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Inhibition of Chitin Synthetase from *Saccharomyces cerevisiae* by a New UDP-GlcNAc Analogue

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The synthesis and biological evaluation of a new UDP-GlcNAc competitor (I), designed to mimic the transition state of the sugar donor in the enzymatic reaction catalysed by chitin synthetase, is described. Compound (I) was found to competitively inhibit chitin synthetase from *Saccharomyces cerevisiae* with respect to UDP-GlcNAc, but displayed minimal antifungal activity.

Keywords: Chitin synthetase, Inhibition, Transition state analogue, Glycosyltransferase, Azasugar nucleotide, UDP-GlcNAc analogue

INTRODUCTION

Chitin synthetases are membrane-bound glycosyltransferases which catalyse the transfer of *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc to a growing chain of β-1→4-linked *N*-acetylglucosamine residues.[1] Because of the importance of chitin as an integral constituent of yeast and fungal cell walls and its widespread occurrence in a large variety of pathogenic fungi, inhibition of the biosynthesis of chitin provides an attractive therapeutic target.[2] The best known and most effective inhibitors of chitin synthetases with marked antifungal activity are the naturally occurring Polyoxins and Nikkomycins. The gross structural similarity of these peptidyl nucleoside antibiotics to UDP-GlcNAc has been pointed out to explain their high potency to bind to the catalytic site of chitin synthetases.[3] Therefore, extensive efforts have been made to modify the basic structure of polyoxins and nikkomycins to improve their inhibitory potency and their antifungal properties.[4]

In the challenging area of glycosyltransferase inhibition, it has been well established that better inhibition is observed for transition state or bisubstrate analogues. However, for chitin synthetase little work has been done to synthesise[5] and screen inhibitors that resemble the active structure of UDP-*N*-acetylglucosamine involved in the transition state of this enzyme-catalysed reaction. Knowledge regarding of the active site of chitin synthetase is rather limited, however

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the putative mechanism postulated[6] for the enzymatic process involved in glycosyl transfer reactions using Leloir donors might be exploited in the design of such inhibitors (Figure 1).

In this paper we report the synthesis together with enzymatic and antifungal evaluation of a new UDP-GlcNAc competitor (**I**) designed to mimic the transition state of the sugar donor in the enzymatic reaction (Figure 1). This model includes the following structural features: (a) the five-membered ring azasugar, stereochemically related to L-xylose, would mimic the half chair conformation and the charge of the glycosyl cation[7] as well as a possible contact of the *N*-acetyl group with the enzyme, (b) a malonyl group is used as surrogate for pyrophosphate to mimic the UDP-GlcNAc-Mn^{2+} complex.

The combination of similar moieties has already been considered in the design of β-1,4-galactosyltransferase inhibitors.[8]

Benzyl groups were kept on the azasugar moiety of the tested inhibitor (**I**) in order to facilitate the binding in the hydrophobic area of the active site of the enzyme[9] as well as to favour penetration into cells.

MATERIALS AND METHODS

General

Digitonin, trypsin, soybean trypsin inhibitor, UDP-GlcNAc, Nikkomycine Z and 2,3,5-tri-*O*-benzyl-D-arabinofuranose were obtained from Sigma. *N*-Acetyl-D-glucosamine, uridine, pyrrolidinopyridine (PPY) and dicyclohexylcarbodiimide (DCC) were purchased from Acros. [$UDP^{14}C$]-GlcNAc (289 mCi/mmol) was obtained from NEN. Vinylmagnesium bromide was obtained from Aldrich and hydroxybenzotriazole (HOBT) from Janssen. *Saccharomyces cerevisiae* strains were

