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Synthesis and antifungal properties of 6-amino-6-deoxyinulin, a kind of precursors for facile chemical modifications of inulin

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ABSTRACT

Inulin, a kind of abundant, water-soluble, renewable polysaccharide, is mainly extracted from such low-requirement crops as Jerusalem artichoke, chicory, and yacon. The objective of this study was to modify inulin at its primary hydroxyls to give 6-amino-6-deoxyinulin, allowing for the facile chemical manipulation of inulin to encourage the employment of this currently underutilized biodegradable and environmentally benign resource. Additionally, its antifungal properties against two strains of phytopathogens, *Cladosporium cucumerinum* (Ell.) et Arthur and *Fusarium oxysporum* sp. *Cucumis sativus* L., were also evaluated by hypha measurement *in vitro* and the inhibitory indices against these two fungi were 60.1% and 53.3% at 1000 μg/mL, respectively. Because 6-amino-6-deoxyinulin is easy to prepare and exhibits improved potential activities, this material may represent an attractive new platform for chemical modifications of inulin.

1. Introduction

As source of renewable and sustainable materials, polysaccharides have been the subject of intense research in years (Habibi & Dufresne, 2008). An important reason for this is the continuously increasing demands for biodegradable and environmentally benign polymeric material. Inulin consists primarily of β-fructosyl fructose units – always presented in furanose form – usually with a glucopyranose unit reducing end (GFn) (Rogge & Stevens, 2004). This polysaccharide has exhibited many interesting properties like beneficial nutritional attributes for human health, moderate average degree of polymerization and readiness of being obtained (Beylot, 2006; Causey, Feirtag, Gallaher, Tungland, & Slavin, 2000). Although many probable applications of this abundant natural resource have been discussed as an interesting biomacromoleculars, its tedious (polyol) functionality has inevitably limited the studies on chemical modifications and utilization (Stevens, Meriggi, & Booten, 2001). It is safe to propose that a reactive precursor would steer the chemical manipulations of this polysaccharide toward a good direction of facility. 6-Amino-6-deoxyinulin is an ideal candidate to be synthesized. First, amino groups are active enough and could play important parts during chemical reactions (Guo, Liu, Chen, Ji, & Li, 2006), for instance through amino groups Roman synthesized fluorescently labeled cellulose nanocrystals (Dong & Roman, 2007). Second, aminoglycosides always exhibit excellent bioactivities, for example via binding to the RNA, some aminoglycosides could work as antibiotics (Ma, Baker, Joseph, & McCammon, 2002). Given these characteristics of inulin and the project, we started to synthesize 6-amino-6-deoxyinulin. Moreover, its antifungal properties against two strains of phytopathogens were also estimated by hypha measurement *in vitro*, respectively.

To prepare 6-amino-6-deoxyinulin, 6-azido-6-deoxyinulin was an excellent intermediate of the project as azide could conveniently transform to amino groups by way of reduction. Meanwhile, 6-azido-6-deoxyinulin could obtain through S_N reaction by azide ion displacing appropriate leaving groups which could be chlorine or tosyl groups. Methods of gently replacing primary other than second hydroxyl groups in carbohydrates by chlorine have already elaborated by Evans in 1968 (Evans, Long, & Parrish, 1968) and this have built one rock on which 6-amino-6-deoxyinulin could synthesized without suffering from protection and deprotection issues. When leaving groups come to tosyls groups, inulin should be fully tosylated at C-6, which may also lead to little C-4 tosylation, however, as long as the reaction temperature was controlled, azide ion could specifically substitute C-6 tosyl groups (Liu & Baumann, 2002). After azide replacing C-6 tosyl groups, those C-4 tosyl groups could be completely removed to release hydroxyl groups.

For above reasons the domain of this work mainly includes followings: (1) total activation of primary hydroxyl groups of inulin by chlorine or tosyl groups; (2) quantitative and specific nucleophilic substitution of the leaving groups at C-6 with azide ion; (3) transformation of azide to amino groups to give 6-amino-6-deoxyinulin;

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(4) when leaving groups were tosyl groups, few tosyl groups at C-4 should be totally removed to release C-4 hydroxyl groups. In addition, useful information was reported to increase the possibility of employing 6-amino-6-deoxyinulin as antifungal agents.

