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DESIGN OF INULIN ACETATES WITH POTENTIAL ANTIMICROBIAL ACTIVITY

N. T. PETKOVA[1], Y. D. TUMBARSKI[2], I. I. IVANOV[1] & P. P. DENEV[1]

[1]Department of Organic Chemistry and Inorganic Chemistry, [2]Department of Microbiology; University of Food Technologies, Plovdiv, Bulgaria

Summary

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Inulin acetates with three different degrees of polymerisation (DP) were designed and their structure was proven by infrared spectroscopy and nuclear magnetic resonance. For the first time the influence of the length of the carbohydrate chain and the acetyl residues on the growth of different microorganisms were evaluated. Inulin acetates in concentration of 1 mg/mL inhibited the fungi *Aspergillus niger* and *Fusarium oxysporum*. The highest antifungal activity was demonstrated by the short-chain inulin acetates (DP=7–9) in concentration of 5 mg/mL against the fungus *Beauveria bassiana*. All tested inulin acetates were inactive against Gram-positive and Gram-negative bacteria, but stimulated the growth of *Bacillus methylotrophicus* BM47. These results revealed the potential application of inulin acetates in pharmacy, agriculture and food technology.

Key words: antimicrobial activity, inulin acetates, NMR spectra

INTRODUCTION

Inulin is a natural storage polysaccharide from fructan family (Stevens *et al.*, 2001). The presence of free hydroxyl groups in inulin reveals their possible modification by converting to ester or carboxyl groups. The potential use of inulin acetates as drug carriers and vaccine adjuvant has been recently published (Poulain *et al*., 2003; Starbird *et al.*, 2007; Kumar *et al*., 2016). Therefore, the acetylation of inulin presents a modern concept in design of new pharmaceutical formulations. The advantages of the inulin acetates over some biodegradable polymers include hydrolysis of ester bonds to inulin and acetic acids without enzymatic (Wu & Lee, 2000) and biodegradation of inulin acetates by inulinase (Damian *et al*., 1999).

At present there are no enough scientific data for the antimicrobial activity of inulin acetates and their application in the field of medicine, veterinary medicine and agriculture. Therefore, the aim of the present study was to evaluate the antimicrobial potential of newly designed inulin acetates against some pathogenic microorganisms.

MATERIAL AND METHODS

Reagents

Chicory inulin with different degree of polymerisation (DP) – short-chain inulin

fructooligosacharides (FOSs) – Frutafit CLR (DP=7–9), medium-chain Frutafit HD (DP=9–12) and long-chain Frutafit TEX (DP=22) were purchased from Sensus (Roosendaal, the Netherlands). Frutafit CLR and Frutafit HD contain 7–9 and 9–12 fructose monomers, respectively. Frutafit TEX is characterised with DP = 22. All reagents were of analytical grade.

Synthesis of inulin acetates

Inulin acetates were synthesised by the reaction of 10.0 g inulin with 30 mL acetic anhydride, 3.0 g sodium acetate as a catalyst under direct heating upon boiling for 60 min. Then the reaction mixture was poured into a 200 mL water-ice mixture, stirred vigorously and left at –18 °C for a night. Inulin acetates were precipitated in an excess of cold water as a white solid, filtered and then washed again with cold water. The acetyl ester was recrystallized by 95% ethanol and re-precipitation with water, and then was dried in a vacuum-oven to the constant weight.

Melting point and water activity

The melting point of the samples was determined with an apparatus BŰCHI 510 (Germany). The water activity (a_w) was measured by AquaLab Pre (Labcell Ltd., UK).

Fourier transformed infrared spectroscopy (FTIR)

The infrared spectra were recorded on a Nicolet FTIR Avatar Nicolet (Thermo Science, USA) spectrometer using KBr pellets with 132 scan and the absorption was reported in wavenumbers (cm^{-1}) in the range of 4000–400 cm^{-1}. Degree of acetylation of inulin acetates was calculated by relative intensity of IR bands at 1745 cm^{-1} divided to $_{C=O}$/1020 $cm^{-1}{}_{C-O}$ (Starbird *et al.,* 2007).

Nuclear magnetic resonance (NMR)

^{13}C NMR spectra were recorded on a Bruker Advance III 500 MHz spectrometer using $CDCl_3$ as a solvent. All chemical shifts were reported in ppm with reference to TMS.

