Application of Squaric Acid to The Preparation of Bioactive Compounds

By

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ABSTRACT

Nucleosides and nucleoside analogues exhibit a broad spectrum of biological activities including antiviral, anticancer, antibacterial and antiparasitic activities, which generally result from their ability to inhibit specific enzymes. Nucleoside analogues can interact with cellular enzymes involved in the biosynthesis or degradation of RNA (ribonucleic acid) and/or DNA (deoxyribonucleic acid) or with specific viral enzymes to result in their biological activities and therapeutic effects. In addition, another possible target is their incorporation into DNA/RNA which could affect replication and transcription. They have been beneficial to the development of new pharmaceuticals. Squaric acid and its derivatives have been successfully used as a bioisosteric group in various biomedicinal areas. The aim of this research proposal was to apply squaric acid analogues to the design and synthesis of novel nucleoside analogues.

Three squaric acid-based new nucleoside analogues were made starting from dimethyl squarate. The compounds were 4-amino-3-[((1R,3S)-3-hydroxymethyl-4-cyclopentene)-1-amino]-3-cyclobutene-1,2-dione, 4-methoxy-3-[((1R,3S)-3-hydroxymethyl-4-cyclopen tene)-1-amine]-3-cyclobutene-1,2-dione, and 4-hydroxy-3-[((1R,3S)-3-hydroxymethyl-4-cyclopentene)-1-amine]-3-cyclobutene-1,2-dionate, sodium salt. A key step in their synthesis was the reaction of (1R, 4S)-(-)-4-(hydroxymethyl)cyclopent-2-en-1-ylamine with 4-amino-3-methoxy-3-cyclobutene-1,2-dione, or 3,4-dimethoxy-3-cyclobutene-1,2-dione, followed by hydrolysis to give the above compounds.

They were sent to the Developmental Therapeutics Program (DTP) of the National Cancer Institute (NCI, USA) to screen and test *in vitro* for their potential anticancer activity in cellular assays. Little to modest antitumour activity was detected for these compounds. Meanwhile, their cytotocity to HeLa cells was investigated as well. However, no significant effect was observed by these three compounds. Also, these compounds were sent out to the National Institute of Allergy and Infectious Diseases (NIAID, USA) to test their antiviral activity against various viruses. These tests are in progress.

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To my family

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LIST OF ABBREVIATIONS

AACF	Antimicrobial Acquisition and Coordinating Facility
AICAR	5-Amino-1-β-D-ribofuranosylimidazole-4-carboxamide
AMPA	2-Amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid
Ara-G	9-beta-D-Arabinofuranosylguanine
5-Azacytidine	4-Amino-1-β-D-ribofuranosyl-1,3,5-s-triazin-2-one
Boc	*tert*-butoxycarbonyl
Boc_2O	Di-*tert*-butyldicarbonate
CANs	Carbocyclic nucleoside analogues
Carbovir	2',3'-Didehydro-2',3-dideoxyguanosine
CNS	Central nervous system
COSY	Correlation spectroscopy
Dimethyl squarate	3,4-Dimethoxy-3-cyclobutene-1,2-dione
DMAP	4-(N,N-dimethylamino)pyridine
DMF	Dimethylformamide
2,2-DMP	2,2-Dimethoxypropane
DNA	Deoxyribonucleic acid
DTP	Developmental Therapeutics Program
EAA	Excitatory amino acids
EDTA	Ethylenediamine tetraacetic acid
EI	Electron impact

ESI	Electrospray ionization
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FT-IR	Fourier transform infrared spectrometer
GABA	γ-Aminobutyric acid
h	hour
HBSS	Hank's balanced salt solution
HIV	Human immunodeficiency virus
HMQC	Heteronuclear multiple quantum correlation
HPV	Human papilloma virus
HRMS	High-resolution mass spectrum spectrum
IMP	Inosine 5'-monophosphate
IR	Infrared spectrometry
LRMS	Low-resolution mass spectrum
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCI	National Cancer Institute
NCI60	NCI 60 human tumour cell line anticancer drug screen
NIAID	National Institute of Allergy and Infectious Diseases
NMDA	*N*-methyl-D-aspartic acid
NMO	N-methylmorpholine N-oxide
NMR	Nuclear magnetic resonance
NPTX-8	Nephilatoxin-8
NSAIDs	Non-steroid anti-inflammatory drugs

PBS	Phosphate buffered saline
PhTX	Philanthotoxin
R_f	Retention factor
Ribavirin	1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
Squaric acid	3,4-Dihydroxy-3-cyclobutene-1,2-dione
TAR	Transactivation responsive
Tat	Transactivator of transcription
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMS	Tetramethylsilane
TsOH	*p*-Toluenesulphonic acid
Ziagen	(1*S,cis*)-4-[2-amino-6-(cyclopropylamino)-9*H*-purin-9-yl]-2-cyclopentene-1-methanol sulfate (salt)

CHAPTER 1

INTRODUCTION

1.1 Bioisosterism in drug design

Drug design is of critical importance in human health care. It is a process whereby the design and synthesis of a compound are undertaken and the biological and physical properties of a lead compound are studied [1]. A lead compound should be well understood as to its desired pharmacological activity and also as to its undesirable side effects, metabolic pathways, and structural factors related to its physicochemical characteristics which can limit the compound's bioavailability [2]. Bioisosterism is a method used in medicinal chemistry for the rational modification of a lead compound in order to improve its pharmacological activity, reduce its adverse effects or even optimize the biological activity and the pharmacokinetics that the lead compound might have. Such modifications may enhance the safety and therapeutic efficacy of the compound under investigation [3].

1.1.1 A primer on bioisosterism

The antecedent of bioisosterism is isosterism, which was first reported by Langmuir in 1919 regarding the similarities of various physicochemical properties of atoms, groups, radicals, and molecules [4]. Some of these groups are listed in Table 1 [3].

Table 1. Groups of Isosteres as Identified by Langmuir [3]

groups	isosteres
1	H^-, He, Li^+
2	O^{2-}, F^-, Ne, Na^+, Mg^{2+}, Al^{3+}
3	S^{2-}, Cl^-, Ar, K^+, Ca^{2+}
4	Cu^{2-}, Zn^{2+}
↓	↓
8	N_2, CO, CN^-
9	CH_4, NH_4^+
10	CO_2, N_2O, N_3^-, CNO^-
↓	↓
20	MnO_4^-, CrO_4^{2-}
21	SeO_4^{2-}, AsO_4^{3-}

In 1925, Grimm further broadened this concept of isosteres with his Hydride Displacement Law (From the left to the right in Table 2) [2]. This law states that one hydrogen atom (H) with a pair of electrons (i.e. hydride) is added after another atom and gives a new isoelectronic pseudoatom with properties with the same physical characteristics of the elements and groups in the vertical columns behind the original atom [5].

Table 2. Grimm's Hydride Displacement Law [2]

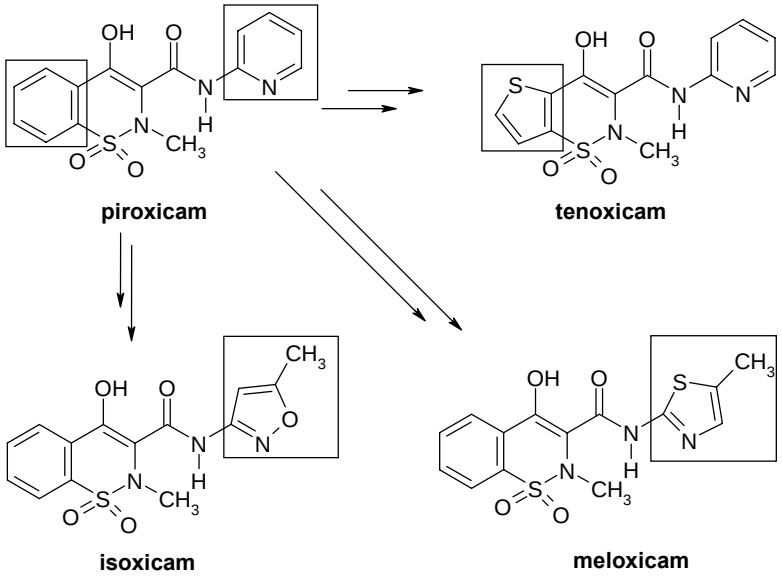
	Group 4A	Group 5A	Group 6A	Group 7A	Group 8A	
No. of e^-	6	7	8	9	10	
	C	N	O	F	Ne	Na
	$\xrightarrow{H^-}$	CH	NH	OH	FH	–
		$\xrightarrow{H^-}$	CH_2	NH_2	OH_2	FH_2^+
			$\xrightarrow{H^-}$	CH_3	NH_3	OH_3^+
				$\xrightarrow{H^-}$	CH_4	NH_4^+

A further extension of the concept of pseudoatoms came about in 1932 by Hans Erlenmeyer. He redefined isosteres as atoms, ions, and molecules with the same number of electrons at the valence level [3] and created a concept of electronically equivalent rings, later broadened to the term ring bioisosterism [2].

During the early 1950s, Harris L. Friedman foresaw the application of the concept of isosterism to the design of bioactive molecules [6]. He coined the term bioisosterism to include all atoms and molecules which fit the broadest definition of isosteres and exercise their biological activity, no matter whether they act as agonists or antagonists (an agonist being a compound that interacts with a receptor eliciting a response and an antagonist one that prevents the action of agonists at the receptor) [7]. However, Thornber found that the term bioisosterism introduced by Friedman was to describe the phenomenon in which structurally related substances have similar or antagonistic biological properties [5]. So a loose flexible term for bioisosteres was defined as: “Bioisosteres are subunits or groups or molecules which possess physicochemical properties of similar biological effects” [5]. In 1991, this definition was broadened by Burger as “Bioisosteres are compounds or groups that possess near-equal molecular shapes and volumes, approximately the same distribution of electrons, and which exhibit similar physical properties such as hydrophobicity. Bioisosteric compounds affect the same biochemically associated systems as agonists or antagonists and thereby produce biological properties that are related to each other” [7].

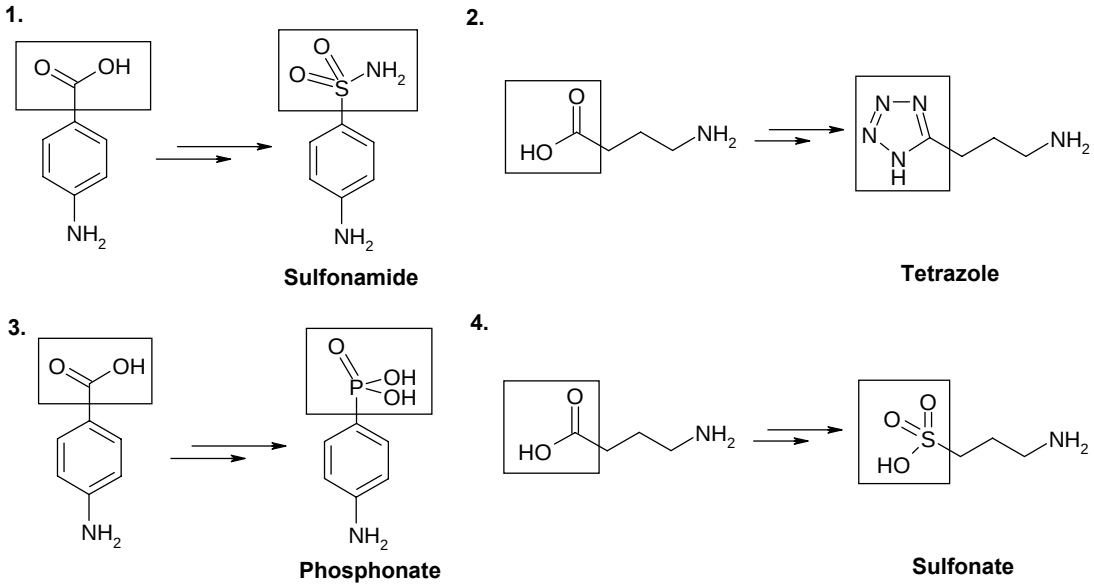
1.1.2 Some important bioisosteric groups

Bioisosteres have been classified as either classical or non-classical. Classic bioisosteres have been divided into: (A) monovalent atoms or groups; (B) divalent atoms or groups; (C) trivalent atoms or groups; (D) tetravalent atoms; and (E) ring equivalents [3]. They are successfully used in the structural design of new drugs. For example, ring bioisosterism is one of the most useful approaches in the drug design of different therapeutic classes [7]. Binder and coworkers had successfully explored new non-steroid anti-inflammatory drugs (NSAIDs) of the oxican group by application of ring bioisosterism (Scheme 1.1) [2]. Piroxicam is a prototype NSAID belonging to the oxicam group. The benzothiazinic nucleus of piroxicam was replaced by the thienothiazinic moiety to synthesize tenoxicam. Isoxicam and meloxicam possess a 5-methylisoxazole ring and 5-methyl-2-thiazolyl ring respectively, as an equivalent of the pyridine ring of piroxicam and tenoxicam [8]. So the ring bioisosteres of the benzothiazinic nucleus may have the same or similar pharmacotherapeutic profiles as piroxicam and tenoxicam, while other bioisosteres at the pyridine ring may have the same or similar pharmacotherapeutic activity as isoxicam and meloxicam.



Scheme 1.1

Non-classic bioisosteres are those groups which practically do not fit the steric and electronic definitions of classical isosteres. Moreover, they do not have the same number of atoms as the substituent or moiety. Non-classic bioisosteres can be divided into groups: (A) cyclic *vs* noncyclic; (B) functional groups; (C) retroisosterism [2]. They have a significant predominance in drug design because of their utility in distinct therapeutic categories such as selective receptor antagonist or agonist drugs, enzyme inhibitors or anti-metabolites [3]. Consider the bioisosterism of functional groups as an example. Numerous functional groups are known for their bioisosteric replacement of the carboxylate group such as sulfonamide [3], tetrazole [2], phosphonate [7], and sulfonate [7] groups (Scheme 1.2). The similarities of these bioisosteres are based on electronic and conformational aspects, as well as physicochemical properties such as acidity and lipophilicity.


1.
O
OH
NH2
O
O
S
NH2
NH2
Sulfonamide
2.
O
HO
NH2
N
N
N
N
H
NH2
Tetrazole
3.
O
OH
NH2
O
P
OH
OH
NH2
Phosphonate
4.
O
HO
NH2
O
O
S
HO
NH2
Sulfonate

Scheme 1.2

1.2 Squaric acid analogues as bioisosteric groups

Squaric acid (3,4-dihydroxy-3-cyclobutene-1,2-dione) is an intriguing multi-functional organic molecule which has a cyclobutenyl ring system [9]. It has 2π electrons in its completely deprotonated form and a negative charge on each of the carbonyl oxygen atoms in its resonance form (Figure 1) [10, 11]. It has been suggested that this resonance structure serves as a good electrostatic mimic for negatively charged groups such as the carboxylate group and phosphate group [12]. Furthermore, reaction of squaric acid **1** with alcohols produces esters **2**. This reaction occurs readily in the presence of an acid catalyst like the usual esterification of carboxylic acids. Replacing the squaric acid esters **2** by amines gives the corresponding diamides **3** or monoamide monoesters **4** (Figure 1). These have been applied to enhance the biological activity of several drugs and to functionalize organic molecules [10]. As a result, derivatives of squaric acid have attracted attention as bioisosteric replacements in bioorganic and medicinal chemistry.

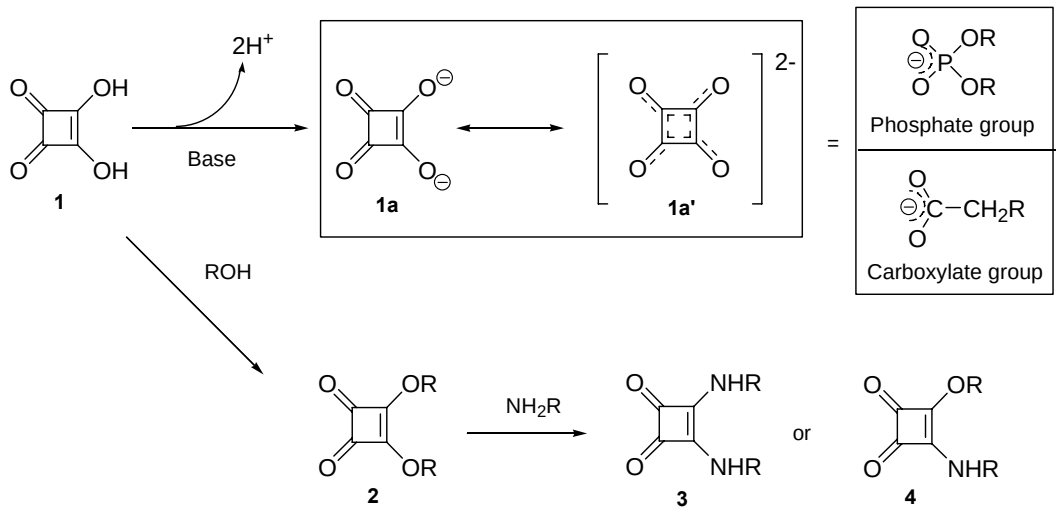


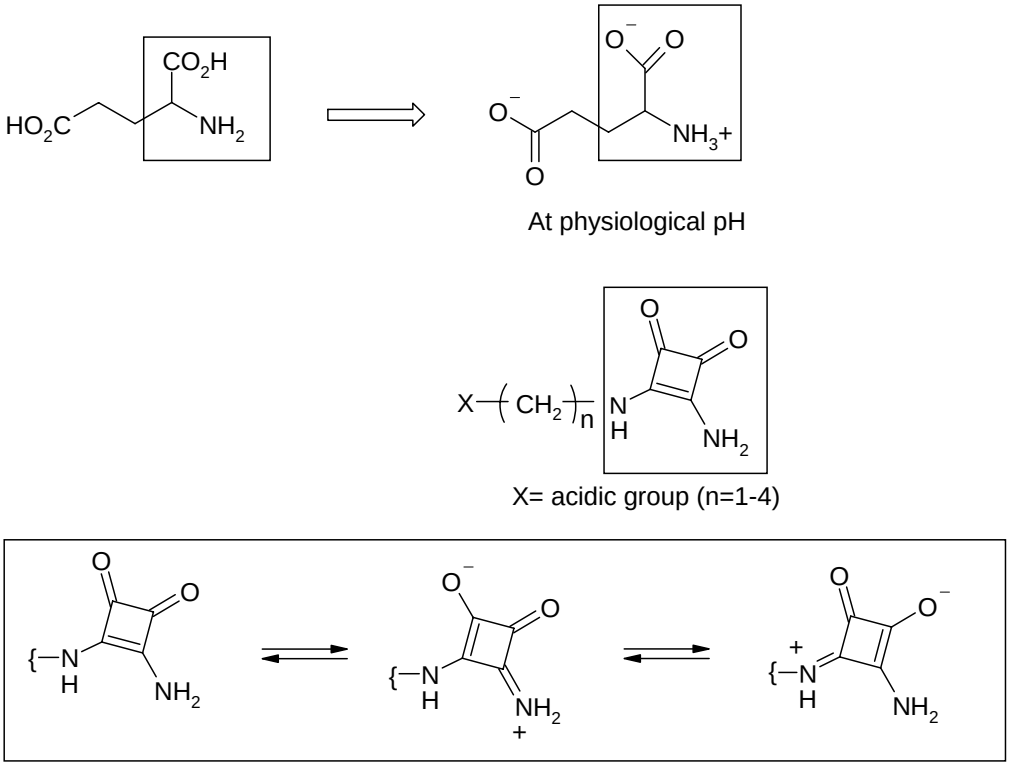
Figure 1: Structures of squaric acid (1), squarates (2), squaryldiamides (3) and squaric monoamide monoesters (4); and resonance structures of squaric acid showing the electrostatic similarity to phosphate group and carboxylate group [10, 13].

1.2.1 Literature review of biomedical applications of squaric acid analogues

Since squaric acid was first synthesized by Cohen *et al.* in 1959 [14], a number of derivatives have been generated and some of their properties have been studied in different areas in recent years. A number of pharmaceutical applications of squaric acid diesters are known. For instance, the di-*n*-butylester of squaric acid is a potent allergen and has been used in the treatment of *alopecia areata*, a non scarring form of hair loss, and in the immunotherapy of warts in children [15-18].

A number of other squaric acid amide-based pharmaceutically active compounds have been studied. For example, squaric acid derivatives have been used to mimic carboxylates or the α-aminocarboxylic acid unit for various receptor molecules by several investigators. Kinney and coworkers replaced the entire α-amino carboxylic acid in various *N*-methyl-D-aspartic acid (NMDA) antagonists with a squaric acid derivative

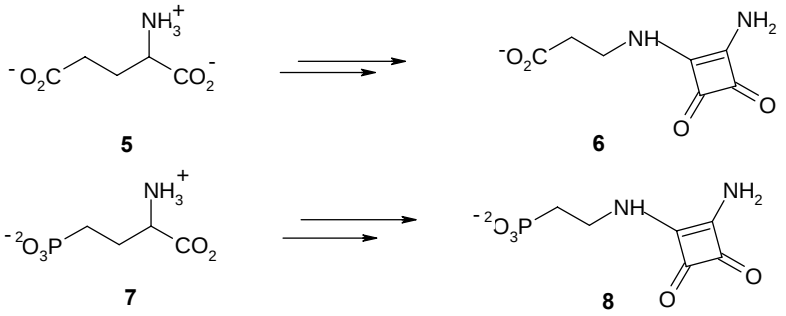
(3,4-diamino-3-cyclobutene-1,2-dione) (Scheme 1.3) [19]. At physiological pH, the α-amino carboxylic acid group is present as its zwitterion. The 3,4-diamino-3-cyclobutene-1,2-dione moiety contains a dipole possessing a partial negative charge on the carbonyls and a partial positive charge on the nitrogen atoms (Scheme 1.3). For these stated reasons, Kinney chose the 3,4-diamino-3-cyclobutene-1,2-dione as a possible electronic mimic of the α-amino carboxylic acid to improve bioavailability and brain penetration of NMDA antagonists [19].



