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Synthesis and Evaluation of Inhibitors for *Escherichia coli* Carbamyl Phosphate Synthetase

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The design, synthesis, and evaluation of potential multisubstrate analog inhibitors for *Escherichia coli* carbamyl phosphate synthetase (CPS) are described. The inhibitors, which combine structural features of glutamine plus ammonia or of glutamine plus a mimic of the electrophilic ammonia acceptor, were designed to probe the spatial relationship between the substrate binding sites on the two subunits of the enzyme. Of the inhibitors described, 2-amino-3-[(*N*-phosphorylglycyl)amino]propanoate, **2a**, with a K_i value of 60 μM, represents the most potent reversible inhibitor yet reported for *E. coli* CPS. The synthetic route to the inhibitors utilized a convenient protection strategy whose refinement was described previously for manipulating ω-amino- or ω-carboxyl-substituted α-amino acids (J. M. Scholtz and P. A. Bartlett, 1988, *Synthesis*, 542).

INTRODUCTION

Carbamyl phosphate is an intermediate in both the arginine and the pyrimidine biosynthetic pathways. In *Escherichia coli* carbamyl phosphate for both pathways is synthesized from HCO_3^-, ATP, and glutamine by a single enzyme, glutamine-dependent carbamyl phosphate synthetase (CPS)[1] (*1*). This enzyme, encoded by the *car*AB operon (*2*), exists as a heterodimer with subunits of molecular weight 42 and 120 kDA (*3, 4*). The small, glutamine-binding subunit catalyzes cleavage of the amide moiety and transfer of the resulting ammonia to the large, synthetase subunit. The latter subunit contains the binding sites for the substrates HCO_3^- and MgATP, as well as those for the allosteric effectors ornithine, IMP, and UMP (*3–6*). In addition to formation of carbamyl phosphate, the large subunit also catalyzes two partial reactions: bicarbonate-dependent ATP cleavage and ATP synthesis from carbamyl phosphate and ADP (*3, 5, 7*). The large subunit can be prepared as an active, NH_3-utilizing CPS either by mild dissociation of the holoenzyme (*5*) or by selective expression of the *car*B gene product (*7*).

The stoichiometry of the overall reaction (Eq. [1]) was established by Anderson and Meister (*8, 9*), who showed that 2 mol of MgATP and 1 mol each of HCO_3^-

[1] Abbreviations used: CP, carbamyl phosphate; CPS, carbamyl phosphate synthetase; EDTA, ethylenediaminetetraacetic acid; PK, pyruvate kinase; LDH, L-lactate dehydrogenase; GDH, L-glutamate dehydrogenase; Gln, L-glutamine; Glu, L-glutamate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TFA, trifluoroacetic acid.

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and glutamine are consumed for each mole of carbamyl phosphate formed (Eq. [1]):

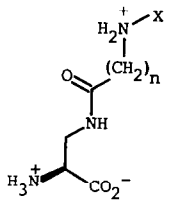
$$2\ \text{MgATP} + \text{HCO}_3^- + \text{L-Gln} \rightarrow 2\ \text{MgADP} + P_i + \text{L-Glu} + \text{CP} \qquad [1]$$

Substantial experimentation has established that the reaction proceeds in three steps (*10–13*) (Eq. [2]):

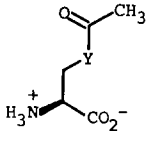
$$\begin{aligned} \text{HCO}_3^- + \text{MgATP} &\rightleftharpoons {}^-\text{O}_2\text{COPO}_3^{2-} + \text{MgADP} \\ {}^-\text{O}_2\text{COPO}_3^{2-} + \text{Gln (NH}_3) &\longrightarrow {}^-\text{O}_2\text{CNH}_2 + \text{Glu} + P_i \\ {}^-\text{O}_2\text{CNH}_2 + \text{MgATP} &\rightleftharpoons \text{H}_2\text{NCO}_2\text{PO}_3^{2-} + \text{MgADP} \end{aligned} \qquad [2]$$

The kinetic mechanism has also been determined (*14*). Bicarbonate is activated by 1 mol of ATP with the formation of carboxyphosphate, which is coupled with ammonia in the second step to give enzyme-bound carbamate. This intermediate is finally phosphorylated by the second mole of MgATP to form carbamyl phosphate (*8, 14*). In the absence of a crystal structure of CPS, information on the spatial relationship between the various binding sites is limited to NMR relaxation (*15, 16*) and fluorescence energy transfer studies (*17*). These have established the distances between various metal binding sites, protein sulfhydryl groups, and specific atoms in glutamate and ATP; however, there is no direct evidence for the relationship between the sites at which ammonia is produced and utilized.