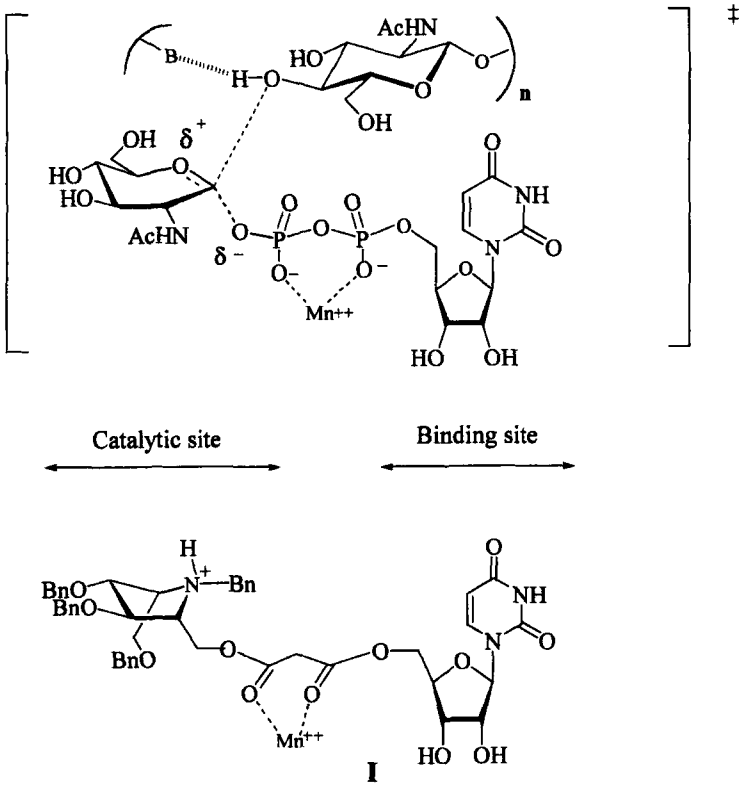


FIGURE 1 Structural analogy between the postulated transition state of the enzymatic reaction and compound (**I**).

a gift from BIOSPRINGER, Maisons-Alfort, France and were kept at −80° before use. Buffers used for biological evaluation contained: Buffer A, 30 mM Tris-chloride, 6 mM $Mg(Ac)_2$, 10 mM EDTA, pH 7; Buffer B, 30 mM Tris-chloride, pH 7.5.

Synthesis of UDP-GlcNAc Analogue (I)

For the synthesis of (**I**) the appropriate 5′-*O*-malonylester of uridine (**3**) and azasugar (**5**) had to be synthesised first.

Synthesis of uridinyl malonate (3):

Synthesis of (**3**) was performed using the method devised by one of us[10] and published later by R. Wang *et al.*[8] Heating 2′,3′-*O*-isopropylideneuridine (**1**) (2.00 g, 7.04 mmol) and Meldrum's acid (**2**) (1.10 g, 7.6 mmol) at 110 °C in a small amount of DMSO (2 mL) for 10 min led to the malonyl ester (**3**) (1.75 g) in a 75% isolated yield (Scheme 1). Compound (**3**) is thermally sensitive but can be kept, after chromatographic purification on a hydrophobic HP20 support, at −20°C for months.

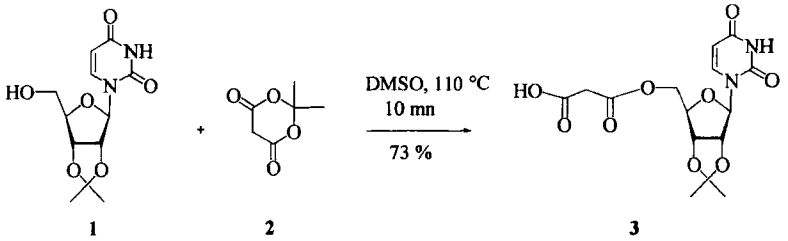
Synthesis of polyhydroxypyrrolidine (5):

Number of synthetic methods leading to five-membered aza-sugars have been devised, most of them starting from natural sugars.[11] We prepared (**5**) according to the method described by Nicotra for the synthesis of (**4**), based on the stereoselective addition of vinylmagnesium bromide to *N*-benzyl-2,3,5-tri-*O*-benzyl-D-arabinofuranosylamine.[12]

