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RESEARCH ARTICLE

2-Picoline-borane: A non-toxic reducing agent for oligosaccharide labeling by reductive amination

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Analysis of *N*-glycans is often performed by LC coupled to fluorescence detection. The *N*-glycans are usually labeled by reductive amination with a fluorophore containing a primary amine to allow fluorescence detection. Moreover, many of the commonly applied labels also allow improved mass spectrometric detection of oligosaccharides. For reductive amination, the amine group of the label reacts with the reducing-end aldehyde group of the oligosaccharide to form a Schiff base, which is reduced to a secondary amine. Here, we propose the use of 2-picoline-borane as the reducing agent, as a non-toxic alternative to the extensively used, but toxic sodium cyanoborohydride. Using dextran oligosaccharides and plasma *N*-glycans, we demonstrate similar labeling efficacies for 2-picoline-borane and sodium cyanoborohydride. Therefore, 2-picoline-borane is a non-toxic alternative to sodium cyanoborohydride for the labeling of oligosaccharides.

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1 Introduction

Protein-linked glycans are involved in important biological processes such as cell-cell interaction, receptor activation and molecular trafficking [1], and recent research into the role of glycans in drug therapy and the search for glycans as biomarkers in specific diseases have created the need for fast and sensitive high-throughput glycoanalytical methods [2, 3]. Such methods comprise HPLC [4, 5] and CE [6] coupled to UV, fluorescence or mass spectrometric detection. Alternatively, MS may be used as a stand-alone technique [7, 8]. Many approaches involve glycan derivatization for the introduction of a UV-absorbing or fluorescent tag [9, 10]. Moreover, these derivatization reactions often result in enhanced ionization efficiencies [11].

An easy and widely used labeling technique involves coupling of an oligosaccharide to an amine-substituted chromophore or fluorophore by reductive amination. In a reversible reaction, the open-ring form of the carbohydrate reacts with the amine group and eliminates water to form a Schiff base. In a second, irreversible reaction, the Schiff base is reduced to form a secondary amine (Fig. 1). The reducing agent that is most widely used in this reaction is sodium cyanoborohydride ($NaBH_3CN$). A major drawback of this reagent is that upon hydrolysis, it readily forms the toxic, volatile compound hydrogen cyanide. Therefore, the alternative reducing agent sodium triacetoxyborohydride ($NaBH(OAc)_3$) has been introduced for the reductive amination of carbohydrates with 4-amino-N-[2-(diethylamino)ethyl] benzamide, and this approach was hypothesized to be suitable for all amine-labels [11]. This reducing agent, however, is not broadly applied in glycan analysis.

We recently developed a high-throughput sample preparation method for *N*-glycan analysis that involves

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Abbreviations: 2-AA, 2-aminobenzoic acid; **2-AB,** 2-aminobenzamide; **HILIC,** hydrophilic interaction chromatography; **$NaBH_3CN$,** sodium cyanoborohydride; **$NaBH(OAc)_3$,** sodium triacetoxyborohydride

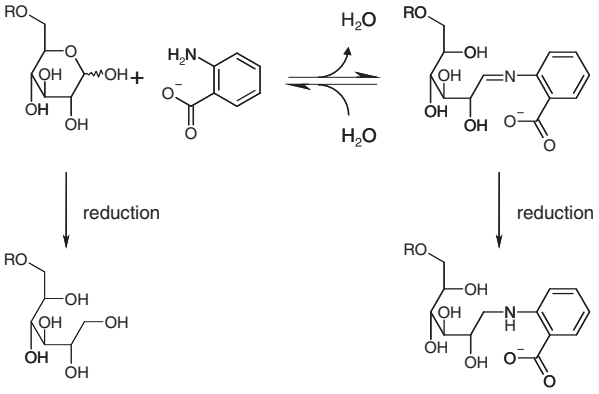

Figure 1. Reaction mechanisms of reductive amination of glucose oligomers derived from dextran with 2-AA label. Reduction of non-labeled glucose oligomers is observed as a side reaction.

reductive amination with 2-aminobenzoic acid (2-AA) in aqueous conditions [5]. To avoid the generation of the acutely toxic compound hydrogen cyanide, we were looking for a substitute for the reducing agent $NaBH_3CN$. 2-Picoline-borane has been described as a reducing agent for the reductive amination of aldehydes and ketones [12]. Here, we show that 2-picoline-borane can be applied as an equally efficient but non-toxic alternative to $NaBH_3CN$ in labeling of oligosaccharides with 2-AA and 2-aminobenzamide (2-AB) by reductive amination.

