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Analogues of *Iso*-Azepinomycin as Potential Transition-State Analog Inhibitors of Guanase: Synthesis, Biochemical Screening, and Structure-Activity Correlations of Various Selectively Substituted Imidazo[4,5-*e*][1,4]diazepines

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Abstract

Guanase is an important enzyme of the purine salvage pathway of nucleic acid metabolism and its inhibition has beneficial implications in viral, bacterial, and cancer therapy. The work described herein is based on a hypothesis that azepinomycin, a heterocyclic natural product and a purported transition state analog inhibitor of guanase, does not represent the true transition state of the enzymecatalyzed reaction as closely as does *iso*-azepinomycin, wherein the 6-hydroxy group of azepinomycin has been translocated to the 5-position. Based on this hypothesis, and assuming that *iso*-azepinomycin would bind to guanase at the same active site as azepinomycin, several analogs of *iso*-azepinomycin were designed and successfully synthesized in order to gain a preliminary understanding of the hydrophobic and hydrophilic sites surrounding the guanase binding site of the ligand. Specifically, the analogs were designed to explore the hydrophobic pockets, if any, in the vicinity of N1, N3, and N4 nitrogen atoms as well as O⁵ oxygen atom of *iso*-azepinomycin. Biochemical inhibition studies of these analogs were performed using a mammalian guanase. Our results indicate that (1) increasing the hydrophobicity near O⁵ results in a negative effect, (2) translocating the hydrophobicity from N3 to N1 also results in decreased inhibition, (3) increasing the hydrophobicity near N3 or N4 produces significant enhancement of inhibition, (4) increasing the hydrophobicity at either N3 or N4 with a simultaneous increase in hydrophobicity at O⁵ considerably diminishes any gain in inhibition made by solely enhancing hydrophobicity at N3 or N4, and (5) finally, increasing the hydrophilic character near N3 has also a deleterious effect on inhibition. The most potent compound in the series has a K_i value of 8.0 ± 1.5 M against rabbit liver guanase.

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Supplementary Data Supplementary data on details of enzyme kinetics including associated tables and graphs (12 pages) are available from the online version of the journal.

Keywords

Organic synthesis and medicinal chemistry; Inhibitors of guanase; Imidazo[4,5-e][1,4]diazepines; *Iso-Azepinomycin* analogs; Biochemical inhibition studies; Structure-activity relationship (SAR) studies

1. Introduction

Guanase or guanine deaminase (EC 3.5.4.3) is an important metalloenzyme in the salvage pathway of purine metabolism, catalyzing the hydrolytic deamination of guanine to xanthine.^{1, 2} Inhibition of guanase has beneficial implications in viral, bacterial, and cancer therapy.³⁻⁸ While many studies of guanase inhibition have been reported both from our lab⁹⁻¹⁶ and others,¹⁷⁻²⁷ it is still one of the least explored enzymes of nucleic acid metabolism, although it has been isolated from rabbit liver,²⁸ human liver,²⁹ and rat brain³⁰ many years ago.

A variety of pathogens are dependent upon the host's purine salvage pathway for the nucleotides necessary for their survival, as they do not possess a *de novo* purine biosynthetic route.³¹ It was further demonstrated that inhibition of guanase could be caused selectively in pathogens without seriously affecting the nucleic acid biosynthesis of the host mammals that largely employ the *de novo* pathway.³¹ A potent guanase inhibitor will also be an excellent tool for studying a number of disease-related metabolic processes in which the enzyme is involved either as an indicative or a causative factor. There are several documented reports on detection of abnormally high levels of serum guanase activity in patients with liver diseases like hepatitis. Increased levels of hepatitis C have been observed when patients with HCV are transfused with blood containing high levels of serum guanase activity.^{8, 32} It is further known that high serum guanase activity is a clear biochemical indicator of organ rejection in liver transplant patients.³³ In addition, patients with multiple sclerosis have significantly elevated levels of guanase activity in their cerebrospinal fluid, and a clear correlation was established between the extent of disability and the level of guanase activity.⁵

Another important aspect of guanase activity is its involvement in cancerous tissues. It has long been known that carcinogenic processes and the activities of some enzymes in cancer tissues and cells are strongly interrelated. In this regard, it is important to consider reports of abnormal levels of guanase activity in various cancer tissues in lung,³⁴ kidney,⁴ and breast cancer tissues.³ It is suggested that this difference in activity is a physiological attempt of the cancer cell to regulate the guanine and /or xanthine level, which are needed by cancer cells to accelerate their salvage metabolic pathway activity. The alternate *de novo* metabolic pathway is mostly employed by normal cells for replication. So, a guanase inhibitor could discriminate the growth of cancer cells without affecting the normal cells. In view of these reasons, it is timely and important to search for a suitable guanase inhibitor that may assist in exploring guanase as a novel chemotherapeutic target as well as in understanding the specific physiological and biochemical role played by guanase in a number of metabolic disorders in which guanase is suspected to be involved.

2. Hypothesis, Specific Aims, and Rationale

Azepinomycin is a naturally occurring moderate inhibitor of guanase, isolated from the culture filtrates of *Streptomyces sp.* MF718-03.³⁵ It is also a purported transition state analog inhibitor of the enzyme-catalyzed reaction with an IC_{50} of ~ 5 M in tissue cultures,³⁵ and a K_i of ~ 2.5 M against an isolated mammalian enzyme from rabbit liver.¹² However,

when one closely looks at the reaction catalyzed by guanase, which is the hydrolysis of guanine to xanthine via the aminol intermediate (see Figure 1), it is apparent that the carbon and nitrogen atoms involved in the hydrolysis reaction are C2 and N3, respectively, and not C2 and N1.

The hydrolysis apparently involves the imine nitrogen N3 instead of the lactam nitrogen N1. Therefore, if one extrapolates this to azepinomycin as the purported transition state analog and *iso*-azepinomycin as a hypothetical transition state analog (Figure 1), it is the latter that more closely resembles the transition state as it can be regarded as an aminol formed from the hydrolysis of an imine precursor.

Therefore, the work described herein is based on the above hypothesis that *iso*-azepinomycin could be a better analog than azepinomycin in mimicking the true transition state of the guanase-catalyzed deaminase reaction of guanine to xanthine. However, we realized early on that there was one potentially serious stability problem in realizing such a compound. This is because of the potentially facile equilibrium existing between an imine and an aminol in *iso*-azepinomycin. Such an equilibrium is considerably less likely in azepinomycin as it involves the lactam nitrogen, which cannot provide as efficient anchimeric assistance as an amino nitrogen of the previous case in elimination of a water molecule to form an imine. Nonetheless, we were optimistic that the corresponding alkoxy derivatives of *iso*-azepinomycin, especially with long alkyl chains, would have reasonable stability to explore some important preliminary information on the hydrophobic or hydrophilic residues surrounding the ligand in the enzyme active site.

Therefore, assuming that *iso*-azepinomycin would bind to guanase at the same active site as azepinomycin, the present work is aimed at gaining a preliminary understanding of the hydrophobic and hydrophilic sites surrounding the guanase binding site of *iso*-azepinomycin. This is a starting point in any new drug design targeting an enzyme. In particular we intend to explore the hydrophobic or hydrophilic pocket, if any, in the vicinity of N1, N3, and N4 nitrogen atoms as well as O⁵ oxygen atom of *iso*-azepinomycin. To this end, we set out to design and synthesize eleven analogs of *iso*-azepinomycin (see Figure 2)

The biochemical inhibition data of the proposed analogs **1–4** against guanase are anticipated to throw some light on the hydrophobic environment, if any, in the vicinity of N3 and O⁵ of the ligand in the enzyme binding site, while analogs **5–7** would serve as probes to map spatial and electronic environments beyond where the phenyl group is seated on the protein. Likewise, inhibition data of analogs **8** and **9** would provide important information on the hydrophobic pockets, if any, in the vicinity of N1 and N4, while compound **10** would assess if the enhanced hydrophobicity at both N1 and N4 would have an additive or deleterious effect on the enzyme inhibition. If the hydrophobic effect indeed turns out to be additive, then analog **11** would determine if further enhancement of hydrophobicity at O⁵ would fair well for the overall enzyme inhibition.

3. Chemistry

Synthesis of the target analogs **1–4** is outlined in Scheme 1. The synthesis commenced with 1-benzyl-5-nitroimidazole-4-carboxylic acid (**12**),³⁶ which was converted into the desired amide (**13**) in 86% yield through activation with 1,1'-carbonyldiimidazole (CDI), followed by treatment with aminoacetaldehyde dimethyl acetal. The nitro group of **13** was reduced under neutral reaction conditions using Pd-C/H₂ in methanol to obtain **14** in 54% yield. The reaction of **14** with 1N aqueous HCl at room temperature overnight resulted in the complete conversion to the ring closed product **1** as revealed by ¹H NMR analysis. While compound **1** was reasonably stable in an aqueous acidic environment, it was quite unstable and

decomposed under neutral reaction conditions. Thus, all our attempts to isolate **1** in a pure solid form were futile, although we were able to obtain the necessary ^1H and ^{13}C NMR data, as well as its high resolution mass spectral data, from the crude reaction mixture in D_2O , all of which were consistent with structure **1**. Apparently, the hydroxy group of **1** at position 5 is very labile and presumably undergoes facile elimination reaction via anchimeric assistance from the adjacent NH at the 4-position to form a highly reactive iminium ion **15**. The latter can readily undergo nucleophilic addition reactions with reactive species present in the medium, including but not limited to self condensation reaction with another molecule of **1**, to form an intractable material. Support for this notion was obtained by treatment of the crude acidic reaction mixture of **1**, obtained from **14**, with methanol, ethanol or butanol at room temperature for 6 hours, which gave the corresponding target compounds **2**, **3**, or **4**, respectively. It was also possible to obtain the target compounds through direct reaction of **14** with the corresponding alcohols in 1N HCl at 50°C for 24 hours. The purification of the products was, however, a little tricky as they readily underwent exchange reactions with methanol when it was used either alone or as part of a mixture of an eluting solvent in silica gel column chromatography. Unfortunately, the target compounds could not be eluted off the column without using at least a minimum amount of a protic solvent such as methanol. The use of a mixture of chloroform: methanol: ammonium hydroxide (5:2:0.2) was preferred among all the eluting solvent systems attempted afforded methyl analog **2** (65% yield), ethyl analog **3** (40%) and butyl analog **4** (22%).