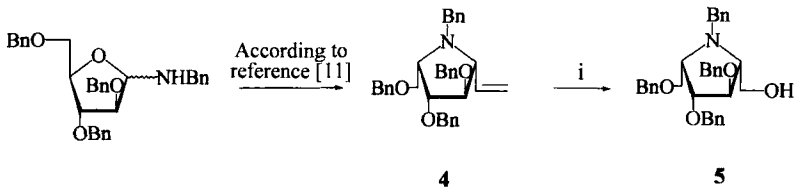
Oxidation of the double bond to the corresponding aldehyde was carried out by ozonolysis of the sulfate salt of vinyl derivative (**4**) in methylene chloride at −70 °C; subsequent reduction with $NaBH_4$ gave the target molecule (**5**) in a 65% overall yield (Scheme 2).

Synthesis of (I):

Compound (**I**) was obtained by coupling the uridinyl malonate (**3**) (0.17 g, 0.46 mmol) with the protected polyhydroxypyrrolidine (**5**) (0.2 g, 0.38 mmol) (Scheme 3). This reaction was best achieved using DCC (0.095 g, 0.46 mmol) and HOBT (0.052 g, 0.38 mmol) as coupling reagents,



SCHEME 1 Synthesis of the acid (**3**).



i) H_2SO_4, O_3; Me_2S, Na_2CO_3, then $NaBH_4$, EtOH.

SCHEME 2 Synthesis of the polyhydroxypyrrolidine (**5**).

in the presence of a catalytic amount of pyrrolidinopyridine (0.01 g) in CH_2Cl_2 (1.5 mL) and DMF (0.7 mL). To complete the reaction, an excess of acid (**3**) and DCC had to be added gradually to the reaction mixture since degradation products of (**3**) appeared during the reaction. Deprotection of the acetonide in 80% formic acid (7 mL) gave compound (**I**)[13] as a yellow oil (0.203 g, 64% yield from **5**).

Cell Permeabilization

S. cerevisiae cells were permeabilized with a mixture of toluene-ethanol, essentially as described by Masson *et al.*[14] An aliquot of 1 g (wet weight) of freshly grown yeast cells was suspended and preincubated in 20 mL of Buffer A (see general) for 5 min. Then, 4 mL of a 1:4 solution of toluene-ethanol was added and the suspension shaken vigorously for 5 min at the same temperature. After cooling on ice and centrifugation at 5000 g (4 °C), the resulting pellet was washed twice with 20 mL of buffer A and finally resuspended in 1 mL of Buffer B for chitin synthetase activation.

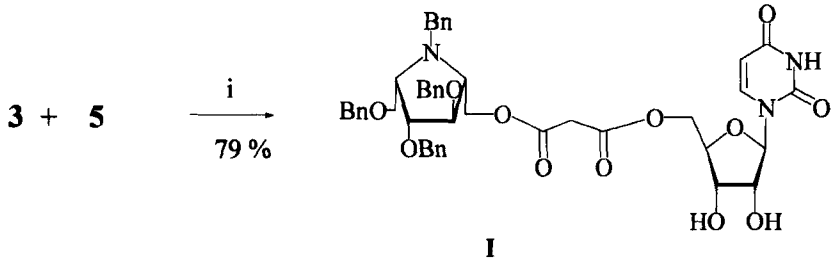
Activation of Chitin Synthetase with Permeabilized Cells

Chitin synthetase 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 as previously reported.[15] In our experiments, an aliquot of permeabilized cell suspension (to a final concentration of 0.26 g/mL) was incubated for 15 min in buffer B containing 55 mM GlcNAc, digitonin (5.5 mg/mL) and trypsin (2.1 mg/mL). Activation was stopped by adding soybean trypsin inhibitor (3.1 mg/mL). The resulting preparation was kept at 0 °C for assay.

