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Glycosyl Azides – An Alternative Way to Disaccharides

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This paper is dedicated to the memory of Prof. Zoltán Györgydeàk, who greatly contributed to the chemistry of glycosyl azides.

Abstract: Glycosyl azides are shown to be efficient donors for β-galactosidases, β-glucosidases and α-mannosidases. Only α-galactosidases do not cleave the respective glycosyl azide **1** and, moreover, they exhibit competitive inhibition (especially α-galactosidase from *Talaromyces flavus*). High water solubility and ready synthesis of glycosyl azides enable transglycosylation reactions even with difficult acceptors like *N*-acetyl-D-mannosamine in good yields. The versatility of glycosyl azides was demonstrated in the synthesis of five disaccharides – two of them are described for the first time. All the reactions were highly regioselective, yielding β(1→6) isomers. β-Galactosidase from *E. coli* proved to have the best synthetic capabilities. The present study shows that glycosyl azides are a valuable alternative to common *p*-nitrophenyl glycoside donors and in many synthetic reactions.

Keywords: azides; enzyme catalysis; glycosylation; modified substrate

Introduction

Transglycosylation reactions are a well known method of carbohydrate synthesis and glycosidases are widely used for this aim.[1] An efficient transfer of the glycone donor moiety to an acceptor requires a relatively high donor concentration, which limits the risk of side hydrolysis. The hydrophobic leaving groups, although providing better substrate cleavage, often cause solubility problems. Therefore, new effective and highly soluble glycosyl donors are still sought after: 3-nitro- and 5-nitro-2-pyridyl glycosides,[2] vinyl glycosides,[3] and also donors other than *O*-glycosides, e.g., glycosyl fluorides,[4] which exhibit many common properties to glycosyl azides.[5]

Glycosyl azides combine some advantages of the above glycosyl donors, such as the small size of a leaving group, a strong nucleophilic character and delocalized π-electron density. Additionally, they are perfectly water-soluble and, contrary to, e. g., many glycosyl fluorides,[4] indefinitely stable even in acidic solutions, which enables reactions with enzymes like fungal glycosidases and α-mannosidase from jack beans. Despite the close chemical resemblance to glycosyl fluorides, no "wrong anomer hydrolysis"[6] was observed during the cleavage of glycosyl azides. Azides are cleaved more slowly than the corresponding *p*-nitrophenyl glycosides[7] and glycosyl fluorides[8] (considering k_{cat}, K_M), however, they do not inhibit the enzyme at higher concentrations and afford good transglycosylation yields, probably due to the reduction of water activity by the high donor concentration. Their chemical behaviour was thoroughly described by Györgydeàk and Thiem.[9]

The cleavage of glycosyl azides was probably first studied by Sinnott with β-galactosidase from *E. coli*,[10] later also with β-glucosidase from *Agrobacterium* sp.[11] β-D-Glucopyranosyl azide was used as a donor in a coupled screening for thioglycoligase activity.[12] We have used 2-acetamido-2-deoxy-β-D-glucopyranosyl and -galactopyranosyl azide in transglycosylation reactions with β-*N*-acetylhexosaminidases.[7]

In this paper we exploit the versatility of glycosyl azides as donors in transglycosylation reactions with a representative sample of various glycosidases: α- and β-galactosidases, β-glucosidases, and α-mannosidases. Three enzyme classes, β-galactosidases, β-glucosidases, and α-mannosidases, proved to be good catalysts for synthesis with glycosyl azides. Five disaccharidic structures were selectively prepared, two of which are de-

scribed for the first time. The reactions had good yields (up to 37%) and a high regioselectivity [exclusive formation of β(1→6) isomers]. The α-galactosidases tested did not cleave α-D-galactopyranosyl azide (**1**). On the contrary, an inhibition effect of the azide was observed ($K_i = 0.25$ mM with α-galactosidase from *Talaromyces flavus*).

Results and Discussion

Preparation and Screening of Glycosyl Azides

Glycosyl azides **1**–**4** (Figure 1) were prepared in good yields according to the published procedures.[13–18]

The potential of azides **1**–**4** to act as substrates for the respective glycosidases was assayed. Azides **1**–**4** were subjected to a hydrolytic screening comprising 43 fungal, microbial, animal and plant glycosidases. The total hydrolysis rates of glycosyl azides **1**–**4** were compared to those of the respective *p*-nitrophenyl glycosides **5**–**8**. The results for azides **2** and **3** are given in Table 1. Azide **4** was cleaved by both tested α-mannosidases (from jack beans and from *Aspergillus oryzae* CCF 1066; 5% referred to **8**). On the contrary, azide **1** was cleaved by none of the α-galactosidases tested (<1% referred to **5**). In summary, the respective azides were cleaved by most tested β-galactosidases, β-glucosidases and α-mannosidases (related to common rates for modified substrates),[19] contrary to α-galactosidases.

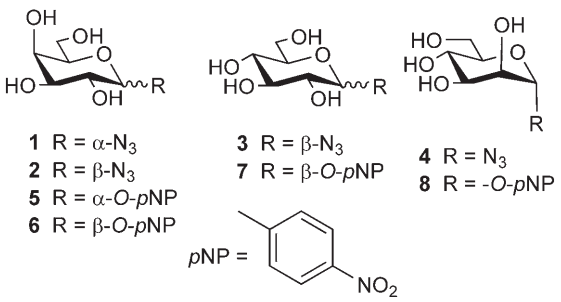


Figure 1. Glycosyl azides **1**–**4** and respective *p*-nitrophenyl glycosides **5**–**8**.

To account for the surprising results that none of the tested α-galactosidases cleaved azide **1**, the inhibition of α-galactosidases by this azide was studied. All examined enzymes were considerably inhibited by azide **1**. For example, the residual activity of the most inhibited α-galactosidase from *Talaromyces flavus* CCF 2686 declined to just 35% in the presence of azide **1** (2 mM). This inhibition was determined to be competitive, with $K_i = 0.25 \pm 0.05$ mM. For comparison, $K_M = 0.54 \pm 0.07$ mM for this enzyme with standard substrate **5**. No inhibition was detected with other glycosidase classes and the respective azides.