A logical starting material for the synthesis of target compounds **5**, **6**, and **7** would be an analog of **12** containing a methoxy or fluoro substituent at the *para* position of the phenyl ring. Surprisingly, a thorough examination of the literature revealed that despite their simple structures, neither **16a** nor **16b** (Scheme 2) was known. Therefore, compounds **16a** and **16b** were synthesized starting from 4(5)-methyl-5(4)-nitro-1*H*-imidazole (**17**), employing procedures well-established in our lab.³⁷

The styrylation of **17** using benzaldehyde and piperidine gave the 4(5)-styryl-5(4)-nitroimidazole (**18**) as a yellow solid in 77% yield. The latter was then benzylated using *p*-methoxybenzyl chloride or *p*-fluorobenzyl chloride and potassium carbonate in DMF at 85°C to yield a mixture of regioisomers, namely, 1-*p*-methoxybenzyl-5-nitro-4-styrylimidazole, **19a** (50% yield) and 1-*p*-methoxybenzyl-4-nitro-5-styrylimidazole, **20a** (41%), or 1-*p*-fluorobenzyl-5-nitro-4-styrylimidazole, **19b** (63%) and 1-*p*-fluoro-benzyl-4-nitro-5-styrylimidazole, **20b** (32%), respectively. The two isomers in each case were separated by silica gel column chromatography and were distinguished from each other by ^1H NMR chemical shifts of the N-alkyl and H-2 protons, both of which are slightly more deshielded in **19** as compared with those of **20**. This is due to the combined inductive and resonance effects of the nitro group attached at position 4 or 5 of the imidazole ring. As shown in Figure 3, the electron-withdrawing nitro group in **19** is conjugated to position-2 of the imidazole ring, thus causing H-2 to be deshielded. Such a conjugation is not possible in **20**. Likewise, the electron-withdrawing inductive effect of the nitro group upon the benzyl CH_2 is expected to be more pronounced in **19** as compared with **20**, due to close proximity of the two functional groups in the former. This regioisomeric structural assignment based on the ^1H NMR data has further been corroborated by single crystal X-ray diffraction analysis of 1-methyl-4-nitro-5-styrylimidazole reported from this laboratory.³⁸ Finally, oxidation of the **19a** and **19b** with aqueous potassium permanganate gave the desired **16a** and **16b**, in 61% and 58% yields, respectively.

1-*p*-Methoxy- or 1-*p*-fluorobenzyl-5-nitroimidazole-4-carboxylic acid (**16a** or **16b**) was further condensed with aminoacetaldehyde dimethylacetal, employing CDI as a condensing agent (Scheme 3) to obtain the amide **21a** or **21b** in 83% or 85% yield, respectively. The nitro group in each was then reduced under neutral conditions using $\text{Pd-C}/\text{H}_2$ in methanol to

obtain the corresponding amine **22a** or **22b** in 76% or 58% yield, respectively. Reaction of **22a** or **22b** with 1N aqueous HCl at room temperature overnight resulted in the complete conversion to a mixture of the ring closed compounds **23a** or **23b** and **5** or **7**, respectively. When the latter mixture was treated with excess methanol and stirred at room temperature for several hours, the target **5** or **7** was formed exclusively. Similarly, when the mixture **23** and **5** was treated with excess ethanol, the corresponding ethyl analog **6** was formed. Apparently, the reaction proceeds through an iminium ion intermediate **24**, which undergoes nucleophilic addition reactions with methanol and ethanol to form the observed target products **5** and **6**, respectively. Compound **5**, **6** or **7** could also be directly synthesized from **22** by reaction with corresponding methanol or ethanol using 1N aqueous HCl at 50 °C for 2 hours. Purification of the resulting analogs using column chromatography was somewhat tricky as they readily underwent exchange reactions when different alcohols other than what is dictated by the alkoxy substituent in the product was used for elution. The product would not move down the silica gel column unless an appropriate combination of alcohol and ammonium hydroxide, along with chloroform or any other non-protic solvent, was used for elution. The use of chloroform: methanol: ammonium hydroxide (10:1:0.05) or chloroform: ethanol: ammonium hydroxide (10:1:0.05) solvent system, respectively, afforded **5** or **6** in 57% or 36% yield, respectively, while the use of chloroform: methanol: ammonium hydroxide (12:1:0.05) gave **7** in 42% yield.

Synthesis of target **8** (Scheme 4) commenced with **20a**, whose synthesis has been described earlier. Compound **20a** was oxidized to the corresponding carboxylic acid **25** using the same procedure as described earlier for the conversion of **19** to **16** (*cf.* Scheme 2)

Compound **25** was carried through the same sequence of steps as described earlier for the conversion of **16** to targets **5–7** (*cf.* Scheme 3). The ¹H NMR of the crude solid isolated from the reaction of **27** with aqueous HCl overnight indicated complete conversion to **28**, but no attempts were made to purify the product because of the known instability and prior experience with difficulties associated with purification of such compounds in our lab, including but not limited to **23**. The target **8** was obtained in a pure form after column chromatography in 52% yield.

Synthesis of target **9** proceeded from **18**, which was converted to the desired starting material *N*-(2,2-dimethoxyethyl)-5-nitro-1*H*-imidazole-4-carboxamide (**30**)³⁹ via 5-nitro-1*H*-imidazole-4-carboxylic acid (**29**),³⁷ employing the procedures reported from our lab several years ago.^{37, 39} Compound **30** was reduced by catalytic hydrogenation over Pd-C at 60 psi to the amine **31** in 50% yield. The 5-amino group of **31** was benzylated by sequential reactions with benzaldehyde, catalyzed by TFA, followed by reduction with sodium cyanoborohydride to obtain **32** in 43% yield. Compound **32** was ring-closed to form **33** upon reaction with 1N HCl at room temperature for 8 hours. Without further purification, the crude solid **33** obtained after evaporation of the solvent was stirred with excess methanol using catalytic amounts of 1N HCl for 8 hours at room temperature, which gave the desired **9** in 38% yield.

Synthesis of targets **10** and **11** was accomplished (Scheme 6) starting from **27** (*cf.* Scheme 4), which was converted into the corresponding *N*-benzyl derivative **34** in 93% yield using an analogous procedure as described above for the conversion of **31** to **32**. Treatment of **34** with 1N HCl at room temperature overnight, evaporation of volatiles, followed by reaction of the residue with methanol or ethanol, catalyzed by 1N HCl at room temperature for 8 hours, provided the target analog **10** or **11** in 38% or 31% yield, respectively.

All isolable intermediates and products were fully characterized by ¹H NMR, ¹³C NMR, and MS data, coupled with elemental microanalyses and/or HRMS data

4. Biochemistry

Guanase inhibition studies of target compounds **2–11** were conducted using guanase from rabbit liver (MP Biochemicals) at 25 °C and pH 7.4 by spectroscopically measuring the rate of hydrolysis of the substrate guanine at λ_{max} 245 nm. Stock solutions of the substrate, enzyme and inhibitors were prepared using 0.05 M Tris buffer (pH 7.4). The enzyme kinetics were followed by measuring decrease in absorbance per minute of the substrate guanine at λ_{max} 245 nm. The change in optical density per unit time is a measure of the guanase activity. A total of 5–6 different concentrations of the substrate, ranging from 5 to 40 μM , was employed for each inhibitor concentration that was either 10, 20, or 30 μM . The Lineweaver-Burk plots (1/V versus 1/S) were generated and used to calculate K_M , V_{max} , and K_i .²⁴ Our biochemical results showed that K_M of enzyme with guanine as substrate is 5.5×10^{-6} M. A representative Lineweaver-Burk plot is shown for compound **2** (Figure 4). The K_i data for all the target compounds are collected in Table 1, and the detailed kinetic data, graphs, and calculations associated with biochemical inhibition studies all target compounds (**2–11**) have been provided in the Supplementary Data available from the online version of the Journal.

5. Conclusions

As stated under Rationale (see section 2 above), the scope of the present work is limited to exploring the hydrophobic environment, if any, near N1, N3, N4, and O⁵ sites of *iso*-azepinomycin. Based on the observed enzyme inhibition data, we conclude that (a) increasing the hydrophobicity near O⁵ has a negative effect on inhibition as revealed by the K_i data of targets **2–4**, (b) further enhancement of hydrophobicity beyond the phenyl group at N3, as in target **5**, favors inhibition, but any simultaneous enhancement of hydrophobic character at O⁵, as in target **6**, would diminish the gain made by increased hydrophobicity at N3, (c) increasing the hydrophilic character with concurrent decrease in hydrophobicity, for example with a fluoro substituent attached to the phenyl group at N3, as in target **7**, brings down the potency of inhibition, (d) enhanced hydrophobicity at N1, as in target **8**, also has deleterious effect on inhibition, (e) on the other hand, increasing the hydrophobicity at N4, as in target **9**, has a large positive effect on inhibition, (f) simultaneous increase of hydrophobicity at both N4 and N1, as in target **10**, diminishes any gain made by increasing the hydrophobicity only at N4, and finally (g) any simultaneous increase of hydrophobicity near O⁵, results in large negative effect on inhibition

In summary, our results suggest that there is a large hydrophobic pocket in the vicinity of N3 and N4 as revealed by the guanase inhibition data of compounds **5** and **9**. It is not yet clear, however, if simultaneous increase of hydrophobicity at both N3 and N4 would have additive or diminished effect on inhibition. The best compound in the series so far is compound **9** containing a phenyl substituent at N4, which shows a K_i of ~ 8 M. Although the inhibitory potency of **9** is somewhat lower than the natural guanase inhibitor azepinomycin with a K_i of ~ 2.5 M, it is not too unreasonable to hope that further structure-activity relationship studies on **5** and **9**, for example, by further increasing hydrophobicity at their respective N3 and/or N4 position, would lead to more potent inhibitors of guanase, and not to mention that much yet needs to be explored about the possible hydrophilic pocket surrounding N1 and the hydrophilic/ hydrophobic pockets surrounding N7 and O⁸ sites of *iso*-azepinomycin.

6. Experimental

6.1 General

The ¹H and ¹³C NMR spectra were recorded on a JEOL-400 NMR spectrometer, operating at 400MHz for ¹H and 100 MHz for ¹³C NMR, respectively. Thin layer chromatography

was performed on Merck Kieselgel 60 F₂₅₄ (0.2 mm thickness). Flash chromatography was performed using 32–63 mesh silica gel. Mass spectra were recorded on a Bruker Daltonics Esquire-3000 LC-Quadrupole ion trap spectrometer. High resolution mass spectra were recorded on a Bruker Daltonics (Billerica, MA) Apex IV FTICR. Melting points were recorded on Hoover capillary melting point apparatus and are uncorrected. All the reagents were purchased from Sigma-Aldrich or Fisher Pharmaceuticals. Anhydrous solvents were purchased in the highest purity available and used without further purification or drying. Bulk solvents were purchased from Pharmco. Deuterated solvents were purchased from Cambridge Isotope Labeling Co. All reactions sensitive to air or moisture were performed in an inert atmosphere of nitrogen.

