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Chapter 2

Ring-Expanded ('Fat') Purines and their Nucleoside/Nucleotide Analogues as Broad-Spectrum Therapeutics

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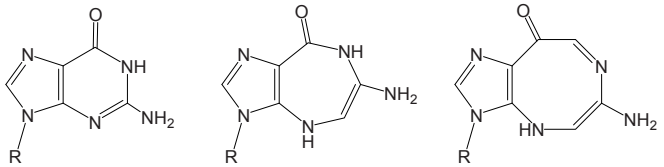
2.1. INTRODUCTION

Purine is a bicyclic, 5:6-fused, aromatic, heterocyclic compound with a 5-membered imidazole ring fused to a 6-membered pyrimidine ring <B-80MI1>. Although purine itself has never been found in nature, substituted purines like adenine and guanine or their respective nucleoside derivatives, adenosine and guanosine, are the most ubiquitous class of nitrogen heterocycles and play crucial roles in wide variety of functions of living beings <B-67MI93; B-67MI287; B-71MI57; 71NCI184; B-77MI130; 04CBD361>. As nucleotides (AMP,GMP), they are the building blocks of nucleic acids (RNA/DNA) <B-87MI269 pp.; B-87MI280; 90NN297; B-95MI89>. They serve as energy cofactors (ATP, GTP) <B-07MI365>, as part of coenzymes (NAD/FAD) <B-95MI89> in oxidation-reduction reactions, as important second messengers in many intracellular signal transduction processes (cAMP/cGMP) <06MMB369; 08JIP1028>, or as direct neurotransmitters by binding to purinergic receptors (adenosine receptors) <09AP415>. Therefore, it is not surprising that analogues of purines have found utility both as chemotherapeutics (antiviral, antibiotic, and anticancer agents) <99MI62; 05MI9; 05DA983; 08CCT21> and pharmacodynamic entities (regulation of myocardial oxygen consumption and cardiac blood flow) <01TCM259; 03CTM369; 07AJC1507; 08JPl4993>. While they can act as substrates or inhibitors of enzymes of purine metabolism (ADA, Guanase, HGPRTase, PNPase, etc) to render their chemotherapeutic action <79BP1057; 80IC257; 82IJB153; 93AR1809; 97AAC1686; 06OBC1131; 06SH22>, their ability to act as agonists or antagonists of A_1/A_{2A} receptors is the basis for modulation of pharmacodynamic property <01TCM259; 03CTM369; 07AJC1507; 08JP4993>. In addition, they can be excellent probes for elucidation of biochemical mechanisms (*e.g.* fluorescent ε-adenosine) <06YH1457> and biophysical characteristics of nucleic acids (*e.g.* 8-bromoguanosine) <03JA2390; 07CB23>. This review concerns a family of ring-expanded purines, informally referred to as 'fat' or *f*-purines, as well as their nucleoside/nucleotide analogues (RENs/RENTs), which have broad applications in chemistry, biology, and medicine <02CTMC1093>.

2.2. SIGNIFICANCE OF 'FAT' PURINES AND THEIR NUCLEOSIDE/ NUCLEOTIDE ANALOGUES

The definition of 'fat' or *f*-purines is explained (Scheme 1), using guanine (G) as an example. 'Fat' purines are largely 5:7-fused ring systems containing an imidazole ring fused to a variety of 7-membered nitrogen heterocycles with amino and/or carbonyl functional groups, and thus can be structurally regarded as ring-expanded purines. When the ring fusion goes beyond the 5:7-fused systems, such as 5:8, 5:9 or 5:10, we informally refer to them as 'super fat' (*sf*) purines. The corresponding ring-expanded nucleoside and nucleotide analogues are often referred to as RENs and RENTs.

Scheme 1



R=H, Ribofuranosyl or 2'-Deoxyribofuranosyl

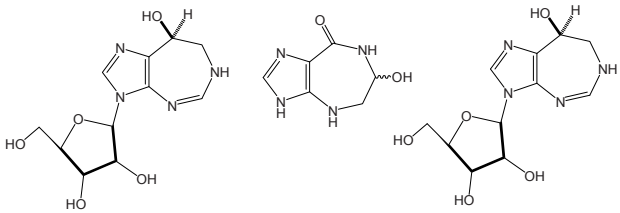
'Fat' purines and their nucleoside and nucleotide analogues are of chemical, biochemical, biophysical and medicinal interest. From a chemical standpoint, their synthesis, structure, stability, acid-base properties, aromaticity, and tautomeric equilibria, are worth exploring. From a biochemical perspective, they are an abundant source of substrates or inhibitors of enzymes of purine metabolism, as well as of those requiring energy cofactors. From a biophysical point of view, they are potentially excellent probes for nucleic acid structure, function, and metabolism. From a medicinal stance, they proved to be anticancer and antiviral agents <02CTMC1093>. They may also have other therapeutic uses, for example, *f*-purines can be regarded as analogues of the extensively-explored benzodi- and triazepines, a family of powerful pharmaceuticals acting on the central nervous system <00PZ871; 08CDM827>.

Concerning their *chemistry*, a number of *f*-purines and their nucleoside/nucleotide analogues (RENs & RENTs) have proved to be synthetically challenging. Although most of them are stable once synthesized, the steps leading to their synthesis are, more often than not, plagued with opportunistic rearrangements as we repeatedly discovered over the years <87CSR533; 88H(27)31; 88JOC382; 88JOC5309; 88S242; 95NN325; 97H(45)857; 02T9567>. It is also the experience of others who worked in similar areas since a large number of alleged seven and larger ring heterocycles were later found to be only 5- or 6-ring systems <84JHC1807; 84JHC1817; 84S1065 and the references cited therein: 85JHC753; 90JHC343>. Therefore, their structures must only be assigned with due caution, preferably by single-crystal X-ray diffraction analyses, unequivocal syntheses or extensive spectroscopic analysis <87CSR533; 88JOCl382; 90S1095; 94NN2307>. Secondly, the presence of several N atoms in the heterocyclic ring makes it difficult to predict and plan the specific site of glycosylation *a priori*, and normally more than one regioisomer is obtained <91NN819; 92NN1175; 92NN1137>. Syntheses of 2'-deoxy analogues are often complicated further by the formation of both α- and β-anomers for each regioisomer <92NN1175>.

The *theoretical* significance of *f*-purines concerns their novel physicochemical properties, including aromaticity, thermodynamic stability, reactivity, acid-base properties, and tautomeric equilibria.

Biochemically, because of their structural similarity to natural counterparts, *f*-purines and their nucleoside/-tide analogues (RENs and RENTs) are potentially rich sources of substrates or inhibitors of enzymes of purine metabolism <B-67MI287; 70PMC69; 71ARB811; B-95MI89>, and of those enzymes requiring energy cofactors such as GTP or ATP. Important precedents for potential substrate/inhibitory activity of ring-expanded nucleosides are provided by nature itself in the form of three naturally-occurring antitumor antibiotics, coformycin <74JA4326; 74JA4327; 76ACR1206; 83NN479> and pentostatin (2'-deoxy- coformycin) <74JHC641; 79JA6127; 82JOC3457; 83JHC629; 84B904; 87B5636>, which contain the 5:7-fused imidazo[4,5-*d*][1,3]diazepine ring system, and are the strongest known inhibitors of adenosine deaminase (ADA) <77BP359; B-78MI159>, and azepinomycin <87JAN1461; 88H(27)1163>, a non-nucleoside containing the imidazo[4,5-*e*][1,4]diazepine ring skeleton, which is reported to inhibit guanine deaminase (guanase) <87JAN1461; 88H(27)1163> (Scheme 2). All three antibiotics possess a tetrahedral geometry at the hydroxyl junction of their seven-membered ring and hence are regarded as transition state analogue inhibitors of ADA and guanase <B-78MI159; 87JAN1461; 88H(27)1163>. Not surprisingly, the majority of work done by others in this area has been targeted to either coformycins or their synthetic analogues <83TL4789; 85JHC349; 86JOC1050>, with only a few exceptions <67JAN227; 75HCA2192; 80DAN591; 88JHC1179; 89JMS175; 90J(P1)173; 90JMC2818; 98B11949>. By contrast, the RENs and RENTs under study in the Author's laboratory cover a broad range of both planar <94NN2307> and puckered molecules <92NN1175>, and as such, can potentially act as substrates and/or inhibitors of a wide variety of enzymes of purine metabolism, besides ADA and guanase.

Scheme 2



Biophysically, nucleotides that incorporate steric bulk in their skeleta, as compared with their natural counterparts, offer a unique means of exploring steric and conformational constraints of formation of nucleic acid double helices. Steric bulk is often associated with considerable structural deviations from the natural array. The replacement of a natural nucleotide by a ring-expanded nucleotide (RENT) affects the base-pairing and stacking interactions of nucleic acids. This would have consequent impact upon helical structure, stability, and conformations of single- and double-stranded RNA and DNA helices. Biophysical studies employing RENTs should aid in understanding better the factors and parameters that govern the formation, structure and stability of nucleic acid double helices <B-87MI269; B-87MI280>. Furthermore, because of their unique geometric features coupled with novel electronic, ionizational, and conformational characteristics, RENTs are also potentially excellent probes for nucleic acid triple helices <B-99MI233; 02COC1333; 08BIE1117>. Therefore, the study of primary and secondary structures of RENs, RENTS, and their polynucleotide derivatives is of both interest and importance <90JOC5882>. The investigations of primary structures and properties includes topology,

tautomerism, acid-base properties, aromaticity, etc. of heterocyclic rings, and *syn*/*anti* base-ribose conformations, *endo/exo* sugar puckers, and α/β-anomeric configurations, etc. of nucleosides <90JOC5882>. Investigations of secondary structures includes helical formation, stability, and conformations of polynucleotides composed of RENTs, and their potential to form double helices by complementary base-pairing with appropriate pyrimidine partners, and by vertical stacking interactions. The secondary structures of nucleic acids are, in large part, governed by the primary structures of the component nucleotides <09JA3791>.

