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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 17 (2007) 6602–6606

Rational design of *N*-alkyl derivatives of 2-amino-2-deoxy-D-glucitol-6P as antifungal agents

Anna Melcer,[a] Izabela Łącka,[b] Iwona Gabriel,[b] Marek Wojciechowski,[b] Beata Liberek,[a] Andrzej Wiśniewski[a] and Sławomir Milewski[b,*]

[a]*Department of Chemistry, University of Gdańsk, 18 Sobieskiego Street, 80-952 Gdańsk, Poland*
[b]*Department of Pharmaceutical Technology & Biochemistry, Gdańsk University of Technology, 11/12 Narutowicza Street, 80-952 Gdańsk, Poland*

Received 14 August 2007; revised 13 September 2007; accepted 18 September 2007
Available online 25 September 2007

Abstract—*N*-Alkyl and *N*,*N*-dialkyl derivatives of 2-amino-2-deoxy-D-glucitol-6P (ADGP) were synthesized and found to inhibit growth of human pathogenic fungi (MICs in the 0.08–0.625 mg mL^{-1} range for the most active compounds). It was thus shown that N-alkylation of ADGP provides novel inhibitors of a fungal enzyme, glucosamine-6P synthase, exhibiting higher antifungal activity than the parent compound, due to the increased lipophilicity and better uptake by fungal cells.

Emerging challenge of systemic fungal infections, especially in immunocompromised patients, and a limited repertoire of effective antifungals stimulate a quest for novel targets and drug candidates. Enzymes involved in biosynthesis of the fungal cell wall components are of a special interest in this respect. Glucosamine-6P (GlcN-6P) synthase catalyzes the first committed step in the biosynthetic pathway leading to the formation of UDP-GlcNAc, a sugar nucleotide precursor providing D-glucosamine for the formation of chitin and mannoproteins.[1] The enzyme was proposed as a target for antifungal chemotherapy[2] and a search for its selective inhibitors as potential antifungals has been continued. So far, two main groups of such compounds were identified: L-glutamine analogs and mimics of a putative *cis*-enolamine transition state intermediate but none of them demonstrated high antifungal activity, due to the inefficient uptake by the fungal cells. On the other hand, some of the L-glutamine analogs, namely *N*-acyl derivatives of L-2,3-diaminopropanoic acid, gave rise to oligopeptides exhibiting high antifungal in vitro and in vivo activity[3] but poor serum stability and unfavorable pharmacokinetic properties.[4]

2-Amino-2-deoxy-D-glucitol-6P (ADGP) and 2-amino-2-deoxy-D-mannitol-6P (ADMP) are known as the strongest inhibitors of fungal GlcN-6P synthase, belonging to the second group, with inhibitory constants at micromolar level.[5,6] Our previous studies showed that N-acylation of the 2-amino group and/or esterification of the phosphate increased lipophilicity of the ADGP molecule and facilitated uptake of the inhibitor into fungal cells.[7] Unfortunately, N-acylation of ADGP strongly decreased GlcN-6P synthase-inhibitory potential of this molecule, thus suggesting that presence of the 2-amino group, positively charged at physiological conditions, may be of crucial importance for high enzyme-inhibitory activity.[6] On the other hand, dimethyl and diethyl esters of ADGP demonstrated improved antifungal in vitro activity,[7] but were unstable in human serum, one may therefore predict their poor in vivo efficiency.

