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Glycamine formation via reductive amination of oligosaccharides with benzylamine: efficient coupling of oligosaccharides to protein †

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ABSTRACT

The conventional reagents for the reductive amination of sugars, ammonium salts or ammonia, require relatively harsh conditions such as high temperatures or high concentrations. In addition, they give substantial amounts of dimeric byproducts. We have developed a method of using benzylamine as a donor to achieve near quantitative amination of reducing oligosaccharides. Benzylamine reacts with reducing oligosaccharides faster and yields less dimeric byproduct than ammonium ion, rendering it especially advantageous for preparative operation. In combination with a heterobifunctional reagent, 5-[*N*-(2,2-dimethoxyethyl)carbamoyl]pentanoyl azide, [Lee et al. *Biochemistry*, 28 (1989) 1856–1861], we could couple a nearly maximal number of phosphorylated mannopentaose molecules to ribonuclease A via its primary amino groups.

INTRODUCTION

Carbohydrate–protein interactions play crucial roles in various biological events such as metastasis[1], fertilization[2,3], protein trafficking[4], microbial infection[5], and cell adhesion[6–8]. Therefore, it is of great interest to elucidate specificities and binding modes of carbohydrate–protein interactions by definitive means. Moreover, in many such interactions, the clustering of ligands and receptors leads to a higher affinity binding[9]. Synthetic glycoconjugates are powerful tools not only for elucidating binding specificity[10], but also for providing controllable clustering of glycosides[9]. Their advantages lie in their relative ease of production as well as their definitive carbohydrate structures.

† Dedicated to Professor C.E. Ballou.

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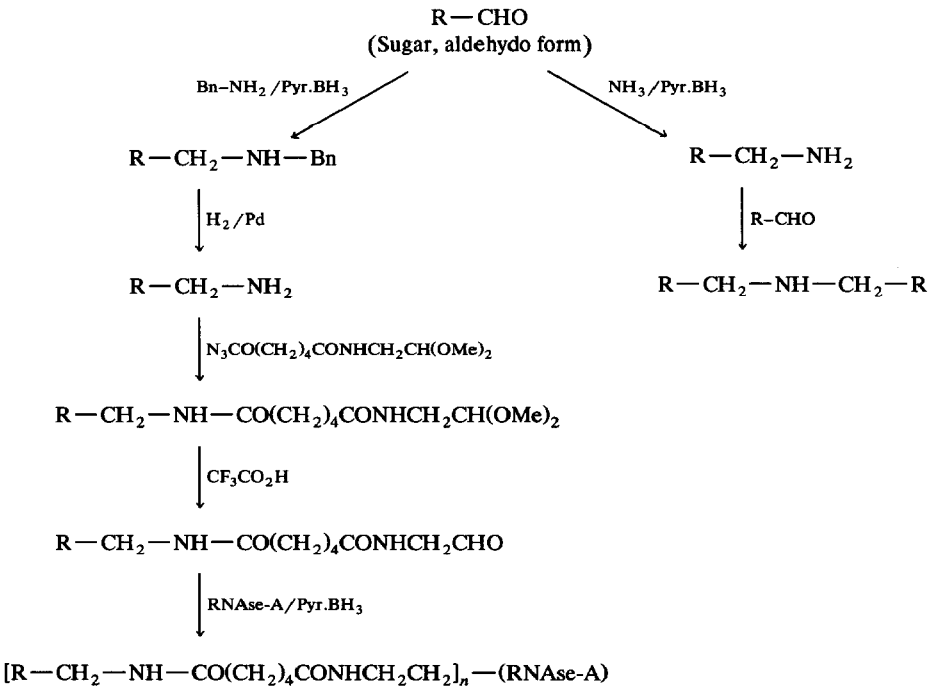
When attaching glycopeptides to a protein, the amino groups of the peptide moieties can be utilized for conjugation via homobifunctional[11] or heterobifunctional coupling reagents[12]. In conjugating oligo- or poly-saccharides that have no amino groups, however, the only readily available site for conjugation is the carbonyl group of the reducing sugar unit. Unfortunately, the carbonyl group of most sugars, being in the cyclic hemiacetal form in aqueous solution, is not very reactive. Conjugating reducing oligosaccharides to a protein by direct reductive amination typically requires many days, even when a large excess of the reducing oligosaccharide is used[13]. On the other hand, when an unmasked aldehydic group is introduced at the terminal position of a spacer attached to an oligosaccharide, reductive amination proceeds smoothly, reaction being completed in 24 h[14]. A method for introducing an unmasked aldehyde group into an oligosaccharide is to reduce the sugar with $NaBH_4$ and then selectively oxidize the oligosaccharide alditol with periodate[15].