2. Materials and methods

2.1. Materials

Inulin was purchased from Wede Biological Corp. (Beijing. China) and was employed without further purification. Its average degree of polymerization was around 20 fructosyl fructose units. p-Toluenesulfonyl chloride, lithium chloride, ethylenediamine (Et_3N), methylsulfonyl chloride, sodium azide and triphenylphosphine (Ph_3P) were from the Sigma–Aldrich Chemical Co. The other reagents were analytical grade and were purified and dried by standard procedures. FT-IR spectrometers were recorded on a Jasco-4100 (Tokyo, Japan, provided by JASCO China (Shanghai), Co., Ltd., Shanghai, China) and the NMR was recorded on a Bruker AVIII-500 spectrometer (Fällanden, Switzerland, provided by Bruker BioSpin CN/Bruker (Beijing) Tech. and Serv. Co., Ltd., Beijing, China).

2.2. Synthesis

2.2.1. 6-Chloro-6-deoxyinulin (CDINL)

Inulin (5 g) was dissolved in anhydrous N,N-dimethylformamide (DMF) (50 mL) by stirring at 70 °C. To this homogenous solution, maintained at 70 °C, methylsulfonyl chloride (25 mL) was added dropwise over 15 min. After stirred at this temperature for 16 h, the mixture was concentrated to a syrup under reduced pressure. The syrup was dissolved in ethanol, treated with sodium ethoxide and the resulting suspension was stirred for 8 h at room temperature. The precipitate was collected, washed by ethanol, and freeze dried.

2.2.2. Tosylated inulin (TSINL)

Inulin (1.62 g, 10 mmol of fructose equivalents) and 0.63 g lithium chloride (dried at 100 °C overnight in vacuum) were dissolved in DMF at 70 °C with stirring under a nitrogen atmosphere until homogenous. The solution was cooled to 0 °C, and a 1.5-fold unit of Et_3N was added. Then a solution of p-toluenesulfonyl chloride (2.85 g) in DMF was added dropwise. After stirred at 0 °C for 36 h under nitrogen atmosphere, the reaction mixture was poured into 450 mL acetone and the tosylated inulin crystallized easily, which was filtered off and washed carefully with acetone. After dialyzed against deionized water for 3 days, it was freeze dried.

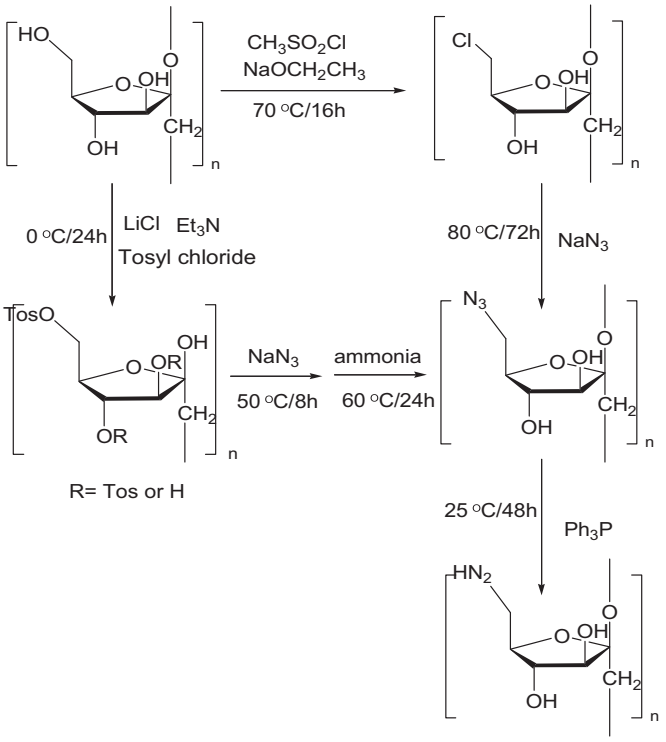
2.2.3. 6-Azido-6-deoxyinulin (AZDINL)

CDINL or TSINL (10 mmol of the furanose rings equivalents) was dissolved in 50 mL dimethysulfoxide (DMSO) at room temperature, and sodium azide (10 mmol) in 15 mL DMSO was added in. After stirred at the desired temperature for stated hours (Scheme 1), the mixture was cooled to room temperature and slowly poured to 400 mL acetone. The precipitate was collected, washed by ethanol and acetone, dialyzed against deionized water for 3 days and dried in vacuum.

