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Short communication

Selective derivatization of nucleotide diphosphate (NDP)-4-keto sugars for electrospray ionization-mass spectrometry (ESI-MS)

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ABSTRACT

Nucleotide diphosphate (NDP) sugars are widely present in antibiotics and glycoconjugates, such as protein- and lipid-linked oligosaccharides, where they act as substrates for glycosyltransferase in eukaryotes and prokaryotes. Among NDP sugars, NDP-4-keto sugars are key intermediates in the synthesis of structurally diverse NDP sugars with different functional groups. However, the structural identification of the NDP-4-keto sugars via mass spectrometry (electrospray ionization-mass spectrometry (ESI-MS)) continues to be a challenge because of the carbonyl group in these sugars interferes with ionization process. In this study, we evaluated various hydroxylamine compounds for the derivatization of NDP-4-keto sugars, so that the detection of the sugars by ESI-MS is more efficient. As a result, *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine was found to be the most effective tagging molecule for the detection of NDP-4-keto sugars without being interfered by original MS. This method can be used for identifying NDP-4-keto sugars such as thymidine diphosphate (TDP)-, adenosine diphosphate (ADP)-, uridine diphosphate (UDP)-, and cytosine diphosphate (CDP)-4-keto sugars as well as new NDP-4-keto-dehydratases.

1. Introduction

Combinatorial biosynthesis, a technique involving the use of a sugar moiety and an aglycon of an antibiotic for producing diverse glycosylated antibiotics, has been proposed to speed up the development of antibiotics [1,2]. To make combinatorial biosynthesis possible, nucleotide diphosphate (NDP) sugars (activated sugars) must be used as substrates for glycosyltransferase. Among NDP sugars, NDP-4-keto sugars such as TDP-4-keto-6-deoxy-D-glucose (TDP-4K6D) [3,4] (a key intermediate of TDP-D-olivose, TDP-L-oleandrose, TDP-L-rhamnose, and TDP-L-oliose) and GDP-4-keto-6-deoxy-D-mannose (GDP-4K6D) (a key intermediate of GDP-β-L-fucose, GDP-6-deoxy-L-glucose, GDP-colitose, and GDP-perosamine) are used in the synthesis of antibiotics and glycoconjugates [5–10]. A typical method for NDP-4K6D synthesis involves the use of a two-enzyme system containing NDP-glucose synthase and NDP-D-glucose 4,6-dehydratase and glucose-1 phosphate and nucleotide triphosphate (NTP) as substrates [11]. Various enzymatic processes have been developed for the production of sugar nucleotides from inexpensive and widely available substrates via efficient and economical pathways following the de novo (or le Loir) synthesis of carbohydrates [9,12,13].

In sugar-nucleotide synthesis, high-performance liquid chromatography (HPLC) has generally been used to detect the products and the enzyme activity of NDP-4K6D [4,9]. However, this method requires an authentic NDP-4-keto molecule, which is not easily available, and a high concentration of salt (<30 mM) or ion-pairing reagents. Consequently, the HPLC column is easily contaminated

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and an additional desalting step is needed to identify NDP-4K6D [4,9,12]. Alternatively, mass spectrometry (MS)-based methods have been used to identify the reaction products. However, in most cases, it is difficult to analyze carbonyl compounds in a reaction mixture because of their polar, reactive, and semi-volatile nature; further, high concentration of samples is needed to obtain an MS signal [14,15].

On the basis of previous research in which hydroxylamine reagents were used to stabilize carbonyl compounds and increase their sensitivity in MS analysis [16,17], available derivatization reagents such as hydroxylamine, *O*-methylhydroxylamine, *O*-ethylhydroxylamine, *O*-allyl hydroxylamine, *O*-*tert*-butylhydroxylamine, *O*-phenylhydroxylamine, *O*-4-(nitrophenyl) hydroxylamine, and *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine were evaluated for the formation of solution-ionizable analyte derivatives with NDP-4-keto sugar. This derivatization has never been applied to the synthesis of NDP-sugar-related molecules. Taken together, we identified the most efficient tagging molecule to detect NDP-4-keto sugar and obtained the peak for derivatized NDP-4-keto-sugar without interference by original MS using *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine. This method can be applied to other NDP sugars such as adenosine diphosphate (ADP) sugar, uridine diphosphate (UDP) sugar, and cytosine diphosphate (CDP) sugar, which are common in plants, and can be used to identify new NDP-4-keto-dehydratases.