Medicinal significance of *f*-purines, RENs and RENTs is rooted in their structural similarity to natural purines. A vast majority of them have proved to be anticancer or antiviral agents <02CTMC1093>. In searching for a molecular basis of their broad spectrum biological activities, we performed molecular modeling studies, which indeed have provided some very interesting mechanistic notions (Figure 1). Shown in Figure 1A is the Watson-Crick base-pairing array for a representative REN containing an *f*G as a base (shown in magenta),

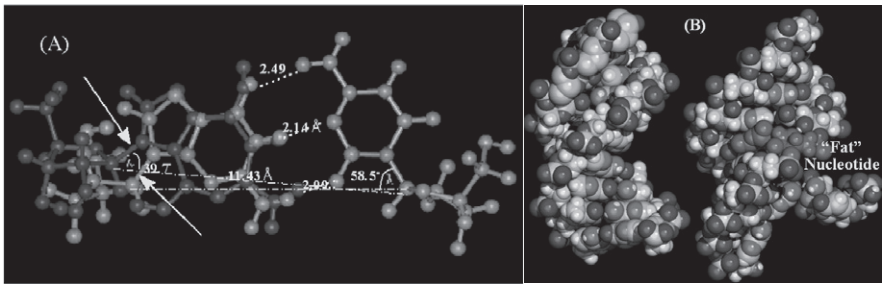


Figure 1: (A) Superimposition of *f*G…C base-pair array (magenta) over natural G…C base-pair (yellow). (B) Comparison of a B-DNA double-helix containing 10 natural nucleotide pairs with that containing the same nucleotides except for an *f*G residue replacing a G at position 5. [Molecular modeling studies were performed using QUANTA/CHARMm, available from Accelrys Software, Inc., San Diego. Extensive energy minimization was carried out on the two duplexes, employing Adopted Basis Newton-Raphson (ABNR) protocol to the point of convergence with an RMS gradient <0.001]

which is base-paired with natural C (cytosine, shown in yellow). For comparison, this *f*G...C pair was superimposed on the natural G...C pair (shown in yellow). As can be seen, while *f*G..C has a favorable base-pair array that is reasonably comparable to that of its natural counterpart, some conspicuous differences do exist. One of these is a considerable deviation of the base-sugar bond of *f*G from that of G, as shown by arrows. Second is the angle λ, comprising the base-sugar bond of *f*G and the anomeric carbon of C in a double helix, which is only 39.7° as opposed to the typical λ values of 53-58° for a natural B-DNA double helix <B-87MI269; B-87Journal280 >. Third, the interstrand distance *r* is 11.43 Å for the *f*G...C pair as contrasted with a typical *r* value of 10.8-11 Å in a B-DNA duplex <B-87MI269; B-87MI280>. Such significant deviations of λ and *r* from the natural array would have dire consequences on the duplex stability of nucleic acids.

In order to further explore the above possibility by molecular modeling, we built a heterooligomer duplex containing 10 natural nucleotide pairs in a random sequence to form a B-DNA (Figure 1B, left). To compare and contrast, another copy of the same B-DNA was built, but keeping only 9 out of 10 nucleotide base-pairs of the original B-DNA and in the same sequence, but replacing G with an *f*G at position 5 in the complementary strand. Extensive energy minimization performed on the two duplexes revealed that the incorporation of an *f*G into DNA causes considerable distortion of the double helix with severe disruption of base-pair hydrogen bonding, ultimately leading to the 'unzipping' of the double-helix starting from the deviant *f*G residue (Figure 1B, right). The implications are that the incorporation of *f*G into the growing DNA chain during transcription or reverse transcription would potentially (a) hinder incorporation of subsequent nucleotides, (b) cause base-pair disruption, mismatch, or frameshift, and/or (c) prevent formation or cause distortion of the double helix. Any one, or a combination, of these factors would lead to inhibition of viral or tumor replication.

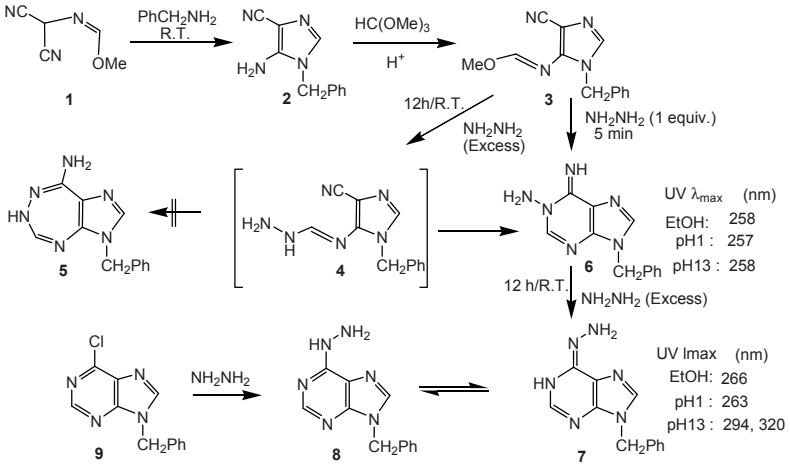
Irrespective of their biological significance, RENs and RENTs offer important platforms for explorations of some fundamental scientific principles pertaining to nucleic acid structure, formation, stability and function. For example, it would be of great interest to explore if there is enough flexibility in the double-helix to accommodate both structural deviations arising from aberrant base-pair array and the increased cross-sectional widths of RENTs. Would the incorporation of such nucleotides increase the stability of a double-helix, and to what extent? Would the increased surface area of RENTs enhance the intrastrand stacking interactions? Since vertical stacking interactions are now widely believed <B-74MI526> to override the complementary base-pair interactions in their respective contributions toward the double helical stability, would the appropriate RENT-based nucleic acids be more stable than the natural? Investigations such as these would be highly interesting and rewarding.

2.3. CHEMISTRY

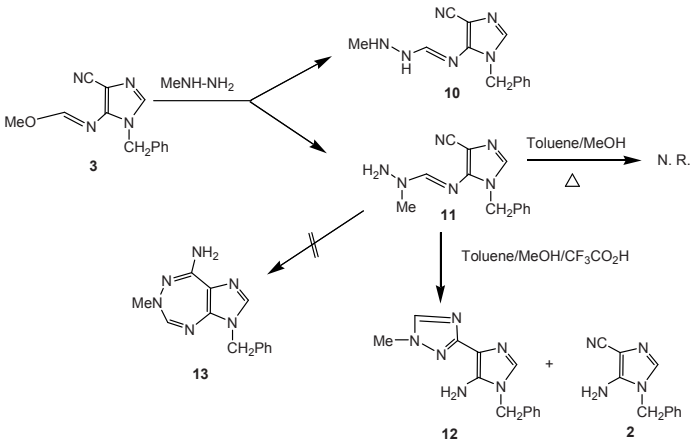
As mentioned earlier, synthesis of 5:7-fused *f*-purines often poses a synthetic challenge especially when they are potentially anti-aromatic by Hückel rules <08JPC(A)13231>. They may prefer to form an aromatic 5:6-fused system over anti-aromatic 5:7. Even if they do form a 5:7-system, they are often prone to easy opportunistic rearrangements or ring-transformations to form either a 5:6- or 5:5-fused rings <88JOC382; 88JOC5309>. A specific example is provided in Scheme 3, which shows that an attempted synthesis of the intended 5:7-fused 'fat' adenine (*f*A) target **5** yielded only the 5:6-fused N^6-hydrazinopurine **8**, the structure of which was confirmed by unequivocal synthesis by reaction of 6-chloropurine **9** with hydrazine <88JOC382>.

In order to avoid the undesired ring-closure of the intermediate **4** to form **6** instead of **5** in the above scheme, we prepared the *N*-methyl derivative of **4** from **3** using methylhydrazine (Scheme 4) <88JOC382>. The two isomers obtained, **10** and **11**, were separated, and the ring-closure of **11** was attempted, using trifluoroacetic acid catalyst in methanol <88JOC382>. The product obtained was an unfused 5:5 ring system **12** instead of the desired **13** <88JOC382>. The structure of **12** was confirmed by an unequivocal synthesis (Scheme 5) involving initial demethylation of **12** to form **14**, using boron tribromide, followed by ring-closure with trimethyl orthoformate to form **15** <88JOC382>. The latter was identical with the product obtained by

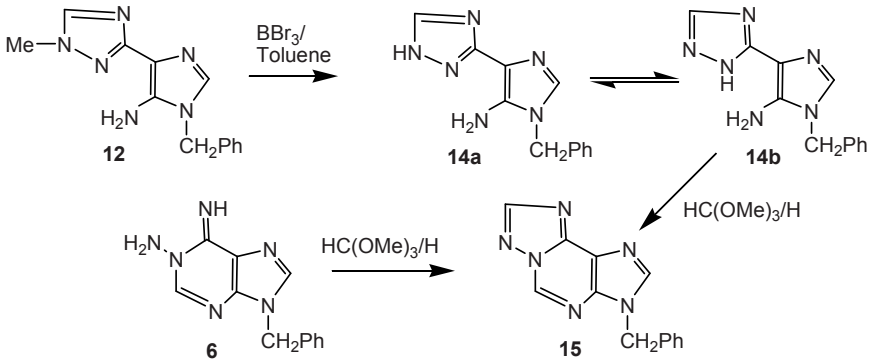
ring-closure of **6** with trimethyl orthoformate. A tentative mechanism for the formation of **12** and **2** from **11** is outlined in Scheme 6 <88JOC382>.



