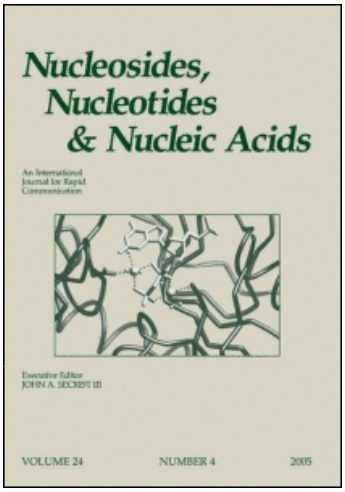

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ANALOGUES OF AZEPINOMYCIN AS INHIBITORS OF GUANASE

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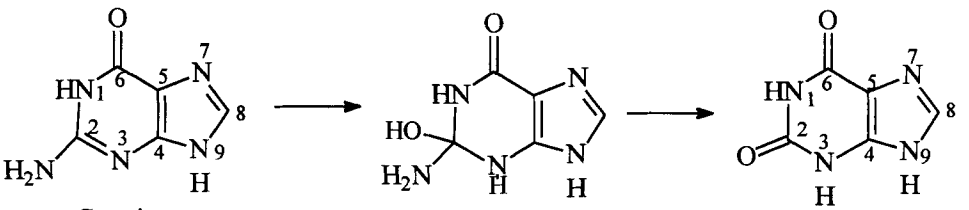
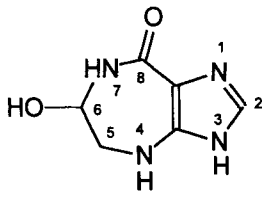
Abstract: Synthesis and biochemical screening against guanase of analogues of the naturally occurring guanase inhibitor azepinomycin (**2**) are reported. Compound 6-amino-5,6,7,8,-tetrahydro-4*H*-imidazo[4,5-*e*][1,4]diazepine-5,8-dione (**3**) was synthesized in six steps commencing with 1-benzyl-5-nitroimidazole-4-carboxylic acid (**5**). Compound **3** and its synthetic precursor 3-benzyl-6-(*N*-benzyloxycarbonyl)amino-5,6,7,8-tetrahydro-4*H*-imidazo[4,5-*e*][1,4]diazepine-5,8-dione (**12**) were screened against rabbit liver guanase. Both were found to be moderate inhibitors of the enzyme with K_i's in the range of 10^{-4} *M*.

Guanase (guanine deaminase or guanine aminohydrolase, EC 3.5.4.3) is an important enzyme in the purine salvage pathway, and catalyzes the hydrolysis of guanine to xanthine via the tetrahedral intermediate (**1**).[1] Since xanthine is an important biosynthetic precursor to other purine-based nucleotides, the inhibition of guanase has beneficial implications in cancer chemotherapy. There have been recent reports of significantly increased guanase activity in lung and gastric cancer tissues.[2] Abnormally high levels of serum guanase activity have also been reported



in patients with liver diseases like hepatitis.[3] Such a high activity is believed to be a biochemical indicator of rejection in liver transplant recipients.[4] There are also documented cases in which patients developed non-A/non-B hepatitis upon transfusion with blood containing high levels of serum guanase activity, with a linear correlation between the number of incidences of posttransfusional hepatitis and the extent of guanase activity in the donor blood.[5] It has been further shown that patients with multiple sclerosis (MS) exhibit significantly elevated levels of guanase activity in their cerebral spinal fluids (CSF), and that a close correlation exists between the extent of disability and the level of CSF guanase activity.[6] In light of these observations, a potent guanase inhibitor would be useful (a) in exploring biochemical mechanisms of the above metabolic disorders, (b) in understanding the specific physiological role played by guanase, and (c) as a potential chemotherapeutic agent that could selectively inhibit the growth of rapidly proliferating cancerous cells via depletion of the purine nucleotide pool. However, despite its obvious metabolic significance, guanase is one of the least-understood enzymes of the purine metabolism, and there have been only a few and scattered reports, if any, on its inhibition studies. We report herein the synthesis and biochemical screening of 6-amino-5,6,7,8,-tetrahydro-4*H*-imidazo[4,5-*e*][1,4]-diazepine-5,8-dione (**3**) and its synthetic precursor 3-benzyl-6-(*N*-benzyloxy carbonyl)amino-5,6,7,8-tetrahydro-4*H*-imidazo[4,5-*e*][1,4]diazepine-5,8-dione (**12**) as analogues of azepinomycin (**2**), a naturally occurring antitumor antibiotic which is also known to be a moderate guanase inhibitor (IC_{50} = ~ 10^{-5} M).[7]