6.2. Organic Synthesis

6.2.1. 1-Benzyl-*N*-(2,2-dimethoxyethyl)-5-nitro-1*H*-imidazole-4-carboxamide

(13)—A mixture of **12** (2.0 g, 8.1 mmol) and 1, 1'-carbonylimidazole (2.0 g, 12.3 mmol) in dry THF (60 mL) was refluxed in a flame-dried three-neck flask kept under a nitrogen atmosphere. When the mixture turned clear it was allowed to cool to room temperature. Amino acetaldehyde dimethylacetal (1 mL, 10 mmol) was added to the cooled solution and stirred for 2 h. The reaction was monitored for completion. The solvent was rotary evaporated to dryness and ice-water was added and stirred overnight. Then the product was extracted with chloroform and washed with water. The organic layer was rotary evaporated and purified by silica-gel column chromatography with EtOAc : Hex (1:1) as the eluting solvent system. The appropriate fraction was collected to give **13**. Yield 2.31 g, 86%; ¹H NMR (DMSO-*d*₆) δ 8.5 (t, 1H, NH, exchangeable with D₂O), 8.24 (s, 1H, Imid-H₂), 7.3 – 7.16 (m, 5H, Ar-H), 5.5 (s, 2H, Ar-H), 4.4 (t, 1H, acetal-H), 3.29 (d, *J* = 5.52 Hz, 2H, CH₂), 3.24 (s, 6H, 2×CH₃); ¹³C-NMR (CDCl₃) δ 40.9, 51.5, 54.5, 102.6, 127.8, 129.2, 129.4, 133.2, 136.8, 159.4; MS (ESI) *m/z* 335 (MH⁺); *Anal.* Calcd for C₁₅H₁₈N₄O₅: C, 53.89, H, 5.43, N 16.76; found C, 53.86, H, 5.42, N, 16.80.

6.2.2. 5-Amino-1-benzyl-*N*-(2,2-dimethoxyethyl)-1*H*-imidazole-4-carboxamide

(14)—A mixture of **13** (2.3 g, 6.7 mmol) and Pd-C (10%) (200 mg) in absolute methanol (50 mL) was hydrogenated in a Parr hydrogenator at 40 psi for 5 h. The reaction mixture was filtered through Celite, and the filtrate evaporated to dryness on a rotary evaporator. The yellow semi-solid residue was triturated with diethyl ether to obtain **14** as a yellowish white solid. Yield 1.1 g, 54%; ¹H NMR (DMSO-*d*₆) δ 7.33–7.16, 7H), 5.7 (s, 2H, NH₂, exchangeable with D₂O), 5.04 (s, 2H, CH₂), 5.0 (s, 2H, NH₂, exchangeable with D₂O), 4.1–4.0 (q, 4H, 2×CH₂), 3.65 (s, 3H, OMe), 4.43 (t, 1H, acetal-H), 3.24 (d, *J* = 5.52 Hz, 2H, CH₂), 3.20 (s, 6H, 2×CH₃); ¹³C-NMR (CDCl₃) δ 40.1, 47.4, 54.3, 103.1, 115.2, 126.8, 128.6, 129.4, 130.4, 134.6, 141.9, 165; MS (ESI) *m/z* 305 (MH⁺); *Anal.* Calcd for C₁₅H_{20.5}N₄O_{3.25}: C, 59.20, H, 6.62, N, 18.41; found C, 58.68, H, 6.64, N, 18.31.

6.2.3. 3-Benzyl-5-hydroxy-4,5,6,7-tetrahydroimidazo[4,5-*e*] [1,4]diazepin-8(3*H*)-one (1)

(1)—Compound **14** (0.8g, 2.6 mmol) was taken in a clean flask and 20 mL of 1N HCl was added and stirred at room temperature for 10 h. The color of the solution turned from red to light yellow. The acidic solution was concentrated under vacuum and then was neutralized to pH 7. The neutral solution was kept in the cold (4 °C) for complete precipitation. The precipitate was then gravity filtered and washed with water. Mass spectral and ¹H NMR data of the aqueous filtrate shows exclusive formation of **1**. The latter, however, decomposes in neutral aqueous medium over time. The following are the spectral and analytical data of the crude reaction mixture containing **1**: ¹H NMR (D₂O) δ 3.25 (d, *J* = 14.64 Hz, 1H), 3.66 (m, 1H), 5.32 (d, *J* = 5.52 Hz, 2H), 5.37 (d, *J* = 5.52 Hz, 1H), 7.49–7.34 (m, 5H), 8.51 (s, 1H); ¹³C-NMR (D₂O) δ 44.4, 47.1, 74.4, 112.0, 126.9, 128.3, 129.2, 135.0,

135.6, 140.8, 168.7; HRMS (FAB) Calcd. for $C_{13}H_{14}O_2$: m/z 259.1190 (MH^+); obsd. m/z 259.1188.

6.2.4. 3-Benzyl-5-methoxy-4,5,6,7-tetrahydroimidazo[4,5-e] [1,4]diazepin-8(3H)-one (2)—Compound **14** (0.2g, 0.6 mmol) was taken in a clean flask to which was added 1mL of 1N HCl and 20 mL of methanol and the mixture was stirred overnight at 50 °C for 24 h. The resulting solution was evaporated and purified by column chromatography, using chloroform: methanol: ammonium hydroxide (5:2:0.2) as the eluting solvent system. Evaporating the appropriate fractions gave **2** as a light yellow colored solid. Yield 0.46g, 65%, mp ~185°C, R_f =0.46 (chloroform: methanol: ammonium hydroxide:5:2:0.2); 1H NMR (DMSO- d_6) δ (2.98, dd, J = 3.64 Hz, 1H), 3.1 (s, 3H), 3.37 (m, 1H), 4.6 (t, J = 5.2 Hz, 1H), 5.08 (d, J = 16.08 Hz, 1H), 5.11 (d, J = 16.08 Hz, 1H), 7.01 (m, NH, exchangeable with D_2O), 7.15–7.35 (m, Aromatic + imidazole H's, 5H+1H), 7.41 (m, NH, exchangeable with D_2O); ^{13}C -NMR (DMSO- d_6) δ 44.2, 47.3, 54.2, 80.7, 113.3, 128.1, 128.4, 129.1, 134.8, 136.9, 139.5, 167.4; MS (ESI) m/z 273 (MH^+); *Anal.* Calcd for $C_{14}H_{16.25}N_4O_{2.125}$: C 61.75, H 5.92, N 20.52; found C 61.12, H 5.90, N 20.10; HRMS (FAB) Calcd. for $C_{14}H_{17}N_4O_2$: 273.1346 (MH^+); obsd. m/z 273.1345.

6.2.5. 3-Benzyl-5-ethoxy-4,5,6,7-tetrahydroimidazo[4,5-e] [1,4]diazepin-8(3H)-one (3)—Compound **14** (0.2g, 0.6 mmol) was taken in a clean flask to which was added 1mL of 1N HCl and 20 mL of ethanol and stirred overnight at 50 °C for 24 h. The resulting solution was evaporated and the residue was purified by column chromatography, using chloroform:methanol:ammonium hydroxide (5:2:0.2) as the eluting solvent system. Evaporating the appropriate fractions gave **3** as a light yellow solid. Yield 0.07 g, 40%, R_f 0.61 (chloroform:methanol: ammonium hydroxide: 5:2:0.2); 1H NMR (DMSO- d_6) δ 1.00 (t, 3H), 2.97 (d, 1H), 3.34–3.41 (m, 2H+1H), 4.76 (t, 1H), 5.04–5.16 (m, 2H), 7.19–7.41 (m, 5H+1H+NH), 7.71 (s, NH, exchangeable with D_2O); ^{13}C -NMR (DMSO- d_6) δ 15.5, 44.0, 46.6, 61.4, 80.2, 114.3, 127.6, 128.0, 129.0, 133.7, 137.2, 139.3, 166.5; MS (ESI) m/z 287 (MH^+); HRMS (FAB) Calcd. for $C_{15}H_{19}N_4O_2$: 287.1502 (MH^+); obsd. m/z 287.1500.

6.2.6. 3-Benzyl-5-butoxy-4,5,6,7-tetrahydroimidazo[4,5-e] [1,4]diazepin-8(3H)-one (4)—Compound **14** (0.2 g, 0.6 mmol) was taken in a clean flask to which was added 1mL of 1N HCl and 20 mL of butanol and the mixture was stirred overnight at 50 °C for 24 h. The resulting solution was evaporated and the residue was purified by column chromatography, using chloroform: methanol:ammonium hydroxide (5:1:0.1) as the eluting solvent system. Evaporating the appropriate fractions gave **4** as a light yellow solid. Yield 0.04 g, 22%; R_f =0.75 (chloroform: methanol: ammonium hydroxide: 5:2:0.2); 1H NMR (DMSO- d_6) δ 0.79 (t, 3H), 1.37–1.17 (m, 4H), 2.98 (d, 1H), 3.42–3.27 (m, 2H+1H), 4.72 (t, 1H), 5.08–5.14 (m, 2H), 7.16–7.39 (m, 5H+1H+NH), 7.54 (s, NH, exchangeable with D_2O); ^{13}C -NMR (DMSO- d_6) δ 14.2, 19.3, 31.6, 44.1, 46.7, 65.8, 80.1, 114.2, 127.6, 128.0, 129.0, 133.7, 137.2, 139.3, 166.7; MS (ESI) m/z 315 (MH^+); HRMS (FAB) Calcd. for $C_{17}H_{23}N_4O_2$: 315.1815 (MH^+); obsd. m/z 315.1811.

6.2.7. 5(4)-Nitro-4(5)-styrylimidazole (18)—To a stirred suspension of 4(5)-methyl-5(4)-nitroimidazole (5 g, 0.04 mol) in benzaldehyde was added piperidine (3.95 g, 0.04 mol). The mixture was heated to 110 °C for 2.5 h. When the reaction was complete and solid started separating, 100 mL ethanol was added to the reaction mixture. The mixture was allowed to cool to room temperature and filtered to obtain a yellow solid which was washed with ethanol and dried to get a pure compound. Yield 6.5 g, 77%; mp: 258–260 °C; 1H NMR (DMSO- d_6) δ 7.90 (s, 1H, Imid-H), 7.68–7.36 (m, 7H, CH=CH, Ar-H); ^{13}C -NMR (DMSO- d_6) δ 143.73, 136.33, 136.10, 135.30, 130.93, 129.76, 129.65, 127.54, 114.64; HRMS (FAB), Calcd. for $C_{11}H_9N_3O_2$: 216.0767 (MH^+); obsd. m/z 216.0769.