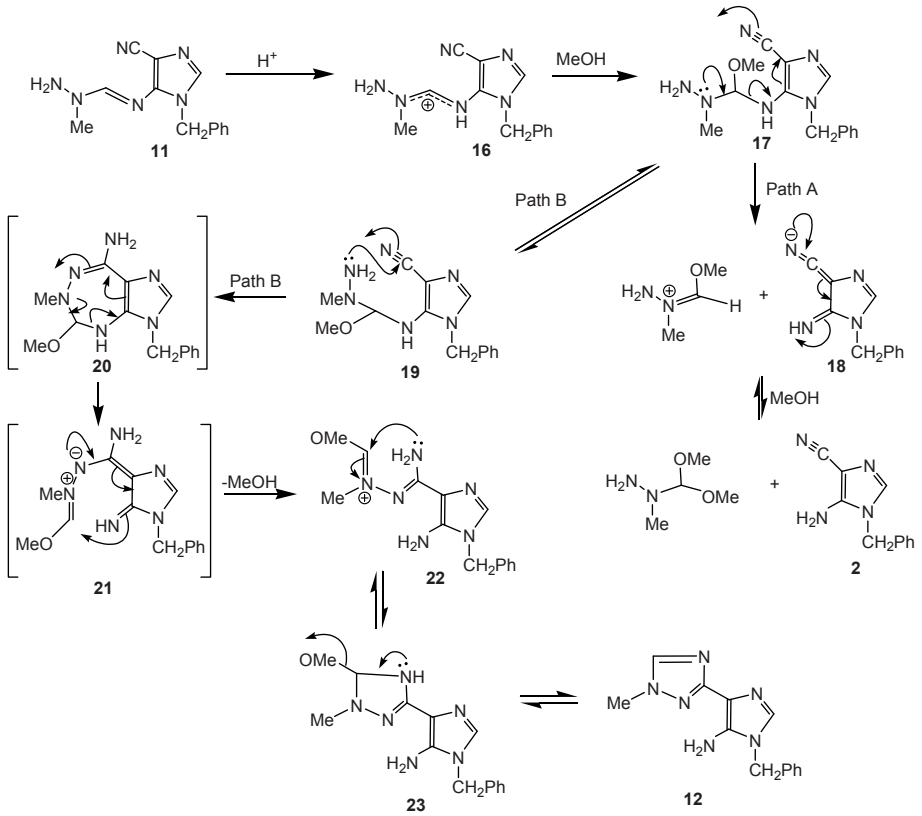
Scheme 3



Scheme 4



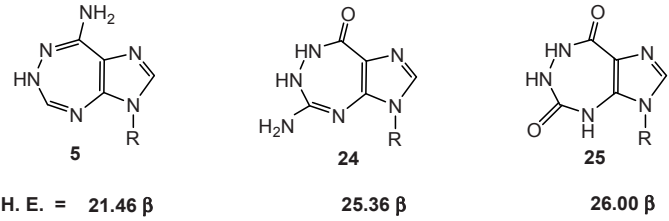
Scheme 5



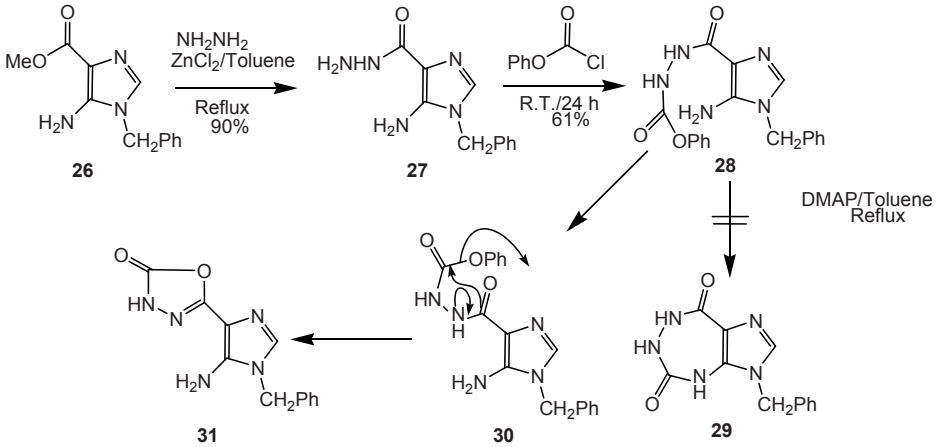
Scheme 6

Molecular modeling studies (Biosym/INSIGHT II/DISCOVER, available from Accelrys Software, Inc., San Diego) revealed that the target **5** exists essentially as a planar structure with only a slight deviation of planarity of its 7-membered ring NH, suggesting that the compound is anti-aromatic by the Hückel rule of [4n+2]π electrons <08JPC(A)13231>. Furthermore, Hückel energy calculations <98QXX119>, showed that the introduction of one or two carbonyl groups in the 7-membered ring would considerably enhance the stability of the 5:7-fused system. Thus the calculated Hückel energy (H.E.) increased in the order of adenine analogue **5** < guanine analogue **24** <xanthine analogue **25** (Scheme 7).

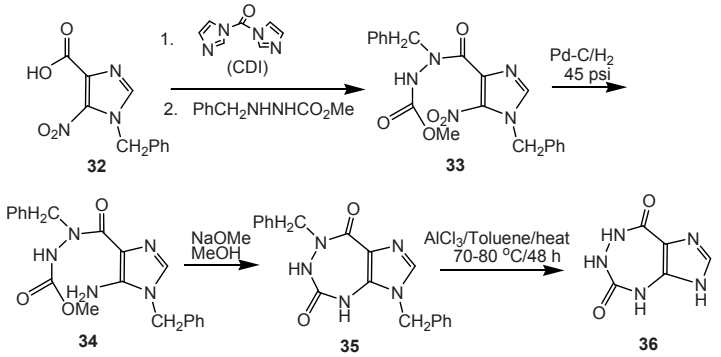
Scheme 7



Encouraged by the above result, we undertook the synthesis of **25** (Scheme 8) <89MI135>. However, the precursor **28** did not yield the desired final product **29**, but instead formed another 5:5-unfused imidazolyl oxadiazepinone (**31**) <90S1095>. The problem was ultimately solved by synthesizing **34** (Scheme 9) with a removable benzyl group to prevent the undesired ring-closure <90S1095>. The latter did form a 5:7-fused intermediate **35** upon treatment with sodium methoxide in methanol. Finally, both benzyl protecting groups of **35** were removed by heating with aluminum chloride in toluene to yield the parent 5:7-fused imidazo[4,5-*e*][1,2,4]triazepin-5,8-dione **36** <90S1095>. The compound was a highly stable solid with considerable aqueous solubility, but with little, if any, solubility in most organic solvents.

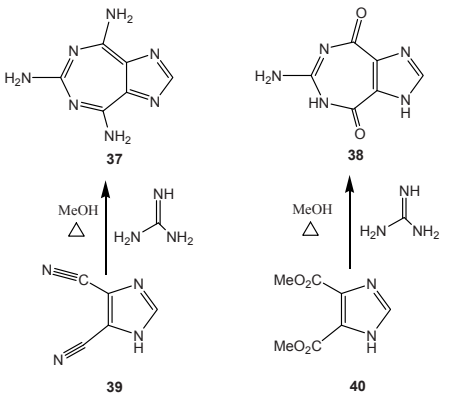


Scheme 8



Scheme 9

In light of the observed stability of **36**, which was consistent with the Hückel energy calculations described earlier, we sought to synthesize completely aromatic and planar 5:7-fused *f*-purine analogues by introduction of appropriate number of amino, imino, and/or carbonyl groups into the 7-membered ring of the heterocycle. The two compounds of interest in this regard were the triamino- and monoamino-dicarbonyl compounds **37** and **38**, respectively, containing imidazo[4,5-*e*][1,3]diazepine ring system (Scheme 10). They were conveniently synthesized by one-step condensation of the corresponding 4,5-dicyano- or -dimethoxycarbonyl derivatives, **39** and **40**, with guanidine <94NN2307; 03JMC4149>. The structure of **37** was confirmed by single-crystal X-ray diffraction analysis <95MI69> as well as by ^{15}N-labeling studies <94NN2307>. Both **37** and **38** are stable solids.



Scheme 10

Unlike the planar, potentially anti-aromatic 5:7-fused systems described earlier, the synthesis of non-planar, non-aromatic 5:7-fused systems were relatively easier. Thus, we synthesized the imidazo[4,5-*e*][1,4]diazepine-5,8-dione **45** (Scheme 11) <90JHC2189; 90NN913> and

imidazo[4,5-*d*][1,3]diazepine-5,8-dione **49** (Scheme 12) <91NN1693>. Structures of both **45** (as a 3-benzyl derivative) <90JHC2189> and **49** (as the parent as well as 3-benzyl derivative) <91NN693> were confirmed by single-crystal X-ray diffraction studies analyses <90JHC2189; 91NN1693>. As anticipated, the 7-membered ring in each was found to be puckered. Successful synthesis of an amino-dicarbonyl compound **57**, containing the imidazo[4,5-*e*][1,4]diazepine ring system, resembling the structure of guanine (a 'fat' guanine), is outlined in Scheme 13 <98NN1141; 99NN835>.

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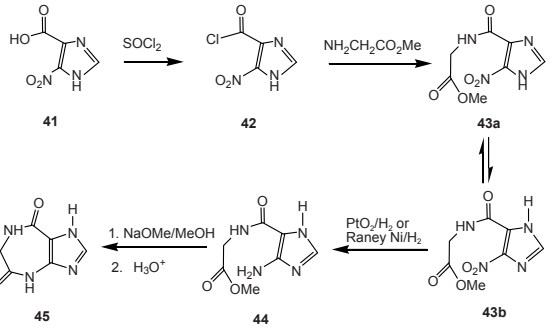
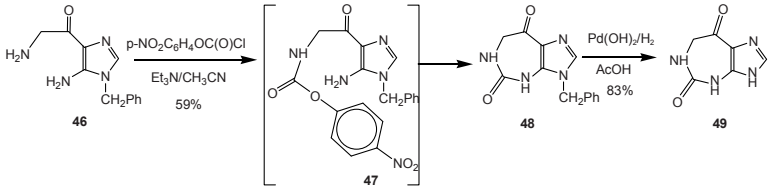
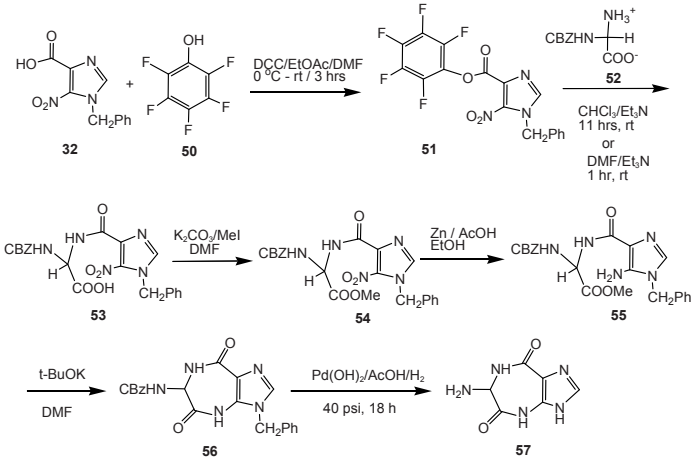