Taking into account the above-mentioned results we reasoned that N-alkylation of ADGP should afford derivatives demonstrating substantially higher antifungal in vitro activity than the parent molecule and high stability in serum, provided their GlcN-6P synthase-inhibitory potential is not diminished, relative to ADGP. In the first stage of our project we performed in silico docking simulations. Structures of *N*-monoalkyl- (C_2–C_6) and *N*,*N*-dialkyl- (C_2–C_6) derivatives of ADGP were docked into an active site of the hexose-

Keywords: Antifungals; Aminoglucitol derivatives; Glucosamine-6-phosphate synthase.
* Corresponding author. Tel.: +48 58 3472107; fax: +48 58 3471144; e-mail: milewski@chem.pg.gda.pl

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doi:10.1016/j.bmcl.2007.09.072

phosphate isomerase domain (ISOM) of *Candida albicans* GlcN-6P synthase.[8] Inspection of the structures of resulting enzyme: ligand complexes clearly showed that the ligands can easily accommodate at the active site, and all favorable ligand: active site residues interactions—noted in the known crystal structure of the bacterial GlcN-6P synthase:ADGP complex[9]—can be preserved. Figure 1 shows that presence of alkyl substituents does not disturb ligand binding, as the alkyl chain(s) can either fit near the hydrophobic entrance to the ISOM binding pocket—shielded by Val709 and Val501 residues—or even protrude out of the pocket. Unfortunately, a three-dimensional structure of the whole fungal GlcN-6P synthase is not known, so that one cannot predict how the *N*-alkyl chains may interact with the remaining part of the enzyme, namely the glutamine amide hydrolyzing domain (GAH) and the putative interdomain linker. However, taking into account the known structure of bacterial GlcN-6P synthase[10] and some sequence homology between the prokaryotic and eukaryotic version of the enzyme monomer subunits, it seems possible that the *N*-alkyl substituents may also participate in the favorable interactions with residues constituting the hydrophobic intramolecular channel connecting active centers of ISOM and GAH. Evidence supporting presence of such channel in *C. albicans* enzyme has been shown recently.[11]

Results of the docking simulations suggested that *N*-alkyl derivatives of ADGP should at least preserve the enzyme-inhibitory potential of ADGP and encouraged us to elaborate synthetic procedures for unequivocal synthesis of *N*-alkyl and *N*,*N*-dialkyl derivatives of ADGP. *N*,*N*-Dialkyl derivatives of ADGP were synthesized by reductive alkylation involving the reaction of ADGP **2** with a suitable aldehyde in the presence of $NaCNBH_3$ in the mixture of CH_3CN–$H_2O \sim 3{:}1$ (Scheme 1).[12] In

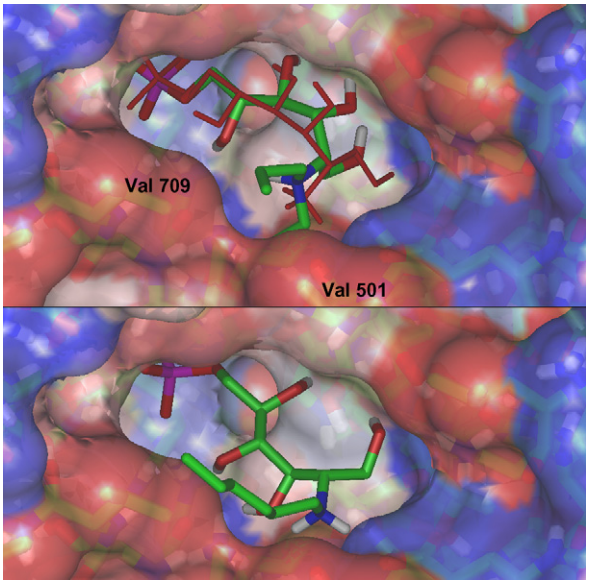
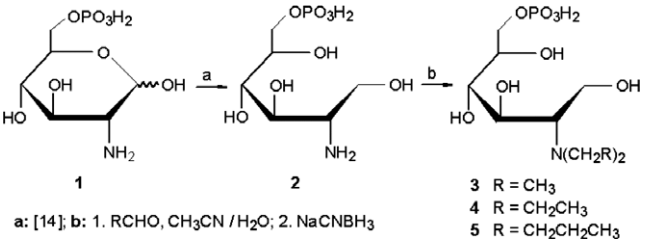


Figure 1. *N*,*N*-Dipropyl- (top) and *N*-hexyl-ADGP (bottom) docked into an active site of the ISOM domain of *C. albicans* GlcN-6P synthase. Red wire model represents a reference ADGP conformation, based on the geometry of its complex with a bacterial enzyme (1 mos).