2 Materials and methods

2.1 Materials

DMSO, ammonium hydroxide, formic acid, Nonidet P-40 (NP-40), 2-AA, 2-AB, $NaBH_3CN$, $NaBH(OAc)_3$ and 2-picoline-borane were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). SDS was bought from United States Biochemicals (Cleveland, OH). PNGaseF was obtained from Roche Diagnostics (Mannheim, Germany). Glacial acetic acid was from Merck (Darmstadt, Germany). ACN was purchased from Biosolve (Valkenswaard, The Netherlands). Dextran 10.000 was obtained from Pharmacia/GE Healthcare (Uppsala, Sweden).

2.2 Preparation of oligosaccharides

A glucose ladder was produced by acid hydrolysis of 10 mg of dextran 10 000 in 1 mL of 1 M TFA (1 h at 80°C). The sample was subsequently diluted using 4 mL of water. N-glycans from human plasma were prepared as described previously [5]. Briefly, proteins from 10 μL of plasma were denatured after addition of 20 μL 2% SDS by incubation at 60°C for 10 min. Subsequently, 10 μL 4% NP-40 and 0.5 mU of PNGase F in 10 μL 5 × PBS were added to the samples. The samples were incubated overnight at 37°C for *N*-glycan release.

2.3 Labeling of oligosaccharides

Labeling of oligosaccharides was performed as published [13] with slight modifications: 50 μL of oligosaccharide solutions (either glucose ladder or plasma *N*-glycans without prior purification) were mixed with 25 μL of a freshly prepared solution of label (2-AA or 2-AB; 48 mg/mL in DMSO containing 15% glacial acetic acid). About 25 μL aliquots of freshly prepared reducing agent solutions (1 M $NaBH_3CN$, 2-picoline-borane, or $NaBH(OAc)_3$ in DMSO) were added, followed by 5 min of shaking and incubation at 65°C for 2 h. To allow comparison to other studies, 2-AB labeling was also performed under water-free conditions, as previously published [13]. Briefly, 50 μL of glucose ladder samples was brought to dryness using vacuum centrifugation. Oligosaccharide samples were then mixed with 25 μL of a freshly prepared solutions of 96 mg/mL 2-AB in DMSO containing 15% glacial acetic acid. 25 μL aliquots of freshly prepared reducing agent solution (2 M $NaBH_3CN$, 2-picoline-borane, or $NaBH(OAc)_3$ in DMSO) were added, followed by 5 min of shaking and incubation at 65°C for 2 h. The reaction mixture was allowed to cool down to room temperature. Samples were diluted 1:3 v/v with ACN prior to analysis by hydrophilic interaction chromatography with fluorescence detection (HILIC-HPLC-FL).

2.4 Porous graphitic carbon-SPE

Free label and reducing agent were removed from the samples using porous graphitic carbon (PGC) SPE. The carbograph SPE cartridges (Grace, Breda, The Netherlands) were conditioned with 2 mL of 80% ACN in water, followed by equilibration with 3 mL of water. Samples were mixed with 300 μL of water and were loaded on the cartridges. After washing with 4 mL water, oligosaccharides were eluted using 1 mL of 50% ACN containing 0.1% TFA. Eluates were diluted 1:1 v/v with ACN prior to analysis by HILIC-ESI-IT-MS (MS).

2.5 HILIC-HPLC-FL

Labeled *N*-glycans were analyzed using HILIC-HPLC with fluorescence detection. The Ultimate LC system (Dionex, Sunnyvale, CA, USA) consisted of a Famos autosampler, a Switchos module with a loading pump, and an ultimate pump module. The system was connected to a fluorescence detector (FP-2020 plus; Jasco, Easton, MD, USA), which was operated at excitation wavelength 360 nm and emission

wavelength 420 nm. The system was controlled by Chromeleon software and equipped with a 2.0 mm × 10 mm TSK gel-Amide 80 trapping column and a 2.0 mm × 250 mm TSK gel-Amide 80 analytical column (Tosoh Biosciences, Stuttgart, Germany).

