substituted pyrazolo[3,4-*d]*pyrimidine derivatives were more potent at the $A_3$ receptor than benzyl substituted pyrazolo[3,4-*d*]pyrimidine derivatives except when the amine at C-4 was cyclohexane methylamine and all compounds had a higher potency at the $A_1$ receptor than at the $A_3$ receptor. Some of these compounds were re-synthesized, purified and tested at the $A_1$ and $A_3$ receptors.

## 5.4 Experimental

Melting points were recorded on a Gallenkamp digital melting point apparatus and are uncorrected. Infra-red absorption spectra were obtained on a Perkin Elmer FT-IR spectrophotometer using sodium chloride plates. $^{1}H$ and $^{13}C$ nuclear magnetic resonance (n.m.r) spectra were recorded on a Varian Gemini-200 (200 MHz) spectrometer, Varian Unity-400 (400 MHz) spectrometer or Varian Unity Plus-600

(600 MHz) spectrometer. All samples were dissolved in deuteriochloroform ($CDCl_3$) containing tetramethylsilane (TMS) as an internal reference unless otherwise stated. The signals are recorded in terms of chemical shift in parts per million (ppm) downfield from TMS ($\delta = 0$) for protons or $CDCl_3$ ($\delta = 77$) for carbon atoms. The signals are recorded in terms of chemical shift ($\delta_H$), relative integral, multiplicity, coupling constants (*J* Hz) and assignments, in that order. The following abbreviations for multiplicity are used: s = singlet, dd = doublet of doublets, dt = doublet of triplets, m = multiplet. $^1H$ and $^{13}C$ spectra were assigned with the aid of HMQC (Heteronuclear Multiple-Quantum Coherence), HMBC (Heteronuclear Multiple-Bond Coherence) and $^1H$-$^1H$ COSY (Correlation Spectroscopy). Electrospray mass spectra (ESMS) were recorded on a Fisons VG Platform mass spectrometer with MassLynx Data System software.

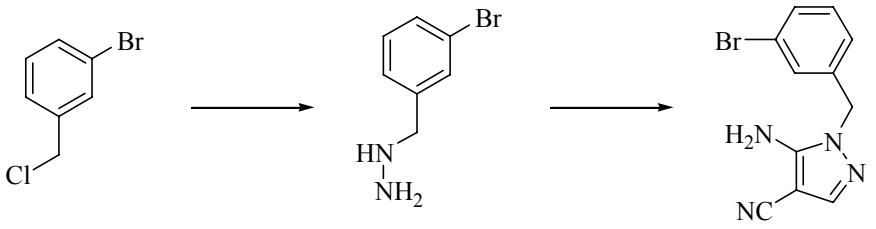
Microanalytical data was obtained from University of Queensland Microanalytical Service.

Analytical thin layer chromatography (TLC) was performed using precoated (0.2 mm) Merck silica gel plates (Merck Kieselgel 60 $F_{254}$). Flash chromatography was carried out using Merck Kieselgel 60 (230-400 mesh) with the indicated solvents.

All solvents for chromatography were distilled before use, unless otherwise stated. Ether refers to diethyl ether and hexane refers to the fraction of b.p. 60-80 °C. Mixed solvent compositions are quoted as v/v.

Solvents and reagents were purified according to the standard techniques of Perrin, Perrin and Amarego.[6]

**1-(3-bromobenzyl)-5-amino-4-cyanopyrazole (27)**




**26** **27**

**Method A**: To a refluxing solution of hydrazine monohydrate (46.44 g, 45.0 ml, 0.93 mol) in ethanol (150 ml), a solution of 3-bromobenzyl chloride (20.26 g, 12.6 ml, 0.099 mol) in ethanol (50 ml) was added dropwise over a period of 1 h. The reaction mixture was refluxed for an additional 3 h before the solvent was removed *in vacuo*. The remaining pale yellow liquid was extracted with ether (3 x 50 ml). The combined organic layers were dried ($MgSO_4$) and concentrated *in vacuo* to yield crude oil. The crude oil was purified by distillation under reduced pressure through a Vigreux column to yield 3-bromobenzylhydrazine **(26)** (16.50 g, 83%) as a clear oil, bp 99-100 °C, at 0.6 mm Hg. The 3-bromobenzylhydrazine **(26)** was unstable and hence it was not fully characterised; $\delta_H$ (400 MHz, DMSO-$d_6$) 3.46 (br, 3H, NH-$NH_2$), 3.73 (s, 2H, $CH_2$), 7.27 (t, $J$ = 7.6 Hz, 1H, 5'-H), 7.31 (dt, $J_{ortho}$ = 7.6 Hz, $J_{meta}$ = 1.2 Hz, 1H, 6'-H), 7.42 (dt, $J_{ortho}$ = 7.6 Hz, $J_{meta}$ = 1.2 Hz, 1H, 4'-H) and 7.52 (s, 1H, 2'-H); ESMS (PI) 201 and 203. calcd for ($C_7H_9{}^{79}BrN_2$ + 1[H]) and ($C_7H_9{}^{81}BrN_2$ + 1[H]) respectively. Found 201 and 203.

**Method B:** To a stirred solution of ethoxymethylenemalononitrile (9.9 g, 0.081 mol) in ethanol (100 ml) under argon, a solution of 3-bromobenzylhydrazine **(26)** (16.30 g, 0.081 mol) in ethanol (20 ml) was added dropwise. The resultant mixture was refluxed over 2 h and a deep red colour solution was produced. The reaction mixture was left to cool to room temperture and yellow crystalline material precipitated. Precipitation was further enhanced by cooling in the fridge overnight before it was filtered and the solid was washed with cold ethanol. The crude solid was purified by flash chromatography (50% ethyl acetate-hexane) to afford 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27)** (12.34 g, 55%) as white solid, mp 101.5 ± 0.5 °C; $\delta_H$ (400 MHz, DMSO-$d_6$) 5.17 (s, 2H, $CH_2$), 6.78 (s, 2H, $NH_2$), 7.16 (d, $J$ =

8.0 Hz, 1H, 6'-H), 7.30 (t, $J$ = 8.0 Hz, 1H, 5'-H), 7.38 (t, $J_{meta}$ = 1.6 Hz, 1H, 2'-H), 7.49 (d, $J$ = 8.0 Hz, 1H, 4'-H) and 7.61 (s, 1H, 3-H); $\delta_C$ (100 MHz, DMSO-$d_6$) 49.3 ($CH_2$), 72.3 (C-4), 115.1 (CN), 121.7 (C-3'), 126.4 (C-6'), 130.1 (C-2'), 130.4 (C-4'), 130.7 (C-5'), 139.3 (C-1'), 140.8 (C-3) and 151.7 (C-5); $\nu_{max}$ (NaCl plates)/cm$^{-1}$ 3400 ($NH_2$) and 2200 (CN). Anal. calcd for $C_{11}H_9BrN_4$: C, 47.68; H, 3.27; N, 20.22C. Found C, 47.80; H, 3.17; N, 20.30%; ESMS (NI) 275 and 277. calcd for ($C_{11}H_9{}^{79}BrN_4$ - 1[H]) and ($C_{11}H_9{}^{81}BrN_4$ - 1[H]) respectively. Found 275 and 277.

**1-benzyl-5-amino-4-cyanopyrazole (48)**

Method B was used to prepare crude solid from benzylhydrazine. The crude solid was purified by flash chromatography (50% ethyl acetate-hexane) to afford 1-benzyl-5-amino-cyanopyrazole **(48)** (65 % yield) as white solid, mp 181.5 ± 0.5 °C; $\delta_H$ (400 MHz, DMSO-$d_6$) 5.12 (s, 2H, $CH_2$), 6.69 (s, 2H, $NH_2$), 7.10-7.35 (m, 5H, ArH) and 7.55 (s, 1H, 3-H); $\delta_C$ (100 MHz, DMSO-$d_6$)) 50.6 ($CH_2$), 72.9 (C-4), 115.9 (CN), 127.9 (C-2', C-6'), 128.1 (C-4'), 129.1 (C-3', C-5'), 137.4 (C-1'), 141.2 (C-3) and 152.3 (C-5); ESMS (NI) 197. calcd for ($C_{11}H_{10}N_4$ - 1[H]). Found 197.

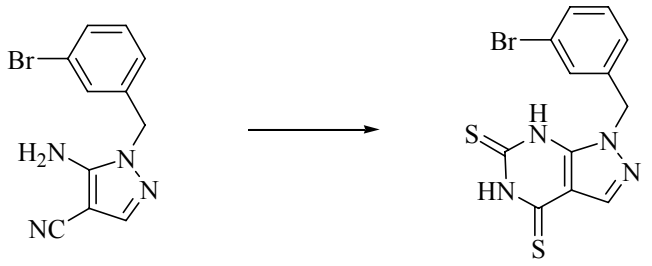
**1-(3-nitrobenzyl)-5-amino-4-cyanopyrazole (49)**

Method A was used to prepare crude 3-nitrobenzyl hydrazine from 3-nitrobenzyl bromide. Method B was used to prepare crude solid from 3-nitrobenzyl hydrazine. The crude solid was purified by flash chromatography (6.5% methanol-DCM) to afford 1-(3-nitrobenzyl)-5-amino-4-cyanopyrazole **(49)** (55% yield over 2 steps) as light yellow solid, mp 152.0 ± 0.5 °C; $\delta_H$ (400 MHz, DMSO-$d_6$) 5.26 (s, 2H, $CH_2$), 6.79 (s, 2H, $NH_2$), 7.54-7.64 (m, 2H, ArH), 7.59 (s, 1H, 3-H), 8.00-8.14 (m, 2H, ArH); $\delta_C$ (100 MHz, DMSO-$d_6$) 49.8 ($CH_2$), 73.0 (C-4), 115.7 (CN), 122.0-148.5 (6 x Ar-C + C-3), and 152.4 (C-5); Anal. calcd for $C_{11}H_9N_5O_2$: C, 54.32; H, 3.73; N, 28.79. Found C, 54.14; H, 3.65; N, 28.41%; ESMS (NI) 242. calcd for ($C_{11}H_9N_5O_2$ - 1[H]). Found 242.

**1-(3-Iodobenzyl)-5-amino-4-cyanopyrazole (50)**

Method A was used to prepare crude 3-iodobenzyl hydrazine from 3-iodobenzyl bromide. Method B was used to prepare crude solid from 3-iodobenzylhydrazine. The crude solid was purified by flash chromatography (45% ethyl acetate-hexane) to afford 1-(3-iodobenzyl)-5-amino-4-cyanopyrazole **(50)** (58% yield over 2 steps) as white solid, mp 108.9 ± 0.5 °C; $\delta_H$ (400 MHz, DMSO-$d_6$) 5.07 (s, 2H, $CH_2$), 6.70 (s, 2H, $NH_2$), 7.08-7.12 (m, 2H, ArH) and 7.49-7.64 (m, 3H, ArH + 3-H); $\delta_C$ (100 MHz, DMSO-$d_6$) 49.8 ($CH_2$), 72.9 (C-4), 94.5 (C-3'), 115.7 (CN), 127.0-142.0 (5 x Ar-C + C-3) and 152.3 (C-5); Anal. calcd for $C_{11}H_9IN_4$: C, 40.76; H, 2.80; N, 17.29. Found C, 40.52; H, 2.59; N, 17.10%; ESMS (NI) 323. calcd for ($C_{11}H_9IN_4$ - 1[H]). Found 323.

**1-(3-bromobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione (28)**




**27** **28**

**Method C**: To a solution of 1-(3-bromobenzyl)-5-amino-4-cyanopyrazole **(27)** (8.32 g, 30.0 mmol) in dry DMF (50 ml) under argon was added potassium-O-ethylxanthogenate (9.62 g, 60.0 mmol). The reaction mixture was heated to 140 °C for 2 h under argon. The initial orange and opaque solution turned to dark brown after 2 h. The reaction mixture was allowed to cool to room temperature and the solvent was removed under reduced pressure to afford crude brown oil. NaOH (2.0 M, 30 ml) was added to the remaining brown oil and stirred at room temperature for 45 min. The basic solution was filtered to give a transparent orange filtrate. HCl (2.0 M) was added dropwise to the filtrate until the neutral pH was reached. A creamy coloured precipitate was formed upon the neutralisation of the filtrate. The crude product was collected by suction filtration and recrystallised from DMSO and water to afford 1-(3-

bromobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(28)** (10.18 g, 96%) as light cream solid, mp > 230 °C; $\delta_H$ (400 MHz, DMSO-$d_6$) 5.26 (s, 2H, $CH_2$), 7.08 (d, $J$ = 8.0 Hz, 1H, 6'-H), 7.22 (t, $J$ = 8.0 Hz, 1H, 5'-H), 7.29 (s, 1H, 2'-H), 7.40 (d, $J$ = 8.0 Hz, 1H, 4'-H), 7.80 (s, 1H, 3-H), 8.18 (br, 1H, NH) and 11.25 (br, 1H, NH); $\delta_C$ (100 MHz, DMSO-$d_6$) 49.2 ($CH_2$), 112.7 (C-3a), 122.4 (C-3'), 126.9 (C-6'), 130.5 (C-2'), 130.9 (C-4'), 131.4 (C-5'), 137.4 (C-3), 140.8 (C-1'), 150.4 (C-7a), 176.4 (C-4) and 179.0 (C-6); ESMS (NI) 351 and 353. calcd for ($C_{12}H_9{}^{79}BrN_4S_2$ - 1[H]) and ($C_{12}H_9{}^{81}BrN_4S_2$ - 1[H]) respectively. Found 351 and 353.

**1-benzyl-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione (26)**

Method C was used to prepare 1-benzyl-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(26)** from 1-benzyl-5-amino-cyanopyrazole **(48)**. Yield (90%) as light cream solid, mp > 230 °C; $\delta_H$ (400 MHz, DMSO-$d_6$) 5.46 (s, 2H, $CH_2$), 6.60-7.60 (m, 6H, ArH + NH), 7.99 (s, 1H, 3-H) and 13.23 (br, 1H, NH); ESMS (NI) 273. calcd for ($C_{12}H_{10}N_4S_2$ - 1[H]). Found 273.

**1-(3-nitrobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione (51)**

Method C was used to prepare 1-(3-nitrobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(51)** from 1-(3-nitrobenzyl)-5-amino-cyanopyrazole **(49)**. Yield (92%) as light yellow solid, mp > 230 °C; $\delta_H$ (400 MHz, DMSO-$d_6$) 5.44 (s, 2H, $CH_2$), 7.50-7.90 (m, 4H, ArH), 7.97 (s, 1H, 3-H), 8.06 (br, 1H, NH) and 11.57 (br, 1H, NH); $\delta_C$ (100 MHz, DMSO-$d_6$) 49.0 ($CH_2$), 112.9 (C-3a), 122.5-148.5 (6 x Ar-C + C-3), 150.8 (C-7a), 174.9 (C-4) and 179.1 (C-6); ESMS (NI) 318. calcd for ($C_{12}H_9N_5O_2S_2$ - 1[H]). Found 318.

**1-(3-iodobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione (52)**

Method C was used to prepare 1-(3-iodobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(52)** from 1-(3-nitrobenzyl)-5-amino-cyanopyrazole **(50)**. Yield (88%) as light cream solid, mp > 230 °C; $\delta_H$ (400 MHz, DMSO-$d_6$) 5.23 (s, 2H, $CH_2$), 7.08-7.64 (m, 4H, ArH), 7.79 (s, 1H, 3-H), 8.16 (br, 1H, NH) and 11.07 (br, 1H, NH); $\delta_C$

(100 MHz, DMSO-$d_6$) 49.1 ($CH_2$), 95.6 (C-3'), 112.7 (C-3a), 127.0-141.0 (5 x Ar-C + C-3), 150.1 (C-7a), 176.1 (C-4) and 179.1 (C-6); ESMS (NI) 399. calcd for ($C_{12}H_9IN_4S_2$ - 1[H]). Found 399.

## 5.5 References


(1) Quinn, R. J.; Dooley, M. J.; Escher, A.; Harden, F. A.; Jayasuriya, H. A computer generated model of adenosine receptors rationalising binding and selectivity of receptor ligands. *Nucleosides & Nucleotides* **1991**, *10*, 1121-1124.

(2) Dooley, M. J.; Quinn, R. J. The three binding domain model of adenosine receptors: molecular modeling aspects. *Journal of Medicinal Chemistry* **1992**, *35*, 211-216.

(3) Poulsen, S.-A.; Quinn, R. J. Pyrazolo[3,4-d]pyrimidines: C4, C6 substitution leads to adenosine A1 receptor selectivity. *Bioorganic & Medicinal Chemistry Letters* **1996**, *6*, 357-360.

(4) Chebib, M.; Quinn, R. J. 1-Phenylpyrazolo[3,4-d]pyrimidines as adenosine antagonists: the effects of substituents at C4 and C6. *Bioorganic & Medicinal Chemistry* **1997**, *5*, 311-322.