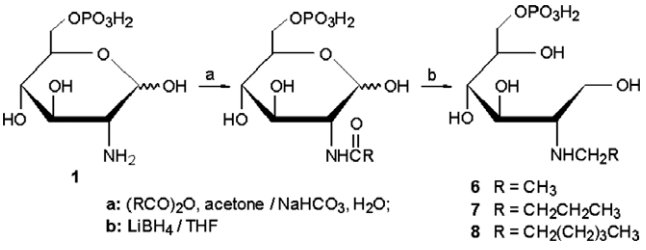


Scheme 1. Synthesis of *N*,*N*-dialkyl derivatives of ADGP.

our previous studies we showed that reductive alkylation of D-glucosamine under such conditions leads to the formation of *N*-alkyl and *N*,*N*-dialkyl derivatives of D-glucosamine.[13] Now we found that the reductive alkylation of ADGP **2**, obtained from GlcN-6P **1** by the method of Bearne,[14] afforded unequivocally the respective *N*,*N*-dialkyl derivatives, provided at least a threefold molar excess of aldehyde with respect to **2** was used. Thus, the procedure shown in Scheme 1 was applied for the preparation of *N*,*N*-dialkyl ADGP derivatives **3**–**5**.

N-Monoalkyl derivatives of ADGP were synthesized by the two-step procedure[15] shown in Scheme 2, starting from **1**, N-acylated in the first step with a respective carboxylic acid anhydride. Subsequently, the resulting *N*-acyl-GlcN-6P was treated with $LiBH_4$ in THF. Under these conditions both an amide and an aldehyde groups were reduced, thus affording *N*-monoalkyl ADGP derivatives **6**–**8**. Identity of compounds **3**–**8** was confirmed by IR, ^1H NMR, and MS.[16]

ADGP **2** and its *N*-alkyl and *N*,*N*-dialkyl derivatives **3**–**8** were tested for physicochemical and biological properties, including apparent lipophilicity, enzyme-inhibitory potential, uptake by fungal cells, and antifungal in vitro activity. Affinity of the examined compounds to the artificial biological membrane immobilized on the bed of the IAM PC DD2 column, especially designed for investigation of low and moderately lipophilic compounds, was quantified and retention parameters k^0_{IAM} were determined as a measure of apparent lipophilicity.[17] Benzene and GlcN-6P, taken as reference compounds, demonstrated the k^0_{IAM} values 0.8893 and −0.7583, respectively. Data shown in Table 1 indicate that ADGP exhibited the k^0_{IAM} value close to 0.0 and all the mono and dialkyl derivatives of ADGP demonstrated higher k^0_{IAM} values than the parent compound. As expected, the affinity to the immobilized artificial membrane depended on length and number of *N*-alkyl



Scheme 2. Synthesis of *N*-monoalkyl derivatives of ADGP.

Table 1. Data characterizing lipophilicity, uptake by *C. albicans* cells, and inhibition of *C. albicans* GlcN-6P synthase by ADGP and its *N*-alkyl derivatives

Compound	Lipophilicity $\log k^0_{IAM}$	Initial uptake rate[a] (nmol/min/mg dry weight)	Inhibition of GlcN-6P synthase[a]	
			IC_{50} (μM)	K_i (μM)
2	−0.0437	1.1 (±0.2)	230 (±15)	35 (±4)
3	0.3895	3.9 (±0.6)	40 (±8)	8 (±1)
4	0.4625	4.7 (±0.5)	52 (±6)	10 (±2)
5	0.5190	5.0 (±0.8)	82 (±8)	12 (±3)
6	0.2283	2.9 (±0.4)	180 (±9)	26 (±5)
7	0.3567	3.4 (±0.2)	115 (±7)	20 (±3)
8	0.4222	4.8 (±0.6)	290 (±21)	49 (±6)

