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N^3-OXOACYL DERIVATIVES OF L-2,3-DIAMINOPROPANOIC ACID AND THEIR PEPTIDES; NOVEL INHIBITORS OF GLUCOSAMINE-6-PHOSPHATE SYNTHASE

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Novel inhibitors **1–4** of glucosamine-6-phosphate synthase from *Candida albicans* have been designed based on acylation of the N3 amino group of L-2,3-diaminopropanoic acid with the corresponding ketoacids. These inhibitors have been shown to alkylate the fungal enzyme in a time-dependent manner. Compound **3** containing *trans*-β-benzoyl acrylic acid as an acyl residue was found to be the most potent inhibitor in the series. Dipeptides composed of the active inhibitors and norvaline demonstrated potent antifungal activity against selected strains of *Candida* spp. and *Saccharomyces cerevisiae*. Their activity was reversed upon addition of N-acetylglucosamine to the medium.

Keywords: Glucosamine-6-phosphate synthase; Oxoacyl derivatives of L-2,3-diaminopropanoic acid; Dipeptides; Antifungal activity

INTRODUCTION

Glucosamine-6-phosphate synthase (L-glutamine : D-fructose-6-phosphate amidotransferase; GlcN-6-P synthase; EC 2.6.1.16) catalyses the first step in hexosamine biosynthesis, converting fructose-6-phosphate (Fru-6-P) into

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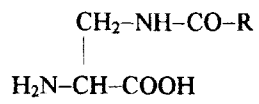
glucosamine-6-phosphate using glutamine as the ammonia source:[1]

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

Glucosamine-6-phosphate is then converted to uridine 5′-diphospho-N-acetylglucosamine (UDP-GlcNAc) which is an activated precursor of a number of amino sugar-containing macromolecules, including chitin and mannoproteins in fungi, peptidoglycan and lipopolysaccharides in bacteria and glycoproteins in mammals.[2] Accordingly, GlcN-6-P synthase offers a potential target for antibacterial and antifungal agents.[3] GlcN-6-P synthase belongs to a group of sixteen glutamine-dependent amidotransferases;[4] only the selective inactivators of this enzyme may be of chemotherapeutic value. Glutamine amidotransferases in general are rapidly inactivated by thiol reagents e.g. iodoacetamide and glutamine analogues, 6-diazo-5-oxo-L-norleucine (DON), azaserine, 6-chloro-5-oxo-L-norleucine.[5] A number of glutamine analogues act as inhibitors and inactivators of GlcN-6-P synthase of fungal and bacterial origin.[6] Our laboratory has developed a series of N3 acylated L-2,3-diaminopropanoic acid derivatives that have been shown to be selective and irreversible inhibitors of GlcN-6-P synthase of bacterial and fungal origin.[7-10] Of the novel designed glutamine analogues, only N^3-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid (FMDP)[8] and N^3-D-*trans*-epoxysuccinamoyl-L-2,3-diaminopropanoic acid (EADP)[11] were shown to be selective and effective inactivators of fungal GlcN-6-P synthase. However, due to the presence of methyl ester (FMDP) and primary amide (EADP) groups, these inhibitors are prone to hydrolysis and are chemically unstable when tested *in vivo*. The free acids formed upon hydrolysis are practically useless as enzyme inhibitors. These observations prompted us to design more chemically stable compounds which did not possess hydrolysable groups. Herein, we report the synthesis of novel glutamine analogues **1**, **2**, **3** and **4**, containing a keto function instead of a methyl



1 R = $CH{=}CH{-}CO{-}CH_3$ - **AADP** **3**R = $CH{=}CH{-}CO{-}C_6H_5$ - **BADP**

2 R = $CH{-}CH{-}CO{-}CH_3$ - **AEDP**

4 R = $CH{-}CH{-}CO{-}C_6H_5$ - **FEDP**

13 Nva-AEDP **14** Nva-BADP **15** Nva-FEDP

ester or primary amide. We also report the evaluation of these compounds as GlcN-6-P synthase inhibitors, synthesis of dipeptides **13–15** as novel inhibitors and the testing of their *in vitro* antifungal activity.