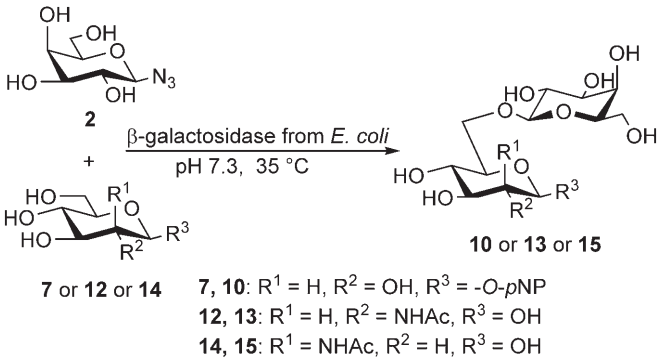
Enzymes suitable for the catalysis of transglycosylation reactions must meet the following criteria: (i) reasonable reaction rate (and thus low enzyme consumption); (ii) efficient product formation (and thus high yield). As for condition (i), it is noteworthy that the fastest hydrolyzing enzymes do not always give the highest yield in transglycosylations, however, a certain cleavage rate (optimum *ca.* 5% cleavage and more, compared to standard substrate) is necessary in order to reduce the enzyme consumption and keep the viscosity of the solution at an acceptable level. Therefore, the best enzymes from the hydrolytic screening were selected to test their synthetic potential with azides **2**–**4**, and to optimize the reaction conditions on an analytical scale. The transglycosylation screening comprised β-galactosidases from *B. circulans*, *E. coli*, and bovine testes, β-glucosidases from *A. oryzae* CCF 1602, *A. sojae* CCF 3060 and *P. chrysogenum* CCF 1269, and both α-mannosidases (from jack beans and *A. oryzae* CCF 1066) with various acceptors: glucoside **7**, GlcNAc (**12**), and ManNAc (**14**). As a result, the following reactions were proposed for the semi-preparative scale: (i) azide **2** with glucoside **7** or GlcNAc (**12**) or ManNAc (**14**) and β-galactosidase from *E. coli* (Scheme 1); (ii) azide **3** with GlcNAc (**12**) and β-glucosidase from *Penicillium chrysogenum* CCF 1269 (Scheme 2); (iii) azide **4** and α-mannosidase from jack beans (Scheme 3; autocondensation reaction).

Table 1. Cleavage of glycosyl azides **2** and **3** by respective glycosidases.

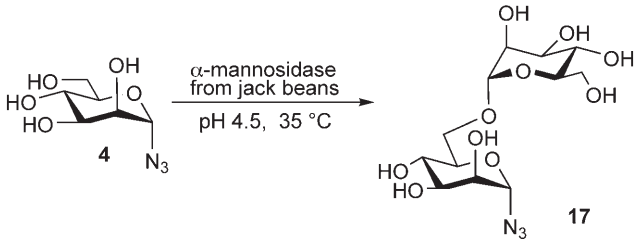
Azide **2** and β-galactosidases[a]	Cleavage	Azide **3** and β-glucosidases[a]	Cleavage
Escherichia coli[b]	++	*A. oryzae* CCF 1602,[b] *A. sojae* CCF 3060[b]	++
A. oryzae CCF 1602, *A. sojae* CCF 3060, *A. terreus* CCF 2539, *Bacillus circulans*,[b] bovine liver, bovine testes[b]	+	*A. terreus* CCF 55, *A. terreus* CCF 58, *A. terreus* CCF 2539, *P. brasilianum* CCF 2155, *P. chrysogenum* CCF 1269[b]	+
A. oryzae (Sigma), *Kluyveromyces lactis*	–	almonds, *A. flavipes* CCF 2026, *P. daleae* CCF 2365	–

[a] The ratio of hydrolyses of azides **2** and **3** related to *p*-nitrophenyl glycosides **6** and **7**, respectively, was determined as the ratio of total hydrolysis times (assayed under the same conditions and analyzed by TLC). The ratio of hydrolyses was 3–7% (++), 3–1% (+) or lower than 1% (–).

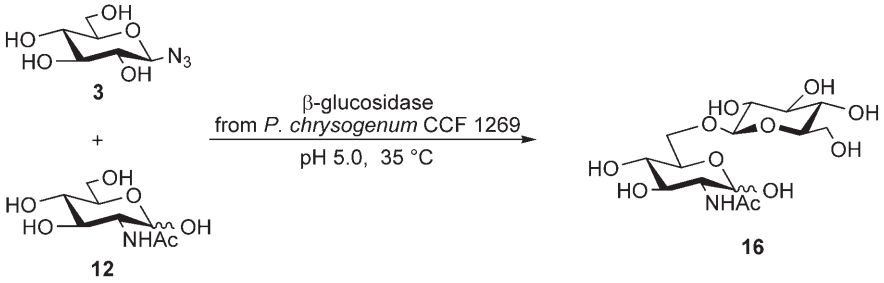
[b] Best cleaving enzymes used in analytical transglycosylations.



Scheme 1. Transglycosylation reactions with azide donor **2**.



Scheme 3. Transglycosylation reaction with azide donor **4**.



Scheme 2. Transglycosylation reaction with azide donor **3**.

The above synthetic reactions were performed on a semi-preparative scale under optimized conditions. Products **10**, **13**, **15**, **16**, and **17** were obtained in good yields (up to 37%, for details see Schemes 1–3 and the Experimental Section) and with a high regioselectivity (exclusive formation of β(1→6) bonds). Products **15** and **17** were synthesized for the first time. All products were fully characterized (NMR, MS, optical rotation) and in the case of known compounds, data were compared to the literature values. β-Galactosidase from *E. coli* proved to be a particularly efficient catalyst in synthetic reactions. No traces of autocondensation products were observed in the reactions with azides **2** and **3**. This can be explained by a huge molar excess of acceptor (5–6:1) as well as by a too low concentration of donor in reaction mixture ($\leq$100 mM) for autocondensation reactions.[7]

Conclusions

This work clearly demonstrates the potential of glycosyl azides as donors in transglycosylation reactions with various glycosidases. The screening for hydrolytic cleavage revealed that the respective glycosyl azides are fairly well cleaved by β-galactosidases, β-glucosidases and α-mannosidases, contrary to the tested α-galactosidases, which were substantially inhibited by the azide **1**. Representative transglycosylation reactions were performed with the selected enzymes on a semi-preparative scale giving good yields (up to 37%) and exclusive regioselectivity [β(1→6) bonds]. Five disaccharides, two of which are novel, were synthesized under the catalysis by β-galactosidase from *E. coli*, β-glucosidase from *P. chrysogenum* CCF 1269 and α-mannosidase from jack beans.