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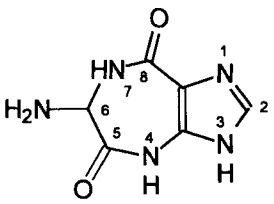

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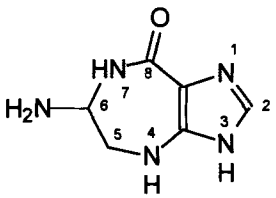


4

MATERIALS AND METHODS

(a) Organic Synthesis: ^{1}H NMR spectra were recorded on a General Electric QE-300 (300 MHz) instrument. The spectral data are reported in the

following format: chemical shift (all relative to Me_4Si as an internal reference standard unless otherwise indicated), multiplicity (s = singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quartet, m = multiplet, b = broad), integration, coupling constants, exchangeability after D_2O addition, and assignment of resonances. Elemental Microanalyses were performed by Atlantic Microlab, Inc., Norcross, Georgia. Ultraviolet spectra were recorded on a Gilford response UV/VIS spectrophotometer. The mass spectra were recorded at the Mass Spectral Facility, Department of Biochemistry, Michigan State University. Thin layer chromatography was performed on Merck Kieselgel 60 GF_{254} plates (0.2 mm thickness). Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Anhydrous THF was freshly distilled from sodium. Anhydrous solvents DMF, and ether were purchased from Aldrich Chemical Co.

*Pentafluorophenyl 1-Benzyl-5-nitroimidazole-4-carboxylate (**7**).* To a stirred ice cooled solution of **5**[8] (600 mg, 2.4 mmol) and pentafluorophenol (**6**) (442 mg, 2.4 mmol) in a mixture of ethyl acetate (10 mL) and DMF (6 mL), dicyclohexylcarbodiimide (495 mg, 2.4 mmol) was added with stirring, and the stirring was continued for 3 hours at 0 °C, followed by 1 hour at room temperature. The Dicyclohexylurea that was formed was filtered off and the solvent was evaporated *in vacuo*. The residue was triturated with *n*-hexane and the off-white solid that separated was filtered to obtain 950 mg (95%) of **7**, mp 115 - 117 °C; ^1H NMR (DMSO-d_6) δ 8.2 (s, 1H, -CH), 7.3 (m, 5H, Ar-H), 5.2 (s, 2H, -CH_2 of benzyl); ^{13}C NMR (DMSO-d_6) δ 140.77, 134.50, 129.11, 127.56, 127.34, 50.90, 50.43; MS (EI/70 eV) *m/z* 414 (MH^+); *Anal.* Calcd for $C_{17}H_8N_3O_4F_5$: C, 49.41; H, 1.95; N, 10.17. Found: C, 49.94; H, 2.20; N, 10.28.

*2-[N-(Benzyloxycarbonyl)amino]-2-[N-(1-benzyl-5-nitroimidazolyl-4-carbonyl)amino]acetic Acid (**9**).* A mixture of compound **8**[9] (224 mg, 1 mmol), **7** (413 mg, 1 mmol), and triethylamine (0.2 mL, 1.4 mmol) was stirred in DMF (5 mL) for 2 hours. After addition of 1N HCl (5 mL), the solvent was rotary evaporated under reduced pressure. The residue was first washed with ether to remove the pentafluorophenol that was formed, and then with water to wash off the triethylamine hydrochloride. The solid remained was filtered and vacuum dried over P_2O_5 to get 300 mg, (85%) of **9**. The product was recrystallized from

DMF/water, mp. 92-95 °C; ^{1}H NMR (DMSO-d_6) δ 8.94 (d, J_{NH-CH} = 8.1 Hz, 1H, -NHCO), 8.25 (s, 1H, -CH), 8.09 (d, J_{NH-CH} = 7.8 Hz, 1H, -NHCO), 7.3-7.1 (m, 10H, Ar-H), 5.58 (dd, 1H, -CH, singlet upon D_2O exchange), 5.51 (s, 2H, -NCH_2), 5.04 (s, 2H, -OCH_2); MS (FAB) *m/z* 454 (MH^+).