Scheme 11

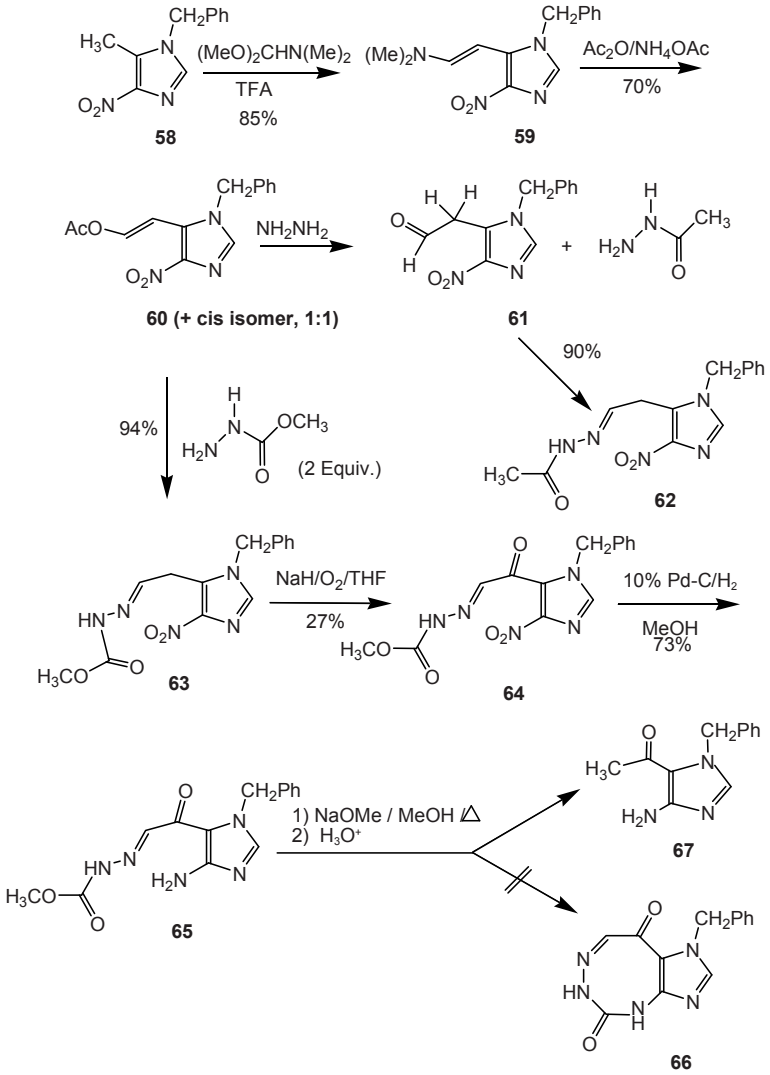


Scheme 12

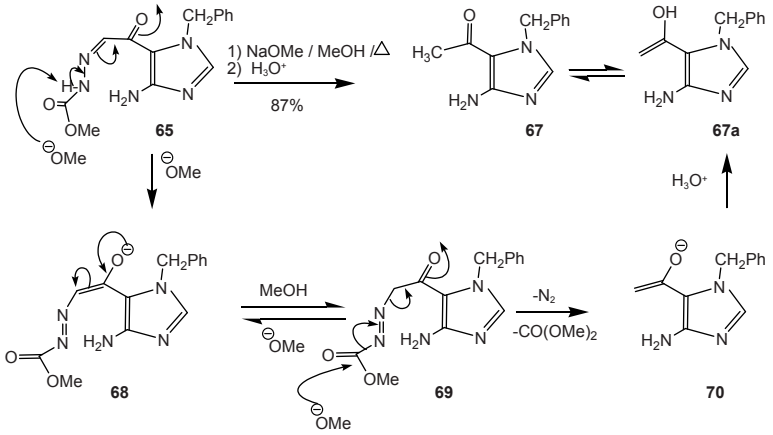


Scheme 13

Our initial efforts to synthesize a 5:8-fused, ring-expanded purine analogue (a super 'fat' or *sf* purine) were focused on imidazo[4,5-*e*][1,2,4]triazocine-5,9-dione **66** (Scheme 14) <95NN325; 97H(45)857>. While we succeeded in synthesizing the necessary precursor **65**, the attempted ring-closure of the latter to the target **66** resulted instead in the formation of 5-acetyl-4-amino-1-benzylimidazole **67** <97H(45)857>. A tentative mechanism for the formation of **67** from **65** is outlined in Scheme 15 <97H(45)857>.

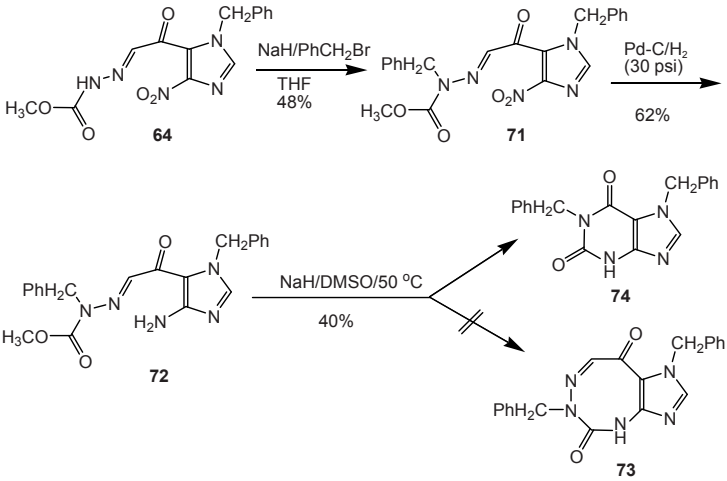


Scheme 14



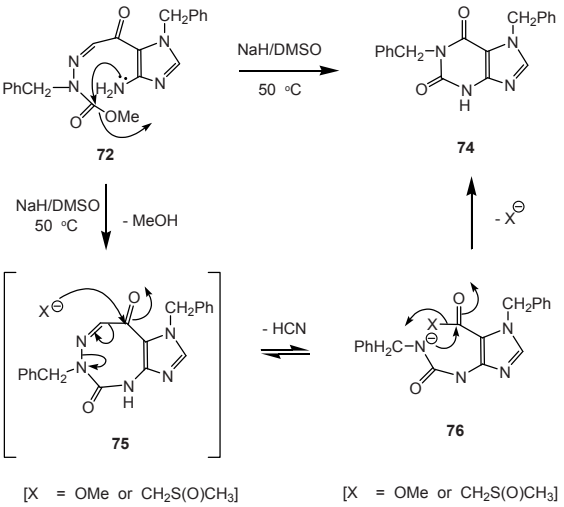
Scheme 15

In order to avoid the undesired degradation of **65** to **67**, it was deemed necessary to protect the free side-chain NH with a removable protecting group. Thus the benzyl derivative **72** was prepared from **64** (Scheme 16), and treated with sodium hydride in DMSO at 50 ºC. The result, once again, was the undesired rearrangement to form a xanthine derivative **74** <95NN325>, and not **73**. The structure of **74** was confirmed by single-crystal X-ray diffraction analysis <02T9567>.



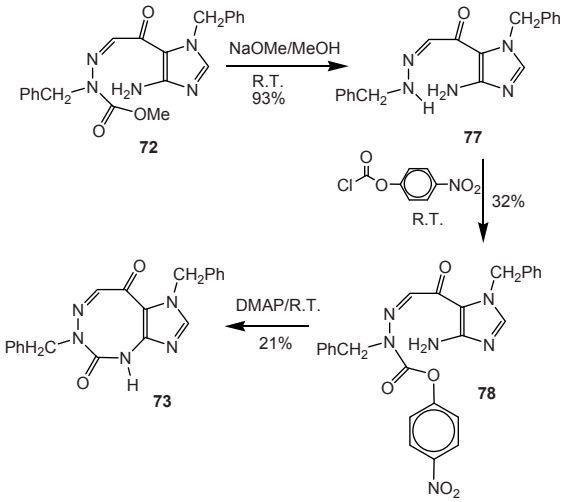
Scheme 16

A tentative mechanism for the formation of **74** from **72** is outlined in Scheme 17<02T9567>.



Scheme 17

Finally, the desired **73** was successfully obtained, albeit in low yield, by replacing the side-chain methoxycarbonyl group of **72** with a highly reactive *p*-nitrophenoxycarbonyl group of **78**, followed by ring-closure under mild basic conditions (Scheme 18) <02T9567>. Compound **73** is a stable, yellow solid, and its structure was consistent with the spectroscopic and analytical data.



Scheme 18

2.4. BIOCHEMICAL AND BIOPHYSICAL CHEMISTRY

2.4.1. Adenosine Deaminase

Coformycin and its 2'-deoxy analogue called pentostatin are the two natural antitumor compounds, and are the strongest known inhibitors of adenosine deaminase (ADA) with K_is ranging 10^{-11}-10^{-13} *M* <77BP359; B-78MI159>. Despite their highly potent ADA inhibitory activity, they have not been clinically as successful as anticipated, granted that the benefits of using pentostatin for the treatment of leukemias and lymphomas, in particular acute lymphocytic leukemia (ALL) and hairy cell leukemia, are notable <75BJC544; 78JCI710; 91BJC903; 92PR459; 99CCR65>. Severe toxicities have limited their effective use as broad-spectrum therapeutics <81BLD91; 81BLD406; 81CCP193; 81PAO487>. It is now widely believed that the basis for the toxicities is the prolonged, extremely tight-binding, nearly irreversible inhibition of ADA, an essential metabolic enzyme, by coformycin and pentostatin, which mimic the transition state of the enzyme-catalyzed reaction, thus necessitating the synthesis of a new enzyme molecule each time for recovery from the toxic effects <79FAB670>. Therefore, it is desirable to have ADA inhibitors that are somewhat less tight-binding, easily reversible, but still highly potent with a shorter duration of action and faster enzyme recovery <B-02MI133>. In an effort to find such inhibitors, we carefully examined the reported X-ray crystal structure of a complex of ADA with pentostatin (also called 2'-deoxycoformycin or dCF) (Figure 2), <96JMC277; 98B8314> which revealed that its tight-binding characteristics arises from multiple hydrogen bonding of its sugar hydroxyls with the amino acid residues of the protein, coupled with a coordinate bond of its heterocyclic 8-OH group with an active site zinc ion. A notable feature in the crystal structure is the observed *anti* sugar conformation, in which the sugar was oriented away from the heterocyclic 7-membered ring. This suggested to us that if the conformation is forced to the predominent *syn* orientation (sugar facing the 7-membered ring) as in

82 because of the anticipated intramolecular hydrogen bonding between the sugar and the heterocycle as shown, some of the hydrogen bonds observed with the *anti* orientation would be lost, in addition to perhaps weakening the coordinate bond of 8-OH with the zinc ion, thus leading to less tight-binding and more easily reversible inhibition characteristics. Such a high *syn* orientation has been confirmed both in the crystal state (X-ray) and in solution (NOE studies) in related ring-expanded nucleoside analogues containing the imidazo[4,5-*e*][1,4]diazepine <90JOC5882; 91NN819>. The observed large NOE (>30%) between H-2 of the imidazole ring and H-1' of the sugar moiety of **82**, the synthesis of which is outlined in Scheme 19 <97NN1053>, corroborated its predominantly *syn* orientation in solution. Indeed, when tested for inhibitory activity against ADA from calf intestinal mucosa, **82** exhibited a K_i of 2.02 ± 0.5 x 10^{-5}, which is several orders of magnitude lower activity than coformycin, as anticipated <97BBR88>. Furthermore, nucleoside **84**, which lacked the crucial 8-OH group for forming a coordination bond with the active site zinc was completely devoid of activity, supporting the significance of 8-OH in enzyme binding <97BBR88>. Efforts are underway to optimize the inhibition potency of **82**, while still maintaining its semi-tight binding characteristics with a reversible enzyme inhibition property <04JMC1044; 04NNN263>.

