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Negative ion ultraviolet matrix-assisted laser desorption ionization mass spectrometry and post source decay of glycosyl esters of nucleoside pyrophosphates*

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Abstract

Six different glycosyl esters of nucleoside pyrophosphates (monosaccharide nucleotides) were analyzed by means of matrix-assisted laser desorption/ionisation reflectron time-of-flight mass spectrometry (MALDI/RTof MS) in the negative ion mode. Several matrices were evaluated and 3-hydroxypicolinic acid as well as α -cyano-4-hydroxycinnamic acid (CHCA) turned out to be the matrices of choice applying the thin layer technique to obtain maximum sensitivity for deprotonated molecular ion detection and maximal fragmentation particular with CHCA. The determination of the molecular mass with a mass accuracy below 0.1% was feasible with sample amounts in the lower femtomole range applying a MALDI desk-top mass spectrometer. A further important refinement of this technique was the use of post source decay (PSD) fragment ion analysis with a curved field reflector (which means no stepping of the reflector voltage). Detailed structural information of the six selected monosaccharide nucleotides could be obtained with PSD and differences in the fragmentation pattern were used to distinguish them. This method (based on molecular mass and PSD fragment ion analysis) has been applied to verify the presence of a glycosyl ester of nucleoside pyrophosphate in samples from *Saccharomyces cerevisiae*.

Keywords

MALDI; post source decay; mass spectrometry; carbohydrate biosynthesis; glycosyl ester of nucleoside pyrophosphate; negative ions; ToF

Introduction

Glycosyl esters of nucleoside pyrophosphates ("monosaccharide nucleotides or sugar nucleotides") are fundamental for many biochemical processes in animals, plants as well as in microorganisms.¹⁻⁵ They function as donors for glycosyl residues where a donor of

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glycosyl residues is required (i.e. glycogen biosynthesis, cellulose biosynthesis). The development of sensitive detection methods of these activated sugars helps to understand different metabolic processes. The glycogen metabolom, for instance, is highly dependent on the presence of monosaccharide nucleotides. Uridine-[5']-diphospho-[1]- α -D-glucose (UDP- α -GLC) is the activated form of glucose and functions as donor of glucose in the biosynthesis of glycogen. Monosaccharide nucleotides are of great importance for the synthesis of cell walls of micro-organisms and plants.³ An improved detection method of these activated sugars will help to the better understanding of such very complex metaboloms.⁶ Classical analysis uses different hydrolytic treatments which include alkaline hydrolysis or enzymatic cleavage with specific exoglycosidases and pyrophosphatases.¹ The obtained degradation products are then compared with authentic monosaccharide and glycosyl phosphate standards (thin layer chromatography, high-performance liquid chromatography, high-performance anion-exchange chromatography with pulsed amperometric detection, nuclear magnetic resonance etc.).^{1,7} These methods require large amounts of sample, which are not easily obtainable from biological sources. Fast atom bombardment mass spectrometry (FABMS),⁸⁻¹¹ which was found to give good results in the detection and characterization of monosaccharide nucleotides, improved the analysis of this class of compounds. The development of MALDI^{12,13} and ESI¹⁴ mass spectrometry for the analysis of nucleic acids was hampered by the polyanionic character of these classes of compound but nevertheless both desorption/ionization techniques developed to a certain level. To our knowledge, only ESI tandem mass spectrometry has been used for the detailed characterization of glycosyl esters of nucleoside pyrophosphates so far.¹⁵ MALDI post source decay (PSD) mass spectrometry¹⁶ was only explored for (to a certain extent) related compound classes, namely fatty acyl coenzyme A ester¹⁷ and dinucleoside polyphosphates.¹⁸ Due to the fact that MALDI-ToF mass spectrometry usually exhibits a better sensitivity than ESI mass spectrometry, the usefulness of MALDI in combination with PSD fragment ion analysis¹⁶ with a coaxial/umbrella-like curved field reflector¹⁹ was investigated in detail. This method was then applied to samples isolated from baker's yeast which require ultimate sensitivity. The noteworthy property of this type of reflector¹⁹⁻²² is that it brings into simultaneous focus product ions created over a broad m/z range and obviates the need for stepping the reflectron voltage to reconstruct the PSD spectrum.