The present study shows that glycosyl azides are a valuable alternative to common *p*-nitrophenyl glycoside donors in many synthetic reactions, even with difficult acceptors like *N*-acetyl-D-mannosamine. Their main advantages are high water solubility, indefinite stability and ready synthesis.

Experimental Section

Materials

All reagents and solvents used were commercially available and of analytical grade. β-Galactosidases from *Aspergillus oryzae*, *Escherichia coli*, *Kluyveromyces lactis* and bovine liver, as well as β-glucosidase from almond, α-galactosidase from *Coffea arabica* and α-mannosidase from jack beans, were purchased from Sigma. β-Galactosidase from *Bacillus circulans* was purchased from Daiwa Kasei K. K. (Osaka, Japan) under the trade name Biolacta FN5. The fungal strains producing α-galactosidases (EC 3.2.1.22), β-galactosidases (EC 3.2.1.23), β-glucosidases (EC 3.2.1.21), and α-mannosidases (EC 3.2.1.24) originated from the Culture Collection of Fungi (CCF), Department of Botany, Charles University in Prague, or from the Culture Collection of the

Institute of Microbiology (CCIM), Prague. The strains were cultivated as described previously.[20] β-Galactosidase from bovine testes was a crude preparation obtained by the method of Hedbys et al.;[21] fresh bovine testes came from a local slaughterhouse and were stored at −20 °C.

Methods

Thin-layer chromatography was performed on precoated Merck silica gel DC-Alufolien Kieselgel 60 F_{254} plates. The spots were visualized by charring with 5% H_2SO_4 in EtOH. Flash chromatography was performed on silica gel 60 (40–63 μm, Merck). Gel chromatography was performed on Bio-Gel® P-2 (Bio-Rad, U.S.A.). ^{1}H and ^{13}C NMR spectra were recorded on a Varian INOVA-400 spectrometer (399.87 MHz and 100.55 MHz, respectively) at 30 °C in the indicated solvents. Chemical shifts are expressed in δ scale and were measured with digital resolution justifying the reported values to three or two decimal places, respectively. HMQC and HMBC readouts are accurate at two decimal places for ^{1}H NMR chemical shifts and at one decimal place for ^{13}C NMR shifts. Residual signal of solvents was used as an internal standard ($CDCl_3$: $\delta_H = 7.265$ ppm, $\delta_C = 77.00$ ppm, D_2O: $\delta_H = 4.508$ ppm). Carbon chemical shifts in D_2O were referred to acetone ($\delta_C = 30.50$ ppm). The reported assignment is based on gCOSY, HOM2DJ, HSQC, HMQC, HMBC, and 1D-TOCSY experiments. The anomeric configuration of mannopyranosides was assigned on the basis of direct coupling constant $^1J_{C\text{-}1,H\text{-}1}$, which was measured by a proton-coupled HMQC experiment. Positive-ion MALDI mass spectra were measured on a Bruker BIFLEX II reflectron time-of-flight mass spectrometer (Bruker-Franzen, Bremen, Germany) equipped with a nitrogen laser (337 nm) and a gridless delayed extraction ion source. A solution (10 mg mL^{-1}) of 2,5-dihydroxybenzoic acid (Sigma) in 50% acetonitrile/0.1% trifluoroacetic acid was used as MALDI matrix. The matrix solution (1 μL) was premixed with a sample (1 μL), loaded on the target and allowed to dry at ambient temperature. Spectra were externally calibrated using the monoisotopic $[M+H]^+$ signal of the matrix and angiotensin II (Sigma). Optical rotation was measured on a Perkin–Elmer 241 polarimeter at 589 nm.

Synthesis of Substrates

α-D-*Galactopyranosyl azide* (**1**) was a kind gift from Prof. Z. Györgydeàk, Debrecen University, Hungary.

β-D-*Galactopyranosyl azide* (**2**) was prepared according to Korytnyk and Mills[13] or Györgydeàk and Szilàgyi.[14]

β-D-*Glucopyranosyl azide* (**3**) was prepared according to Soli et al.[15] or Ogawa et al.[16] The ^{1}H and ^{13}C NMR data for compounds **1–3** were consistent with the structures (see the literature,[14,16] respectively).

α-D-*Mannopyranosyl azide* (**4**): 1,2,3,4,6-Penta-*O*-acetyl-D-mannose (**4a**) was prepared according to Watt and Williams.[17] 2,3,4,6-Penta-*O*-acetyl-α-D-mannopyranosyl azide (**4b**) was prepared according to Ponpipom et al[18] as a yellowish honey. ^{1}H NMR ($CDCl_3$; $^1J_{C\text{-}1,H\text{-}1} = 171$ Hz): $\delta = 1.971$, 2.031, 2.085, 2.144 (each s, 3H, 4×Ac), 4.13[a] (m, 1H, H-5), 4.144 (m, 1H, H-6u), 4.28[a] (m, 1H, H-6d), 5.130 (dd, $J_{1,2} = 1.9$ Hz, $J_{2,3} = 3.0$ Hz, 1H, H-2), 5.223 (dd, $J_{2,3} = 3.0$ Hz, $J_{3,4} = 10.4$ Hz, 1H, H-3), 5.261 (dd, $J_{3,4} = 10.4$ Hz, $J_{4,5} = 9.7$ Hz, 1H, H-4), 5.369 (d, $J_{1,2} = 1.9$ Hz, 1H, H-1); ^{13}C NMR ($CDCl_3$): $\delta = 20.47$, 20.55, 20.58, 20.68 (4×Ac), 62.11 (C-6), 65.62 (C-4), 68.20 (C-3), 69.13 (C-2), 70.62 (C-5), 87.41 (C-1), 169.52, 169.61, 169.72, 170.45 (4×CO). [a] Readout from HOM2DJ; [b] some acetyls were not found with certainty.

