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Synthesis of 2-deoxy-α-D-*arabino*-hexopyranosyl phosphate and 2-deoxy-maltooligosaccharides with phosphorylase

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Abstract

A convenient one-step synthesis of 2-deoxy-α-D-*arabino*-hexopyranosyl phosphate on a millimolar scale is described by reaction of potato phosphorylase with D-glucal at equimolar phosphate concentration. Furthermore, in the presence of catalytic amounts of phosphate, a 2-deoxy-maltooligosaccharide is obtained from maltotetraose and D-glucal. The water-insoluble oligosaccharide was isolated and characterized by ^{1}H and ^{13}C NMR spectroscopy. An average dp of 20 was thus determined.

Key words: Enzymatic synthesis; Phosphorylase; 2-Deoxy-α-D-*arabino*-hexopyranosyl phosphate; 2-Deoxyamylose

1. Introduction

Glycosyl phosphates are of considerable interest as intermediates for the synthesis of nucleotide sugars which in turn are substrates for glycosyl transferases and participate in the synthesis of oligosaccharide structures in glycoproteins and glycolipids. In particular, α-D-glucopyranosyl phosphate is a substrate for various phosphorylases [1,2]. In the course of investigations of glycogen phosphorylase, several deoxy and deoxyfluoro derivatives of glucosyl phosphate have been synthesized [3] and tested as substrates or inhibitors. In order to obtain information

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about the reaction mechanism, the enzyme was crystallized with an inhibitor bound to the active site simulating the proposed enzyme–substrate intermediate.

Several methods for the preparation of glycosyl phosphates have been introduced: melting of per-*O*-acetylated sugars with phosphoric acid [4], phosphorylation of protected sugars with diphenyl phosphorochloridate and 4-dimethylaminopyridine [5] or with a phosphoramidite [6] via the phosphite intermediate, and glycosylation employing trichloroacetimidates [7]. These methods were also applied to the preparation of 3-, 4-, and 6-deoxyglycosyl phosphates [8,9]; however, 2-deoxyglycosyl phosphates were too labile, due to the lack of inductive effect from the 2-hydroxyl group, and could not be isolated. Recently, a glycosylation with *S*-(2-deoxyhexosyl) dithiophosphates was described [10], which among other compounds gave dibenzyl (tri-*O*-acetyl-2-deoxy-α-D-*arabino*-hexopyranosyl) phosphate.

In these cases the product still carries protecting groups, which have to be removed. In an enzymic process, on the other hand, the free sugar can be obtained directly. Percival and Withers prepared 2-deoxy-α-D-*arabino*-hexopyranosyl phosphate ('2-deoxyglucosyl phosphate') in a two-step synthesis [11], which required a nucleotide sugar and rather expensive enzymes. This is now achieved in one step and with a cheaper method. Klein et al. had previously reported on the reaction of phosphorylase with D-glucal [12]. During their investigation of a reaction mechanism for this process, which was later suggested by the same group [13], they proved the formation of 2-deoxyglucosyl phosphate from radioactively labelled D-glucal on an analytical scale and by NMR measurements of the reaction mixture. Also a precipitate of oligosaccharides was observed, but no products have been isolated or characterized; yields were only estimated from the incorporation of radioactivity. In this paper, reaction conditions are described which allow the synthesis of each of the two products on a preparative scale.

The synthesis of 2-deoxyamylose derivatives is of interest for studies of enzymic action and for structural investigations. Previously, the partial chemical deoxygenation of amylose at the 6-position has been described [14,15]. Withers reported the enzymic chain elongation of glycogen with 3- and 4-deoxyglucosyl phosphate; however, only an average of up to 1.5 units were transferred [16]. The 2-deoxyamylose prepared from D-glucal contains a long chain of 10 to 20 2-deoxyglucose residues attached to the maltotetraose.

2. Results and discussion

The phosphorylase reaction is dependent on a primer with at least four glucosyl units [17]. Therefore, maltotetraose was used as a primer in the oligosaccharide synthesis in order to keep the glucosyl portion as small as possible (Fig. 1). After one day of incubation of D-glucal and the tetrasaccharide with phosphorylase and 0.1 equiv of inorganic phosphate, a white polysaccharide began to settle. After six days, no tetrasaccharide could be detected by TLC and the precipitate was isolated by centrifugation and washed several times with water. In addition, small amounts of 2-deoxy-α-D-*arabino*-hexopyranosyl phosphate were formed and remained in the supernatant phase.

