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Investigation of Mechanism of Nitrogen Transfer in Glucosamine 6-Phosphate Synthase with the Use of Transition State Analogs

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Several structural analogs of putative tetrahedral intermediates of the reaction catalyzed by the glutamine amide transfer domain of *Candida albicans* glucosamine 6-phosphate synthase have been designed and synthesized. Esters and amides of γ-phosphonic and γ-sulfonic analogs of glutamine and glutamic acid were tested as potential inhibitors of the enzyme. N-substituted amides **9** and **15** were found to be the strongest inhibitors in the series. Structure–activity relationship studies led to conclusions supporting the possibility of a direct nucleophilic attack of the glutamine amide nitrogen on an electrophilic site of the enzyme-bound fructose 6-phosphate as the most likely mechanism of nitrogen transfer in glucosamine 6-phosphate synthase.

INTRODUCTION

Glucosamine 6-phosphate synthase, EC 2.6.1.16, is a member of the family of enzymes transferring an amino group from the γ-amide function of L-glutamine to different acceptor molecules. The amidotransferase family comprises two subfamilies known as G-type and F-type, depending on location of the catalytic cysteinyl residue (*1*). GlcN-6-P[2] synthase belongs to the latter subfamily, together with three other proteins, namely phosphoribosylpyrophosphate (PRPP) amidotransferase, glutamate synthase, and asparagine synthetase (*1*), however differs from other members in its apparent inability to use exogenous ammonia as an alternative nitrogen donor (*2*) and catalyzes almost exclusively an irreversible reaction:

L-glutamine + D-fructose 6-phosphate → D-glucosamine 6-phosphate + L-glutamate.

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[2] Abbreviations used: GlcN-6-P, D-glucosamine 6-phosphate; Fru-6-P, D-fructose 6-phosphate; γ-Glu(P), (3-amino-3-carboxypropyl)-phosphonic acid; GLUPA, γ-glutamyl-*p*-nitroanilide; A_2pr, 2,3-diaminopropanoic acid; THF, tetrahydrofurane; DMF, dimethylformamide; DMSO, dimethylsulfoxide; MNDO, modified neglect of diatomic overlap.

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In the absence of Fru-6-P, the enzyme is capable of catalyzing the glutamine hydrolysis, but the velocity of this reaction is several orders of magnitude lower than that of GlcN-6-P formation (*3*).

The molecular mechanism of the complex reaction catalyzed by GlcN-6-P synthase has been extensively investigated and special attention has been paid to the mechanism of nitrogen transfer (*4–8*). There is little doubt that in G amidotransferases the amide nitrogen labilization is mediated by a catalytic triad Cys–His–Asp in a manner similar to that of cysteine proteases (*9*). The presence of such a system, previously postulated also for F amidotransferases (*10*), was ruled out and Cys-1 was identified as the only catalytic residue participating in nitrogen transfer in PRPP amidotransferase (*11*) and GlcN-6-P synthase (*12*).

Badet-Denisot *et al.* in a recent paper proposed three possible mechanisms of nitrogen transfer in GlcN-6-P synthase (*7*). The authors finally excluded a possibility of nitrogen labilization as a result of cyclization of glutamine into pyroglutamic acid, but two other possibilities can be still considered (Fig.1):

—a ''classical'' cleavage of the glutamine amide bond upon the action of Cys-1 nucleophile, followed by hydrolysis of the acyl-enzyme intermediate (Mechanism 1), based on the previous proposition of Chmara *et al.* (*4*). A transient release of ammonia which is subsequently transferred to Fru-6-P in an unknown way is likely to occur in this mechanism.

—a direct nucleophilic attack of the glutamine amide nitrogen on an electrophilic site of the enzyme-bound acceptor (Mechanism 2) in a manner similar to that proposed previously for asparagine synthetase (*13*). In this mechanism Cys-1 participates only in releasing of glutamate.