2. Materials and methods

2.1. Strains and materials

The chemical reagents used in this study, hydroxylamine, *O*-methylhydroxylamine, *O*-ethylhydroxylamine, *O*-allyl hydroxylamine, *O*-*tert*-butylhydroxylamine, *O*-phenylhydroxylamine, *O*-4-(nitrophenyl)hydroxylamine, *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine, and GDP-D-mannose were purchased from Sigma (MO, USA). TDP-D-glucose (TDP-Glc) was supplied by GeneChem (Daejeon, Korea). TDP-4K6D was synthesized enzymatically from thymidine triphosphate (TTP) and glucose-1-phosphate and purified by a previously reported method (97% purity confirmed by HPLC) [12]. The GDP-mannose-4,6-dehydratase gene was amplified using the chromosomal DNA of *Helicobacter pylori* as a template [18] and cloned into pET28a (Novagen, USA). Proteins were prepared in *E. coli* BL21(DE3). For the production of GDP-4K6D, purified GDP-pyrophosphorylase (1.85 μg) was mixed with Tris–HCl buffer (0.05 M, pH 7.5) and GDP-mannose (5 mM) and incubated at 37 °C for 1 h.

2.2. Derivatization method for TDP-4K6D

The derivatization of TDP-4K6D with different reagents was performed as previously reported [15,19]. The process, in brief, is as follows. Solutions of TDP-4K6D (0.5 mM, 100 μl) and the derivatizing agent (50 mM, 10 μl) in deionized water were mixed. The mixed samples were preheated at 40 °C for 10 min with stirring at 1100 rpm and then were transferred to vials.

2.3. Analytical method

ESI-MS was performed using an LCQ Deca XP ion trap mass spectrometer (Thermo Electron Corp, USA) with standard Thermo ESI source in negative ion mode at a spray voltage of 5 kV. Samples were infused into the mass spectrometer with a syringe pump at a rate of 5 μl min^{-1} and nebulized with dry nitrogen gas [20]. The heated capillary was maintained at 270 °C. The maximum ion collection time was set to 50 ms, and five microscans were averaged per scan. The sample of NDP-4-keto sugar (2 mM) was analyzed by HPLC using an RS-tech C18 column (250 mm × 4.6 mm; particle size: 5 μm). A mixture of 30 mM potassium phosphate and 5 mM tetrabutylammonium bisulfate in water was used as the mobile phase at a flow rate of 1 ml min^{-1}, and the diode array detector was set at 260 nm.

