Dansyl *N*-Acetyl Glucosamine as a Precursor of Fluorescent Chitin: A Method to Detect Fungal Cell Wall Inhibitors

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The increasing medical need for active, specific and safe new antifungal agents in the last decade is mainly related to the growing population of immunocompromised patients[1,2]. The search and the discovery of new antifungal agents that specifically inhibit fungal cells is recognized as an important scientific challenge. Although fungi have biochemical targets common to other eukaryotic cells, their cell wall contains structural elements not shared with mammalian cells. The inhibition of the biosynthesis of these different structural elements not only should affect the fungal growth but has the intrinsic promise for non toxic antifungal agents. Three major cell wall structural elements such as glucan, chitin and mannan are generally considered as therapeutic targets[3,4]. As a part of our program for the discovery of new antifungal agents, we were interested in searching for inhibitors of chitin synthase avoiding radiolabeled precursors. Three different chitin synthase genes (CHS1, CHS2, CHS3) have been identified in *C. albicans* and their role in growth and morphogenesis have been investigated[5,6]. Chitin synthase is responsible for the polymerization of the monomeric unit *N*-acetylglucosamine leading to the linear homopolymer chitin. Inhibition of chitin polymerization affects septum formation, cell wall maturation and bud ring formation damaging the cell growth. Here, we suggest a method for the screening of fungal cell wall inhibitors, that requires the use of fluorescent *N*-acetyl glucosamine (DNAG) as a monomeric precursor of chitin.

Hydroxyl groups of carbon 1 and 4 of NAG are involved in formation of (1,4)-β-glycosidic bonds during polymerization. To avoid interferences in the polymerization reaction, the fluorescent *N*-acetylglucosamine derivative (**4**) (DNAG) was prepared from the *N*-acetylglucosamine (**1**) specifically labelled at carbon 6 with the fluorescent dansyl compound as shown in Fig. 1. The temporary protection of the anomeric hydroxyl group was achieved by reacting (**1**) in a dioxane-water mixture (1 : 1 v/v) in the presence of triethylamine and catalytic amount of dimethylamino pyridine to give the corresponding carbonate (**2**) in 70% yield after purification on silica gel. The ^{1}H NMR (DMSO-d_6-Bruker AMX600) of compound (**2**) showed the anomeric proton at 5.78 ppm as a doublet with a constant coupling of 3.5 Hz typical for the α configuration. The CI-MS (Finningan TSQ 700) spectrum of (**2**) was in accordance with the proposed structure (*m/z* 322 MH^+). The treatment of the carbonate (**2**) with 5-dimethylamino-naphtalene-1-sulfonyl chloride (Dansyl chloride) in pyridine solution at room temperature provided the 6-*O* dansyl compound (**3**) as a yellow solid in 75% yield. The FAB-MS spectrum of (**3**) (*m/z* 555 MH^+) confirmed the monosubstitution and ^{1}H NMR (DMSO-d_6) studies were in agreement with the regiospecific sulfonylation at carbon 6. Removal of the temporary protection by trifluoroacetic acid yielded the desired 6-*O*-dansyl-*N*-acetylglucosamine (**4**) (α/β anomeric mixture) as confirmed by ^{1}H NMR and MS studies. The ability of DNAG to be incorporated into the *C. albicans* cell wall was assessed.

The *C. albicans* strain ATCC 10231 was grown in Minimal 40 (Difco), in Minimal 40 with 1% *N*-acetylglucosamine (NAG) or 1% DNAG as carbon source, at 32°C overnight to about 5×10^8 cells/ml. The growth was quantified by monitoring the OD 600 by a Shimadzu UV-2100 spectrophotometer. No effect on the *C. albicans* growth rate was observed due to presence of NAG or DNAG. Cells grown in the presence of DNAG resulted to be fluorescent.

To localize the fluorescence we followed the protoplast regeneration in the presence of DNAG. First, *C. albicans* cells were converted to protoplasts following the published procedure[7]. Briefly, 50 ml cells were centrifuged for 10 minutes at 400 ~ 500 g. Cells were resuspended in 20 ml of sorbitol 1.0 M, Tris-HCl 100 mM, EDTA 5 mM, β-mercaptoethanol 15 mM (STEM) pH 7.4. 50000 U Lyticase (Sigma) and 5000 U chitinase were added and the suspension incubated at room temperature for 1.5 hours. After this time, the protoplast formation was higher than 95% by measuring the decrease in turbidity at 600 nm. The suspension was centrifuged for 5 minutes at 200 g and the pellet resuspended in 2 ml of 10 mM Tris-HCl pH 7.0, Sorbitol 100 mM and EDTA 5 mm (STE).

Protoplasts were then resuspended in the regeneration

Fig. 1. Scheme of the DNAG synthesis.

