Solid-Phase Oligosaccharide and Glycopeptide Synthesis Using Glycosynthases

Jakob F. Tolborg,[†,‡] Lars Petersen,[†,‡] Knud J. Jensen,*[,†,§] Christoph Mayer,[⊥] David L. Jakeman,[⊥] R. Antony J. Warren,[∥] and Stephen G. Withers*[,⊥]

Department of Chemistry, Building 201, Kemitorvet, Technical University of Denmark, DK-2800, Kgs. Lyngby, Denmark, and Protein Engineering Network of Centres of Excellence, Department of Chemistry and Department of Microbiology, University of British Columbia, 2036 Main Mall, Vancouver, British Columbia V6T 1Z1, Canada

kjj@kvl.dk

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Enzymatic approaches for the preparation of oligosaccharides are interesting alternatives to traditional chemical synthesis, the main advantage being the regio- and stereoselectivity offered without the need for protecting groups. The use of solid-phase techniques offers easy workup procedures and the prospect of automatability. Here, we report the first application of glycosynthases to solid-phase oligosaccharide synthesis by use of the 51 kDa serine and glycine mutants of *Agrobacterium* sp. *β*-glucosidase, Abg E358S and E358G. Acceptors were linked to PEGA resin through a backbone amide linker (BAL), and using these mutated enzymes, a galactose moiety was transferred from a donor sugar, α-D-galactosyl fluoride, with high efficiency (>90%) together with excellent recovery of material. Furthermore, it was demonstrated that a resin-bound model glycopeptide was also an acceptor for the glycosynthase.

Oligosaccharides play numerous roles in biological recognition processes through, for example, their location on cell surfaces. A better understanding of these recognition events will assist in the design of new drug candidates against a wide range of illnesses including cancer and HIV.[1] A problem in studying carbohydrate interactions is the limited access to well-defined oligosaccharides and purification of oligosaccharides from natural sources is tedious. While the synthesis of complex oligosaccharides is quite feasible, no general glycosylation protocol has yet been developed, making each structure a challenging synthetic target.[2] There is a need for parallel and combinatorial synthesis of oligosaccharides for the study of carbohydrate interactions, and the field of solid-phase oligosaccharide synthesis has gained much attention over the past decade.[3]

Enzymatic approaches for the preparation of oligosaccharides are interesting alternatives to traditional chemical synthesis, the main advantage being the regio- and stereoselectivity offered without the need for protecting groups. Glycosidases have been employed for this task but typically give low yields due to competing product hydrolysis. Glycosyl transferases have seen only limited use until recently due to problems with their solubility and availability, though recombinant DNA technology is quickly solving this problem. Solution-phase enzymatic glycosylations[4] using glycosyl transferases have received increasing attention, and following early work by Zehavi,[5] the groups of Wong,[6] Meldal and Palcic,[7] Thiem,[8] Köpper,[9] Lee,[10] and Norberg[11] have successfully applied these enzymes to solid-phase oligosaccharide synthesis.[12]

A glycosynthase is a mutated retaining glycosidase with the active-site carboxylate nucleophile replaced by a non-nucleophilic amino acid side chain.[13] The glycosidase thus loses its ability to hydrolyze glycosidic bonds, but it can instead catalyze the glycosylation of sugar acceptors using glycosyl fluoride donors (Scheme 1). The first glycosynthase was reported by Withers, Warren, and co-workers in 1998,[13] and others have since been reported.[14,15] Here, we present the first application of

[†] Technical University of Denmark.
[‡] The first two authors contributed equally to this work.
[§] Present address: Department of Chemistry, Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg, Denmark.
[⊥] Department of Chemistry, University of British Columbia.
[∥] Department of Microbiology, University of British Columbia.

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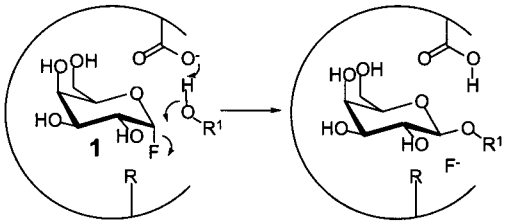
Scheme 1. Generalized Mechanism of Glycosynthases Using α-D-Galactosyl Fluoride, 1, as Donor[a]



[a] R is the side chain of the mutated residue (R: H in Abg E358G; CH_2OH in Abg E358S; $(CH_2)_2COO^-$ in wild type).[13]

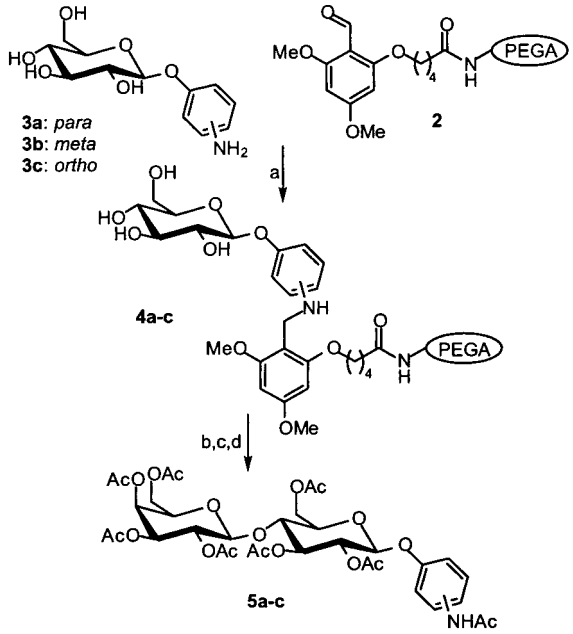
glycosynthases to solid-phase oligosaccharide synthesis by use of the 51 kDa serine and glycine mutants of *Agrobacterium* sp. β-glucosidase, Abg E358S,[15] and E358G[16] to transfer a galactose moiety from the donor sugar α-D-galactosyl fluoride (**1**) with high efficiency.