Various phosphonic, sulfonic and boronic derivatives have been previously proposed as analogs of tetrahedral intermediates formed transiently during different enzymatic reactions involving hydrolysis of amide or ester bonds, especially those catalyzed by proteases and esterases. Since presence of tetrahedral intermediates is also postulated in both possible mechanisms of nitrogen transfer carried out by GlcN-6-P synthase, we synthesized several γ-phosphono- and γ-sulfonic analogs of glutamine and glutamic acid—potential mimics of putative tetrahedral intermediates I, II, and III. The synthesis and inhibitory properties of these compounds toward *Candida albicans* GlcN-6-P synthase are described in the present paper.

MATERIALS AND METHODS

Reagents

L-Methionine sulfoxide **10,** L-methionine sulfone **11,** and L-*SR*-methionine sulfoximine **13** were purchased from Sigma. Other reagents were of the finest grade commercially available. Diazomethane was generated from *N*-nitroso-*N*-methylurea and used immediately for esterification.

Synthetic Chemistry

General. Thin-layer chromatography (TLC) was performed on Kieselgel 60 plates (Merck) using following solvent systems: solvent A (iPrOH:NH_4OH:H_20, 8:1:1), solvent B (nBuOH:AcOEt:AcOH:H_2O, 1:1:1:1), solvent C ($CHCl_3$:MeOH, 9:1),

