Journal of Enzyme Inhibition and Medicinal Chemistry, October 2005; 20(5): 439–447

Glutamine analogues containing a keto function – novel inhibitors of fungal glucosamine-6-phosphate synthase

ALEKSANDRA WALKOWIAK, ROLAND WAKIEĆ, MARIA M. BONTEMPS-GRACZ, & RYSZARD ANDRUSZKIEWICZ

Department of Pharmaceutical Technology and Biochemistry, Gdańsk University of Technology, 11/12 Narutowicza Street, 80-952 Gdańsk, Poland

(Received 30 May 2005; in final form 20 June 2005)

Abstract

A series of novel inhibitors of glucosamine-6-phosphate synthase, analogues of AADP and BADP, have been synthesized and their inhibitory, lipophilic and antifungal properties have been tested. The improvement in lipophilicity has not much affected the antifungal activity of the new compounds. Dipeptides containing norvaline and selected inhibitors have shown substantial activity against *S. cerevisiae* and *C. glabrata* and only poor activity against *C. albicans* strain. These peptides do not seem to be toxic towards human cells.

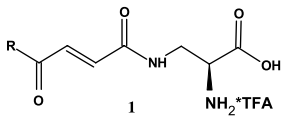
Keywords: *Antifungal agents, glucosamine-6-phosphate synthase inhibitors, AADP analogues, BADP analogues*

Introduction

Glucosamine-6-phosphate synthase (GlcN-6-P synthase – EC 2.6.1.16) is a key enzyme that catalyses the formation of D-glucosamine-6-phosphate (GlcN-6-P) from D-fructose-6-phosphate using L-glutamine as the ammonia source [1]. GlcN-6-P is then transformed into uridine-5′-N-acetylglucosamine (UDP-GlcN) which is an important aminosugar utilized in the biosynthesis of various cellular structures – lipopolisaccharides and peptidoglycan in bacteria, mannan and chitin in fungi, and glycoproteins in mammals. The enzyme may be considered as a selective molecular target since substantial differences in physiological consequences of GlcN-6-P synthase inhibition is observed in these organisms. In fungal cells, the GlcN-6-P synthase inhibition resulted in cell agglutination and lysis whereas in mammalian cells temporary inhibition of the enzyme is not lethal due to the rapid turnover of the mammalian gene encoding GlcN-6-P synthase. Therefore GlcN-6- synthase has been proposed as a potential target for design of antibacterial and especially antifungal agents. In antifungal chemotherapy, on the contrary to antibacterial chemotherapy there is still lack of safe and effective drugs [2]. GlcN-6-P synthase is inactivated by a number of electrophilic reagents, however, only selective and irreversible inhibitors may be of chemotherapeutic value. Of the GlcN-6-P synthase inhibitors, analogues of one of the enzyme substrates – glutamine, e.g. N^3-(4-methoxyfumaroyl)-(S)-2,3-diaminopropanoic acid (FMDP), N^3-(*trans*-epoxysuccinamoyl)-(S)-2,3-diaminopropanoic acid (EADP) have been shown to be strong and selective inactivators of fungal GlcN-6-P synthase [3,4]. Although these inhibitors displayed excellent inhibitory properties, they exhibit poor activity against whole cells. High polarity of the inhibitors effectively hampered their transport inside the cells. However, these inhibitors in the latent form – most commonly as peptides [5,6] can be recognized by peptide permeases and transported into the cells. Lipophilic FMDP-peptides [7] and diffusible FMDP analogues [8] also exerted antifungal activity. Development of inhibitor with enhanced lipophilicity that can enter the fungal cells by free diffusion may also be another possible solution to the

Correspondence: Ryszard Andruszkiewicz, Department of Pharmaceutical Technology and Biochemistry, Gdańsk University of Technology, 11/12 Narutowicza Street, 80-952 Gdańsk, Poland. Tel: 48 58 3471493. Fax: 48 58 3471144. E-mail: ryszarda@chem.pg.gda.pl

ISSN 1475-6366 print/ISSN 1475-6374 online © 2005 Taylor & Francis
DOI: 10.1080/14756360500272690



where R is:

phenyl (**BADP**)	2,4,6-trimethylphenyl (**1g**)	4-chloro-3-nitrophenyl (**1p**)
methyl (**AADP**)	2-chlorophenyl (**1h**)	naphthyl (**1q**)
methoxyl (**FMDP**)	3-chlorophenyl (**1i**)	2-furyl (**1r**)
4-methylphenyl (**1a**)	4-chlorophenyl (**1j**)	2-pyrrolyl (**1s**)
4-ethylphenyl (**1b**)	2-bromophenyl (**1k**)	*tert*-butyl (**1t**)
4-*n*-butylphenyl (**1c**)	3-bromophenyl (**1l**)	ethyl (**1u**)
4-*tert*-butylphenyl (**1d**)	4-bromophenyl (**1m**)	*iso*-propyl (**1w**)
4-decylphenyl (**1e**)	4-fluorophenyl (**1n**)	*n*-butyl (**1x**)
2,4-dimethylphenyl (**1f**)	3,4-dichlorophenyl (**1o**)	

Figure 1. Structures of new inhibitors.

GlcN-6-P synthase inhibitor transport problem. Previously, we have synthesised novel inhibitors and inactivators of fungal GlcN-6-P synthase containing a keto function, i.e. N^3-*trans*-4-oxo-2-pentenoyl-L-2,3-diaminopropanoic acid (AADP) and N^3-*trans*-4-oxo-4-phenyl-2-butenoyl-L-2,3-diaminopropanoic acid (BADP) and demonstrated their inhibitory activity against fungal GlcN-6-P synthase [9]. Based on this observation, we decided to synthesise a series of novel BADP and AADP analogues with enhanced lipophilicity and to evaluate these compounds as GlcN-6-P synthase inhibitors (Figure 1).