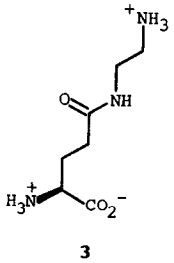
In designing potential multisubstrate analogs as inhibitors for CPS, we sought, on the one hand, to take advantage of the enzyme's known affinity for glutamine antagonists (*18*) and, on the other, to gain specificity for this particular glutamine-dependent amidotransferase by incorporating amino or anionic groups to bind in the ammonia acceptor or carboxyphosphate sites, respectively. We envisaged that such inhibitors could also provide further information on the distance between the various sites on the small and large subunits of CPS. The inhibitors we describe are derivatives of 2,3-diaminopropanoic acid (**1, 2,** and **4**) and glutamic acid (**3** and **5**). In the series of amino analogs (**1a, 1b,** and **3**), the amino group is extended from one to three bond lengths relative to the amide nitrogen of glutamine, and it is incorporated as a basic amine in order to best mimic ammonia. Addition of ammonia to carboxyphosphate presumably proceeds via the tetrahedral adduct **6.** To incorporate a partial mimic of this structure in our inhibitors, we



1: X = H
2: X = $PO_3^=$
a: n = 1
b: n = 2



4: Y = NH
5: Y = CH_2



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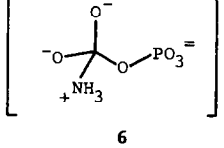

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synthesized the phosphoramidate derivatives, **2.** Finally, to explore the possibility that a ketone moiety at the δ-carbonyl position of glutamine would undergo a favorable, hemithioketal interaction with the catalytic thiol moiety of the small subunit, we synthesized the methyl ketone **5** and, for comparison, the amide **4.**

EXPERIMENTAL PROCEDURES

Synthetic Chemistry

General

Methylene chloride, acetonitrile, diisopropylethylamine, and triethylamine were distilled from calcium hydride and used immediately or stored over 4-Å molecular sieves under nitrogen. Tetrahydrofuran, 1,2-dimethoxyethane, and diethyl ether were distilled from sodium/benzophenone. Tetrabenzyl pyrophosphate was prepared by the method of Khorana and Todd (*19*). All other reagents were used as obtained commercially. Gaseous nitrogen was dried by passage over Drierite and potassium hydroxide columns. Molecular sieves were activated prior to use by heating to 150°C at 0.2 mm for 18 h and were stored under vacuum. Infrared spectra were obtained in chloroform and NMR spectra in deuterochloroform, unless otherwise indicated. ^{31}P NMR spectra are referenced to external trimethylphosphate (sealed capillary) at δ 3.086. Unless otherwise indicated, reaction workups culminated in washing the organic layer with 5% aqueous sodium bicarbonate, water, 0.5 N HCl, and brine, drying over magnesium sulfate, and evaporating the solvent under reduced pressure. Chromatography refers to silica gel chromatography as described by Still *et al.* (*20*).

General Procedure for Hydrogenolytic Deprotection: Method A

The carbobenzoxy substrate (100 mg) is dissolved in a 4:1:1 mixture of methanol:water:ethyl acetate (10 ml) containing 1 N HCl (1 ml). Pd/C (40 mg, 10%) is added with stirring and the reaction is stirred under 1 atm of hydrogen gas at room temperature. After 2 h, the hydrogen atmosphere is replaced with nitrogen, the catalyst is removed by filtration through Celite, and the solvents are removed under reduced pressure followed by lyophilization to afford the dihydrochloride salts as white powders.

(S)-2-Amino-3-(glycylamino)propanoic acid, dihydrochloride salt, ***1a.*** Hydrogenolysis via Method A; yield: 98%; mp 218–221°C; ir (KBr) 3600–2800, 2900, 1740, 1700, 1550, 1400, 1335, 1210, 1180 cm^{-1}; ^1H NMR (D_2O) δ 3.61–3.74 (ddd, 2, J = 4.3, 5.6, 15.1), 3.67 (s, 2), 4.04 (dd, 2, J = 5.6, 4.3); ^{13}C NMR (D_2O) δ 43.5, 48.1, 55.7, 174.6, 178.2; *Anal.* Calcd for $C_5H_{13}N_3O_3Cl_2$: C, 25.64; H, 5.59; N, 18.02. Found: C, 25.51; H, 5.44; N, 18.11.

(S)-2-Amino-3-[(3-aminopropanoyl)amino]propanoic acid, dihydrochloride Salt, ***1b.*** Hydrogenolysis via Method A; yield: 97%; mp 210–211°C; ir (KBr) 3600–2800, 2950, 1765, 1705, 1500, 1450, 1335, 1210 cm^{-1}; ^1H NMR (D_2O) δ 3.52 (d, 2, J = 4.9), 3.61–3.74 (ddd, 2, J = 4.4, 5.2, 14.2), 3.87 (d, 2, J = 5.0), 4.04 (dd, 2, J =

5.6, 4.3); ^{13}C NMR (D_2O) δ 44.0, 46.4, 48.4, 55.1, 175.4, 179.4; *Anal.* Calcd for $C_6H_{15}N_3O_3Cl_2$: C, 29.02; H, 6.08; N, 17.00. Found: C, 28.71; H, 6.24; N, 16.85.

General Procedure for Hydrogenolytic Deprotection of Phosphoramidates: Method B

The fully protected phosphoramidate (0.125 mmol) is dissolved in 2:1 THF: H_2O (10 ml). Li_2CO_3 (27.8 mg, 0.375 mmol) and 10% Pd/C (12.3 mg) are added, and the reaction mixture is stirred under 1 atm of hydrogen gas at room temperature. After 8 h, the catalyst is removed by filtration through Celite and the solvents are removed under reduced pressure followed by lyophilization to afford a white powder which is a mixture of product and $LiHCO_3$. The composition of this mixture is determined by phosphorus combustion analysis.