Sample-containing wells in a 800 μL, V-bottom polypropylene 96-well plate (Westburg, Leusden, The Netherlands) were sealed with silicon lids (Labservices, Breda, The Netherlands), and placed in the autosampler. A 20 μL aliquot was injected using a full loop injection procedure. The labeled oligosaccharides were transfered to the trapping column and washed using ACN: 50 mM ammonium formate (pH 4.4; 80:20, v/v) for 3 min at a flow rate of 150 μL/min. Subsequently the trapping column was switched in line with the analytical column which was equilibrated with 70% ACN (solvent A), 30% ammonium formate (50 mM, pH 4.4 (solvent B) at a flow rate of 150 μL/min. A linear gradient was applied with Solvent B increasing from 30% (0 min) to 60% (87 min) followed by 5 min isocratic elution at 60% solvent B and reequilibration of the column at 30% solvent B for 15 min.

2.6 HILIC-ESI-IT-MS(/MS)

HILIC-nanoLC-ESI-ion trap (IT)-MS/MS was performed on aAmide-80 column (3 μm particles; 75 μm × 150 mm; Tosoh Biosciences) using an Ultimate 3000 nanoLC system (Dionex) equipped with a guard column (5 μm Amide-80 170 μm × 10 mm). Samples were brought to an ACN content of 75%, and 10 μL sample were transferred to the guard column, which was washed for 5 min with ACN:50 mM ammonium formate (pH 4.4, 90:10 v/v). The guard column was then brought in line with the nano column which was operated at a flow rate of 400 nL/min and equilibrated with 17.5% solvent A (50 mM ammonium formate, pH 4.4) and 82.5% solvent B (ACN:50 mM ammonium formate, pH 4.4, 80:20 v/v). After an immediate step to 25% solvent A, a linear gradient was applied to 40% solvent A within 30 min, followed by a 4 min wash at 100% solvent A and reequilibration at 17.5% solvent A for another 25 min. The nanoLC system was directly coupled to an Esquire High Capacity Trap (HCTultra) ESI-IT-MS (Bruker Daltonics) equipped with an online nano-spray source operating in the positive-ion mode. For electrospray (900–1200 V), capillaries (360 μm od, 20 μm id with 10 μm opening) from New Objective (Cambridge, MA, USA) were used. The solvent was evaporated at 165°C with a nitrogen stream of 6 L/min. Ions from *m/z* 400 to *m/z* 2000 were registered. Automatic fragment ion analysis was enabled for precursors between *m/z* 1170 and *m/z* 1180, resulting in tandem mass spectra of the reduced Glc_7species.

2.7 Data processing

For the HILIC-HPLC-FL data, peak intensities in milli volts were determined for five peaks from the glucose standard. Results from duplicate analyses were averaged. The differences between replicates were less than 10%. The HILIC-ESI-IT-MS data were analyzed using Data analysis version 4.0 software (Bruker Daltonics). Peak intensities of proton, ammonium, sodium and potassium ions from Glc_4 to Glc_8 were determined for both labeled and non-labeled species. Both single and double charged ions were considered. Intensities of all adducts were summed, and the relative abundances of labeled *versus* non-labeled glycans were calculated.

3 Results

The reducing agents $NaBH(OAc)_3$ and 2-picoline-borane were evaluated for their ability to serve as substitutes for $NaBH_3CN$ in the labeling of *N*-glycans by reductive amination. Therefore, dextran-derived glucose oligomers and *N*-glycans from human plasma were labeled using the aforementioned three reducing agents. Glucose oligomers were labeled with both the amines 2-AA and 2-AB, whereas plasma-derived *N*-glycans were labeled with 2-AA only. All samples were analyzed using HILIC-HPLC-FL. A selection of samples, *i.e.*, 2-AA labeled glucose ladder and 2-AB-labeled glucose ladder (non-aqueous labeling), were additionally purified by graphitized carbon-SPE and analyzed by HILIC-LC-ESI-IT-MS.

