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SYNTHESIS OF THE DISACCHARIDE 6-*O*-β-D-GALACTOPYRANOSYL-2-ACETAMIDO-2-DEOXY-D-GALACTOSE USING IMMOBILIZED β-GALACTOSIDASE

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SUMMARY: The disaccharide 6-*O*-β-D-galactopyranosyl-2-acetamido-2-deoxy-D-galactose has been synthesized by transfer of the β-D-galactopyranosyl residue from lactose to 2-acetamido-2-deoxy-D-galactose utilizing the transferase activity of β-galactosidase from E. coli. To make the enzyme reusable, it was applied in an immobilized form covalently bound to Sepharose CL-4B. The yield of the disaccharide was about 20 %, calculated on the amount of acetamido-deoxy-D-galactose added. The disaccharide could also be obtained by reversal of the hydrolytic activity of the enzyme, using D-galactose and 2-acetamido-2-deoxy-D-galactose as substrate. The yield in this reaction, however, was only 2-3 % under the conditions applied.

There is a growing appreciation of the potential of biologically active carbohydrates in biology and medicine. Much of this interest stems from observations on the role of glycoconjugates in biological receptor interactions e.g. cell surface receptors on bacteria, viruses, mycoplasma, protozoans, hormone receptors and recognition phenomena in plants and the like (1). To name one example, adherence of bacteria to mammalian mucosal surfaces has been gaining recognition as an important initial step in the pathogenesis of bacterial infections (2). Prevention of infections based on the use of carbohydrate-containing substances that inhibit initial

Abbreviations: β-D-Gal*p*-(1-6)-D-GalNAc = 6-*O*-β-D-galactopyranosyl-2 acetamido-2-deoxy- D-galactose
β-D-Gal*p*-(1-6)-D-Gal = 6-*O*-β-D-galactopyranosyl-D-galactose
β-D-Gal*p*-(1-6)-D-Glc = 6-*O*-β-D-galactopyranosyl-D-glucose
N-acetyl-galactosamine = 2-acetamido-2-deoxy-D-galactose
hex*p*-(1-6)-hexNAc = 6-*O*-hexopyranosyl-2-acetamido-2-deoxy-hexose

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attachment of the bacteria e.g. by competition thus appears to have great potential. As a result of these insights, there is a growing need for cheap, synthetically produced oligo- and disaccharides. However, as normal organic-chemical methods are cumbersome and give a low (or no) yield of the desired product, in part due to the large number of isomers possible, the enzymatic approach is attractive. Its usefulness in fact could be even greater than that of for e.g. the synthesis of peptides (3,4), lipids (5) or other esters (6) which already are being prepared enzymatically using normal hydrolytic enzymes.

As an example we have chosen here the enzymatic synthesis of the disaccharide 6-*O*-β-D-galactosyl-2-acetamido-2-deoxy-D-galactose which, to our knowledge, has not previously been synthesized and properly characterized. This disaccharide has primarily been obtained by utilizing the transferase activity (7,8) of the enzyme β-galactosidase (E.C. 3.2.1.23) from E. coli with lactose and 2-acetamido-2-deoxy-D-galactose as substrates. The yield was typically 20 %. The disaccharide could also be obtained, although in low yield, by reversing the normal hydrolytic reaction of the enzyme. In this case D-galactose and 2-acetamido-2-deoxy-D-galactose were used as substrates. In both cases β-galactosidase was employed in its immobilized state because of its inherent advantage in usage and preparation.

MATERIALS AND METHODS

Materials: E. coli β-galactosidase (E.C. 3.2.1.23) grade 6 (80 U/mg), lactose, D-galactose, 2-acetamido-2-deoxy-D-galactose and o-nitrophenyl-β-D-galactoside were from Sigma (St. Louis, Mo., U.S.A.). Sepharose CL-4B was from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Tresyl chloride was from Fluka AG (Buchs, Switzerland). All other chemicals were of analytical grade and used as supplied.

