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New structural insights into the oligosaccharide phosphate fraction of *Pichia* (*Hansenula*) *holstii* NRRL Y2448 phosphomannan

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ABSTRACT

The oligosaccharide phosphate fraction (OPF) obtained from mild acid hydrolysis of *P. holstii* NRRL Y-2448 phosphomannan is the starting material for the preparation of the Phase III anticancer drug candidate PI-88. The OPF was for the first time successfully separated by preparative ion exchange chromatography and the major oligosaccharides isolated and characterized by NMR spectroscopy. The components were also acetylated and subjected to LC-MS analysis. These studies revealed that the OPF also contained all-α(1 → 3)-linked oligosaccharides in addition to the known α(1 → 3)/(1 → 2)-linked species, most likely formed by hydrolysis of the latter. Contrary to previous assumptions, the only phosphorylated disaccharide present is α(1 → 3)-linked. In addition, it was determined that a glycosylamine derivative previously isolated is, in fact, a manufacturing byproduct formed from exposure to aqueous ammonium bicarbonate during chromatographic purification. Based on these findings a new generic structure for PI-88 is proposed which more accurately reflects its composition.

1. Introduction

The exopolysaccharide produced by the yeast *Pichia (Hansenula) holstii* NRRL Y-2448 when grown in a culture medium containing excess orthophosphate, is made up of side-chains of repeating phosphorylated oligosaccharides attached to a highly branched phosphomannan core [1]. Mild hydrolytic cleavage of the glycosyl phosphate linkages in the side chains releases the low molecular weight oligosaccharide phosphate fraction (OPF), which may account for 90% of the total polysaccharide [2]. The OPF has been a useful tool to study Man-6P receptors [3–5]. The OPF is also the synthetic precursor [6,7] of the anticancer drug candidate PI-88 [8,9] (also known as muparfostat), which was recently in Phase III clinical trials for post-resection hepatocellular carcinoma [10–12].

The phosphorylated side chains that make up the OPF have been intensively investigated [2,6,13]. The major component (~60%) is the α(1 → 3)/(1 → 2)-linked pentasaccharide phosphate **3** (Fig. 1), which has been identified by NMR and MS [2]. The tetrasaccharide phosphate **5** accounts for ~30% and the remainder is made up of hexa-, tri- and disaccharide phosphates. The isolation of the individual oligosaccharide phosphates of the OPF, including **3**, has proven to be elusive by size exclusion chromatography (SEC) and until this study none had been isolated in pure form. The linkage patterns of the minor components were thus inferred from the structures of the neutral oligosaccharides, obtained by hydrolysis of phosphomannan with aqueous HF [2] or by enzymatic dephosphorylation of OPF [2,13], which were isolated and characterized by NMR spectroscopy. HPLC evidence suggests that the neutral α(1 → 2)-linked disaccharide **10** constitutes the major non-reducing end side chain-terminating unit which is not phosphorylated *in vivo* [6].

In an earlier study [13], coelution of the dephosphorylated OPF with the authentic neutral oligosaccharide samples on HPLC analysis provided evidence for the linkage patterns of the parent phosphosugars. However, the HPLC conditions employed were unsuitable for complete resolution of the components. Capillary electrophoresis (CE) analysis of the OPF (Fig. 2) yielded peaks broadly corresponding to six phosphorylated oligosaccharides which were assigned the structures **1**, **3**, **5**, **8**, **9** and **15** [13].

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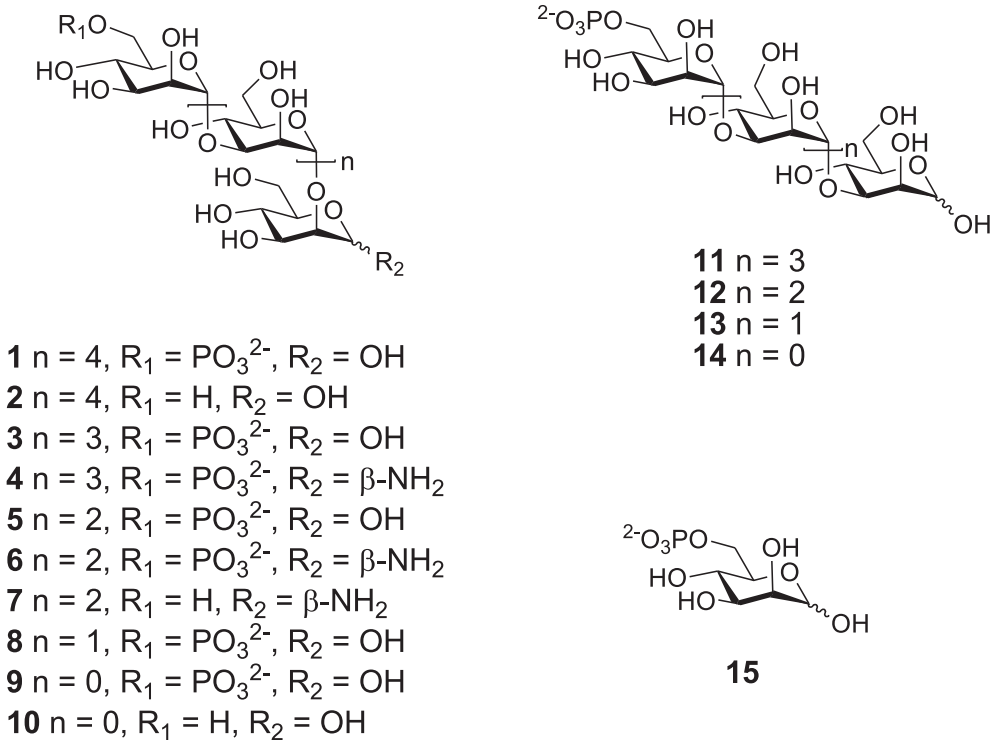