Scheme 1.3

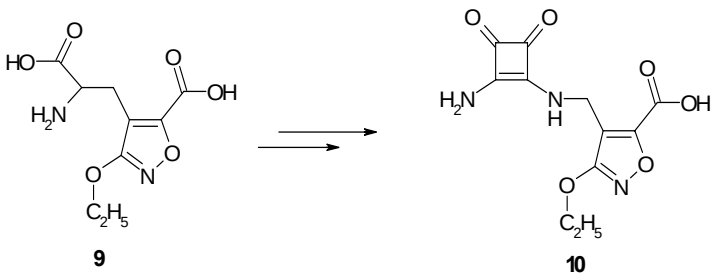
As shown in Scheme 1.4, the α-amino carboxylic acid of compound **5** (glutamic acid) was replaced by 3,4-diamino-3-cyclobutene-1,2-dione to produce compound **6** [19]. Examining the functional activities of compound **5** and **6** showed that compound **6** was a

weak functional NMDA antagonist relative to compound **5**. However, optimizing NMDA receptor potency of several phosphonic acid derivatives including compounds **7** and **8**, demonstrated that compound **8** had extremely potent NMDA antagonist properties [19].



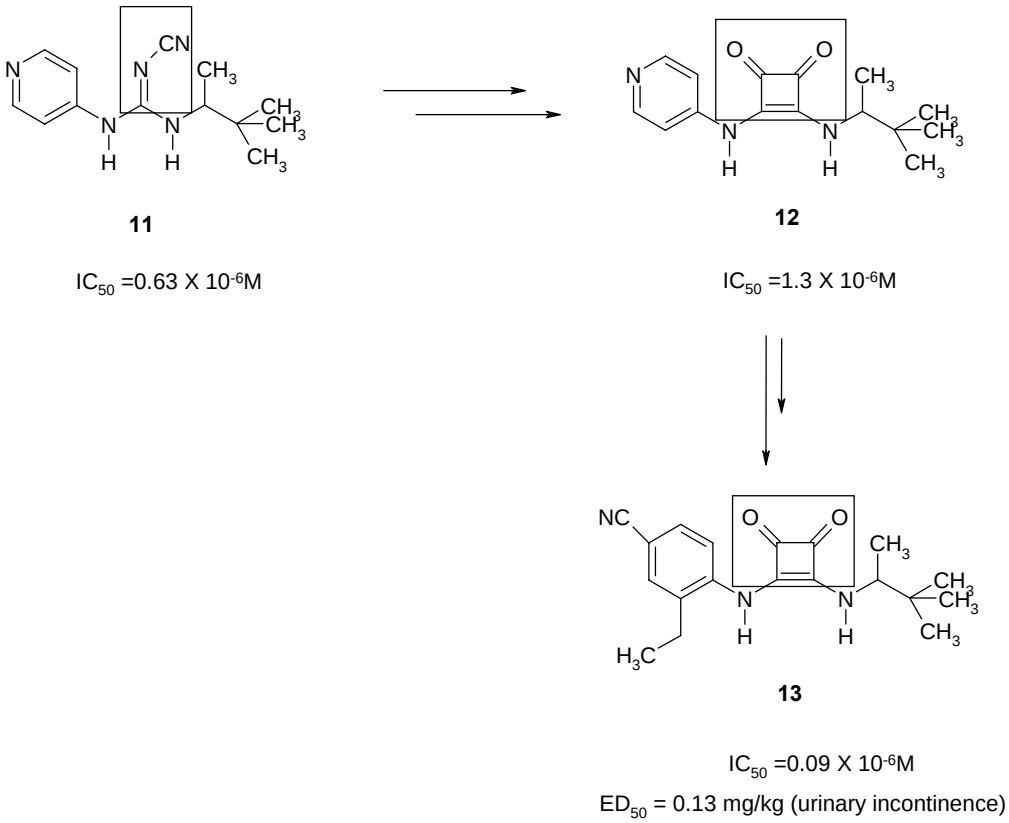
Scheme 1.4

Benny and co-workers [20] also used the 3,4-diamino-3-cyclobutene-1,2-dione group to substitute the α-amino carboxylic acid unit of 2-amino-3-(5-carboxy-3-ethoxy-4-isoxazolyl)propionic acid **9** (Scheme 1.5). The receptor binding of both compounds was studied using rat brain membranes. Compound **9** is a potent inhibitor of [^{3}H]AMPA (2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid) receptor binding and showed agonist potency, while compound **10** was completely devoid of an effect on AMPA receptors and had only weak affinity for NMDA receptor sites, reflecting a weak NMDA antagonist effect.



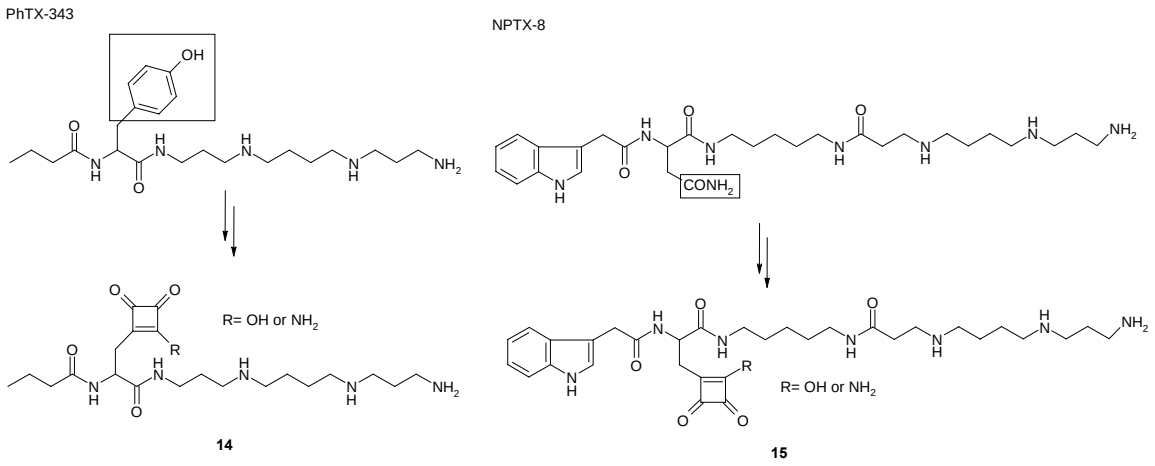
Scheme 1.5

The 3,4-diamino-3-cyclobutene-1,2-dione group has been also recognized as an isostere for functional groups, such as cyanoguanidine. In the quest for new selective agonists of potassium channels, Butera and coworkers carried out modifications in the structure of anti-hypertensive lead compound pinacidil **11** (Scheme 1.6). They described the replacement of the *N*-cyanoguanidine template, present in pinacidil, with a 1,2-diaminocyclobutene-3,4-dione moiety which afforded a novel series of potent bladder-selective agonists of the K_{ATP} channel **12** and **13**, as a novel drug candidate to treat urge urinary incontinence [21, 22].



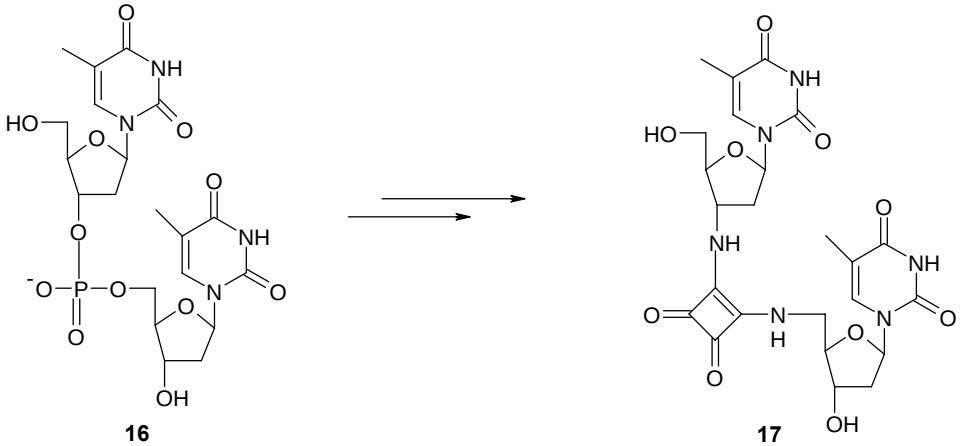
Scheme 1.6

Shinada replaced the phenol group of tyrosine within philanthotoxin (PhTX) and the amide group of glutamine within nephilatoxin-8 (NPTX-8) with squaric acid derivatives as shown in Scheme 1.7 [9]. The paralytic activity was examined for PhTX-343, NPTX-8 and analogues **14** and **15**. The paralytic activity of compound **14** (R=OH or NH_2) was 10-100-fold less potent than that of PhTX-343. On the other hand, the paralytic activity of compound **15** (R=OH or NH_2) showed more potent paralytic activity than that of natural NPTX-8, even much more potent than those of PhTX-343. Among them, the glutamine-type **15** (R=NH_2) was found to be the most potent analog. These compounds were further shown to be selective potent antagonists of ionotropic glutamate receptors.



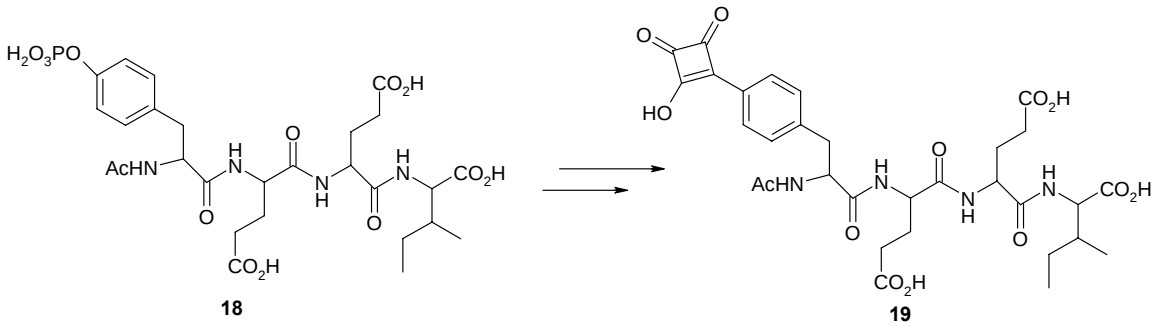
Scheme 1.7

Derivatives of squaric acid have also been used to mimic the phosphate group in phosphotyrosine residues, and that these compounds can be used to design effective inhibitors of protein tyrosine phosphatases [11]. Sekine and co-workers have used a diamide of squaric acid to replace a phosphate diester linkage in an oligodeoxynucleotide **16** (Scheme 1.8) [10]. The modified oligodeoxynucleotide **17** exhibited properties such as increased binding affinity for the target nucleic acid, a resistance to degradation by nucleases, and increased membrane permeability [10].



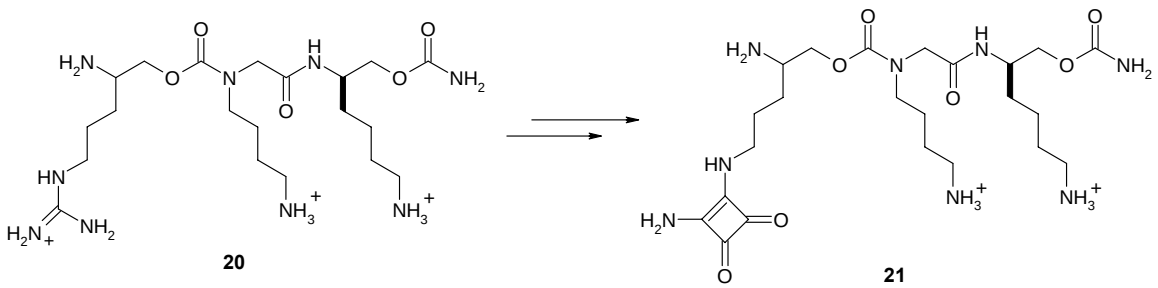
Scheme 1.8

Beaulieu and co-workers replaced phosphate in a peptide-based ligand **18** for an SH2 domain with squaric acid (Scheme 1.9) [23]. Unfortunately, the resultant compound **19** had severely attenuated binding affinity within the phosphate binding pocket of the $p56^{lck}$ SH2 domain.



Scheme 1.9

Recently, Chi-Wan Lee and co-workers discovered a novel peptidomimetic containing the squaryldiamide moiety as a new potential bioisosteric group for guanidine that binds transactivation responsive (TAR) RNA with high affinity (Scheme 1.10) [24]. Compound **20** (inhibitor TR87) showed potent, sustained anti-HIV-1 activity that did not significantly affect cell viability in single-round replication assays. The squaryldiamide derivative **21** was tested as a transactivator of transcription (Tat)-TAR inhibitor and was able to bind TAR RNA with high affinity. In summary, the squaryldiamide could be used as a potential bioisostere of unsubstituted guanidine in peptidomimetics. It was easily incorporated into a carbonate monomer which could then be coupled to produce peptidomimetics for Tat-TAR antagonists [24].



Scheme 1.10

1.2.2 Previous work in our laboratory

Dr. P. Chan and co-workers in the Honek laboratory have used derivatives of squaric acid to substitute the carboxylate group to synthesize several novel *N*-(hydroxydioxocyclobutenyl)-containing analogues of *γ*-amino-butyric acid and L-glutamate [13]. Glutamate is an excitatory amino acid (EAA), which is a critical neurotransmitter in the central nervous system (CNS) [25]. The EAA neurotransmitter effects are mediated by three heterogeneous classes of ionotropic receptors named NMDA, AMPA, and kainic acid receptors and a number of subtypes of metabotropic receptors [26]. The subtypes of these receptors have been associated with certain neurologic and psychiatric diseases and have been suggested to be potential targets for the treatment of such diseases.

The three *N*-(hydroxydioxocyclobutenyl)-containing analogues **22**, **23**, and **24** (Figure 2) were successfully synthesized by suitable protection of the diamine or diamino acid followed by reaction with diethyl squarate. These analogues were screened as displacers in several receptor binding assays. The diaminopropane analogue **23** showed poor affinity for various binding sites. The diaminoethane analogue **22** exhibited little affinity to $GABA_A$ and $GABA_B$ (GABA: *γ*-aminobutyric acid) binding sites. In contrast, the

glutamate analogue **24** exhibited potent binding site affinities compared to the GABA analogues.

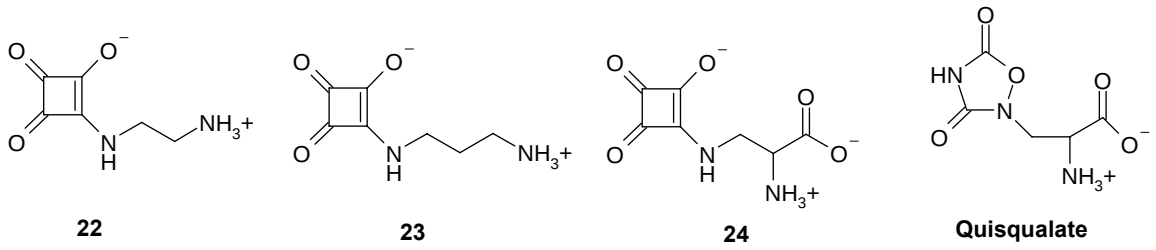


Figure 2: The structure of three *N*-(hydroxydioxocyclobutenyl)-containing analogues and Quisqualate [13]

Moreover, pharmacological actions and quisqualate (Figure 2) sensitization of neurons (QUIS-effect) by these three compounds **22**, **23**, and **24** were studied [13]. For pharmacological information, the compounds were examined for agonist potency for CA1 pyramidal neurons in a rat hippocampal slice preparation. The results were that compounds **22** and **23** showed little activity in this assay, while compound **24** rivaled AMPA as one of the most potent agonists for depolarizing pyramidal neurons in media in which kainate/AMPA receptors were active but NMDA receptors were inhibited. It was less potent for depolarizing pyramidal neurons in another media in which kainate/AMPA receptors were inhibited but NMDA receptors were active. For the QUIS-effect, compounds **22**, **23**, and **24** did not induce sensitization of CA1 pyramidal neurons and did not inhibit the sensitization induced by exposure to L-quisqualic acid.

1.3 Nucleoside analogues

Nucleosides and nucleotides are of fundamental importance for all living systems, such as structural modules of nucleic acids, cofactors, and messenger substances [27, 28]. Therefore, it is not surprising that nucleoside analogues have attracted considerable attention, mainly as antitumor, antiviral, antimicrobial [29], and immunosuppressive [30] agents in pharmacology.

While certain nucleoside analogues are incorporated into nucleic acids as chain terminators, thereby interrupting the replication of cancer cells or a virus, others are designed to block certain enzymes necessary for cancer or viral reproduction. Numerous modifications to heterocyclic nucleobase moieties as well as the sugar ring of the nucleosides have been applied for many years to increase their chemotherapeutic activities [31]. These studies have resulted in the development of many clinically useful drugs.

1.3.1 Furanose-derived nucleoside analogues

The base moiety of nucleosides has been replaced by five-membered heterocycles and these nucleosides exhibit a wide range of biological activities. Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, Figure 3) was the first synthetic nucleoside exhibiting a broad spectrum of antiviral activities against many RNA and DNA viruses [32]. There are many other nucleoside analogues of certain five-membered heterocycles, such as tiazofurin, bredinin, pyrazomycin, and pyrrole dicarboxamide (Figure 3). These compounds are structurally similar to 5-amino-1-β-D-ribofuranosylimidazole-4-carboxamide (AICAR), which is a key intermediate in purine nucleotide biosynthesis

[33]. Thus, all of these nucleosides are potent inhibitors of inosine 5'-monophosphate (IMP) dehydrogenase. The latter is one of the key rate-controlling enzymes for de novo guanine nucleotide biosynthesis and has been considered as a target enzyme for anticancer chemotherapy as well as antiviral and antiparasitic chemotherapy [34].

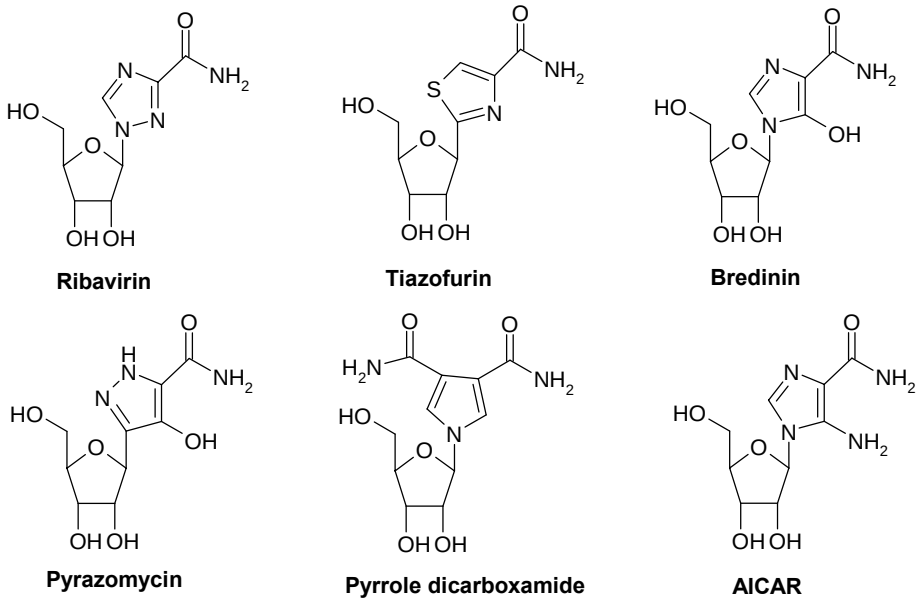


Figure 3: Nucleoside analogues containing certain five-membered heterocycles

Few studies have been done for pyrimidine-modified nucleosides, but these compounds have exhibited anticancer, antiviral and antibacterial activities. For example, 5-azacytidine (4-amino-1-β-D-ribofuranosyl-1,3,5-s-triazin-2-one, Figure 4) inhibits Gram-negative bacteria and is active against T-4 lymphomas and L-1210 leukaemia. It has been used clinically in the treatment of acute leukemia [28]. The compounds 6-azacytidine and 3-deazacytidine (Figure 4) have been prepared and also show anticancer and antiviral activity [35].

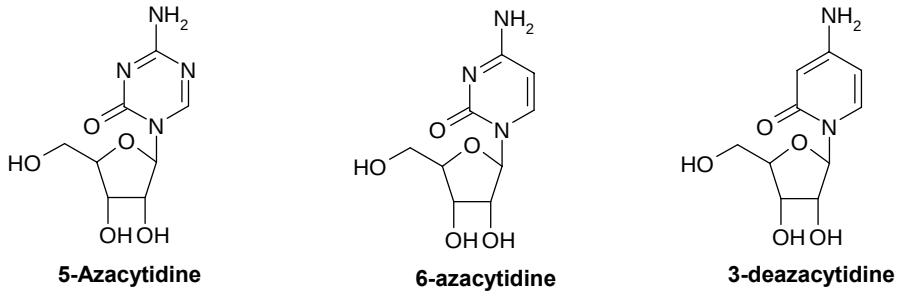


Figure 4: Some pyrimidine-modified nucleosides

A wide range of purine-modified nucleosides have been prepared and their biological activities have been examined. Modifications of adenosine in the heterocyclic base moiety have led to many other biologically active molecules. Tubercidin (Figure 5), the naturally occurring pyrrolo[2,3-d]pyrimidine analogue of adenosine, exhibits significant antineoplastic and antiviral properties [36]. The compound 2-azaadenosine (Figure 5) exhibits modest in vivo activity against L1210 cells [37]. The analogue 1-deazaadenosine (Figure 5) has been used as an inhibitor of blood platelet aggregation and adenosine deaminase and shows potential antitumour activity [38, 39]. The compound 3-deazaadenosine (Figure 5) acts as an alternate substrate competing with adenosine and 3-deazaguanine (Figure 5) exhibits potential cytotoxic activity in vitro against a variety of tumor cells, such as L1210, HeLa, and human KB cells [40, 41]. In 2005, nelarabine (Figure 5) was approved by the US Food and Drug Administration (FDA) for oncology indication. It is a prodrug of the antimetabolite 9-beta-D-Arabinofuranosylguanine (ara-G) for acute lymphocytic leukemia [42].