(5) Poulsen, S.-A.; Quinn, R. J. Synthesis and Structure-Activity Relationship of Pyrazolo[3,4-d]pyrimidines: Potent and Selective Adenosine A1 Receptor Antagonists. *Journal of Medicinal Chemistry* **1996**, *39*, 4156-4161.

(6) Armarego, W. L. F.; Perrin, D. D. *Purification of Laboratory Chemicals, Fourth Edition*, 1997; 512 pp.

# CHAPTER 6:

## Structure-Activity relationships of selected compounds

### 6.1 Introduction

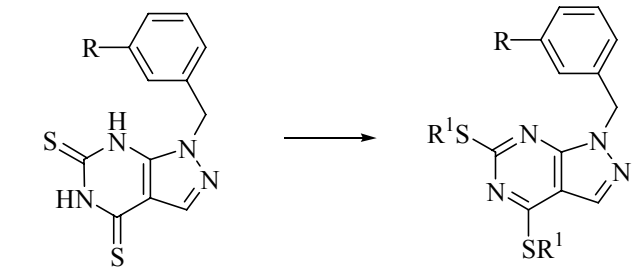
In previous chapter, a library of 256 crude 6-alkylthio-4-alkyl(aryl)amino-pyrazolo[3,4-*d*]pyrimidine derivatives with the correct mass were synthesized and tested at human $A_1$, $A_{2A}$ and $A_3$ adenosine receptors at the same concentration. Based on the result obtained, most of these crude compounds were active at the human $A_1$ adenosine receptor at micromolar concentration. Since each substituent interacted with the binding sites of the receptor, the size and shape of each substituent at N-1, C-4 and C-6 of the pyrazolo[3,4-*d*]pyrimidine would have an effect on the binding affinity. In this study, 14 compounds with good and low biologically active were individually re-synthesized for characterization and structure-activity relationship studies. These compounds had various structure size and shape for each substituent.

### 6.2 Results and Discussion

#### 6.2.1 Synthesis of 4,6-bis-alkylthio-pyrazolo[3,4-*d*]pyrimidine

Ten 4,6-bis-alkylthio-pyrazolo[3,4-*d*]pyrimidine **(437-447)** were synthesized from the corresponding pyrazolo[3,4-*d*]pyrimidines-4,6-dithiones in a mix-solvent of sodium hydroxide (2.0 M) and dioxane (1:1) at room temperature. Bis-methylthio-pyrazolo[3,4-*d*]pyrimidines **(437-439)** were obtained in 1 h. Bis-(1-ethylpyrrolidinethio)-pyrazolo[3,4-*d*]pyrimidine **(440-441)**, bis-(1-ethylpiperidinethio)-pyrazolo[3,4-*d*]pyrimidine **(442-443)**, and bis-(4-(2-ethyl)morpholinethio)-pyrazolo[3,4-*d*]pyrimidine **(444)** were obtained in 4 h at room temperature. Bis-(2-methyltetrahydropyranthio)-pyrazolo[3,4-*d*]pyrimidine **(445-447)**

were prepared by warming the reaction mixture to 50 $^{o}$C and stirred for 24 h since the alkyl side chain contained only one carbon spacer, hence it would hinder the second alkylation at the C-4 position. The general synthetic route for the syntheses of these 10 bis-alkylated pyrazolo[3,4-*d]*pyrimidines is outlined in **Scheme 6.1**.



R = H **(26)**, Br **(28)**
I **(52)**

$R^1$= Methyl;
R = H **(437)**, Br **(438)**, I **(439)**
$R^1$= 1-ethylpyrrolidine;
R = H **(440)**, R = I **(441)**
$R^1$= 1-ethylpiperidine;
R = H **(442)**, Br **(443)**
$R^1$= 4-(2-ethyl)morpholine;
R = H **(444)**
$R^1$= 2-methyltetrahydropyran;
R = H **(445)**, Br **(446)**, I **(447)**

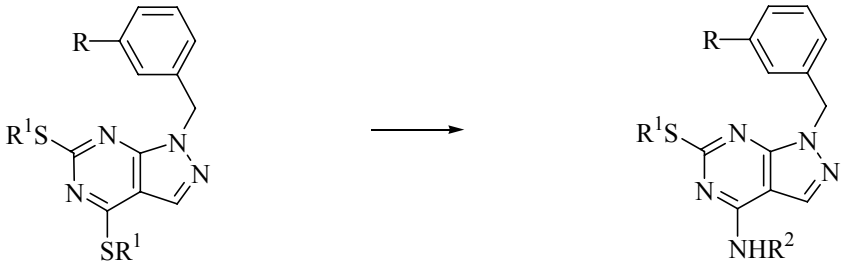
**Scheme 6.1**: *Reagents and conditions:* Alkylhalide, NaOH (2.0 M): Dioxane (1:1), RT-50 $^{o}$C, 1.0-24 h, 55-75%.

The NMR assignments for these compounds are recorded in the experimental section **(Section 6.4)**. The $^{1}$H n.m.r spectra of these bis-alkylated products showed the presence of two $SCH_2$ protons at $\delta_H$ 3.30-3.60 ppm ($\delta_H$ 2.60-2.70 ppm in place of $SCH_3$ of bis-methylthio-pyrazolo[3,4-*d*]pyrimidines **(437-439)**) and their $^{13}$C n.m.r spectra showed the presence of two $SCH_2$ carbons at $\delta_C$ 26.0-30.0 ppm ($\delta_C$ 12.0-15.0 ppm in place of $SCH_3$ of bis-methylthio-pyrazolo[3,4-*d*]pyrimidines **(437-439)**).

### 6.2.2 Synthesis of 6-alkylthio-4-alkylamino-pyrazolo[3,4-*d*]pyrimidine

Since all 4,6-bis-alkylthio-pyrazolo[3,4-*d*]pyrimidines were soluble in dioxane, the target compounds **(165, 166, 273, 274, 310, 315, 333, 334, 421, 422, 423, 433, 434** and **435)** were prepared by refluxing them with the desired nucleophilic amine substituents in dioxane at 100 $^{o}$C. Cyclopropyl amine and butyl amine were added every 1 h and 4 h respectively since they were very volatile and had a low boiling points. Reaction mixtures containing bulky exo-2-aminonorbornane were

refluxed at this temperature for 4 days. The general synthetic route for the syntheses of these target compounds was summarized in **Scheme 6.2**.



$R^1$= Methyl;
R = H **(437)**, Br **(438)**, I **(439)**
$R^1$= 1-ethylpyrrolidine;
R = H **(440)**, R = I **(441)**
$R^1$= 1-ethylpiperidine;
R = H **(442)**, Br **(443)**
$R^1$= 4-(2-ethyl)morpholine;
R = H **(444)**
$R^1$= 2-methyltetrahydropyran;
R = H **(445)**, Br **(446)**, I **(447)**

$R^2$ = 4-(2-ethyl) morpholine;
$R^1$= 2-methyltetrahydropyran;
R = I **(165)**, R = H **(166)**
$R^2$ = Exo-2-norbornane;
$R^1$= Methyl; R = I **(273)**, H **(274)**
$R^2$ = Cyclopropyl;
$R^1$= 4-(2-ethyl)morpholine; R = H **(310)**
$R^2$ = Cyclopropyl;
$R^1$ = 1-ethylpiperidine; R = Br **(315)**
$R^2$ = Cyclopropyl;$R^1$= 1-ethylpyrrolidine;
R = I **(333)**, R **=** H **(334)**
$R^2$ = Butyl; $R^1$= 2-methyltetrahydropyran;
R = I **(421)**, R = H **(422)**, R = Br **(423)**
$R^2$ = Butyl; $R^1$= methyl;
R = I **(433)**, H **(434)**, Br **(435)**

**Scheme 6.2**: *Reagents and conditions:* Nucleophilic amine, dioxane, 100 $^{o}$C, 8-96 h, 45-65%.

The NMR assignments for these target compounds are recorded in the experimental section **(Section 6.4)**. The structures of these products showed the absence of $SCH_2$ protons and carbons at C-4 and its related alkyl side chain (one $SCH_3$ proton and one carbon were lost for 4,6-bis-methylthio compound **(437-439)**) in the $^{1}$H n.m.r and $^{13}$C n.m.r spectrum respectively. The change of the electronic properties at C-4 carbon from sulphur to nitrogen changed the chemical shifts of C-3a, C-4 and C-7a. The C-3a and C-4 in the target compounds, 6-alkylthio-4-alkylamino-pyrazolo[3,4-*d*]pyrimidines, were shifted to a higher field relative to C-3a and C-4 in

the starting materials, 4,6-bis-alkylthio-pyrazolo[3,4-*d*]pyrimidine whereas C-7a was shifted to a lower field.

### 6.2.2 Radio-ligand binding results

14 target compounds and 2 of 4,6-bis-alkylthio-pyrazolo[3,4-*d*]pyrimidines, 1-benzyl-4,6-bis-methylthio-pyrazolo[3,4-*d*]pyrimidine **(437)** and 1-benzyl-4,6-bis-(2-methyltetrahydropyranthio)-pyrazolo[3,4-*d*]pyrimidine **(445)** were tested for binding affinities at the human $A_1$ and $A_3$ adenosine receptors against [$^3$H]DPCPX and [$^3$H]NECA. In the $A_3$ receptor binding assays, all the compounds showed inhibition values less than 40% at 30 μM and were therefore considered inactive. In the $A_1$ receptor binding assays, 5 out of 14 target compounds and two 4,6-bis-alkylthio-pyrazolo[3,4-*d*]pyrimidines showed inhibition values less than 40% at 30 μM and were therefore considered inactive. The $IC_{50}$ of the remaining target compounds were obtained by competitive binding against [$^3$H]DPCPX at the human $A_1$ adenosine receptor **(Figure 6.1, 6.2** and **6.3)**. The corresponding $K_i$ values were then determined using the $K_d$ value of 3.9 for [$^3$H]DPCPX **(See section 7.5)**. The binding results for these compounds are recorded in **Table 6.1**.

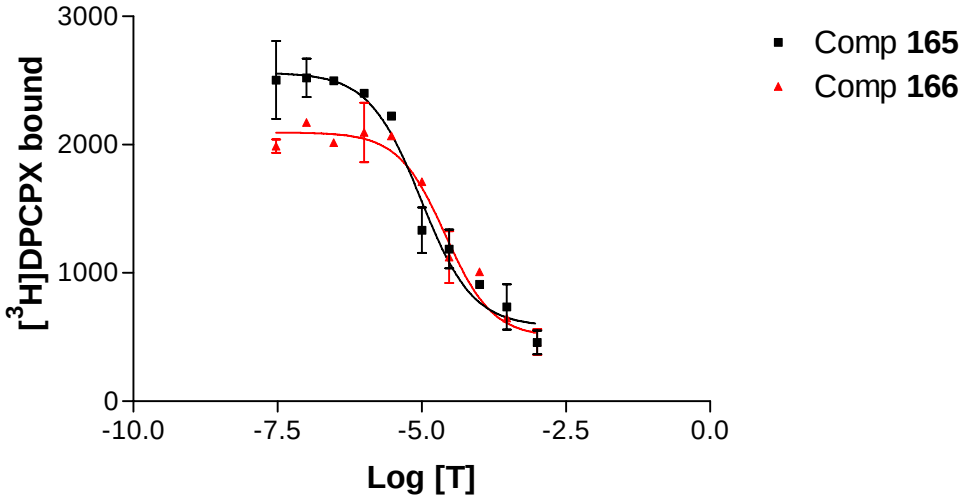



**Figure 6.1**: The curves of competition binding of the target compounds **165** and **166**.

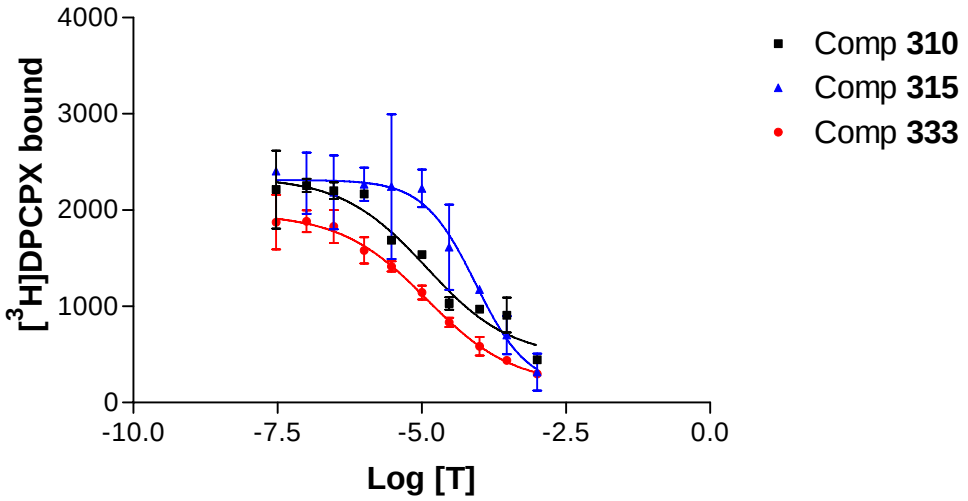



**Figure 6.2**: The curves of competition binding of the target compounds **310, 315** and **333**.

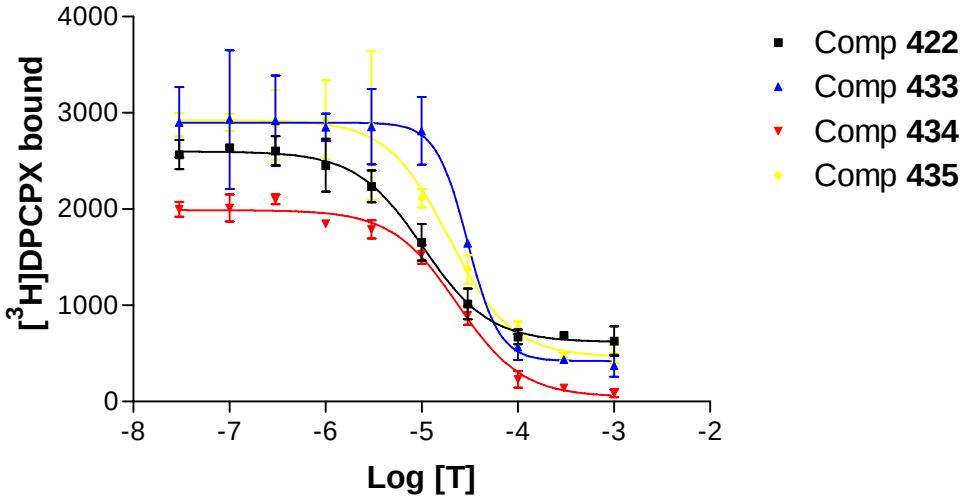
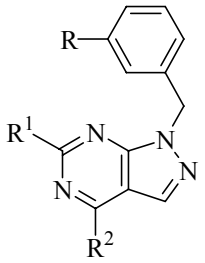
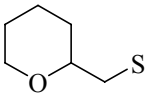
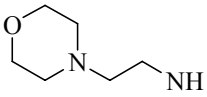
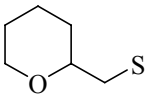
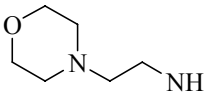
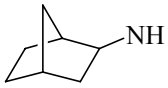
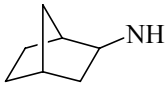
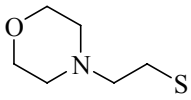
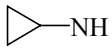
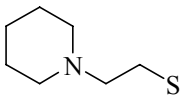
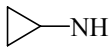
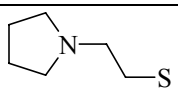
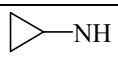
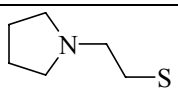
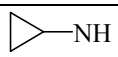
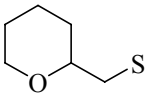
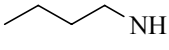
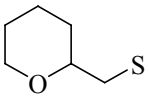
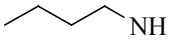
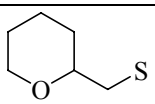
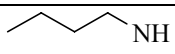
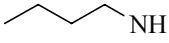


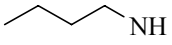
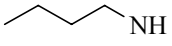
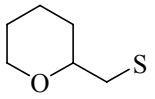
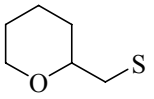

**Figure 6.3**: The curves of competition binding of the target compounds **422, 433, 434** and **435**.

**Table 6.1**: Binding results of **(T)** expressed as $K_i$ in μM at human $A_1$ adenosine receptor.



**(T)**

| Compound | R | $R^1$ | $R^2$ | $K_i$ (μM)[a] |
|---|---|---|---|---|
| **165** | I |  |  | 6.48 ± 1.0 |
| **166** | H |  |  | 17.1 ± 2.0 |
| **273** | I | $SCH_3$ |  | Inactive |
| **274** | H | $SCH_3$ |  | Inactive |
| **310** | H |  |  | 8.0 ± 1.5 |
| **315** | Br |  |  | 57.4 ± 3.0 |
| **333** | I |  |  | 8.19 ± 1.5 |
| **334** | H |  |  | Inactive |
| **421** | I |  |  | Inactive |
| **422** | H |  |  | 6.85 ± 1.0 |
| **423** | Br |  |  | Inactive |
| **433** | I | $SCH_3$ |  | 20.0 ± 1.3 |

| | | | | |
|---|---|---|---|---|
| **434** | H | $SCH_3$ |  | 15.53 ± 1.5 |
| **435** | Br | $SCH_3$ |  | 12.9 ± 2.0 |
| **437** | H | $SCH_3$ | $SCH_3$ | Inactive |
| **445** | H |  |  | Inactive |

[a] Displacement of [$^3$H]DPCPX binding at human $A_1$ receptor expressed in CHO cells. Data was the average of two independent experiments in duplicate. $K_d$ of [$^3$H]DPCPX was 3.9 nM. $K_i$ values were obtained from Cheng-Prusoff equation.