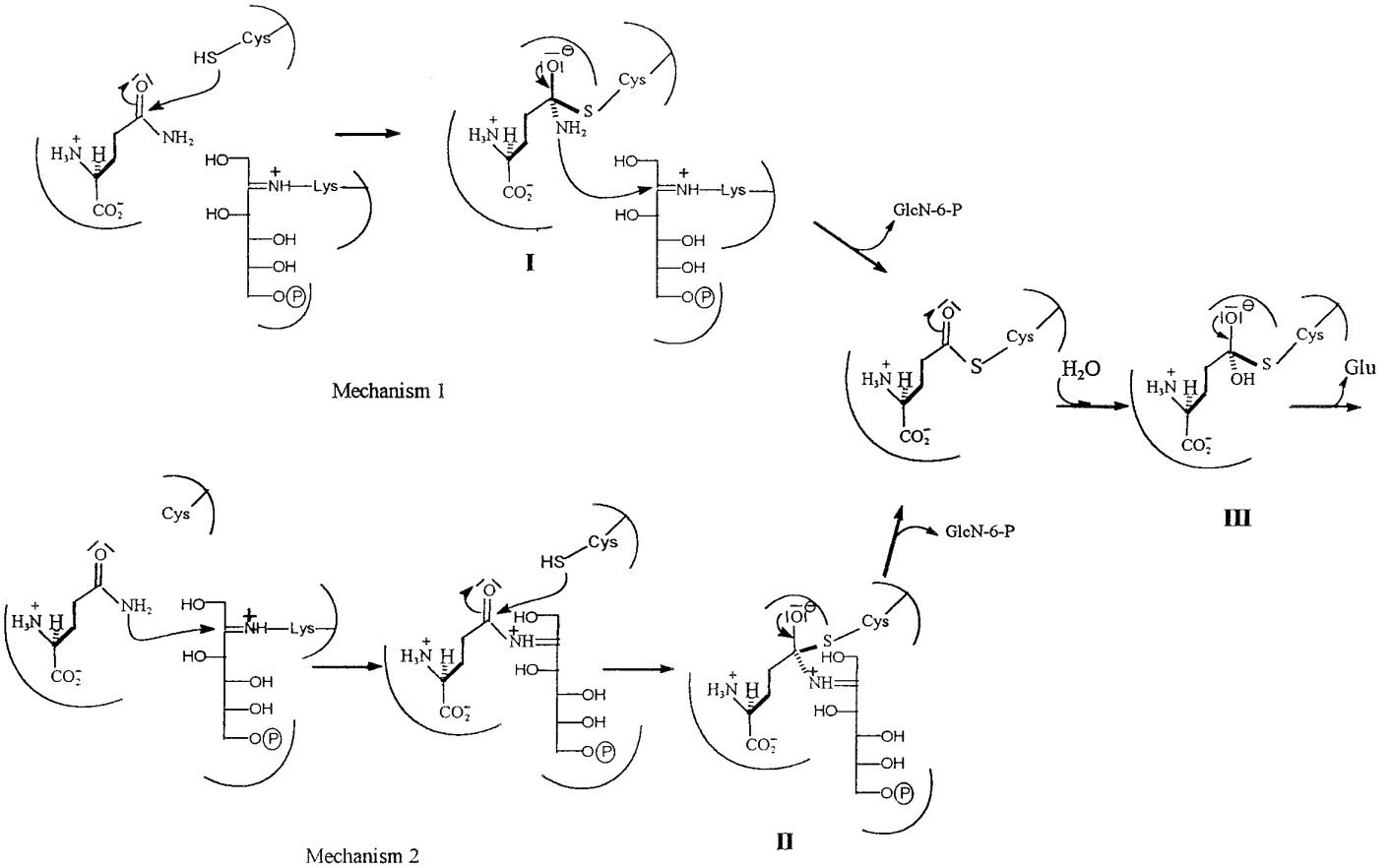

FIG. 1. Possible alternative mechanisms of nitrogen transfer in GlcN-6-P synthase. Putative tetrahedral intermediates are numbered with Roman numerals: **I, II,** and **III.** Configurations of the γ-glutamyl carbon atom in tetrahedral intermediates, shown for the clarity of the scheme, are purely hypothetical and do not have to reflect the actual situation.

solvent D (nBuOH:Pyr:AcOH:H_2O, 6:2:3:3), or solvent E (nPrOH:H_2O, 1:1). Amino acids were visualized with ninhydrin. Melting points are uncorrected. ^{1}H-NMR and ^{31}P-NMR spectra were recorded on a Gemini Varian 500 MHz spectrometer.

DL-[3-Amino-3-carboxypropyl]phosphonic acid **1** was synthesised according to the previously described procedure (*14*). Acylation of **1** with benzyloxycarbonyl chloride under conditions described elsewhere (*15*) afforded DL-[3-[(benzyloxycarbonyl)amino]-3-carboxypropyl]phosphonic acid **1a.** Analytical results for compounds **1** and **1a** (TLC, mp, and NMR) were satisfactory and consistent with literature data.

Synthesis of dimethyl-DL-[*3*-[(*benzyloxycarbonyl*)*amino*]-*3-methoxycarbonylpropyl*]*phosphonate* ***2a.*** An ice-cooled solution of 1 mmol of **1a** in 5 ml of methanol was treated portionwise with an etheral solution of diazomethane, until the solution became slightly yellow. Volatile components of the mixture were removed under reduced pressure. The residue was dissolved in 10 ml of ethyl acetate and washed subsequently with 5% aqueous HCl solution and water. The organic layer was dried over anhydrous $MgSO_4$. Removal of the drying agent and the solvent afforded ester **2a** as an oil. Yield, 88%; TLC (solvent C) R_f = 0.5; ^{1}H-NMR ($CDCl_3$) δ: 1.70–2.30 (m, 4H), 3.72, 3.73 (two d, 6H, J = 11 Hz), 3.75 (s, 3H), 4.30–4.50 (m, 1H), 5.12 (s, 2H), 5.55 (d, 1H), 7.35 (s, 5H).

Synthesis of dimethyl-DL-[*3-amino-3-methoxycarbonylpropyl*]*phosphonate* ***2.*** A 0.25-mmole of **2a** sample was dissolved in 5 ml of methanol and 15 mg of 10% Pd/C was added. The mixture was then stirred under H_2 gas for 2 h at room temperature. After filtration and removal of the solvent under reduced pressure, a viscous oily product **2** was obtained. Yield, 90%; TLC (solvent A) R_f = 0.57; ^{1}H-NMR (CF_3COOD) δ: 2.15–2.70 (m, 4H), 3.92–4.02 (m, 9H), 4.53 (t, 1H).