In many applications, it is desirable to convert the reducing group into an amino group. Glycamines (aminoalditols) have been used for monosaccharide analysis by cation exchange chromatography[16], and other amines have been conjugated to oligosaccharides solely for HPLC analysis (e.g., 2-aminopyridine, ethyl 4-aminobenzoate, or hydroxylamine derivatives). However, these derivatizations do not lead to useful ligands for carbohydrate-binding proteins. Although conjugation with 4-trifluoroacetamidoaniline[17] is a useful alternative for eventual coupling to proteins, the introduction of an aromatic ring can cause undesirable antigenicity or nonspecific interactions.

In our attempt to prepare neoglycoproteins from ribonuclease A and mannopentaose phosphate from yeast mannan[18], neither direct reductive amination nor reductive amination after selective periodate oxidation gave satisfactory results. It was surmised that the sugar moieties adjacent to the aldehydic group might be hindering the coupling reaction. Therefore, we opted to attach a long spacer arm, by means of the heterobifunctional reagent used successfully before[12], to the glycamine derived from the reducing oligosaccharide (Scheme 1). We have previously used a combination of galactose oxidase and reductive amination with benzylamine to convert the 6-OH of a Gal residue to a 6-NH_2 group[19], and now have employed the same approach of using an available aldehydo group for the production of glycamines. We describe here a high-yielding procedure for glycamine formation with benzylamine, applicable even to unstable oligosaccharides in minute quantities. The glycamines formed by this scheme can be very efficiently coupled to a protein.

EXPERIMENTAL

General methods.—Carbohydrates were determined by the phenol–H_2SO_4 reaction[20], using glucose (for laminarihexaose) and mannose (for mannopentaose phosphate) as standards. The color yield of mannose 6-phosphate was 95% that of



Scheme 1.

mannose. Protein was determined by fluorescamine assay after an 11-h hydrolysis in 2 M NaOH at 100°C[21], using ribonuclease A as standard.

Elemental analysis was done by Galbraith Inc. (Knoxville, TN). ^{1}H NMR spectra were obtained in D_2O solutions using acetone as an internal standard ($\delta = 2.25$ ppm) on a Bruker AMX 300 spectrometer.

All evaporations were carried out under reduced pressure, using either a rotary evaporator or a Speed-Vac®. Thin-layer chromatography was done on silica Gel 60 F_{254} (Merck, Darmstadt, Germany) layers precoated on aluminum sheets. Carbohydrates were detected by charring after spraying with 15% H_2SO_4 in aq 50% EtOH.

Materials.—Borane–pyridine complex, palladium (10% on activated carbon), CF_3CO_2H (98%), and *tert*-butyl nitrite were from Aldrich Chemical Co. (Milwaukee, WI). Fluorescamine, α-lactose, and ribonuclease A were from Sigma Chemical Co. (St. Louis, MO). Benzylamine was from J.T. Baker Inc. (Phillipsburg, NJ), and laminarihexaose (fine) was purchased from Seikagaku America Inc., (Rockville, MD). Mannopentaose phosphate (MPP) was prepared by the established method[22] from phosphomannan from *Hansenula hostii* (Y-2448) donated by Dr. Morey Slodki of the Northern Regional Research Center, USDA (Peoria, IL).