(S)-2-Amino-3-[(N-phosphorylglycyl)amino]propanoic acid, trilithium salt, ***2a.*** Hydrogenolysis of **10a** (below) via Method B above; P analysis, 6.34% P, 53.1% of the mixture is **2a;** ir (KBr) 3600–2800, 2900, 1740, 1700, 1550, 1400, 1335, 1210, 1180 cm^{-1}; ^1H NMR (D_2O) δ 3.61–3.74 (ddd, 2, J = 4.5, 5.3, 14.5), 3.76 (d, 2, J = 12.8), 4.04 (dd, 2, J = 5.6, 4.3); ^{13}C NMR (D_2O) δ 44.5, 49.5, 55.1, 175.2, 177.7; ^{31}P NMR (D_2O) δ 8.50; HRMS (FAB, MH^+) Calcd for $C_5H_9N_3PO_6Li_3$: 258.9348. Found: 258.9344.

(S)-2-Amino-3-[[3-(phosphorylamino)propanoyl]amino]propanoic acid, trilithium salt, ***2b.*** Hydrogenolysis of **10b** (below) via Method B above; P analysis, 5.87% P, 51.7% of the mixture is **2b;** ir (KBr) 3600–2800, 2900, 1730, 1690, 1540, 1420, 1335, 1210 cm^{-1}; ^1H NMR (D_2O) δ 3.65–3.73 (ddd, 2, J = 4.5, 5.3, 14.5), 3.76 (dd, 2, J = 5.9, 13.9), 3.88 (dd, 2, J = 6.0, 13.2), 4.08 (dd, 2, J = 5.6, 4.12); ^{13}C NMR (D_2O) δ 45.7, 49.1, 52.8, 55.8, 173.4, 178.9; ^{31}P NMR (D_2O) δ 9.10; HRMS (FAB, MH^+) Calcd for $C_6H_{11}N_3PO_6Li_3$: 272.9619. Found: 272.9625.

(S)-3-Carbobenzoxy-N-[2-[N-(carbobenzoxy)amino]ethyl]-5-oxo-4-oxazolidinepropanamide. A solution of (*S*)-3-carbobenzoxy-5-oxo-4-oxazolidinepropanoic acid **11** (*21*) (2.67 g, 9.10 mmol), *N*-hydroxybenzotriazole (1.23 g, 9.10 mmol), and **12** (see below) (1.77 g, 9.10 mmol) in distilled DMF (25 ml) was stirred under an atmosphere of nitrogen at 0°C. Dicyclohexylcarbodiimide (1.88 g, 9.10 mmol) was added, and the solution was stirred overnight at 0°C. The dicyclohexylurea was removed by filtration, and the resulting solution was partitioned between methylene chloride and 1 N HCl. The organic layer was washed with additional HCl, water, sat $NaHCO_3$, water, and brine. After drying over $MgSO_4$, the solvent was removed by evaporation at reduced pressure to afford 4.07 g (94%) of a viscous oil. After purification by flash chromatography, 3.52 g (83%) of the amide was obtained as a white solid; mp 105–106°C; ir 3460, 3370, 3010, 2950, 1805, 1725, 1715, 1660, 1520, 1455, 1420, 1360, 1260, 1170, 1130, 1060 cm^{-1}; ^1H NMR δ 2.22–2.28 (m, 4), 3.25–3.31 (m, 4), 4.34 (t, 1, J = 5.5), 5.09 (s, 2), 5.17 (s, 2), 5.20 (d, 2, J = 4.8), 5.50 (d, 1, J = 4.1), 6.15–6.25 (br s, 1), 7.33 (s, 5), 7.36 (s, 5); ^{13}C NMR δ 26.4, 31.0, 39.9, 40.7, 66.6, 68.0, 78.0 127.9, 128.0, 128.2, 128.4, 128.5, 128.6, 135.1, 136.3, 153.0, 157.0, 171.8; *Anal.* Calcd for $C_{24}H_{27}N_3O_7$: C, 61.39; H, 5.79; N, 8.94. Found: C, 61.11; H, 5.70; N, 9.04.

(S)-N^5-(2-Aminoethyl)glutamine, dihydrochloride salt, ***3.*** Hydrogenolysis of

the above compound via Method A; yield: 96%; mp 205–207°C; ir (KBr) 3400–2800, 2990, 1750, 1590, 1540, 1410, 1320, 1265, 1155 cm^{-1}; ^1H NMR (D_2O) δ 2.05 (m, 2), 2.36 (m, 2), 2.94 (t, 2, J = 5.8), 3.31 (t, 2, J = 5.9), 3.89 (t, 2, J = 6.5); ^{13}C NMR (D_2O) δ 27.6, 33.3, 39.1, 41.5, 54.4, 173.6, 177.4; *Anal.* Calcd for $C_7H_{17}N_3O_3Cl_2$: C, 32.05; H, 6.53; N, 16.09. Found: C, 32.50; H, 6.41; N, 16.31.