α-D-Mannopyranosyl azide (**4**) was prepared from **4b** by deacetylation according to Zemplen (MeONa/dry MeOH) as a yellowish honey in 14% overall yield. In our hands, both **4b** and **4** were repeatedly isolated in a mixture with an unidentified impurity, inert to enzymatic reactions (*ca.* 10–30%), different from the β-anomer (as compared to the published ^{13}C NMR spectrum).[22]

Enzymatic Methods

Enzyme activity assay: The reaction mixture containing the respective substrate (substrate **5** for α-galactosidases, **6** for β-galactosidases, **7** for β-glucosidases, or **8** for α-mannosidases; starting concentration 2 mM) and the respective enzyme (0.02–0.03 U mL^{-1}) in citrate/phosphate buffer (0.05 M, pH 5.0, for fungal enzymes or pH indicated by producer for commercial enzymes) or phosphate buffer (0.05 M, pH 7.3, for *E. coli* β-galactosidase) was incubated with shaking at 35 °C (or at the temperature indicated by producer for commercial enzymes) for 10 min. Liberated *p*-nitrophenol was determined spectrophotometrically (420 nm) under alkaline conditions (0.1 M Na_2CO_3). One unit of enzymatic activity released 1 μmol of *p*-nitrophenol per min under the above conditions.

Hydrolytic screening of glycosyl azides 1–4 with glycosidases: The reaction mixture, composed of glycosyl azide **1–4** or standard *p*-nitrophenyl glycoside **5–8** (20 mM, starting concentration) and the respective glycosidase (0.54 U mL^{-1}) in citrate/phosphate buffer (0.05 M, pH 5.0, for fungal enzymes or the pH indicated by the producer for commercial enzymes) or phosphate buffer (0.05 M, pH 7.3, for *E. coli* β-galactosidase), was shaken at 35 °C and monitored by TLC (2-propanol:H_2O:aqueous NH_4OH, 7:2:1). The total hydrolysis time was indicated as the time when no more starting material was detectable in the reaction mixture. The enzymes were compared according to the ratio of total hydrolysis times for **1–4** and **5–8**, respectively. α-Galactosidases, tested with azide **1** and standard **5**, were obtained from the following sources: *Aspergillus flavipes* CCF 2026, *A. flavus* CCF 1129, *A. oryzae* CCF 1602, *A. parasiticus* CCF 3058, *A. phoenicis* CCF 61, *A. sojae* CCF 3060, *A. tamarii* CCF 3058, *A. terreus* CCF 55, *A. terreus* CCF 2539, *Circinella muscae* CCF 1568, *Chaetomium globosum*, coffee beans, *Micromucor ramannianus* CCF 1022, *Penicillium brasilianum* CCF 2155, *P. chrysogenum* CCF 1269, *P. commune* CCF 2962, *P. daleae* CCF 2365, *P. multicolor* CCF 2244, *P. ochrochloron* CCF 2379, *Talaromyces flavus* CCF 2324, *T. flavus* CCF 2686, *Trichoderma harzianum* CCF 2687. β-Galactosidases and β-glucosidases, tested with azide **2** and standard **6** or with azide **3** and standard **7**, respectively, are listed in Table 1. α-Mannosidases from *Aspergillus oryzae* CCF 1066 and from jack beans were tested with azide **4** and standard **8**.

Enzyme inhibition assay: The reaction mixture containing the standard *p*-nitrophenyl glycoside **5–8**, the respective azide **1–4** (both 2 mM) and the respective glycosidase (0.02–0.03 U mL^{-1}) in citrate/phosphate buffer (0.05 M, pH 5.0, for fungal enzymes or the pH indicated by the producer for

commercial enzymes) or phosphate buffer (0.05 M, pH 7.3, for *E. coli* β-galactosidase) was incubated under shaking at 35 °C for 10 min and assayed spectrophotometrically as described above. Assayed α-galactosidases as listed above, assayed β-galactosidases and β-glucosidases as listed in Table 1. Azide **4** was tested with α-mannosidases from *Aspergillus oryzae* CCF 1066 and from jack beans. The residual activity was determined as the ratio of hydrolysis rates of standard substrate **5**–**8** in the presence of the respective azide **1**–**4** and without it. Kinetic constants (K_M, K_i, V_{max}) for α-galactosidase from *Talaromyces flavus* CCF 2686 were determined from initial hydrolysis rates at various starting concentrations of the standard substrate **5** (0.4–2 mM) with azide **1** (0–2 mM) using SigmaPlot 2001 (SPSS Science, U.S.A.). Initial hydrolysis rates were determined at 30 °C and pH 4 in 1 mm path-length cells. The released *p*-nitrophenol was continuously monitored at 348 nm (the pH-independent isosbestic point of ionized *p*-nitrophenol). Under these conditions, the difference of absorption coefficients of *p*-nitrophenol and the substrate was 2698 M^{-1} cm^{-1}.[23]

Analytical Transglycosylation Reactions

β-*Galactosidases:* The reaction mixture containing β-galactosidase from *B. circulans*, *E. coli* or bovine testes (0.5 U or 0.7 U), azide **2** (50 mM or 100 mM, respectively), the acceptor [glucoside **7** (300 mM), GlcNAc (500 mM) or ManNAc (500 mM)] in citrate/phosphate buffer (50 mM; pH 6.5 for *B. circulans* enzyme, pH 4.5 for bovine testes enzyme) or phosphate buffer (0.05 M, pH 7.3, for *E. coli* enzyme) was incubated with shaking at 35 °C for 24 h.

β-*Glucosidases:* The reaction mixture containing β-glucosidase from *A. oryzae* CCF 1602, *A. sojae* CCF 3060 or *P. chrysogenum* CCF 1269 (0.5 U), azide **3** (100 mM), the acceptor (500 mM, GlcNAc or ManNAc) in citrate/phosphate buffer (0.05 M, pH 5.0) was incubated with shaking at 35 °C for 24 h.

