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Note

Polymer-supported enzymic synthesis on a preparative scale

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A number of feasible approaches for the solid-phase synthesis of oligosaccharides, using protecting group chemistry, have been published in recent years [1–4]. Nevertheless, a widespread use of these approaches suffers from the fact that there is no chemical glycosylation method that is generally applicable for the synthesis of any glycosidic linkage. Enzymic glycosylation, on the other hand, represents a strategy applicable for any glycosidic linkage once the appropriate enzyme is available. Glycosylations using glycosyltransferases have proved to be an attractive alternative to protecting group chemistry in numerous examples [5,6]; the method is therefore ideally suited to apply to a solid-phase synthesis. An additional reason for the application of solid-phase synthesis in enzymic glycosylations is the ease of work-up and separation in the solid-phase approach. This is especially advantageous in enzymic systems that use complex mixtures of a multitude of enzymes and cofactors for regeneration of substrates.

A method for the attachment and release of the saccharide acceptor onto the polymer suitable for enzymic glycosylations has been developed by Zehavi et al. [7,8]. This approach utilises a photocleavable bond for the linkage of the acceptor to the polymer and was used with several enzyme systems [9]. The incorporation yields obtained in these reactions vary with the polymers and the enzymic systems applied. In recent studies [10], we have developed some insoluble polymers that carry feasible spacers for the attachment of the saccharide. These polymers were effective supports for enzymic glycosylation with galactosyltransferase, which was demonstrated by small-scale synthesis using radioactively labelled donors. The use of radioactive labelling is very advisable in the development of polymeric supports because of the high costs of cofactors and enzymes. In order to demonstrate the feasibility of the solid-phase approach, we now report a polymer-supported enzymic synthesis on a preparative scale, including the photochemical release and isolation

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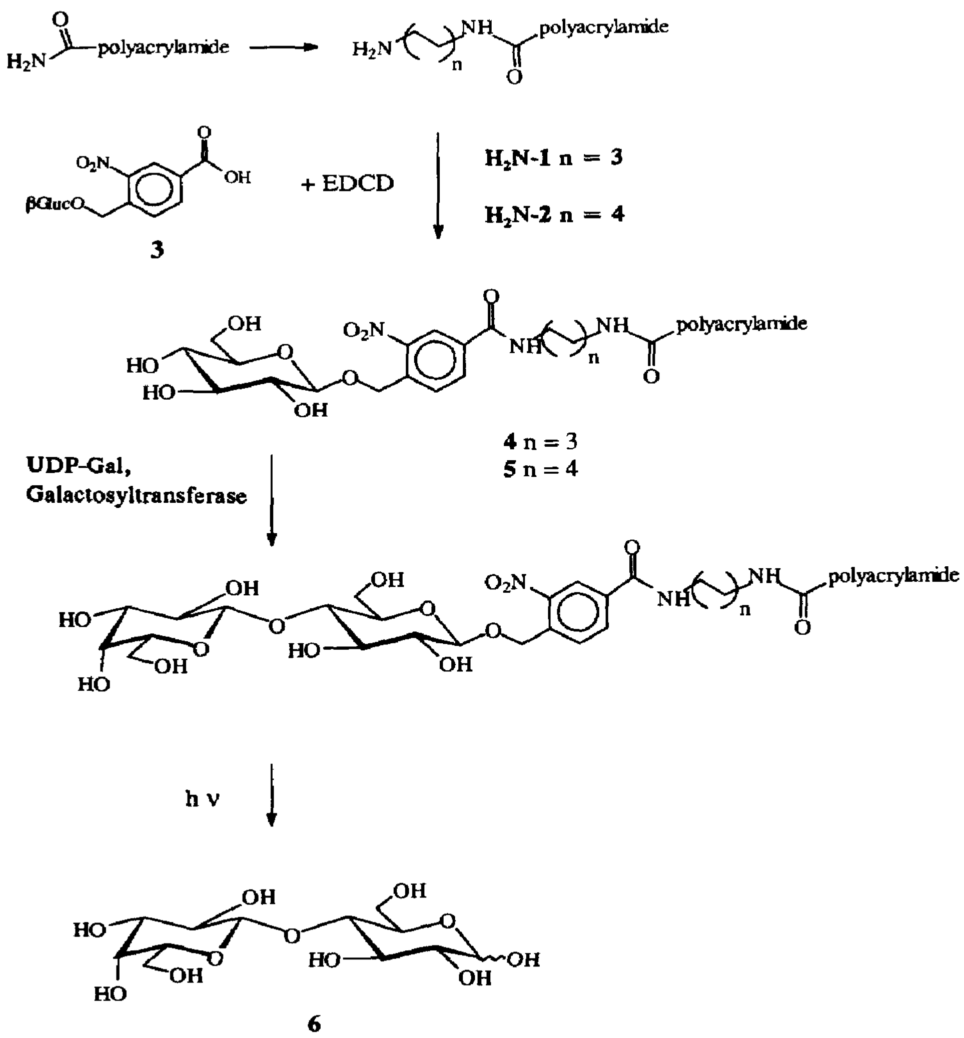