Immobilization of β-galactosidase: Immobilization was done according to the tresyl chloride method (9,10). Wet Sepharose CL-4B, 5 g, was washed with water, 30:70, 50:50 and 70:30 of acetone:water (v/v), acetone, and finally with dried acetone (dried over molecular sieve). The gel, 4.2 g, was rapidly transferred to a small beaker containing 1.3 ml dry acetone and 95 µl dry pyridine. Tresyl chloride, 125 µl, was added under vigorous magnetic stirring of the gel suspension, and the reaction was allowed to continue for 15 minutes. The gel was then washed with acetone, 70:30, 50:50, 30:70 of acetone:5 mM HCl, and finally with 5 mM HCl. The activated gel was stored at 4 °C until used.

Activated gel, 5.1 g wet weight, was washed with 0.2 M Na-phosphate buffer, pH 8.0, and mixed with 475 U β-galactosidase in 6 ml 0.2 M Na-phosphate buffer, pH 8.0. The suspension was gently shaken for 2 h at 24 °C. After the reaction was completed, the gel was first washed with 0.2 M Na-phosphate buffer, pH 8.0, and then with 0.2 M Tris-HCl, pH 8.0, in which the gel was suspended for 30 minutes to quench the non-coupled tresyl groups. The gel was then washed with 0.5 M NaCl, water and finally 0.1 M Na-phosphate buffer, pH 7.0, containing 2 mM $MgCl_2$. (Tresyl chloride activated Sepharose is now also commercially available from Pharmacia, Uppsala, Sweden.)

Enzyme assays: β-Galactosidase activity was determined from initial rate measurements of o-nitrophenyl galactoside hydrolysis at 24 °C. The hydrolysis was followed spectrophotometrically at 420 nm, where the product o-nitrophenol absorbs. The molar absorption coefficient at 420 nm in phosphate buffer, pH 7.0, was determined separately as 2050 M^{-1} cm^{-1}. The coefficient is very pH-sensitive in this region. Assay mixtures contained 1.0 mM o-nitrophenyl galactoside and 2 mM $MgCl_2$ in 0.10 M Na-phosphate buffer, pH 7.0. Enzyme was added in an amount to give an increase in absorbance of approximately 0.5 A/minute.

When immobilized enzyme was assayed, a spectrophotometric cell equipped with a magnetic stirring device was used to keep the particles suspended. Normally 2-5 mg of gel was added to 2.0 ml of assay mixture.

The activity that will hydrolyze 1 µmol of substrate per minute under the above conditions is defined as 1 unit (U).

Preparation of β-D-Gal*p*-(1-6)-D-GalNAc: Immobilized enzyme, 1.4 g corresponding to 70 U was suspended in 17 ml 0.1 M K-phosphate buffer, pH 6.5, containing 0.22 M 2-acetamido-2-deoxy-D-galactose and 0.45 M lactose. The mixture was gently shaken at room temperature, and intermittently analyzed with HPLC. The reaction was stopped after 30 h by centrifugation and separation of the gel. The supernatant was then boiled for 3 minutes to denaturate possible traces of enzyme, and freeze dried.

Reversed hydrolysis: Immobilized enzyme, 5 U, was suspended in 0.5 ml 0.1 M K-phosphate buffer, pH 6.5, containing 0.31 M galactose and 0.31 M 2-deoxy-2-acetamido-D-galactose. The mixture was gently shaken at room temperature for 24 h before the reaction was stopped and treated as above.

Isolation of β-D-Gal*p*-(1-6)-D-GalNAc: Part of the freeze dried reaction mixture (2.79 g) was gel chromatographed on a Sephadex G-15 (3 x 100 cm) column and the peak containing the disaccharides was pooled. The disaccharide pool was further purified by preparative paper chromatography on Whatman 3MM using ethyl acetate:acetic acid:water (3:1:1 v/v) as eluant. The band with $R_{lactose}$ = 1.5 was collected. Analysis of the oligosaccharides in this fraction, as permethylated alditols, showed the presence of small amounts of neutral disaccharides. After acethylation, further purification of the GalNAc-containing disaccharide was performed on a silica gel column using ethyl acetate as eluant. Fractions containing the major component were pooled and deacetylated using 0.25 M ammonia in water:methanol (2:1 v/v). The deacetylated product was finally passed through a Sephadex G-15 column (1 x 20 cm) and lyophilized. 120 mg was isolated.