[a] Values are means of three experiments, standard deviation is given in parentheses.

substituents. However, all the compounds **3**–**8** could be considered as moderately lipophilic and thus potentially able to cross the real cell membrane by free diffusion. This assumption was confirmed by the results of determination of initial uptake rates of compounds **2**–**8** by *C. albicans* cells, shown in Table 1. The rates, determined under conditions described previously,[7] were much higher for *N*-alkyl ADGP derivatives, in comparison to that of ADGP. Kinetic studies on the uptake of *N*-alkyl derivatives of ADGP provided evidence for its passive character, including a linear dependence of uptake rates on initial compound concentration and lack of any effect of metabolic inhibitors, such as NaN_3 (detailed results not shown). It is worth mentioning therefore that *N*-acyl ADGP derivatives were effectively taken up by *C. albicans* cells by a passive transport mechanism, while the ADGP molecule was slowly accumulated by the way of an unidentified active transport system.[7] The novel *N*-alkyl ADGP derivatives **3**–**8** thus closely resemble their *N*-acyl counterparts in terms of lipophilicity and uptake properties.

Compounds **2**–**8** were tested for their GlcN-6P synthase- and phosphoglucose isomerase-inhibitory activities.[18] Both these enzymes utilize D-fructose-6-phosphate as a substrate and catalyze ketose–aldose isomerization, according to the very similar mechanisms involving, respectively, *cis*-enolamine or *cis*-endiol intermediate. *N*-alkyl ADGP derivatives **3**–**8** appeared relatively strong inhibitors of *C. albicans* GlcN-6P synthase, as under in vitro conditions, 50% inhibition of enzyme activity (IC_{50}) was observed in the 40–290 μM range. Data shown in Table 1 reveal that the inhibitory potential in terms of the IC_{50} values of nearly all the *N*-alkyl derivatives, except the *N*-hexyl-ADGP **8**, was even higher than that of ADGP. Especially low IC_{50} values were found for *N*,*N*-dialkyl compounds **3**–**5**. This is in contrast to the inhibitory potential of *N*-acyl ADGP derivatives, for which we previously found the IC_{50} values in the 6–19 mM range.[6] Good inhibitory potential of compounds **3**–**8** thus confirmed the suggestion resulting from the docking simulations described above. Inhibition of *C. albicans* GlcN-6P synthase by ADGP and its *N*-alkyl derivatives was competitive in respect to D-Fru-6P and non-competitive in respect to L-Gln. The order of inhibitory potential reflected by K_i values determined from kinetic data was similar to that found for the IC_{50} data. Interestingly, Floquet et al. reported recently that K_i values of ADGP, *N*-acetyl-ADGP and *N*-ethyl-ADGP toward *Escherichia coli* GlcN-6P synthase were 25, 28, and 500 μM, respectively, so that in the case of the bacterial enzyme, *N*-acetyl-ADGP was as effective as ADGP, while *N*-ethyl-ADGP was much poorer.[19] It seems therefore possible that despite the close sequence homology between *E. coli* and *C. albicans* ISOM domains and conservation of all residues important for catalytic activity, there could be a difference in spatial arrangements of catalytic residues of bacterial and fungal enzyme versions, especially in the region containing residues interacting with the 2-amino group of ADGP and its derivatives. None of the studied compounds at concentrations up to 20 mM inhibited activity of yeast phosphoglucose isomerase, thus confirming the previously suggested structural requirements for effective transition state analog inhibitors of this enzyme.[6]

Compounds **2**–**8** were tested for their antifungal in vitro activity against 3 strains of human pathogenic yeast from the genus *Candida* and 2 recombinant strains of *Saccharomyces cerevisiae*: AD and ADCDR1. The AD cells are sensitive to antifungals, due to the deletion of the seven genes encoding main drug exporting membrane proteins of *S. cerevisiae*,[20] while the ADCDR1 cells were constructed by transformation of AD with a plasmid containing a gene encoding Cdr1p, the main drug exporter of *C. albicans*.[21] Minimum inhibitory concentration (MIC) values determined by a serial dilution microplate method[22] for compounds **2**–**8** and a well-known antifungal drug fluconazole as a reference are summarized in Table 2.