Scheme 1.

of the product. As a model glycosylation, we chose the β-(1 → 4)-galactosyl transfer to D-glucose catalysed by β-(1 → 4)-galactosyltransferase.

In a first step, the hexyl- and octyl-amino spacers were attached to poly(acrylamide) gel beads by direct treatment with the alkanediamines [8,11]. Incorporation of the glucosyl acceptor **3** [8,12], into polymers H_2N-**1** and H_2N-**2** was accomplished in a second step by 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDCD)-mediated amidation. Polymers **4** and **5** were thus isolated carrying a carbohydrate loading of 0.07 and 0.03 mmol/g, respectively. The remaining amino groups in both polymers were acetylated to prevent interaction of the enzyme with these groups (Scheme 1).

The newly synthesised polymers H_2N-**1**, H_2N-**2**, **4**, and **5** differ from previously prepared supports [10] by a higher degree of cross-linking in the backbone polymer. The degree of cross-linking obviously has a strong effect on the availability of carbohydrate acceptor on the polymer and consequently influences the effectiveness of the enzymic reaction. We therefore tested both polymers **4** and **5** in a small-scale reaction with radioactively labelled

Table 1
Characteristic data of polymer acceptors of galactosyltransferase

Polymer	Spacer	Bound Glc (mmol/g)	Substitution of NH_2 by glucoside (%)	Hydrated bed volume (mL/g)	Enzymic Gal transfer (%)
4	Hexylamine	0.07	27	26	15.0 [a]
5	Octylamine	0.03	30	18	25.3 [a]; 51 [b]

[a] Incorporation yield of a small-scale synthesis with radioactively labelled and molar amounts of UDP-Gal.
[b] Isolated yield of a preparative-scale synthesis with in situ generation of UDP-Gal.

UDP-D-galactose and galactosyltransferase. Incorporations of 15 and 25%, respectively, were obtained for polymers **4** and **5**. These are relatively high yields for the reaction conditions applied. Other physical characteristics which influence the enzymic reaction are depicted in Table 1. Both polymers exhibit good swelling properties and the amount of attached carbohydrate is in a range that allows an efficient enzymic transfer [10]. The availability of amino groups on polymers H_2N-**1** and H_2N-**2** is reflected by the yield of incorporation of the glucoside. Again, the incorporation in both polymers is satisfactory with respect to expected availability in the following enzymic reaction [10].

Higher yields for the enzymic glycosylation, however, were obtained when the reaction was carried out with in situ generation of UDP-D-galactose and optimised reaction conditions on polymer **5**. Thus, a synthesis on a scale of 100 mg of glycoside was performed. After enzymic galactosylation, the carbohydrate product was set free by irradiation and separated from monosaccharides by gel chromatography. The product lactose (**6**) was isolated in an overall yield of 51%. This yield is comparable to yields obtainable in enzymic glycosylations carried out on solutions and shows the feasibility of a polymer-supported enzymic synthesis, an approach that is expected to be also practicable with other glycosyltransferases.