Gas-liquid chromatography and mass spectrometry: Monosaccharides were analyzed as alditol acetates by gas-liquid chromatography (GLC) (11) and mass spectrometry (MS) (12) after hydrolysis with 90 % aqueous formic acid at 100 °C for 5 h followed by hydrolysis with 0.25 M sulphuric acid at 100 °C for 18 h. Methylation analyses were performed as previously described (13). GLC was carried out on a Perkin-Elmer 3920 gas chromatograph equipped with a flame ionization detector. Separations were performed on (a) a SE-30 W.C.O.T. vitreous silica capillary column (25 m x 0.2 mm) at 180 °C for

partially methylated alditol acetates and at 180-330 °C for permethylated oligosaccharide alditols, (b) a Silar 10 glass capillary column (4 m x 0.25 mm) at 170-210 °C for alditol acetates. GLC-MS was performed on a Finnigan 4021 instrument fitted with the appropriate column. The spectra were recorded at 70 eV with an ionization current of 0.3 mA and an ion-source temperature of 280 °C. The spectra were processed on an on-line computer system (Nova 3, Data General).

RESULTS AND DISCUSSION

Immobilization of β-galactosidase. β-Galactosidase was immobilized on the macroporous support Sepharose CL-4B using the tresyl chloride coupling method. The method has been shown to constitute a very mild immobilization procedure (9,10), which is in line with the present findings. More than 50 % of the added activity was thus found immobilized on the gel. Preliminary kinetic analysis indicated that at least part of the lost activity was due to limiting mass transfer rates in the gel.

A key aspect of using immobilized enzymes instead of free enzymes is their easy reuse. This requires, however, that the immobilization method gives a preparation stable enough to make reuse meaningful. Although no extensive studies were carried out, it was observed that β-galactosidase immobilized by the tresyl chloride method could be used several times without any noticeable loss of activity.

Preparation of β-D-Galp-(1-6)-D-GalNAc. Immobilized β-galactosidase was used to prepare β-D-Gal*p*-(1-6)-D-GalNAc from lactose and N-acetyl-galactosamine in a transferase reaction that effected the transfer of a galactosyl moiety from lactose to N-acetyl-galactosamine. Several competing reactions are occurring in a transferase system as shown in Fig. 1. A high concentration of lactose will saturate the enzyme and convert it into a galactosyl-enzyme complex. The galactosyl-enzyme complex may now be attacked by water, which will split off galactose and return the enzyme to its native state. Alternatively, N-acetyl-galactosamine may attack the galactosyl-enzyme to give the desired compound Gal-GalNAc.

It is interesting to note that the yield of Gal-GalNAc could be as high as 20 % (based on the amount of GalNAc added) in spite of the fact that only

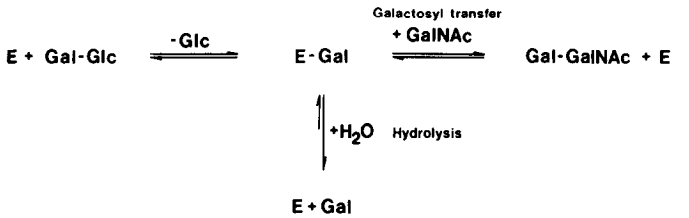

Figure 1: Simplified scheme of the proposed mechanism of β-galactosidase, showing the transfer and the hydrolysis reactions. See text for further details. E = β-galactosidase.

a two-fold excess of lactose was used. This must mean that the rate constant for the transferase reaction step was much higher than the rate constant for the hydrolytic reaction step. Otherwise the high water concentration should have caused an almost complete breakdown into monosaccharides.

