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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),

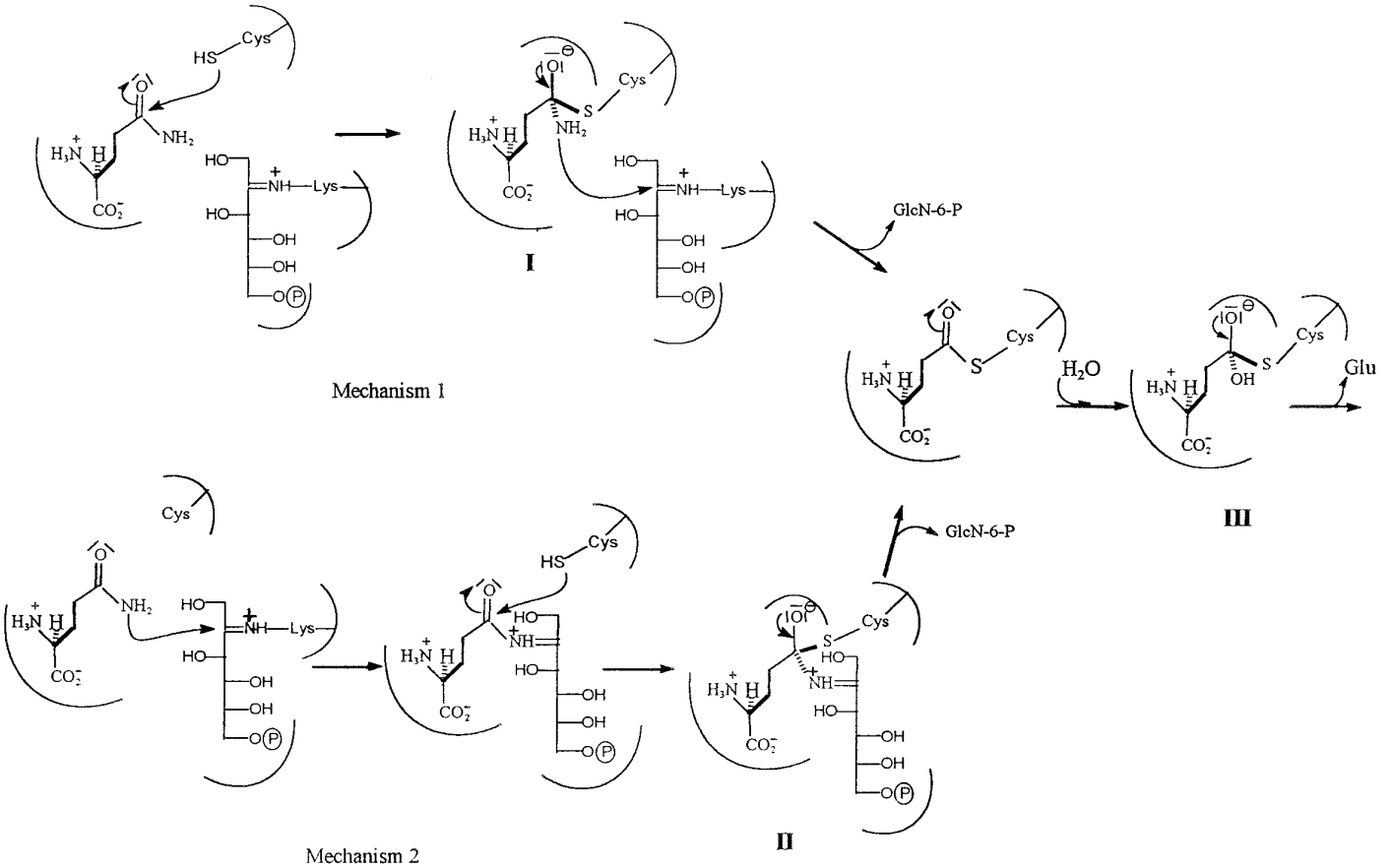


FIG. 1. Possible alternative mechanisms of nitrogen transfer in GlcN-6-P synthase. Putative tetrahedral intermediates are numbered with Roman numerals: **I, II,** and **III.** Configurations of the γ-glutamyl carbon atom in tetrahedral intermediates, shown for the clarity of the scheme, are purely hypothetical 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).