We have also determined the inhibitory activity of the new inhibitors against glucosamine-6-phosphate synthase, their affinity to biological membranes and their antifungal activity. Moreover, we have synthesised three peptides (**7b, s, u**) containing novel inhibitors (**1 b, s, u**) and norvaline in the N-terminal position, and tested their *in vitro* activity. Previously, we have demonstated that peptides containing norvaline and GlcN-6-P synthase inhibitors displayed excellent antifungal activity [5,6].

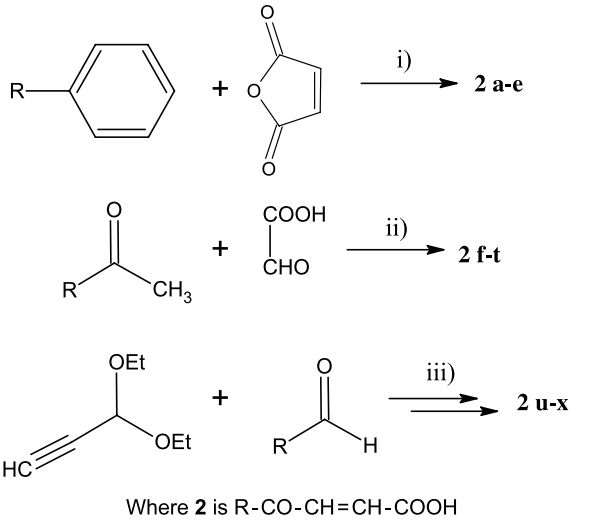
Experimental

Chemistry

*(E)-4-oxo-4-(2′-pyrrolyl)-2-butenoic acid (**2s**)*. A solution of 2-acetylpyrrole (1.0 g; 9.2 mmol), glyoxylic acid monohydrate (1.69 g, 9.2 mmol) in 2 M NaOH (20 ml) and methanol (6 ml) was heated for 5 h at 50°C. The reaction mixture was then acidified with 2 M HCl and evaporated to a small volume. The concentrated solution was extracted with diethyl ether (5 × 30 ml), dried over $MgSO_4$ and the solvents were evaporated leaving an oily residue which was crystallized from diethyl ether /hexane to yield **2s** as a solid (0.45 g, 30%). ^{1}H NMR (500 MHz, $CDCl_3$) δ = 6.65–6.66 (m, 1H), 7.01 (d, 1H), 7.42 (d, 1H), 7.73 (m, 1H), 7.86 (d, 1H). MS(FAB) *m/z:* 166 (MH^+)

*(E)-3-(2′-ethyl-1′,3′-dioxolan-2′-yl)-2-propenoic acid (**3u**)*. To a solution of (*E*)-4-oxo-2-hexenoic acid (0.40 g, 3.2 mmol) in benzene (20 ml) (CARE–Carcinogenic) triethylorthoformate (1.74 ml, 32 mmol), ethylene glycol (2.6 ml, 16 mmol) and *p*-toluenesulfonic acid (0.08 g, 0.4 mmol) were added. The mixture was heated at 35°C for 24 h, then diluted with ethyl acetate (20 ml) and concentrated NaCl solution (20 ml) and extracted with ethyl acetate (3 × 50 ml). The combined organic phase was dried over $MgSO_4$, the solvents were evaporated and the residue was dissolved in methanol (40 ml) and treated with 2 M KOH solution (10 ml) at room temperature. After 24 h the solution was acidified to pH 4 with conc. HCl end extracted with CH_2Cl_2. The organic phase was dried, the solvents were evaporated and the residue was purified by column chromatography on silica gel gel ($CHCl_3$–AcOH, 60:1) to give **3u** (0.35 g, 75%) as white crystals. M.p. = 63–64°C; ^{1}H NMR (500 MHz, $CDCl_3$) δ = 0.94 (t, 3H), 1.78 (q, 2H), 3.88–3.93 (m, 2H), 3.96–4.00 (m, 2H), 6.10 (d, 1H), 6.84 (d, 1H). MS(FAB) *m/z*: 173 (MH^+)

*N-succinimidoyl ester of (E)-4-(4′-methyl)phenyl-4-oxo-2-butenoic acid (**4a**)*. DCC (0.45 g, 2.2 mmol) dissolved in THF (10 ml) was slowly added to a solution of (*E*)-4-(4′-methyl)phenyl-4-oxo-2-butenoic acid **2a** (0.38 g, 2 mmol) and *N*-hydroxysuccinimide (0.23 g, 2 mmol) in freshly distilled THF (10 ml) at 0°C. After 24 h the precipitate formed was filtered off, the solution was concentrated and a few drops of acetic acid were added and the mixture kept for 24 h.



Scheme 1. Syntheses of (*E*)-4-oxo-2-butenoic acids (**2**). i) $AlCl_3$; ii) H^+ for **2 f–q** and **2 t**; 1. H^+2. OH^- for **2 r**; OH^- for **2 s**; iii) 1. BuLi, 2. H_2O, 3. MnO_2, 4. HBr_{aq}/dioxane.

Table I. Properties of (*E*)-4-oxo-2-butenoic acids.