HILIC-HPLC-FL chromatograms of the glucose ladder after labeling using different reducing agents and labels are depicted in Fig. 2A–C. From these data, average peak heights of the Glc_4–Glc_8 oligomers were determined (Table 1). The observed intensities for fluorescently labeled glucose oligomers were very similar for 2-picoline-borane and $NaBH_3CN$, whereas the use of $NaBH(OAc)_3$ resulted in much lower intensities. This holds true for 2-AA labeling as well as 2-AB labeling under both aqueous and non-aqueous conditions. Notably, the labeling of glucose oligomers with 2-AB was just as efficient under aqueous as under non-aqueous conditions, for both $NaBH_3CN$ and 2-picoline-borane.

To determine labeling efficacies, two glucose oligomer ladders with 2-AA and 2-AB label (non-aqueous labeling) were purified by graphitized carbon SPE and analyzed by HILIC-ESI-IT-MS, which allowed the parallel registration of labeled glucose oligomers, unlabeled species, as well as side products of the labeling reaction. As an example of the obtained data, mass spectra of the 2-AA-labeled Glc_7, as well as the remaining Glc_7 are depicted in Fig. 3. $NaBH_3CN$ and 2-picoline-borane show nearly complete labeling, with high-intensity signals for the proton adducts (*m/z* 1274.3) as well as sodium adducts (*m/z* 1296.3) of the 2-AA labeled Glc_7 (Fig. 3B and D), whereas only low amounts of unlabeled Glc_7 were detected (ammonium adducts at *m/z* 1170.3 and sodium adducts at *m/z* 1175.3 in Fig. 3A and C). In contrast, hardly any labeled Glc_7 was observed for $NaBH(OAc)_3$ (Fig. 3F), with most of the Glc_7 being present in unlabeled form (Fig. 3E). Glc_4–Glc_7 were monitored by HILIC-ESI-IT-MS in labeled as well as unlabeled form, while glucose oligomers with a higher degree of

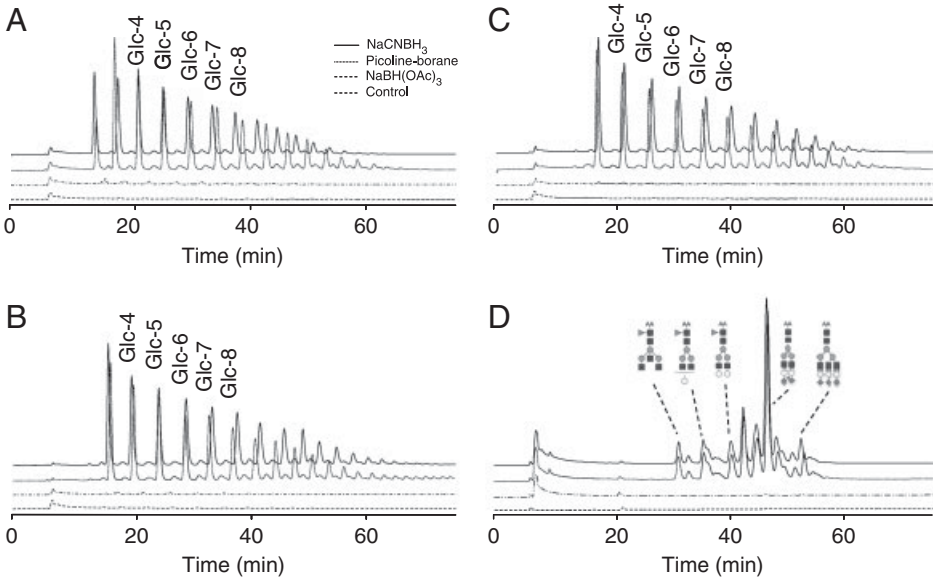

Figure 2. HILIC-HPLC-FL chromatograms of labeled oligosaccharides, where different reducing agents were applied. Chromatograms of glucose oligomer standard with (A) 2-AA-label, (B) 2-AB-label using non-aqueous conditions and (C) 2-AB-label using aqueous conditions. Chromatogram of *N*-glycans from human plasma with 2-AA label (D). Chromatograms present in the same panel are scaled in the same manner. Compositions and structural schemes are given in terms of N-acetylglucosamine (square), mannose (dark circle), galactose (light circle), sialic acid (diamond) and fucose (triangle).