When TSINL was starting material, the resulting product should be treated by 28% ammonia at 60 °C for 24 h to remove those few side C-4 tosyl groups.

2.2.4. 6-Amino-6-deoxyinulin

AZDINL (10 mmol of the furanose rings equivalents) and Ph_3P (20 mmol) were dissolved in 200 mL anhydrous DMSO and stirred for 24 h at room temperature. Water (1 mL) was added to the mixture and the solution was stirred for a further 24 h. The reaction mixture was poured into 450 mL acetone and the product was collected, washed 3 times by ethanol and acetone, and dried in vacuum.



Scheme 1. Synthetic pathway of 6-amino-6-deoxyinulin.

2.3. Antifungal ability assays

Two strains of pathogenic fungi associated with plant diseases were assembled for this study, which were *Cladosporium cucumerinum* (Ell.) et Arthur (ATCC 16402) and *Fusarium oxysporum* sp. *Cucumis sativus* L. (ATCC 7808). Prior to antifungal ability assays, the synthesized 6-amino-6-deoxyinulin was Soxhlet extracted with dichloromethane for 24 h to remove the probable remained Cl^-, Ts, N_3^- trace, otherwise which may influence the antifungal activity.

The test of fungistatic activity was carried out according to Jasso de Rodríguez (Jasso de Rodríguez, Hernández-Castillo, Rodríguez-García, & Angulo-Sánchez, 2005). Briefly, samples of inulin and 6-amino-6-deoxyinulin were dissolved in distilled water at a concentration of 5 mg/mL. Then, each samples were added to sterile petri dishes (9-cm diameter) containing sterilized potato dextrose agar (PDA) to give a final concentration at 50, 500 and 1000 μg/mL. The plates were inoculated with 5-mm-diameter plugs taken from the margins of 3 or 5 days old colonies of the stated fungi on PDA. Three replicates for each sample concentration were tested. Control plates were also inoculated with the fungi. When it came to control plates, identical volume distilled water substituted samples. All plates were incubated in the dark at 27 °C. The radial colony growth was measured when the mycelium of fungi reached the edges of the control plate. The antifungal index was calculated as follows:

$$\text{antifungal index}\ (\%) = \frac{D_b - D_a}{D_b} \times 100$$

where D_a is the diameter of the growth zone in the test plates and D_b is the diameter of the growth zone in the control plate. All data are expressed as means ± SD. Data were analyzed by an analysis of variance ($P < 0.05$) and the means were separated by Duncan's multiple range test. The results were processed by the computer programs: Excel and SPSS.

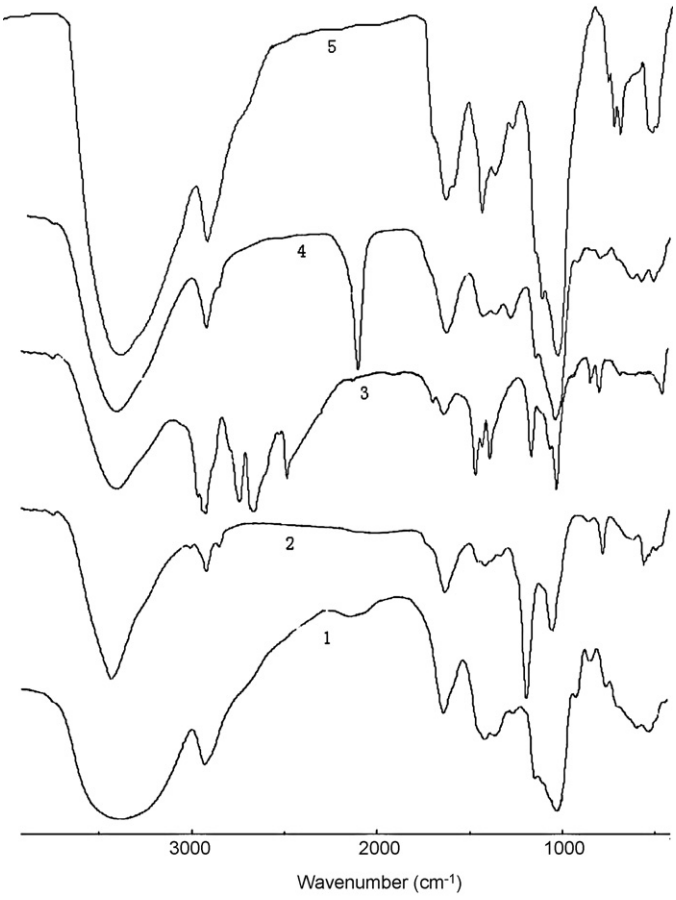


Fig. 1. FT-IR spectra of inulin (1), CDINL (2), TSINL (3), AZDINL(4) and 6-amino-6-deoxyinulin (5).

