Journal of Enzyme Inhibition and Medicinal Chemistry, April 2005; 20(2): 123–127

Potent inhibition of chitin synthase by an azasugar—investigation of synergistic effect with UDP

MOUNIRA DJEBAILI & JEAN-BERNARD BEHR

Laboratoire Réactions Sélectives et Applications UMR 6519, UFR Sciences-CNRS, BP 1039, 51687 Reims Cedex 2, France

(Received 20 June 2004; in final form 24 August 2004)

Abstract
We identified 6-deoxy-homoDMDP as a potent inhibitor of chitin synthase ($K_i = 38\,\mu M$), displaying an uncompetitive inhibition pattern. Dual inhibition was also performed with the enzymatic reaction product uridine 5′-diphosphate in order to evaluate the concurrent effect of both inhibitors. An interaction coefficient α of 0.9 was found, revealing synergistic inhibition.

Keywords: *Chitin synthase, uncompetitive, glycosyltransferase, inhibition, azasugar*

Introduction

Chitin, a homopolymer of β-1,4-*N*-acetylglucosamine residues, is an essential component of the cell membrane of yeast and fungi. Since chitin is absent from vertebrates, inhibition of enzymes implicated in its biosynthetic pathway has been envisaged as a therapeutic option [1]. Chitin synthase (CS, EC 2.4.1.16) is involved in the last step of this biological process and catalyzes the transfer of *N*-acetylglucosamine (GlcNAc) units from uridine 5′-diphospho-*N*-acetylglucosamine (UDP-GlcNAc) to the growing chitin chain (Figure 1) accompanied by release of UDP (uridine 5′-diphosphate, **1**) [2]. The naturally occurring polyoxins and nikkomycins are potent CS inhibitors with marked antifungal activity [3].

To date there is no structural information available for CS that go beyond the primary level. As a consequence, the exact catalytic mechanism of this processive glycosyltransferase is largely unknown. However, recent structure prediction studies including hydrophobic cluster analysis [4], homology modelling [5] or mutational analysis [6] have permitted further insights to be gained into the nature of the CS active site.

In addition, the design of new synthetic Transition State mimetics or bisubstrate analogues for chitin synthase is a recent approach developed by us [7–9] and others [10,11] which could promote a better understanding of this biological process. In particular five-membered ring azasugars were synthesized with the aim of mimicking the half chair conformation and the charge (through protonation at physiological pH) of the putative glycosyl cation involved at the Transition State of the catalytic reaction shown in Figure 1 [12].

Most of the pyrrolidinols we assayed on CS activity were modest competitive inhibitors with K_i's in the millimolar range [13]. However, this screening permitted us to identify azasugar **2** as a new and very potent inhibitor of chitin synthase, displaying an unusual uncompetitive pattern. We report here the biological evaluation of azasugar **2** on chitin synthase activity, the rationalisation and general rate equations describing a synergistic effect between a competitive and an uncompetitive inhibitor as well as the investigation of a possible synergistic effect between **2** and UDP **1**, the byproduct formed during the catalytic process.

Materials and methods

Reagents

6-Deoxy-homoDMDP was synthesized and purified as described [14]. Digitonin, trypsin, soybean trypsin inhibitor, UDP-GlcNAc were obtained from Sigma.

Correspondence: J.-B. Behr, Laboratoire Réactions Sélectives et Applications UMR 6519, UFR Sciences-CNRS, BP 1039, 51687 Reims Cedex 2, France. Tel: 33 326 913 238. Fax: 33 326 913 166. E-mail: jb.behr@univ-reims.fr

ISSN 1475-6366 print/ISSN 1475-6374 online © 2005 Taylor & Francis Ltd
DOI: 10.1080/14756360400014622

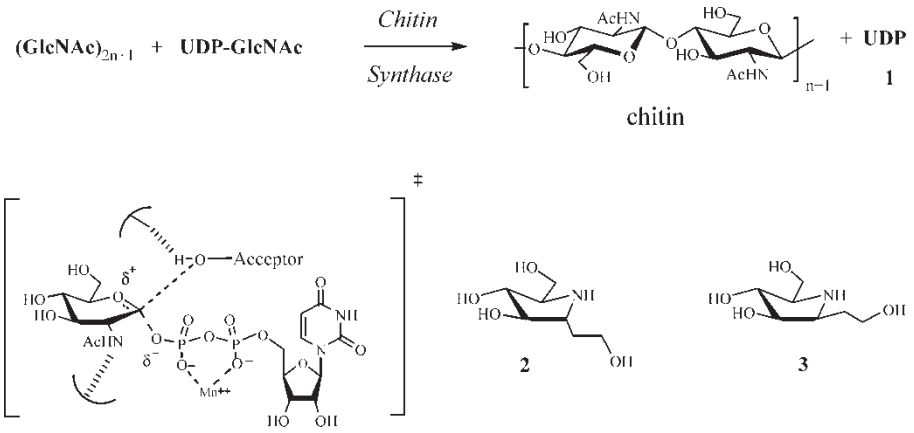


Figure 1. Chitin Synthase catalyses the transfer of GlcNAc residues from UDP-GlcNAc to the growing chitin chain.