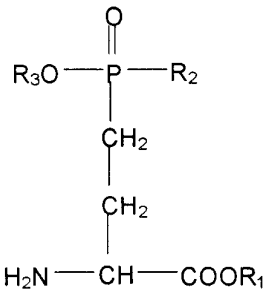
Amidohydrolase activity of the enzyme was determined using 1 mM GLUPA as a substrate, in the same buffer as shown above. Fru-6-P (7.5 mM) was either present or omitted from incubation mixtures and GlcN-6-P synthase was present at 1–2 μM. Formation of *p*-nitroaniline was followed spectrophotometrically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

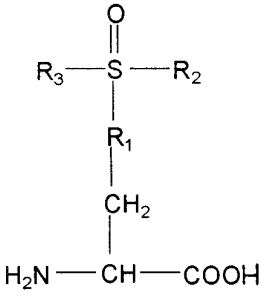
All the measurements were done in triplicate. Inhibitory constants were determined from the secondary plots of k_{app} versus inhibitor concentration, derived from Lineweaver–Burk plots.

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





1 $R_1 = H$; $R_2 = OH$; $R_3 = H$
2 $R_1 = CH_3$; $R_2 = OCH_3$; $R_3 = CH_3$
3 $R_1 = H$; $R_2 = OCH_3$; $R_3 = CH_3$
4 $R_1 = CH_3$; $R_2 = OH$; $R_3 = CH_3$
5 $R_1 = H$; $R_2 = OH$; $R_3 = CH_3$
6 $R_1 = CH_3$; $R_2 = NH_2$; $R_3 = CH_3$
7 $R_1 = CH_3$; $R_2 = NHC_2H_5$; $R_3 = CH_3$
8 $R_1 = CH_3$; $R_2 = N(CH_3)_2$; $R_3 = CH_3$
9 $R_1 = CH_3$; $R_2 = N(C_2H_5)_2$; $R_3 = CH_3$

10 $R_1 = CH_2$ $R_2 = O$ $R_3 =$ none
11 $R_1 = CH_2$ $R_2 = O$ $R_3 = CH_3$
12 $R_1 = NH$ $R_2 = O$ $R_3 = CH_3$
13 $R_1 = CH_2$ $R_2 = NH$ $R_3 = CH_3$
14 $R_1 = CH_2$ $R_2 = O$ $R_3 = NH_2$
15 $R_1 = CH_2$ $R_2 = O$ $R_3 = N(C_2H_5)_2$

FIG. 2. Structures of transition state analogs tested as potential inhibitors of GlcN-6-P synthase.

conditions to afford, after subsequent *N*-deprotection, the diester **3.** Alternatively, treatment of dimethyl phosphonate **2a** with *t*-butylamine under reflux, followed by acidification, resulted in selective hydrolysis to monomethyl phosphonate **4a.** This compound was either *N*-deprotected to give diester **4,** saponified, and *N*-deprotected to afford monoester **5,** or used as a starting substrate for the synthesis of methyl γ-Glu-(P) amidates **6–9.** For this purpose, **4a** was first converted into phosphonochloridate upon treatment with oxalyl chloride. Aminolysis of this compound followed by *N*-deprotection yielded required products **6–9.**

All our attempts to prepare P-unprotected phosphonamidates were unsuccessful due to an extreme instability of these compounds, resulting in a rapid decomposition in aqueous solutions. This is in agreement with previous literature reports confirming the sensitivity of the phosphonamidate bond to hydrolysis, especially under acidic conditions (*20*, *21*). However, other authors demonstrated good enzyme inhibitory properties of monomethyl phosphonamidates designed as potential transition state analog inhibitors of HIV-1 protease (*22*).