Experimental

Chemicals

Water, used throughout this study, was purified by an Elgastat water purification system (Bucks, UK) exhibiting a conductivity of $18 \text{ M}\Omega^{-1}\text{cm}^{-1}$. Trifluoroacetic acid (HPLC Spectro Grade, TFA) was purchased from Pierce (Rockford, IL, USA). Acetonitrile (LiChrosolv) was obtained from Merck (Darmstadt, Germany). The matrices (analytical grade) 2,5-dihydroxy benzoic acid (DHB), α -cyano-4-hydroxycinnamic acid (CHCA) and 3-hydroxypicolinic acid (3-HPA) were obtained from Sigma-Aldrich (St Louis, MO, USA). Sinapinic acid (SA) was purchased from Fluka (Buchs, Switzerland) and all MALDI matrices were used without any further purification. Standard peptides for mass spectrometric calibration were oxytocin, LH-RH (human), angiotensin I and III (human),

substance P, somatostatin (all from Sigma–Aldrich, St Louis, MO, USA) and LWMRFA (Serva, Heidelberg, Germany). All other solvents and reagents were analytical grade quality.

Standard compounds

The following glycosyl esters of nucleoside pyrophosphates were obtained from Sigma–Aldrich (St Louis, MO, USA) as sodium salts and used without any further purification step: uridine-[5′]-diphospho-[1]-α-D-glucose (UDP-α-GLC), uridine-[5′]-diphospho-[1]-α-D-glucuronic acid (UDP-α-GLCA), uridine-[5′]-diphospho-[1]-α-D-*N*-acetylglucosamine (UDP-α-GLCNAC), uridine-[5′]-diphospho-[1]-α-D-mannose (UDP-α-MAN), guanosine-[5′]-diphospho-[1]-α-D-mannose (GDP-α-MAN) and guanosine-[5′]-diphospho-[1]-α-D-glucose (GDP-α-GLC). All compounds were dissolved in water at a standard concentration of 800 fmol μL⁻¹ except in case of the determination of the limit of detection (dilution of the standard solution).

MALDI mass spectrometry

MALDI mass spectra from the glycosyl esters of nucleoside pyrophosphates were obtained by means of a Kompact MALDI IV linear (0.7 m)/reflectron (1.2 m) time-of-flight (ToF) mass spectrometer (Shimadzu Biotech Kratos Analytical, Manchester, UK). A nitrogen laser with a pulse width of 3 ns was used to generate photons at 337 nm wavelength. In all experiments, the ions generated were extracted in the continuous mode by an electric field of 24 kV. Each sample was measured in the linear and reflectron modes as well as positive and negative ion modes across the whole sample well. In general, 40 to 100 single unselected mass spectra (*m/z* range 200 to 2000) were averaged and an external calibration based on standard peptides was applied. All mass spectra were smoothed using the manufacturer-supplied Savitsky–Golay algorithm. Average mass values are reported throughout the whole paper.

PSD fragment ion analysis

PSD spectra in the negative ion mode were generated on the above mentioned curved field reflectron (in a coaxial configuration) mass spectrometer with an annular dual microchannel plate detector. The ion gate was set to a width of ±15 Da for the selected precursor ion and a 10% higher laser irradiance (compared to the standard negative ion reflectron mode) was applied. During all PSD experiments, the pressure was typically 2 × 10⁻⁶ mbar or less. The instrument was calibrated in the PSD mode by recording time/mass data for the well-defined product ions obtained from a PSD spectrum of the peptide angiotensin III and fitting these data to an empirical-derived calibration correction curve. PSD spectra were acquired by averaging the unselected spectra of 150 to 400 laser pulses. Signal smoothing and baseline subtraction were performed in all cases.

Sample preparation

For matrix evaluation, the following matrix solutions were prepared: (a) 3-HPA was dissolved (saturated solution) in a mixture of 70% acetonitrile and 30% water (v/v, the water contained 0.1% TFA) and the solution was sonicated for 2–3 min. Then the saturated solution was centrifuged and the supernatant used for sample preparation. (b) CHCA was

dissolved (saturated solution) in a mixture of 99% acetone and 1% water (v/v, the water contained 0.1% TFA), sonicated for 2–3 minutes, centrifuged and the remaining supernatant applied for further sample preparation. (c) SA (10 mg mL^{-1}) was dissolved in acetonitrile/water (containing 0.1% TFA) mixture (7/3, v/v). (d) DHB (10 mg mL^{-1}) was dissolved in acetonitrile/water (containing 0.1% TFA) mixture (1/1, v/v).