UDP trisodium salt hydrate was from Aldrich and *N*-acetyl-D-glucosamine from Acros. [UDP^{14}C]-GlcNAc (289 mCi/mmol) came from NEN.

Yeast strains and culture

Saccharomyces cerevisiae X2180 strains were used in this study. Yeast strains were grown essentially as described by Orlean [15], at 30°C, in YEPG medium containing 1% yeast extract, 2% bactopeptone and 2% glucose. Overnight cultures were diluted in 400 mL fresh medium to an A_{600} of 0.15 and harvested when they reached an A_{600} of 1.2. These cells were used for the permeabilization procedure.

Permeabilization procedure

Saccharomyces cerevisiae cells were permeabilized by osmotic shock according to the recent procedure described by Cabib [16]. An aliquot of 1 g (wet weight) of freshly grown yeast cells was suspended and preincubated with shaking for 30 min at 30°C in 1.4 mL of EDTA (0.1 M), 24 µL of 2-mercapto-ethanol and water to a final volume of 3.5 mL.

The cells were harvested by centrifugation (12 000 × *g*, 10 min) and washed with 5 mL of 0.8 M sorbitol. After centrifugation, the pellet was suspended in 0.57 mL of citrate-phosphate buffer (pH 6.3), 67 µL of 0.1 M EDTA, 0.64 mL of 1.6 M sorbitol and 0.8 M sorbitol to a final volume of 6.7 mL. The suspension was incubated with shaking (30 min, 30°C) and centrifuged as above. The cells were then suspended in 30 mL of cold 0.05 M Tris-HCl (pH 7.5), kept on ice for 5 min and centrifuged (12 000 × *g*, 10 min). The resulting pellet was finally resuspended in 1.6 mL of 0.05 M Tris-HCl containing 33% glycerol before chitin synthase activation.

Chitin synthetase assay

The activity of Chitin Synthase I was assayed specifically at pH 6.5 by measuring the rate of formation of [^{14}C]-chitin from UDP-*N*-acetyl-[^{14}C]-glucosamine, according to the standard method reported by Choi and Cabib, with some modifications. [17] Chitin synthase I is present as its zymogen in the permeabilized cells and must be activated prior to assay by partial proteolysis with trypsin and treatment with digitonin.

In our experiments, an aliquot of permeabilized cell suspension (to a final concentration in the activation assay of 78 mg/mL) was incubated for 15 min in 30 mM Tris-HCl (pH 6.5) containing 55 mM GlcNAc, digitonin (5.2 mg/mL) and trypsin (1.0 mg/mL). Activation was stopped by adding soybean trypsin inhibitor (1.5 mg/mL). The resulting preparation was generally kept at 0°C for assay but may also be stored at −80°C for some days without loss in activity.

Assays were than carried out at 30°C in a volume of 60 µL which contained, in addition to the activated permeabilized cells preparation (40 µL), the following final concentrations of components: 30 mM Tris-HCl (pH 6.5), 3 mM $Mg(Ac)_2$ and variable concentrations of UDP-[^{14}C]-GlcNAc (0.25–1 mM, 20 000 cpm) for K_i determinations or 1 mM UDP-[^{14}C]-GlcNAc for IC_{50} determination and investigation of the synergistic effect.

Reaction was initiated by addition of the cell suspension. After 30 min incubation the reaction was stopped with 1 mL trichloroacetic acid and 950 µL of the resulting suspension was filtered through a glass-fibre filter (Whatman GF-C). The filter was washed 3 times with 1 mL 60% aqueous EtOH and dried. The ^{14}C-chitin formed was quantitated by liquid scintillation counting in 4 mL of ultima flow AP (Packard) scintillation fluid.