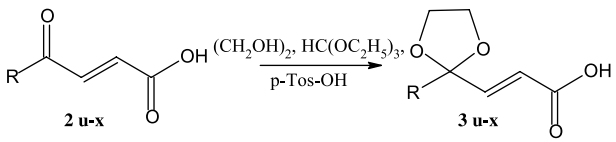
Co	R=	Method of synthesis (lit.)	Yield [%]	Crystal. solvents	Mp. [°C] (lit.)
2a	4-$CH_3C_6H_4$	A1[14]	75	toluene	135–137 (139–140)[15]
2b	4-$C_2H_5C_6H_4$	A2[15]	80	cyclohex/toluene	95–98 (105–106)[15]
2c	4-*n*-$C_4H_9C_6H_4$	A2	82	cyclohex/toluene	76–78 (90–91)[15]
2d	4-*tert*-$C_4H_9C_6H_4$	A2	63	cyclohex/toluene	119–123 (123–125)[13]
2e	4-*n*-$C_{10}H_{21}C_6H_4$	A2	79	cyclohex/toluene	79–81 (82–83)[15]
2f	2,4-$(CH_3)_2C_6H_3$	B1[18]	39	cyclohex/toluene	105–106 (113–114)[16]
2g	2,4,6-$(CH_3)_3C_6H_2$	B1	52	hex/diethyl ether⋆	139–141
2h	2-ClC_6H_4	B1	24	hex/benzene	78–81
2i	3-ClC_6H_4	B1	23	hex/benzene	151–153
2j	4-ClC_6H_4	B1	50	toluene/ethyl acetate	154–156 (156–157)[15]
2k	2-BrC_6H_4	B1	28	hex/benzene	76–80
2l	3-BrC_6H_4	B1	54	hex/benzene	145–147
2m	4-BrC_6H_4	B1	36	hex/benzene	150–153 (154–155)[13]
2n	4-FC_6H_4	B1	23	benzene	130–132 (133–135)[16]
2o	3,4-$(Cl)_2C_6H_3$	B1	32	hex/benzene	138–141
2p	3-Cl–4-$NO_2C_6H_3$	B1	34	hex/benzene	154–156
2q	2-$C_{10}H_7$	B1	56	hex/benzene	158–161
2r	2-C_4H_3O	B2[18]	10	2-propanol	153–155 (158–160)[18]
2s	2-C_4H_3N	B3	30	hex/diethyl ether	> 160 (dec.)
2t	$(CH_3)_3C$	B1	14	hex/benzene	87–92 (68–86)[21]
2u	CH_3CH_2	C[19]	47	chloroform/acetic acid⋆	105-108 (110–112)[22]
2w	$(CH_3)_2CH$	C	27	chloroform/acetic acid⋆	81–83 (83)[23]
2x	$CH_3(CH_2)_3$	C	27	chloroform/acetic acid⋆	98–100 (98–99,5)[24]

⋆ Purified by column chromatography on silica gel.

Then the rest of the dicyclohexylurea was filtered off and the residue was crystallised from THF/diethyl ether giving **4a** (0.46 g, 80%) as yellow crystals. M.p. = 137–141°C; ^{1}H NMR (200 MHz, $CDCl_3$) δ = 2.48 (t, 3H), 2.93 (s, 4H), 7.04–7.11 (d, 1H), 7.34–7.38 (d, 2H), 7.92–7.96 (d, 2H), 8.12–8.20 (d, 1H). MS(FAB) *m/z* 288 (MH^+)

*(2S)-2-[(N-tert-butoxycarbonyl)amino]-3-{[(2E)-4-oxo-4-(4-methylphenyl)-2-butenoyl]amino}propanoic acid (**5a**).* (2*S*)-3-amino-2-[(N-*tert*-butoxycarbonyl) amino]propanoic acid (2.04 g, 10 mmol) and $NaHCO_3$ (0.84 g, 10 mmol) were dissolved in water-methanol solution (1:1, 20 ml). Then the *N*-succinimidoyl ester of (*E*)-4-(4′-methyl)phenyl-4-oxo-2-butenoic acid (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 evaporated leaving an oily residue which was crystallised from ethyl ether/hexane to give **5a** (3.0 g, 63%) as a white solid. ^{1}H NMR (200 MHz, $CDCl_3$) δ = 1.46 (s, 9H) 2.44 (s, 3H), 3.77–3.94 (m, 2H), 4,40–4.44 (m, 1H), 6.0–6.1 (m, 1H), 6.98–7.06 (d, 1H), 7.30 (d, 2H), 7.90–7.99 (m, 3H). MS(FAB) *m/z*: 377 (MH^+)

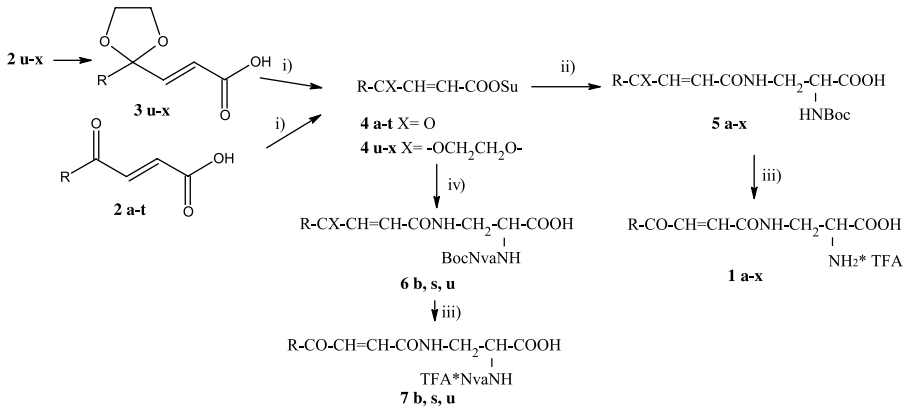
*(2S)-2-amino-3-{[(2E)-4-oxo-4-(4′-methyl)phenyl-2-butenoyl]amino}propanoic acid trifluoroacetate (**1a**).* Compound **5a** was dissolved in cold trifluoroacetic acid (10 ml) and kept for 1 h. TFA was removed *in vacuo*, the residue was triturated with diethyl ether and the precipitate was filtered off and dried over KOH pellets to give **1a** (2.13 g, 55%) as an amorphous powder $[\alpha]_{589}^{20} = -10.1°$ (c = 1, DMSO), ^{1}H NMR (200 MHz, DMSO-d_6) δ = 2.48 (s, 3H), 3.4–3.8 (m, 3H), 6.96 (d, 1H), 7.39 (d, 2H), 7.76 (d, 1H), 7.93 (d, 1H), 8.8–8.9 (m, 1H). MS(FAB) *m/z*: 252 (MH^+).