*Methyl 2-[N-(Benzyloxycarbonyl)amino]-2-[N-(1-benzyl-5-nitroimidazolyl-4-carbonyl) amino] acetate (**10**).* A mixture of compound **9** (800 mg,1.76 mmol) and potassium carbonate (365 mg, 2.46 mmol) in 4 mL of DMF was stirred in a 25 mL round-bottom flask. MeI (0.31 mL, 5 mmol) was added to the stirring solution and continued stirring for 4 hours. The reaction mixture was diluted with water (25 mL) and extracted with chloroform (3 x 15 mL). The chloroform layer was dried over $MgSO_4$, filtered, the filtrate evaporated, and the residue poured over ice. The white solid separated was filtered and dried under vacuum to get 700 mg (85%) of **10**, mp 75-76 °C; ^{1}H NMR (DMSO-d_6) δ 8.94 (d, J_{NH-CH} = 8.1 Hz, 1H, -NHCO), 8.25 (s, 1H, -CH), 8.09 (d, J_{NH-CH} = 7.8 Hz, 1H, -NHCO), 7.3-7.1 (m, 10H, Ar-H), 5.58 (dd, 1H, -CH, singlet upon D_2O exchange), 5.51 (s, 2H, -NCH_2), 5.04 (s, 2H, -OCH_2), 3.69 (s, 3H, -OMe); MS (FAB) *m/z* 468 (MH^+); *Anal.* Calcd for $C_{22}H_{21}N_5O_7$: C, 56.53; H, 4.53; N, 14.98. Found: C, 56.63; H, 4.55; N, 15.02.

*Methyl 2-[N-(Benzyloxycarbonyl)amino]-2-[N-(5-amino-1-benzylimidazolyl-4-carbonyl) amino]acetate (**11**).* Compound **10** (200 mg, 0.43 mmol) was taken in a mixture of 15 mL EtOH and 1.5 mL AcOH in a 50-mL round-bottom flask. Zinc powder (800 mg) in 3 mL AcOH was added to the flask and the mixture was heated at 60-70 °C for 30 minutes. The TLC analysis (10:1 $CHCl_3$:MeOH) indicated completion of the reaction showing a single spot slightly slower moving than the starting material. The mixture was filtered through a pad of Celite™ and the solution was repeatedly co-evaporated under reduced pressure with ethanol to remove acetic acid. The residue was loaded on a silica gel column and the column was eluted with a mixture of 200:1 $CHCl_3$:MeOH. Pooling and evaporation of the appropriate eluent fractions under reduced pressure afforded 125 mg (65%) of **11**, mp. 176-178 °C; ^{1}H NMR (DMSO-d_6) δ 8.12 (d, 1H, NH, exchangeable with D_2O), 7.83 (d, 1H, NH,exchangeable with D_2O), 7.3-7.1 (m, 11H, imidazole & Ar-H's), 5.97 (s, 2H, NH_2, exchangeable with D_2O), 5.79 (d-d, 1H, -CH), 5.09 (s, 2H, OCH_2),

5.04 (s, 2H, NCH_2), 3.6 (s, 3H, OCH_3); MS (FAB) *m/z* 438 (MH^+); *Anal.* Calcd for $C_{22}H_{23}N_5O_5$: C,60.41; H, 5.30; N, 16.01. Found: C, 60.48; H, 5.33; N, 16.10.

*3-Benzyl-6-(N-benzyloxycarbonyl)amino-5,6,7,8-tetrahydro-4H-imidazo[4,5-e][1,4]diazepine-5,8-dione (**12**).* Dry DMF (5 mL) was placed in a flame-dried round-bottom flask kept under N_2 atmosphere. Potassium *tert*-butoxide (40.39 mg, 0.36 mmol) was added to the flask and the mixture was stirred for 1 minute. Compound **11** (100 mg, 0.24 mmol) was added and the stirring was continued. After 30 minutes the TLC analysis (4:1 $CHCl_3$:MeOH) showed two spots, one on the base line and the other slightly above it, indicating completion of the reaction. The reaction mixture was neutralized with 0.1N HCl at 0 °C. The filtrate was rotary evaporated and the residue was loaded on a silica gel column. The column was eluted using 20:1 $CHCl_3$:MeOH. Appropriate UV-absorbing fractions were pooled and evaporated, and the residue was triturated with diethyl ether, which gave pale yellow, shiny powder. Yield 50 mg (51%), mp. 245-246 °C; ^{1}H NMR (DMSO-d_6) δ 11.23 (br, s, 1H, -CONH), 7.97 (d, *J* = 1.5 Hz, 1H, -NHCO), 7.73 (d, *J* = 2.7 Hz, 1H, -NHCO), 7.66 (s, 1H, imidazole-H), 7.41-7.19 (m, 10H, Ar-H), 5.26 (s, 2H, OCH_2), 5.1 (d-d, *J* = 1.5 and 2.7, 1H, -CH), 5.05 (s, 2H, -NCH_2); UV (MeOH) λ_{max} 256.5 nm, MS (FAB) *m/z* 406 (MH^+); *Anal.* Cacld for $C_{21}H_{19}N_5O_4$: C, 61.48; H, 4.64; N, 17.08. Found: C, 61.45; H, 4.83; N, 17.01.