Synthesis of dimethyl-DL-[*3*-[(*benzyloxycarbonyl*)*amino*]-*3-carboxypropyl*]*phosphonate* ***3a.*** Selective saponification of carboxyl methyl ester **2a** was accomplished by the slow addition of an equimolar amount of 1 M NaOH to the ice-cooled ethanolic solution of **2a.** The mixture was then left for 1 h at room temperature. Subsequently, the solvent was removed under reduced pressure; the remaining aqueous phase was acidified with 6 M HCl at 0°C and extracted several times with ethyl acetate. Combined organic extracts were dried over $MgSO_4$. Removal of the drying agent and the solvent afforded a crystalline product **3a.** Yield, 80%; mp 120–122°C; TLC (solvent A) R_f = 0.5; ^{1}H-NMR ($CDCl_3$) δ 1.75–2.30 (m, 4H), 3.73, 3.76 (two d, 6H, J = 11 Hz), 4.30–4.50 (m, 1H), 5.11 (s, 2H), 5.71 (d, 1H), 7.35 (s, 5H).

Synthesis of dimethyl-DL-[*3-amino-3-carboxypropyl*]*phosphonate* ***3.*** Hydrogenolysis of **3a** under conditions described above for the conversion of **2a** into **2** afforded viscous oily product **3.** Yield, 99%; TLC (solvent A) R_f = 0.20, (solvent B) R_f = 0.34; ^{1}H-NMR (CF_3COOD) δ: 1.80–2.50 (m, 4H), 3.66 (d, 6H, J = 11 Hz), 4.31 (m, 1H)

Synthesis of methyl-DL-[*3*-[(*benzyloxycarbonyl*)*amino*]-*3-methoxycarbonylpropyl*]*phosphonic acid* ***4a.*** Dimethyl-DL-[3-[(benzyloxycarbonyl]-3-methoxycarbonylpropyl]phosphonate **2a** (0.5 mmol) was dissolved in *t*-butylamine (8 ml) and heated at refluxed temperature for 4 days. The reaction mixture was concentrated *in vacuo* to provide the product as a white salt (208 mg). The salt was dissolved in methanol

and acidified with cation exchange resin (Dowex 50WX8, H^+ form), 200–400 mesh. The Dowex resin was removed by filtration and the filtrate was concentrated *in vacuo* to afford oily product **4a.** Yield, 90%; TLC (solvent A) R_f = 0.5; ^{1}H-NMR ($CDCl_3$) δ: 1.65–2.35 (m, 4H,), 3.70 (d, 3H, J = 11 Hz), 3.75 (s, 3H), 4.35–4.50 (m, 1H), 5.11 (s, 2H), 5.40–5.70 (bs, 1H), 7.35 (s, 5H).

Synthesis of methyl-DL-[*3-amino-3-methoxycarbonylpropyl*]*phosphonic acid* ***4.*** Hydrogenolysis of **4a** under conditions described above for conversion of **2a** into **2** afforded **4** as a viscous oily product. Yield, 89%; TLC (solvent A) R_f = 0.15; ^{1}H-NMR (CF_3COOD) δ: 1.80–2.40 (m, 4H), 3.63 (d, 3H, J = 11 Hz), 3.72 (s, 3H), 4.23 (m, 1H).

Synthesis of methyl-DL-[*3-*[(*benzyloxycarbonyl*)*amino*]*-3-carboxypropyl*]*phosphonic acid* ***5a.*** *t*-Butylamine salt of **4a** (0.5 mmol) was dissolved in 1 ml 1 M NaOH and the solution heated for 30 min. Afterward the mixture was acidified with cation exchange resin (Dowex 50WX8, H^+ form). The resin was removed by filtration and the filtrate was concentrated *in vacuo*. The residue was crystallized from ethyl acetate/ether. Yield, 90%; TLC (solvent A) R_f = 0.20; mp 109–110°C; ^{1}H-NMR (DMSO) δ: 1.50–2.00 (m, 4H), 3.53 (d, 3H, J_{PH} = 11 Hz), 3.95–4.12 (m, 1H), 5.04 (s, 2H), 7.36 (s, 5H), 7.70 (d, 1H, J = 8 Hz).

