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Glycinamide Ribonucleotide Analogue Probes for Glycinamide Ribonucleotide Transformylase[1]

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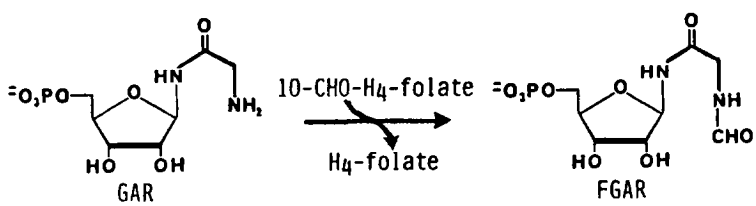
Glycinamide ribonucleotide (GAR) transformylase catalyzes the conversion of glycinamide ribonucleotide and 10-formyltetrahydrofolate to formylglycinamide ribonucleotide and tetrahydrofolate. This reaction constitutes the third step in *de novo* purine biosynthesis. A series of glycinamide ribonucleotide analogues, in which the glycinamide side chain (R = CH_2NH_2) has been replaced by R = CH_2Br, CH_2Cl, CH_2CN, CHN_2, $CHClCH_2NH_2$, and  , has been prepared. All of these analogues were inhibitors of GAR transformylase, competitive against GAR, but none of these proved to be enzyme inactivators. Neither R = $CHClCH_2NH_2$ nor R =  served as substrates for the enzyme-catalyzed transformylation reaction.

Glycinamide ribonucleotide (GAR)[3] transformylase catalyzes the third step in *de novo* purine biosynthesis, the conversion of glycinamide ribonucleotide (GAR) and 10-formyltetrahydrofolate to formylglycinamide ribonucleotide (FGAR) and tetrahydrofolate (Eq. [1]).



[1]

This activity was first isolated by Warren and Buchanan (*1*), who identified the β-anomer of GAR as the nucleotide substrate. Further studies on the nucleotide

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[3] Abbreviations used: GAR, glycinamide ribonucleotide; FGAR, formylglycinamide ribonucleotide; H_4-folate, tetrahydrofolate; C-GAR, carbocyclic glycinamide ribonucleotide; GAR-OH, α,β-hydroxyacetamide ribonucleotide; FGAM, formylglycinamidine ribonucleotide; BSA, bovine serum albumin; TFA, trifluoroacetic acid; DCC, dicyclohexylcarbodiimide; DBU, 1,8-bicyclo[5.4.0]undec-7-ene; TMSBr, bromotrimethylsilane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NCS, *N*-chlorosuccinimide; Bz, benzoyl; Bzl, benzyl; TLC, thin-layer chromatography.

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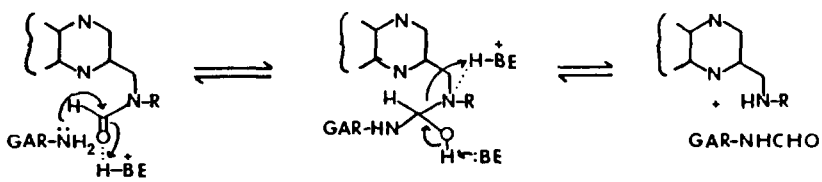
substrate specificity of GAR transformylase have been limited to our determination that the carbocyclic analogue of GAR (C-GAR) is an alternate substrate for the enzyme (*2*) and that hydroxyacetamide ribonucleotide (GAR-OH) is an inhibitor of the enzyme, competitive against GAR (*3*).

Initial velocity and dead-end inhibition studies (*3*) indicated that the kinetic mechanism of GAR transformylase is ordered-sequential, with the folate substrate binding first. Furthermore, the pH optimum of the reaction, between pH 7.9 and 8.3 (*3*), suggests that the neutral amine is the active substrate species, even though the pK_a of the amino group of GAR is 8.15 (*4*). Based on these results, and the well-defined chemistry of amide aminolysis (*5*), we have proposed a chemical mechanism for the transformylation reaction which invokes the participation of enzyme residues, as general acid–base catalysts, in the formation and subsequent decomposition of the proposed tetrahedral intermediate (Eq. [2]).



[2]

In order to test the proposed mechanism, to further define the nucleotide substrate specificity of GAR transformylase, and to provide potential affinity labels for the enzyme, we have prepared a series of GAR analogues in which the glycine aminomethylene side chain has been replaced by electrophilic, or latently electrophilic, moieties. The syntheses of these analogues, patterned after the syntheses of FGAR analogues (*6*), and their interactions with purified GAR transformylase are reported herein.

MATERIALS AND METHODS

1-*O*-Acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose, 4-nitrophenethyl alcohol, TMSBr, DBU, CsF, TFA, dibenzyl phosphate, dibenzyl phosphite, anhydrous acetonitrile, 4-(4-nitrobenzyl)pyridine, azidotrimethylsilane, 2,2-dimethoxypropane, potassium phthalimide, 2-chloroacrylonitrile, $POCl_3$, 10% Pd on carbon, vanillin, diphenyl phosphite, *N*-chlorosuccinimide, CaH_2, bromoacetic acid, chloroacetic acid, cyanoacetic acid, and di-*tert*-butyl dicarbonate were purchased from Aldrich. BSA, Hepes, and QAE–Sephadex A-25 were obtained from Sigma. Reagent grade solvents and salts, Analtech silica gel G preparative TLC plates (2000μm), Universal Adsorbents TLC grade silica gel (5–20 μm), and cellulose TLC plates (Eastman 13254) were obtained from Fisher. Acetone was dried over and distilled from $CaSO_4$, pyridine was distilled from CaH_2, methanol was distilled from sodium, and CH_2Cl_2 was distilled from P_2O_5. GAR was synthesized according to Chettur and Benkovic (*7*). Dibenzyl phosphorochloridate was prepared as described by Smith and Griffin (*8*). Dibenzyl phosphorofluoridate was prepared from tetrabenzyl pyrophosphate (*9*) according to Watanabe *et al.* (*10*). Bis[2-(*p*-nitrophenyl)ethyl] phosphorochloridate was prepared by a modification of the