Scheme 2. Syntheses of keto protected derivatives of alkenoic acids.

Table II. Properties of keto protected derivatives of alkenoic acids.

Comp	Yield (%)	M.p. (°C)
3u	75	63–64
3w	80	60–61
3x	72	82–84



Scheme 3. Syntheses of new compounds. i) HOSu, DCC, ii) 1. $NH_2CH_2CH(NHBoc)COONa$, 2. H^+, iii) TFA, iv) 1. $NH_2CH_2CH(BocNvaNH)COONa$, 2. H^+.

*(2S)-2-[(N-tert-butoxycarbonyl)norvalyl]amino-3-{[(E)-4-oxo-4-(4′-ethyl)phenyl-2-butenoyl]amino}-propanoic acid (**6b**)*. Compound **6b** was synthesized from (2*S*)-3-amino-2-{[(*N*-*tert*-butoxycarbonyl)norvalyl]amino}propanoic acid **5a** (0.303 g, 1 mmol) and the *N*-succinimidoyl ester of (*E*)-4-oxo-4-(4′-ethyl)phenyl-2-butenoic acid (**4b**) (0.301 g, 1 mmol). It was purified on silica gel ($CHCl_3$–MeOH–AcOH, 15:1:0.05) to afford **6b** (0.25 g, 51%) as an oily residue. ^{1}H NMR (500 MHz, $CDCl_3$) δ = 0.90–0.95 (m, 3H), 1.25 (t, 3H), 1.40–1.44 (m, 11H), 1.52–1.75 (m, 2H), 2.70 (q, 2H), 3.14–3.29 (m, 2H), 3.99–4.02 (m, 1H), 4.32–4.35 (m, 1H), 7.05 (d, 1H), 7.30 (m, 2H), 7.91–7.94 (m, 3H). MS(FAB) *m*/*z*: 490 (MH^+)

*(2S)-2-(norvalyl)amino-3-{[(E)-4-oxo-4-(4′-ethyl)phenyl-2-butenoyl]amino}propanoic acid trifluoroacetate (7**b**)*. Compound **6b** was treated with trifluoroacetic acid (10 ml) for 0.5 h at 0°C. TFA was evaporated *in vacuo* and the residue was triturated with ethyl ether, the precipitate was filtered off, dried over KOH pellets giving **7b** (0.23, 91%) as an amorphous powder, $[\alpha]_{589}^{20} = -1.1$ (c = 1, H_2O), ^{1}H NMR (200 MHz, D_2O) δ = 0.83–0.93 (m, 3H), 1.14 (t, 3H), 1.21–1.41 (m, 2H), 1.76–1.84 (m, 2H), 2.59–2.73 (m, 2H), 3.7–3.9 (m, 2H), 3.95–4.05 (m, 1H), 4.6–4.7 (m, 1H), 6.88 (d, 1H), 7.37–7.39 (m, 2H), 7.77 (d, 1H), 7.87–7.90 (m, 2H). MS(FAB) *m*/*z*: 351(MH^+).

Biological preparation of the crude enzyme (cell free extract) and determination of glucosamine-6 phosphate synthase activity. Candida albicans glucosamine-6-phosphate synthase cell free extract was prepared by the previously described procedure [10]. The standard incubation mixture contained: D-fructose-6-phsphate (15 mM), L-glutamine (10 mM), EDTA (1 mM), potassium phosphate buffer (25 mM, pH 7.0), inhibitor at an appropriate concentration and enzymatic protein (0.005–0.01 mg ml^{-1}) in a total volume of 0.4 ml. The mixtures were incubated at 37°C for 30 min. The reaction was stopped by heating at 100°C for 1 min. The activity of glucosamine-6-phosphate synthase was determined according to the modified Elson-Morgan method [11].

Inactivation of glucosamine-6-phosphate synthase. Incubation mixtures containing: glucosamine-6-phosphate synthase from *C. albicans* (0.005–0.01 mg), albumin (1 mg/ml), phosphate buffer (25 mM, pH 7.0) EDTA (1 mM) and inactivators at various concentration in a total volume of 1 ml, were incubated at 25°C. After 5, 10, 15 and 30 min, aliquots (200 μl) were withdrawn from the reaction mixture and applied to the top of a small 1-ml column packed with Sephadex G-25 (equilibrated with 25 mM potassium phosphate buffer pH 7.0) and centrifuged (500 × g for 1 min at 4°C). Under these condition the unbound inhibitor was separated from the enzyme and the protein was recovered in clean test-tubes. Appropriate aliquots were used for the determination of the residual activity of glucosamine-6-phosphate synthase using the standard method. The GlcN-6-P synthase activity for various inhibitory concentrations was plotted versus time and the kinetic parameters were determined following the literature procedure [12].

Determination of the affinity to the artificial biological membrane. Interactions between the test compounds and immobilised artificial biological membrane were investigated using HPLC column IAM PC DD2 (Regis Technologies, Inc., Morton Grove, IL, USA) [13]. The column dimensions were 3 cm × 4.6 mm, particle diameter 300 Å. The chromatographic system consisted of a Model L-6200 A, Model L-4250 UV/VIS detector and Model D-2500 integrator (all from Merck-Hitachi, Vienna, Austria). Potassium phosphate buffer (0.1 M) was used as the mobile phase; the injection volume was 10 μl; the flow rate

Table III. Properties of N-succinimidoyl active esters.