Results and discussion

The reaction rate of chitin synthase observed with the *in situ* assay described here, showed normal Michaelis-Menten kinetics. A K_m value of 0.47 mM was

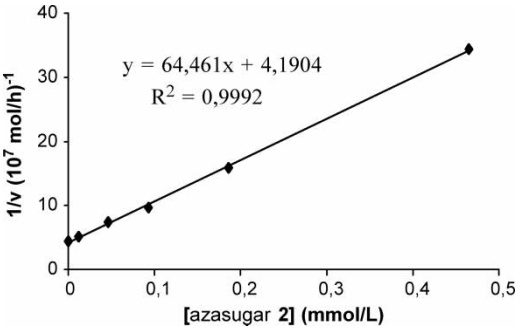


Figure 2. IC_{50} determination (Dixon plots) for azasugar **2**.

calculated from a double reciprocal plot for UDP-GlcNAc, which was in good agreement with published data [17]. To verify the reliability of the *in situ* assay used in these experiments, the inhibitory potency of the standard Nikkomycin Z was tested and a K_i of 0.34×10^{-6} M was found (lit.[3] $0.1–3 \times 10^{-6}$M).

The inhibition potency of 6-deoxy-homoDMDP was examined under the same conditions and an IC_{50} value of 65 μM was determined by the Dixon method, as shown in Figure 2.

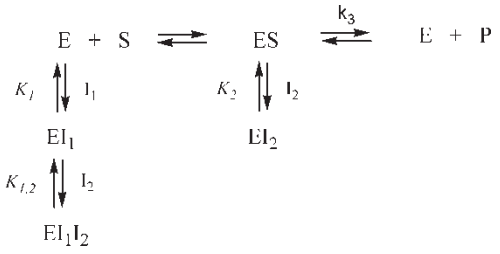
Compound **2** was significantly more effective than the other azasugars tested in our laboratories, exceeding the parent compound's activity by two orders of magnitude. Although of minimal structural complexity azasugar **2** was as active as more elaborate natural or synthetic CS inhibitors [1]. The stereochemical integrity of **2** was essential for enzyme binding since the C-2 modified derivative **3** exhibited only weak inhibition on chitin synthase ($IC_{50} = 2.6$ mM).

Unexpectedly, further studies showed that **2** behave kinetically as an uncompetitive inhibitor ($K_i = 38$ μM) as illustrated in Figure 3. To our knowledge, this is the first example of a CS inhibitor with such an inhibition pattern.

Concerning other glycosyl-transfer enzymes, a number of azasugars have been found to interfere with the biosynthesis of carbohydrates. In general, they competitively inhibit the corresponding glycosyltransferases with moderate affinities (K_i's in the millimolar range) [18], except one example of an uncompetitive inhibitor of β-1,4-galactosyltransferase ($K_i = 61$ μM) recently described [19]. Thus, the inhibition potency of 6-deoxy-homoDMDP determined in this study, places **2** among the most potent azasugar-type inhibitors of a glycosyltransferase reported so far.

Synergy of azasugar **2** in combination with UDP was then evaluated for the inhibition of chitin synthase. UDP is a known competitive inhibitor of CS and initial experiments permitted us to determine the K_i value of UDP ($K_i = 0.27$ mM, plot not shown).

The second set of experiments focused on the dual effect between UDP **1** and the uncompetitive inhibitor **2**; the presence of one inhibitor may increase (or decrease) the affinity of the other. The reaction sequence describing inhibition of an enzyme in the simultaneous presence of two competitive inhibitors has been analyzed by Yonetani and Theorell [20]. Only a few examples in the literature relate to a possible interaction of a competitive and an uncompetitive inhibitor [21]. In our study, the combinative inhibition of chitin synthase by UDP (I_1, competitive) and azasugar **2** (I_2, uncompetitive) might be rationalized as follows:



Thus, the following rate equations might be used to relate the kinetic expressions to the concentrations of various species.