The samples were prepared by the thin layer technique²³ in case of the matrices 3-HPA and CHCA. First, a droplet of $0.5 \mu\text{L}$ of matrix solution (supernatant) was deposited on the pre-cleaned stainless steel target and the solvent was evaporated at room temperature by means of a stream of air. On top of the microcrystalline matrix layer, $0.5 \mu\text{L}$ of the aqueous sample solution was deposited and the solvent was again evaporated by a stream of air. In the case of SA and DHB, samples were generated by the volume technique²⁴ mixing the aqueous sample solution and matrix solution in an Eppendorf tube in the ratio 1 : 1 (v/v) and an aliquot was deposited onto the stainless steel MALDI target.

Briefly, the sample from *Saccharomyces cerevisiae* was prepared after cell isolation by aqueous trichloroacetic acid extraction, gel chromatography and subsequent RP HPLC.^{5,25} Fractions of interest were monitored at 254 nm, collected and lyophilized. The fractions were redissolved in $7 \mu\text{L}$ water containing 0.1% TFA and an aliquot of $0.5 \mu\text{L}$ was used directly for MALDI mass spectrometric analysis with the matrix CHCA (thin layer technique).

Results and discussion

Selection of the matrix

The MALDI mass spectra were obtained in both positive and negative ion modes, but it turned out, independent of the selected matrix, that the positive ion mode generated a complex molecular ion region of sodiated, potassiated and mixed molecular ions. This turned out not to be very helpful, particular if real-world samples are analyzed and only low quantities are available. In contrast, the negative ion mode showed the presence of an intense signal that arises from the deprotonated free acid molecule, $[\text{M} - \text{H}]^-$. Therefore, all further investigations were performed in the negative ion mode due to the fact that a sensitivity gain of factor 17 was achieved compared to the positive ion mode.

3-HPA was described in the literature^{26–30} as a good, reproducible and extremely sensitive matrix for oligonucleotides. Oligonucleotides and monoglycosyl esters of nucleoside pyrophosphates share a similar chemical structure, so 3-HPA²⁶ was evaluated as a matrix in combination with the thin layer technique for the selected glycosyl ester of nucleoside pyrophosphate compounds and it proved to be a very efficient matrix to generate abundant deprotonated molecular ions. There were no disturbing background peaks in the m/z region (m/z 560–650) of the molecular ions.

CHCA,³¹ applied using the thin layer sample preparation technique,²³ turned out to be an excellent MALDI matrix for the generation of deprotonated molecular ion species, $[\text{M} - \text{H}]^-$ with a higher sensitivity than 3-HPA. We could obtain excellent mass spectra down in the low femtomole range, as shown in Figure 1(b) for GDP- α -D-GLC (75 femtomole on target

with a S/N ratio of 5/1). The selected compounds exhibited a limit of detection (S/N ratio of 5/1 and based on six independent analyses, standard deviation across all analytes $\pm 22\%$) range between 60 fmol to 100 fmol. Furthermore, the PSD spectra (Figure 2, UDP- α -D-GLCA) based on $[M - H]^-$ as the precursor ion, exhibited more abundant fragment ions than with the 3-HPA matrix. This corroborates a similar described observation for peptides (“hot, CHCA” vs “cold, DHB, 2,6-dihydroxyacetophenone or 3-HPA” MALDI matrix).³²

The application of the MALDI matrix sinapinic acid^{33,34} and 2,5-dihydroxy benzoic acid^{24,35,36} by means of the volume technique turned out to give positive results but required two orders of magnitude more material and the background ions in the deprotonated molecular ion region were much more abundant. For the PSD mode, no advantage was observed.

In general, the thin-layer technique with CHCA turned out to be the best for this compound class and it was noticed that pre-mixing the matrix-solution and the aqueous solution of standards led to much weaker signals.

Molecular mass determination

All MALDI mass spectra obtained in the negative ion mode showed the presence of an intense signal [as an example of 750 femtomole deposited on the sample plate see Figure 1(a)] that arises from the deprotonated free acid molecule and sometimes, if at all, a weak signal for the monosodiated species $[M - 2H + Na]^-$ (only in the case of large amounts of sample). In very few cases, a small signal from the disodiated species, $[M - 3H + 2Na]^-$ of the monoglycosyl ester of nucleoside pyrophosphates could be found, and again, only if a larger amount was applied. With the used desk-top MALDI mass spectrometer a detection limit (S/N ratio of 5/1) in the low femtomole range (Figure 1(b)) for each compound was obtained in the reflectron mode by applying the thin layer technique (see Figure 1 for GDP- α -D-GLC, 75 femtomole deposited on the thin layer of matrix in a well (2×1 mm) on the stainless steel target). A mass accuracy for the molecular mass determination between -0.06 and $+0.10\%$ was achieved with external peptide calibration (Table 1) which is quite good for a table-top ToF instrument.