An important first consideration in these studies was the choice of resin, which must swell extensively in aqueous buffers to provide access to the enzyme. This requirement is met by poly(ethylene glycol) polyacrylamide copolymers (PEGA).[17] The low-loading (0.2 mmol/g) PEGA resin has excellent swelling abilities in water (31.7 mL/g) and a good permeability toward macromolecules (60−70 kDa).[17c] Meldal et al. have demonstrated that an *N*-glycodipeptide bound to a PEGA support was a substrate for a glycosyl transferase yielding a disaccharide glycodipeptide.[7]

The second key consideration is the choice of linker by which the acceptor sugar is attached to the resin. Jensen and co-workers recently introduced the backbone amide linker (BAL)[18] to solid-phase oligosaccharide synthesis.[19] In this strategy, an amino group in the ligand is linked to resin-bound *o*-PALdehyde (**2**) via reductive amination to form a TFA-stable secondary amine. After completion of all reactions, the linker can be cleaved by N-acylation to increase the acid-lability of the handle, followed by treatment with TFA−H_2O (19:1) to release the modified ligand.[19]

Attempts to use a glucosamine derivative anchored through the 2-amino group by a BAL handle as acceptor were unsuccessful, presumably due to unfavorable steric interactions and for recognition of the 2-position by the enzyme. Likewise, cellobiosylamine attached via the squarate linker[20] used by Blixt and Norberg[11] was also a poor acceptor. Instead, on the basis of the previously noted preference of Abg for aryl glycoside acceptors, readily available aminophenyl glycosides such as **3a** linked via a squarate linker were synthesized and shown to function as useful acceptors. However, their release from the resin proved more difficult than anticipated. Instead, **2** was used as a handle for the attachment of three regioisomers of aminophenyl β-D-glucoside (**3a**−**c**, Scheme 2). Sugars linked in this way could indeed be released by standard protocols, i.e., acetylation followed by treatment with TFA−H_2O (19:1). Although phenylamino derivatives could be released without prior acylation, which gave a free amine useful for immobilization or conjugation, the acetylation step was maintained for analytical reasons.

Scheme 2. Solid-Phase Attachment of Aminophenyl Glucosides and Glycosylations Using Glycosynthases[a]



[a] Key: (a) $NaBH_3CN$, AcOH−MeOH (1:99); (b) Abg glycosynthase (1.0 mg/mL), α-D-galactosyl fluoride (20 mM), NaP_i buffer (100 mM); (c) Ac_2O−pyridine (2:1); (d) TFA−H_2O (19:1).

Preliminary results indicated that **4b** (ortho) and **4c** (meta) regioisomers were inferior (often with no or very low degrees of glycosylation) to **4a** (para) as resin-bound acceptors, thus qualifying the latter only for further study. The sodium phosphate buffer concentration was kept at 100 mM, pH 7.0; lowering the concentration or removing the buffer led to significant premature cleavage by liberated hydrogen fluoride during glycosylation. Likewise, the presence of additional salts (e.g., NaCl) was observed to reduce glycosylation rates as much as 30%, most likely because high salt concentrations reduce swelling of the resin. For the same reason, enzyme stock solutions were desalted immediately prior to use. The optimal concentration of the glycosyl donor **1** was found to be 20 mM and an enzyme concentration of 1.0 mg/mL was used throughout all experiments. The use of downloaded resins carrying only ∼30% of the maximum acceptor-loading did increase the degree of conversion; however, it lowered the capacity of the resin and still failed to provide complete glycosylation.

Handling of the resin, as well as accurate measurement of resin loading, was facilitated by drying of the resin

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Table 1. Enzymatic Glycosylation of Dried vs Nondried Resin[a]

entry	acceptor	enzyme	product[b] (%)	recovery[c] (%)
1	**4a**, nondried	Abg E358S	63	NA[d]
2	**4a**, nondried	Abg E358G	79	NA[d]
3	**4a**, dried	Abg E358S	63	91
4	**4a**, dried	Abg E358G	80	92

[a] Enzyme (1.0 mg/mL) and **1** (20 mM) in NaP_i (100 mM, pH 7.0), 24 h. [b] Amount of disaccharide relative to combined amounts of monosaccharide and disaccahride. [c] Total amount of mono- and disaccharide compared to determined loading. [d] Not available, as weighing of swelled PEGA resin is inaccurate for small amounts.

prior to use. If PEGA resin is dried after a normal washing procedure, it becomes very sticky and difficult to handle. For this reason, PEGA is purchased swelled in methanol and the manufacturer recommends handling the resin only swelled in an appropriate solvent depending on the reaction in which it is to be used. However, this approach is not well suited for glycosylation reactions where small amounts of resin typically are weighed off numerous times. If the swelled resin either loses solvent due to evaporation or takes up water from the air caused by hygroscopic resin or solvent, the effective loading will vary to an unsatisfactory degree. Therefore, it was desirable to develop a way of handling the resin in the dry state. Poly(ethylene glycol) (PEG) is insoluble in diethyl ether, a fact that is exploited in the use of MPEG soluble supports, and we reasoned that the PEGA resin could be crystallized or collapsed in a controlled fashion by treatment with diethyl ether. After a normal washing procedure, successively increasing concentrations of diethyl ether in DCM (1 × 5 min with 25%, 50%, and 75% solutions) caused the resin to collapse gradually. Finally, it was washed with neat diethyl ether (2 × 5 min each) and dried in vacuo to constant weight. This resin was slightly sticky, but firm, beadshaped, and easier to handle than PEGA in the swelled state, thus small quantities (10−20 mg) were readily handled with plastic spatulas. The loadings of resin-bound acceptors were established to be 0.15 mmol/g (80%) for **4a** and 0.12 mmol/g (65%) for both **4b** and **4c** based on RP-HPLC standard curves.