Chitin Synthetase Assay

Enzyme activity was measured by a radioactive assay using UDP-*N*-acetyl-[^{14}C]-glucosamine as described by Ruiz *et al.*[16] Assays were 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-chloride, 36 mM GlcNAc, 5 mM $Mg(Ac)_2$ and variable concentrations of UDP-[^{14}C]-GlcNAc (0.1–2 mM, 20 000 cpm).

Reaction was initiated by addition of the cell suspension. After 60 min incubation the reaction was stopped with 1 mL of 60% aqueous ethanol[17] and the resulting suspension was filtered through a glass-fibre filter (Whattman 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.



I

i) DCC, HOBT, PPY, DMF/CH_2Cl_2, **3**; HCOOH 80 %.

SCHEME 3 Synthesis of the transition state analogue (**I**).

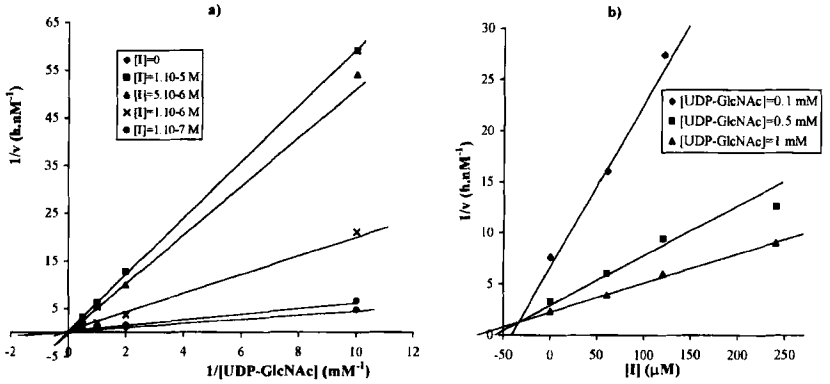


FIGURE 2 K_i determination for inhibitors of chitin synthetase from *S. cerevisiae* permeabilized cells; (a) Lineweaver-Burk plots of chitin synthetase inhibition by Nikkomycin Z; (b) Dixon plots of chitin synthetase inhibition by transition state analogue (**I**). Linear regression analyses were used to determine intercept values for calculation of the kinetic constants.

Inhibition Study

The same assay was used for evaluation of the inhibitory effect of (**I**) present in the incubation mixture at concentrations varying from 60 to 240 µM. All Kinetic constants were determined from Dixon or Lineweaver-Burk plots. The computer program described by Cleland[18] (Ultra-fit software Macintosh) was used for data analysis.

RESULTS AND DISCUSSION

The reaction rate of chitin synthetase, studied in the *in situ* assay described, showed normal Michaelis-Menten kinetics with UDP-GlcNAc as the variable substrate and GlcNAc (36 mM) as the fixed substrate. A K_m value for UDP-GlcNAc of 0.5 mM was calculated from a double reciprocal plot.

Nikkomycin Z is the most potent inhibitor of yeast chitin synthetase. To verify the reliability of the *in situ* method used in our experiments, its inhibitory potency was tested in a competitive way and a K_i of 0.78×10^{-6} M was found (literature[19] cites $0.1–3 \times 10^{-6}$M) (Figure 2).

The inhibitory potency of (**I**) was examined under the same conditions and the K_i value determined after kinetic analysis was 35×10^{-6} M (Figure 2). Pyrrolidine (**5**) (after deprotection) and acid (**3**) were tested in a similar manner and no inhibition was observed for concentrations of up to 1 mM, which underlines the importance of the combination of both moieties in the structural feature of (**I**) for a good interaction with enzyme.