Antifungal activity of ADGP (**2**) is very poor, since a growth inhibitory effect was observed at concentrations as high as 5–10 mg mL^{-1}. MIC values determined for *N*-alkyl ADGP derivatives **3**–**8** were generally an order or even nearly two orders of magnitude lower and the lowest values 0.08–0.625 mg mL^{-1} were observed for *N*,*N*-dialkyl compounds **3** and **4**. Presence of 10 mM *N*-acetyl-D-glucosamine in the growth medium completely abolished the antifungal activity of investigated compounds, thus indicating that GlcN-6P synthase is their primary target in fungal cells. Comparison of the MIC values determined against the recombinant *S. cerevisiae* cells AD and ADCDR1 clearly indicates that presence of the Cdr1p, mediating multidrug resistance, does not affect antifungal activity of both ADGP and its *N*-alkyl derivatives, while strongly decreases that of fluconazole. This result suggests that ADGP and its derivatives are probably not extruded from yeast cells by the Cdr1p drug transporter.

Compounds **2**–**8** were stable in human serum, as no products of their possible degradation were detected by HPLC[23] upon their incubation for 72 h at 37 °C in 5% human serum (details not shown).

According to our best knowledge, *N*-alkyl derivatives of ADGP constitute the first reported example of N-alkyl-

Table 2. Antifungal in vitro activity of ADGP and its *N*-alkyl derivatives

Compound	MIC (mg mL^{-1})				
	Candida albicans	*Candida glabrata*	*Candida tropicalis*	*Saccharomyces cerevisiae* AD	*Saccharomyces cerevisiae* ADCDR1
2	5	10	10	5	5
3	0.31	0.625	0.625	0.31	0.31
4	0.16	0.625	0.625	0.08	0.16
5	0.312	1.25	2.5	1.25	1.25
6	1.25	2.5	2.5	2.5	2.5
7	0.625	1.25	1.25	1.25	1.25
8	0.625	0.625	0.31	0.31	0.31
FLUC[a]	0.008	0.016	0.016	0.004	0.032

[a] FLUC, fluconazole.

ated aminohexitol phosphates demonstrating antimicrobial activity. In the literature, there are also very few examples of structurally related N-alkylated amino sugars exhibiting any type of biological activity.[24,25] Structures of our novel compounds have been rationally designed, taking into account results of in silico docking simulations and predictions concerning expected improvement of the uptake parameters upon introduction of alkyl substituents at the 2-amino group of ADGP. This approach has appeared successful, although the antifungal activity of the obtained compounds is not high enough to consider them true drug candidates.