However, it is noteworthy that drying the resin prior to derivatization with acceptor glycoside was observed to reduce rates of enzymatic glycosylation significantly (<20% galactosylation of **4a** within 24 h). This is believed to arise from irreversible changes or aggregation within the resin structure. However, when the resin was dried after reductive amination, no significant difference was observed between permanently swelled (nondried) and dried resin (Table 1). Thus, drying of resin could be performed without affecting the efficiency of glycosylation. The recovery of oligosaccharides from the latter was virtually quantitative, reflecting the high attach-release efficiency of the linker.

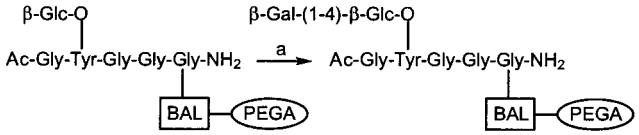
The above results also show that the glycine mutant, Abg E358G, in each case gave superior yields in the glycosylation step than did the serine mutant Abg E358S. This is an interesting observation since it was expected that the serine side chain via hydrogen bonding could mediate the leaving of fluoride anions from the donor (Scheme 1). It seems probable that, by analogy with results on the E358D mutant,[21] interactions of the departing fluoride with Tyr298 in the glycine mutant might be the source of the improved glycosylation efficiency. Further studies indicated that reactions in alkaline buffers were optimal, possibly due to the acid sensitivity of Abg nucleophile mutants. Thus, galactosylation of resin bound **4a** with Abg E358G at pH 9.0 and room temperature proceeded with a high degree of substrate conversion (83−90% within 48 h) followed by excellent recovery of the product (>90%).

Table 2. Enzymatic Glycosylation of 4a Using Abg E358G[a]

entry	acceptor	reaction time (h)	product[b] (%)	recovery[c] (%)
1	**4a**, dried	24	83	98
2	**4a**, dried	48	90	90
3	**4a**, dried	72	91	90

[a] Abg E358G (1.0 mg/mL) and **1** (20 mM) in NaP_i (100 mM, pH 9.0). [b] Disaccharide to monosaccharide. [c] Total amount of mono- and disaccharide compared to determined loading.

Scheme 3. Structure and Enzymatic Glycosylation of Model Glycopeptide[a]



[a] Key: (a) Abg E358G (1.0 mg/mL), **1** (20 mM), NaP_i (100 mM pH 9.0).

Next, we extended our investigations to glycopeptide acceptors. For this purpose, a resin-bound pentapeptide with a β-D-glucosylated tyrosine,[22] a model for glycogenin,[23] was synthesized on PEGA resin using a BAL strategy.[18] The peptide was assembled from Fmoc-protected amino acid pentafluorophenyl esters[24] and standard peptide coupling reagents.

The resin-bound glycopeptide was then galactosylated using conditions given in Table 2 (Scheme 3). Upon per-O-acetylation and cleavage, HPLC indicated a 52% conversion to the disaccharide glycopeptide after 48 h, which was verified by NMR and HRMS.

In conclusion, we have shown that the Abg E358G glycosynthase can efficiently catalyze glycosylation of resin-bound acceptor glycosides. Furthermore, it was shown that glycopeptides can act as acceptors after assembly of the peptide, thus demonstrating a synthetically advantageous tolerance of acceptor structure. However, further work with a broader range of glycopeptides is needed to demonstrate the generality of this approach. Likewise, glycosynthases hold great promise for solid phase oligosaccharide synthesis due to the facility of the technique and the fact that simple and readily available glycosyl fluorides are used as donors.

Experimental Section

General Procedures. Melting points were uncorrected. Solvents were distilled and/or stored over 3 or 4 Å molecular

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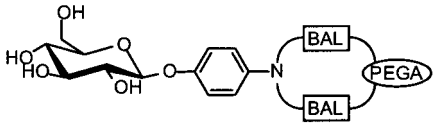

Figure 1. Reductive amination in DMF on a PEGA resin resulted in double alkylation of the amine.

sieves as appropriate. ^{1}H and ^{13}C NMR spectra were recorded on either a Varian Unity Inova 500 operating at 499.87 MHz for ^{1}H and 125 MHz for ^{13}C equipped with a *z*- (single axis) PFG inverse detection C−H−P probe or a Bruker AV-400 operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Chemical shift (δ) values are in ppm (residual solvent peak); coupling constants (J) are in Hz. All assignments were supported by 2D homonuclear chemical-shift correlation spectroscopy (gCOSY). Elemental analysis, MS, and HRMS were conducted at the Department of Chemistry, University of British Columbia. Thin-layer chromatography was performed on Merck silica gel 60 F_{254} plates, and spots were visualized by UV light at 254 nm and/or spraying with 10% aq. H_2SO_4 followed by heating. Amines were visualized with ninhydrin in EtOH. HPLC analysis were carried out on a Waters system (600 control unit, two-channel UV/vis detector or 996 PDA, 717 Plus autosampler, Millenium32 control software) on a Waters Nova-Pak C18 column (3.9 × 150 mm; 4 mm particle size), Waters XTerra C18 cartridge (3.0 × 50 mm, 3.5 mm particle size), or a Phenomenex Kingsorb C18 column (4.6 × 150 mm; 3 mm particle size) using a gradient of CH_3CN in water. Integrations were performed at 215 and 265 nm or at individual λ_{max} as indicated. Purity of compounds was determined from integrations at 215 nm. PEGA resin was obtained from Polymer Laboratories. Water used for buffers and washing procedures was deionized and filtrated. $NaBH_3CN$ (95% purity) was purchased from Aldrich. Enzyme stock solutions were kept refrigerated (5 °C). Buffers were made by mixing solutions of Na_2HPO_4 and $NaH_2PO_4{\cdot}H_2O$ (J. T. Baker).