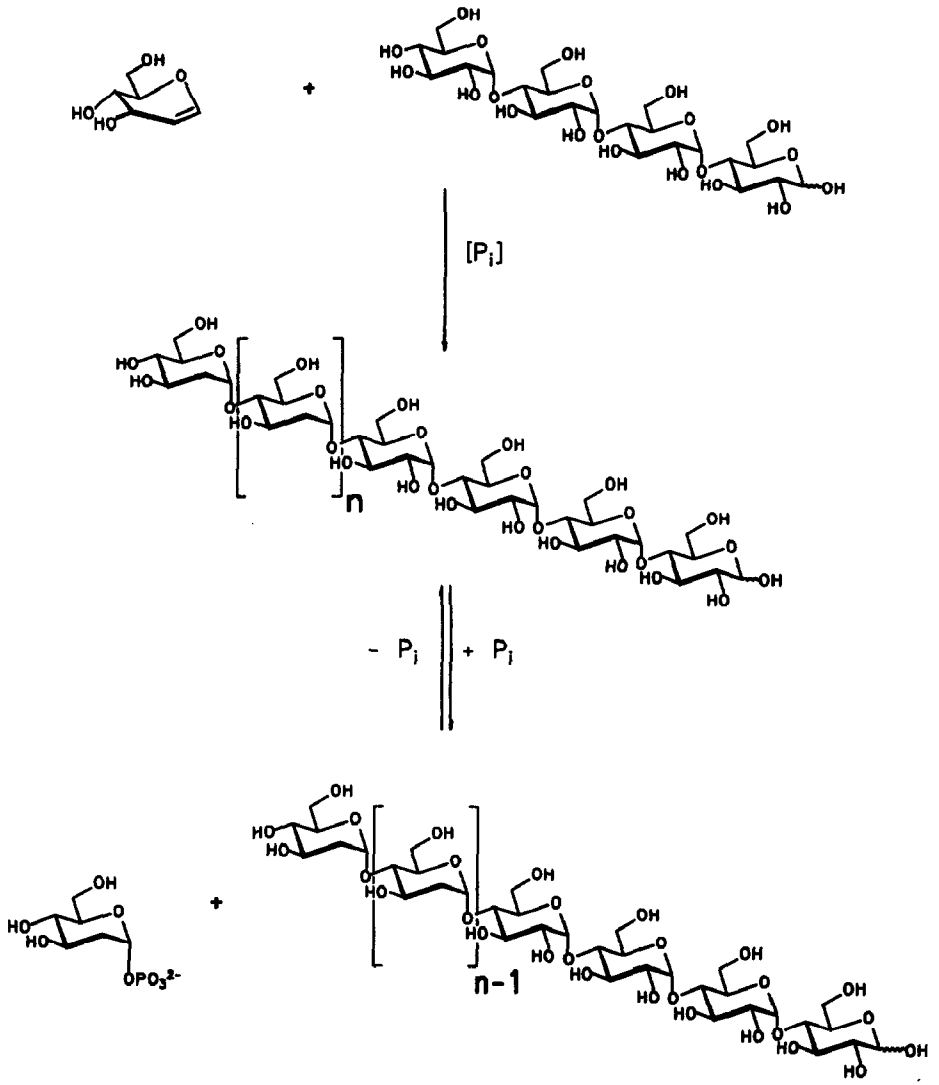

Fig. 1. Phosphorolytic formation and degradation of modified maltooligosaccharides containing 2-deoxy-D-*arabino*-hexopyranosyl residues.

The polysaccharide was insoluble in water, poorly soluble in dimethyl sulfoxide, but easily soluble in 0.5 M sodium hydroxide. In a qualitative analysis, the precipitate was hydrolyzed in dilute hydrochloric acid (pH 3) and 2-deoxy-D-*arabino*-hexopyranose was isolated as the main product.

The average dp was determined by integration of the NMR peaks. NMR spectra were recorded in Me_2SO-d_6; for the ^1H-spectra a trace of D_2O was added to suppress the OH-signals. By comparison with a maltotetraose spectrum in the same solvent, the peak at 5.02 ppm could be assigned to H-1 of the glycosidic

bonds of the tetraose unit; the doublets at 4.92 and 4.33 ppm correspond to H-1 of the reducing end in the α and β configuration, respectively. At 5.40 ppm, a large signal of H-1 of 2-deoxy-α-D-*arabino*-hexose appeared. In the ^{13}C NMR spectrum, only signals of the 2-deoxy-α-D-glucosyl residues can be seen due to the low solubility of the material in Me_2SO and because only a relatively small portion of the molecule consists of glucosyl residues.