6.2.8. 1-(4-Methoxybenzyl)-5(4)-nitro-4(5)-styryl-1H-imidazole (19a & 20a)—To a solution of **18** (4 g, 18 mmol) in hot DMF was slowly added anhydrous potassium carbonate (3.73 g, 27 mmol). To this solution was added *p*-methoxybenzyl chloride (3.38 g, 21 mmol). The mixture was heated at 85 °C for 5 h, cooled to room temperature and filtered. The filtrate was concentrated under reduced pressure to remove DMF. The residual thick solution was poured into ice and extracted with chloroform. The organic layer was dried over anhydrous sodium sulfate and was further purified by column chromatography using 400:1 chloroform/methanol as eluting solvent system to obtain pure compounds:

Compound 19a: Yield 3.0 g, 50%; mp: 105–108 °C; R_f =0.5 (chloroform/methanol 400:1); ^1H NMR (CDCl_3) δ 7.79–7.70 (m, 2H, Ar-H), 7.60–7.57 (m, 2H, Ar-H), 7.50 (s, 1H, Imid-H), 7.17–7.14 (m, 2H, Ar-H), 6.89–6.85 (m, 2H, CH=CH), 5.44 (s, 2H, benzylic- CH_2), 3.78 (s, 3H, $-\text{OCH}_3$); ^{13}C -NMR (CDCl_3): δ 159.99, 144.08, 140.45, 137.28, 136.32, 133.98, 129.38, 129.17, 128.89, 127.65, 126.36, 117.88, 114.63, 55.43, 51.68; HRMS (FAB) Calcd. for $\text{C}_{19}\text{H}_{17}\text{N}_3\text{O}_3$: 335.1263 (MH^+); obsd m/z 336.1328; *Anal.* Calcd. for $\text{C}_{19}\text{H}_{17}\text{N}_3\text{O}_3$: C, 68.05, H, 5.11, N, 12.53; found, C, 68.35, H, 5.09, N, 12.37.

Compound 20a: Yield 2.5 g, 41%; mp: 115–119 °C; R_f =0.23; ^1H NMR (CDCl_3) δ 7.43–7.33 (m, 7H, Ar-H), 7.11–7.07 (m, 3H, Ar-H, imid-H), 6.93–6.91 (m, 2H, CH=CH), 5.23 (s, 2H, benzylic- CH_2), 3.84 (s, 3H, $-\text{OCH}_3$); ^{13}C -NMR (CDCl_3) δ 160.04, 138.45, 136.37, 135.64, 130.21, 129.59, 129.43, 129.03, 128.72, 128.49, 126.10, 114.94, 113.03, 55.48, 50.33; HRMS (FAB) Calcd. for $\text{C}_{19}\text{H}_{17}\text{N}_3\text{O}_3$: 336.1342 (MH^+); obsd. m/z 336.1345.

6.2.9 (E)-1-(4-Fluorobenzyl)-5-nitro-4-styryl-1H-imidazole (19b) and (E)-1-(4-fluorobenzyl)-4-nitro-5-styryl-1H-imidazole (20b)—To a solution of **18** (4 g, 18.5 mmol) in hot DMF was slowly added anhydrous potassium carbonate (3.828 g, 27.7 mmol). To this solution was added *p*-fluorobenzyl chloride (3.2 g, 22.2 mmol). The mixture was heated at 85 °C for 5 h, cooled to room temperature and filtered. The filtrate was concentrated under reduced pressure to remove DMF. The thick solution was poured onto ice and extracted with chloroform. The organic layer was dried over anhydrous sodium sulfate and the two regioisomers were separated by column chromatography using 400:1 chloroform/methanol as eluting solvent system to obtain pure compounds.

Compound 19b: Yield 3.8g, 63%; mp: 134–137 °C; R_f =0.71 (chloroform/methanol, 100:1); ^1H NMR (CDCl_3) δ 7.80–7.70 (m, 2H, Ar-H), 7.60–7.58 (m, 2H, Ar-H), 7.55 (s, 1H, Imid-H), 7.38–7.31 (m, 3H, Ar-H), 7.20–7.16 (m, 2H, Ar-H), 7.06–7.01 (m, 2H, CH=CH), 5.47 (s, 2H, benzylic- CH_2); ^{13}C -NMR (CDCl_3) δ 164.20, 161.73, 144.31, 140.63, 137.72, 136.33, 130.54, 129.65, 129.39, 129.03, 127.78, 117.82, 116.52, 116.30, 51.41; HRMS (FAB) Calcd. for $\text{C}_{19}\text{H}_{17}\text{N}_3\text{O}_3$: 324.1142 (MH^+); obsd. m/z 324.1144 (MH^+); *Anal.* Calcd. for $\text{C}_{19}\text{H}_{17}\text{N}_3\text{O}_3$: C, 66.87, H, 4.36, N, 13.00; found, C, 67.07, H, 4.35, N, 13.04.

Compound 20b: Yield: 1.2 g, 32%; R_f =0.56 (chloroform/methanol, 100:1); (^1H NMR (CDCl_3) δ 7.39 (s, 1H, Imid-H), 7.33–7.28 (m, 4H, Ar-H), 7.07–7.02 (m, 4H, Ar-H), 5.24 (s, 2H, benzylic- CH_2), 2.88–2.80 (d, 1H, CH=CH, J = 18.76 Hz), 2.53–2.47 (d, 1H, CH=CH, J = 18.76 Hz). ^{13}C -NMR (CDCl_3) δ 164.11, 161.63, 138.59, 136.40, 129.71, 129.06, 128.81, 128.72, 127.21, 116.79, 116.69, 116.57, 112.81, 49.97.

6.2.10. 1-(4-Methoxybenzyl)-5-nitro-1H-imidazole-4-carboxylic Acid (16a)—

Finely powdered **19a** (3.0 g, 9 mmol) was placed in a 250 mL round-bottomed flask equipped with a magnetic stirring bar. Ice-cold water (36 mL) was added, followed by 2N sodium hydroxide (7.5 mL) and crushed ice (75 g). The temperature inside the flask was

maintained between 0–5 °C. Finely powdered potassium permanganate (6.0 g) was added slowly over a period of 1 h and the reaction mixture was stirred vigorously for 36 h. The dark brown reaction mixture was filtered *in vacuo* and the solid sludge (manganese dioxide) was thoroughly washed with hot water. The light yellow filtrate was acidified with concentrated hydrochloric acid to pH 1, when a light yellow solid precipitated out. The solid was filtered, air dried for 24 h, and suspended in ether with stirring for a few minutes to dissolve the by-product, benzoic acid. An off-white solid which remained was filtered *in vacuo*, washed with ether and dried over phosphorous pentoxide for 24 h to obtain **16a** as a pure compound. Yield 1.5 g, 61%; mp: 162–166 °C; ¹H NMR (DMSO-*d*₆) δ 8.22 (s, 1H, Imid-H), 7.19–7.14 (m, 2H, Ar-H), 6.94–6.91 (m, 2H, Ar-H), 5.43 (s, 2H, benzylic-CH₂), 3.73 (s, 3H, -OCH₃); ¹³C-NMR (DMSO-*d*₆) δ 162.87, 159.71, 127.42, 129.57, 114.76, 140.41, 135.00, 138.12, 55.66, 50.31; HRMS (FAB) Calcd. for C₁₂H₁₁N₃O₅: 277.0699 (MH⁺); obsd *m/z* 278.0778; *Anal.* Calcd. for C₁₂H₁₁N₃O₅: C, 51.99, H, 4.00, N, 15.16; found, C, 51.63, H, 4.03, N, 14.82.

6.2.11. 1-(4-Fluorobenzyl)-5-nitro-1*H*-imidazole-4-carboxylic Acid (16b)—Finely powdered **19b** (5 g, 15 mmol) was placed in a 250 mL round bottomed flask equipped with a magnetic stirring bar. Ice-cold water (60 mL) was added, followed by 2N sodium hydroxide (12.5 mL) and crushed ice (75 g). The temperature inside the flask was maintained between 0–5 °C. Finely powdered potassium permanganate (10 g) was added slowly over a period of 1 h and the reaction mixture was stirred vigorously for 36 h. The dark brown reaction mixture was filtered *in vacuo* and the solid sludge (manganese dioxide) was thoroughly washed with hot water. The light yellow filtrate was acidified with concentrated hydrochloric acid to pH 1, when a light yellow solid precipitated out. The solid was filtered, air dried for 24 h, and suspended in ether with stirring for few minutes to dissolve the by-product, benzoic acid. An off-white solid which remained was filtered *in vacuo*, washed with ether and dried over phosphorous pentoxide for 24 h to obtain pure **16b**. Yield: 2.4 g, 58%; mp: 168–170 °C; ¹H NMR (DMSO-*d*₆) δ 8.24 (s, 1H, Imid-H), 7.31–7.27 (m, 2H, Ar-H), 7.23–7.19 (m, 2H, Ar-H), 5.50 (s, 2H, benzylic-CH₂); ¹³C-NMR (DMSO-*d*₆) δ 162.90, 161.90, 140.68, 136.50, 134.49, 131.95, 130.22, 116.37, 116.15, 50.00; *Anal.* Calcd. for C₁₂H₁₁N₃O₅: C, 49.82, H, 3.04, N, 15.84; found, C, 50.05, H, 3.01, N, 15.59.

6.2.12. 1-(4-Methoxybenzyl)-*N*-(2,2-dimethoxyethyl)-5-nitro-1*H*-imidazole-4-carboxamide (21a)—A mixture of **16a** (2 g, 7.2 mmol) and 1,1'-carbonyldiimidazole (1.16 g, 7.2 mmol) in dry THF (10 mL) was refluxed in a flame-dried three-neck flask kept under a nitrogen atmosphere. When the mixture turned clear it was allowed to cool to room temperature. Aminoacetaldehyde dimethylacetal (1.17 mL, 10.8 mmol) was added to the cooled solution and allowed to stir for 2 h. The reaction was monitored by TLC for completion. The solvent was rotary evaporated to dryness; ice-cold water was added and the mixture was stirred overnight. Then the product was extracted with chloroform and washed with water and dried over anhydrous magnesium sulfate. The organic layer was evaporated to obtain a thick oily syrup. The latter was further subjected to silica gel column chromatography using 1:1 hexanes/ethyl acetate as the eluting solvent system to obtain **21a** as a pure compound. Yield: 2.2 g, 83%; *R*_f = 0.73; ¹H NMR (CDCl₃) δ 7.37 (s, 1H, Imid-H), 7.18 (t, 1H, -CONH) 7.14–7.09 (m, 2H, Ar-H), 6.89–6.85 (m, 2H, Ar-H), 5.29 (s, 2H, benzylic-CH₂), 4.46–4.43 (t, 1H, -CH, *J*_{1,2} = 5.04 Hz, *J*_{2,3} = 5.6 Hz), 3.78 (s, 3H, -OCH₃), 3.56–3.53 (t, 2H, CH₂, *J*_{1,2} = 5.96 Hz, *J*_{2,3} = 5.48 Hz), 3.39 (s, 6H, 2×OCH₃); ¹³C-NMR (CDCl₃) δ 160.31, 159.48, 136.60, 136.54, 133.94, 129.68, 124.99, 114.84, 102.62, 55.45, 54.58, 51.24, 40.96; HRMS (FAB) Calcd. for C₁₆H₂₀N₄O₆: 365.1455 (MH⁺); obsd. *m/z* 365.1447; *Anal.* Calcd. for C₁₆H₂₀N₄O₆: C, 52.74, H, 5.53, N, 15.38; found, C, 52.46, H, 5.47, N, 15.57.