(S)-3-Acetamido-2-aminopropanoic acid, ***4.*** A 20-ml round-bottomed flask was charged with 4-aminomethyl-3-carbobenzoxy-5-oxazolidinone, trifluoroacetate salt (1.76 g, 4.83 mmol, prepared as described below), and CH_2Cl_2 (5 ml) with stirring under an atmosphere of nitrogen. After cooling to 0°C, acetyl chloride (3.43 ml, 48.3 mmol) and triethylamine (1.49 ml, 10.6 mmol) were added in succession. The reaction mixture was stirred at 0°C for 2 h and allowed to warm to room temperature for an additional 2 h. The sat $NaHCO_3$ (10 ml) was added, the mixture was worked up, and the crude product was purified by chromatography (8% EtOAc/CH_2Cl_2) to give 1.14 g (81%) of the acetamide as a yellow oil: ir 3060, 2960, 1805, 1725, 1705, 1670, 1535, 1455, 1260, 1220, 1140, 1035, 740, 700 cm^{-1}; ^1H NMR δ 1.93 (s, 3), 3.65 (m, 1), 3.90 (m, 1), 4.30 (t, 1, J = 4.9), 5.19 (s, 2), 5.22 (br s, 1), 5.45 (br s, 1), 6.1–6.4 (br s, 1), 7.36 (s, 5); ^{13}C NMR δ 22.9, 39.7, 54.9, 68.2, 78.1, 128.3, 128.6, 128.7, 135.2, 152.8, 170.7, 171.8; *Anal.* Calcd for $C_{14}H_{16}N_2O_5$: C, 57.51; H, 5.52; N, 9.62. Found: C, 57.35; H, 5.61; N, 9.88.

This material was hydrogenolyzed by Method A above to give **4** in 98% yield: mp 175–178°C; ir (KBr) 3420–2850, 2995, 1745, 1580, 1480, 1405, 1300, 1280, 1140 cm^{-1}; ^1H NMR (D_2O) δ 1.88 (s, 3), 3.45 (m, 1), 3.6–3.8 (m, 2), 4.67 (HOD); ^{13}C NMR (D_2O) δ 23.2, 40.8, 55.1, 169.1, 171.4; *Anal.* Calcd for $C_5H_{10}N_2O_3$: C, 41.06; H, 6.89; N, 19.24. Found: C, 40.92; H, 6.81; N, 19.51.

(S)-2-Amino-5-oxo-hexanoic acid, ***5.*** A solution of (*S*)-3-carbobenzoxy-4-(3-oxobutyl)-oxazolidine-5-one **13** (*21*) (195.1 mg, 0.67 mmol) in 30% HBr/HOAc (1.5 ml) was stirred at room temperature for 1 h. The solution was partitioned between H_2O and EtOAc, and the aqueous layer was washed with additional EtOAc and lyophilized to afford **5** as a slightly yellow powder; yield: 93%; mp 201–203°C; ir (KBr) 3500–2800, 2990, 1690, 1600, 1400, 1215, 1130 cm^{-1}; ^1H NMR (D_2O) δ 2.1–2.6 (m, 2), 2.32 (s, 3), 3.00 (t, 2, J = 7.8), 4.67 (HOD), 4.93 (t, 1, J = 8.1); ^{13}C NMR (D_2O) δ 21.0, 26.6, 41.0, 69.4, 174.2, 200.9; *Anal.* Calcd for $C_6H_{12}NO_3Br$: C, 31.87; H, 5.35; N, 6.22. Found: C, 32.02; H, 5.22; N, 6.34.

General Method for the Synthesis of ***8*** *and* ***9***

(*S*)-3-Carbobenzoxy-4-[(*N*-t-butoxycarbonyl)aminomethyl]-5-oxazolidinone (*21*) (1.40 g, 4.0 mmol) is dissolved in CH_2Cl_2 and cooled to 0°C with stirring under an atmosphere of nitrogen. Trifluoroacetic acid (1.54 ml, 20 mmol) is added in one portion and the solution is stirred for 2 h. The ice bath is removed and the solution is allowed to warm to room temperature over 30 min. The volatile materials are removed by concentration under reduced pressure and the oily residue is triturated with diethyl ether to give the TFA salt as a white, hygroscopic powder which is used directly in the next step of the sequence.

The TFA salt and potassium bicarbonate (820 mg, 8.20 mmol) are stirred in acetonitrile (25 ml) under an atmosphere of N_2. The appropriate *N*-hydroxysuc-

cinimide ester (of Cbz-Gly, Cbz-β-Ala, Boc-Gly, or Boc-β-Ala) (4.10 mmol) is added and the mixture is stirred at room temperature for 5–12 h. After the reaction mixture is diluted with sat NH_4Cl (15 ml) and carried through the standard aqueous workup, the amide **8** or **9** is purified by chromatography to give a white crystalline compound.

(S)-3-Carbobenzoxy-4-[N-[N-(t-butoxycarbonyl)glycyl]aminomethyl]-5-oxazolidinone, ***8a.*** Yield: 89%; mp 132–134°C; ir 3430, 3330, 3025, 2990, 1805, 1715, 1520, 1310, 1200, 1140 cm^{-1}; ^1H NMR δ 1.45 (s, 9), 3.65 (m, 1), 3.72 (d, 2, $J = 5.5$), 4.05 (br m, 1), 4.35 (br s, 1), 5.21 (s, 2), 5.26 (m, 1), 5.43 (br s, 2), 7.05 (br s, 1), 7.37 (s, 5); ^{13}C NMR δ 28.2, 39.1, 44.1, 55.1, 68.0, 77.2, 80.1, 128.2, 128.5, 128.6, 135.2, 152.6, 156.0, 170.6; *Anal.* Calcd for $C_{19}H_{25}N_3O_7$: C, 56.01; H, 6.18; N, 10.31. Found: C, 55.94; H, 6.19; N, 10.40.