*6-Amino-5,6,7,8,-tetrahydro-4H-imidazo[4,5-e][1,4]diazepine-5,8-dione (**3**).* Compound **12** (200 mg) was dissolved in 50 mL of glacial acid, and $Pd(OH)_2$-C(20%) (200 mg) was added to this solution under nitrogen. The mixture was hydrogenated at 45 psi in a Parr hydrogenator for 18 hours. The reaction mixture was filtered through a pad of Celite™ and the filtrate was rotary evaporated to get a gummy residue. The latter upon trituration with ether afforded a grey powder which was filtered and dried to obtain 40 mg (45%) of **3**. The grey solid obtained was washed with distilled methanol, followed by distilled water to obtain pure **3** (*Note*: the compound could not be recrystallized as it decomposes upon heating), mp 242-245 °C; ^{1}NMR (DMSO-d_6) δ 12.89 (br, s, 1H, NH, ex./w D_2O), 10.81 (s, 1H, NH, ex./w D_2O), 7.70 (s, 1H, CH), 7.46 (d, *J*=3.3Hz, 1H, NH, ex./w D_2O), 4.51 (m, 1H, CH, s in D_2O), 3.4-3.29 (br, 2H, NH_2, ex./w D_2O), 3.313 (s, 3H, OCH_3 of

adventitious MeOH); ^{13}C NMR (DMSO-d_6) δ 169.59 (CO), 159.68 (CO), 140.71 (C-3a), 137.07 (C-2), 112.31 (C-8a), 61.91 (C-NH_2); HRMS (FAB) Calcd for $C_6H_7N_5O_2$, *m/z* 182.0679, observed *m/z* 182.0688; *Anal.* Calcd. for $C_6H_7N_5O_2 \cdot CH_3OH \cdot ½ H_2O$: C, 37.81; H, 5.40; N, 31.50. Found: C, 37.51; H, 4.34; N, 31.81. The HPLC chromatogram of **3** [C_{18} column, eluting solvent: a mixture of MeOH (90%)-H_2O (9.75%)-AcOH (0.25%)] exhibited a single, sharp peak with a retention time of 3.21 min.

(b) Biochemical Screening against Guanase (guanine deaminase): Guanase from rabbit liver (Sigma) was employed in the described biochemical studies. All studies were carried out at 25 °C by spectroscopic measurements of the rate of hydrolysis of the substrate guanine at λ_{max} 245 nm. The following are the principle and procedure employed for the assay: (1) *Principle:* Guanine + H_2O ----> Xanthine + NH_3. The change in optical density at λ_{max} 245 nm per unit time is a measure of the guanase activity. The Michaelis constant (K_M) of guanase is 1.10-1.20x10^{-5} *M*. (2) *Solutions:* (a) Tris 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 1*N* NaOH, and then diluted to 1000 mL using deionized, distilled water). (b) Guanine solution (15 mg of guanine was dissolved in 1 mL of 1N NaOH and diluted to 100 mL using deionized distilled water. Above solution was filtered after some time to get a clear solution and the concentration of this stock solution was calculated from its UV absorbance). (c) Substrate concentration in each assay was in the range of 4-28 μM (taking calculated volume of above guanine solution and then using the buffer solution to dilute to 1 mL). (d) Inhibitor solution, 3.32 x 10^{-2} *M* (*e.g.* 6 mg of **3** dissolved in 1 mL of DMSO). (e) Inhibitor concentration in each assay was 30 or 40 μM (taking calculated amount of above inhibitor solution and diluting it to the required concentration using the buffer solution). (f) Enzyme solution, 0.1278 units/mL (133.1 μL of rabbit liver guanine deaminase suspension (Sigma) with a specific activity of 0.96 units/mL was diluted to 1mL using cold buffer solution. 60 μL of this solution was used in each assay, which equals 0.0077 unit). Guanine deaminase from rabbit liver was purchased from SIGMA as a suspension in 3.2 *M* $(NH_4)_2SO_4$, pH 6.0, acitvity = 12 mg/mL, 0.08 units/mg. (3)