α-*Mannosidases:* The reaction mixture containing azide **4** (100 mM, 200 mM, 300 mM, or 500 mM) and α-mannosidase from jack beans (0.75, 0.95, 1.1 or 1.5 U, respectively) in citrate/phosphate buffer (0.05 M, pH 4.5) was incubated with shaking at 35 °C for 24 h. The course of all analytical reactions was monitored by TLC (2-propanol:H_2O:aqueous NH_4OH, 7:2:1).

p-Nitrophenyl β-D-Galactopyranosyl-(1→6)-β-D-glucopyranoside (10)

β-D-Galactopyranosyl azide (**2**; 5 mg, 24.4 μmol) and *p*-nitrophenyl β-D-glucopyranoside (**7**; 43.4 mg, 144 μmol) were dissolved in phosphate buffer (480 μL, 0.05 M, pH 7.3). β-Galactosidase from *E. coli* (3 U) was added and the reaction mixture was shaken (850 rpm) at 35 °C and monitored by TLC (2-propanol: H_2O:aqueous NH_4OH, 7:2:1). The reaction was stopped after 2 h by heating (100 °C, 5 min) and centrifuged (13,500 rpm, 10 min). Purification by gel chromatography (Bio-Gel® P-2; mobile phase H_2O, flow rate 13.8 mL h^{-1}, ambient temperature) afforded compound **10** as white solid; yield: 1.1 mg, (2.4 μmol; 37 % related to consumed donor **2**; donor **2** was partially recovered: 3.7 mg). ^{1}H NMR (D_2O): $\delta = 3.316$ (dd, $J_{1',2'} = 7.6$ Hz, $J_{2',3'} = 9.9$ Hz, 1 H, H-2′), 3.373 (m, 1 H, H-4), 3.375 (dd, $J_{2',3'} = 9.9$ Hz, $J_{3',4'} = 3.3$ Hz, 1 H, H-3′), 3.384 (ddd, $J_{4',5'} = 1.0$ Hz, $J_{5',6'u} = 4.5$ Hz, $J_{5',6'd} = 7.6$ Hz, 1 H, H-5′), 3.425 (m, 2 H, H-2, H-3), 3.489 (dd, $J_{5',6'u} = 4.5$ Hz, $J_{6'u,6'd} = 11.6$ Hz, 1 H, H-6′u), 3.533 (dd, $J_{5',6'd} = 7.6$ Hz, $J_{6'u,6'd} = 11.6$ Hz, 1 H, H-6′d), 3.669 (dd, $J_{3',4'} = 3.3$ Hz, $J_{4',5'} = 1.0$ Hz, 1 H, H-4′), 3.69[a] (m, 1 H, H-5), 3.70[a] (m, 1 H, H-6u), 4.010 (dd, $J_{5,6d} = 4.7$ Hz, $J_{6u,6d} = 14.5$ Hz, 1 H, H-6d), 4.190 (d, $J_{1',2'} = 7.6$ Hz, 1 H, H-1′), 5.070 (d, $J_{1,2} = 7.7$ Hz, 1 H, H-1), 7.076 (m, 2 H, H-*ortho*), 8.065 (m, 2 H, H-*meta*); ^{13}C NMR (D_2O, HMQC and HMBC readouts): $\delta = 61.1$ (C-6′), 68.5 (C-6), 68.8 (C-4′), 69.3 (C-4), 70.7 (C-2′), 72.8 (C-3′), 72.9 (C-2), 75.1 (C-5′), 75.4 (C-3), 75.5 (C-5), 99.5 (C-1), 103.4 (C-1′), 116.8 (C-*ortho*), 126.2 (C-*meta*), 142.8 (C-*para*), 161.6 (C-*ipso*).[a] Readout from HMQC. The spectroscopic data of **10** were in accord with the literature values.[24]

β-D-Galactopyranosyl-(1→6)-2-acetamido-2-deoxy-D-glucopyranose (13)

β-D-Galactopyranosyl azide (**2**; 24.8 mg, 0.121 mmol) and 2-acetamido-2-deoxy-D-glucopyranose (**12**; 132.8 mg, 0.600 mmol) were dissolved in phosphate buffer (1.2 mL, 0.05 M, pH 7.3). β-Galactosidase from *E. coli* (6 U) was added and the reaction mixture was shaken (850 rpm) at 35 °C and monitored by TLC (2-propanol:H_2O:aqueous NH_4OH, 7:2:1). After 2.5 h, another portion (2 U) of enzyme was added. The reaction was stopped after 4.5 h by heating (100 °C, 5 min) and centrifuged (13,500 rpm, 10 min). Purification by gel chromatography (Bio-Gel® P-2; mobile phase H_2O, flow rate 13.8 mL h^{-1}, ambient temperature) afforded compound **13** as a white solid; yield: 6.8 mg (0.018 mmol; 28 % related to consumed donor **2**; donor **2** was partially recovered: 11.6 mg). According to NMR data, **13** was a mixture of two anomers ($\alpha/\beta = 1.5$).

α-Anomer of **13**: ^{1}H NMR (D_2O): $\delta = 1.823$ (s, 3 H, 2-Ac), 3.333 (dd, $J_{2',3'} = 9.9$ Hz, $J_{1',2'} = 7.8$ Hz, 1 H, H-2′), 3.359 (dd, $J_{4,5} = 10.1$ Hz, $J_{3,4} = 8.9$ Hz, 1 H, H-4), 3.432 (dd, $J_{2',3'} = 9.9$ Hz, $J_{3',4'} = 3.4$ Hz, 1 H, H-3′), 3.470 (ddd, $J_{5',6'd} = 7.8$ Hz, $J_{5',6'u} = 1.5$ Hz, $J_{4',5'} = 1.0$ Hz, 1 H, H-5′), 3.520 (dd, $J_{6'd,6'u} = 11.7$ Hz, $J_{5',6'u} = 1.5$ Hz, 1 H, H-6′u), 3.539 (dd, $J_{2,3} = 10.6$ Hz, $J_{3,4} = 8.9$ Hz, 1 H, H-3), 3.578 (dd, $J_{6'd,6'u} = 11.7$, $J_{5',6'd} = 7.8$ Hz, 1 H, H-6′d), 3.668 (dd, $J_{2,3} = 10.6$ Hz, $J_{1,2} = 3.5$ Hz, 1 H, H-2), 3.675 (dd, $J_{6d,6u} = 11.4$ Hz, $J_{5,6u} = 5.1$ Hz, 1 H, H-6u), 3.700 (dd, $J_{3',4'} = 3.4$, $J_{4',5'} = 1.0$ Hz, 1 H, H-4′), 3.794 (ddd, $J_{4,5} = 10.1$ Hz, $J_{5,6u} = 5.1$ Hz, $J_{5,6d} = 2.1$ Hz, 1 H, H-5), 3.952 (dd, $J_{6d,6u} = 11.4$ Hz, $J_{5,6d} = 2.1$ Hz, 1 H, H-6d), 4.211 (d, $J_{1',2'} = 7.8$ Hz, 1 H, H-1′), 4.977 (d, $J_{1,2} = 3.5$ Hz, 1 H, H-1); ^{13}C NMR (D_2O, HMQC readouts): $\delta = 21.3$ (2-Ac), 54.4 (C-2), 61.4 (C-6′), 69.1 (C-6, C-4′), 70.3 (C-4), 71.1 (C-3, C-5), 71.2 (C-2′), 73.1 (C-3′), 75.5 (C-5′), 91.3 (C-1), 103.7 (C-1′).