procedure of Himmelsbach *et al.* (*11*) in which chlorination of the intermediate phosphite was achieved with NCS, rather than sulfuryl chloride. 2-Chloro-β-alanine was prepared according to a Mitsui Toatsu Chemicals Inc. patent abstract (*12*) and was N-protected as the *tert*-butyl carbamate as described by Moroder *et al.* (*13*), except that 0.5 M sodium bicarbonate (pH 10) was substituted for 1 N NaOH. Standard barium precipitation techniques (*14*) were employed to separate inorganic and organic phosphates. TLC analysis of the nucleotide products was performed on cellulose (Eastman 13254) with either (A) *n*-butanol : acetic acid : H_2O (5 : 2 : 3) or (B) isopropanol : H_2O : 10 N HCl (66 : 33 : 1) as eluant. Phosphorus-containing compounds were visualized with molybdate spray reagent and either ninhydrin for amino compounds or 4-(4-nitrobenzyl)pyridine (*15*) for alkylating agents. Solution concentrations of nucleotides were determined by phosphate assays (*16*), and, in general, organic phosphate constituted ≥90% of the weighed amount. Protein concentration was determined according to Sedmack and Grossberg (*17*), with BSA as standard. GAR transformylase was purified from fresh chicken liver as described previously (*18*). NMR spectra were recorded on a Bruker AC-E 300-MHz spectrometer, and IR spectra were obtained on a Perkin–Elmer 1600 FT-IR.

GAR transformylase assay. GAR transformylase activity was assayed at 25°C with the alternate folate analogue substrate, 10-formyl-5,8-dideazafolate, as described previously (*3*). The reaction solutions contained 50 mM Hepes, pH 7.5, 50 μM 10-formyl-5,8-dideazafolate, 0.25 mM α,β-GAR, and enzyme in a final volume of 1.0 ml. The reaction was followed by monitoring the production of 5,8-dideazafolate at 295 nm ($\Delta\varepsilon$ = 18.9 mM^{-1}). For the steady-state inhibition studies, the concentration of α,β-GAR was varied from 0.3 to 3 times its K_m, in the absence and the presence of increasing concentrations of GAR analogue. The initial velocity data were analyzed with Cleland's programs (*19*) for competitive (Eq. [3]), noncompetitive (Eq. [4]), and uncompetitive (Eq. [5]) inhibition,

$$v = \frac{V_A}{K_A(1 + I/K_{is}) + A}, \quad [3]$$

$$v = \frac{V_A}{K_A(1 + I/K_{is}) + A(1 + I/K_{ii})}, \quad [4]$$

$$v = \frac{V_A}{K_A + A(1 + I/K_{ii})}, \quad [5]$$

where K_{ii} and K_{is} are the inhibition constants obtained from the intercepts and the slopes, resepctively.

Assay of ***8*** *and* ***13*** *as substrates.* These assays were performed as described for the standard GAR transformylase assay, except that twice the amount of enzyme was used. The concentrations of **8** (see Scheme I) ranged from 25 to 500 μM, and those of **13** (see Scheme II) ranged from 50 to 525 μM.

Inactivation assays. In general, these studies were conducted by incubating enzyme, 50 μM 10-formyl-5,8-dideazafolate, and analogue (≥40 times K_i) in 10 mM Hepes, pH 7.5, in a final volume of 20 μl at 25°C. The reaction was initiated with enzyme, aliquots (2 μl) were removed and added to the standard assay solution at

25 s and 5, 10, 15, 20, and 30 min, and GAR transformylase activity was monitored as described above. A control, omitting analogue, was performed for each study.

Preparation of **4.** The preparation of 2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl azide (**1,** Scheme I), its subsequent reduction to amine **2,** coupling with the appropriate carboxylic acid to afford amides **3,** and the deblocking to provide triols **4** were conducted according to the procedure of Schendel and Stubbe (*6*), with the substitution of 10% Pd on carbon for Pt_2O in the catalytic hydrogenation. Intermediates **3** were purified on silica gel as described by Harwood (*20*). The yields of **4** from **1** ranged from 61% (**4d**) to 85% (**4c**). The products were obtained as α,β (1 : 1) anomeric mixtures, whose proton NMRs were consistent with the indicated structures.