Spectrophotometric measurements: Wavelength, 245 nm; final volume, 1.0 mL; light path, 1 cm; temp. 25 °C; read against air. (4) *Procedure*: A mixture of calculated amount of guanine solution (6 different substrate concentrations in the range of 4-28 μM) and calculated amount of inhibitor solution (the inhibitor concentration was constant for each series) 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 concentraton of enzyme used was kept constant. After adding 60 μL of enzyme solution to the above cuvette, the solution was quickly mixed by stirring with a plastic rod. The reaction was followed at 25 °C by measuring the decrease in absorbance at 245 nm using a Guilford UV spectrophotometer. The data were plotted using a data analysis and graphics program, Grafit™, version 3.0.

RESULTS AND DISCUSSION

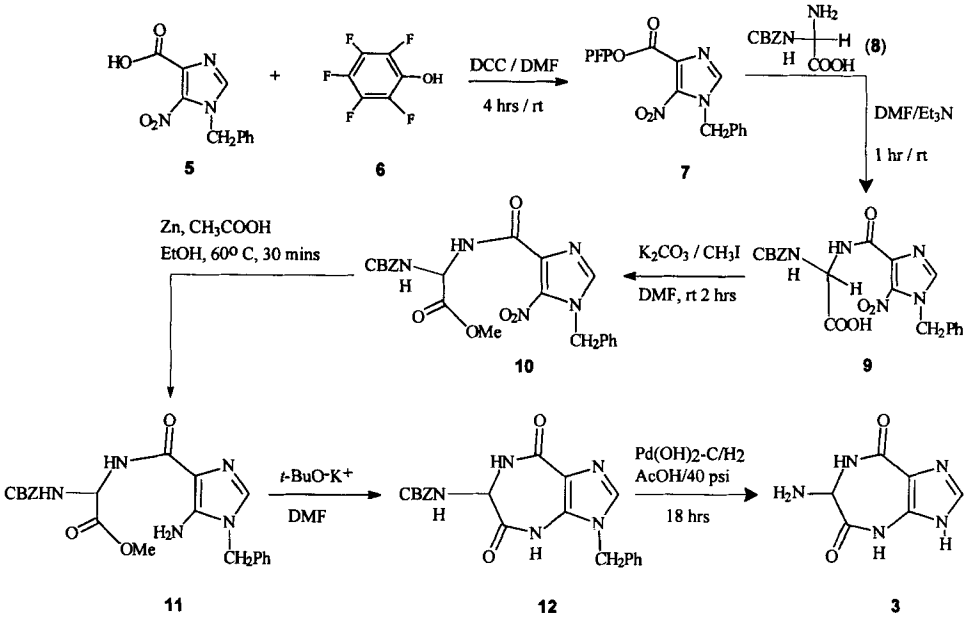
The modest inhibition of guanase by azepinomycin calls for the latter's structural modifications to determine its structure-activity relationship so as to enhance its therapeutic efficacy. This is especially necessary in view of the lack of crystal structure of guanase to-date. One of the logical structural modifications is to introduce an amino group in place of the hydroxy at position 6 of azepinomycin, which would give compound **4**. In view of the fact that the hydrolytic intermediate **1**, and hence the transition state leading to it, contain both the amino and hydroxy groups at the tetrahedral junction where the concerned hydrolysis takes place, substitution of NH_2 for OH in azepinomycin, followed by investigation of biochemical consequence on enzyme inhibition would be of interest. Ideally, an analogue of azepinomycin containing both the NH_2 and OH groups at position 6 is desirable since such a compound would best mimic the intermediate **1**. However, owing to the anticipated instability, its synthesis would be difficult as it most likely will yield the corresponding oxo or imino product resulting from elimination of ammonia or water. So, this leaves one to choose between an amino or a hydroxy group at position 6. How does one know which of the two is more important in closely mimicking the transition state structure? Since the two functional groups are isosteric to each other, the choice is less obvious. However, since the amino group can potentially

form two hydrogen bonds with the protein as contrasted with a single H-bond of the hydroxy functionality, there could be considerable differences in the energetics and stability of the protein-ligand complexes formed from the two groups. This, of course, would be based on an assumption that the OH or the NH_2 group at position 6 of **2** or **4** would be involved in interaction with the enzyme. In view of the fact that the analogous hydroxy group at position 8 of coformycin, a naturally occurring inhibitor of adenosine deaminase (ADA)[10]--another enzyme of purine metabolism that is functionally similar to guanase--is known to be involved in coordination with a zinc ion at the active site of ADA,[11] the above assumption is not unreasonable.