The binding studies showed that 4,6-bis-alkylthio-pyrazolo[3,4-*d*]pyrimidines, 1-benzyl-4,6-bis-methylthio-pyrazolo[3,4-*d*]pyrimidine **(437)** and 1-benzyl-4,6-bis-(2-methyltetrahydropyranthio)-pyrazolo[3,4-*d*]pyrimidine **(445)** were inactive at human $A_1$ adenosine receptor whereas the corresponding 6-alkylthio-4-alkylamino-pyrazolo[3,4-*d*]pyrimidines were either active **(165, 166, 422, 433-435)** or inactive **(273, 274, 421, 423)**. These results were consistent with the hypothesis, based on the previous studies at the rat $A_1$ adenosine receptor[1-3] which indicated that an amino substitutent at C-4 position of pyrazolo[3,4-*d*]pyrimidine was more potent than the corresponding alkylthio substituent at C-4 position. In the previous work, phenethyl at N-1 and phenyl at N-1 were more potent than the benzyl at N-1 at the rat $A_1$ receptor.[1-3]

Nine of sixteen pyrazolo[3,4-*d*]pyrimidines substituted at C-4, C-6 and N-1 showed low potency at the human $A_1$ adenosine receptor with the $K_i$ values in the micromolar range. **Table 1** shows that there is no trends of $K_i$ values in the trisubstituted pyrazolo[3,4-*d*]pyrimidines. Comparison of the values of $K_i$ of compounds **165 and 333 (**3-iodobenzyl at N-1) with those of compound **166 and 334** (benzyl at N-1) showed that the potency increased at least 3-fold when 3-iodobenzyl group was at N-1 position. But when the $K_i$ values of compounds **421** (3-iodobenzyl at N-1) **and 422** (benzyl at N-1) were compared, the benzyl group at the N-1 position increased the potency. The comparision of the $K_i$ values of compounds **433** (3-iodobenzyl at N-1) **and 434** (benzyl at N-1) also could not suggest which hydrophobic side chain at N-1, benzyl or 3-iodobenzyl was important for potency of the trisubstituted pyrazolo[3,4-*d*]pyrimidines.

Similarly, comparison of the $K_i$ value of compound **166** (ethylmorpholineamino at C-4) with that of compound **422** (butylamino at C-4) showed that ethylmorpholineamino at C-4 was unfavorable compared with butylamino at C-4. Compound **166** (ethylmorpholineamino at C-4) was nearly 3 times less potency than compound **422** (butylamino at C-4). But the ethylmorpholineamino at C-4 had the higher potency than butylamino at C-4 when comparing the $K_i$ value of compound **165** (ethylmorpholineamino at C-4) with that of compound **421** (butylamino at C-4). Compounds **165 and 421** had an iodobenzyl substitutent at N-1 compared to compound to compounds **166 and 422** with a benzyl substitutent at N-1.

Similar results were also obtained when comparing the $K_i$ value of compound **421** (2-methyltetrahydropyran at C-6) with that of compound **433** (methyl at C-6) and comparing the $K_i$ value of compound **423** (2-methyltetrahydropyran) with that of compound **435** (methyl at C-6) where the substitutent at N-1 was 3-iodobenzyl or 3-bromobenzyl, the introduction of methyl at C-6 increased potency. However the benzyl series (compare $K_i$ values of compound **422 and 434**) behaved differently, the potency was decreased by 2-fold when the 2-methyltetrahydropyran at C-6 was replaced by methyl at C-6.

In summary, compound **165** was the most active compound in this series of trisubstituted pyrazolo[3,4-*d*]pyrimidines with a $K_i$ value of 6.48±1.0 μM at the human $A_1$ adenosine receptor.

## 6.3 Conclusion

Trisubstituted pyrazolo[3,4-*d*]pyrimidines with different benzyl substitutents at N-1 and various substitutents at C-4 and C-6 were not very active at the human $A_1$ as predicted from previous knowledge. The most active compound in the trisubstituted pyrazolo[3,4-*d*]pyrimidines was **165** with a $K_i$ value of 6.48±1.0 μM at human $A_1$ adenosine receptor. They were inactive at the human $A_3$ adenosine receptors. The design resulted in the desired reduction of $A_1$ affinity but did not give the desired $A_3$ affinity. The modeling hypothesis needs to be revisited.

The results of the present study did not confirm the hypothesis which proposed that the pyrazolo[3,4-*d*]pyrimidines with benzyl substitutents such as benzyl or 3-iodobenzyl at N-1 would generate potent and selective $A_3$ antagonists.

The great differences in the human $A_1$ potency of these benzyl substituted pyrazolo[3,4-*d*]pyrimidines with that of the rat $A_1$ potency of phenyl substituted pyrazolo[3,4-*d*]pyrimidines[1-3] could be explained by the differences in the dimensions of the active sites at the human and at the rat $A_1$ adenosine receptors. Ferrarini *et al.* reported that their compound, 1,8-naphthyridine derivative, had a $K_i$ value of 0.15 nM at the bovine adenosine $A_1$ receptor[4] but when retested at human $A_1$ adenosine receptor,[5] this compound had a $K_i$ value of 300 nM **(Figure 6.4)**.

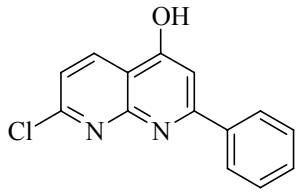



**Figure 6.4**: 1,8-naphthyridine derivative.

The double substitutions of phenyl group at N-1 and amide side chains at C-6 with benzyl group at N-1 and hydrophobic side chains at C-6 may also contribute to the loss of the potency at the human $A_1$ adenosine receptor. Since the potency of these trisubstituted pyrazolo[3,4-*d*]pyrmidines were in micromolar range at the human $A_1$ adenosine receptor, these compounds were not tested at the rat brain $A_1$ adenosine receptor to confirm these explainations.

## 6.4 Experimental

Melting points were recorded on a Gallenkamp digital melting point apparatus and are uncorrected. Infra-red absorption spectra were obtained on a Perkin Elmer FT-IR spectrophotometer using sodium chloride plates. $^1H$ and $^{13}C$ nuclear magnetic resonance (n.m.r) spectra were recorded on a Varian Gemini-200 (200 MHz) spectrometer, Varian Unity-400 (400 MHz) spectrometer or Varian Unity Plus-600

(600 MHz) spectrometer. All samples were dissolved in deuteriochloroform ($CDCl_3$) containing tetramethylsilane (TMS) as an internal reference unless otherwise stated. The signals are recorded in terms of chemical shift in parts per million (ppm) downfield from TMS ($\delta = 0$) for protons or $CDCl_3$ ($\delta = 77$) for carbon atoms. The signals are recorded in terms of chemical shift ($\delta_H$), relative integral, multiplicity, coupling constants (*J* Hz) and assignments, in that order. The following abbreviations for multiplicity are used: s = singlet, dd = doublet of doublets, dt = doublet of triplets, m = multiplet. $^1H$ and $^{13}C$ spectra were assigned with the aid of HMQC (Heteronuclear Multiple-Quantum Coherence), HMBC (Heteronuclear Multiple-Bond Coherence) and $^1H$-$^1H$ COSY (Correlation Spectroscopy). Electrospray mass spectra (ESMS) were recorded on a Fisons VG Platform mass spectrometer with MassLynx Data System software.

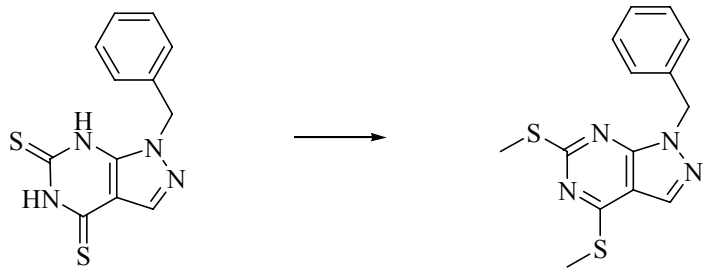
Microanalytical data was obtained from University of Queensland Microanalytical Service.

Analytical thin layer chromatography (TLC) was performed using precoated (0.2 mm) Merck silica gel plates (Merck Kieselgel 60 $F_{254}$). Flash chromatography was carried out using Merck Kieselgel 60 (230-400 mesh) with the indicated solvents.

All solvents for chromatography were distilled before use, unless otherwise stated. Ether refers to diethyl ether and hexane refers to the fraction of b.p. 60-80 °C. Mixed solvent compositions are quoted as v/v.

Solvents and reagents were purified according to the standard techniques of Perrin, Perrin and Amarego.[6]

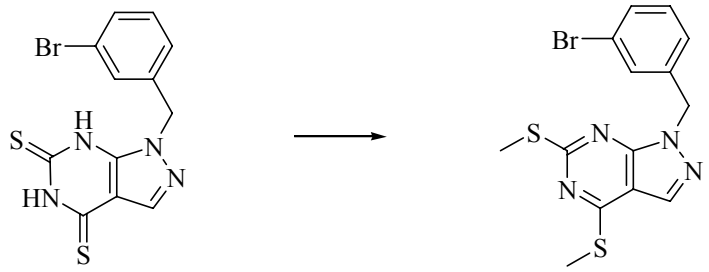
**1-benzyl-4,6-bis-methylthio-pyrazolo[3,4-*d*]pyrimidine (437)**



**26** **437**

**Method A**: To a solution of 1-benzyl-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(26)** (0.25 g, 0.911 mmol) in 2.0 M NaOH (10 ml) : Dioxane (10 ml) was added iodomethane (0.25 ml, 4.0 mmol). After stirring at room temperature for 1 h, the reaction mixture was extracted with ethyl acetate (3 x 30 ml), dried ($MgSO_4$) and the solvent removed in vacuo. The resultant residue was purified by flash chromatography (10% ethyl acetate-hexane) to yield 1-benzyl-4,6-bis-methylthio-pyrazolo[3,4-*d*]pyrimidine **(437)** (179 mg, 65%) as white solid, mp 86.0 ± 0.5 °C; $\delta_H$ (400 MHz) 2.58 (s, 3H, $SCH_3$), 2.64 (s, 3H, $SCH_3$), 5.52 (s, 2H, $CH_2$), 7.20-7.40 (m, 5H, Ar-H) and 8.19 (s, 1H, 3-H); $\delta_C$ (100 MHz) 12.2 ($SCH_3$), 14.5 ($SCH_3$), 50.7 ($CH_2$), 109.5 (C-3a), 128.4 (C-4', C-2', C-6'), 129.2 (C-3', C-5'), 132.8 (C-3), 137.3 (C-1'), 152.0 (C-7a), 165.5 (C-4) and 168.7 (C-6); ESMS (PI) 303. calcd for ($C_{14}H_{14}N_4S_2$ + 1[H]). Found 303.

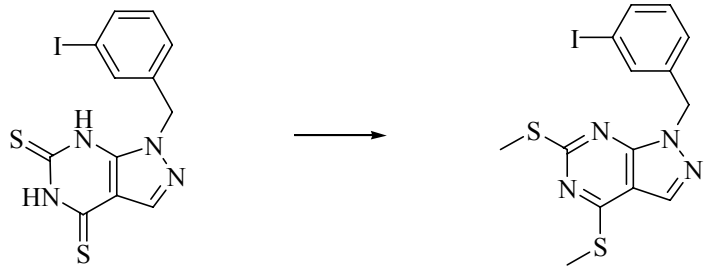
**1-(3-bromobenzyl)-4,6-bis-methylthio-pyrazolo[3,4-*d*]pyrimidine (438)**



**28** **438**

Method A was used to prepare 1-(3-bromobenzyl)-4,6-bis-methylthio-pyrazolo[3,4-*d*]pyrimidine **(438)** from 1-(3-bromobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(28)**. Yield (68 %) as white solid, mp 110.8 ± 0.5 °C; $\delta_H$ (400 MHz) 2.61 (s, 3H, $SCH_3$), 2.66 (s, 3H, $SCH_3$), 5.48 (s, 2H, $CH_2$), 7.14 (t, $J_{ortho}$ = 8.0 Hz, 1H, 5'-H), 7.22 (dt, $J_{ortho}$ = 8.0 Hz, $J_{meta}$ = 1.2 Hz, 1H, 6'-H), 7.37 (dt, $J_{ortho}$ = 8.0 Hz, $J_{meta}$ = 1.2 Hz, $J_{meta}$ = 2.0 Hz, 1H, 4'-H), 7.46 (t, $J_{meta}$ = 2.0 Hz, 1H, 2'-H) and 7.91 (s, 1H, 3-H); $\delta_C$ (100 MHz) 12.2 ($SCH_3$), 14.6 ($SCH_3$), 50.4 ($CH_2$), 109.7 (C-3a), 122.9 (C-3'), 127.0 (C-6'), 130.5 (C-5'), 131.3 (C-4'), 131.5 (C-2'), 132.5 (C-3), 138.6 (C-1'), 151.9 (C-7a), 165.5 (C-4) and 169.3 (C-6); Anal. calcd for $C_{14}H_{13}BrN_4S_2$: C, 44.10; H, 3.44; N, 14.69. Found C, 44.12; H, 3.35; N, 14.47%; ESMS (PI) 381 and 383. calcd for ($C_{14}H_{13}{}^{79}BrN_4S_2$ + 1[H]) and ($C_{14}H_{13}{}^{81}BrN_4S_2$ + 1[H]). Found 381 and 383.

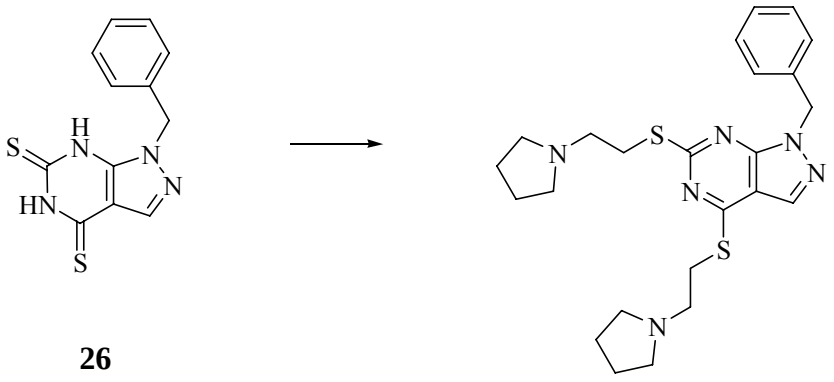
**1-(3-iodobenzyl)-4,6-bis-methylthio-pyrazolo[3,4-*d*]pyrimidine (439)**



**52** **439**

Method A was used to prepare 1-(3-iodobenzyl)-4,6-bis-methylthio-pyrazolo[3,4-*d*]pyrimidine **(439)** from 1-(3-iodobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(52)**. Yield (62 %) as white solid, mp 133.5 ± 0.5 °C; $\delta_H$ (400 MHz) 2.62 (s, 3H, $SCH_3$), 2.66 (s, 3H, $SCH_3$), 5.45 (s, 2H, $CH_2$), 7.01 (t, $J$ = 8.0 Hz, 1H, 5'-H), 7.26 (d, $J$ = 8.0 Hz, 1H, 6'-H), 7.58 (d, $J$ = 8.0 Hz, 1H, 4'-H), 7.70 (s, 1H, 2'-H) and 7.91 (s, 1H, 3-H); $\delta_C$ (100 MHz) 12.1 ($SCH_3$), 14.7 ($SCH_3$), 50.2 ($CH_2$), 94.7 (C-3'), 109.7 (C-3a), 127.6 (C-6'), 130.6 (C-5'), 132.5 (C-3), 137.3 (C-4'), 137.4 (C-2'), 138.7 (C-1'), 152.1 (C-7a), 165.3 (C-4) and 169.3 (C-6); Anal. calcd for $C_{14}H_{13}IN_4S_2$: C, 39.26; H, 3.06; N, 13.08. Found C, 38.84; H, 3.30; N, 11.94%; ESMS (PI) 429. calcd for ($C_{14}H_{13}IN_4S_2$ + 1[H]). Found 429.