β-Anomer of **13**: ^{1}H NMR (D_2O): $\delta = 1.821$ (s, 3 H, Ac), 3.309 (m, 2 H, H-3, H-4), 3.338 (dd, $J_{2',3'} = 9.9$ Hz, $J_{1',2'} = 7.8$ Hz, 1 H, H-2′), 3.409 (m, 1 H; H-5), 3.435 (dd, $J_{2',3'} = 9.9$ Hz, $J_{3',4'} = 3.4$ Hz, 1 H, H-3′), 3.463 (dd, $J_{2,3} = 10.3$ Hz, $J_{1,2} = 8.4$ Hz, 1 H, H-2), 3.478 (ddd, $J_{5',6'd} = 7.8$ Hz, $J_{5',6'u} = 1.5$ Hz, $J_{4',5'} = 1.0$ Hz, 1 H, H-5′), 3.530 (dd, $J_{6'd,6'u} = 11.7$ Hz, $J_{5',6'u} = 1.5$ Hz, 1 H, H-6′u), 3.578 (dd, $J_{6'd,6'u} = 11.7$ Hz, $J_{5',6'd} = 7.8$ Hz, 1 H, H-6′d), 3.637 (dd, $J_{6d,6u} = 11.5$ Hz, $J_{5,6u} = 5.9$ Hz, 1 H, H-6u), 3.700 (dd, $J_{3',4'} = 3.4$ Hz, $J_{4',5'} = 1.0$ Hz, 1 H, H-4′), 4.005 (dd, $J_{6d,6u} = 11.5$ Hz, $J_{5,6d} = 2.1$ Hz, 1 H, H-6d), 4.228 (d, $J_{1',2'} = 7.8$ Hz, 1 H, H-1′), 4.506 (d, $J_{1,2} = 8.4$ Hz, 1 H, H-1); ^{13}C NMR (D_2O, HMQC readouts): $\delta = 21.3$ (2-Ac), 57.0 (C-2), 61.4 (C-6′), 69.1 (C-6, C-4′), 70.2 (C-4), 71.2 (C-2′), 73.1

(C-3′), 74.2 (C-3), 75.2 (C-5), 75.5 (C-5′), 95.4 (C-1), 103.7 (C-1′). The spectroscopic data of **13** were in accord with the literature values.[25]

β-D-Galactopyranosyl-(1→6)-2-acetamido-2-deoxy-D-mannopyranose (15)

β-D-Galactopyranosyl azide (**2**; 51 mg, 0.249 mmol) and 2-acetamido-2-deoxy-D-mannopyranose (**14**; 266 mg, 1.202 mmol) were dissolved in phosphate buffer (1.8 mL, 0.05 M, pH 7.3). β-Galactosidase from *E. coli* (26 U) was added and the reaction mixture was shaken (850 rpm) at 35 °C and monitored by TLC (2-propanol: H_2O:aqueous NH_4OH, 7:2:1). The reaction was stopped after 4.5 h by heating (100 °C, 2 min) and centrifuged (13,500 rpm, 10 min). Purification by gel chromatography (Bio-Gel® P-2; mobile phase H_2O, flow rate 13.8 mL h^{-1}, ambient temperature) afforded compound **15** as a white solid; yield: 6.7 mg (0.017 mmol; 14 % related to consumed donor **2**; donor **2** was partially recovered: 26 mg). $[\alpha]_D^{23}$: +10.0 (*c* 0.12 in water). According to NMR data, **15** was a mixture of two anomers ($\alpha/\beta = 1.22$).

α-Anomer of **15**: ^{1}H NMR (D_2O; $^1J_{C\text{-}1,H\text{-}1} = 176$ Hz): $\delta =$ 1.828 (s, 3H, Ac), 3.349 (dd, $J_{1',2'} = 7.8$ Hz, $J_{2',3'} = 9.9$ Hz, 1H, H-2′), 3.445 (dd, $J_{2',3'} = 9.9$ Hz, $J_{3',4'} = 3.4$ Hz, 1H, H-3′), 3.483 (ddd, $J_{4',5'} = 1.0$ Hz, $J_{5',6'u} = 4.2$ Hz, $J_{5',6'd} = 7.9$ Hz, 1H, H-5′), 3.489 (dd, $J_{3,4} = 9.9$ Hz, $J_{4,5} = 10.0$ Hz, 1H, H-4), 3.528 (dd, $J_{5',6'u} = 4.2$ Hz, $J_{6'u,6'd} = 11.6$ Hz, 1H, H-6′u), 3.585 (dd, $J_{5',6'd} =$ 7.9 Hz, $J_{6'u,6'd} = 11.6$ Hz, 1H, H-6′d), 3.707 (dd, $J_{3',4'} = 3.4$ Hz, $J_{4',5'} = 1.0$ Hz, 1H, H-4′), 3.727 (dd, $J_{5,6u} = 4.8$ Hz, $J_{6u,6d} =$ 11.3 Hz, 1H, H-6u), 3.794 (ddd, $J_{4,5} = 10.0$ Hz, $J_{5,6u} = 4.8$ Hz, $J_{5,6d} = 2.0$ Hz, 1H, H-5), 3.834 (dd, $J_{2,3} = 4.7$ Hz, $J_{3,4} = 9.9$ Hz, 1H, H-3), 3.946 (dd, $J_{5,6d} = 2.0$ Hz, $J_{6u,6d} = 11.3$ Hz, 1H, H-6d), 4.101 (dd, $J_{1,2} = 1.6$ Hz, $J_{2,3} = 4.7$ Hz, 1H, H-2), 4.235 (d, $J_{1',2'} = 7.8$ Hz, 1H, H-1′), 4.896 (d, $J_{1,2} = 1.6$ Hz, 1H, H-1); ^{13}C NMR (D_2O, HMQC and HMBC readouts): $\delta = 22.0$ (Ac), 53.4 (C-2), 61.1 (C-6′), 66.7 (C-4), 68.7 (C-3, C-6), 68.8 (C-4′), 70.8 (C-2′), 71.0 (C-5), 72.8 (C-3′), 75.4 (C-5′), 93.2 (C-1), 103.3 (C-1'), 174.8 (CO).