PSD fragment ion analysis in the negative ion mode

The MALDI mass spectrometric apparatus applied in this work allowed the generation of PSD spectra without any stepping of the reflector voltage, which reduced the time of data acquisition and calibration as well as the number of laser shots, due to the use of a coaxial curved field reflector. All PSD spectra (see as example the PSD spectrum in Figure 2) were derived from $[M - H]^-$ as the precursor ion (Table 1), exhibiting the highest number and abundance of fragment ions with the CHCA matrix and the use of a 10% higher laser irradiance than the threshold level for the formation of deprotonated molecular ions.

First, the six different compounds, differing either structurally in the nucleoside (UDP or GDP) or in the glycosyl (glucose, glucuronic acid, mannose, *N*-acetyl glucoseamine) part, exhibited in the negative ion PSD spectra, to a certain extent, similar but also differing fragmentation patterns. Table 2 shows the structurally relevant PSD fragment ions obtained

applying a curved field reflector without the necessity of stepping the reflector voltage. It is obvious that the compounds with the same nucleoside residue (activator) have a very similar fragmentation pattern. Fragment ions such as the nucleoside monophosphate ion, $[\text{NMP-H}]^-$, or the nucleoside diphosphate ion, $[\text{NDP-H}]^-$, exhibit the same m/z values (for example, m/z 323 and m/z 403 in the case of UDP) and these ions were accompanied by ions showing the neutral loss of water ($[\text{NMP-H}_2\text{O-H}]^-$ m/z 305 and $[\text{NDP-H}_2\text{O-H}]^-$ m/z 385 in the case of UDP and m/z 344 and m/z 424 in the case of GDP). In the case of GDP-containing compounds, the fragment ion $[\text{NDP-H}]^-$ was missing. Further structural relevant fragments, $[\text{Ribosyl-5'-P}_2\text{O}_5\text{-H}]^-$ (m/z 273) and $[\text{HP}_2\text{O}_6]^-$ (m/z 159), were often detected with high abundance as has been described in detail,¹⁰ but sometimes missing completely (for example, UDP- α -D-GLCNAC). Now, a further three types of fragment ions could be detected which allow the determination of the different glycosyl residues, which is of major importance for biological questions – $[\text{Glycosyl-NDP-H}_3\text{PO}_4\text{-H}]^-$, $[\text{Glycosyl-1''-P}_2\text{O}_6]^-$ and $[\text{Glycosyl-1''-PO}_3]^-$. In all investigated cases, the latter two types of fragment ion were determined with significant abundance. The presence of $[\text{Glycosyl-NDP-H}_3\text{PO}_4\text{-H}]^-$ (the loss of one phosphate group from the inner part of the deprotonated molecule) was determined in the case of UDP- α -D-GLC and UDP- α -D-Man and indicates the occurrence of molecular rearrangements during the desorption/ionization process or the post source flight. A similar observation was described by Wolucka *et al.*¹⁰ for FAB tandem MS, indicating a similarity between the two SIMS-based techniques, FAB and MALDI. The fragment ions $[\text{Glycosyl-1''-P}_2\text{O}_6]^-$ and $[\text{Glycosyl-1''-PO}_3]^-$ are of great importance (see as an example, Figure 2, m/z 335 and m/z 255) because these types of fragment ion were diagnostic for the glycosyl residue of the glycosyl esters of nucleoside pyrophosphates. Of course, a differentiation, for example, between a mannose and glucose residue on the same nucleoside was not possible, although differences in abundance were observed. The combination of the molecular mass of monoglycosyl esters of nucleoside pyrophosphates derived from the deprotonated molecule and characteristic fragments $[\text{NMP-H}]^-$, $[\text{Glycosyl-1''-P}_2\text{O}_6]^-$ and $[\text{Glycosyl-1''-PO}_3]^-$ allowed the unambiguous and sensitive identification of these classes of compound.