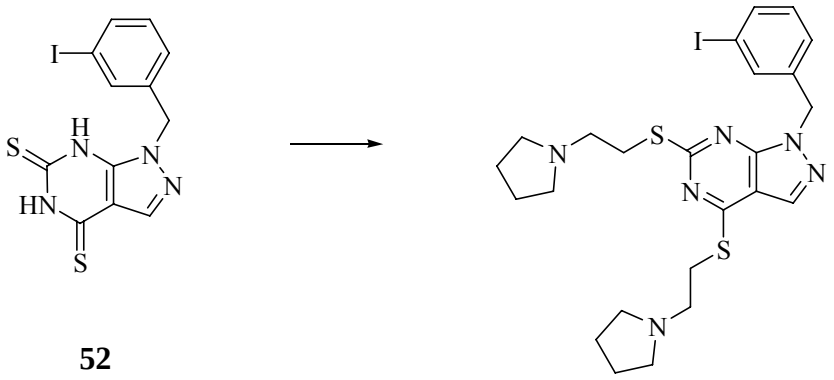
**1-benzyl-4,6-bis-(1-ethylpyrrolidinethio)-pyrazolo[3,4-*d*]pyrimidine (440)**



**26** **440**

**Method B**: To a solution of 1-benzyl-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(26)** (0.200 g, 0.729 mmol) in 2.0 M NaOH (10 ml) : Dioxane (10 ml) was added 1-(2-chloroethyl)pyrrolidine hydrochloride (0.545 g, 3.2 mmol). After stirring at room temperature for 4 h, the reaction mixture was extracted with ethyl acetate (3 x 30 ml), dried ($MgSO_4$) and the solvent removed in vacuo. The resultant residue was purified by flash chromatography (10% methanol-chloroform) to yield 1-benzyl-4,6-bis-(1-ethylpyrrolidinethio)-pyrazolo[3,4-*d*]pyrimidine **(440)** (188 mg, 55%) as yellow oil; $\delta_H$ (600 MHz, DMSO-$d_6$) 1.66 (q, 4H, $J$ = 3.6 Hz, 2 x $CH_2$), 1.68 (q, 4H, $J$ = 3.6 Hz, 2 x $CH_2$), 2.45-2.55 (m, 8H, 4 x $NCH_2$), 2.75 (t, 4H, $J$ = 7.2 Hz, 2 x $NCH_2$), 3.32 (t, 2H, $J$ = 7.2 Hz, $SCH_2$), 3.47 (t, 2H, $J$ = 7.2 Hz, $SCH_2$), 5.54 (s, 2H, $CH_2$), 7.21-7.34 (m, 5H, Ar-H) and 8.21 (s, 1H, 3-H); $\delta_C$ (100 MHz, DMSO-$d_6$) 23.7 (2 x $CH_2$), 23.8 (2 x $CH_2$), 28.3 ($SCH_2$), 29.8 ($SCH_2$), 50.7 ($CH_2$), 53.9 (2 x $NCH_2$), 54.0 (2 x $NCH_2$), 54.8 ($NCH_2$), 55.3 ($NCH_2$), 109.6 (C-3a), 128.2 (C-2', C-6'), 128.4 (C-4'), 129.3 (C-3', C-5'), 132.9 (C-3), 137.3 (C-1'), 152.0 (C-7a), 165.3 (C-4) and 168.2 (C-6); ESMS (PI) 469. calcd for ($C_{24}H_{32}N_6S_2$ + 1[H]). Found 469.

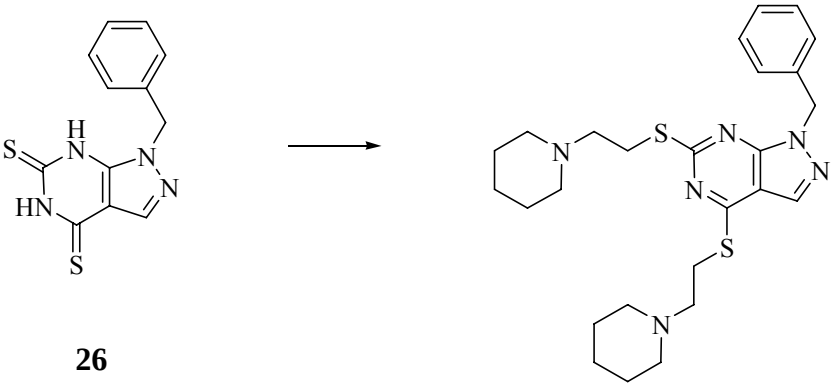
**1-(3-iodobenzyl)-4,6-bis-(1-ethylpyrrolidinethio)-pyrazolo[3,4-*d*]pyrimidine (441)**



**52** **441**

Method B was used to prepare 1-(3-iodobenzyl)-4,6-bis-(1-ethylpyrrolidinethio)-pyrazolo[3,4-*d*]pyrimidine **(441)** from 1-(3-iodobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(52)**. Yield (56 %) as yellow oil; $\delta_H$ (600 MHz) 1.64-1.72 (m, 8H, 4 x $CH_2$), 2.46-2.56 (m, 8H, 4 x $NCH_2$), 2.72-2.80 (m, 4H, 2 x $NCH_2$), 3.33 (t, 2H, *J* = 7.2 Hz, $SCH_2$), 3.48 (t, 2H, *J* = 7.2 Hz, $SCH_2$), 5.52 (s, 2H, $CH_2$), 7.12 (t, *J* = 7.8 Hz, 1H, 5'-H), 7.19 (d, *J* = 7.8 Hz, 1H, 6'-H), 7.65 (d, *J* = 7.8 Hz, 1H, 4'-H), 7.67 (s, 1H, 2'-H) and 8.24 (s, 1H, 3-H); $\delta_C$ (100 MHz) 22.5 (2 x $CH_2$), 22.6 (2 x $CH_2$), 27.2 ($SCH_2$), 28.5 ($SCH_2$), 48.7 ($CH_2$), 52.8 (2 x $NCH_2$), 52.9 (2 x $NCH_2$), 53.8 (2 x $NCH_2$), 94.6 (C-3'), 108.6 (C-3a), 126.5 (C-6'), 130.3 (C-5'), 132.1 (C-3), 135.8 (C-4'), 135.9 (C-2'), 137.8 (C-1'), 150.9 (C-7a), 164.7 (C-4) and 167.7 (C-6); ESMS (PI) 595. calcd for ($C_{24}H_{31}IN_6S_2$ + 1[H]). Found 595.

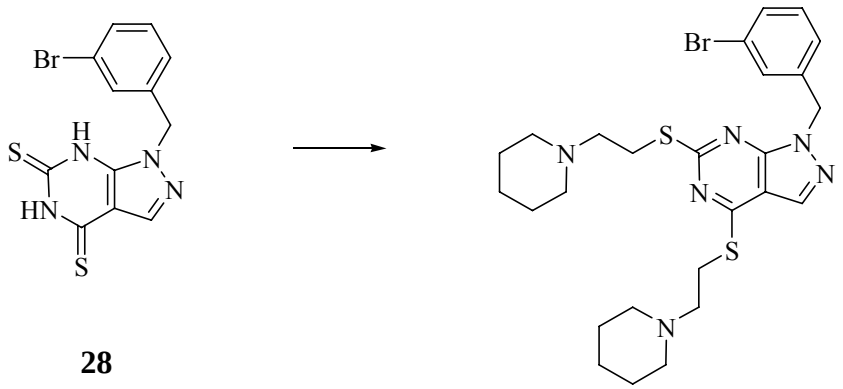
**1-benzyl-4,6-bis-(1-ethylpiperidinethio)-pyrazolo[3,4-*d*]pyrimidine (442)**



**26** **442**

Method B was used to prepare 1-benzyl-4,6-bis-(1-ethylpiperidinethio)-pyrazolo[3,4-*d*]pyrimidine **(442)** from 1-benzyl-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(26)** and 1-(2-chloroethyl)piperidine monohydrochloride. Yield (60%) as yellow oil; $\delta_H$ (600 MHz) 1.33-1.44 (m, 4H, 2 x $CH_2$), 1.50-1.60 (m, 8H, 4 x $CH_2$), 2.36-2.53 (m, 8H, 4 x $CH_2$), 2.60-2.72 (m, 4H, 2 x $CH_2$), 3.26-3.34 (m, 2H, $SCH_2$), 3.40-3.46 (m, 2H, $SCH_2$), 5.48 (s, 2H, $CH_2$), 7.10-7.35 (m, 5H, Ar-H) and 7.87 (s, 1H, 3-H); $\delta_C$ (100 MHz) 24.4 ($CH_2$), 24.5 ($CH_2$), 26.0 (2 x $CH_2$), 26.1 (2 x $CH_2$), 26.4 ($SCH_2$), 28.2 ($SCH_2$), 50.8 ($CH_2$), 54.5 (2 x $NCH_2$), 54.6 (2 x $NCH_2$), 58.1 ($NCH_2$), 58.6 ($NCH_2$), 109.7 (C-3a), 128.0 (C-2', C-6'), 128.1 (C-4'), 128.9 (C-3', C-5'), 132.4 (C-3), 136.6 (C-1'), 152.1 (C-7a), 165.0 (C-4) and 168.5 (C-6); Anal. calcd for $C_{26}H_{36}N_6S_2$: C, 62.87; H, 7.30; N, 16.92. Found C, 61.90; H, 7.67; N, 16.34%; ESMS (PI) 497. calcd for ($C_{26}H_{36}N_6S_2$ + 1[H]). Found 497.

**1-(3-bromobenzyl)-4,6-bis-(1-ethylpiperidinethio)-pyrazolo[3,4-*d*]pyrimidine (443)**

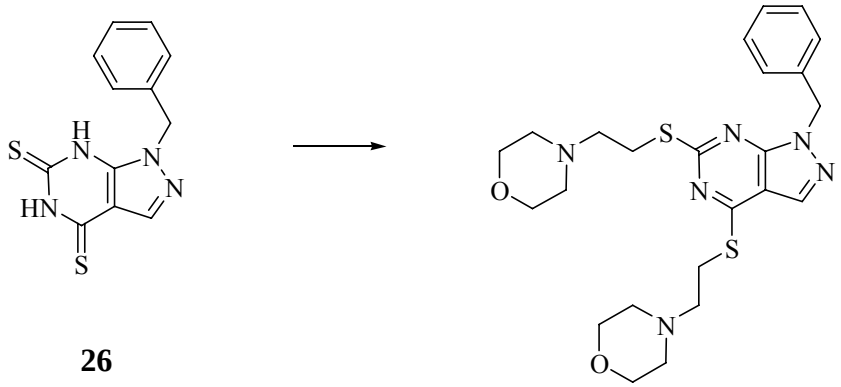


**28** **443**

Method B was used to prepare 1-(3-bromobenzyl)-4,6-bis-(1-ethylpiperidinethio)-pyrazolo[3,4-*d*]pyrimidine **(443)** from 1-(3-bromobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(28)** and 1-(2-chloroethyl)piperidine monohydrochloride. Yield (75 %) as yellow oil; $\delta_H$ (400 MHz) 1.34-1.50 (m, 4H, 2 x $CH_2$), 1.54-1.70 (m, 8H, 4 x $CH_2$), 2.42-2.62 (m, 8H, 4 x $CH_2$), 2.68-2.80 (m, 4H, 2 x $NCH_2$), 3.34-3.40 (m, 2H, $SCH_2$), 3.45-3.54 (m, 2H, $SCH_2$), 5.48 (s, 2H, $CH_2$), 7.13 (t, $J$ = 7.6 Hz, 1H, 5'-H), 7.15 (d, $J$ = 7.6 Hz, 1H, 6'-H), 7.36 (dt, $J_{ortho}$ = 7.6 Hz, $J_{meta}$ = 2.0 Hz, 1H, 4'-H), 7.38 (s, 1H, 2'-H) and 7.90 (s, 1H, 3-H); $\delta_C$ (100 MHz) 24.1 ($CH_2$),

24.2 ($CH_2$), 25.6 (2 x $CH_2$), 25.7 (2 x $CH_2$), 26.1 ($SCH_2$), 27.8 ($SCH_2$), 50.2 ($CH_2$), 54.5 (2 x $NCH_2$), 54.6 (4 x $NCH_2$), 57.8 ($NCH_2$), 58.3 ($NCH_2$), 109.8 (C-3a), 122.9 (C-3'), 126.6 (C-6'), 130.5 (C-5'), 130.9 (C-2'), 131.3 (C-4'), 132.7 (C-3), 138.8 (C-1'), 152.2 (C-7a), 165.1 (C-4) and 168.6 (C-6); ESMS (PI) 575 and 577. calcd for ($C_{26}H_{35}{}^{79}BrN_6S_2$ + 1[H]) and ($C_{26}H_{35}{}^{81}BrN_6S_2$ + 1[H]). Found 575 and 577.

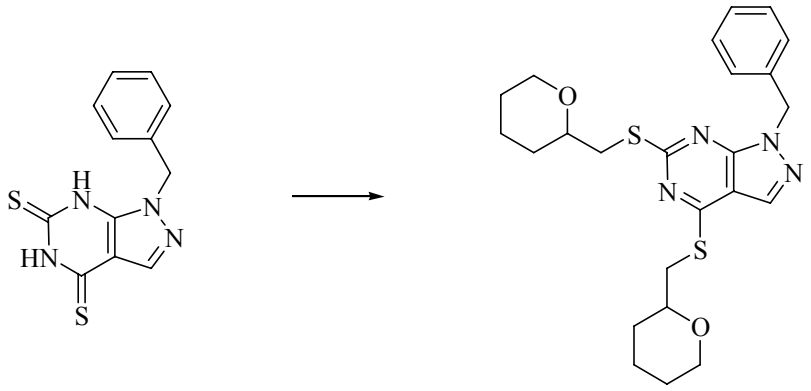
**1-benzyl-4,6-bis-(4-(2-ethyl)morpholinethio)-pyrazolo[3,4-*d*]pyrimidine (444)**



**26** **444**

Method B was used to prepare 1-benzyl-4,6-bis-(4-(2-ethyl)morpholinethio)-pyrazolo[3,4-*d*]pyrimidine **(444)** from 1-benzyl-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(26)** and 4-(2-chloroethyl)morpholine hydrochloride. Yield (58%) as yellow oil; $\delta_H$ (400 MHz) 2.42-2.55 (m, 8H, 4 x $NCH_2$), 2.64-2.74 (m, 4H, 2 x $NCH_2$), 3.30 (t, 2H, $J$ = 7.2 Hz, $SCH_2$), 3.43 (t, 2H, $J$ = 7.2 Hz, $SCH_2$), 3.62-3.71 (m, 8H, 4 x $OCH_2$), 5.48 (s, 2H, $CH_2$), 7.15-7.30 (m, 5H, Ar-H) and 7.88 (s, 1H, 3-H); $\delta_C$ (100 MHz) 26.1 ($SCH_2$), 28.0 ($SCH_2$), 50.9 ($CH_2$), 53.6 (2 x $NCH_2$), 53.7 (2 x $NCH_2$), 57.7 ($NCH_2$), 58.1 ($NCH_2$), 67.0 ($OCH_2$), 67.1 ($OCH_2$), 109.8 (C-3a), 128.0 (C-2', C-6'), 128.2 (C-4'), 128.9 (C-3', C-5'), 132.4 (C-3), 136.5 (C-1'), 152.1 (C-7a), 165.9 (C-4) and 168.4 (C-6); Anal. calcd for $C_{24}H_{32}N_6O_2S_2$: C, 57.57; H, 6.44; N, 16.79. Found C, 57.21; H, 6.76; N, 16.95%; ESMS (PI) 501. calcd for ($C_{24}H_{32}N_6O_2S_2$ + 1[H]). Found 501.