Comp.	Yield (%)	M. p. (°C)	Comp.	Yield (%)	M. p. (°C)
4a	80	137–141	**4m**	66	150–155
4b	70	130–132	**4n**	60	160–162,5
4c	63	86–90	**4o**	70	140–143
4d	78	145–147	**4p**	52	176–181 (dec.)
4e	90	95–98	**4q**	77	181–183
4f	53	134–137	**4r**	81	172–178 (de.)
4g	90	157–161	**4s**	57	166–170 (dec.)
4h	56	92–95	**4t**	89	125–128
4i	83	127–131	**4u**	86	147–149
4j	67	141–144	**4w**	74	152–155
4k	81	97–100	**4x**	46	82–84
4l	82	145–149			

was 1 ml/min; the samples were detected at 220 nm. The dead volume of the column was determined by the retention time of citric acid (aqueous solution, 50 mg/ml) and was used to calculate capacity factors $k'_{IAM} = (t_r - t_0)/t_0$.

Antifungal susceptibility tests. Minimal inhibitory concentrations (MIC's) of the examined compounds were determined by the serial twofold dilution microtiter plate method, in the minimal liquid Yeast Nitrogen Base (YNB) medium without amino acids and ammonium sulphate containing 2% glucose and L-proline (4 mg ml^{-1}). Wells containing serially diluted test compounds and control were inoculated with 10^4 cells ml^{-1} of an overnight culture of fungal cells and the microtiter plates were incubated for 24 h at 30°C. Fungal growth was measured using the microplate reader (Labsystems, Multiscan Bichromatic) at $\lambda = 595$ nm. The MIC was defined as the inhibitor concentration preventing at least 80% of fungal growth, as compared to the inhibitor-free control.

Disc diffusion competition assay. The minimal solid YNB medium without amino acids and ammonium sulphate containing 2% glucose, 4 mg ml^{-1} of L-proline and 2% agar was liquefied by warming to 100°C and then chilled to 50°C. The semi-liquid medium was inoculated with 3×10^5 cells ml^{-1} of an overnight culture of fungal cells and poured into Petri dishes and left for solidification. Sterile filter discs, 6 mm diameter, were saturated with 10 µl portion of sterile aqueous solutions containing either 200 µg of GlcNAc or 100 µg of one of the test compounds. The discs were placed onto the agar medium surface. In each plate, the GlcNAc saturated disc was flanked by four discs containing test compounds placed at a distance of 20 mm. Plates were incubated at 30°C for 48 h. Growth inhibition zones appearing around the discs containing the test compounds were evaluated and observed distortions were assessed quantitatively.

Cell lines. Human promyelocytic leukemia sensitive cell line HL-60 (Kansas State University, Manhattan, KS, USA) was grown in RPMI 1640 medium supplemented with 10% FBS penicillin G (100 000 units/L), streptomycin (100 mg/L). The cell line was grown in a controlled (air-5% CO_2) humidified atmosphere at 37°C and was transplanted three times a week. For the experiments the cells in logarithmic growth were suspended in the growth medium to give a final required density.

In vitro *cytotoxic evaluation*. Cells of the required density were seeded and different concentrations of the test compounds were added. The experiments were carried out in a controlled (air-5% CO_2) humidified atmosphere at 37°C. The exposure time was 72 h. The cytotoxic activity (IC_{50} values) of the compounds was defined as their *in vitro* concentrations causing 50% inhibition of cell growth after continuous exposure to the test compound, as measured by cell counting with a Z2 Cell Analyzer

Table IV. Properties of protected inhibitors.

Comp.	R=	Yield (%)	M. p. (°C)
5a	4-$CH_3C_6H_4$	63	125–128
5b	4-$C_2H_5C_6H_4$	49	128–132
5c	4-n-$C_4H_9C_6H_4$	60	129–131
5d	4-tert-$C_4H_9C_6H_4$	64	127–130
5e	4-n-$C_{10}H_{21}C_6H_4$	53	134–136
5j	4-ClC_6H_4	67	141–144 (dec.)
5n	4-FC_6H_4	60	136–140
5r	2-C_4H_3O	20	oil (chloroform/methanol)*
5s	2-C_4H_3N	66	oil (chloroform/methanol)*
5u	CH_3CH_2	65	oil (chloroform/acetic acid)*
5w	$(CH_3)_2CH$	74	oil (chloroform/acetic acid)*
5x	$CH_3CH_2CH_2CH_2$	80	oil (chloroform/acetic acid)*

* Purified by column chromatography on silica gel

Table V. Properties of final inhibitors.

Comp.	R=	Yield (%)	$[\alpha]_{589}^{20}$
1a	4-$CH_3C_6H_4$	75	−10.1
1b	4-$C_2H_5C_6H_4$	80	−19.3
1c	4-*n*-$C_4H_9C_6H_4$	79	−31.5
1d	4-*tert*-$C_4H_9C_6H_4$	80	−41.7
1e	4-*n*-$C_{10}H_{21}C_6H_4$	86	−26.7
1f	2,4-$(CH_3)_2C_6H_3$	53	−44.7
1g	2,4,6-$(CH_3)_3C_6H_2$	87	−35.3
1h	2-ClC_6H_4	75	−13.3
1i	3-ClC_6H_4	29	−24.8
1j	4-ClC_6H_4	75	−36.7
1k	2-BrC_6H_4	57	−10.0
1l	3-BrC_6H_4	36	−31.3
1m	4-BrC_6H_4	45	−30.1
1n	4-FC_6H_4	80	−37.6
1o	3,4-$(Cl)_2C_6H_3$	45	−13.2
1p	3-Cl-4-$NO_2C_6H_3$	19	−14.9
1q	2-$C_{10}H_7$	67	−21.3
1r	2-C_4H_3O	45*	−32.1
1s	2-C_4H_3N	70	−213
1t	$(CH_3)_3C$	70	−28.2
1u	CH_3CH_2	90	−60.4
1w	$(CH_3)_2CH$	82	−36.2
1x	$CH_3CH_2CH_2CH_2$	89	−40.3

(Beckman Coulter). Results are given as the mean of at least three independent experiments ± standard error of the mean (SEM).

