Carbocyclic Substrates for de Novo Purine Biosynthesis*

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The carbocyclic analogues of phosphoribosylamine, glycaminamide ribonucleotide, and formylglycinamide ribonucleotide have been prepared as the racemates. Carbocyclic phosphoribosylamine was utilized as a substrate by the monofunctional glycaminamide ribonucleotide synthetase from Escherichia coli as well as the glycaminamide ribonucleotide synthetase activity of the eucaryotic trifunctional enzyme of de novo purine biosynthesis. Furthermore, carbocyclic glycaminamide ribonucleotide was processed in the reverse reaction catalyzed by these enzymes. In addition, carbocyclic formylglycinamide ribonucleotide, which was accepted as a substrate by the aminoimidazole ribonucleotide synthetase to carbocyclic formylglycinamide ribonucleotide, was converted by E. coli formylglycinamidase ribonucleotide synthetase to carbocyclic formylglycinamide ribonucleotide, which was also catalyzed by these enzymes. This study has afforded carbocyclic substrate analogues, in particular for the chemically labile phosphoribosyl amine, for the initial steps of de novo purine biosynthesis.

Interest in de novo purine biosynthesis has undergone a resurgence caused, in part, by the recent demonstration that this pathway is an effective target for cancer chemotherapy (Beardsley et al., 1986). Also contributing to this renewed interest is the fact that in eukaryotes most of the enzymatic activities that constitute the pathway are biosynthesized as multifunctional proteins (Patey and Shaw, 1973; Woodward, 1978; Benkovic, 1984; Daubner et al., 1986; Schrimsher et al., 1986). This multifunctionality raises the possibility of substrate channeling.

One factor that has hampered mechanistic studies on the early enzymes of the pathway is the difficulty in preparing substrates and substrate analogues for these enzymes, in particular the chemically labile 5-phosphoribosylamine (PRA).† (Schenkdel et al., 1988) which is the first substrate in the pathway. Indeed, the instability of PRA has led to the suggestion that PRA is channeled from phosphoribosyl-1-pyrophosphate amidotransferase to glycaminamide ribonucleotide (GAR) synthetase (reaction 2 in Scheme 1) to initiate the de novo pathway (Shen et al., 1990).

We demonstrated previously that the carbocyclic analogue of GAR (C-GAR), in which the ribose ring is replaced by a cyclopentane ring, serves as an efficient substrate for the GAR transformylase activity (reaction 3 in Scheme 1) of the mammalian trifunctional enzyme (Caperelli and Price, 1988), which also catalyzes the GAR synthetase (reaction 2 in Scheme 1) and aminoimidazole ribonucleotide (AIRM) synthetase (reaction 5 in Scheme 1) reactions. Given this positive result we decided to prepare the carbocyclic analogues of PRA and formylglycinamide ribonucleotide (FGAR) and evaluate their ability to serve as substrates for their respective enzymes, GAR synthetase and formylglycinamide ribonucleotide (FGAM) synthetase. We were particularly interested in C-PRA, given the chemical instability of PRA itself. The synthesis of these analogues and their preliminary enzymatic characterization are reported herein.

**EXPERIMENTAL PROCEDURES**

**Materials and Methods**

Trizma (Tris base), HEPES, QAE-Sephadex A-25, ADP, ATP, NADH, NADP, phosphoenolpyruvate, glycine, N-formylglycine, pyruvate kinase, lactate dehydrogenase, hexokinase, and glucose-6-phosphate dehydrogenase were purchased from Sigma. Silica gel (60–200 mesh), di-tert-butyl dicarbonate, 2,2-dimethoxy propane, CF COOH, LDA (1.5 M in cyclohexane), Cs-glycine, CDCl3, D2O, and 10% Pd on carbon were from Aldrich. TLC grade silica and Kodak silica gel TLC plates (no. 13181) were obtained from Fisher. All other chemicals and solvents were reagent grade. Escherichia coli GAR synthetase, FGAM synthetase, and FGAM were generously provided by Dr. JoAnne Stubbe (Massachusetts Institute of Technology). The trifunctional enzyme was purified from chicken liver as described previously (Young et al., 1984). Standard barium precipitation techniques (Cardini and Leloir, 1957) were employed to isolate organic phosphates. Nucleotide solution concentrations were determined by phosphate assay (Ames and Dubin, 1960). Dibenzyl phosphorochloridate was prepared according to Smith and Griffin (1961), 4-bromo-2,3-di-hydroxy-1-β-cyclopentanemethanol (1) was prepared according to Kam and Oppenheimer (1981), and GAR was prepared as described by Chetverin and Benkovic (1977). Dry column chromatography was performed with TLC grade silica gel as described by Harwood (1985). H and 31P NMR spectra were recorded on a Bruker AC-300 spectrometer at 300.13 and 121.49 MHz, respectively. Proton spectra were referenced to internal Me₄Si (CDCl3) or HOD at δ 4.65 (D₂O), and phosphorus spectra were referenced to external triphenylphosphine (5 ppm).