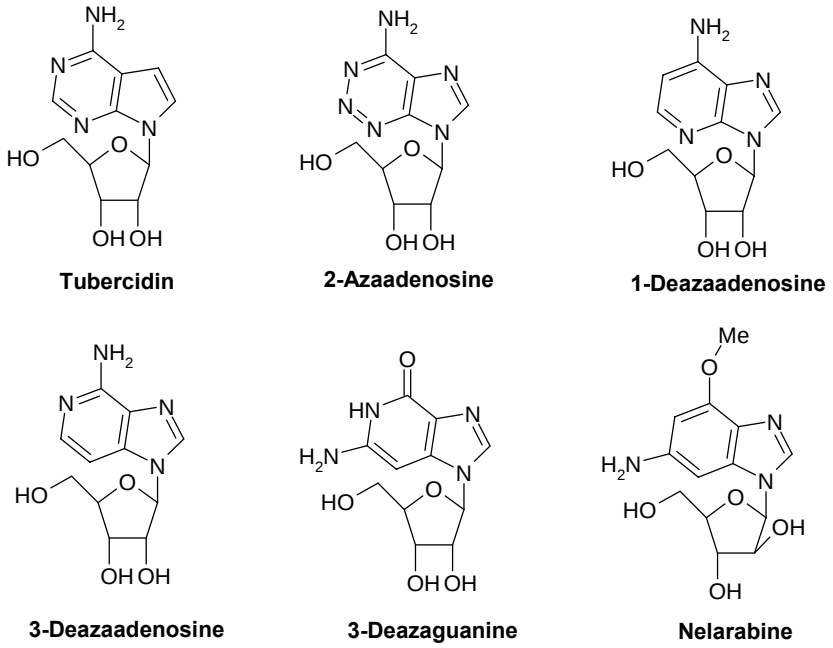


Figure 5: Some purine-modified nucleosides

1.3.2 Carbocyclic nucleoside analogues

Due to their interesting biological activity, carbocyclic nucleoside analogues (CANs) have also received much attention in recent years. CANs, which have no conventional glycoside linkage between the base and the carbocycle replacing the sugar of true nucleosides, have an attractive *in vivo* stability advantage over the 2',3'-dideoxynucleosides, as well as being more lipophilic and hence potentially more readily absorbed because of the replacement of the endocyclic oxygen by a methylene group [27].

The first member of this class was the carbocyclic analogue of adenosine described by Shealy in 1966 [43], and the interest was spurred by the discovery of the antibiotic and antitumoural activities of the natural products aristeromycin [44] and neplanocin A [45] (Figure 6). Since then, a large number of synthetic CANs have been prepared and tested [46]. Some of them were discovered with important therapeutic properties. For example,

3-deazaristeromycin, an analogue of aristeromycin (Figure 6), shows interesting antiviral activities [47].

Prominent synthetic CANs with anti- human immunodeficiency virus (HIV) activity are the 2',3'-unsaturated compounds, such as 2',3'-didehydro-2',3-dideoxyguanosine (carbovir) [48], amino-carbovir and abacavir (Ziagen) (Figure 6). The latter has better oral bioavailability and better penetration into the central nervous system than the other two and is currently being used in combination with other antiretroviral drugs to treat HIV infection in adults [49-52].

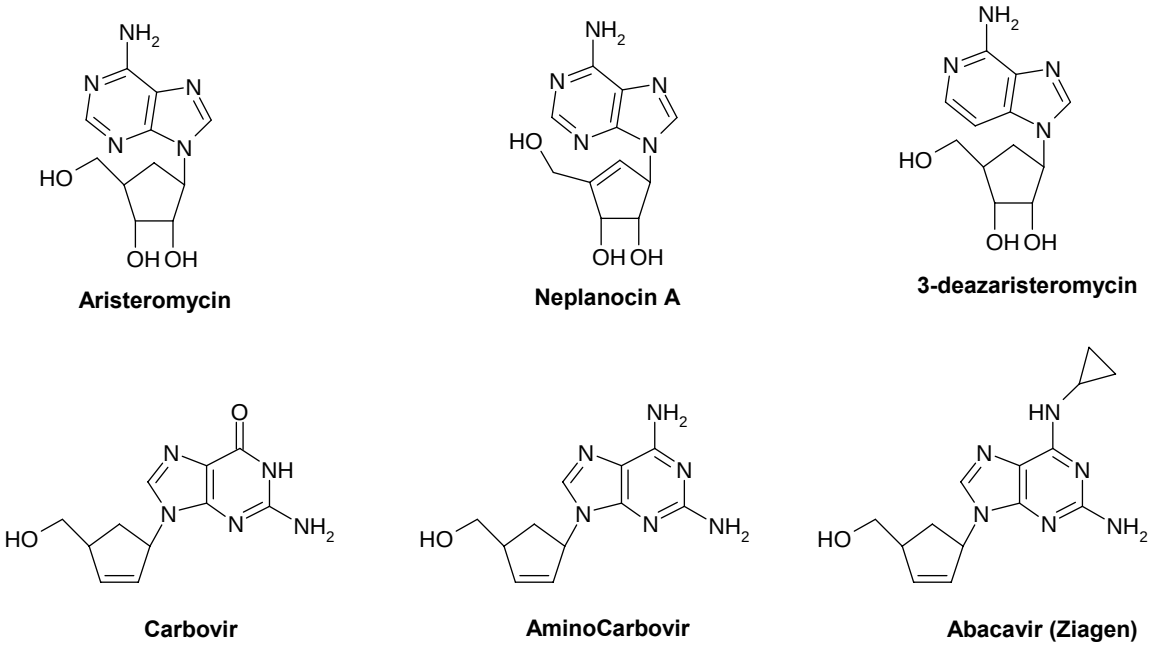


Figure 6: Carbocyclic Nucleoside Analogues

1.4 Statement of goal

There are two fundamental approaches for the construction of nucleoside analogues: 1) convergent attachment of an intact sugar or carbocyclic ring with an appropriately functionalized heterocyclic base by substitution and 2) convergent attachment of an intact heterocyclic base with an appropriately functionalized sugar or carbocyclic ring by substitution. We proposed to try both approaches to synthesize some novel nucleoside analogues. The target compounds of nucleoside analogues for this project are shown in Figure 7.

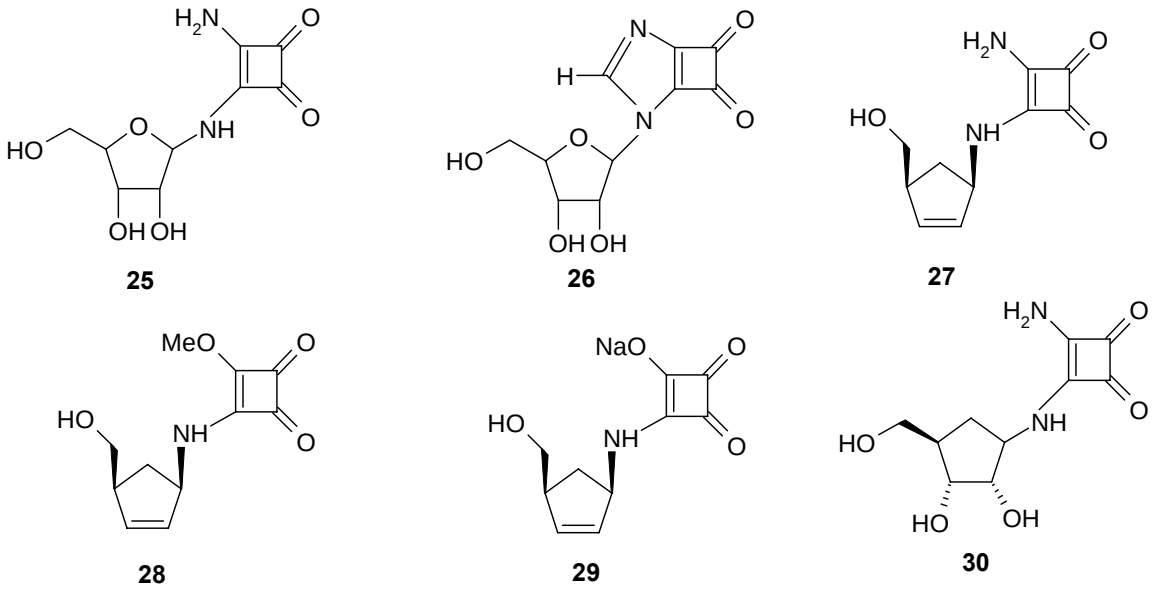


Figure 7: The target compounds of nucleoside analogues to be synthesized

Even though some carbocyclic nucleoside analogues are already in clinical use, this class of compounds still possesses a huge and largely unexploited potential for the development of new pharmaceuticals. Nucleoside analogues with a four-membered

heterocyclic as the base moiety have not yet been studied. So we proposed to use the carbocyclic ring as the sugar part of the nucleosides and to substitute the heterocyclic base by squarate analogues to synthesize novel types of carbocyclic nucleoside analogues.

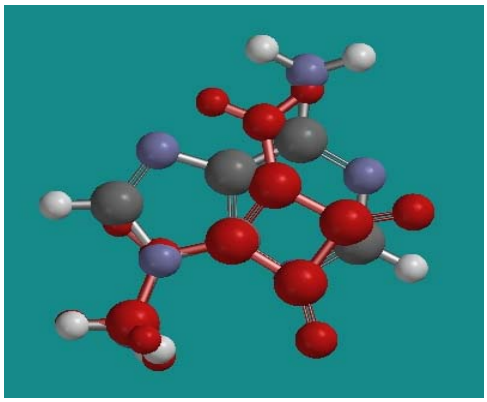
After synthesizing all the target compounds, we screened and tested their potential anticancer and antiviral activities at cellular levels.

CHAPTER 2:

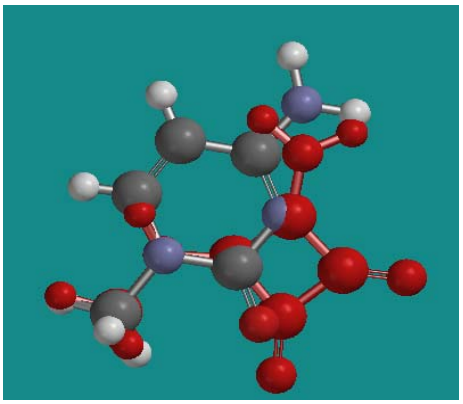
DESIGN AND SYNTHESIS OF NUCLEOSIDE ANALOGUES

2.1 Molecular modeling

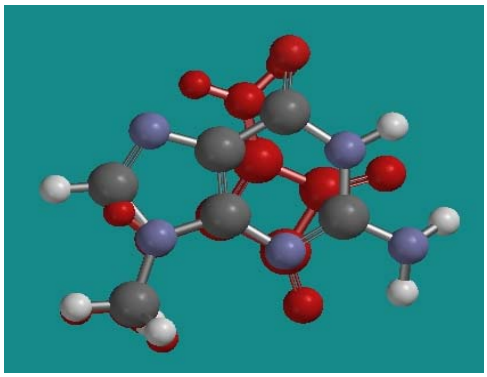
Molecular modeling is a theoretical method and computational technique to model or mimic the behaviour of molecules [53]. We used the program Spartan for Windows' 06 to show several potential alignments of *N*-methyldiaminosquarate with several nucleic acid bases (Figure 8). From Figure 8, we can see that there is greater atomic overlap of *N*-methyldiamino squarate with 9-methyladenine and 9-methylguanine than with 1-methylcytosine and 1-methyluracil. This might indicate that the structure of squaramide could resemble adenine or guanine. So it is of significant interest to utilize the monoamino squarate group as a base and modify the saccharide portion for nucleoside analogues to exploit the nucleoside platform as a source for new cellular probes and possibly drug candidates.



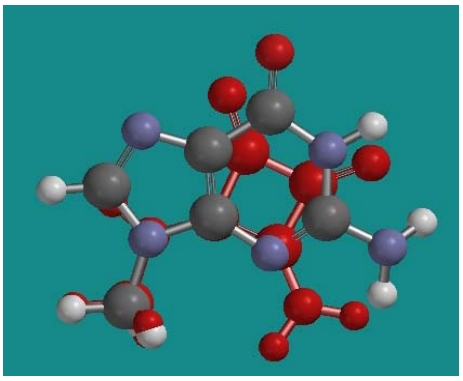
9-Methyladenine



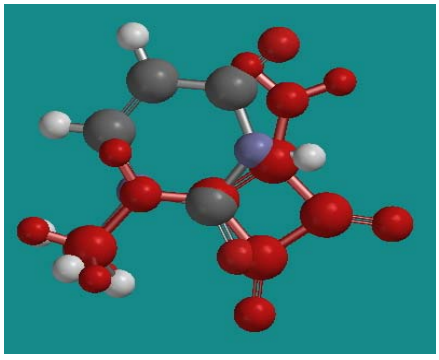
1-Methylcytosine



9-Methylguanine A



9-Methylguanine B



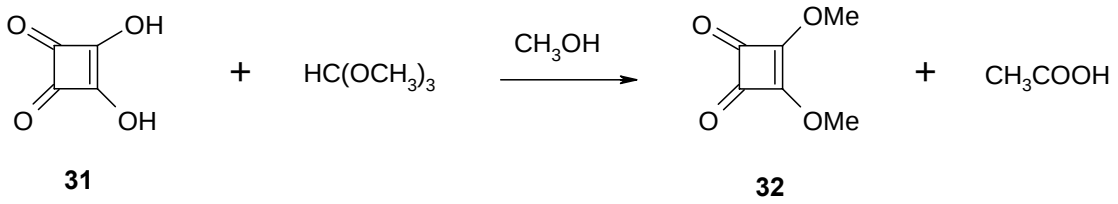
1-Methyluracil

Figure 8: Alignments of *N*-methyldiamino squarate (in red) with several nucleic acid bases such as 9-methyladenine, 9-methylguanine and 1-methyluracil.

2.2 Synthesis of starting materials

2.2.1 Synthesis of 3,4-dimethoxy-3-cyclobutene-1,2-dione (32)

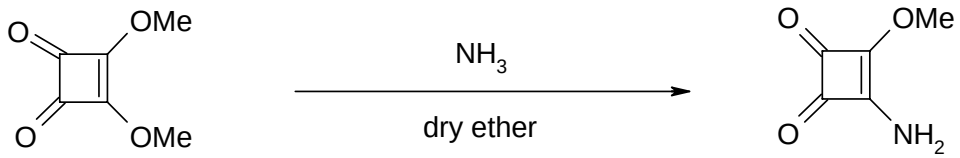
The whole synthesis begins with the commercially available 3,4-dihydroxy-3-cyclobutene-1,2-dione **31** (squaric acid) (Scheme 2.1), which is converted to its dimethyl ester **32** by refluxing a methanol solution of squaric acid in the presence of excessive trimethyl orthoformate [54]. The result was the formation of the dimethyl ester on a multigram scale in 89% yield. This route is convenient, safe and inexpensive for the preparation of dialkyl squarates and is suitable for large scale synthesis.



Scheme 2.1

2.2.2 Synthesis of 4-amino-3-methoxy-3-cyclobutene-1,2-dione (33)

Dimethyl squarate **32** was treated with ammonia gas to replace one of the methoxyl groups by an amino group [55] (Scheme 2.2). The product **33** is a monoamino compound, which is quite insoluble in ether (0.02%) [55]. Therefore treatment of compound **32** in dry ether with ammonia gas led to precipitation of monoamino compound **33** which allowed for facile isolation from starting material and the diamino side product in 81% yield.

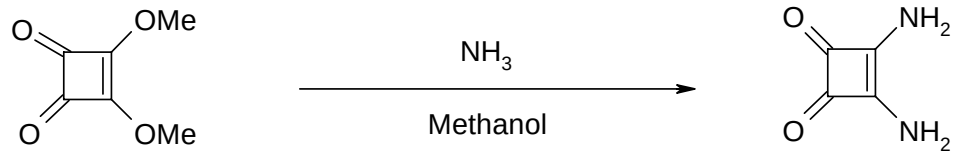


32 **33**

Scheme 2.2

2.2.3 Synthesis of 3, 4-diamino-3-cyclobutene-1,2-dione (34)

The ammonolysis in the previous reaction also provided the diamino compound **34** in low yield. In order to improve the yield, the solvent methanol instead of ether was used in this experiment, in which the monoamino compound **33** is soluble allowing for further addition of the second ammonia to form the diamino product (Scheme 2.3).

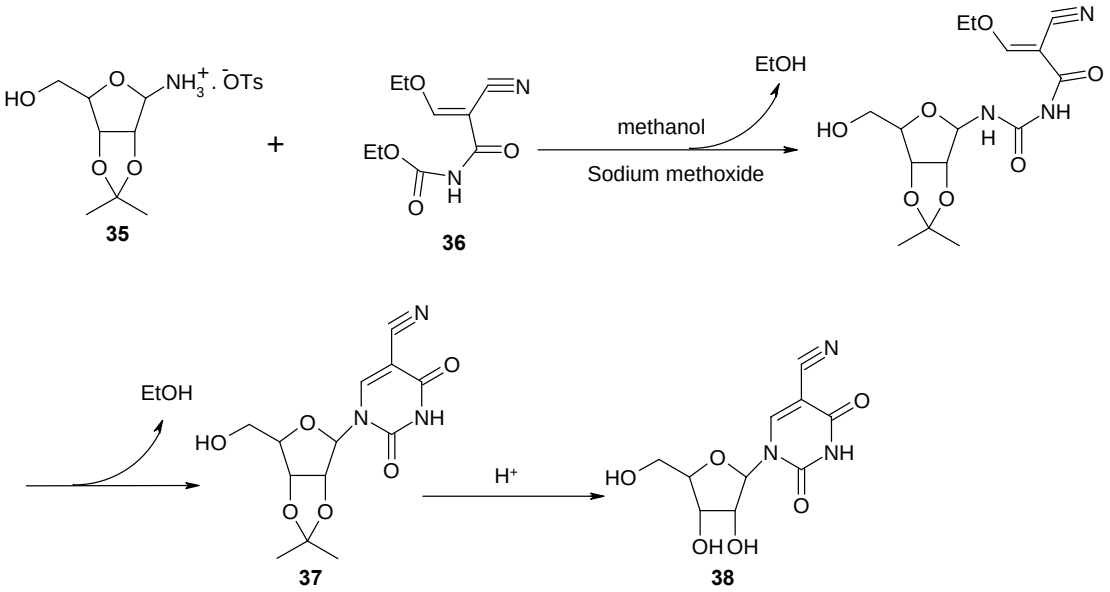


32 **34**

Scheme 2.3

2.3 Synthesis of 4-amino-3-[(β-D-ribofuranosyl)-β-1-amino]-3-cyclobutene-1,2-dione (25)

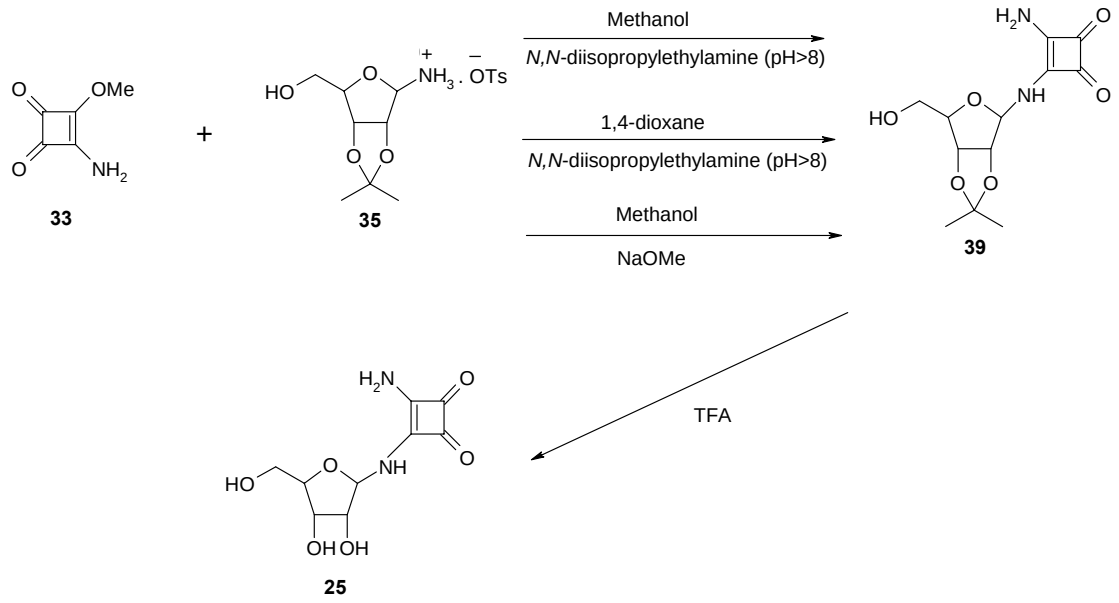
In the literature, the reaction of the 2,3-O-isopropylidene-β-D-ribofuranosylamine **35** with the ethoxy-carbamate **36** in the presence of sodium methoxide in methanol gave the 5-cyanoisopropylideneuridine **37**, which subsequently reacted with trifluoroacetic acid (TFA) giving the corresponding 5-cyanouridine **38** (Scheme 2.4) [56].



Scheme 2.4

So in this study, we took product **33** at hand and reacted with commercially available 2,3-O-isopropylidene-β-D-ribofuranosylamine p-toluenesulfonate salt **35** in refluxing methanol or 1,4-dioxane (Scheme 2.5). The second methoxyl group of squarate is known to be active towards amines under basic conditions [57], thus *N,N*-diisopropylethylamine was used as a base to adjust the ionization state of the mixture. The mixture was stirred

at 68 °C-70 °C for 2 days to afford 4-amino-3-[(2,3-O-isopropylidene-β-D-ribofuranosyl)amino]-3-cyclobutene-1,2-dione **39** as a brownish-yellow oil.



Scheme 2.5

Mass spectrometric analysis from this reaction was in agreement with the expected product mass characteristics (Calcd. For $C_{12}H_{16}O_6N_2$ ($M + H^+$): 285.1086. Found 285.1048) (Figure 9). However, the yield was found to be low and there were a number of side products in the mixture based on observation of the thin layer chromatography (TLC) which was found to be complex and many spots had similar retention factor (R_f) values. It was extremely difficult to obtain the pure product **39** by flash chromatography.