Fig. 1. Oligosaccharide structures.

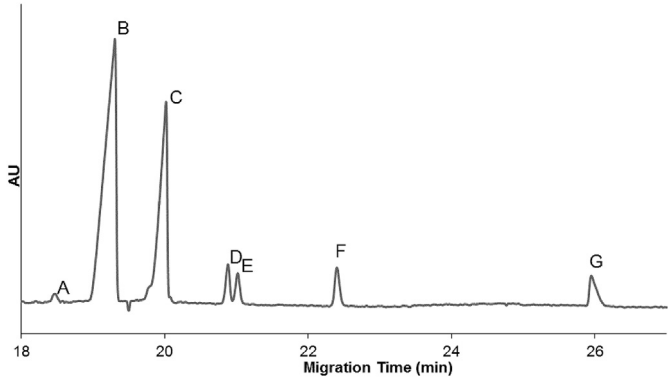

Fig. 2. Electropherogram from CE analysis of OPF, reproduced with permission from Ferro et al. [13].

However, the trisaccharide peak was split into two peaks (D and E, Fig. 2), and the shoulder on the leading edge of peak C is suggestive of partial resolution of another unknown component. Repeated chromatographic purification (SEC) of the neutral oligosaccharide fraction resulted in the isolation of tetrasaccharylamine **7**, as its peracetylated derivative, along with the neutral hexasaccharide **2**. No other glycosylamine components were observed in that study, suggesting that tetrasaccharylamine **7** was a real component and not an artifact from chromatography with aqueous ammonium bicarbonate eluants. It was thus speculated that peaks D and E were either trisaccharide **8** or tetrasaccharylamine **6**.

The previous studies provided strong evidence for the structures of the major components of the OPF, however, the structures of the minor components were not conclusively established. Given the importance of the OPF for the manufacture of PI-88, the current study sought to more firmly establish the structures of the minor components, to further investigate the presence of the glycosylamines, and to attempt to obtain individual oligosaccharide phosphates in sufficient quantity for characterization and further study.

2. Results and discussion

2.1. Preparative HPLC purification of major OPF components

Previous attempts at OPF fractionation using SEC or ion exchange chromatography with detection by refractive index failed to resolve the components. Preliminary analytical weak anion exchange HPLC analysis of the OPF utilising gradient elution coupled with detection by evaporative light scattering (ELS) indicated that some separation of the components was possible. The OPF was thus fractionated on a preparative scale by HPLC on a DEAE column eluted with a gradient of aqueous ammonium acetate, which was employed instead of ammonium bicarbonate in order to minimize the formation of glycosylamines. The chromatographic resolution observed was preserved on scales of at least 1.5 g of OPF per injection. On this basis, approximately 64 g of OPF was roughly fractionated using the gradient elution, and enriched fractions of each oligosaccharide chain length were obtained. These were rechromatographed using smaller injection sizes to improve resolution, and employing isocratic mobile phase conditions. A representative chromatogram obtained using the optimised gradient conditions is given in Fig. 3, which features seven peaks labelled **A-G**. The seven resolved components were analysed using direct infusion mass spectrometry in negative ion mode, and the identities of these peaks are reported in Table 1. The purity of each repurified fraction was determined by CE and this indicated that the penta-, tetra- and disaccharide components (94%, 93% and 80% pure, respectively) contained one dominant component. However, the trisaccharide fraction (peak D) showed two major components in an approximately 2.4:1 ratio.

Peak A in Fig. 3, initially assumed to correspond to the