The high yield of the desired compound was thus kinetically governed and it was important to follow the reaction to find out at what time the yield was at optimum. To this end monitoring with HPLC was found to be very satisfactory, especially since an analysis could be carried out in a couple of minutes. Although the resolution was inferior to that of GC with permethylated sugars, it was sufficient to accurately determine the correct time of terminating the reaction. Fig. 2 shows a series of analyses belong-

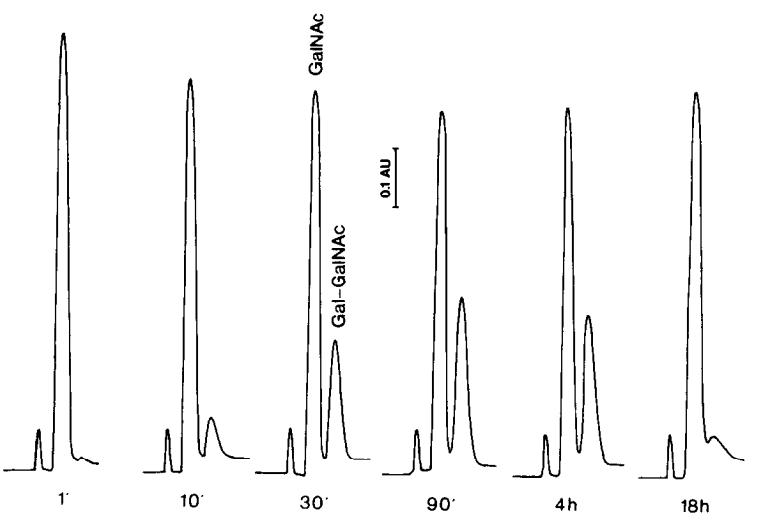

Figure 2: HPLC-analysis of a typical transfer experiment. Samples were taken intermittently from the reaction mixture and separated on a LiChrosorb-NH_2 column (Merck; 5 µm particles, 25 cm) and detected at 235 nm. Flow rate 1 ml/min. Solvent acetonitrile:water, 3:1. Separation time 11 min.

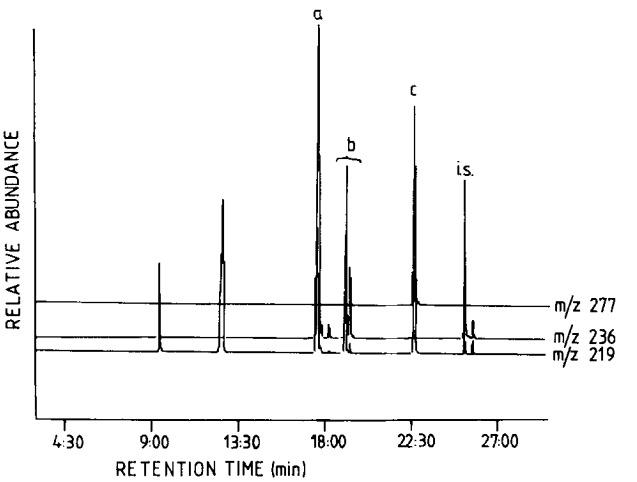

Figure 3: Gas-liquid chromatography of the product mixture from a transfer experiment. See text for further details.

ing to one transferase experiment (not the same as the one described in the experimental section). A UV-detector was used to detect acetamido-group containing sugars. Fig. 2 shows that the yield of Gal-GalNAc was highest at 90 minutes and then slowly diminished.