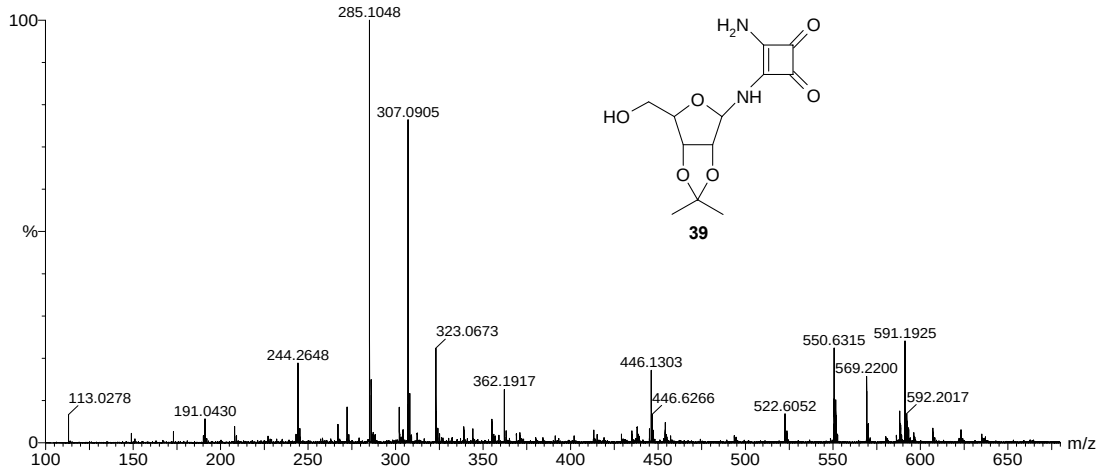


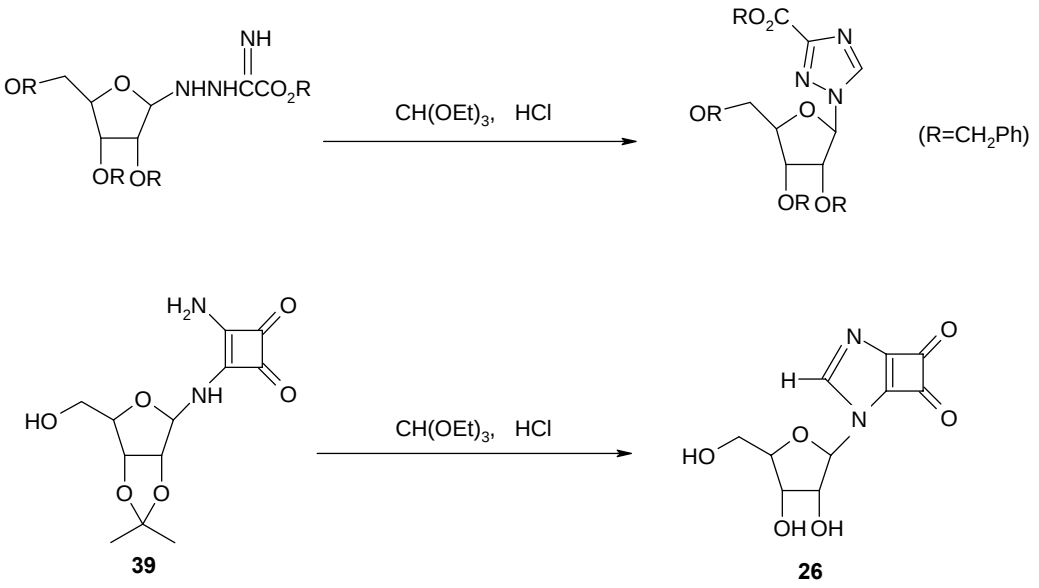
Figure 9: Mass spectrum (ESI+) for compound **39**

We assumed that the reaction time was not long enough. So we tried the reaction stirring for a longer time, 3 days and 7 days. But they were still the same as presented. Another possibility was that the base *N,N*-diisopropylethylamine was not strong enough. So we tried the base sodium methoxide (Scheme 2.5). The yield was also very low and it was difficult to purify the products.

There are some possible reasons why the mixture was complex: (1) The monoester monoamino squarate **33** may react by itself to make a diamino disquarate ($C_8H_2O_4N_2$, Calcd. for (M + H+) 190.0093. Found 190.0430 in the mass spectrum as shown in Figure 9). (2) The ribosylamine was unstable which might allow it to react with the solvent methanol or the hydroxyl group of the ribosylamine reacted with the monoamino squarate **33**. (3) The 2,3-O-isopropylidene group could be removed to uncover two hydroxyl groups and then these might have reacted with monoester monoamino squarate **33**. The complex mixtures of products and lack of sufficient material led us to abandon our attempts to prepare the product 4-amino-3-[(β-D-ribofuranosyl)-β-1-amino]-3-cyclobutene-1,2-dione **25**.

2.4 Synthesis of 2-(β-D-ribofuranosyl)-3H-2,4-diazabicyclo[3,2,0] nona-1,3-diene (26)

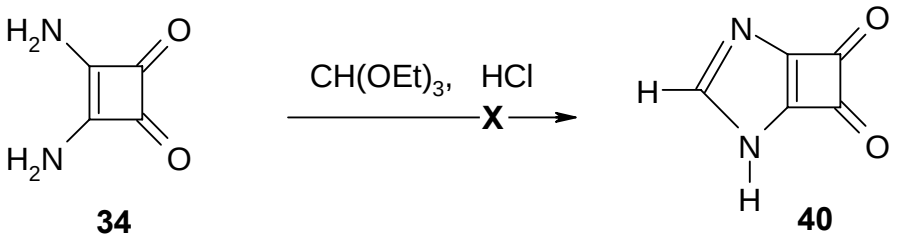
Triethyl orthoformate has been used in the formation of heterocyclic systems (Scheme 2.6) [58]. We attempted to undertake similar reactions for compound **39** by attempting a ring closure reaction with triethyl orthoformate in the presence of hydrochloric acid in a sealed flask for 12 hours (Scheme 2.6), to form compound **26**.



Scheme 2.6

Unfortunately, we could not get the pure protected compound **39** to generate the product **26**. The yield of impure compound **39** was low as well. So we collected the impure compound **39** several times and tried to generate the product **26** as presented above. However, we did not obtain the compound **26** under these conditions based on the mass spectrum. No more impure compound **39** was available to investigate this reaction. So we tried to model the ring closure of diamino squarate first (Scheme 2.7), but no

reaction occurred at all. It may be because the diamide **34** is highly insoluble and presumably strongly hydrogen bonded. Another factor in the lack of success in this reaction might be the presence of increased ring strain in the product, which would be a five membered ring fused to a four membered ring, versus, for example adenine, a five membered ring fused to a six membered ring system. Molecular modeling of the product expected from reaction of **34** with triethyl orthoformate (Scheme 2.7) at the RHF/6-31* level using Spartan '06 software indicates the product has a N-C-N bond angle of 114.56 degrees whereas the bond angle for formamidine itself ($HC(=N)NH_2$) is approximately 125.3 degrees. It may be that this bond angle difference results in a higher energy for the squaric acid bicyclic base therefore making it energetically more difficult to close the ring. A decrease in the reactivity of the two amino groups in compound **34** due to the delocalization of electron density from the nitrogens to the carbonyl oxygens compared to unconjugated amino groups might also contribute to this lack of reactivity. No previous literature on compound **34** was found.



34 **40**

Scheme 2.7

2.5 Synthesis of 4-amino-3-[((1R, 3S)-3-hydroxymethyl-4-cyclopen tene)-1-amino]-3-cyclobutene-1,2-dione (27)

Our synthesis of compound **27** started from commercially available (1R)-(-)-2-azabicyclo[2.2.1]hept-5-en-3-one **41**. The synthesis of this compound **41** is shown in Figure 10 [59, 60]. It starts from cyclopentadiene and arylsulfonyl cyanide. They react at room temperature for 30 min to generate 3-benzyl-2-azabicyclo[2.2.2]hepta-2,5-diene, which is hydrolyzed with acetic acid and water to (+/-)-lactam ((+/-)-2-azabicyclo[2.2.2]hept-5-en-3-one) [59]. Then enantiospecific and enantiocomplementary hydrolyase of (+/-)-lactam are catalysed by whole cell preparations of microbial strains ENZA-1 (*Rhodococcus equi*) and ENZA-20 (*Pseudomonas solanacearum*) [60]. From the fermentation using the ENZA-1, (+)-lactam is obtained. From an equally facile bioconversion ENZA-20 produces (-)-lactam (compound **41**, > 98% optical purity) [60]. The racemate and each of the enantiomers (≥ 98% purity with ≥ 99% (HPLC) optical purity) of the lactam are available commercially from Sigma-Aldrich.

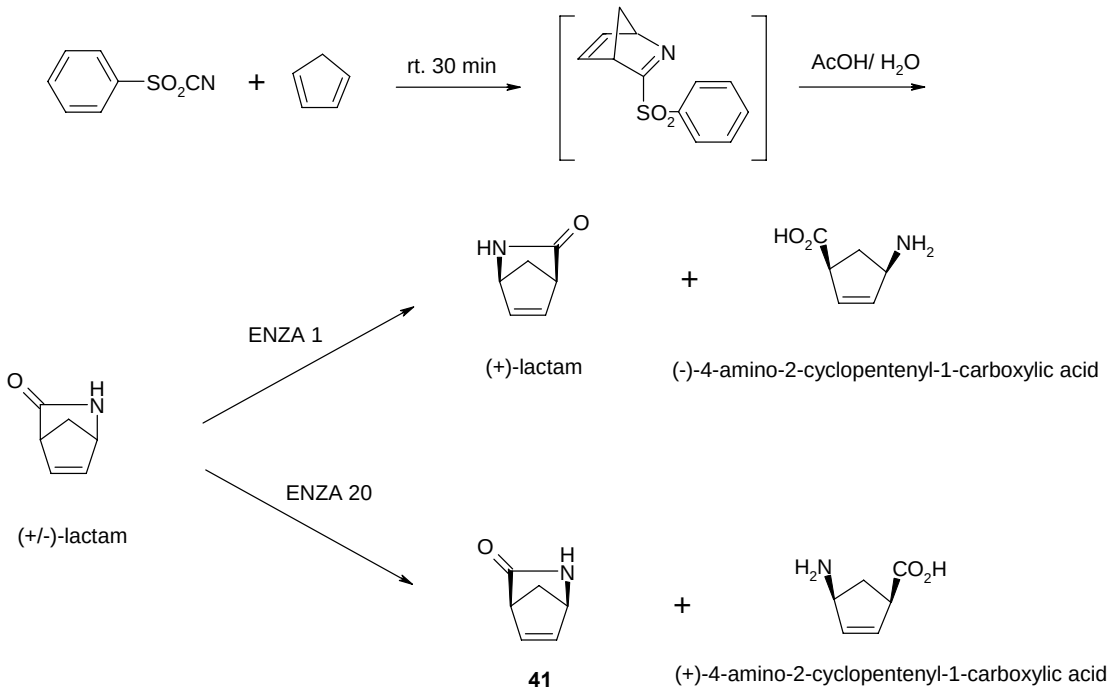
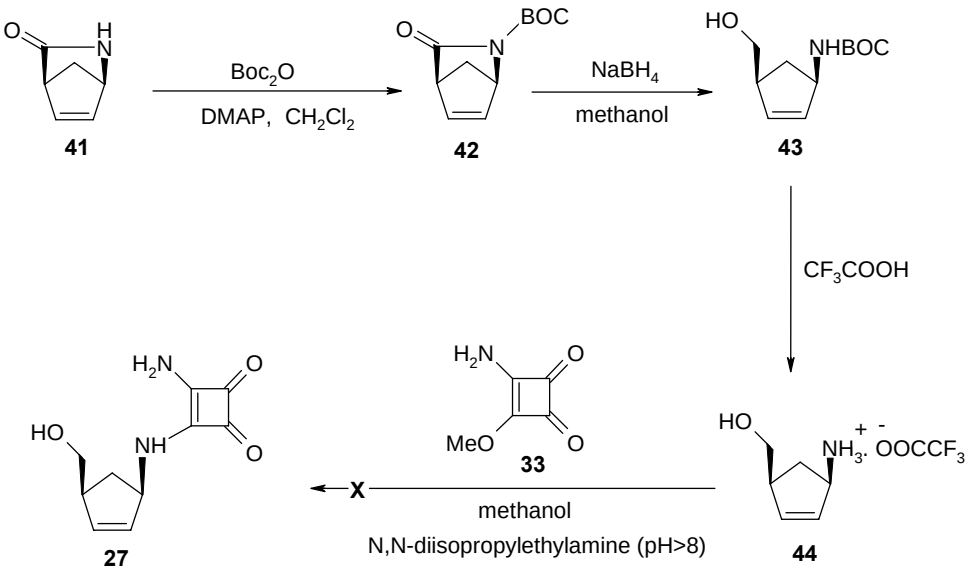


Figure 10: Reaction scheme for synthesis of starting material (1R)-(-)-2-azabicyclo [2.2.1]hept-5-en-3-one **41** [59, 60]

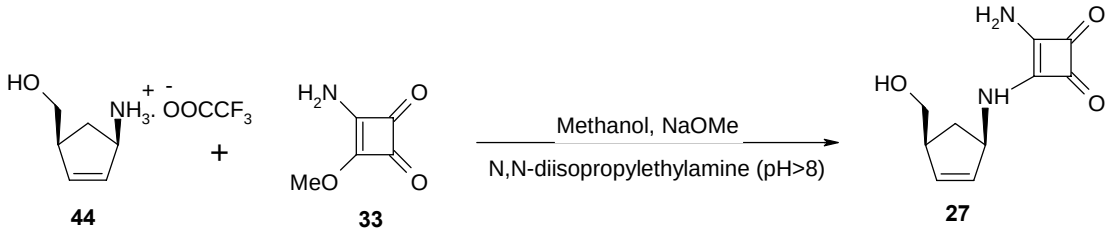
There are several established routes to give cyclopentenylamine from the bicyclic lactam **41** [61-63]. We made the pure amine alcohol **44** from lactam **41** in three, high-yielding steps (Scheme 2.8) based on the method reported by Taylor *et al.* [63]. To cleave the amido bond, the *tert*-butoxycarbonyl (Boc) group was introduced to the 2-position of **41** by the reaction of lactam **41** with di-*tert*-butyldicarbonate (Boc_2O) in the presence of catalytic 4,4-dimethylaminopyridine (DMAP). The resulting compound **42** (71% yield) was then treated with sodium borohydride to give the N-Boc protected cyclopentene derivative **43** in 61% yield from **41**. The Boc amino alcohol **43** is a stable solid from which the pure amino alcohol **44** was generated under mild conditions using a slight excess of trifluoroacetic acid.



Scheme 2.8

Transformation of **44** to **27** was carried out by a usual manner as shown in Scheme 2.8. As mentioned above, the second methoxyl group of squarate is active towards amines in basic condition. So addition of N,N-diisopropylethylamine (pH > 8) as a base to a solution of the cyclopentenylamine **44** and 4-amino-3-methoxy-3-cyclobutene-1,2-dione **33** in methanol should produce **27**. However, we could not obtain compound **27** under these conditions. Most of the cyclopentenylamine **44** was found to be unreactive under these conditions. So we were aware that the N,N-diisopropylethylamine was not strong enough to readily remove the trifluoroacetic group on **44** under the above preliminary experimental conditions.

Another stronger base was needed for the reaction. So a quantitative amount of sodium methoxide with **44** was added to the TFA salt **44** in order to completely remove the trifluoroacetate group and N,N-diisopropylethylamine was also added to adjust the pH of the mixture to pH 8 ~ 9, giving the compound **27** in a yield of 49% (Scheme 2.9).



44 33 27

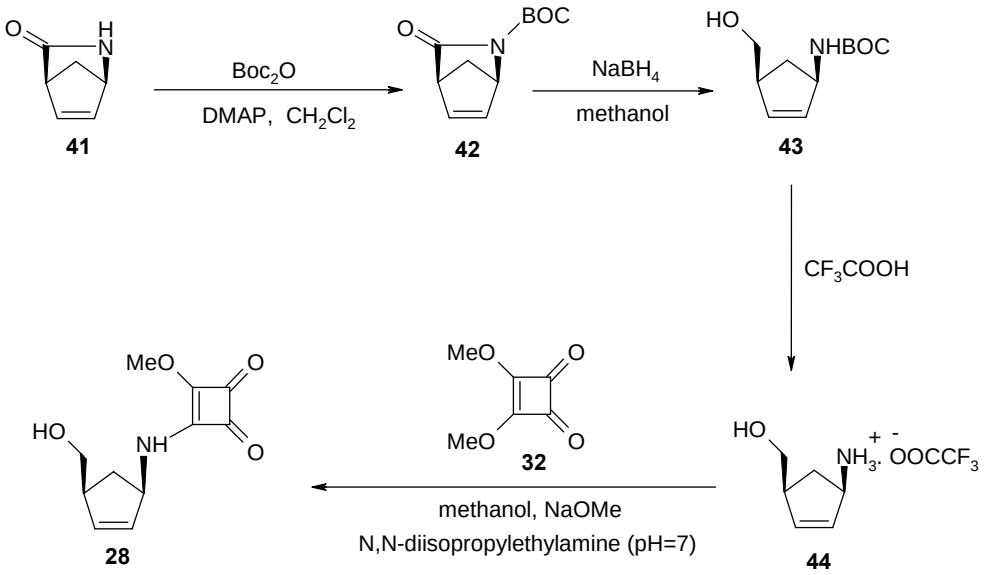
Scheme 2.9

The structure of compound **27** is very novel. So we tried to crystallize compound **27** for X-ray to investigate the structure of this compound. It was crystallized from methanol or ethanol for 1 week. But it was disappointing that we could not achieve the crystal of compound **27** under these conditions.

2.6 Synthesis of 4-methoxy-3-[((1R, 3S)-3-hydroxymethyl-4-cyclopen tene)-1-amine]-3-cyclobutene-1,2-dione (28)

We combined the cyclopentene ring as the sugar part and the monoester monoamino squarate as the base to make the squarate monoester nucleoside analogue. We also started from the commercially available (1R)-(-)-2-azabicyclo[2.2.1]hept-5-en-3-one **41** to give the pure amino alcohol **44** under conditions similar to those previously presented (Scheme 2.10). In order to avoid both methoxy groups reacting with the cyclopentenylamine **44**, the pH of the mixture was controlled at around 7.0. Under these conditions using microwave irradiation at 100 ^{o}C for 60 min, compound **28** was generated in a yield of 67% based on starting compound **44**. Microwave irradiation was applied using a Biotage Initiator 8 instrument. The advantages of microwave irradiation

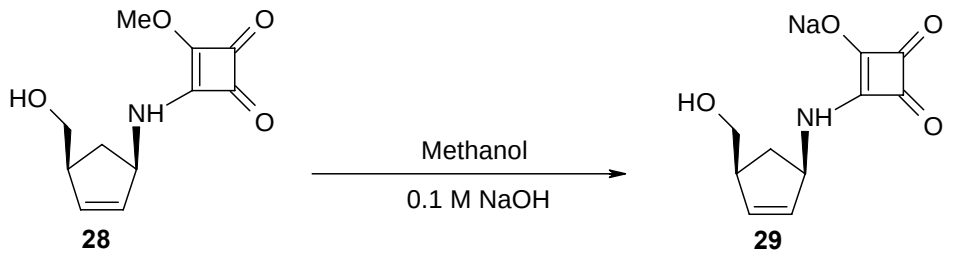
include not only improving classical reactions, shortening reaction times, improving yields, and suppressing byproduct formation as compared with conventional thermal heating but also promoting new reaction types for drug discovery and process chemistry [64-68].



Scheme 2.10

2.7 Synthesis of 4-hydroxy-3-[((1R, 3S)-3-hydroxymethyl-4-cyclopen tene)-1-amine]-3-cyclobutene-1,2-dionate, sodium salt (29)

The first four steps of the synthesis of this compound are the same as presented in Scheme 2.10. Treatment of compound **28** with 0.1 M sodium hydroxide (1 equivalent) under ice-cooling for 3 hours and then at room temperature for 7 hours under argon gave the salt **29** in high yield 80% (Scheme 2.11).



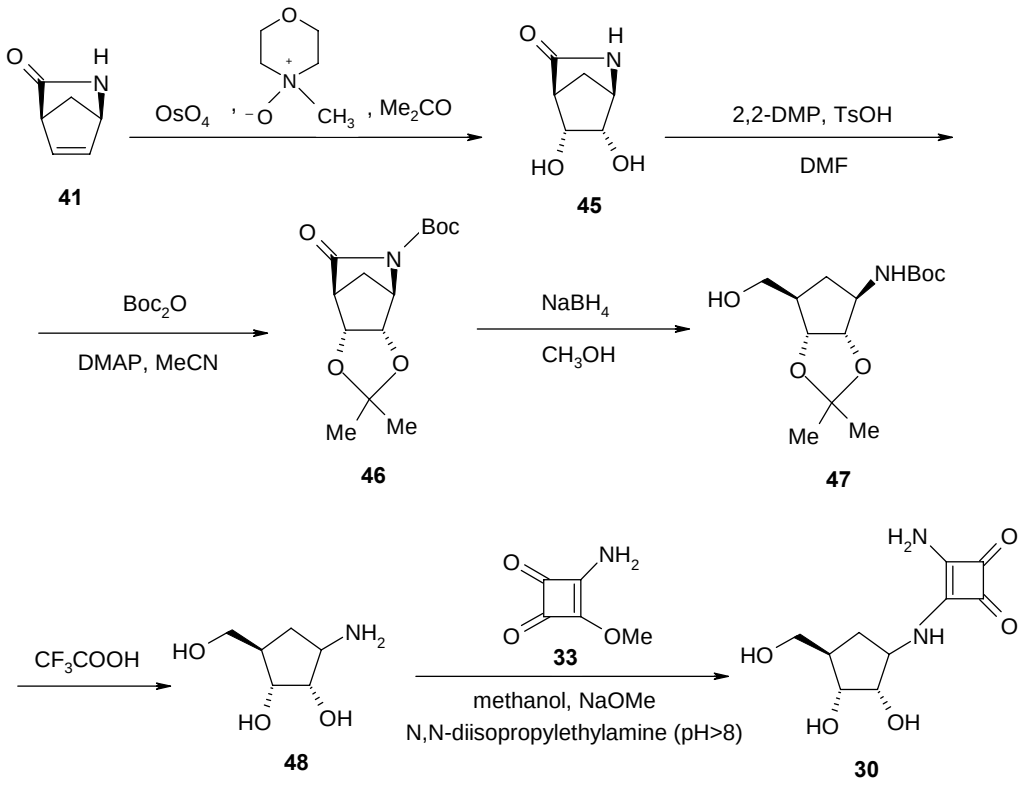
Scheme 2.11

2.8 Synthesis of (1R, 2S, 3R, 5R)-4-amino-3-[(5-hydroxymethyl-cyclo pentane-1,2-diol)-3-amino]-3-cyclobutene-1,2-dione (30)

The starting material (1R)-(-)-2-azabicyclo[2.2.1]hept-5-en-3-one **41** was subjected to *cis*-dihydroxylation utilizing catalytic quantities of osmium tetroxide, in the presence of N-methylmorpholine N-oxide (NMO) to regenerate the OsO_4 (acetone/H_2O, room temperature) [69]. The glycolization product **45**, obtained in 95% yield, was protected by its addition to a stirred solution of *p*-toluenesulphonic acid (TsOH) monohydrate in 2,2-dimethoxypropane (2,2-DMP) and dimethylformamide (DMF) at room temperature for 20 hours. This was then followed by the addition of acetonitrile, N, N-4-dimethylaminopyridine (DMAP) and di-*tert*-butyl dicarbonate (Boc_2O) to the solution. The solution was stirred at room temperature for 15 hours in attempts to prepare the protected compound **46** (Scheme 2.12) [70].