Preparation of **5a.** Phosphorylation of **4a** was accomplished with $POCl_3$ in triethyl phosphate as described previously (*6*) to afford **5a** in 26% yield. TLC (A) indicated coincident phosphate- and 4-(4-nitrobenzyl)pyridine-reactive material at R_f 0.44 and an additional, less intense, phosphate spot at R_f 0.29. NMR (D_2O) δ 5.79 (d, α-H-1′, $-CH_2OH$), 5.78 (d, J = 4.5 Hz, α-H-1′, $-CH_2Br$), 5.56 (d, β-H-1′, $-CH_2OH$), 5.54 (d, J = 4.8 Hz, β-H-1′, $-CH_2Br$), 4.28 (m), 4.19 (m), 4.06 (s, $-CH_2OH$, α-anomer), 4.03 (s, $-CH_2OH$, β-anomer), 3.82 (s, $-CH_2Br$, α-anomer), 3.80 (s, $-CH_2Br$, β-anomer), 3.72 (m). The ratio of total anomeric protons to the other protons was 1 : 7. GAR-OH constituted approximately 40% of the mixture. The ratio of β-**5a** : α-**5a** was approximately 55 : 45, while the ratio of β-GAR-OH : α-GAR-OH was approximately 2 : 1. Attempted separation of **5a** and GAR-OH on QAE–Sephadex A-25 was unsuccessful, as were attempts to preclude and/or minimize its formation.

Preparation of **5e.** Direct diazotization of GAR to **5e** employed the procedure of Curphey and Daniel (*21*). The barium salt of GAR (23 μmol) was dissolved in H_2O (0.5 ml) and the solution was adjusted to pH 2.9 by dropwise addition of 0.5 M aqueous chloroacetic acid. The solution, protected from light with Al foil, was cooled in an ice bath and 5 M $NaNO_2$ (69 μl, 345 μmol, 15 eq) was added in small portions over a 30-min period. After a total of 1.5 h at 0–5°C, the pale yellow solution was added to 5 vol of absolute EtOH and the pH was adjusted to pH 8.5. The resulting suspension was kept at −20°C for several hours to complete precipitation. The solid was collected by centrifugation, washed with EtOH and ether, and dried *in vacuo* over P_2O_5 to afford 9.3 mg, 21.5 μmol, 94%, of a cream-colored product. TLC (B) indicated loss of the ninhydrin-reactive material (GAR) at R_f 0.42. IR (KBr) 2120 cm^{-1}. NMR (D_2O) δ 5.59 (d, J = 4.7 Hz, α-H-1′), 5.34 (d, J = 5.5 Hz, β-H-1′), 4.22–4.07 (m), 3.96 (br s), 3.68 (m). The anomeric ratio was 1 : 1, and the ratio of anomeric protons to other protons was 1 : 6.

Preparation of **9.** A solution of **4b** (650 mg, 2.89 mmol) in pyridine (2.0 ml) was frozen in a dry ice/acetone bath. Dibenzyl phosphorochloridate (1.66 g, 5.6 mmol) (*8*) was added to the frozen solution and the resulting mixture was shaken at room temperature until it was homogeneous. It was then kept in the dry ice/acetone bath for 1 h and at -20°C overnight. H_2O (2.0 ml) was added and the resulting solution was stirred for 15 min at 25°C. Volatiles were evaporated and the residue was purified on a silica gel preparative TLC (20 cm × 20 cm × 2000 μm) developed with $CHCH_3$: MeOH (9 : 1). The band with R_f 0.5 was scraped from the plate.

Elution from the silica gel with MeOH, followed by evaporation of solvent, afforded 470 mg, 0.969 mmol, 33%, of a clear oil.

Preparation of ***5b.*** A solution of **9** (440 mg, 0.91 mmol) in MeOH (10 ml)–H_2O (5 ml) was hydrogenated (5 psi) over 10% Pd on carbon (100 mg) for 1 h at room temperature. After filtration and evaporation of volatiles, the residue was dissolved in H_2O (25 ml) and the pH was adjusted to 8.5 with saturated aqueous $Ba(OH)_2$. Barium phosphate was allowed to precipitate at 4°C for several hours. The precipitate was removed by filtration, the filtrate was treated with absolute EtOH (5 vol) (*14*) and the pH was readjusted to 8.5 if necessary. Organic phosphate was allowed to precipitate at −20°C overnight. The precipitate was collected by centrifugation, washed with EtOH and ether, and dried to yield 185 mg, 0.42 mmol, 47%, of a white solid. TLC (A) showed two sets of coincident phosphate- and 4-(4-nitrobenzyl)pyridine-reactive material with R_f 0.44 and R_f 0.21 (most likely diphosphorylated material). A portion of this mixture (120 mg) was chromatographed on a 1 × 7 cm column of QAE–Sephadex A-25 eluted with a 100 ml gradient of triethylammonium bicarbonate (0–0.4 M) (pH 7.5) and 53 mg of the Ba salt of **5b** was recovered. TLC (A) showed coincident phosphate and 4-(4-nitrobenzyl)pyridine reactive material at R_f 0.44. NMR (D_2O) δ 5.64 (d, J = 4.5 Hz, α-H-1′), 5.37 (d, J = 4.8 Hz, β-H-1′), 4.16 (m), 4.11 (s, $-CH_2Cl$, α-anomer), 4.08 (s, $-CH_2Cl$, β-anomer), 4.04 (m), 3.73 (m). The ratio of total anomeric protons to total other protons was 1 : 7, and the ratio α-H-1′ : β-H-1′ was 1.4 : 1.