Selectively protected diamino acid, methyl N^2-tert-butoxycarbonyl-L-2,3-diaminopropanoate, was acylated with methanesulfonyl chloride. Saponification of the methyl ester and deprotection of the α-amino function with TFA afforded N^3-methanesulfonyl-L-2,3-diaminopropanoic acid **12.**

The sulfonamides **14** and **15** were prepared from commercially available (DL)-homocysteic acid which gave corresponding acid dichloride (both carboxyl and sulfonyl groups were converted) by heating with thionyl chloride. Immediate treatment of the dichloride with an excess of either aqueous ammonia or *N*,*N*-diethyl-

TABLE 1
Inhibition of GlcN-6-P Synthase by γ-Glu(P) and Its Ester Derivatives

Compound	GlcN-6-P synthetic activity		Amidohydrolase activity,
	IC_{50} (mM)	K_i (mM)	IC_{50} (mM)
1	26	12 (6.0)	22
2	>50	ND	>50
3	>50	ND	>50
4	2.6	1.2 (0.6)	2.1
5	2.0	0.9 (0.45)	1.8

Note. ND, not determined. In parentheses K_i values corrected for one active enantiomer. SD was ±5%.

amine solution at elevated temperature led to the formation of required products **14** or **15** in moderate yields. Under these conditions sulfonyl chloride was readily converted into sulfonamide, whereas carboxyl chloride was mainly hydrolyzed. Crude preparations, slightly contaminated by disubstitution products, were finally purified by ion-exchange chromatography.

Inhibition Studies

Compounds **1–15** were tested as potential inhibitors of GlcN-6-P-forming and amidohydrolyzing activities of pure *C. albicans* GlcN-6-P synthase. Results obtained for the former activity were essentially the same when either of two assay systems was used: fixed point colorimetric determination of GlcN-6-P or continuous coupled quantitation of glutamate, so that a more convenient and cheaper colorimetric determination was routinely used. The enzyme exhibited K_M = 1.56 mM for L-glutamine, $k_{cat} = 1150\ min^{-1}$, and $k_{cat}/K_M = 12.290\ M^{-1}\ s^{-1}$. Amidohydrolase activity of GlcN-6-P synthase was measured with an artificial substrate, γ-glutamyl *p*-nitroanilide. The kinetic parameters of this reaction were as follows: K_M for GLUPA, 0.67 mM, $k_{cat} = 1.52\ min^{-1}$, $k_{cat}/K_M = 38\ M^{-1}\ s^{-1}$ (in the presence of 7.5 mM Fru-6-P); k_{cat} decreased to $1.09\ min^{-1}$ when Fru-6-P was omitted from incubation mixtures.

Cumulative data including IC_{50} and K_i values are summarized in Tables 1–3. Inhibitory constants were determined for those compounds which were found active in initial screening. Since most of the tested compounds were racemic mixtures, K_i values determined in kinetic experiments were corrected, taking into account a highly probable action of enantiomer L as an actual inhibitor. Such an assumption is fully justified in light of the fact that D-glutamine is an extremely poor substrate for GlcN-6-P synthase (data not shown). Generally none of the studied compounds showed as high inhibitory potency as could be expected for a proper transition state analog. Inhibitory constants for the most active compounds **9** and **15** were only less than an order of magnitude lower than the K_M for L-glutamine. All the active compounds inhibited GlcN-6-P synthase competitively with respect to L-Gln. The representative graph obtained for compound **9** is shown in Fig. 3. On the other hand the inhibition with respect to Fru-6-P was either uncompetitive or mixed.

TABLE 2
Inhibition of GlcN-6-P Synthase by Amide Derivatives of γ-Glu(P)

Compound	GlcN-6-P synthetic activity		Amidohydrolase activity, IC_{50} (mM)
	IC_{50} (mM)	K_i (mM)	
6	25	11 (2.75)	30
7	7.8	4.4 (1.1)	7.1
8	4.4	1.3 (0.325)	6.1
9	3.6	0.94 (0.235)	3.2

Note. In parentheses K_i values corrected for one, possibly active diastereoisomer. SD was ±5%.