**Enzyme Assays**

All assays were performed at 25°C unless otherwise noted. Activity is expressed as μmol of product/min/mg of enzyme.

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‡ The abbreviations used are: PRA, 5-phosphoribosylamine; GAR, glycaminamide ribonucleotide; C-GAR, carbocyclic glycaminamide ribonucleotide; AIR, aminoimidazole ribonucleotide; FGAR, formylglycinamide ribonucleotide; C-PRA, carbocyclic phosphoribosylamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LDA, lithium diisopropylamidophosphorochloridate; CD20, benzoyloxy carbonyl; C-FGAR, carbocyclic formylglycinamide ribonucleotide; C-FGAM, carbocyclic formylglycinamidase ribonucleotide; C-AIR, carbocyclic aminoimidazole ribonucleotide; THF, tetrahydrofuran; BOC, tert-butoxycarbonyl.
GAR Synthetase

GAR synthetase was assayed in the forward direction with C-PRA essentially as described by Shen et al. (1990) with the following modifications: the 1-ml assay solution contained 2.7 mM glycine and 1.7 mM MgATP; 100 mM HEPES buffer was used for reaction at pH 7.5; and the reaction was initiated by the addition of a small volume of enzyme after preincubation of the other components at 25 °C for 5 min.

In the reverse direction, GAR synthetase was assayed with C-GAR using a coupled assay for ATP production (Shen et al., 1990) with the following modifications: the 1-ml assay solution contained 0.5 mM MgADP and 2 mM Pi; 100 mM HEPES buffer was used for assays at pH 7.5; and the reaction was initiated by the addition of a small volume of enzyme to the other reaction components that had been preincubated at 25 °C for 5 min.

For comparative purposes, the kinetic constants for the GAR synthetase activity of the trifunctional enzyme with PRA and GAR in the forward and reverse directions, respectively, were also determined. In the forward direction the procedure of Shen et al. (1990) was followed except that a 1-ml reaction in a 1-cm light path cuvette was used. The procedure of Shen et al. (1990) was used for assays in the reverse direction except that the reaction was run at 25 °C. For both directions the kinetic constants were obtained by varying the concentration of one substrate while maintaining the concentrations of the other two substrates at saturating levels. All initial velocity data were analyzed according to Equation 1 (Cleland, 1979).

\[ v = \frac{VA}{K_v + A} \]  

(1)

Synthetic Procedures

Preparation of 2—To a stirred solution of 1 (1.0 g, 5.4 mmol), Kam and Oppenheimer (1981) in 1:l dioxane, 1 N NaOH (30 ml) was added di-tert-butyl dicarbonate (1.19 g, 5.46 mmol). The resulting mixture was stirred at 25 °C for 3 h. The solvent was evaporated, and the residue was suspended in absolute ethanol. This suspension was filtered, and the filtrate was dried over MgSO4. Evaporation of the solvent left a residue that was dissolved in H2O. The aqueous solution was treated with 2 equivalents of Ba(OAc)2, and the pH was adjusted to 8.5 with 0.5 N NaOH. The suspension was chilled (4 °C) for several h, and the precipitate was removed by filtration. The filtrate was reduced in volume and treated with 5 volumes of absolute ethanol. The resulting suspension was kept at −20 °C for several h, and the precipitate was collected by centrifugation and dried to yield 6 (55 mg, 0.097 mmol, 40%) as a white powder. 1H NMR (D2O) 5.82 ppm.