Preparation of ***6.*** To a cold (ice bath), stirred solution of **4d** (254 mg, 0.717 mmol) in pyridine (4.1 ml) was added dropwise 1.4 ml of a 1 M solution of bis[2-(*p*-nitrophenyl)ethyl] phosphorochloridate (*11*) in benzene. The resulting solution was stirred at 0–5°C for 8 h and kept at −20°C overnight. $CHCl_3$ (20 ml) was added and the resulting solution was extracted with 0.1 M potassium phosphate, pH 7.2 (3 × 15 ml). The organic layer was dried (Na_2SO_4), and the volatiles were evaporated. The residue was purified by silica gel preparative TLC (20 cm × 20 cm × 2000 μm) developed with $CHCl_3$: MeOH (9 : 1). The band of R_f 0.46 was extracted with EtOH and the volatiles were evaporated to afford 180 mg, 0.246 mmol, 34% of clear, colorless oil. NMR ($CDCl_3$) δ 8.13 (m, 4H), 7.38 (m, 4H), 5.72 (dd, α-H-1′, 0.4H), 5.48 (dd, β-H-1′, 0.6H), 4.4–3.9 (m, 10H), 3.6 (m, 2H), 3.05 (m, 4H), 1.40 (s, 9H).

Preparation of ***7.*** A solution of **6** (100 mg, 0.137 mmol) in 50% aqueous TFA (2.5 ml) was stirred at room temperature for 1 h. Volatiles were evaporated to afford the TFA salt of **7** in quantitative yield. This material was carried through to the next step without further purification.

Preparation of ***8.*** A solution of **7** (102 mg, 0.137 mmol) in 10 ml of 0.5 M DBU in pyridine was stirred at room temperature for 24 h. Then 5 ml of 1 M acetic acid in pyridine was added and the volatiles were evaporated. The residue was dissolved in H_2O, treated with 1 M $Ba(OAc)_2$ (0.275 ml), and the pH was adjusted to 8.5. This solution was treated with absolute EtOH and the resulting suspension was kept at −20°C for several hours. The precipitate was collected by centrifugation, washed with EtOH and ether, and dried *in vacuo* to afford 44 mg, 0.100 mmol, 74%, of a white powder. TLC (A) showed coincident phosphate-, ninhydrin-, and 4-(4-nitrobenzyl)pyridine-reactive (*22*) material at R_f 0.32. NMR (D_2O) δ 5.59 (d,

J = 4.2 Hz, α-H-1′, 0.5H), 5.33 (d, J = 5.2 Hz, β-H-1′, 0.5H), 4.12 (m, 2H), 3.97 (m, 1H), 3.69 (m, 2H), 2.54, (m, aziridine methine, 0.5H), 2.47 (m, aziridine methine, 0.5H), 1.77 (m, aziridine methylene, 2H), 1.74 (s, acetate). The ratio of α-H-1′ : β-H-1′ was 1 : 1. ^{13}C NMR (D_2O) δ 173.97 (amide), 160.39 (amide), 83.37 (C-4′, $J_{C\text{-}4'\text{-}P}$ = 8.2 Hz), 81.46 (C-4, $J_{C\text{-}4'\text{-}P}$ = 7.7 Hz), 79.99 (C-1′), 73.75 (C-1′), 71.08, 71.02, 70.82, 69.94, 63.63 (C-5′, $J_{C\text{-}5'\text{-}P}$ = 4.7 Hz), 63.42 (C-5′, $J_{C\text{-}5'\text{-}P}$ = 4.2 Hz), 29.39 (aziridine methine), 25.40, and 25.29 (aziridine methylene) (relative to external dioxane at 66.50 ppm).

Preparation of ***10***. A suspension of **4c** (380 mg, 1.76 mmol) in acetone (22 ml) was treated with 2,2-dimethoxypropane (6.6 ml, 53.8 mmol) and 816 mg of Dowex 50W-X8, H^+ at room temperature for 2.5 h. The resin was filtered from the clear solution. The filtrate was evaporated and the residue chromatographed (*20*) on 6 g of silica gel eluted with ethyl acetate–hexanes to afford 320 mg, 1.25 mmol, 71%, of a crystalline product. NMR (acetone-d_6) δ 5.83 (dd, $J_{1'\text{-}2'}$ = 4.2 Hz, $J_{1'\text{-}NH}$ = 9.2 Hz, α-H-1′), 5.66 (br d, $J_{1'\text{-}NH}$ = 7.8 Hz, β-H-1′), 4.8 (m, β-H-2′,3′), 4.59 (m, α-H-2′,3′), 4.18 (m, H-4′), 3.72 (m, H-5′), 3.62 (s, $-CH_2CN$), 1.43 (s, β-acetonide methyl), 1.41 (s, α-acetonide methyl), 1.31 (s, α-acetonide methyl), 1.27 (s, β-acetonide methyl). The ratio of β-H-1′ : α-H-1′ was 1.8 : 1.

Preparation of ***11***. A stirred solution of **10** (115 mg, 0.451 mmol) in acetonitrile (2 ml) was treated with dibenzyl phosphorofluoridate (252 mg, 0.9 mmol) and CsF (205 mg, 1.35 mmol) (*10*) at room temperature for 18 h. Solvent was evaporated and the residue was dissolved in ethyl acetate (30 ml). The ethyl acetate solution was washed with H_2O (3× 20 ml) and dried (Na_2SO_4), and the residue, after evaporation of volatiles, was chromatographed (*20*) on 5 g of silica gel with ethyl acetate–hexanes to yield 137 mg, 0.266 mmol, 59%, of a clear oil. NMR ($CDCl_3$) δ 7.38 (br s, phenyl) 7.37 (br s, phenyl), 5.71 (m, α-H-1′), 5.68 (d, J = 6.6 Hz, β-H-1′), 5.07 (m, benzylic methylene), 4.5 (m, H-2′,3′), 4.33 (m, H-4′) 4.03 (m, H-5′s), 3.21 (s, $-CH_2CN$), 1.55 (s, α-acetonide methyl), 1.51 (s, β-acetonide methyl), 1.32 (α-acetonide methyl), 1.30 (s, β-acetonide methyl). The ratio of β-H-1′ : α-H-1′ was 3.5 : 1.