Compounds **1–5** (Table 1) could be considered as structural analogs of putative tetrahedral intermediate **III.** In this hypothetical compound, one of the oxygen atoms linked to phosphorus is negatively charged. Among the γ-Glu(P) esters tested, compounds **4** and **5** are the best mimics of this structure and the best inhibitors of the enzyme. Presence of two potentially negatively charged oxygen atoms (compound **1**) or absence of any charge (compounds **2** and **3**) markedly lowers the inhibitory potency. Nevertheless, the inhibitory constant for **5** is much higher than could be expected for a transition state analog. Undoubtedly, the best mimic of **III** known so far is a covalent adduct formed upon the reaction of γ-glutamic semialdehyde with the active site cysteine, described previously by Badet-Denisot *et al.* (*7*) and Bearne and Wolfenden (*23*). Inhibitory constant of this compound against *Escherichia coli* GlcN-6-P synthase was as low as 3×10^{-8} M (*23*). It is therefore not very surprising that our phosphonate and sulfonate analogs, being unable to react with cysteine, show much lower affinity to the enzyme active site. However, chemical reactivity is not an absolute requirement for an effective transition state analog inhibitor mimicking a tetrahedral intermediate, as was pre-

TABLE 3
Inhibition of GlcN-6-P Synthase by Sulfonic Analogs of Glutamate and Glutamine

Compound	GlcN-6-P synthetic activity		Amidohydrolase activity, IC_{50} (mM)
	IC_{50} (mM)	K_i (mM)	
10	>50	ND	>50
11	>50	ND	>50
12	>50	ND	>50
13	2.8	1.5 (0.75)	2.1
14	5.5	2.8 (1.4)	7.2
15	1.6	0.6 (0.3)	1.2

Note. ND, not determined. In parentheses K_i values corrected for one active enantiomer. SD was ±5%.

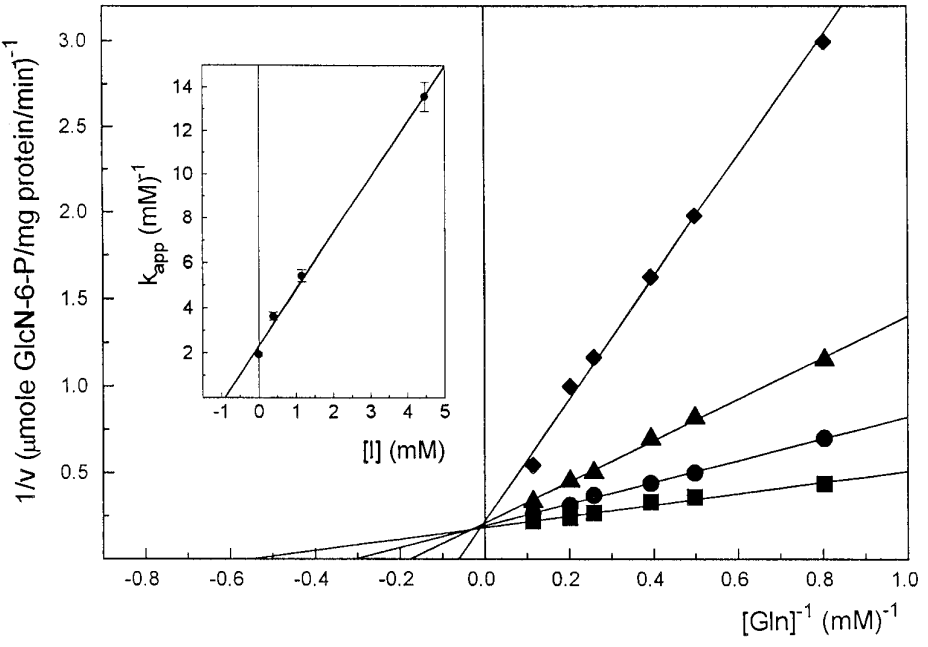
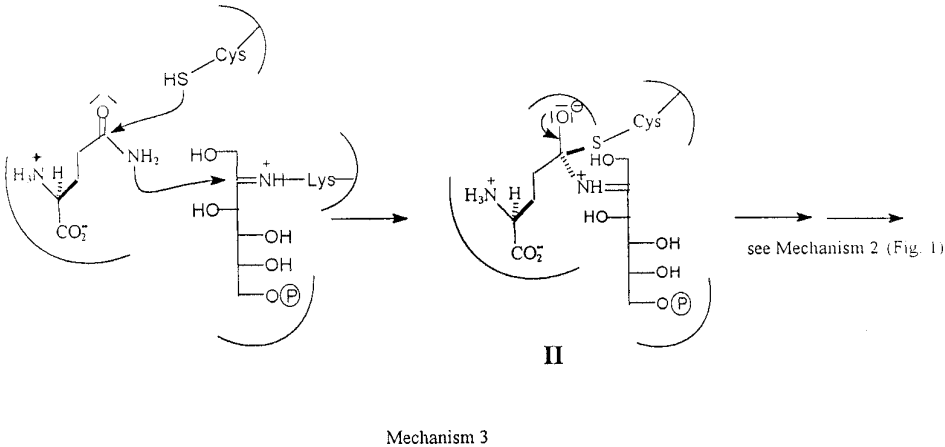