Synthesis of methyl-DL-[*3-amino-3-carboxypropyl*]*phosphonic acid* ***5.*** Hydrogenolysis of **5a** under conditions described above for conversion of **2a** into **2** afforded **5** as a viscous oily product. Yield, 99%; TLC (solvent B) R_f = 0.20; ^{1}H-NMR (DMSO + CF_3COOD) δ: 1.50–2.20 (m, 4H), 3.58 (d, 3H, J_{PH} = 11 Hz), 3.90–4.30 (m, 1H).

General procedure for the synthesis of methyl DL-[*3-*[(*benzyloxycarbonyl*)*amino*]*-3-methoxycarbonylpropyl*]*-SR-phosphonamidates:* ***6a, 7a, 8a, 9a.*** Oxalyl chloride (0.066 g, 0.52 mmol) was added dropwise to a solution of **4a** (115 mg, 0.334 mmol) and DMF (1.25 μl, 0.017 mmol) dissolved in CH_2Cl_2 at 0°C. The solution was stirred at 0°C for 20 min and then warmed to room temperature and stirred for 1.5 h. The reaction mixture was concentrated, dissolved in toluene (2 ml), and then reconcentrated to remove the volatile reagents. This left the phosphonochloridate as a yellow oil which was used immediately in a reaction with ammonia or amine.

The amine reagent, dimethylamine, ammonia (both as a 2 M solution in THF), ethyl amine, or diethylamine (4 mmol), cooled to 0°C, was added to the phosphonochloridate (1 mmol) dissolved in 5 ml of CH_2Cl_2. The reaction mixture was allowed to warm to room temperature and stirred overnight. The resulting solution was concentrated *in vacuo*. The oily residue was dissolved in ethyl acetate and washed successively with 5% $NaHCO_3$, 5% $KHSO_4$, and water, dried with $MgSO_4$, filtered, and concentrated to afford the product.

General procedure for the synthesis of methyl DL-[*3-amino-3-methoxycarbonylpropyl*]*-SR-phosphonamidates:* ***6, 7, 8, 9.*** Hydrogenolysis of **6a, 7a, 8a, 9a** under conditions described above for conversion of **2a** into **2,** afforded **6, 7, 8,** or **9** as viscous oily products.

6a: DL-Methyl [3-[(benzyloxycarbonyl)amino]-3-methoxycarbonylpropyl-*SR*-phosphonamidate. Yield, 55%; TLC (solvent C) R_f = 0.35; ^{1}H-NMR ($CDCl_3$) δ: 1.70–2.60 (m, 6H), 3.71, 3.72 (two d, 3H, J_{PH} = 11 Hz), 3.79 (s, 3H), 4.40–4.60 (m, 1H), 5.14 (s, 2H), 5.65–5.80 (br, 1H), 7.38 (s, 5H).

6: DL-Methyl [3-amino-3-methoxycarbonylpropyl]-*SR*-phosphonamidate. Yield quant.; TLC (solvent A) R_f = 0.45, (solvent B), R_f = 0.32; ^{31}P-NMR (CD_3OD) δ: 39.56, 39.54; ^{1}H-NMR (CD_3OD) δ: 1.70–2.20 (m, 4H), 3.67 (d, 3H, J = 11 Hz), 3.78 (s, 3H), 3.60–3.80 (m, 1H).

7a: DL-Methyl *N*,*N*-dimethyl-[3-[(benzyloxycarbonyl)amino]-3-methoxycarbonylpropyl]-*SR*-phosphonamidate. Yield 70.5%; TLC (solvent C) R_f = 0.55; ^{31}P-NMR ($CDCl_3$) δ: 37.46, 37.43; ^{1}H-NMR ($CDCl_3$) δ: 1.60–2.10 (m, 4H), 2.67 (d, 6H, J_{PH} = 8,9 Hz), 3.57, 3.58 (two d, 3H, J_{PH} = 11 Hz), 3.75 (s, 3H), 4.30–4.50 (m, 1H), 5.12 (s, 2H), 5.63–5.72 (bd, 1H), 7.35 (s, 5H).