Preparation of Resins. PEGA resin (loading of 0.20 mmol/g; swollen in MeOH; 9.41 wt %) was washed with 2 × DMF, 2 × DIEA−DMF (1:49), 1 × DMF. *o*-PALdehyde was coupled using a standard peptide coupling protocol by dissolving *o*-PALdehyde, BOP, and HOBt (2.0 equiv each) in DMF. DIEA (5 equiv) was added, and the mixture was allowed to stand for 5 min and then added to the above resin. After the mixture was shaken for 16 h (400 min^{-1}), the resin was washed with 3 × DMF, 3 × DCM−MeOH (2:1), and 5 × DCM. A Kaiser test (ninhydrin) at this point was clearly negative, but an acetylation with Ac_2O−pyridine (2:1) for 16 h was maintained nevertheless. After another washing (3 × DMF, 3 × DCM−MeOH (2:1), 5 × DCM), one of three regioisomers of aminophenyl β-D-glucopyranoside (**3a**−**c**, 5 equiv) was coupled by reductive amination in the presence of $NaBH_3CN$ (10 equiv) in AcOH−MeOH (1:99). After shaking for 16 h, resins were washed again (3 × DMF, 3 × DCM−MeOH (2:1), and 5 × DCM) and collapsed with diethyl ether in DCM as in the above-mentioned sequences.

Notes on Reductive Amination. Reductive aminations are generally easier to control when the aldehyde is attached to a solid support, due to site isolation on the resin preventing double alkylation of the amine. However, because reactive sites in PEGA resins are at the ends of flexible PEG chains, site-isolation is much less pronounced compared to polystyrene resins. Use of DMF as solvent for the reductive amination resulted in a yield of only 43% (**3a**). Presumably, this is due to double alkylation (Figure 1), as repeating the reductive amination did *not* increase the yield. However, substituting DMF for methanol as solvent increased the yield to 80%. It is known that methanol suppresses double alkylation in solution-phase reductive aminations.[18a]

Determination of Loadings. Resin (10−20 mg) was weighed off in 3 mL disposable syringes fitted with a polypropylene filter and a Teflon stopcock. The resin was acetylated in Ac_2O−pyridine (2:1) for 16 h or treated directly with TFA−water (19:1) for 2 × 30 min and washed with DCM (5×), and the combined filtrates were concentrated to dryness. The residue was dissolved into a total of 5.0 mL of acetonitrile or 0.1% aqueous TFA for the free amine. A sample of this solution was analyzed by HPLC giving the calculated loadings presented in Table 3. The procedure was repeated once to produce the second set of loadings, i.e., the amount of acceptor remaining after the first cleavage.

Table 3. Determined Loading of Acceptor after 2 × 30 min (Cleavage with TFA−Water (19:1))

			loading (mmol/g)		
entry	acceptor	λ_{max} (nm)	first cleavage	second cleavage	total[a] (%)
1	**4a**	215	*b*	*b*	*b*
		268	0.113	0.045	0.158 (87)
2	**4a** (acetylated)	245	0.133	0.014	0.147 (81)
		280	0.132	0.013	0.145 (80)
3	**4b** (acetylated)	243	0.117	0.002	0.119 (66)
		278	0.114	0.001	0.115 (65)
4	**4c** (acetylated)	243	0.097	0.020	0.117 (65)
		278	0.098	0.021	0.119 (66)

[a] Calculated from an initial loading of 0.2 mmol/g giving a maximum possible acceptor loading of 0.181 mmol/g. [b] Standard curve not linear at 215 nm caused by absorbance of injection peak.

These data showed that cleavage times of 60 min instead of 30 min were required. However, determined loadings for **4b** (meta) and **4c** (ortho) were lower than expected. This can be explained by lower nucleophilicity, caused by steric encumbrance, of the amine (**3b** and **3c**) as compared to **3a** (para). However, **4a** (para) shows loadings in the expected range (∼80%).

Choice of Cleavage Conditions. Although the resin-bound phenylamino glucoside could be released without prior acetylation as previously shown, the acetylation was performed for three reasons. First, the rate of cleavage was lower for the nonacetylated than for the acetylated system. For the acetylated system, a smaller amount (10% of total) of product was observed in the second cycle, whereas for the nonacetylated system a significant amount (28% of total) was observed. Long cleavage times seemed to give small amounts of byproducts, and a significant amount of an unpolar compound was formed in the nonacetylated system. Second, because of the polar and basic nature of the free amine, an eluent system containing 0.1% TFA was required to obtain integratable peaks. However, the free amine eluted in the injection peak, thus hampering accurate integration at 215 nm. More accurate results were obtained at 268 nm, a local λ_{max} for 4-aminophenyl β-D-glucopyranoside, but the absorbance at this wavelength was much lower, thus demanding injection of larger amounts of compound. Third, the stability of this free amine was also questionable as seen in workup problems and seemingly low yield for the synthesis.

Solid-Phase Glycosylations. Resin (10−20 mg) was weighed off in 3 mL disposable syringes fitted with a polypropylene filter and a Teflon stopcock. Enzyme was prepared using a Pharmacia Hi-Trap desalting column equilibrated in the required buffer, thus eliminating salts from stock solution. Donor (α-D-galactosyl fluoride, **1**) was weighed off in 1.5 mL centrifuge tubes, dissolved in the required buffer, and added to desalted enzyme solution (final 1.0 mg/mL). This reaction mixture (total 1.0 mL) was then added to the resin. Reaction mixtures were shaken at 400 min^{-1} for 24 h or as indicated and then washed with 5 × water, 3 × DMF, 3 × DCM−MeOH (2:1), and 5 × DCM. The mixtures were acetylated (Ac_2O−pyridine 2:1, containing a catalytic amount of DMAP) for 16 h, washed again (3 × DMF, 3 × DCM−MeOH (2:1), 5 × DCM), and finally cleaved (TFA−H_2O 19:1, 60 min) and analyzed as above.