β-Anomer of **15**: ^{1}H NMR (D_2O; $^1J_{C\text{-}1,H\text{-}1} = 161$ Hz): $\delta =$ 1.867 (s, 3H, Ac), 3.34[a] (m, 1H, H-5), 3.356 (dd, $J_{1',2'} =$ 7.8 Hz, $J_{2',3'} = 9.9$ Hz, 1H, H-2′), 3.36[a] (m, 1H, H-4), 3.445 (dd, $J_{2',3'} = 9.9$ Hz, $J_{3',4'} = 3.4$ Hz, 1H, H-3′), 3.486 (ddd, $J_{4',5'} =$ 1.0 Hz, $J_{5',6u'} = 4.2$ Hz, $J_{5',6d'} = 7.9$ Hz, 1H, H-5′), 3.530 (dd, $J_{5',6u'} = 4.2$ Hz, $J_{6u',6d'} = 11.6$ Hz, 1H, H-6u′), 3.585 (dd, $J_{5',6d'} =$ 7.9 Hz, $J_{6u',6d'} = 11.6$ Hz, 1H, H-6d′), 3.610 (dd, $J_{2,3} = 4.5$ Hz, $J_{3,4} = 9.7$ Hz, 1H, H-3), 3.692 (dd, $J_{5,6u} = 4.7$ Hz, $J_{6u,6d} =$ 11.4 Hz, 1H, H-6u), 3.707 (dd, $J_{3',4'} = 3.4$ Hz, $J_{4',5'} = 1.0$ Hz, 1H, H-4′), 3.999 (dd, $J_{5,6d} = 1.5$ Hz, $J_{6u,6d} = 11.4$ Hz, 1H, H-6d), 4.235 (dd, $J_{1,2} = 1.7$ Hz, $J_{2,3} = 4.5$ Hz, 1H, H-2), 4.243 (d, $J_{1',2'} = 7.8$ Hz, 1H, H-1′), 4.812 (d, $J_{1,2} = 1.7$ Hz, 1H, H-1); ^{13}C NMR (D_2O, HMQC and HMBC readouts): $\delta = 22.1$ (Ac), 54.2 (C-2), 61.1 (C-6′), 66.6 (C-4), 68.6 (C-6), 68.8 (C-4′), 70.8 (C-2′), 72.0 (C-3), 72.8 (C-3′), 75.4 (C-5, C-5′), 93.1 (C-1), 103.3 (C-1′), 175.7 (CO),[a] Readout from HMQC; MS (MALDI-TOF): $m/z = 406.2$ $[M+Na]^+$.

β-D-Glucopyranosyl-(1→6)-2-acetamido-2-deoxy-D-glucopyranose (16)

β-D-Glucopyranosyl azide (**3**; 51 mg, 0.249 mmol) and 2-acetamido-2-deoxy-D-glucopyranose (**12**; 267 mg, 1.207 mmol) were dissolved in citrate/phosphate buffer (2.4 mL, 0.05 M, pH 5.0). β-Gluctosidase from *Penicillium chrysogenum* CCF 1269 (12 U) was added and the reaction mixture was shaken (850 rpm) at 35 °C and monitored by TLC (2-propanol:H_2O:aqueous NH_4OH, 7:2:1). The reaction was stopped after 5 h by heating (100 °C, 2 min) and centrifuged (13,500 rpm, 10 min). Purification by gel chromatography (Bio-Gel® P-2; mobile phase H_2O, flow rate 14.7 mL h^{-1}, ambient temperature) afforded compound **16** as a white solid; yield: 17 mg (0.044 mmol; 28 % related to consumed donor **3**; donor **3** was partially recovered: 19 mg). For NMR characterization, compound **16** was peracetylated (Ac_2O/dry py, DMAP, 24 h) and purified by silica gel chromatography (CH_2Cl_2/MeOH, 20:1) to afford peracetate **16a**. According to NMR data, **16a** was a mixture of two anomers ($\alpha/\beta = 1.22$).

α-Anomer of **16a**: ^{1}H NMR ($CDCl_3$): $\delta = 1.934$ (s, 3H, 2-*N*Ac), 1.999 (s, 3H, Ac), 2.020 (s, 3H, Ac), 2.034 (s, 3H, Ac), 2.047 (s, 3H, Ac), 2.056 (s, 3H, Ac), 2.089 (s, 3H, Ac), 2.188 (s, 3H, Ac), 3.508 (dd, $J_{6d,6u} = 10.9$ Hz, $J_{5,6u} = 6.6$ Hz, 1H, H-6u), 3.679 (ddd, $J_{4',5'} = 9.9$ Hz, $J_{5',6'd} = 4.6$ Hz, $J_{5',6'u} =$ 2.4 Hz, 1H, H-5′), 3.895 (dd, $J_{5,6d} = 10.9$ Hz, $J_{5,6d} = 2.0$ Hz, 1H, H-6d), 3.945 (ddd, $J_{4,5} = 10.5$ Hz, $J_{5,6u} = 6.6$ Hz, $J_{5,6d} =$ 2.0 Hz, 1H, H-5), 4.113 (dd, $J_{6'd,6'u} = 12.3$ Hz, $J_{5',6'u} = 2.4$ Hz, 1H, H-6′u), 4.283 (dd, $J_{6'd,6'u} = 12.3$ Hz, $J_{5',6'd} = 4.6$ Hz, 1H, H-6′d), 4.426 (ddd, $J_{2,2\text{-}NH} = 11.1$ Hz, $J_{2,3} = 8.9$ Hz, $J_{1,2} = 3.7$ Hz, 1H, H-2), 4.522 (d, $J_{1',2'} = 8.0$ Hz, 1H, H-1′), 4.954 (dd, $J_{2',3'} =$ 9.6 Hz, $J_{1',2'} = 8.0$ Hz, 1H, H-2′), 5.013 (dd, $J_{4,5} = 10.5$ Hz, $J_{3,4} = 9.3$ Hz, 1H, H-4), 5.063 (dd, $J_{4',5'} = 9.9$ Hz, $J_{3',4'} = 9.5$ Hz, 1H, H-4′), 5.197 (dd, $J_{2',3'} = 9.6$ Hz, $J_{3',4'} = 9.5$, 1H, H-3′), 5.212 (dd, $J_{2,3} = 11.1$ Hz, $J_{3,4} = 9.3$ Hz, 1H, H-3), 5.546 (d, $J_{2,2\text{-}NH} = 8.9$ Hz, 1H, 2-NH), 6.147 (d, $J_{1,2} = 3.7$ Hz, 1H, H-1); ^{13}C NMR ($CDCl_3$, HMQC readouts): $\delta = 23.9$–24.5 (7 × Ac), 26.7 (2-*N*Ac), 54.7 (C-2), 65.4 (C-6′), 72.0 (C-4, C-4′), 71.8 (C-6), 74.4 (C-3), 74.6 (C-5, C-2′), 75.6 (C-5′), 76.3 (C-3′), 94.0 (C-1), 104.3 (C-1′).