FIG. 3. Competitive inhibition of GlcN-6-P synthase by compound **9** in respect to L-glutamine. Inhibitor concentrations were as follows: (■) none; (●) 0.5 mM; (▲) 1.0 mM; (◆) 4.5 mM. (Inset) A secondary plot of $k_{app} = f([I])$.

viously demonstrated by a high inhibitory activity of L-methionine sulfone with respect to glutamate synthase (*24*). Nevertheless, it seems sure that the presence of **III** in a molecular mechanism of nitrogen transfer in GlcN-6-P synthase cannot be questioned. It should be noted, however, that this intermediate is common for both alternative mechanisms shown in Fig. 1.

Rather unexpected results were obtained for amide derivatives of γ-Glu(P). Data obtained for these compounds, summarized in Table 2, are corrected, taking into account a highly probable possibility that only one of the four possible diastereoisomers could be an actual inhibitor. The closest structural analog of a putative tetrahedral intermediate **I,** i.e., compound **6,** appeared to be the poorest inhibitor of GlcN-6-P synthase. Presence of a steric hindrance at the amide nitrogen enhanced the inhibitory potency of γ-Glu(P) amidates, resulting in a relatively low inhibitory constant exhibited by the most substituted compound **9.** The same inhibitory potency order was observed when γ-Glu(P) amidates **6–9** were tested as inhibitors of amidohydrolase activity of GlcN-6-P synthase.

This tendency was also confirmed for γ-sulfonic analogs of glutamate and glutamine (Table 3). Homocysteine sulfonamide **14** was previously reported to be a strong inhibitor of glutamate synthase, an amidotransferase belonging to the same subfamily as GlcN-6-P synthase. Masters and Meister demonstrated the high inhibitory potency of this compound ($K_i = 3.6$ μM) against the *S. cerevisiae* enzyme and



Mechanism 3

FIG. 4. "Concerted attack" as the most probable mechanism of initiation of nitrogen transfer in GlcN-6-P synthase.

suggested its close structural analogy to the putative tetrahedral transition state (*24*). In our hands this compound was only a moderate inhibitor of *C. albicans* GlcN-6-P synthase, poorer than methionine sulfoximine **13.** N-substituted homocysteine sulfonamide **15** was again the best inhibitor in the series, thus confirming the tendency observed for γ-Glu(P) amidates **6–9.** No inhibitory effect was demonstrated for methionine sulfoxide **10,** methionine sulfone **11,** and $A_2prSO_2CH_3$ **12,** potential analogs of transition state **III,** having no negatively charged oxygen atoms linked to sulfur. Relatively good inhibitory activity of methionine sulfoximine **13** is therefore worth mentioning. Although in this compound no substituents are present at the sulfur-linked nitrogen, hybridization of the latter atom is the same as that of the respective nitrogen in the putative transition state **II.**