EXPERIMENTAL

Chemistry

N^2-tert-butoxycarbonyl,N^3-DL-trans-3(1-oxoethyl)oxirane-2-carbonyl-L-2,3-diaminopropanoic Acid 6

N^2-*tert*-butoxycarbonyl, N^3-*trans*-4-oxo-2-pentenoyl-L-2,3-diaminopropanoic acid[8] **5** (2.24 g, 7.45 mmol) and sodium bicarbonate (0.62 g, 7.45 mmol) were dissolved in water (5 ml) and cooled to 0°C. Then hydrogen peroxide (0.7 ml, 50% soln, 12.7 mmol) and sodium carbonate (0.45 g, 4 mmol) were added to the mixture and stirred for 3 h. The aqueous layer was acidified with 1 M $KHSO_4$ and extracted with ethyl acetate (3 × 25 ml). The organic extract was dried and concentrated, then chromatographed on silica gel (ethyl acetate–MeOH, 10 : 1) to afford **6** (1.46 g, 62%) as a colorless solid. M.p. 103–105°C. NMR (DMSO-d_6) $\delta = 1.37$ (9H, s), 2.07 (3H, s), 3.3–3.4 (2H, m), 3.53 (1H, d, $J = 2.0$ Hz), 3.76 (1H, d, $J = 2.0$ Hz), 3.8–3.9 (1H, m), 6.7 (1H, d), 8.27 (1H, m); $[\alpha]_{578}$ +3.2° ($c = 1$, MeOH). Found: C, 49.12; H, 6.20; N, 8.61. $C_{13}H_{20}N_2O_7$ requires C, 49.36; H, 6.32; N, 8.86%.

N^2-tert-butoxycarbonyl,N^3-trans-4-oxo-4-phenyl-2-butenoyl-L-2,3-diaminopropanoic Acid 8

N^2-*tert*-butoxycarbonyl-L-2,3-diaminopropanoic acid[12] **7** (2.04 g, 10 mmol) and $NaHCO_3$ (0.84 g, 10 mmol) were dissolved in a water–methanol solution (1 : 1, 20 ml). Then N-succinimidoyl *trans*-4-oxo-4-phenyl-2-butenoate (2.73 g, 10 mmol) was added with stirring at 0°C and the reaction mixture was left overnight. The solvents were removed *in vacuo*. The residue was dissolved in 10 ml of water, acidified with 1 M $KHSO_4$ to pH 2 and extracted with ethyl acetate (3 × 30 ml). The organic phase was dried and the solvent was concentrated leaving an oily residue which was crystallized from ethyl acetate–ethyl ether to give **8** as a white solid (3.0 g, 84% yield). M.p. 125–126°C. NMR ($CDCl_3$) $\delta = 1.39$ (9H, s), 3.75–3.9 (2H, m), 4.47 (1H, m), 6.1 (1H, br.s), 7.1 (1H, d, $J = 15.5$ Hz), 7.45–7.70 (3H, m), 7.9 (2H, m), 8.1 (1H, d, $J = 15.5$ Hz), 9.4–9.7 (1H, br.s). $[\alpha]_{578}$ −23.7° ($c = 1$,

MeOH). Found: C, 59.85; H, 5.87; N, 8.09. $C_{18}H_{22}N_2O_6$ requires C, 59.66; H, 6.07; N, 7.73%.

N^2-tert-butoxycarbonyl,N^3-DL-trans-3(1-oxobenzyl)oxirane-2-carbonyl-L-2,3-diaminopropanoic Acid 9

Starting from N^2-*tert*-butoxycarbonyl, N^3-*trans*-4-oxo-phenyl-2-butenoyl-L-2,3-diaminopropanoic acid **8** (3.04 g, 8.4 mmol), sodium bicarbonate (0.7 g, 8.4 mmol), sodium carbonate (0.46 g, 4.3 mmol), hydrogen peroxide (0.8 ml, 50% soln. 14.5 mmol) and using ethyl acetate–MeOH (20 : 1) for column chromatography, compound **9** was prepared as described for the preparation of **6**. Yield 2.3 g, 64%. M.p. 143–145°C. NMR (DMSO-d_6) $\delta = 1.32$ (9H, s), 3.3–3.55 (2H, m), 3.64 (1H, d, $J = 2.0$ Hz), 3.9 (1H, m), 4.6 (1H, d, $J = 2$ Hz), 6.45 (1H, m), 7.5–7.8 (3H, m), 7.95–8.1 (2H, m), 8.3–8.5 (1H, m). $[\alpha]_{578}$ +6.2° ($c = 1$, MeOH). Found: C, 57.32; H, 5.60; N, 7.63. $C_{18}H_{22}N_2O_7$ requires C, 57.14; H, 5.82; N, 7.40%.