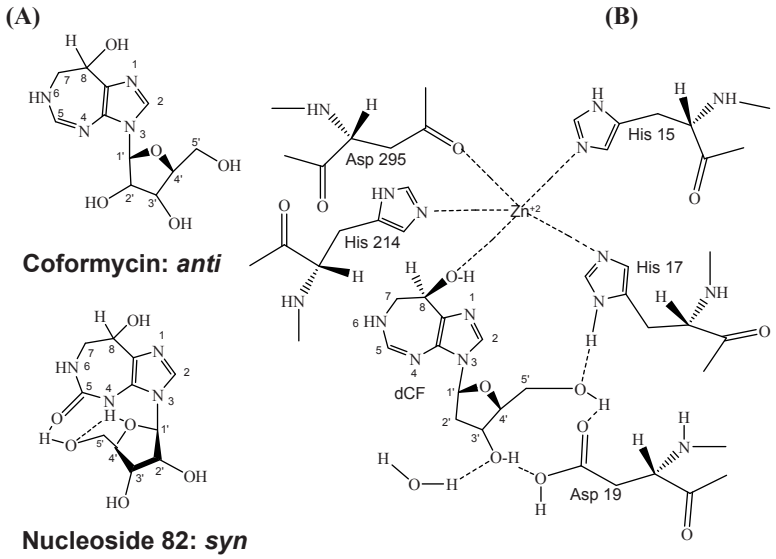
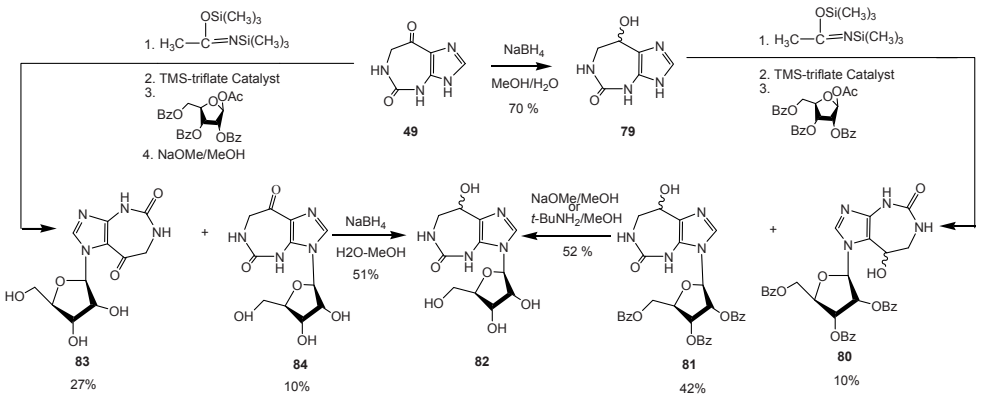


Figure 2: (A) *Anti/Syn* conformations of coformycin and **82**. (B) Observed *anti* conformation of 2'-deoxycoformycin (dCF or pentostatin) in the crystal X-ray structure of a complex of ADA with dCF



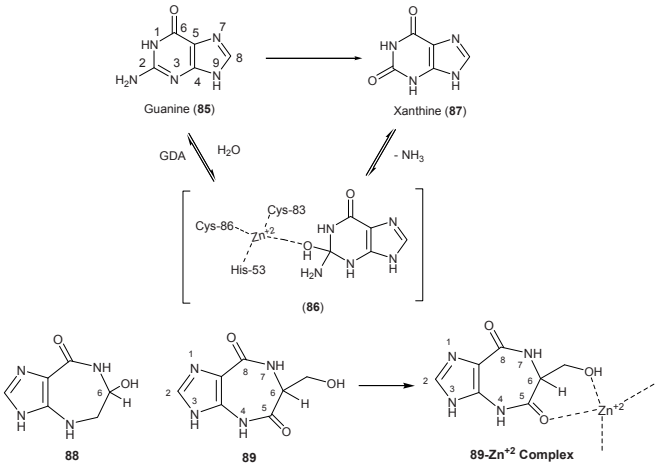
Scheme 19

2.4.2. Guanine Deaminase (Guanase)

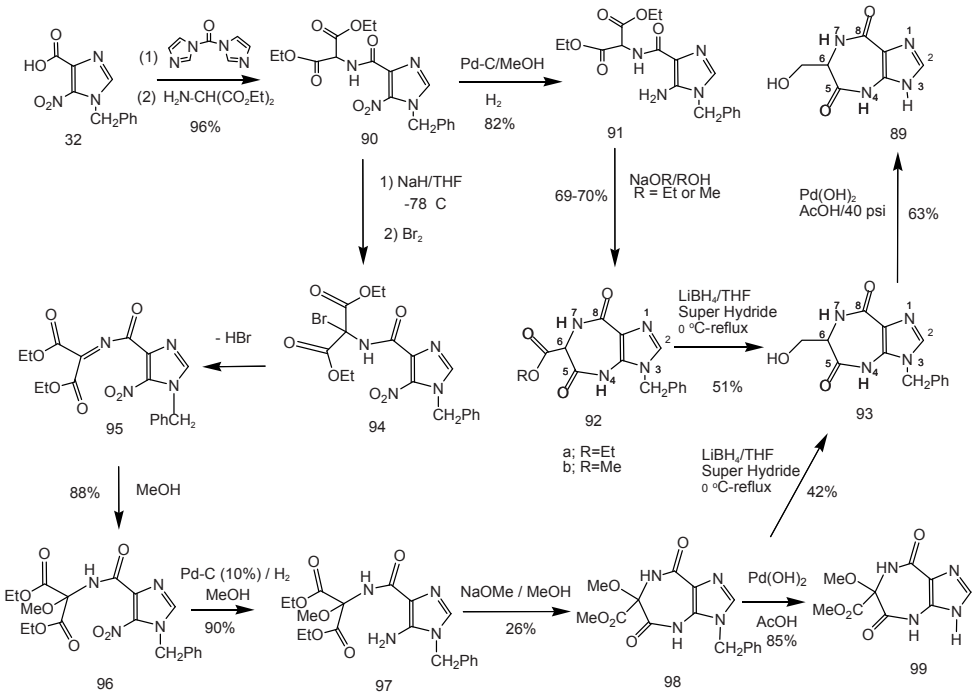
Guanine deaminase (GDA) or guanase (EC 3.5.4.3) is an enzyme that catalyzes the hydrolytic deamination of guanine to xanthine. This enzyme has been found in human liver, brain, and kidney <65JLCM355>. There have been reports of abnormally high levels of serum guanase activity in patients with liver diseases <88H383; 89JJM22; 89RB1392>, and so, the elevated enzyme activity has been suggested as a marker of hepatitis and hepatoma <89JJM22>. Furthermore, such a high guanase activity is believed to be a biochemical indicator of rejection in liver transplant recipients <89TP2315>. Increased levels of guanase have also been detected in cancerous kidney and breast cancer tissues <96BCT189; 97CI212; 99JBC8175; 02NS15>. In addition, patients with multiple sclerosis exhibit significantly elevated levels of guanase activity in their cerebral spinal fluids <89RS854>. These observations suggest that a potent guanase inhibitor is necessary for exploring the biochemical mechanisms of the above metabolic disorders as well to understand the specific physiological role played by guanase, not to mention its potential therapeutic use in treating these disorders. While many studies on guanase inhibition have been reported in the literature, <53JBC89; 67BJ691; 67JHC1101; 68JMC644; 70CPB392; 71CPB1737; 76JMC62; 77IJB27; 79JMC944; 80PZ16> including our own <94TL6831; 95NN455; 98BMCL3649; 98NN1141; 99NN835; 01BMCL2893>, a potent guanase inhibitor with a submicromolar K_i has yet to be realized.

Guanase catalyzes the hydrolysis of guanine **85** to xanthine **87** (Scheme 20) via the tetrahedral intermediate **86** (Scheme 20)<74JBC3862>. The X-ray crystal structure of GDA <04JBC35479> from *Bacillus subtilis* suggested that the enzyme-catalyzed reaction is assisted by an active site zinc ion (Zn^{+2}) as in ADA, leading to the speculation that the known GDA inhibitor such as azepinomycin **88** may act like a transition state mimic just as coformycins do in case of ADA inhibition described earlier. However, only a moderate inhibition of guanase by **88** has been reported <87JAN1461>, which suggested somewhat weaker binding of the inhibitor to the enzyme via zinc metal coordination. We hypothesized that this might be due to possible facile elimination of the crucial OH group of **88** as water, assisted by the anchimeric ring NH group. Therefore moving the OH group one carbon away from the ring, as in **89**, would alleviate this problem. We further hypothesized that the introduction of a carbonyl group at position-5 of

the heterocycle, coupled with the presence of a hydroxymethyl group at position-6, as in **89**, would allow excellent coordination of the inhibitor with Zn^{+2} to form a stable, 6-membered ring structure as shown. The latter would use two of the four metal coordination sites, while the other two would be occupied by two of the three original amino acid residues at the enzyme active site. With this rationale, we synthesized **89** <06BMCL5551> (Scheme 21), starting from **32**.



Scheme 20



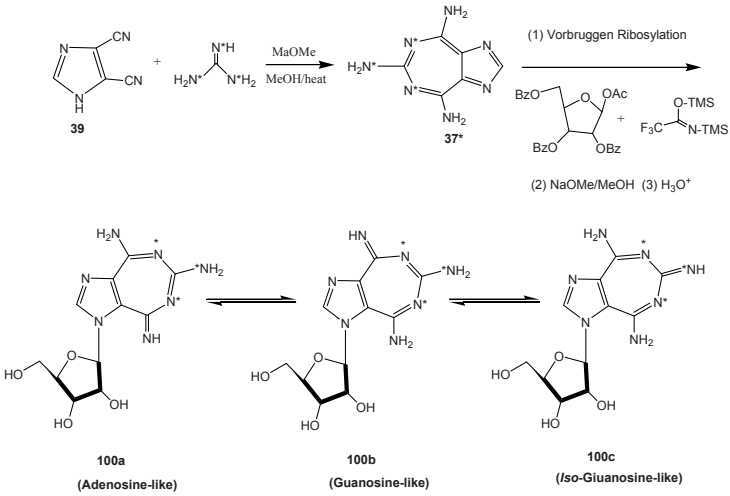
Scheme 21

The target **89**, along with **93** and **99**, were screened against rabbit liver guanase, and were found to possess K_i values of 5.36±0.14 x 10^{-5} *M*, 2.01±0.16 *M*, and 5.4 x 10^{-4} M, respectively. <06BMCL5551> The values are roughly comparable to the reported IC_{50} value of 0.5 x 10^{-5} M for azepinomycin. Further SAR studies are currently in progress in order to enhance the potency without compromising the reversible enzyme binding characteristics of these compounds.