Preparation of 3—To an acetone (10 ml) solution of 2 (190 mg, 0.77 mmol) was added 2,2-dimethoxypropane (0.5 ml, 423 mg, 4 mmol) and a trace of p-toluenesulfonic acid. The solution was stirred at 25 °C for 3 h and neutralized by the addition of a few drops of 6 N HCl. Evaporation of the solvent left a residue that was dissolved in CH2Cl2. The CH2Cl2 solution was washed with H2O and dried over MgSO4. The crude product, in CH2Cl2 (5 ml), was treated with di-tert-butyl dicarbonate (200 mg, 0.9 mmol), at 25 °C for 16 h. The crude product, after evaporation of solvent, was purified by dry column chromatography (ethyl acetate:hexanes; 3:1) to yield 3 (198 mg, 0.69 mmol, 89%) as a clear oil. 1H NMR (CDCl3) 6.9 Hz, 1H), 7.33 (bs, 10 H), 4.96 (d, 2H), 3.76 (m, 1H, H-3); 5.58 (m, 1H, H-5), 5.55 (m, 1H, H-6), 3.45 (s, 2H, NHCOENHCH2), 2.11 (s, 1H, H-1), 1.76 (m, 1H, H-5); 13C NMR (CDCl3) 6 12.67.

Preparation of 4—A solution of 5 (100 mg, 0.24 mmol) and 10% Pd on carbon (20 mg) in 95% ethanol (15 ml) was stirred under an atmosphere of H2 for 5 h at 25 °C. Filtration and evaporation of solvent left a residue that dissolved in H2O. The aqueous solution was treated with 2 equivalents of Ba(OAc)2, and the pH was adjusted to 8.5 with 0.5 N NaOH. The suspension was chilled (4 °C) for several h, and the precipitate was removed by filtration. The filtrate was reduced in volume and treated with 5 volumes of absolute ethanol. The resulting suspension was kept at −20 °C for several h, and the precipitate was collected by centrifugation and dried to yield 5 (65 mg, 0.097 mmol, 40%) as a white powder. 1H NMR (D2O) 5.85 ppm.

Preparation of 5—To a stirred solution of 5 (100 mg, 0.24 mmol) and 10% Pd on carbon (20 mg) in 95% ethanol (15 ml) was stirred under an atmosphere of H2 for 5 h at 25 °C. Filtration and evaporation of solvent left a residue that dissolved in H2O. The aqueous solution was treated with 2 equivalents of Ba(OAc)2, and the pH was adjusted to 8.5 with 0.5 N NaOH. The suspension was chilled (4 °C) for several h, and the precipitate was removed by filtration. The filtrate was reduced in volume and treated with 5 volumes of absolute ethanol. The resulting suspension was kept at −20 °C for several h, and the precipitate was collected by centrifugation and dried to yield 6 (55 mg, 0.097 mmol, 40%) as a white powder. 1H NMR (D2O) 5.85 ppm.

Preparation of 6—A suspension of 5 (100 mg, 0.24 mmol) and 10% Pd on carbon (20 mg) in 95% ethanol (15 ml) was stirred under an atmosphere of H2 for 5 h at 25 °C. Filtration and evaporation of solvent left a residue that dissolved in H2O. The aqueous solution was treated with 2 equivalents of Ba(OAc)2, and the pH was adjusted to 8.5 with 0.5 N NaOH. The suspension was chilled (4 °C) for several h, and the precipitate was removed by filtration. The filtrate was reduced in volume and treated with 5 volumes of absolute ethanol. The resulting suspension was kept at −20 °C for several h, and the precipitate was collected by centrifugation and dried to yield 7 (150 mg, 0.23 mmol, 51%) as a pale yellow oil. 1H NMR (CDCl3:CD3OD; 1:1) 5.97 ppm.

Preparation of 7—To an ice-cooled solution of Cbz-glycine (108 mg, 0.52 mmol) in ethyl acetate (10 ml) was added triethylamine (52 mg, 0.52 mmol) and isobutyl chloroformate (71 mg, 0.52 mmol). Then a solution of 6 (200 mg, 0.49 mmol) in ethyl acetate (4 ml) was added to the mixture, and stirring was continued at 25 °C overnight.

Preparation of 9—A solution of 5 (150 mg, 0.37 mmol), N-formylglycine (67 mg, 0.56 mmol), and dicyclohexylcarbodiimide (115 mg, 0.56 mmol) in THF (15 ml) was stirred at 25 °C for 5 h. The dicyclohexyl urea was filtered off, and the filtrate was concentrated and filtered. Evaporation of the solvent left a residue that was purified by dry column chromatography (CH2Cl2:CH3OH:CH3ONa 9:1:1) to yield 9 (198 mg, 0.69 mmol, 89%) as a clear oil. 1H NMR (CDCl3) 6 12.67.

Preparation of 10—A suspension of 9 (50 mg, 0.11 mmol) as described above, afforded 10 (37 mg, 0.082 mmol, 82%) as the barium salt. 1H NMR (D2O) 6 12.65.