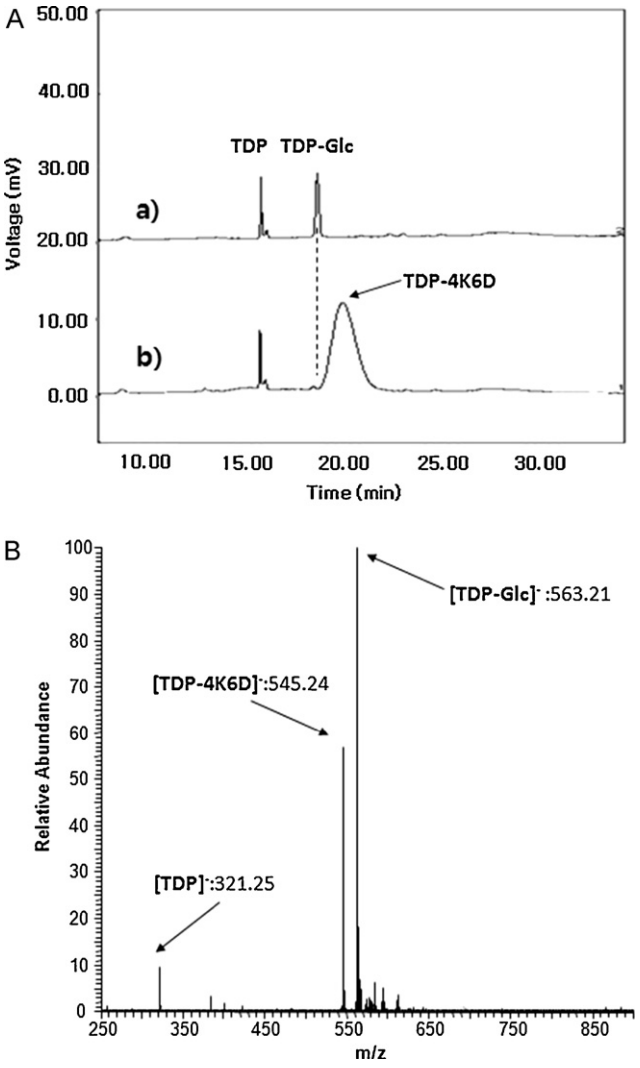


Fig. 1. HPLC analysis of TDP-sugars (A) and ESI-MS analysis of purified TDP-4K6D (0.45 mM) (B) before derivatization. For HPLC analysis, TDP (0.05 mM) and TDP-Glc (0.05 mM) (a) and TDP-4K6D (0.45 mM) (b) were used. Small peak due to TDP-Glc could be detected in the chromatogram (b).

3. Results and discussion

3.1. Selective derivatization of various hydroxylamines on TDP-4K6D

Although highly purified TDP-4K6D was used as previously described [12] and the relative molar ratio of TDP-4K6D as confirmed by HPLC was much higher than that of TDP-glucose (>97%) (Fig. 1A), ESI-MS profiling showed that TDP-4K6D was not easily detected in the spectral analysis (Fig. 1B). To overcome this issue, a derivatization method for MS was applied on the basis of the reaction of ketones (NDP-4K6D) with hydroxylamine to afford oximes [21]. This is the first step in the selective derivatization of TDP-4K6D, which is described in Fig. 2A. To examine the selective derivatization of the carbonyl group of