N^3-trans-4-oxopentenoyl-L-2,3-diaminopropanoic Acid Trifluoroacetate 1

Compound **5** (0.6 g, 2 mmol) was dissolved in cold trifluoroacetic acid (10 ml) and anisole (1 ml) and kept for 2 h. TFA was removed *in vacuo*, the residue was triturated with ethyl ether and the precipitate was filtered off and dried *in vacuo* over KOH pellets. Yield 0.56 g, 89% as an amorphous powder. NMR (D_2O) $\delta = 2.23$ (3H, s), 3.56–3.75 (2H, m), 3.92–4.02 (1H, m), 6.8 (2H, ABq, $J = 16.0$ Hz). $[\alpha]_{578}$ −24.2° ($c = 1$, DMF). Found: C, 37.81; H, 4.28; N, 9.05. $C_8H_{12}N_2O_4CF_3COOH$ requires C, 38.21; H, 4.14; N, 8.91%.

N^3-DL-trans-3(1-oxoethyl)oxirane-2-carbonyl-L-2,3-diaminopropanoic Acid Trifluoroacetate 2

This was prepared from **6** as described for compound **1**. Yield 1.25 g, 86% as an amorphous powder. NMR (D_2O) $\delta = 2.1$ (3H, s), 3.40 (1H, d, $J = 1.9$ Hz), 3.6–3.65 (2H, m), 3.68 (1H, d, $J = 1.9$ Hz), 4.6–4.9 (1H, m). $[\alpha]_{578}$ −14.2° ($c = 1$, DMF). Found: C, 36.22; H, 4.12; N, 8.68. $C_8H_{12}N_2O_5CF_3COOH$ requires C, 36.36; H, 3.93; N, 8.48%.

N^3-trans-4-oxo-4-phenyl-2-butenoyl-L-2,3-diaminopropanoic Acid Trifluoroacetate 3

This compound was prepared from **8** as described for compound **1**. Yield 0.69 g, 91%. M.p. 103–105°C. NMR (D_2O) $\delta = 3.4$–3.7 (2H, m), 3.9–4.1 (1H, m), 7.2 (1H, d, $J = 16.0$ Hz), 7.3–7.6 (3H, m), 7.9–8.2 (2H, m and 1H, d, $J = 16.0$ Hz). $[\alpha]_{578}$ −27.5° ($c = 1$, DMF). Found: C, 47.59; H, 4.12; N, 7.62. $C_{13}H_{14}N_2O_4CF_3COOH$ requires C, 47.87; H, 3.98; N, 7.44%.

N^3-DL-trans-3(1-oxobenzyl)oxirane-2-carbonyl-L-2,3-diaminopropanoic Acid Trifluoroacetate 4

This was obtained from **9** as described for **1**. Yield 1.45 g, 88% M.p. 145–148°C. NMR (D_2O) $\delta = 3.55$ (1H, d, $J = 2.0$ Hz), 3.6–3.8 (2H, m), 3.9–4.0 (1H, m), 4.6 (1H, d, $J = 2.0$ Hz), 7.4–7.7 (3H, m), 7.8–8.8 (2H, m). $[\alpha]_{578}$ −35.2° ($c = 1$, DMF). Found: C, 45.77; H, 3.68; N, 7.31. $C_{13}H_{14}N_2O_5CF_3COOH$ requires C, 45.92; H, 3.82; N, 7.14%.

N^2-(N-tert-butoxycarbonyl-L-norvalyl),N^3-DL-trans-3(1-oxoethyl) oxirane-2-carbonyl-L-2,3-diaminopropanoic Acid 10