4-Aminophenyl β-D-Glucopyranoside 3a. 4-Nitrophenyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (3.75 g, 8 mmol) was deprotected by Zemplén deacetylation (MeOH, 150 mL, and cat. NaOMe) overnight. The solution was neutralized with AcOH until pH 5, Pd/C was added (ca. 280 mg, 10% Pd based on dry weight, contains 50% water), and the mixture was

hydrogenated overnight at 1 atm. Upon filtration through Celite and concentration, the crude syrup turned brown on standing. The coloration was removed by flash chromatography on silica gel, but returned upon evaporation of appropriate fractions. The syrup was crystallized from EtOH, and a brownish solid was collected by filtration (0.87 g, 40%). Product was pure by TLC (R_f 0.55; DCM−acetone−MeOH 6:2:2) and gave a bright pink color with ninhydrin.

An aliquot was treated with Ac_2O−pyridine (3:1) and characterized as the peracetate: colorless foam; no clear mp (in agreement with ref 25); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.67 (1H, s), 7.28 (2H, ddd, J = 2.1, 3.2, 9.0 Hz), 6.90 (2H, J = 2.1, 3.2, 9.0 Hz), 5.25 (1H, t, J = 9.4 Hz), 5.20 (1H, dd, J = 7.5, 9.4 Hz), 5.12 (1H, dd, J = 9.2, 9.8 Hz), 4.97 (1H, d, J = 7.5 Hz), 4.25 (1H, dd, J = 5.3, 12.3 Hz), 4.11 (1H, dd, J = 2.3, 12.3 Hz), 3.80 (1H, ddd, J = 2.3, 5.2, 9.9 Hz), 2.10 (3H, s), 2.03 (3H, s), 2.02 (3H, s), 2.00 (3H, s), 1.98 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.5, 170.1, 169.4, 169.3, 168.4, 153.3, 133.6, 121.5, 117.6, 99.5, 72.6, 71.9, 71.1, 68.2, 61.8, 24.2, 20.6 (two C), 20.5 (two C); MS m/z calcd for $C_{22}H_{28}NO_{11}$ 482.17 (M + H), found 482.2; UV 245 nm (ϵ 17.6 × 10^3 M^{-1} cm^{-1}). Anal. Calcd for $C_{22}H_{27}NO_{11}$: C, 54.88; H, 5.65; N, 2.91. Found: C, 55.06; H, 5.69; N, 3.05.

3-Aminophenyl β-D-Glucopyranoside 3b. 2,3,4,6-Tetra-*O*-acetyl-α-D-glucosyl bromide (1.23 g, 3.00 mmol) in acetonitrile (5 mL) was added to a suspension of 3-nitrophenol (0.501 g, 3.60 mmol), silver carbonate (0.496 g, 1.8 mmol), and DIEA (0.627 mL, 3.60 mmol) in acetonitrile (10 mL). After reaction overnight under Ar, the reaction mixture was concentrated. The residue was dissolved in EtOAc (100 mL), extracted with 1 M H_2SO_4 (2 × 25 mL), 0.5 M NaOH (2 × 15 mL), and brine (2 × 25 mL), dried ($MgSO_4$), and concentrated. One recrystallization from EtOH−H_2O (2:1) yielded 0.846 g (60%) of a colorless solid (98% pure by RP-HPLC, 215 nm). This material was deprotected and hydrogenated as described for **3a**, which upon concentration yielded an oil that crystallized from EtOH and was lyophilized (petroleum ether) to give an essentially quantitative yield containing a small amount of sodium acetate. The product was pure by TLC (R_f 0.57; DCM−acetone−MeOH 6:2:2) and gave a bright pink color with ninhydrin.

An aliquot was treated with Ac_2O−pyridine (3:1) and characterized as the peracetate: colorless foam; no clear mp; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 (2H, m), 7.18 (1H, t, J = 8.1 Hz), 7.04 (1H, J = 7.9 Hz), 6.71 (1H, d, J = 7.9 Hz), 5.29−5.20 (2H, m), 5.14 (1H, dd, J = 9.2, 9.6 Hz), 5.07 (1H, d, J = 7.4 Hz), 4.27 (1H, dd, J = 4.7, 12.3 Hz), 4.15 (1H, dd, J = 1.5, 12.3 Hz), 3.89−3.80 (1H, m), 2.13 (3H, s), 2.04 (3H, s), 2.03 (3H, s), 2.01 (3H, s), 2.00 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.6, 170.2, 169.4, 169.3, 168.3, 157.2, 139.2, 129.7, 114.3, 112.7, 108.7, 99.0, 72.8, 72.1, 71.1, 68.2, 61.8, 24.6, 20.6 (four C); MS m/z calcd for $C_{22}H_{28}NO_{11}$ 482.17 (M + H), found 482.2; UV 243 nm (ϵ 14.8 × 10^3 M^{-1} cm^{-1}), 278 nm (ϵ 3.42 × 10^3 M^{-1} cm^{-1}). Anal. Calcd for $C_{22}H_{27}NO_{11}$: C, 54.88; H, 5.65; N, 2.91. Found: C, 54.59; H, 5.78; N, 2.98.

2-Aminophenyl β-D-Glucopyranoside 3c. 2-Nitrophenyl β-D-glucopyranoside (Sigma) was hydrogenated as described for **3a**. After removal of catalyst and solvent, an essentially quantitative yield of a white solid was obtained directly, again containing small amounts of salts. Product was pure by TLC (R_f 0.45; DCM−acetone−MeOH 6:2:2) and gave a bright pink color with ninhydrin.