(S)-3-Carbobenzoxy-4-[N-[3-(t-butoxycarbonylamino)propanoyl]aminomethyl]-5-oxazolidinone, ***8b.*** Yield: 92%; mp 147–148°C; ir 3425, 3325, 3015, 2900, 1805, 1720, 1500, 1420, 1380, 1300, 1245, 1080 cm^{-1}; ^1H NMR δ 1.43 (s, 9), 3.32–3.41 (br m, 2), 3.45–3.55 (br m, 2), 3.62–3.71 (m, 1), 4.35 (m, 1), 5.15–5.25 (m, 4), 5.47 (br s, 1), 6.95 (br s, 1), 7.37 (s, 5); ^{13}C NMR δ 28.1, 35.7, 36.4, 39.0, 54.8, 67.8, 77.8, 78.9, 128.0, 128.3, 128.4, 135.1, 152.5, 155.8, 171.4, 172.3; *Anal.* Calcd for $C_{20}H_{27}N_3O_7$: C, 56.97; H, 6.45; N, 10.01. Found: C, 56.94; H, 6.39; N, 10.04.

(S)-3-Carbobenzoxy-4-[N-[N-(carbobenzoxy)glycyl]aminomethyl]-a5-oxazolidinone, ***9a.*** Yield: 94%; mp 129–130°C; ir 3420, 3330, 3010, 2980, 1805, 1720, 1515, 1420, 1365, 1220, 1115, 1025 cm^{-1}; ^1H NMR δ 1.43 (s, 9), 3.55–3.70 (m, 1), 3.72 (d, 2, $J = 5.5$), 3.90–4.05 (m, 1), 4.34 (br s, 1), 5.21 (s, 2), 5.28 (d, 1, $J = 4.0$), 5.45–5.55 (br s, 2), 7.10 (br m, 1), 7.37 (s, 5); ^{13}C NMR δ 28.2, 39.1, 44.1, 55.1, 68.0, 77.6, 80.2, 128.2, 128.4, 128.5, 135.5, 152.6, 156.1, 170.6; *Anal.* Calcd for $C_{22}H_{23}N_3O_7$: C, 59.83; H, 5.25; N, 9.56. Found: C, 59.71; H, 5.21; N, 9.42.

(S)-3-Carbobenzoxy-4-[N-[3-(carbobenzoxyamino)propanoyl]aminomethyl]-5-oxazolidinone, ***9b.*** Yield: 94%; mp 142–144°C; ir 3420, 3335, 3010, 2985, 1805, 1720, 1515, 1420, 1220, 1115 cm^{-1}; ^1H NMR δ 3.65–4.05 (br m, 6), 4.38 (t, 1, $J = 5.9$), 5.10 (s, 4), 5.18–5.25 (m, 1), 5.48 (br s, 2), 6.80 (br s, 1), 7.37 (s, 5), 7.39 (s, 5); ^{13}C NMR δ 39.1, 44.3, 46.0, 55.0, 67.3, 68.2, 77.6, 128.2, 128.4, 128.5, 135.5, 136.0, 141.7, 169.8; *Anal.* Calcd for $C_{23}H_{25}N_3O_7$: C, 60.63; H, 5.53; N, 9.26. Found: C, 60.81; H, 5.64; N, 9.18.

General Method for N-Phosphorylation

The ω-t-butoxycarbonyl compound **8a** or **8b** (0.30 mmol) is dissolved in CH_2Cl_2 (20 ml) with stirring at room temperature. HCl gas, generated in a separate flask from NaCl and H_2SO_4, is bubbled through the solution for 30 min. The HCl gas inlet tube is removed and the solution is stirred for an additional 15 min while a white precipitate forms. The volatile materials are removed by evaporation under reduced pressure to give a white, hygroscopic powder, which is used immediately in the next step.

A 50-ml round-bottomed flask is charged with distilled CH_3CN (20 ml), powdered 4-Å molecular sieves (500 mg), and the amine hydrochloride salt (0.30

mmol). After stirring for 30 min at room temperature under an atmosphere of N_2, diisopropylethylamine (125 μl, 0.73 mmol) is added, followed by a solution of tetrabenzyl pyrophosphate (265 mg, 0.44 mmol) in CH_3CN (5 ml). After stirring for 12–18 h, the reaction mixture is partitioned between 0.1 N HCl and EtOAc, and the aqueous layer is extracted with additional EtOAc. The combined organic layers are washed with H_2O, sat $NaHCO_3$, H_2O, and brine and dried over Na_2SO_4, and the solvents are removed by evaporation at reduced pressure to give an oil which is purified by silica gel chromatography.

(S)-3-Carbobenzoxy-4-[N-[N-[dibenzyloxyphosphinyl)glycyl]aminomethyl]-5-oxazolidinone, ***10a.*** Yield: 71%; ir 3460, 3020, 2990, 1801, 1740, 1715, 1500, 1440, 1420, 1370, 1195 cm^{-1}; ^1H NMR δ 3.5–3.7 (br m, 4), 4.22 (br s, 1), 4.55 (br s, 1), 5.14 (s, 2), 5.24 (d, 4, J = 13.1), 5.43 (br s, 1), 5.54 (br s, 1), 7.3–7.5 (m, 15); ^{31}P NMR δ −0.50; ^{13}C NMR δ 39.8, 42.0, 57.5, 67.0, 67.9, 68.3, 78.5, 126–128 (>10 lines), 135.1, 135.4, 135.5, 166.0, 168.8, 172.7; *Anal.* Calcd for $C_{28}H_{40}N_3PO_8$: C, 58.20; H, 6.98; N, 7.28; P, 5.36. Found: C, 57.86; H, 7.09; N, 6.99; P, 5.11.