Identification and characterization of products. Fig. 1 gives a simplified version of the events in a transferase experiment. The figure does, for example, not take into account the possible formation of oligomers (7,8). To carefully characterize the product pattern from a transferase experiment, qualitative and quantitative analyses were carried out by gas chromatography - mass spectrometry of permethylated sugars. Fig. 3 gives the result. Peak a in the figure had the same retention time and gave the same mass spectrum as the permethylated alditol of lactose. The components in the major peaks in the peak cluster b were identified as the permethylated alditol of the disaccharides β-D-Gal*p*-(1-6)-D-Gal and β-D-Gal*p*-(1-6)-D-Glc by comparison of retention times and mass spectra of authentic standards. The component in peak c was identified by its mass spectrum as the permethylated alditol of a hex*p*-(1-6)-hexNAc disaccharide (Fig. 4). The yield was calculated with the aid of an internal standard (i.s.) which was the permethylated alditol of maltotriose. No evidence for other linkage types than (1-6) was observed.

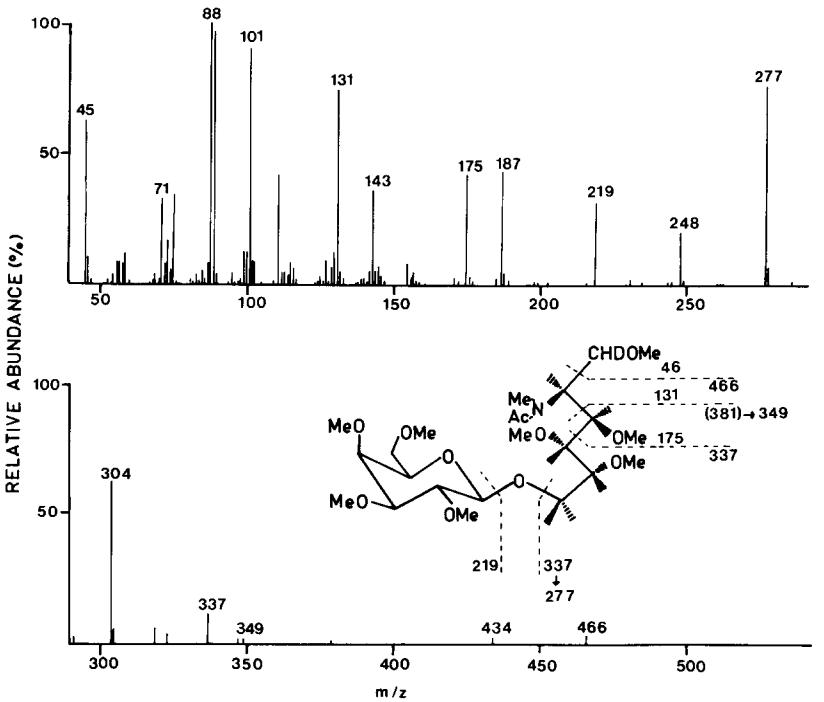

Figure 4: Mass spectrum of peak c in Fig. 3 (β-D-Gal*p*-(1-6)-D-GalNAc).

In order to characterize the hex*p*-(1-6)-hexNAc disaccharide formed, it was purified by gel chromatography, paper chromatography and chromatographed on silica gel. The pure disaccharide was analyzed by sugar and methylation analyses which proved the structure D-Gal*p*-(1-6)-D-GalNAc. The anomeric configuration of the D-Gal*p* residue is β which was evident from the optical rotation $\alpha_D = 26^0$. The proposed structure was further substantiated by proton NMR.

Reversed hydrolysis. Attempts to use the immobilized enzyme in a reversed hydrolysis reaction were less satisfactory since a yield of only 2-3 % of the disaccharide β-D-Gal-(1-6)-D-GalNAc was obtained, obviously due to the unfavourable equilibrium of the reaction. Studies to improve the yield by lowering the water content, or by other means, are now in progress.

Concluding remark. The study shows that at least the transferase activity and possibly the reversed hydrolysis activity of immobilized β-galactosidase can be used for the preparative scale synthesis of Gal-GalNAc. Compared to conventional organic-chemical synthesis, the enzymatic

route is rapid and gives a high yield of product. The transfer reaction may be beneficial in other synthetic applications.

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