$$[E_T] = [E] + [ES] + [EI_1] + [EI_2] + [EI_1I_2] \quad (1)$$

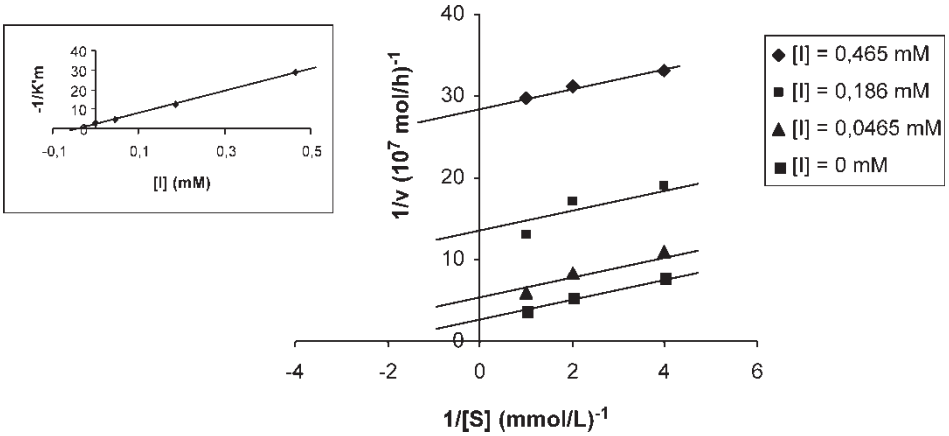


Figure 3. K_i determination (Lineweaver-Burk plots) for azasugar **2**.

where $[E_T]$ is the total concentration of all enzyme-containing species. According to the reaction sequence above, some of these terms can be expressed as concentrations of the appropriate molecular species to give,

$$[E_T] = [E] + [ES] + \frac{[E][I_1]}{K_1} + \frac{[ES][I_2]}{K_2} + \frac{[EI_1][I_2]}{K_{1,2}} \quad (2)$$

$$K_m = \frac{[E][S]}{[ES]} \quad (3)$$

Equations (2) and (3) give,

$$[E_T] = [ES] + [E] \times \left(1 + \frac{[I_1]}{K_1} + \frac{[I_2]}{K_2} \times \frac{[S]}{K_m} + \frac{[I_1][I_2]}{K_1 \times K_{1,2}}\right) \quad (4)$$

$$[E_T] = [ES] + \frac{K_m[ES]}{[S]}\left(1 + \frac{[I_1]}{K_1} + \frac{[I_2]}{K_2} \times \frac{[S]}{K_m} + \frac{[I_1][I_2]}{K_1 \times K_{1,2}}\right) \quad (5)$$

Since $vi = k_3[ES]$, and $V_{max} = k_3[E_T]$, rearrangement of (5) gives

$$\frac{[E_T]}{[ES]} = \frac{V_{max}}{Vi} = \frac{[S] + K_m\left(1 + \frac{[I_1]}{K_1} + \frac{[I_2]}{K_2} \times \frac{[S]}{[K_m]} + \frac{[I_1][I_2]}{K_1 \times K_{1,2}}\right)}{[S]} \quad (6)$$

To evaluate a possible synergistic effect, it might be convenient to introduce a coefficient α defined as, $K_{1,2} = \alpha K_2$. Thus,

$\alpha = 1$, means that the EI_1 (Enzyme-UDP) induces the same binding of the uncompetitive I_2 (azasugar **2**) as the natural ES complex.

$\alpha < 1$ reflects a synergistic effect in that the binding of azasugar **2** is more favorable with the E-UDP complex than with the ES complex.

$\alpha > 1$ means that the binding of the competitive inhibitor UDP **1** leads to a poorer affinity of the uncompetitive inhibitor **2**, which reflects antisynergy.

Substituting and rearranging Equation (6) gives

$$\frac{1}{vi} = \frac{1}{V_{max}} + \frac{K_m\left(1 + \frac{[I_2][S]}{K_2 K_m}\right)}{[S]V_{max}} + \frac{K_m\left(1 + \frac{[I_2]}{\alpha K_2}\right)}{[S]V_{max}K_1}[I_1] \quad (7)$$

Equation (7) indicates that, if 1/vi is plotted against $[I_1]$ at fixed $[I_2]$, straight lines will be obtained (Figure 4) which intersect at an abscissa value of $(-\alpha\ [S]\ K_1/K_m)$. Using the values of K_1 and K_m determined in the first set of experiments, this provides the possibility for a graphical straightforward method of determination of α, related to K_1. As displayed in Figure 4a, the interaction constant α was graphically determined to be 0.9.

A more accurate possibility for the determination of α, related to K_2, is to consider the slope s of each line which is given by,

$$s = \frac{K_m}{[S]V_{max}K_1}\left(1 + \frac{[I_2]}{\alpha K_2}\right) \quad (8)$$

A secondary plot of s versus $[I_2]$ (Figure 4b) gives a straight line with an intercept at the $[I_2]$ axis of $(-\alpha\ K_2)$. Using this method, the same value of α ($\alpha = 0.9$) was obtained, indicating a synergistic effect.