Results and discussion

Synthesis

The novel GlcN-6-P synthase inhibitors (indicated by Arabic numbers) were synthesised starting from suitably substituted at the C4 position (E)-4-oxo-2-butenoic acids, which were obtained using three different routes (Scheme 1).

Derivatives with the alkyl group in the *para* position of the phenyl ring, **2 a–e,** were obtained applying the Friedel-Crafts alkylation reaction of an appropriate alkylbenzene with maleic acid anhydride according to the published procedures [14–16] and described as Method A (see Table I).

All the other (*E*)-4-oxo-4-phenyl-2-butenoic acids **2 f**–**q** and compounds where the phenyl ring was replaced by furyl **2 r**, pyrrolyl **2 s** or *tert*-butyl **2 t** group were prepared by condensation of an appropriate methyl ketone with glyoxylic acid (Method B, Table I) [17,18]. The three acids **2 u**–**x** were prepared via condensation of the appropriate aldehyde with propionlaldehyde diethyl acetal followed by oxidation and hydrolysis (Method C, Table I)[19]. The keto function in these acids was first converted into a diacetal by reacting with ethylene glycol [20] to obtain the protected compounds **3 u**–**x** for further syntheses (Scheme 2 and Table II).

The crystalline acids were then activated with *N*-hydroxysuccinimide (HOSu) and dicyclohexylcarbodiimide (DCC) [25] according to the procedure described for the preparation of **4a** to give the active esters in good yields (Scheme 3 and Table III) and condensed with N^2-*tert*-butoxycarbonyl-(*S*)-2,3-diaminopropanoic acid to afford the protected inhibitors (Table IV).

Deprotection of the amino group and the keto function was performed simultaneously in anhydrous trifluoroacetic acid, and the final compounds were precipitated with diethyl ether (Table V).

The furyl derivative **1r** was additionally purified on Sephadex LH-20 in methanol. Three inhibitors **1 b, 1s** and **1u** were then combined with the N-hydoxysuccinimide ester of N-(*tert*-butoxycarbonyl)-L-norvaline to obtain the protected dipeptides **6b, 6s** and **6u** (Scheme 3 and Table VI).

Final deprotection of the Boc groups afforded dipeptides **7 b**, **7s** and **7u** in good yields (Table VI). All new compounds were fully characterised by ^{1}H NMR and mass spectrum analyses.

Table VI. Properties of new peptides.

Comp.	Yield (%)	Comp.	Yield (%)	$[\alpha]_{589}^{20}$
6b	51	**7b**	91	−1.4
6s	45	**7s**	90	+2.2
6u	61	**7u**	89	−4.2

GlcN-6-P synthase inhibition studies

All the newly synthesised inhibitors as well as the parent compounds AADP and BADP were tested against purified glucosamine-6-phosphate synthase from *Candida albicans* [26]. Their ability to inhibit this enzyme was measured by determining a concentration which caused 50% inhibition of the enzyme[27]. Kinetic parameters of the inactivation by compounds **1a**–**x**, AADP and BADP are summarized in Table VII. For all the tested compounds, a time-dependent loss of enzyme activity (plots not shown) was observed. Therefore, these novel compounds are irreversible agents.

It is apparent from inspection of the Table that as the size of the substituents on the aromatic ring increases, the K_{inact} values, in general for most of the compounds, also increase, suggesting there is a steric effect involved in binding to the active site, since K_{inact} value reflects affinity of an inhibitor to the enzyme active site. Inhibitors with longer aliphatic chains were found to be poorly soluble in water making determination of K_{inact} for compound **1e** impossible. The only analogue with a 4-ethyl substituent, **1b,** showed inhibitory potency against glucosamine-6-phosphate synthase comparable to that of the parent compound BADP. Compounds with *ortho* substituted phenyl ring seems to be less reactive (higher T values) than *para* analogues. On the other hand, analogues with an aliphatic chain, **1t** and **1w,** displayed lowest inhibitory activity in the series although their affinity to the active site was markedly

improved in comparison to other inhibitors with aliphatic chains. The inactivation potencies (k_2/K_{inact}) of the examined inhibitors were also lowered in comparison to BADP. Probably, the compounds with a substituted phenyl ring may not be properly fitted into the enzyme binding pocket, and may disturb the enzyme conformation before its inactivation. These discrepancies observed between reactivity and affinity should be explained in further studies.

Membrane affinity

For all these compounds their ability to diffuse through biological membrane was measured. This property was investigated using a HPLC chromatographic column IAM (immobilized artificial membrane) PC DD 2 with stationary phase mimicking the cell membrane. The retention times were measured and affinity to the biological membrane was expressed as $\log k'_{IAM}$ (Table VII). These values describe not only lipophilic properties but also hydrogen bond formation or electrostatic interactions. The highest $\log k'_{IAM}$ values were determined for compounds with linear aliphatic chain substituents, however, these compounds show only moderate affinity to the biological membrane.

Antifungalal activity

Minimal inhibitory concentration (MIC's) were determined in YNB medium with glutamine and proline as carbon source. Table VIII presents activity against a few species. Most of the synthesised inhibitors exhibited rather poor antifungal activity with MIC's in the range from 62.5 μg/ml to 1000 μg/ml and their activity is not correlated with apparent lipophilicity. Compound **1c** with a n-butyl substituent on the aromatic ring and with $\log k'_{IAM}$ 1.078 displayed almost the same antifungal activity as the lead compound (BADP) with a lower $\log k'_{IAM}$. Much lower $\log k'_{IAM}$ would hamper diffusion of the inhibitor into the microbial cells and higher $\log k'_{IAM}$ would cause the retainment of the tested compound in the cytoplasmic membrane. On the other hand inhibitors with aliphatic chains e.g. **1x** with $\log k'_{IAM}$ 0.324 containing a n-butyl group and AADP itself with $\log k'_{IAM}$ as low as −1.415 showed also similar antifungal properties. Unexpectedly high activity of AADP against *S. cerevisiae* suggests rather different than the facilitated diffusion transport mechanism in this case. All compounds may be considered as only moderately lipophilic and therefore may not be able to cross the cell membrane by free diffusion. Furthermore, these inhibitors are polar compounds still bearing a net charge, and probably this factor also influences their diffusion across the membrane. Inhibitor **1 e** containing a large substituent on the aromatic ring, is only sparingly soluble in buffers used in the experiments, so making $\log k'_{IAM}$ determination impossible.