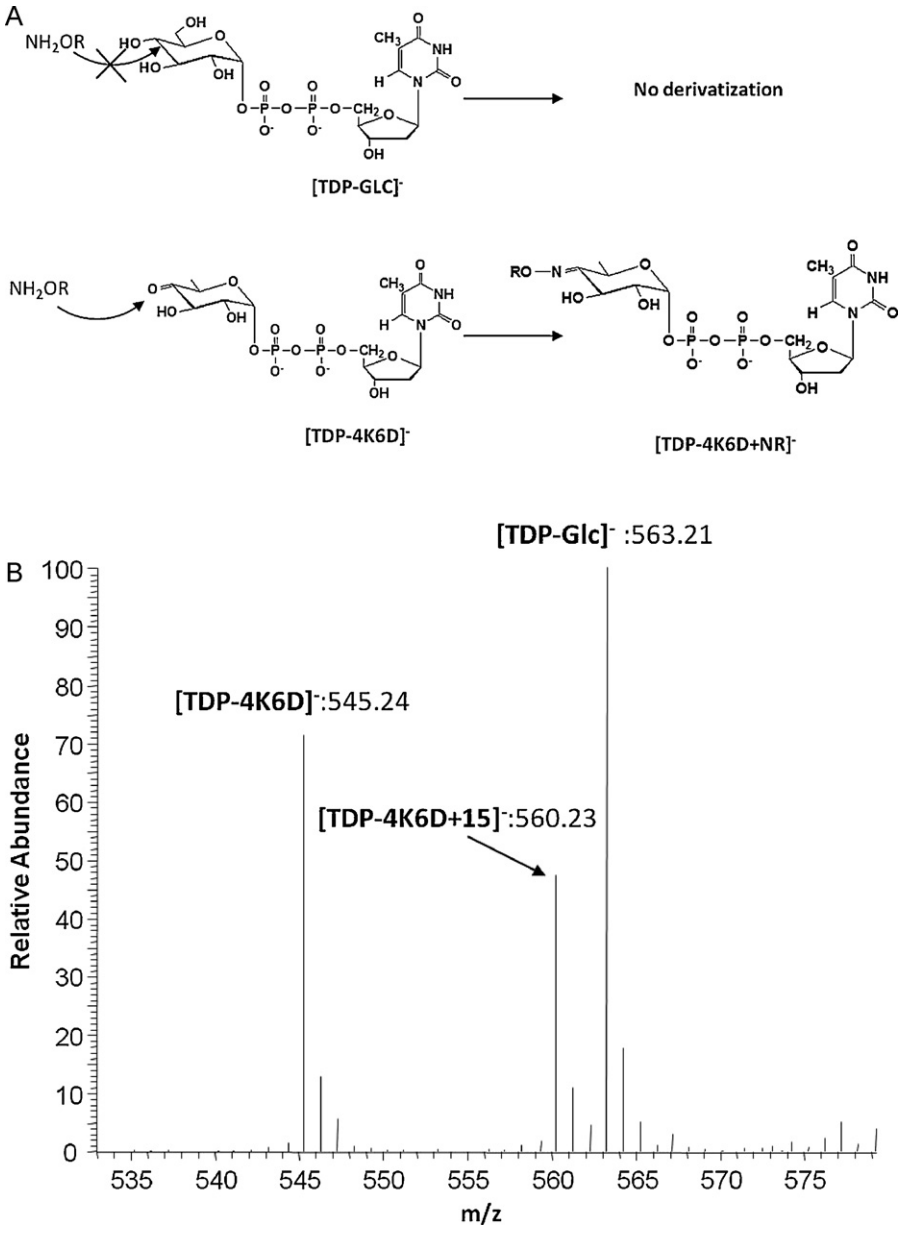


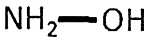
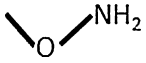
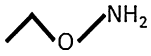
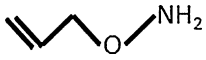
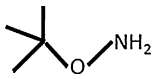
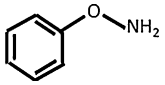
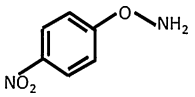
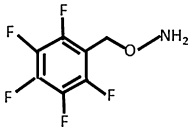
Fig. 2. Scheme of selective derivatization of TDP-4K6D with derivatization molecule (A) and ESI-MS analysis of the derivatization of TDP-4K6D with hydroxylamine.

TDP-4K6D, hydroxylamine was initially used for the derivatization of TDP-4K6D. Peaks due to TDP-4K6D (m/z = 545.2), TDP-Glc (m/z = 563.2), and TDP-4K6D-NH (m/z = 560.2) were identified (Fig. 2B) in the spectrum. This suggested that hydroxylamine can be covalently attached to TDP-4K6D, although some rearrangements may be possible with TDP-4K6D + NR [16]. There were two additional carbonyl groups in the pyrimidine moiety of TDP-GLC (Fig. 2A). However, hydroxylamine did not attach to them, and no peak due to TDP-Glc-NH (m/z = 578.2) was identified. This suggested that hydroxylamine can selectively derivatize the carbonyl group of a sugar moiety. Although the reaction of TDP-4K6D with hydroxylamine indicated the selectivity of hydroxylamine, the intensity of the TDP-4K6D-NH peak (m/z = 560.2) was less than that of the TDP-Glc peak, which was not strong evidence for confirming the suitability of hydroxylamine as a derivatization molecule. Therefore, additional hydroxylamine derivatives need to be screened for the selective derivatization of TDP-4K6D.