(S)-3-Carbobenzoxy-4-[N-[3-[N-(dibenzyloxyphosphinyl)amino]propanoyl]-aminomethyl]-5-oxazolidinone, ***10b.*** Yield: 71%; ir 3450, 3025, 2990, 1805, 1735, 1720, 1510, 1480, 1370, 1195 cm^{-1}; ^1H NMR δ 3.5–3.9 (br m, 6), 4.34 (br s, 1), 4.50 (br s, 1), 5.19 (s, 2), 5.22 (d, 4, J = 12.5), 5.48 (br s, 1), 5.34 (br s, 1), 7.3–7.5 (m, 15); ^{31}P NMR δ −0.65; ^{13}C NMR δ 36.6, 39.6, 43.8, 56.5, 67.8, 67.9, 68.6, 78.9, 126–128 (>10 lines), 135.1, 135.4, 135.5, 166.8, 169.6, 175.9; *Anal.* Calcd for $C_{29}H_{42}N_3PO_8$: C, 58.85; H, 7.15; N, 7.13; P, 5.40. Found: C, 59.16; H, 7.11; N, 6.89; P, 5.15.

2-[(Carbobenzyloxy)amino]ethylamine, ***12.*** A solution of distilled ethylenediamine (5.0 ml, 75 mmol) in distilled methylene chloride (50 ml) was stirred at 0°C under an atmosphere of nitrogen and benzyl chloroformate (5.4 ml, 40 mmol) was added via syringe over a 45-min period. After addition was complete, the solution was allowed to warm to room temperature over 1 h and then partitioned between methylene chloride and 1 N HCl. The organic layer was washed with additional 1 N HCl and the aqueous layers were combined, neutralized with KOH, and extracted with three portions of methylene chloride. The combined organic layers were washed with water and brine and dried over $MgSO_4$, and the solvent was removed by cold evaporation at reduced pressure to give 1.81 g (23%) of a colorless liquid which was used immediately in the next reaction of the sequence; ^1H NMR δ 1.33 (s, 2), 2.75 (t, 2, J = 5.9), 3.18 (dt, 2, J = 5.8, 6.1), 5.07 (s, 2), 5.60 (br s, 1), 7.32 (s, 5).

Enzymology

General

All cofactors, auxiliary enzymes, substrates, buffers, and biochemical reagents were of the highest grade available from commercial sources and were used without further purification. The chromatographic media were obtained from Bio-Rad or Sigma and were used as described by the suppliers. Centrifugation was accomplished with a Sorvall RC-5C and an SS-34 rotor. Buffers were prepared and

adjusted to the indicated pH at room temperature. Protein concentration was determined by the method of Lowry *et al.* (*22*) using bovine serum albumin as the standard.

Enzyme Purification

CPS was isolated from *E. coli B* (ATCC 11303, 3/4 log phase, minimal medium) by a modification of the published procedure (*23*). In our hands, the 40–65% ammonium sulfate fraction is unstable to storage in the reported buffer solution. In contrast, enzyme stored at 4°C in a buffer consisting of Hepes (200 mM, pH 7.65), EDTA (0.5 mM), dithiothreitol (1 mM), dimethyl sulfoxide (30%, v/v), and glycerol (5%, v/v) retains activity for up to 1 month.

Enzyme Assays

All assays were conducted at 37°C in 1.0-cm cuvettes (1.0 ml total volume) using a Cary 219 spectrophotometer equipped with a circulating constant temperature water bath. Absorbances were monitored at 340 nm and the initial rate data were analyzed with the OLIS data system. One unit of CPS activity is defined as the amount of enzyme required to produce 1 μmol of CP/min/mg of protein (mM min^{-1} mg^{-1}).

Assay Based on ADP Formation

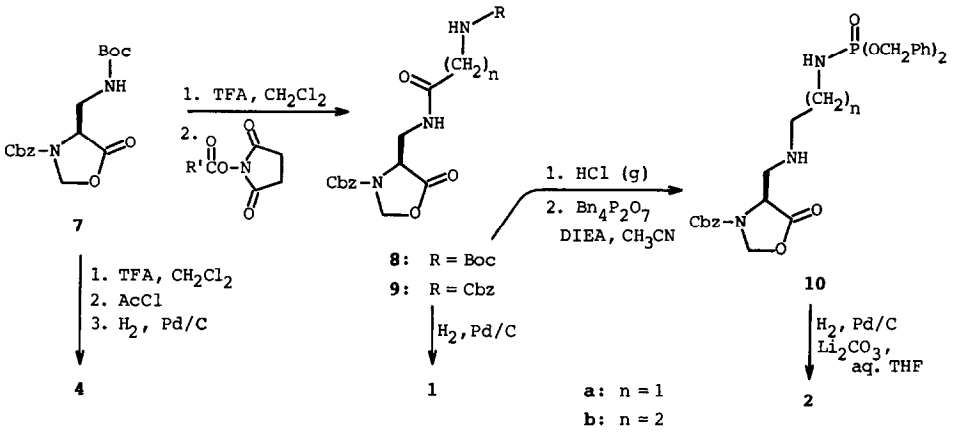
CPS activity can be measured by following the amount of ADP produced by a standard coupled assay utilizing PK and LDH. This assay has been described previously (*23*) and was employed both for inhibitor studies and for routine activity measurements during the isolation sequence. A typical assay contained Hepes (200 mM, pH 8.0), KCl (100 mM), $MgCl_2$ (25 mM), L-ornithine (10 mM), NADH (0.15 mM), PEP (1.0 mM), PK (1.0 U/ml), LDH (1.0 U/ml), ATP (10 mM), $KHCO_3$ (20 mM), and varying amounts of glutamine, NH_4Cl, CPS (usually 1.5–2.0 μg of protein), and inhibitor, depending on the particular study. The reaction was initiated by addition of CPS.