1. Experimental

General methods.—Scintillation counting was performed with a Beckman LS 7800 or a Packard Tricarb 460 C instrument, using standard scintillation cocktails. IR spectra of KBr discs were recorded on a Nicolet MX-S FT-IR spectrometer. Colorimetric measurements were performed with a Zeiss PMQ II instrument. Cross-linked poly(acrylamide) gel beads (Bio-Gel P-100, 100–200 mesh) were purchased from Bio-Rad Laboratories. Galactosyltransferase (EC 2.4.1.22) and UDP-D-galactose were obtained from Sigma Chemical Co., UDP-D-[$U^{14}C$]galactose was purchased from Amersham International plc. The ^1H and ^{13}C NMR spectra of synthesised lactose were recorded on a Bruker ARX 500 spectrometer at 500.13 and 121.5 MHz, respectively.

Reactions using polymers.—Glassware used for treatment of the polymers was siliconised. Prior to any treatment, the polymers were preswollen in water with gentle stirring for at least 4 h. The 2-nitrobenzyl glucoside **3** and its polymer derivatives are light-sensitive and were treated and stored under protection from light. The presence of amino groups on the polymer was qualitatively tested by treatment with ninhydrin or sodium 2,4,6-trinitrobenzenesulfonate (TNBS) [13]. The amount of accessible amino groups on the polymer was determined by titration according to literature procedures [11].

Determination of polymer-bound sugar.—Two to four batches of ca. 20 mg of polymer in 2 mL of water were irradiated for 20 h in an RPR-100 apparatus (Rayonet, The Southern New England Ultraviolet Company, Hamden, CT 06514, USA) with RPR 3500 Å lamps in Pyrex glassware at 320 nm. The solution was filtered and D-glucose was determined by the glucose oxidase test [14].

Aminohexyl-polymer H_2N-1.—Dry poly(acrylamide) polymer (15 g) was slowly added with stirring to 1,6-hexanediamine (750 g) preheated at 90°C. Gentle stirring was continued at 90°C for 3 h and 40 min. The mixture was then cooled in an ice bath, followed by the addition of ice (500 mL). The polymer was filtered off and washed alternately with water and 0.1 M NaCl until no amine was detected in the eluate. The polymer was lyophilised to yield 12.6 g of polymer H_2N-**1**, which contained 0.26 mequiv NH_2/g; hydrated bed volume, 41 mL/g; IR: ν_{max}^{KBr} 3190 (NH), 1570, 1360, and 1320 cm^{-1} (CONH).

Aminooctyl-polymer H_2N-2.—Dry poly(acrylamide) polymer (20 g) was treated with 1,8-octanediamine (700 g) at 90°C for 5 h. It was worked up as described for H_2N-**1**. The polymer was re-suspended in water (200 mL) and 1,8-octanediamine (100 g). The pH was adjusted to 4.7 and EDCD (4.7 g) in water (50 mL) was added with gentle stirring during 20 min. Stirring was continued for 20 h, and the polymer was filtered off and washed with dil HCl and water. Final lyophilisation afforded 16.7 g of H_2N-**2**; 0.1 mequiv NH_2/g; hydrated bed volume, 25 mL/g; IR: ν_{max}^{KBr} 3200 (NH), 1570, 1370, and 1330 cm^{-1} (CONH).