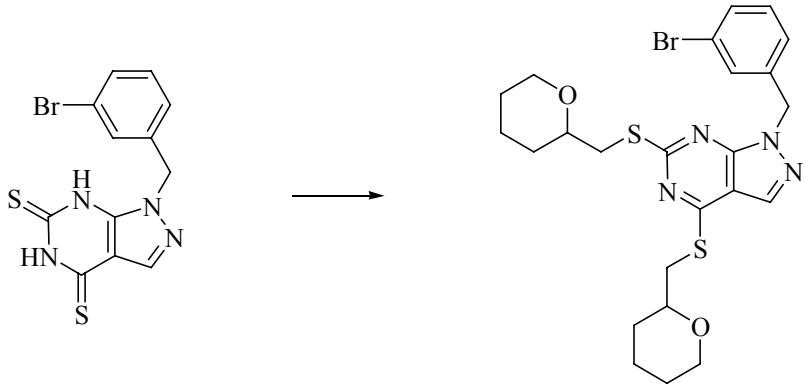
**1-benzyl-4,6-bis-(2-methyltetrahydropyranthio)-pyrazolo[3,4-*d*]pyrimidine (445)**



**26** **445**

**Method C**: To a solution of 1-benzyl-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6dithione **(26)** (0.242 g, 0.882 mmol) in 2.0 M NaOH (10 ml) : Dioxane (10 ml) was added 2-(bromomethyl)tetrahydropyran (0.5 ml, 3.9 mmol). After stirring at 50 °C for 24h, the reaction mixture was extracted with ethyl acetate (3 x 30 ml), dried ($MgSO_4$) and the solvent removed in vacuo. The resultant residue was purified by flash chromatography (10% ethyl acetate-hexane) to yield 1-benzyl-4,6-bis-(2-methyltetrahydropyranthio)-pyrazolo[3,4-*d*]pyrimidine **(445)** (232 mg, 56%) as white solid, mp 82.0 ± 0.5 °C; $\delta_H$ (600 MHz) 1.30-2.00 (m, 12H, 6 x $CH_2$), 3.25-3.40 (m, 3H, 1 x $SCH_2$ + 1 H of $SCH_2$), 3.45-3.46 (td, 2 x 1H, $J_{6a,5}$ = 12 Hz, $J_{6a,6e}$ = 1.8 Hz, $H_{6a}$), 3.50-3.7 (m, 3H, 2 x CH + 1 H of $SCH_2$), 4.00-4.10 (m, 2 x 1H, $H_{6e}$), 5.53 (s, 2H, $CH_2$), 7.20-7.40 (m, 5H, Ar-H) and 7.94 (s, 1H, 3-H); $\delta_C$ (100 MHz) 23.4 ($CH_2$), 23.5 ($CH_2$), 26.0 ($CH_2$), 26.1 ($CH_2$), 31.2 ($CH_2$), 31.4 ($CH_2$), 34.5 ($SCH_2$), 36.8 ($SCH_2$), 51.1 ($CH_2$), 68.9 ($CH_2$), 69.0 ($CH_2$), 76.6 (CH), 76.8 (CH), 109.8 (C-3a), 128.2 (C-4'), 128.3 (C-2', C-6'), 128.9 (C-3', C-5'), 132.4 (C-3), 136.5 (C-1'), 151.9 (C-7a), 165.0 (C-4) and 168.3 (C-6); Anal. calcd for $C_{24}H_{30}N_4O_2S_2$: C, 61.25; H, 6.42; N, 11.90. Found C, 61.04; H, 6.52; N, 11.70%; ESMS (PI) 471. calcd for ($C_{24}H_{30}N_4O_2S_2$ + 1[H]). Found 471.

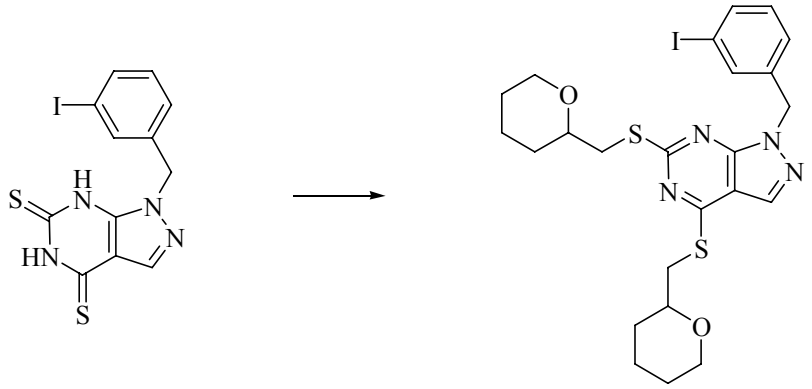
**1-(3-bromobenzyl)-4,6-bis-(2-methyltetrahydropyranthio)-pyrazolo[3,4-*d*]-pyrimidine (446)**



**28** **446**

Method C was used to prepare 1-(3-bromobenzyl)-4,6-bis-(2-methyltetrahydropyranthio)-pyrazolo[3,4-*d*]pyrimidine **(446)** from 1-(3-bromobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(28)**. Yield (60 %) as white solid, mp 83.5 ± 0.5 °C; $\delta_H$ (600 MHz) 1.30-1.90 (m, 12H, 6 x $CH_2$), 3.25-3.38 (m, 3H, 1 x $SCH_2$ + 1 H of $SCH_2$), 3.44 (t, 2 x 1H, $J_{6a,5}$ = 12 Hz, $H_{6a}$), 3.54-3.67 (m, 3H, 2 x CH + 1 H of $SCH_2$), 3.97-4.04 (m, 2 x 1H, $H_{6e}$), 5.47 (s, 2H, $CH_2$), 7.16 (t, *J* = 7.8 Hz, 1H, 5'-H), 7.23 (d, *J* = 7.8 Hz, 1H, 6'-H), 7.38 (d, *J* = 7.8 Hz, 1H, 4'-H), 7.45 (s, 1H, 2'-H) and 7.93 (s, 1H, 3-H); $\delta_C$ (100 MHz) 23.4 ($CH_2$), 23.5 ($CH_2$), 25.9 ($CH_2$), 26.0 ($CH_2$), 31.2 ($CH_2$), 31.4 ($CH_2$), 34.5 ($SCH_2$), 36.8 ($SCH_2$), 50.3 ($CH_2$), 68.9 ($CH_2$), 69.0 ($CH_2$), 76.5 (CH), 76.7 (CH), 109.8 (C-3a), 122.9 (C-3'), 126.9 (C-6'), 130.5 (C-4'), 131.2 (C-5'), 131.3 (C-2'), 132.6 (C-3) 138.7 (C-1'), 152.0 (C-7a), 165.1 (C-4) and 168.6 (C-6); Anal. calcd for $C_{24}H_{29}BrN_4O_2S_2$: C, 52.45; H, 5.32; N, 10.20. Found C, 52.29; H, 5.31; N, 10.09%; ESMS (PI) 549 and 551. calcd for ($C_{24}H_{29}{}^{79}BrN_4O_2S_2$ + 1[H]) and ($C_{24}H_{29}{}^{81}BrN_4O_2S_2$ + 1[H]). Found 549 and 551.

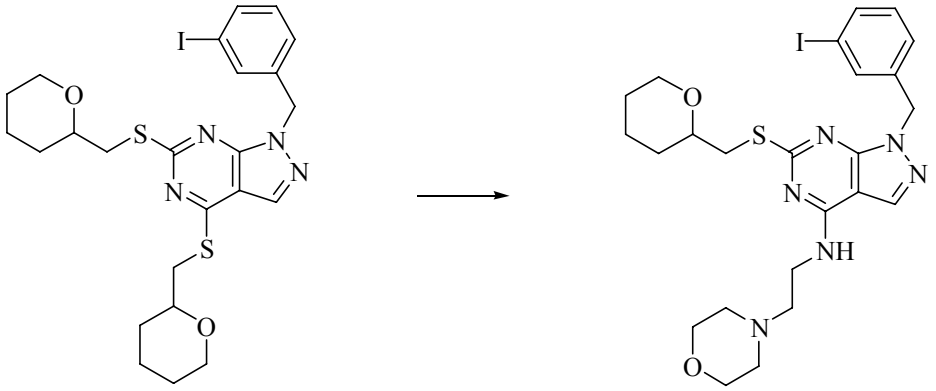
**1-(3-iodobenzyl)-4,6-bis-(2-methyltetrahydropyranthio)-pyrazolo[3,4-*d*]-pyrimidine (447)**



**52** **447**

Method C was used to prepare 1-(3-iodobenzyl)-4,6-bis-(2-methyltetrahydropyranthio)-pyrazolo[3,4-*d*]-pyrimidine **(447)** from 1-(3-iodobenzyl)-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione **(52)**. Yield (57 %) as white solid, mp 97.4 ± 0.5 °C; $\delta_H$ (400 MHz) 1.30-1.90 (m, 12H, 6 x $CH_2$), 3.23-3.39 (m, 3H, 1 x $SCH_2$ + 1 H of $SCH_2$), 3.44 (td, 2 x 1H, $J_{6a,5}$ = 11.6 Hz, $J_{6a,6e}$ = 2.4 Hz $H_{6a}$), 3.53-3.69 (m, 3H, 2 x CH + 1 H of $SCH_2$), 3.98-4.05 (m, 2 x 1H, $H_{6e}$), 5.46 (s, 2H, $CH_2$), 7.03 (t, *J* = 7.6 Hz, 1H, 5'-H), 7.27 (d, *J* = 7.6 Hz, 1H, 6'-H), 7.60 (d, *J* = 7.6 Hz, 1H, 4'-H), 7.67 (s, 1H, 2'-H) and 7.95 (bs, 1H, 3-H); $\delta_C$ (100 MHz) 23.4 ($CH_2$), 23.5 ($CH_2$), 25.9 ($CH_2$), 26.0 ($CH_2$), 31.2 ($CH_2$), 31.4 ($CH_2$), 34.5 ($SCH_2$), 36.8 ($SCH_2$), 50.2 ($CH_2$), 68.9 ($CH_2$), 69.0 ($CH_2$), 76.5 (CH), 76.7 (CH), 94.7 (C-3'), 110.0 (C-3a), 127.6 (C-6'), 130.6 (C-5'), 132.7 (C-3), 137.1 (C-4'), 137.3 (C-2'), 138.7 (C-1'), 148.6 (C-7a), 165.3 (C-4) and 168.6 (C-6); Anal. calcd for $C_{24}H_{29}IN_4O_2S_2$: C, 48.32; H, 4.90; N, 9.39. Found C, 47.81; H, 4.90; N, 9.06%; ESMS (PI) 597. calcd for ($C_{24}H_{29}IN_4O_2S_2$ + 1[H]). Found 597.

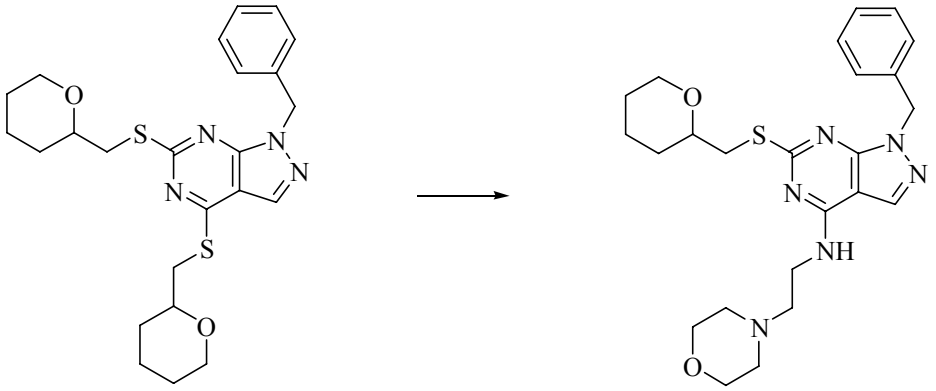
**1-(3-iodobenzyl)-6-(2-methyltetrahydropyranthio)-4-(2-ethyl)morpholineamino-pyrazolo[3,4-*d*]pyrimidine (165)**



**447** **165**

**Method D**: To a solution of 1-(3-iodobenzyl)-4,6-bis-(2-methyltetrahydropyranthio)-pyrazolo[3,4-*d*]-pyrimidine **(447)** (50 mg, 0.082 mmol) in dioxane (4.0 ml) was added 4-(2-Aminoethyl)morpholine (55 µl, 0.42 mmol) and the mixture was heated to 100 °C for 24 h. The reaction mixture was concentrated and purified by flash chromatography (10% methanol-chloroform) to yield 1-(3-iodobenzyl)-6-(2-methyltetrahydropyranthio)-4-(2-ethyl)morpholineamino-pyrazolo-[3,4-*d*]pyrimidine **(165)** (24 mg, 49%) as yellow oil; $\delta_H$ (400 MHz, DMSO-$d_6$) 1.14-1.74 (m, 6H, 3 x $CH_2$), 2.34-2.52 (m, 6H, 3 x $NCH_2$), 3.02-3.10 (dd, 1H, $J_{6a,5}$ = 6.8 Hz, $H_{6a}$), 3.18-3.58 (m, 9H, 2 x $CH_2O$ + $CH_2$ + $SCH_2$ + CH), 3.77-3.84 (dd, 1H, $J_{6e,5}$ = 11.2 Hz, $J_{6e,6a}$ = 2.0 Hz, $H_{6e}$), 5.33 (s, 2H, $CH_2$), 7.07 (t, $J$ = 7.6 Hz, 1H, 5'-H), 7.13 (d, $J$ = 7.6 Hz, 1H, 6'-H), 7.55 (s, 1H, 2'-H), 7.58 (d, $J$ = 7.6 Hz, 1H, 4'-H), 8.00 (s, 1H, 3-H) and 8.32 (t, $J$ = 5.6 Hz, 1H, NH); $\delta_C$ (100 MHz, DMSO-$d_6$) 23.5 ($CH_2$), 26.1 ($CH_2$), 31.3 ($CH_2$), 36.4 ($SCH_2$), 37.9 ($NHCH_2$), 49.6 ($NCH_2$), 54.1 (2 x $NCH_2$), 57.9 ($NCH_2$), 66.8 (2 x $CH_2$), 68.2 ($CH_2$), 77.1 (CH), 95.5 (C-3'), 98.9 (C-3a), 127.6 (C-6'), 131.4 (C-5'), 133.1 (C-3), 136.7 (C-4'), 136.9 (C-2'), 140.5 (C-1'), 154.0 (C-7a), 156.1 (C-4) and 168.9 (C-6); ESMS (PI) 595. calcd for ($C_{24}H_{31}IN_6O_2S$ + 1[H]). Found 595.

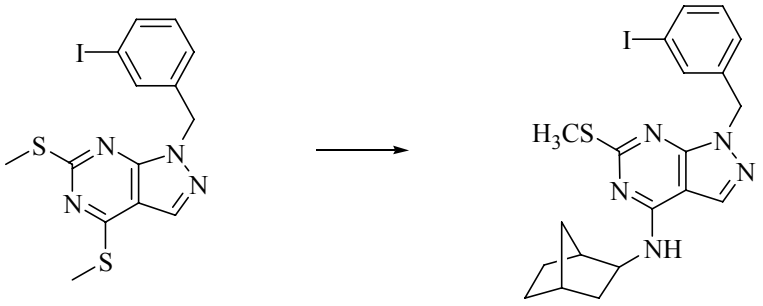
**1-benzyl-6-(2-methyltetrahydropyranthio)-4-(2-ethyl)morpholineamino-pyrazolo[3,4-*d*]pyrimidine (166)**



**445** **166**

Method D was used to prepare 1-benzyl-6-(2-methyltetrahydropyranthio)-4-(2-ethyl)morpholineamino-pyrazolo[3,4-*d*]pyrimidine **(166)** from 1-benzyl-4,6-bis-(2-methyltetrahydropyranthio)-pyrazolo[3,4-*d*]pyrimidine **(445)**. Yield (57 %) as yellow oil; $\delta_H$ (400 MHz, DMSO-$d_6$) 1.12-1.72 (m, 6H, 3 x $CH_2$), 2.34-2.51 (m, 6H, 3 x $NCH_2$), 3.02-3.10 (dd, 1H, $J_{6a,5}$ = 6.8 Hz, $H_{6a}$), 3.17-3.57 (m, 9H, 2 x $CH_2O$ + $CH_2$ + $SCH_2$ + CH), 3.77-3.84 (dd, 1H, $J_{6e,5}$ = 11.2 Hz, $J_{6e,6a}$ = 2.4 Hz, $H_{6e}$), 5.36 (s, 2H, $CH_2$), 7.12-7.29 (m, 5H, Ar-H), 7.99 (s, 1H, 3-H) and 8.29 (t, $J$ = 5.6 Hz, 1H, NH); $\delta_C$ (100 MHz, DMSO-$d_6$) 23.5 ($CH_2$), 26.1 ($CH_2$), 31.3 ($CH_2$), 36.4 ($SCH_2$), 37.9 ($NHCH_2$), 50.4 ($NCH_2$), 54.1 (2 x $NCH_2$), 57.9 ($NCH_2$), 66.8 (2 x $CH_2$), 68.2 ($CH_2$), 77.1 (CH), 99.0 (C-3a), 128.1 (C-6', C-4', C-2'), 129.1 (C-3', C-5'), 132.9 (C-3), 138.0 (C-1'), 154.0 (C-7a), 156.1 (C-4) and 168.7 (C-6); ESMS (PI) 469. calcd for ($C_{24}H_{32}N_6O_2S$ + 1[H]). Found 469.