Assay Based on Glutamate Formation

An alternative assay developed to measure glutaminase activity directly employs GDH as a coupling enzyme. A typical assay contained Hepes (200 mM, pH 8.0), KCl (100 mM), $MgCl_2$ (25 mM), L-ornithine (10 mM), NADP (0.25 mM), GDH (2.0 U/ml), ATP (10 mM), $KHCO_3$ (20 mM), and varying amounts of glutamine, NH_4Cl, CPS (usually 1.5–2.0 μg protein), and inhibitor, depending on the particular study. The reaction was initiated by addition of CPS.

Inhibitor Studies

The inhibition constants (K_i values) for the compounds listed in Table 1 were determined using the programs developed by Cleland (*24*). In all cases, the inhibi-



SCHEME 1

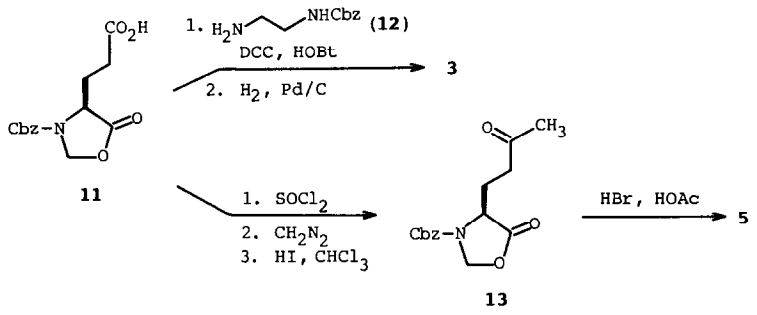
tors were found to be competitive with glutamine. The errors in the K_i values given in Table 1 are all ±5–7% (standard errors).

RESULTS AND DISCUSSION

Synthetic Chemistry

The synthetic route utilized in the preparation of the amides **1** and **2** is depicted in Scheme 1. The differentially protected 2,3-diaminopropanoic acid derivative **7** was prepared from commercially available *N*-Cbz-L-aspartic acid as described previously (*21*). The terminal Boc group was removed with TFA and the amine was acylated with the appropriately protected *N*-hydroxysuccinimide esters of glycine or β-alanine (*25*). The Cbz groups of **9a** and **9b** were removed by hydrogenolysis, which also resulted in ring opening of the oxazolidinone to afford two of the targets, **1a** and **1b,** directly. The phosphoramidates **10** were prepared from the Boc derivatives **8** by selective deprotection and phosphorylation with tetrabenzyl pyrophosphate (*19*). The final hydrogenolytic deprotection was carried out under basic conditions (Li_2CO_3 in aqueous THF) in order to avoid cleavage of the acid-sensitive P–N bonds in **2a** and **2b.**

Three additional compounds were synthesized and tested as potential inhibitors. N^5-(2-Aminoethyl)glutamine, **3,** was prepared from the oxazolidinone derived from glutamic acid, **11** (*21*). A standard DCC coupling of the latter material with the mono-Cbz derivative of ethylenediamine, **12,** followed by hydrogenolysis, afforded **3.** In order to assess the difference between an amide or a ketone linkage in the δ position of the glutamate moiety, and specifically with the hope that the ketone could form a hemithioketal in the active site of the enzyme, the two methyl compounds **4** and **5** were prepared and evaluated. The acetamide **4** was synthesized as depicted in Scheme 1 for the amino-substituted derivatives. The protected methyl ketone **13** has been described previously (*21*); it was depro-



SCHEME 2

tected under nonreductive conditions (HBr in acetic acid) to preclude cyclization to the methylproline analog.

Enzymology

The published procedure for isolation of CPS from *E. coli* (*23*) was modified to reduce the instability problem we encountered on trying to store the partially purified enzyme in the reported buffers. As described under Experimental Procedures, in a buffer system consisting of EDTA (0.5 mM), dithiothreitol (1 mM), dimethyl sulfoxide (30%, v/v), and glycerol (5%, v/v), enzyme activity was stable for up to 1 month at 4°C. This buffer was shown not to affect the kinetic characteristics of the enzymatic reaction, as determined by the K_m and V_{max} values.

The conventional assay for CPS activity involves a determination of the ADP produced in the steps in which bicarbonate and carbamate are activated (*23*). However, CPS has a slow, bicarbonate-dependent ATPase activity which is independent of glutamine hydrolysis or carbamyl phosphate formation, and it was not clear a priori whether this background rate would interfere with our evaluation of glutamine–carboxyphosphate multisubstrate analogs. It was therefore desirable to develop an alternative method of determining CPS activity, one which would measure directly the amount of glutamate produced in the reaction. As described under Experimental Procedures, a coupled assay was developed utilizing glutamate dehydrogenase.