Reductive amination of saccharides.—The benzylamine reagent for reductive amination was prepared by mixing equimolar quantities of benzylamine and glacial acetic acid. Laminarihexaose or lactose, the benzylamine reagent, and pyridine–

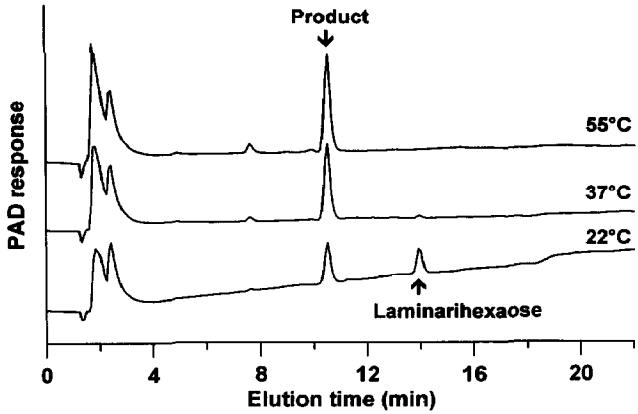


Fig. 1. HPEAC analysis of laminarihexaose reductively aminated with benzylamine. Laminarihexaose (20 mM) was treated with the benzylamine reagent (500 mM) and pyridine–borane at the indicated temperature for 18 h. The first 2 peaks were identified as pyridine and benzylamine.

borane were dissolved in 25% MeOH in water at final concentrations of 20, 500 (in both benzylamine and acetic acid), and 100 mM, respectively, unless otherwise noted. Ammonium acetate was used at a final concentration of 1 M. The preparative reaction of MPP was done at final concentrations of 400 mM MPP, 500 mM benzylamine reagent, and 1 M pyridine–borane in 34% MeOH–water.

Preparative reductive amination of lactose with benzylamine.—Lactose (100 mM), the benzylamine reagent (500 mM), and pyridine–borane (500 mM) were reacted in 10 mL of aq 25% MeOH at 55°C for 14 h. The mixture was cooled to room temperature, extracted with $CHCl_3$ to remove most of the excess benzylamine and pyridine–borane, and then fractionated on a Sephadex LH-20 column (1.4 × 42 cm) eluted with aq 50% MeOH, with monitoring of the effluent by absorbance at 254 nm and with the phenol–H_2SO_4 reagent[20].

HPAEC and HPCEC analyses.—The yield of each reaction of laminarihexaose and MPP was examined by the combination of high performance anion exchange chromatography (HPAEC) and pulsed amperometric detection[23]. A Carbopac PA-1 column (4.6 × 250 mm) was eluted with a mixture of 100 mM NaOH with NaOAc, the latter increasing linearly from 100 to 300 mM in 20 min (Fig. 1). The relative response factor of reductively aminated laminarihexaose was virtually identical to that of laminarihexaose. The yields of reactions with lactose were determined by high performance cation exchange chromatography (HPCEC) using a cation-exchange column, CHO-611 carbohydrate (6.5 × 150 mm) from Interaction (Mountain View, CA), under isocratic elution with 4 mM NaOH[24]. For simultaneous analysis of mannose and mannose 6-phosphate, the Carbopac PA-1 column was eluted with 14 mM NaOH and 10 mM NaOAc for 5 min isocratically, followed by a linear increase of NaOAc to 500 mM in the subsequent 25 min.

Activation of the heterobifunctional reagent.—A previously developed heterobifunctional reagent, 5-[*N*-(2,2-dimethoxyethyl)carbamoyl]pentanoyl azide[12], was

used to conjugate glycamine produced as above to ribonuclease A. The conversion of the acyl hydrazide form of the reagent to acyl azide was performed by in situ production of nitrous acid[25]. Briefly, the acyl hydrazide, 9.1 mg (37 μmol), was dissolved in 500 μL of DMF and chilled to −25°C. Trifluoroacetic acid (23 μL; 300 μmol) and 5 mg (44 μmol) of *tert*-butyl nitrite in 50 μL of DMF were added to the hydrazide solution and the mixture was stirred in an ice bath for 30 min. Assay with trinitrobenzenesulfonate[26] confirmed the total disappearance of the hydrazide group during the reaction. Triethylamine (80 μL) was added to neutralize the mixture, and the acyl azide thus formed was used immediately for coupling with glycamine.