3. Results and discussion

3.1. Chemical syntheses and characterization

A reactive precursor of chemical manipulations of inulin, 6-amino-6-deoxyinulin, have been prepared, during which several modified intermediates were involved (Scheme 1). Each step of the syntheses was followed by FT-IR and ^{1}H NMR spectroscopy. The FT-IR of inulin, CDINL, TSINL, AZDINL and 6-amino-6-deoxyinulin is shown in Fig. 1 and ^{1}H NMR spectra of them are in Fig. 2. The FT-IR spectrum of inulin shows peaks of saccharide at 852 cm^{-1}, 1029 cm^{-1} and 3041 cm^{-1}. In CDINL spectra, new strong peaks at 786 cm^{-1} and 1199 cm^{-1} appeared compared that of inulin, which were assigned to the vibration of the CH_2-6-Cl (Cimecioglu, Ball, Kaplan, & Huang, 1994). Characteristic peaks of $\upsilon(SO_2)_{sym}$ and $\upsilon(SO_2)_{asym}$ were observed at 1172 cm^{-1} and 1396 cm^{-1} in TSINL spectra (Zhong et al., 2007). The obvious peak at 2105 cm^{-1} in AZDINL spectra is the typical absorption of C–N_3 and when C-6-azido was reduced to C-6-NH_2, a new sorption band at about 1600 cm^{-1} appeared and the strong band at 2105 cm^{-1} for C–N_3 disappeared (Cimecioglu et al., 1994). As marked in Fig. 2, the signals of protons in ^{1}H NMR spectra are well attributed to the structure of the inulin derivatives. Moreover, from the integral ratio of the protons at amino groups (1.8–1.9 ppm) and the furanose rings' protons (3.0–3.8 ppm) the degree of substitution of the 6-amino-6-deoxyinulin was calculated to be 100%. And this was further proved by the ^{13}C NMR of inulin and 6-amino-6-deoxyinulin. Fig. 3 gives the ^{13}C NMR of inulin and 6-amino-6-deoxyinulin. It is evident that the chemical shifts of ^{13}C NMR of inulin are all above 60.1, which is consistent with