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8. The structure of the receptor (ISOM domain of *C. albicans* GlcN-6P synthase) was homology modeled on the basis of the available crystal structure of *E. coli* enzyme (1jxa) which was used as a template. The structures of ligands studied were built by means of InsightII molecular modeling package from Accelrys,[26] as described earlier.[6] The putative geometries of the ligand–enzyme complexes were then obtained by docking each of the ligands to the modeled receptor using the AutoDock program.[27] Lamarckian genetic algorithm (LGA) was used as the search method and ligands were flexible during the calculations with all single bonds marked for rotation. The crystal geometry of ADGP complexed with *E. coli* GlcN-6P synthase ISOM domain (1 mos) was used as the reference. For each ligand studied, geometry of the ligand best resembling the reference was, in all cases, found either as the best scored solution or within the top five solutions found by AutoDock.
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12. General procedure for N,N-dialkylation of ADGP: ADGP **2** (200 mg, 0.76 mmol) and appropriate aldehyde (2.28 mmol) were stirred in the mixture of acetonitrile and water (3:1, 14 mL) at rt for 30 min. Then $NaCNBH_3$ (132 mg, 2.1 mmol) was added and stirring was continued for 1 h at rt. The end of reaction was detected by TLC ($MeOH/NH_3/H_2O$, 6:2:1). The reaction mixture was concentrated and chromatographed on silica gel ($MeOH/NH_3/H_2O$, 6:2:1).
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15. General procedure for N-monoalkylation of ADGP: GlcN-6P **1** (200 mg, 0.76 mmol) and $NaHCO_3$ (300 mg, 3.6 mM) were dissolved in a smallest possible amount of water (~1.5 mL) and 25% acetone solution of appropriate anhydride (1.5 mM) was added dropwise. The reaction mixture was stirred at rt for 24 h. Then isopropanol (20 mL) was added and stirring was continued for a few min. The reaction mixture was concentrated and purified on a Dowex 50 WX4 (H^+) ion-exchange resign. Obtained *N*-acyl derivative of GlcN-6P (0.66 mmol) was dissolved in dry THF (5 mL) and $LiBH_4$ (216 mg, 9.9 mmol) was added. The reaction mixture was stirred at rt for 2 h. When the end of reduction was detected by TLC ($MeOH/CHCl_3$, 1:1), methanol (5 mL) was added and the reaction mixture was kept at rt for a few hours. Then it was concentrated and purified on a Dowex 50 WX4 (H^+) ion-exchange resign.
16. *Compound* **3***:* Yield 86%, colorless syrup. IR: *ν* 3460, 3140 (O–H), 3040, 2806 (C–H), 2349 (R_3N–H^+) cm^{-1}; ^{1}H NMR (400 MHz, D_2O): *δ* 4.33 (d, *J* = 8.8 Hz, 1H), 4.04 (m, 4H), 3.80–3.85 (m, 3H), 3.59 (m, *J* = 6.8 Hz, 2H, CH_2), 3.43 (m, 2H, CH_2), 1.41 (t, *J* = 7.2 Hz, 6H, $2CH_3$). MALDITOF: *m/e* 317.1 ($M^{+\cdot}$), 318.1 ($M^{+\cdot}$+1), 339.0 ($M^{+\cdot}$−1+Na), 361.0 ($M^{+\cdot}$−2+2 Na).
Compound **4***:* Yield 78%, colorless syrup. IR: *ν* 3500, 3235 (O–H), 3043, 2816 (C–H), 2349 (R_3N–H^+) cm^{-1}; ^{1}H NMR (400 MHz, D_2O): *δ* 4.21 (d, 1H), 3.96–4.02 (m, 3H), 3.78–3.86 (m, 2H), 3.64 (dd, *J* = 10.8, 6.4 Hz, 1H), 3.51 (dd, *J* = 10.8, 7.2 Hz, 1H), 3.26 (m, 2H, CH_2), 3.14 (m, 2H, CH_2), 1.78 (m, 4H, $2CH_2$), 1.01 (t, *J* = 7.3 Hz, 6H, $2CH_3$). MALDITOF: *m/e* 345.2 ($M^{+\cdot}$), 346.2 ($M^{+\cdot}$+1).
Compound **5***:* Yield 90%, colorless syrup. IR: *ν* 3234 (O–H), 3070, 2964, 2936 (C–H), 2347 (R_3N–H^+) cm^{-1}; ^{1}H NMR (500 MHz, D_2O): *δ* 4.14 (d, *J* = 8.79 Hz, 1H), 3.80–3.89 (m, 4H), 3.64–3.68 (m, 3H), 3.30 (m, 2H, CH_2), 3.13 (m, 2H, CH_2), 1.66 (m, 2H, CH_2), 1.57 (m, 2H, CH_2), 1.25 (m, *J* = 7.32 Hz, 4H, $2CH_2$), 0.80 (t, *J* = 7.32 Hz, 6H, $2CH_3$). MALDITOF: *m/e* 371.5 ($M^{+\cdot}$−2), 394.5 ($M^{+\cdot}$−2+Na).