An aliquot was treated with Ac_2O−pyridine (3:1) and characterized as the peracetate: colorless crystals; mp 181−183 °C (lit.[25] mp 185−187 °C); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.36 (1H, dd, J = 1.5, 8.1 Hz), 7.82 (1H, s), 7.04 (1H, dt, J = 1.5 Hz, 8.1 Hz), 6.97 (1H, dt, J = 1.5 Hz, 8.1 Hz), 6.91 (1H, dd, J = 1.5, 8.1 Hz), 5.34 (1H, dd, J = 9.0, 9.8 Hz), 5.28 (1H, dd, J = 7.5, 9.8 Hz), 5.12 (1H, dd, J = 9.1, 9.3 Hz), 5.00 (1H, d, J = 7.6 Hz), 4.31 (1H, dd, J = 5.4, 12.4 Hz), 4.14 (1H, dd, J = 2.4, 12.4 Hz), 3.88 (1H, ddd, J = 2.3, 5.4, 12.4 Hz), 2.18 (3H, s), 2.05 (6H, 2 × s), 2.03 (6H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.4, 170.4, 169.9, 169.4, 168.6, 145.0, 128.9, 123.8, 123.4, 120.3, 113.7, 99.8, 72.1, 71.8, 71.4, 68.2, 61.7, 24.5, 20.8, 20.6, 20.5 (two C); MS m/z calcd for $C_{22}H_{28}NO_{11}$ 482.17 (M + H), found 482.4; UV 243 nm (ϵ 13.9 × 10^3 M^{-1} cm^{-1}), 278 nm (ϵ 3.85 × 10^3 M^{-1} cm^{-1}). Anal. Calcd for $C_{22}H_{27}NO_{11}$: C, 54.88; H, 5.65; N, 2.91. Found: C, 55.06; H, 5.87; N, 2.87.

Standard Curves. The above peracetates were used as basis for quantification of resin loadings and solid-phase glycosylations monitored at individual λ_{max}. An aliquot (20 mg) of each was dissolved in acetonitrile (10.0 mL total volume) and diluted 10 times. From this solution different volumes were injected and corresponding areas recorded. The relationship between injected nmol and area was linear in the range up to two absorbance units, and calculated correlation coefficients were 0.9998 or better.

4-Acetamidophenyl 2,3,6-Tri-*O*-acetyl-4-(2,3,4,6-tetra-*O*-acetyl-β-D-galactosyl)-β-D-glucopyranoside 5a. 4-Aminophenyl β-D-glucopyranoside **3a** (27.2 mg, 1.0 mmol) and α-D-galactosyl fluoride **1** (21.9 mg, 1.2 mmol) were dissolved in NaP_i buffer (100 mM, pH 8.0, 1232 mL), Abg E358S stock solution (5.6 mg/mL, 268 μL; total 1.5 mg enzyme) was added, and the solution was shaken for 17 h at 400 min^{-1}. TLC (EtOAc−MeOH−H_2O 7:2:1): R_f 0.32 (gave a bright pink color with ninhydrin). No trace of starting material could be observed (R_f 0.80). The reaction mixture was concentrated to dryness, acetylated with Ac_2O−pyridine (2:1), poured over crushed ice, and extracted with CH_2Cl_2 (2 × 50 mL). Combined organic phases were washed with 1 M H_2SO_4 (50 mL) and aq $NaHCO_3$ (50 mL), dried ($MgSO_4$), and concentrated to a glass (67 mg). The product was separated from a byproduct, formed during the prolonged acetylation, by flash chromatography on silica gel using EtOAc/petroleum ether (PE). The compound with R_f 0.67 (EtOAc) was isolated and crystallized from ether/PE, giving 33.1 mg (43%) of title compound as a colorless solid: no clear mp (approximately 110−130 °C); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.41 (1H, d, J = 9.0 Hz), 7.09 (1H, s; NH), 6.95 (1H, d, J = 9.0 Hz), 5.36 (1H, dd, J = 0.7, 3.4 Hz; H-4′), 5.28 (1H, t, J = 9.0 Hz; H-3), 5.15 (1H, dd, J = 8.1, 9.0 Hz; H-2), 5.13 (1H, dd, J = 7.7, 10.2 Hz; H-2′), 4.99 (1H, d, J = 8.1 Hz; H-1), 4.97 (1H, dd, J = 3.4, 10.2 Hz, H-3′), 4.52 (1H, d, J = 7.7 Hz; H-1′), 4.56 (1H, dd, J = 2.1, 11.0 Hz; H-6_a), 4.17−4.15 (2H, m; H-6_b, H-6_a′), 4.10 (1H, dd, J = 7.7, 11.5 Hz; H-6_b′), 3.89 (1H, t, J = 9.4 Hz; H-4), 3.88 (1H, dd, J = ∼1.0, 7.7 Hz; H-5′), 3.76 (1H, ddd, J = 1.5, 2.3, 5.9 Hz; H-5), 2.17, 2.16, 2.09, 2.08, 2.07, 2.07, 2.06, 1.973 (total: 24H, 8 × s); ^{13}C NMR (100 MHz, $CDCl_3$) d 170.4, 170.3, 170.1, 170.0, 169.7, 169.6, 169.1, 168.2, 153.5, 133.4, 121.5, 117.7, 101.1, 99.3, 76.2, 72.9, 72.8, 71.5, 71.0, 70.8, 69.1, 66.6, 62.0, 60.8, 24.5, 20.8−20.5 (m); MS m/z calcd for $C_{34}H_{43}NNaO_{19}$ 792.2 (M + Na), found 791.8. Anal. Calcd for $C_{34}H_{43}NO_{19}$: C, 53.06; H, 5.63; N, 1.82. Found: C, 52.96; H, 5.81; N, 1.90.

Title compound was identical with product from solid-phase glycosylations with respect to HPLC retention times.