*4-[N-(**1** and **1Ac**)Carbamoyl]-2-nitrobenzyl β-D-glucopyranoside* (**4**).—A suspension of 4-carboxy-2-nitrobenzyl β-D-glucopyranoside [8,12] (**3**; 2.65 g, 7.4 mmol) and preswollen polymer H_2N-**1** (14.6 g, 1.4 mmol NH_2) in water was adjusted to pH 4.7, and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDCD, 200 mg, 1.04 mmol) was added. Addition of the same amount of EDCD was repeated twice after 1 and 7 h, accompanied by re-adjustment of the pH to 4.7 if necessary. After a total of 12 h of gentle stirring, the mixture was centrifuged and washed with water, dil HCl, dil aq Na_2CO_3 and water. In a volume of approximately 10 mL, the wet polymer was treated with $NaHCO_3$ (500 mg) and Ac_2O (0.5 mL) at 4°C with gentle stirring overnight. It was centrifuged, washed thoroughly, and finally lyophilised to give **4** (17.2 g), which released 0.03 mmol glucose/g upon irradiation (30% yield for substitution of amino groups and release); hydrated bed volume, 18 mL/g.

*4-[N-(**2** and **2Ac**)Carbamoyl]-2-nitrobenzyl β-D-glucopyranoside* (**5**).—Glucoside **3** [8,11] (5.97 g, 16.6 mmol) and preswollen polymer H_2N-**2** (11.8 g, 3.0 mmol NH_2) were treated with two portions of EDCD as described for **4**, and the product was acetylated, worked-up, and lyophilised to yield polymer **5** (8.5 g), which released 0.07 mmol glucose/g upon irradiation (27% yield of substitution of amino groups and release); hydrated bed volume, 26 mL/g.

Radioactively probed galactosyltransferase reaction.—Under protection from light, polymer **4** (190 mg, 5.6 μmol of bound glucose) was preswollen in cacodylate buffer (4 mL, 25 mM sodium cacodylate, pH 7, containing 0.3 mM $MnCl_2$, 3 mg of α-lactalbumin, and 0.1% 2-mercaptoethanol) and preincubated at 38°C. UDP-D-Galactose (sodium salt, 6 mg, 9.8 μmol), UDP-D-[U-^{14}C]galactose (ca. 0.6 μCi), and galactosyltransferase (EC

2.4.1.22; 50 μL, 1 U) in water (150 μL) were added and the suspension was incubated with gentle shaking for 2 days. The polymer was then filtered off, washed extensively until the eluates were free from radioactivity, lyophilised, and finally dried over P_2O_5 in vacuo. Radioactivity was measured by scintillation counting and showed 15.0% incorporation of galactose. Similar treatment of **5** (81 mg, 5.6 μmol of bound glucose) gave 25.3% incorporation.

Preparative scale galactosyltransferase reaction.—Under protection from light, polymer **5** (15.8 g, 0.47 mmol glucose content) was preswollen in cacodylate buffer (500 mL, 25 mM sodium cacodylate, pH 7, containing 0.3 mM $MnCl_2$, 0.1% 2-mercaptoethanol, and 90 mg of NaN_3), incubated at 37°C and set under Ar. UDP-D-Glucose (332 mg, 0.51 mmol), α-lactalbumin (15 mg), UDP-D-galactose 4-epimerase (EC 5.1.3.2, 10 U in 100 μL of cacodylate buffer) and galactosyltransferase (EC 2.4.1.22, 5 U) were added and kept with shaking at 37°C for 6 days. The reaction was surveyed by photometric assay of UDP [15]. The polymer was filtered off and washed extensively with water and alternately with 0.2 M NaCl. The polymer was then irradiated for 30 h as described in "*Determination of polymer-bound sugar*", and the solution was filtered and evacuated to yield 152 mg of raw material, which was separated on Bio-Gel P-200 into glucose and lactose (**6**; 82 mg, 51%). The latter was identical to a commercial product (TLC, NMR spectroscopy, and optical rotation).

Note added in proof

Since submission of this manuscript a paper describing a combination of chemical and enzymic solid-phase synthesis of glycopeptides has been published: M. Schuster, P. Weng, J.C. Paulson, and C.-H. Wong, *J. Am. Chem. Soc.*, 116 (1994) 1135–1136.

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