Inhibition Results

The results of the enzymatic evaluation of the inhibitors are shown in Table 1. While all of the inhibitors were found to be competitive with glutamine, they were not competitive with the bicarbonate- or ammonia-dependent reactions. Under the standard conditions, the bicarbonate-dependent ATPase activity proved to be insignificant. Thus in each case the K_i values determined from the two assays were identical within experimental error.

The inhibition results show some interesting trends. First, the longer inhibitors **1b** and **2b** are not as effective as the shorter analogs **1a** and **2a**, suggesting that the optimal distance between the binding sites on the two subunits has already been

TABLE 1

Inhibition of CPS by Multisubstrate Analogs[a]

Compound	K_i (mM)	
	PK assay	GDH assay
1a	0.21	0.28
1b	2.9	2.1
2a	0.061	0.066
2b	1.6	nd
3	1.4	2.0
4	2.3	nd
5	1.9	nd

[a] Determined at pH 8.0, 37°C, as described in the text; nd, not determined.

realized in the latter compounds. This conclusion is supported by the weak inhibition found for analog **3** as well. Moreover, the *N*-phosphoryl derivatives **2a** and **2b** are better inhibitors than the amino compounds, despite their considerably greater polarity. In fact, the shorter phosphoramidate **2a** is the most potent, reversible inhibitor so far reported for CPS. It is appealing to suggest that the phosphoramidate moiety is occupying the binding site of the electrophilic ammonia acceptor, and that the zwitterionic phosphoramidate mimics to some degree the tetrahedral adduct between ammonia and carboxyphosphate, **7**, as intended. However, the lack of competition between these inhibitors and ammonia or bicarbonate would argue against this interpretation.

Finally, the keto analog **5** is not bound more tightly to CPS than the corresponding amide, **4**, despite the potential for the former to form an adduct with the active site thiol. As a result of this observation, we did not attempt to synthesize the ketones corresponding to **1** or **2**.

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REFERENCES

1. PIÉRARD, A., AND WILSON, J. M. (1964) *Biochem. Biophys. Res. Commun.* **15**, 76–81.
2. MERGEAY, M., GIGOT, D., BECKMANN, J., GLANSDORFF, N., AND PIÉRARD, A. (1974) *Mol. Gen. Genet.* **133**, 299–316.
3. TROTTA, P. P., BURT, M. E., HASCHEMEYER, R. H., AND MEISTER, A. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 2599–2603.
4. MATTHEWS, S. L., AND ANDERSON, P. M. (1972) *Biochemistry* **11**, 1176–1183.

5. TROTTA, P. P., PINKUS, L. M., HASCHEMEYER, R. H., AND MEISTER, A. (1974) *J. Biol. Chem.* **249,** 492–499.
6. ANDERSON, P. M. (1977) *Biochemistry* **16,** 587–593.
7. RUBINO, S. D., NYUNOYA, H., AND LUSTY, C. J. (1987) *J. Biol. Chem.* **262,** 4382–4386.
8. ANDERSON, P. M., AND MEISTER, A. (1965) *Biochemistry* **4,** 2803–2809.
9. ANDERSON, P. M., AND MEISTER, A. (1966) *Biochemistry* **5,** 3157–3163.
10. POWERS, S. G., AND MEISTER, A. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 3020–3024.
11. WIMMER, W. J., ROSE, I. A., POWERS, S. G., AND MEISTER, A. (1979) *J. Biol. Chem.* **254,** 1854–1859.
12. RAUSHEL, F. M., AND VILLAFRANCA, J. J. (1979) *Biochemistry* **18,** 3424–3429.
13. RAUSHEL, F. M., AND VILLAFRANCA, J. J. (1980) *Biochemistry* **19,** 3170–3174.
14. RAUSHEL, F. M., ANDERSON, P. M., AND VILLAFRANCA, J. J. (1978) *Biochemistry* **17,** 5587–5591.
15. RAUSHEL, F. M., ANDERSON, P. M., AND VILLAFRANCA, J. J. (1983) *Biochemistry* **22,** 1872–1876.
16. KASPRAZYK, P. G., WHALEN-PEDERSON, E., ANDERSON, P. M., AND VILLAFRANCA, J. J. (1985) *Bioorg. Chem.* **13,** 98–109.
17. KASPRAZYK, P. G., ANDERSON, P. M., AND VILLAFRANCA, J. J. (1983) *Biochemistry* **22,** 1877–1882.
18. MEISTER, A., AND POWERS, S. G. (1978) *Adv. Enzyme Regul.* **162,** 289–315, and references cited therein.
19. KHORANA, H. G., AND TODD, A. R. (1953) *J. Chem. Soc.*, 2257–2260.
20. STILL, W. C., KAHN, M., AND MITRA, A. (1978) *J. Org. Chem.* **43,** 2953–2955.
21. SCHOLTZ, J. M., AND BARTLETT, P. A. (1989) *Synthesis*, 542–544.
22. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A.L., AND RANDALL, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
23. KASEMAN, D. S., AND MEISTER, A. (1985) *in* Methods in Enzymology (Meister, A., Ed.), Vol. 113, pp. 305–336, Academic Press, San Diego, CA.
24. CLELAND, W. W. (1979) *in* Methods in Enzymology (Purich, D. L., Ed.), Vol. 63, pp. 103–138, Academic Press, San Diego, CA.
25. ANDERSON, G. W., ZIMMERMAN, J. E., AND CALLAHAN, F. M. (1969) *J. Amer. Chem. Soc.* **86,** 1839–1842.