Peptide Synthesis. PEGA resin (3.21 g, swelled in MeOH, 9.41% w/w) was washed and derivatized with PALdehyde as described above. Without drying after washing, the resin was subjected to reductive amination with glycine amide hydrochloride (133 mg, 1.20 mmol, 20 equiv) in the presence of $NaBH_3CN$ (78 mg, 1.20 mmol, 20 equiv) in AcOH−MeOH (1:99). After being shaken for 24 h, the resin was washed (3 × DMF, 3 × DCM−MeOH (2:1), and 5 × DCM). The second amino acid was coupled as the symmetrical anhydride: Fmoc-Gly-OH (178 mg, 0.60 mmol, 10 equiv) and dicyclohexyl carbodiimide (62 mg, 0.30 mmol, 5 equiv) were dissolved in freshly distilled DCM (8 mL). After being stirred for 15 min, the reaction mixture was filtered and concentrated. Resin was washed with freshly distilled DCM (3×), the symmetrical anhydride in freshly distilled DCM (4 mL) was added, and the mixture was shaken for 90 min followed by washing (3 × DCM). The sequence was repeated once followed by washing (3 × DCM and 3 × DMF). Remaining amino groups were capped by acetylation (5 mL DMF−Ac_2O−DIEA 18:1:1) for 16 h, followed by washing (3 × DCM and 3 × DMF). An aliquot of resin was dried and treated with piperidine−DMF (1:4) for Fmoc-quantification[18a] (λ_{max} 301 nm, ϵ = 7800 cm^{-1} M^{-1}) giving

(25) Wagner, G. *Pharmazie* **1960**, *15*, 609−614.

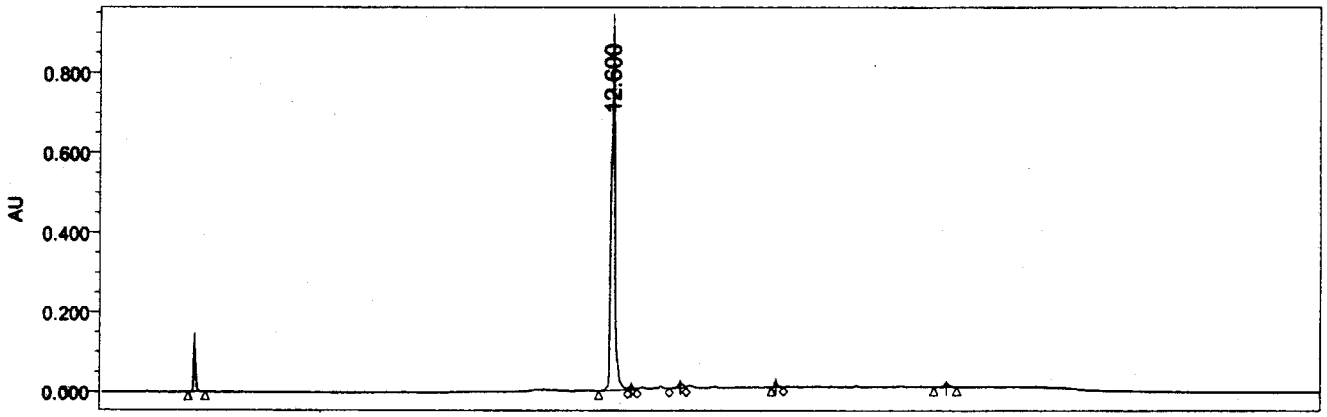

Figure 2. RP-HPLC of peracetylated monosaccharide glycopeptide (t_R = 12.60 min, 215 nm) (column: Phenomenex Kingsorb C18).

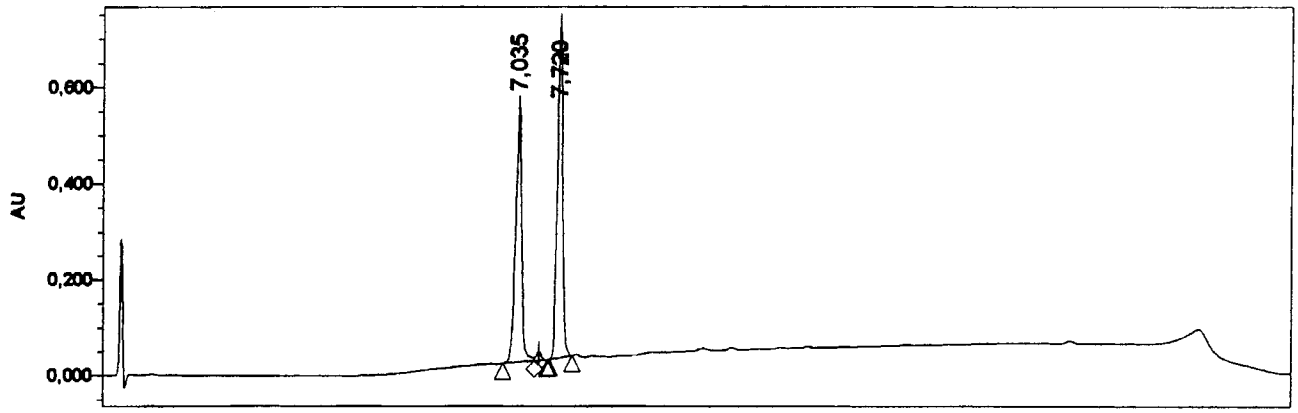

Figure 3. RP-HPLC of peracetylated mono- and disaccharide glycopeptide (t_R = 7.04 and 7.72 min, respectively, 215 nm). t_R = 7.04 min is identical with t_R = 12.60 in Figure 2 (column: Waters XTerra).

a loading of 0.089 mmol/g (50% of maximum loading). The remaining resin was deprotected with piperidine−DMF (1:4, 2 × 5 min. and 1 × 15 min) and then washed (5 × DMF). The third amino acid (Gly) was coupled as the pentafluorophenyl (Pfp) ester: Fmoc-Gly-OPfp (85 mg, 0.18 mmol, 3.0 equiv) and HOBt (25 mg, 0.18 mmol, 3.0 equiv) were dissolved in dry DMF (2 mL) and added to the resin swelled in DMF. After being shaken at 400 min^{-1} for 100 min, the resin was drained, washed twice with DMF, and deprotected as above.