To a cooled solution of **2** (0.82 g, 2.5 mmol) and $NaHCO_3$ (0.42 g, 5 mmol) in water (5 ml), the N-hydroxysuccinimide ester of N-(*tert*-butoxycarbonyl)-L-norvaline (0.78 g, 2.5 mmol) dissolved in MeOH (5 ml) was added. The mixture was kept overnight, the solvent was evaporated, and the residue was dissolved in water (5 ml) acidified with 1 M $KHSO_4$, and extracted with ethyl acetate (3 × 20 ml). The organic extract was dried over $MgSO_4$ and evaporated to give **10** (0.75 g, 72%) as a colorless solid. M.p. 180–182°C. NMR (DMSO-d_6) $\delta = 0.8$ (3H, t, $J = 7.0$ Hz), 1.2–1.4 (2H, m and 9H, s), 1.5–1.7 (2H, m), 2.05 (3H, s), 3.3–3.4 (2H, m), 3.56 (1H, d, $J = 1.8$ Hz), 3.74 (1H, d, $J = 1.8$ Hz), 3.8 (1H, m), 3.9 (1H, m). $[\alpha]_{578}$ −7.1° ($c = 1$, MeOH). Found: C, 51.82; H, 7.22; N, 10.22. $C_{18}H_{29}N_3O_8$ requires C, 52.05; H, 6.98; N, 10.12%.

N^2-(N-tert-butoxycarbonyl-L-norvalyl),N^3-trans-4-oxo-4-phenyl-2-butenoyl-L-2,3-diaminopropanoic Acid 11

This compound was prepared from **3** and the N-hydroxysuccinimide ester of N-(*tert*-butoxycarbonyl)-L-norvaline using the procedure for the preparation of **10**. Yield 0.86 g, 62%. M.p. 150–155°C. NMR (DMSO-d_6) $\delta = 0.8$ (3H, t, $J = 7.0$ Hz), 1.25–1.45 (2H, m, and 9H, s), 1.5–1.7 (2H, m), 3.4–3.6 (2H, m), 4.0–4.2 (1H, m), 4.2–4.3 (1H, m), 7.1 (1H, d, $J = 15.5$ Hz), 7.2–7.4 (1H, br.s), 7.45–7.70 (3H, m), 7.9 (2H, m), 8.1 (1H, d, $J = 15.5$ Hz), 8.3–8.4 (1H, br.s), 8.4–8.6 (1H, br.s). $[\alpha]_{578}$ −17.8° ($c = 1$, MeOH). Found: C, 59.66; H, 6.91; N, 9.03. $C_{23}H_{31}N_3O_7$ requires C, 59.86; H, 6.73; N, 9.11%.

N^2-(N-tert-butoxycarbonyl-L-norvalyl),N^3-DL-trans-3(1-oxobenzyl) oxirane-2-carbonyl-L-2,3-diaminopropanoic Acid 12

This was prepared from compound **4** and the N-hydroxysuccinimide ester of N-(*tert*-butoxycarbonyl)-L-norvaline by the procedure used for the synthesis of **10**. Yield 0.89 g, 75%. M.p. 172–175°C NMR (DMSO-d_6) $\delta = 0.8$ (3H, t, $J = 7.0$ Hz), 1.2–1.4 (2H, m, and 9H, s), 1.5–1.7 (2H, m), 3.3–3.4

(2H, m), 3.6 (1H, d, $J = 1.8$ Hz), 3.8–3.9 (1H, m), 4.1-4.2 (1H, m, z), 4.6 (1H, d, $J = 1.8$ Hz), 7.1 (1H, m), 7.5–7.6 (3H, m), 7.75 (1H, m), 8.0–8.1 (2H, m), 8.4-8.5 (1H, m). $[\alpha]_{578}$ −5.2° ($c = 1$, MeOH). Found: C, 57.66; H, 6.62; N, 8.66. $C_{23}H_{31}N_3O_8$ requires C, 57.86; H, 6.49; N, 8.80%.

N^2-L-norvalyl-N^3-DL-trans-3(1-oxoethyl)oxirane-2-carbonyl-L-2,3-diaminopropanoic Acid Trifluoroacetate 13

This compound was prepared from **10** as described for **1**. Yield 0.35 g, 79% M.p. 135–137°C NMR (D_2O) $\delta = 0.8$ (3H, m), 1.2–1.3 (2H, m), 1.4–1.7 (2H, m), 2.1 (3H, s), 3.3–3.4 (2H, m), 3.55 (1H, d, $J = 1.8$ Hz), 3.75 (1H, d, $J = 1.8$ Hz), 3.95 (1H, m), 4.1 (1H, m). $[\alpha]_{578}$ +8.1° ($c = 1$, DMF). Found: C, 36.42; H, 4.68; N, 9.83. $C_{13}H_{21}N_3O_6CF_3COOH$ requires C, 36.36; H, 4.89; N, 9.79%.