Table 1. Intensities of dextran derived glucose oligomers labeled with the aid of different reducing agents and their labeling efficiency

Reducing agent	Glucose oligomer	2-AA	2-AB, non-aqueous	2-AB, aqueous
$NaBH_3CN$	Glc_4	598 (100%)	585 (96%)	568 (n.d.)
	Glc_5	501 (100%)	476 (97%)	463 (n.d.)
	Glc_6	433 (100%)	427 (98%)	404 (n.d.)
	Glc_7	374 (86%)	352 (98%)	342 (n.d.)
	Glc_8	313 (n.d)	310 (n.d.)	287 (n.d.)
2-Picoline-borane	Glc_4	789 (100%)	594 (99%)	601 (n.d.)
	Glc_5	605 (100%)	492 (98%)	501 (n.d.)
	Glc_6	486 (100%)	399 (98%)	428 (n.d.)
	Glc_7	476 (90%)	385 (98%)	364 (n.d.)
	Glc_8	376 (n.d.)	328 (n.d.)	317 (n.d.)
$NaBH(OAc)_3$	Glc_4	22 (0%)	13 (4%)	7 (n.d.)
	Glc_5	18 (4%)	10 (6%)	6 (n.d.)
	Glc_6	16 (1%)	7 (6%)	5 (n.d.)
	Glc_7	13 (1%)	5 (4%)	4 (n.d.)
	Glc_8	10 (n.d.)	2 (n.d.)	3 (n.d.)
Control (no reducing agent)	Glc_4	0 (0%)	0 (0%)	0 (n.d.)
	Glc_5	0 (0%)	0 (0%)	0 (n.d.)
	Glc_6	0 (0%)	0 (0%)	0 (n.d.)
	Glc_7	0 (0%)	0 (0%)	0 (n.d.)
	Glc_8	0 (n.d.)	0 (n.d.)	0 (n.d.)

Intensities are shown in mV. n.d., not determined.

polymerization were not fully covered within the time window of the LC-MS analysis and, therefore, not used for determination of the labeling efficacy. In accordance with the HILIC-HPLC-FL data, the LC-MS data for labeled *versus* unlabeled Glc_4, Glc_5, Glc_6 and Glc_7, which are summarized in Table 1, indicate efficient labeling for both $NaBH_3CN$ and 2-picoline-borane, whereas $NaBH(OAc)_3$ appeared to be a very inefficient reducing agent.

Interestingly, in the case of both $NaBH_3CN$ and 2-picoline-borane, additional peaks at m/z 1172.3 and m/z 1177.3 were detected that co-eluted with the non-labeled Glc_7. (Fig. 3A and C). These peaks correspond to ammonium and sodium adducts, respectively, of the reduced form of Glc_7 as shown by HILIC-ESI-IT-MS/MS analysis of the sodium adduct (m/z 1177.3; Fig. 4). The B_6-ion at m/z 995.3 resulted from the loss of the innermost residue (−182 Da), indicating that this residue is a reduced glucose. The masses of the observed Y-ions were likewise 2 Da higher than in the case of a native glucose oligomer, which again is consistent with the