Compared with Nikkomycin Z or Polyoxin D, the strength of binding of (**I**) to chitin synthetase appeared less effective (Table I). However, (**I**) exhibits a K_i value in the range for those of other peptide-nucleosides of the same family and synthetic analogues.[20]

Thus, the preliminary results obtained here are most promising and it is therefore of interest to synthesize analogues of (**I**) combining other azasugars of different stereochemistry linked to a uridine moiety with other pyrophosphate mimics. The synthetic method used in this study should provide a versatile and efficient route to such series of inhibitors.

TABLE I K_i values for different nucleoside derivatives as inhibitors of chitin synthetase

Compound	K_i (10^{-6} M)	Reference
Nikkomycin Z	0.1–3.0	19 (and references cited therein)
Polyoxin D	0.37	14
Compound (**I**)	35.0	

Compound (**I**) was also tested against a panel of several pathogenic fungi[21] (*Cryptococcus neoformans*, *Trichophyton mentagrophytes*, *Microsporum canis*, *Aspergillus fumigatus*, *Candida albicans*, *Saccharomyces cerevisiae*). No activity up to the maximum concentration tested of 128 µg/mL was found for (**I**), whereas the standard compound terbinafine used as reference in the inhibition growth study showed the normal activity expected.

Acknowledgements

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[13] NMR data for (**I**) ^{1}H NMR (250 MHz, D_2O) δ ppm, J Hz: 7.40 (d, 1H, $J_{6,5}$ 8.1, H-6), 7.28–7.15 (m, 20H, Ph), 5.80 (d, 1H, $J_{1',2'}$ 3.1, H-1'), 5.70 (d, 1H, J 8.1, H-5), 4.50–4.35 (m, 6H, 6 CH_2Ph), 4.30 (s, 2H, H-5'a,b), 4.15 (m, 2H, H-2' and H-4'), 4.05–3.90 (m, 5H, H-3', H-4'', 2 CH_2O, 1 NCH_2Ph), 3.82–3.70 (m, 3H, H-3'',H-6''a, 1 NCH_2Ph), 3.48 (dd, 1H, J 4.7, 9.3, H-6''b), 3.38 (dt, 1H, J 4.7, 4.7, 7.5, H-5''), 3.18 (s, 2H, (C=O)CH_2(C=O)), 3.15 (t, 1H, J 9.0, H-2''). ^{13}C NMR ($CDCl_3$, 125 MHz) 165.9, 165.8, 163.7, 151.0 (4 C=O), 140.0 (C-6), 138.7–137.9 (C-Ph), 129.5–127.1 (C-Ph), 102.6 (C-5), 90.3 (C-1'), 82.3, 81.9 and 81.5 (C-3'',C-4'', C-3'), 74.3 (C-4'), 73.3, 72.3 and 71.0 (3 CH_2Ph), 69.7 (C-2'), 69.4 (C-6''), 67.6 (C-2''), 66.5 (CH_2O), 66.0 (C-5''), 64.0 (C-5'), 59.4 (NCH_2Ph), 41.0 ((C=O)CH_2(C=O)). L.C.M.S. (EI): 835 $(M)^{\bullet+}$. Found: C, 66.37; H, 5.78; N, 5.34. $C_{46}H_{49}N_3O_{12}$, requires C, 66.10; H, 5.91; N, 5.03%.
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[21] MIC's were determined in broth macrodilution assays according to guidelines of the NCCLS M27-A protocol, with slight modifications.[22] Inocula for assays were prepared from stocks frozen at −80 °C by dilution in growth medium to give a final viable cell count of 2.5×10^3 CFU/mL. Each assay was performed with a duplicate series of drug dilutions. In brief, the assays were done in RPMI 1640 medium buffered to pH 7.0 with MOPS buffer, incubated at 35 °C for 48 h. *Cryptococcus neoformans* and *Aspergillus fumigatus* were tested in the same assay, except that incubations were for 72 h. The dermatophytes *T. mentagrophytes* and *M. canis* were incubated at 30 °C for 7 days. The MIC was defined as the lowest concentration of the drug causing 80% inhibition of fungal growth in comparison with untreated controls.
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