Compound **6***:* Yield 88%, colorless syrup. IR: *v* 3296 (O–H), 2943, 2883 (C–H), 2348 (R_2HN–H^+) cm^{-1}; ^1H NMR (400 MHz, D_2O): δ 4.18 (m, 1H), 4.12 (m, J = 6.8 Hz, 2H), 3.91 (dt, J = 12.4, 3.6 Hz, 1H), 3.80 (dd, 1H), 3.74 (dt, 1H), 3.68 (m, 1H), 3.61 (m, 2H, CH_2), 3.55 (m, 1H), 1.58 (t, J = 6.4 Hz, 3H, CH_3). MALDITOF: *m/e* 288.2 ($M^{+\cdot}$).
Compound **7***:* Yield 77%, colorless syrup. 3200 (O–H), 2934 (C–H), 2397 (R_2HN–H^+) cm^{-1}; ^1H NMR(400 MHz, D_2O): δ 4.04–4.18 (m, 3H), 3.96 (m, 1H), 3.86 (m, 1H), 3.73 (m, 1H), 3.64 (m, 1H), 3.57 (m, 2H, CH_2), 3.55 (m, 1H), 1.55 (m, 4H, $2CH_2$), 0.86 (t, 3H, CH_3). MALDITOF: *m/e* 317.0 ($M^{+\cdot}$), 339.0 ($M^{+\cdot}-1+Na$).
Compound **8***:* Yield 94%, colorless syrup. IR: *v* 3334 (O–H), 2957, 2931, 2860 (C–H), 2336 (R_2HN–H^+) cm^{-1}; ^1H NMR (400 MHz, D_2O): δ 4.08–4.20 (m, 3H), 3.90 (dt, 1H, J = 10.4 Hz, J = 3.2 Hz), 3.82 (m, 1H), 3.75 (m, 1H), 3.64 (m, 1H), 3.60 (m, 2H, CH_2), 3.55 (m, 1H), 1.57 (m, 4H, 2 CH_2), 1.28 (m, 4H, $2CH_2$) 0.86 (t, 3H, CH_3). MALDI-TOF: *m/e* 344.1 ($M^{+\cdot}-1$), 367.1 ($M^{+\cdot}-1+Na$).

17. Interactions between the examined compounds and the IAM PC DD2 column (Regis Technologies, Inc., Morton Grove, IL, USA) were studied under conditions described previously.[7] Briefly, the column loaded with a sample was developed with the solvent system acetonitrile/50 mM potassium phosphate buffer, pH 7.2 (50:50, 30:70, 20:80, 10:90, 5:95 v/v) containing 0.1% acetone, flow rate 1 mL min^{-1}. Retention times of the studied compounds (t_R) were determined for different compositions of the mobile phase and used to calculate the capacity parameters $k'_{IAM} = (t_R - t_0)/t_0$. Values of $\log k'_{IAM}$ were plotted against the acetonitrile content in the mobile phase and the resulting linear plots were extrapolated to give the k^0_{IAM} value for the hypothetical purely aqueous mobile phase.
18. *Candida albicans* GlcN-6P synthase was purified to near homogeneity, as described previously.[28] Enzyme activity was determined by the modified Elson–Morgan method.[29] Reactions were carried out in 25 mM phosphate buffer, pH 6.9, containing 1 mM EDTA, 1 mM DTT, D-Fru-6P (0.5–7.5 mM), L-glutamine (0.625–10 mM), GlcN-6P synthase (0.1–0.2 μM), and a given inhibitor in appropriate concentration. For determination of IC_{50} values concentrations of both substrates (D-Fru-6P and L-Gln) were 10 mM. Data were plotted as Michaelis–Menten graphs and kinetic data were subjected to the non-linear regression analysis. The IC_{50} and K_i were determined in triplicate, to give the mean values ± SD. Activity of yeast phosphoglucose isomerase (Sigma) was determined using the Glc-6P dehydrogenase-coupled assay.[30]
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