It was disappointing that we did not get the protected compound **46**. The spots on the TLC were numerous and the TLC was itself very complex. We tried 1:10:1, 1:10:1.2, 1:1:1.2 ratio of compound **45**, 2, 2-DMP, and TsOH. The spots on the TLC were the same and still complicated. Also, the amount of added catalyst would affect the reaction no matter it is too much or not enough. We tried 1:2 amount of catalytic DMAP with

Boc_2O which was the same as that for preparing the N-Boc protected cyclopentene derivative **43**. The DMAP might be too much for this reaction. We will continue to try different ratios to find the best conditions for this reaction and then finish the following steps as shown in scheme 2.12 to get the final product **30**.



Scheme 2.12

2.9 Experimental procedures

2.9.1 General experimental

Reagent grade solvents were used throughout the course of this work. Reagents were purchased commercially from Chemstore, at University of Waterloo and used without further purification. MeOH was HPLC grade.

Solvent evaporation was carried out under reduced pressure using a Buchi rotary evaporator with a Wheaton water aspirator). Aqueous solutions were dried *in vacuo* by a rotary evaporator with a vacuum pump or on a lyophilizer under reduced pressure.

For thin layer chromatography (TLC) analysis, Merck silica gel 60 F_{254} aluminum sheets were used. The developed sheets were viewed under UV light or stained with iodine, ninhydrin, vanillin, phosphomolybdic acid stain or potassium permanganate [71]. At an early stage, flash chromatography was performed using silica gel 60 (70-230 mesh). At the later stage, flash chromatography was carried out using the Discovery-Scale FLASH™ Chromatography Systems and Modules (Biotage, USA) and using the FLASH Purification Cartridges (Biotage, USA) instead of using the silica gel packed by myself. The latter FLASH chromatography systems allowed for faster flow rates and provided increased throughput and higher resolution. The solvent mixtures used as eluent are indicated in each case. Moreover, the experimental reactions were performed using the Initiator™ Microwave Synthesis System Initiator 8 (Biotage, USA) at the later stage, which is able to quickly achieve temperatures and pressures beyond traditional reflux heating.

Proton (^{1}H) and carbon (^{13}C) proton decoupled nuclear magnetic resonance (NMR) spectra were obtained on Bruker AM-300 and AM-500 instruments. Chemical shifts (δ)

for ^{1}H NMR spectra run in $CDCl_3$, DMSO-d_6, CD_3OD, D_2O are reported in ppm relative to the internal standard tetramethylsilane (TMS) ($\delta = 0$). For ^{13}C NMR spectra, chemical shifts are reported in ppm relative to $CDCl_3$ ($\delta = 77.0$ for central peak), DMSO-d_6 ($\delta = 39.5$ for central peak), CD_3OD ($\delta = 49.5$ for central peak) and D_2O ($\delta = 0$ for external standard TMS) [72]. Coupling signals were assigned based on correlation spectroscopy (COSY) and heteronuclear multiple quantum correlation (HMQC) data. Mass spectra were obtained at the WATSPEC Mass Spectrometry Facility, Department of Chemistry, University of Waterloo. Low-resolution (LRMS) and high-resolution (HRMS) electron impact (EI) mass spectra were recorded on a JEOL HX 110 double focusing mass spectrometer. Electrospray Ionization (ESI) mass spectra were obtained with a Waters/Micromass QTOF Ultima Global mass spectrometer. Melting points were measured on a Mel-Temp Melting Point Apparatus and are uncorrected. Fourier transform infrared spectra were recorded on a Perkin-Elmer 1600 FT-IR using solid samples with potassium bromide (KBr) (IR grade).

2.9.2 Materials

The following chemicals were purchased from the Sigma-Aldrich Company, Canada: (1R)-(-)-2-azabicyclo[2.2.1]hept-5-en-3-one, *N,N*-diisopropylethylamine, triethylamine, di-*tert*-butyldicarbonate, dimethylformamide, 3,4-dihydroxy-3-cyclobutene-1,2-dione, 4-dimethylaminopyridine, sodium borohydride, 2,2-dimethoxypropane, sodium methoxide, 4-methylmorpholine *N*-oxide, trimethyl orthoformate, osmium tetroxide, and 2,3-O-isopropylidene-β-D-ribofuranosylamine *p*-toluenesulfonate salt.

Deuterated solvents were purchased from Cambridge Isotope Laboratories, USA. The following were purchased from EMD Chemical Inc., USA: anhydrous diethyl ether, acetone, and N, N-dimethylformamide.

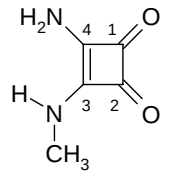
The following chemicals were purchased from Fisher Scientific, Canada: acetic acid, magnesium sulfate, hydrochloric acid, Celite 545®.

The following chemicals were purchased from Caledon Laboratories LTD, Canada: trifluoroacetic acid, sodium hydroxide.

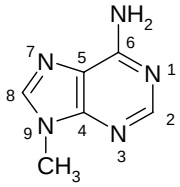
The following chemical was purchased from J. T. Baker Chemical Co., USA: *p*-toluenesulphonic acid monohydrate.

2.9.3 Reaction conditions and experimental data

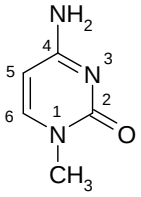
Molecular modeling



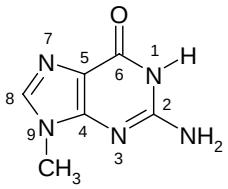
N-methyl-diaminosquaramide



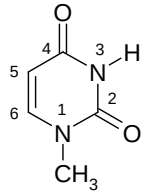
9-methyladenine



1-methylcytidine



9-methylguanine

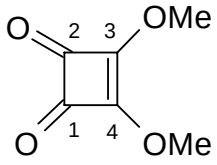


1-methyluracil

Molecular modeling was carried out by using Spartan 06, version 1.0.3 for Mac (Wavefunction Inc., Irvine California) molecular modeling software. Molecules were drawn using the graphical user interface. The above molecules were used in calculations as simpler analogs of the intact nucleosides which would have required the presence of the ribose ring: 9-methyladenine, 1-methylcytidine (4-amino-2-hydroxy-1-methylpyrimidine), 9-methylguanine, 1-methyluracil and N-methyl-diaminosquaramide. These compounds were then geometry optimized at the RHF/3-21G* level. The resulting structures were then superimposed such that the three CH_3-N-C heavy atoms of N-methyl-diaminosquaramide were overlapped (using the "superimpose" command in Spartan) with the CH_3-N9-C4 of 9-methyladenine, the CH_3-N1-C2 of 1-methylcytidine,

the CH_3-N9-C4 of 9-methylguanine (showing two orientations of the squaramide), and the CH_3-N1-C2 of 1-methyluracil.

3,4-Dimethoxy-3-cyclobutene-1,2-dione (32)



To a 50 ml round-bottomed flask was added 3,4-dihydroxy-3-cyclobutene-1,2-dione (squaric acid, 2.053 g, 18 mmol), methanol (18 ml), and trimethyl orthoformate (4 ml, 36.5 mmol). The reaction mixture was refluxed at 56 °C for 24 hours. The crude product was then concentrated under reduced pressure. The pale yellow solid was dissolved in methylene chloride and purified on a silica gel column (EtOAc: Hexanes 1:2) to give dimethyl squarate (2.29 g, 89.4%) as a white solid.

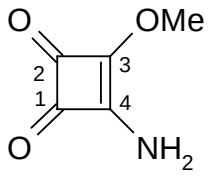
mp 55-57 °C (lit. 56-58 °C) [55];

^{1}H NMR (300 MHz, CD_2Cl_2) δ 4.34 (6H, s, OCH_3)

^{13}C NMR (75 MHz, CD_3OD) δ 189.35 (C2, C=O), 189.35 (C1, C=O), 184.35 (C3), 184.35 (C4), 60.29 (OCH_3), 60.29 (OCH_3)

LRMS (EI): 142.03 (100), 114.03 (18), 99.01 (16), 86.01 (54), 67.99 (8), 56.25 (7)

4-Amino-3-methoxy-3-cyclobutene-1,2-dione (33)



Dimethyl squarate **32** (0.903 g, 6 mmol) was dissolved in 150 ml of dry diethyl ether under ice-cooling and was added ammonia gas until precipitation was completed (~ 30 mins). The mixture was boiled gently for 10 mins and the ether was decanted. The residue was then washed with ether, dried *in vacuo*, and crystallized from acetone-petroleum ether (1:1) to give the product (0.617g, 81%) as a pale yellow solid.

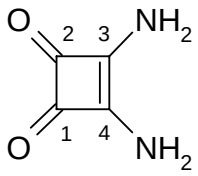
mp 200-202 °C (lit. ~202 °C) [55];

^{1}H NMR (300 MHz, acetone-d_6) δ 4.31 (3H, s, OCH_3), 7.39 (2H, brs, NH_2);

^{13}C NMR (75 MHz, DMSO- d_6) δ 190.25 (C2, C=O), 183.63 (C1, C=O), 178.50 (C3), 174.27 (C4), 60.17 (OCH_3). The ^{13}C NMR spectrum recorded was in complete agreement with the theoretical prediction (δ190.41 (C2, C=O), 185.06 (C1, C=O), 179.50 (C3), 175.25 (C4), 60.34 (OCH_3))

LRMS (EI): 127.04 (100), 99.05 (12), 71.03 (35), 56.29 (36)

3, 4-diamino-3-cyclobutene-1,2-dione (34)



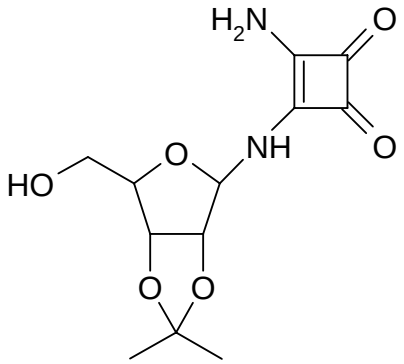
Dimethyl squarate **32** (0.142 g, 1 mmol) was dissolved in 25 ml of dry methanol under ice-cooling and was added ammonia gas until precipitation was completed (~ 20 mins). The mixture was warmed for 1h, the methanol was decanted. The residue was washed with acetone and ether, and then dried *in vacuo* to give the product (0.092 g, 82%) as a yellow powder.

It does not melt, turning dark over 248 °C, no visible gas evolution when heated to 380 °C (lit. no noticeable gas evolution when heated to 350 °C) [55];

^{13}C NMR (75 MHz, DMSO- d_6) δ 184.22 (C2, C=O), 184.22 (C1, C=O), 170.74 (C3), 170.74 (C4)

LRMS (EI): 112.01 (100), 84.00 (12), 56.30 (36)

4-amino-3-[(2,3-O-isopropylidene-β-D-ribofuranosyl)amino]-3-cyclobutene-1,2-dione (39)



To a solution of 2,3-O-isopropylidene-β-D-ribofuranosylamine p-toluenesulfonate salt (0.036 g, 0.1 mmol) (commercially available from Sigma-Aldrich Company) in 4 ml methanol, the squarate **33** (0.013 g, 0.1 mmol) was added and *N,N*-diisopropylethylamine (1.1 ml) was added to adjust the pH of the mixture to 8 ~ 9. The mixture was stirred at 68 °C-70 °C for 2 days, or 3 days, or 7 days and then condensed under *in vacuo*, giving a brownish-yellow residue.

Methanol was substituted by 1, 4-dioxane. The other conditions were the same as above.

To a solution of 2,3-O-isopropylidene-β-D-ribofuranosylamine p-toluenesulfonate salt (0.108 g, 0.3 mmol) (commercially available from Sigma-Aldrich Company) in 5 ml methanol, the squarate **33** (0.038 g, 0.3 mmol) was added and sodium methoxide (0.016 g, 0.3 mmol) was added. The mixture was stirred at 68 °C-70 °C for 2 days, or 3 days, or 7 days and then condensed under *in vacuo*, giving an orange residue. The residue from 7-

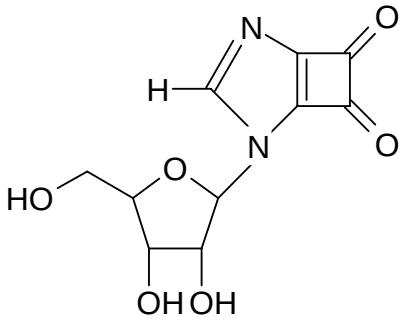
day reaction was purified on a silica column (EtOAc: methanol = 50:1) to give an orange solid.

LRMS FAB: m/z Calcd. For $C_{12}H_{16}O_6N_2$ ($M + H^+$): 285.1086. Found 285.1048. However, the purified product was found to be a mixture based on observation of the TLC and mass spectrum. There are three spots on TLC and these three spots had similar R_f values. Extensive attempts were made to purify these but these attempts were not successful. Therefore it was used directly for the next reaction.

To a solution of 2,3-O-isopropylidene-β-D-ribofuranosylamine p-toluenesulfonate salt (0.108 g, 0.3 mmol) (commercially available from Sigma-Aldrich Company) in 7 ml methanol, the squarate **33** (0.038 g, 0.3 mmol) was added and sodium methoxide (0.016 g, 0.3 mmol) was added. The mixture was stirred at 68 °C-70 °C for 1 hour. More sodium methoxide (0.016 g, 0.3 mmol) was added and the mixture was stirred at 68 °C-70 °C for 2 days, or 7 days and then condensed under *in vacuo*, giving an orange residue.

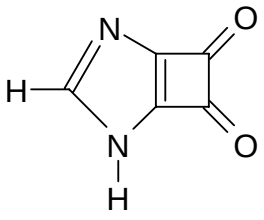
In all cases, the TLC results were extremely complex and numerous compounds were present. But mass spectrometric analysis of the residue from each reaction was in agreement with the expected product mass characteristics.

2-(β-D-ribofuranosyl)-3H-2,4-diazabicyclo[3,2,0]nona-1,3-diene (26)



To triethyl orthoformate (1 ml) was added the impure compound **39** (22 mg, 0.08 mmol) and half drop of conc. hydrochloric acid. The mixture was stirred in a sealed flask at room temperature for 1 day and then concentrated to give an oil that was further purified by column chromatography ($CHCl_3$: CH_3OH = 99:1) to give the products. Mass spectrometric analyses of each product were not in agreement with the expected product mass characteristics.

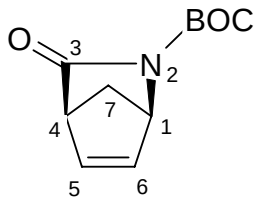
3H-2,4-diazabicyclo[3,2,0]nona-1,3-diene (40)



To triethyl orthoformate (4 ml) was added the diamino squarate **34** (0.112 g, 1 mmol) and 2 drops of conc. hydrochloric acid. The mixture was stirred in a sealed flask at room temperature for 1 day. Most of the yellow diamino squarate powder was still left in the solution.

The same amount of reagents and reactant was performed for this reaction. The mixture was run under microwave conditions for 1 h at 100 °C. After the reaction, the mixture became paste and was hard to dissolve in any solvent system. No indication of product was observed.

(1R, 4S)-(-)-2-*tert*-butoxycarbonyl-2-azabicyclo[2.2.1]hept-5-en-3-one (42)



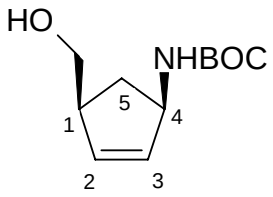
(1R)-(-)-2-Azabicyclo[2.2.1]-hept-5-en-3-one (0.546 g, 5 mmol) (commercial from Sigma-Aldrich Company, ≥ 98% purity with ≥ 99% (HPLC) optical purity) was dissolved in CH_2Cl_2 (10 ml). To this solution were added successively triethylamine (0.7 ml, 5 mmol), di-*tert*-butyl dicarbonate (2.183 g, 10 mmol), and 4-dimethylaminopyridine (0.611 g, 5 mmol). The mixture was stirred for 24 hours at room temperature. The solvent was removed *in vacuo*. To the resulting residue was added water (5 ml) and ether

(5 ml). The organic layer was collected, dried (anhydrous $MgSO_4$) and concentrated *in vacuo*. The residue was purified by column chromatography (hexane: EtOAc = 5:1) to give the product (0.740 g, 71%) as colorless prisms.

mp 85-86 °C (lit. 84-86 °C) [63]

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.48 (9H, s, Boc, $C(CH_3)_3$), 2.14 and 2.34 (each 1H, AB type, C_7-H_2), 3.36 (1H, br s, C_4-H), 4.93 (1H, m, C_1-H), 6.65 (1H, dm, C_5-H), 6.88 (1H, dd, J = 5.3 Hz, 2.2 Hz, C_6-H). The ^{1}H NMR spectrum recorded was in complete agreement with the data reported in the literature [73].

(1S, 4R)-(-)-(4-*tert*-butoxycarbonylaminocyclopent-2-en-1-yl)carbinol (43)



The product **42** (2.220 g, 10.6 mmol) from the previous reaction was dissolved in methanol (21 ml). $NaBH_4$ (1.203 g, 31.8 mmol) was added with stirring under ice cooling. Stirring was continued for 30 mins at 0 ^{o}C, and then stirred at room temperature for 1 h. The mixture was neutralized with 10% HCl in H_2O and then concentrated *in vacuo*. The residue was purified by silica gel chromatography (50% EtOAc/hexane) to give 1.95 g (86.4%) of colorless prisms.

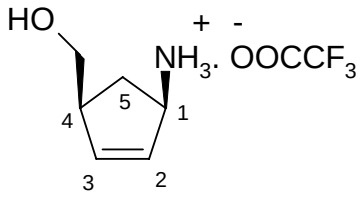
mp 58-62 °C (lit. 56-60 °C) [73]

^{1}H NMR (300 MHz, CD_3OD) δ 1.23 (1H, m, C_5-H), 1.41 (9H, s, Boc, $C(CH_3)_3$), 2.41 (1H, m, C_5-H), 2.74 (1H, s, C_1-H), 3.46 and 3.48 (2H, d, J = 6.0, CH_2OH), 4.55 (1H, s, C_4-H), 5.67 (1H, s, C_3-H), 5.80 (1H, s, C_2-H). The ^{1}H NMR spectrum recorded was in complete agreement with the data reported in the literature [63].

^{13}C NMR (75 MHz, MD_3OD) δ 156.35 (BOC, C = O), 142.33 (BOC, C), 134.05 (C3), 132.50 (C2), 78.53 (CH_2OH), 64.94 (C4), 55.96 (C5), 34.20 (C1), 27.28 (BOC, CH_3)

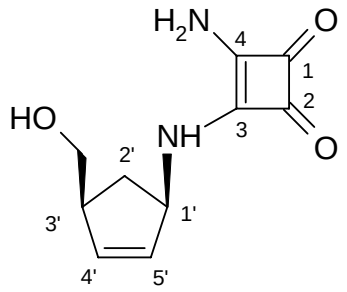
LRMS FAB: m/z Calcd. For $C_{11}H_{19}NO_3$ ($M + H^+$) 214.1443. Found 214.1391

(1R, 4S)-(-)-4-(hydroxymethyl)cyclopent-2-en-1-ylamonium trifluoroacetate (44)



To trifluoroacetic acid (TFA) (2 ml) was added **43** (426 mg, 2 mmol) under ice-cooling. The mixture was stirred at room temperature for 2 h, and then evaporated *in vacuo* to give a brown oil (428.80 mg, 94%). The residue was used directly for the next reactions without further purification [73].

4-Amino-3-[((1R,3S)-3-hydroxymethyl-4-cyclopentene)-1-amino]-3-cyclobutene-1,2-dione (27)



To a solution of **44** (428.8 mg, 1.88 mmol) in methanol (4 ml), squarate **33** (238.8 mg, 1.88 mmol) and sodium methoxide (101.5 mg, 1.88 mmol) were added. The pH of the mixture was adjusted to 9.0 ~ 10 by *N, N*-diisopropylethylamine (1.2 ml). Then the mixture was run under microwave conditions for 1 h at 100 °C. After the reaction, precipitation occurred and was filtered to obtain the crude product (233.0 mg). The crude product was recrystallized from methanol to provide the product (192.3 mg, 49%) as orange powder.

The orange powder (15 mg) was dissolved in boiled methanol or ethanol to make the supersaturated solution, which was then quickly filtrated while it was hot. The filtrated solution was cooled down to room temperature and then was crystallized at room temperature for 1 week. No crystal was achieved under these conditions.

mp 278 °C

^{1}H NMR (300 MHz, DMSO-d_6) δ 1.28-1.36 (1H, m, C_2'-H), 2.33-2.43 (1H, m, C_2'-H), 2.67-2.69 (1H, m, C_3'-H), 3.28 and 3.36 (2H, d, CH_2OH), 4.67 (1H, s, C_1'-H), 5.02 (1H, brs, OH) 5.72-5.74 (1H, d, J = 6.0, C_4'-H), 5.88-5.90 (1H, d, J = 6.0, C_5'-H), 7.40 (2H, brs, NH_2)

^{13}C NMR (75 MHz, DMSO-d_6) δ 183.62 (C2, C=O), 183.24 (C1, C=O), 169.58 (C3), 167.97(C4), 136.73 (C_5'), 132.55 (C_4'), 64.83 (CH_2OH), 59.60 (C_1'), 47.76 (C_3'), 35.57 (C_2')

Here are the predicted ^{13}C NMR values using the ACD/Labs' ^{13}C and ^{1}H NMR prediction software: δ 193.4 (C2, C=O), 193.4 (C1, C=O), 152.0 (C3), 144.5 (C4), 136.5 (C_5'), 136.5 (C_4'), 67.5 (CH_2OH), 64.5 (C_1'), 42.0 (C_3'), 38.3 (C_2'). But here is the literature [74] and it is in agreement with this literature.