The fourth amino acid (glucosylated Tyr) was again coupled as the Pfp-ester: Fmoc-Tyr(Bz_4-β-D-Glc)-OPfp[22] (134 mg, 0.12 mmol, 2.0 equiv) and HOBt (16 mg, 0.12 mmol, 2.0 equiv) were dissolved in dry DMF (2 mL) and added to the resin. After being shaken at 400 min^{-1} for 16 h, the resin was drained, washed twice with DMF, and deprotected as above. The fifth and last amino acid (Gly) was coupled as *N*-Ac-Gly-OH using BOP/HOBt: *N*-Ac-Gly-OH (35 mg, 0.30 mmol, 5.0 equiv), BOP (132 mg, 0.30 mmol, 5.0 equiv), and HOBt (40 mg, 0.30 mmol, 5.0 equiv) were dissolved in dry DMF (2.5 mL). After 5 min, DIEA was added (103 mL, 0.60 mmol, 10 equiv), and the mixture was added to the resin. After being shaken for 16 h, the resin was drained and washed (3 × DMF, 3 × DCM−MeOH (2:1), and 5 × DCM). An aliquot was cleaved (TFA−H_2O 19:1, 1 h): RP-HPLC (215 nm) indicated purity ∼85%, and the identity of the product was established by HR-LSIMS (positive mode): calcd for $C_{53}H_{53}N_6O_{16}$ (M + H), 1029.3518, found 1029.3522. Remaining resin was washed (3 × MeOH) and deprotected by repeated treatments with NaOMe in MeOH (∼1 mg/mL) until no methyl benzoate could be detected in the liquid. Resin was washed (2 × MeOH, 3 × DMF, 3 × DCM−MeOH (2:1), and 5 × DCM). A sample was cleaved and analyzed by HR-LSIMS (positive mode): calcd for $C_{25}H_{37}N_6O_{12}$ (M + H), 613.2469, found 613.2470. To improve the chromatographic properties of the sample, another aliquot of resin was acetylated (Ac_2O−pyridine 2:1, 16 h) and cleaved. RP-HPLC (Figure 2) indicated purity ∼94% and the identity of the product was again established by HR-LSIMS (positive mode): calcd for $C_{33}H_{45}N_6O_{16}$ (M + H), 781.2892, found 781.2885. At this point, the remaining resin was collapsed with increasing amounts of diethyl ether in DCM as described above and dried in vacuo.

The remainder of the nonacetylated resin above (199.4 mg) was subjected to enzymatic glycosylation using desalted Abg E358G (1.0 mg/mL) and 20 mM α-D-galactosyl fluoride (36.4 mg, 0.20 mmol) in 100 mM NaP_i at pH 9.00 (total 10.0 mL) for 48 h. After washing, acetylation, and cleavage as above, the residue was treated with dry diethyl ether and concentrated to obtain ∼19 mg of crude product. RP-HPLC (Figure 3) indicated 52% conversion and the identity of the product was established by HR-LSIMS (positive mode): calcd for $C_{45}H_{61}N_6O_{24}$ (M + H), 1069.3737, found 1069.3731. Purification of the mixture by preparative HPLC yielded 3.8 mg of monosaccharide glycopeptide and 6.3 mg of disaccharide glycopeptide. This corresponds to yields of 28 and 33%, respectively, or totally 61% based on Fmoc-quantification of second amino acid.

Each of the two compounds was pre-exchanged in D_2O (2 × 30 min followed by concentration to dryness) and finally dissolved in D_2O (0.7 mL) for NMR spectroscopy:

Monosaccharide glycopeptide: ^{1}H NMR (500 MHz, D_2O) δ 7.18 (2H, d, J = 8.5 Hz), 6.98 (2H, d, J = 8.5 Hz), 5.41 (1H, d, J = 8.1 Hz; H-1b), 5.39 (1H, t, J = 9.4 Hz; H-3), 5.19 (1H, dd, J = 7.7, 8.1 Hz; H-2), 5.14 (1H, t, J = 9.4 Hz; H-4), 4.55 (1H, dd, J = 6.4, 8.5 Hz; Tyr-Ha), 4.35 (1H, dd, J = 4.3, 12.8 Hz; H-6_a), 4.18 (1H, dd, J = 2.1, 12.8 Hz; H-6_b), 4.16 (1H, ddd, J = 2.1, 4.3, 9.4 Hz; H-5), 3.92−3.73 (8H, m; 8 × Gly-Ha), 3.09

(1H, dd, $J = 6.4, 14.1$ Hz; Tyr-Hb), 2.94 (1H, dd, $J = 8.5, 14.1$ Hz; Tyr-Hb), 2.06, 2.05, 2.04, 2.03, 1.94 (total 5 × acetyl-CH_3)

Disaccharide glycopeptide: ^{1}H NMR (500 MHz, D_2O, complex spectrum, selected data only) δ 7.17 (2H, d, $J = 8.5$ Hz), 6.96 (2H, m), 5.34 (1H, d, $J = 7.7$ Hz; H-1b), 5.27 (1H, t, $J = 9.0$ Hz; H-3), 4.55 (1H, dd, $J = 6.4, 8.5$ Hz; Tyr-Ha), 4.10 (1H, dd, $J = 9.0, 9.8$ Hz; H-4), 3.98 (1H, ddd, $J = 2.1, 6.0, 9.8$ Hz; H-5), 3.93−3.64 (8H, m; Gly-CH_2), 3.09 (1H, dd, $J = 6.4, 14.1$ Hz; Tyr-Hb), 2.93 (1H, dd, $J = 8.5, 14.1$ Hz; Tyr-Hb), 2.18−1.93 (24H, m; CH_3).

The glucosyl moiety must be galactosylated on the 4-position on the basis of the above data. The H-5 has a large coupling constant with H-4, and the latter is shifted upfield compared to other signals, which are acetylated: ^{13}C NMR (125 MHz, D_2O, selected data) δ 100.9 (β-anomer), 99.1 ((β-anomer, second anomer signal split into two signals due to rotamers).

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