Preparation of 11—A suspension of 10 (37 mg, 0.082 mmol, 82%) as the barium salt. 1H NMR (D2O) 6 12.65.

RESULTS AND DISCUSSION

Previous results from this laboratory (Caprelli and Price, 1988) demonstrated that the carbocyclic analogue of GAR, in which the ribose ring is replaced by a cyclopentane ring, is a
substrate for the GAR transformylase activity of the trifunctional enzyme of de novo purine biosynthesis. Furthermore, we showed that only one enantiomer of the racemic mixture was processed by the enzyme. These results prompted us to examine whether carbocyclic analogues of the substrates for the early activities of de novo purine biosynthesis would serve as substrates for or inhibitors of their respective enzymes. We were particularly interested in the carbocyclic analogue of PRA because of the lability of the natural substrate (Schendel et al., 1988). An additional advantage is that the synthetic route to these analogues affords the epimer corresponding to the β-anomer of the nucleotide substrates.

Preparation of Analogues—Our approach to the syntheses of C-PRA, C-FGAR, and a modified route to C-GAR is outlined in Scheme 2. The key intermediate in this approach is the dibenzyl ester of C-PRA, 5, which we anticipated could be converted readily to C-GAR and C-FGAR. In order to obtain 5, protecting groups for the amine and the secondary hydroxyls had to be selected so that they could be removed without affecting the benzyl groups. We chose tert-butyloxy-carbonyl (BOC) for amine protection and the acetonide for hydroxy protection since they could be removed simultaneously under acidic conditions.

4-β-Amino-2α,3α-dihydroxy-1β-cyclopentanemethanol hydrochloride 1 (Kam and Oppenheimer, 1981) was converted to 2 in 96% yield upon treatment with di-tert-butyl dicarbonate according to Moroder et al. (1976). Treatment of 2 with acetone, 2,2-dimethoxypropane, and a trace of p-toluenesulfonyl acid afforded 3. During this protection step, partial hydrolysis (approximately 10% by TLC) of the BOC group was observed, and the crude product was again treated with di-tert-butyl dicarbonate to afford 3 in 90% yield. Attempts to reverse the order of addition of the blocking groups was unsuccessful because of the insolubility of 1 in acetone.

Phosphorylation of 3 to 4, in 75% yield after chromatography, was accomplished with dibenzyl phosphoroophorochloridate and LDA. Treatment of 4 with aqueous CF₃COOH effected simultaneous deprotection of the amine and the hydroxyl groups, to afford 5 in 85% yield after chromatography. Hydrogenolysis of the benzyl protecting groups, followed by barium precipitation (Cardini and Leloir, 1957) of the organic phosphate, provided C-PRA (6) in 40% yield. The proton NMR of 6 (see "Materials and Methods") was very similar to that of 1, with the following exceptions: the C-6 protons were not resolved, and H-4 was shifted upfield (3.30 ppm) relative to H-4 of the amine hydrochloride 1 (3.54 ppm) (Kam and Oppenheimer, 1981). C-PRA (6) displayed a single phosphorus resonance at 12.67 ppm.

Coupling 5 with the mixed anhydride formed from Cbz-glycine and isobutyl chloroformate afforded 7 in 51% yield. Hydrogenolysis of the benzyl and Cbz protecting groups and barium precipitation afforded C-GAR (8) in 45% yield. As noted previously (Capperelli and Price, 1988), the proton NMR of C-GAR is almost identical to that of 1, with the exception of the resonance at 3.45 ppm contributed by the glycine methylene protons. A single phosphorus resonance was observed at 12.69 ppm.

Since we were concerned about possible deformylation of N-formylglycine under the conditions of mixed anhydride reaction, 5 and N-formylglycine were coupled using dicyclohexylcarbodiimide. This procedure provided 9 in 66% yield after chromatography. Treatment of 9 with barium acetate and dicyclohexylcarbodiimide in 45% yield. The proton NMR showed the formyl proton as a doublet, J = 6.9 Hz, at 7.77 ppm. Debenzylolation and barium precipitation provided C-FGAR (10) in 82% yield. Although the formamide aldehydic proton was not detected in the proton NMR (D₂O), presumably because of hydration, the downfield shift of the side chain methylene protons from 3.45 ppm (C-GAR) to 3.84 ppm (C-FGAR) suggests that the terminal amino group is indeed formylated.