LRMS (EI): 208.13(100), 177.10 (10), 150.08 (36), 133.10 (8), 112.04 (13), 94.08 (16), 79.04 (57), 56.32 (50)

HRMS FAB: m/z Calcd. For $C_{10}H_{12}N_2O_3$ ($M + H^+$) 209.0926. Found 209.0926

IR (KBr) 3500-3000 cm^{-1} (OH), 3295.8, 3169.0 cm^{-1} (NH_2), 2939.6 cm^{-1} (C-H), 1806.1 cm^{-1} (C=O), 1642.4 cm^{-1} (C=O), 1569.5 cm^{-1} (C=C), 1518.7 cm^{-1} (C=O), 1478.1 cm^{-1} (C-N), 699.7 cm^{-1} (broad N-H)

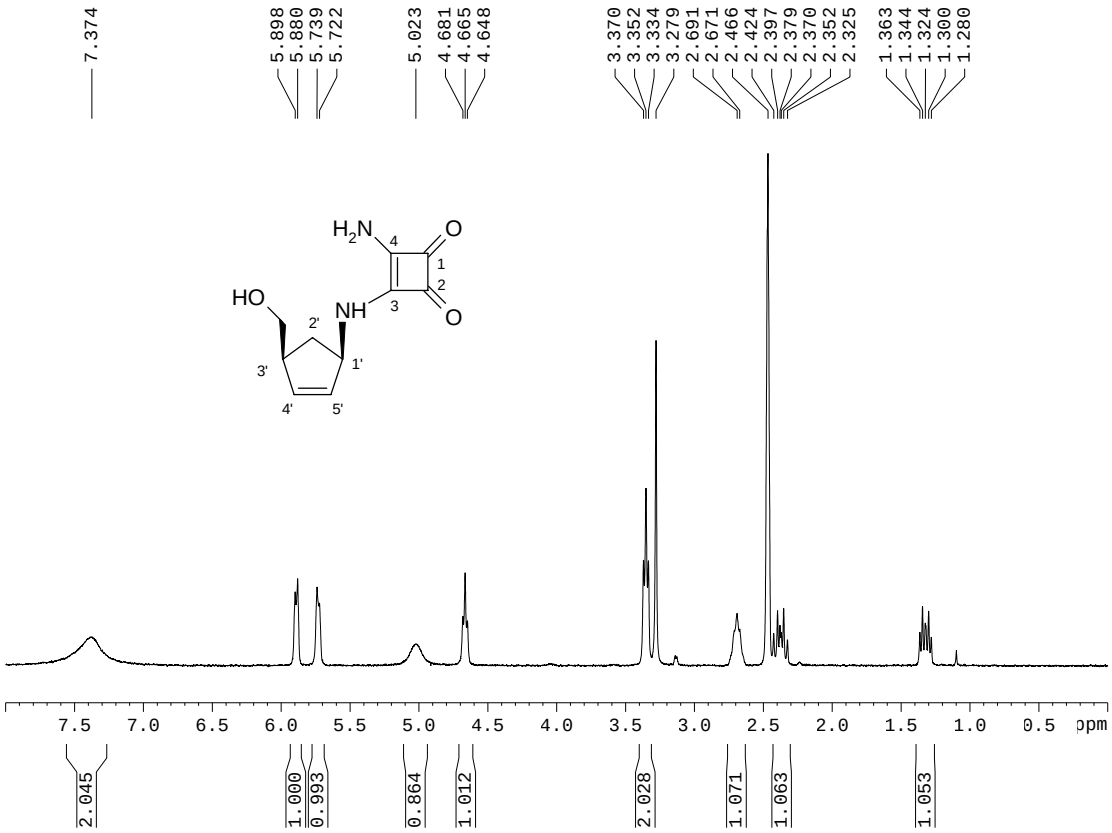


Figure 11: ^{1}H NMR data for compound **27**

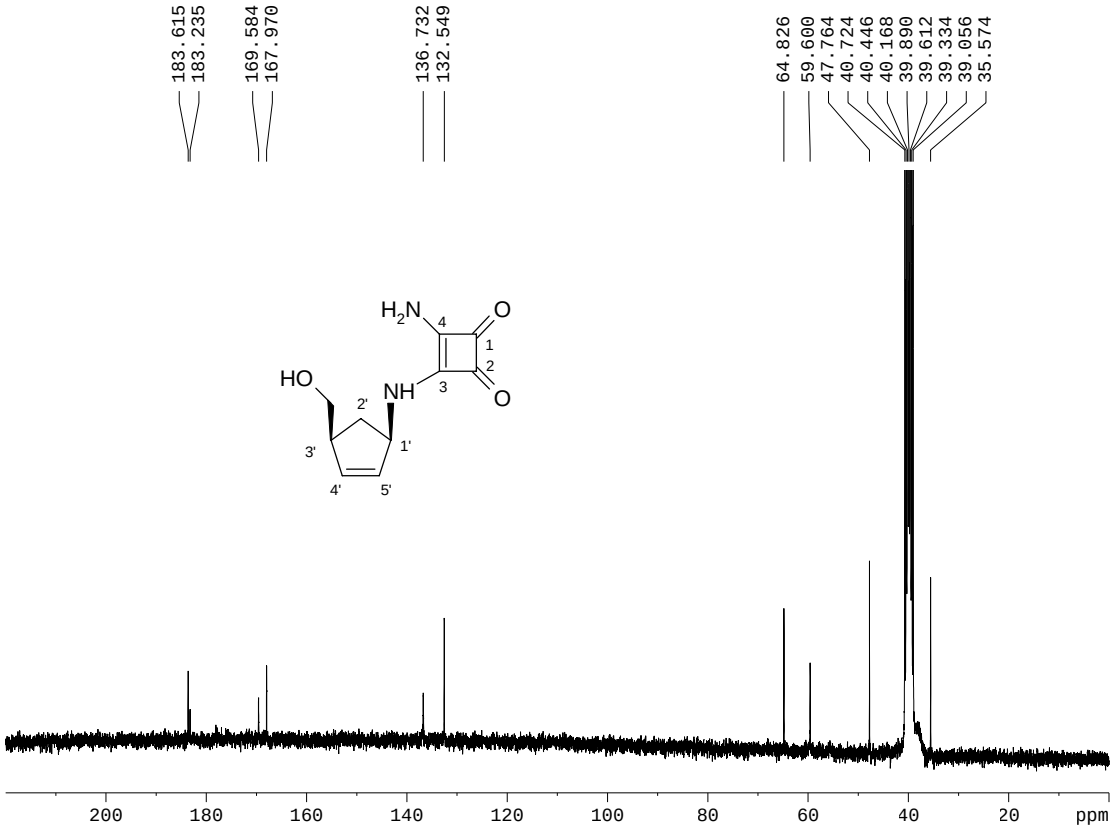


Figure 12: ^{13}C NMR data for compound **27**

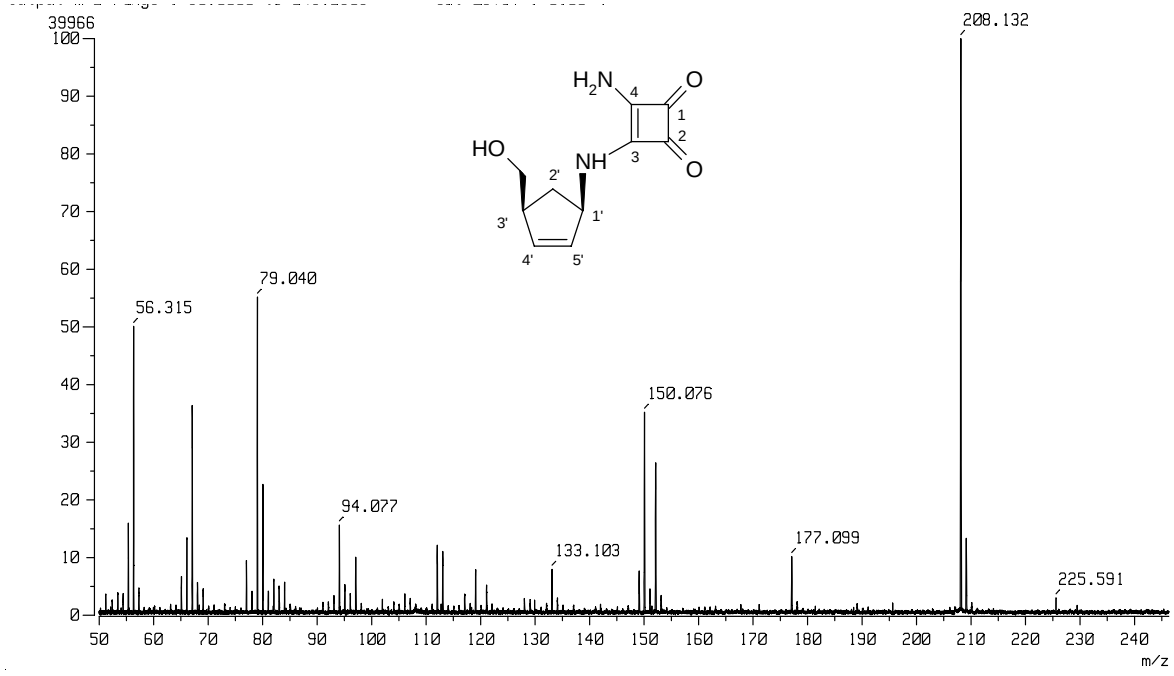
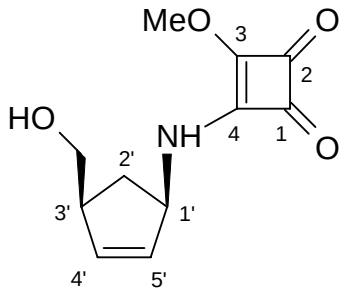


Figure 13: Mass spectrum (EI+) for compound **27**

3-Methoxy-4-[((1R,3S)-3-hydroxymethyl-4-cyclopentene)-1-amino]-3-cyclobutene-1,2-dione (28)



To a solution of **44** (638.6 mg, 2.8 mmol) in methanol (5 ml), dimethyl squarate **32** (398 mg, 2.8 mmol) and sodium methoxide (151.2 mg, 2.8 mmol) were added. The pH of the mixture was adjusted to 7.0 by *N, N*-diisopropylethylamine (1 ml). The reaction was run under microwave conditions for 60 mins at 100 °C. Then the mixture was concentrated *in vacuo*. The residue was purified on a silica column ($CHCl_3$: CH_3OH = 95:5) to give the product (421.5 mg, 67%) as pale yellow oil.

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.36-1.40 (1H, m, C_2'-H), 2.29-2.39 (1H, m, C_2'-H), 2.68 (1H, m, C_3'-H), 3.30-3.34 (2H, m, CH_2OH), 4.24-4.27 (3H, d, *J* = 9.0, OCH_3), 4.63-4.66 (1H, m, C_1'-H), 5.07 (1H, brs, OH) 5.69 (1H, s, C_4'-H), 5.88-5.89 (1H, m, C_5'-H)

^{13}C NMR (75 MHz, DMSO-d_6) δ 189.51 (C1, C=O), 182.46 (C2, C=O), 177.51 (C3), 171.36(C4), 136.63 (C_5'), 131.77 (C_4'), 64.90 (CH_2OH), 60.22 (C_1'), 59.93 (OCH_3), 47.85 (C_3'), 35.05 (C_2'). The ^{13}C NMR spectrum recorded was compared to literature [74]. It compares favorably.

LRMS (EI): 223.10 (100), 192.08 (24), 165.05 (50), 149.06 (7), 134.06 (20), 128.04 (8), 97.07 (11), 79.02 (65), 67.07 (36), 66.07 (19)

HRMS (EI): m/z Calcd. For $C_{11}H_{13}NO_4$ M^+ 223.2253. Found 223.0849

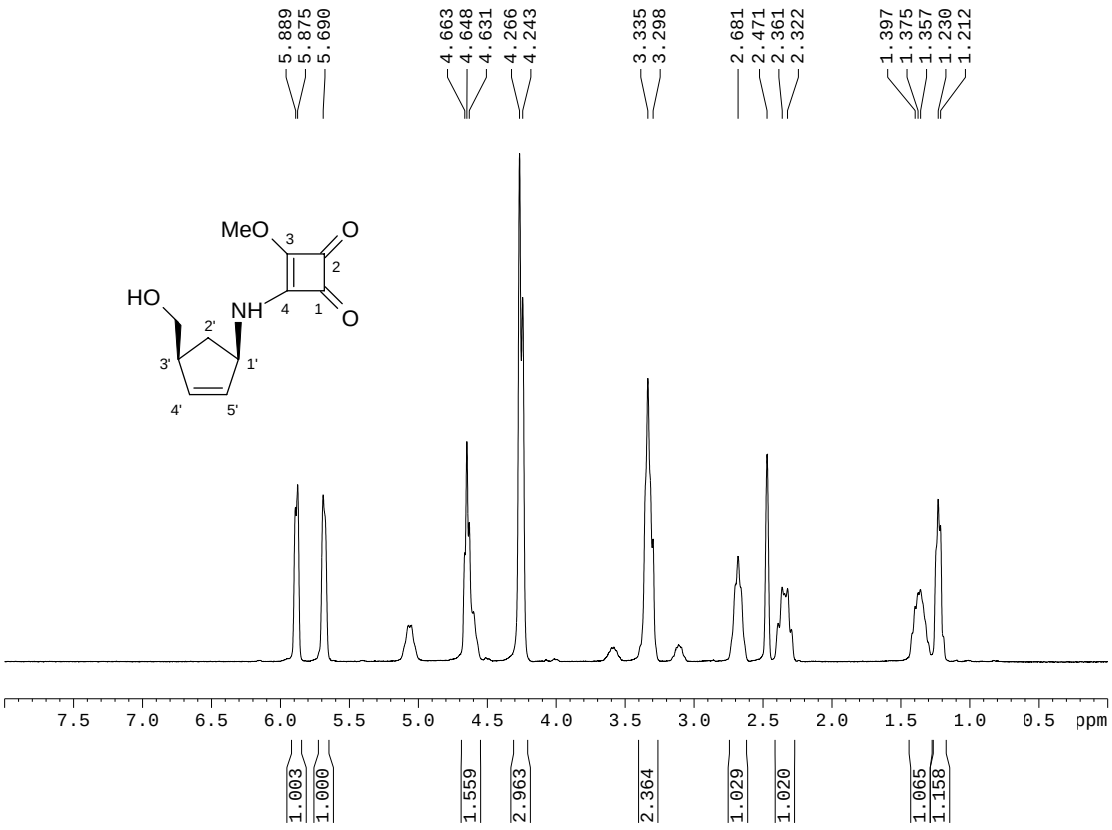


Figure 14: ^{1}H NMR data for compound **28**

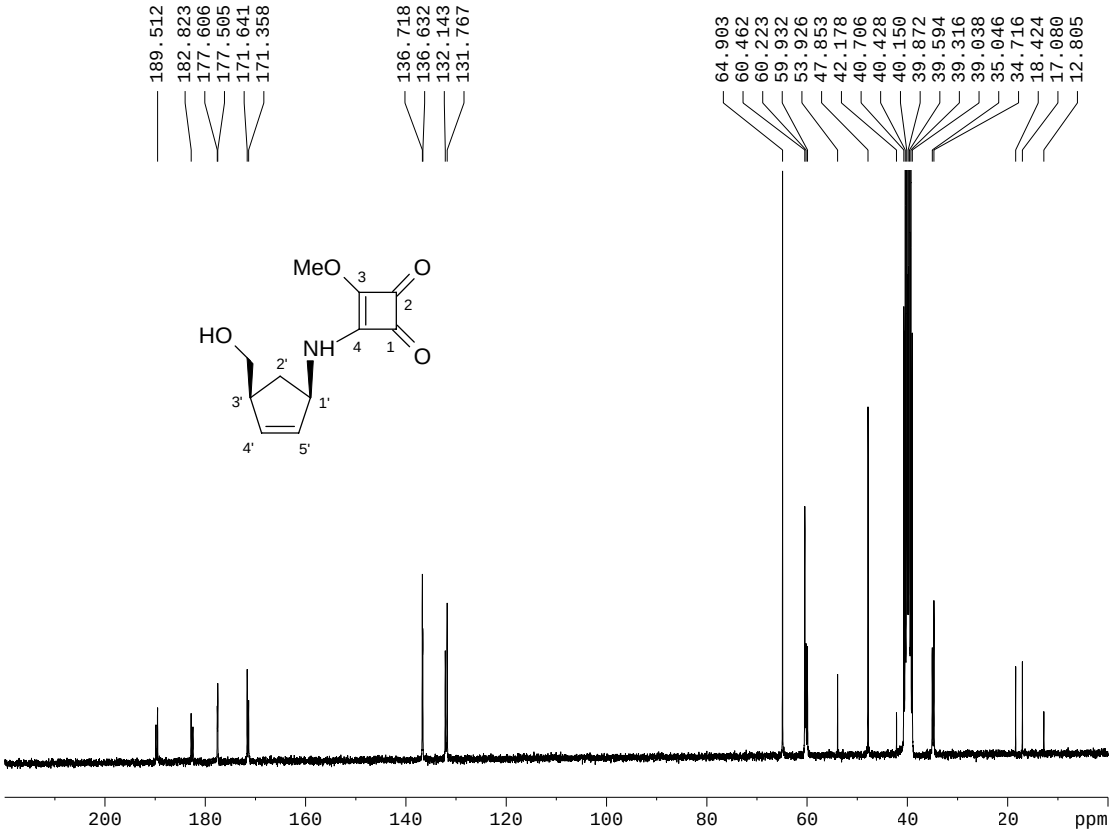


Figure 15: ^{13}C NMR data for compound **28**

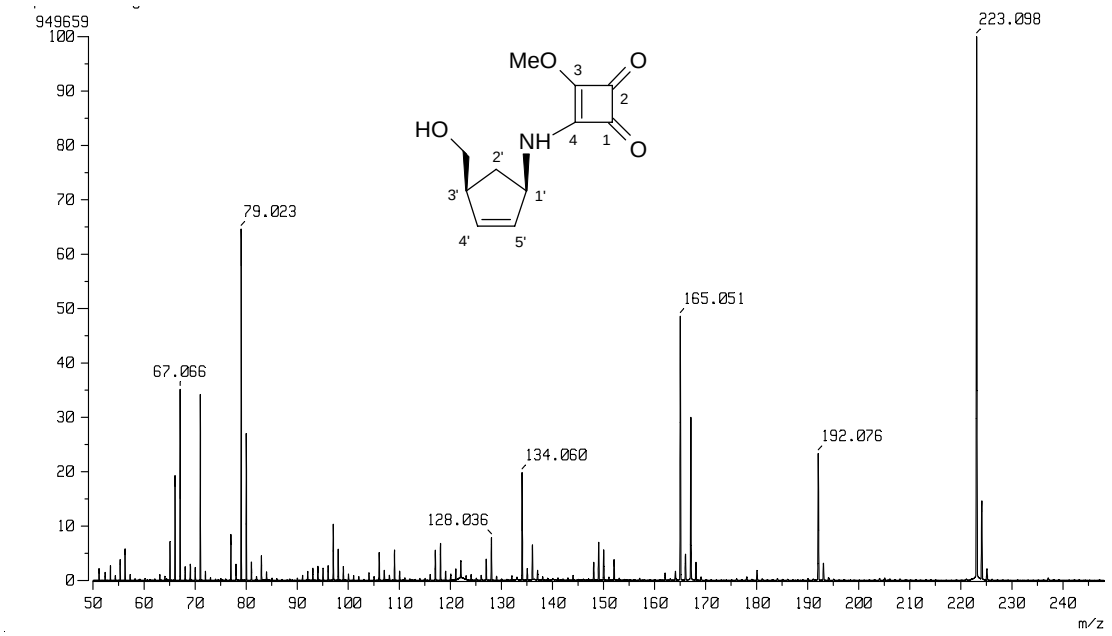
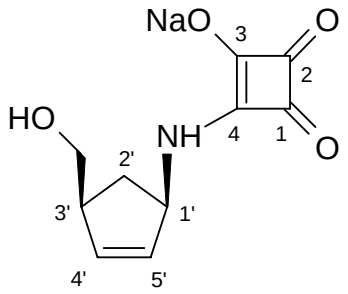


Figure 16: Mass spectrum (EI+) for compound **28**

3-Hydroxy-4-[((1R,3S)-3-hydroxymethyl-4-cyclopentene)-1-amino]-3-cyclobutene-1,2-dionate, sodium salt (29)



The product **28** (223 mg, 1 mmol) from the previous reaction was dissolved in cooled methanol (10 ml). To this solution was added dropwise cooled 0.1 M sodium hydroxide (10 ml). The mixture was stirred at 4 °C for 3 hours and then stirred at room temperature for another 7 hours. After removal of the solvent *in vacuo*, the residue was subjected to flash chromatography ($CHCl_3$: CH_3OH = 10: 1), giving the product (185.4 mg, 80%) as yellow powder.

mp 160 °C

^{1}H NMR (300 MHz, DMSO-d_6) δ 1.30-1.33 (1H, m, C_2'-H), 2.31-2.33 (1H, m, C_2'-H), 2.67 (1H, m, C_3'-H), 3.36 and 3.38 (2H, d, CH_2OH), 4.66-4.68 (1H, m, C_1'-H), 5.11-5.12 (1H, brs, OH) 5.69-5.70 (1H, m, C_4'-H), 5.78-5.79 (1H, m, C_5'-H), 6.72-6.74 (1H, brs, NH)

^{13}C NMR (75 MHz, DMSO-d_6) δ 199.56 (C1, C=O), 199.56 (C2, C=O), 188.88 (C4), 181.31 (C3), 134.75 (C_5'), 134.26 (C_4'), 65.08 (CH_2OH), 58.77 (C_1'), 47.75 (C_3'), 35.74

(C_2'). These ^{13}C NMR values are in agreement with those for the similar compounds from the literature [75].

