

Chitin Synthase, a Fungal Glycosyltransferase that Is a Valuable Antifungal Target

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Abstract: During the last 30 past years, more life-threatening fungal infections have appeared due to the increasing frequency of patients with weakened immune systems. Inhibition of fungal enzymes involved in the biosynthesis of sterols is considered to be a safe and effective option for antifungal therapy. However, the intensive use of sterol-biosynthesis inhibitors for years has resulted in resistance development. Consequently, the search for alternative therapeutics must be intensified. In this context, the biosynthesis of chitin, an essential component of the fungal cell wall that is absent in mammalian cells, was envisioned as a safe and selective therapeutic target. We present here recent successes in the inhibition of chitin synthase, the enzyme involved in the last step of the biosynthesis of chitin.

Keywords: Antifungal · Glycosyltransferase · Iminosugars · Inhibition · Nikkomycin

Introduction

Fungal cells are protected from the external environment by the cell wall, an initial barrier made mostly of carbohydrates.^[1] This cell wall is essential for the maintenance of cell shape, prevention of lysis and regulation of biochemical exchange with the environment.^[2] The composition of the cell walls is made up of three biopolymers: glucans, mannoproteins and chitin.^[3] Chitin is a linear polysaccharide composed of 2-acetamido-2-deoxy-D-glucose (GlcNAc) units joined through β -(1,4) glycosidic linkages (Fig. 1).^[4] It is indispensable for fungal survival. Cells in which the formation of chitin is disrupted are affected by osmotic sensitivity, abnormal morphology and growth arrest.^[5] Since chitin is absent from vertebrates, inhibition of its biosynthesis has been considered as a safe and selective option for the development of antifungal agents.^[6]

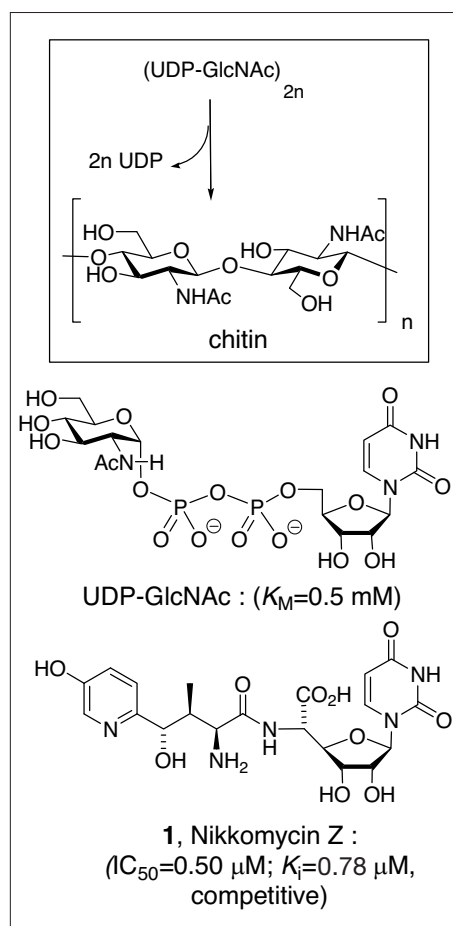


Fig. 1. Biosynthesis of chitin from UDP-GlcNAc.

UDP-GlcNAc as the obligate substrate. In *Saccharomyces cerevisiae*, three distinct chitin synthase activities (ScCSI, ScCSII and ScCSIII) have been described, which differ in function, localization, cation specificity, optimum pH and even sensitivity to inhibitors.^[8] The chemical transformation catalyzed by CS can be seen as a repetitive transfer of GlcNAc residues from the activated donor UDP-GlcNAc to the growing chitin chain (Fig. 1), with concomitant release of UDP.