Finally, we tested our MALDI mass spectrometric approach on an isolated monoglycosyl ester of nucleoside pyrophosphate in the negative ion mode by means of a curved field reflector. The sample was isolated and purified from *Saccharomyces cerevisiae* and MALDI sample preparation was done by the thin layer technique with the CHCA matrix. Figure 3 exhibits the resulting PSD spectrum and, based on the deprotonated molecule (m/z 606) as well as characteristic fragment ions (m/z 403, m/z 362, m/z 323 and m/z 282), the compound was identified as UDP- α -D-GLCNAC.

Conclusions

The deprotonated molecular ions and the characteristic fragmentation pattern in the negative ion mode, which is rarely applied, allowed the unambiguous and sensitive identification of glycosyl esters of nucleoside pyrophosphate by means of MALDI PSD MS applying a curved field reflector desk-top mass spectrometer. The application of the analytical technique was demonstrated on a sample from *S. cerevisiae*.

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Figure 1.

Molecular ion region (deprotonated molecular ion at m/z 604) of the negative ion MALDI mass spectrum of GDP- α -D-GLC (750 (a) and 75 (b) femtomole sample amount deposited, thin layer technique, matrix: CHCA, 100 laser pulses).

Figure 2.

Negative ion MALDI curved field reflector PSD spectrum of the deprotonated molecular ion at m/z 579 from UDP-α-D-Glucose (400 fmol sample amount deposited, thin layer technique, matrix: CHCA, 400 laser pulses).

Figure 3.

Negative ion MALDI curved field reflector PSD spectrum of the deprotonated molecular ion at m/z 606 from UDP- α -D-GLCNAC isolated from *Saccharomyces cerevisiae* (thin layer technique, matrix: CHCA, 150 laser pulses).

Table 1

Molecular ions and m/z values detected in the negative ion MALDI mass spectra of the selected glycosyl ester of nucleoside pyrophosphate (GLC, glucose; GLCA, glucuronic acid; GLCNAC, *N*-acetyl glucosamine; MAN, mannose; Glycosyl-NDP, glycosyl ester of nucleoside pyrophosphate)

Glycosyl-NDP	$[M - H]^-$	$[M - H]^-$	Deviation in %	$[M + Na - 2H]^-$	$[M + Na - 2H]^-$	Deviation in %
	Calculated (av.)	Found		Calculated (av.)	Found	
UDP- α -D-GLC	565.3	565.6	+0.05	587.3	587.1	-0.03
UDP- α -D-GLCA	579.3	579.7	+0.06	601.3	601.9	+0.10
UDP- α -D-GLCNAC	606.5	606.9	+0.07	628.5	628.1	-0.06
UDP- α -D-MAN	565.3	565.8	+0.08	587.3	587.8	+0.08
GDP- α -D-MAN	604.3	604.6	+0.05	626.3	626.8	+0.08
GDP- α -D-GLC	604.3	604.7	+0.06	626.3	626.9	+0.10

Table 2

Major fragment ions found in the negative ion MALDI PSD spectra of the six reference compounds: m/z values (+, detected; –, not detected). Glycosyl-NDP, glycosyl ester of nucleoside pyrophosphate; NDP, nucleoside diphosphate; NMP, nucleoside monophosphate; l, low abundance; h, high abundance

Fragment ions obtained from $[M-H]^-$ ion	UDP- α -D-GLC (565)		UDP- α -D-MAN (565)		UDP- α -D-GLCA (579)		UDP- α -D-GLCNAC (606)		GDP- α -D-GLC (604)		GDP- α -D-MAN (604)	
	Found	Mass value	Found	Mass value	Found	Mass value	Found	Mass value	Found	Mass value	Found	Mass value
[Glycosyl-NDP- $H_3PO_4-H]^-$	+	467	+	467	–	481	–	508	–	506	–	506
[NDP- $H]^-$	+	403	+	403	+	403	+	403	–	442	–	442
[NDP- $H_2O-H]^-$	+	385	+	385	+	385	–	385	+	424	+	424
[NMP- $H]^-$	+	323	+	323	+	323	+	323	+	362	+	362
[NMP- $H_2O-H]^-$	+	305	+	305	+	305	–	305	+	344	+	344
[Glycosyl- $l''-PO_3]^-$	+	241	+	241	+	255	+	282	+	241	+	241
[Glycosyl- $l''-P_2O_6]^-$	+	321	+	321	+	335	+	362	+	321	+	321
[Ribosyl- $5'-P_2O_5-H]^-$	+	273	+	273	+	273	–	273	–	273	–	273
[$HP_2O_6]^-$	–	159	–	159	+	159	–	159	+	159	+	159