Test microorganisms

Nine test microorganisms including Gram-positive bacteria (*Bacillus subtilis* 46/H1, *Bacillus methylotrophicus* BM47), Gram-negative bacteria (*Escherichia coli* ATCC8739 and *Salmonella abony*), yeasts (*Candida tropicalis* and *Saccharomyces cerevisiae*) and fungi (*Aspergillus niger, Fusarium oxysporum* and *Beauveria bassiana)* from the collection of the Department of Microbiology at University of Food Technologies, Plovdiv, Bulgaria, were used.

Culture media

LBG agar medium was used for cultivation of the test bacteria and yeasts. LBG medium (10 g tryptone, 5 g yeast extract, 10 g NaCl and 10 g glucose dissolved in 1 L of deionized water) was prepared, the final pH was adjusted to 7.5 and then 15 g/L agar before autoclaving (for 20 min at 121 °C) was added.

Malt extract agar (MEA). This medium was used for cultivation of the test fungi. Ingredients per 1 L of deionized water were 20 g malt extract, 20 g dextrose, 6 g peptone and 15 g agar. The final pH was corrected to 5.5 and the medium was autoclaved for 15 min at 121 °C.

Antimicrobial assay

The antimicrobial activity of inulin acetates was determined by using the conventional agar-well diffusion method (Tumbarski et *al*., 2017). The test bacteria (except for *B. subtilis* 46/H1 and *B. methylotrophicus* BM47) and yeasts were cul-

tured on LBG agar medium at 37 ºC for 24 h. *B. subtilis* 46/H1 and *B. methylotrophicus* BM47 were cultured on LBG agar medium at 30 ºC for 24 h. The test fungi were grown on MEA at 30 ºC for 7 days or until sporulation. The inocula of bacteria/yeasts were prepared by homogenization of a small amount of biomass in 5 mL of sterile 0.5% NaCl. The inocula of fungi were prepared by addition of 5 mL of sterile 0.5% NaCl into the tubes. After vigorous shaking they were filtered and replaced in another tubes before use. The number of viable cells and fungal spores was determined by Thoma's haemocytometer. Their final concentrations were adjusted to 1.0×10^8 cfu/mL (for bacterial/yeasts cells) and 1.0×10^5 cfu/mL (for fungal spores) and then inoculated in preliminarily melted and tempered at 45–48 ºC LBG agar media. The inoculated media were transferred in quantity of 20 mL in sterile Petri dishes (d=10 cm) and allowed to solidify. Then six wells (d=6 mm) per dish were cut.

Inulin acetates were dissolved in aqueous methanol (80%) and pipetted in quantity of 60 μL into the agar wells in two replicates. As controls 80% methanol as well as the antibiotics Ampicillin (10 μg/mL) and Nystatin (40 μg/mL) were used. The inoculated Petri dishes were incubated at 37 ºC (for test bacteria/yeasts) and at 30 ºC (for *B. subtilis* 46/H1, *B. methylotrophicus* BM47 and fungi). The antimicrobial activity was determined by measuring the diameter of the inhibition zones around the wells at the 24^{th} and 48^{th} h of incubation. Sensitive were the microorganisms with inhibition zones of 18 mm or more; moderately sensitive – with inhibition zones from 12 to 18 mm; resistant – with inhibition zones up to 12 mm or completely missing.

RESULTS

The designed inulin acetates were odorless white powders with bitter taste, soluble in acetone, DMSO, 95% ethanol, methanol and insoluble in water. The highest yield was obtained for medium-chain inulin acetates – 80%. The hydrophobic character can be explained with the substitution of free OH groups with acetyl residues. The introduction of new acetyl groups in inulin chain led to new physicochemical properties. The lower melting temperatures (below 80 ºC) and lower water activity were observed for inulin esters. The degree of acetylation was in range from 2.2 to 3.0 and these esters were characterised as highly acetylated (Table 1).

The appearance of new functional groups in inulin chain was confirmed by infrared spectroscopy (Fig. 1). Inulin acetates showed strong new bands at 1745 cm^{-1} attributed to stretching vibration of C=O groups from ester, 1370 cm^{-1} bending of C–H and 1220 cm^{-1} due to stretching vibration of C–O. These three typical bands were due to ester bonds of acetyl residues to the inulin chains. The strong bands at 3335 cm^{-1} typical for the stretching vibrations of OH groups decreased significantly in the inulin acetates spectra. The presence of bands at 817 cm^{-1} in both spectra proved that resulting ester contained β-D-fructose residues linked 1→2 glycoside bonds.