Though all enzymes of the chitin biosynthetic pathway could serve as targets in antifungal drug design, most studies concerned chitin synthase,^[9] by analogy with what is found in Nature. Indeed, the 'natural' control of chitin metabolism is achieved by the antifungal antibiotics nikkomycins and polyoxins that specifically inhibit chitin synthase with K_i values in the low micromolar range.^[10] The structural resemblance of these peptidyl nucleosides with UDP-GlcNAc has been pointed out to explain their high potency to bind to the catalytic site of chitin synthase. Nikkomycin Z (**1**, NiZ), one of the most potent CS inhibitors ($K_i = 0.78$ μ M),^[11] showed good *in vitro* activity against a number of pathogenic fungi (MIC = 12 μ g/mL for *Candida albicans*, MIC = 0.78 μ g/mL for *Blastomyces dermatidis*) and to date, this compound is the only CS inhibitor that underwent clinical trials.^[12] However, despite excellent *in vitro* results, nikkomycins and polyoxins display restricted ability to stop fungal growth *in vivo* due to either inefficient transport or degradation before reaching the target enzyme. The search for new CS inhibitors with enhanced antifungal activity follow three strategies: i) identification of new inhibitors from natural sources by screening programs,^[13] ii)

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Formation of chitin is a highly complex and interconnected series of biochemical reactions that has been intensively studied in the yeast *Saccharomyces cerevisiae*.^[7] The last step of this process is governed by an enzymatic activity called chitin synthase (CS, EC 2.4.1.16), which uses

inhibition based on substrate analogues using **1** as the lead compound or iii) inhibition based on mechanism with the design of transition-state mimetics or bisubstrate analogues. Recent results following the two last options are discussed here.

The Development of Synthetic NiZ Analogues

The design of analogues that could function as inhibitors of chitin synthase focused on structural features of either the natural substrate UDP-GlcNAc or the NiZ core and aimed to address the issues limiting *in vivo* activity: hydrolytic degradation and poor uptake. The most simple non-hydrolyzable analogue of UDP-GlcNAc was prepared by the Finney^[14] and Kirk^[15] groups and features a methylene in place of the glycosidic oxygen (compound **2**, Fig. 2). Phosphonate **2** was a very weak inhibitor of chitin synthase ($K_i > 10$ mM) having much lower affinity for the active site than UDP-GlcNAc itself ($K_M = 0.5$ mM). Interestingly, the replacement of the sugar residue in **2** by a hetaryl moiety (compound **3**) restored the inhibitory potency ($IC_{50} = 0.82$ mM).^[16] The quinoline group certainly assumes complementarity between the hydrophobicity of CS and its inhibitors, as exemplified by NiZ. Using **3** as a template, we prepared analogues featuring other nonhydrolyzable phosphate mimics such as tartrate, malonate or monosaccharidic residues (Fig. 3).^[17] Whereas quinoline-malonate **4** ($IC_{50} = 2.9$ mM) or tartrate **5** ($IC_{50} = 2.0$ mM, $K_i = 0.79$ mM, competitive) display weaker activity than the corresponding phosphonate, the inhibition potencies of monosaccharidic derivatives **6** depend on the substitution pattern ($IC_{50} = 0.80$ – 3.2 mM). The 1,3 substituted glucose nucleoside **6a** was the most active of the series, which displayed $K_i = 0.25$ mM with a competitive inhibition pattern.

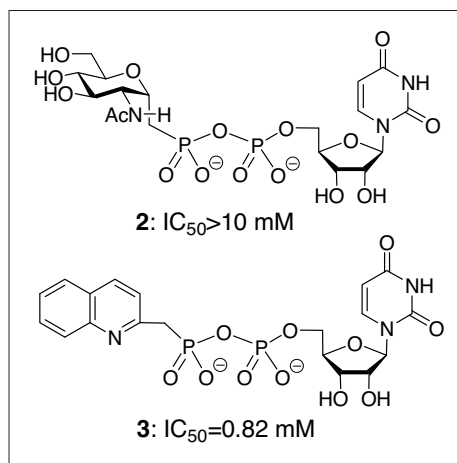


Fig. 2. Inhibition of chitin synthase by substrate analogues **2** and **3**.