Preparation of ***5c***. A solution of **11** (68.5 mg, 0.133 mmol) in CH_2Cl_2 (2 ml) was treated with 0.2 M TMSBr in CH_2Cl_2 (1.46 ml, 0.292 mmol) at 0–5°C for 2.5 h. The reaction solution was added to a cold mixture of CH_2Cl_2 (70 ml) and H_2O (70 ml) with stirring continued for 1 h at 0–5°C. The layers were separated and the aqueous solution was neutralized with ethanolic triethyl amine. Volatiles were evaporated to afford a quantitative yield of the triethylammonium salt. This salt (60 mg, 0.11 mmol) was dissolved in 20% formic acid–10% ethylene glycol–70% H_2O (0.62 ml) and the solution was stirred at room temperature for 72 h. The solution was diluted with H_2O, extracted with ether, adjusted to pH 8.5 with saturated aqueous $Ba(OH)_2$, and treated with absolute EtOH (5 vol). Precipitation was allowed to proceed at −20°C overnight. The precipitate was collected by centrifugation, washed with EtOH and ether, and dried *in vacuo* to afford 33 mg, 0.077 mmol, 70%, of white solid. Material from two preparations (66 mg) was combined and chromatographed on a 0.5 × 15 cm column of QAE–Sephadex A-25 eluted with a 50 ml gradient of triethylammonium bicarbonate (0–0.4 M) (pH 7.5) and 42 mg of the Ba salt of **5c** was recovered. TLC (A) R_f 0.31, (B) R_f 0.66. NMR (D_2O) δ 5.59 (d, J = 4.5 Hz, α-H-1′), 5.31 (d, J = 5.2 Hz, β-H-1′), 4.19–4.06 (m), 3.97 (m),

3.67 (m). The anomeric ratio was 1 : 1, and the ratio of anomeric protons to other protons was 1 : 7.

Preparation of ***12.*** A solution of **6** (50 mg, 0.68 mmol) in 5 ml of 0.5 M DBU in pyridine was stirred at room temperature for 24 h. Then 2.5 ml of 1 M acetic acid in pyridine was added and the volatiles were evaporated. The residue was dissolved in H_2O and applied to a 1 × 7 cm column of QAE–Sephadex A-25. The column was washed with H_2O (40 ml) and eluted with 0.3 M triethylammonium bicarbonate (pH 7.5). Phosphate containing fractions were combined and the solvent was evaporated to afford 31 mg of a pale yellow oil (containing approximately 50% triethylammonium acetate by NMR) which was taken through the next step.

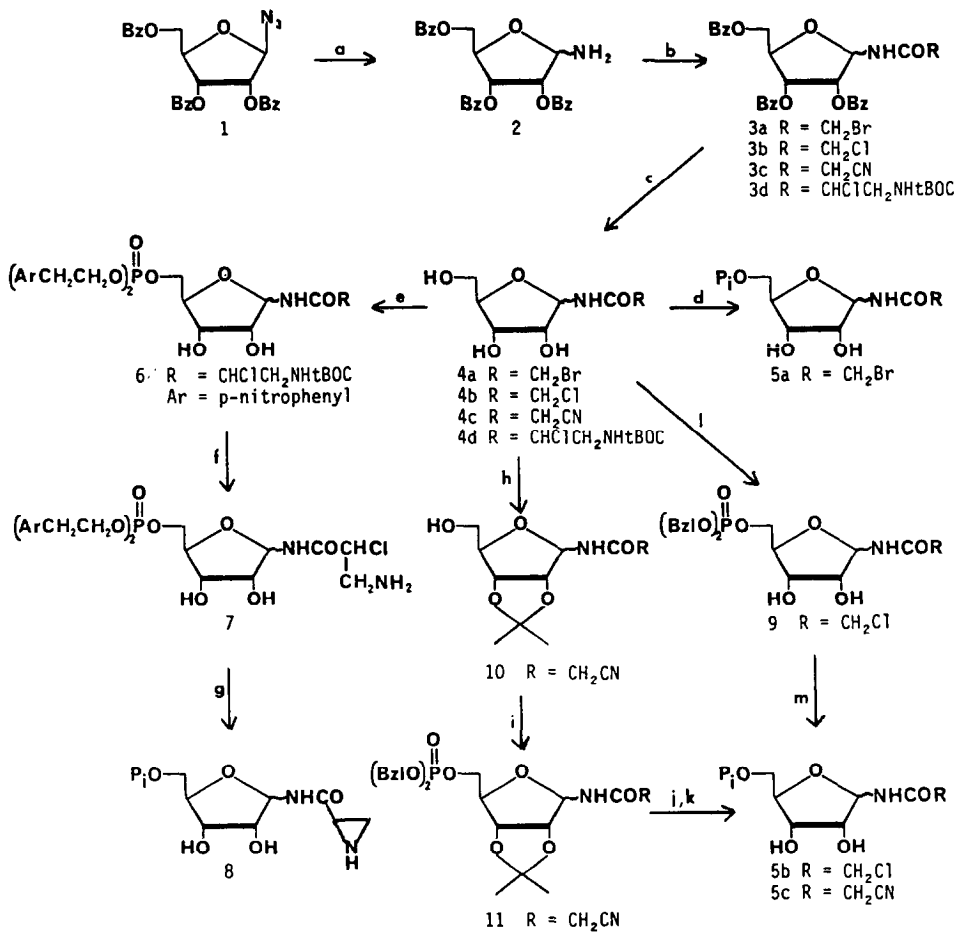
Preparation of ***13.*** The oil was dissolved in 50% aqueous TFA (1.5 ml) and the solution was stirred at room temperature for 6 h. Volatiles were evaporated and the residue was dissolved in H_2O (2 ml). This solution was treated with 1 M $Ba(OAc)_2$ (78 μl) and adjusted to pH 8.5. The resulting suspension was filtered and the filtrate was diluted with absolute EtOH (5 vol). After several hours at −20°C, the precipitate was collected by centrifugation, washed with EtOH and ether, and dried *in vacuo* to afford 8 mg of white solid. TLC (A) coincident phosphate-, ninhydrin-, and 4-(4-nitrobenzyl)pyridine-reactive material at R_f 0.26. NMR (D_2O) δ 5.56 (d, J = 4.7 Hz, α-H-1′), 5.30 (d, J = 4.9 Hz, β-H-1′), 4.19 (t, J = 4.8 Hz), 4.1 (m), 3.96 (m) 3.65 (m). The ratio of α-H-1′ : β-H-1′ was 1.2 : 1, and the ratio of anomeric protons to other protons was 1 : 8.