N^2-L-norvalyl-N^3-trans-4-oxo-4-phenyl-2-butenoyl-L-2,3-diaminopropanoic Acid Trifluoroacetate 14

Compound **14** was synthesised from **11** by the method used for **1**. Yield 0.64 g, 82%. M.p. 120–125°C. NMR (D_2O) $\delta = 0.85$ (3H, m), 1.2–1.3 (2H, m), 1.4–1.7 (2H, m), 3.3–3.4 (2H, m), 3.95–4.1 (1H, m) 4.2–4.3 (1H, m), 7.1 (1H, d, $J = 15.5$ Hz), 7.4–7.6 (2H, m), 7.9 (2H, m), 8.1 (1H, d, $J = 15.5$ Hz), 8.3–8.4 (1H, m). $[\alpha]_{578}$ +4.8° ($c = 1$, DMF). Found: C, 45.62; H, 4.97; N, 8.88. $C_{18}H_{23}N_3O_5CF_3COOH$ requires C, 45.47; H, 4.84; N, 8.84%.

N^2-L-norvalyl-N^3-DL-trans-3(1-oxobenzyl)oxirane-2-carbonyl-L-2,3-diaminopropanoic Acid Trifluoroacetate 15

This was obtained from **12** as described for **1**. Yield 0.85 g, 92%. M.p. 154–156°C. NMR (D_2O) $\delta = 0.8$ (3H, m), 1.2–1.3 (2H, m), 1.4–1.7 (2H, m), 3.4–3.5 (2H, m), 3.55 (1H, d, $J = 2.0$ Hz), 3.9–4.0 (1H, m), 4.1–4.2 (1H, m), 4.6 (1H, d, $J = 2.0$ Hz), 7.4–7.6 (2H, m), 7.9 (2H, m), 8.3–8.4 (1H, m). $[\alpha]_{578}$ +2.4° ($c = 1$, DMF). Found: C, 44.78; H, 4.77; N, 8.72. $C_{18}H_{23}N_3O_6CF_3COOH$ requires C, 44.90; H, 4.68; N, 8.55%.

Purification of the Enzyme and Determination of Glucosamine-6-phosphate Activity

Candida albicans glucosamine-6-phosphate synthase overproduced by *Saccharomyces cerevisiae* YRSC-65 was purified to apparent homogeneity by the method described recently by Milewski *et al.*[13] Glc-6-P synthase activity was assayed as described previously, using 15 mM D-fructose-6-phosphate, 10 mM L-glutamine, 50 mM potassium phosphate buffer

(pH 6.5), inhibitor at appropriate concentration and 0.1–0.2 μM Glc-6-P synthase in a total volume of 2 ml incubation mixture. The concentration of glucosamine-6-phosphate was determined by the modified Elson–Morgan procedure.[14]

Inactivation of Glucosamine-6-phosphate Synthase

Standard incubation mixtures containing 5 μg of Glc-6-P synthase, 50 mM potassium phosphate buffer (pH 6.5), 1 mM EDTA, 15 mM D-fructose-6-phosphate and inactivators at various concentrations in a total volume of 1 ml were incubated at 25°C. To follow inactivation of the enzyme, 200 μl aliquots were withdrawn from the mixtures and applied at the top of small, 1 ml columns packed with Sephadex G-25 (equilibrated previously with 50 mM potassium phosphate buffer pH 6.5) and centrifuged ($500 \times g$ for 1 min at 4°C). Under these conditions the unbound inhibitor was separated from the enzyme and the protein was recovered in test tubes. Appropriate effluent aliquots were used for the determination of the residual enzyme activity. All the measurements were done in triplicate.

Molecular Modelling

The molecular modelling was done using software programs from Molecular Simulations Inc. Calculations were performed with the Discover® program, using CVFF forcefield, and graphical displays were printed out from the Insight®II molecular modelling system. Since the X-ray structure of the complete GlcN-6-P synthase is not available so far, the geometry of the protein-inhibitor complex was modelled on the basis of the single glutamine binding domain structure 1GDO available in the Protein Data Bank.[15] The original X-ray structure ligand was removed, and the BADP molecule was docked in its place. This complex was then fully relaxed and the energy was optimized.