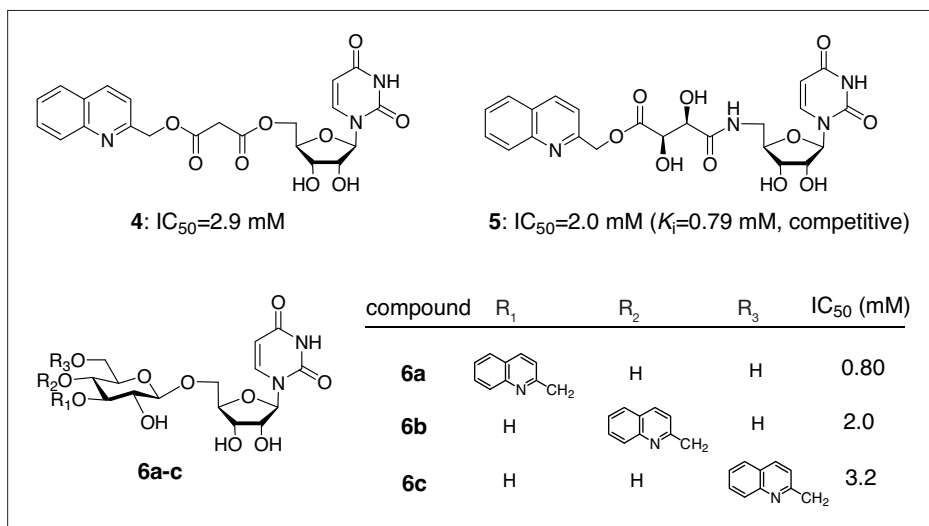
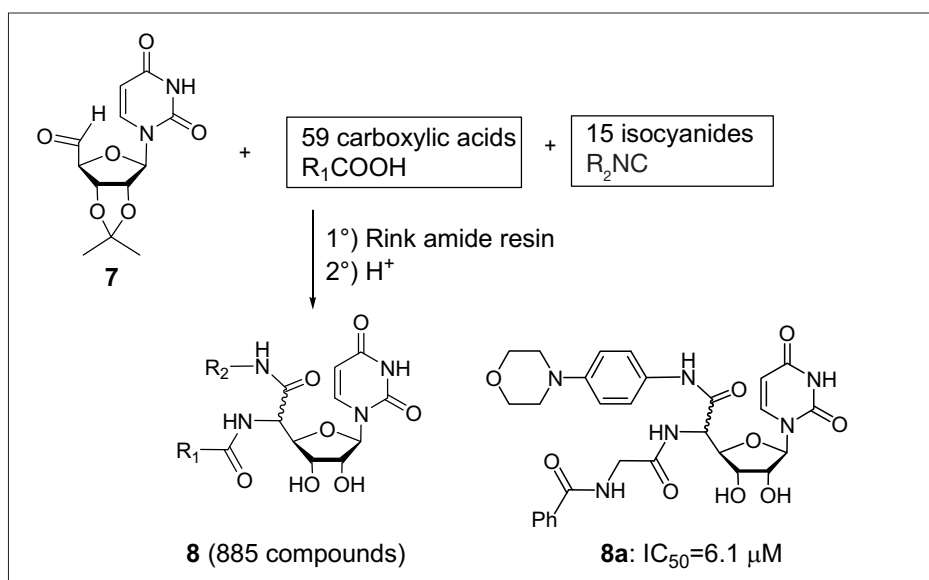


Fig. 3. Inhibition of chitin synthase by hetaryl nucleosides **4**–**6**.