RESULTS

Optimizing the conditions of the reductive amination with benzylamine.—For the reductive amination reaction of unprotected aldehydes with the ε-NH_2 groups of proteins, neutral to mildly acidic conditions were found to be optimal[27]. However, for reducing oligosaccharides the pH optimum was reported[28] to be 8–9. The reaction between laminarihexaose (20 mM) and the benzylamine reagent (500 mM) in fact gave product in 83, 86, 83 and 73% yields at pH 6, 7, 8, and 9, respectively, after 48 h at room temperature, corresponding more closely to the optimal pH range for the reaction between unprotected aldehyde and ε-NH_2 groups. Although more concentrated reagents are likely to give faster reaction rates and higher yields, too large an excess of benzylamine would make the subsequent purification more difficult. Preliminary results indicated that a 500 mM concentration of the benzylamine reagent gave a good yield within a reasonable reaction time, and thus this concentration was chosen for further experiments below. As expected, the rate of reaction was considerably accelerated at 37 or 55°C (Fig. 2). At 55°C, more than 95% of the oligosaccharide was aminated in 6 h as compared with 33% at room temperature. Furthermore, even when lower concentrations of the oligosaccharide were used, (such as 10 or 5 mM), a 95% yield was attained by a 12-h reaction at 55°C (Table I).

Comparison with reductive amination with NH_4OAc.—We confirmed the inefficiency of reductive amination with ammonium acetate by careful analysis of reaction products as shown in Table II. The reaction rate of laminarihexaose (20 mM) with benzylamine was much faster than with ammonium acetate, especially at room temperature, which makes the benzylamine method particularly useful for some heat-sensitive carbohydrates such as phosphorylated or sialylated oligosaccharides. Moreover, dimeric byproduct was not detectable after an 18-h incubation at 55°C when benzylamine was used (Fig. 1), whereas it was considerable (11.4%) when ammonium acetate was used (Scheme 1). The inefficiency of the ammonium acetate reaction at room temperature could not be significantly improved by increasing the concentration of laminarihexaose or by longer incubation periods (Table III). On the other hand, the overall yield of product increased at the higher concentrations of laminarihexaose.

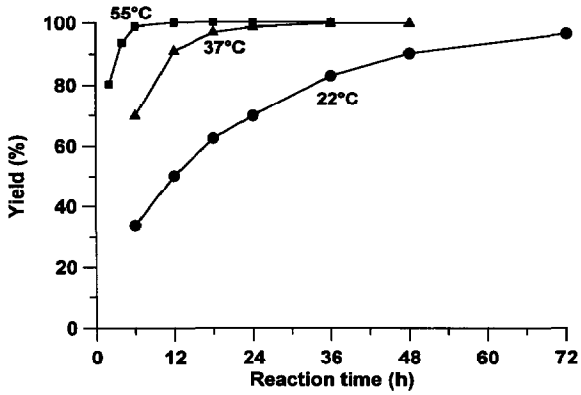


Fig. 2. Time course of the reductive amination of laminarihexaose with benzylamine at various temperatures. Laminarihexaose (20 mM) was reacted with 100 mM pyridine–borane and 500 mM benzylamine reagent at each temperature as described in the Experimental section. The yield was determined by HPAEC.

When lactose was aminated reductively, the reaction rate was notably slower and production of a dimeric byproduct was more noticeable than with laminarihexaose, regardless of whether ammonium acetate or the benzylamine reagent was used (Table IV). However, the benzylamine reagent reacted with 100 mM lactose at 55°C to yield 80% of the desired compound in 36 h, while ammonium acetate led to more than 85% of the dimeric byproduct under comparable conditions. The

TABLE I

Efficiency of amination of laminarihexaose

Reaction time at 55°C (h) [a]	Yields [b] of glycamine (%) at the laminarihexaose concentrations shown				
	20 mM	10 mM	5 mM	2.5 mM	1.25 mM
6	99	91	83	64	43
12	[c]	99	95	85	66

[a] Laminarihexaose at each concentration was reacted with 500 mM benzylamine and 100 mM pyridine–borane. [b] The yields were determined by HPAEC analysis. [c] Not determined.

TABLE II

Comparison of benzylamine and ammonium acetate as reagents for reductive amination

Reagents [a]	Yields [b] at the temperatures shown		
	22°C	37°C	55°C
1 M NH_4OAc	6	28	74
500 mM Benzylamine	63	97	100

[a] Laminarihexaose (20 mM) was mixed with either ammonium acetate or benzylamine and 100 mM pyridine–borane, and reacted at the indicated temperature for 18 h at pH 7. [b] Determined by HPEAC analysis.