7: DL-Methyl *N*,*N*-dimethyl-[3-amino-3-methoxycarbonylpropyl]-*SR*-phosphonamidate. Yield, 95%; TLC (solvent A) R_f = 0.55, (solvent B) R_f = 0.38; ^{31}P-NMR (CD_3OD) δ: 40.09, 40.02; ^{1}H-NMR (CD_3OD) δ: 1.70–2.10 (m, 4H), 2.69, 2.70 (two d, 6H, J_{PH} = 9 Hz), 3.59 (d, 3H, J_{PH}= 11 Hz), 3.74 (s, 3H), 3.50–3.80 (m, 1H).

8a: DL-Methyl *N*-ethyl-[3-[(benzyloxycarbonyl)amino]-3-methoxycarbonylpropyl]-*SR*-phosphonamidate. Yield, 70%; TLC (solvent C), R_f = 0.46; ^{1}H-NMR ($CDCl_3$) δ: 1.13, 1.25 (two t, 3H, J = 7 Hz), 1.65–2.30 (m, 4H), 2.90 (dq, 2H, J_{PH} = 11 Hz, J_{HH} = 7 Hz), 3.62, 3.64 (two d, 3H, J_{PH} = 11 Hz), 3.75 (s, 3H), 4.35–4.50 (m, 1H), 5.11 (s, 2H), 5.70–5.85 (br, 1H), 7.35 (s, 5H).

8: DL-Methyl *N*-ethyl-[3-amino-3-methoxycarbonylpropyl]-*SR*-phosphonamidate. Yield quant.; TLC (solvent A), R_f = 0.55, (solvent B) R_f = 0.42; ^{1}H-NMR (CD_3OD) δ: 1.14, 1.30 (two t, 3H, J = 7Hz), 1.65–2.20 (m, 4H), 2.95, 4.15 (two dq, 2H), 3.62 (d, 3H, J_{PH} = 11 Hz), 3.74 (s, 3H), 3.50–3.75 (m, 1H).

9a: DL-Methyl *N*,*N*-diethyl-[3-[(benzyloxycarbonyl)amino]-3-methoxycarbonylpropyl]-*SR*-phosphonamidate. Yield, 51%; TLC (solvent C), R_f = 0.6; ^{31}P-NMR ($CDCl_3$) δ: 36,67; ^{1}H-NMR ($CDCl_3$) δ: 1.09, 1.30 (two t, 6H, J = 7 Hz), 1.60–2.20 (m, 4H), 3.06, 4.10 (two dq, 4H, J_{PH} = 11 Hz, J_{HH} = 7 Hz), 3.55, 3.56 (two d, 3H, J = 11 Hz), 3.75 (s, 3H), 4.30–4.50 (m, 1H), 5.12 (s, 2H), 5.75 (br d), 7.35 (s, 5H).

9: DL-Methyl *N*,*N*-diethyl-[3-amino-3-methoxycarbonylpropyl]-*SR*-phosphonamidate. Yield, 95%; TLC (solvent A), R_f = 0.64, (solvent B), R_f = 0.48; ^{31}P-NMR (CD_3OD) δ: 39.26, 39.15; ^{1}H-NMR (CD_3OD) δ: 1.13, 1.33 (two t, 6H), 1.65–2.10 (m, 4H), 3.05, 4.10 (two dq, 4H, J_{HH} = 6 Hz, J_{PH} = 12 Hz), 3.55 (d, 3H, J = 11 Hz), 3.73 (s, 3H), 3.50–3.80 (m, 1H).

*Synthesis of N^3-methanesulfonyl-*L*-2,3-diaminopropanoic acid trifluroacetate* ***12.*** Methyl N^2-tert-butoxycarbonyl-L-2,3-diaminopropanoate (*16*) (1 mmol) was dissolved in 5 ml of THF and the solution was cooled to 0°C. Triethylamine (0.14 ml) was added, followed by methanesulfonyl chloride (0.07 ml). The reaction mixture was stirred for 6 h at room temperature and then diluted with 100 ml of ethyl acetate. The organic layer was washed with 10% $KHSO_4$, brine, dried over $MgSO_4$, filtered, and evaporated to give 0.27 g (88%) of oily methyl N^2-tert-butoxycarbonyl-N^3-methanesulfonyl-L-2,3-diaminopropanoate. Saponification of the methyl ester with 1 M NaOH (0.9 ml, 2 h) and removal of the *t*-butoxycarbonyl protecting group with cold trifluoroacetic acid (5 ml), afforded an oily product which on trituration with ethyl ether gave N^3-methanesulfonyl-L-2,3-diaminopropanoic acid trifluroacetate as an amorphous powder. Yield, 40%; TLC (solvent D), R_f = 0.52; ^{1}H-NMR (D_2O) δ: 3.2 (s, 3H), 3.3–3.5 (m, 2H), 4.2 (m, 1 H).