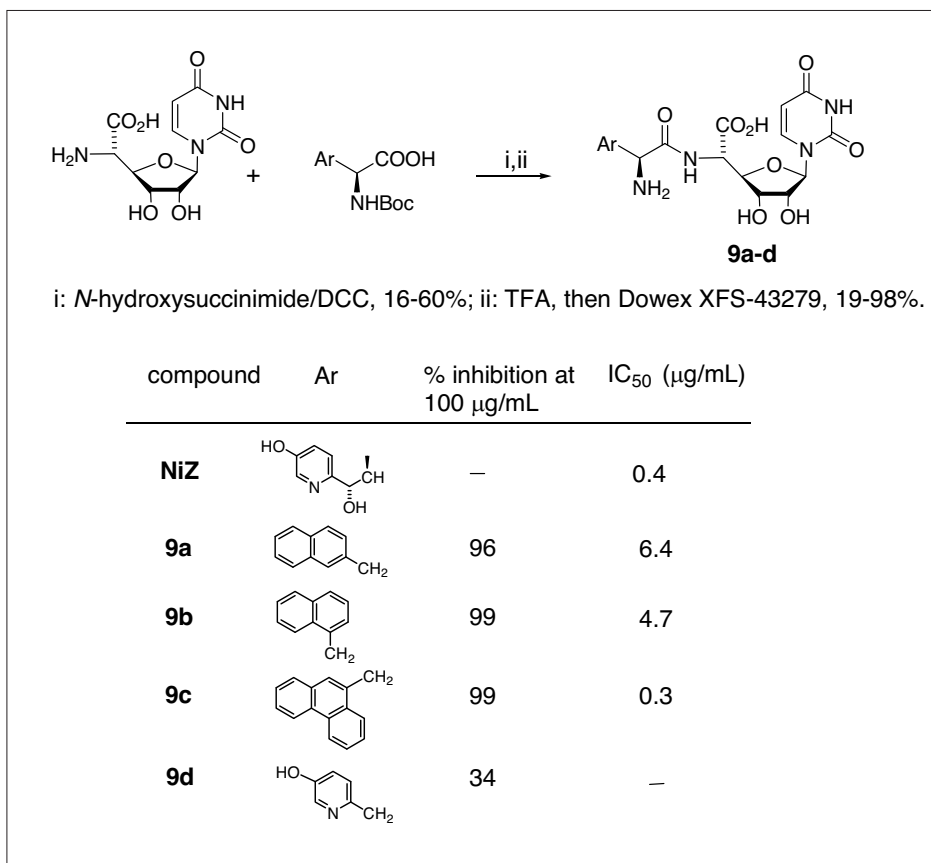
Scheme 1. Combinatorial synthesis of NiZ analogues **8**.

Surprisingly, the replacement of the quinoline moiety in the structure of **4** or **5** by a hydroxypyridine to resemble NiZ did not improve the binding interactions.^[17]

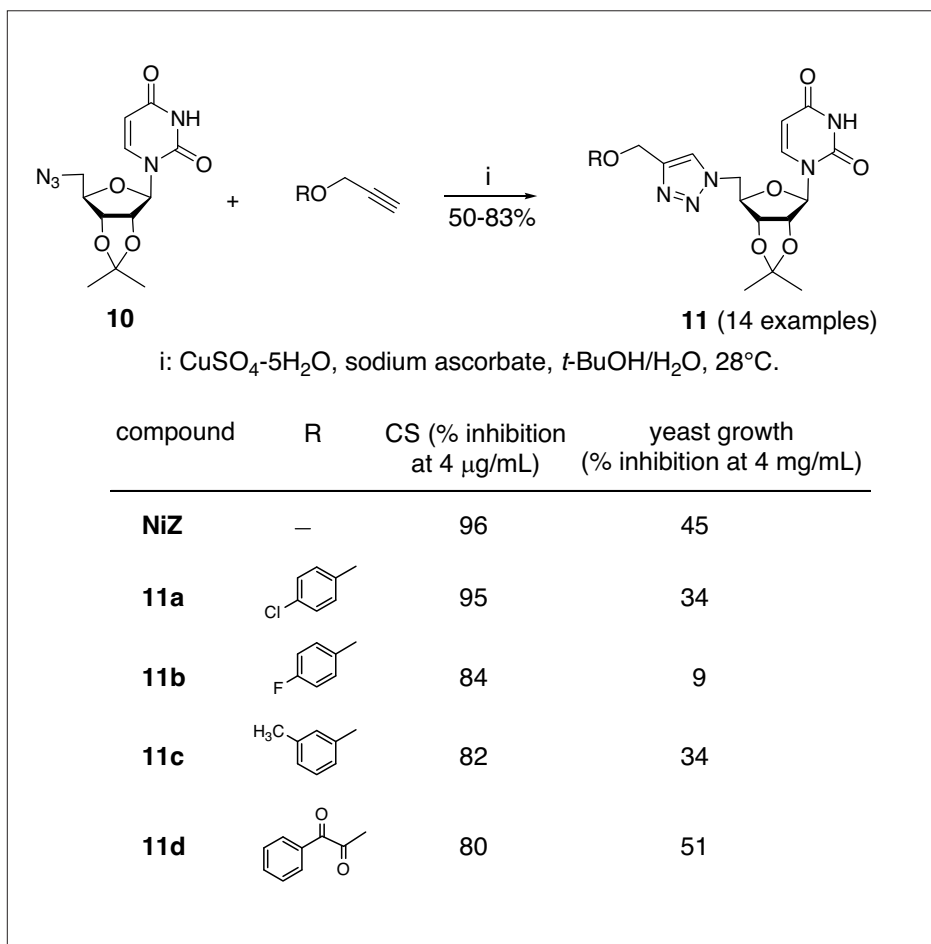
A combinatorial synthesis of a large series of peptidyl-nucleosides has been described for the construction of library of NiZ analogues. The Ugi condensation of the uridyl aldehyde **7** with a combination of various carboxylic acids and isocyanides gave 885 bis-amides of type **8** (Scheme 1).^[18] Some hits were identified, which were as active as NiZ against CS from *Candida albicans*, compound **8a** ($IC_{50} = 6.1$ μM) being the most promising candidate for therapeutic application. Based on a receptor site model calculation for NiZ, a study by Obi and coworkers aimed at designing a new series of NiZ analogues with simplified chemical structure.^[19] Molecular modeling has stressed out the existence of a binding pocket