TABLE III

Reductive amination using higher concentrations of laminarihexaose

Reagent	Incubation time [a] (h)	Yields [b] of glycamine (%) at the laminarihexaose concentrations shown		
		20 mM	100 mM	400 mM
1 M NH_4OAc	18	5	8	2
	36	9	12 [c]	7
500 mM Benzylamine	18	45	70	74
	36	75	90	89

[a] Laminarihexaose was mixed with either ammonium acetate or benzylamine and 5-fold excess of pyridine–borane. The reaction was carried out at room temperature for the indicated duration. [b] Determined by HPAEC analysis as described in the Experimental section. [c] The dimeric byproduct (2%) was detected under this condition, while < 2% of byproduct was detected in the other runs.

predominant formation of the dimeric byproduct of lactose measured by HPAEC was confirmed by thin layer chromatography (2:1:1, 1-butanol–acetic acid–water), where the dimeric byproduct migrated more slowly (R_f 0.14) than the desired compound (R_f 0.4).

Production and characterization of benzylaminated lactose and laminarihexaose. —We used lactose as a model substrate to study the exact course of the reductive amination. Lactose was conjugated to benzylamine as described in the Experimental section. The reaction mixture gave a single spot (R_f 0.4) on thin layer chromatography in 2:1:1 1-butanol–acetic acid–water. The pure product was obtained after fractionation on a Sephadex LH-20 column (1.4 × 43 cm) in aq 50% methanol. The ^1H NMR spectrum showed the ratio of phenyl protons to protons

TABLE IV

Reductive amination of lactose with ammonium acetate or benzylamine

Reagent	Lactose (mM)	Reaction time [a] (h)	Yields [b] (%) of glycamine (byproduct) at the temperatures shown		
			22°C	37°C	55°C
1 M NH_4OAc	20	18	15 (< 2)	13	23 (24)
	20	36	16 (12)	27 (9)	44 (49)
	100	18	4 (< 2)	9 (6)	8 (86)
	100	36	7 (2)	10 (72)	ND [c]
	400	18	2 (< 2)	3 (45)	3 (90)
	400	36	2 (67)	7 (93)	ND
500 mM	20	18	23 (< 2)	75 (2)	91 (2)
Benzylamine	20	36	35 (< 2)	95	ND
	100	18	20 (< 2)	35 (7)	67 (5)
	100	36	79 (6)	63 (11)	80 (21)
	400	18	10 (8)	29 (29)	36 (31)
	400	36	44 (30)	48 (29)	60 (41)

[a] Lactose in the indicated concentration was reacted with either ammonium acetate or benzylamine in the presence of a 5-fold excess (with respect to lactose) of pyridine–borane. [b] Determined by HPLC with a cation-exchange column as described in the Experimental section. [c] Not determined.

from lactose and methylene groups to be 5.1 to 17, indicating that one molecule of lactose had been conjugated to one molecule of benzylamine. Anal. Calcd for $C_{19}H_{37}NO_{13}$: C, 46.8%; H, 7.65%; N, 2.87%. Found: C, 46.65%; H, 7.11%; N 2.69%

Benzylaminated laminarihexaose was purified on a Bio-Gel P-4 column (1.5 × 92 cm) in 0.1 M acetic acid and subjected to ^1H NMR analysis in D_2O, which indicated a reasonable ratio of 5.1 to 43 for phenyl protons to protons from the hexaose and methylene group.