RESULTS AND DISCUSSION

GAR transformylase catalyzes the formylation of GAR to produce FGAR, with 10-formyltetrahydrofolate serving as the formyl group donor (*23*) (Eq. [1]). Based on initial velocity and dead-end inhibition studies (*3*), which indicated that the kinetic mechanism is ordered-sequential with the folate substrate binding first, we suggest that the chemical mechanism involves direct transfer of the formyl group from 10-formyltetrahydrofolate to the primary amino group of GAR. Furthermore, we invoke the participation of enzyme residues, acting as general acid–base catalysts, in the formation and decomposition of the proposed tetrahedral intermediate (Eq. [2]). Therefore, in order to test this mechanism, to further investigate the nature of the GAR binding site, and to provide potential affinity labels for GAR transformylase, we prepared a series of GAR analogues as outlined in Schemes I and II.

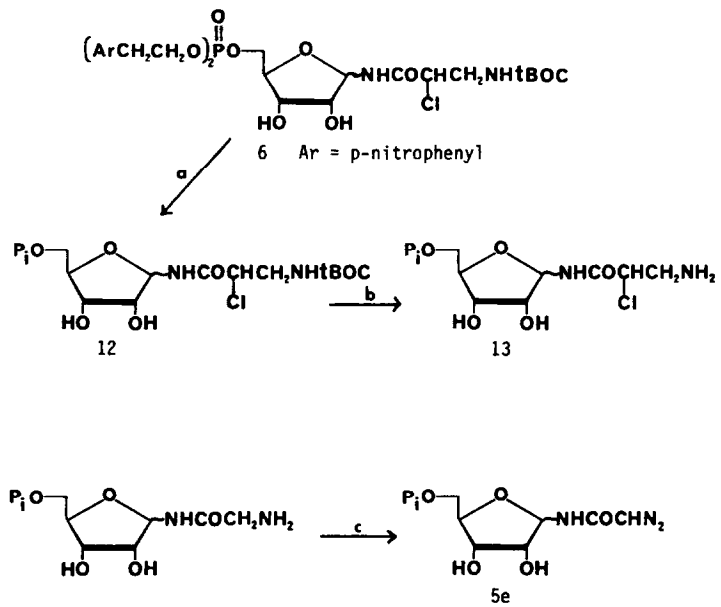
Preparation of GAR analogues. The synthetic procedure to triols **4** (Scheme I), based on the procedure of Schendel and Stubbe (*6*) for the synthesis of FGAR analogues, provided the required nucleosides, as an α,β mixture, in reasonable yield (61–85%). As noted by these authors, phosphorylation of the nucleosides to the nucleotides presents a significant problem, although they obtained modest yields (20–30%) of nucleotide with $POCl_3$ in triethyl phosphate.

We attempted to improve the yield of this step through the use of monofunctional phosphorylating reagents. This approach also had the advantage of allowing chromatographic purification of the intermediate phosphotriesters. We were, however, only moderately successful with this approach, although all of target nucleotides



SCHEME I. (a) 10% Pd on carbon, H_2 (5 psi), 25°C, 1–2 h; (b) RCOOH, DCC, acetone, 25°C, 3 h; (c) CH_3ONa, CH_3OH, 25°C, 1 h; (d) $POCl_3$, $(EtO)_3PO$,)–5°C, 2 h; (e) bis[2-(*p*-nitrophenyl)ethyl]phosphorochloridate, pyridine, 0–5°C (8 h), −20°C (12 h); (f) 50% aqueous TFA, 25°C, 1 h; (g) DBU, pyridine, 25°C, 24 h; (h) acetone, 2,2-dimethoxypropane, Dowex 50W-X8 H^+, 25°C, 2.5 h; (i) dibenzyl phosphorofluoridate, CsF, acetonitrile, 25°C, 18 h; (j) TMSBr, CH_2Cl_2, 0–5°C, 2.5 h; H_2O, 0–5°C, 1 h; (k) HCOOH : $HOCH_2CH_2OH$: H_2O (20 : 10 : 70), 25°C, 72 h; (l) dibenzyl phosphorochloridate, pyridine, −78°C (1 h), −20°C (15 h); (m) 10% Pd on carbon, H_2 (5 psi), CH_3OH–H_2O, 1 h.