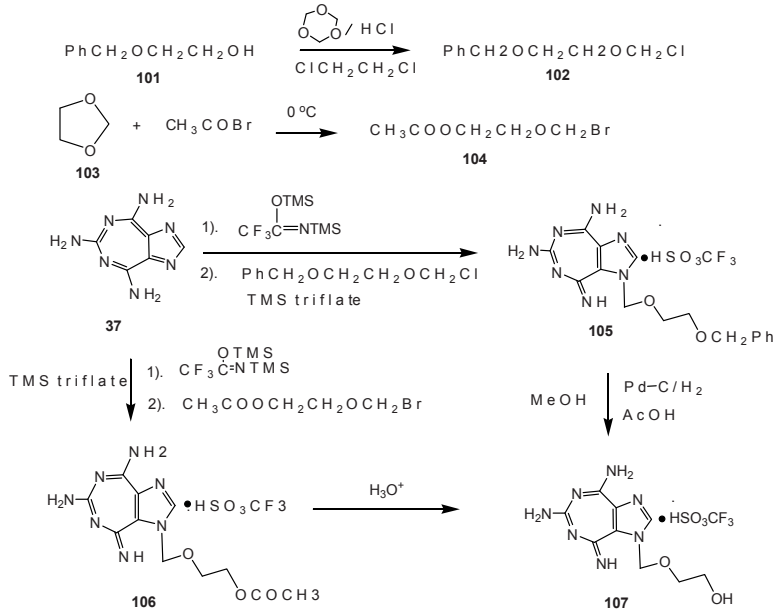
2.4.3. Dual Inhibition of Adenosine Deaminase (ADA) and Guanine Deaminase (GDA)

The planar, aromatic heterocycle **37** was of interest for exploration of tautomeric equilibria especially when converted into its nucleoside analogue, as one of the three amino groups would necessarily exist as an imino functionality. Depending upon which one of the three amino groups would assume an imino structure, the nucleoside analogue **100** (Scheme 22) can mimic adenosine, guanosine or iso-guanosine. To resolve this issue, we labeled all three nitrogen atoms of guanidine with N^{15} and carried out the condensation with 4,5-dicyanoimidazole **39** to obtain the triply labeled **37*** <94NN2307>. The latter, upon standard Vorbrüggen ribosylation <94NN2307> provided the triply labeled **100**. The ^{15}N NMR of **100** showed two singlets at ~211 δ (ppm) corresponding to the two labeled ring nitrogen atoms and one triplet (J_{*N-H}= 80 Hz) in the 120-123 δ region corresponding to the labeled exonuclear NH_2 group, suggesting that the molecule exists as **100a** or **100b** or as an equilibrium mixture of the two, but never as **100c**. The possible existence of both adenosine- and guanosine-like tautomers in solution for **100** gave rise

to the notion that it might be possible to design an inhibitor that could simultaneously inhibit both ADA and GDA. But there was an important difference in substrate requirements of the two enzymes that needed to be reconciled. While the natural substrate for ADA is a nucleoside adenosine, that for GDA is a heterocyclic base guanine. In order to circumvent this problem, we decided to attach an acyclic sugar moiety to **37**. Synthesis of such an acyclic nucleoside **107** is outlined in Scheme 23 <01BMCL2893>. Both nucleoside **100** and acyclic nucleoside **107** were bichemically assayed, as before, against both ADA and GDA, and as anticipated, nucleoside **100** was found to be a competitive inhibitor of ADA (K_i = 3.85 x 10^{-5} *M*)<95MI69>, but not GDA, whereas acyclic nucleoside **107** was found to be a competitive inhibitor of both ADA (K_i = 1.52±0.34 x 10^{-4} *M*) and GDA (K_i = 2.97±0.25 x 10^{-5} *M*) <01BMCL2893>.



Scheme 22



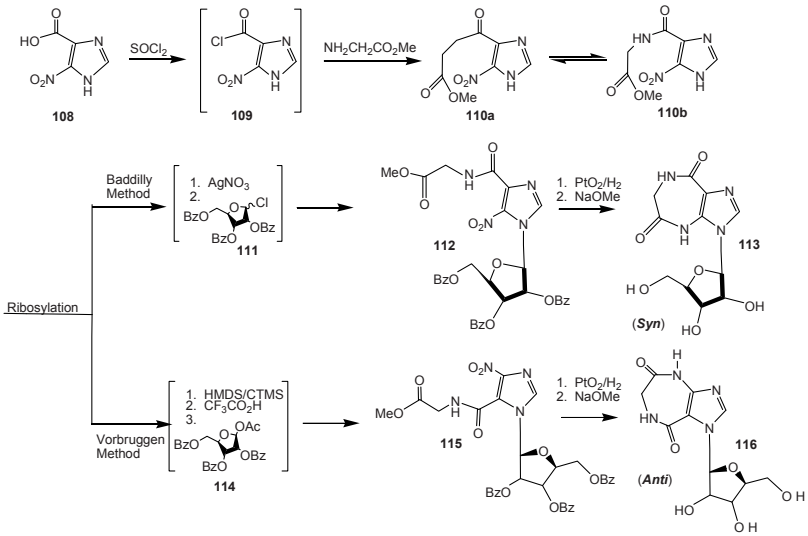
Scheme 23

2.4.4. Polynucleotide Phosphorylase

Polynucleotide phosphorylase from *E. coli* is a primer-independent polymerase that converts any nucleoside 5'-diphosphates into the corresponding homopolymers in the presence of Mn^{+2} <78B3677; 79IBM194; 80NAR1675>. With no requirement for a primer, it was obviously an attractive enzyme for synthesis of 'fat' polymers from ring-expanded nucleotides for exploration of some fundamental biophysical properties of nucleic acids, in particular the spatial and conformational constraints for formation of nucleic acid double helices as well as the factors governing their helical structure, stability and conformation. Also, in light of the long-suspected correlation between conformational properties of nucleosides and their observed biological activities, systematic studies continue to be warranted for exploring the little understood interrelationships of nucleoside base-sugar conformations, sugar pucker, ease of *in vivo* phosphorylation, enzymic polymerization, and biological activity. To this end, we synthesized two ring-expanded nucleoside (REN) analogues **113** and **116**, containing the imidazo[4,5-*e*][1,4]diazepine ring system (Scheme 24) <90JOC5882>. The heterocyclic bases of both RENs were anticipated to be non-planar and non-aromatic but with opposite base-sugar conformations, **113**:*syn* and **116**:*anti*, and thus were considered to be good probes for the intended biophysical evaluations.

When the above two target nucleosides **113** and **116** were screened against murine leukemia virus (MuLV) in tissue culture systems, **116** showed potent antiviral activity with an IC_{50} (inhibitory concentration of the compound required to reduce the viral pathogenicity by 50%) in micromolar range, while **113** was totally devoid of activity <90JOC5882>. This interesting

biological result further elevated our interest in the biophysical properties of these isomeric nucleoside analogues. The single-crystal X-ray structure analysis of the two nucleosides (Figure 3) <90JOC5882> revealed that the sugar of **113** existed in a *syn* orientation as dictated by a strong hydrogen bond between the bottom NH of the 7-membered ring the 5'-O of the sugar ring, while the sugar orientation of **113** was *anti*. This led to a speculation that if the compounds existed in the same preferred conformation in solution, **113** would be less prone to phosphorylation *in vivo* than **116** since kinases, the enzymes responsible for *in vivo* phosphorylation of nucleosides to nucleotides, are known to prefer *anti* orientation <01B11037>. So, if the actual reactive species *in vivo* is a nucleotide, that would explain the difference in observed biological activities of the two nucleosides. In an attempt to throw more light on the subject, we first investigated the solution conformations of the two RENs using the ^{1}H NMR-based Nuclear Overhauser Effect (NOE) <91NN819> and Circular Dichroism studies <89BBC106>. The observed NOE between the imidazole H-2 and the sugar H-1' of **113** was >35% <91NN819> suggesting a highly *syn* base-sugar conformation, while the same in **116** was 0% consistent with an anti conformation favored by kinases.



Scheme 24

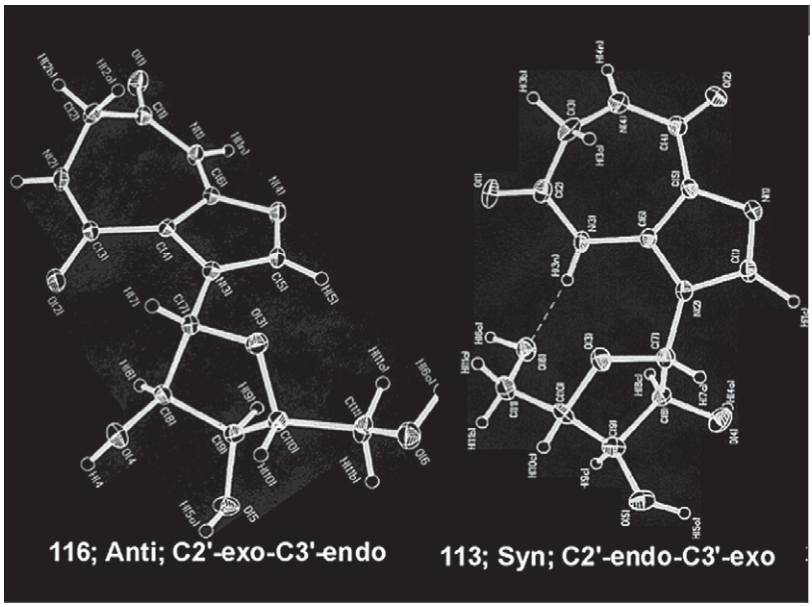
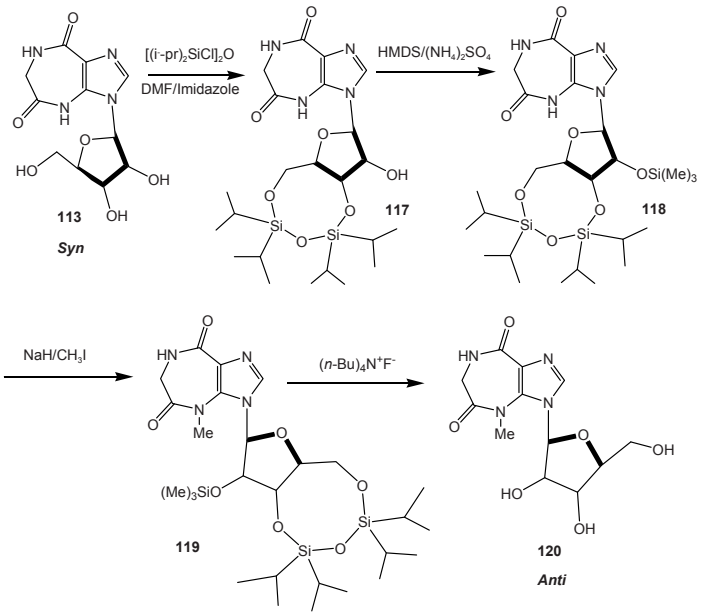


Figure 3: Single-crystal X-ray structures of ring-expanded nucleosides **113** and **116** <90JOC5882>