The chain length distribution could be assigned following separation of the per-*O*-methylated oligosaccharides by HPLC on an RP-18 column. Further methods for a more detailed analysis of this novel class of polymers in which the 2-deoxyglycosidic bond is hydrolyzed much more facilely than the normal glycosidic bond have been developed and are described elsewhere [18].

Independent of the ratio glucal:primer, the average dp was always in the range of 20 to 22. Obviously the polysaccharide became insoluble at a certain chain length and was then withdrawn from the enzymic glycosylation process.

The 2-deoxy-α-D-*arabino*-hexopyranosyl phosphate was formed in a reverse reaction by phosphorolysis of the polysaccharide. A direct phosphorylation of the glucal without primer could be excluded, since a portion of the enzyme was purified until it was free of endogenous primer. Then only after addition of a primer was formation of 2-deoxyglucosyl phosphate observed. These results are consistent with those of Klein et al. [12]. For the large scale production of the 2-deoxyglucosyl phosphate, an enzyme extract which has not been completely purified is sufficient, a fact that facilitates the synthesis considerably.

Instead of the rather expensive maltotetraose, soluble starch can be used as a primer. With an excess of inorganic phosphate, α-D-glucopyranosyl phosphate was formed in small amounts and could not be separated from 2-deoxyglucosyl phosphate. However, using one equiv of phosphate, only 2-deoxyglucosyl phosphate was obtained. It can be supposed that the reaction with D-glucal is faster than the phosphorolytic degradation of starch. Alternatively, the phosphorolysis of 2-deoxyamylose could be faster than that of amylose. Under these conditions no polysaccharide precipitates. Thus, 1 mmol of the 2-deoxyglucosyl phosphate was produced in 50% yield. If the reisolated glucal is considered the yield would correspond to 81%.

In contrast to previous reports the product turned out to be relatively stable in solution. Even after heating the mixture to 100°C for 10 min in order to denature the protein, 2-deoxyglucosyl phosphate could be isolated. Nevertheless, to avoid any loss of product, in subsequent experiments protein was removed by ultrafiltration. The sugar phosphate could then be isolated as the sodium salt from an ion-exchange column and desalted by gel chromatography. The pure product, however, proved to be rather unstable even under an argon atmosphere. Therefore, no correct elemental analysis could be obtained.

It can be concluded that the reaction is controlled via the phosphate concentration to favour one of the two products from the same substrates and enzyme. Interestingly, the phosphorylase accepts an amylose consisting completely of 2-deoxy-α-D-*arabino*-hexosyl residues in the binding region of the enzyme. So far, only amylose derivatives with a small degree of substitution could be used as substrates

[14]. In the case of 2-deoxyoligomers no hydrogen bonds with a 2-OH group can exist. For maltooligosaccharides and amylose the formation of O-2–O-3′ hydrogen bonds has been demonstrated in the polyiodide complex [19] and when bound to an allosteric site of phosphorylase. The same interactions are proposed for amylose in aqueous solution [20]. It has now been shown that a secondary structure with O-2–O-3′ hydrogen bonds is not essential for the recognition of the enzyme.

3. Experimental

General methods.—Maltotetraose, soluble starch, and tri-*O*-acetyl-D-glucal were purchased from Merck, Darmstadt. *N*-Morpholinepropanesulfonic acid (MOPS) was from Serva. TLC was performed on Silica Gel 60 F_{254} from Merck in 2:2:1 2-propanol–acetone–1 M lactic acid with detection by spraying with 10% H_2SO_4 in EtOH and charring. The buffer solution contained 20 mM MOPS, 20 mM 2-mercaptoethanol, 10 mM sodium tartrate, 3 mM EDTA, and 0.1% sodium azide, pH 6.8. Ultrafiltration was performed in a stirred ultrafiltration cell (Amicon 8050) with a Diaflo YM-10 membrane (10 000 MW cutoff).

Phosphorylase preparation.—Glycogen phosphorylase was prepared from potato juice by solid ammonium sulphate (SAS) precipitation according to the method of Ziegast and Pfannemüller [21]. The crude enzyme after the second SAS precipitation was dialyzed three times against buffer (20 mM Tris, 20 mM NaCl, 15 mM EDTA, 0.1% NaN_3, pH 6.9), thus improving the activity from 0.21 to 1.32 U/mg protein. A small portion of enzyme was completely freed of primer by incubation with alpha-amylase and subsequent FPLC on Fractogel EMD TMAE-650 (S) (1 × 15 cm). The column was equilibrated with 5 mM Tris, pH 8.4 and eluted with a gradient of 0 to 0.4 M NaCl in the same buffer. Phosphorylase is reported to be stable in a pH range of 5.9 to 8.7 for at least 17 h [22]. Eluted protein was detected by UV-absorption and phosphorylase activity measurements.