were prepared. Analogue **5b,** prepared previously by Schendel and Stubbe (*6*), was obtained by phosphorylation with dibenzyl phosphorchloridate, followed by hydrogenolysis of the benzyl groups. Since this procedure resulted in some diphosphorylated material, we decided to pursue the use of the less reactive dibenzyl phosphorofluoridate–CsF reagent (*10*). Attempted phosphorylations of **4a** and **4c** were unsuccessful, presumably due to the limited solubility of the nucleosides in acetonitrile, the required solvent. Conversion of the nucleosides to the 2′,3′-acetonides obviated this problem and **10** was sequentially converted to **11** and **5c.**



SCHEME II. (a) DBU, pyridine, 25°C, 24 h; (b) 50% aqueous TFA, 25°C, 6 h; (c) $ClCH_2COOH$, $NaNO_2$, 0–5°C, 1.5 h.

We were, however, unable to convert **4a** to **5a** by this procedure. It has been postulated (*10*) that a nucleophilic phosphorus intermediate is generated under the reaction conditions and this may have reacted deleteriously with the electrophilic methylene group of **4a.** Conversion of **4a** to **5a** was accomplished, albeit in poor yield and contaminated with its hydrolysis product GAR-OH, with $POCl_3$ in triethyl phosphate (*6*). Diazotization (*21*) of GAR led to **5e** (Scheme II), as evidenced by the diazo absorption band at 2120 cm^{-1}, the loss of ninhydrin reactivity, and the loss of the resonance for the glycine methylene protons of GAR in the proton NMR spectrum. *p*-Nitrophenethyl was chosen as the phosphate blocking group in the synthesis of **8,** since the basic conditions used for its elimination, DBU in pyridine (*11*), should also effect ring closure of **7** to **8.** Indeed, **8** was obtained in 74% yield from **7.** The proton and ^{13}C NMR data (see Materials and Methods) are consistent with the proposed structure. We have tentatively assigned the downfield aliphatic doublet at δ 83.43 to C-4′ of the α-anomer, since C-4′ of the α-anomer is more likely to experience deshielding by the aziridine than is C-4′ of the β-anomer. Interestingly, the C-5′s of the two anomers were resolved, in contrast to what was observed with the FGAR analogues (*6*). From **6** we were also able to prepare **13** by reversing the order of the deblocking steps.

The proton NMR data for these analogues are summarized in Table 1. The assignments for the anomeric protons are based on those of Schendel and Stubbe (*6*) for FGAR analogues in which they determined that the downfield doublet with the smaller 1′–2′ coupling constant represents the H-1′ proton of the α-anomer. Assignments of other resonances (see Materials and Methods), for example, the

TABLE 1

Chemical Shifts for Anomeric Protons of GAR Analogues[a]

Compound	R	Anomer	H-1′ (ppm)	$J_{1'-2'}$ (Hz)
5a	CH_2Br	α	5.78	4.5
		β	5.54	4.8
5b	CH_2Cl[b]	α	5.64	4.5
		β	5.37	4.8
5c	CH_2CB	α	5.59	4.5
		β	5.31	5.2
5e	CH=N=N	α	5.59	4.7
		β	5.34	5.5
8		α	5.59	4.2
		β	5.33	5.1
13	$CHClCH_2NH_2$	α	5.56	4.7
		β	5.30	4.9

[a] Relative to HOD at 4.67 ppm.
[b] Prepared previously by Schendel and Stubbe (*6*).

side chain methylenes of **5a, 5b,** and GAR-OH, could be made when the two anomers were present in unequal amounts.

Assay of GAR analogues. The steady-state inhibition of GAR transformylase by these analogues was determined using the standard assay and the results are summarized in Table 2. The Lineweaver–Burk plot of the initial velocity data obtained with **5b** (Fig. 1) is representative of these data. All of the analogues proved to be good inhibitors of the enzyme, competitive against GAR, indicating that these structural modifications do not adversely affect binding to the GAR binding site. Furthermore, the fact that the α-haloamide analogues **(5a, 5b)** are excellent inhibitors, suggests that hydrogen bonding to the side chain terminus is not a prerequisite for productive binding. As noted above, **5a** contained GAR-OH ($K_i = 4.16 \pm 0.29$ μM (*3*)) at approximately 40% of the mixture. Consequently, the K_i obtained for this analogue is not an accurate value.

TABLE 2

Inhibition Patterns and Constants for GAR Analogues

Compound	R	Pattern[a]	K_{is} (μM)
5a	CH_2Br	C	9.19 ± 0.40
5b	CH_2Cl	C	7.03 ± 0.45
5c	CH_2CN	C	8.84 ± 0.39
5e	CH=N=N	C	12.85 ± 0.80
8		C	7.65 ± 0.80
13	$CHClCH_2NH_2$	C	14.06 ± 0.77

[a] Pattern obtained from Lineweaver–Burk plots of the initial velocity data. C, competitive.