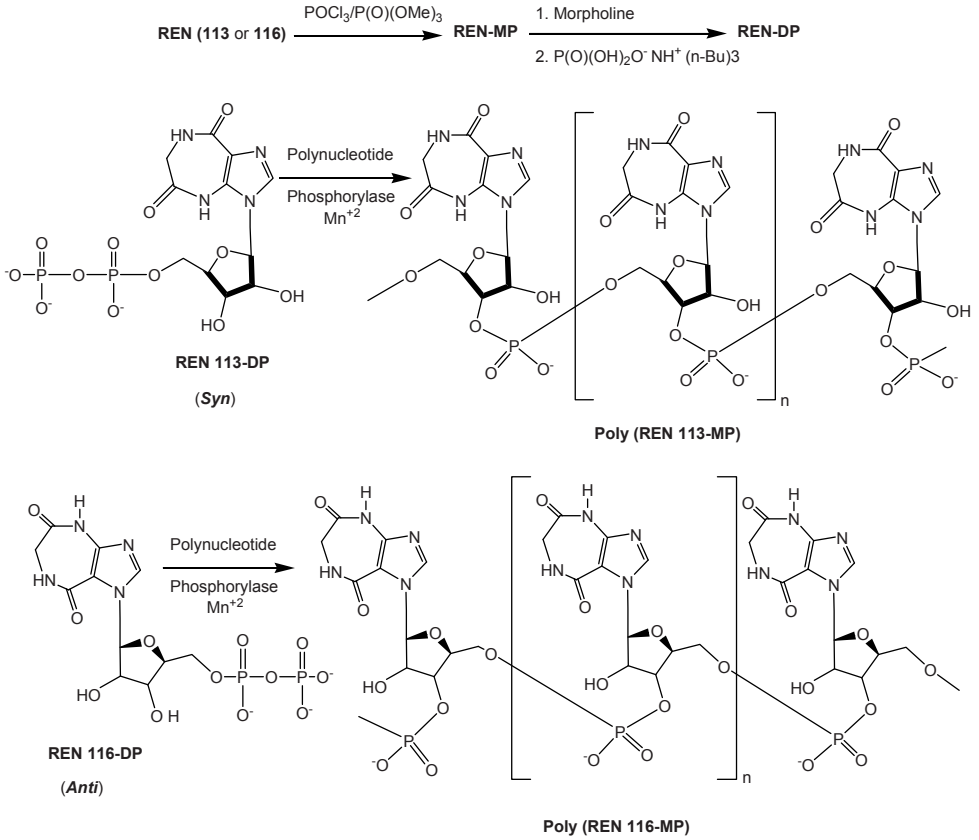
To confirm that the *syn/anti* conformation indeed plays a role in the observed biological activity, we decided to eliminate the H-bond responsible for the *syn* conformation by synthesizing the N^8-methyl derivative of **113**, anticipating that the bulky methyl group would not only remove the H-bonding but would also force the conformation to become predominantly *anti*. The synthesis of the N^8-methyl derivative (**120**) is outlined in Scheme 25 <91NN819>. Indeed, when **120** was assayed against MuLV as before, its antiviral activity was even slightly better than that of **116**, thus corroborating the speculated correlation between conformation and biological activity.



Scheme 25

We then proceeded to evaluate the difference in conformational features of the nucleic acid homopolymers derived from the two isomeric RENs. To this end, we chemically synthesized the necessary 5'-diphosphate derivatives of **113** and **116** employing standard phosphorylation procedures <90JOC5882>, and subjected the REN diphosphates **113-DP** and **116-DP** to polymerization with *E.coli* polynucleotide phosphorylase in the presence of Mn^{+2} to form the corresponding homopolymers **poly[113-MP]** and **poly[116-MP]** (Scheme 26) <90JOC5882>. Both diphosphates were found to be substrates for the enzyme, although the yield of the homopolymer obtained from the *syn* isomer was nearly 50% less than from the *anti* isomer. The extent of polymerization was determined by (a) the assessment of the inorganic phosphate release from the polymerization reaction, (b) the UV absorbance of the extensively dialyzed product, and (c) determination of the lengths of the homopolymers by gel electrophoresis which indicated polymerization in the 50-mer range <90JOC5882>. The secondary structures of the homopolymers were determined by UV and CD spectral measurements <90JOC5882>. A considerable hyperchromic effect was observed upon increase in temperature with the homopolymer **poly[116-MP]** obtained by the *anti* isomer, suggesting that it possesses significant secondary structure. In the presence of stoichiometric amounts of spermine, a cation with +4 charge, this homopolymer displayed a very broad absorbance-temperature profile, with a T_m of ~40 ºC and hyperchromicity of ~30-35%, suggesting the presence of intrastrand secondary structure <90JOC5882>. By contrast, the results obtained with the homopolymer **poly[113-MP]** obtained from the *syn* isomer, gave little indication of secondary structure without spermine, and even with spermine, the observed Tm of ~28 ºC and hyperchromicity of 17% or less were considerably lower than those of **poly[116-MP]**. The CD spectra of the two homopolymers corroborated the above findings by exhibiting a significant Cotton effect only for **poly[116-MP]**

with a maximum at 283 nm, crossover at 271, and a minimum at 260 nm <90JOC5882>. Upon addition of a stoichiometric level of spermine, there was a further change in the spectrum with a doubling of the absolute value of the minimum, suggesting a helical structure. By contrast, poly[113-MP] exhibited neither the Cotton effect nor noticeable spectral perturbation upon addition of spermine <90JOC5882>.



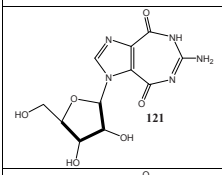
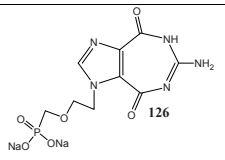
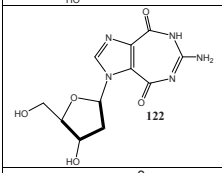
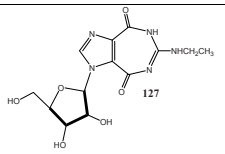
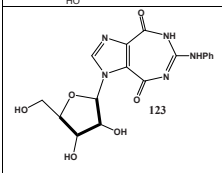
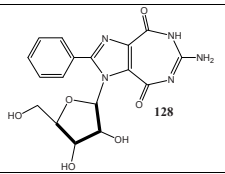
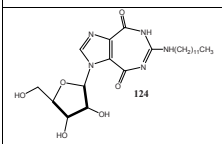
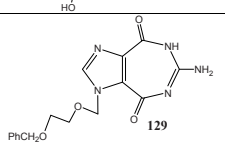
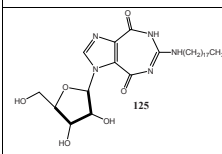
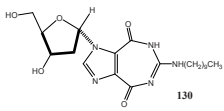
Scheme 26

2.5. BIOLOGICAL ACTIVITY

RENs and RENTs, and some of their heterocyclic aglycons turned out to be a goldmine of antiviral and anticancer agents <02CTMC1093; B-04MI135>. A host of RENs exhibited potent *in vitro* broad-spectrum antiviral activity (Table I) against a dozen different viruses <01MI31; 02BMCL3391; 02CTMC1093; 03JMC4149; 03JMC4776; 04AR209; B-04MI135; 05BMCL5397; 06MI231; 07BMC4933; 07BMCL2225; 08JMC5043>, including the hepatitis B virus (HBV), hepatitis C virus (HCV), Epstein-Barr virus (EBV), varicella zoster virus (VZV),

respiratory syncytial virus (RSV), herpes simplex virus types 1 and 2 (HSV-1 & HSV-2), measles virus (MV), rhino virus (RV), influenza A & B viruses (IAV & IBV), Japanese encephalitis virus (JEV), West Nile virus (WNV), severe acute respiratory syndroma (SARS) corona virus, and human immunodeficiency virus (HIV).

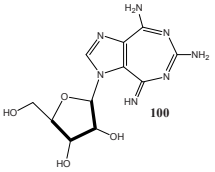
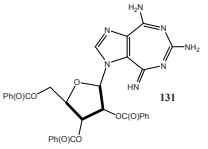
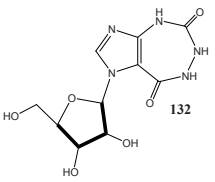
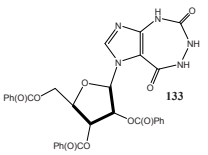
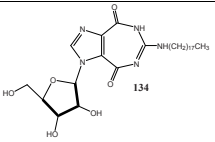
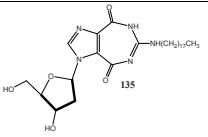
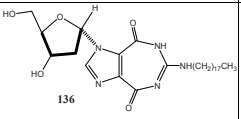
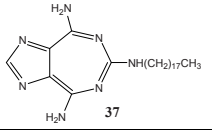
Table 1: Antiviral Activity of Some Representative RENS

REN Structure	Virus	Antiviral Activity (μ*M*)	REN Structure	Virus	Antiviral Activity (μ*M*)
 **121**	HBV	*EC_{50}= 0.13 SI > 18,500	 **126**	HSV	*EC_{50}= 1.4 SI > 200
 **122**	EBV	EC_{50}= 8.8 SI > 193	 **127**	Rhino	EC_{50}= 0.20 SI >500
 **123**	RSV	EC_{50}= 0.10 SI > 1000	 **128**	Measles	EC_{50}= 0.50 SI > 200
 **124**	VZV	EC_{50}= 2.5 SI >100	 **129**	Influenza B	EC_{50}= 65 SI > 150
 **125**	HCV	EC_{50}= 18 SI > 35	 **130**	WNV	EC_{50}=0.51 SI > 195

*EC_{50}=effective concentration of the compound to reduce viral pathogenicity by 50%; SI=selectivity index=ratio of cytotoxic concentration of the compound to kill 50% of the normal cells to that required to kill 50% of the viral cells

RENs have also exhibited potent *in vitro* anticancer activity against 50 different human tumor cell lines, a few of which are listed in Table II, including but not limited to those of the tumors of the breast, lung, prostate, colon, kidney, CNS, skin, ovary, and leukemia.

Table 2: Anticancer Activity of Some Representative RENS

REN Structure	Tumor (Cell Line)	Antitumor Activity (GI_{50})* μM	REN Structure	Tumor (Cell Line)	Antitumor Activity (GI_{50})* μM
 **100**	Leukemia (CCRF-CEM) Lung (non-SC) (A549/ATCC) Colon (Colo 205) CNS (SF-295) Melanoma (M14) Ovarian (IGROV1) Renal (UO-31) Prostate (PC-3/Du-45) Breast (MCF-7)	0.12 6.44 1.49 6.03 1.91 5.13 6.4 4.46/8.79 1.47	 **131**	Leukemia (CCRF-CEM) Lung (non-SC) (A549/ATCC) Colon (Colo 205) CNS (SF-295) Melanoma (M14) Ovarian (IGROV1) Renal (UO-31) Prostate (Du-45) Breast (MCF-7)	2.49 3.43 4.43 1.67 1.71 3.61 6.99 3.05 1.79
 **132**	Leukemia (K-562) Lung (non-SC) (HOP-62)) Colon (Colo 205) CNS (SF-295) Melanoma (M14) Ovarian (IGROV1) Renal (UO-31) Prostate (PC-3/Du-45) Breast (MCF-7)	1.79 0.41 1.20 1.02 1.81 3.59 5.54 1.61/8.83 1.46	 **133**	Leukemia (CCRF-CEM) Lung (non-SC) (HOP-92) Colon (Colo 205) CNS (SF-295) Melanoma (M14) Ovarian (IGROV1) Renal (UO-31) Prostate (PC-3/Du-45) Breast (MCF-7)	1.32 < 0.01 3.78 3.38 6.79 3.54 4.0 2.25/4.01 3.99
 **134**	Lung (non-SC) (A549) (H-460) Breast (MCF-7) Prostate (PC-3)	4.30 3.0 5.5 3.5	 **135**	Lung (non-SC) (A549) (H-460) Breast (MCF-7) Prostate (PC-3)	2.0 1.5 2.5 2.0
 **136**	Lung (non-SC) (A549) (H-460) Breast (MCF-7) Prostate (PC-3)	IC_{50} (μM) 9.1 6.5 19 0.8	 **37**	Lung (non-SC) (A549) (H-460) Breast (MCF-7) Prostate (PC-3)	IC_{50} (μM) 1.70 1.45 2.15 2.01