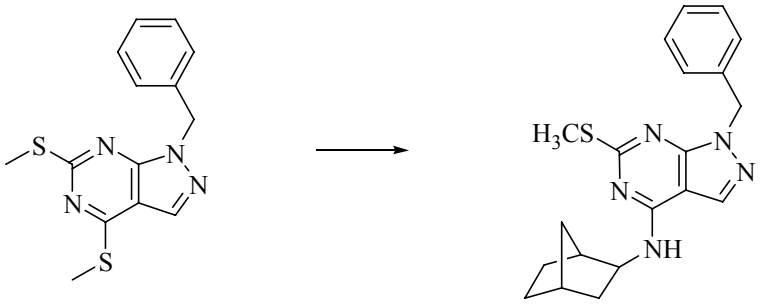
**1-(3-iodobenzyl)-6-methylthio-4-(exo-2-norbornylamino)-pyrazolo[3,4-*d*]-pyrimidine (273)**



**439** **273**

**Method E**: To a solution of 1-(3-iodobenzyl)-4,6-bis-methylthio-pyrazolo[3,4-*d*]pyrimidine **(439)** (69 mg, 0.161 mmol) in dioxane (4.0 ml) was added exo-2-aminonorbornane (95 µl, 0.80 mmol) and the mixture was heated to 100 °C for 4 days. The reaction mixture was concentrated and purified by flash chromatography (2.5% methanol-DCM) to yield 1-(3-iodobenzyl)-6-methylthio-4-(exo-2-norbornylamino)-pyrazolo[3,4-*d*]-pyrimidine **(273)** (36 mg, 45%) as light yellow solid, mp 99.0 ± 0.5 °C; $\delta_H$ (400 MHz) 1.10-1.70 (m, 7H, norbornyl-H), 1.84-1.96 (m, 1H, norbornyl-H), 2.26-2.40 (m, 2H, norbornyl-H), 2.56 (s, 3H, $SCH_3$), 3.50-3.90 (br, 1H, norbornyl-H), 5.39 (s, 2H, $CH_2$), 6.00-6.50 (br, 1H, NH), 7.00 (t, *J* = 7.6 Hz, 1H, 5'-H), 7.25 (d, *J* = 7.6 Hz, 1H, 6'-H), 7.56 (d, *J* = 7.6 Hz, 1H, 4'-H), 7.68 (s, 1H, 2'-H), and 7.80 (s, 1H, 3-H); $\delta_C$ (100 MHz) 14.4 ($SCH_3$), 26.4 ($CH_2$), 28.5 ($CH_2$), 35.8 ($CH_2$), 36.0 (CH), 41.0 ($CH_2$), 42.6 (CH), 50.1 ($CH_2$), 56.8 (CH), 94.6 (C-3' + C-3a), 127.7 (C-6'), 130.5 (C-5'), 133.9 (C-3), 137.2 (C-4'), 137.4 (C-2'), 138.9 (C-1'), 154.5 (C-7a), 155.4 (C-4) and 168.4 (C-6); ESMS (PI) 492. calcd for ($C_{20}H_{22}IN_5S$ + 1[H]). Found 492.

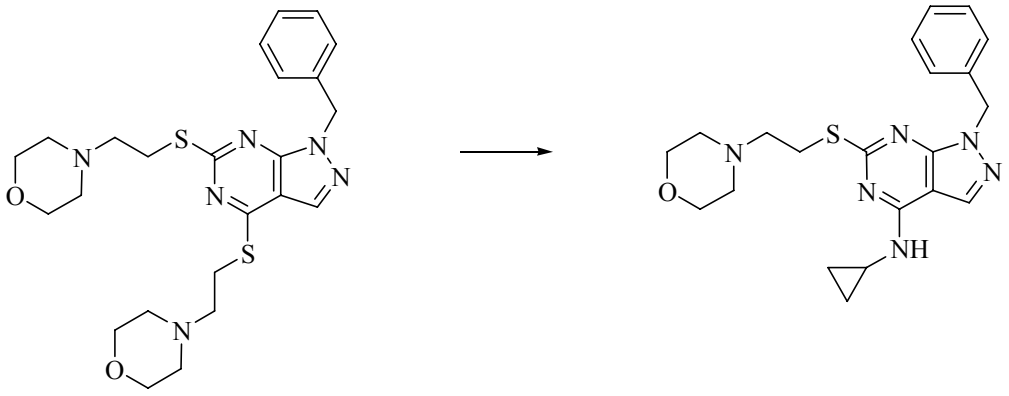
**1-benzyl-6-methylthio-4-(exo-2-norbornylamino)-pyrazolo[3,4-*d*]pyrimidine (274)**



**437** **274**

Method E was used to prepare 1-benzyl-6-methylthio-4-(exo-2-norbornylamino)-pyrazolo[3,4-*d*]pyrimidine **(274)** from 1-benzyl-4,6-bis-methylthio-pyrazolo[3,4-*d*]pyrimidine **(437)**. Yield (65 %) as white solid, mp 135.0 ± 0.5 °C; $\delta_H$ (400 MHz) 1.10-1.35 (m, 4H, norbornyl-H), 1.35-1.65 (m, 3H, norbornyl-H), 1.85-1.95 (m, 1H, norbornyl-H), 2.27-2.37 (m, 2H, norbornyl-H), 2.55 (s, 3H, $SCH_3$), 3.60-3.90 (br, 1H, norbornyl-H), 5.46 (s, 2H, $CH_2$), 5.60-6.00 (br, 1H, NH), 7.17-7.36 (m, 5H, Ar-H) and 7.78 (s, 1H, 3-H); $\delta_C$ (100 MHz) 14.3 ($SCH_3$), 26.4 ($CH_2$), 28.4 ($CH_2$), 35.7 ($CH_2$), 35.9 (CH), 41.2 ($CH_2$), 42.6 (CH), 50.8 ($CH_2$), 56.4 (CH), 97.4 (C-3a), 128.0 (C-4'), 128.4 (C-2', C-6'), 128.8 (C-3', C-5'), 133.5 (C-3), 136.9 (C-1'), 154.7 (C-7a), 156.0 (C-4) and 168.9 (C-6); Anal. calcd for $C_{20}H_{23}N_5S$: C, 65.72; H, 6.34; N, 19.16. Found C, 64.80; H, 6.41; N, 19.31%; ESMS (PI) 366. calcd for ($C_{20}H_{23}N_5S$ + 1[H]). Found 366.

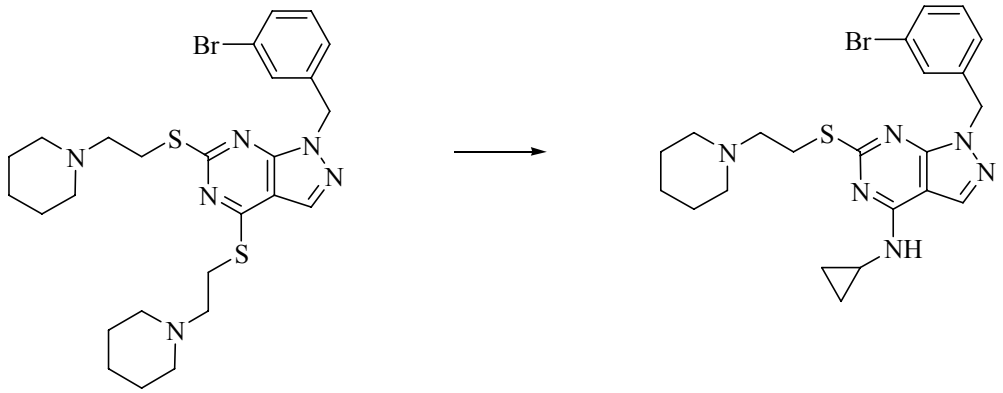
**1-benzyl-6-(4-(2-ethyl)morpholinethio)-4-cyclopropylamino-pyrazolo[3,4-*d*]-pyrimidine (310)**



**444** **310**

**Method F**: To a solution of 1-benzyl-4,6-bis-(4-(2-ethyl)morpholinethio)-pyrazolo[3,4-*d*]pyrimidine **(444)** (65 mg, 0.13 mmol) in dioxane (4.0 ml) was added cyclopropylamine (45 µl, 0.65 mmol). The mixture was heated to 100 °C and monitored by electrospray mass spectrometer. Cyclopropylamine (45 µl, 0.65 mmol) was added every hour until no starting material was detected. After 8 h, the reaction mixture was concentrated and purified by flash chromatography (2.5% methanol-DCM) to yield 1-benzyl-6-(4-(2-ethyl)morpholinethio)-4-cyclopropylamino-pyrazolo[3,4-*d*]-pyrimidine **(310)** (26 mg, 49%) as yellow oil; $\delta_H$ (400 MHz) 0.68-0.76 (br, 2H, $CH_2$), 0.90-0.96 (dd, 2H, $J$ = 5.2 Hz, $J$ = 6.8 Hz, $CH_2$), 2.48-2.68 (br, 4H, 2 x $NCH_2$), 2.79 (t, 2H, $J$ = 7.2 Hz, $NCH_2$), 2.82-2.92 (m, 1H, CH), 3.26-3.34 (m, 2H, $SCH_2$), 3.66-3.80 (br, 4H, 2 x $OCH_2$), 5.47 (s, 2H, $CH_2$), 6.10-6.60 (br, 1H, NH), 7.16-7.34 (m, 5H, Ar-H) and 8.05 (s, 1H, 3-H); $\delta_C$ (100 MHz) 9.1 (2 x $CH_2$), 25.1 (CH), 27.1 ($SCH_2$), 50.6 ($CH_2$), 53.3 (2 x $NCH_2$), 58.3 ($CH_2$), 66.5 ($OCH_2$), 98.4 (C-3a), 128.0 (C-2', C-6', C-4'), 128.8 (C-3', C-5'), 132.3 (C-3), 136.9 (C-1'), 155.1 (C-7a), 158.9 (C-4) and 167.9 (C-6); ESMS (PI) 411. calcd for ($C_{21}H_{26}N_6OS$ + 1[H]). Found 411.

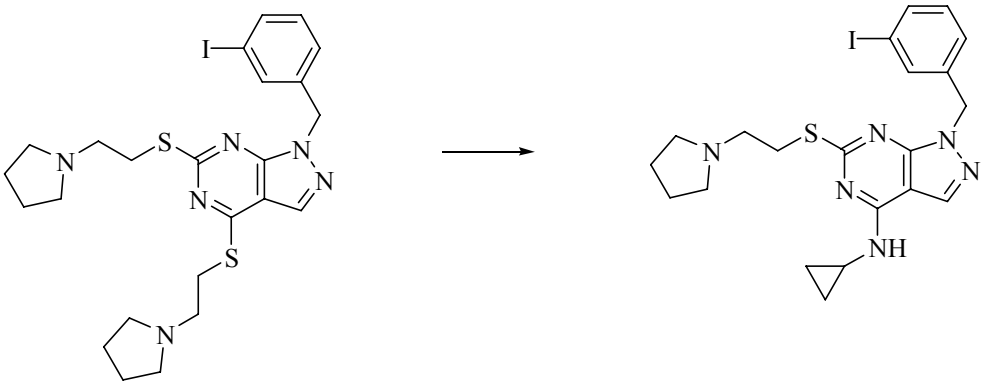
**1-(3-bromobenzyl)-6-(1-ethylpiperidinethio)-4-cyclopropylamino-pyrazolo[3,4-*d*]-pyrimidine (315)**



**443** **315**

Method F was used to prepare 1-(3-bromobenzyl)-6-(1-ethylpiperidinethio)-4-cyclopropylamino-pyrazolo[3,4-*d*]-pyrimidine **(315)** from 1-(3-bromobenzyl)-4,6-bis-(1-ethylpiperidinethio)-pyrazolo[3,4-*d*]pyrimidine **(443)** and cyclopropylamine. Yield (55 %) as yellow oil; $\delta_H$ (400 MHz) 0.66-0.80 (br, 2H, $CH_2$), 0.88-0.96 (dd, 2H, $J$ = 5.2 Hz, $J$ = 6.8 Hz, $CH_2$), 1.40-1.50 (br, 2H, $CH_2$), 1.64-1.74 (br, 4H, 2 x $CH_2$), 2.55-2.68 (br, 4H, 2 x $NCH_2$), 2.80-2.90 (m, 3H, $NCH_2$ + CH), 3.27-3.34 (m, 2H, $SCH_2$), 5.43 (s, 2H, $CH_2$), 6.70-7.00 (br, 1H, NH), 7.13 (t, $J$ = 7.6 Hz, 1H, 5'-H), 7.17 (d, $J$ = 7.6 Hz, 1H, 6'-H), 7.34 (d, $J$ = 7.6 Hz, 1H, 4'-H), 7.39 (s, 1H, 2'-H) and 8.05 (s, 1H, 3-H); $\delta_C$ (100 MHz) 8.8 (2 x $CH_2$), 23.0 ($CH_2$), 23.8 (2 x $CH_2$), 25.1 (CH), 26.2 ($SCH_2$), 49.8 ($CH_2$), 53.8 (2 x $NCH_2$), 57.5 ($NCH_2$), 98.5 (C-3a), 122.9 (C-3'), 126.7 (C-6'), 130.4 (C-5'), 130.9 (C-4'), 131.0 (C-2'), 134.7(C-3), 139.3(C-1'), 155.3 C-7a), 157.0 C-4) and 167.8 (C-6); ESMS (PI) 487 and 489. calcd for ($C_{22}H_{27}{}^{79}BrN_6S$ + 1[H]) and ($C_{22}H_{27}{}^{79}BrN_6S$ + 1[H]). Found 487 and 489.

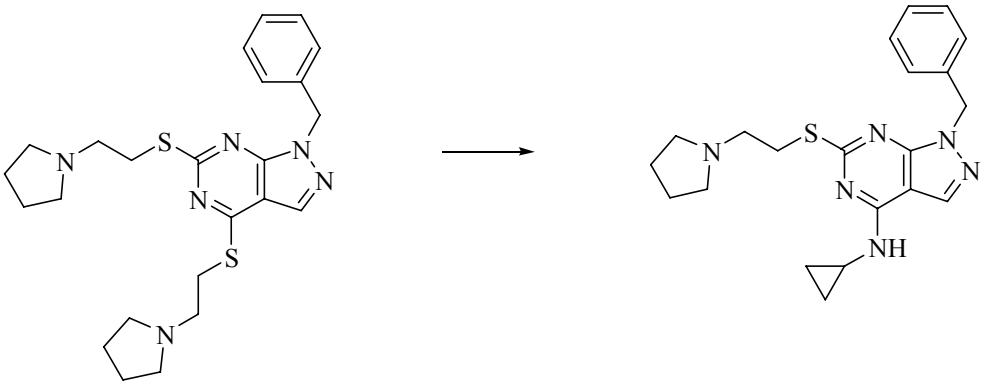
**1-(3-iodobenzyl)-6-(1-ethylpyrrolidinethio)-4-cyclopropylamino-pyrazolo[3,4-*d*]-pyrimidine (333)**



**441** **333**

Method F was used to prepare 1-(3-iodobenzyl)-6-(1-ethylpyrrolidinethio)-4-cyclopropylamino-pyrazolo[3,4-*d*]-pyrimidine **(333)** from 1-(3-iodobenzyl)-4,6-bis-(1-ethylpyrrolidinethio)-pyrazolo[3,4-*d*]pyrimidine **(441)** and cyclopropylamine. Yield (50 %) as yellow oil; $\delta_H$ (400 MHz) 0.68-0.72 (br, 2H, $CH_2$), 0.90-0.96 (m, 2H, $CH_2$), 1.86-1.94 (br, 4H, 2 x $CH_2$), 2.78-2.92 (br, 5H, 2 x $NCH_2$ + CH), 2.98-3.04 (m, 2H, $NCH_2$), 3.32-3.40 (m, 2H, $SCH_2$), 5.40 (s, 2H, $CH_2$), 6.70-7.00 (br, 1H, NH), 6.99 (t, $J_{ortho}$ = 7.6 Hz, 1H, 5'-H), 7.21 (d, $J_{ortho}$ = 7.6 Hz, 1H, 6'-H), 7.55 (dt, $J_{ortho}$ = 7.6 Hz, $J_{meta}$ = 1.2 Hz, 1H, 4'-H), 7.61 (t, $J_{meta}$ = 1.6 Hz, 1H, 2'-H) and 8.06 (br, 1H, 3-H); $\delta_C$ (100 MHz) 8.9 (2 x $CH_2$), 23.6 (2 x $CH_2$), 25.1 (CH), 28.3 ($SCH_2$), 49.8 ($CH_2$), 54.1 (2 x $NCH_2$), 55.7 ($NCH_2$), 94.7 (C-3'), 98.5 (C-3a), 127.4 (C-6'), 130.5 (C-5'), 134.5 (C-3), 136.9 (C-2'), 137.0 (C-4'), 139.2 (C-1'), 153.5 (C-7a), 158.9 (C-4) and 170.8 (C-6); ESMS (PI) 521. calcd for ($C_{21}H_{25}IN_6S$ + 1[H]). Found 521.