Synthesis of DL-*3-amino-3-carboxypropanesulfonamide* ***14.*** DL-Homocysteic acid (5 mmol) and thionyl chloride (5 ml) were heated together under reflux for 3 h. An excess of thionyl chloride was evaporated and the residue containing the crude homocysteic acid chloride was stirred with a cold 25% aqueous ammonia (25 ml) for 6 h. The reaction mixture was concentrated under reduced pressure and the crude product was purified on a Dowex 50WX8 ion exchange resin (H^+ form). The column was washed with water and then with 0.5 M ammonia solution. Fractions containing the title compound were collected and evaporated to dryness to give the pure product. Yield, 50%; mp 245–250°C [lit. 247°C (*17*)]; TLC (solvent E), $R_f = 0.5$; ^{1}H-NMR (D_2O) δ: 2.0–2.4 (m, 2H), 2.9–3.2 (m, 2H), 4.2 (t, 1H).

Synthesis of DL-*3-amino-3-carboxy-N,N-diethyl-propanesulfonamide* ***15.*** The crude homocysteic acid chloride obtained as described above was treated with a cold 50% aqueous *N,N*-diethyl amine. Reaction conditions and product isolation was performed analogously as for the compound **14.** Final product was obtained as an amorphous powder. Yield, 35%; TLC (solvent D), $R_f = 0.6$; ^{1}H-NMR (D_2O) δ: 1.1 (m, 6H), 2.2 (m, 2H), 2.9 (m, 2H), 3.3 (m, 4H), 4.2 (m, 1H).

Enzymology

Candida albicans GlcN-6-P synthase overproduced by *Saccharomyces cerevisiae* YRSC-65 was purified to apparent homogeneity as described previously (*18*). Two assays of GlcN-6-P synthase activity were used: (a) determination of GlcN-6-P formation by the modified Elson–Morgan procedure (*19*) and (b) continuous quantitation of glutamate in a coupled assay with glutamate dehydrogenase in the presence of acetylpyridine adenine dinucleotide (*2*). In both assays, reactions were carried out in 0.1 M Tris–HCl buffer, pH 6.85, containing 1 mM EDTA and 1 mM dithiothreitol. Reaction mixtures contained 0.1–0.2 μM GlcN-6-P synthase and 7.5 mM Fru-6-P. Glutamine concentrations were either fixed (10 mM, for IC_{50} determinations) or variable (0.5–5 mM, for K_i determinations).

Amidohydrolase activity of the enzyme was determined using 1 mM GLUPA as a substrate, in the same buffer as shown above. Fru-6-P (7.5 mM) was either present or omitted from incubation mixtures and GlcN-6-P synthase was present at 1–2 μM. Formation of *p*-nitroaniline was followed spectrophotometrically at 420 nm.

All the measurements were done in triplicate. Inhibitory constants were determined from the secondary plots of k_{app} versus inhibitor concentration, derived from Lineweaver–Burk plots.

RESULTS AND DISCUSSION

Synthesis

Structures of compounds synthesized by us and commercially available methionine derivatives tested as potential inhibitors of GlcN-6-P synthase are shown in Fig. 2. *N*-benzyloxycarbonyl derivative of γ-Glu(P) was converted into *N*-protected methyl triester **2a** using diazomethane. Hydrogenolysis of **2a** yielded directly methyl triester **2.** Carboxyl methyl ester in **2a** could be selectively saponified under alkaline