Table VII. Inhibitory and inactivatory data for new compounds in respect to *C. albicans* GlcN-6-P synthase and affinity to biological membranes.

Compound	R=	IC_{50} [mM]	T [min]	k_2 $\left[\frac{1}{min}\right]$	K_{inact} [μM]	k_2/K_{inact} $\left[\frac{1}{M \cdot s}\right]$	$\log k'_{IAM}$
1a	4-methylphenyl	0.45	0.30	2.31	921	41.8	−0.09
1b	4-ethylphenyl	0.2	0.77	0.904	136	111	0.277
1c	4-*n*-butylphenyl	0.3	0.94	0.735	890	13.8	1.08
1d	4-*tert*-butylphenyl	1.5*	0.60	1.16	940	20.5	0.807
1e	4-*n*-decylphenyl	2.1*	–	–	–	–	–
1f	2.4-dimethylphenyl	0.45	1.80	0.384	1 880	3.4	0.236
1g	2.4.6-trimethylphenyl	1.5	1.00	0.693	80 800	0.1	0.410
1h	2-chlorophenyl	0.4	3.50	0.198	4 040	0.8	−0.211
1i	3-chlorophenyl	0.2	1.00	0.693	1 730	6.7	0.028
1j	4-chlorophenyl	0.4	0.97	0.717	1 360	8.8	0.055
1k	2-bromophenyl	0.2	5.10	0.136	18 700	0.1	−0.095
1l	3-bromophenyl	0.3	0.50	1.39	1 080	21.4	0.170
1m	4-bromophenyl	0.45	0.63	1.11	1 360	13.6	0.203
1n	4-fluorophenyl	0.65	1.63	0.425	2 230	3.2	−0.409
1o	3.4-dichlorophenyl	0.6	1.20	0.578	3 320	2.9	0.581
1p	4-chloro-3-nitrophenyl	0.4	0.48	1.430	590	40.4	0.159
1q	2-naphthyl	0.3	1.05	0.657	640	17.0	0.755
1r	2-furyl	0.65	0.625	1.11	7 830	2.4	−1.11
1s	2-pyrrolyl	1.65	2.12	0.326	13 500	0.4	−0.813
1t	*tert*-butyl	4.0	1.49	0.467	1 480	5.3	−0.883
1u	ethyl	0.6	1.75	0.396	6 060	1.1	−1.24
1w	*iso*-propyl	3.0	1.55	0.448	3 000	2.5	−1.11
1x	*n*-butyl	1.0	1.1	0.630	40 000	0.3	0.325
BADP	phenyl	0.2	0.38	1.84	327	93.8	−0.477
AADP	methyl	0.65	1.5	0.462	13 500	0.6	−1.42

* Difficult to measure due to poor water solubility.

Table VIII. Antifungal activity of new inhibitors.

Compound	MIC [μg/ml]			
	S. cerevisiae	*C. glabrata*	*C. albicans*	*C. crusei*
1a	125	250	250	250
1b	62.5	250	250	125
1c	62.5	250	125	125
1d	62.5	250	250	250
1e	> 500	> 500	> 500	> 500
1g	62.5	250	125	250
1f	125	250	125	250
1h	125	250	250	250
1i	62.5	250	250	250
1j	125	250	250	250
1k	62.5	250	125	250
1l	62.5	250	250	250
1m	62.5	250	250	125
1n	125	250	250	250
1o	62.5	250	250	250
1p	125	250	250	250
1q	62.5	250	250	250
1r	125	> 1000	> 1000	–
1s	> 250	> 250	> 250	–
1t	500	> 1000	> 1000	–
1u	125	> 1000	> 1000	–
1w	250	> 1000	> 1000	–
1x	> 250	> 250	> 250	–
BADP	–	250	125	250
AADP	15.64	> 1000	> 1000	–

Table IX. Antifungal activity of new peptides.

Peptide	MIC [μg/ml]		
	S. cerevisiae	*C. glabrata*	*C. albicans*
7b	> 1000	31.25	> 1000
7s	31.25	15.63	> 1000
7u	31.25	7.81	> 1000
LysNvaFMDP	31.25	7.81	3.13

The peptides **7b, 7s** and **7u** with new inhibitors were tested against a few fungal stains (Table IX). Such compounds are transported into the cell not by diffusion but through peptide permeases [28]. For comparative purposes LysNvaFMDP, known as one of the most active peptide-containing strong GlcN-6-P synthase inhibitor, was also used. Surprisingly, the new peptides show high activity against *S. cerevisiae* and *C. glabrata*, but no activity against *C. albicans* was detected. Such results suggest that the new peptides are not transported into *C. albicans* cells via peptide permeases.

The activity of the new inhibitors and their peptides was also tested against GlcN-6-P synthase in cell free extract (Table X). For the compounds with the aromatic phenyl rings i.e., **1b** and **7b** no significant differences in activities were observed. On the other hand, the inhibitory activity of the peptides **7s** and **7u** were much higher then those of inhibitors **1s** and **1u** respectively.