In Vitro Activity

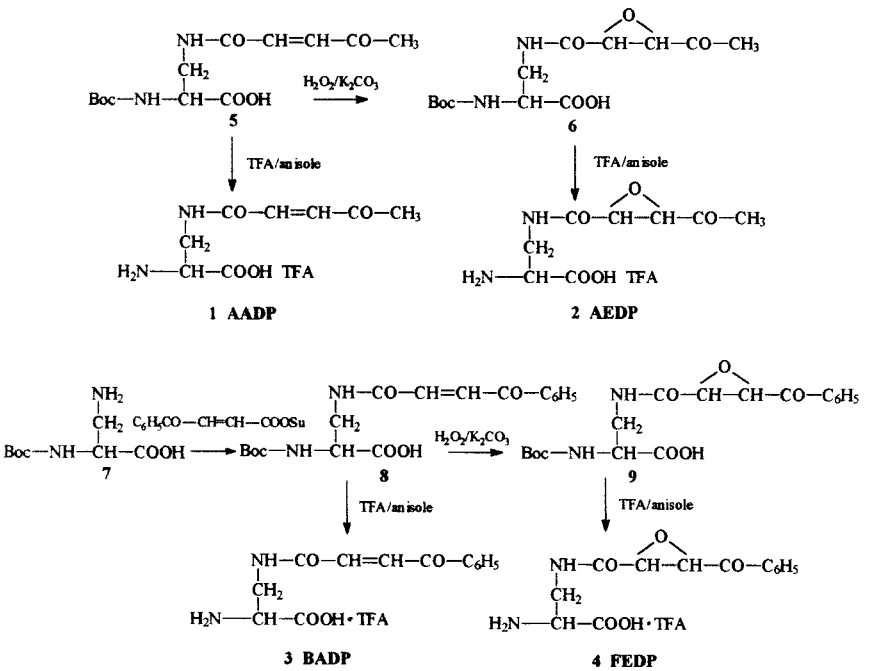
Minimal inhibitory concentrations (MIC) were determined by a serial dilution method on YNB modified medium containing 1.7 g of YNB without amino acids and ammonium sulfate (Difco), 0.4 g of sodium glutamate, 10 mg of L-histidine, 20 mg of L-methionine, 20 mg of L-tryptophan and 10 mg of D-glucose in 1000 ml of distilled water. The medium was inoculated with *Candida albicans* ATTC 26278 cells from overnight culture on

the same medium to a concentration of 10^4 cfu/ml and incubated at 30°C for 48 h. Results were determined using a turbidimetric method at 660 nm.

RESULTS AND DISCUSSION

Synthesis

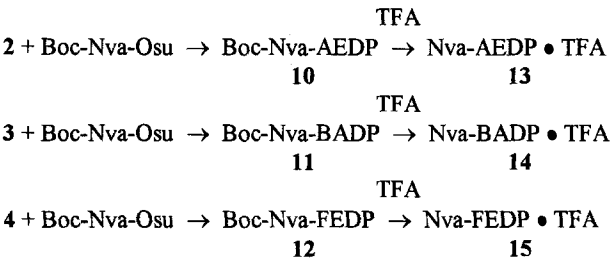
The synthesis (Scheme 1) started with N^2-*tert*-butoxycarbonyl,N^3-*trans*-4-oxo-2-pentenoyl-L-2,3-diaminopropanoic acid **5** and N^2-*tert*-butoxycarbonyl-L-2,3-diaminopropanoic acid **7** prepared by known procedures.[8,12] Then *trans*-β-benzoylacrylic acid[16] was activated with dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (HOSu)[17] to give the active ester in good yield (95%). The active ester was used for the acylation of **7** to obtain the corresponding L-2,3-diaminopropanoic acid derivative **8** in moderate yield. After deprotection of the Boc groups with anhydrous trifluoroacetic acid (TFA) in the presence of anisole, the compounds **1** and **3** were tested as GlcN-6-P synthase inhibitors and used for the preparation of peptides. Conversion of the α,β-unsaturated compounds **5** and **8** to the epoxy acids



SCHEME 1 Synthesis of novel GlcN-6-P synthase inhibitors **1–4**.