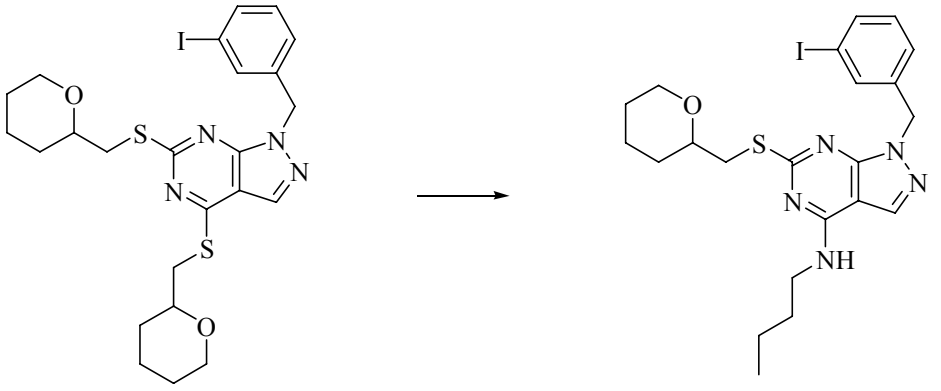
**1-benzyl-6-(1-ethylpyrrolidinethio)-4-cyclopropylamino-pyrazolo[3,4-*d*]-pyrimidine (334)**



**440** **334**

Method F was used to prepare 1-benzyl-6-(1-ethylpyrrolidinethio)-4-cyclopropylamino-pyrazolo[3,4-*d*]-pyrimidine **(334)** from 1-benzyl-4,6-bis-(1-ethylpyrrolidinethio)-pyrazolo[3,4-*d*]pyrimidine **(440)** and cyclopropylamine. Yield (45%) as yellow oil; $\delta_H$ (400 MHz) 0.66-0.80 (br, 2H, $CH_2$), 0.88-0.96 (dd, 2H, $J$ = 5.2 Hz, $J$ = 6.8 Hz, $CH_2$), 1.86 (t, 4H, 2 x $CH_2$), 2.74-2.90 (m, 5H, 2 x $NCH_2$ + CH), 2.94-3.04 (m, 2H, $NCH_2$), 3.28-3.37 (m, 2H, $SCH_2$), 5.47 (s, 2H, $CH_2$), 6.70-7.00 (br, 1H, NH), 7.14-7.34 (m, 5H, Ar-H) and 8.05 (s, 1H, 3-H); $\delta_C$ (100 MHz) 8.9 (2 x $CH_2$), 23.6 (2 x $CH_2$), 25.1 (CH), 28.3 ($SCH_2$), 50.6 ($CH_2$), 53.9 (2 x $NCH_2$), 55.7 ($NCH_2$), 98.4 (C-3a), 127.9 (C-4'), 128.1 (C-2', C-6'), 128.8 (C-3', C-5'), 134.2 (C-3), 137.0 (C-1'), 155.2 (C-7a), 159.0 (C-4) and 167.5 (C-6); ESMS (PI) 395. calcd for ($C_{21}H_{26}N_6S$ + 1[H]). Found 395.

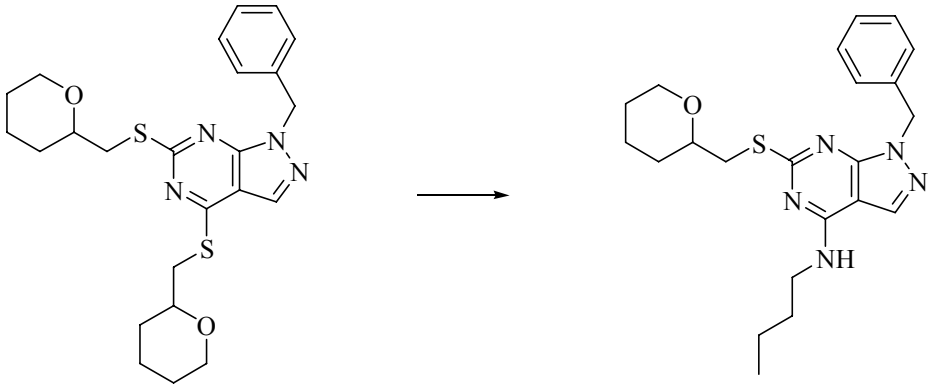
**1-(3-iodobenzyl)-6-(2-methyltetrahydropyranthio)-4-butylamino-pyrazolo[3,4-*d*]pyrimidine (421)**



**447** **421**

**Method G**: To a solution of 1-(3-iodobenzyl)-4,6-bis-(2-methyltetrahydropyranthio)-pyrazolo[3,4-*d*]pyrimidine **(447)** (70 mg, 0.117 mmol) in dioxane (4.0 ml) was added butylamine (58 µl, 0.586 mmol). The mixture was heated to 100 °C and monitored by electrospray mass spectrometer. Butylamine (58 µl, 0.586 mmol) was added every 4 hour until no starting material was detected. After 8 h, the reaction mixture was concentrated and purified by flash chromatography (2.5% methanol-DCM) to yield 1-(3-iodobenzyl)-6-(2-methyltetrahydropyranthio)-4-butylamino-pyrazolo[3,4-*d*]pyrimidine **(421)** (38 mg, 60%) as white solid, mp 97.4 ± 0.5 °C; $\delta_H$ (400 MHz) 0.91 (t, 3H, $J$ = 7.6 Hz, $CH_3$), 1.20-1.84 (m, 10H, 5 x $CH_2$), 3.25 (d, 2H, $J$ = 5.6 Hz, $SCH_2$), 3.39 (td, 1H, $J_{6a,5}$ = 11.6 Hz, $J_{6a,6e}$ = 2.0 Hz, $H_{6a}$), 3.48-3.58 (m, 3H, $NCH_2$ + CH), 3.92-3.99 (dd, 1H, $J_{6e,5}$ = 11.2 Hz, $J_{6e,6a}$ = 2.0 Hz, $H_{6e}$), 5.36 (s, 2H, $CH_2$), 5.70-6.00 (br, 1H, NH), 6.98 (t, $J$ = 7.6 Hz, 1H, 5'-H), 7.21 (d, $J$ = 7.6 Hz, 1H, 6'-H), 7.54 (d, $J$ = 7.6 Hz, 1H, 4'-H), 7.60 (s, 1H, 2'-H) and 7.76 (br, 1H, 3-H); $\delta_C$ (100 MHz) 14.0 ($CH_3$), 20.3 ($CH_2$), 23.6 ($CH_2$), 26.1 ($CH_2$), 31.3 ($CH_2$), 31.7 ($CH_2$), 36.5 ($SCH_2$), 49.9 (2 x $NCH_2$), 68.9 ($CH_2$), 77.2 (CH), 94.6 (C-3' + C-3a), 127.5 (C-6'), 130.5 (C-5'), 131.1 (C-3), 137.0 (C-4'), 137.1 (C-2'), 139.2 (C-1'), 154.5 (C-7a), 161.4 (C-4) and 169.3 (C-6); Anal. calcd for $C_{22}H_{28}IN_5OS$: C, 49.16; H, 5.25; N, 13.03. Found C, 49.46; H, 5.39; N, 12.93%; ESMS (PI) 538. calcd for ($C_{22}H_{28}IN_5OS$ + 1[H]). Found 538.

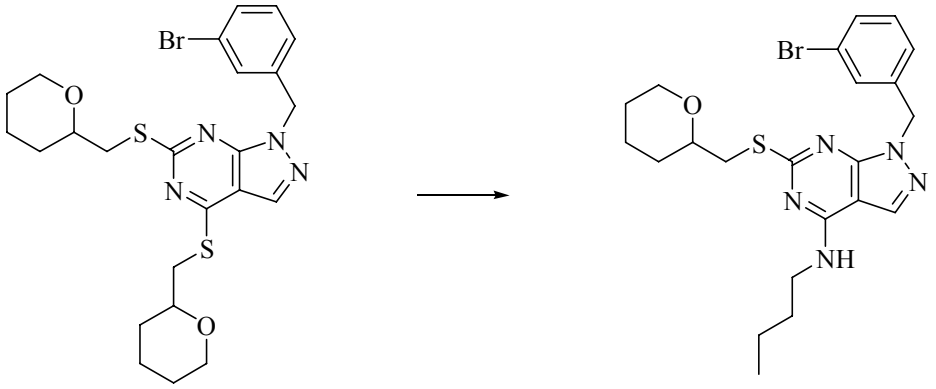
**1-benzyl-6-(2-methyltetrahydropyranthio)-4-butylamino-pyrazolo[3,4-*d*]-pyrimidine (422)**



**445** **422**

Method G was used to prepare 1-benzyl-6-(2-methyltetrahydropyranthio)-4-butylamino-pyrazolo[3,4-*d*]-pyrimidine **(422)** from 1-benzyl-4,6-bis-(2-methyltetrahydropyranthio)-pyrazolo[3,4-*d*]pyrimidine **(445)**. Yield (58%) as white solid, mp 94.0 ± 0.5 °C; $\delta_H$ (400 MHz) 0.91 (t, 3H, $J$ = 7.6 Hz, $CH_3$), 1.20-1.84 (m, 10H, 5 x $CH_2$), 3.26 (m, 2H, $SCH_2$), 3.38 (td, 1H, $J_{6a,5}$ = 11.6 Hz, $J_{6a,6e}$ = 2.4 Hz, $H_{6a}$), 3.48-3.58 (m, 3H, $NCH_2$ + CH), 3.93-4.00 (m, 1H, $H_{6e}$), 5.43 (s, 2H, $CH_2$), 5.70-6.10 (br, 1H, NH), 7.16-7.32 (m, 5H, Ar-H) and 7.75 (br, 1H, 3-H); $\delta_C$ (100 MHz) 13.9 ($CH_3$), 20.3 ($CH_2$), 23.6 ($CH_2$), 26.1 ($CH_2$), 31.3 ($CH_2$), 31.7 ($CH_2$), 36.5 ($SCH_2$), 40.8 ($NHCH_2$), 50.8 ($NCH_2$), 68.9 ($CH_2$), 77.2 (CH), 98.7 (C-3a), 127.9 (C-4'), 128.2 (C-2', C-6'), 128.8 (C-3', C-5'), 130.9 (C-3), 137.0 (C-1'), 154.3 (C-7a), 156.2 (C-4) and 169.1 (C-6); Anal. calcd for $C_{22}H_{29}N_5OS$: C, 64.20; H, 7.10; N, 17.02. Found C, 64.40; H, 7.31; N, 16.91%; ESMS (PI) 412. calcd for ($C_{22}H_{29}N_5OS$ + 1[H]). Found 412.

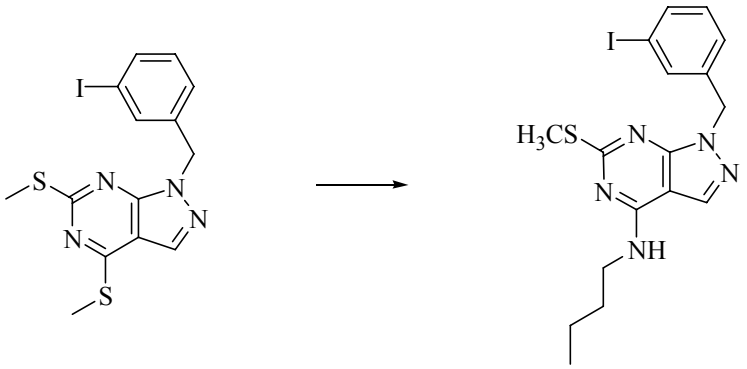
**1-(3-bromobenzyl)-6-(2-methyltetrahydropyranthio)-4-butylamino-pyrazolo-[3,4-*d*]pyrimidine (423)**



**446** **423**

Method G was used to prepare 1-(3-bromobenzyl)-6-(2-methyltetrahydropyranthio)-4-butylamino-pyrazolo-[3,4-*d*]pyrimidine **(423)** from 1-(3-bromobenzyl)-4,6-bis-(2-methyltetrahydropyranthio)-pyrazolo[3,4-*d*]pyrimidine **(446)** and butylamine. Yield (64 %) as white solid, mp 83.5 ± 0.5 °C; $\delta_H$ (400 MHz) 0.92 (t, 3H, $J$ = 7.6 Hz, $CH_3$), 1.24-1.85 (m, 10H, 5 x $CH_2$), 3.26 (d, 2H, $J$ = 5.6 Hz, $SCH_2$), 3.39 (td, 1H, $J_{6a,5}$ = 11.6 Hz, $J_{6a,6e}$ = 2.4 Hz, $H_{6a}$), 3.46-3.62 (m, 3H, $NCH_2$ + CH), 3.92-4.01 (m, 1H, $H_{6e}$), 5.40 (s, 2H, $CH_2$), 6.30-6.65 (br, 1H, NH), 7.12 (t, $J$ = 8.0 Hz, 1H, 5'-H), 7.19 (d, $J$ = 8.0 Hz, 1H, 6'-H), 7.35 (d, $J$ = 8.0 Hz, 1H, 4'-H), 7.39 (s, 1H, 2'-H) and 7.77 (br, 1H, 3-H); $\delta_C$ (100 MHz) 13.9 ($CH_3$), 20.3 ($CH_2$), 23.5 ($CH_2$), 26.1 ($CH_2$), 31.3 ($CH_2$), 31.6 ($CH_2$), 36.5 ($SCH_2$), 50.1 (2 x $NCH_2$), 68.9 ($CH_2$), 77.2 (CH), 98.7 (C-3a), 122.9 (C-3'), 126.9 (C-6'), 130.4 (C-5'), 131.2 (C-4', C-2'), 133.4 (C-3), 139.0 (C-1'), 154.3 (C-7a), 156.3 (C-4) and 169.5 (C-6); Anal. calcd for $C_{22}H_{28}BrN_5OS$: C, 53.87; H, 5.75; N, 14.28. Found C, 53.84; H, 5.85; N, 14.19%; ESMS (PI) 490 and 492. calcd for ($C_{22}H_{28}{}^{79}BrN_5OS$ + 1[H]) and ($C_{22}H_{28}{}^{81}BrN_5OS$ + 1[H]). Found 490 and 492.

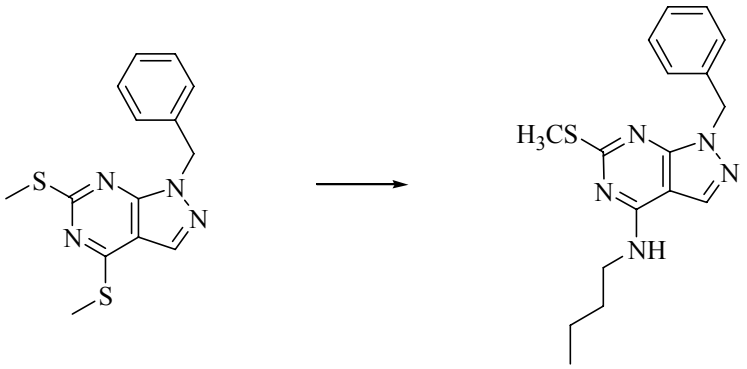
**1-(3-iodobenzyl)-6-methylthio-4-butylamino-pyrazolo[3,4-*d*]pyrimidine (433)**



**439** **433**

Method G was used to prepare 1-(3-iodobenzyl)-6-methylthio-4-butylamino-pyrazolo[3,4-*d*]pyrimidine **(433)** from 1-(3-iodobenzyl)-4,6-bis-methylthio-pyrazolo[3,4-*d*]pyrimidine **(439)** and butylamine. Yield (50%) as white solid, mp 117.5 ± 0.5 °C; $\delta_H$ (400 MHz) 0.94 (t, 3H, $J$ = 7.6 Hz, $CH_3$), 1.20-1.80 (m, 4H, 2 x $CH_2$), 2.57 (s, 3H, $SCH_3$), 3.48-3.68 (m, 2H, $CH_2$), 5.39 (s, 2H, $CH_2$), 7.00 (t, $J$ = 8.0 Hz, 1H, 5'-H), 7.25 (d, $J$ = 8.0 Hz, 1H, 6'-H), 7.57 (d, $J$ = 8.0 Hz, 1H, 4'-H), 7.68 (s, 1H, 2'-H) and 7.79 (br, 1H, 3-H); Anal. calcd for $C_{17}H_{20}IN_5S$: C, 45.04; H, 4.45; N, 15.45. Found C, 45.02; H, 4.69; N, 14.62%; ESMS (PI) 454. calcd for ($C_{17}H_{20}IN_5S$ + 1[H]). Found 454.