Moreover, dipeptide NvaFMDP was also tested, but it showed relatively poor activity in cell-free extract, probably due to the instability of its methyl ester group, that resulted in the formation of inactive inhibitor. FMDP itself, however, under the experimental condition displayed strongest inhibitory potencies. Such results suggest that in the cell free extract either peptides (but not inhibitors) were activated or, after the hydrolysis of the peptide bond, the inhibitor was in a more preferable conformation for inactivation of GlcN-6-P synthase. For the peptides **7 s** and **7 u** experiments on Petri plates were carried out to examine reversal of inhibition in presence of N-acetylglucosamine. If the enzyme Glc-6-P synthase was the only molecular target for the new peptides, addition of the enzymatic reaction product would result in complete prevention of anticandidal activity of the tested peptide, as took place for LysNvaFMDP. However, for the new peptides **7s** and **7u** no such effect was observed, which suggests that glucosamine-6-phosphate synthase is not the only molecular target for the new peptides.

Cytotoxic evaluation

Peptides **7 s** and **7 u** were tested against human tumour cells (Table XI) and no toxic effects were observed even at high concentrations. Therefore, these compounds may be regarded as non-toxic to normal human cells.

Conclusions

We have synthesised twenty-three novel GlcN-6-P synthase inhibitors with enhanced lipophilicity in comparison to BADP and AADP. These compounds displayed however, weak or medium inhibitory properties. Peptides containing the novel inhibitors (with pyrrolyl **7 s** and ethyl **7 u** substituents adjacent to the keto group) were highly active against *C. glabrata* and *S. cerevisiae* strains. High anticandidal activity and lack of disturbance of the growth in the inhibition zone in the presence of GlcNAc suggested that GlcN-6-P synthase is not the only molecular target for the new peptides, as it is for FMDP peptides. Further studies to elucidate the mechanism of the antifungal action of the new compounds are in progress.

Acknowledgements

This work was supported by the Ministry of Science and Information Society Technologies, Grant No. 2PO5F 014 26 and in part Grant No. 3PO5F02324.

Table X. Peptides and inhibitors activity in cell-free extracts.

Peptide	IC_{50} (μM) in cell-free extract	Inhibitor	IC_{50} (μM) in cell-free extract	IC_{50} (μM) for purified enzyme
7b	150	**1b**	160	200
7s	160	**1s**	1600	1650
7u	12	**1u**	360	600
NvaFMDP	160*	FMDP	1	2

* Difficult to measure, probably due to hydrolysis of methyl ester group present in a peptide molecule, resulting in the loss of inhibitory activity.

Table XI. Activity of tested peptides against HL-60 human promyelocytic leukaemia cells.

Compound	HL-60 $IC_{50} \pm$ SEM (μg/ml)
7s	30% inhibition at 250 μg/ml
7u	232 ± 2
NvaFMDP	15% inhibition at 250 μg/ml

References

[1] Milewski S. Biochim Biophys Acta 2002;1597:173–192.
[2] Andriole VT. J Antimicrob Chemother 1999;44:151–162.
[3] Andruszkiewicz R, Chmara H, Milewski S, Borowski E. J Int Peptide Protein Res 1986;27:449–453.
[4] Andruszkiewicz R, Milewski S, Borowski E. J Enz Inhib 1995;9:123–133.
[5] Andruszkiewicz R, Chmara H, Milewski S, Borowski E. J Med Chem 1987;30:1715–1719.
[6] Andruszkiewicz R, Milewski S, Zieniawa T, Borowski E. J Med Chem 1990;33:132–135.
[7] Andruszkiewicz R, Zieniawa T, Walkowiak A. J Enz Inhib Med Chem 2005;20:115–121.
[8] Zgódka D, Jędrzejczak R, Milewski S, Borowski E. Bioorg Med Chem 2001;9:931–938.
[9] Andruszkiewicz R, Jędrzejczak R, Zieniawa T, Wojciechowski M, Borowski E. J Enz Inhib 2000;15:429–441.
[10] Milewski S, Kuszczak D, Jędrzejczak R, Smith RJ, Brown AJP, Gooday GW. J Biol Chem 1999;274:4000–4008.
[11] Kenig M, Vandamme E, Abraham EP. J Gen Microbiol 1976;94:46–54.
[12] Meloche HP. Biochemistry 1967;6:2273–2280.
[13] Ong S, Liu H, Pidgeon C. J Chromatogr A 1996; 728:113–128.
[14] Kauer JC. Org Synth Wiley, New York 1963,vol. III,109–112.
[15] Kirchner FK, Bailey JH, Cavallito CJ. J Am Chem Soc 1949;71:1210–1213.
[16] Papa D, Schwenk E, Villani F, Klingsberg E. J Am Chem Soc 1948;70:3356–3360.
[17] Scheffold R, Dubs P. Helv Chim Acta 1967;50:798–808.
[18] Bianchi M, Butti A, Christidis Y, Perronnet J, Barzaghi F, Cesena R, Nencioni A. Eur J Med Chem 1988;23:45–52.
[19] Obrecht D, Weiss B. Helv Chim Acta 1989;72:122–171.
[20] Kobayashi Y, Nakano M, Kumar GB, Kishihara K. J Org Chem 1998;63:7505–7515.
[21] Sugiyama N, Kataoka H, Kashima C, Yamada K. Bull Chem Soc Japan 1969;42:1098–1100.
[22] Kawashima M, Sato T, Fujisawa T. Bull Chem Soc Japan 1988;61:3255–3264.
[23] Ellington EV, Hassall CH, Plimmer JR, Seaforth CE. J Chem Soc 1959;80–85.
[24] Ernest I, Jelínková H. Collect Czech Chem Commun 1959;24:3341–3347.
[25] Anderson GW, Zimmerman JE, Callahan FM. J Am Chem Soc 1964;86:1839–1842.
[26] Sachadyn P, Jędrzejczak R, Milewski S, Kur J, Borowski E. Protein Exp Purif 2000;19:343–349.
[27] Chmara H, Andruszkiewicz R, Borowski E. Biochem Biophys Res Commun 1984;120:865–872.
[28] Milewski S, Andruszkiewicz R, Borowski E. FEMS Microbiol Lett 1988;50:73–78.