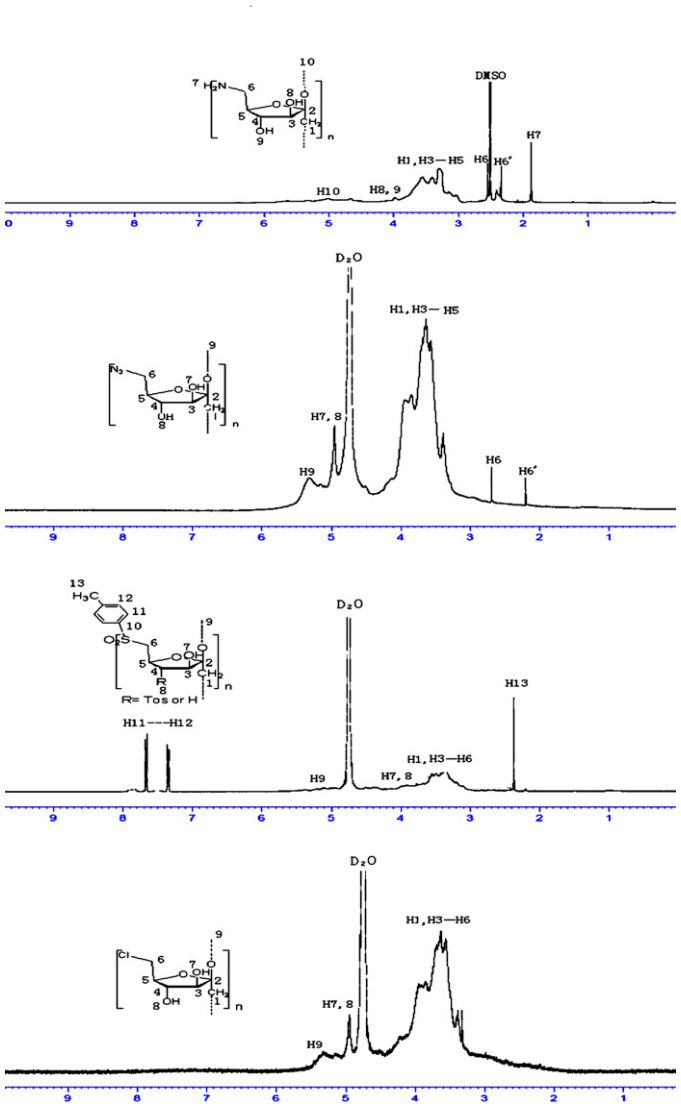


Fig. 2. ^{1}H NMR spectra of CDINL, TSINL, AZDINL and 6-amino-6-deoxyinulin.

the Rogge's report (Rogge & Stevens, 2004). In the ^{13}C NMR of the product, chemical shift for C-6-OH (60.1 ppm) disappears and new signal for C-6-NH_2 appears at 41.3 ppm. So, all C-6-OH groups of inulin were quantitatively transformed to C-6-NH_2. The results mentioned above evidently substantiated the obtainment of 6-amino-6-deoxyinulin.

Selective activations of hydroxyl groups of carbohydrates establish the rocks on which we could build useful derivatives accordingly (Evans et al., 1968). To our knowledge, however, the literature on direct C-6 activation of inulin is limited. Fortunately, there are examples for other polysaccharides. In this respect, we started the syntheses from activating the primary hydroxyls by chlorination or tosylation at high degree of substitution, respectively. Because of high degree of selectivity, chlorination at the primary carbon of inulin was taken as one of the initial activation strategies. Nevertheless, it took so long as to 72 h for the 6-chloro inulin to undergo nucleophilic substitution to give 6-azdio inulin derivative and to have a better leaving group than chlorine helping to shorten the reaction time, we synthesized tosylated inulin. With AZDINL in hand, we attempted to reduce azido groups to amino groups in an elegant way. Generally, azides are reduced to corresponding amides by lithium aluminium hydride or catalytic hydrogenation, which were also our initial choice. One of the main

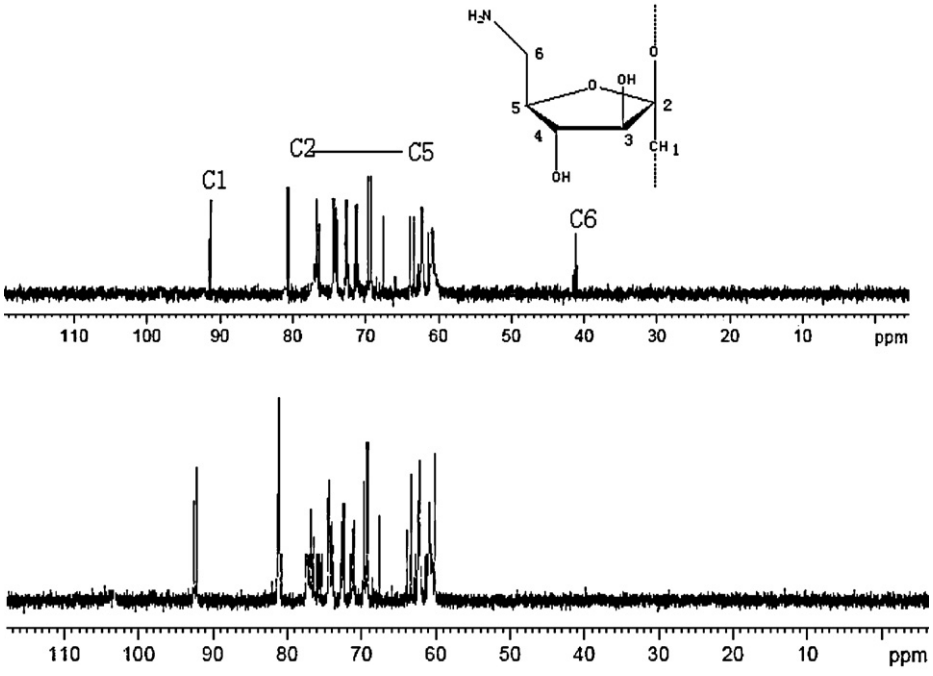


Fig. 3. ^{13}C NMR spectra of inulin and 6-amino-6-deoxyinulin, solvent: D_2O.

drawbacks in these reactions is their requiring of drastic conditions. So, we avoided the use of lithium aluminium hydride or catalytic hydrogenation and it is turned out that as proposed by Cimecioglu in 1994 (Cimecioglu et al., 1994), triphenylphosphine could perform the reaction efficiently.