Reductive amination of mannopentaose phosphate.—MPP was reductively aminated with benzylamine by the same procedure as used for laminarihexaose. The reaction, however, was performed at room temperature to avoid dephosphorylation. Also, 400 mM oligosaccharide was employed to accelerate the reaction. After 48 h, when MPP had completely disappeared by HPAEC analysis, a byproduct, most likely MPP dimerized through benzylamine, was detected to the extent of 2.0–6.5%. The mixture was dried with a Speed-Vac, dissolved in aq 50% methanol, and subjected to hydrogenolysis in a Brown hydrogenator[29] using palladium on activated carbon as a catalyst. Analysis by HPAEC showed that the elution time of the product (MPP-amine) was significantly decreased, presumably due to the removal of benzyl group. After evaporating the solvent methanol, the MPP-amine was purified on a BioGel P-4 column (1.5 × 92 cm) in water to remove remaining excess reagents and dimerized MPP (the first peak in Fig. 3). The fractions collected were positive to both the phenol–sulfuric acid and the trinitrobenzensulfonate reactions, which indicated the presence of a primary amino group associated with carbohydrate. The overall yield was 74% based on phenol–sulfuric acid analysis. In contrast, the reaction of 1.9 M ammonium acetate and 100 mM of

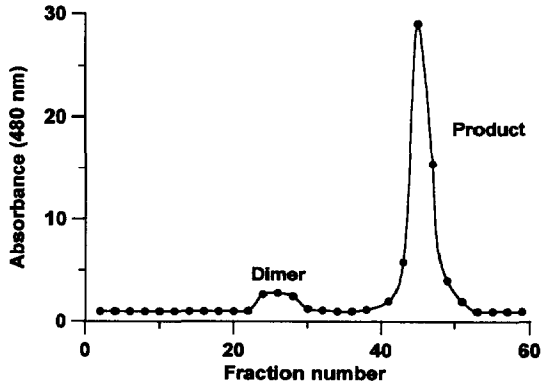


Fig. 3. Gel filtration chromatography of aminated mannopentaose phosphate. Mannopentaose phosphate was reductively aminated with the benzylamine reagent and hydrogenolyzed to give a primary amine. The mixture was fractionated on a BioGel P-4 column (1.5×92 cm) in water. Each fraction (2.5 mL) was analyzed for its carbohydrate content by the phenol–sulfuric acid method.

MPP resulted in less than 50% yield even with incubation at 37°C, a condition which causes loss of the phosphate group (data not shown).

Coupling to the heterobifunctional spacer.—The acyl hydrazide form of the heterobifunctional reagent, carrying a masked aldehyde group at its distal end[12], was converted to acyl azide (see Scheme 1) as described in the Experimental section. Immediately after the acyl azide was formed, sufficient mixture was added to the MPP-amine in water to give a 2.4–11.7 fold excess of reagent. The reaction was completed in 14 h at 4°C as determined by HPAEC. The product eluted earlier than the MPP-amine. The spacer-extended MPP-amine was purified on a Sephadex G-10 column (2.5 × 92 cm) in 0.1 M acetic acid, effluent being monitored by absorbance at 214 nm. The first peak was positive to the phenol–sulfuric acid assay (i.e., carbohydrate-containing), and was pooled and evaporated to dryness.

The pooled intermediate was treated with aq 50% trifluoroacetic acid for 5 h at room temperature to unmask the aldehyde group[12]. When the mixture was evaporated on a Speed-Vac and analyzed by HPAEC, the elution time was found to be longer than that of the acetal form.

Conjugation of MPP to ribonuclease A.—To a solution of 3.3 μmol of the spacer-extended MPP-amine, 75 nmol of ribonuclease A and 3.3 μmol of pyridine–borane were added and the mixture was made to 50 mM with phosphate buffer, pH 7.5 (final volume 300 μL). Since the ribonuclease A molecule possesses 11 primary amino groups including the *N*-terminal group, there was a 4-fold excess of aldehyde per amino group. The reaction was allowed to proceed for 2 days at room temperature. Doubling the amount of aldehydic reagent did not improve the efficiency of coupling (Table V). When the mixture was fractionated on a Sephadex G-50 column (2.5 × 90 cm) eluted with 0.5 mM phosphate buffer, pH 7.0, containing 50 mM Na_2SO_4, it gave two broad protein peaks (monitored by $A_{280\ nm}$) as well as a peak attributable to pyridine. The fluorescamine assay for determining protein content and the phenol–sulfuric acid assay of the saccharides revealed that the earlier peak contained 15.1 mol of MPP per mol of ribonuclease. Interestingly, the later peak showed 19.1 mol of MPP per mol of ribonuclease, nearly the maximal