with strong hydrophobic character, close to the catalytic site. Thus, 23 novel NiZ analogues **9** with aromatic groups at the β -position were prepared, some of them displayed strong anti-CS activity (selected data in Scheme 2).

A library of 1,4-disubstituted-1,2,3-triazolyluridine derivatives was realized recently using Cu(I)-catalyzed 1,3-dipolar cycloaddition of the azido-uridine **10** with propargylated aromatics (Scheme 3).^[20] The design of these targets was mainly based on the replacement of the peptide linker of NiZ with a triazole moiety. Chitin synthase activity was evaluated for *Benjaminiella poitrasii* cells. Surprisingly, the isopropylidene-protected nucleosides **11** displayed potent anti-CS activity (80–95% inhibition at 4 $\mu g/mL$) and good antifungal potency against human and plant pathogens. In an analogous approach, Plant and coworkers designed unprecedented NiZ



Scheme 2. Synthesis and chitin synthase inhibition potencies of NiZ analogues 9.



Scheme 3. Synthesis and biological evaluation of triazoles 11.

analogues bearing an isoxazole ring as the phosphate surrogate.^[21] Unfortunately, their inhibitory activities toward CS have not been reported yet.

The Design of Mechanism-based Inhibitors

Like other polymerizing transferases, chitin synthase is an integral membrane protein for which little information is available concerning its structure and mode of action. However, by analogy with other glycosyltransferases or with glycosidases, a direct S_N2-type transfer seems to be a reasonable hypothesis. It requires the intervention of a metallic cation (Mg²⁺, Mn²⁺, Ni²⁺) as an activating Lewis acid (Fig. 4).^[22]

The design of transition state mimetics or bisubstrate analogues has been applied with success to glycosyltransferases in the *gluco*, *galacto* or *fuco* series.^[23] The goal is to simulate in a stable mimetic, most functionalities (charge, shape, polarity) of one or both substrates in the transition state of the enzymatic reaction. Since the topology of the active site has evolved to best complement the structure of the reaction transition state, such an analogue should largely surpass a conventional substrate or a substrate mimic in affinity.^[24] In our group several of transition state mimics for chitin synthase were designed that incorporate single or multiple features of UDP-GlcNAc and/or chitin under formation (Fig. 4). On this basis, we synthesized polyhydroxypyrrolidines **12a,b**,^[25,26] difluoromethyl-phosphonoiminosugar **13**,^[27] pseudodisaccharides **14a,b** and pyrrolizidine **15**.^[28] The five-membered ring iminosugar mimics the half chair conformation and the charge (through protonation at physiological pH) of the glycosyl cation, whereas the phosphonate moiety and the additional GlcNAc residue promote supplementary binding interactions by mimicking either the pyrophosphate group or the growing chitin chain. Iminosugar **12b** and phosphonoiminosugar **13** are weak inhibitors of CS, with IC₅₀s above 2.6 mM. Surprisingly, the presence of a supplementary GlcNAc residue, expected to mimic the acceptor of the glycosyltransfer reaction, led to a reduction of the inhibition of the enzyme (IC₅₀ = 5.5 mM for **14a**, whereas IC₅₀ = 2.6 mM for the corresponding pyrrolidinol **12b**). Interestingly, compound **12a**, an iminosugar found in Nature, is significantly more effective (IC₅₀ = 65 μM , K_i = 38 μM).^[29] In spite of minimal structural complexity, iminosugar **12a** was as active as more elaborated natural or synthetic CS inhibitors. It is worth noting that the stereochemical integrity of **12a** is essential for binding since the C(2) epimer **12b** exhibited only weak inhibition