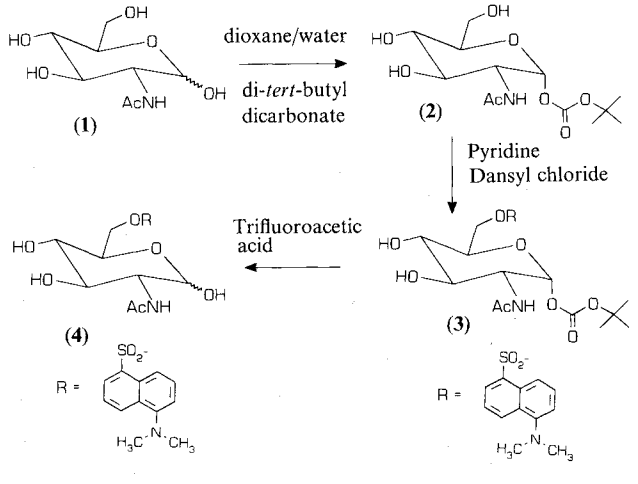
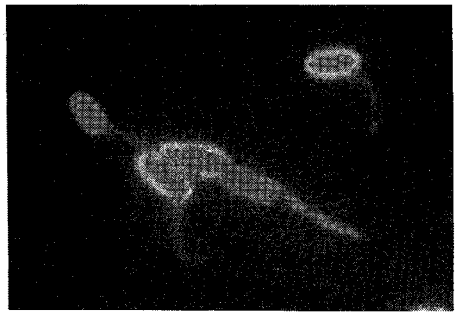


Fig. 2. Fluorescent candida cells obtained by regenerating protoplast in the presence of DNAG.



medium, Minimal 40 supplemented with 0.5 M sorbitol as osmotic stabilizer and *N*-acetylglucosamine (NAG) or 6-*O*-dansyl-*N*-acetylglucosamine (DNAG) 1 mg/ml. Cells were regenerated after overnight incubation at 20°C.

As shown by the photography (Fig. 2) the protoplasts regenerated in the presence of DNAG had the fluorescence localized in the cell wall.

No fluorescence was observed when protoplast were regenerate in the presence of polyoxin D, a specific inhibitor of all three chitin synthase enzymes.

It has been reported[8,9] that fluorescent brightener interacts with various polysaccharides and is absorbed on to chitin and cellulose through hydrogen bonds and dipolar interactions.

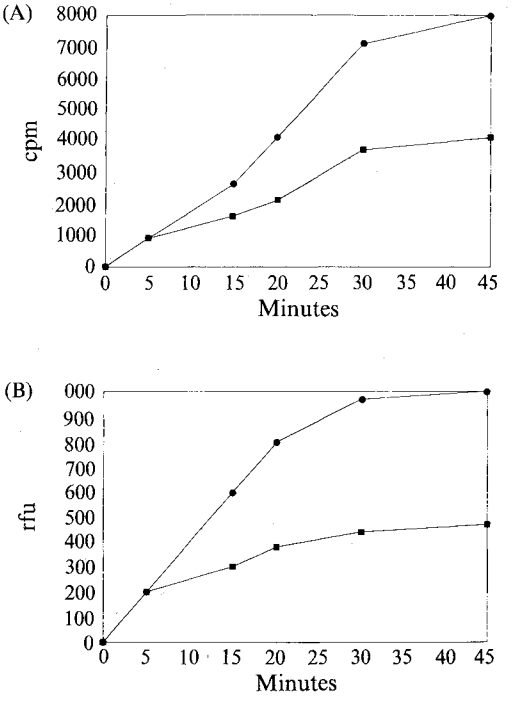
We found that all the fluorescence incorporated into cells (about 0.1% of the fluorescence administered) was associated with the alkali-insoluble fraction containing the glucan-chitin biopolymers as for the radioactive administered. To discriminate between aspecific (absorption) and selective (inclusion into chitin) incorporation into the cell wall, we followed incorporation of radioactive and fluorescent NAG in *C. albicans* cell wall. The assay was performed as described[10]. Briefly, *C. albicans* was grown at 32°C to an absorbance of 0.6 at 600 nm in a defined medium containing 3.5 g/liter NH_4Cl, 0.5 g/liter KH_2PO_4 and $MgSO_4$; 0.1 g/liter $CaCl_2$, 0.1 g/liter NaCl, 3% (v/v) glycerol, 0.1% (w/v) glucose, 0.2% (w/v) Difco casamino acids and 0.1% (w/v) inositol.

Chitin synthesis was measured by using 0.15 μg/ml 6-*O*-dansyl-*N*-acetylglucosamine (DNAG) and 1.25 μg /ml NAG as precursor in the fluorescent assay and 4 μCi/ml ^{3}H,*N*-acetylglucosamine (^{3}H-NAG) and 1.4 μg/ml NAG in the radiolabelled control experiment. After 5 minutes incubation the cell culture was divided into two aliquots. Polyoxin D was added to one of them to a final concentration of 0.2 mg/ml.

Samples (200 μl) were taken at time intervals (minute) and pelleted with 200 μl 10% TCA. The pellet was then treated for 10 minutes with hot 5% KOH . The incorporated fluorescence was determined by diluting the hot alkali-insoluble material in water and measuring the light emitted at 535 nm with an excitation wavelength of

Fig. 3. Time course incorporation of ^{3}H-NAG (A) and DNAG (B) in the alkali insoluble *C. albicans* fraction. Effect of 0.2 mg/ml of polyoxin D.

● Control, ■ polyoxin D.



360 nm in a Perkin Elmer apparatus. The incorporation of ^{3}H-NAG into chitin was determined by measuring the radioactivity present in the hot alkali-insoluble material.

As shown in Fig. 3 the incorporation of both radioactive and fluorescent precursors decreases in the presence of polyoxin D, a specific inhibitor of chitin synthase, suggesting a selective incorporation of DNAG into chitin biopolymer rather than a nonspecific absorption in the cell wall matrix. On the basis of the above results, fluorescent DNAG is eligible for the application in non radioactive high throughput screening to detect new cell wall-specific antifungal agents.

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