3.2. Comparison of derivatizing reagents

To identify a molecule that efficiently derivatizes TDP-4K6D and gives highly intensive peaks, eight different molecules *O*-methylhydroxylamine, *O*-ethylhydroxylamine, *O*-allyl hydroxyl amine, *O*-*tert*-butylhydroxylamine, *O*-phenylhydroxylamine, *O*-4-(nitrophenyl)hydroxylamine, and *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine were examined (Table 1). TDP-4K6D was derivatized with each reagent, as explained in Section 2. The peak intensities of TDP-4K6D and its derivatives were compared with the peak intensity of TDP-Glc, and their relative ratios were calculated. After ESI-MS analysis, the peaks of the TDP-4K6D derivatives, except for that of the *O*-*tert*-butylhydroxylamine derivative, were confirmed to have the expected m/z values, which were calculated on the basis of the corresponding molecular weights. The peak due to TDP-4K6D-NC_4H_9 (m/z = 616.24), which is the *O*-*tert*-butylhydroxylamine derivative, was not observed, indicating that

Table 1
Derivatization of TDP-4K6D with different reagents.

Structure	Name (NH_2OR)	Δm[a]	Relative ratio[b]			Absolute intensity[d]
			TDP-Glc	TDP-4K6D	Derivative[c]	
–	No derivatization	0	1	0.56 ± 0.01	0	1.8
	Hydroxyl amine	14.95	1	0.71 ± 0.01	0.43 ± 0.00	1.5
	*O*-Methyl Hydroxyl arnine	29.52	1	0.66 ± 0.00	1.15 ± 0.01	1.7
	*O*-Ethyl hydroxy lamine	43.54	1	0.70 ± 0.00	2.53 ± 0.00	2.2
	*O*-Aillyl hydroxyl arnine	55.54	1	0.71 ± 0.00	3.00 ± 0.04	l.8
	*O*-Terf-butyl hydroxyl arnine	71.14	1	0.53 ± 0.01	0	1.9
	*O*-Phenyl hydroxyla mine	91.59	1	0.53 ± 0.20	12.59 ± 2.03	2.6
	*O*-4-Nitrophenyl hydroxyl arnine	150.51	1	0.79 ± 0.01	7.96 ± 0.67	2.5
	*O*-(Z,3,4,5,6 pentafluoro benzyl] hydroxyl arnine	195.57	1	0.76 ± 0.02	11.46 ± 0.27	6.4

[a] Δm is the mass increase by derivatization.
[b] Relative ratio was calculated from the intensity of TDP-Glc to TDP-4K6D derivatives.
[c] Derivatives of TDP-4K6D with reagents.
[d] Total current ions ($\times 10^8$) detected by ESI-MS.

O-*tert*-butylhydroxylamine did not react with TDP-4K6D (data not shown). The relative peak ratio of *O*-phenylhydroxylamine was the highest (1:12.6 (TDP-Glc:Derivative)) (Fig. 3A), and that of *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine was the second highest (1:11.5 (TDP-Glc:Derivative)) (Fig. 3B). Although the *O*-phenylhydroxylamine derivative gave a more intense peak than the TDP-Glc peak, there was an unknown peak at m/z = 443.22 (Fig. 3A), which was not identified and seemed to interfere with the peaks due to other NDP sugars. Considering these results, *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine, was selected for further use [19]. It enhanced detection limit from 0.39 mM without derivatization up to 0.004 mM with derivatization (Supplementary Fig. 1). Oximes that were formed from *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine show relatively specific mass spectra as well as high sensitivity in different detection systems, such as electron impact (EI) and negative-ion chemical ionization mass spectrometry (NCI-MS), and give better results for aqueous samples [17,22].