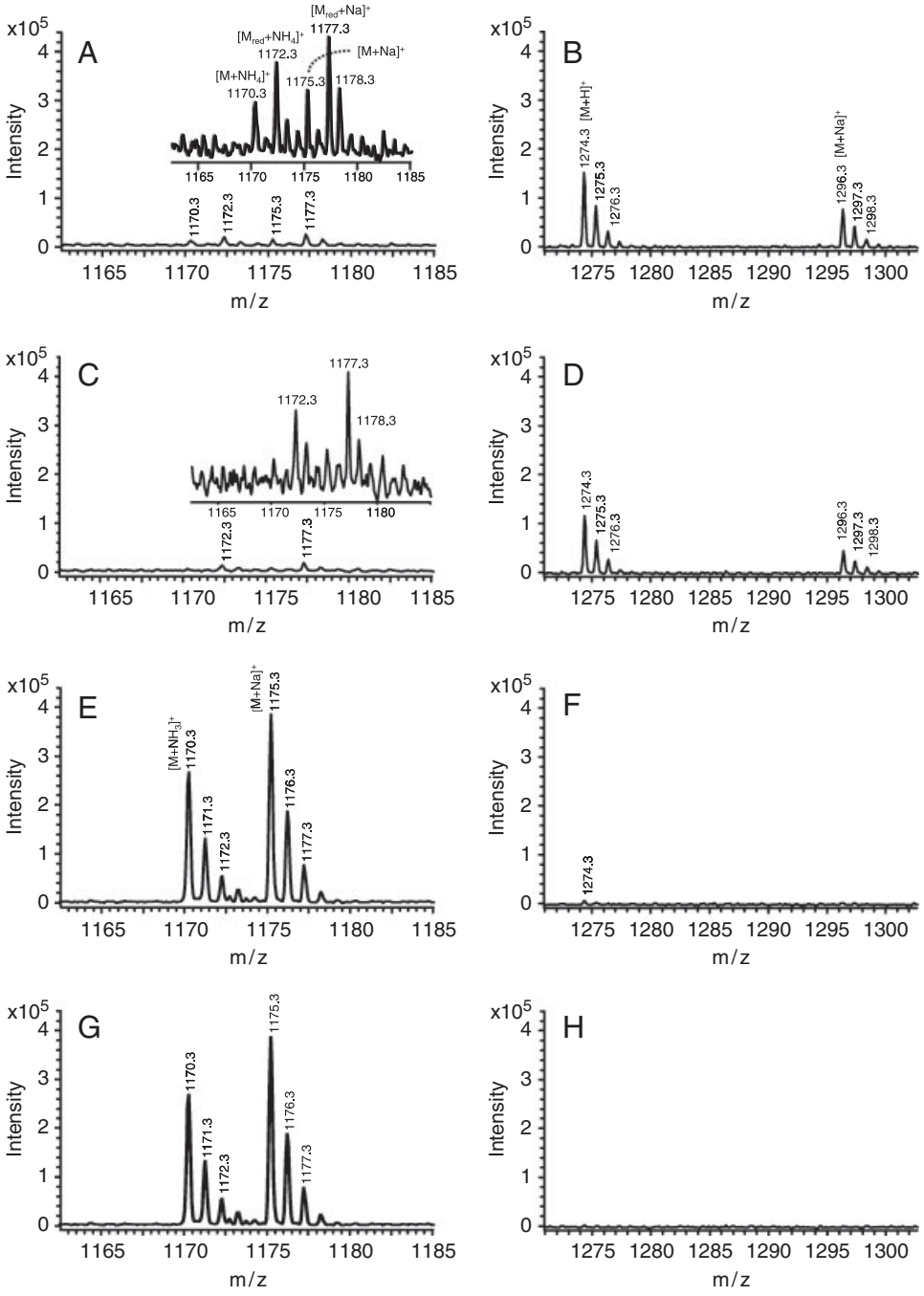

Figure 3. HILIC-ESI-IT-MS spectra of 2-AA-labeled and non-labeled glycans from Glc_7. Mass spectra of Glc_7 derivatized using 2-AA and $NaBH_3CN$ (A, B), 2-picoline-borane (C, D), $NaBH(OAc)_3$ (E, F) and DMSO (G, H). Both non-labeled (A, C, E and G) as well as labeled glycans (B, D, F and H) are shown. All spectra are depicted at the same intensity, and can thus be compared immediately. For non-labeled glycans ammonium as well as sodium adducts are depicted, whereas for labeled glycans both proton and sodium adducts are included.

reduction of the innermost glucose. These results thus indicate that the reducing agents $NaBH_3CN$ and 2-picoline-borane not only reduce the Schiff-base, but may also reduce the native glycan. This reduction is depicted in the reaction mechanism in Fig. 1.

To test the alternative reducing agents on more relevant biological samples, *N*-glycans derived from human plasma were labeled using $NaBH_3CN$, 2-picoline-borane and $NaBH(OAc)_3$. HILIC-HPLC-FL chromatograms as depicted in Fig. 2D, showed similar labeling performance of $NaBH_3CN$ and 2-picoline-borane. Again, $NaBH(OAc)_3$ appeared to be much less efficient.