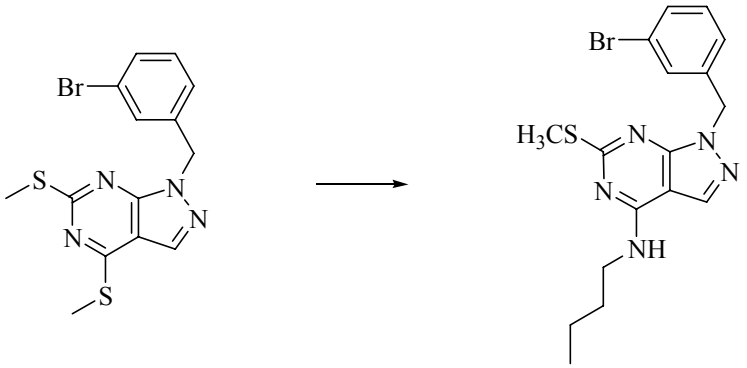
**1-benzyl-6-methylthio-4-butylamino-pyrazolo[3,4-*d*]pyrimidine (434)**



**437** **434**

Method G was used to prepare 1-benzyl-6-methylthio-4-butylamino-pyrazolo[3,4-*d*]pyrimidine **(434)** from 1-benzyl-4,6-bis-methylthio-pyrazolo[3,4-*d*]-pyrimidine **(437)** and butylamine. Yield (58%) as white solid, mp 84.5 ± 0.5 °C; $\delta_H$ (400 MHz) 0.92 (t, 3H, $J$ = 7.2 Hz, $CH_3$), 1.40 (s, 2H, $J$ = 7.2 Hz, $CH_2$), 1.63 (q, 2H, $J$ = 7.2 Hz, $CH_2$), 2.55 (s, 3H, $SCH_3$), 3.54 (m, 2H, $CH_2$), 5.46 (s, 2H, $CH_2$), 5.75-6.15 (br, 1H, NH), 7.19-732 (m, 5H, Ar-H) and 7.76 (br, 1H, 3-H); Anal. calcd for $C_{17}H_{21}N_5S$: C, 62.36; H, 6.46; N, 21.39. Found C, 62.20; H, 6.52; N, 21.39%; ESMS (PI) 328. calcd for ($C_{17}H_{21}N_5S$ + 1[H]). Found 328.

**1-(3-bromobenzyl)-6-methylthio-4-butylamino-pyrazolo[3,4-*d*]pyrimidine (435)**




**438** **435**

Method G was used to prepare 1-(3-bromobenzyl)-6-methylthio-4-butylamino-pyrazolo[3,4-*d*]pyrimidine **(435)** from 1-(3-bromobenzyl)-4,6-bis-methylthio-pyrazolo[3,4-*d*]pyrimidine **(438)** and butylamine. Yield (60%) as white solid, mp 113.5 ± 0.5 °C; $\delta_H$ (400 MHz) 0.92 (t, 3H, $J$ = 7.2 Hz, $CH_3$), 1.40 (s, 2H, $J$ = 7.2 Hz, $CH_2$), 1.62 (q, 2H, $J$ = 7.2 Hz, $CH_2$), 2.55 (s, 3H, $SCH_3$), 3.54 (m, 2H, $CH_2$), 5.41 (s, 2H, $CH_2$), 5.70-6.10 (br, 1H, NH), 7.12 (t, $J$ = 7.6 Hz, 1H, 5'-H), 7.21 (d, $J$ = 7.6 Hz, 1H, 6'-H), 7.34 (d, $J$ = 7.6 Hz, 1H, 4'-H), 7.44 (s, 1H, 2'-H) and 7.76 (br, 1H, 3-H; Anal. calcd for $C_{17}H_{20}BrN_5S$: C, 50.25; H, 4.96; N, 17.24. Found C, 50.20; H, 4.97; N, 17.21%; ESMS (PI) 406 and 408. calcd for ($C_{17}H_{20}{}^{79}BrN_5S$ + 1[H]) and ($C_{17}H_{20}{}^{81}BrN_5S$ + 1[H]). Found 406 and 408.

## 6.5 References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(5) Ferrarini, P. L.; Mori, C.; Manera, C.; Martinelli, A.; Mori, F. et al. Study on affinity profile toward native human and bovine adenosine receptors of a series of 1,8-naphthyridine derivatives. *Journal of Medicinal Chemistry* **2004**, *47*, 3019-3031.

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# CHAPTER 7

## Cell cultures, membrane preparation and radioligand binding assays

### 7.1 Introduction

The human $A_1$, $A_{2A}$ and $A_3$ receptors stably expressed in CHO (Chinese hamster ovary) cells were grown and the membranes were prepared for radioligand binding assays using the literature procedure.[1] All synthesized compounds in this study were evaluated for their affinities against the radioligands at the adenosine $A_1$, $A_{2A}$ and $A_3$ receptors. The percentage activity for each crude compound at these adenosine receptors was calculated using the equation in the **Figure 7.1**. The dissociation constants of the purified compounds ($K_i$ values) at $A_1$ and $A_3$ receptors were determined from radioligand binding data using the Cheng-Prusoff equation **(Figure 7.2)**.[2]

$$\% \text{ Activity} = 100\% - \left(\frac{\text{Unk} - \text{NSB}}{\text{TB} - \text{NSB}}\right) \text{ x } 100\%$$

Unk is the counted filter bound radioactivity for the binding in the presence of test compound.

NSB (non-specific binding) is the counted filter bound radioactivity for the binding in the presence of unlabeled compound.

TB (total binding) is the counted filter bound radioactivity for the binding of labeled compound to the receptor.

**Figure 7.1**: Percentage activity equation.

$$K_i = \frac{IC_{50}}{1 + \frac{L}{K_d}}$$

$K_i$ is the dissociation constant of the test compound

$IC_{50}$ is the concentration of test compound to cause 50% inhibition of labeled compound

$K_d$ is the dissociation constant of the labeled compound

L is the concentration of labeled compound.

**Figure 7.2**: Cheng-Prusoff equation.[2]

## 7.2 Materials

The human $A_1$, $A_{2A}$ and $A_3$ receptors stably expressed in CHO (Chinese hamster ovary) cells was obtained from Klotz, K. N, Institute of Pharmacology and Toxicology, University of Wurzburg, Germany. Cell culture media, fetal calf serum and penicillin (100 unit/ml) and streptomycin (100 µg/ml) were purchased from Biowhittaker. L-glutamine and geneticin were purchased from Gibco-life technologies. [$^3$H]NECA and [$^3$H]DPCPX were purchased from Amersham pharmacia biotech. All other materials were purchased from Sigma-Aldrich.

## 7.3 Cell culture

Frozen CHO cells stably transfected with human $A_1$ receptor were thawed in a warm water bath at 37 °C. Dulbecco's Modified Eagles Medium with nutrients F12 without nucleosides (DMEM/F12) (45 ml) containing 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 µg/ml), L-glutamine (2 mM) and geneticin (0.2 mg/ml) was added. Cells and media were transferred to a 250 ml culture flask and placed in an incubator with 5% $CO_2$/ 95% $O_2$ air at 37 °C. Once the cells were adhered to the surface of the flask and 80-90% confluent (1-1.5 days), the media was poured off (NB: don't pour medium over cells) and 0.02% EDTA in PBS (16 ml) was

added. The flask was placed back in an incubator for 10-15 min. The flask was agitated slightly and cells came off surface into EDTA/PBS. Cells in PBS (16 ml) was split into 8 x 250 ml culture flasks and media (45 ml) was added to each flask. Two and half day later, the cells in each flask were 80-90% confluent. Cells in each flask were collected into EDTA/PBS (20 ml/ flask) as before and then were split into 10 x 250 ml culture flasks. Media (45 ml) was added to each flask and 80 flasks of cells were left to grow. After 3 days, they were 80-90 % confluent. They were then collected in EDTA/PBS (15 ml/ flask) and pooled into 24 x 50 ml centrifuge tubes. They were spun for 10 mins at 1600 rpm and at 4 °C to remove EDTA/PBS. Cell pellets were kept at 0-4 °C for the membrane preparation.

CHO cells pellets stably transfected with human $A_{2A}$ and $A_3$ receptors were also prepared using the same procedure.

### 7.4 Membrane preparation

The $A_1$ cell pellets were suspended in an ice-cold hypotonic buffer (100 ml) (5 mM/ Tris/HCl, 2 mM EDTA, pH 7.4). The cell suspension was transferred to 6 x 25 ml high centrifuge tubes and homogenized on ice with ultra-turrax polytron twice at full speed (2 x 15 seconds). The homogenate was centrifuged for 35 min at 48000 rpm at 4 °C. The membrane pellets were re-suspended in 50 mM Tris/HCl buffer at pH 7.4 (120 ml). The membranes were stored at -80 °C for protein estimation and for binding assays.

The $A_{2A}$ and $A_3$ cell cell membranes were prepared using the same procedure. For $A_{2A}$ adenosine receptors, the membrane pellets were re-suspended in 50 mM Tris/HCl and 10 mM $MgCl_2$ buffer at pH 7.4 (140 ml). For $A_3$ adenosine receptors, the membrane pellets were re-suspended in 50 mM/HCl, 10 mM $MgCl_2$ and 1 mM EDTA buffer at pH 7.4 (140 ml).

### 7.5 Protein estimation

Bovine serum albumin (BSA) (200 mg) was dissolved in phosphate buffered saline (PBS) (25 ml) to give a stock concentration of 8 mg/ml. Each standard solution (160 µl) was prepared as in **Table 7.1**. Each standard (40 µl) was dispensed into each well. 10% sodium dodecyl sulfate (SDS) in water (10 µl) was added to all experimental wells to digest the protein. Freshly prepared BCA protein estimation kit reagents A & B (A/B:50/1) (200 µl) was added to each well. The plate was incubated for 30 mins at 37 °C. The optical density of each well was measured at wavelength of 540 nm.

**Table 7.1**: Preparation of the standard for protein estimation.

| Standards | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| mg/ml | 6.25 | 4.69 | 3.75 | 3.13 | 2.5 | 2.19 | 1.88 | 1.25 | 0.63 | 0.31 | 0.16 | 0 |
| mg/ml | 1 | 0.75 | 0.6 | 0.5 | 0.4 | 0.35 | 0.3 | 0.2 | 0.1 | 0.05 | 0.03 | 0 |
| $V_{BSA}$ (µl) | 125 | 94 | 75 | 63 | 50 | 44 | 38 | 25 | 13 | 6 | 3 | 0 |
| $V_{H20}$ (µl) | 35 | 66 | 85 | 98 | 110 | 116 | 123 | 135 | 148 | 154 | 157 | 160 |

The curve of standard BSA concentration versus absorbance **(Figure 7.3)** was used to calculate the protein concentration of receptor in the buffer.

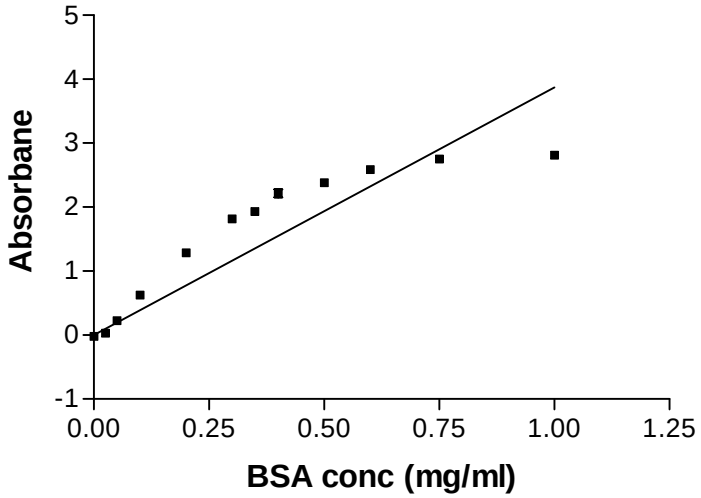


**Figure 7.3**: The graph of BSA concentration versus absorbance.

In the same plate, each dilution of unknown (160 µl) was prepared as in **Table 7.2** to give a dilution factor for each dilution. Similarly, each standard (40 µl) was dispensed into each well. 10% sodium dodecyl sulfate (SDS) in water (10 µl) was added to all experimental wells to digest the protein. The homogenate mixture went clear after addition of SDS. Freshly prepared BCA protein estimation kit reagents A & B (A/B:50/1) (200 µl) was added to each well. The plate was incubated for 30 mins at 37 °C. The optical density of each well was measured at a wavelength of 540 nm. Using the graph of standard BSA concentration versus absorbance, the protein concentration of the $A_1$, $A_{2A}$ and $A_3$ receptors could be determine from the value of the absorbance of each well.

**Table 7.2**: Preparation of the unknown for protein estimation.

| Dilution of unknown | **1** | **2** | **3** | **4** | **5** | **6** |
|---|---|---|---|---|---|---|
| Dilution factor | 0.625 | 0.313 | 0.125 | 0.106 | 0.081 | 0.063 |
| Dilution factor | 1/10 | 1/20 | 1/50 | 1/60 | 1/80 | 1/100 |
| $V_{homogenate}$ (µl) | 100 | 50 | 20 | 17 | 13 | 10 |
| $V_{H20}$ (µl) | 60 | 110 | 140 | 143 | 148 | 150 |

The protein concentration of the $A_1$, $A_{2A}$ and $A_3$ receptors were 0.95 mg/ml, 1.53 mg/ml and 0.309 mg/ml.

## 7.6 Radioligand binding

### 7.6.1 $A_1$ receptor binding assay

All synthesized compounds were tested for their ability to inhibit the binding of the $A_1$ antagonist labeled ligand [$^3$H]DPCPX ([*propyl*-$^3$H]8-cyclopentyl-1,3-dipropylxanthine) to the receptor using the literature procedure.[1] The assays were carried out in 96-well microtitre plates. Each assay contained membrane protein (25 µg, adenosine deaminase (0.2 U/ml), test compound in DMSO with a final DMSO concentration of 1% and 2 nM [$^3$H]DPCPX in a total volume of 200 µl binding buffer of 50 mM Tris/HCl pH 7.4. Nonspecific binding was determined in the presence of

100 μM R-PIA (R-$N^6$-phenylisopropyladenosine). The assay was incubated for 3h at room temperature and then filtered through presoaked filtermats B in 0.1% polyethyleneimine (PEI) using the Brandel cell harvester. The filtermat was dried at 60 °C for 1 h. It was then soaked with scintillant fluid (Wallac scintillation products) in a sealed bag. The filter bound radioactivity was counted using a Micro Beta® Trilux Wallace scintillation counter. The assays for crude products were performed in duplicate and the percentage activity was calculated using the equation in the **Figure 7.1**. The assays for purified compounds were performed twice in duplicate. All binding data was analyzed using a non-linear regression program (GraphPad Prism). $K_i$ value for purified compound was calculated using the Cheng-Prusoff equation **(Figure 7.2)**.

### 7.6.2 $A_{2A}$ receptor binding assay

All synthesized compounds were tested for their ability to inhibit the binding of the $A_{2A}$ agonist labeled ligand [$^3$H]NECA (5'-N-Ethylcarboxamido[8(n)-$^3$H]adenosine) to the receptor using the literature procedure.[1] The assays were carried out in 96-well microtitre plates. Each assay contained membrane protein (50 μg), adenosine deaminase (0.2 U/ml), test compound in DMSO with a final DMSO concentration of 1% and 50 nM [$^3$H]NECA in a total volume of 200 μl binding buffer of 50 Tris/HCl and 10 mM $MgCl_2$ buffer at pH 7.4. Nonspecific binding was determined in the presence of 100 μM NECA. The assay was incubated for 3h at room temperature and then filtered through presoaking filtermats B in 0.1% polyethyleneimine (PEI) using the Brandel cell harvester. The filtermat was dried at 60 °C for 1 h. It was then soaked with scintillant fluid (Wallac scintillation products) in a sealed bag. The filter bound radioactivity was counted using a Micro Beta® Trilux Wallace scintillation counter. The assays for crude products were performed in duplicate and the percentage activity was calculated using the equation in the **Figure 7.1**.

### 7.6.3 $A_3$ receptor binding assay

All synthesized compounds were tested for their ability to inhibit the binding of the $A_{2A}$ agonist labeled ligand [$^3$H]NECA (5'-N-Ethylcarboxamido[8(n)-

$^{3}$H]adenosine) to the receptor using the literature procedure.[1] The assays were carried out in 96-well microtitre plates. Each assay contained membrane protein (60 µg), adenosine deaminase (0.2 U/ml), test compound in DMSO with a final DMSO concentration of 1% and [$^{3}$H]NECA (40 nM) in a total volume of 200 µl binding buffer of 50 mM/HCl, 10 mM $MgCl_2$ and 1 mM EDTA at pH 7.4. Nonspecific binding was determined in the presence of 100 µM NECA. The assay was incubated for 3h at room temperature and then filtered through presoaking filtermats B in 0.1% polyethyleneimine (PEI) using the Brandel cell harvester. The filtermat was dried at 60 °C for 1 h. It was then soaked with scintillant fluid (Wallac scintillation products) in a sealed bag. The filter bound radioactivity was counted using a Micro Beta® Trilux Wallace scintillation counter. The assays for crude products were performed in duplicate. The assays for purified compounds were performed twice in duplicate and the percentage activity was calculated using the equation in the **Figure 7.1**.

## 7.7 References


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