TABLE V

Conjugation of mannopentaose phosphate (MPP) to ribonuclease A

MPP/RNAse A [a] (mole ratio)	Composition of conjugate eluted from a G-50 column [b] (mol MPP/mol protein [c])	
	Early eluate	Late eluate
44	15	20
88	13	19

[a] MPP, modified with the heterobifunctional spacer by reductive amination, was reacted with RNAse A as described in the Experimental section. [b] Mixture was fractionated on a Sephadex G-50 column in 50 mM Na_2SO_4. [c] The proportions of carbohydrate and protein were determined by the phenol–sulfuric acid and fluorescamine methods, respectively.

number that would result from each amino group reacting with two molecules of aldehyde[30], and being converted into a tertiary amino group. The late elution of this component may be rationalized by the hydrophobic interaction between the spacer groups and the column medium.

The presence of a phosphate group on conjugated MPP was confirmed by monosaccharide analysis by HPAEC after hydrolysis in 2 M trifluoroacetic acid at 100°C for 4 h[23]. Standard mannose and mannose 6-phosphate were treated under the same conditions to obtain correction factors. Hydrolysis under the employed conditions converted 8% of mannose 6-phosphate into mannose. Taking into account the extent of dephosphorylation (8%) and the degradation of mannose (9%) during the hydrolysis, the ratio of mannose to mannose 6-phosphate was calculated to be 1:3.2, which was consistent with the expected value (1:3), and suggests negligible (< 7%) dephosphorylation during the reaction sequences.

DISCUSSION

The preparation of neoglycoproteins containing acid- or alkali-sensitive groups such as phosphate or sialic acids requires a judicious choice of reaction conditions. When glycopeptides are used, there are several mild chemical methods for conjugating to protein[11,31]. When oligosaccharides are to be coupled, it is expedient, as mentioned in the Introduction, to convert them into glycamines. The conventional reaction of glycamine formation, using ammonium salts[17,32] as donors, however, does not proceed efficiently under mild conditions even at higher concentrations of oligosaccharides. Additionally, as we confirmed, such conditions often produce substantial amounts of dimeric byproducts (e.g., for lactose as shown in the present work). The method presented here utilizes benzylamine as an amine source to improve the reaction rate at any temperature and to decrease byproduct formation. A method for the large-scale preparation of 1-aminoalditols from cellobiose, lactose, and maltose utilizing benzylamine has been reported recently[33].

The higher efficiency of this method is due to the ease of formation of Schiff's bases by benzylamine, attributable to the lower basicity of this amine compared with ammonia (therefore a higher concentration of unprotonated amine), and to the difficulty of tertiary amine formation. Although it requires an extra step of hydrogenation to unmask the amino group, the advantages of the method outweigh this minor inconvenience.

We also attempted to use dibenzylamine as the amino group donor to suppress byproduct formation even further. However, this avenue was not pursued because dibenzylamine is not readily soluble in the aqueous media usually required for large or charged oligosaccharides.

Once the amino group is introduced into the target oligosaccharide, conjugation to a protein becomes quite easy. In the present work, we used our previously developed heterobifunctional reagent[12] to provide some spacing between the protein and the oligosaccharide as well as to provide a reactive unmasked aldehyde

group for effective reductive amination. Indeed, we could obtain nearly a maximal incorporation (to the tertiary amine stage) of mannopentaose phosphate into ribonuclease A with only a 4-fold excess of the aldehyde-containing reagent with respect to protein primary amino groups. Furthermore, the fact that an 8-fold excess of derivatized oligosaccharide did not improve the coupling efficiency suggests that the aldehydic group in this heterobifunctional reagent is extremely reactive and allows almost quantitative coupling under mild conditions.

The mildness of the reaction conditions described here is attested by the fact that the HPAEC analysis of hydrolyzed neoglycoprotein formed from mannopentaose phosphate and ribonuclease revealed that 94% of the phosphate group was preserved.

Although we report here only a limited exploration of the coupling of oligosaccharides to ribonuclease A by introducing an amino group at the reducing end of the sugars with benzylamine, the methodology used should be applicable to the conjugation of many other oligosaccharides (including sialyl oligosaccharides) to proteins.

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