3.3. Application of *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine for GDP-4K6D detection in reaction mixture

For further application of *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine to the selective derivatization of NDP-4K6D, the reaction of GDP-mannose to GDP-4K6D-mannose was monitored. The enzymatic reaction was performed as explained in Section 2. After the reaction was terminated, the reaction mixture

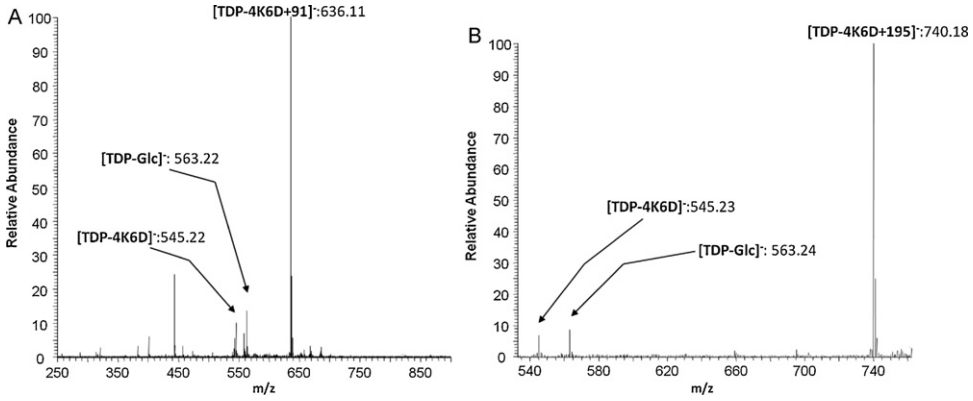


Fig. 3. ESI-MS analysis of the derivatization of TDP-4K6D with *O*-phenylhydroxylamine (A) and *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (B).

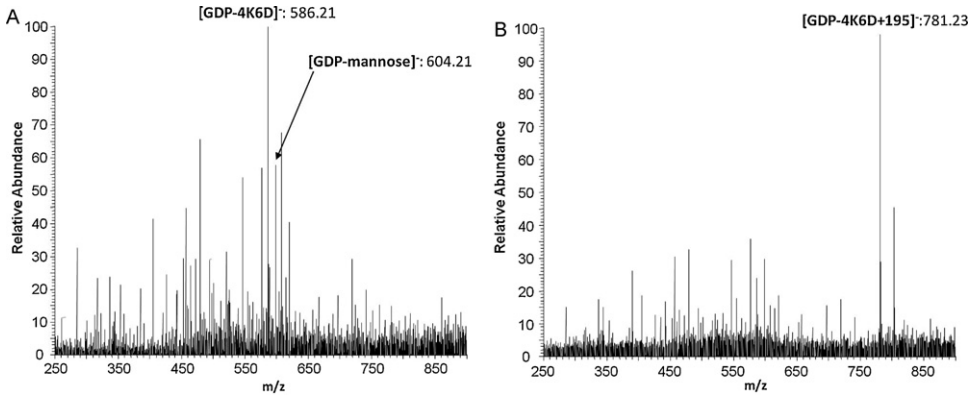


Fig. 4. Application of selective derivatization method for ESI-MS analysis of GDP-4K6D reaction mixture without (A) and with (B) *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine.

was boiled and centrifuged to remove the enzymes, and then, the residual mixture of GDP-mannose and GDP-mannose-4,6-dehydratase was directly analyzed by ESI-MS. The enzymatic reaction was performed as explained in Section 2, and the reaction mixture of GDP-mannose with GDP-mannose-4,6-dehydratase was directly applied to ESI-MS after stopping the reaction with boiling and centrifugation to remove enzymes. Unlike TDP sugars, the peak due to GDP-4K6D showed better intensity in the spectrum than did the peak due to GDP-mannose (Fig. 4A). However, there were several noise peaks around the GDP-4K6D peak, and the signal-to-noise ratio was quite low. By applying the derivatization method to the GDP-4K6D reaction mixture, an intense derivative peak (m/z = 781.23) was observed (Fig. 4B), confirming the presence of GDP-4K6D, which is the product GDP-mannose-4,6-dehydratase.

In conclusion, to detect the NDP-4K6D sugar by ESI-MS, we employed a selective derivatization method using a hydroxylamine derivative. Among the eight different candidates studied, *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine dramatically improved the sensitivity of NDP-4K6D. Therefore, this method can be applied to an unpurified reaction mixture and used to measure the enzyme activity of NDP-4K6D.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2012.02.045.

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