6 and **9** (as racemic mixtures) was accomplished in about 70% yield by epoxidation with 50% hydrogen peroxide in the presence of K_2CO_3.[18] Earlier attempts to obtain activated epoxyketoacid esters from the corresponding epoxyketoacids failed due to partial polymerization reaction. Deprotection of the Boc groups with TFA afforded compounds **2** and **4**.

For the synthesis of dipeptides (Scheme 2) compounds **2–4** were acylated with the N-hydroxysuccinimide ester of *tert*-butoxycarbonyl-L-norvaline to give the protected dipeptides **10–12**, which were deprotected by TFA to yield the dipeptides **13–15** in good yields. All new compounds were fully characterized by ^{1}H NMR and elemental analyses.



SCHEME 2 Synthesis of dipeptides **13–15**.

GlcN-6-P Synthase Inhibition Studies

Compounds **1–4** together with FMDP and EADP were tested as inhibitors and inactivators of pure *Candida albicans* GlcN-6-P synthase overproduced by *Saccharomyces cerevisiae* YRSC-65f.[13]

The cumulative data (Table I) confirm that substitution of the methyl ester in FMDP with methylketone in AADP **1** drastically reduced inhibitory

TABLE I Inhibitory and inactivatory data for compound **1–4** in respect to *C. albicans* GlcN-6-P synthase

Compound	*Inhibition*	*Inactivation*			
	IC_{50} (μM)	K_{inact} (μM)	T (min)	k_2 (min^{-1})	k_2/K_{inact} ($M^{-1}s^{-1}$)
FMDP	4.0	2.1	2.97	0.233	1850
EADP	58	83	1.20	0.572	115
AADP **1**	1400	7500	7.5	0.092	0.205
AEDP **2**	2800	13100	1.20	0.573	0.730
BADP **3**	200	8.3	1.15	0.597	1200
BADP (Gln)*	—	140	0.9	0.764	91
BADP (Fru-6-P)*	—	12.8	9.0	0.079	104
FEDP **4**	380	2210	1.06	0.689	5.200

*Inactivation performed in the presence of either 10 mM L-Gln or 10 mM D-Fru-6-P.

activity (IC_{50}), affinity of an inhibitor to the enzyme active site (K_{inact}) and reactivity (k_2) towards the enzyme. The phenyl ketone analogue BADP **3** showed affinity and reactivity comparable to that of FMDP. Moreover, its inactivatory potency (k_2/K_{inact}) was lowered approximately by 30% in comparison to FMDP. As expected, L-Gln efficiently protected enzyme against inactivation (Figure 1).

Unexpectedly, Fru-6-P strongly reduced reactivity of an inhibitor towards the enzyme without affecting affinity. Fru-6-P usually promotes inactivation of an enzyme by modification of enzyme conformation, thus it facilitates binding of inhibitors to the glutamine binding site.[7,9] To explain this phenomenon we examined the structure of the GlcN-6-P synthase–BADP inhibitor complex by means of molecular modelling. Our calculations revealed that since the keto moiety is relatively immobile, the only possible conformation that allows BADP molecule to fit into the glutamine binding pocket is the conformation with the phenyl ring sticking out from the glutamine binding site (Figure 2).

In the native enzyme, glutamine and Fru-6-P binding sites are spatially located close to each other. Thus in the presence of Fru-6-P, BADP molecule may not be suitably fitted into the glutamine binding site to adopt the most suitable conformation for enzyme inactivation. Presumably, the inhibitor phenyl ring may be principally involved in the steric interaction with Fru-6-P. Substitution of a primary amide (EADP) with a methyl

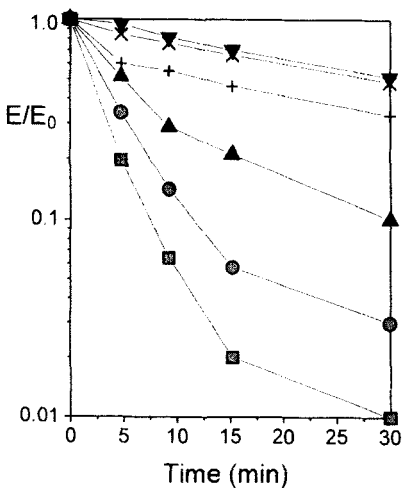


FIGURE 1 Inactivation of *C. albicans* GlcN-6-P synthase with BADP and protective effect of substrates: 10 μM BADP (■); 5 μM BADP (●); 1 mM Fru-6-P (▲); 10 mM Fru-6-P (▼); 1 mM Gln (+); 10 mM Gln (×).