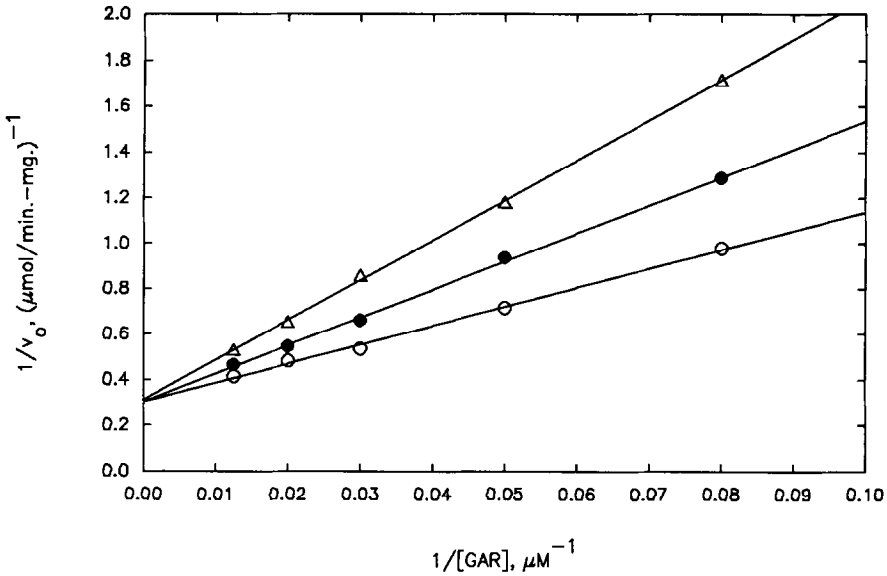


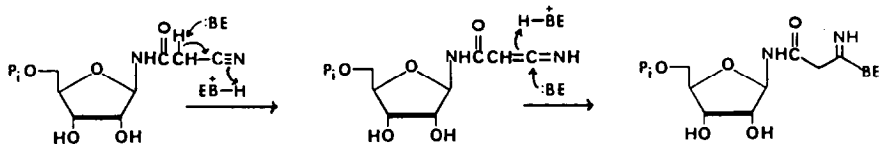
FIG. 1. Lineweaver–Burk plot of initial velocity data for inhibition of GAR transformylase by **5b** as a function of GAR concentration. Concentrations of **5b** were 0 (○), 4 (cs), and 8 (△) μM. The straight lines were generated with Cleland's (*19*) program for competitive inhibition.

Both **8** and **13** were tested as substrates for GAR transformylase with the standard assay. Neither of these analogues showed any substrate activity at concentrations up to 20 times the K_m for GAR. From these results we can conclude that these modifications are not compatible with productive enzyme processing, but which structural change is primarily responsible cannot be pinpointed at this time. For example, with **8,** either the three-membered heterocycle or the secondary amine (vs the primary amine in GAR) could be the major contributor, while with **13,** either side chain length or stereoeffects and/or electronic effects introduced by the chlorine could be the major contributor. Further studies are in progress to distinguish between these factors.

All of the analogues were tested for their ability to inactivate GAR transformylase. These studies were conducted in the presence of saturating 10-formyl-5,8-dideazafolate, based on our previously determined (*3*) kinetic mechanism for this enzyme. Analogues **5e** and **8** were designed as potential mechanism-based inactivators in the hope that protonation by the proposed active site acid would produce reactive intermediates capable of alkylating the conjugate base. Disappointingly, neither of the analogues inactivated the enzyme even at concentrations greater than 100 times their respective K_i's. Several explanations can be offered to account for these results: (1) the proposed mechanism is not valid; (2) neither of these analogues can adopt a conformation compatible with protonation by the active site acid; or (3) the length of the side chain in these analogues is insufficient to place the protonation site in proximity to the proton donor. The latter explanation will be tested through the use of appropriate GAR analogues.

The α-haloamide analogues **5a** and **5b** also failed to inactivate the enzyme. This was surprising in view of the fact that 10-bromoacetyl-5,8-dideazafolate has been shown to inactivate GAR transformylase (*18*). Again, the precise, required alignment of analogue electrophile and enzyme nucleophile has not, apparently, been achieved with these analogues. More precisely, we believe that side chain length is the primary factor responsible for the inability of these analogues to act as affinity labels. We are currently testing this hypothesis.

If GAR binds to the enzyme as the ammonium species, deprotonation by an active site base would be a prerequisite to nucleophilic addition of the amino group of GAR to the formamide carbonyl carbon. This base could, in principle, abstract a proton from the side chain methylene of nitrile analogue **5c** to produce, by isomerization, an electrophilic ketenimine analogue capable of active site alkylation (Eq. [6]).



[6]

Again, this analogue failed to inactivate the enzyme. Either general base catalysis is not required during the initial stages of the transformylation reaction, i.e., GAR binds as the neutral amine as suggested previously (*3*) or the orientation of this functional group relative to the base is not optimized in this case.

Although the GAR analogues synthesized to data have not provided us with mechanism-based inactivators or affinity labels for GAR transformylase, they have provided us with "molecular rulers" to begin to define the constraints at the GAR binding site, as well as providing us with competitive inhibitors for this enzyme. The results obtained with these analogues have led us to a better understanding of what might be required, in terms of side chain length, for successful mechanism-based inhibition and affinity labeling. We are engaged in testing this proposal.

Summary. A series of GAR analogues has been prepared and evaluated with GAR transformylase. All of the analogues proved to be inhibitors of the enzyme, competitive against GAR, but none were capable of inactivating the enzyme. Although this study did not result in successful affinity labels for GAR transformylase, these analogues have afforded some information about the requirements of the GAR binding site of GAR transformylase.

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