				Ovarian (OVCAR-3)	1.17

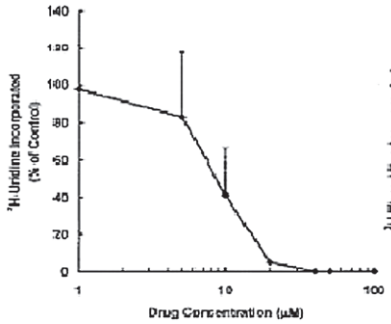
*GI_{50}=concentration of the compound required to reduce the tumor growth by 50%

In view of the observed broad spectrum antiviral and anticancer activities of RENs as listed in Tables 1 and 2, exploring their mechanism of biological activity became an instant interest. To this end, we decided to focus on a few important viruses and cancers using representative RENs. Our mechanistic efforts in the cancer area predominantly involved breast and prostate cancers <01NNN1043; 02MI392>, the leading causes of cancer mortality in American females <B-06MI103> and males <07CCR1889>, respectively. Mechanistic investigations in the viral area mainly targeted *flavi* viruses, including the hepatitis C (HCV), West Nile (WNV) and Japanese encephalitis (JEV) viruses<03JMC4149; 03JMC4776>, and to some extent on hepatitis B (HBV) <02AR159> and human immunodeficiency (HIV) viruses <08JMC5043>. These are the viruses of current global health priority <06LNZ797; 06PWPS6; 08AIDS7; 08L500>.

With regard to prostate cancer, we explored the *in vitro* biological effects of RENs **100** and **131** (the latter is perceived to be a prodrug of the former) (see Table 2) in two androgen-independent human prostate cancer cell lines, PC-3 and DU-145 <01NNN1043>. The studies concentrated on exploring the dose-dependent induction of apoptosis (programmed cell death) in treated cancer cells. The dose response profile of the cytotoxic effects of nucleosides against human prostate cancer cells was investigated. Specifically, PC-3 and DU-145 cells were each treated with increasing concentrations of **100** or **131** (0 - 50 μM) for 2 days, and the cell viability was determined using the Trypan Blue Exclusion assay <09TXL13>. The results in each case indicated that the treatment of exponentially growing culture of cells with **100** or **131** for two days leads to marked loss of cell viability in a dose-dependent manner. We further investigated the time course of cytotoxicity of **100** and **131** against androgen-independent prostate cancer cells. After 6 days of treatment of PC-3 and DU-145 cells each with 30 μM concentrations of **100** or **131**, ~98% cell killing was observed <01NNN1043>.

In an attempt to understand the potent anti-breast cancer activity of RENs **100** and **131** in MCF-7 cells (see Table 2), we discovered that these compounds stop cell proliferation, while not seemingly reducing the cell number in treated cultures <02MI392>. We performed further mechanistic studies using the prodrug **131** and found that the compound significantly decreases both RNA and DNA synthesis in MCF-7 cells (see **Figures 4** and **5**) <02MIP392>. It strongly inhibits the cell proliferation, whereas the cell growth assays demonstrated that **131** (**MB-1**) only slightly decreased the cell number after incubation for either 6 or 24 hours with the compound. The decrease was equivalent to that observed when the cells were incubated with the IC_{50} concentration for Ara-C. Regardless of the exposure time, combining Ara-C with **131** (10 μM) does not enhance the cell killing, which suggested that both Ara-C and **131** act on the same mechanistic pathway <02MIP392>.

The observed inhibition of both RNA and DNA synthesis by REN **131** pointed to some intriguing possibilities for its mechanism of action including, but not limited to, (a) it could be phosphorylated *in vivo* by kinases to its 5'-triphosphate derivative, and subsequently incorporated into nucleic acids during transcription of a DNA/RNA template by a polymerase, and cause double-helical distortion, ultimately leading to chain termination, (b) **131** could bind to an active or allosteric site of the polymerase, either as a nucleoside or nucleotide, and cause competitive or non-competitive inhibition.



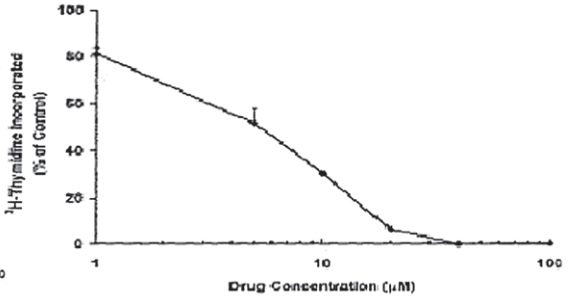
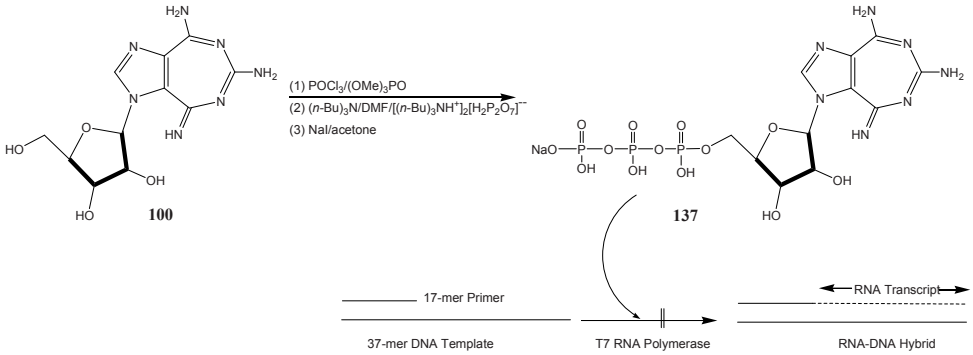


Figure 5 (left)**:** Effect of REN **131**on MCF-7 cell RNA synthesis. **Figure 6** (right)**:** Effect of REN **131** on MCF-7 cell DNA synthesis. MCF-7 cells (5 x 10^4) were seeded onto 60 mm cell culture plates in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS. The cells growing in log-phase were exposed to increasing concentrations of **131** for 24 hours at 37 ºC. The cells were then labeled with [^{3}H] thymidine (1 μCi/mL of medium) and incubated for 4 hours. The cells were lysed by 0.1% SDS/PBS and and the level of RNA/DNA synthesis was determined by quantifying the amount of [^{3}H] thymidine present in RNA/DNA by liquid scintillation counting.

In order to throw more light on the above subject, we chemically converted **100** (the reactive species of **131** *in vivo*) to its 5'-triphosphate derivative **137** (Scheme 27), and biochemically screened for inhibition of nucleic acid polymerase activity, employing synthetic DNA oligonucleotide templates and the bacteriophage T7 RNA polymerase as a representative Polymerase <99BMC2931; 99NN837>. Our results suggest that **137** is a moderate inhibitor of T7 RNA polymerase, and that the 5'-triphosphate moiety of **137** appears to be essential for inhibition as nucleoside **100** alone failed to inhibit the polymerase reaction <99BMC2931>.

Our mechanistic explorations in the viral field largely focused on *Flaviviridae*, including HCV, WNV, and JEV. We have discovered that the mechanism of action of this family of viruses involves inhibition of viral NTPase/helicase <03JMC4149; 03JMC4776>. Helicases are responsible for unwinding duplex RNA and DNA structures by disrupting the hydrogen bonds that keep the two strands together <88NAT22; 98STR89>. This process is dependent upon the necessary energy released by hydrolysis of a molecule of nucleoside-5'-triphosphate (NTP) such as ATP or GTP <98JV6758>. Our studies pointed to the existence of an allosteric binding site on the viral NTPases/helicases that can be occupied by nucleoside/nucleotide-type molecules such as RENs and inhibit the viral helicase activity <03JMC4149; 03JMC4776>. We also found that at low ATP concentrations, the REN-5'-triphosphates bind to the enzyme's NTP binding site and competitively inhibit the viral NTPase activity <03JMC4149; 03JMC4776>. For example, at an ATP concentration equal to 1 x 10^{-5} K_M value of the enzyme, the REN-triphosphate **137** inhibited the WNV NTPase reaction with an IC_{50} value of 0.15 μM <03JMC4149; 03JMC4776>.



Scheme 27

Recently, we have discovered that a number of RENs inhibit both HIV and HCV <08ICAR (LB18); 08MI12>. The leading RENs in this regard are **135** and **136** (see Table 2), containing a long hydrophobic chain at position-6 of the heterocyclic ring, which practically wiped out the HIV replication in T cells and monocyte-derived macrophages in micromolar concentrations <08JMC5043>. They were found to potently inhibit the ATP dependent helicase activity of human RNA helicase DDX3 <08JMC5043> as well as NTPase/helicase activity of HCV <08MI18>. Furthermore, neither of the two RENs exhibited toxicity at therapeutic doses in *ex vivo* cell culture or *in vivo* in mice <08JMC5043>. The dual inhibition of HIV and HCV by RENs has important implications in treating HIV patients infected with HCV. A vast majority of such patients in the US and the Western hemisphere ultimately die of liver cirrhosis and liver carcinoma <05JGH739; 05JH341; 06AIDS49>. This is because the currently available separate therapies for HIV and HCV have unfavorable drug interactions, which lead to exacerbation of both HCV and HIV. Therefore, the drugs that would act against both HCV and HIV are imminently needed, and so, dual-active RENs such as **135 and 136** have good prospects in this regard.

RENs **100**, **121**, and **132** were found to be potent and selective inhibitors of replication of the hepatitis B virus (HBV) in cultured human hepatoblastoma 2.2.15 cells <02AR159>. The most active REN **121** inhibited the synthesis of intracellular HBV replication intermediates and extracellular virion release with 50% effective concentration (EC_{50}) of 0.604 and 0.131μM, respectively, with selectivity indices of >18,500 and >4000, respectively <02AR159>. The toxicity of all three compounds was also measured in rapidly growing human foreskin fibroblast (HFF) cells and Daudi cells by cell proliferation assay. Once again, negligible or no toxicity was observed in both cells <02AR159>. It was also interesting to note that all three RENs, unlike the industrial standard 3TC (2',3'-dideoxy-L-3'-thiacytidine) used for anti-HBV activity, resulted in the reduction of viral protein synthesis, especially that of the core antigen <02AR159>.

2.6. CONCLUSION

Ring-expanded heterocycles, nucleosides, and nucleotides possess excellent promise as broad-spectrum therapeutics against both cancers and viruses. The promising *in vitro* data, particularly against prostate and breast cancers, and against viruses, in particular HBV, HCV,

WNV, and HIV, support their further development as drug candidates via extensive animal and clinical studies.

2.7. ACKNOWLEDGMENTS

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