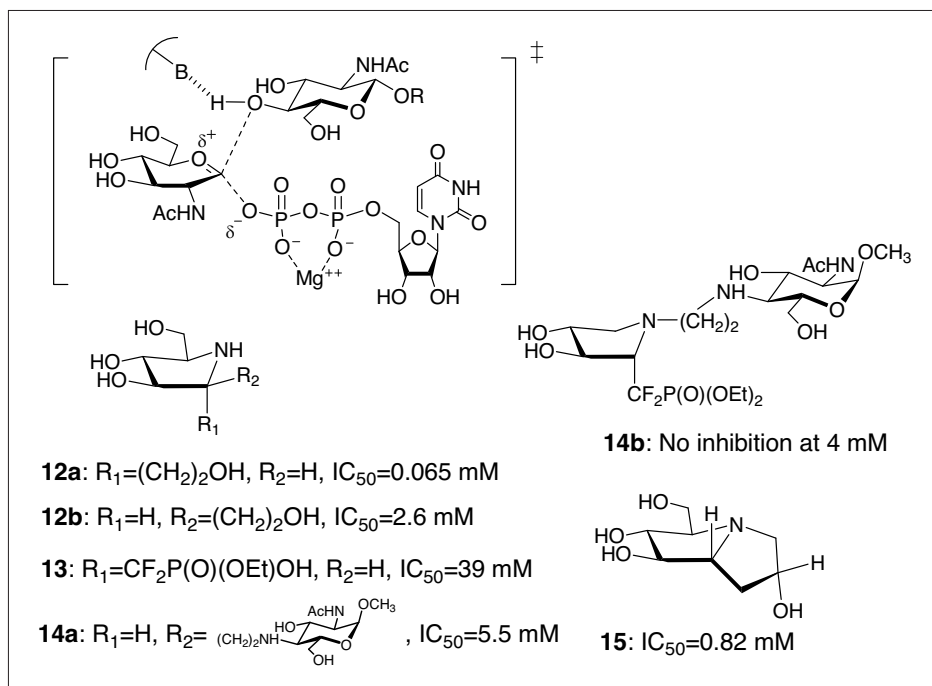


Fig. 4. Design of transition-state analogues for chitin synthase.