We believe that N-substituted γ-Glu(P) amidates **7, 8, 9,** and homocysteine *N*,*N*-diethyl sulfonamide **15** show less structural analogy to the transition state **I** than the respective compounds **6** and **10.** On the other hand, *N*-substitution makes them more resembling an amino acid part of the intermediate **II.** The probability of existence of intermediate **I** was also previously questioned using semiempirical quantum mechanical MNDO energy calculations (*8*). In our opinion, results presented above provide new data supporting the possibility of a direct nucleophilic attack of the glutamine amide nitrogen according to mechanism 2 (Fig. 1) as a more likely way of nitrogen transfer in GlcN-6-P synthase. However, the glutamine amide nitrogen is undoubtedly a poor nucleophile. Its nucleophilicity may be sufficient to attack aspartyl-AMP in asparagine synthetase (*13*), but not necessarily other, less electrophilic acceptors. Therefore, we believe that the most likely pathway of nitrogen transfer in GlcN-6-P synthase should be initiated with a concerted attack according to mechanism 3 (Fig. 4). Approaching of the Cys-1 to glutamine amide carbonyl would enhance the nucleophilicity of the amide nitrogen and facilitate its

simultaneous attack on the neighbouring electrophilic centre. It should be noted that a 20-Å-long, solvent inaccessible channel, connecting the glutamine and the phosphoribosylpyrophosphate binding sites, has been recently revealed in phosphoribosylpyrophosphate amidotranferase. This channel permits an interdomain transfer of the NH_3 intermediate (*25*). However, a very close proximity between the N-terminal cysteine located at the glutamine binding site and the Fru-6-P domain has been demonstrated in two independent studies on affinity labeling of the bacterial GlcN-6-P synthase with *N*-iodoacetylglucosamine 6-phosphate and anhydro-1,2-hexitol 6-phosphate (*26*, *27*). Such a proximity should obviously facilitate the direct nucleophilic attack of the glutamine amide nitrogen on an electrophilic site of the enzyme-bound Fru-6-P.

Apparent inability of exogenous ammonia utilization, unique among amidotransferases (*2*), and marginal amidohydrolase activity of the enzyme in an absence of Fru-6-P (*3*, this work) further support this assumption. At last, it should be noted that the presence of the acceptor molecule, i.e., Fru-6-P, which is known to bind to the enzyme active site before glutamine (*5*) and to bring about the conformational change in the enzyme molecule (*12*), strongly enhances the velocity of L-Gln hydrolysis (*12*) but has very little effect on hydrolysis rate of γ-glutamyl *p*-nitroanilide (this work). This is obvious that in the latter case, *p*-nitroaniline cannot be transferred to the acceptor.

All these data taken together seem to favor mechanism 3. The recently revealed molecular structure of the glutamine binding domain of *E. coli* GlcN-6-P synthase does not provide any information undermining our hypothesis. Two amino acid residues, Asn 98 and Gly 99, were unequivocally identified as those involved in stabilization of oxyanionic tetrahedral intermediates (*12*). Respective residues, Asn 124 and Gly 125, are present in the *C. albicans* GlcN-6-P synthase, as can be deduced from the gene sequence (*28*). These residues could be therefore involved in stabilization of **II** and **III.**

Undoubtedly further studies are necessary to reach the conclusive evidence. One of the possible approaches could be a design and synthesis of other structural analogs of intermediate **II** that could be better mimics than bisubstrate analogs designed and synthesized by Badet-Denisot *et al.* (*7*) and our compounds **9** and **15.** These studies are in progress in our laboratory.

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