6.2.13. 1-(4-Fluorobenzyl)-N-(2,2-dimethoxyethyl)-5-nitro-1H-imidazole-4-carboxamide (21b)—A mixture of **16b** (3 g, 11.3 mmol) and 1,1'-carbonyldiimidazole (3.08 g, 11.3 mmol) in dry THF (10 mL) was refluxed in a flame-dried three-neck flask kept under nitrogen atmosphere. When the mixture turned clear it was allowed to cool to room temperature. Aminoacetaldehyde dimethylacetal (1.83 mL, 17 mmol) was added to the cooled solution and the mixture was allowed to stir for 2 h. The reaction was monitored for completion. The solvent was removed by rotary evaporation, ice-cold water was added and the resulting mixture was stirred overnight. Then the product was extracted with chloroform and washed with water and dried over anhydrous magnesium sulfate. The organic layer was evaporated to obtain a thick oily syrup. This was further subjected to silica gel column chromatography using 1:1 hexanes/ethyl acetate as the eluting solvent system to obtain pure **21b**. Yield: 3.4 g, 85%; mp: 86–89 °C; R_f = 0.63 (hexanes/ethyl acetate, 1:1); ^1H NMR (CDCl_3) δ 7.43 (s, 1H, Imid-H), 7.19–7.14 (m, 3H, Ar-H, -CONH), 7.07–7.03 (m, 2H, Ar-H), 5.35 (s, 2H, benzylic- CH_2), 4.47–4.44 (t, 1H, -CH, $J_{1,2}$ = 5.04 Hz, $J_{2,3}$ = 5.48 Hz), 3.57–3.55 (t, 2H, - CH_2 , $J_{1,2}$ = 1.84 Hz, $J_{2,3}$ = 5.96 Hz), 3.40 (s, 6H, 2 $\times$ OCH₃); ^{13}C -NMR (CDCl_3) δ 159.32, 136.63, 134.17, 129.91, 129.83, 129.10, 116.69, 116.48, 102.59, 54.59, 50.86, 40.97; HRMS (FAB) Calcd. for $\text{C}_{16}\text{H}_{20}\text{N}_4\text{O}_6$: 353.1255 (MH^+); obsd. m/z 353.1257.

6.2.14. 5-Amino-1-(4-methoxybenzyl)-N-(2,2'-dimethoxyethyl)-1H-imidazole-4-carboxamide (22a)—A mixture of **21a** (2 g, 5.48 mmol) and Pd-C (0.2 g) in absolute methanol (30 mL) was hydrogenated in a Parr hydrogenator at 55 psi for 5 h. The reaction mixture was filtered through Celite, and the filtrate was evaporated to dryness on a rotary evaporator to obtain a thick oily syrup. This was further triturated with hexane to obtain **22a** as a pure white compound. Yield: 1.4 g, 76%; mp: 62–65 °C; R_f = 0.14 (hexanes/ethylacetate, 1:1); ^1H NMR (CDCl_3) δ 7.08–7.05 (m, 2H, Ar-H), 7.01 (s, 1H, Imid-H), 6.86–6.85 (m, 3H, Ar-H), 6.81 (t, 1H, -CONH) 4.86 (s, 2H, benzylic- CH_2), 4.61 (s, 2H, NH_2), 4.44–4.41 (t, 1H, -CH, $J_{1,2}$ = 5.48 Hz, $J_{2,3}$ = 5.52 Hz), 3.78 (s, 3H, -OCH₃), 3.52–3.48 (t, 2H, - CH_2 , $J_{1,2}$ & $J_{2,3}$ = 5.96 Hz), 3.38 (s, 6H, 2 $\times$ OCH₃); ^{13}C -NMR (CDCl_3) δ 165.02, 159.81, 141.99, 130.34, 128.39, 126.39, 115.28, 114.77, 103.14, 55.45, 54.54, 47.15, 40.17; HRMS (FAB) Calcd. for $\text{C}_{16}\text{H}_{22}\text{N}_4\text{O}_4$: 335.1713 (MH^+); obsd. m/z 335.1705; *Anal.* Calcd. for $\text{C}_{16}\text{H}_{22}\text{N}_4\text{O}_4 \cdot 0.25 \text{H}_2\text{O}$: C, 56.71, H, 6.69, N, 16.53; found, C, 56.71, H, 6.81, N, 16.41.

6.2.15. 5-amino-1-(4-Fluorobenzyl)-N-(2,2-dimethoxyethyl)-1H-imidazole-4-carboxamide (22b)—A mixture of **21b** (3 g, 8.5 mmol) and Pd-C (0.3 g) in absolute methanol (50 mL) was hydrogenated in a Parr hydrogenator at 40 psi for 5 h. The reaction mixture was filtered through Celite, and the filtrate was concentrated on a rotary evaporator to obtain a thick oily syrup. This was further triturated with hexane to obtain **22b** as a pure white compound. Yield: 1.6 g, 58%; mp: 58–60 °C; R_f = 0.41 (hexanes/ethyl acetate, 1:1); ^1H NMR (CDCl_3) δ 7.13–7.02 (m, 5H, Ar-H, Imid-H), 6.82 (t, 1H, -CONH), 4.92 (s, 2H, benzylic- CH_2), 4.62 (s, 2H, NH_2), 4.44–4.41 (t, 1H, -CH, $J_{1,2}$ & $J_{2,3}$ = 5.48 Hz), 3.52–3.49 (t, 2H, - CH_2 , $J_{1,2}$ & $J_{2,3}$ = 5.48 Hz), 3.39 (s, 6H, 2 $\times$ OCH₃); ^{13}C -NMR (CDCl_3) δ 164.94, 141.74, 130.40, 130.24, 128.73, 116.56, 116.34, 115.54, 103.14, 54.33, 46.81, 40.18; HRMS (FAB) Calcd. for $\text{C}_{16}\text{H}_{22}\text{N}_4\text{O}_4$: 323.1513 (MH^+); obsd. m/z 323.1515.

6.2.16. 3-(4-Methoxybenzyl)-5-methoxy-4,5,6,7-tetrahydro imidazo[4,5-e][1,4]diazepin-8(3H)-one (5)—1N HCl (2 mL) was added to compound **22a** (0.1 g, 0.3 mmol), and the mixture was stirred overnight. Solvent was removed on a rotary evaporator. To the residue was added methanol (2 mL) and about 0.1 mL of 1N HCl, and the mixture was stirred at room temperature for about 6 h. The reaction was complete based on the TLC analysis using 8:1:0.01 chloroform/methanol/ammonium hydroxide. Volatiles were removed when a thick oily liquid was obtained. This was further purified by column chromatography using 12:1:0.05 chloroform/methanol/ammonium hydroxide solvent system to obtain **5** as a

pure white solid. Yield: 0.05 g, 57%; mp: 155–158 °C; R_f =0.19 (chloroform/methanol/ammonium hydroxide, 10:1:0.05); ^1H NMR (DMSO- d_6) δ 7.41–7.40 (d, J =4 Hz, 1H, -NH), 7.30 (s, 1H, Imid-H), 7.18–7.15 (m, 2H, Ar-H), 7.01–6.99 (m, 1H, -CONH), 6.92–6.89 (m, 2H, Ar-H), 5.04–5.00 (d, J =16 Hz, 1H, benzylic-CH₂), 4.98–4.95 (d, J =12 Hz, 1H, benzylic-CH₂), 4.63–4.61 (t, 1H, -CH, $J_{1,2}$ = 5.48 Hz, $J_{2,3}$ = 5.04 Hz), 3.72 (s, 3H, -OCH₃) 3.70 (s, 1H, CH), 3.13 (s, 3H, -OCH₃), 2.99–2.94 (dd, 1H, -CH); ^{13}C -NMR (DMSO- d_6) δ 163.40, 159.25, 138.0, 131.0, 129.90, 129.16, 114.90, 114.51, 82.29, 55.65, 53.69, 40.39, 40.59; HRMS (FAB) Calcd. for C₁₅H₁₈N₄O₃: 303.1451 (MH⁺); obsd. m/z 303.1446.

6.2.17. 3-(4-Methoxybenzyl)-5-ethoxy-4,5,6,7-tetrahydroimidazo[4,5-e]

[1,4]diazepin-8(3H)-one (6)—1N HCl (2 mL) was added to **22a** (0.1 g, 0.3 mmol) and the mixture was stirred over-night. Solvent was removed on a rotary evaporator. To this was added ethanol (2 mL) and about 0.1 mL of 1N HCl and stirred at room temperature for about 6 h. The reaction was complete based on the TLC analysis using 10:1:0.01 chloroform/ethanol/ammonium hydroxide. Volatiles were removed to obtain a thick, oily liquid. This was further purified by column chromatography using 10:1:0.05 chloroform/ethanol/ammonium hydroxide solvent system to obtain **6** as a pure white solid. Yield: 0.034 g, 35.66%; ^1H NMR (DMSO- d_6) δ 7.34 (d, 1H, NH, J = 5.04 Hz), 7.29 (s, 1H, imidazole-H), 7.16–7.14 (m, 2H, Ar-H), 6.99–6.98 (m, 1H, -CONH), 6.91–6.87 (m, 2H, Ar-H), 5.03–4.93 (dd, 2H, benzylic-CH₂), 4.74–4.71 (t, 1H, -CH, $J_{1,2}$ = 5.04 Hz, $J_{2,3}$ = 5.04 Hz), 3.71 (m, 4H, -OCH₃ & CH), 3.45–3.36 (m, 2H, -OCH₂), 2.99–2.95 (dd, 1H, -CH), 1.01 (t, 3H, -CH₃, $J_{1,2}$ = 7.36 Hz, $J_{2,3}$ = 6.84 Hz); ^{13}C -NMR (DMSO- d_6) δ 166.22, 159.26, 138.89, 133.26, 129.20, 128.92, 114.49, 108.70, 80.30, 61.39, 55.64, 46.08, 43.75, 15.47; HRMS (FAB) Calcd. for C₁₆H₂₀N₄O₃: 317.1608 (MH₊); obsd. m/z 317.1608.