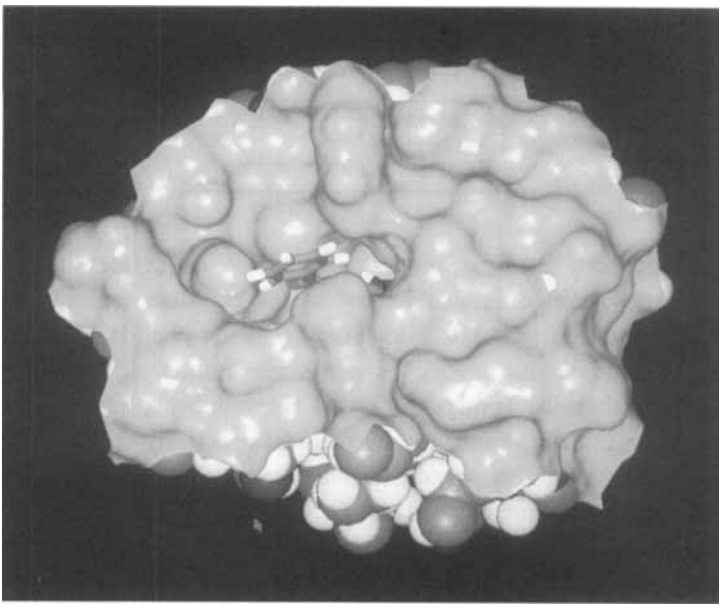


FIGURE 2 Potential complex of BADP in the GlcN-6-P synthase glutamine binding site. See Color Plate I.

ketone, AEDP **2**, strongly reduces affinity of an inhibitor, but does not affect reactivity. The epoxy analogue FEDP **4** containing a phenyl ketone, however, displays lowered affinity to the enzyme and a somewhat higher reactivity than that of EADP. GlcN-6-P synthase was inhibited by the novel glutamine analogues **1–4** competitively in respect to L-Gln and uncompetitively in respect to Fru-6-P. Although, the molecular mechanism of enzyme inactivation have not yet been established, it might be expected that compound **1** and **2** can react as Michael acceptors with the enzyme Cys-1 thiol group and that compounds **3** and **4** may also irreversibly alkylate the enzyme thiol group via nucleophilic epoxy ring opening.

Anticandidal Activity

All inhibitors exerted only poor antifungal activity but application of the "warhead delivery" concept,[19,20] i.e. incorporation of the inhibitor into a peptide structure has afforded a series of peptides with good antifungal activity when tested against *C. albicans* ATCC 26278 strain.[21] The data presented in Table II clearly shows that their anti-candidal potency was dependent on inhibitor structure. Thus, the peptide **15** was the most active and peptide **14** displayed weak activity. This is in direct contrast to the

TABLE II Anticandidal activity of compounds **13–15**

Dipeptide	*MIC* (μg/mL)
Nva-AEDP **13**	2.5
Nva-BADP **14**	7.5
Nva-FEDP **15**	1.0

inhibitory properties of compounds **3** and **4**. Inhibitory potency of BADP **3** was several orders of magnitude higher than that of FEDP **4**. Presumably, the transport rate into fungal cells via peptide permeases and the cleavage rate of the peptide by peptidases influenced their activities. It is believed that the transport rate of norvaline peptides[22] is a more important factor determining the anti-candidal activity than intracellular hydrolysis of peptides. It should also be noted that fungal growth was increased upon addition of N-acetylglucosamine (5 mM) to the medium containing peptides **13–15** (data not shown). This suggests that GlcN-6-P synthase is probably the only target for the action of the peptides.

CONCLUSIONS

We have developed novel, chemically stable, GlcN-6-P synthase inhibitors. The compound containing a phenyl ketone (BADP) shows exceptionally strong inactivatory properties out of the compounds tested. Its high affinity for the active site of the enzyme is comparable to that of FMDP, one of the most effective inactivators of GlcN-6-P known to date. This compound is of interest as a novel, chemically stable tool in GlcN-6-P synthase studies. Peptides with the novel inhibitors demonstrated also high anti-candidal activity. Further work is underway to study the molecular mechanism of the enzyme inactivation and anti-fungal activity of the peptides.

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