Thus, the presence of one inhibitor slightly increases the affinity of the other one. A profound synergistic inhibition of recombinant human α-1,3-fucosyltransferase by a competitive azasugar-type inhibitor and the corresponding nucleoside diphosphate product has already been observed [22]. In this example, it was assumed that both competitive inhibitors formed a complex in the active site to closely mimic the transition state of the glycosyl transfer reaction. However, in our case, the synergistic

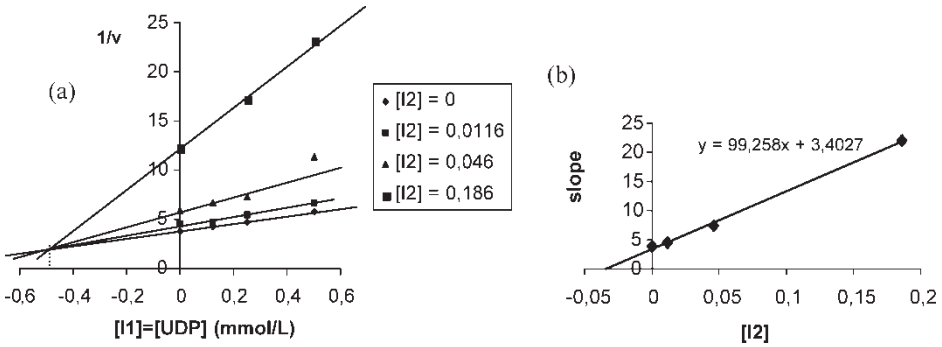


Figure 4. Multiple inhibition studies of Chitin Synthase with competitive inhibitor **1** and uncompetitive inhibitor **2**.

effect between azasugar **2** and UDP might be rationalised in a different manner. Our results might indicate that the conformational changes that take place in the enzyme after binding of either the natural substrate UDP-GlcNAc or the competing inhibitor UDP **1** are slightly different. The interactions that should stabilise the uncompetitive inhibitor **2** in its binding site thus revealed are more favourable with the E-UDP complex than with the natural ES complex. Nevertheless, these results provide encouragements for the development of other iminosugar-based CS inhibitors.

Acknowledgements

We would like to thank Professeur G. Guillerm for his continuous interest in our chitin synthase project.

References

[1] Behr J-B. Curr Med Chem (Anti Infective Agents) 2003;2:173–189.
[2] Merz RA, Horsch M, Nyhlen LE, Rast DM. EXS 1999;87:9–23, (Chitin and Chitinases).
[3] Zhang D, Miller MJ. Curr Pharm Des 1999;5:73.
[4] Saxena IM, Brown RM, Fèvre M, Geremia RA, Henrissat B. J Bacteriol 1995;177:1419–1424.
[5] Horsch M, Sowdhamini R. In: Muzzarelli RAA, editor. In Chitin enzymology., Atec Edizioni; 1996. vol. 2 p 447–458.
[6] Yabe T, Yamada-Okabe T, Nakajima T, Sudoh M, Arisawa M, Yamada-Okabe H. Eur J Biochem 1998;258:941–957.
[7] Behr J-B, Gautier-Lefebvre I, Mvondo-Evina C, Guillerm G. J Enz Inhib 2001;16:107.
[8] Behr J-B, Gourlain T, Helimi A, Guillerm G. Bioorg Med Chem Lett 2003;13:1713–1716.
[9] Gautier-Lefevre I, Behr J-B, Guillerm G, Ryder N. Bioorg Med Chem Lett 2000;10:1483–1486.
[10] Yeager AR, Finney NS. J Org Chem 2004;69:613–620.
[11] Devel L, Vidal-Cros A, Thellend A. Tetrahedron Lett 2000;41:299–301.
[12] Behr J-B, Mvondo Evina C, Phung N, Guillerm G. J Chem Soc Perkin Trans I 1997;:1597–1599.
[13] Unpublished results.
[14] Behr J-B, Guillerm G. Tetrahedron: Asymmetry 2002;13: 111–113.
[15] Orlean P. J. Biol. Chem. 1987;262:5732–5739.
[16] Crotti LB, Drgon T, Cabib E. Anal Biochem 2001;298:8–15.
[17] Choi W-J, Cabib E. Anal Biochem 1994;219:368.
[18] Compain P, Martin OR. Bioorg Med Chem 2001;9:3077.
[19] Saotome C, Kanie Y, Kanie O, Wong C-H. Bioorg Med Chem 2000;8:2249–2261.
[20] Yonetani T, Theorell H. Arch Biochem Biophys 1964; 106:243–251.
[21] Chou T-C, Talalay P. J. Biol Chem 1977;252:6438–6442.
[22] Wong C-H, Dumas DP, Ichikawa Y, Koseki K, Danishefsky SJ, Weston BW, Lowe JB. J Am Chem Soc 1992;114:7321–7322.