The structure of inulin acetates was additionally confirmed by the ^{13}C NMR spectrum.^{13}C NMR of medium-chain inulin acetates (DP = 9–12) contained shifts as follow: δ 170.15 (t, J = 50.1 Hz), 103.73 (s), 77.53 (d, J = 41.6 Hz), 77.33 (s), 77.07 (s), 76.82 (s), 75.79 (s), 75.53 – 74.62 (m), 63.84 (s), 62.66 (s), 21.64 – 21.29 (m), 20.73 (d, J = 12.9 Hz), 20.65

(t, J = 16.5 Hz), 20.43 (s). The long-chain inulin acetates contained the chemical shifts: δ 170.67, 170.09, 169.78, 103.73, 77.69, 77.30, 77.05, 76.79, 75.81, 75.51, 63.84, 62.69, 20.79, 20.66 and 20.43. Three chemical shifts characteristic for acetyl carbonyl was appeared at *δ* 170.15. All the methyl carbons from the acetyl residue appeared at *δ* 20.5 (3×CO**C**H_3). Carbon atoms of the inulin moiety were found in the range of 62.66~103.73 ppm.

The results from the antimicrobial screening showed that all of the inulin acetates in concentration of 1 mg/mL and 5 mg/mL possessed low inhibitory effect on the plant pathogenic fungi *Asp. niger* and *Fus. oxysporum*. The highest antifungal activity was demonstrated by the short-chain inulin acetates (DP = 7–9) in concentration of 5 mg/mL against the fungus *B. bassiana*. In contrast, the other inulin acetates did not affect the mycelial growth of the same test microorganism. The yeasts *C. tropicalis* remained unaffected by both concentrations of the tested inulin acetates. However, the medium-chain inulin acetates (DP=9–12) in both concentrations inhibited the growth of *S. cerevisiae*, while the rest of the inulin acetates did not show antimicrobial activity. No inhibition of the Gram-positive and Gram-negative bacteria was detected. Applied as a control, the solvent (80% methanol) did not show antimicrobial activity. Nystatin showed activity only against the fungus *Asp. niger*, while ampicillin inhibited the growth of *E. coli* ATCC 8739 and *B. subtilis* 46/H1 (Table 2).

DISCUSSION

The inulin acetates in the current study were synthesised without solvents. The main advantage was that toxic solvents, as DMF or pyridine were not used in comparison with other studies (Damian *et al*., 1999; Wu & Lee, 2000). The melting points of inulin acetates in range of 87-92 °C were also reported (Wu & Lee, 2000; Poulain *et al*., 2003). The lower water activity of inulin acetates suggests antimicrobial effect. The structure of inulin acetates was characterised with similar FTIR and NMR spectra (Damian *et al*., 1999; Poulain *et al*., 2003; Starbird *et al.*, 2007).

To the best of our knowledge, this is the first scientific report which presents data for antimicrobial activity of synthesized inulin acetates against different pathogenic microorganisms. Moreover, a relationship between the structure (length of the chain) of inulin acetates and their antimicrobial activity was established. The short- and medium-chain inulin acetates possessed a stronger antimicrobial effect than the long-chain inulin acetates. This could be explained with the more difficult diffusion of the long-chain inulin acetates in the agar medium.

A promising antimicrobial activity of undecylenate and laurinate esters of inulin and sucrose was also demonstrated (Petkova *et al.*, 2016; Vassilev *et al*., 2016). No inhibition against Gram-positive and Gram-negative bacteria was observed a well as in the current investigation with inulin acetates. Important finding in the current study was the stimulation of growth of *Bacillus methylotrophicus* strain BM47. The probable reason was that the inulin acetates provided a substrate for its growth.

The antifungal activity of inulin acetates reveals a new aspect of their potential application as bioproducts against some plant pathogens. The inulin acetates with antifungal activity could be successfully used in agriculture for development of new environment-friendly formulations

for plant protection, in order to avoid the use of chemical pesticides. The increasingly proven harm from the chemical pesticides resulted in a growing interest in considering the compounds of biological origin as alternative plant-protecting agents against a broad spectrum of microorganisms. An additional investigation will be needed in this field of application of inulin acetates and this could be object of future experimental work.

CONCLUSION

Inulin acetates with different length of carbohydrate chain were designed and a screening for *in vitro* antimicrobial activity was performed. The synthesised inulin acetates with their antifungal activity could find wide and successful application in the field of agriculture as a mean for plant protection and biocontrol. Their potential use as alternatives of conventional chemical pesticides could prevent the pollution of the environment and thus to protect animal and human health.

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