Enzyme Studies—C-PRA was assayed for substrate activity with the monofunctional GAR synthetase (reaction 2 in Scheme 1) isolated from E. coli (Shen et al., 1990) and with the GAR synthetase activity of the trifunctional enzyme isolated from chicken liver (Young et al., 1984), using a coupled assay for ADP production (Shen et al., 1990), at both pH 7.5 and pH 8.0. The kinetic constants obtained from these studies are summarized in Table I, along with the data obtained with PRA for comparison. Our results indicate clearly that C-PRA is accepted as a substrate by both enzymes although with reduced efficiency compared with PRA. However, given the lability of PRA (Schendel et al., 1988), C-PRA should prove to be a useful alternate substrate for this enzyme. It is also worth noting that for both enzymes, the Kₘ for C-PRA decreased approximately 4-fold on increasing the pH from 7.5 to 8.0. This suggests that the neutral amine is the

![Scheme 2](Image)

**Scheme 2.** Reagents: a, di-tert-butyl dicarbonate, NaOH, dioxane/H₂O; b, 2,2-dimethoxypropane, H⁺, acetone; c, LDA, dibenzyl phosphoroophoricodichloridate, THF; d, 50% aqueous CF₃COOH, e, H₂, 10% Pd on carbon, ethanol; Fb(OAc)₃; f, Cbz-glycine, isobutyl chloroformate, ethyl acetate; g, N-formylglycine, dicyclohexylcarbodiimide, THF.

**Table 1**

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>pH</th>
<th>Substrate</th>
<th>Vₘₐₓ</th>
<th>Kₘ</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>units/mg</td>
<td>mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chicken liver</td>
<td>7.5</td>
<td>C-PRA</td>
<td>3.82 ± 0.38</td>
<td>1.46 ± 0.27</td>
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<tr>
<td>Chicken liver</td>
<td>8.0</td>
<td>C-PRA</td>
<td>1.95 ± 0.16</td>
<td>0.37 ± 0.08</td>
</tr>
<tr>
<td>Chicken liver</td>
<td>8.0</td>
<td>PRA</td>
<td>1.13 ± 0.08</td>
<td>0.028 ± 0.004</td>
</tr>
<tr>
<td>E. coli</td>
<td>7.5</td>
<td>C-PRA</td>
<td>4.76 ± 1.25</td>
<td>5.82 ± 2.00</td>
</tr>
<tr>
<td>E. coli</td>
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<td>3.58 ± 0.26</td>
<td>1.32 ± 0.18</td>
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<td>0.03 ± 0.003</td>
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</table>

* Obtained at 18 °C.
* Specific activity obtained at 37 °C.
* Data from Shen et al. (1990).
active component. In contrast, the $V_{\text{max}}$ is less sensitive to the pH change. It is reduced about 2-fold for the chicken liver enzyme and approximately 25% for the E. coli enzyme upon increasing the pH from 7.5 to 8.0. C-GAR was tested as a substrate for the reverse reaction catalyzed by GAR synthetase, using a coupled assay for ATP (Shen et al., 1990). This analogue was processed by both enzymes, albeit considerably less efficiently than GAR (Table I).

We attempted to assay C-FGAR as a substrate for E. coli FGAM synthetase (reaction 4 in Scheme 1) using a coupled assay for ADP (Schendel et al., 1989); however, the residual ATPase activity in this preparation precluded this approach.

We next decided to try the AIR synthetase coupled assay (Schendel and Stubbe, 1986). This assay requires not only that C-FGAR be processed by FGAM synthetase, but that the C-FGAM produced serve as a substrate for AIR synthetase. At 125 $\mu$M C-FGAR we obtained a specific activity that was 43% of the specific activity obtained with saturating FGAR. Clearly we have not fully characterized the FGAM synthetase reaction with C-FGAR as substrate, but this result does indicate that both FGAM synthetase and AIR synthetase accept the carbocyclic analogues, C-FGAR and C-FGAM, respectively, as substrates.

Our preliminary results indicate the carbocyclic analogues of the early intermediates of de novo purine biosynthesis are accepted as substrates by their respective enzymes. However, the efficiency of their utilization, especially the processing of C-GAR in the reverse direction by GAR synthetase, is reduced compared with the natural substrates. One possible explanation for this is that the unnatural enantiomer in these racemic preparations is an inhibitor of the enzyme. We are continuing our efforts to develop carbocyclic analogues for de novo purine biosynthesis using a stereospecific route to the enantiomer of the carbocyclic analogues corresponding to d-ribose (Wolfe et al., 1990). In conjunction with this, we have recently developed a new synthesis of 2,3-O-cyclohexylidene-D-ribonolactone from D-ribose.²

REFERENCES