3.2. Antifungal activity

The antifungal activities of inulin and 6-amino-6-deoxyinulin against *C. cucumerinum* (Ell.) et Arthur are shown in Fig. 4 and against *F. oxysporum* sp. *C. sativus* L. in Fig. 5. From these two figures we could conclude the results as follows: firstly, inulin does not inhibit the growth of those phytopathogen fungi at the tested concentrations. Secondly, compared with inulin, after the introduction of amino groups, 6-amino-6-deoxyinulin has evident antifungal activities against the tested phytopathogen fungi and the inhibitory indices of which against these two fungi were 60.1% and 53.3% at 1000 μg/mL, respectively. Thirdly, 6-amino-6-deoxyinulin exhibits concentration-dependent inhibitory effect on the hyphal growth of those two strains fungi. It is reasonable to propose that the obtained antifungal activities of 6-amino-6-deoxyinulin may benefit from amino groups on the inulin backbone. It has been reported that

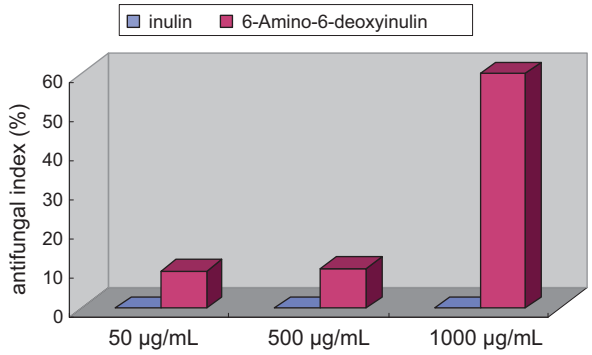


Fig. 4. The antifungal activity of inulin and 6-amino-6-deoxyinulin against *Cladosporium cucumerinum* (Ell.) et Arthur.

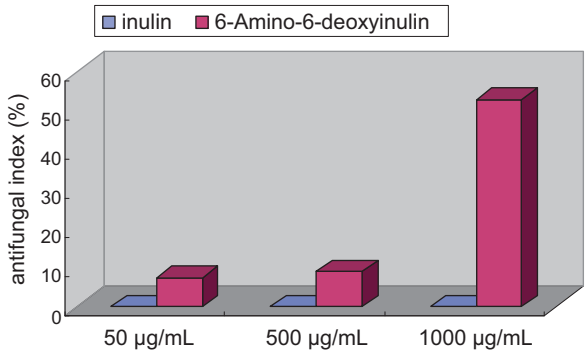


Fig. 5. The antifungal activity of inulin and 6-amino-6-deoxyinulin against *Fusarium oxysporum* sp. *Cucumis sativus* L.

amino groups of aminoglycosides may interact with anionic components of the cell wall, such as glucan, mannan, protein and lipid, which in turn leads to the forming of an impervious layer around the cell, which could prevent the transport of essential nutrients from entering the cell, such as glucose and may also disturb the cell wall thereby causing severe leakage of cell constituents and ultimately leading to cell death (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004).

4. Conclusion

In conclusion, the preparation of 6-amino-6-deoxyinulin, a kind of precursors for facile chemical modifications of inulin, has been established and the investigation of its potential antifungal activities against two strains phytopathogen fungi at series concentrations has also been reported in this paper. For the synthesis, we have thoroughly activated inulin at C-6-OH by chlorination or tosylation. Then, azide ion nucleophilic substituted the leaving groups at C-6 position of inulin. And finally, triphenylphosphine elegantly reduced the azido groups on inulin backbones to amino groups to give us 6-amino-6-deoxyinulin. With active amino groups, the product is a suitable precursor for facile chemical

manipulation of inulin. For the investigation of scavenging ability against hydroxyl radicals, the data obtained *in vitro* models clearly suggested the antifungal potency of the substances. The mechanism of the obtained antifungal activities was also discussed in this paper. This may be probably the results of the grafted NH_2 groups, which could interact with anionic components of the cell wall and influence the growth of the cell.

Acknowledgements

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