Another consideration for increasing the azepinomycin potency concerns carbon-5. What, if any, role does this carbon play in the overall enzyme inhibition? This is a conspicuous extra carbon as compared to the 5:6-fused structure of **1**. Should this carbon be in a fully saturated form as in **2** or **4**, or would an oxo group at the same position as in **3** increase or decrease the inhibition potency? Substitution of a C=O group in place of a CH_2 can potentially lead to any of the all three possible effects--positive, negative, or neutral--on enzyme binding, depending upon the relative location of the functional group in the enzyme active site. As a first step toward search of answers to these various questions, we report here the synthesis and guanase inhibition studies of **3**.

Compound **3** was synthesized (**Scheme I**) in six steps, commencing with 1-benzyl-5-nitroimidazole-4-carboxylic acid.[8] Compound **5** was condensed with pentafluorophenol (**6**) to produce the reactive anhydride (**7**), which upon reaction with the protected 2-aminoglycine (**8**),[9] gave **9**. The latter was converted into the ester **10** by base-catalyzed methylation with methyl iodide. Reduction of **10** with zinc and acetic acid (to produce **11**), followed by ring-closure with potassium *t*-butoxide in dimethylformamide (to produce **12**), and debenzylation with palladium hydroxide-charcoal/H_2 in acetic acid afforded **3**.

Compound **3**, along with its synthetic precursor **12**, were biochemically screened against rabbit liver guanase at 25 °C and pH 7.4 by spectrophotometrically monitoring the rate of hydrolysis of the substrate guanine at λ_{max} 245 nm. A total of six different concentrations of the substrate, ranging 4-28 μM, was employed for each inhibitor concentration that was either 30 or 40 μM,



Scheme 1

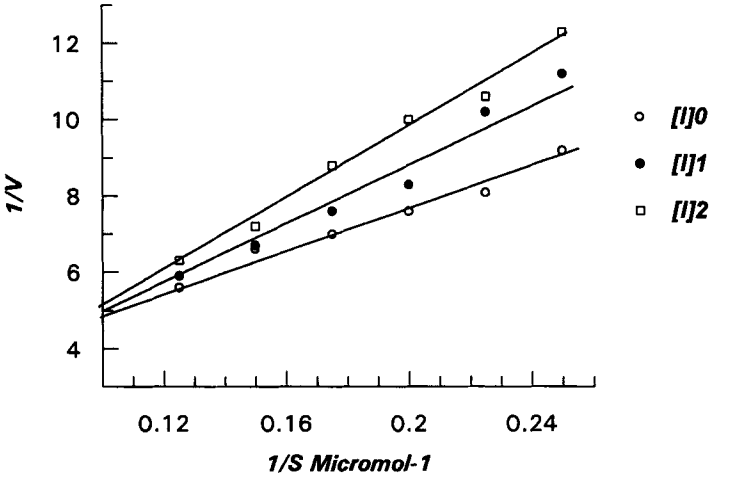


Figure 1: Lineweaver-Burk plots showing inhibition of guanase by Compound **3**.

while the amount of enzyme in each assay was 7.67×10^{-3} unit. The plots of [V] versus [S] were generated, which indicated that both **3** and **12** are moderate inhibitors of guanase, with K_i's equal to 2.27×10^{-4} *M* and 3.6×10^{-4} *M*, respectively. Compound **3** was also further analyzed for the mode of inhibition, employing Lineweaver-Burk plots (1/*V* *vs* 1/S) (see **Figure 1**), which clearly pointed to competitive inhibition.

CONCLUSION

In an effort to explore the structure-activity relationship of azepinomycin **2**, compound **3** has been successfully synthesized. Biochemical screening of **3**, along with its synthetic precursor **12**, against rabbit liver guanase showed only moderate inhibition by both compounds. It is not yet clear if the observed low K_i of **3** is due to replacement of the 6-OH group of azepinomycin **2** with an NH_2 group or due to introduction of a C=O functionality in place of the 5-CH_2. Compound **4** which lacks this C=O group at position 5 is anticipated to throw more light on this aspect, and such an endeavor is currently in progress.

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