6.2.18. 3-(4-Fluorobenzyl)-5-methoxy-4,5,6,7-tetrahydroimidazo[4,5-e]

[1,4]diazepin-8(3H)-one (7)—To **22b** (0.1 g, 0.3 mmol) was added 1N HCl (2 mL), and the mixture was stirred overnight. Solvent was removed by rotary evaporation. To the residue was added methanol (2 mL) and about 0.1 mL of 1N HCl, and the mixture was stirred at room temperature for about 6 h. The reaction was complete based on the TLC analysis using 8:1:0.01 chloroform/methanol/ammonium hydroxide. Volatiles were removed and the resulting reaction mixture was further purified by silica gel column chromatography using 12:1:0.05 chloroform/methanol/ammonium hydroxide solvent system to obtain **7** as a pure white solid. Yield: 0.038 g, 42%; mp: 192–195 °C; R_f =0.24 (chloroform/methanol/ammonium hydroxide, 10:1:0.05); ^1H NMR (MeOH- d_4) δ 7.47 (s, 1H, Imid-H), 7.27–7.23 (m, 2H, Ar-H), 7.16–7.10 (m, 2H, Ar-H), 5.24–5.5.20 (d, J =16 Hz, 1H, benzylic-CH₂), 5.15–5.11 (d, J =9 Hz, 1H, benzylic-CH₂), 4.78 (d, J =5.52 Hz, 1H, -CONH), 3.68–3.62 (dd, 1H, -CH), 3.38 (s, 3H, -OCH₃), 3.22 (s, 2H, CH₂), 3.19–3.15 (d, J =4 Hz, 1H, -NH); ^{13}C -NMR (MeOH- d_4) δ = 167.54, 163.64, 161.33, 139.54, 133.68, 128.79, 128.70, 115.46, 81.90, 48.08, 46.09, 43.51; HRMS (FAB) Calcd. for C₁₅H₁₈N₄O₃: 291.1251 (MH⁺); obsd. m/z 291.1238.

6.2.19. 1-(4-Methoxybenzyl)-4-nitro-1H-imidazole-5-carboxylic Acid (25)

—Finely powdered **20a** (4 g, 11 mmol) was placed in a 250 mL round bottomed flask equipped with a magnetic stirring bar. Ice-cold water (36 mL) was added, followed by 2N sodium hydroxide (10 mL) and crushed ice (75 g). The temperature inside the flask was maintained between 0–5 °C. Finely powdered potassium permanganate (8 g) was added slowly over a period of 1 h and the reaction mixture was stirred vigorously for 36 h. The dark brown reaction mixture was filtered *in vacuo* and the solid sludge (manganese dioxide) was thoroughly washed with hot water. The light yellow filtrate was acidified with concentrated hydrochloric acid to pH 1, when a light yellow solid precipitated out. The solid was filtered, air dried for 24 h, and suspended in ether with stirring for few minutes to dissolve the by-product, benzoic acid. An

off-white solid which remained was filtered *in vacuo*, washed with ether and dried over phosphorous pentoxide for 24 h to obtain pure **25**. Yield: 1.8 g, 54%; mp: 153–156 °C; ¹H NMR (DMSO-*d*₆) δ 8.12 (s, 1H, Imid-H), 7.22–7.19 (m, 2H, Ar-H), 6.94–6.90 (m, 2H, Ar-H), 5.35 (s, 2H, benzylic-CH₂), 3.73 (s, 3H, -OCH₃); ¹³C-NMR (DMSO-*d*₆): δ 159.95, 159.70, 138.11, 129.78, 128.10, 114.72, 55.68, 50.01; HRMS (FAB) Calcd. for C₁₂H₁₁N₃O₅: 278.0771 (MH⁺); obsd. *m/z* 278.0775.

6.2.20. 1-(4-Methoxybenzyl)-N-(2,2-dimethoxyethyl)-4-nitro-1H-imidazole-5-carboxamide (26)—A mixture of **25** (2 g, 7.2 mmol) and 1,1'-carbonyldiimidazole (1.16 g, 7.2 mmol) in dry THF (10 mL) was refluxed in a flame-dried three-neck flask kept under nitrogen atmosphere. When the mixture turned clear it was allowed to cool to room temperature. Aminoacetaldehyde dimethylacetal (1.17 mL, 10.8 mmol) was added to the cooled solution which was allowed to stir for 2 h. The reaction was monitored for completion. The solvent was removed by rotary evaporation and to the residue was added ice-cold water (10 mL) and the mixture was stirred overnight. Then the product was extracted with chloroform and washed with water and dried over anhydrous magnesium sulfate. The organic layer was evaporated to obtain a thick oily syrup. This was further subjected to column chromatography using 1:1 hexanes/ethyl acetate as the eluting solvent system to obtain pure **26**. Yield: 2.3 g, 87%; mp: 125–127 °C; *R*_f 0.68 (hexanes/ethyl acetate, 1:1); ¹H NMR (CDCl₃) δ 7.75 (t, 1H, -CONH), 7.40 (s, 1H, Imid-H), 7.17–7.15 (m, 2H, Ar-H), 6.88–6.85 (m, 2H, Ar-H), 5.40 (s, 2H, benzylic-CH₂), 4.43–4.40 (t, 1H, -CH, *J*_{1,2} = 5.48 Hz, *J*_{2,3} = 5.04 Hz), 3.78 (s, 3H, -OCH₃), 3.56–3.53 (t, 2H, -CH₂, *J*_{1,2} = 5.48 Hz, *J*_{2,3} = 5.52 Hz), 3.39 (s, 6H, 2×OCH₃); ¹³C-NMR (CDCl₃): δ 160.10, 157.63, 140.50, 136.69, 129.89, 126.28, 123.76, 114.63, 102.18, 55.42, 54.49, 51.51, 41.46; HRMS (FAB) Calcd. for C₁₆H₂₀N₄O₆: 365.1455 (MH⁺); obsd. *m/z* 365.1444.

6.2.21. 4-Amino-1-(4-Methoxybenzyl)-N-(2,2'-dimethoxyethyl)-1H-imidazole-5-carboxamide (27)—A mixture of **26** (2 g, 5.48 mmol) and Pd-C (0.2 g) in absolute methanol (30 mL) was hydrogenated in a Parr hydrogenator at 40 psi for 5 h. The reaction mixture was filtered through Celite, and the solvent was removed on a rotary evaporator to yield a thick oily syrup. This was further triturated with hexane to obtain **27** as a white solid. Yield: 1.2 g, 65%; mp: 95–98 °C; *R*_f 0.13 (hexanes/ethylacetate, 1:2); ¹H NMR (CDCl₃) δ 7.16 (s, 1H, Imid-H), 7.13–7.09 (m, 2H, Ar-H), 6.85–6.81 (m, 2H, Ar-H), 6.77 (t, 1H, -CONH), 5.33 (s, 2H, benzylic-CH₂), 4.37–4.35 (t, 1H, -CH, *J*_{1,2} & *J*_{2,3} = 5.04 Hz), 3.95 (s, 2H, NH₂), 3.76 (s, 3H, -OCH₃), 3.46–3.44 (t, 2H, -CH₂, *J*_{1,2} = 5.92 Hz, *J*_{2,3} = 5.04 Hz), 3.33 (s, 6H, 2×OCH₃); ¹³C-NMR (CDCl₃): δ 161.49, 159.49, 149.20, 137.01, 128.94, 128.45, 114.33, 109.21, 102.75, 55.36, 54.35, 50.08, 40.36; HRMS (FAB) Calcd. for C₁₆H₂₂N₄O₄: 335.1713 (MH⁺); obsd. *m/z* 335.1704.

6.2.22. 5-Methoxy-1-(4-methoxybenzyl)-4,5,6,7-tetrahydroimidazo[4,5-e][1,4]diazepin-8(1H)-one (8)—To **27** (0.1 g, 0.3 mmol) was added 1N HCl (2 mL) and the mixture was stirred overnight. Solvent was removed by rotary evaporation. To the residue was added methanol (2 mL) and about 0.1 mL of 1N HCl, and the resulting mixture was stirred at room temperature for 5h. The reaction was complete based on the TLC analysis using 8:1:0.01 chloroform/methanol/ammonium hydroxide. Volatiles were removed and the resulting reaction mixture was further purified by column chromatography using 12:1:0.05 chloroform/methanol/ammonium hydroxide solvent system to obtain **8** as a pure white solid. Yield: 0.047 g, 52%; mp: 138–141 °C; *R*_f 0.38 (chloroform/methanol/ammonium hydroxide, 10:1:0.05); ¹H NMR (DMSO-*d*₆) δ 7.63 (s, 1H, Imid-H), 7.51–7.50 (d, *J* = 5.04 Hz, 1H, -NH), 7.19–7.13 (m, 3H, -CONH & Ar-H), 6.86–6.83 (m, 2H, Ar-H), 5.61–5.57 (d, *J* = 14.64 Hz, 1H, benzylic-CH₂), 5.14–5.10 (d, *J* = 14.64 Hz, 1H, benzylic-CH₂), 4.55–4.52 (t, 1H, -CH, *J*_{1,2} & *J*_{2,3} = 5.04 Hz), 3.70 (s, 3H, -OCH₃), 3.18 (s, 3H, -OCH₃),

2.86–2.81 (dd, 1H, -CH); ^{13}C -NMR (DMSO- d_6): δ 164.18, 159.05, 149.36, 140.49, 131.08, 129.33, 114.27, 103.67, 82.35, 55.56, 53.48, 48.65, 43.20; HRMS (FAB) Calcd. for $\text{C}_{15}\text{H}_{18}\text{N}_4\text{O}_3$: 303.1451 (MH^+); obsd. m/z 303.1448.

6.2.23. 5-Amino-*N*-(2,2-dimethoxyethyl)-1*H*-imidazole-4-carboxamide (31)—A mixture of **30** (0.5 g, 2 mmol) and Pd-C (0.05 g) in absolute methanol (10 mL) was hydrogenated in a Parr hydrogenator at 60 psi for 5 h. The reaction mixture was filtered through Celite, and the filtrate was concentrated on a rotary evaporator to obtain **31** as a thick oily syrup. Yield: 0.2 g, 50%, R_f 0.51 (chloroform/methanol, 10:1); ^1H NMR (CDCl_3) δ 7.02 (s, 1H, Imid-H), 6.98 (t, 1H, -CONH), 5.14 (s, 2H, NH_2), 4.39–4.36 (t, 1H, -CH, $J_{1,2} = 5.48$ & $J_{2,3} = 5.04$ Hz), 3.48–3.45 (t, 2H, -CH $_2$, $J_{1,2} = 5.96$ & $J_{2,3} = 5.48$ Hz), 3.32 (s, 6H, $2 \times \text{OCH}_3$); ^{13}C -NMR (CDCl_3): δ 165.14, 144.35, 129.17, 111.46, 103.2, 54.45, 40.48; HRMS (FAB) Calcd. for $\text{C}_8\text{H}_{15}\text{N}_4\text{O}_3$: 215.1138 (MH^+); obsd. m/z 215.1135.