Phosphorylase assay.—Phosphorylase activity was measured by determination of the inorganic phosphate liberated from D-glucopyranosyl phosphate during the formation of polysaccharides. The photometric phosphate assay is based on the method of Fiske and Subbarow [23].

2-Deoxy-α-D-arabino-hexopyranosyl phosphate, disodium salt.—D-Glucal (290 mg, 1.98 mmol), KH_2PO_4 (260 mg, 1.91 mmol), and starch (60 mg) were dissolved in buffer (20 mL) and incubated with potato phosphorylase (3 mL, 53 U) for six days at 30°C. Then protein was removed by ultrafiltration, and the filtrate was lyophilized and desalted on Fractogel TSK-HW-40 S (2 × 100 cm). The product was isolated as the disodium salt from an ion-exchange column (Fractogel TSK-DEAE 650 S, 3 × 30 cm) with gradient elution of 0–0.2 M $NaHCO_3$, and desalted again by gel filtration. 2-Deoxy-α-D-*arabino*-hexopyranosyl phosphate (285 mg, 0.99 mmol; 50%) was obtained as a white powder [D-glucal (112 mg, 0.76 mmol; 39%) was reisolated; based on reacted D-glucal the yield was 81%]; $[\alpha]_D^{20}$ + 59° (*c* 0.55, H_2O); ^1H NMR data (400 MHz, D_2O): δ 5.42 (ddd, 1 H, $J_{1,P}$ 7.5, $J_{1,2ax}$ 1.5, $J_{1,2eq}$ 1.0 Hz, H-1), 3.90 (ddd, 1 H, $J_{2ax,3}$ 11.5, $J_{3,4}$ 9.0, $J_{2eq,3}$ 5.0 Hz, H-3), 3.76 (m, 2 H, H-5, H-6a), 3.62

(dd, 1 H, $J_{6a,6b}$ 12.5, $J_{5,6b}$ 6.0 Hz, H-6b), 3.22 (dd, 1 H, $J_{4,5}$ 10.0, $J_{3,4}$ 9.0 Hz, H-4), 2.09 (ddd, 1 H, $J_{2ax,2eq}$ 12.7, $J_{2eq,3}$ 5.0, $J_{1,2eq}$ 1.0 Hz, H-2*eq*), 1.56 (dddd, 1 H, $J_{2ax,2eq}$ 12.7, $J_{2ax,3}$ 11.5, $J_{2ax,P}$ 3.5, $J_{1,2ax}$ 1.5 Hz, H-2*ax*); ^{13}C NMR (100 MHz, D_2O): δ 93.14 (C-1), 73.03, 71.67, 68.49 (C-3, C-4, C-5), 61.31 (C-6), 38.57 (d, $^3J_{2,P}$ 6.1 Hz, C-2).

2-Deoxy-maltooligosaccharides.—D-Glucal (1.0 g, 6.8 mmol), KH_2PO_4 (88 mg, 0.65 mmol), and maltotetraose (70 mg, 0.1 mmol) in buffer (30 mL) were incubated for four days at 30°C with potato phosphorylase (3 mL, 53 U). After centrifugation, the precipitate was washed with water three times and then lyophilized to yield 144 mg of oligosaccharides as a white powder. The average dp determined by integration of the H-1 signals of the NMR spectrum was 20.7 which corresponds to a yield of 46.4% with respect to the maltotetraose employed. ^{1}H NMR data (400 MHz, Me_2SO-d_6): δ 5.40 (m, 16.7 H, H-1 of 2-deoxy-D-*arabino*-hexopyranose), 5.02 (m, 3 H, H-1 of D-glucopyranose), 4.92 (d, 0.3 H, $J_{1,2}$ 3.5, H-1 of reducing α-D-glucopyranose), 4.33 (d, 0.7 H, $J_{1,2}$ 7.6, H-1 of reducing β-D-glucopyranose), 3.75 (m, 16.7 H, H-3), 3.59–3.45 (m, 50.1 H, H-5, H-6a, H-6b), 3.30 (m, 16.7 H, H-4), 1.96 (m, 16.7 H, H-2*eq*), 1.48 (m, 16.7 H, H-2*ax*); ^{13}C NMR (100 MHz, Me_2SO-d_6): δ 96.89 (C-1), 75.39, 72.11, 68.68 (C-3, C-4, C-5), 60.88 (C-6), 38.65 (C-2). Only signals of the 2-deoxy-D-*arabino*-hexosyl units could be detected in the ^{13}C NMR spectrum.

Acknowledgments

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