4 Discussion

We here describe the use of a novel, non-toxic reducing agent 2-picoline-borane for the reductive amination of oligosaccharides. This reducing agent has previously been reported for the amination of several aldehydes and ketones

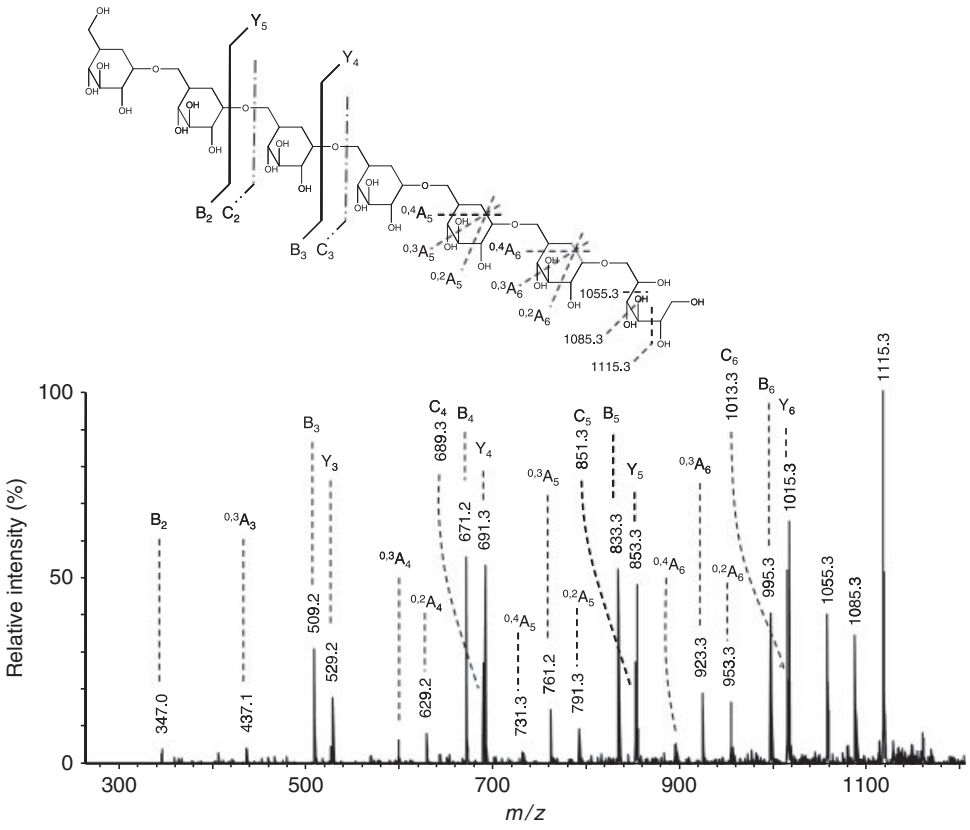

Figure 4. HILIC-ESI-IT-MS/MS of reduced Glc_7. Fragmentation was performed on the signal at *m/z* 1177.3 which represents the sodium adduct of reduced Glc_7. Fragment ions were annotated according to Domon and Costello [14].

[12], a reaction widely applied within the field of organic chemistry. 2-Picoline-borane showed labeling efficacies which were very similar to those observed for the extensively used reducing agent $NaBH_3CN$. Using the alternative reducing agent $NaBH(OAc)_3$, which has been described in literature for the reductive amination of oligosaccharides [11], we could not obtain satisfying yields. In this study, however, we applied a lower concentration of reducing agent than originally published [11]. Moreover, originally a two-step reaction was performed: the Schiff base was first produced, whereas the reducing agent was only introduced after 1 hour. These changes might have caused the low yields of labeled glycans we obtained. As a two step reaction is not favorable in high-throughput analysis, we did not consider it in this study.

Although 2-picoline-borane is approximately four times more expensive than $NaBH_3CN$, the use of 2-picoline-borane is advantageous. Notably, the application of this much less toxic compound is environmentaly friendly and reduces the health risks for the researchers, as no hydrogen cyanide is released upon contact with water, as is the case with $NaBH_3CN$. Especially in a research environment where glycan analysis is performed in a high-throughput application, relatively large amounts of the acute toxic compound hydrogen cyanide are generated. An additional advantage is that the reductive amination could be performed equally well in aqueous as well as non-aqueous conditions, which should allow the reduction of organic solvents in labeling procedures.

Although 2-picoline-borane might now be readily applied for the labeling of oligosaccharides, further research towards optimal labeling conditions, such as concentration and reaction temperature, might increase its potential. Overall, 2-picoline-borane would appear to be an efficient, non-toxic and environmentaly-friendly alternative for the use of $NaBH_3CN$ as reducing agent in the reductive amination of oligosaccharides, which lends itself well to application in high-throughput procedures.

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The authors have declared no conflict of interest.

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