6.2.24. 5-(Benzylamino)-*N*-(2,2-dimethoxyethyl)-1*H*-imidazole-5-carboxamide (32)—To **31** (0.1 g, 0.5 mmol) in methanol was added benzaldehyde (0.06 g, 0.6 mmol) and trifluoroacetic acid (0.07 g, 0.6 mmol) under a nitrogen atmosphere and the mixture was stirred at room temperature overnight. When the starting material was consumed as determined by TLC analysis using a 10:1 chloroform/methanol solvent system, sodium cyanoborohydride (0.04 g, 0.6 mmol) was added to the reaction mixture. The reaction was stirred for about 8 h and the reaction was completed based on the TLC analysis using 10:1 chloroform/methanol solvent system. Solvents were removed in vacuo and the product was isolated by silica gel column chromatography using 15:1 chloroform/methanol solvent system to obtain **32** as a white solid. Yield: 0.08 g, 43.3%; R_f 0.13 (chloroform/methanol, 10:1); ^1H NMR (CDCl_3): δ 7.37–7.33 (m, 5H, Ar-H), 6.95 (s, 1H, Imid-H), 6.79 (t, 1H, CONH), 4.45–4.42 (t, 1H, CH, $J_{1,2} = 5.04$ & $J_{2,3} = 5.48$ Hz), 4.39 (s, 2H, benzylic CH $_2$), 3.53–3.5 (t, 2H, CH $_2$, $J_{1,2} = 5.48$ Hz, $J_{2,3} = 5.96$ Hz), 3.38 (s, 6H, -OCH $_3$), 3.33 (s, 1H, NH); ^{13}C -NMR (CDCl_3): δ 163.7, 138.26, 130.1, 129.15, 128.94, 128.47, 127.96, 127.15, 103.19, 54.36, 48.7, 40.25; HRMS (FAB) Calcd. for $\text{C}_{15}\text{H}_{20}\text{N}_4\text{O}_3$: 305.1608 (MH^+); obsd. m/z 305.1599.

6.2.25. 4-Benzyl-5-methoxy-4,5,6,7-tetrahydroimidazo[4,5-*e*][1,4]diazepin-8(3*H*)-one (9)—To **32** (0.05 g, 0.16 mmol) was added 1N HCl (2 mL) and the mixture was stirred overnight. When the starting material was consumed as determined by TLC analysis using 10:1:0.01 chloroform/methanol/ammonium hydroxide as eluent, the solvent was removed in vacuo. Methanol (2 mL) and 0.1 mL of 1N HCl were added and the mixture was stirred for 6 h. Volatiles were removed and the resulting reaction mixture was further purified by column chromatography using 30:1:0.01 chloroform/methanol/ammonium hydroxide solvent system to obtain **9** as a pure white solid. Yield: 0.016 g, 37.5%; R_f 0.36 (chloroform/methanol/ammonium hydroxide, 10:1:0.01); ^1H NMR (DMSO- d_6) δ 12.12 (s, 1H, Imid-NH), 7.44 (s, 1H, Imid-H), 7.30–7.26 (m, 5H, Ar-H), 7.2 (m, 1H, -CONH), 5.14–5.10 (d, 1H, benzylic CH, $J_{1,2} = 15.6$ Hz), 4.71–4.69 (d, 1H, benzylic CH, $J_{1,2} = 5.96$ Hz), 4.56–4.52 (d, 1H, -CH, $J_{1,2} = 16.04$ Hz), 3.53–3.50 (m, 1H, -CH), 3.17 (s, 3H, -OCH $_3$), 2.98–2.95 (dd, 1H, -CH); ^{13}C -NMR (DMSO- d_6) δ 163.72, 147.16, 140.17, 136.03, 128.72, 127.99, 127.19, 105.89, 87.30, 54.13, 53.65, 42.57; HRMS (ESI) Calcd. for $\text{C}_{14}\text{H}_{16}\text{N}_4\text{O}_2$: 273.1346 (MH^+); obsd. m/z 273.1340.

6.2.26. 1-(4-Methoxybenzyl)-4-(benzylamino)-*N*-(2,2-dimethoxyethyl)-1*H*-imidazole-5-carboxamide (34)—To **27** (0.1 g, 0.3 mmol) in methanol was added benzaldehyde (0.038 g, 0.36 mmol) and trifluoroacetic acid (0.04 g, 0.36 mmol) under a nitrogen atmosphere and the mixture was stirred at room temperature overnight. When the starting material was consumed as determined by TLC analysis using 1:1 hexanes/ethyl

acetate solvent system, sodium cyanoborohydride (0.028 g, 0.45 mmol) was added to the reaction mixture. The reaction mixture was stirred for about 8 h when the reaction was completed based on the TLC analysis using 1:1 hexanes/ethyl acetate solvent system. Solvents were removed *in vacuo* and the product was isolated by column chromatography using 2:1 hexanes/ethyl acetate solvent system to obtain **34** as a white solid. Yield: 0.12 g, 93%; R_f 0.12 (hexanes/ethyl acetate, 2:1); ^1H NMR (CDCl_3) δ 7.39 (s, 1H, imid-H), 7.36–7.25 (m, 5H, Ar-H), 7.13–7.11 (m, 2H, Ar-H), 6.87–6.84 (m, 2H, Ar-H), 6.69 (t, 1H, CONH), 5.34 (s, 2H, benzylic CH_2), 4.44 (s, 2H, benzylic CH_2), 4.31–4.28 (t, 1H, CH, $J_{1,2}$ & $J_{2,3}$ = 5.04 Hz), 3.77 (s, 3H, $-\text{OCH}_3$), 3.46 (s, 1H, NH), 3.43–3.40 (t, 2H, CH_2 , $J_{1,2}$ = 5.48 Hz, $J_{2,3}$ = 5.52 Hz), 3.24 (s, 6H, $-\text{OCH}_3$); ^{13}C -NMR ($\text{DMSO}-d_6$) δ 160.98, 159.82, 150.76, 139.04, 136.22, 128.92, 128.65, 128.02, 127.45, 127.25, 114.60, 108.52, 102.67, 55.41, 54.39, 50.67, 49.62, 40.42; HRMS (FAB) Calcd. for $\text{C}_{23}\text{H}_{28}\text{N}_4\text{O}_4$: 425.2183 (MH^+); obsd. m/z 425.2187.

6.2.27. 1-(4-Methoxybenzyl)-4-benzyl-5-methoxy-4,5,6,7-tetrahydroimidazo[4,5-e][1,4]diazepin-8(1H)-one (10)—To **34** (0.12 g, 0.28 mmol) was added 1N HCl (2 mL) and the mixture was stirred overnight. When the starting material was consumed as determined by TLC analysis using 10:1:0.01 chloroform/methanol/ammonium hydroxide as the solvent system, the solvent was removed *in vacuo*. Methanol (2 mL) and 0.1 mL of 1N HCl were added and the mixture was stirred for 6 h. Volatiles were removed and the resulting reaction mixture was further purified by silica gel column chromatography using 50:1:0.05 chloroform/methanol/ ammonium hydroxide solvent system to obtain **10** as a pure white solid. Yield: 0.042 g, 38%; R_f 0.38 (chloroform/methanol/ammonium hydroxide, 10:1:0.01); ^1H NMR (CDCl_3) δ 7.28–7.24 (m, 8H, imid-H and Ar-H), 6.84–6.82 (m, 2H, Ar-H), 5.58–5.55 (m, 2H, $-\text{CONH}$ and $-\text{CH}$), 5.38–5.25 (dd, 2H, benzylic CH_2), 4.60–4.51 (dd, 2H, benzylic CH_2), 3.76 (s, 3H, $-\text{OCH}_3$), 3.46–3.39 (m, 1H, $-\text{CH}$), 3.27 (s, 3H, $-\text{OCH}_3$), 3.15–3.09 (dd, 1H, $-\text{CH}$); ^{13}C -NMR (CDCl_3) δ 164.62, 159.34, 150.07, 139.12, 138.63, 129.53, 129.01, 128.62, 127.76, 127.24, 114.15, 104.68, 87.31, 55.35, 54.82, 55.33, 50.32, 42.85; HRMS (ESI) Calcd. for $\text{C}_{22}\text{H}_{24}\text{N}_4\text{O}_3$: 393.1921 (MH^+); obsd. m/z 393.1920.

6.2.28. 1-(4-Methoxybenzyl)-4-benzyl-5-ethoxy-4,5,6,7-tetrahydroimidazo[4,5-e][1,4]diazepin-8(1H) (11)—To **34** (0.1 g, 0.23 mmol) was added 1N HCl (2 mL) and the mixture was stirred over-night. When the starting material was consumed as determined by TLC analysis using 10:1:0.01 chloroform/methanol/ammonium hydroxide solvent system, the solvent was removed *in vacuo* and to this residue was added ethanol (2 mL) and 0.1 mL of 1N HCl and the mixture was stirred for 6 h. The solvent was evaporated to dryness and the product was isolated by silica gel column chromatography using chloroform/ethanol/ ammonium hydroxide (25:1:0.1) solvent system to obtain **11** as an off-white solid. Yield: 0.03 g, 31%; ^1H NMR ($\text{DMSO}-d_6$) δ 7.70 (s, 1H, imid-H), 7.35 (m, 1H, $-\text{CONH}$), 7.28–7.17 (m, 7H, Ar-H), 6.87–6.85 (m, 2H, Ar-H), 5.16–5.15 (dd, 2H, benzylic CH_2), 5.02–4.67 (dd, 2H, benzylic CH_2), 4.58–4.54 (d, 1H, $-\text{CH}$, J = 15.56 Hz), 3.72 (s, 4H, $-\text{OCH}_3$, $-\text{CH}$), 3.48–3.36 (m, 2H, $-\text{OCH}_2$), 2.94–2.89 (dd, 1H, $-\text{CH}$), 1.05 (t, 3H, $-\text{CH}_3$); ^{13}C -NMR ($\text{DMSO}-d_6$) δ 164.24, 159.11, 149.43, 140.02, 139.63, 130.89, 129.43, 128.70, 127.98, 127.16, 114.33, 104.27, 86.72, 61.73, 55.56, 52.79, 48.92, 41.97, 15.78; HRMS (ESI) Calcd. for $\text{C}_{23}\text{H}_{26}\text{N}_4\text{O}_3$: 407.2077 (MH^+); obsd. m/z 407.2084.

6.3. Biochemical Studies

Target analogs of *iso*-azepinomycin were screened *in vitro* as inhibitors of guanase from rabbit liver in Tris buffer (pH 7.4) at 25° C by spectrophotometric measurements of the rate of hydrolysis of the substrate guanine at λ_{max} 245 nm. Stock solutions of the substrate, enzyme and inhibitors were prepared using 0.05 M Tris buffer (pH 7.4). The enzyme kinetics were followed by measuring the change in optical density (decrease in absorbance)

per minute of the substrate guanine. By keeping the concentration of the inhibitor constant, and varying the concentration of the substrate, a set of kinetic data was obtained. Additional sets of data were generated using different concentrations of the inhibitor.