LRMS FAB: m/z Calcd. For $C_{10}H_{10}NO_4 + Na^+$ ($M + H^+$) 232.0586. Found 232.0509

HRMS FAB: m/z Calcd. For $C_{10}H_{10}NO_4 + Na^+$ ($M - Na^+ - H^+$) 208.0610. Found 208.0617

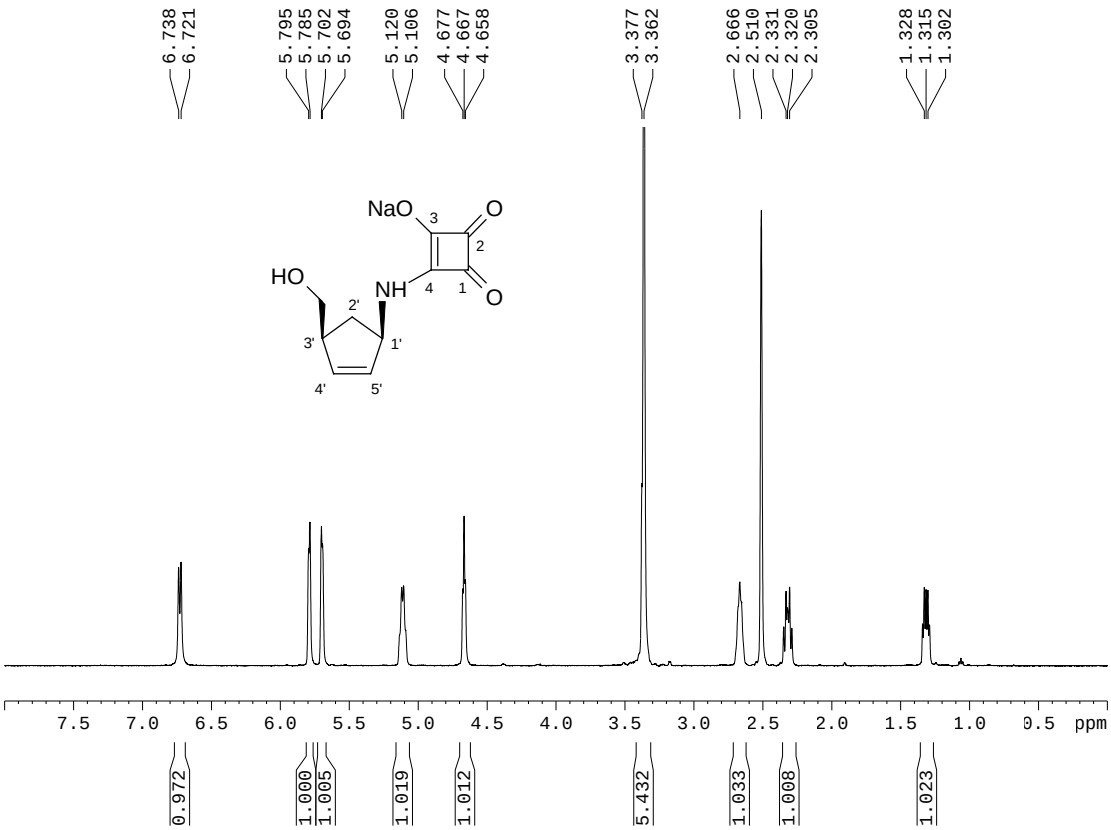


Figure 17: ^1H NMR data for compound **29**

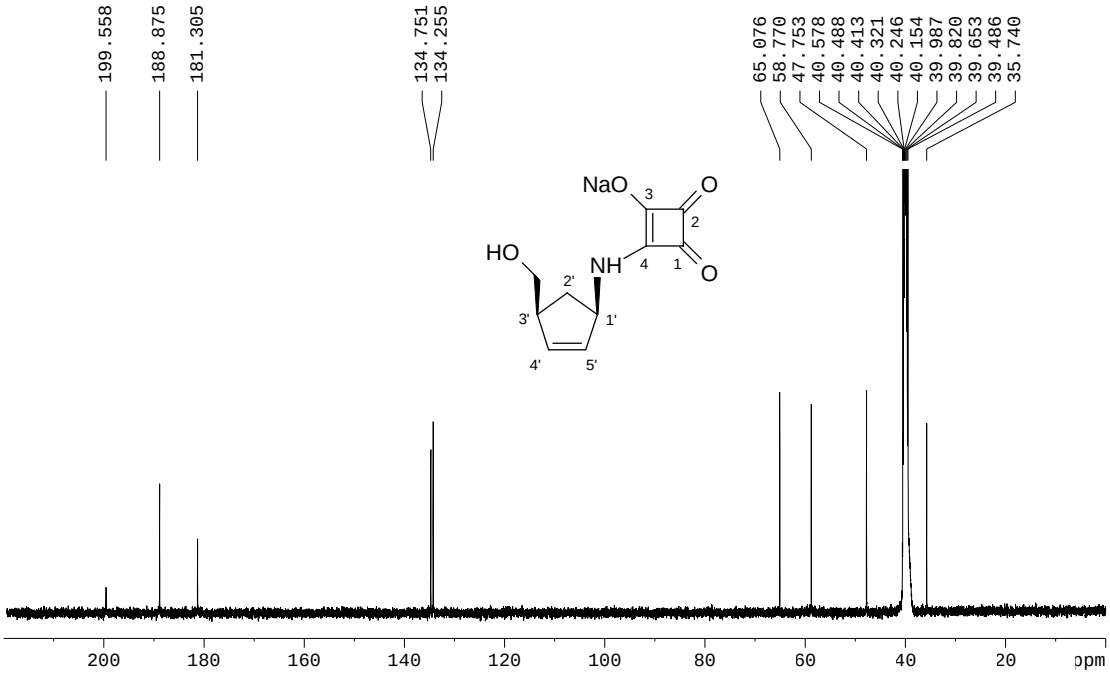


Figure 18: ^{13}C NMR data for compound **29**

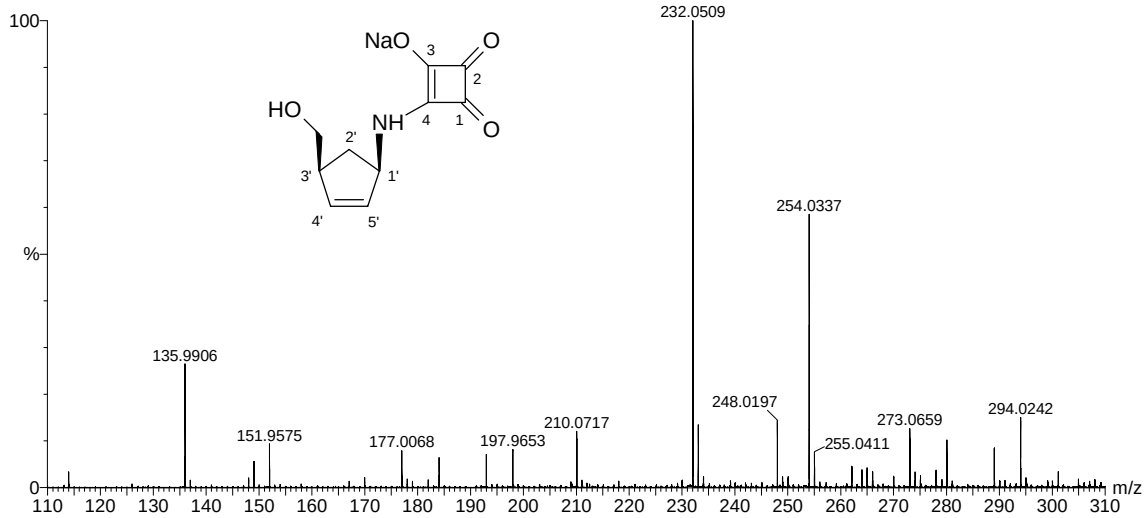
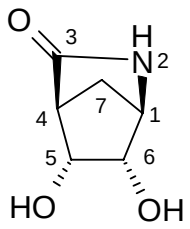


Figure 19: Mass spectrum (ESI+) for compound **29**

(1R, 4S, 5R, 6R)-5,6-dihydroxy-2-azabicyclo[2.2.1]heptan-3-one (45)

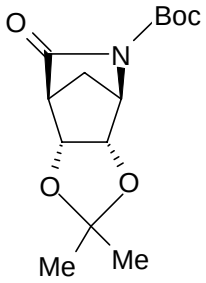


To a solution of (1R)-(-)-2-azabicyclo[2.2.1]hept-5-en-3-one (0.546 g, 5 mmol) (commercial from Sigma-Aldrich Company, ≥ 98% purity with ≥ 99% (HPLC) optical purity) and N-methylmorpholine N-oxide (1.375 g, 10 mmol) in acetone/water (8:1, 100 ml), osmium tetroxide (65 mg, 0.05 mmol) was added and the mixture was stirred for 4 h. The mixture was diluted with acetone (75 ml) and an excess of solid sodium hydrogen sulfite was added in order to destroy the oxidant. The suspension was filtered through Celite and the Celite was washed with an additional 25 ml of acetone. The filtrate was concentrated under reduced pressure. The crude product was purified by column chromatography ($CHCl_3$: CH_3OH = 10:1) to give a white solid (679.5 mg, 95%).

^{1}H NMR (300 MHz, D_2O) δ 1.92 (2H, s, C_5-H, C_6-H), 2.47 (1H, s, C_4-H), 3.63 (1H, s, C_1-H), 3.85-3.92 (2H, dd, J = 13.9 Hz, 5.9 Hz, C_7-H_2).

LRMS (EI): 143.13 (22), 125.12 (8), 114.12 (2.5), 96.11 (7), 81.06 (5), 83.6 (100), 55.40 (46), 54.44 (6)

N-Boc, 2,3-isopropylidene-protected 45 (46)



The product **45** (143.1 mg, 1 mmol), 2,2-dimethoxypropane (1.1 ml, 10 mmol), *p*-toluenesulphonic acid (190.2 mg, 1 mmol) were dissolved in dimethylformamide (4 ml). The mixture was stirred at room temperature for 20 hours and then concentrated *in vacuo*. Without further purification, the residue was dissolved in acetonitrile (4 ml). To this solution were added di-*tert*-butyl dicarbonate (436.5 mg, 2 mmol) and 4-dimethylaminopyridine (122.2 mg, 1 mmol). The solution was stirred at room temperature for 15 hours and then concentrated *in vacuo*, furnishing the dark residue.

The ratio of compound **45**, 2,2-DMP, TsOH was changed to 1:10:1.2 or 1:1:1.2. The other conditions are the same as presented above.

CHAPTER 3

BIOACTIVITY STUDIES

3.1 Anticancer activity studies

3.1.1 Human tumour cell line screen

The three compounds **27**, **28**, **29** were sent to the Developmental Therapeutics Program (DTP) of the National Cancer Institute (NCI, USA) to have a complete screen of their anticancer activity. Compound screening at DTP has focused on the response of a panel of 60 human tumour cell lines [76], representing nine distinct tumour types: leukemia, colon, lung, central nervous system (CNS), renal, melanoma, ovarian, breast and prostate. The NCI 60 human tumour cell line anticancer drug screen (NCI60) was developed in the late 1980s as an *in vitro* drug-discovery tool with a rich source of information about the mechanisms of growth inhibition and tumour-cell kill [77]. The three synthetic compounds were analyzed for selective growth inhibition or cell killing of particular tumour cell lines. The screening begins with the evaluation of all compounds against the 60 cell lines at a single dose of 10 μM. The output from the single dose screen is a biological response pattern and is reported as a mean graph, which is available for analysis in pattern recognition algorithms (COMPARE program) [78]. Using these algorithms, it is possible to assign a putative mechanism of action to a test compound, or to determine that the response pattern is unique and not similar to that of any of the standard prototype compounds included in the NCI database. In addition, following

characterization of various cellular molecular targets in the 60 cell lines, it may be possible to select compounds most likely to interact with a specific molecular target. After the analysis by the COMPARE program, compounds which exhibit significant growth inhibition might be evaluated against the 60 cell panel at five concentration levels.

3.1.1.1 Results and discussions

The NCI60 screening data for compounds **27, 28, 29** against 60 different human tumour cell lines are presented in Figures 20, 21, 22 respectively. By convention, bars to the left indicate resistance, and bars to the right indicate sensitivity. As shown in Figure 20, non-small cell lung cancer (EKVX), colon cancer, breast cancer, renal cancer (ACHN), melanoma (SK-MEL-28), and CNS cancer cells were relatively unaffected by compound **27**. But non-small cell lung cancer (A549/ATCC, HOP-62 and NCI-H522), ovarian cancer, leukemia, renal cancer (UO-31) and melanoma exhibit some sensitivity to compound **27** at the 10 micromolar level (Figure 20). Among these somewhat sensitive cell lines, renal cancer (UO-31) cell line expresses the highest growth inhibition (22%) by compound **27**. The others are inhibited by 9% ~ 18%.

The profiles of cell line responses for compound **28** are similar to those for compound **27**. Compound **28**, at the 10 micromolar level, appears to have moderate growth inhibition on non-small cell lung cancer (NCI-H522), colon cancer (KM12), renal cancer (UO-31), and melanoma (UACC-257) cells, whose growth inhibition rates are 18%, 17%, 16% and 16% respectively (Figure 21).

Compound **29**, at the 10 micromolar level, appears to have moderate growth inhibition against CNS cancer (SF-295) cells, which was inhibited up to 36% of growth. Non-small

cell lung cancer (NCI-H522) showed 20% growth inhibition. Several other cell lines exhibited growth inhibition ranging from 1% to 12% (Figure 21).

As a result, several of the 60 human tumour cell lines could be inhibited to some extent at a single dose of 10 μM of compounds **27**, **28**, **29**. However, they did not exhibit significant growth inhibition and no further testing is recommended by the National Cancer Institute.