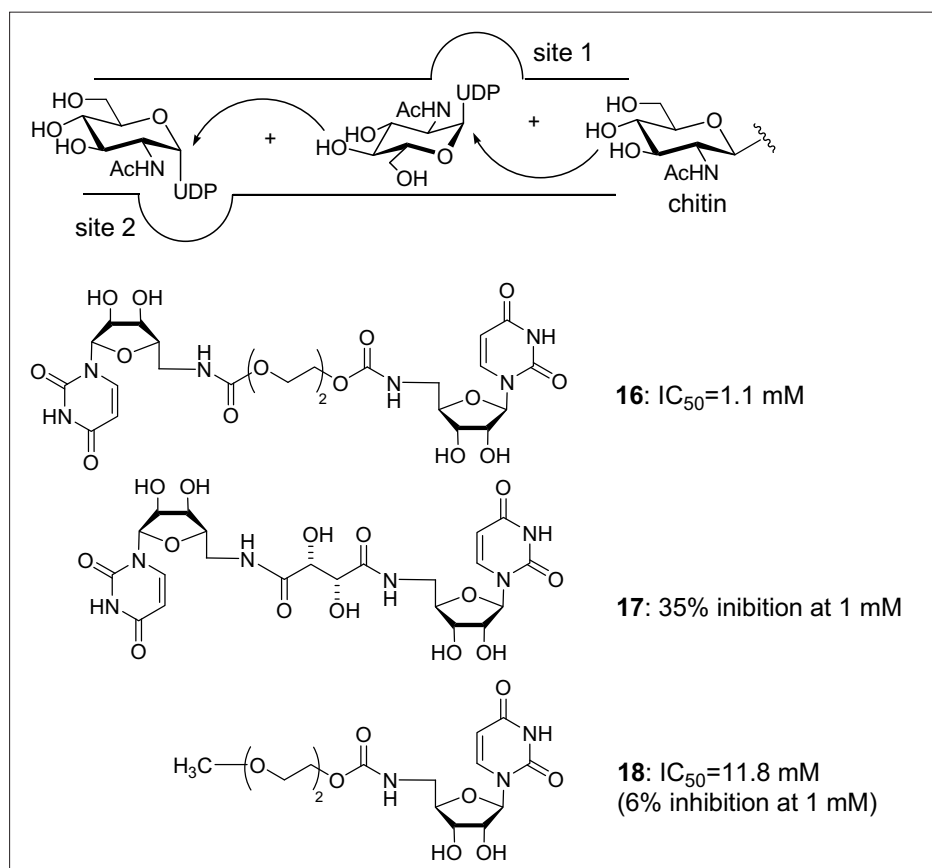


Fig. 5. Design of dimeric inhibitors for chitin synthase.

towards chitin synthase.

New polyhydroxy-pyrrolizidines were designed next as constrained analogues of 6-deoxy-homoDMDP **12a**, to improve binding through pre-orientation of the essential hydroxyethyl substituent. Enzymatic assays revealed that 7-deoxycasuarine

15 was the best inhibitor of the series ($IC_{50} = 820$ μ M) displaying a non-competitive inhibition pattern, whereas the diastereoisomeric pyrrolizidines had IC_{50} values in the range 4.3–18.9 mM.^[28]

Attempts to prepare mechanism-based inhibitors of CS have been reported by

other groups.^[30] The Finney group has focused on the design of dimeric analogues that assume a two-active-site mechanism. Indeed, the opposed orientation of two adjacent monosaccharidic units in the structure of chitin (each sugar is rotated $\approx 180^\circ$ relative to the preceding one in the chain) have suggested that a processive transferase such as chitin synthase operates by transferring two sugar units simultaneously with alternating up/down configuration. One possible explanation is that CS or an associated protein pre-assembles the required disaccharide donor (UDP-chitinose), allowing extension by two residues at a time. However, this hypothesis was excluded by running experiments with radio-labelled UDP-chitinose.^[31] An alternative proposal is that CS has two active sites in close proximity, one for each sugar orientation (Fig. 5).^[32] To probe this hypothesis, Finney and coworkers prepared a series of dimeric inhibitors that contain two uridiny moieties linked by an aliphatic tether.^[33] Though the compounds displayed weak inhibition potencies it appeared clearly that bivalent inhibitors such as **16** or **17** displayed activity approximately 10-fold higher than that of the corresponding monomer **18**, providing some evidence for the two-active-site mechanism.

Conclusion

Chitin synthase, the enzyme that converts UDP-GlcNAc to chitin, is a member of a class of enzymes known as polymerizing (or processive) glycosyltransferases. Since chitin is an essential component of the fungal cell wall, that is absent in mammals, CS has been recognized as a promising antifungal target. The natural peptidyl nucleosides nikkomycins and polyoxins are competitive chitin synthase inhibitors with K_i s in the micromolar range, which display strong *in vitro* antifungal activity. The search for new CS inhibitors allowed identification of candidates with excellent affinities for CS. To date, no detailed information is available on the structure of CS or its accurate mechanism of action, impeding the rational design of more effective inhibitors. Alternatively, the design of future inhibitors should explore new mechanistic proposals or should focus on identifying new functional groups that improve binding with the enzyme.

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