The following are the principles and procedures employed for the biochemical assay: (1) The change in optical density at λ_{max} 245 nm per unit time is the measure of the guanine deaminase activity. The Michaelis constant (K_M) of the guanine was determined for each of the target compounds. (2) *Solutions*: (a) Tris-HCl buffer, pH= 7.4, 0.05 M (7.88 g of Tris-HCl was dissolved in 950 mL of deionized distilled water, the pH was adjusted to 7.4 using 1N NaOH, and then diluted to 1000 mL using deionized distilled water). (b) Guanine solution: 30 – 40 mg of guanine was dissolved in 1N NaOH solution and diluted to 100 mL using 0.05 M Tris-buffer solution. The solution was filtered to get clear solution and the concentration of the stock solution was calculated from its UV absorbance. (c) Substrate concentration in each assay was in the range of 5–40 μM . (d) Inhibitor solution were made either in DMSO or methanol. (e) The inhibitor concentration in each assay was 10 – 20 μM . (f) In our laboratory, guanase of activity 0.1278 U/mL was used for biochemical studies. A 60 μL volume of the enzyme solution used in each assay corresponds to 0.0077 units of the enzyme. Guanine deaminase from rabbit liver was purchased from M P Biochemicals as a suspension in 3.2 M $(\text{NH}_4)_2\text{SO}_4$, pH 6.0, activity = 10mg/mL, 0.06 U/mg and 0.6 U/mL. 0.1278 U/mL equals to 2.13 mg/mL in protein concentration. To calculate the volume of enzyme to be used from the stock enzyme solution, the following formula was employed: 10 mg/mL $\times$ (volume of stock enzyme solution) mL = 2.13 mg/mL $\times$ (volume of the enzyme solution to be made) mL. (3) *Spectrophotometric measurements*: Wavelength, 245 nm, final volume 1.0 mL, light path 1 cm, temperature 25°C, read against air. The enzymatic hydrolysis was ~2 mins long. The data collected from the linear part of the plot, ranging from 0.1 – 0.6 sec, corresponds to the initial velocity. The mean of the initial velocity (V) was calculated from two data sets and was used to generate values of $1/V$ in the Y-axis of Lineweaver-Burk plot. (4) *Procedure*: A mixture of calculated amount of guanine solution and calculated amount of inhibitor solution were mixed in a 1 mL cuvette and diluted with Tris buffer to make up to a volume of 940 μL . The Tris buffer was used as reference. In each case the concentration of the enzyme used was kept constant. After adding 60 μL of the enzyme solution to the cuvette, the solution was mixed thoroughly. The biochemical reaction was completed in about 2 mins. For lower concentrations of the substrate, the reaction was even faster. The reaction mixture was quickly and carefully mixed to make sure there were no bubbles. The reaction was followed at 25°C by measuring the decrease in absorbance at $\lambda = 245$ nm using Cary-50 UV spectrophotometer. The data were plotted as $1/V$ vs $1/[S]$ (standard Lineweaver-Burk plots) using Microsoft Word Excel 2007. Computed K_i values have been collected in Table 1 in the text. The details of enzyme kinetics on all target compounds have been provided in the Supplementary Data.

Supplementary Material

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References

1. Chang YJ, Huang CH, Hu CY, Liaw SH. Acta Crystallogr., Sect. D: Biol. Crystallogr. 2004; D60:1152. [PubMed: 15159585]
2. Yuan G, Bin JC, McKay DJ, Snyder FF. J Biol Chem. 1999; 274:8175. [PubMed: 10075721]

3. Canbolat O, Durak I, Cetin R, Kavutcu M, Demirci S, Ozturk S. Breast Cancer Res Treat. 1996; 37:189. [PubMed: 8750586]
4. Durak L, Beduk Y, Kavutcu M, Szer O, Yaman O, Ozturk HS, Canbolat O, Ulutepe S. Cancer Invest. 1997; 15:212. [PubMed: 9171854]
5. Kaneshige Y, Matsumoto H, Chiba S, Hashimoto S, Noro H. Rinsho Shinkeigaku (Clin. Neurol.). 1989; 29:854.
6. Kuzmits R, Seyfried H, Wolf A, Muller MM. Enzyme. 1980; 25:148. [PubMed: 6105073]
7. Paletzki RF. Neuroscience (Oxford, United Kingdom). 2002; 109:15.
8. Shiota G, Fukada J, Ito T, Tsukizawa M, Yamada M, Sato M. Jpn. J. Med. 1989; 28:22. [PubMed: 2542677]
9. Bhan A, Hosmane RS. Tetrahedron Lett. 1994; 35:6831.
10. Bhan A, Hosmane RS. Nucleosides Nucleotides. 1995; 14:455.
11. Chakraborty S, Shah NH, Fishbein JC, Hosmane RS. Bioorg. Med. Chem. Lett. 2011; 21:756. [PubMed: 21183343]
12. Chakraborty S, Shah NH, Fishbein JC, Hosmane RS. Bioorg. Med. Chem. Lett. 2012; 22:7214. [PubMed: 23084905]
13. Rajappan V, Hosmane RS. Bioorg. Med. Chem. Lett. 1998; 8:3649. [PubMed: 9934488]
14. Rajappan VP, Hosmane RS. Nucleosides Nucleotides. 1998; 17:1141. [PubMed: 9708314]
15. Rajappan VP, Hosmane RS. Nucleosides Nucleotides. 1999; 18:835. [PubMed: 10432688]
16. Wang L, Hosmane RS. Bioorg. Med. Chem. Lett. 2001; 11:2893. [PubMed: 11677121]
17. Dietrich LS, Shapiro DM. J Biol Chem. 1953; 203:89. [PubMed: 13069490]
18. Kanzawa F, Hoshi A, Kureitani K. Chem. Pharm. Bull. 1971; 19:1737. [PubMed: 5122677]
19. Kanzawa F, Hoshi A, Nishimoto T, Kureitani K. Chem. Pharm. Bull. 1970; 18:392. [PubMed: 5442773]
20. Kumar S, Josan V, Sanger KCS, Tewari KK, Krishnan PS. Biochem. J. 1967; 102:691. [PubMed: 16742482]
21. Lucacchini A, Bazzichi L, Biagi G, Livi O, Segnini D. Ital. J. Biochem. 1982; 31:153. [PubMed: 6897062]
22. Lucacchini A, Montali U, Segnini D. Ital. J. Biochem. 1977; 26:27. [PubMed: 266488]
23. Lucacchini A, Segnini D, Da Settimo A, Livi O. Ital. J. Biochem. 1979; 28:194. [PubMed: 583599]
24. Meyer RB Jr, Skibo EB. J. Med. Chem. 1979; 22:944. [PubMed: 490539]
25. Saxena AK, Ahmad S, Shanker K, Bhargava KP, Kishor K. Pharmazie. 1980; 35:16. [PubMed: 7384171]
26. Saxena AK, Ahmad S, Shanker K, Kishor K. Pharmacol. Res. Commun. 1984; 16:243. [PubMed: 6718460]
27. Silipo C, Hansch C. J. Med. Chem. 1976; 19:62. [PubMed: 1246055]
28. Lewis AS, Glantz MD. J Biol Chem. 1974; 249:3862. [PubMed: 4857982]
29. Gupta NK, Glantz MD. Arch. Biochem. Biophys. 1985; 236:266. [PubMed: 3966794]
30. Mansoor M, Kalyankar GD, Talwar GP. Biochim. Biophys. Acta. 1963; 77:307. [PubMed: 14090447]
31. Somoza JR, Skillman AG Jr, Munagala NR, Oshiro CM, Knegt RMA, Mpoke S, Fletterick RJ, Kuntz ID, Wang CC. Biochemistry. 1998; 37:5344. [PubMed: 9548915]
32. Ito S, Tsuji Y, Kitagawa N, Akihiko I, Syundo J, Tamura Y, Kishi S, Mori H. Hepatology (Baltimore, Md.). 1988; 8:383.
33. Cray GS, Yasmineh WG, Snover DC, Vine W. Transplant. Proc. 1989; 21:2315. [PubMed: 2652749]
34. Greengard O, Head JF, Goldberg SL. Cancer Res. 1980; 40:2295. [PubMed: 6248202]
35. Isshiki K, Takahashi Y, Iinuma H, Naganawa H, Umezawa Y, Takeuchi T, Umezawa H, Nishimura S, Okada N, Tatsuta K. J. Antibiot. 1987; 40:1461. [PubMed: 3680014]
36. Bhan A, Hosmane RS, Zhang H, Hosmane NS. Synth. Commun. 1995; 25:2723.
37. Hosmane RS, Bhan A. J. Heterocycl. Chem. 1990; 27:2189.

38. Vaidya VP, Hosmane RS, Siriwardane U, Zhang H, Hosmane NS. Struct. Chem. 1993; 4:339.
39. Bhan A, Hosmane RS. Nucleosides Nucleotides. 1992; 11:1175.

Figure 1. Comparison of the intermediate of the guanase-catalyzed hydrolysis reaction with the purported and proposed transition state analog inhibitors azepinomyacin and iso-azepinomyacin

Figure 2.
The target analogs of *iso*-azepinomyacin

Figure 3.
Resonance effect in the two regioisomeric products **19** and **20**

Figure 4.
Lineweaver-Burk plot for guanase inhibition study of target compound 2.

Scheme 1.
Synthesis of the target analogs **1–4**

Scheme 2.
Synthesis of the Key Intermediates **16a** and **16b**

Scheme 3.
Synthesis of the target analogs **5**, **6** and **7**

Scheme 4.
Synthesis of target analog **8**

Scheme 5.
Synthesis of target **9**

Scheme 6.
Synthesis of targets **10** and **11**

Table 1

Biochemical inhibition (K_i) data of target compounds **2–11** against rabbit liver guanase. For comparison, the inhibition data of azepinomycin, reported by us recently,¹² has been included.

General Chemical Structure	Target Compounds	K_i (μ M)
1 ; R=H 2 ; R=CH ₃ 3 ; R=CH ₂ CH ₃ 4 ; R=CH ₂ CH ₂ CH ₂ CH ₃	-- 40±1 47±2 54±3
5 ; R=CH ₃ ; R'=OCH ₃ 6 ; R=CH ₂ CH ₃ ; R'=OCH ₃ 7 ; R=CH ₃ ; R'=F	21±2 33.4±2.5 39.7±2
8	47.5±1
9	8.8±0.8
10 ; R=CH ₃ 11 ; R=CH ₂ CH ₃	26.5±1.5 43.8±0.6
Azepinomycin	2.5±0.6