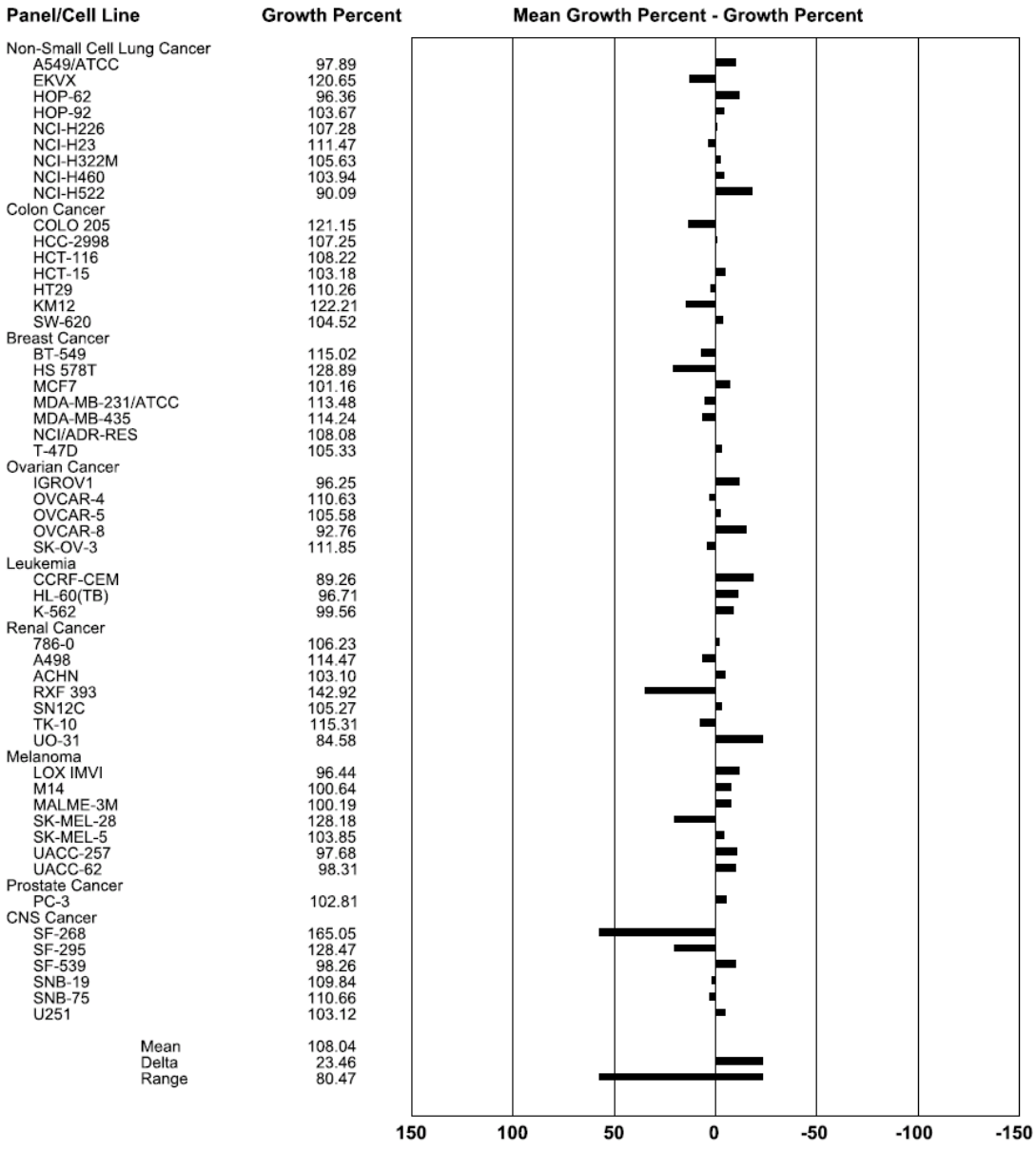


Figure 20: Screening data for Compound **27** from the NCI60

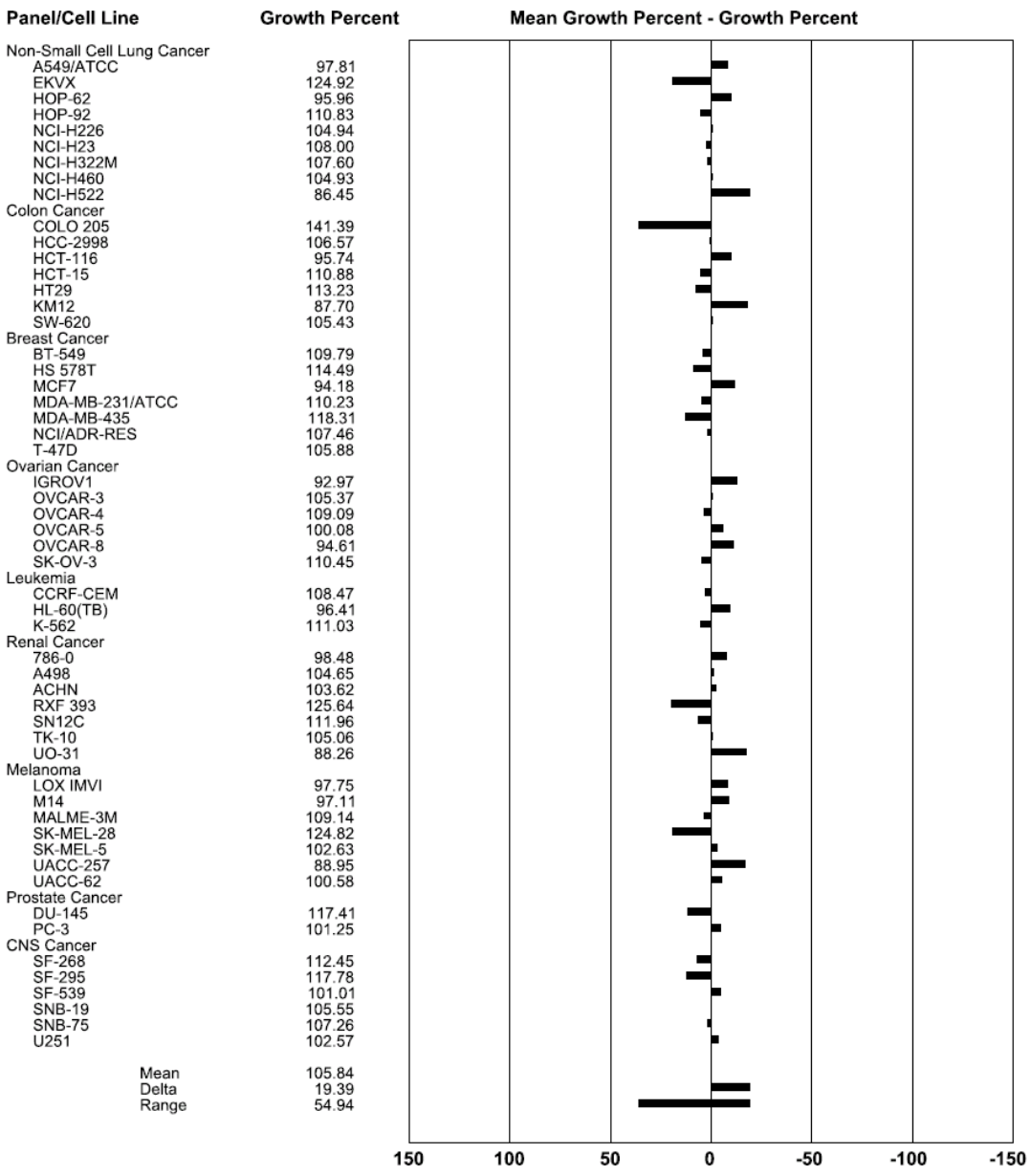


Figure 21: Screening data for Compound **28** from the NCI60

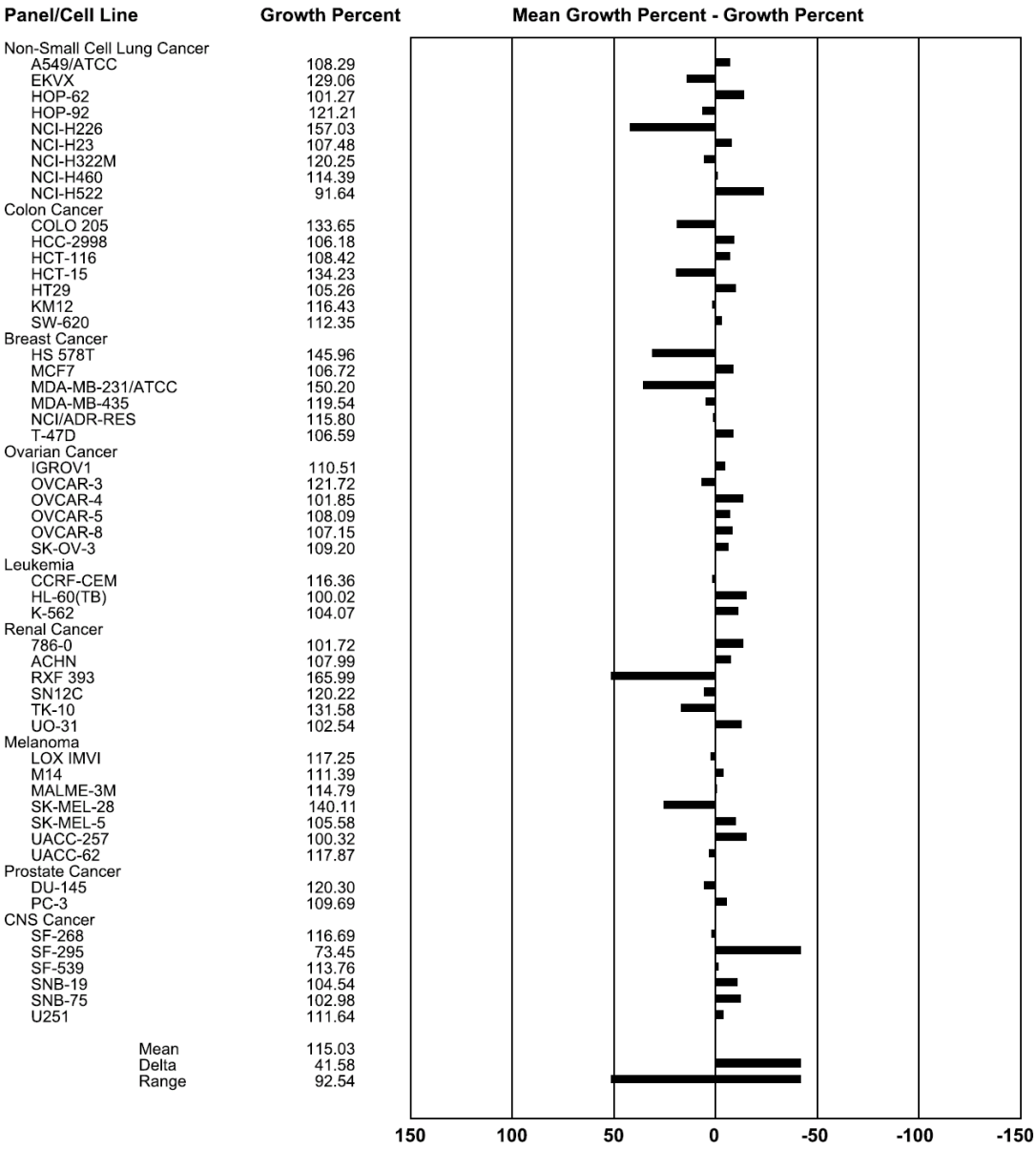


Figure 22: Screening data for Compound **29** from the NCI60

3.1.2 Cell viability and proliferation

We were interested in undertaking some preliminary tumour cell viability studies in the presence of our three nucleoside analogues while waiting for anticancer testing by the NCI. *In vitro* and *living cell* measurements of cell death were conducted in Dr. Qing-Bin Lu's laboratory at the University of Waterloo. The colorimetric assay kits were used for the analysis of cell viability. Vybrant® MTT cell proliferation assay kit is a non-hazardous, precise, rapid, colorimetric, straightforward and lack of any radioisotope assay for monitoring cell viability [79]. So Vybrant® MTT cell proliferation assay kit (V-13154) was used to monitor the growth rate of a cell population and the amount of live cells. Unlike other colorimetric assays, the MTT assay can be used with all cell types. It is based on the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), which measures only living cells and the results can be read on standard microplate absorbance readers at 570 nm [80]. This colorimetric assay involves the conversion of the yellow tetrazolium salt MTT to the blue formazan derivative by mitochondrial enzymes in viable cells. It is a sensitive assay with excellent linearity up to approximately 10^6 cells per well.

3.1.2.1 Materials and methods

3.1.2.1.1 Reagents

Eagle's minimum essential medium with phenol red, Eagle's minimum essential medium without phenol red, MEM non-essential amino acid solution (100x), Dulbecco's phosphate buffered saline (PBS), and L-glutamine were purchased from the Sigma-Aldrich Company, Canada. Vybrant® MTT cell proliferation assay kit (including MTT

and sodium dodecyl sulfate (SDS)) and penicillin-streptomycin were purchased from Invitrogen™ Corporation, USA. Fetal bovine serum (FBS) was purchased from ATCC, USA. Trypsin EDTA, 1x (0.25% trypsin/2.21 mM EDTA in Hank's balanced salt solution (HBSS) without sodium bicarbonate, calcium & magnesium) was purchased from Mediatech, Inc., USA. Reagent grade hydrochloric acid was purchased from Fisher Scientific Co., Canada.

3.1.2.1.2 Growth of HeLa cell line

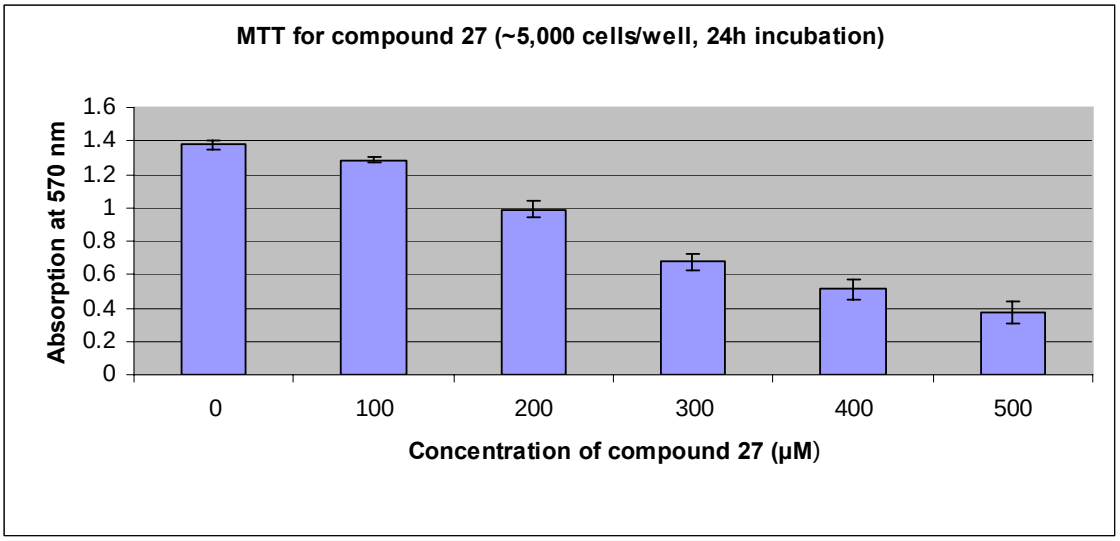
HeLa (Human epithelial cervical adenocarcinoma cell line) cells were routinely grown in Eagle's minimum essential medium with phenol red containing 1% of MEM non-essential amino acid solution, 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin as antibiotics. Cultures were maintained in BD falcon tissue culture flasks in a humidified atmosphere of 95% air/ 5% carbon dioxide at 37 ^{o}C CO_2 Incubator (Thermo Electron Corporation). Cells were removed by trypsinization and subcultured according to the following procedure. Medium was decanted, and the cells were rinsed once with 2 ml of sterile phosphate-buffered saline (PBS) (pH = 7.4). The cells were then overlaid for 1-3 min with 0.5 ml of trypsin. Cells were passaged at 85-90% of confluency in BD falcon tissue culture flasks. All cell counts were performed on a Hemacytometer purchased from Bright-Line, USA. Compound **27**, **28**, **29** were prepared in Eagle's minimum essential medium with phenol red at the following concentrations: 100, 200, 300, 400, 500 μM.

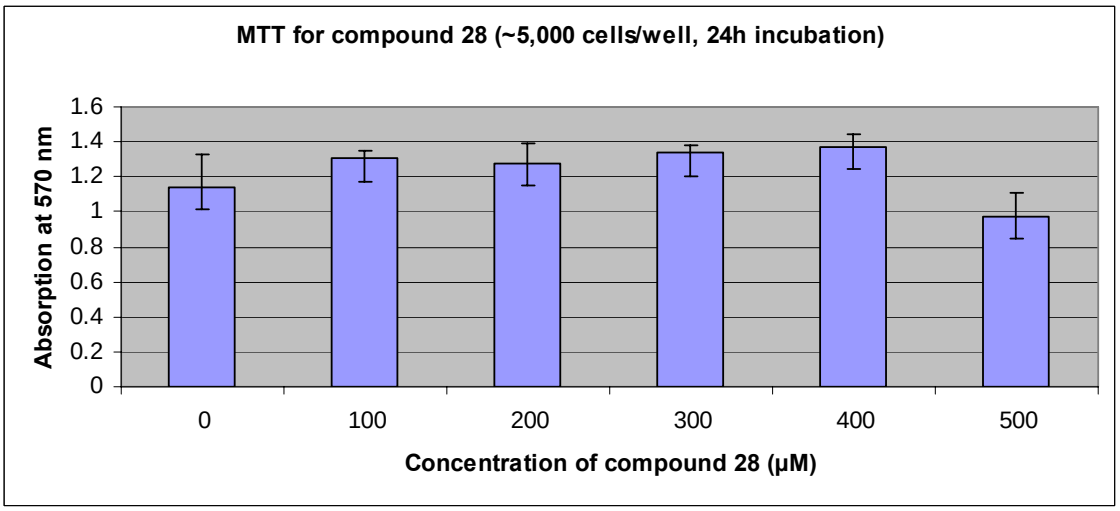
3.1.2.1.3 MTT assay

After 85-90% of cell confluency, the cells were trypsinized as described above and seeded at a concentration of 2.5 x 10^4 cells/ml. Cells (200 µl) were introduced into each well of 96-well culture plates. After 24 hours incubation, the old medium was removed and different concentrations of compound **27**, **28**, **29** were added in triplicate. The plate was incubated at 37 ^{o}C for 24 hours. Then the medium containing the compounds were removed. Eagle's minimum essential medium (100 µl) without phenol red containing 0.1 % of 200 mM L-glutamine and 10 µl of 12 mM MTT in sterile PBS were added to each well. After an incubation time of 4 hours, SDS-HCl (100µl , 1 gm SDS/ 10 ml of 0.01 M HCl) was added to each well and the plate was incubated for another 12 hours. Absorption of each well was assayed by a scanning multiwell spectrophotometer at 570 nm (Thermo Labsystems, Canada).

3.1.2.2 Results and discussions

As shown in Figure 23, HeLa cells were unaffected by compounds **28**, **29** at 100, 200, 300, 400, and 500 µM but was sensitive to compound **27** at 300 µM. Cisplatin is a chemotherapy drug for the treatment of various types of cancers. For comparison, almost all HeLa cells are dead by cisplatin at 50 µM (Figure 24). The concentrations we tested were much higher than 50 µM. Since there was no significant effect on the HeLa cell by compounds **27**, **28**, **29**, no further experiments at lower concentrations were followed.





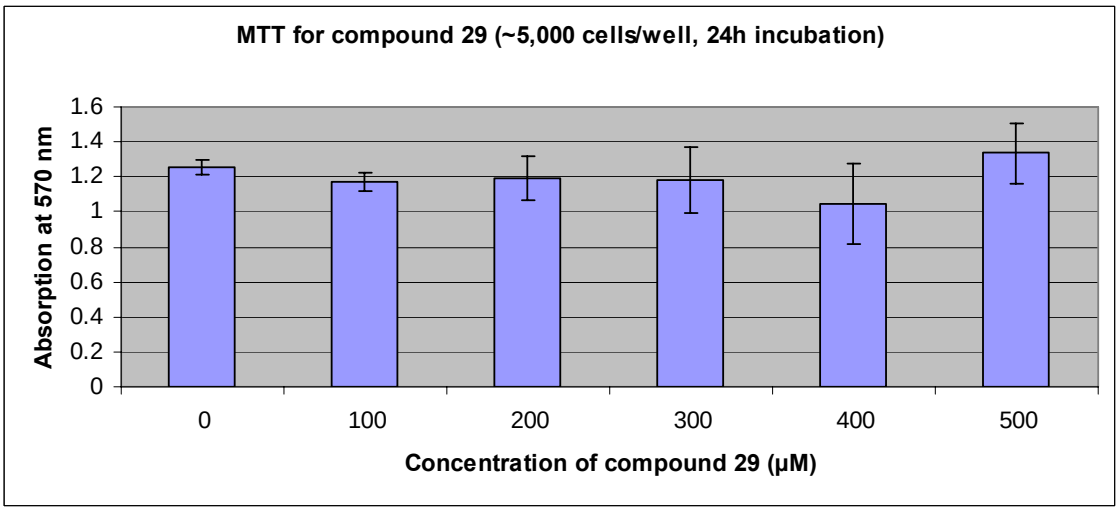


Figure 23: MTT data for compounds 27, 28, 29 at 100, 200, 300, 400, and 500 μM

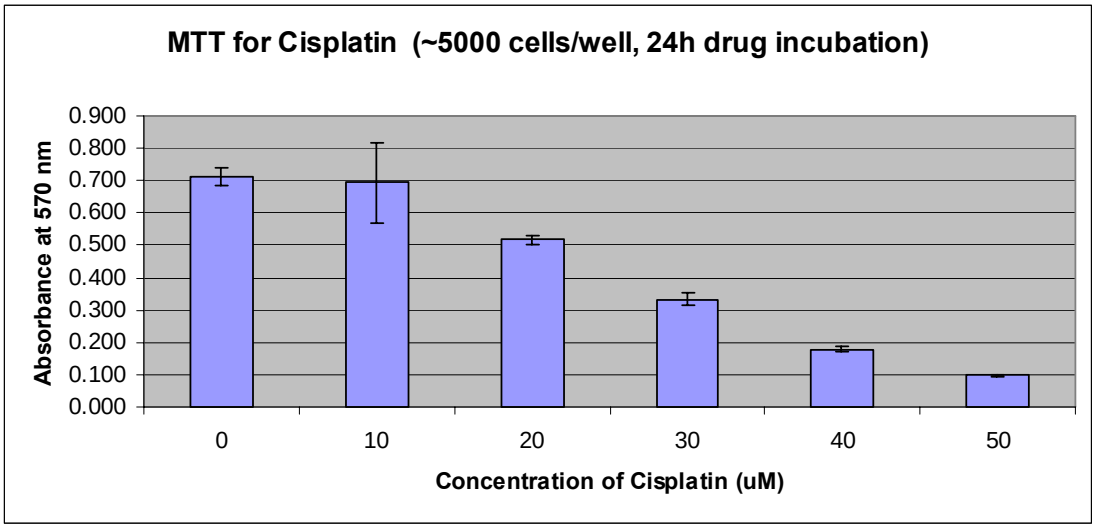


Figure 24: MTT data for Cisplatin at 10, 20, 30, 40, and 50 μM

3.2 Antiviral activity studies

The National Institute of Allergy and Infectious Diseases (NIAID) has established the Antimicrobial Acquisition and Coordinating Facility (AACF) to provide free and confidential services for submitting compounds to be evaluated for antiviral activity. The AACF uses animal models for evaluating antiviral agents against the following viruses: SARS, Respiratory panel (Flu A, Flu B, RSVS, PIV, Measles, HRV, Adeno) and the Biodefense panel (Rift Valley Fever, Tacaribe, VEE, Yellow Fever, WNY, Dengue) [81], Pox panel (Cowpox, Vaccinia), Herpes panel (HSV-1, HSV-2, VZV, EBV, CMV), Hepatitis B virus, Hepatitis C virus [82], Human papilloma virus (HPV). The three synthesized compounds **27**, **28**, **29** have been sent to AACF to screen their potential antiviral activity against all the above viruses. This was determined after discussions with Dr. Tseng at NIAID. Confidentiality agreements were signed between the University of Waterloo and NIAID to ensure structure confidentiality in the event that the

patenting of the compounds would be of interest. Compounds demonstrating reasonable antiviral and cytotoxicity profiles could be candidates for several additional follow-up analyses.

CHAPTER 4

SUMMARY AND FUTURE WORK

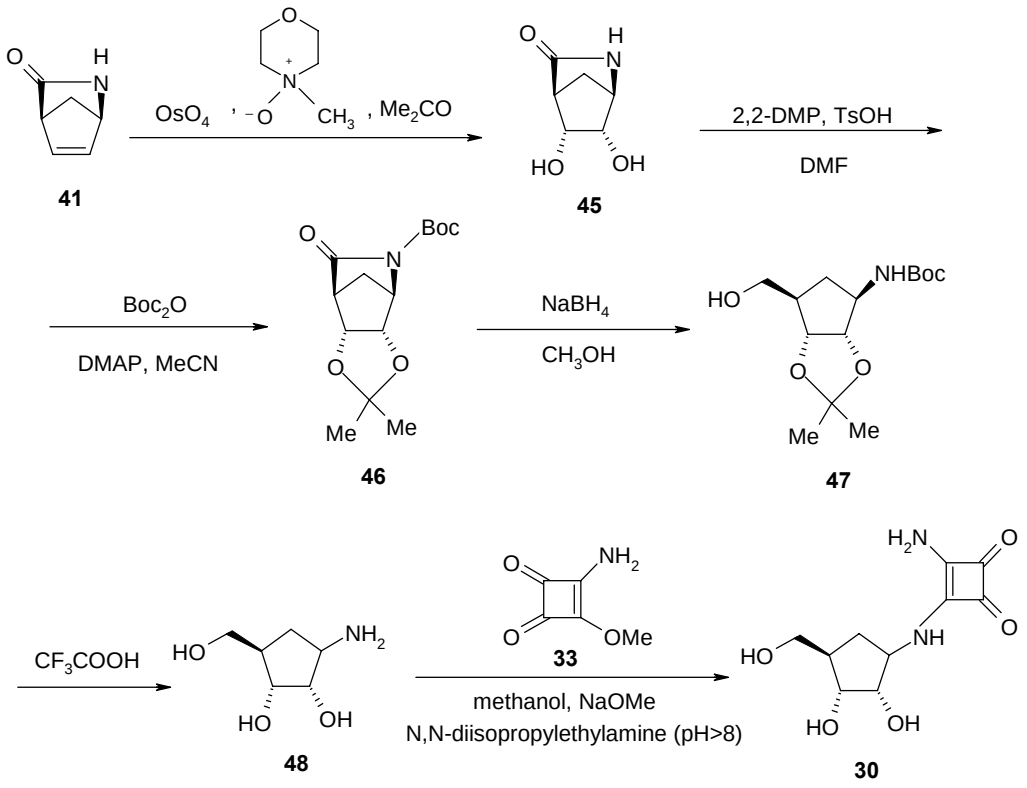
4.1 Summary

The objectives of this project were to explore the application of squaric acid analogues to the synthesis of novel nucleoside analogues and to examine their potential bioactivities. The syntheses of three nucleoside analogues containing the squaric acid moiety were completed. Their synthesis involved the reaction of (1R)-(-)-4-(hydroxymethyl)cyclopent -2-en-1-ylamine with either 4-amino-3-methoxy-3-cyclobutene-1,2-dione or react with 3,4-dimethoxy-3-cyclobutene-1,2-dione, followed by hydrolysis. These three compounds were sent to NCI to screen for their potential anticancer activities. Little to modest antitumour activity was detected for these compounds. In addition, a study of their cytotoxity to HeLa cells was performed as well. However, no significant effect was observed by these compounds. Furthermore, these three compounds are currently being tested for their potential antiviral activity against various viruses by NIAID.

4.2 Future work

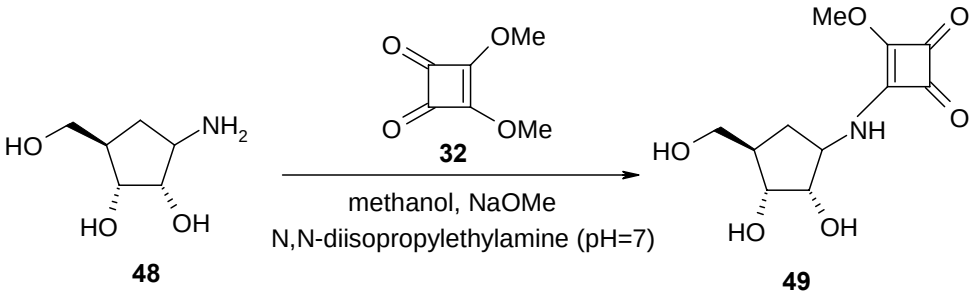
We will continue our attempts to try and crystallize compounds **27**, **28**, **29** for X-ray to investigate the structure of these three novel compounds.

We will also try to generate compound **46** by changing the ratios of reagents. Once the protected compound **46** is furnished, it may undergo the reductive cleavage of the lactam using sodium borohydride in methanol to give the protected alcohol **47**. Removal of both isopropylidene and Boc protecting groups by refluxing in trifluoroacetic acid (TFA, CF_3COOH) should give the free cyclopentylamine **48** [70]. The condition of the last step will be the same as that of making the compound **27**. And it is hoped that this approach will afford (1R, 2S, 3R, 5R)-4-amino-3-[(5-hydroxymethyl-cyclopentane-1,2-diol)-3-amino]-3-cyclobutene-1,2-dione **30** (Scheme 4.1).



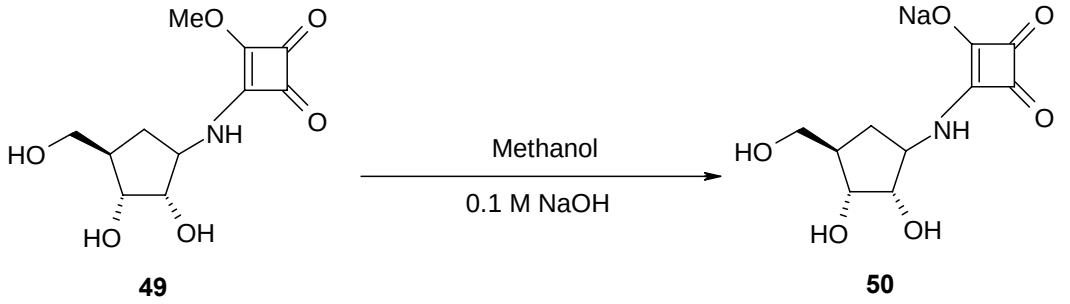
Scheme 4.1

Another nucleoside analogue **49** like compound **28** could be synthesized by treatment of **48** with squarate diester **32** using sodium methoxide and N,N-diisopropylethylamine (Scheme 4.2).



Scheme 4.2

If compound **49** is successfully obtained, we will also attempt to conduct the reaction of **49** with sodium hydroxide to generate the salt **50** (Scheme 4.3).



Scheme 4.3

Once achieving these three compounds, we will send them to NCI and NIAID to screen their potential anticancer and antiviral activities respectively.

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