β-Anomer of **16a**: ^{1}H NMR ($CDCl_3$): $\delta = 1.924$ (3H, s, 2-*N*Ac), 2.002 (s, 3H, Ac), 2.020 (s, 3H, Ac), 2.044 (s, 3H, Ac), 2.056 (s, 6H, 2 × Ac), 2.096 (s, 3H, Ac), 2.113 (s, 3H, Ac), 3.592 (dd, $J_{6d,6u} = 11.2$ Hz, $J_{5,6u} = 6.1$ Hz, 1H, H-6u), 3.672 (ddd, $J_{4',5'} = 9.6$ Hz, $J_{5',6'd} = 4.6$ Hz, $J_{5',6'u} = 2.2$ Hz, 1H, H-5′), 3.749 (dd, $J_{4,5} = 9.6$ Hz, $J_{5,6u} = 6.1$ Hz, $J_{5,6d} = 2.4$ Hz; 1H, H-5), 3.906 (dd, $J_{6d,6u} = 11.2$ Hz, $J_{5,6d} = 2.4$ Hz, 1H, H-6d), 4.128 (dd, $J_{6'd,6'u} = 12.4$ Hz, $J_{5,6'u} = 2.2$ Hz, 1H, H-6′u), 4.232 (ddd, $J_{2,3} = 10.3$ Hz, $J_{2,2\text{-}NH} = 9.6$ Hz, $J_{1,2} = 8.8$ Hz, 1H, H-2), 4.264 (dd, $J_{6'd,6'u} = 12.4$ Hz, $J_{5',6'd} = 4.6$ Hz, 1H, H-6′d), 4.551 (d, $J_{1',2'} = 8.0$ Hz, 1H, H-1′), 4.970 (dd, $J_{4,5} = 9.6$ Hz, $J_{3,4} =$ 9.6 Hz, 1H, H-4), 4.976 (dd, $J_{2',3'} = 9.5$ Hz, $J_{1',2'} = 8.0$ Hz, 1H, H-2′),5.063 (dd, $J_{4',5'} = 9.6$ Hz, $J_{3',4'} = 9.6$ Hz, 1H, H-4′), 5.101 (dd, $J_{2,3} = 10.3$ Hz, $J_{3,4} = 9.6$ Hz, 1H, H-3), 5.190 (dd, $J_{3',4'} =$ 9.6 Hz, $J_{2',3'} = 9.5$ Hz, 1H, H-3′), 5.517 (d, $J_{H,2\text{-}NH} = 9.6$ Hz, 1H, 2-NH), 5.664 (d, $J_{1,2} = 8.8$, 1H, H-1); ^{13}C NMR ($CDCl_3$, HMQC readouts): $\delta = 23.9$–24.5 (7 × Ac), 26.7 (2-*N*Ac), 56.8 (C-2), 65.5 (C-6'), 71.6 (C-6), 72.0 (C-4, C-4′), 74.6 (C-2'), 75.6 (C-5′), 76.3 (C-3, C-3′), 77.8 (C-5), 96.2 (C-1), 104.4 (C-1′). The spectroscopic data of **16a** were in accord with the structure.[26]

α-D-Mannopyranosyl-(1→6)-α-D-mannopyranosyl azide (17)

α-D-Mannopyranosyl azide (**4**; 170 mg, 0.829 mmol) was dissolved in citrate/phosphate buffer (1.2 mL, 0.05 M, pH 4.5). α-Mannosidase from jack beans (24 U) was added and the reaction mixture was shaken (850 rpm) at 35 °C and monitored by TLC (2-propanol:H_2O:aqueous NH_4OH, 7:2:1). The reaction was stopped after 4.5 h by heating (100 °C, 2 min) and centrifuged (13,500 rpm, 10 min). Purification by gel chromatography (Bio-Gel® P-2; mobile phase H_2O, flow rate 7.6 mL h^{-1}, ambient temperature) afforded compound **17** as a white hygroscopic solid; yield: 6.5 mg, 0.018 mmol; 6% related to consumed donor **4**; donor **4** was partially recovered: 108 mg). $[\alpha]_D^{23}$: +120.0 (*c* 0.1 in water); ^{1}H NMR (D_2O; $^1J_{C\text{-}1,H\text{-}1}$ = 172 Hz, $^1J_{C\text{-}1',H\text{-}1'}$ = 172 Hz): δ = 3.424 (dd, *J* = 9.1 Hz, *J* = 9.6 Hz, 1H, H-4′), 3.461 (m, 1H, H-5′), 3.51[a] (m, 2H, H-3, H-4), 3.53[a] (m, 1H, H-6′u), 3.59[a] (m, 1H, H-6u), 3.61[a] (m, 1H, H-3′), 3.65[a] (m, 1H, H-6′d), 3.651 (dd, $J_{1,2}$ = 1.8 Hz, $J_{2,3}$ = 2.8 Hz, H-2), 3.707 (m, 1H, H-5), 3.74[a] (m, 1H, H-6d), 3.75[a] (m, 1H, H-2′), 4.691 (d, $J_{1',2'}$ = 1.7 Hz, 1H, H-1′), 5.220 (d, $J_{1,2}$ = 1.8 Hz, 1H, H-1); ^{13}C NMR (D_2O, HMQC readouts): δ = 61.15 (C-6'), 65.77 (C-6), 66.50 (C-4), 66.94 (C-4′), 69.97 (C-2), 70.17 (C-2′), 70.28 (C-3), 70.75 (C-3′), 72.97 (C-5′), 73.05 (C-5), 90.05 (C-1), 99.63 (C-1′), [a] Readout from HSQC; MS (MALDI-TOF): *m/z* = 390.1 $[M+Na]^+$, 406.2 $[M+K]^+$.

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